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Journal of Nutritional Biochemistry 16 (2005) 538–546
Journal of Nutritional Biochemistry

Effects of docosahexaenoic acid on the survival and neurite outgrowth of rat cortical neurons in primary cultures

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Received 31 December 2004

Abstract

Effects of docosahexaenoic acid (DHA) on survival and neurite outgrowth were investigated in primary cultures of rat cortical neurons. Cell cultures were prepared from cortex on embryonic day 18 (E-18) for treatment with a series of DHA concentrations (12.5, 25, 50, 75, 100 and 200 μM). Docosahexaenoic acid (25–50 μM) significantly enhanced neuronal viability, but lower concentration of DHA (12.5 μM) did not show an obvious effect. In contrast, higher concentrations of DHA (100–200 μM) exerted the significant opposite effects by decreasing neuronal viability. Furthermore, treatment with 25 μM DHA significantly prevented the neurons from death after different culture days in vitro (DIV). Moreover, measurements from the cultures exposed to 25 μM DHA immediately after plating showed significant increases in the percentage of cells with neurites, the mean number of neurite branches, the total neuritic length per cell and the length of the longest neurite in each cell after 24 and 48 h in vitro (HIV). The DHA-treated neurons had greater growth-associated protein-43 (GAP-43) immunoactivity and higher phosphatidylserine (PS) and phosphatidylethanolamine (PE) contents, but lower phosphatidylcholine (PC) content than control neurons. The significant increased DHA contents were also observed in both PE and PS in the treated neurons. These findings suggest that optimal DHA (25 μM) may have positive effects on the survival and the neurite outgrowth of the cultured fetal rat cortical neurons, and the effects probably are related to DHA-stimulating neuron-specific protein synthesis and its enhancing the discrete phospholipid (PL) content through enrichment of DHA in the PL species.

Keywords: Docosahexaenoic acid (DHA); Cortical neurons; Survival; Neurite outgrowth

1. Introduction

Docosahexaenoic acid (DHA, 22:6n-3) is one of the major polyunsaturated fatty acids (PUFAs) found in the mammalian brain and the most abundant n-3 PUFA in the neural membrane [1,2]. Docosahexaenoic acid delivery to the central nervous system (CNS) is most efficient at times prior to and coinciding with synaptogenesis when plasma DHA is primarily esterified in phospholipids (PLs) [3]. Current information from studies in animal and human models indicates that DHA is essential for optimal development and function of the brain [4,5]. When n-3 fatty acid-deficient diets are fed to animals for two to three generations, a marked decrease in nervous system DHA results. Loss of brain DHA results in the loss of many behavioral and cognitive functions in animals [6–8]. It has been demonstrated that the intelligence quotients of children fed with breast milk (rich in DHA) exceed those of children fed with formula milk [9]. When human infants had DHA-deficient diets, impaired cortical development and neuro-developmental quotient have been noted [10–12].

Most of these studies have required long-term treatment of whole animals or humans in order to study the effects of DHA on the structural and functional changes in neurons of the CNS since mature neurons will not survive for long periods in vitro. Investigation of the DHA on developing neurons can, however, be conducted in cell culture. Cell culture makes individual living neuron accessible and permits direct observation of growing neurons and their neurite formation. The cell culture model can also be used to investigate direct effects of DHA on special target neurons apart from endogenous endocrine or metabolic influences.

Although it has been reported that DHA has positive effects on the neurite outgrowth of rat clonal pheochromocytoma PC12 cells [13] and hippocampal cells in vitro [14],

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doi:10.1016/j.jnutbio.2005.02.002

little is known about the effect of DHA on neurite outgrowth in developing cortical neurons. The cerebral cortex mediates complex integrative functions such as abstract reasoning, planning, language and sensory perception, and is generally held to be the site of long-term memory storage. Information derived from the external environment, from homeostatic systems and from internal conscious and unconscious events all converge onto the cerebral cortex for processing and storage. Results from several laboratories, utilizing human experimental paradigms, have shown that each region of the cerebral cortex is capable of performing perceptual, motor and mnemonic operations [15].

Because cerebral cortex plays the crucial role in the memory function and the cerebral neurite elongation involves an expansion of the cell surface membrane, and newly synthesized membrane materials such as PLs and proteins must be supplied to the growing neurite [16], in the present study, therefore, the cerebral cortical cells from embryonic day 18 (E-18) rat embryos were selected to examine the neuronal cell viability, the neurite outgrowth and the changes in the growth-associated protein-43 (GAP-43) immunoactivity, the discrete PL contents and the fatty acid composition of the PL species after different periods of DHA exposure in vitro, which makes us to investigate the effects of DHA on the cortical neuron development and differentiation.

