Arachidonic acid diet attenuates brain Aβ deposition in Tg2576 mice

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Abstract

The amyloid β-protein (Aβ) is believed to play a causative role in the development of Alzheimer's disease (AD). Because the amyloid precursor protein (APP), a substrate of Aβ, and β-secretase and γ-secretase complex proteins, which process APP to generate Aβ, are all membrane proteins, it is possible to assume that alterations in brain lipid metabolism modulate APP and/or Aβ metabolism. However, the role of polyunsaturated fatty acids in Aβ metabolism remains unknown. We report here that 9-months-treatment of Tg2576 mice with arachidonic acid (ARA)-containing (ARA+) diet prevented brain Aβ deposition in 17-month-old Tg2576 mice. APP processing to generate soluble APPα, CTF-β, and Aβ synthesis was attenuated in Tg2576 mice fed with the ARA+ diet. These findings suggest that ARA+ diet could prevent Aβ deposition through the alteration of APP processing in Tg2576 mice.

1. Introduction

Accumulation of the amyloid β-protein (Aβ), which is the major component of senile plaques in the brains of Alzheimer’s disease (AD) patients, was hypothesized to initiate a pathologic cascade that eventually leads to AD, and several lines of evidence have converged recently to demonstrate that the soluble oligomers of Aβ, but not monomers or insoluble amyloid fibrils, may be responsible for synaptic dysfunction in the brains of AD patients and AD animal models (Hardy and Selkoe, 2002). Aβ is generated from the amyloid precursor protein (APP) through its sequential proteolytic cleavage catalyzed by β- and γ-secretases. β-Site APP-cleaving enzyme (BACE) is a typical aspartyl protease, and γ-secretase is an aspartyl protease complex composed of four...
individual proteins (presenilin, nicastrin, APH-1, and PEN-2), with presenilin carrying the protease active site and working intramembrane site (Haass, 2004). The nonamyloidogenic pathway is mediated by α-secretase, which is a member of the disintegrin and metalloprotease (ADAM) family. This secretase produces a soluble fragment of APP (sAPPα), which is considered to have neurotrophic and neuroprotective properties (Thornton et al., 2006).

Since APP, BACE, and proteins consisting of the γ-secretase complex are membrane proteins, it is reasonable to assume that APP metabolism is modulated by membrane lipid composition. In support of this notion, it has been shown that the proteolytic activities of β-secretase and γ-secretase are modulated by membrane lipids, neutral glycosphingolipids, anionic glycosphosphatidyls, and cholesterol (Ehelt et al., 2003; Sawamura et al., 2004; Simons et al., 1998). In addition to glycosphingolipids, glycosphospholipids, and cholesterol, neural tissues of mammals contain the highest concentrations of polyunsaturated fatty acids (PUFAs), particularly arachidonic acid (ARA) and docosahexaenoic acid (DHA) (Diaz et al., 2005). Both ARA and DHA are major constituents of cell membranes, and thus play important roles in the preservation of physiological and psychological functions, and the development and maintenance of the central nervous system (Jauw and Dangour, 2006).

Low levels of ARA and DHA have been shown in the brain membrane of AD patients compared with that of normal controls (Prasad et al., 1998). Recently, DHA has been extensively investigated for its potential neuroprotective properties (Hooijmans et al., 2009; King et al., 2006). Several studies have shown that dietary intake of DHA decreases brain Aβ levels in APP transgenic mice (Calon et al., 2004; Green et al., 2007; Hooijmans et al., 2009; Oksman et al., 2006; Sahlin et al., 2007). However, the effect of another group of fatty acids, n-6 PUFAs, particularly ARA, on the impairment of learning memory and pathophysiology of AD remains unknown.

In this study, we examined the effects of dietary supplementation with ARA or DHA on the cognitive function of Tg2576 mice. In addition, we determined whether the ARA+ or DHA+ diet alters Aβ levels and APP processing in vivo and in vitro.

2. Results

2.1. Effects of ARA+ and DHA+ diets on Aβ deposition and Aβ levels

In Tg2576 mice, the level of Aβ begins to increase at 6–7 months and the subsequent Aβ deposition is observed at approximately 9 months of age. Between 15 and 23 months of age, Aβ deposition level in the Tg2576 brain increases with age, which is similarly observed in the AD brain (Kawarabayashi et al., 2001). To investigate the effect of dietary PUFA on Aβ deposition and Aβ levels, Tg2576 mice at 9 months of age, at which brain Aβ deposition starts to occur, were fed with a diet containing PUFA supplementation and maintained for 8 months until 17 months of age. Seventeen-month-old Tg2576 mice were killed and the levels of Aβ and Aβ deposition in the brain were determined.

