acetylcholinesterase activity and evoke oxidative stress, although it increases the disorder of the global and annular lipids of rat synaptic plasma membranes.

Keywords: Docosahexaenoic acid; Acetylcholinesterase; Synaptic plasma membrane; Membrane disorder; Rat cortex

Introduction

Cholinesterase inhibition is targeted for the efficacious treatment approach of Alzheimer's disease (AD) (Giacobini et al., 2002), seen frequently associated with cholinergic dysfunctions including increased acetylcholinesterase (AChE) activity (Sáez-Valero et al., 2002), decreased choline acetyltransferase activity (Nitta et al., 1994, 1997; Nabeshima and Nitta, 1994) or impaired acetylcholine release (Itoh et al., 1996). Investigation of the effects of dietary docosahexaenoic acid (DHA, C22:6, n-3) on the activity of synaptic plasma membrane-bound AChE is of special significance, because (i) AChE is one of the key enzymes of cholinergic synapses in the CNS, (ii) the lipids of synaptic plasma membrane (SPM) contain large amounts of polyunsaturated fatty acids, especially DHA (Salem et al., 1986; Salem, 1989; Neuringer et al., 1994), and (iii) both cholinergic functions (Blockland, 1995) and DHA (Gamoh et al., 1999, 2001) play an important role in formation and/or retention of memory, which deteriorates in age-related pathologies such as Alzheimer's disease, which is characterized by a deficit in brain DHA concentration (Soderberg et al., 1991) and decrease in membrane fluidity (Scheuer et al., 1996; Muller et al., 1998). A decrease in DHA content in the neuronal membrane results in a decrease in membrane disorder or fluidity (Samuel et al., 1982) and synaptic plasma membrane-bound AChE enzyme activity is sensitive to a deficiency of n-3 fatty acids (Foot et al., 1983).

We (Hossain et al., 1998, 1999c; Gamoh et al., 1999, 2001; Hashimoto et al., 2002) and others (Bourre et al., 1990; Green and Yavin, 1995; Makrides et al., 1996; Lim and Suzuki, 2001) showed that cerebral enrichment of DHA could be dependent on the supply in the diet. It is thus likely that chronic administration of DHA would affect the membrane fluidity and/or order of the synaptic area, where the AChE remains embedded and/or attached (Fernandez et al., 1996). DHA increases the fluidity of the plasma membranes of endothelial cells (Hashimoto et al., 1999b), liver cells (Hashimoto et al., 2001) and platelets (Hossain et al., 1999b) and is involved in modulation of the activity of several membrane-bound enzymes including Na^+-K^+-ATPase (Ruiz-Gutierrez et al., 1993), 5′-nucleotidase, adenylate cyclase (Brown and Subbaiah, 1994) and Mg^{2+}-ATPase (Hashimoto et al., 2001). If DHA exerts a similar influence on fluidity in the SPM, it could be expected that DHA would also affect the activity of SPM-bound AChE. Alterations of brain synaptic plasma membrane fluidity by ethanol (Tanii et al., 1995), benzene (Engelke et al., 1992), toluene (Edelfors and Ravn-Jonsen, 1992) and several anaesthetics (Ondrias et al., 1983; Sidek et al., 1984; Mazzanti et al., 1986) may influence the activity of SPM-bound enzyme such as AChE. Dietary cholesterol, which inversely relates to membrane fluidity (Hashimoto et al., 1999a,b; Hossain et al., 1999a,b), affects synaptosomal AChE activity (Sanchez-Yague et al., 1991). On the other hand, DHA may increase the susceptibility of the membrane to oxidative stress (Halliwell, 1992), which alters membrane fluidity (Hashimoto et al., 1999a). Oxidative damage to the cerebral cortex causes deficits in learning and memory (Akita et al., 1997). We therefore investigated the question of whether chronic administration of DHA does in fact modify membrane fluidity, oxidative status and SPM-bound AChE activity in the rat cerebral cortex.

Materials and methods

Materials

All the standard fatty acids, 1,6-diphenyl-1,3,5-hexatriene (DPH), 1-[4-(trimethylamonium)-phenyl]-6-phenyl-1,3,5-hexatriene-DPH (TMA-DPH), pyrene, 2′,7′-dichlorofluorescein (DCF) and BW284c51 were purchased from Sigma (St. Louis, MO., USA), and dichlorofluorescin diacetate (DCFH-DA) from Molecular Probes (Eugene, OR., USA).

Animals and diets

All the rats were cared for and killed in accordance with the procedures outlined in the Guidelines for Animal Experimentation of Shimane Medical University, compiled from the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science. Wistar rats (generation 1, G1) (Jcl: Wistar; Clea Japan, Osaka, Japan) were kept in an environmentally controlled room at 23 ± 2 °C and relative humidity of 50 ± 10%, with automatic lighting from 08:00 to 20:00, and fed an F1® diet containing no fish products (Funabashi Farm Co., Chiba, Japan), with water ad libitum. Second generation rats (G2) were bred from the G1 rats, and 10–15 litters of G2 rats were crossed to derive third generation (G3) rats. The siblings were not of the same group rather they were spread across the dietary group. The G3 rats were on the same F1® diets. Twenty G3 male rats at the age of 10 weeks (body weight 220–250 g) were randomly divided into two groups: the DHA group was orally fed DHA-95E (an ethyl-ester all-cis 4,7,10,13,16,19-docosahexaenoate with a purity of >95%; 300 mg/kg·day; Harima Chemicals, Inc., Tokyo, Japan) gently emulsified in a 5% gum Arabic solution in ice-cold water before administration, and the control group was fed an equal volume of 5% gum Arabic solution. Both F1® pellet feeding and subsequent DHA administration were carried out for a period of 12 weeks (Table 1).

Preparation of brains

After sodium pentobarbital (65 mg/kg BW) intraperitoneal anesthesia, the rat cerebral cortex was separated from the whole brain on ice, blotted gently by filter paper to remove blood and extraneous tissue fragments, weighed and then homogenized in ice-cold 0.32 M sucrose buffer (pH 7.4) containing 5 mM HEPES, 0.5 mM EDTA and the following protease inhibitors: 0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin, 0.5 μg/ml aprotinin, 0.2 mM phenylmethylsufonyl fluoride (PMSF), with a Polytron homogenizer (PCU 2-110, Kinematica GmbH, Steinhofhale, Switzerland). The homogenates were adjusted to a final concentration of 100 mg tissue/ml of buffer and immediately subjected to the assays described below and/or stored at − 80 °C after N_2 flash and bath until the assay could be performed.

Preparation of synaptosomal plasma membrane

Synaptosomal plasma membrane (SMP) was prepared according to procedures described previously (Mason et al., 1999) with slight modification. The cortex homogenate was centrifuged at 578 g for 10 min, the supernatant was again centrifuged at 17,300 g for 10 min, and the resulting pellet was suspended in 0.32 M sucrose buffer and then layered over 7.5 and 13% Ficoll solutions (wt/vol; Ficoll/sucrose buffer) containing 0.5 mM EDTA and the above protease inhibitors. The gradients were

Table 1
Composition and fatty acid composition of F1 diet[1]

	[2]F_1 (g/100 g)	Fatty acid	[2]F_1 (g/100 g)
Corn grain	19.1	Myristic acid ($C_{14:0}$)	0.09 ± 0.09
Wheat bran	21.8	Palmitic acid ($C_{16:0}$)	15.6 ± 0.53
Wheat flour	35.8	Palmitoleic acid ($C_{17:1, n-7}$)	ND
Soybean meal	8.0	Stearic acid ($C_{18:0}$)	5.41 ± 0.09
Casein	4.0	Oleic acid ($C_{18:1, n-9}$)	21.1 ± 0.17
Dry skim milk	3.8	Linoleic acid ($C_{18:2, n-6}$)	52.4 ± 0.80
Soybean oil	1.5	Linolenic acid ($C_{18:2, n-6}$)	4.49 ± 0.13
[3]Mineral mixture	1.0	Arachidic acid ($C_{20:0}$)	0.14 ± 0.09
[4]Vitamin mixture	1.0	Ecosenoic acid ($C_{20:1, n-9}$)	0.33 ± 0.13
[5]Amino acid mixture	1.0	Arachidonic acid ($C_{20:4, n-6}$)	ND
[5]DL-methionine	0.1	Eicosapentaenoic acid ($C_{20:5, n-3}$)	0.06 ± 0.06
[6]Calcium carbonate	0.9	Docosapentaenoic acid ($C_{22:5, n-3}$)	ND
		Docosahexaenoic acid ($C_{22:6, n-3}$)	ND
		Lignoceric acid ($C_{24:0}$)	0.11 ± 0.07

[1] Values are mean ± SEM, n = 4; ND = not detected.

[2] The F1 standard diet containing no fish products contains [(in g/100 g : protein, 21.3; fat 5.1; fiber 3.1; carbohydrate, 5; nonnitrigen, 57.5; and total energy, 17.7 J/g] and was purchased from Funabashi Farm, Chiba, Japan.

[3] Mineral mixture (g/Kg) (as formulated by Takeda Kagaku Shiryo, Tokyo, Japan): $MnSO_4$, 15.7; $FeSO_4$, 23.8; $CoSO_4$, 0.7; $CuSO_4$, 1.0; Ca $(IO_3)_2$, 0.5; $MgCO_3$, 3.0; Na Cl, 300.0;$CaCO_3$, 655.3.

