Effects of Dietary Docosahexaenoic Acid Connecting Phospholipids on the Learning Ability and Fatty Acid Composition of the Brain

Seiichi Hiratsuka1, Kyoko Koizumi1, Tomoko Ooba2 and Hidehiko Yokogoshi3

1 Shizuoka Prefectural Research Institute of Fishery, Yaizu, Shizuoka 425–0033, Japan
2 Maruhachi Maramatsu, Inc., Yaizu, Shizuoka 425–0025, Japan
3 Graduate School of Food and Nutritional Sciences, The University of Shizuoka, Shizuoka 422–8526, Japan

(Received December 19, 2008)

Summary The effects of dietary docosahexaenoic acid (DHA, C22:6n-3) connecting phospholipids on the learning ability and fatty acid composition of the brain were investigated in hypercholesterolemic mice. ICR mice were subjected to a very low level of n-3 fatty acids through two generations. At 4 wk of age, the F1 generation, n-3 fatty acid deficient male mice were provided with an experimental diet containing four kinds of lipids (safflower oil: Saf, DHA connecting triacylglycerols: DHA-TG, DHA connecting phospholipids: DHA-PL, soybean phospholipids: Soy-PL) for 5 wk. Another group of ICR mice were obtained and fed a commercial diet (CE-2, CLEA Japan, Inc.) as a control. The learning and memory abilities of the mice were evaluated by the modified avoidance procedure. The learning and memory ability level was significantly higher in mice fed the DHA-PL diet than in those fed the Saf and Soy-PL diets, and was the same level as the control. The DHA levels of phosphatidylethanolamine in the brain were significantly higher in the mice fed the two types of DHA-containing diets than in those fed the Saf and Soy-PL diets and was not significantly different between DHA-TG and DHA-PL. The dimethylacetal levels in the brain were significantly higher in the mice fed the DHA-PL diet than in those fed the Saf and DHA-TG diets. These results suggest that the dietary DHA connecting phospholipids have the effect of improving memory learning, and may be related to the both the DHA and plasmalogen levels in the brain.

Key Words docosahexaenoic acid, phospholipid, learning ability, fatty acid, plasmalogen

The n-3 polyunsaturated fatty acids (PUFA), especially docosahexaenoic acid (DHA), have various physiological functions, such as prevention of cardiovascular disease (1), lowering of plasma lipids (2) and improving memory learning (3). Most of these studies used the DHA connecting triacylglycerols (DHA-TG) or their ethyl esters; however, it has recently been reported that the DHA connecting phospholipids (DHA-PL) have some peculiar functions such as anti-inflammatory actions (4), improving the deformability of human erythrocytes in microchannels (5), and promoting the cell differentiation of erythroleukemia cancer cells (6).

In our previous study (7), which compared the effects of the PL and TG types of dietary DHA on the lipid peroxidation of the brain in STZ-induced diabetic mice, the DHA-PL diet group showed antioxidant activity in the brain lipid, and the plasmalogen level of the brain was higher than that of the DHA-TG group. Therefore, we suggested that the effect of the antioxidant activity of the brain lipid was related to the plasmalogen concentration.

There are several reports that the plasmalogen concentration in blood is related to aging, dementia and hyperlipidemia. The ethanolamine plasmalogen levels of the serum have been shown to be lower in dementia patients of the Alzheimer’s type (8) and significant negative correlations between age and the plasmalogen level of the erythrocyte membrane and plasma phospholipids have been obtained (9). In addition, a negative correlation of serum triacylglycerol (TG) with the plasmalogen level was also detected in patients with impaired carbohydrate or lipid metabolism (10). Perkings et al. (11) reported that serum antioxidant levels were associated with poor memory performance in the elderly. Oxidative stress has been implicated both in the aging process and in the pathological changes associated with Alzheimer’s disease (12–14). Therefore, if DHA-PL intake enhances the plasmalogen level and prevents oxidative stress in the brain, it is suggested that DHA-PL intake may improve learning and memory abilities.

In this study, the effect of dietary DHA-PL on learning and memory abilities, and fatty acid and plasmalogen levels were compared to dietary DHA-TG and soybean phospholipid (Soy-PL) in mice. We used hypercholesterolemic mice in this study because Hossain et al. (15) reported that the lipid peroxidation of the brain was increased in the hypercholesterolemic rats. In addition, the plasmalogen may be related to oxidative stress.
MATERIALS AND METHODS

