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Protective effect of chronic ethyl docosahexaenoate administration on brain injury in ischemic gerbils

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Abstract

There is evidence that the excessive generation of reactive oxygen free radicals contributes to the brain injury associated with cerebral ischemia. In the present study, the protective effect of chronic administration of ethyl docosahexaenoate (E-DHA) against oxidative brain injury was evaluated in the gerbil model of transient cerebral ischemia. Weanling male gerbils were orally pretreated with either E-DHA (200 mg/kg) or vehicle, once a day, for 10 weeks and subjected to bilateral occlusion of common carotid arteries for 10 min. At the different reperfusion times, E-DHA pretreatment significantly inhibited the increases in the production of brain salicylate-derived 2,5-dihydroxybenzoic acid (2,5-DHBA) and content of brain malonildialdehyde (MDA). The superoxide dismutase (SOD) activity was not modified; however, pretreatment with E-DHA significantly prevented the level of brain-reduced glutathione (GSH) and activities of brain glutathione peroxidase (GSH-P_X) and catalase (CAT) from declines caused by cerebral ischemia. Moreover, ischemia and reperfusion-induced delayed neuronal loss in the hippocampus CA1 sector and locomotor hyperactivity were also significantly attenuated by pretreatment with E-DHA. These results suggested that the neuroprotective effect of E-DHA might be due to its antioxidant property.

Keywords: Ethyl docosahexaenoate (E-DHA); Cerebral ischemia and reperfusion; Brain injury; Antioxidant; Gerbil

1. Introduction

Cerebral ischemia or stroke, one of the leading causes of death and long-term disability in aged populations, often results in irreversible brain damage and subsequent loss of neuronal function. There is no proven efficient treatment for this condition, primarily because the pathophysiology involved is not yet well understood (Read et al., 1999). It has been shown that a series of events, including massive release of excitatory amino acids, intracellular calcium overload, and free radical generation, has been involved in the pathogenesis of stroke-induced neuronal injury (Siesjö, 1992; Parnham and Sies, 2000). Many recent evidences have suggested that the excessive generation of oxygen free radicals such as superoxide anion, hydroxyl radical, and hydrogen peroxide during reperfusion plays a major role in brain injury associated with stroke (Ikeda and Long, 1990; Chan, 1996). Because of the brain's low concentrations of antioxidant substance reduced glutathione (GSH) (Sinet et al., 1980) and low activities of antioxidative enzymes including superoxide dismutase (SOD), glutathione peroxidase (GSH-P_X), and catalase (CAT) (Mizuno and Ohta, 1986), it is exceptionally vulnerable to ischemia and reperfusion-induced oxygen free radicals, which cause oxidative damage to brain lipids, proteins, and nucleic acids, leading to brain dysfunction and cell death (Braughler and Hall, 1989; Oliver et al., 1990; Chen et al., 1997).

Docosahexaenoic acid (DHA; 22:6 $n-3$), one of the $n-3$ polyunsaturated fatty acid ($n-3$ PUFA) found plentifully in marine products such as fish oils, is important for normal cerebral development and brain function of vertebrates (Menon and Dhopeshwarkar, 1983). Recently, it has been reported that ethyl docosahexaenoate (ethyl DHA, E-DHA) can enhance free radical scavenging and decrease lipid

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peroxidation in the rat fetal brain (Green et al., 2001). Furthermore, DHA, when administered, produced antithrombotic effects with reduced ischemic brain damage in a middle cerebral artery thrombosis rat model (Umemura et al., 1995) and reduced the spatial cognitive deficit caused by transient forebrain ischemia in rats (Okada et al., 1996). However, the protective effects of DHA on the oxidative brain injury induced by ischemia and reperfusion were not examined in these studies.

The aim of this study, therefore, is to investigate the effects of chronic E-DHA administration against oxidative stress, which occurs during the reperfusion of the ischemic gerbil brain. The following biochemical, behavioral, and morphological parameters were evaluated: (1) salicylate (SA)-derived 2,5-dihydroxybenzoic acid (2,5-DHBA) formation; (2) lipid peroxidation production; (3) GSH level; (4) activities of antioxidative enzymes CAT, GSH-P_X, and SOD; (5) locomotor activity; and (6) neuronal cell loss in the hippocampal CA1 region.

