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Protective effect of docosahexaenoic acid against brain injury in ischemic rats☆

Hung-Chuan Pan[a,b], Tsung-Kuei Kao[c], Yen-Chuan Ou[d], Dar-Yu Yang[e], Yu-Ju Yen[f], Chun-Chiang Wang[f], Yu-Han Chuang[f], Su-Lan Liao[f], Shue-Ling Raung[f], Ching-Wen Wu[f], An-Na Chiang[g], Chun-Jung Chen[f,h,i,*]

[a]*Chung Hwa University of Medical Technology, Tainan 717, Taiwan*
[b]*Department of Neurosurgery, Taichung Veterans General Hospital, Taichung 407, Taiwan*
[c]*Department of Nursing, Tajen University, Pingtung 907, Taiwan*
[d]*Division of Urology, Taichung Veterans General Hospital, Taichung 407, Taiwan*
[e]*Chang Bing Show Chwan Memorial Hospital, Changhua 542, Taiwan*
[f]*Department of Education and Research, Taichung Veterans General Hospital, Taichung 407, Taiwan*
[g]*Institute of Biochemistry and Molecular Biology, National Yang-Ming University, Taipei 112, Taiwan*
[h]*Center for General Education, Tunghai University, Taichung 407, Taiwan*
[i]*Institute of Medical Technology, National Chung-Hsing University, Taichung 402, Taiwan*

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Abstract

Evidence suggests that inactivation of cell-damaging mechanisms and/or activation of cell-survival mechanisms may provide effective preventive or therapeutic interventions to reduce cerebral ischemia/reperfusion (I/R) injuries. Docosahexaenoic acid (DHA) is an essential polyunsaturated fatty acid in the central nervous system that has been shown to possess neuroprotective effects. We examined whether different preadministrative protocols of DHA have effects on brain injury after focal cerebral I/R and investigated the potential neuroactive mechanisms involved. Sprague–Dawley rats were intraperitoneally pretreated with DHA once 1 h or 3 days being subjected to focal cerebral I/R or daily for 6 weeks before being subjected to focal cerebral I/R. Reduction of brain infarction was found in all three DHA-pretreated groups. The beneficial effect of DHA on the treatment groups was accompanied by decreases in blood–brain barrier disruption, brain edema, malondialdehyde (MDA) production, inflammatory cell infiltration, interleukin-6 (IL-6) expression and caspase-3 activity. Elevation of antioxidative capacity, as evidenced by decreased MDA level and increased superoxide dismutase activity and glutathione level, was detected only in the chronic daily-administration group. The two single-administration groups showed increased phosphorylation of extracellular-signal-regulated kinase (ERK). Elevation of Bcl-2 expression was detected in the chronic daily-administration and 3-day-administration groups. In vitro study demonstrated that DHA attenuated IL-6 production from stimulated glial cells involving nuclear factor κB inactivation. Therefore, the data suggest that the neuroprotective mechanisms of DHA pretreatment are, in part, mediated by attenuating damaging mechanisms through reduction of cytotoxic factor production and by strengthening survival mechanisms through ERK-mediated and/or Bcl-2-mediated prosurvival cascade.

Keywords: Apoptosis; Cerebral ischemia/reperfusion; DHA; Inflammation; Neuroprotection; Oxidative stress

1. Introduction

Stroke, mainly resulting from interruption of cerebral blood circulation, is a devastating pathology characterized by the sudden development of neurological deficits due to neuronal dysfunction and/or destruction. There are multiple interrelated mechanisms that cause progressive brain damage

☆ Hung-Chuan Pan and Tsung-Kuei Kao contributed equally to this work.

* Corresponding author. Department of Education and Research, Taichung Veterans General Hospital, Taichung 407, Taiwan. Tel.: +886 4 23592525x4022; fax: +886 4 23592705.
E-mail address: cjchen@vghtc.gov.tw (C.-J. Chen).

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during the initial hours of ischemic stroke. Cerebral ischemia/reperfusion (I/R) triggers a complex series of biochemical and molecular changes that impairs neurological functions through breakdown of cellular integrity mediated by excitotoxic signaling, ionic imbalance, oxidative stress, inflammation and others. [1–10]. These intricate processes result in disruption of cellular homeostasis leading to cellular alterations, which might trigger activation of cell-survival and/or cell-damaging mechanisms. The damaging mechanisms are thought to substantially contribute to the pathogenesis of the disease, proceeding through necrosis or apoptosis, depending upon the severity and duration of the ischemic insult. Generally, the survival mechanisms are endogenous defense systems that restrain the enlargement of injury and promote repair processes. Studies have shown that the cell-survival/cell-damaging mechanisms may provide effective preventive or therapeutic interventions to reduce cerebral I/R injuries. Inhibition of reactive oxygen species generation, inflammatory cell activation, proinflammatory cytokine production and apoptotic gene induction provides neuroprotective effects against cerebral I/R injury [11–16]. Studies demonstrating the neuroprotective effects of neurotrophic factors, free radical scavenging enzymes, angiogenic factors and the Bcl-2 antiapoptotic gene family in animal models of focal brain ischemia support the possibility of the therapeutic implication of cell-survival mechanisms in cerebral I/R injury [11–20].

Fatty acids are important for the histological, anatomical and biochemical integrity of the brain, and fatty acid composition influences various physiological and biochemical processes. It is widely accepted that long-chain saturated fatty acids increase the risk for the development of cardiovascular diseases, whereas polyunsaturated fatty acids (PUFAs) tend to decrease it. Concerning neurological disorders, case–control studies have shown that, compared with controls, stroke patients have lower proportions of PUFAs and higher proportions of saturated fatty acids [21,22]. A lower content of PUFAs in patients increases the risk and mortality of stroke [23,24]. In ischemic patients and animals, an increase in arachidonic acid and other PUFAs has been detected in cerebrospinal fluid (CSF) and brain tissues [25,26]. These observations have implications for the involvement of fatty acids in the initiation and/or progression of postischemic brain injury. Recently, we found that acute posttreatment with PUFAs after ischemia remarkably exacerbated cerebral I/R injury partly through augmented oxidative burden [27]. However, there is now considerable research describing the beneficial effects of PUFAs on the prevention of cerebral I/R injury in both human and animal studies. Most neuroprotective effects of PUFAs are demonstrated in chronic dietary supplementation prior to cerebral I/R involving improvement of hemodynamics, blockade of glutamatergic transmission, reduction of eicosanoid production, enhancement of antioxidative capacity and/or induction of chaperon proteins [17,26,28–35].