[4] Vitamin mixture (g/kg) as formulated by Takeda Kagaku Shiryo: retinal, 1,000,000 IU/kg; vitamin C oil, 200,000 IU/kg; *dl*-α-tocopherol acetate, 5.0; menadione, 1.0; thiamine nitrate, 0.7; riboflavin, 0.8; pyridoxine hydrchloride, 1.0; nicotinamide, 4.0; calcium pantothenate, 1.7; choline chloride, 65.0; cyanocobalamine, 0.5; biotin, 0.015; saccharine sodium, 8.5; mil S-Na_2 [natural spice (in g/100 g: carbohydrate 8; protein 16; lactate 52; fat, 18.5)], 100.0; glucose 90.0.

[5] Amino acid mixture (g/kg) as formulated by Takeda Kagaku Shiryo; dl-methionine, 300.0; l-lysine hydrochloride, 300.0; defatted rice bran, 400.0.

[6] Wako Pure Chemicals (Osaka, Japan).

centrifuged at 80,000 g for 30 min. The interface between 7.5 and 13% Ficoll enriched in synaptosome was carefully removed, placed in the same sucrose buffer and again centrifuged at 17,300 g for 15 min. The synaptosomal pellet was resuspended in the sucrose buffer and centrifuged again by the above procedure to remove the remaining Ficoll. The pellets suspended in an appropriate volume of cold Tris-HCl buffer (pH. 7.4) containing 137 mM NaCl, 5.4 mM KCl and 11 mM glucose were lysed by freeze-thaw cycles using liquid N2 to isolate SPM. The lysed suspensions were centrifuged at 41,000 g for 20 min. The synaptosomal pellet was resuspended in cold double distilled water and centrifuged at 41,000 g for 30 min. Finally, the SPM pellet was resuspended in Tris-HCl buffer (pH. 7.4) with 137 mM NaCl, 5.4 mM KCl and 11 mM glucose.

Measurements of annular and average/global membrane order

When excited at its own wavelength of 334 nm, a pyrene molecule in close proximity to another pyrene molecule (monomer, M) forms a monomer-monomer dimer (excimer, E). The ratio of E/M fluorescence intensity can therefore be used as an index of lateral diffusion (lateral mobility of the membrane environment); thus, the higher the ratio, the higher the membrane lateral mobility. In principle, pyrene diffuses randomly at all nonpolar regions into the bilayer membrane, irrespective of the

presence of any microdomains. Accordingly, the resulting E/M ratio gives an average or global membrane lateral mobility of the membrane. When a fluorescence emission is created through energy transfer from the tryptophan of the membrane protein, only the pyrene molecules localized in the annular lipid are excited and the molar E/M molar ratio gives the lateral mobility of the annular lipids (Mason et al., 1999; Avdulov et al., 1997).

Briefly, 100 μL of SPM suspension containing100 μg of protein was suspended in 2.0 ml of 25 mM Tris-HCl buffer (pH. 7.4) containing 137 mM NaCl, 5.4 mM KCl and 11 mM glucose, incubated in the dark at 37 °C for 30 min, transferred to a cuvette and injected with 2 μl of 10 mM pyrene. After incubation for 5 min, the pyrene was excited at a wavelength of 286 nm, and the fluorescence emission spectrum was recorded from 320 to 530 nm. After tracing, the pyrene was excited at 334 nm and the intensity profile was again recorded from 320 to 530 nm. The E/M ratio obtained at 286 nm wavelength was taken as the annular fluidity, and the ratio at 334 nm as the global (bulk) fluidity, where E is the fluorescence intensity of pyrene excimer at 480 nm and M the fluorescence intensity of pyrene monomer at 373 nm.

The global membrane order of BCM was also determined by measuring the polarization of the fluorescence probe 1,6-diphenyl-1,3,5-hexatriene (DPH) and its 1-[4-trimethylamonium]-phenyl]-6-phenyl-1,3,5-hexatriene-DPH (TMA-DPH) as described previously (Hashimoto et al., 1999a,b).

Acetylcholinesterase assay

SPM-bound AChE was determined by measuring the hydrolysis of acetylthiocholine. Total cholinesterase (ChE) activity was measured by the method of Ellman et al. (1961) at 25 °C. The standard 1.0 ml reaction mixture for the assay contained 100 mM phosphate buffer (pH 8.0), 1 mM $MgCl_2$, 0.50 mM acetylthiocholine, 0.125 mM 5,5′-dithiobis-2-nitrobenzoic acid and 100 ~ 150 μg SPM protein. The blank consisted of solutions without the SPM protein. The reaction was recorded at 412 nm by using a Hitachi U-2000 spectrophotometer and the rate was calculated as μmoles of substrate hydrolyzed per min per mg protein. AChE activity was defined as the difference of total ChE activity between in the absence and presence of 0.1 mM BW284c51 (specific AChE inhibitor) as described (Lassiter et al., 1998).

The temperature dependence of AChE also was measured over a temperature range 5 to 45 °C. The samples of individual groups were mixed (to have a large volume) and assayed at 2.5 °C intervals, keeping the samples at a constant temperature by a thermostatted cell holder coupled to a circulating water bath. Lines were fitted to the data points in Arrhenius plots by regression analysis.

Reactive oxygen species assay

The fluorescent probe 2′,7′-dichlorofluorescin diacetate (2′,7′-DCFH-DA) was used for the assessment of reactive oxygen species (ROS) as described previously (Hashimoto et al., 2001). Briefly, 200 μg of the protein of the homogenate was diluted in 1.5 ml of 25 mM Tris-HCl buffer (pH 7.4) containing 137 mM NaCl, 5.4 mM KCl and 11 mM glucose, and then incubated with DCFH-DA (at a final concentration of 100 μM) in methanol for 1 h at 37 °C. The dye-loaded samples were centrifuged at 12,500 g for 10 min at 4 °C. The pellet was vortexed in the same Tris-HCl buffer (pH 7.4) at ice-cold temperature and again incubated for 30 min at 37 °C. Fluorescence was measured with a Hitachi 850 spectrofluorometer at wavelengths of 488 nm for excitation and 525 nm for emission. The temperature

of the cuvette holder overlying a magnetic stirrer was maintained at 37 °C. ROS were determined from the standard curve for DCF.

Lipid peroxide and reduced glutathione assay

Lipid peroxide content (LPO) was estimated by the thiobarbituric acid reactive substances (TBARS) test of Ohkawa et al. (1979), as previously described (Hashimoto et al., 1999a,b). Malonaldehyde levels were calculated relative to a standard preparation of 1,1,3,3-tetraethoxypropane. Reduced glutathione (GSH) levels were determined by the method of Hissin and Hilf (1976). GSH levels were determined from the standard curve for GSH and expressed as nanomoles per mg protein.

Lipid analyses

Liver fatty acid profile was measured from liver homogenate. Briefly, 10% homogenate of the liver was prepared in chilled buffer containing 300 mM mannitol, 5 mM EGTA, 18 mM Trsi-HCl, 1 mM PMSF, (pH 7.4) with a polytron homogenizer (PCU 2-110, Kinematica GmbH, Steinhofhale, Switzerland).

SPM total lipids were extracted by the method of Folch et al. (1957) using a chloroform/methanol mixture (2:1, v/v). The solvent was evaporated by blowing N_2 gas at 25 °C. An appropriate volume of Tris-HCl buffer was added to the dried lipid and it was resuspended with a bath sonicator at ice-cold temperature. The suspension was used to estimate phospholipid content by measuring inorganic phosphate (Pi) using an inorganic phosphorus kit (Sigma, St. Luis, USA). Phospholipid content of the SPM was calculated as 25 times the Pi in the Folch extract (Shattil. et al., 1975).

SPM cholesterol was measured using gas chromatography on a Model 5890 plus gas chromatograph (Hewlett-Packard, Avondale, PA, USA) equipped with a flame ionization detector and an automatic sampler (Model 7673), as previously described (Naeemi et al., 1995) with a few modifications. Briefly, 50 μl of SPM suspension (100–150 μg protein) containing 50 μl of α-cholestane (1.0 μg/μl) in ethanol as an internal standard was dissolved in saturated methanolic KOH and incubated at 80 °C for 30 min. After cooling, 1.0 ml of saturated NaCl solution and 200 μl of cyclohexane were added to the mixture. The mixture was vigorously shaken and centrifuged at 2000 g, and the upper organic layer was directly supplied to gas chromatography using a 30 m × 0.25 mm inner diameter fused silica column coated with methyl siloxane to a film thickness of 0.25 μM (HP-1, Hewlett-Packard), helium with flow rate of 1.5 ml/min as the carrier gas, the oven temperature programmed from 180 °C to 280 °C at 20 °C/min and at 280 °C for 10 min, and the injector splitter at 20 min/min and temperature 290 °C.

The liver homgenate and SPM fatty acid profile were determined by one step analysis of Lepage and Roy (1986) using gas chromatography as described previously (Hashimoto et al., 1999a,b).

Protein concentration was estimated by the method of Lowry et al. (1951).

Statistical analysis

Results were expressed as the mean ± SE. Statistical analysis was performed by Student's *t*-test. Correlation coefficients were determined by simple regression analyses. All data were analyzed using

MindVision software, StatView® 4.01 (Abacus Concepts, Inc., Berkeley, CA, USA). A level of $p < 0.05$ was considered statistically significant.

Results

SPM lateral mobility

Lateral mobility of both the annular and global regions (which include both annular and nonannular regions) of the cerebral cortex SPM were measured by pyrene excimer fluorescence spectroscopy (Fig. 1A and 1B). Dietary DHA administration significantly increased the lateral mobility of both the annular and global regions of the SPM.

DPH-polarization value reflects the average membrane order of the mid-acyl chain region of the phospholipid bilayer (Hashimoto et al., 1999a,b). Membrane disorder is inversely related to fluorescence polarization. DPH-determined average membrane disorder of the SPM of DHA-fed rats increased to a significantly greater extent than that of the SPM of control rats (Fig. 1C). Differences in SPM membrane acyl chain order between the DHA-fed and control rats were not revealed to the same extent by TMA-DPH, a cationic derivative of DPH that measures the fluidity at the lipid-water interfacial region of the bilayer membrane and provides information on the fatty acyl carbonyls just below the polar head group region (Prendergast et al., 1981; Kuhry et al., 1983) (Fig. 1D).