2. Materials and methods

2.1. Primary cultures of cortical neurons and treatment

Cortical neurons were prepared from fetal Sprague–Dawley rat pups at E-18, as previously described with slight modifications [17]. Briefly, the whole brain was removed aseptically and a small piece of cortical tissue was dissected out. After removing the blood vessels and meninges, the cortical tissues were incubated for approximately 60 min in minimum essential medium (MEM, GIBCO) containing 0.25% trypsin–0.1% DNAse. The cell pellet obtained by centrifugation was resuspended in MEM containing 5% fetal bovine serum and plated at a density of 5×10^5 cells/ml on the poly-L-lysine-coated 6-well (2 ml/well) plates for the cell viability, morphometric analysis, cellular PL content and fatty acid composition assays or 1×10^6 cells/ml on the 96-well (100 μl/well) plates for the GAP-43 protein determination. One hour after plating, unattached cells and debris were removed by replacing the initial medium with fresh neurobasal medium containing B27 supplements (GIBCO), glutamine (0.5 mM), cytosine arabinoside (5 μM) and glutamate (25 μM). Glial and fibroblast proliferation was prevented by the presence of cytosine arabinoside (5 μM) in the medium. Cortical cells were maintained at 37°C in a humidified 5% CO_2–95% air incubator.

Cells were randomly divided into control and DHA-treated (DHA) groups. Docosahexaenoic acid (12.5, 25, 50, 75, 100 and 200 μM) from Sigma (St. Louis, MO) was added to the neurobasal medium after plating. The DHA supplement was preconjugated with fraction V bovine serum albumin (BSA, Sigma) at a 4:1 molar ratio (DHA/BSA). The medium was changed every 3 days.

2.2. Cell viability

Cell viability was assessed by means of trypan blue (TB) exclusion technique after 24 h or 1, 3, 7, 14 and 21 days of DHA exposure. The cells were incubated with 1.5% TB for 10 min at room temperature, and the cultures were then washed. For qualitative observation of viable and nonviable cells, cultures were fixed so that attached cells could be viewed under light microscope. The nonstained cells with intact soma and neurites were regarded as viable. Only dark-stain cells possessing fragmented neurites were considered nonviable. The viability of the cultures was calculated as the percentage of the ratio of the number of unstained cells (viable cells) to the total number of cells counted (viable cells plus nonviable cells). In each experiment, 210 cells on three wells were counted at random.

2.3. Immunohistochemistry

Cultures were rinsed with 0.01 M phosphate-buffered saline (PBS, pH 7.4) and fixed with 4% paraformaldehyde+0.5% glutaraldehyde in 0.01 M PBS for 30 min at room temperature. Cultures were washed with PBS containing 0.25% Tween-20 (TBS) and incubated with 3% H_2O_2 solution for 5 min in order to inhibit endogenous peroxidase. After three washes with TBS, cultures were incubated with a blocking solution of 10% normal goat serum for 15 min, followed by incubation overnight at 4°C with primary antibodies to microtubule-associated protein 2 (MAP2, Sigma) and neuron-specific enolase (NSE; DACO, Santa Barbara, CA, USA). After washing with TBS, cultures were processed with an ExtrAvidin Biotin staining kit (Sigma) and visualized with 0.05% 3,3-diaminobenzidine (DAB, Sigma) and 0.01% H_2O_2 following the manufacturer's instructions.

2.4. Cell counting and morphometric measurements

The immunostained neurons from control and DHA groups after 24 and 48 h in vitro (HIV) were examined for neurite formation. A neurite was identified as a process greater than one-cell body diameter in length and possessing a terminal growth cone. The percentage of cells with neurites was calculated by counting 100 cells per well in triplicate wells.

At each of two time points (24 and 48 HIV), 100 labeled neurons selected randomly from 20 fields on each of the two wells were photographed with an Olympus photomicroscope (PM-10AD) at 400× for morphometric analyses. In each experiment, therefore, a total of 200 neurons from control cultures and 200 neurons from DHA cultures were analyzed. Neurons were photographed and measured at 24 and 48 HIV in three experiments. The number of neurite, the total neuritic length and the length of the longest neurite

were measured on film negatives using the Macintosh-based image analysis system, NIH Image. In each cell, all primary neurites and their higher order branches were measured from their points of origin to their free tips to determine the total neuritic length and the length of the longest neurite. Neurites less than one-cell diameter in length were excluded from these measurements.