A growing body of in vitro cell studies indicates that PUFAs such as docosahexaenoic acid (DHA) possess a diversity of biological activities that affect cell viability and protect against stress insults. DHA activates positive regulators of cell survival by up-regulating Akt, extracellular-signal-regulated kinase (ERK) and/or Bcl-2. Besides, DHA also has a negative effect on damaging factor production such as inflammatory cytokines and free radicals [36–39]. Although the modulatory effects of DHA on cell-survival/cell-damaging molecules have been demonstrated in cell studies, involvement of the inactivation of damaging mechanisms and/or the activation of survival mechanisms in DHA-mediated ischemic neuroprotection was largely unknown. Therefore, the present investigation was designed to evaluate the effect of pretreatment with DHA on postischemic brain injury via focal cerebral-I/R-injured animals and to attempt to determine whether the cell-survival/cell-damaging mechanisms contribute to DHA's effects. If this proved to be the case, we would be interested in identifying the therapeutic mechanisms in both continuous daily administration and single administration prior to ischemia. In the present study, ERK, Bcl-2, oxidative stress and cytotoxic interleukin-6 (IL-6) would be the evaluating targets of potential survival/damaging molecules.

2. Materials and methods

2.1. Animal and cerebral I/R

The Animal Experimental Committee of Taichung Veterans General Hospital approved the protocol of the animal study. Male Sprague–Dawley rats were divided into three administration groups: single administration 1 h before the experiment, single administration 3 days before the experiment and daily administration for 6 weeks. Intraperitoneal administration of saline and DHA (100 and 500 nmol/kg) was carried out and modified in accordance with the method used in a previous report [30]. Briefly, rats were anesthetized with chloral hydrate (400 mg/kg ip). A midline cervical incision on the ventral side was first performed to isolate the bilateral common carotid arteries (CCAs). A craniectomy slightly anterior to the right foramen ovale was performed to expose the middle cerebral artery (MCA) without destroying the zygomatic arch. Transient focal cerebral ischemia was produced by ligation of the right MCA and bilateral CCAs for 90 min, followed by 24 h of reperfusion, as described previously [40]. In animals receiving sham operations, all surgical procedures performed were the same as above, but no arterial occlusion was performed.

2.2. Quantification of brain infarction

After 24 h of reperfusion, the brains were quickly removed and chilled in cold phosphate-buffered saline (PBS) for 5 min, and 2-mm coronal slices were cut using a tissue slicer. Slices were immersed in a PBS solution

containing 2% triphenyltertrazolium chloride (TTC) at 37°C for 30 min, after which sections were fixed in 10% phosphate-buffered formalin for 45 min [40]. TTC is reduced by certain enzymes in normal tissues to a deep-red, fat-soluble, light-sensitive compound that turns into deep-red normal tissue and thereby clearly delineates ischemic areas. The infarct areas were measured with a computer image analysis system (IS1000; Alpha Innotech Corporation).

2.3. Measurement of blood–brain barrier permeability

Blood–brain barrier (BBB) permeability was assessed by measurement of Evans blue content in the brain. Briefly, Evans blue (4%, 1 ml/kg) was injected 24 h after reperfusion via the tail vein. Three hours after the Evans blue injection, animals were perfused with heparinized saline solution. Ipsilateral and contralateral cortical tissues were dissected, weighed, homogenized in 500 μl of PBS and centrifuged. Supernatants were diluted with 500 μl of 100% trichloroacetic acid (TCA) overnight at 4°C. After centrifugation at 12,000 rpm for 30 min, Evans blue was quantified in the samples (absorbance at 620 nm). A standard Evans blue curve was generated using a standard solution.

2.4. Water content

The brain samples were dried in an oven at 110°C for 24 h, and the water content of these samples was then measured by the wet/dry weight method as follows: water content (%)=[wet weight−dry weight]/wet weight×100 [7].

2.5. Caspase activity assay

Samples were homogenized on ice in a lysis buffer containing 20 mM HEPES (pH 7.4), 4 mM EDTA, 1 mM ethyleneglycotetraacetic acid, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT) and a protease inhibitor cocktail. A 50-μl aliquot of supernatants was incubated with an equal volume of the reaction buffer containing 20 mM HEPES (pH 7.4), 4 mM EDTA, 0.2% CHAPS, 10 mM DTT and the fluorogenic peptide substrate Ac-DEVD-AMC. Enzymatic release of free AMC was measured at an excitation of 380 nm and at an emission of 460 nm. Arbitrary activity was expressed as fluorescent change per amount of protein [40].

2.6. Myeloperoxidase activity assay

Protein extracts were harvested from cortical tissues with potassium phosphate buffer containing 0.5% hexadecyltrimethyl-ammonium bromide [7]. The protein concentration in the supernatant was determined by Bradford assay. In brief, protein extracts (100 μl) were mixed with 2.9 ml of the assay solution. Optical absorbance was determined at 470 nm for 1 min with a spectrophotometer. Arbitrary activity was expressed as the value of OD_{470} per amount of protein. The assay solution consisted of H_2O (26.9 ml), 0.1 M sodium phosphate buffer (pH 7.0; 3.0 ml), 0.1 M H_2O_2 (0.1 ml) and guaiacol (0.048 ml).

2.7. Determination of IL-6

IL-6 levels in the tissue extracts and supernatants were detected by ELISA, following the instructions provided by the manufacturer (R&D Systems).

2.8. Measurement of lipid peroxidation

A thiobarbituric-acid-reactive substance (TBARS) assay kit was use to measure lipid peroxidation (ZeptoMetrix). In brief, brain tissues were homogenized with 0.1 M sodium phosphate buffer (pH 7.4). One hundred microliters of homogenate was mixed with 2.5 ml of reaction buffer (kit provided) and heated at 95°C for 60 min. After the supernatants had been cooled, their absorbance was measured at 532 nm using a spectrophotometer. TBARS is expressed in terms of malondialdehyde (MDA) equivalents.

2.9. Measurement of superoxide dismutase activity

Total superoxide dismutase (SOD) activity was carried out as previously reported [41]. The protein extracts (5 μl) were placed into a 96-well plate and mixed with 200 μl of buffer [23 mM $NaHCO_3$, 14.4 mM NaOH, 1.2 mM xanthine and 0.1 mM iodonitrotetrazolium violet]. Twenty-five microliters of xanthine oxidase diluted 1:500 in water (55 U/L) was added, and the mixture was incubated at room temperature for 30 min. Absorbance was measured at 490 nm. Arbitrary enzymatic activity was expressed as the ratio of changes in optical absorbance to changes in protein content.

2.10. Measurement of reduced glutathione

Glutathione (GSH) was determined using a commercially available GSH assay kit (Cayman). Briefly, brain tissues were weighed and homogenized with 0.1 M sodium phosphate buffer (pH 7.4). The homogenates were then centrifuged with 5% TCA to remove the proteins. A 50-μl aliquot of homogenate was mixed with 150 μl of reaction buffer (kit provided). The mixture was vortexed, and the absorbance was read at 405 nm within 30 min. The content was calculated using a standard solution of GSH.