Effect of DHA administration on SPM-bound acetylcholinesterase

AChE activity in the SPM of DHA-fed rats remained at the same level as that in the SPM of the control rats (Fig. 2).

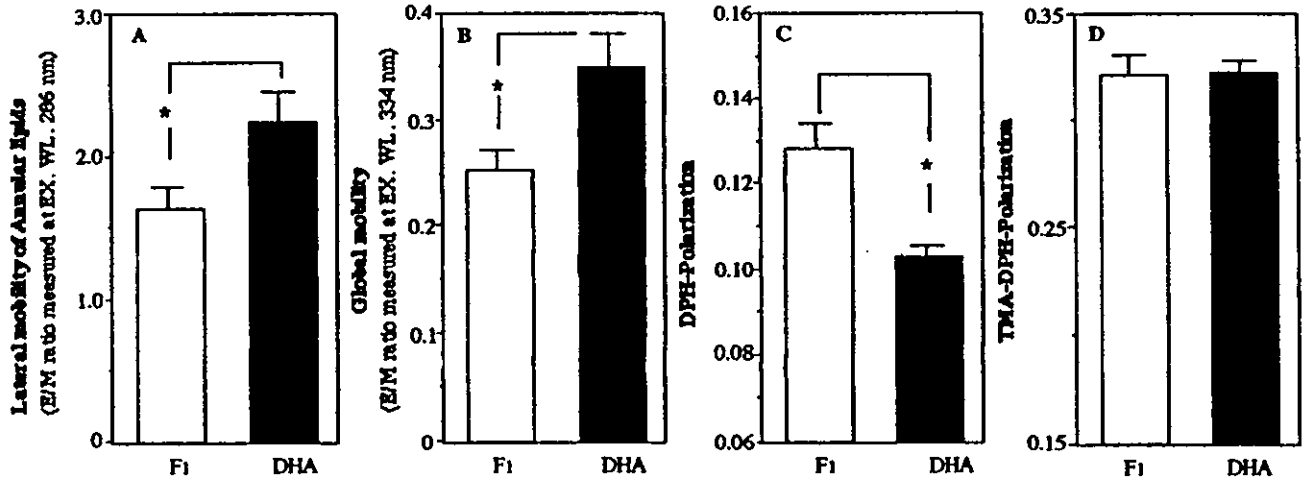

Fig. 1. Effects of DHA administration on membrane order of rat synaptic plasma membrane (SPM). Pyrene-determined annular lipid lateral mobility is shown in (A), average or global lateral mobility in (B), and average membrane order as determined by the fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH) in (C) and of 1-[4-(trimethylamonium)-phenyl]-6-phenyl-1,3,5-hexatriene-DPH (TMA-DPH) in (D). Results are mean ± SE for 10 to 12 rats each with duplicate determinations. See Materials and methods section for details. *$p < 0.05$, unpaired student's t test; □ = F1, control rats; ■ = F1 + DHA, DHA-administered rats.

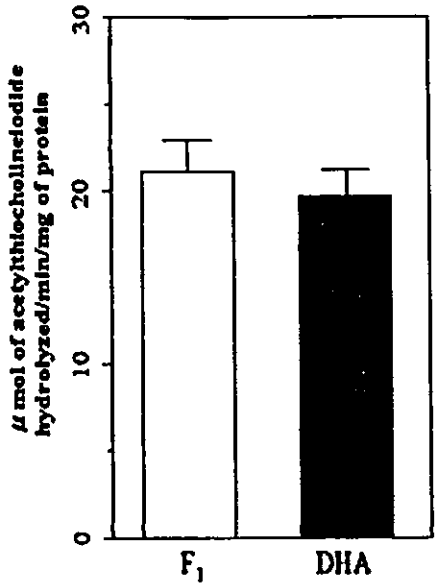

Fig. 2. Effects of DHA administration on synaptic plasma membrane (SPM)-bound acetylcholinesterase (AChE) activities. AChE activity is defined as the difference between total choline esterase activity occurring in the absence and presence of 0.1 mM BW284c51 (specific AChE inhibitor). Results are mean ± SE for 10 to 12 rats each with duplicate determinations. See Materials and methods section for details. □ = F1, control rats; ■ = F1+ DHA, DHA-administered rats. No significant difference was found between the groups.

Effect of DHA on oxidative stress

Lipid peroxidation alters membrane fluidity (Aksentsev et al., 1995; Urano et al., 1997). We therefore measured the LPO (TBARS) level in the cerebral cortex SPM fraction and the ROS level in the cortex whole homogenate of the DHA-fed and control rats to determine whether dietary administration of DHA either ameliorates or exacerbates the oxidative status of the nerve terminals (Fig. 3). In the DHA-fed rats, dietary DHA administration reduced both the LPO level in the SPM (Fig. 3A) and the ROS level in the cortex homogenate to a significantly greater extent than in the control rats (Fig. 3B).

The GSH levels in the cortex homogenate of DHA rats significantly increased over that of the control rats (DHA-fed rats: 4.0 ± 0.20 nmol/mg protein; control rats: 3.2 ± 0.15 nmol/mg protein).

Effect of dietary DHA administration on fatty acid profiles of liver, and fatty acid profiles, cholesterol and phospholipid composition of the SPM

Table 2 shows the fatty acid profiles of liver homogenate. After DHA feeding, n-6 arachidonic acid significantly decreased with a concomitant increase of n-3 eicosapentaenoic, docosapentaenoic and docosahexaenoic acid in the liver tissues. Among the n-3 PUFAs, DHA was doubled. This was consistent with the report of Bourre et al. (1990). Palmitic, steraric, oleic, linoleic and linolenic acids were not altered. Finally, the changes in the fatty acid profiles brought about a significant change in the total unsaturation index (USI), n-3 USI and the ratio of n-3 USI/n-6 USI of the liver of DHA-fed rats.

The DHA content of the SPM increased concomitantly with a decrease in arachidonic acid (AA) content in the DHA-fed rats, leading to an increase in the DHA/AA molar ratio (Table 3). Oleic acid

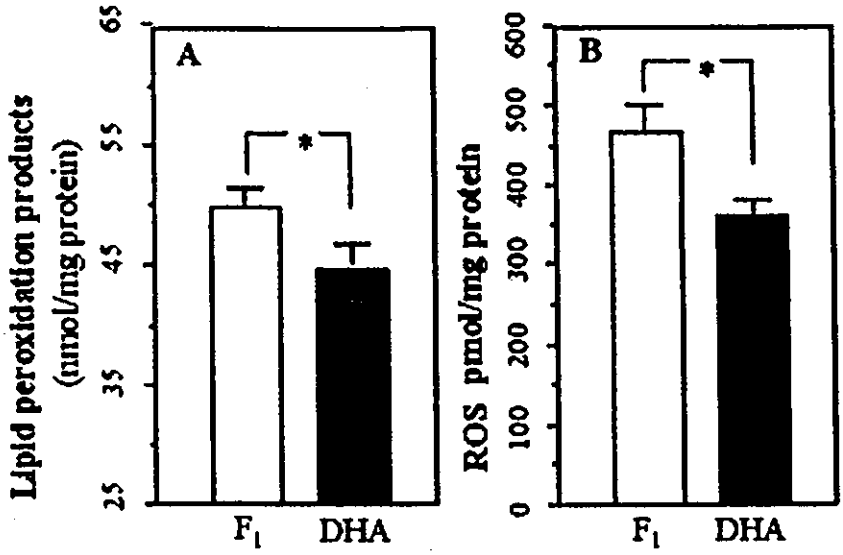

Fig. 3. Effects of DHA administration on synaptic plasma membrane lipid per-oxidation, as measured by thiobarbituric acid reactive substances (A), and on the basal levels of reactive oxygen species (ROS) in the cortex whole homogenate (B). Results are mean ± SE for 10 to 12 rats each with duplicate determinations. See Materials and methods section for details. * $p < 0.05$, unpaired student's t test; □ = F1, control rats; ■ = F1 + DHA, DHA-administered rats.

increased significantly upon DHA administration. Docosapentaenoic acid was present as trace amount, however, it increased significantly in the SPM of DHA rats. The presence of eicosapentaenoic acid in the synaptic membrane is often disputed. Thus, it remains to be clearly known whether it is the retro-conversion product of synaptic DHA or whether it comes from extra-neuronal sources and are misidentified as contaminating n-3 fatty acids. Other fatty acids increased slightly, but not significantly,

Table 2
Effect of dietary administration of DHA on the Liver Fatty acid Profile (μg/mg of protein)

	F_1	F_1 + DHA
Palmitic acid ($C_{16:0}$)	28.0 ± 1.70	33 ± 1.30
Stearic acid ($C_{18:0}$)	19.0 ± 1.15	20.0 ± 0.5
Oleic acid ($C_{18:1,\ n\text{-}9}$)	11.0 ± 1.50	11.0 ± 0.9
Linoleic acid ($C_{18:2,\ n\text{-}6}$)	26.5 ± 2.50	30.0 ± 1.2
Linolenic acid ($C_{18:2,\ n\text{-}6}$)	0.45 ± 0.05	0.50 ± 0.04
Arachidonic acid ($C_{20:4,\ n\text{-}6}$)	25.5 ± 1.00	20.0 ± 0.50*
Eicosapentaenoic acid ($C_{20:5,\ n\text{-}3}$)	0.47 ± 0.04	2.05 ± 2.05*
Docosapentaenoic acid ($C_{22:5,\ n\text{-}3}$)	1.40 ± 0.04	1.97 ± 0.09*
Docosahexaenoic acid ($C_{22:6,\ n\text{-}3}$)	8.25 ± 0.25	16.9 ± 0.40*
n-3 USI	0.55 ± 0.03	0.93 ± 0.01*
USI	1.22 ± 0.009	1.55 ± 0.012*
n-6 USI	0.56 ± 0.01	0.53 ± 0.001
n-3 USI/n-6 USI	1.01 ± 0.08	1.75 ± 0.05*

Results are mean ± SE with n = 10 rats from each group. Unsaturation index (USI) was calculated as [Σ(mole% of each polyunsaturated fatty acid × number of double bonds)]/100. n-3 USI = [3 × ($C_{18:3}$) + 5 × ($C_{20:5}$) + 6 ($C_{22:5}$) + 6 × ($C_{22:6}$)]/100, and n-6 USI = [2 × ($C_{18:2}$) + 4 × ($C_{20:4}$)]/100.