2. Materials and methods

2.1. Materials

E-DHA (ethyl all *cis*-4,7,10,13,16,19-docosahexaenoic acid; 98% pure) was obtained from Harima Chemicals (Tokyo, Japan). SA, 2,5-DHBA, malonildialdehyde (MDA), thiobarbituric acid (TBA), 5,5′ -dithiobis-2-nitrobenzoic acid (DTNB), reduced β-nicotinamide adenine dinucleotide phosphate (NADPH), reduced GSH, glutathione reductase (GSSG-R), H_2O_2 stock solution, cumene hydroperoxide sodium azide, ethylenediamine tetraacetic acid (EDTA), and pyrogallol were purchased from Sigma (St. Louis, MO, USA). All other reagents or chemicals were of the highest grade commercially available.

2.2. Animals and treatments

Weanling male Mongolian gerbils (21 days old; Experimental Animal Center of Zhejiang Medical University, China) were randomly divided into two groups. One group (E-DHA group) was orally treated with E-DHA emulsified in 5% gum Arabic solution at 200 mg/kg (1 ml/kg), once a day, for 10 weeks (the dose and timing of treatment were according to Okada et al., 1996; Gamoh et al., 1999, respectively); the other group (vehicle group) was treated with a similar volume of vehicle alone. Before and after ischemia or reperfusion, gerbils were housed six to a cage at the constant room temperature of 21–22 °C under a light/dark cycle of 12/12 h (700 a.m./7:00 p.m.). The animals were allowed free access to pelleted food and drinking water. All gerbils were cared for and killed in accordance with the internationally accepted principles and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.3. Surgical preparation

At the termination of treatment, the gerbil (40–60 g) was anesthesized by inhalation of 2% halothane in 30% oxygen/70% nitrous oxide; the total period of anesthesia was about 18 min. A midline ventral incision was made in the neck and both common carotid arteries were exposed, separated carefully from the vagus nerve, and occluded with Heifetz aneurysm clips for 10 min (Nowak, 1991). Blood flow during the reperfusion after remove of the clips was visually confirmed. Rectal and brain temperatures (measured by rectal probe and tympanic probe, respectively) were maintained at 37.0±0.5 °C during the ischemia and the early postischemia period by placing the animal in a heated box and using a controlled heating lamp (Minamisawa et al., 1990). The same surgically operated animals without carotid occlusion served as sham animals. The gerbils were divided into four subgroups: Sham (vehicle, n=36), E-DHA (sham, n=36), I/R (ischemia/reperfusion+vehicle, n=36), and I/R+E-DHA (ischemia/reperfusion+E-DHA, n=36). A total of 180 gerbils were used and 36 animals were excluded from the study due to death or poor reflow.

2.4. Detection and quantitation of hydroxyl radicals

Salicylic acid reacts with hydroxyl radical (·OH) to produce 2,3-dihydroxybenzoic acid (2,3-DHBA) or 2,5-DHBA, which can be quantified by high-performance liquid chromatography (HPLC) with electrochemical detection using a modification of the technique described by Hall et al. (1993). SA (sodium salt), 100 mg/kg (dissolved in 0.9% saline, 100 mg/ml), was administered (i.p.) 10 min before gerbil decapitation. The brain was quickly removed within 1 min of reperfusion and then frozen at −70 °C until the time of assay. Frozen brain tissue was homogenized with 4× (wt/vol) 10% trichloroacetic acid. The homogenate was kept on ice for 10 min and then centrifuged at 15,000 rpm for 2 min at 4 °C. The supernatant (20 μl) was injected into the HPLC apparatus, composed of a model PM-80 pump (BAS) and a model LC-4B electrochemical detector (BAS). The column was a phase II ODS 100×3.2 mm column with 3 μm particle size (MF-6213; BAS). The mobile phase contained 0.03 M citric acid, 0.03 M sodium citrate, and 1.8% glacial acetic acid, pH 3.5, and the flow rate was 0.40 ml/min. All samples were measured by comparison to external standards of 2,3-DHBA and 2,5-DHBA and SA. The column effluent flowed into an electrochemical detector set at 625 mV and then flowed into a UV detector (U-3000; Hitachi) set at 295 nm. The 2,3-DHBA and 2,5-DHBA compounds were detected electrochemically, and SA was detected spectrophotometrically (UV). 2,3-DHBA levels were found to be less than those of 2,5-DHBA in the samples (Hall et al., 1993); therefore, we only focused on the latter here. This system is capable of detecting hydroxylation products of SA at the nanomolar concentration level. The data were expressed as the ratio of 2,5-DHBA/SA×10^{-6} in order to

eliminate the influence of variations in the brain uptake of SA.