2.11. Western blot analysis

To isolate protein extracts, cortical tissue samples were homogenized in potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethyl ammonium bromide and then sonicated with three bursts of 10 s [42]. The protein concentration in the supernatant was determined by Bradford assay. Protein extracts (50 μg) were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto a blotting membrane. After the membranes had been blocked with nonfat milk, they were incubated with phospho-ERK, ERK, Bcl-2 and β-tubulin antibodies (Santa Cruz Biotechnology) overnight at 4°C. Finally, the membrane was incubated with horseradish-peroxidase-conjugated secondary antibody. The membranes were developed using ECL Western blot reagents. The intensity of protein

bands was determined with a computer image analysis system (IS1000; Alpha Innotech Corporation).

2.12. Cell cultures

Glial cells were prepared from cerebral cortices of 1-day-old Sprague–Dawley rats [42]. In brief, the dissociated cells were resuspended with Dulbecco's modified Eagle's medium/10% fetal bovine serum. The medium was replenished 4 days after plating and changed every 3 days. The resultant glial cultures (~85% of astrocytes and ~15% of microglia) were used for the experiments after 14–16 days in vitro.

2.13. Preparation of nuclear extracts and electrophoretic mobility shift assay

The isolation of nuclear extract and electrophoretic mobility shift assay (EMSA) was conducted as described previously [42]. The oligonucleotides specific for nuclear factor κB (NF-κB) (5′-AGTTGAGGGGACTTTCC-CAGGC) were synthesized and labeled with biotin. Nuclear extract (5 μg) was used for EMSA. The binding reaction mixture included 1 μg of poly(dI–dC), 0.1 μg of poly-L-lysine and 100 fmol of biotin-labeled DNA probe in a 20-μl binding buffer [10 mM HEPES (pH 7.6), 50 mM NaCl, 0.5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM dithiothreitol and 5% glycerol]. The DNA/protein complex was analyzed on 6% native polyacrylamide gels.

2.14. Statistical analysis

Data are expressed as mean±standard deviation. For comparison, statistical significance was determined by one-way analysis of variance followed by Dunnett's test. $P \leq .05$ was considered statistically significant.

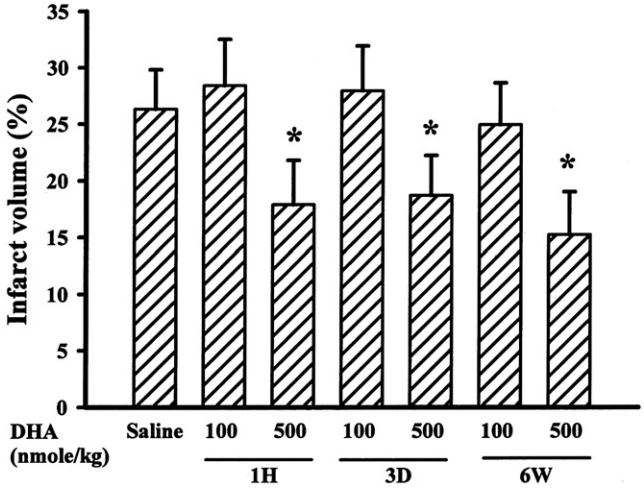

Fig. 1. Effect of DHA administration on cerebral-I/R-induced brain infarction. Rats were subjected to 90-min ischemia followed by 24-h reperfusion and received a single administration [1 h (1H) and 3 days (3D) prior to ischemia] or daily administration of saline or DHA (100 and 500 nmol/kg) for 6 weeks (6W). The average percentage of infarct volume in each ipsilateral hemisphere is shown. *P<.05 versus saline; n=8 (each group).

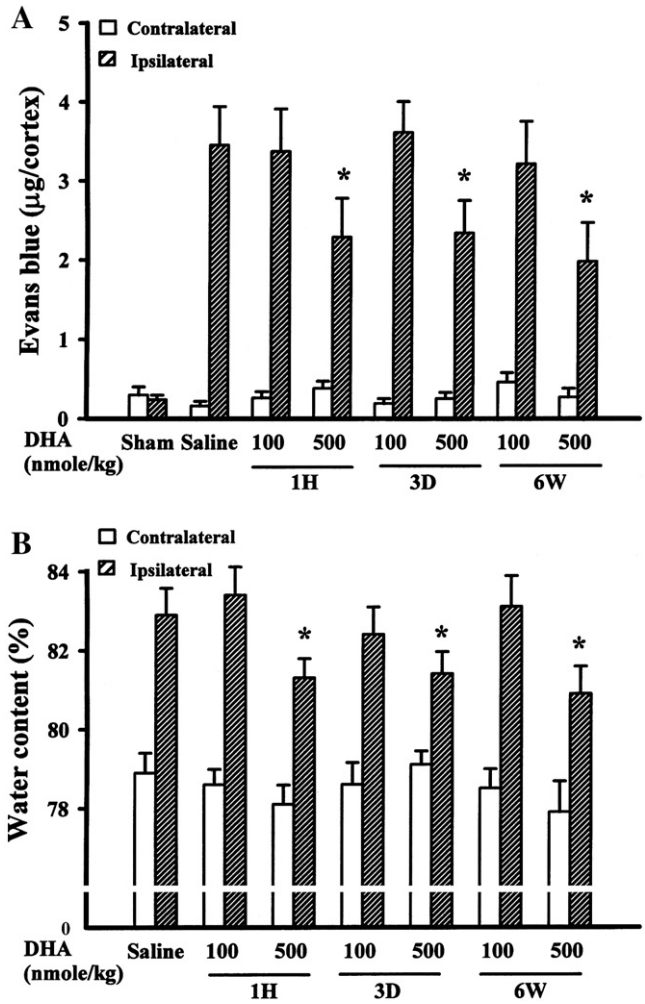

Fig. 2. Effects of DHA administration on cerebral-I/R-induced BBB disruption and brain edema. Rats were subjected to 90-min ischemia followed by 24-h reperfusion and received a single administration [1 h (1H) and 3 days (3D) prior to ischemia] or daily administration of saline or DHA (100 and 500 nmol/kg) for 6 weeks (6W). (A) The integrity of BBB was determined by measuring the content of Evans blue in each contralateral and ipsilateral cortical tissue (sham and ischemic animals). *P<.05 versus saline (ipsilateral); n=6 (each group). (B) The content of brain water was determined in each contralateral and ipsilateral cortical tissue. *P<.05 versus saline (ipsilateral); n=8 (each group).