Diets. Ovaries of the skipjack tuna Euthynnus pelamis were obtained from a katsuobushi factory. The skipjack ovary lipids were extracted using Bligh and Dyer’s method modified by Hanson and Olley (16), and then the phospholipid was separated using a Sep-pak silica cartridge (10 g; Waters, Tokyo, Japan). The lipid class composition of the phospholipid obtained, measured according to the method of Bartlett (17), was 34.3% phosphatidylcholine (PC), 34.2% lysoPC, 14.8% sphingomyelin, 10.7% phosphatidylethanolamine (PE), and 6.0% others. DHA oil was purchased from Nihon Suisan, Inc. (Tokyo, Japan) and high oleic safflower oil (Saf) was purchased from the Ajinomoto Co., Inc. (Tokyo, Japan). Soybean phosphatidylcholine and sn-glycero-3-phosphocholine were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The skipjack ovary phospholipid (DHA-PL) and DHA oil (DHA-TG) were mixed with safflower oil to give a DHA content of 2% of the total lipids. DHA-PL and soybean phosphatidylcholine (Soy-PL) were also mixed with safflower oil to give a phospholipid content of 11.9% of the total lipids. The fatty acid content of the experimental lipids is shown in Table 1. A total of 4.8 g sn-glycero-3-phosphocholine was added to the safflower and DHA-TG diets to give equivalent phospholipids. The DHA-PL diet included 103 mg/100 g lipid of dimethylacetal from the plasmalogens. The α-tocopherol content in each diet was adjusted to 30 mg/kg. The lipid peroxide levels of all experimental diets were less than 0.3 meq/kg (data not shown). The composition of the experimental diet was as follows (g/kg diet): casein, 230; cornstarch, 430; sucrose, 200; AIN-76 mineral mixture, 35; AIN-76 vitamin mixture, 10; cellulose powder, 20; DL-methionine, 3; choline bitartrate, 2; lipid, 50; cholesterol, 15; sodium cholate, 5. Sucrose was purchased from the Maruhana Nichiro Foods, Inc. (Tokyo, Japan). DL-Methionine and choline bitartrate were from Wako Pure Chemical Industries, Ltd.

Animals. All animals used in the experiments were maintained in a controlled temperature room at 23 ± 1°C, and 50–60% relative humidity with a 07:00–19:00 light cycle. All animal procedures were performed in accordance with the guidelines of the Shizuoka Prefectural Research Institute of Fishery for the ethical treatment of laboratory animals. Female ICR mice (F0) at 3 wk of age were obtained from Japan SLC, Inc. (Shizuoka, Japan). The mice were fed the n-3 fatty acid deficient diet (safflower oil) for 6 wk and were mated at 9 wk of age. The male mice obtained were fed a commercial diet (CE-2, CLEA Japan, Inc.). At 4 wk of age, the male pups (F1) were divided into four dietary groups of twelve mice each (Saf, DHA-TG, DHA-PL, Soy-PL). Other 4-wk-old male ICR mice were obtained and fed a commercial diet as a control. The learning and memory ability test was started at 8 wk of age and the open field test was performed at 9 wk of age. After the open field test, all mice were fasted for 18 h, and then anesthetized with diethyl ether. Whole blood was collected from a cervical wound and the brains were removed and placed in cold methanol containing 0.03% butylated hydroxytoluene (BHT). The blood serum, livers and brains were stored at −70°C until analysis.

Learning and memory ability test. The learning and memory ability test of the mice at 8 wk of age was evaluated using the modified avoidance procedure with a shuttle box (step-through) that consisted of two equal compartments (15×15×15 cm) connected by a guillotine door. One of the compartments was lit while the other was dark. In the first trial, each mouse was placed in the dark compartment, and after a brief orientation period (10 s), the guillotine door was raised to allow the mouse to freely explore the setup. After 5 min, once the mouse had entered the dark compartment, the door was lowered and an electric shock was applied to the floor grids for 2 s. After 1 wk, the same procedure except for the electric shock, was carried out again (the second trial). The total time the mouse spent in the dark compartment and the total number of crossings from one compartment to the other were recorded.

Open-field test. The open-field box was a 40 cm square with walls 30 cm high made of glass. The floor was divided into 16 squares by black lines. A mouse was placed in the corner of the open-field and observed for 5 min. The movement and time in the central area (central 4 squares; 20×20 cm) were recorded.

Serum cholesterol and triacylglycerol. The concentrations of the total cholesterol, HDL-cholesterol and triacylglycerol were enzymatically determined using a commercial kit (Cholesterol E-test, HDL-cholesterol E-test, triacylglycerol E-test, Wako Pure Chemical Industries, Ltd.). The LDL-cholesterol concentration was calculated.

---

Table 1. Fatty acid contents of dietary lipids.