2.5. Measurement of lipid peroxidation

For assessing the level of oxidative stress, the MDA, an indicator of lipid peroxidation, was estimated 60 min following reperfusion after ischemia. Gerbils were decapitated under light ether anesthesia. The brain was quickly removed and then frozen at −70 °C for biochemical analysis. The MDA was measured in the brain homogenate using a modified TBA test as described by Ohkawa et al. (1979). Briefly, brain tissues were homogenized with 10× (wt/vol) 0.1 M sodium phosphate buffers (pH 7.4). An amount of 0.1 ml of homogenate was added to the test tube containing 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid solution, pH 3.5, and 1.5 ml of 0.8% TBA solution. The mixture was added and diluted to 4.0 ml with distilled water and heated at 100 °C for 60 min. After cooling on ice, 4.0 ml of *n*-butanol:pyridine (15:1, vol/vol) was added to the mixture and shaken vigorously for extraction. The organic layer was withdrawn after centrifugation at 4000 rpm for 10 min, and the absorbance was measured at 532 nm using a spectrophotometer. The results were expressed as nanomoles of MDA per milligram of protein. Protein concentrations were determined by the method of Lowry et al. (1951), using bovine serum albumin as the standard.

2.6. Estimation of antioxidant markers

To estimate the antioxidant capacity, the level of reduced GSH, and the activities of antioxidant enzymes GSH-P_X, CAT, and SOD were assayed 60 min after reperfusion and ischemia. The brain was quickly removed after light ether anesthesia and then frozen at −70 °C for analysis.

2.7. Determination of reduced GSH

The GSH was determined according to the method of Ellman (1959) with slight modifications. Briefly, brain tissues were weighed and homogenized with 10× (wt/vol) 0.1 M sodium phosphate buffer (pH 7.4). The homogenates were then centrifuged with 5% trichloroacetic acid to remove the proteins. To 0.1 ml of homogenate, 2 ml of phosphate buffer (pH 8.4), 0.5 ml of DTNB, and 0.4 ml of double distilled water were added. The mixture was vortexed and the absorbance read at 412 nm within 15 min. The results were expressed as micrograms per gram of tissue.

2.8. Assays of antioxidant enzymes

For assaying the activities of antioxidant enzymes GSH-P_X, CAT, and SOD, the brain was weighed and homogenized with a buffer consisting of 10 mmol/l sucrose, 10 mmol/l Tris–HCl, and 0.1 mmol/l EDTA (pH 7.4), and then centrifuged at 4000 rpm for 15 min (4 °C). The supernatant was used for bioassays. The GSH-P_X activity was assayed as described by Mohandas et al. (1984). The reaction mixtures (1 ml) containing 50 mM potassium phosphate (pH 7.0), 1 mM sodium azide, 2 mM GSH, 0.2 mM NADPH, 1 U/ml GSSG-R, 1.5 mM cumene hydroperoxide, and 20–100 μl of samples were incubated at 25 °C for 5 min. The reaction was initiated by the addition of cumene hydroperoxide. The kinetic change was spectrophotometrically recorded at 340 nm (25 °C) for 3 min. The GSH-P_X activity was expressed as a unit, which is defined as the unit (micromoles of oxidized NADPH per minute) per milligram of protein. The CAT activity was assayed using the method described by Claiborne (1986). The reaction mixture (1 ml) consisted of 50 mM potassium phosphate (pH 7.0), 19 mM H_2O_2, and 20–50 μl of sample. The reaction was initiated by the addition of H_2O_2, and absorbance changes were measured at 240 nm (25 °C) for 30 s. The molar extinction coefficient for H_2O_2 is 43.6 M^{-1} cm^{-1}. The CAT activity was expressed as the unit that is defined as micromoles of H_2O_2 consumed per minute per milligram of protein. The SOD activity was assayed using pyrogallol according to Shukla et al. (1987). The essay mixture contained 50 mM Tris–HCl buffer (pH 8.2), 1 mM EDTA, and 20–50 μl of sample. The reaction was initiated by the addition of pyrogallol (final concentration of 0.2 mM), and the absorbance was measured kinetically at 420 nm (25 °C) for 3 min. The SOD activity was expressed as the unit that is defined as the amount that reduced the absorbance change by 50% and results were normalized on the basis of total protein content (U/mg protein).

