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Journal of Nutritional Biochemistry 21 (2010) 345–350
Journal of Nutritional Biochemistry

Docosahexaenoic acid supplementation of primary rat hippocampal neurons attenuates the neurotoxicity induced by aggregated amyloid beta protein$_{42}$ and up-regulates cytoskeletal protein expression☆

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Received 21 October 2008; received in revised form 13 January 2009; accepted 13 January 2009

Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized by extracellular deposits of fibrillar aggregates of amyloid-β peptide (Aβ). Levels of docosahexaenoic acid (DHA, 22:6n-3), the major fatty acid component of the neuronal membrane, are reduced in the AD hippocampus. We hypothesized that hippocampal neurons with reduced DHA levels would be more susceptible to aggregated Aβ-induced death and that this might be overcome by increasing hippocampal neuronal DHA levels. Embryonic Day 18 rat hippocampal cells were cultured in neurobasal medium with B27 supplemented with 0–100 μM DHA for 8 days, then were treated with 5 μM aggregated Aβ_{42} for 1 day. We found that supplementation with 5–10 μM DHA, which resulted in hippocampal neuron DHA levels of 12–16% of total fatty acids, was optimal for primary hippocampal neuronal survival, whereas supplementation with 5 or 25 μM DHA attenuated aggregated Aβ_{42}-induced neurotoxicity and protected hippocampal neurons, with 25 μM DHA being more effective. DHA supplementation also resulted in significant up-regulation of expression of tyrosine tubulin and acetylated tubulin. We suggest that hippocampal neuronal DHA levels may be critical for AD prevention by attenuating the neurotoxicity induced by Aβ and in maintaining hippocampal neuron survival.

Keywords: Alzheimer's disease; Docosahexaenoic acid; Cytoskeleton; Amyloid beta peptide; Hippocampus; Neuron protection

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized by dementia. The main pathology of AD is extracellular deposits of amyloid-β peptide (Aβ) as fibrillar aggregates in senile plaques and the intracellular accumulation of hyperphosphorylated tau proteins as neurofibrillary tangles [1–3]. Aβs, consisting of 39, 40, 42 or 43 amino acids, are metabolic products of Aβ precursor protein, a neuronal cell transmembrane protein [4]. Aβ_{40} is the most abundant form, but neurotoxicity is mainly induced by Aβ_{42} [5,6].

Docosahexaenoic acid (DHA, 22:6n-3) is essential for normal neurological function [7,8]. DHA and arachidonic acid (20:4n-6) are the major polyunsaturated fatty acids in neuronal membranes [9]. Most DHA accumulation in the brain takes place during brain development in the perinatal period [10–13]. Brain DHA levels decrease with aging [14,15] and are reduced by as much as 50% in the hippocampus of AD patients compared to those in non-AD of the same age [16–18], but are unchanged in the cortex, thalamus, occipital lobes, or white or gray matter [16,19,20]. It is not known whether hippocampal neurons with reduced DHA levels are more susceptible to aggregated Aβ_{42}-induced neurotoxicity, but we hypothesized that higher DHA levels in hippocampal neurons would protect against neurotoxicity.

Evidence is accumulating that DHA supplementation restores reduced hippocampal DHA levels and rescues hippocampal neuron function. DHA-enriched fish oil supplementation of DHA-deficient rats restores hippocampal DHA levels and enhances reference and working memory performance [21]. In studies of DHA supplementation in an AD mouse model or in aged animals, learning memory performance was improved by DHA [22–25]. Progression in AD patients and cognitive decline in elderly men are delayed by fish oil consumption [26–28]. Moreover, evidence is accumulating for an effect of DHA in protecting neurons against apoptosis induced by serum starvation or oxidative stress [22,29–32], but the mechanism of neuronal protection by DHA is not well understood.