| Fatty acid (g/100 g lipid) | Safflower | DHA-TG | DHA-PL | Soy-PL |
|---------------------------|-----------|--------|--------|--------|
| n-3/n-6                   | 0.0       | 0.6    | 0.0    | 0.0    |
| Dimethylacetal (mg/100 g lipid) |           |        |        |        |
| C16:0                     | 5.2       | 7.1    | 6.5    | 5.7    |
| C18:0                     | 2.0       | 2.0    | 2.1    | 1.9    |
| C16:1                     | 0.0       | 1.1    | 0.0    | 0.0    |
| C18:1n-9                  | 72.3      | 67.0   | 69.3   | 67.7   |
| C18:1n-7                  | 0.0       | 0.7    | 0.0    | 0.1    |
| C18:2n-6                  | 14.1      | 12.4   | 12.8   | 17.1   |
| C18:3n-3                  | 0.0       | 0.0    | 0.0    | 0.4    |
| C20:4n-6                  | 0.0       | 0.2    | 0.3    | 0.0    |
| C20:5n-3                  | 0.0       | 0.4    | 0.3    | 0.0    |
| C22:6n-3                  | 0.0       | 2.0    | 2.0    | 0.0    |
| Total fatty acid contents | 93.6      | 93.4   | 93.4   | 92.9   |
| n-3/n-6                   | 0.00      | 0.19   | 0.18   | 0.02   |
| sn-Glycero-3-phosphocholine (g/100 g lipid) | 4.8       | 4.8    | 0.0    | 0.0    |
| PL ratio (%)              | 0.0       | 0.0    | 11.9   | 11.9   |

---

Effect of Docosahexaenoic Acid on Learning Ability
as follows:

\[
\text{LDL cholesterol} = \text{total cholesterol} - \text{HDL-cholesterol} - \text{triacylglycerol} / 5.
\]

**Fatty acid and dimethylacetal.** The total lipids in the brain and liver were extracted according to Bligh and Dyer’s method modified by Hanson and Olley (16). The phospholipid classes of the total lipids in the brain were separated by thin-layer chromatography (TLC) on silica gel plates with chloroform/methanol/water (65 : 35 : 6 by vol.) as the developing solvent. Each band was detected under saturated iodine gas, and scraped off from the PE and PC band on the plate. The separated PE and PC were converted to fatty acid methyl ester (FAME, from diacyl type) and dimethylacetal (DMA, from plasmalogen type) by direct transesterification with methanol containing 1% concentrated hydrochloric acid for 2.5 h at 85˚C as previously described (18). The FAME and DMA were separated by gas chromatography using a flame ionization detector (GC-14A; Shimadzu Corporation, Kyoto, Japan). The chromatograph was equipped with a fused silica capillary column, TC-WAX (30 m x 0.25 mm i.d.; GL Science Co., Ltd., Tokyo, Japan). The carrier gas was helium with a split injection of 50 : 1. The temperature profiles were as follows: initial temperature, 170˚C; heating rate, 1˚C/min; final temperature, 225˚C (final time, 15 min); injector temperature, 250˚C; and detector temperature, 270˚C. The FAME and DMA were identified by comparison of their retention times with standards, and also by gas chromatography-mass spectrometry (GC-MS). The GC-MS analysis was done after oxazoline derivatization (19) by a Shimadzu QP-5000 GC-MS system using a Supelco Omegawax 320 column (30 m x 0.32 mm). The temperature was set at 260˚C (column), 200˚C (interface), and 200˚C (ionization chamber). The ionization voltage was 70 eV. The DMA were identified by the characteristic peak of the DMA fragment at m/z 75 (CH (OCH$_3$)$_2$).

**Statistical analysis.** All results were expressed as the means±SE, and statistical significance was determined by a Student’s t-test and one-way analysis of variance (ANOVA) using SPSS 11.5J for Windows. When the F-test was significant, comparisons between the groups were done using Tukey’s multiple range test. The significance level was set at p<0.05.

### RESULTS

**Body weight and food consumption**

There were no significant differences in the average final body weights (36.0–37.1 g). The average food consumptions were between 3.8 and 4.0 g/d by the mice in the five groups and there were no significant differences among the groups (data not shown).

**Relative liver weight, lipid contents of brain and liver, serum cholesterol and triacylglycerol**

The relative liver weight, the lipid contents of the brain and liver, and the concentrations of serum cholesterol and triacylglycerol of the mice are shown in Table 2. The relative liver weights, liver lipid contents, serum

|                  | Saflower | DHA-TG | DHA-PL | Soy-PL | Control |
|------------------|----------|--------|--------|--------|---------|
| **Relative liver weight (%)** | 9.7±0.4* | 9.7±0.4* | 8.9±0.4* | 9.6±0.5* | 4.0±0.1 |
| **Lipid content (%)**          |          |        |        |        |         |
| Brain             | 10.0±0.1 | 10.1±0.2 | 9.8±0.2 | 9.7±0.2 | 9.7±0.2 |
| Liver             | 21.7±1.0* | 18.1±0.6* | 18.9±0.7* | 19.6±0.7* | 5.3±0.1 |
| **Serum cholesterol (mg/dL)** |          |        |        |        |         |
| Total             | 420.7±23.5* | 402.9±20.9* | 356.8±19.2* | 414.2±16.1* | 124.9±7.2 |
| HDL               | 89.4±6.2  | 90.8±5.4  | 91.2±7.8  | 84.6±7.1  | 87.0±4.9 |
| LDL               | 321.3±28.6*a | 302.6±17.5*ab | 236.8±23.2*b | 317.0±18.8*a | 38.4±5.2  |
| **Serum triacylglycerol (mg/dL)** | 41.5±3.8 | 42.7±4.0 | 53.1±8.0 | 46.2±3.9 | 61.9±6.7 |