3. Results

3.1. Effect on postischemic injury

In general, brain infarction detected by TTC stain is a common morphological evidence of cellular death [5]. The 90-min ischemia/24-h reperfusion course resulted in 27.3±3.5% brain infarction in the ischemic hemisphere. Single administration of DHA 1 h or 3 days prior to occlusion or daily administration of DHA for 6 weeks prior to occlusion all caused reduction in postischemic brain infarction in a concentration-dependent manner (Fig. 1). The neuroprotective effects of these three preadministration groups were not significantly different. Increased vascular

permeability and disruption of the BBB could be initiating factors for the development of cerebral infarction and critical factors for the determination of postischemic severity. To elicit the effect of DHA administration on BBB permeability, we quantified the extravasation of Evans blue in the brain parenchyma as an indicator of BBB breakdown. In the sham-operated and contralateral cortical tissues, the content of Evans blue was minimal and was unchanged by DHA administration (Fig. 2A). However, levels of Evans blue increased dramatically following cerebral I/R insult in the ipsilateral cortex. Single administration of DHA 1 h or 3 days prior to occlusion or daily administration of DHA for 6 weeks prior to occlusion all decreased the extravasation of Evans blue in a concentration-dependent manner (Fig. 2A). Increased vascular permeability, which results in edema formation, is a major complication in strokes [43]. Brain water content, as the index of cerebral edema, was higher in the ipsilateral cortical tissues than in the contralateral sides. Single administration of DHA 1 h or 3 days prior to occlusion or daily administration of DHA for 6 weeks prior to occlusion produced a significant decrease in postischemic cerebral edema (Fig. 2B). These results indicate that DHA could alleviate postischemic brain injury if acutely or chronically administered before ischemia.

3.2. Effect on postischemic apoptosis

The occurrence of apoptosis is of paramount importance for ischemic progression and is a determining factor for postischemic severity. Biochemical features of apoptosis include internucleosomal DNA fragmentation, proapoptotic gene expression and caspase activation [44,45]. Caspase-3 is the most abundant caspase and serves as a downstream executioner for caspase. Cerebral I/R caused a significant increase in caspase-3 activity in the ipsilateral cortex, but not in the contralateral cortex. Single administration of DHA 1 h or 3 days prior to occlusion or daily administration of DHA for 6 weeks prior to occlusion attenuated cerebral-I/R-induced caspase-3 activity in ischemic animals (Fig. 3). These results indicate that DHA could decrease postischemic apoptosis in the brain if acutely or chronically administered before ischemia.

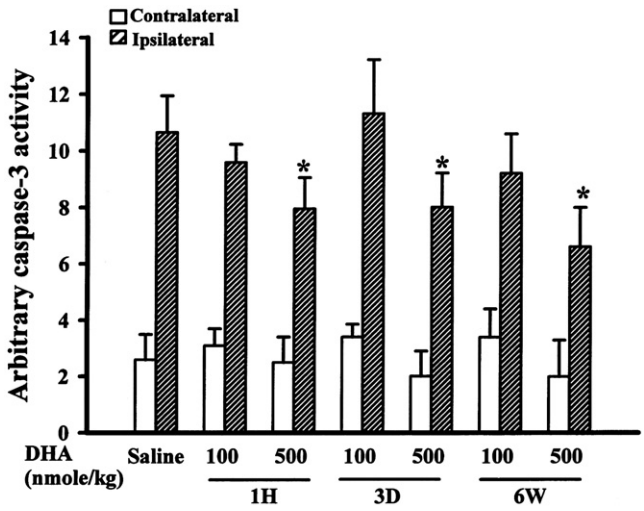

Fig. 3. Effect of DHA administration on cerebral-I/R-induced caspase-3 activity. Rats were subjected to 90-min ischemia followed by 24-h reperfusion and received a single administration [1 h (1H) and 3 days (3D) prior to ischemia] or daily administration of saline or DHA (100 and 500 nmol/kg) for 6 weeks (6W). Total proteins were isolated from the contralateral and ipsilateral cortical tissues and subjected to enzymatic assay of caspase-3 activity. *P<.05 versus saline (ipsilateral); n=6 (each group).

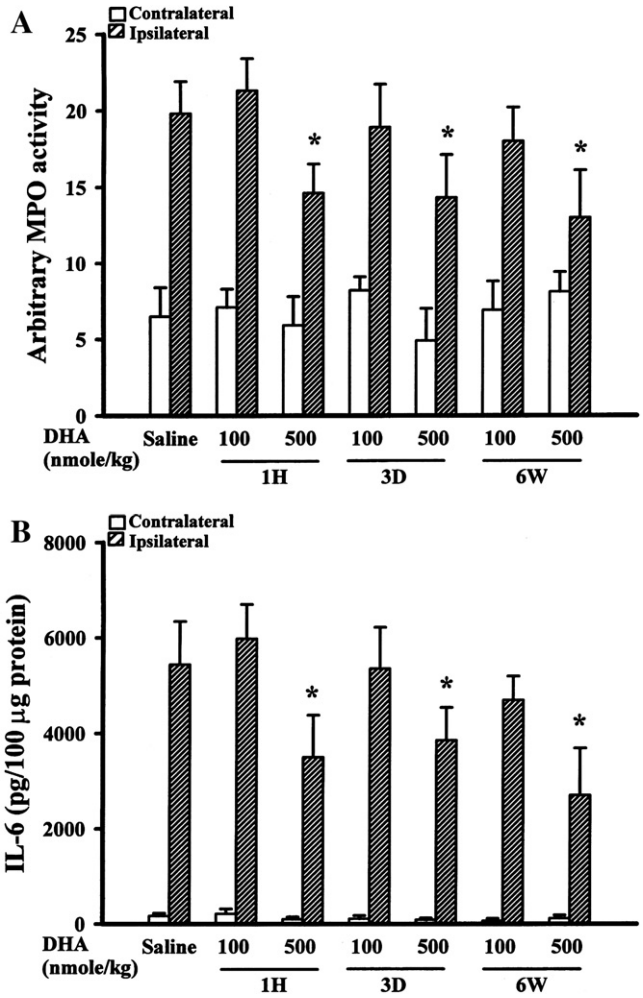

Fig. 4. Effect of DHA administration on cerebral-I/R-induced inflammatory changes. Rats were subjected to 90-min ischemia followed by 24-h reperfusion and received a single administration [1 h (1H) and 3 days (3D) prior to ischemia] or daily administration of saline or DHA (100 and 500 nmol/kg) for 6 weeks (6W). Total proteins were isolated from the contralateral and ipsilateral cortical tissues and subjected to enzymatic assay of MPO activity (A) and ELISA for the measurement of IL-6 (B). *P<.05 versus saline (ipsilateral); n=6 (each group).

3.3. Effect on postischemic inflammation

Inflammation is characterized by the accumulation of inflammatory cells and mediators. The activation of resident microglia and the infiltration and accumulation of neutrophils/

leukocytes are postulated to be cytotoxic to brain tissues and the main cause of postischemic injury [7,11–15]. Biochemical assay of myeloperoxidase (MPO) activity is a common assessment for the measurement of neutrophils/leukocytes accumulation. MPO activity was significantly increased in the ipsilateral cortex (Fig. 4A). In addition, cerebral I/R caused an elevated production of the proinflammatory cytokine IL-6 in the ipsilateral hemisphere (Fig. 4B). Single administration of DHA 1 h or 3 days prior to occlusion or daily administration of DHA for 6 weeks prior to occlusion suppressed cerebral-I/R-induced MPO activity and IL-6 production. These results indicate that DHA could suppress postischemic brain inflammation if acutely or chronically administered before ischemia.