Sagittal brain sections were stained with antibodies specific for Aβ1-40 and Aβ1-42 to detect human Aβ deposition. In control-diet-fed Tg2576 mice, Aβ depositions containing Aβ1-40 and Aβ1-42 were observed in cortical and hippocampal regions (Fig. 1A). In the Tg2576 mice fed with ARA+-diet, widespread reductions in the number of Aβ1-40 and Aβ1-42 deposits were observed, whereas this is not the case for the brain of Tg2576 mice fed with DHA+-diet (Fig. 1A). The number of Aβ1-40 deposits in the cortex was significantly decreased in ARA+-diet-fed mice compared with control diet-fed mice (Fig. 1B). The number of Aβ1-42 deposits in the cortex and the number of Aβ1-40 and Aβ1-42 deposits in the hippocampus tended to be lower in the ARA+-diet-fed mice than in the control diet-fed mice, although this difference was not statistically significant (Fig. 1A and C). The Aβ1-40- and Aβ1-42-immunopositive areas were decreased in the brain of ARA+-diet-fed mice; however, the decrease was not statistically significant (Fig. 1D and E). In the cortex and hippocampus of mice fed with DHA+-diet, Aβ1-40 and Aβ1-42 deposit levels did not change compared with those of mice fed with control diet (Fig. 1A–D). We also determined the effect of PUFA diet on amyloid deposition assayed by thioflavin-S staining. The number of thioflavin-S-positive amyloid deposits in the cortex was significantly decreased in mice fed with ARA+-diet compared with that of mice fed with control diet (Fig. 2A and B). The number of thioflavin-S-positive amyloid deposits in the hippocampus of mice fed with DHA+-diet was smaller than that of mice fed with control diet; however, the difference did not reach a statistically significant level (Fig. 2C).

Next, we performed experiments to determine the effect of PUFA diet on Aβ level in Tg2576 mouse brains by ELISA. Supporting the histological findings, ELISA showed that insoluble Aβ1-40 level was significantly decreased (35% lower) in the cortex of the mice fed with the ARA+-diet compared with that of mice fed with control diet (Fig. 3C). However, insoluble Aβ1-42 level was unchanged in the cortex of the mice fed with the ARA+-diet compared with that of mice fed with control diet (Fig. 3D). Soluble Aβ1-40 level also tended to decrease in the cortex of mice fed with the ARA+-diet compared with that of mice fed with control diet (p=0.11 for Fig. 3A). In the cortex and hippocampus of mice fed with DHA+-diet, both soluble and insoluble Aβ levels were unchanged compared with those of mice fed with the control diet.

2.2. Effects of dietary ARA on the levels of sAPPs, CTFs, cellular APP, APP processing enzymes, and Aβ-degrading enzymes

There may be several reasons why Aβ levels and Aβ deposition level were lower in the cortex of mice fed with the ARA+-diet. One possibility is that the ARA+-diet reduced Aβ production through the modulation of APP processing. To elucidate this possibility, we examined whether the ARA+-diet has any effects on APP processing and Aβ degradation. We first measured α-, β-, and γ-secretase expression levels in the cortex of PUFA-supplemented Tg2576 mice. ADAM10, BACE1, PS-1, and nicastrin expression levels were unchanged by the dietary PUFA supplementation (Fig. 4A). We next
measured the expression levels of APP metabolites, secreted forms of APPs (sAPPα and sAPPβ), and C-terminal fragments of APP (CTF-α and CTF-β). The expression levels of these metabolites reflect the APP processing activity. The sAPPα and sAPPβ expression levels were significantly lower in the cortex of mice fed with the ARA+ diet (Fig. 4B). The expression level of membrane-bound APP tended to increase in the ARA+ diet group. The CTF-α and CTF-β expression levels were also reduced in the cortex of mice fed with the ARA+ diet (Fig. 4C). These APP metabolite expression levels were unchanged in the cortex of mice fed with the DHA+ diet. Because Aβ levels are also modulated by degradation of Aβ peptides, we next measured the expression levels of Aβ degradation enzymes, neprilysin and IDE. The expression levels of these enzymes were unchanged by the dietary PUFA supplementation (Fig. 4D). These results suggest that the ARA+ diet reduces Aβ levels and Aβ deposition level by suppression of APP processing.