Several reports suggest that Aβ induces neuron death by cytoskeleton perturbation [33,34]. Immunofluorescence studies on primary cortical neurons have demonstrated that DHA prevents

☆ This work was supported by the National Science Council of Taiwan (grants NSC93-2320-B-002-026 and NSC-94-2320-B-002-104).
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doi:10.1016/j.jnutbio.2009.01.012

neuron death induced by soluble $A\beta_{40}$ oligomer by preventing cytoskeleton perturbation [29]. As it has been reported that Aβ-induced neurotoxicity is mainly caused by $A\beta_{42}$ [5,6,35] and requires the fibrillar aggregated form [36], we examined whether DHA protected mature primary hippocampal neurons against aggregated $A\beta_{42}$ and the effect of DHA on cytoskeleton protein expression. In addition, it is important to know the optimal hippocampal DHA levels for neuronal survival under normal conditions and for protection against $A\beta_{42}$-induced neurotoxicity.

Embryonic Day 18 (E18) rat hippocampal cells were plated on poly-L-lysine-coated plates and cultured in neurobasal medium with B27 supplemented with 0–100 μM DHA for 8 days, then were treated with 5 μM aggregated $A\beta_{42}$ for 1 day. The optimal DHA concentration and hippocampal DHA level for neuron survival in the normal physiological and pathological states were determined and cytoskeletal protein expression was examined. We also determined whether the supplemented DHA was incorporated into hippocampal neurons.

2. Materials and methods

2.1. Primary hippocampal cultures

Pregnant Sprague-Dawley rats at 14 days of gestation were obtained from the National Laboratory Animal Center, Taipei, Taiwan, and were housed in a humidity-controlled room at 24±1°C on a 12-h light–dark cycle with free access to tap water and chow diet (5001, LabDiet). The protocols and animal treatments used in this study were approved by the Animal Care and Use Committee of the National Taiwan University College of Medicine. Culture media were purchased from Gibco Invitrogen (Grand Island, NY, USA), and, unless otherwise specified, all chemicals were from Sigma (St. Louis, MO, USA).

The method used for hippocampal primary culture was that described previously [37] with some modifications. In brief, the hippocampi were removed from the E18 brain, freed of meninges and dissected in calcium-/magnesium-free Hanks balanced salt solution (HBSS) containing 10 mM HEPES and 1 mM MEM sodium pyruvate. The neurons were dissociated by incubation with 0.25% trypsin at 37°C for 15 min, followed by two additions of trypsin inhibitor (1 mg/ml in HBSS) for 5 min and trituration with a fire-polished pasture pipette. The neuronal suspension was then plated onto poly-L-lysine-coated (0.5 mg/ml in 0.1 M borate buffer) coverslips, plates or dishes at a low density of 150 cells/mm^2 in neurobasal medium containing serum-free B27 (neurobasal medium/B27, 50:1), 0.025 mM glutamate, 0.5 mM glutamax I, 0.025 mM β-mercaptoethanol, l00 U/ml of penicillin and 100 μg/ml of streptomycin, supplemented with 0, 1.5, 5, 10, 25, 50, 75 or 100 μM B27-bound DHA. Three days after plating, 5 μM 1-β-D-arabinofuranosylcytosine was added to the culture medium to inhibit the growth of non-euronal cells. Six days after plating, one-third of the culture medium was replaced by fresh neurobasal medium containing B27, 0.5 mM glutamax I, l00 U/ml of penicillin and 100 μg/ml of streptomycin, supplemented with the same concentrations of B27-bound DHA as above. These conditions yielded cultures consisting of up to 95% neurons [37–39].

2.2. Preparation of aggregated $A\beta_{42}$ and B27-bound DHA

The preparation of aggregated $A\beta_{42}$ was modified from a previous method [40]. Synthetic $A\beta_{42}$ (1–42) (Sigma) was dissolved in phosphate-buffered saline (PBS) at 0.3 mg/ml, shaken at 37°C for 2 days and incubated at 37°C for another 2 days for $A\beta_{42}$ aggregation. Aggregated $A\beta_{42}$ was added to the culture medium at a final concentration of 5 μM.

B27-bound DHA was prepared by stirring 18.7 μl (16.8 mg) of pure DHA (Nu-Chek Prep, Elysian, MN, USA) with 10 ml of B27, which is rich in bovine albumin [41], for 4 h and was stored as aliquots in a −80°C freezer.