Values are the means±SE of twelve mice. Asterisks (*) indicate significant differences from the control group at p<0.05 using a Student’s t-test, and the means without the same letters (a, b) are significantly different at p<0.05 based on Tukey’s multiple range test.
total and LDL cholesterols were significantly higher in the mice fed the four experimental diets (hypercholes-
terol diets) than in those fed the control diet, and the
LDL cholesterol concentration was significantly lower
in the mice fed the DHA-PL diet than in those fed the
Saf and Soy-PL diets. There were no significant differ-
ces in relative liver weights, liver lipid contents or
serum total cholesterol concentrations among the four
experimental diet groups. There were no significant dif-
ferences in the brain lipid contents, serum HDL choles-
terol or triacylglycerol concentrations among the five
groups.

Learning and memory abilities
The total mean time the mice from each group spent
in the dark compartment during the 5 min test is
shown in Fig. 1. In the first trial, there were no signifi-
cant differences in the times among the five groups.
In the second trial, the total time the DHA-PL diet mice
spent in the dark compartment was significantly shorter than that of the Saf and Soy-PL diet groups, and
that of the Saf, DHA-TG and Soy-PL groups were signif-
ificantly longer than that of the control. The total num-
ter of times a mouse crossed from one compartment to
the other during the 5 min test is shown in Fig. 2. In the
first trial, the total number of crossings from one com-
partment to the other in the four experimental diet
groups was more than that of the control and there
were no significant differences between the four ex-
perimental diet groups. In the second trial, there were no
significant differences among the five diet groups.

Open field test
There were no significant differences in movement
times (Saf: 58.7±3.4, DHA-TG: 51.9±2.1, DHA-PL:
53.8±3.0, Soy-PL: 58.0±3.4, Control: 53.0±3.0) or
time spent in the central area (Saf: 17.2±2.5, DHA-TG:

![Diagram](image)

**Fig. 2** The mean number of times the mice of each
group crossed from one compartment to the other
during the 5 min test (left: first; right: second). Bars rep-
resent the means±SE of twelve mice. Asterisks (*) indi-
cate significant differences from the control at p<0.05
using a Student’s t-test, and the means without the
same letter are significantly different at p<0.05 based
on Tukey’s multiple test.

|  |  |
|---|---|
| **Table 3. Fatty acid and dimethylacetal compositions of phosphatidylethanolamine in brain of mice.**<sup> (wt%)</sup> |
|  | Saflower | DHA-TG | DHA-PL | Soybean-PL | Control |
|---|---|---|---|---|---|
| **Fatty acid** |
| C16:0 | 5.19±0.12 a | 4.93±0.08 ab | 4.65±0.07 b | 5.02±0.12 ab | 4.66±0.10 |
| C18:0 | 17.95±0.24 | 17.66±0.17 | 17.45±0.18 | 17.42±0.19 b | 17.44±0.26 |
| C18:1n-9 | 11.01±0.24 ab | 11.08±0.19 a | 10.45±0.12 ab | 10.33±0.17 b | 10.41±0.20 |
| C18:1n-7 | 2.55±0.07 a | 2.39±0.05 bd | 2.07±0.02 bc | 2.33±0.07 d | 1.97±0.05 |
| C20:1n-9 | 2.94±0.10 | 3.08±0.11 | 2.98±0.06 | 2.92±0.08 | 3.03±0.07 |
| C20:4n-6 | 13.16±0.17 a | 9.98±0.11 b | 9.77±0.10 b | 12.41±0.13 c | 9.85±0.12 |
| C22:4n-6 | 6.68±0.12 a | 4.76±0.10 b | 4.71±0.08 b | 6.31±0.06 a | 4.34±0.06 |
| C22:5n-6 | 12.53±0.16 a | 4.74±0.20 b | 4.88±0.12 b | 11.10±0.24 c | 0.00 |
| C22:6n-3 | 6.08±0.24 a | 18.52±0.35 b | 18.42±0.22 b | 7.50±0.38 c | 22.75±0.35 |
| SAFD | 23.14±0.32 | 22.59±0.23 | 22.11±0.20 | 22.44±0.25 | 22.10±0.30 |
| MOFA | 16.50±0.32 | 16.35±0.32 | 15.50±0.16 | 15.58±0.28 | 15.41±0.29 |
| PUFA | 38.43±0.26 | 38.00±0.34 | 37.77±0.28 | 37.35±0.35 | 36.94±0.33 |
| Others | 1.51±0.31 | 1.82±0.32 | 1.54±0.26 | 1.88±0.31 | 1.38±0.26 |
| **Dimethylacetal** |
| C16:0DMA | 5.57±0.20 | 5.52±0.11 | 5.91±0.08 | 6.06±0.19 | 6.18±0.11 |
| C18:0DMA | 8.22±0.30 a | 8.84±0.20 ab | 9.65±0.10 b | 9.39±0.19 b | 10.73±0.17 |
| C18:1n-9DMA | 3.16±0.09 a | 3.20±0.06 ab | 3.66±0.06 b | 3.50±0.08 b | 3.58±0.07 |
| C18:1n-7DMA | 3.47±0.11 | 3.52±0.06 a | 3.86±0.10 a | 3.81±0.10 ab | 3.68±0.09 |
| Total DMA | 20.42±0.67 a | 21.24±0.39 ab | 23.08±0.29c | 22.76±0.49 bc | 24.17±0.38 |
| C16:0DMA/FA | 1.09±0.05 a | 1.13±0.03 ab | 1.27±0.02 b | 1.22±0.05 ab | 1.33±0.03 |
| C18:0DMA/FA | 0.46±0.02 a | 0.50±0.01 ab | 0.55±0.01 b | 0.54±0.01 b | 0.62±0.02 |
| C18:1n-9DMA/FA | 0.29±0.01 a | 0.30±0.01 ab | 0.35±0.01 c | 0.34±0.01 bc | 0.35±0.01 |
| C18:1n-7DMA/FA | 1.38±0.06 a | 1.62±0.04 ab | 1.86±0.05 c | 1.65±0.06 bc | 1.87±0.04 |