2.9. Locomotor activity

Locomotor activity was evaluated using an open field test similar to that described elsewhere (Dowden et al., 1999). Testing was performed on days 3 and 5 postischemia in a sound-proof room where distinctive features of the room and lighting conditions were kept constant throughout the experiment. Without prior habituation, the gerbils were placed in an open field apparatus (72×76×57 cm). The floor of the open field was divided into 25 equal squares and the numbers of squares entered per minute over a 10-min test session were recorded by a visual tracking system (HVS Systems Kingston, UK). The total number of squares entered throughout the 10-min test session was then used for analysis. The test was always performed between 10:00 and 12:00 a.m.

2.10. Histological examination

Following the final open field test on day 5 postischemia, the gerbils were anesthesized with sodium pentobarbital (50 mg/kg, i.p.) and perfused transcardially with cold saline followed by 4% paraformaldehyde in phosphate-buffered

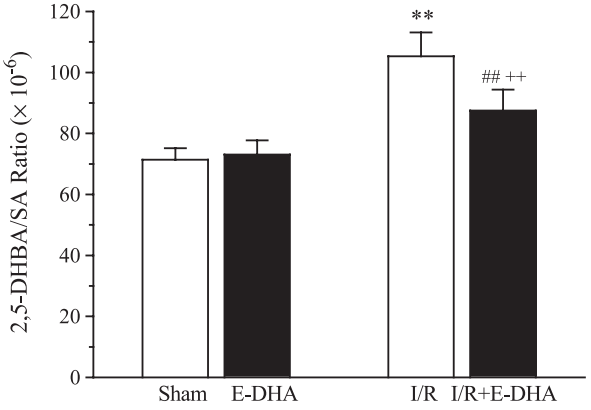

Fig. 1. Effects of E-DHA pretreatment on hydroxyl radical formation (2,5-DHBA/SA ratio) at 1 min of reperfusion after 10 min of transient cerebral ischemia. Values are expressed as the mean±S.E.M. of six animals. **$p<0.01$, I/R vs. Sham; $^{\#\#}p<0.01$, I/R+E-DHA vs. I/R; $^{++}p<0.01$, I/R+E-DHA vs. E-DHA.

saline (pH 7.4). The brains were removed from the skull and fixed in the same fixative for 24 h. After dehydration with graded concentration of alcohol, the brains were embedded in paraffin. Coronal sections (5 μm), which included the dorsal hippocampus, were obtained stereologically from 1.7 mm posterior to bregma and stained with hematoxylin and eosin for light microscope examinations. The hippocampal CA1 damage was determined by counting the surviving pyramidal neurons (sharply delineated nucleus with ellipsoid or round shape, clear distinguishable nucleolus located centrally within the nucleus, nucleus slightly darker than surrounding neurophils, neuronal cytoplasm clearly demarcated from surrounding neurophils, and less than a third of the neuron surrounded by confluent vacuolization) (Stummer et al., 1994). The mean number of CA1 pyramidal neurons per millimeter for both hemispheres in a section of dorsal hippocampus was calculated for each group of gerbils. The sections were evaluated by an investigator blinded to the experimental conditions.

2.11. Statistical analysis

Data were reported as mean±S.E.M. Statistical analysis was performed using ANOVA (2×2 ANOVA for biochemical parameters: $OH^{\cdot}$, MDA, GSH, GSH-P_X, CAT, SOD, and histological data; 2×2×2 ANOVA for locomotor behavior). Individual comparisons were carried out using Newman–Keuls post-hoc test with significance levels set as $p<0.05$.

3. Results

3.1. Effect of E-DHA on hydroxyl radical production

As shown in Fig. 1, there was no significant difference ($p>0.05$) in the brain 2,5-DHBA/SA ratio between Sham group and E-DHA group. Ten minutes of cerebral ischemia resulted in a significant 48% ($p<0.01$) postischemic elevation in the ratio of I/R group to Sham group, but only 20% elevation ($p<0.05$) in I/R+E-DHA group over E-DHA group was observed at 1 min of reperfusion. The postischemic increase in the brain 2,5-DHBA/SA ratio was significantly suppressed ($p<0.05$) in I/R+E-DHA group compared with I/R group, which indicated that the E-DHA pretreatment significantly inhibited the hydroxyl radical formation following ischemia.

3.2. Effect of E-DHA on level of MDA

No significant difference ($p>0.05$) in the brain MDA level was found between the Sham group and the E-DHA group. The level of postischemic brain MDA observed at 60 min of reperfusion was significantly increased ($p<0.01$) in I/R as compared to Sham, but the postischemic-enhanced MDA level was reduced significantly ($p<0.01$) in I/R+E-DHA group compared with I/R group (Table 1).