2.3. Quantification of neuronal survival

Neuronal survival was measured as described previously [42]. In brief, phase-contrast photomicrographs were taken on Day 8 or 9 of culture, at which time the cells are considered to be mature neurons [43]. Cells with intact neurites and an intact soma were considered viable, while those with fragmented neurites or a shrunken soma were considered nonviable. For each determination of neuron survival, six to nine fields (10× objective) in triplicate wells were counted and the average survival result taken. In each field of controls without DHA supplementation (0 μM), an average of 150 viable neurons was counted. Neuronal survival after $A\beta_{42}$ treatment was also quantified by measuring mitochondrial dehydrogenase activity using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay [44]. MTT (0.5 mg/ml) was added on Day 9 of culture after 8 h of treatment with $A\beta_{42}$, and incubation continued at 37°C for 4 h, then the formazan crystals were solubilized in dimethyl sulfoxide and the absorbance at 570 nm read on a spectrophotometer. In a pilot study, the results for neuronal survival obtained by cell counting as described above, cell counting using trypan blue exclusion or the MTT reduction assay were similar.

2.4. Fatty acid analysis

Three wells of primary hippocampal cells cultured on six-well plates for 9 days were pooled as one sample. The medium was removed and the cells washed three times with PBS, then trypsinized, homogenized for total lipid extraction according to the method of Bligh and Dyer [45], and dried down under nitrogen, then were converted to their methyl esters and analyzed by Hewlett-Packard 5890 gas chromatography using flame ionization detection [21].

2.5. Immunocytochemistry

Hippocampal neurons were plated onto poly-L-lysine-coated coverslips (Bellco, Vineland, NJ, USA) in 12-well plates in medium supplemented with 0, 5 or 25 μM DHA, and cultured for 8 days, then 5 μM $A\beta_{42}$ was added for 8 h. The neurons were then fixed in acetone at −20°C for 5 min, washed with PBS, immunolabeled with primary mouse monoclonal anti-α-tubulin antibody (1:500, Sigma) in PBS at 37°C for 90 min, rinsed in PBS for 3×5 min, incubated for 1 h at 37°C with rhodamine-conjugated antimouse IgG antibody (1:50 Chemicon) and rinsed in PBS for 3×5 min.

2.6. Western blot analysis of cytoskeletal protein expression

Hippocampal neurons were plated onto 10-cm dishes in medium supplemented with 0, 5 or 25 μM DHA, and cultured for 9 days, then were scraped off the dishes and sonicated in lysis buffer (10 mM Tris-HCl buffer, pH 7.3, containing 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and a protease inhibitor cocktail mix; Complete, Roche, Basel, Switzerland). The homogenate was centrifuged at 14,000×*g* for 15 min at 4°C, and the supernatant collected as "soluble protein," while the pellet was resuspended in 5% sodium dodecylsulphate (SDS) and 6 M urea in 10 mM Tris buffer (pH 7.5), the suspension centrifuged and the supernatant collected as "insoluble protein." The soluble and insoluble protein were used for Western blotting, the protein concentration being measured using a Bradford protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The proteins were denatured by heating at 95°C for 5 min in SDS sample buffer and aliquots of 1 μg of protein separated by 10% SDS-polyacrylamide gel electrophoresis, and electrotransferred at 90 V to polyvinylidene difluoride membranes (Bio-Rad) for 1.5 h, followed by blocking with 5% nonfat milk in 0.1% Tween 20 in Tris-buffered saline (blocking buffer) for 1 h at room temperature. The membranes were then subjected to immunoblotting with primary antibodies in blocking buffer at 4°C overnight. The mouse monoclonal antibodies used were antityrosine tubulin (clone TUB-1A2, 1:6000, Sigma), antiacetylated tubulin (clone 6-11B-1, 1:6000, Sigma), anti-β-actin (clone AC-74, 1:5000, Sigma) and anti-GAPDH (1:2000, Novus Biologicals, Inc., Littleton, CO, USA). GAPDH was used as the loading control. Bound antibody was detected using horseradish peroxidase-conjugated antimouse IgG antibody in blocking buffer, followed by an enhanced chemiluminescence Western blot detection reagent (ECL, Amersham Biosciences, Buckinghamshire, England). Quantification of the data was performed using a UVP BioDoc-IT Imaging system.