Values are the means±SE of twelve mice. Underlining indicates significant difference from the control group at p<0.05 using a Student’s t-test, and the means without the same letters (a, b, c) are significantly different at p<0.05 based on Tukey’s multiple range test.
Table 1. Fatty acid composition of phosphatidylcholine in brain of mice. (wt%)

| Fatty acid | Safflower | DHA-TG | DHA-PL | Soybean-PL | Control |
|-----------|-----------|--------|--------|------------|---------|
| C16:0     | 36.94±0.71| 35.13±0.71| 34.87±0.73| 35.10±0.97| 34.51±0.79 |
| C18:0     | 20.48±0.17| 21.10±0.26| 20.84±0.22| 21.01±0.32| 21.94±0.36 |
| C18:1n-9  | 20.96±0.37| 21.77±0.38| 21.52±0.40| 20.62±0.34| 20.89±0.37 |
| C18:1n-7  | 6.20±0.11 a| 5.59±0.07 b| 5.67±0.06 bc| 5.92±0.07 ac| 5.10±0.07 |
| C20:1n-9  | 1.63±0.05| 1.65±0.03| 1.67±0.05| 1.61±0.04| 1.53±0.04 |
| C20:3n-6  | 4.92±0.26 ab| 4.73±0.17 a| 4.51±0.14 a| 5.58±0.21 b| 5.26±0.18 |
| C22:4n-6  | 1.23±0.07 a| 0.91±0.02 b| 0.95±0.04 b| 1.41±0.06 a| 0.89±0.05 |
| C22:5n-6  | 5.16±0.23 a| 2.13±0.07 b| 2.39±0.10 b| 5.13±0.21 a| 0.00 |
| C22:6n-3  | 1.68±0.09 a| 6.22±0.26 b| 6.21±0.21 b| 2.42±0.17 a| 8.65±0.22 |
| Others    | 0.80±0.22| 0.77±0.21| 1.37±0.16| 1.20±0.27| 1.23±0.2 |
| SAFA      | 57.42±0.49| 56.23±0.56| 55.71±0.62| 56.11±0.72| 56.45±0.54 |
| MOFA      | 28.79±0.38| 29.01±0.44| 28.86±0.48| 28.15±0.37| 27.52±0.42 |
| PUFA      | 12.99±0.49| 14.00±0.46| 14.06±0.42| 14.54±0.56| 14.80±0.35 |

Values are the means±SE of twelve mice. Underlining indicates significant difference from the control group at p<0.05 using a Student’s t-test, and the means without the same letters (a, b, c) are significantly different at p<0.05 based on Tukey’s multiple range test.

16.1±1.5, DHA-PL: 19.0±3.4, Soy-PL: 17.8±3.0, Control: 18.7±3.0) among the five diet groups.

**Fatty acid and dimethylacetal (DMA) of the phospholipids in the brain**

The fatty acid and dimethylacetal compositions of PE and PC in the brain of the mice are shown in Tables 3 and 4, respectively. The levels of the polyunsaturated fatty acid (PUFA) tended to be higher in the PE than in the PC. In both the PE and PC, the fatty acid compositions of the brains of the mice were affected by their diets; that is, the levels of C20:4n-6, C22:4n-6 and C22:5n-6 were significantly higher in mice fed the Saf and Soy-PL diets than in those fed the two types of DHA diets. However, the levels of C22:6n-3 in the Saf and Soy-PL diet groups were significantly lower than those in the two types of DHA diet groups. The C16:0DMA, C18:1n-9DMA and C18:1n-7DMA levels were significantly higher in the mice fed the DHA-PL diet than in those fed the Saf diet. The total DMA level was significantly higher in mice fed the DHA-PL diet than in those fed the Saf and DHA-TG diets. The ratios of C16:0 and C18:0DMA to the corresponding fatty acid methyl esters (DMA/FAME) were significantly higher in mice fed the DHA-PL diet than in those fed the Saf diet. The DMA/FAME of C18:1n-9 and C18:1n-7 were significantly higher in mice fed the DHA-PL diet than in those fed the Saf and DHA-TG diets.