3.4. Effect on postischemic oxidative stress

Oxidative stress has been implicated in the pathogenesis of cerebral injury after I/R [9,16,17,43]. The elevated generation of reactive oxygen free radicals could result in production of lipid peroxidation products such as MDA and/or depletion of antioxidant molecules such as reduced GSH. No significant difference in brain MDA levels was found between vehicles and DHA treatment in contralateral tissues, except for chronic administration of a higher concentration of DHA (Fig. 5). The level of postischemic brain MDA significantly increased in the ipsilateral cortex as compared to the contralateral side in ischemic animals. Single administration of DHA 1 h or 3 days prior to occlusion or daily administration of DHA for 6 weeks prior to occlusion reduced postischemia-enhanced MDA levels (Fig. 5). These results indicate that DHA could alleviate oxidative burden in

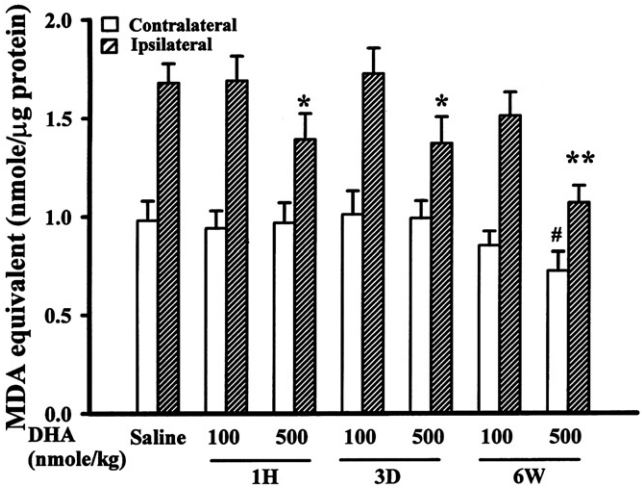

Fig. 5. Effect of DHA administration on cerebral-I/R-induced lipid peroxidation. Rats were subjected to 90-min ischemia followed by 24-h reperfusion and received a single administration [1 h (1H) and 3 days (3D) prior to ischemia] or daily administration of saline or DHA (100 and 500 nmol/kg) for 6 weeks (6W). The content of brain MDA was determined in each contralateral and ipsilateral cortical tissue. *P<.05 and **P<.01 versus saline (ipsilateral); $^{\#}P$<.05 versus saline (contralateral); n=6 (each group).

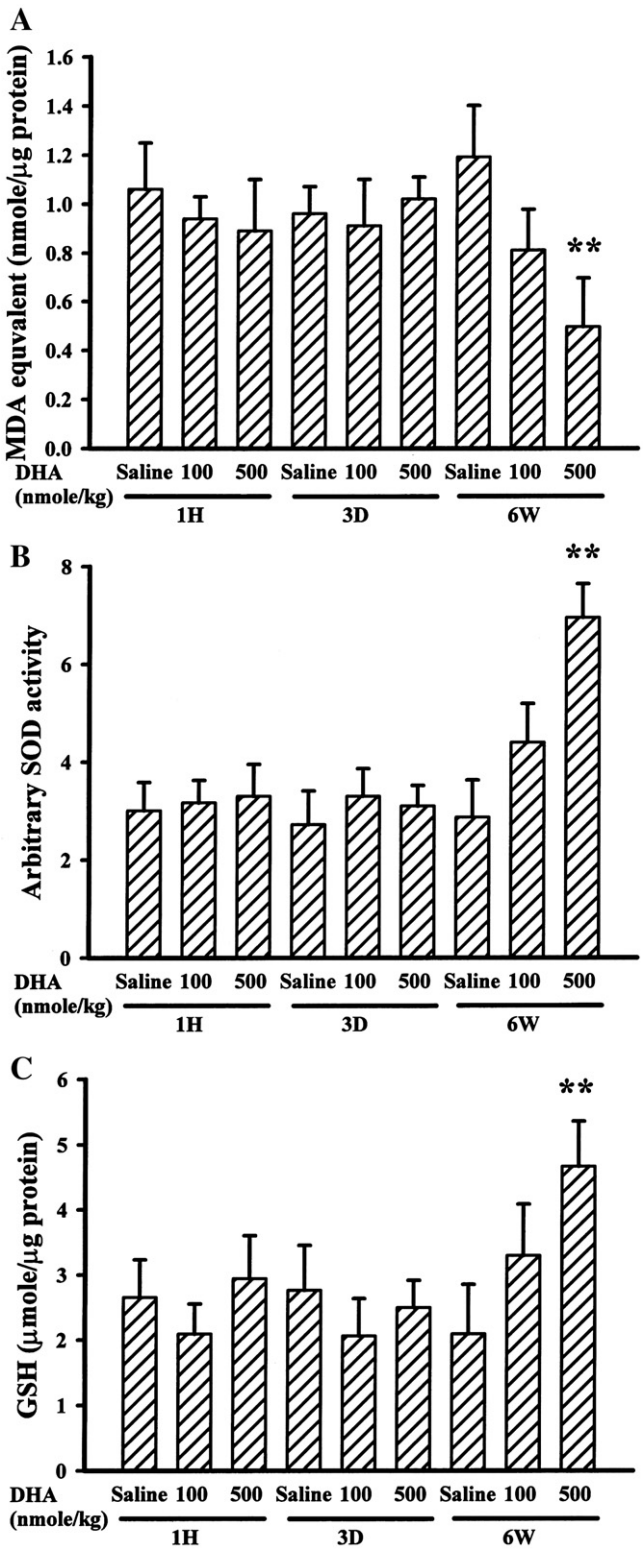

Fig. 6. Effect of DHA administration on oxidative stress in brain tissues. Tissue homogenates were isolated from the right cortical tissues in rats that received a single administration [1 h (1H) and 3 days (3D) prior to sacrifice] or daily administration of saline or DHA (100 and 500 nmol/kg) for 6 weeks (6W). The content of brain MDA (A), SOD activity (B) and GSH (C) were measured. **P<.01 versus saline; n=6 (each group).

