Effect of Dietary Docosahexaenoic Acid Connecting Phospholipids on the Lipid Peroxidation of the Brain in Mice

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(Received April 24, 2008)

Summary The effect of dietary docosahexaenoic acid (DHA, C22:6n-3) with two lipid types on lipid peroxidation of the brain was investigated in streptozotocin (STZ)-induced diabetic mice. Each group of female Balb/c mice was fed a diet containing DHA-connecting phospholipids (DHA-PL) or DHA-connecting triacylglycerols (DHA-TG) for 5 wk. Safflower oil was fed as the control. The lipid peroxide level of the brain was significantly lower in the mice fed the DHA-PL diet when compared to those fed the DHA-TG and safflower oil diets, while the α-tocopherol level was significantly higher in the mice fed the DHA-PL diet than in those fed the DHA-TG and safflower oil diets. The DHA level of phosphatidylethanolamine in the brain was significantly higher in the mice fed the DHA-PL diet than in those fed the safflower oil diet. The dimethylacetal levels were significantly higher in the mice fed the DHA-PL diet than in those fed the safflower oil and DHA-TG diets. These results suggest that the dietary DHA-connecting phospholipids have an antioxidant activity on the brain lipids in mice, and the effect may be related to the brain plasmalogen.

Key Words docosahexaenoic acid, phospholipid, lipid peroxidation, brain, plasmalogen

The n-3 polyunsaturated fatty acids (PUFA), especially docosahexaenoic acid (DHA), have various physiological functions, such as the prevention of cardiovascular disease (1), lowering of plasma lipids (2, 3) and improving memory learning (4). Most of these studies used the DHA-connecting triacylglycerols (DHA-TG) or their ethyl esters (DHA-EE); however, it has recently been reported that the DHA-connecting phospholipids (DHA-PL) have some peculiar functions such as anti-inflammatory actions (5, 6), improving the deformability of human erythrocytes in microchannels (7), antitumor activity against Meth-A fibrosarcoma (8), and promoting the cell differentiation of erythroleukemia cancer cells (9).

On the other hand, the PUFA-connecting lipids have been considered to be very susceptible to peroxidation because of their high degree of unsaturation (10–12). However, recent in vivo studies have shown that the dietary n-3 PUFA including DHA prevents oxidative stress (13–16). Furthermore, Song and Miyazawa (17) reported the effect of dietary DHA oil with different lipid types on lipid peroxidation. In their studies, lipid peroxidation in the plasma and liver was significantly lower in rats fed the DHA-PL diet than in those fed the DHA-TG and DHA-EE diets. Therefore, it was suggested that the lipid peroxidative level is different depending on the DHA molecular form.

Lipid peroxidation occurring in the human body has been considered to cause various disorders including atherosclerosis, diabetes and burn injury (18). In diabetic patients, the serum lipid peroxide level was reported to have increased (19). There are several reports of the effects of the streptozotocin treatment on the lipid peroxidation in rats, such as decreasing the ascorbic acid levels in the plasma, liver and kidney (20), increasing the lipid peroxide levels of the retina and kidney (21), and increasing the activity of mitochondrial oxidative markers of the liver and kidney (22).

We have investigated the lipid classes and fatty acids of phospholipids from the gonads of the skipjack tuna, whose commercial value is low because they are by-products from skipjack processing companies, to find an effective way of using them. We concluded that the gonads of the skipjack tuna could become a good source for products from skipjack processing companies, to find an effective way of using them. We concluded that the gonads of the skipjack tuna could become a good source of the sn-2 position combined DHA in phospholipids (23, 24). In this study, the effect of dietary DHA-PL extracted from the skipjack tuna ovaries on the lipid peroxidation of the brain was studied in streptozotocin-induced diabetic mice, and the lipid peroxide level and fatty acid composition of the brain were compared to the dietary DHA-TG.

MATERIALS AND METHODS

Animals. Twenty-four female Balb/c mice (4 wk
The content of the experimental lipids is shown in Table 1. DHA contents are 3% of the total lipids. The fatty acid DHA oil (DHA-TG) were mixed with safflower oil as the controlled temperature of Japan. The skipjack ovary phospholipid (DHA-PL) and Suisan, Inc. (Tokyo, Japan) and the high oleic safflower and 7.5% others. DHA oil was purchased from Nihon sphingomyelin, 7.0% phosphatidylethanolamine (PE), 37.2% phosphatidylcholine (PC), 36.6% lysoPC, 11.7% and then the phospholipid was separated by a Sep-pak treatment buffer (pH 4.5) for 5 d to induce diabetes. The non-STZ old) were obtained from Japan SLC, Inc. (Hamamatsu, Japan), 502 intraperitoneally injected with 40 mg of streptozotocin (STZ) per kg of body weight dissolved in a 50 mM citrate buffer (pH 4.5) for 5 d to induce diabetes. The non-STZ treatment group (Safflower) of eight mice was considered the control. At the end of the experimental period, all mice were fasted for 18 h and anesthetized with diethyl ether. The 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 and brains were stored at −70˚C until analysis.