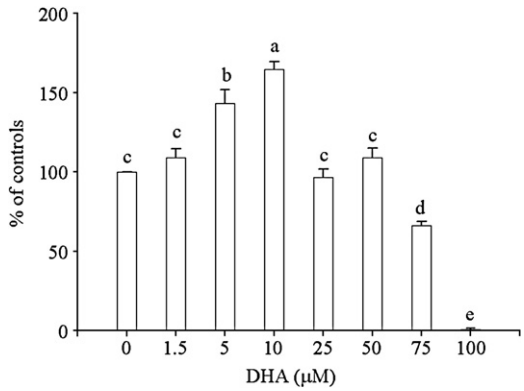

Fig. 1. Effect of DHA supplementation on the survival of primary rat hippocampal neurons. Hippocampal cells were cultured from E18 rat embryos and seeded in six-well plates in neurobasal medium with B27 supplemented with 0–100 μM DHA. At Day 8, phase-contrast photomicrographs were taken in three fields per well for three wells for each treatment. Cells with intact neurites and soma were considered viable. Viable cells in the nine fields were counted and the average used as a determination of cell survival. The cultured neurons without DHA supplementation (0 μM) were used as the 100% controls. The data are the mean±S.E.M. for six independent experiments for each treatment, except for 75 and 100 μM DHA (n=4). The different letters show significant differences analyzed by one-way ANOVA followed by Duncan's multiple range tests.

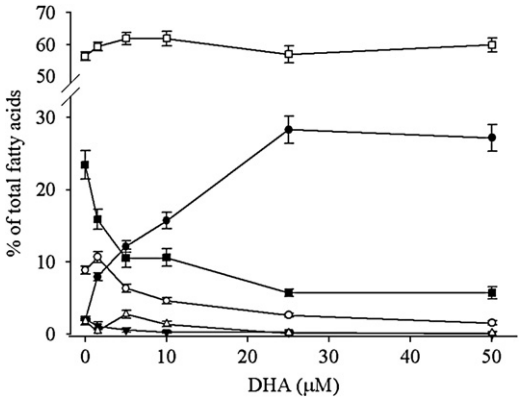

Fig. 2. Effect of DHA supplementation on primary rat hippocampal DHA levels. Hippocampal neurons were seeded in six-well plates and grown for 9 days in neurobasal medium with B27 supplemented with 0, 1.5, 5, 10, 25 or 50 μM DHA. Three wells for each DHA concentration were collected as one sample for fatty acid analysis. The levels of DHA (●), 20:4(n-6) (○), 22:4(n-6) (▼) or 22:5(n-6) (△); the sum of the monounsaturated fatty acid (■); and the sum of the saturated fatty acids (□) are shown as the % of total fatty acids. The data are the mean±S.E.M. for six independent experiments for each treatment.

2.7. Statistical analysis

Data are presented as the mean±S.E.M. Statistical differences between treatments were determined by one-way or two-way ANOVA followed by Duncan's multiple range test using the SAS program (version 9.1.3, SAS Institute, Cary, NC, USA). Differences were considered significant at $P \leq .05$.

3. Results

3.1. Effect of DHA supplementation on hippocampal neuron survival

DHA is essential for normal neurological development. However, the optimal DHA concentration for neurons is still not clear. To address this issue, we examined survival at Day 8 in cultures of rat embryonic E18 hippocampal cells cultured in neurobasal medium containing B27 supplemented with 0, 1.5, 5, 10, 25, 50, 75 or 100 μM DHA. Compared to no DHA supplementation (0 μM), supplementation with 5 or 10 μM DHA resulted in a significant increase in neuronal survival of 43% or 65%, respectively, while supplementation with 1.5, 25 or 50 μM DHA had no effect (Fig. 1). At 75 μM DHA, neuronal survival was significantly decreased to 66% of that in controls, while, at 100 μM DHA, most neurons were dead after 2 days of culture. This suggests that an appropriate DHA concentration is important for neuron survival.