2.5. Detection of GAP-43 immunoactivity

Neurons grown in 96-well plates after 24 and 48 HIV were fixed in 4% polyformaldehyde (100 μl per well) at room temperature for 1 h. After the supernatant was decanted, cells were incubated with methanol at −20°C for 20 min, washed three times in PBS and then incubated with PBS containing 10% fetal bovine serum for 1 h, followed by anti-GAP-43 immunoglobulins (rabbit anti-GAP-43 IgG, 1:1000) at 4°C for 18–24 h. After washing three times with PBS, cells were incubated for 1 h with horseradish peroxidase (HRP)-labeled goat anti-rabbit antibodies (GAR-IgG, 1:750) at 37°C, washed three times in PBS and then incubated for 20 h with 100 μl/well *o*-phenylenediamine at 37°C. The reaction was stopped by H_2SO_4 (2 N). Optical densities were measured at 490 nm using a model 550-μl plate reader (Bio-Rad, USA) [18].

2.6. Lipid extraction and PL separation

Neurons grown in the six-well plates after 24 and 48 HIV were washed twice with 3 ml of PBS containing 0.1% fatty acid-free BSA and harvested with a rubber policeman with 0.5 ml of cold PBS containing 1 mM EDTA at 4°C. The lipids of neurons in each well were extracted according to the method of Folch et al. [19], dissolved in 1 ml $CHCl_3$ and applied to a silicic acid column (UNISIL, 100–200 mesh, Clarkson Chemical, Williamsport, PA, USA) to separate less polar neutral lipids from polar PLs [20]. To isolate the major class of PL, an aliquot (20 μl) of the PL fraction was subjected to one-dimensional thin-layer chromatography (TLC) on a preabsorbent silica gel G plate (Analtech, Newark, DE, USA) for about 150 min, using a mobile phase containing chloroform–triethylamine–ethanol–water (30:30:34:8, by volume) [21]. Phospholipid standards were used to identify the corresponding spots for phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI) and phosphatidylethanolamine (PE) under UV light after spraying with 0.02% 2′ ,7′ -dichlorofluorescein (Sigma) in 95% aqueous ethanol. The relative mobility (Rf) values were 0.08, 0.52, 0.29 and 0.40, respectively. There was no overlapping among the different PL spots. The spots of PC, PS, PI and PE were scrapped from the plates for phosphorus content determination [22] or for fatty acid analysis.

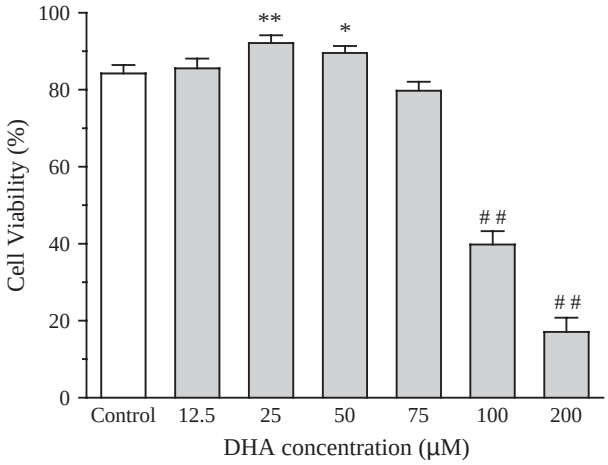

Fig. 1. Effects of different concentrations of DHA on the cell viability after 24 HIV. Cell viability was assessed by means of TB exclusion method. The graphs represent the means±S.E.M. for four independent experiments of DHA groups or for five independent experiments of control groups. *P<.05 versus control, **P<.01 versus control; $^{\#\#}P$<.01 versus control. Statistical analysis was performed by a one-way ANOVA using Student–Newman–Keuls' multiple comparison test.

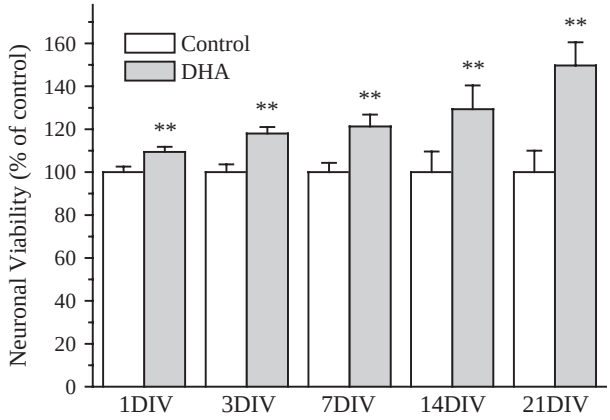

Fig. 2. Effect of DHA (25 μM) against natural neuronal death after different culture days in vitro (DIV). Cell viability was assessed by means of TB exclusion method. The graphs represent the means±S.E.M. for three independent experiments of DHA groups or of control groups. **P<.01 versus control. Statistical analysis was performed by unpaired Student's t test.