Table 1
Effects of E-DHA pretreatment on brain MDA level, GSH content, GSH-P_X, CAT, and SOD activities at 60 min of reperfusion after 10 min of transient cerebral ischemia

	Sham	E-DHA	I/R	I/R+E-DHA
MDA (nmol/mg protein)	9.90±0.87	10.53±0.97	22.18±1.36**	$15.57\pm0.82^{\#\#,++}$
GSH (μg/g wet wt)	2.35±0.13	$2.66\pm0.19^{\S}$	2.08±0.20*	$2.49\pm0.17^{\#\#}$
GSH-P_X (U/mg protein)	4.19±0.34	$4.70\pm0.28^{\S}$	3.55±0.23**	$4.14\pm0.32^{\#\#,+}$
CAT (U/mg protein)	1.01±0.13	$1.25\pm0.15^{\S}$	0.71±0.10**	$0.89\pm0.12^{\#,++}$
SOD (U/mg protein)	8.83±0.50	9.25±0.81	6.93±1.26**	$7.34\pm0.82^{++}$

Values are expressed as the mean±S.E.M. of six animals.
* $p<0.05$, I/R vs. Sham.
** $p<0.01$, I/R vs. Sham.
$^{\#}$ $p<0.05$, I/R+E-DHA vs. I/R.
$^{\#\#}$ $p<0.01$, I/R+E-DHA vs. I/R.
$^{+}$ $p<0.05$, I/R+E-DHA vs. E-DHA.
$^{++}$ $p<0.01$, I/R+E-DHA vs. E-DHA.
§ $p<0.05$, E-DHA vs. Sham.

Table 2
Effects of E-DHA pretreatment on the level of locomotor activity on days 3 and 5 of reperfusion after 10 min of transient cerebral ischemia

	3 days	5 days
Sham	496±86	479±73
E-DHA	473±108	445±86
I/R	988±188**	732±114**
I/R+E-DHA	703±125$^{\#\#,++}$	582±91$^{\#,+}$

Values are expressed as the mean±S.E.M. of six animals.
** $p<0.01$, I/R vs. Sham.
$^{\#}$ $p<0.05$, I/R+E-DHA vs. I/R.
$^{\#\#}$ $p<0.01$, I/R+E-DHA vs. I/R.
$^{+}$ $p<0.05$, I/R+E-DHA vs. E-DHA.
$^{++}$ $p<0.01$, I/R+E-DHA vs. E-DHA.

3.3. Effect of E-DHA on content of GSH

The E-DHA group showed a significant increase ($p<0.05$) in brain GSH content as compared to the Sham group (Table 1). The content of brain GSH at 60 min of reperfusion was depleted significantly ($p<0.05$) in the I/R group compared with the Sham group, but the depletion of postischemic GSH level was significantly prevented ($p<0.01$) in the I/R+E-DHA group as compared to the I/R group.

3.4. Effect of E-DHA on activities of antioxidant enzymes

The E-DHA group also showed a significant elevation in the activities of brain GSH-P_X ($p<0.05$) and CAT ($p<0.05$), but not SOD ($p>0.05$), as compared to the Sham group (Table 1). The activities of GSH-P_X, CAT, and SOD, detected at 60 min of reperfusion, were decreased significantly in the I/R group compared with the Sham group ($p<0.01$). The extent of postischemic declines in the activities of GSH-P_X and CAT was significantly attenuated in the I/R+E-DHA group ($p<0.01$ and $p<0.05$, respec-