3.2. Docosahexaenoic acid incorporation into primary hippocampal neurons

Next, we examined whether the supplemented DHA was incorporated into the hippocampal neurons. After 9 days of cultures with 0–50 μM DHA, mature neurons were collected for fatty acid analysis. As shown in Fig. 2, DHA levels were increased from 2.0% at 0 μM DHA to 7.9%, 12.1% or 15.7% of total fatty acids with DHA supplementation of 1.5, 5 or 10 μM, then plateaued at 28.3% and 27.2% with 25 or 50 μM DHA supplementation, respectively. The increase in DHA levels was accompanied by a decrease in 20:4n-6 levels from 8.8% to 1.5% and a decrease in the sum of the monounsaturated fatty acids from 23.4% to 5.7%. The main reduction in monounsaturated fatty acids was in oleic acid (18:1n-9), others being changed by less than 1.5%. In contrast, the sum of the saturated fatty acids showed little change, ranging from 55.2% to 61.9%. Levels of docosatetraenoic acid (22:4n-6) and docosapentaenoic acid (22:5n-6) were also reduced from 2.1% to 0.0% and from 1.7% to 0.2%, respectively, with increasing DHA supplementation. n-3 Fatty acids other than DHA accounted for less than 1% of the total fatty acids in cultured hippocampal neurons.

3.3. Docosahexaenoic acid attenuates the neurotoxicity induced by aggregated $A\beta_{42}$

Hippocampal cells were seeded in medium supplemented with 0, 5 or 25 μM DHA and cultured for 8 days, then 5 μM $A\beta_{42}$ was added on Day 9 for 0–24 h. On Day 9 immediately before $A\beta_{42}$ treatment (Time 0), survival of neurons supplemented with 5 μM DHA was 39% higher than that of the controls (0 μM DHA supplementation), whereas no change was seen with 25 μM DHA supplementation (Fig. 3). After 4 h of treatment with 5 μM $A\beta_{42}$, 64% of the cultured neurons with no DHA supplementation had died, whereas most neurons with 5 or 25 μM DHA supplementation were viable and showed no $A\beta_{42}$-induced damage. After 8 h of treatment with 0, 5 or 25 μM DHA, survival was reduced to 18%, 48% or 70%, respectively, of that of the Time 0 controls and was further reduced to 5.6%, 10% or 19% after 24 h of treatment.

With the MTT reduction assay, 8 h of treatment with $A\beta_{42}$ was found to induce neuron death (Fig. 4). In the DHA-treated cells, two-way (DHA and Aβ) ANOVA revealed significant main effects of both DHA supplementation ($P<.0001$) and $A\beta_{42}$ treatment ($P<.0001$) with a DHA×Aβ interaction ($P<.0001$). Supplementation with 5 or 25 μM DHA provided significant protection, with, respectively, 47% or 88% of the neurons remaining viable compared to that with 0 μM DHA with no AB_{42} treatment. These results demonstrate that higher DHA supplementation resulting in higher levels of neuronal DHA is required to provide protection of hippocampal neurons against

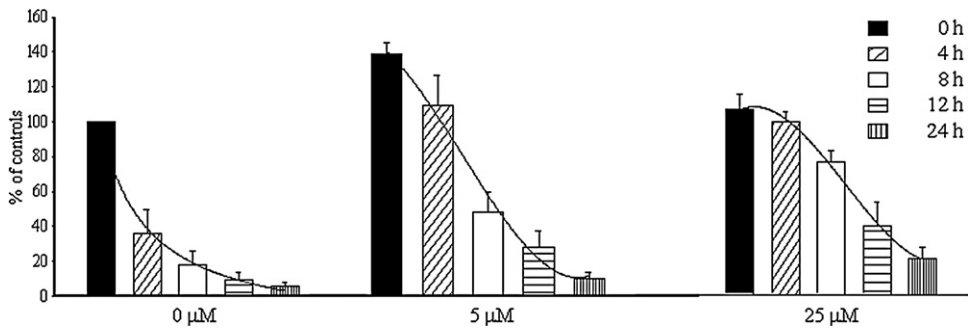

Fig. 3. Effect of DHA supplementation on the survival of primary hippocampal neurons incubated with aggregated $A\beta_{42}$. Cells were seeded in 12-well plates and grown for 8 days in neurobasal medium with B27 supplemented with 0, 5 or 25 μM DHA, then 5 μM $A\beta_{42}$ was added for 24 h. Photographs were taken in a premarked microscopic field immediately before (0 h) and at 4, 8, 12 or 24 h of $A\beta_{42}$ treatment. Surviving neurons were counted and the average of six individual fields (two fields per well and three wells) was used as one determination. The cells without DHA supplementation (0 μM) before $A\beta_{42}$ treatment were used as the controls (100%). The data are the mean±S.E.M. for four independent experiments for each treatment.