**DISCUSSION**

It has been previously reported in several studies that n-3 fatty acid intake improved the learning and memory abilities of rats or mice, shown by maze behavior tests (20, 21), brightness-discrimination learning tests (22), eight-arm radial maze tests (23), and Morris water maze tests (24). In these studies, it was considered that the n-3 fatty acid intake enhanced the brain DHA level, and as a consequence, the fluidity of the synaptic membrane increases and the learning ability improves (25).

In the present study, the effects of the PL and TG types of dietary DHA on the learning and memory abilities of the n-3 fatty acid deficient hypercholesterolemic mice using a shuttle box (step-through) were compared. When the learning ability of mice is measured by an avoidance test using a shuttle box setup, passive or active avoidance methods are often used (26–28). In both avoidance methods, the mouse is first given an electric shock in the dark compartment. In the passive avoidance method, after the mouse is placed in the lit compartment, the time it stays there before moving to the dark compartment is measured. The active avoidance method examines the successful avoidance response in which the mouse moves from the dark compartment to the other compartment within the prescribed time. However, mice, especially young ones, show much more exploratory behavior than rats. Thus, from the results of both avoidance methods, it is difficult to understand whether the mouse behavior is avoidance or exploration. For this reason, the time the mouse stayed in the dark compartment during the 5 min test was measured to provide an index of learning ability in the present study. Based on the results of this study, there were no significant differences in the time the mouse stayed in the dark compartment among the five diet groups in the first trial. After the mouse was given an electric shock, the time spent in the dark compartment by the DHA-PL diet group was significantly shorter than that of the Saf and Soy-PL diet groups, and was not significantly different when compared to the control group whose behavior is considered to be normal. However, the time the DHA-TG diet group spent in the dark compartment was significantly longer than that of the control group. In addition, no significant differences in the total number of crossings from one com-
partment to the other or the locomotion times in the open-field test were observed among the five diet groups. Therefore, the difference in the times spent in the dark compartment is not due to differences in exploratory or motor activities. Thus, we concluded that the DHA-PL diet group has the highest learning ability among the four experimental diet groups.

In the present study, the two types of DHA-containing diet groups were compared, and there were no significant differences in the DHA levels of the PC or PE in the brain, while the total DMA and C18:1(DMA/FAE) levels were significantly higher in the mice fed the DHA-PL diet than in those fed the DHA-TG diet. This suggests that the plasmalogen levels of the PE in the brain of the DHA-PL diet group were higher than those of the DHA-TG diet group. Several studies on the effects of the differences of the DHA molecular types on the DHA level of the serum or brain have been reported. When the rats were injected with the labeled DHA into the tail vein, the serum or brain have been reported. When the rats were injected with the labeled DHA into the tail vein, the lyso PC type of DHA was preferentially recovered in the brain, while the DHA-PL diet than in those fed the DHA-TG diet. This suggests that the DHA level of each organ could not be determined.

In this and our previous study, the plasmalogen levels of brain PE were significantly higher in the mice fed the DHA-PL diet than in those fed the DHA-TG diet. PE in the brain has a relatively high proportion of DHA and its main sub-class is plasmalogen. The function of plasmalogen is not known; however, according to recent studies, plasmalogen in the PE has an antioxidant action in lowering the ability of cholesterol to be oxidized in the phospholipid bilayers as well as in the entire membranes. In addition, it has been reported that the plasmalogen concentration in the blood plasma is significantly reduced by aging and hyperlipidemia and the ethanolamine plasmalogen levels of serum were lower in dementia patients of Alzheimer’s type. Based on these studies, it is considered that plasmalogen has some important functions in vivo, especially antioxidant action for maintaining and improving brain functions. Therefore, it is suggested that the improvement of memory and learning abilities may be related to both the DHA and plasmalogen levels in the brain.

In this study, the DHA-PL diet contained plasmalogen (10.3 mg/kg DMA in the diet). The vinyl ether double bonds in the plasmalogen are known to be sensitive to acid; however, Nishimura et al. demonstrated that dietary plasmalogen was absorbed from the intestine in rats. Thus, it is suggested that plasmalogen in the dietary DHA-PL was supplied to the brain. However, it could be that the normal type (di-acyl) of phospholipids and not the plasmalogen intake may cause the brain plasmalogen levels to increase. The total DMA levels in the brain appeared to be higher in mice fed the phospholipid-containing diet groups (in both DHA-PL and Soy-PL) than in those fed the tricaplylglycerol diet groups (DHA-TG and SaF) in the present study. Nagano and Zoeller reported on the biosynthetic pathway for the ethanolamine plasmalogens. According to the report, the ethanolamine plasmalogens are biosynthesized through seven reactions from the long chain acyl-CoA in the brain. Therefore, it was considered that if ethanolamine is supplied to the brain, the ethanolamine plasmalogen could be biosynthesized by the diacylglycerol ethanolamine phosphotransferase (catalyzing the attachment of the phosphoethanolamine in the sn-3 position) and 15-desaturase (catalyzing the insertion of a double bond between the C1 and C2 of the alkyl chain) in the brain. Further investigations on the relationships between the dietary components and the regulation of the plasmalogen biosynthesis are needed.