*$p < 0.05$ vs. F_1 control. n-3 USI = USI calculated using only n-6 polyunsaturated fatty acids.

Table 3
Effect of dietary administration of DHA on the synaptosomal plasma membrane fatty acid profile and cholesterol-phospholipid composition in rat cerebral cortex

	Control rats (n = 10)	DHA-fed rats (n = 10)
Fatty acid (nmol/mg protein)		
Palmitic acid ($C_{16:0}$)	16.9 ± 0.65	20.4 ± 0.95
Stearic acid ($C_{18:0}$)	61.2 ± 2.30	69.1 ± 2.40
Oleic acid ($C_{18:1,\ n-9}$)	8.10 ± 0.60	10.3 ± 0.70[a]
Linoleic acid ($C_{18:2,\ n-6}$)	1.10 ± 0.08	1.60 ± 0.90
Arachidonic acid ($C_{20:4,\ n-6}$)	15.1 ± 0.70	12.3 ± 0.90[a]
Eicosapentaenoic acid ($C_{20:5,\ n-3}$)	1.05 ± 0.03	0.95 ± 0.06
Docosapentaenoic acid ($C_{22:5,\ n-3}$)	0.45 ± 0.02	0.60 ± 0.05[a]
Docosahexaenoic acid ($C_{22:6,\ n-3}$)	42.0 ± 1.25	48.8 ± 1.50[a]
$C_{22:6,\ n-3}/C_{20:4,\ n-6}$	2.85 ± 0.15	4.20 ± 0.35[a]
Cholesterol-Phospholipid (nmol/mg protein)		
Cholesterol	40.7 ± 3.85	34.5 ± 3.20
Phospholipid	100 ± 6.40	120 ± 6.35[a]
Cholesterol-Phospholipid	0.41 ± 0.25	0.29 ± 0.02[a]

Results are mean ± SE.
[a] $p < 0.05$, significantly different with control rats.

as a result of the dietary administration of DHA. The total cholesterol content of the cerebral cortex SPM was not altered by DHA administration, while the total phospholipid content of the SPM increased significantly in the DHA-fed rats, leading to a significant decrease in the cholesterol/phospholipid molar ratio.

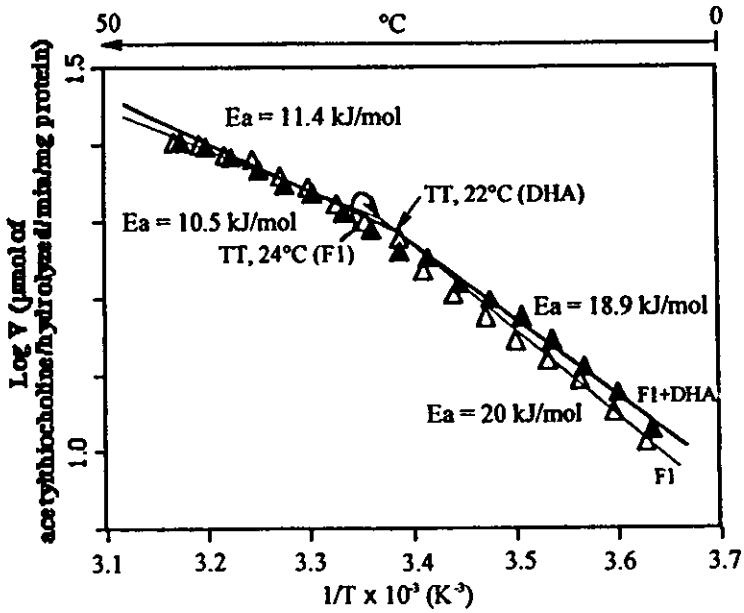

Fig. 4. Arrhenius plots showing the effects of DHA administration on the temperature-dependence of synaptic plasma membrane-bound AChE activity of control (△) and DHA-fed (▲) rats.

Effect of dietary DHA administration on the temperature dependence of AChE activity

The Arrhenius plot showed that the break point for the AChE activity from DHA-fed rats was 22 °C and that of the F1 rats was 24 °C (Fig. 4). The activation energy (Ea) of the enzyme below and above the transition temperatures (TT) were not altered significantly between the two rat groups (Ea (kJ/mol) below the TT: F1 rats, 20.0 and F1 + DHA, 18.9; above the TT: F1, 10.5 and F1 + DHA, 11.5).

Correlation of SPM fatty acids, LPO and membrane order

Pyrene-determined membrane lateral mobility of annular and global regions both correlated positively with the DHA/AA molar ratio of the cerebral cortex SPM (annular mobility, $r = 0.59$, $p < 0.005$; average or global mobility, $r = 0.45$, $p < 0.05$). DPH polarization of the SPM correlated negatively with both DHA content ($r = -0.53$, $p < 0.05$) and the DHA/AA molar ratio ($r = -0.58$, $p < 0.02$). Synaptosomal membrane LPO correlated positively with DPH-polarization ($r = 0.44$, $p < 0.05$) and negatively with pyrene-determined lateral mobility, of both the annular ($r = -0.61$, $p < 0.005$) and global ($r = -0.48$, $p < 0.05$) regions. The DHA/AA molar ratio in the SPM correlated negatively with the level of LPO ($r = -0.39$, $p < 0.05$).

Discussion

The present experiment revealed that DHA administration increases the disorder of the annular and hydrophobic core regions of the cerebral cortex SPM and also reduces the levels of LPO and ROS, while having no effect on SPM-bound AChE activity. Increased DHA content of the cerebral cortex SPM failed to alter the order of the water-lipid interfacial surface region of the bilayer leaflet, as indicated by the absence of change in TMA-DPH fluorescence polarization. This TMA-DPH fluoroprobe result is consistent with our previous demonstration in endothelial cell plasma membranes (Hashimoto et al., 1999b), but it appears, however, to conflict with the results of Yorek et al. (1989), who reported an increase in surface membrane disorder in DHA-enriched retinoblastoma (Y-76) cells. This discrepancy may be based on the differences in cell types, the extent of membrane perturbations and/or the sensitivity of the TMA-DPH probe to the small increases (by 16% only) in DHA content in the SPM of DHA-fed rats observed in our experiment as opposed to higher increases (1400%) in DHA content in the Y-76. AChE remains attached to the surface of the SPM (Fernandez, 1996), and since the surface membrane order of the SPM was not altered, DHA enrichment thus may not affect the activity of the surface-bound AChE.

Mitchell and Litman (1998) reported that polyunsaturated fatty acids particularly DHA confers a greater degree of disorder at the midplane of the bilayer than do monounsaturated and/or saturated fatty acids or even polyunsaturated n-6 arachidonic acid. Conceivably, therefore, DHA administration caused an increase in disorder of the DPH-probed mid-acyl chain hydrophobic regions of the SPM, but the SPM-bound AChE activity was not, however, affected by the increase in hydrophobic core lipid disorder. The extent to which AChE is sensitive to alterations in the hydrophobic lipid core is thus uncertain (Nemat-Gorgani and Meisami, 1979). This finding contrasts with the increased activity of synaptosomal AChE found in rats fed high versus low 18:2/18:3 diets (Foot et al., 1983). Our results also contrast with those of several other studies, where aqueous (Tanii et al., 1995), organic (Edelfors and

Ravn-Jonsen, 1992; Engelke et al., 1992) or anesthetic compounds (Ondrias et al., 1983; Sidek et al., 1984; Mazzanti et al., 1986) were shown to alter AChE activity with concomitant changes in DPH-determined membrane disorder of the SPM. Although it is difficult to account for this discrepancy, it may be considered that these organic molecules were encountered by the acyl chains of the phospholipid bilayer acyl chain as an extrinsic factor, whereas DHA after in vivo administration is intrinsically esterified to the phospholipid glycerol backbone. Following this line of reasoning, the extent of membrane perturbation, regarding the effects on membrane free volume and net effects on activation energy conferred by the organic molecules (viz. alcohol, toluene and anesthetics) and by dietary DHA enrichment would obviously be different and their respective effects on AChE would also be different. This inconsistency led us to measure the mobility of annular lipid region, which remains intimately contacted with the membrane-bound proteins. The random distribution of DPH, TMA-DPH and pyrene provide average values for all microregions of the bilayers even if the bilayers have microregions of different disorder. The changes in the membrane order of the annular region, rather than those of the non-annular region, would thus have a more intense effect, if there is an effect at all, on the properties of the membrane-bound proteins.

The increase in the annular lipid mobility in the DHA-fed rats did not affect the activity of AChE. It can be speculated that the interaction of DHAs of phospholipids in the SPM and AChE did not reach the extent that might lead to a change in the AChE activity.