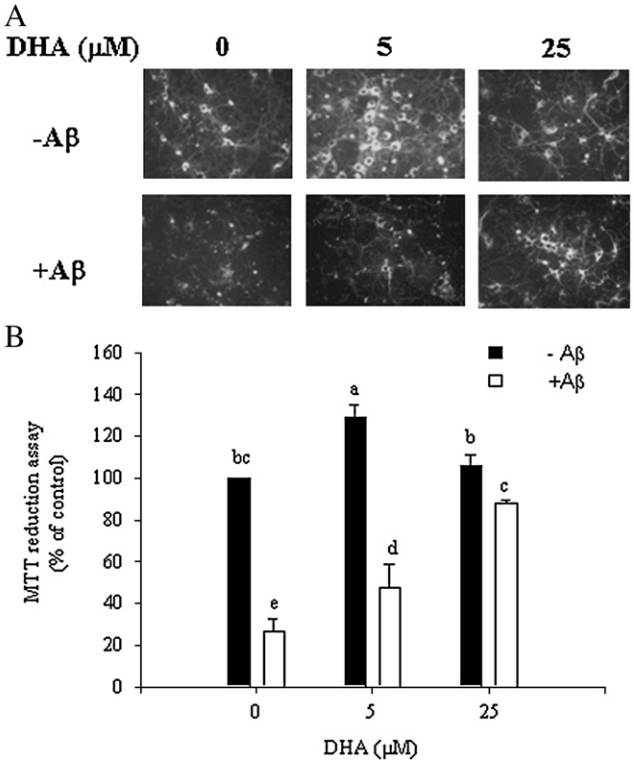

Fig. 4. DHA Attenuates the neurotoxicity induced by aggregated $A\beta_{42}$ in primary mature hippocampal neurons. (A) Neurons were seeded on poly-L-lysine-coated coverslips in 12-well plates and grown in neurobasal medium with B27 supplemented with 0, 5 or 25 μM DHA for 8 days, then 5 μM $A\beta_{42}$ was added for 8 h and the neurons immunolabeled with monoclonal anti-α-tubulin antibody. (B) Cell viability was measured by the MTT reduction assay in neurons in 48-well plates cultured for 8 days, followed by addition of 5 μM $A\beta_{42}$ for 8 h, then incubation with MTT for 4 h. The cells without DHA supplementation (0 μM) with no $A\beta_{42}$ treatment were used as the controls (100%). The data are the mean±S.E.M. for five independent experiments for each treatment. The different letters show significant differences between groups by two-way ANOVA followed by Duncan's multiple range test.

$A\beta_{42}$-induced neurotoxicity. It is interesting to note that, without DHA supplementation, most neurons were still viable after 8 h of incubation with 1 or 3 μM $A\beta_{42}$, but most died after treatment with 10 or 20 μM $A\beta_{42}$ (data not shown).

3.4. Effect of DHA on the expression of cytoskeletal proteins

Next, we examined the effect of DHA on cytoskeletal protein expression. Surprisingly, expression of acetylated tubulin (a marker of stable microtubules), tyrosine tubulin (a marker of dynamic microtubules) and β-actin in the primary hippocampal neurons was up-regulated after 9 days of supplementation with 5 or 25 μM DHA compared to no DHA supplementation (Fig. 5A). Levels compared to control levels (0 DHA) were increased by 46% (P=.0013) or 65% (P=.0003) for acetylated tubulin expression, 19% (P=.0178) or 25% (P=.0412) for tyrosine tubulin expression and 14% (P=.1731) or 20% (P=.0028) for β-actin expression (Fig. 5B).

4. Discussion

This study examined the ability of DHA to increase the survival of mature primary hippocampal neurons. Supplementation of DHA increased hippocampal DHA levels, up-regulated cytoskeletal protein expression and attenuated aggregated $A\beta_{42}$-induced neurotoxicity, which resulted in slowing of neuron death. We propose that optimal DHA supplementation and hippocampal DHA levels may be different in the normal physiological state (5–10 μM and 12–16% of total fatty acids, respectively) and for protection against $A\beta_{42}$-induced damage (25 μM and 28% of total fatty acids) and may be important in AD prevention.