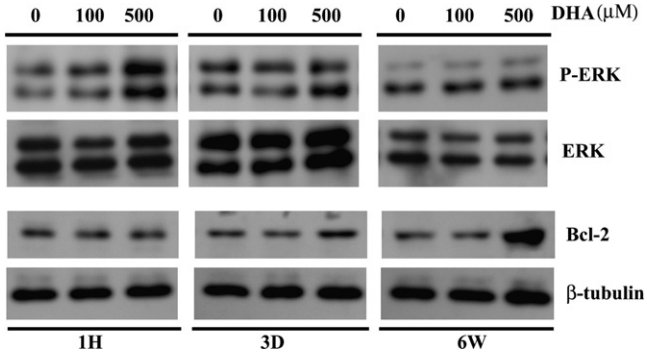

Fig. 7. Effects of DHA administration on ERK and Bcl-2 in brain tissues. Tissue homogenates were isolated from the right cortical tissues in rats that received a single administration [1 h (1H) and 3 days (3D) prior to sacrifice] or daily administration of DHA (0, 100 and 500 nmol/kg) for 6 weeks (6W). The isolated proteins were subjected to Western blot analysis with antibodies against phospho-ERK, ERK, Bcl-2 and β-tubulin. One of six independent experiments is shown.

the ischemic brain if acutely or chronically administered before ischemia.

3.5. Effect on antioxidative capacity

Studies have illustrated that PUFAs have a role in regulating oxidative stress via the modulation of antioxidative defense capacity [17,46,47]. To elicit the potential neuroprotective mechanisms of preadministered DHA, the alteration of cellular oxidative/antioxidative-related molecules such as MDA level, SOD activity and GSH level was evaluated in DHA-treated animals. The content of brain MDA (Fig. 6A) and GSH (Fig. 6C), and SOD activity (Fig. 6B) were maintained within a detected range. Content and activity were not changed in either group of DHA singly administered animals. Remarkably, daily administration of DHA for 6 weeks reduced brain MDA levels (Fig. 6A), increased SOD activity (Fig. 6B) and elevated brain GSH levels (Fig. 6C). These results indicate that chronically — but not acutely — administered DHA could elevate antioxidative defense capacity, leading to reduction of oxidative burden in brain tissues.

3.6. Effect on survival gene expression

Cerebral I/R triggers a complex series of biochemical and molecular changes leading to neuronal injury. Potential neuroprotective intervention includes inactivation of cell-damaging mechanisms and/or activation of cell-survival mechanisms. Studies have shown that activation of ERK and expression of antiapoptotic protein Bcl-2 contribute to the survival of neurons after neurotoxic insult [18,19]. We found that single administration of DHA had little effect on Bcl-2

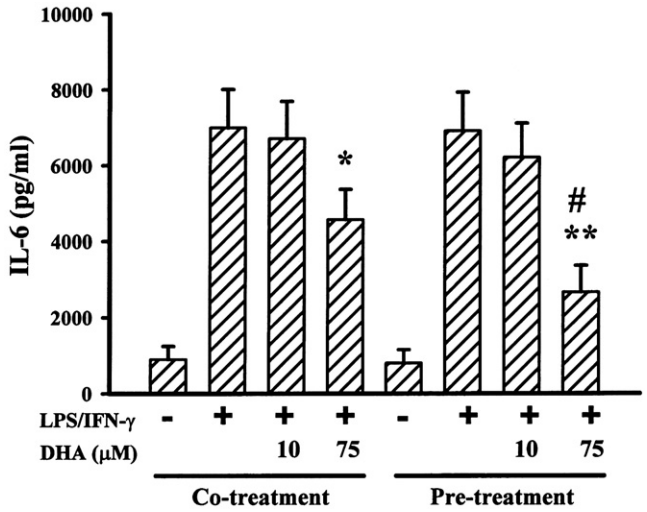

Fig. 8. Effect of DHA on LPS/IFN-γ-induced IL-6 production. Glial cells were pretreated with (pretreatment) or without (cotreatment) various concentrations of DHA for 1 h prior to 24-h stimulation with LPS (10 ng/ml)/IFN-γ (10 U/ml). Supernatants were collected and subjected to ELISA for the measurement of IL-6. *P<.05 and **P<.01 versus each LPS/IFN-γ; $^{\#}P$<.05 versus LPS/IFN-γ+DHA (75 μM) (cotreatment); n=6.

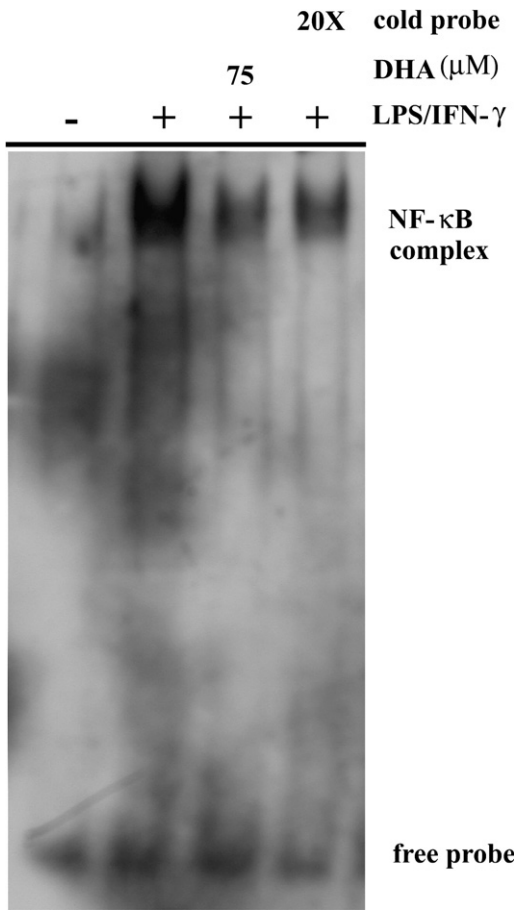

Fig. 9. Effect of DHA on LPS/IFN-γ-induced NF-κB activation. Glial cells were pretreated with or without DHA (75 μM) for 1 h prior to 1-h stimulation with LPS (10 ng/ml)/IFN-γ (10 U/ml). Nuclear extracts were collected and subjected to EMSA for the determination of NF-κB DNA binding activity. Competition was conducted by adding 20× excess nonlabeled probe to the reaction mixtures. One of three independent experiments is shown.

expression, but a higher concentration of DHA caused ERK activation (5.3±1.6-fold, $P<.01$) (Fig. 7). Chronic daily administration of DHA had little effect on ERK activity, but a higher concentration of DHA elevated Bcl-2 expression (7.1±2.6-fold, $P<.01$) (Fig. 7). Interestingly, single administration of DHA (3 days) had dual effects. A higher concentration of DHA induced ERK activation (1.9±0.6-fold, $P<.05$) and increased Bcl-2 expression (2.7±1.3-fold, $P<.05$) (Fig. 7). These results indicate that acutely and chronically administered DHA could elevate cell-survival mechanisms involving ERK activation and Bcl-2 expression in brain tissues.