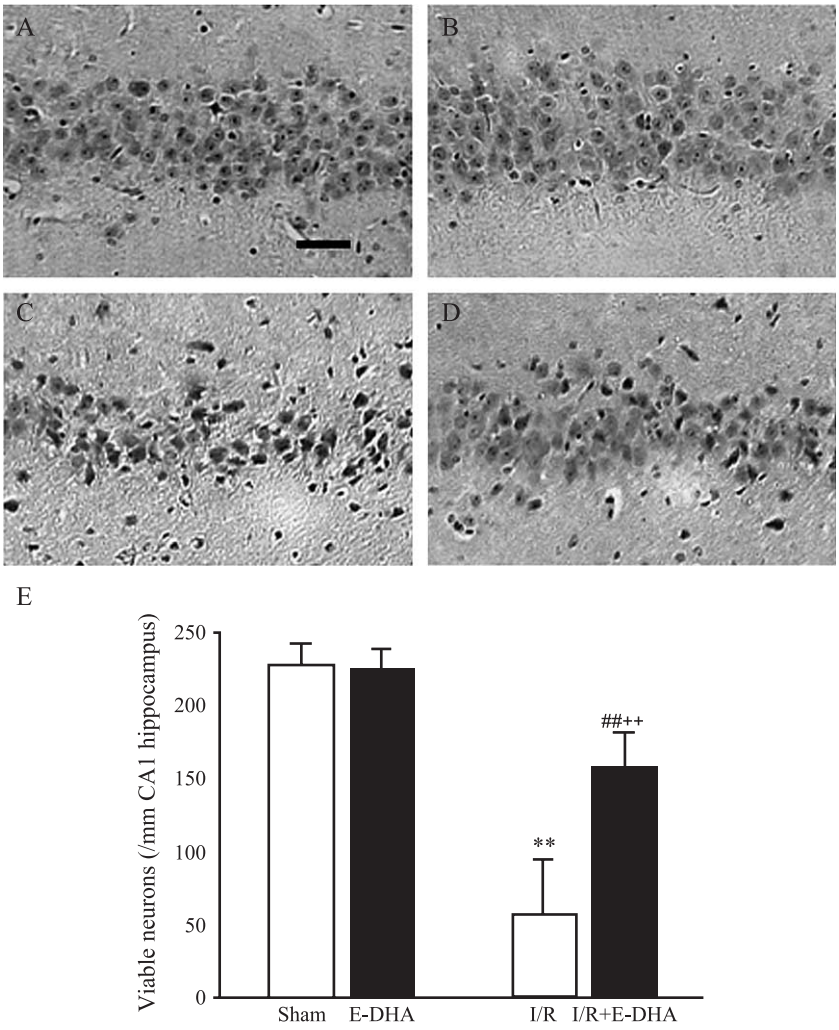

Fig. 2. Effects of E-DHA pretreatment on neuronal damage in the hippocampal CA1 region in gerbils subjected to transient cerebral ischemia and reperfusion. Microphotographs of the hippocampal CA1 regional neuron at 5 days of reperfusion after 10 min of transient cerebral ischemia (hematoxylin and eosin staining). (A) Sham; (B) E-DHA; (C) I/R; and (D) I/R+E-DHA. Magnification (A–D) ×20; scale bar=50 μm. (E) Viable neurons per millimeter of the hippocampal CA1 region. Values are expressed as the mean±S.E.M. of six animals. **$p<0.01$, I/R vs. Sham; $^{\#\#}p<0.01$, I/R+E-DHA vs. I/R; $^{++}p<0.01$, I/R+E-DHA vs. E-DHA.

tively) as compared to the I/R group. But when compared to the I/R group of animals, E-DHA did not induce a significant increase in SOD activity in the I/R+E-DHA group ($p>0.05$).

3.5. Effect of E-DHA on locomotor activity

There was no significant difference ($p>0.05$) found in preischemia basal level between the Sham group (559±81) and the E-DHA group (545±94). When compared to Sham group animals on days 3 and 5 after the onset of reperfusion, the E-DHA group of gerbils also failed to show a significant difference in the level of locomotor activity ($p>0.05$), but the gerbils in I/R group exhibited a significant increase in locomotor activity ($p<0.01$) on all test days (Table 2). The degree of increase in locomotor activity caused by cerebral ischemia and reperfusion was significantly reduced in the I/R+E-DHA group as compared to the I/R group ($p<0.05$).

3.6. Effect of E-DHA on histological changes

Histological examination of the brain demonstrated no significant cell damage in the hippocampal CA1 region in E-DHA group gerbils, but marked neuronal damage in I/R group gerbils when compared with Sham group animals. CA1 pyramidal neurons showed pyknosis, eosinophilia, karyorrhexia, and chromosome condensation in the I/R group. Fig. 2 shows that by 5 days after ischemia, there is a 75% loss of hippocampal CA1 neurons in I/R group, but only a 31% loss in the I/R+E-DHA group compared with the Sham group. This neuronal cell damage caused by cerebral ischemia and reperfusion was significantly reduced in the I/R+E-DHA group as compared to the I/R group ($p<0.01$).