Diets. The ovaries of the skipjack tuna Euthynnus pelamis were obtained from a katsuo-bushi factory. The skipjack ovary lipids were extracted according to Bligh and Dyer’s method modified by Hanson and Olley (25), and then the phospholipid was separated by a Sep-pak silica cartridge (10 g; Waters, Tokyo, Japan). The lipid class composition of the obtained phospholipid was 37.2% phosphatidylcholine (PC), 36.6% lysoPC, 11.7% sphingomyelin, 7.0% phosphatidylethanolamine (PE), and 7.5% others. DHA oil was purchased from Nihon Suisan, Inc. (Tokyo, Japan) and the high oleic safflower oil was purchased from Ajinomoto Corp. (Tokyo, Japan). The skipjack ovary phospholipid (DHA-PL) and DHA oil (DHA-TG) were mixed with safflower oil as the DHA contents are 3% of the total lipids. The fatty acid content of the experimental lipids is shown in Table 1. The α-tocopherol content in each diet was adjusted to 30 mg/kg. The lipid peroxide levels of the experimental diets were between 0.21 and 0.29 meq/kg in the three types of diets (data not shown). The composition of the experimental diet was as follows (g/kg diet): casein, 230; cornstarch, 400; sucrose, 200; AIN-76 mineral mixture, 35; AIN-76 vitamin mixture, 10; cellulose powder, 20; DL-methionine, 3; choline bitartrate, 2; lipid, 100. Sucrose was purchased from the Maruha Corp. (Tokyo, Japan). DL-Methionine and choline bitartrate were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Serum cholesterol, glucose and insulin. The concentrations of total cholesterol, glucose and insulin were enzymatically determined using a commercial kit (Cholesterol E-test, Glucose C II-test; Wako Pure Chemical Industries, Ltd. and Insulin-ELISA; Funakoshi, Inc., Tokyo, Japan).

Fatty acid and dimethylacetal. The total lipids in the brain were extracted according to Bligh and Dyer’s method modified by Hanson and Olley (25). 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 band on the plate. The separated PE was converted to the 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. The FAME and DMA were separated by gas chromatography using a flame ionization detector (GC-14A; Shimadzu Co., Ltd., Kyoto, Japan). The chromatograph was equipped with a fused silica capillary column, TC-WAX (30 m×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 (26) by a Shimadzu QP-5000 GC-MS system using a Supelco Omegawax 320 column (30 m×0.32 mm). The temperature was set at 260˚C (column), 200˚C (interface), and 200˚C (ionization chamber). Ionization voltage was 70 eV.

Lipid peroxide. The lipid peroxide (LOOH) was determined using a fluorometric reagent (diphenyl-1-pyrenylphosphine, DPPP) by the method of Meguro et al. (27). The lipid samples (0.5–1 mg) extracted from the brain and serum by Hanson and Olley (25) were dissolved in 100 μL of chloroform/methanol (2 : 1 by vol.). This solution and 100 μL of DPPP solution (1 mg/10 mL in chloroform/methanol (1 : 1)) were added to a screw-cap test tube. The mixture then reacted in a water bath at 60˚C for 60 min in the dark. After cooling in an ice bath, 3 mL of methanol was added to the mixture and the fluorescence intensity at 380 nm (Ex. at

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Saflower</th>
<th>DHA-TG</th>
<th>DHA-PL</th>
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<tbody>
<tr>
<td>C14:0</td>
<td>—</td>
<td>0.5</td>
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<tr>
<td>C16:0</td>
<td>5.5</td>
<td>7.0</td>
<td>6.1</td>
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<td>C18:0</td>
<td>1.9</td>
<td>1.9</td>
<td>1.8</td>
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<tr>
<td>C18:1n-7</td>
<td>—</td>
<td>0.8</td>
<td>0.5</td>
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<tr>
<td>C18:1n-9</td>
<td>76.1</td>
<td>70.1</td>
<td>65.6</td>
</tr>
<tr>
<td>C18:1n-7</td>
<td>—</td>
<td>0.8</td>
<td>0.6</td>
</tr>
<tr>
<td>C18:2n-6</td>
<td>14.9</td>
<td>13.0</td>
<td>12.4</td>
</tr>
<tr>
<td>C20:4n-6</td>
<td>—</td>
<td>0.2</td>
<td>0.3</td>
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<tr>
<td>C20:5n-3</td>
<td>—</td>
<td>0.5</td>
<td>0.3</td>
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<tr>
<td>C22:6n-3</td>
<td>—</td>
<td>3.0</td>
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n-6/n-3 3.77 3.85
Effect of Docosahexaenoic Acid on Brain Peroxidation

352 nm) was measured. The standard lipid peroxide (PV 18 meq/kg by JOCS method ((28))) was made from the skipjack head oil, which was stored for 1 d at 5°C. 