There is a growing body of evidence that neuron protection is provided by DHA, but not other fatty acids. DHA at concentrations of 1.5–25 μM, either bound to bovine albumin or fetal bovine serum (FBS) or dissolved in alcohol, has been used in most previous studies on neuronal protection. At a concentration of 25 μM, FBS-bound DHA, but not 22:5n-6, 20:4n-6 or oleic acid (18:1n-9), protected against apoptosis induced by 2-day serum starvation in mouse neuroblastoma Neuro 2A cells [30,46] and FBS-bound DHA, but not 20:4n-6 or 18:1n-9, prevented oxidative stress-induced apoptosis in Neuro 2A cells [32]. At 6.7 μM, albumin-bound DHA, but not palmitic acid (16:0), 18:1n-9 or 20:4n-6, prevented oxidative stress-induced apoptosis and apoptosis during development in rat retina photoreceptors [47,48]. At 10 μM in ethanol, 18:3n-3, a DHA precursor, but not 16:0, protected against ischemia-induced hippocampal cell death and prevented kainic acid-induced seizures in rats [31], and, at 1.5 μM, albumin-bound DHA, but not 20:4n-6, 22:5n-6 or 18:1n-9, promoted neurite growth in rat primary hippocampal neurons [49]. Since all the above evidence indicates a specific effect of DHA in neuron protection, we focused on the effect of DHA in the $A\beta_{42}$-induced neurotoxicity of rat primary hippocampal neurons and found that 5 μM DHA bound to B27 protected neurons against aggregated $A\beta_{42}$-induced neuron death and that 25 μM DHA was even more effective. An effect of DHA on neuron protection has also been demonstrated by the findings that 0.5 μM DHA bound to albumin prevents neuronal apoptosis induced by soluble Aβ oligomers in rat cortical neurons [29]; 50 nM neuroprotectin D1, a DHA metabolite, attenuates $A\beta_{42}$-induced neurotoxicity in human neural cells [16]; and 15–150 μM DHA in ethanol protects rat hippocampal cultures against glutamate-induced cytotoxicity [50].

In general, 40–55% or 68–86% of primary hippocampal cells grown, respectively, in neurobasal medium with B27 or in Dulbecco's modified medium with 10% calf serum are dead after 2–3 days in culture, while 90% of the remaining cells develop neuronal

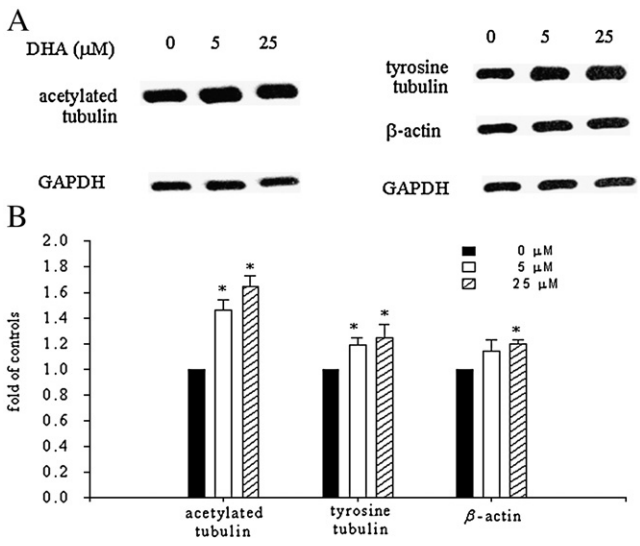

Fig. 5. Effect of DHA supplementation on cytoskeletal protein expression. Hippocampal cells were seeded in 10-cm dishes and grown for 9 days in neurobasal medium with B27 supplemented with 0, 5 or 25 μM DHA, then Western blot analysis was performed on cell lysates using the indicated antibodies. GAPDH was used as the loading control. The levels are expressed relative to those in the 0 μM DHA control. '*' indicates a significant difference compared to controls. The data are the mean±S.E.M. for four independent experiments for each treatment.