Cholesterol decreases the membrane free volume by intimate interaction with the saturated acyl chains (Mitchell and Litman, 1998). A decrease in the cholesterol/phospholipid molar ratio causes an increase in plasma membrane order (North and Fleischer, 1983; Hashimoto et al., 1999a,b; Hossain et al., 1999a). This ratio was also significantly lower in the more disordered synaptic membranes of the DHA-fed rats. The similarity in AChE activity levels in the SPM of the control and DHA rats, despite differences in their cholesterol/phospholipid molar ratio, goes against the assumption that the modulation of rat AChE activity reflects changes in the membrane sterol components rather than changes in its acyl composition (Hrboticky et al., 1989). In addition, an increase in membrane lipid peroxidation has been found to reduce membrane order in the isolated synaptosomes (Aksentsev et al., 1995; Urano et al., 1997), platelets (Hossain et al., 1999b) and endothelial cells (Hashimoto et al., 1999a,b). In the present study, DPH polarization correlated positively and pyrene-determined annular/global lateral mobility correlated negatively with LPO. This suggests that the enhancement of annular lipid and/or global mobility in the SPM of DHA-fed rats resulted from the combined effects of the reduced cholesterol/phospholipid molar ratio and lipid peroxidation, and the increased synaptosomal DHA and DHA/AA molar ratio. These results also indicate that DHA in vivo did not play an oxidative role, in which case SPM disorder would have been reduced rather than increased.

The activity of AChE was not affected by DHA administration. An increased membrane disorder allows the enzyme more conformational freedom, thus decreasing the activation energy. To evaluate this, the enzyme AChE activity was measured at different temperatures and the energy of activation was calculated. In this study, Arrhenius plots of synaptosomal AChE activity were discontinuous, with a 2 °C lower transition temperature in the DHA rats than that in the control rats. Activation energies of AChE of both above and below the transition temperatures were not altered significantly between the SPMs of control and DHA-fed rats. Though a lower transition temperature suggests a more disordered or fluid microenvironment for the AChE in the synaptosomal membrane, such environment, however, could not affect the activity of the enzyme. Thus the behavior of AChE is not consistent with the defined changes in the fatty acid composition of the SPM of DHA vs.

control rats. This may suggest that acyl chain structure of the lipid fatty does not affect the temperature dependence of the AChE activity so long as the lipids that surround and/or hold the enzyme are in the liquid-crystalline state as seen above the transition temperature. The activity of AChE in the DHA-fed rats tended to increase, though not significantly, below the transition temperatures and this may relate to increased membrane free volume and hence smaller activation energy conferred by the DHA acyl chain. Or, in other words, the catalytic unit of the AChE does not interact substantially with the lipid acyl chain regions that undergo phase transition, and is to be classified as extrinsic protein. This behavior of AChE is also consistent with the fact that the activity of this enzyme was not altered even though the SPM annular lipid mobility increased significantly. Our result on the effect of n-3 DHA on the temperature-dependence of AChE is consistent with that of the Horboticky et al. (1989).

A DHA-induced increase in memory related-performance is accompanied by increased levels of acetylcholine (Minami et al., 1997) in the cerebrum, and if DHA could cause an increase in the AChE activity, this increase in the acetylcholine level could not occur. This is because acetylcholine is hydrolysed by AChE, causing its level to decrease. In general, acetylcholine level is decreased and choline levels are increased in memory impairment such as in Alzheimer's disease due to cholinergic dysfunctions including increased AChE activity (Sáez-Valero et al., 2002), decreased choline acyltransferase activity (Nabeshima and Nitta, 1994; Nitta et al., 1994, 1997), and impaired acetylcholine release (Itoh et al., 1996). Furthermore, DHA-induced increases in membrane disorder and antioxidative effects could be extrapolated to at least a partial relationship with increased vesicular neurotransmitter (acetylcholine) release, because an increased proportion of DHA relative to AA, reduced synaptosomal lipid peroxidation and a reduced cholesterol/phospholipid ratio (which all occurred in the present investigation) have been demonstrated to facilitate the increased release of acetylcholine (Urano et al., 1997). Thus the lack of the effects of DHA on the AChE activity may relate with its ability to increase the brain acetylcholine level (Minami et al., 1997) with concurrent increases of cognitive functions after DHA administration (Hashimoto et al., 2002).

Conclusion

Dietary administration of DHA significantly increased the synaptic plasma membrane annular lipid mobility without a concurrent effect on acetylcholinesterase activity. The present observations may have significant physiological implications and may be of importance in understanding the physical and biochemical effects of DHA in connection with the physiological impact of DHA on acetylcholinesterase.

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Chronic Administration of Docosahexaenoic Acid Ameliorates the Impairment of Spatial Cognition Learning Ability in Amyloid β–Infused Rats

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ABSTRACT We investigated whether administration of docosahexaenoic acid (DHA), a major (n-3) fatty acid of the brain, ameliorates the impairment of learning ability in an animal model of Alzheimer's disease (AD), rats infused with amyloid-β (Aβ) peptide (1–40) into the cerebral ventricle. Inbred 3rd generation male rats (20 wk old) fed a fish oil–deficient diet were randomly divided into 4 groups: a vehicle group, an Aβ peptide-infused group (Aβ group), a DHA group, and an Aβ + DHA group. A mini-osmotic pump filled with Aβ peptide or vehicle was implanted in the rats, and they were tested for learning ability–related reference and working memory in an 8-arm radial maze. The rats were then orally fed DHA dissolved in 5% gum Arabic solution at 300 mg/(kg · d) (DHA and Aβ + DHA groups) or vehicle alone (vehicle and Aβ groups) and tested again for learning ability. DHA administered for 12 wk significantly reduced the increase in the number of reference and working memory errors in the Aβ-infused rats, and increased both the cortico-hippocampal level of DHA and the molar ratio of DHA/arachidonic acid, suggesting an amelioration of the impaired spatial cognition learning ability. Furthermore, DHA suppressed the increases in the levels of lipid peroxide and reactive oxygen species in the cerebral cortex and the hippocampus of Aβ-infused rats, suggesting that DHA increases antioxidative defenses. DHA is thus a possible therapeutic agent for ameliorating learning deficiencies due to Alzheimer's disease. J. Nutr. 135: 000–000, 2005.

KEY WORDS: • *docosahexaenoic acid* • *therapeutic agent* • *spatial working memory* • *antioxidative defense* • *Alzheimer's disease*

Docosahexaenoic acid [DHA; 22:6(n-3)],[2] one of the main structural lipids in the mammalian brain, is essential for normal neurological development and for vision (1). Deficiency in this fatty acid is associated with a loss of discriminative learning ability (2,3); thus intake of DHA may restore lost learning ability. Consistent with these findings, we demonstrated that chronic administration of DHA enhances long-term memory in both young (4) and old (5) rats. Treatment with DHA improves the neurological condition in Zellweger's syndrome, a peroxisomal disorder that produces serious mental retardation (6). More interestingly, the DHA level in the hippocampus was found to be very low (7) in patients with Alzheimer's disease (AD), compared with that in brain samples from age-matched human controls. AD is characterized by the formation of neurofibrillary tangles and neuritic plaque of amyloid peptides such as amyloid-β (Aβ) peptide (1–40), as well as by neuronal and memory loss. We reported recently that preadministration of DHA protects against the impairment of learning ability in an animal model of AD, rats infused with Aβ peptide (1–40) into the cerebral ventricle (8). Epidemiologic studies show a relation between sources of dietary fish oil, especially DHA, and AD. Intake of DHA has been associated with reduced risk of AD (9). DHA oil supplementation was shown to improve intellectual function in the elderly (10). We therefore hypothesized that chronic administration of DHA may ameliorate the impairment of learning ability in Aβ-infused rats.

MATERIALS AND METHODS

Animals and diet. Wistar rats (generation 1, G1) (*Jcl*: Wistar; Clea Japan) were housed in a room under controlled temperature (23 ± 2°C), relative humidity (50 ± 10%), and light-dark cycles (light: 0800 to 2000 h; dark: 2000 to 0800 h). Rats consumed a fish oil–deficient diet (F-1®; Funabashi Farm) (Table 1) and water ad libitum. The inbred 3rd generation male rats [n = 38; 20 wk old; 376.3 ± 3.3 g body weight (BW)], fed the same F-1® diet, were randomly divided into 4 groups: a vehicle group (n = 9), an Aβ peptide-infused group (Aβ group) (n = 10), a DHA group (n = 9) and an Aβ + DHA group (n = 10). The rats were handled and killed in accordance with the procedures outlined in the Guidelines for Animal Experimentation of Shimane Medical University, compiled from the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science.

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[2] Abbreviations used: AA, arachidonic acid; Aβ, amyloid-β; AD, Alzheimer's disease; BW, body weight; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; LTP, long-term potentiation; PLSD, protected least significant difference; RME, reference memory error; ROS, reactive oxygen species; USI, unsaturation index; WME, working memory error.

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TABLE 1

Ingredients and fatty acid composition of the F1 diet

	F1[1]	Fatty acid	F1[2]
	g/kg		*g/kg*
Corn grain	191	Myristic acid (14:0)	0.9 ± 0.9
Wheat bran	218	Palmitic acid (16:0)	156 ± 5.3
Wheat flour	358	Palmitoleic acid [17:1(n-7)]	ND
Soybean meal	80	Stearic acid (18:0)	54.1 ± 0.9
Casein	40	Oleic acid [18:1(n-9)]	211 ± 1.7
Dry skim milk	38	Linoleic acid [18:2(n-6)]	524 ± 8
Soybean oil	15	Linolenic acid [18:2(n-3)]	44.9 ± 1.3
Mineral mixture[3]	10	Arachidic acid (20:0)	1.4 ± 0.9
Vitamin mixture[4]	10	Eicosenoic acid [20:1(n-9)]	3.3 ± 1.3
Amino acid mixture[5]	10	Arachidonic acid [20:4(n-6)]	ND
DL-Methionine[6]	1	Eicosapentaenoic acid [20:5(n-3)]	0.6 ± 0.6
Calcium carbonate[6]	9	Docosapentaenoic acid [22:5(n-3)]	ND
		Docosahexaenoic acid [22:6(n-3)]	ND
		Lignoceric acid (24:0)	1.1 ± 0.7

[1] The F1 standard diet, containing no fish products, comprised (g/100 g): protein, 21.3; fat 5.1; fiber 1; carbohydrate, 5; nonnitrogen, 57.5; and total energy, 17.7 J/g and was purchased from Funabashi Farm, Chiba, Japan.