3. Discussion

In this study, we report the novel findings that ARA+ diet reduces Aβ deposition assayed by immunohistochemistry using anti-Aβ antibodies and thioflavin-S staining in 17-month-old Tg2576 mouse cortices. ARA+ diet also reduces insoluble Aβ40 levels demonstrated by ELISA in 17-month-old Tg2576 mouse cortices. In addition, sAPPα, sAPPβ and CTF-α levels are decreased in ARA+ diet mouse cortices. However, ARA+ diet has no effect on the expression levels of ADAM10, BACE1, PS-1 and nicastrin and also amyloid degrading enzymes, neprilysin and IDE in these mouse brains. These lines of evidence suggest that the reduction of Aβ levels by ARA+ diet in Tg2576 mouse brains is mediated by a suppression of proteolytic processing in APP.

A Western diet contains high amounts of n-6 PUFA and low amounts of n-3 PUFA, and the resulting high n-6/n-3 ratio is considered to contribute to cardiovascular disease, inflammation, and cancer (Simopoulos, 2008). It has been shown that a high-n-6/n-3-ratio diet accelerates atherosclerosis and inflammation in several mouse disease models (Wang et al., 2009; Yamashita et al., 2005). It has been shown that cognitive deficits in Tg2576 mice are worsened by a high-n-6/n-3-ratio diet (85:1) compared with a low-ratio diet (5:1) (Calon et al., 2004), and that the n-6/n-3 ratio also affects AD pathophysiology; a high ratio (72:1) induces large amounts of Aβ deposition compared with a low ratio (4:1) (Julien et al., 2010). However, the n-6/n-3 ratio in the diet used in their study was varied; thus, it is difficult to determine the actual effect of target fatty acids such as DHA on AD pathologies. Therefore, in this study, we used the same n-6/n-3 ratio (2:1) in all the experimental diets in order to determine the specific effects of the ARA+ or DHA+ diet on AD pathophysiology, to remove the effect of the difference in the n-6/n-3 fatty acid ratio.

ARA is metabolized to eicosanoids, such as prostaglandins, leukotrienes, and thromboxanes, all of which are potent mediators of inflammation. Cyclooxygenase is essential for the synthesis of prostaglandin E2 (PGE2), which stimulates the
production of Aβ through internalization of PGE_2 receptors (Hoshino et al., 2009). It has been shown that the expression levels of PGE_2 are elevated in the brains of AD patients (Montine et al., 1999), whereas phospholipase A_2 reduction ameliorates cognitive deficits in a mouse model of AD (Sanchez-Mejia et al., 2008). However, the protective effects
of ARA on brain and cognitive functions have also been reported, namely, an ARA+-diet preserves synaptic plasticity in aged rats (Kotani et al., 2003) and dietary supplementation of ARA and DHA improves cognitive dysfunction (Kotani et al., 2006). Most recently, it has been demonstrated that exposing differentiated neuronal cells to type III secretory phospholipase A2 and ARA increases the sAPPα secretion level and decreases the levels of Aβ1-42 in SH-SY5Y cells and that these changes are accompanied by increased membrane fluidity (Fukaya et al., 2007; Ma et al., 2009). Because higher levels of PUFAs in the membrane result in greater membrane fluidity (Hulbert, 2003), the ARA+-diet may increase the fluidity of phospholipid membranes and affect Aβ production by altering the activities of enzymes responsible for Aβ production.

Previous reports have shown that cognitive dysfunction was caused by the extracellular accumulation of 56-kDa soluble Aβ assemblies (Lesne et al., 2006), and that Aβ oligomers and Aβ-derived diffusible ligands may play the crucial role of being the earliest effector that causes synaptic dysfunction and early memory loss associated with dementia in AD (Gong et al., 2003). In our study, the ARA+-diet decreased insoluble Aβ levels in the Tg2576 mouse brain. In our study, ARA levels in mouse brain cortices fed with ARA+-diet are significantly higher than those in control mouse cortices (Table 2), suggesting that altered ARA levels in neural cells in the brain cortices of mice fed with ARA+-diet attenuate Aβ generation in Tg2576 mice.