characteristics and survive for at least 4 weeks [38,51]. In our study, after 8 days of culture in neurobasal medium with B27 at a low density of 150 cells per square millimeter in the absence of DHA supplementation, around 58% of the hippocampal neurons had died and most remaining cells survived for at least up to 4 weeks. Significantly less death was seen with 5–10 μM DHA supplementation from plating. However, increased cell death was seen at 75 μM DHA and even more at 100 μM DHA, showing that an appropriate concentration of DHA is needed to improve hippocampal neuron survival. According to our analysis of neurobasal medium with B27, α-linolenic acid (18:3n-3) was the only n-3 fatty acid at a concentration as high as 4.5 μM in this culture medium, given hippocampal neuronal DHA levels of only 2% of total fatty acids. Other authors have obtained a similar result of DHA levels of 1.1–2.5% of total fatty acids in hippocampal neurons cultured in this neurobasal medium with B27 [39]. The unesterified DHA concentration in rat plasma and brain is reported to be 10.6 μM and 1.3 nmol/g brain, respectively [52], and hippocampal DHA levels in normal rats or primates are 12–14% of total fatty acids [21,53,54]. We found that supplementation with 5–10 μM DHA resulted in hippocampal neuron DHA levels of 12–16% of total fatty acids, which is in the physiological range, and that this was optimal for the survival of hippocampal neurons.

In AD patients, hippocampal DHA levels are reduced to 8% of total fatty acids in the phosphatidylethanolamine fraction compared to 17% in healthy age-matched controls [18]. We suspected that hippocampal neurons with lower DHA levels might be more susceptible to Aβ-induced neurotoxicity. In primary rat hippocampal neurons, most DHA is incorporated into phospholipids, primarily the phosphatidylethanolamine fraction [39]. We found that supplementation with 25 μM DHA resulted in hippocampal neuron DHA levels of 28% of total fatty acids and protected the cells against $A\beta_{42}$-induced neurotoxicity. We suggest that higher hippocampal neuronal DHA levels and higher DHA supplementation are more effective in protecting hippocampal neurons from Aβ-induced damage. Furthermore, we suggest that appropriate DHA requirements or neuronal DHA levels may differ in the normal physiological and pathological states, with 5–10 μM DHA (resulting in DHA levels of 12–16% of total fatty acids) being optimal for maintaining neuron viability under normal conditions and 25 μM DHA (resulting in DHA levels of 28% of total fatty acids) having more potential to protect against Aβ-induced damage. However, the increased hippocampal DHA levels were compensated by reduced 20:4n-6 and 18:1n-9 levels, and it is important to examine whether these reduced levels played a role in these findings. In addition, it cannot be excluded that the neuron protection may result from the increased DHA/20:4n-6 or n-3/n-6 fatty acid ratio.

DHA plays an important role in neuronal protection, but the mechanism is unclear. It is interesting that we found that expression of tyrosine tubulin and acetylated tubulin was significantly up-regulated in DHA-treated hippocampal neurons, suggesting that the increase in cytoskeleton proteins caused by DHA may make the cells more resistant to the destructive effect of aggregated $A\beta_{42}$. This idea is strengthened by the findings that soluble $A\beta_{40}$ oligomer-induced cytoskeleton perturbation in cortical neurons is prevented by DHA [29] and that DHA prevents loss of drebrin, a dendritic spine actin-regulating protein, in the AD mouse [22]. It has also been proposed that DHA (i) increases the activity of the Akt signaling pathway by increasing phosphatidylserine levels, resulting in protection against serum starvation-induced apoptosis [46]; (ii) increases growth-associated protein-43 levels for neurite outgrowth in cortical neurons [55]; (iii) promotes neurite growth in hippocampal neurons [49]; and (iv) increases nerve growth factor levels in the hippocampus [56], all supporting the idea of neuronal protection by DHA.

This study demonstrated that DHA can be incorporated into hippocampal neurons and can increase cytoskeletal protein expression, maintain neuronal viability and attenuate $A\beta_{42}$-induced neurotoxicity. We propose that appropriate hippocampal DHA levels are critical for protection against Aβ-induced neurotoxicity and in maintaining hippocampus viability and neuronal function to prevent AD.

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