[2] Values are mean ± SEM, $n = 4$; ND, not detected.

[3] Mineral mixture (g/kg) as formulated by Takeda Kagaku Shiryo, Tokyo, Japan: $MnSO_4$, 15.7; $FeSO_4$, 23.8; $CoSO_4$, 0.7; $CuSO_4$, 1.0; $Ca(IO_3)_2$, 0.5; $MgCO_3$, 3.0; NaCl, 300.0; $CaCO_3$, 655.3.

[4] Vitamin mixture (g/kg) as formulated by Takeda Kagaku Shiryo: retinal, 300 mg/kg; vitamin D oil, 5 mg/kg; *dl*-α-tocopherol acetate 5.0; menadione, 1.0; thiamine nitrate, 0.7; riboflavin, 0.8; pyridoxine hydrochloride, 1.0; nicotinamide, 4.0; calcium pantothenate, 1.7; choline chloride, 65.0; cyanocobalamin, 0.5; biotin, 0.015; saccharin sodium, 8.5; mil S-Na_2 [natural spice] (g/100 g): carbohydrate 8; protein 16; lactate 52; fat, 18.5], 100.0; glucose 90.0.

[5] Amino acid mixture (g/kg) as formulated by Takeda Kagaku Shiryo: DL-methionine, 300.0; L-lysine hydrochloride, 300.0; defatted rice bran, 400.0.

[6] Wako Pure Chemicals (Osaka, Japan).

Preparation of Aβ-infused rats. The surgical techniques for preparing Aβ-infused rats were essentially the same as those described (8). Briefly, each rat was anesthetized lightly with sodium pentobarbital (50 mg/kg BW, i.p.); the skull was then exposed and 2 holes (right and left, relative to the bregma; 0.8 mm posterior, 1.4 mm lateral) were drilled according to the atlas of Paxinos and Watson (11) using a stereotaxic frame (Narishige). Then, 0.5 μg $AlCl_3$ (in 0.5 μL, intracerebroventricularly, 1 μL/min) was injected through a cannula 3.5 mm into the right ventricle, with a Hamilton syringe. Although the cause of AD is Aβ (1–42), we used Aβ (1–40) because of its better solubility. Moreover, because a small amount of $AlCl_3$ facilitated aggregation of Aβ peptide in vitro, and because the method has limited reproducibility without $AlCl_3$, we used $AlCl_3$ before implanting the osmotic pump for continuous infusion of Aβ. This procedure greatly improved the reproducibility and reliability of producing this animal model of AD, rats with impaired memory. A mini-osmotic pump (Alzet 2002, Durect), containing either Aβ peptide (1–40) solution or vehicle alone was quickly implanted in the back of the rat. The outlet of the pump was inserted 3.5 mm into the left ventricle and attached to the skull with screws and dental cement.

Radial maze-learning ability and DHA administration. The rats were tested for learning ability 4 wk after the implantation of the mini-osmotic pump to verify the memory impairment. Learning-related behavior was assessed using an 8-arm radial maze (Toyo Sangyo) as described (4,5). Briefly, the rats were trained to acquire a reward (food-pellet) at the end of each of 4 arms of an 8-arm radial maze. The performance involved 2 parameters of memory function, i.e., reference memory error (RME), entry into unbaited arms; and working memory error (WME), repeated entry into arms that had already been visited and obtaining the rewards within a trial. Each rat was given 2 daily trials, 6 d/wk for a total of 2.5 wk. The DHA and Aβ + DHA groups were then orally fed DHA-95E [300 mg/(kg · d), an ethyl-ester all-*cis*-4,7,10,13,16,19-docosahexaenoate with a purity of over 95%; Harima Chemicals] gently emulsified in a 5% gum Arabic solution in ice-cold water; the vehicle and Aβ groups were fed an equal volume of vehicle only.

Seven weeks after starting the administration of DHA, the rats were tested again for learning ability using an 8-arm radial maze for a total of 5 wk, to assess the effect of DHA on the impairment of learning ability.

Measurement of fatty acid profiles and oxidative status. After completing the behavioral studies, the rats were anesthetized with sodium pentobarbital (65 mg/kg BW, i.p.), blood was collected, and the cerebral cortex and hippocampus were separated as described (8). The tissues were stored at −80°C by flash-freezing in liquid N_2 until use or immediately homogenized in ice-cold 0.32 mol/L sucrose buffer (pH 7.4) containing 2 mmol/L EDTA, 0.5 mg/L leupeptin, 0.5 mg/L pepstatin, 0.5 mg/L aprotinin, and 0.2 mmol/L phenylmethylsulfonyl fluoride using a Polytron homogenizer (PCU 2–110; Kinematica). The homogenates were immediately subjected to the assays described below or stored at −80°C after liquid N_2 flash and bath until use.

Lipid peroxide concentration was assessed by the TBARS assay, as described (8,12). TBARS levels are expressed as nanomoles of malondialdehyde/mg protein. Malondialdehyde levels were calculated relative to a standard preparation of 1,1,3,3-tetraethoxypropane.

The levels of reactive oxygen species (ROS) were determined as described (8,12). Briefly, 50 μL freshly prepared tissue homogenate was mixed with 4.85 mL of 100 mmol/L potassium phosphate buffer (pH 7.4) and incubated with 2′,7′-dichlorofluorescin diacetate in methanol at a final concentration of 5 μmol/L for 15 min at 37°C. The dye-loaded samples were centrifuged at 12,500 × g for 10 min at 4°C. The pellet was mixed on a vortex at 0°C in 5 mL of 100 mmol/L phosphate buffer (pH 7.4) and incubated again for 60 min at 37°C. Fluorescence was measured with a Hitachi 850 spectrofluorometer (Tokyo, Japan) at wavelengths of 488 nm for excitation and 525 nm for emission. The cuvette holder was maintained at 37°C. ROS were quantified from a dichlorofluorescin standard curve in methanol.

Fatty acid composition was determined by the one-step analysis of Lepage and Roy (13) by GC as described (12,14). Protein concentration was estimated by the method of Lowry et al. (15).

Statistical analysis. Results are expressed as means ± SEM. Behavioral data were analyzed by a 2-factor (group and block) randomized block factorial ANOVA, and all other parameters were analyzed for intergroup differences by 1-way ANOVA. ANOVA was followed by Fisher's PLSD for post-hoc comparisons. Correlation was determined by simple regression analysis. The statistical programs used were GB-STAT™ 6.5.4 (Dynamic Microsystems), and StatView® 4.01 (MindVision Software, Abacus Concepts). Differences with $P < 0.05$ were considered significant.

RESULTS

Body weight. Body weights did not differ among the groups after the administration of DHA for 12 wk (vehicle: 494 ± 8 g; DHA: 481 ± 6 g; Aβ: 484 ± 9 g; Aβ + DHA: 490 ± 10 g).

Effect of DHA administration on radial-maze learning ability. The effects of Aβ peptide (1–40) infused into the rat cerebral ventricle and that of DHA administered to vehicle and Aβ-infused rats for 12 wk on reference and working memory-related learning ability are shown in Figures 1 and 2, respectively. The score is expressed as the mean number of RMEs and WMEs for each group, with data averaged over blocks of 6 trials. The *left panels* in both figures indicate the effect of the infused Aβ peptide (1–40). Randomized 2-factor (block and group) ANOVA to analyze the effect of the infused Aβ revealed significant main effects of both blocks of trials (P

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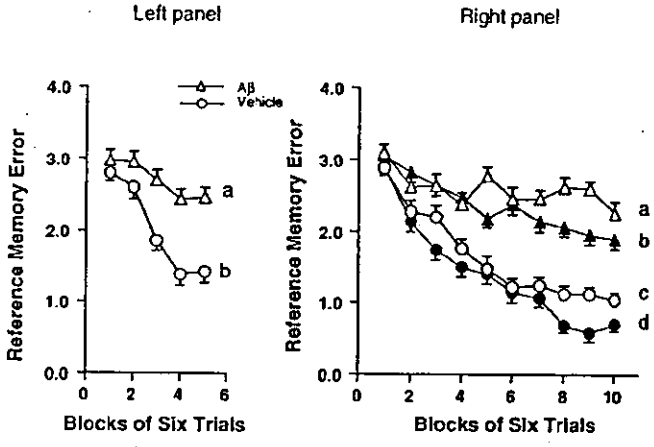

FIGURE 1 Effect of the infusion of amyloid β (Aβ) peptide (1–40) into the rat cerebral ventricle (*left panel*) and the effect of docosahexaenoic acid (DHA) administered to the Aβ-infused rats (*right panel*) on reference memory–related learning ability in the radial maze task. Each value represents the number of RMEs as the mean ± SEM in each block of 6 trials. Groups without a common letter for the main effects of groups are significantly different at $P < 0.05$. *Left panel*: vehicle rats ($n = 19$), Aβ rats ($n = 19$). The significance of differences between the 2 groups was determined by randomized 2-factor (block and group) ANOVA followed by Fisher's PLSD test; main effects of blocks of trials and groups were both significant ($P < 0.0001$), with a significant block × group interaction ($P < 0.0001$) on the number of RMEs. *Right panel*: Aβ rats ($n = 9$), Aβ + DHA rats ($n = 10$), vehicle rats ($n = 9$), DHA ($n = 10$). The significance of differences among the 4 groups was determined by randomized 2-factor (block and group) ANOVA followed by Fisher's PLSD test; main effects of blocks of trials and groups were both significant ($P < 0.0001$), with a significant block × group interaction ($P < 0.0001$) on the number of RME. Details of the subtest analysis between 2 groups of main effects of blocks of trials and groups, and between 2 groups of block × group interaction are shown in Table 2.