2.7. Fatty acid analysis

Individual recovered PLs were transmethylated using BF_3-methanol (14% w/v) at 100°C for 2 h under N_2 [23]. A known amount of heneicosanoic acid (21:0) was added as an internal standard. The resulting fatty acid methyl esters were analyzed by a gas–liquid chromatograph (HP 5890; Hewlett-Packard, Avondale, PA, USA) equipped with a flame-ionization detector and a silica capillary column (30 cm×0.32 mm i.d., SP-2330, Supelco, Bellefonte, PA, USA). The oven temperature was programmed to raise from 170 to 240°C, and detector temperature was set at 270°C. Identification of the fatty acids was made by comparison of retention times with those of known standards run under the same conditions. Peak areas were calculated with a

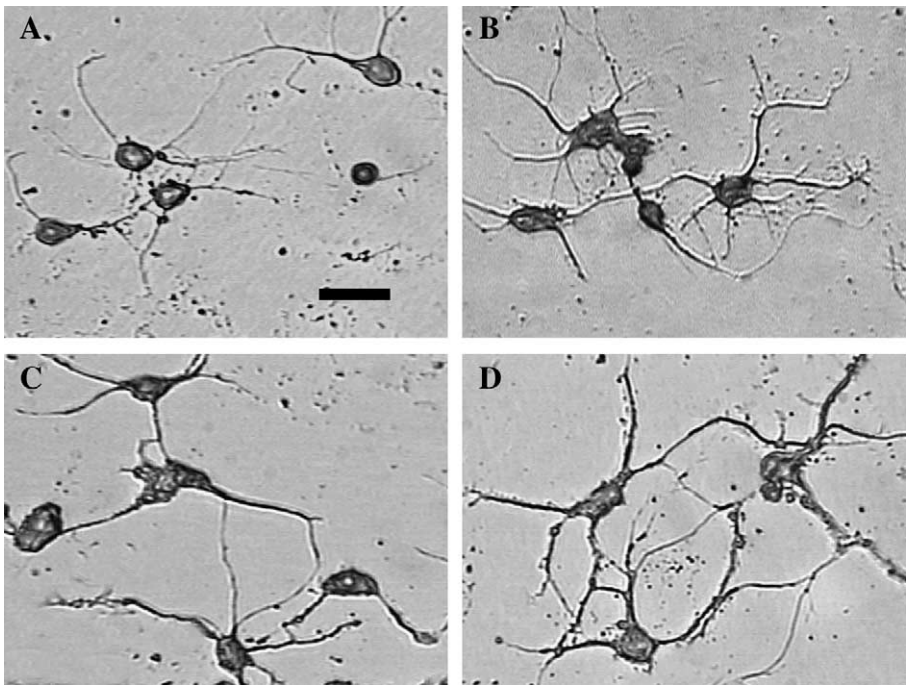

Fig. 3. Bright-field micrographs of cultured cortical neurons stained with NSE and MAP2. Cortical neurons were cultured for 24 h (A, B) and 48 h (C, D) in the absence (A, C) or presence (B, D) of 25 μM of DHA. Note the DHA-associated increases in the neurite length and neurite branches. Bar=25 μm.

Hewlett-Packard HP 3396 series integrator, and the fatty acid concentrations were reported as percent of total fatty acid contents.

2.8. Statistical analyses

Statistical significant was performed by an unpaired Student's *t* test or by a one-way analysis of variance (ANOVA) with Student–Newman–Keuls' multiple comparison test to compare control and treated groups. For each comparison, a *P* value of less than .05 was considered significant. Data were expressed as means±S.E.M. of at least three independent experiments.

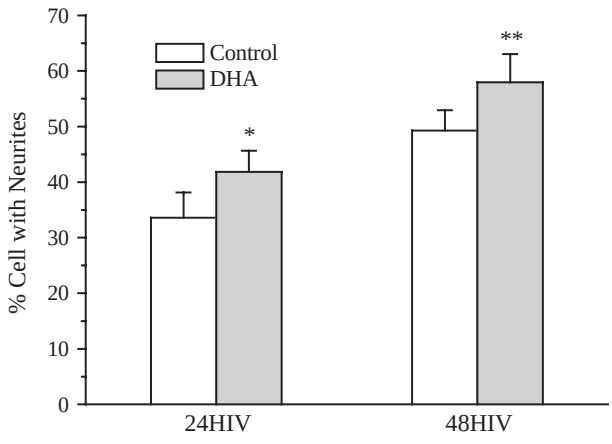

Fig. 4. Effects of DHA (25 μM) on the percentage of cells with neurites after 24 and 48 HIV. The graphs represent the means±S.E.M. for four independent experiments of DHA groups or for five independent experiments of control groups. *$P<.05$ versus control, **$P<.01$ versus control. Statistical analysis was performed by unpaired Student's *t* test.