\( \alpha \)-Tocopherol. The \( \alpha \)-tocopherol concentrations in the brain and serum were analyzed by HPLC. The sample preparations for the HPLC analyses were as follows: 150 \( \mu \)L of chloroform solution extracted from the brain using the Hanson and Olley method (25) was evaporated under nitrogen and the residual fraction was redissolved in 50 \( \mu \)L of iso-octane. To 75 \( \mu \)L of serum was added 250 \( \mu \)L of ethanol and 200 \( \mu \)L of distilled water. After vortexing for 30 s, the mixture was added 750 \( \mu \)L of \( n \)-hexane and then vortexed for 1 min. The mixture was centrifuged at 1,000 \( \times g \) for 10 min and the 500 \( \mu \)L of the supernatant was then collected. The solvent was evaporated under nitrogen and the residual fraction was redissolved in 50 \( \mu \)L of iso-octane for the HPLC analyses. HPLC was carried out using a Waters 510 liquid chromatography system equipped with a \( \mu \)Bondasphere 5 \( \mu \) NH2 100A-column (150×3.9 mm i.d.; Waters). The column was eluted with a mobile phase composed of iso-octane/dioxithan/ethanol (958/40/2 in vol.) at the flow rate of 1.5 mL/min. A fluorescent peak was detected at 325 nm (Ex. at 298) using a Hitachi F-1000 fluorescent spectromonitor (Hitachi, Tokyo, Japan).

Statistical analysis. All results were expressed as the means±SE, and statistical significance was determined by a 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 Duncan’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 weight between the three groups (20.1–20.8 g). The average food consumptions were between 2.3 and 2.4 g/d by the mice in the three groups and there were no significant differences between the groups (data not shown).

Serum total cholesterol, glucose and insulin

The levels of total cholesterol, glucose and insulin in the serum of the mice are shown in Fig. 1. The total cholesterol levels in the serum were significantly lower
in the mice fed the two types of DHA-containing diets than in those fed the safflower oil diet. The glucose levels in the serum were significantly higher in the STZ treatment mice than in the control (non-STZ). There were no significant differences in the glucose or insulin levels between the three groups. The average glucose levels of the three experimental diet groups were around 200 mg/dL. They exhibited a mild elevation in serum glucose, when the dose of STZ was 40 mg/kg daily for 5 d for the Balb/c mice.

Lipid peroxide and α-tocopherol in the brain and serum

The levels of lipid peroxide and α-tocopherol in the brain of the mice are shown in Fig. 2. By the STZ treatment, the peroxide level in the brain increased and the α-tocopherol level decreased. The peroxide level in the brain was significantly lower in the mice fed the DHA-PL diet than in those fed the DHA-TG and safflower oil diets, while the α-tocopherol level was significantly higher in the mice fed the DHA-PL diet than in those fed the DHA-TG and safflower oil diets.

The levels of lipid peroxide and α-tocopherol in the serum of the mice are shown in Fig. 3. By the STZ treatment, the levels of lipid peroxide and α-tocopherol in the serum did not significantly change. The higher tendency in mice fed the two types of DHA-containing diets compared with the safflower oil fed mice was observed in the lipid peroxide level of the serum; however, there were no significant differences among the three diet groups. On the contrary, the α-tocopherol level tended to be lower in the mice fed the two types of DHA-containing diets compared with the safflower oil fed mice, and significantly lower in the mice fed the DHA-TG diet than in those fed the safflower oil diet.

Fatty acid and dimethylacetal (DMA) of PE in the brain

The fatty acid and dimethylacetal compositions of PE in the brain of the mice are shown in Table 2. With the STZ treatment, the fatty acid composition did not significantly change but the C16:0 DMA and total DMA significantly decreased. The levels of C16:0 and C18:0 tended to be lower in the mice fed the DHA-PL diet than in those fed the other two diets. The levels of C20:4n-6 (AA) and C22:4n-6 were significantly lower in the mice fed the two types of DHA diets than in those fed the safflower oil diet. The DHA levels tended to be higher in the mice fed the two types of DHA-containing diets than in those fed the safflower oil diet. The DHA levels tended to be higher in the mice fed the two types of DHA-containing diets than in those fed the safflower oil diet, and significantly higher in the mice fed the DHA-PL diet than in those fed the safflower oil diet. The C16:0 DMA, C18:0 DMA and total DMA levels were significantly higher in the mice fed the DHA-PL diet than in those fed the other two diets. The DHA/AA ratios were significantly higher in the mice fed the two types of DHA-containing diets than in those fed the safflower oil diet. The ratios of C16:0 and C18:1n-7 DMA to the corresponding fatty acid methyl esters (DMA/FAME) were significantly higher in the mice fed the DHA-PL diet than in those fed the other two diets.