< 0.0001) and groups ($P < 0.0001$) with a significant block × group interaction ($P < 0.0001$) on the number of RMEs (Fig. 1, *left panel*). Similarly, a significant main effect of blocks of trials ($P < 0.0001$) and groups ($P < 0.0001$) with a significant block × group interaction ($P = 0.0174$) was observed on the number of WMEs (Fig. 2, *left panel*). These results indicate that Aβ peptide (1–40) infused into the rat cerebral ventricle impaired reference and working memory in the rats, suggesting learning impairment, a well-known characteristic of AD.

The *right panels* in both figures show the effect of DHA administered to vehicle and Aβ-infused rats. Randomized 2-factor (block and group) ANOVA revealed significant main effects of both blocks of trials ($P < 0.0001$) and groups ($P < 0.0001$) on the number of RMEs (Fig. 1, *right panel*), with a significant block × group interaction ($P < 0.0001$). Similarly, significant main effects of both blocks of trials ($P < 0.0001$) and groups ($P < 0.0001$) were observed, but without a significant block × group interaction ($P = 0.0911$) on the number of WMEs (Fig. 2, *right panel*). Subtest analysis (Table 2) of the number of RMEs showed the effect of DHA on Aβ-infused rats [blocks of trials ($P < 0.0001$) and groups ($P = 0.0027$), with a significant block × group interaction ($P = 0.0051$)]; the effect of DHA on vehicle rats [blocks of trials ($P < 0.0001$) and groups ($P = 0.0008$), without a significant block × group interaction]; and the effect of Aβ on vehicle rats [blocks of trials ($P < 0.0001$) and groups ($P < 0.0001$), with a significant block × group interaction ($P < 0.0001$)], demonstrating that the Aβ + DHA and DHA rats had a lower RME score than the Aβ-infused and vehicle rats (Fig. 1, *right panel*). Similarly, subtest analysis (Table 2) of the number of WMEs showed the effect of DHA on Aβ-infused rats [blocks of trials ($P < 0.0001$) and groups ($P = 0.0003$), without a significant block × group interaction]; the effect of DHA on vehicle rats [blocks of trials ($P < 0.0001$), but not groups ($P = 0.0823$), without a significant block × group interaction]; and the effect of Aβ on vehicle rats [blocks of trials ($P < 0.0001$) and groups ($P < 0.0001$), without a significant block × group interaction ($P = 0.067$)], demonstrating that the Aβ + DHA and DHA rats had a lower WME score than the Aβ-infused and vehicle rats (Fig. 2, *right panel*). These analyses suggest that administration of DHA improved reference and working memory-related spatial cognition of Aβ-infused and vehicle rats.

Effect of DHA administration on fatty acid profiles of plasma and brain. The plasma DHA level was significantly higher in both the DHA and Aβ + DHA groups than in the vehicle and Aβ groups and was accompanied by a significant decrease in arachidonic acid (AA), resulting in a significantly higher DHA/AA ratio (Table 3). The plasma levels of eicosapentaenoic acid [20:5(n-3)] and docosapentaenoic acid [DPA, 22:5(n-3)] were also significantly higher in both the DHA and Aβ + DHA groups than in the vehicle and Aβ groups. The increase in plasma (n-3) PUFA led to a higher unsaturation index (USI) of fatty acids in both the DHA and Aβ + DHA groups than in vehicle and Aβ groups (Table 3). T3

The administration of DHA significantly increased the DHA level in the hippocampus and hence the DHA/AA

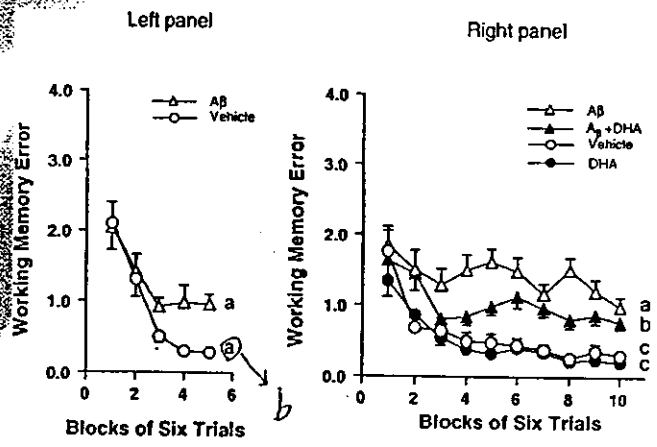

FIGURE 2 Effect of the infusion of Aβ peptide (1–40) into the rat cerebral ventricle (*left panel*) and the effect of DHA administered to the Aβ-infused rats (*right panel*) on working memory–related learning ability in the radial maze task. Each value represents the number of WMEs as the mean ± SEM in each block of 6 trials. Groups without a common letter for the main effects of groups are significantly different at $P < 0.05$. *Left panel*: vehicle rats ($n = 19$), Aβ rats ($n = 19$). The significance of differences between 2 groups was determined by randomized 2-factor (block and group) ANOVA followed by Fisher's PLSD test; significant main effects of blocks of trials ($P < 0.0001$) and groups ($P < 0.0001$) were observed, with a significant block × group interaction ($P = 0.0174$) on the number of WMEs. *Right panel*: Aβ rats ($n = 9$), Aβ + DHA rats ($n = 10$), vehicle rats ($n = 9$), vehicle + DHA ($n = 10$). The significance of differences among the 4 groups was determined by randomized 2-factor (block and group) ANOVA followed by Fisher's PLSD test; the main effects of blocks of trial and groups were both significant ($P < 0.0001$), but without a significant block × group interaction ($P = 0.0911$) on the number of WMEs. Details of subtest analysis between 2 groups of main effects of blocks of trials and groups, and between 2 groups of block × group interaction are shown in Table 2.

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TABLE 2

Statistical comparisons among the vehicle, DHA, Aβ, and Aβ + DHA groups in a randomized 2-factor (block and group) ANOVA followed by Fisher's PLSD test[1]

	Reference memory error			Working memory error		
Group	Block	Group	Block × Group interaction	Block	Group	Group × Block interaction
			P-value			
Aβ vs. Aβ + DHA	<0.0001	0.0027	0.0051	<0.0001	0.0003	0.551
Aβ vs. Vehicle	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.067
Aβ vs. DHA	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.079
Aβ + DHA vs. Vehicle	<0.0001	<0.0001	0.0227	<0.0001	<0.0001	0.083
Aβ + DHA vs. DHA	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.794
Vehicle vs. DHA	<0.0001	0.0008	0.367	<0.0001	0.0823	0.470

[1] Data are presented in Figures 1 and 2; $n = 9$ or 10.

molar ratio in the DHA and Aβ + DHA groups compared with the vehicle and Aβ groups. Furthermore, it tended to increase ($P = x.xx$) the DHA level in the cerebral cortex and significantly increased the DHA/AA molar ratio in the DHA and Aβ + DHA groups (Table 4). The DPA levels in both the cerebral cortex and the hippocampus were significantly higher in both the DHA and Aβ + DHA groups than in the vehicle and Aβ groups. The USI of the cerebral cortex was significantly higher in the DHA and Aβ + DHA groups than in the vehicle and Aβ groups, and that of the hippocampus in the DHA group, but not in the Aβ + DHA group, was significantly higher than in the vehicle group.

Significant positive correlations were observed between cortical and plasma DHA levels [(cortex DHA) = 40.5 (plasma DHA) + 142.4; $r = 0.42$, $P = 0.0085$] and between hippocampal and plasma DHA levels [(hippocampal DHA) = 37.5 (plasma DHA) + 134.6; $r = 0.54$, $P = 0.0004$]. These results suggest a substantial entry of DHA into the cortico-hippocampal regions of the brain.

Effect of DHA administration on the oxidative status of rat brains. TBARS levels in both the cerebral cortex and the hippocampus were higher in the Aβ group than in the vehicle, DHA, or Aβ + DHA group ($P < 0.05$). Similarly, levels of ROS in both tissues were significantly higher in the Aβ group than in any of the other groups (Table 5).

Significant positive correlations were found between learning ability (both RMEs and WMEs) and cortico-hippocampal ROS (Table 6). Likewise, RMEs correlated positively with cortico-hippocampal TBARS, and WMEs correlated with hippocampal, but not with cortex TBARS. On the other hand, there was a significant negative correlation of RMEs with the hippocampal DHA/AA ratio.

DISCUSSION

The present study describes the effects of DHA administration on the learning impairment of Aβ-infused rats produced by infusing Aβ peptide (1–40) into the brain ventricle. The infusion of Aβ impaired both reference and working memory, indicating a deficit in learning ability, a well-known characteristic of Alzheimer's disease. The administration of DHA improved both the reference and the working memory of Aβ-infused rats, clearly indicating that DHA ameliorated the Aβ-induced impairment of spatial cognitive learning ability in Aβ-infused rats.