There is a discrepancy between previous studies and our study in terms of the effects of the DHA+-diet on Aβ levels. In recent studies, the DHA+-diet attenuates AD pathology, decreases Aβ deposition levels, and improves cognitive dysfunction (Calon et al., 2004; Green et al., 2007; Hooijmans et al., 2009; Oksman et al., 2006; Sahlin et al., 2007). Previous study showed a protective effect associated with increased fish consumption (Kalmijn et al., 2004). This may be because the n-6/n-3 ratios in the diets in our study and those in previous studies are quite different. In previous studies, DHA
diets contained high doses of DHA (15.0–25.4% of total fat), and various n-6/n-3 ratios in diets were used (Calon et al., 2004; Green et al., 2007). In contrast, in our study, we used a low-dose DHA diet (4.0% of total fat) and kept the n-6/n-3 ratio constant (2:1) across all experimental diets, and we could not detect decreases in Aβ levels by the DHA+ diet. The fatty acid profiles in general were not markedly changed in the brains of Tg2576 mice fed the ARA+ or DHA+ (Table 2). These lines of evidence suggest that not only the difference in DHA dose but also n-6/n-3 ratio affects membrane profiles and functions of proteins associated with the membrane.

4. Experimental procedures

4.1. Diets and animals

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Center for Geriatrics and Gerontology. The protocol was approved by the Committee on the Ethics of Animal Experiments of the National Center for Geriatrics and Gerontology (Permission Number: Dou23-02-3). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. We prepared three different diets, a modified AIN-76 rodent diet, containing ARA or DHA with a constant n-6/n-3 ratio and a constant proportion of polyunsaturated, monounsaturated, and saturated fatty acids (PMSs). The control diet contained 5% fat prepared by mixing palm oil, soybean oil, and linseed oil, without ARA or DHA. The ARA- or DHA-containing diet contained 5% fat prepared by mixing ARA- or DHA-enriched triacylglycerol with the oils described above. The fatty acid composition, n-6/n-3 ratio, and proportion of PMS of each diet are shown in Table 1. Dietary foods with ARA or DHA were stored at 4°C and protected from light. Mice at 9 months of age were fed with PUFA-containing diet or control diet ad libitum for 8 months. The fatty acid residues in general were not markedly changed in the brains of Tg2576 mice fed the ARA- or DHA-containing diet (Table 2).

4.2. Fatty acid analysis

Total lipids in red blood cells, the liver, and the cortex were extracted using chloroform and methanol (Folch et al., 1957). The phospholipid fraction was separated from the extracted lipids by thin-layer chromatography using silica gel 60 (Merck, Darmstadt, Germany). The solvent system used was n-hexane/diethyl ether (70/30, v/v). The fatty acid residues in the phospholipid fraction were analyzed (Sakuradani et al., 1999). In brief, an internal standard (pentadecanoic acid) was added to each sample and it was incubated in methanolic HCl for 3 h to induce transmethylation of fatty acid residues. Fatty acid methyl esters were extracted with n-hexane and analyzed by capillary gas–liquid chromatography. Analytical conditions were as follows: 1) apparatus, Agilent 6890 (Agilent Technologies Inc., Wilmington, DE); 2) column, SP-2330 (30 m × 0.32 mm × 0.2 mm, Supelco, Inc., Bellefonte, PA); 3) carrier, He (30 cm/s); 4) column temperature, 180°C for 2 min, and increased to 220°C at a rate of +2°C/min.

4.3. Immunohistochemistry and thioflavin-S staining

The left hemispheres of the brains of Tg2576 mice were sagittally cut into 30-μm sections using a freezing microtome (RM 2145; Leica, Wetzlar, Germany). Serial sections were immunostained with anti-Aβ1-40 and anti-Aβ1-42 end-specific polyclonal antibodies (IBL, Gunma, Japan), and immunopositive signals were visualized using an ABC elite kit (Vector Laboratories Inc., Burlingame, CA). Aβ1-40 and Aβ1-42 deposits were quantified as the percentage of immunostained area (positive pixels) divided by the examined area (total pixels). Thioflavin-S staining was performed as described elsewhere (Bussiere et al., 2004). Sections were postfixed in 10% formalin for 10 min, and