**Discussion**

Several studies about the relationships between the n-3 fatty acid and the oxidative stress in vivo have been reported. In the study of rats fed safflower and fish oil
diets for 6 wk, there were no significant differences in the thiobarbituric acid-reactive substances (TBARS), PC-OOH, or PE-OOH in the erythrocyte membranes among the groups (13). Under conditions when the rats underwent ferric nitrilotriacetic acid (Fe-NTA) oxidative stress, the increased level of TBARS in the livers was significantly higher in the fish oil diet group than in the safflower oil diet group; however, the 8-hydroxydeoxyguanosine (8-OHdG) level in the livers of the rats fed safflower oil diet group was remarkably lower than that in the livers of the safflower oil diet group (14). Moreover, when the n-3 fatty acid-deficient old rats were given the DHA-containing diet for 4 mo, the TBARS levels in the rat brains decreased and the activities of their antioxidative enzymes increased (15). These reports suggest that DHA, which may be very susceptible to peroxidation under atmospheric conditions, does not enhance, but appears to protect against oxidative stress in vivo. The reason for this can be considered as follows. Due to the lower oxygen partial pressure in vivo than that under atmospheric conditions, the fatty acids having a higher peroxidizability index are not very significantly induced to undergo peroxidation. On the contrary, the beneficial effects of DHA, such as activating cell functions, may appear.

In the present study, the effects of the PL and TG types of dietary DHA on the lipid peroxide of brain and serum in the STZ-induced diabetic mice were compared. It is known that the lipid peroxidative levels increase in the STZ-induced diabetic rats (20–22, 29). Thus, the mice in this experiment were evaluated under oxidative stress. Based on the results of this experiment, the levels of lipid peroxide and α-tocopherol in the brain of mice fed the DHA-TG diet were the same level as those fed the safflower oil diet; however, in the mice fed the DHA-PL diet, the level of lipid peroxide was lower and the level of α-tocopherol was higher than in those fed the DHA-TG diet. In the serum of mice, the lipid peroxide levels of both types of DHA diet groups had higher tendencies than those of the safflower oil diet group; however, the α-tocopherol level in the mice fed the DHA-PL diet had a higher tendency than in those fed the DHA-TG diet. These results indicated that DHA-PL worked in mice brains as an antioxidant. Song and Miyazawa (17) reported the effect of dietary DHA oil with different lipid types on lipid peroxidation. In their studies, the lipid peroxidation in the plasma and liver was significantly higher than the control (palm oil) in rats fed the DHA-TG or DHA-EE, but not DHA-PL. In general, the turnover in brain cells is slow, so the change in the components in the brain is slight compared with those in the liver or serum. However, in the present study, the dietary DHA-PL changed into the brain peroxidative components.

PE in the brain has a relatively high proportion of DHA and its main sub-class is plasmalogen (30). The function of plasmalogen is not known; however, according to recent studies, plasmalogen in the PE has an antioxidant action in lowering the oxidizability of cholesterol in the phospholipid bilayers as well as the whole membranes (31, 32). In addition, it has been reported that the plasmalogen concentration in the blood plasma is significantly reduced by aging and hyperlipidemia (33). It is suggested on the basis of these studies that plasmalogen has some important functions in vivo. In the present study, the levels of C16:0 DMA, C18:0 DMA and total DMA of the PE in the brain were significantly higher in the mice fed the DHA-PL diet than in those fed the safflower oil and DHA-TG diets. Therefore, it is suggested that the lower peroxidative levels of the mice brain lipid fed the DHA-PL diet might be associated with the plasmalogen in the PE.

Vinyl ether double bonds in the plasmalogen are known to be sensitive to acid; however, Nishimura et al. (34) demonstrated that dietary plasmalogen was absorbed from the intestine in rats. In the present study, the main lipid classes of the dietary phospholipid are PC and lysoPC, and the ratio of PE is low. Therefore, the plasmalogen content of the dietary DHA-PL was considered to be low. In the present study, it could not be clarified which phospholipid class affected the brain plasmalogen content in the dietary DHA-PL group. Further investigations about this will be needed.

In conclusion, the results of this study indicate that the dietary DHA-connecting phospholipids have an antioxidant activity regarding the brain lipid in mice, and the effect may be related to the brain plasmalogen concentration.

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