3. Results

3.1. Effect of DHA on cell viability

Effects of different DHA concentrations on cell viability were assessed after 24 h of exposure. As shown in Fig. 1, no

Table 1
Effects of DHA (25 μM) on the number of neurite branches and neurite length per neuron after 24 and 48 HIV

	24 HIV	48 HIV
Number of neurite branches		
Control	6.7±0.2	7.8±0.4
DHA	8.0±0.7*	9.5±1.1*
Δ%	19.4	21.8
Longest neurites (μm)		
Control	69.2±5.6	104.8±12.4
DHA	87.7±15.8**	134.5±22.1**
Δ%	26.7	27.2
Total neuritic length (μm)		
Control	216.4±27.2	291.5±18.5
DHA	280.6±23.4*	418.0±56.2*
Δ%	29.7	43.4

Values are means±S.E.M. for three independent experiments of DHA groups or of control groups. Statistical analysis was performed by unpaired Student's *t* test. Δ% represents the difference between the values for DHA-treated and control neurons.

* $P<.01$ versus control.

** $P<.05$ versus control.

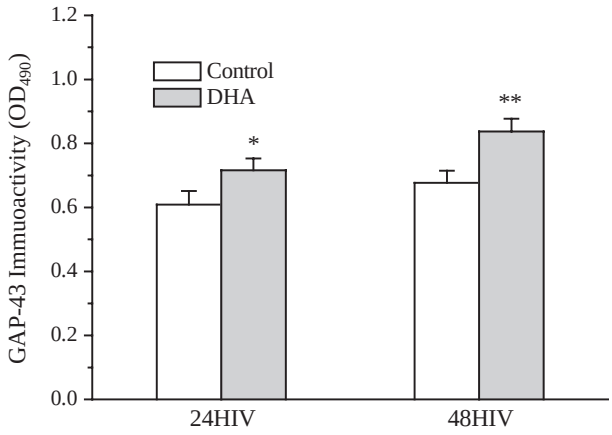

Fig. 5. Effects of DHA (25 μM) on changes in the neuronal GAP-43 immunoactivity determined by ELISA after 24 and 48 HIV. The values are expressed as optical densities measured at 490 nm. The graphs represent the means±S.E.M. for four independent experiments of DHA groups or for five independent experiments of control groups. *$P<.05$ versus control, **$P<.01$ versus control. Statistical analysis was performed by unpaired Student's t test.

significant differences ($P>.05$) were observed in the percentages of viable cells in cultures exposed to control and 12.5 μM DHA. However, cultures treated with 25 and 50 μM DHA had significant increases ($P<.01$ and $P<.05$, respectively) in the percentages of viable cells relative to control cultures. In contrast, the significant decreases ($P<.01$) in the percentages of viable cells were detected at higher concentration (100–200 μM), although 75 μM DHA caused a slight decrease ($P>.05$), as compared with that of control. The effects of DHA against the naturally occurring neuronal death for different days of cultures in vitro were assessed quantitatively for 25 μM DHA. Quantitative studies showed that DHA significantly prevented ($P<.01$) the neurons from death in this system (Fig. 2).

3.2. Effect of DHA on neurite outgrowth

Treatment with 25 μM DHA was associated with the enhanced neurite outgrowth between the 24 and 48 HIV (Fig. 3). A significant increase in the numbers of cells with neurites was found after 24 ($P<.05$) or 48 HIV ($P<.01$) (Fig. 4). Morphometric analyses indicated that DHA treatment was associated with significant increases in the total neuritic length ($P<.01$) and length of the longest neurite ($P<.05$) and in the number of neurite branches ($P<.01$) in treated neurons (Table 1).

During the first 48 HIV, the DHA-treated neurons also showed accelerated neurite elongation compared with the