<table>
<thead>
<tr>
<th>Fatty acid (g/100 g diet)</th>
<th>Control</th>
<th>4% ARA</th>
<th>4% DHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0 palmitic acid</td>
<td>1.33</td>
<td>1.32</td>
<td>1.37</td>
</tr>
<tr>
<td>18:0 stearic acid</td>
<td>0.20</td>
<td>0.22</td>
<td>0.20</td>
</tr>
<tr>
<td>18:1 oleic acid</td>
<td>1.51</td>
<td>1.43</td>
<td>1.53</td>
</tr>
<tr>
<td>18:2 linoleic acid</td>
<td>1.25</td>
<td>1.00</td>
<td>1.21</td>
</tr>
<tr>
<td>18:3 α-linolenic acid</td>
<td>0.62</td>
<td>0.63</td>
<td>0.36</td>
</tr>
<tr>
<td>20:3 Dihomo-γ-linolenic acid</td>
<td>0</td>
<td>0.02</td>
<td>0</td>
</tr>
<tr>
<td>20:4 ARA</td>
<td>0</td>
<td>0.22</td>
<td>0</td>
</tr>
<tr>
<td>22:6 DHA</td>
<td>0</td>
<td>0</td>
<td>0.24</td>
</tr>
<tr>
<td>Others</td>
<td>0.09</td>
<td>0.17</td>
<td>0.10</td>
</tr>
<tr>
<td>%SAT</td>
<td>32.42</td>
<td>34.22</td>
<td>33.32</td>
</tr>
<tr>
<td>%MONO</td>
<td>30.24</td>
<td>28.52</td>
<td>30.53</td>
</tr>
<tr>
<td>%POLY</td>
<td>37.34</td>
<td>37.26</td>
<td>36.15</td>
</tr>
<tr>
<td>n-6 to n-3 ratio</td>
<td>2.01</td>
<td>1.97</td>
<td>2.02</td>
</tr>
</tbody>
</table>

SAT, Saturated; MOMO, monounsaturated; POLY, polyunsaturated.
washed in PBS. After incubation for 10 min in 0.25% potassium permanganate, sections were washed in PBS and incubated in 2% potassium metabisulfite and 1% oxalic acid until they appeared white. Sections were then washed in water and stained for 10 min with a solution of 0.015% Thioflavin-S (Sigma-Aldrich Co., St Louis, MO) in 50% ethanol. Finally, sections were washed in 50% ethanol and then in water. They were dried and mounted onto slides using FluorSave reagent (Calbiochem, La Jolla, CA). Thioflavin-S-stained plaques were counted in four to five sections per left hemisphere of the brain.

4.4. Aβ ELISA

The frozen mouse cortex and hippocampus were first homogenized in 30 volumes of Tris-buffered saline (TBS, 10 mM Tris and 150 mM NaCl, pH 7.5) containing a protease inhibitor cocktail (Protease Inhibitor Cocktail, Roche, Mannheim, Germany) and then centrifuged at 450,000 × g for 20 min at 4 °C, as described elsewhere with some modifications (Kawarabayashi et al., 2001). Supernatants were saved for soluble Aβ analyses. Pellets were resuspended and further homogenized in 30 volumes of 2% SDS with the same protease inhibitors, followed by centrifugation at 15,000 × g for 20 min. SDS supernatants were used for insoluble Aβ determination. Aβ1-40 and Aβ1-42 were assayed using ELISA kits (Wako Pure Chemical Industries, Osaka, Japan).

4.5. Immunoblots

Protein extracts were prepared from the cortex using TBS and SDS solutions as described in the Aβ ELISA section. Protein concentration was determined by the BCA method. Equal amounts of protein samples (3 μg/well) were subjected to SDS-PAGE, and separated proteins were transferred to 0.4 μm Immobilon-P membranes (Millipore, Billerica, MA) and blocked for 1 h in 5% (w/v) nonfat dry milk in TBS supplemented with 0.1% Tween-20. The following antibodies were used: anti-ADAM 10 (Millipore), anti-BACE1 (Cell Signaling Technology, Danvers, MA), anti-nicastrin (Epitomics, Burlingame, CA), anti-presenilin 1 (PS-1, Millipore), anti-N-terminal APP (22C11, Millipore), anti-human sAPPβ with Swedish mutation (IBL, Gunma, Japan), UT-18 (C-terminal fragment of APP antibody (Tomita et al., 1999)), anti-neprilysin (R&D systems), anti-insulin-degrading enzyme (IDE, Covance, Princeton, NJ), and anti-actin (Cell Signaling Technology) antibody. Membranes were labeled with a primary antibody overnight at 4 °C, and incubated with a horse-radish peroxidase-linked secondary antibody for 1 h at room temperature. The bands of interest were analyzed using a luminescent image analyzer LAS-3000 mini (Fujifilm, Tokyo, Japan), and signals were quantified using Multi Gauge software (Fujifilm).

4.6. Statistical analysis

All results were expressed as the mean ± S.E.M. for each group. Statistical analysis was performed using a statistical package, GraphPad prism software (GraphPad Software, San Diego, CA). In Figs. 1–4 and Table 2, the difference between groups was analyzed by one-way ANOVA with Dunnett’s multiple comparison post-hoc test.

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References