In the Aβ-infused rats administered DHA, the increase in

TABLE 3

Plasma fatty acid profile in vehicle, DHA, Aβ, and Aβ + DHA rats[1]

Fatty acid[2]	Vehicle	DHA	Aβ	Aβ + DHA
		μmol/L		
PA, 16:0	1.815 ± 0.120	1.597 ± 0.155	1.846 ± 0.194	1.627 ± 0.088
SA, 18:0	0.570 ± 0.019[a]	0.437 ± 0.021[b]	0.529 ± 0.021[a]	0.440 ± 0.023[b]
OA, 18:1(n-9)	0.879 ± 0.089	0.701 ± 0.081	0.952 ± 0.153	0.698 ± 0.047
LA, 18:2(n-6)	2.012 ± 0.111	1.752 ± 0.168	1.944 ± 0.240	1.796 ± 0.103
LLN, 18:3(n-3)	0.040 ± 0.004	0.029 ± 0.005	0.040 ± 0.007	0.032 ± 0.002
AA, 20:4(n-6)	1.490 ± 0.059[a]	0.554 ± 0.026[b]	1.386 ± 0.048[a]	0.590 ± 0.046[b]
EPA, 20:5(n-3)	0.029 ± 0.003[b]	0.141 ± 0.024[a]	0.025 ± 0.004[b]	0.161 ± 0.019[a]
DPA, 22:5(n-3)	0.028 ± 0.004[b]	0.049 ± 0.009[a]	0.032 ± 0.007[ab]	0.046 ± 0.005[a]
DHA, 22:6(n-3)	0.202 ± 0.014[b]	0.547 ± 0.052[a]	0.208 ± 0.021[b]	0.581 ± 0.035[a]
DHA/AA	0.135 ± 0.005[b]	0.983 ± 0.079[a]	0.149 ± 0.011[b]	1.027 ± 0.095[a]
USI[3]	177.2 ± 2.456[b]	185.2 ± 1.587[a]	174.9 ± 3.395[b]	188.6 ± 2.592[a]

[1] Values are means ± SEM, $n = 9$ or 10. Means in a row with superscripts without a common letter differ, $P < 0.05$.
[2] Abbreviations used: EPA, eicosapentaenoic acid; LA, linoleic acid; LLN, linolenic acid; OA, oleic acid; PA, palmitic acid; SA, stearic acid.
[3] USI was calculated as a function of the sum of the mole percentages of the unsaturated fatty acids times the number of olefinic double bonds.

TABLE 4

Cortico-hippocampal fatty acid levels in vehicle, DHA, Aβ, and Aβ + DHA rats[1]

Fatty acid[2]	Cerebral cortex				Hippocampus			
	Vehicle	DHA	Aβ	Aβ + DHA	Vehicle	DHA	Aβ	Aβ + DHA
	nmol/mg protein							
PA, 16:0	249.9 ± 8.0	242.8 ± 7.9	253.5 ± 8.6	246.5 ± 10.4	248.3 ± 4.0^{a}	248.0 ± 7.4^{a}	229.1 ± 8.6^{b}	238.4 ± 5.7^{ab}
SA, 18:0	220.7 ± 7.6	216.3 ± 6.9	228.8 ± 9.7	219.3 ± 9.2	223.4 ± 4.0	221.9 ± 7.1	215.7 ± 3.9	216.8 ± 5.5
OA, 18:1(n-9)	135.9 ± 8.6	135.0 ± 5.2	150.4 ± 10.4	138.9 ± 7.7	167.6 ± 4.0	175.8 ± 7.7	160.8 ± 4.4	166.7 ± 6.9
LA, 18:2(n-6)	5.2 ± 0.4^{b}	6.8 ± 0.4^{a}	5.8 ± 0.6^{ab}	7.0 ± 0.3^{a}	4.6 ± 0.1^{b}	5.9 ± 0.4^{a}	4.6 ± 0.3^{b}	5.9 ± 0.2^{a}
LLN, 18:3(n-3)	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0^{a}	0.3 ± 0.0^{a}	0.3 ± 0.0^{a}	0.2 ± 0.0^{b}
AA, 20:4(n-6)	105.9 ± 3.7^{a}	93.1 ± 3.0^{b}	104.1 ± 2.8^{a}	92.6 ± 4.6^{b}	113.2 ± 2.9^{a}	102.6 ± 3.1^{c}	109.4 ± 2.6^{b}	101.9 ± 2.2^{c}
EPA, 20:5(n-3)	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	0.5 ± 0.1	0.4 ± 0.0	0.4 ± 0.0
DPA, 22:5(n-3)	1.0 ± 0.1^{b}	1.6 ± 0.1^{a}	1.1 ± 0.1^{b}	1.6 ± 0.1^{a}	1.1 ± 0.1^{b}	1.6 ± 0.1^{a}	0.4 ± 0.0	1.6 ± 0.1^{a}
DHA, 22:6(n-3)	146.8 ± 4.5^{b}	161.7 ± 6.4^{ab}	157.3 ± 8.9^{ab}	166.4 ± 6.2^{a}	143.1 ± 2.9^{b}	159.6 ± 5.8^{a}	140.1 ± 3.7^{b}	153.1 ± 3.8^{ab}
DHA/AA	1.39 ± 0.03^{b}	1.74 ± 0.06^{a}	1.52 ± 0.08^{b}	1.81 ± 0.06^{a}	1.27 ± 0.02^{b}	1.56 ± 0.04^{a}	1.29 ± 0.05^{b}	1.51 ± 0.03^{a}
USI[3]	168.5 ± 0.9^{b}	175.0 ± 1.1^{a}	170.0 ± 1.7^{b}	175.7 ± 1.2^{a}	166.0 ± 0.7^{b}	171.0 ± 1.0^{a}	169.1 ± 2.4^{ab}	171.3 ± 0.5^{a}

[1] Values are means ± SEM, n = 9 or 10. Means in a row for each brain area with superscripts without a common letter differ, $P < 0.05$.
[2] Abbreviations used: EPA, eicosapentaenoic acid; LA, linoleic acid; LLN, linolenic acid; OA, oleic acid; PA, palmitic acid; SA, stearic acid.
[3] USI was calculated as a function of the sum of the mole percentages of the unsaturated fatty acids times the number of olefinic double bonds.

the level of DHA in the hippocampus and cerebral cortex was accompanied by a significant decrease in AA, resulting in a significant increase in the DHA/AA ratio. An increased DHA/AA ratio in the hippocampus is involved in the acquisition of higher reference memory–related learning ability. These observations are in agreement with the results of previous studies demonstrating that the DHA/AA ratio in the rat cortico-hippocampal region is inversely related to RMEs in radial maze tasks (4) and that an increased DHA/AA molar ratio in the cortico-hippocampal region of Aβ-infused rats is associated with increased active avoidance response–related learning ability (8). The DHA/AA ratio in the cerebrum is an indicator of the antioxidative action of DHA in aged rats (16), and the increased cortico-hippocampal DHA/AA ratio in Aβ-infused rats correlates negatively with corresponding levels of apoptotic products (8). Dietary DHA reduces the amount of AA in phospholipids by jointly decreasing its synthesis and simply replacing it physically (17). AA is considered to be an essential precursor of biologically active molecules, as well as a contributor to increased production of lipid peroxide through the cyclooxygenase pathway. This is because some of the AA-cascade products of endoperoxides themselves have free radical characteristics (18). DHA can modulate the inflammation and oxidative stress in which AA and its metabolites participate directly or indirectly (19). Thus, an increase in the DHA/AA ratio might contribute to decreased TBARS levels, because the higher the DHA level in the brain, the lower the AA level and the higher the DHA/AA ratio. An increased DHA/AA ratio in the cortico-hippocampal regions may therefore play an enhanced role against oxidative neuronal damage and impairment of learning and memory after the infusion of Aβ.

The free-radical hypothesis of AD suggests that increased production of lipid peroxide causes deterioration of a wide variety of cellular enzymes, subsequently exacerbating the neurodegenerative processes (20). Chronic treatment with antioxidants such as α-tocopherol, improves cognitive functions in aging (21), a process frequently associated with increased oxidative damage and neurodegenerative diseases including AD. In the present study, DHA administration reduced the increased cortico-hippocampal TBARS and ROS levels in Aβ rats to the levels in vehicle rats. DHA protects the brain against ischemic and excitotoxic damage in rats (22,23); it also acts as an antioxidant in brain tissue under certain circumstances because of the intrinsic potential of brain tissue to generate free radicals (16,24). Thus, DHA likely was more effective against Aβ-induced oxidative stress at the neuronal level.

Epidemiologic studies show a relation between dietary (n-3) fatty acids and AD. High fish consumption, especially

TABLE 5

Oxidative status of cerebral cortex and hippocampus in rats administered vehicle, DHA, Aβ, and Aβ + DHA[1]

	Cerebral cortex		Hippocampus	
	TBARS	Reactive oxygen species	TBARS	Reactive oxygen species
	nmol/mg protein	*pmol/(min · mg protein)*	*nmol/mg protein*	*pmol/(min · mg protein)*
Vehicle	1.055 ± 0.018^{b}	0.657 ± 0.052^{b}	0.970 ± 0.042^{b}	0.647 ± 0.056^{b}
DHA	1.035 ± 0.070^{b}	0.629 ± 0.050^{b}	0.864 ± 0.108^{b}	0.623 ± 0.062^{b}
Aβ	1.390 ± 0.105^{a}	1.023 ± 0.114^{a}	1.204 ± 0.075^{a}	1.045 ± 0.100^{a}
Aβ + DHA	1.055 ± 0.050^{b}	0.727 ± 0.038^{b}	0.988 ± 0.047^{b}	0.648 ± 0.078^{b}

[1] Values are means ± SEM, n = 9 or 10. Means in a column with superscripts without a common letter differ, $P < 0.05$.