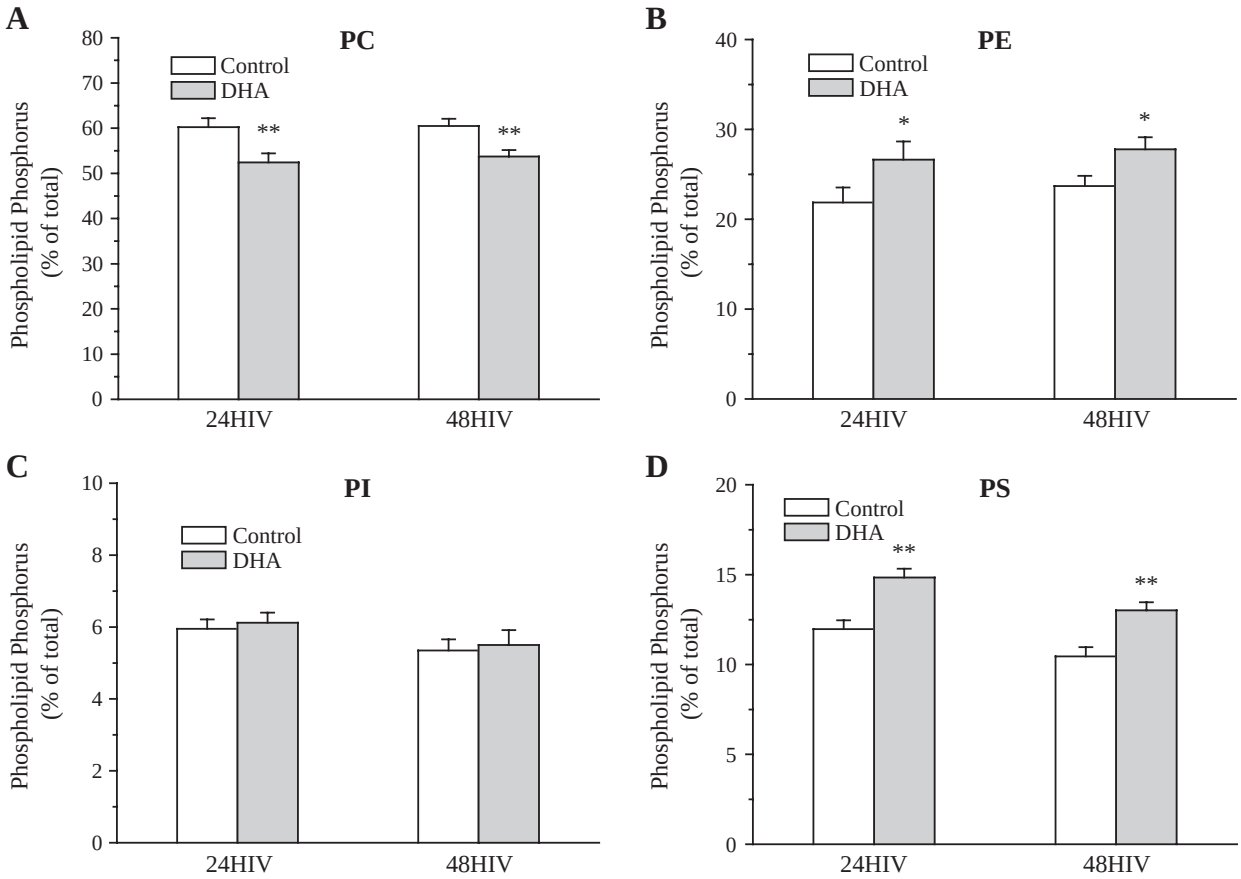

Fig. 6. Effects of DHA (25 μM) on changes in the relative amount of major neuronal PL species after 24 and 48 HIV. The values are expressed as phosphorus weight percentage. The graphs represent the means±S.E.M. for four independent experiments of DHA groups or for five independent experiments of control groups. *$P<.05$ versus control, **$P<.01$ versus control. Statistical analysis was performed by unpaired Student's t test.

controls. The change of increase in total neuritic length in the DHA group was 49.0% between 24 and 48 HIV compared to 34.7% in the controls. The average length of each neurite, however, was not significantly different in the DHA and control groups (data not shown). This apparent discrepancy appeared to result from the fact that neurons in the DHA groups had more neurite branches than those in the control groups, indicating that the DHA-induced increase in total neuritic length resulted predominantly from an increase in the number of neurite branches. Nonetheless, analysis of the length of the longest neurite per cell showed that DHA also promoted elongation of the major neurites.

3.3. Effect of DHA on GAP-43 immunoactivity

Since GAP-43 proteins are distributed mainly in the growth cones, the immunoactivity of GAP-43 can be used as a marker of axonal growth [24]. Fig. 5 depicts the effect of DHA on the GAP-43 immunoactivity. It showed that the DHA-treated neurons had higher immunoactivities of GAP-43 than control after 24 ($P<.05$) and 48 h ($P<.01$) of treatment.

3.4. Effect of DHA on PL content and fatty acid composition

The effects of DHA on the major cellular PLs after 24 and 48 h of treatment are shown in Fig. 6. There were no significant differences in the total PL contents observed between the DHA and control groups after 24 HIV (PL phosphorus, 92.4±6.2 and 92.1±4.5 ng/well, respectively) and 48 HIV (PL phosphorus, 107.5±7.5 and 106.9±8.4 ng/well, respectively). However, within the cellular PLs, the significant increases in PS ($P<.01$) and PE ($P<.05$) contents and the significant decrease in PC ($P<.01$) content were found in the DHA-treated neurons when compared with controls, whereas the increase in PI level was not significant ($P>.05$) (Fig. 6).