3.7. Effect on in vitro IL-6 production

Our findings showed that the neuroprotective effects of acutely and chronically administered DHA were associated with the suppression of overactivated inflammatory reaction (Fig. 4). To further elicit the potential effect of DHA on the activation of inflammatory responses, primary cortical glial cultures were used as cell model. Lipopolysaccharide (LPS)/interferon-γ (IFN-γ) stimulation caused glial cells to release an elevated level of IL-6 (Fig. 8). DHA cotreatment suppressed LPS/IFN-γ-induced IL-6 production in a concentration-dependent manner (Fig. 8). Pretreatment with DHA further diminished IL-6 production (Fig. 8). In addition, DHA pretreatment attenuated LPS/IFN-γ-induced NF-κB DNA binding activity (Fig. 9). These findings suggest that DHA has a role in decreasing cytokine expression in stimulated glial cells involving suppression of NF-κB.

4. Discussion

Studies have revealed that dietary supplementation of fish oil or ω-3 PUFA may be beneficial in ameliorating cerebral ischemic injury. In the present study, we demonstrated that DHA, when administered prior to ischemia, alleviated cerebral ischemic injury by manifesting itself in the reduction of cerebral infarction (Fig. 1). Single administration 1 h or 3 days prior to ischemia or chronic daily administration for 6 weeks prior to ischemia had a beneficial effect on ischemic brain infarction. However, there was no remarkable difference in beneficial effects between the administrative protocols. The postischemic neuroprotection in DHA treatment groups was accompanied by decreases in BBB permeability (Fig. 2A), brain edema (Fig. 2B), caspase-3 activity (Fig. 3), MPO activity (Fig. 4A), proinflammatory cytokine IL-6 production (Fig. 4B) and MDA production (Fig. 5). Six weeks of daily administration of DHA increased SOD activity and GSH content, and decreased MDA production (Fig. 6) in the brain. Single administration of DHA 1 h or 3 days prior to occlusion had little effect on brain levels of SOD, GSH and MDA (Fig. 6). Acute single administration of DHA 1 h prior to occlusion significantly stimulated ERK phosphorylation, but had little effect on Bcl-2 expression in cortical brain tissues. Single administration of DHA 3 days prior to occlusion induced ERK phosphorylation and Bcl-2 expression. Elevated Bcl-2 expression, but not ERK phosphorylation, was detected in chronic daily DHA-administered rats (Fig. 7). The in vitro cell study further demonstrated that DHA modulated inflammatory cell activity. DHA was capable of suppressing NF-κB DNA binding activity (Fig. 9) and IL-6 production from activated glial cells (Fig. 8). Existing evidence indicates that ischemic neuroprotection of PUFAs in chronic dietary supplementation or single injection prior to injury involves stabilization of membrane integrity, improvement of local cerebral blood flow, blockade of glutamatergic transmission, reduction of eicosanoid production, enhancement of antioxidative capacity and/or induction of chaperon proteins [17,26,28–35]. In comparison with those proposed neuroprotective mechanisms, we found that the beneficial effects of DHA preadministration on ischemic brain injury were well associated with the reduction of oxidative burden, elevation of Bcl-2 expression and/or stimulation of ERK activity. Taken together, our experimental findings further support the therapeutic potential of DHA preadministration in postischemic progression. The beneficial effect of these three preadministrative protocols on ischemic injury was mediated by distinct neuroprotective mechanisms.

There is considerable research indicating that repeated administration of fish oil or ω-3 PUFA has a beneficial effect on the ischemic brain. Rodents that receive a fish-oil-supplemented diet for 6 weeks or daily administration of fish oil by gavage for 2 weeks prior to ischemia suffer less brain injury from ischemic insult [17,35]. Daily administration of ω-3 PUFA (150 or 200 mg/kg) by gavage for 4–10 weeks protects against brain ischemic damage [26,32–34]. Intravenous administration of ω-3 PUFA (500 nmol/kg) 3 days prior to ischemia induces ischemic tolerance [30]. Delayed administration of ω-3 PUFA EPA (100 mg/kg) by gavage once a day for 4 weeks improves local cerebral blood flow and metabolism without affecting infarct volumes in ischemic rats [28]. Single injection of linolenic acid (167 nmol/kg icv or 100 nmol/kg iv) 30 min before stress prevents ischemia-induced neuronal loss [29]. However, acute post-treatment with DHA (500 nmol/kg ip) after ischemia remarkably exacerbated cerebral I/R injury [27]. In the present study, we found that intraperitoneal administration of DHA (500 nmol/kg) once 1 h or 3 days prior to ischemia attenuated postischemic brain injury. Daily administration of DHA (500 nmol/kg) for 6 weeks also provided potent tolerance to cerebral I/R injury (Fig. 1). Our findings and those of previous studies suggest that pretreatment with fish oil or ω-3 PUFA has a neuroprotective effect against brain injury in ischemic animals, independently of administrative routes or course. These experimental findings show some similarities with those clinical observations indicating lower proportions of PUFAs in stroke patients, and the reduction parallels stroke severity [21–24]. According to these relevant studies, the neuroactive effects of ω-3 PUFAs could be

demonstrated in different administration routes, including oral gavage or intracerebroventricular, intravenous or intraperitoneal injection [26–30,32–34]. Surprisingly, the administration of DHA (100 nmol/kg ip) was ineffective in modulating postischemic alterations in this study. Cao et al. [33,34] reported that chronic administration of DHA by gavage at doses of 150 and 200 mg/kg, but not 100 mg/kg (280 μmol/kg), showed ischemic protection. Unfortunately, due to instrument and technique limitations, we were unable to determine the exact change in the plasma level of DHA and other PUFAs in our study. Therefore, characterization of the detailed dosage–response effect of DHA on ischemic brain injury would be helpful in resolving these questions.

Progressive expansion of brain infarction and consequent neurological deficits are determined by multiple mechanisms. DHA and other PUFAs are major constituents of neuronal membranes in the central nervous system [29]. After ischemia, an acute and brief increase in arachidonic acid and other PUFAs is detected in CSF and brain tissues [25,26]. During this period, elevation of fatty acids could be one of the consequent results of ischemic-insult-induced membrane disruption or one of the detrimental factors involved in postischemic progression after active release. Acute posttreatment with PUFAs, possibly further elevating free fatty acids, augments postischemic brain injury, indicating the possibility of the latter hypothesis [27]. This augmented effect could be partly mediated by enhancement of oxidative burden. Following delayed administration after the development of brain infarction, ω-3 PUFA had little ability to ameliorate the established infarction, but was able to improve local cerebral blood flow and metabolism through hemodynamic mechanisms [28].