In conclusion, the results of this study indicate that the dietary DHA connecting phospholipids improve memory learning, which may be related to the both DHA and plasmalogen levels in the brain.

REFERENCES

1) Nordoy A, Marchioli R, Arnesen H, Videbaek J. 2001. n-3 polyunsaturated fatty acids and cardiovascular diseases. Lipids 36: S127–129.
2) Yonekubo A, Honda S, Hagiwara M, Okano M, Yamamoto Y. 1990. The effects of dietary fish oil on the serum lipids and tissue fatty acid composition of rats. Agric Biol Chem 54: 1829–1833.
3) Ikemoto A, Ohishi M, Sato Y, Hata N, Misawa Y, Fujii Y, Okuyama H. 2001. Reversibility of n-3 fatty acid deficiency-induced alterations of learning behavior in the rat: level of n-6 fatty acids as another critical factor. J Lipid Res 42: 1655–1663.
4) Morizawa K, Tomobe Y, Tsuchida M, Nakano Y, Hibino H, Tanaka Y. 2000. Dietary oils and phospholipids containing n-3 highly unsaturated fatty acids suppress 2,4-dinitro-1-fluorobenzene-induced contact dermatitis in mice. Jpn Oil Chem Soc 49: 59–65.
5) Hosokawa M, Sato A, Ishigamori H, Kohno H, Tanaka H, Takahashi K. 2001. Synergistic effects of highly unsaturated fatty acid-containing phosphatidylethanolamine on differentiation of human leukemia HL-60 cells by dibutyryl cyclic adenosine monophosphate. Jpn J Cancer Res 92: 666–672.
6) Tochizawa K, Hosokawa M, Kuribara H, Kohno H, Odashima S, Takahashi K. 1997. Effect of phospholipids containing docosahexaenoic acid on differentiation and growth of HL-60 human promyelocytic leukemia cells. J Nutr Sci Vitaminol 54: 382–390.
7) Hiratsuka S, Ishihara K, Kitagawa T, Wada S, Yokogoshi H. 2008. Effect of dietary docosahexaenoic acid connecting phospholipids on the lipid peroxidation of brain in mice. J Nutr Sci Vitaminol 54: 501–506.
8) Goodenowe DB, Cook LL, Liu J, Lu Y, Jayasinghe DA, Ahiahonu PWK, Heath D, Yamazaki Y, Flax J, Krenitsky KP, Sparks DL, Lerner A, Friedland RP, Kudo T, Kamino K, Morihara T, Takeda M, Wood PL. 2007. Peripheral ethanalamine plasmalogen deficiency: a logical causative factor in Alzheimer’s disease and dementia. J Lipid Res 48: 2485–2498.

9) Brosche T. 1997. Plasmalogen phospholipids—facts and theses to their antioxidative qualities. Arch Gerontol Geriatr 25: 73–81.

10) Brosche T. 2001. Plasmalogen levels in serum from patients with impaired carbohydrate or lipid metabolism and elderly subjects with normal metabolic values. Arch Gerontol Geriatr 32: 283–294.

11) Perkings AJ, Hendrie HC, Callahan CM, Gao S, Unverzagt FW, Xu Y, Hail KS, Hui S. 1999. Association of antioxidants with memory in a multiethnic elderly sample using the third national health and nutrition examination survey. Am J Epidemiol 150: 37.

12) Lytras L, Cairns NJ, Jenner A, Jenner P, Halliwell B. 1997. An assessment of oxidative damage to proteins, lipids and DNA in brain from patients with Alzheimer’s disease. J Neurochem 68: 2061–2068.

13) Ceballos PI, Merad BM, Nicole A, Thevenin M, Hellier G, Hossain MS, Hashimoto M, Masumura S. 1998. Influence of molecular forms on distribution of docosahexaenoic acid in the young rat brain. Lipids 33: 219–223.

14) Smith CD, Carney JM, Starke-Reed PE, Oliver CN, Stadtman ER, Floyd RA, Marksbery WR. 1991. Excess brain protein oxidation and enzyme dysfunction in normal aging and in Alzheimer disease. Proc Natl Acad Sci USA 88: 10540–10543.

15) Hossain MS, Hashimoto M, Masumura S. 1998. Influence of docosahexaenoic acid on cerebral lipid peroxide level in ages rats with and without hypercholesterolemia. Neurosci Lett 244: 157–160.