A detailed study of the fatty acid composition of the individual cellular PLs showed that significant increases of DHA contents compared with controls were observed in PS ($P<.01$) and PE ($P<.01$) in the DHA-treated neurons, whereas the increase in PC ($P>.05$) and the decrease in PI ($P>.05$) were not significant after 24 and 48 HIV (Table 2).

Table 2
Effects of DHA (25 μM) on the DHA contents in the major neuronal phospholipid species after 24 and 48 HIV

PL species	DHA content (wt.% of total fatty acids)†	
	24 HIV	48 HIV
PI		
Control	1.83±0.17	1.91±0.14
DHA	1.78±0.15	1.90±0.21
Δ%	−2.73	−0.52
PS		
Control	16.72±0.56	17.04±0.84
DHA	19.55±0.75**	20.02±0.91**
Δ%	16.93	17.49
PC		
Control	1.53±0.13	1.58±0.25
DHA	1.64±0.27	1.69±0.18
Δ%	6.71	6.96
PE		
Control	15.46±0.72	15.13±0.85
DHA	17.52±0.95**	17.70±0.92**
Δ%	13.32	16.99

Values are means±S.E.M. for four independent experiments of DHA groups or for five independent experiments of control groups. Statistical analysis was performed by unpaired Student's t test. Δ% represents the difference between the values for DHA-treated and Control neurons.
$P<.05$ versus control.

† DHA content is expressed as weight percentage of DHA from total fatty acids of individual phospholipid species.

** $P<.01$ versus control.

4. Discussion

The present study indicates that in cultures of cortical neurons, relative low concentration of DHA (12.5 μM) had no effect on neuronal survival after 24 h of treatment. In contrast, the high concentrations of DHA (100–200 μM) were found neurotoxic to cultured fetal cortical cells and significantly decreased the cell viability. However, 25–50 μM DHA promotes significant increases in the cell viability. These results suggest that the effect of DHA on the survival of cortical neurons in cultures is at least associated with its treatment concentration. The pattern of the concentration response indicates that the effect of DHA involves a conditional and highly regulated function such that a narrow window of concentrations constitutes the effective range. Because DHA possesses a high degree of unsaturation, it can increase lipid peroxidation, providing a variety of lipid peroxides and aldehydic breakdown products with pro-oxidant property [25]. Numerous in vivo and in vitro studies on the incidence of DHA supplementation at high doses have reported higher peroxidation and oxidative stress [26,27]. A concomitant supplementation with the antioxidant vitamin E has been proposed to minimize this deleterious side effect [28]. Although highly unsaturated fatty acids are believed to be easy targets of lipid peroxidation, their contribution to lipid peroxidation should be based on a complicated and not yet fully clarified oxidant/antioxidant balance. Recently, Delton-Vandenbroucke et al. [29] compared the effect of a low (10 μM) versus high (100 μM) concentration of DHA on oxidant/antioxidant balance in bovine retinal and bovine aortic endothelial cells and showed that 100 μM DHA elicited a marked oxidative stress in both cells, but 10 μM DHA induced a mild oxidative stress. It is well noted that Green et al. [30] reported treatment with ethyl docosahexaenoate (the ethyl form of DHA with the same action as DHA) enhanced free radical scavenging and decreased lipid peroxidation in the rat fetal brain. In our study, the neurotoxic effect of high concentrations of DHA (100–200 μM) may be related to the DHA-elicited oxidative stress, but the outcome of DHA

concentrations (25–50 μM) may well be protective against free radicals.

One of the critical steps in neuronal differentiation is the outgrowth of neuronal processes, axons and dendrites, because these processes establish a neuron's structural and functional polarity [31,32]. Our further study shows that 25 μM DHA promoted significant increases in total neuritic length, in length of the longest neuritis and in the number of neurite branches after 24 and 48 HIV. The study corroborated recent findings that DHA supplementation increased the individual neurite length as well as the number of branches in hippocampal neuronal culture [14]. The fact that DHA promoted increases in length of the longest neurite of each neuron and in the number of neurite branches suggests that outgrowth of both axons and dendrites was affected. An increase in the number of neurites would appear to reflect an effect of DHA treatment on growth of dendrites since they are the more numerous type of neurite. Correspondingly, an increase in the length of the longest neurite probably reflected a change in the axonal growth since the longest neurite with a prominent growth is usually the growing axon. These results suggest that DHA may affect the growth of dendrites and axons in different ways.