4. Discussion

The present study demonstrated that E-DHA (200 mg/kg/day) pretreatment for 10 weeks produced beneficial effects on postischemic histological, behavioral, and biochemical changes induced by transient cerebral ischemia and reperfusion in gerbils, as evidenced by: (i) evelation of hydroxyl free radical scavenging capacity; (ii) inhibition of lipid peroxidation; (iii) attenuation of the depletion of GSH level; (iv) prevention of decline in the activities of GSH-P_X and CAT, although not affecting SOD activity; (v) reduction of locomotor hyperactivity; and (vi) decrease in the hippocampal neuronal loss.

The generation of oxygen free radicals as well as free radical-mediated oxidative damage of macromolecules (lipids, proteins, and DNA), and neuronal cell death in the processes of cerebral ischemia and reperfusion have been widely studied (Oliver et al., 1990; Chen et al., 1997). Furthermore, the oxygen free radical scavengers have been reported to ameliorate ischemic damage in gerbils and rats (Phillis, 1989; Martz et al., 1989). The hydroxyl radicals ($^{\cdot}OH$) are the most highly reactive among oxygen free radicals. A major source of $^{\cdot}OH$ in biological system is the reactive sequence of superoxide radical ($O_2^{-\cdot}$) with hydrogen peroxide (H_2O_2) in the presence of the iron ion (Fenton reaction) (Ikeda and Long, 1990). The formation of $^{\cdot}OH$ was indicated (Cao et al., 1988) and confirmed (Morimoto et al., 1996) by an increase in cerebral hydroxylated SA in postischemic insult. Our results showed that the level of 2,5-DHBA/SA ratio, which indicates $^{\cdot}OH$ generation, was significantly increased in the brain of the I/R group of animal during the first minute of reperfusion after ischemia. This is consistent with the findings of Hall et al. (1993), who indicated a burst of generation of $^{\cdot}OH$ during the 1-min reperfusion after 10 min of globe ischemia in gerbils. The E-DHA pretreatment significantly reduced the 2,5-DHBA/SA ratio that was increased by ischemia and reperfusion, and thus it is likely that the reduction in lipid peroxidation is attributable to the decrease in hydroxyl radical level in this study. In fact, E-DHA has been reported to enhance free hydroxyl radical scavenging and decrease lipid peroxidation in the rat fetal brain (Green et al., 2001). Although it has been suggested that 2,5-DHBA could be produced by the cytochrome *P*450 system in humans and other mammals (Floyd et al., 1986; Grootveld and Halliwell, 1986), recent experiments clearly demonstrate that both 2,3-DHBA and 2,5-DHBA can be used as indices of $^{\cdot}OH$ formation in the brain (Chiueh et al., 1994).

Another one of the reactive oxygen free radicals is H_2O_2, the product of dismutation of $O_2^{-\cdot}$, which can form $^{\cdot}OH$ via the Fenton reaction. In biological systems, $O_2^{-\cdot}$ can be scavenged mainly by SOD. The toxic H_2O_2 can be decomposed to water by CAT and GSH-P_X. GSH also participates in the reductive detoxification of H_2O_2 (Ikeda and Long, 1990; Chan, 1996). During the ischemia and reperfusion, the H_2O_2 cannot be readily scavenged because of low activities of SOD, CAT, and GSH-P_X, and the low level of GSH (Sinet et al., 1980) in the brain. In the present study, the pretreatment with E-DHA significantly increased the brain GSH-P_X and CAT activities and GSH level, but did not significantly affect the SOD activity, which indicated that E-DHA protects against ischemia and reperfusion-induced increases in H_2O_2 and further lipid peroxidation might be due to increasing H_2O_2-scavenging ability of the brain tissue, without preventing the H_2O_2 formation. Results from the study of Hossain et al. (1999) have suggested that E-DHA pretreatment plays an important role in inducing an antioxidative defense against active oxygen by enhancing the cerebral activities of CAT, GSH-P_X, and GSH.

Brain trauma is linked to the generation of reactive oxygen free radicals and production of lipid peroxidation. The evidence that oxygen free radical and lipid peroxidation mediates oxidative stress-induced neuronal death has been documented (Hall and Braughler, 1989). In this study, during the period of reperfusion following ischemia, we

observed a significant increase in brain MDA level and a significant neuronal death in the CA1 region of the gerbil hippocampus. E-DHA pretreatment significantly suppressed MDA increase and protected against neuronal death. This suggested that the protective effect that E-DHA has on hippocampal damage is related to the inhibitory effect on lipid oxidative damage, which leads to a protective effect on the cell death induced by ischemia and reperfusion.