16) Hanson SWE, Oley J. 1963. Application of the Bligh and Dyer method of lipid extraction to tissue homogenates. J Biol Chem 239: 101–102.

17) Bartlett GR. 1959. Phosphorus assay in column chromatography. J Biol Chem 234: 466–468.

18) Hiratsuka S, Kitagawa T, Matsue Y, Hashidume M, Wada S. 2004. Lipid class and fatty acid composition of plasmalipids from the gonads of skipjack tuna. Fish Sci 70: 903–909.

19) Yu QT, Liu BN, Zhang YJ, Huang ZH. 1989. Location of double bonds in fatty acids of fish oil and rat testis lipids. Gas chromatography-mass spectrometry of the oxoamine derivatives. Lipois 24: 79–83.

20) Lim SY, Suzuki H. 2000. Effect of dietary docosahexaenoic acid and phosphatidylcholine on mouse behavior and fatty acid composition of plasma and brain lipids in mice. Int J Vitam Nutr Res 70: 251–259.

21) Shirai N, Higuchi T, Suzuki H. 2006. Effect of lipids extracted from a salted herring roe food product on maze-behavior in mice. J Nutr Sci Vitaminol 52: 451–456.

22) Gamoh S, Hashimoto M, Sugiuera K, Hossain MS, Hata N, Misawa Y, Masumura S. 1999. Chronic administration of docosahexaenoic improves reference memory-related learning ability in young rats. Neuroscience 93: 237–241.

23) Hashimoto M, Tanabe Y, Fujii Y, Kikuta T, Shibata H, Shido O. 2005. Chronic administration of docosahexaenoic acid ameliorates the impairment of spatial cognition learning ability in amyloid beta-infused rats. J Nutr 135: 549–555.

24) Xiao Y, Wang L, Xu RJ, Chen ZY. 2006. DHA depletion in rat brain is associated with impairment on spatial learning and memory. Biomed Environ Sci 19: 474–480.

25) Suzuki H, Park SJ, Tamura M. 1998. Effect of the long-term feeding of dietary lipids on the learning ability, fatty acid composition of brain stem phospholipids and synaptic membrane fluidity in adult mice: a comparison of sardine oil diet with palm oil diet. Mech Ageing Dev 101: 119–128.

26) Chang YC, Hosoda K, Tsai CJ, Yamamoto S, Wang MF. 2006. Favorable effects of tea on reducing the cognitive deficits and brain morphological changes in senescence-accelerated mice. J Nutr Sci Vitaminol 52: 266–273.

27) Stewart CN, Coursin DB, Bhagavan HN. 1975. Avoidance behavior in vitamin B-6-deficient rats. J Nutr 105: 1363–1370.

28) Ou HP, Wang MF, Yang SC, Yamamoto S, Wang CR. 2007. Effect of Monascus-fermented products on learning and memory in the SAMP8 mice. J Nutr Sci Vitaminol 53: 253–260.

29) Thies F, Delachambre MC, Bentecaj M, Lagarde M, Leerc J. 1992. Unsaturated fatty acids esterified in 2-acetyl-1-lysophosphatidylcholine bound to albumin are more efficiently taken up by the young rat brain than the unesterified form. J Neurochem 59: 1110–1116.

30) Thies F, Pillon C, Moliere P, Lagarde M. Leerc J. 1994. Preferential incorporation of sn-2 lysoPC DHA over unesterified DHA in the young rat brain. Am J Physiol 267: R1273–1279.

31) Tanaka Y, Ohkubo T, Fukuda N, Hibi no H. 2003. Effect of molecular forms on distribution of docosahexaenoic acid into organs in mice. J Oleo Sci 52: 89–97.

32) Song JH, Miyazawa T. 2001. Enhanced level of n-3 fatty acid in membrane phospholipids induces lipid peroxidation in rats fed dietary docosahexaenoic acid oil. Atherosclerosis 155: 9–18.

33) Nagan N, Zeaeller RA. 2001. Plasmalogens: Biosynthesis and functions. Prog Lipid Res 40: 199–229.

34) Maeb A, 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.

35) Maeb A, Ueta N. 2003. Ethanolamine plasmalogen and cholesterol reduce the total membrane oxidizability measured by the oxygen uptake method. Biochem Biophys Res Commun 302: 265–270.

36) Reiss D, Beyer K, Engelmann B. 1997. Delayed oxidative degradation of polyunsaturated diacyl phospholipids in the presence of plasmalogen phospholipids in vitro. Biochem J 323: 807–814.

37) Farooqui AA, Horrocks L. 1998. Plasmalogen-selective phospholipase A2 and its involvement in Alzheimer’s disease. Biochem Soc Trans 26: 243–246.

38) Nishimura M, Wakisaka T, Harra H. 2003. Ingestion of plasmalogen markedly increased plasmalogen levels of blood plasma in rats. Lipids 38: 1227–1235.