Neurite growth requires newly synthesized membrane components, such as PLs and proteins [16]. GAP-43, also known as B-50, is a neuron-specific protein associated with axon growth and growth cone formation that also modulates neurite outgrowth [18,24]. In neuronal tissue culture, GAP-43 has been shown to be concentrated in the growth cones, the axons and the somal plasma membrane, and to be expressed concomitantly with neurite outgrowth [18,33]. In the present study, DHA significantly increased the cellular GAP-43 immunoactivity or GAP-43 content. This is similar to the study of Takahata [34] who recently reported that DHA elevated GAP-43 mRNA in PC 12 cells. The morphological changes observed in our study might be due to the increase of GAP-43 content or neurons by DHA treatment.

Neuronal membranes are largely made of PLs. Growth of axons and dendrites involves the synthesis of PLs [16]. It has been reported that DHA stimulates PL synthesis, particularly PS and PE that are the principal repositories for DHA and critical for optimal neural function and structure [35], in PC12 cells [16]. As a consequence of DHA treatment in this study, the significant changes were encountered in individual PL species in the cultured cortical neurons. Docosahexaenoic acid significantly increased the both PS and PE contents and decreased the PC content, whereas it did not cause a progressive increase in the PI content. The increase in the PS content and the decrease in the PC content are most likely as a result of base exchange activity. Because serine base exchange is the only accepted pathway for PS synthesis in mammals [36,37], it would appear that DHA treatment enhanced the activity of enzyme in this pathway. This can be attributed to the prior alteration of DHA contents in PC. Serine and choline base exchanges, for example, proceed better in the presence of PL possessing unsaturated rather than saturated acyl chains [38]. The changes in DHA concentration in PC in the neurons in our study are in accord with this possibility. The results from Hargreaves and Clandinin [39] showed that PC biosynthesis via the PE methyltransferase pathway was inhibited by n-3 fatty acid, which would also give an explanation for the effects of DHA on the PC decrease found in the present study. Phosphatidylethanolamine can be synthesized from free serine by base exchange, which is stimulated by PS decarboxylase (PSD) through removal of the C1 carboxyl group. The increased PS mediates PSD activity and promotes the serine and ethanolamine base exchanges [40], which seems to be responsible for the increase of PE content observed in this study. The PI fraction was not enriched with DHA in our study, which indicated that DHA treatment might not have any significant effect on the PI biosynthesis.

Although there is not a proportional correlation between PE or PS synthesis and neurite outgrowth, the content of ethanolamine plasmalogen (mainly of PE) was significantly elevated following differentiation of NIE-115 neuroblastoma cells [41], suggesting that PE may play an important role in neurite outgrowth. The increased PS content caused by DHA in neuronal membranes has been shown to promote the activation of Raf-1 and the PI-3 kinase pathways [42,43], which are involved in inducing neurite growth in PC12 and H19-7 hippocampal cell line [44–47]. Additionally, certain unsaturated PS species are located entirely on the interior leaflet of plasma membrane and are essential for the activation of protein kinase C (PKC) [48]. The PKC can stimulate GAP-43 phosphorylation that plays an important role in transducting extracellular signals into directed neurite outgrowth [33,49].

What may be the cellular mechanism of effect of DHA in prevention of the naturally occurring neuronal death in the present study? One of the possibilities is the interference with apoptosis. It has been found that DHA markedly decreases apoptosis of neuron-2A cells in response to serum deprivation [42]. The authors claimed that DHA acted through aminophospholipids, first, through PS to prevent DNA fragmentation. A number of other options may also be considered: (a) DHA promotes, while arachidonic acid (AA) suppresses, neurite outgrowth triggered by nerve growth factor (NGF) [13]. Since NGF supports the development of cortical neurons and DHA seems to be necessary for maintenance of the optimal synthesis of NGF, for example, in the developing hippocampus [50], it is plausible to assume that DHA might act through NGF on the developing cortical neurons. (b) Enhanced free radical scavenging and decreased lipid peroxidation in the rat fetal brains have been found after treatment with ethyl docosahexaenoate [30]. It seems to be that the plasmalogens possess an exceptional potency in scavenging oxygen free radicals [51,52]. It might be more than a simple coincidence that DHA (25 μM) increased the contents of PS and PE, the major amino-

phospholipids in the plasmalogens, in the present study. Further studies are advised to focus on this question.

In summary, DHA-exposed cultures show a concentration-dependent increase in neuronal viability in the present study. Further morphological and biochemical findings suggest that optimal DHA (25 μM) can enhance the percentage of neurite-bearing cells and neurite outgrowth of the cultured fetal rat cortical neurons. The effects of DHA may be due to its promoting GAP-43 protein and PS biosynthesis and its modifying the DHA contents in the PL molecular species. However, the precise mechanisms involved and the functional significance of these effects are currently not well known and needed to elucidate.

Acknowledgment

This study was supported by grants from the National Science Foundation of Jiangsu Province, China (No.95021301).

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