The cessation of cerebral blood flow during ischemic periods leads to energy crisis and necrotic neuronal death, which could initially disturb the homeostasis of the cerebral microenvironment and trigger cellular alterations. Evidence suggests that ischemia-associated and reperfusion-associated alterations play an important role in the extent of postischemic severity. Disruption of BBB and induction of oxidative stress are common alterations that occur during the early stage following cerebral I/R. These early changes can further contribute to secondary brain injury. Secondary brain damage can develop as a consequence of brain edema, oxidative stress, immune cell infiltration, inflammation and apoptosis [11–20]. In this study, the three administrative protocols of DHA all suppressed cerebral-I/R-induced BBB disruption (Fig. 2A), brain edema (Fig. 2B), inflammatory cell accumulation (Fig. 4A), proinflammatory cytokine production (Fig. 4B), oxidative stress (Fig. 5) and apoptosis (Fig. 3). These findings indicate that DHA treatment statistically attenuated cerebral-I/R-induced early and late alterations. Multiple factors, molecules and mechanisms underlie the initiation and progression, as well as the restriction, of postischemic brain injury. Therapeutic agents that either inactivate damaging molecules or activate protective molecules can provide neuroprotection. Numerous studies have shown that dietary supplementation with PUFAs or chronic administration shortly prior to a severe ischemic insult may ameliorate some of the symptoms associated with cerebral I/R by increasing antioxidative capacity, lowering lipid peroxidation, inducing chaperon molecules or stabilizing membrane integrity [17,26,30–34]. Regarding oxidative stress, it is possible that chronic administration of PUFAs may make the brain more vulnerable to lipid peroxidation, thus inducing antioxidative defense capacity and leading to elevated tolerance to and protection against free-radical-induced injury. This assumption was partly supported by this study. Chronic daily administration of DHA for 6 weeks increased SOD activity and elevated reduced GSH, leading to effective reduction of the brain lipid peroxidation product MDA (Fig. 6). In contrast, single administration of DHA had little effect on the modulation of brain antioxidative capacity (Fig. 6). Studies have shown that SOD deficiency exacerbated cerebral infarction [43,48]. Therefore, elevated antioxidative capacity is a neuroprotective mechanism of long-term DHA treatment.

A variety of cellular actions of PUFAs, including protection against apoptotic death and modulation of gene expression, have been demonstrated [17,32,36,38,39,49]. ERK is a member of the family of mitogen-activated protein kinases involved in the diversity of cellular activities, including cell proliferation, differentiation, migration and apoptosis. The activation of the ERK pathway plays a critical role in brain ischemic preconditioning and tolerance, and is involved in neurotrophin-mediated neuron survival [20,50]. Single administration of DHA 1 h prior to occlusion significantly increased ERK phosphorylation in cortical brain tissues, and this effect was also observed 3 days later, but with lower efficacy. Conversely, elevated phosphorylation of ERK was not detected in animals given long-term repeated DHA treatment (Fig. 7). In vitro, DHA possesses the ability to induce ERK activation, leading to modulation of cellular activity [38,39]. These findings indicate that one pretreatment with DHA can induce ischemic tolerance leading to suppression of postischemic brain injury for up to a 3-day window. Blondeau et al. [30] reported that administration of linolenic acid 3 days prior to global ischemia reduced neurodegeneration in the hippocampus associated with the induction of heat shock protein 70. However, the change in heat shock protein 70 expression was not detected in this study (data not shown). In addition to ERK, Bcl-2 is another prosurvival molecule. The Bcl-2 proteins are a family of proapoptotic and antiapoptotic regulators [44,45,51,52]. Antiapoptotic members of the Bcl-2 family, including Bcl-2 and Bcl-xL, are associated with the mitochondrial outer membrane and can inhibit the release of cytochrome *c*. On the other hand, Bax and Bad, which belong to proapoptotic membranes, once activated, translocate to mitochondria and interact with Bcl-2 or Bcl-xL. These interactions in the mitochondria then initiate the opening of mitochondrial permeability transition pores and

the consequent release of cytochrome *c* leading to apoptosis. The down-regulation of Bcl-2 occurs in the ischemic brain [40], and its overexpression reduces postischemic injury [18]. Chronic daily administration of DHA for 6 weeks significantly increased Bcl-2 expression in cortical brain tissues. Single administration of DHA also caused elevated expression of Bcl-2 three days later, but not 1 h after treatment (Fig. 7). In a cell study, German et al. [39] found that DHA treatment induced Bcl-2 expression in neurons. Evidence suggests that multiple signaling molecules modulate Bcl-2 expression, including the ERK signaling pathway [39]. This study did not address whether the induction of Bcl-2 expression by DHA was mediated by activating the ERK pathway. However, our findings indicate that the beneficial effect of DHA treatment on cerebral I/R injury was associated with the induction of prosurvival molecules such as ERK and Bcl-2. The inductive characteristics varied and depended on administrative protocols.

Recent evidence suggests that neuroinflammation and the subsequent release of proinflammatory mediators are involved in the progression of cerebral I/R injury. Activation of inflammatory cells and their subsequent release of cytotoxic factors have been demonstrated in ischemic brain injury. For example, IL-6 is a well-documented member of the proinflammatory cytokines [6]. Accumulating evidence suggests that, in addition to antiapoptotic agents, anti-inflammatory agent treatments reduced the occurrence of apoptosis and ischemic brain injury [11–15]. Here, we found that the neuroprotective effect of DHA was accompanied by decreased immune cell infiltration and IL-6 production (Fig. 4), demonstrating the potential immunosuppressive effect of DHA. In vitro cell study further demonstrated that DHA treatment was capable of suppressing LPS/IFN-γ-stimulated glial cells, as evidenced by the decreased production of IL-6 (Fig. 8). This immunosuppressive action of DHA was partly mediated by down-regulating transcription factor NF-κB (Fig. 9). ω-3 PUFAs, such as DHA and EPA, have long been recognized as being able to modulate the inflammatory response and are widely applied clinically as adjuvant immunosuppressants in the treatment of inflammatory disorders [46,47]. A recent study further suggested that neuroprotectin D1 might be involved in ω-3 PUFA-mediated antiapoptotic, anti-inflammatory and neuroprotective action [53]. It should be noted that DHA pretreatment showed better immunosuppressive effects in cell studies (Fig. 8). Together with other related studies, our findings indicate that DHA pretreatment might induce more immune modulator expressions or stabilize the microenvironment, thus avoiding overactivated immune stimulation leading to suppression of inflammatory response.

Disruption of BBB; induction of oxidative stress, inflammation and apoptosis; and their subsequent cellular alterations have been demonstrated in ischemic brain injury. Accumulating evidence suggests that antioxidants, anti-inflammatory agents, neurotrophins or antiapoptotic agents reduce ischemic brain injury [11–20]. The main findings of this study are that pretreatment with DHA via intraperitoneal injection improved cerebral-I/R-induced alterations and reduced cerebral-I/R-induced brain injury in rats. Related studies and our current experimental findings support a therapeutic potential for DHA in the prevention/treatment of ischemic brain injury. DHA participating in neuroprotection is well paralleled by the induction of prosurvival molecules and the suppression of immune overactivation. Therefore, we suggest that the neuroprotective mechanisms of DHA pretreatment are, in part, mediated by attenuating damaging mechanisms through reduction of cytotoxic cytokine production and by strengthening prosurvival mechanisms through ERK-mediated and/or Bcl-2-mediated antiapoptotic cascade. However, detailed underlying mechanisms require further investigation.

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