The ischemia generally results in significant changes in behavior mainly due to hippocampal damage. It has been established that ischemia-induced locomotor hyperactivity correlates with the severity of CA1 injury (Kuroiwa et al., 1991). Since the open field test has been shown to be a sensitive indicator of an animal's ability to habituate to a novel environment and increased activity in the open field correlates with ischemic injury to CA1 but not to other brain regions and predicts histological outcome as effectively as T-maze tests (Colbourne and Corbett, 1995), the effect that E-DHA pretreatment has on the locomotor activity in open field test after an ischemia and reperfusion-induced change was examined. In the present study, E-DHA significantly inhibited the postischemic increase in locomotor activities measured on days 3 and 5 of reperfusion after ischemia. Hatakeyama et al. (1988) reported that the postischemic lesions in the CA1 region extended laterally after 2 days of reperfusion following 10 min of ischemia and covered the entire CA1 region after 3 days of reperfusion. Our study showed that on day 5 of reperfusion after 10-min ischemia, there is a 75% loss of hippocampal CA1 neurons in the I/R group, but only a 31% loss in the I/R+E-DHA group compared with the Sham group. These results suggested that global ischemia induces CA1 neuronal damage, which consequently results in disruption of hippocampal function (such as spatial mapping) leading to hyperactivity, and it is possible that the protective effect of E-DHA on hippocampal damage might contribute to a beneficial effect on the behavioral deficits caused by ischemia and reperfusion. Our results are in agreement with those reported by Okada et al. (1996), who showed that chronic administration of DHA contributes to protection against neuronal damage in the hippocampal CA1 region and reduced cognitive deficit in ischemic rats.

These findings clearly demonstrate that the protective effects of E-DHA pretreatment on hippocampal damage and behavior deficits induced by ischemia and reperfusion are at least in part the result of its antioxidant property. However, the precise mechanism of this purported neuroprotective effects is still unknown, and, indeed, these findings are paradoxical, given the high oxidizability of E-DHA (Wagner et al., 1994). The explanation of our findings may involve the effects of E-DHA pretreatment on the brain arachidonic acid (AA) content and its oxidation. Under conditions of ischemia and reperfusion, AA oxidation by the prostaglandin synthase complex is a major contributor to oxygen free radical formation in the brain (Hall et al., 1993). Inhibition of the oxygen free radical generation and lipid peroxidation production is observed in the presence of cyclooxygenase inhibitors (Kukreja et al., 1986; McGowan et al., 1994). Because DHA is a strong inhibitor of prostaglandin synthesis (Corey et al., 1983), it could decrease oxygen free radical generation and thus reduce the susceptibility to lipid peroxidation. Furthermore, an increased ischemia-evoked release of membrane AA in the brain is mainly related to increased phospholipase A_2 (PLA_2). It was reported that the activity of PLA_2, a rate-limiting step in the release of AA from membrane phospholipids, could be reduced by DHA (Martin, 1998), which could reduce the AA release and the availability of AA for oxidation. In addition, the ratio of DHA/AA is considered to be a marker for host defense capability against damage induced by oxidants. Dietary DHA facilitates the expulsion of cerebral AA with a concomitant increase in the DHA/AA ratio, and potentiates the CAT and GSH-P_X activities, which might provide a line of defense against the oxidative stress (Hossain et al., 1999). Moreover, by replacing AA with DHA, the relative contents of DHA carrying brain aminophospholipids, such as phosphatidylserines (PS) and phosphatidylethanolamine (PE), are increased after E-DHA administration (Green et al., 2001). The PE and PS have been shown to possess lipid peroxidation-inhibitory capacities and high antioxidant properties (Yoshida et al., 1991; Sindelar et al., 1999), which also offer a possible explanation for the protection of the brain from oxidation found in the present study.

The glutamate is also associated with oxidative stress in neurodegenerative disorder (Siesjö, 1992; Parnham and Sies, 2000). There is now good evidence that ischemia and reperfusion of the brain lead to a rapid increase in the extracellular level of glutamate, which results in activation of glutamate receptors, an increase in intracellular calcium, and, subsequently, the generation of oxygen free radicals (Dawson et al., 1991). Recently, DHA has been reported to have a neuroprotective effect against glutamate neurotoxicity in the cultured hippocampal cells in our laboratory (Wang et al., 2003). The contribution of this possible action of DHA in protecting neuronal injury cannot be ruled out in the present study. Thus, in order to elucidate the precise mechanism of the neuroprotective effects of E-DHA against reperfusion brain injury following ischemia, further investigation will be required.

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