Enriched endogenous omega-3 fatty acids in mice protect against global ischemia injury

Chuanming Luo,1,* Huixia Ren,1,* Jian-Bo Wan,* Xiaoli Yao,1 Xiaojing Zhang,* Chengwei He,* Kwok-Fai So,1 Jing X. Kang,** Zhong Pei,2,† and Huanxing Su2,*

State Key Laboratory of Quality Research in Chinese Medicine,* Institute of Chinese Medical Sciences, University of Macau, Macao, China; Department of Neurology,† National Key Clinical Department and Key Discipline of Neurology, The First Affiliated Hospital Sun Yat-Sen University, Guangzhou 510080, China; Guangdong-Hong Kong-Macau Institute of CNS Regeneration,3 Jinan University, Guangzhou 510632, PR China; and Laboratory for Lipid Medicine and Technology,** the Department of Medicine, Massachusetts General Hospital and Harvard Medical School, Boston, MA.

Abstract  Transient global cerebral ischemia, one of the consequences of cardiac arrest and cardiovascular surgery, usually leads to delayed death of hippocampal cornu Ammonis1 (CA1) neurons and cognitive deficits. Currently, there are no effective preventions or treatments for this condition. Omega-3 (ω-3) PUFAs have been shown to have therapeutic potential in a variety of neurological disorders. Here, we report that the transgenic mice that express the fat-1 gene encoding for ω-3 fatty acid desaturase, which leads to an increase in endogenous ω-3 PUFAs and a concomitant decrease in ω-6 PUFAs, were protected from global cerebral ischemia injury. The results of the study show that the hippocampal CA1 neuronal loss and cognitive deficits induced by global ischemia insult were significantly less severe in fat-1 mice than in WT mice controls. The protection against global cerebral ischemia injury was closely correlated with increased production of resolvin D1, suppressed nuclear factor-kappa B activation, and reduced generation of pro-inflammatory mediators in the hippocampus of fat-1 mice compared with WT mice controls. Our study demonstrates that fat-1 mice with high endogenous ω-3 PUFAs exhibit protective effects on hippocampal CA1 neurons and cognitive functions in a global ischemia injury model.—Luo, C., H. Ren, J-B. Wan, X. Yao, X. Zhang, C. He, K-F. So, J. X. Kang, Z. Pei, and H. Su. Enriched endogenous omega-3 fatty acids in mice protect against global ischemia injury. J. Lipid Res. 2014. 55: 1288–1297.

Transient global ischemia, caused by cardiac arrest or cardiac surgery, initiates selective delayed death of hippocampal cornu Ammonis1 (CA1) pyramidal neurons and cognitive deficits 3–7 d after insult (1–4). Despite tremendous advances made in understanding the pathophysiology of global ischemia injury, the desired preventive or neuroprotective drug for this condition has not been developed.

Several studies suggest that increasing intake of omega-3 (ω-3) PUFAs in the diet improves neurological and histological outcomes in the focal cerebral ischemia model (5–9). However, the exact mechanisms underlying the effects of ω-3 PUFAs on ischemic brain injury remain unknown. It is reported that inflammatory signaling plays important roles in ischemia-induced programmed neuronal death (10). Several studies provide evidence that ω-3 PUFAs are able to modulate inflammatory responses, especially influencing inflammatory gene expression via inhibition of activation of nuclear factor kappa B (NF-κB), one of the most important transcription factors involved in inflammatory responses (9, 11–13). Therefore, it could be interesting to investigate whether ω-3 PUFAs can reduce inflammatory responses after global ischemia/reperfusion injury and thus protect hippocampal neuronal death.

A transgenic mouse expressing the Caenorhabditis elegans fat-1 gene provides a tool to study the role of ω-3 PUFAs in ischemic brain injury (14). The fat-1 gene, which is absent in mammals, encodes ω-3 desaturase that converts ω-6 to ω-3 PUFAs. When fat-1 mice and WT mice are maintained on a diet high in ω-6 PUFAs, fat-1 mice are capable of producing ω-3 PUFAs from the ω-6 type, leading to elevated amounts of ω-3 PUFAs and higher ω-3 PUFAs/ω-6 PUFAs ratio in their tissues compared with the WT mice (14–16). In the present study, we developed transient global brain ischemia induced by occlusion of the bilateral common...
carotid arteries (2-VO) on fat-1 mice. We report here that the enriching endogenous ω-3 PUFAs in brain tissue inhibited inflammation, reduced CA1 neuronal loss, and improved cognitive functions in fat-1 transgenic mice compared with their nontransgenic counterparts.

MATERIAL AND METHODS

Animals and diets

We obtained fat-1 breeders on a C57BL/6 background from Dr. Jing X. Kang (Harvard Medical School) and raised them in the Laboratory Animal Center, University of Macau. Mice were housed in a temperature-controlled, 12:12 light/dark room and were allowed free access to water and food. The heterozygous fat-1 mice and WT littermates were obtained by mating male heterozygous fat-1 mice with female C57BL/6 mice. The fat-1 phenotypes of each animal were characterized using isolated genomic DNA and fatty acid composition analysis from mouse tails. Both the fat-1 and WT mice were fed a modified diet containing 10% corn oil (TROPHIC Animal Feed High-tech Co., Ltd, China), with a fatty acid profile high in ω-6 PUFAs (mainly linoleic acid) and low in ω-3 PUFAs (~0.1% of the total fat supplied).

Fatty acid analysis

To examine whether the expression of the fat-1 gene and the dietary regime altered the PUFA composition in the hippocampus, the naïve (non-ischemic) hippocampus of the two experimental groups was isolated and processed for fatty acid analysis by using GC-MS as described previously (17). Briefly, tissue samples were ground to powder under liquid nitrogen and subjected to fatty acid methylation by 14% boron trifluoride-methanol reagent at 100°C for 1 h. Fatty acid methyl esters were analyzed by an Agilent GC-MS system (Agilent Technologies, Palo Alto, CA) consisting of an Agilent 6890 gas chromatograph and an Agilent 5973 mass spectrometer. Fatty acids were identified in forms of their methyl esters by three means: i) searching potential compounds from NIST MS Search 2.0 database, ii) comparing retention time with those of reference compounds (Nu-Chek Prep, Elysian, MN) eluted under the identical chromatographic condition, and iii) comparing their mass spectra plots with those of authentic standards. Quantification was performed by normalizing individual peak area as the percentage of total fatty acids.

Transient global ischemia model

All studies were conducted in accordance with prevailing laws on animal experiments and were approved by the ethical committee of the University of Macau. A total of 60 mice fed a high ω-6 PUFA diet were used in the study. Among them, 48 mice (24 fat-1 mice and 24 WT mice with a high ω-6 PUFA diet) were subjected to transient cerebral ischemia produced by 20 min bilateral common carotid artery occlusion (2-VO) with small vascular clips and 12 mice (6 fat-1 mice and 6 WT mice) served as sham-operated controls (sham). In addition, an extra group consisting of 12 WT mice with a normal diet served as baseline controls in the present study. The animals were deeply anesthetized with 0.035ml/10g 10% chloral hydrate and the rectal temperature was maintained at 37 ± 0.5°C with a regulated heating pad (TR-200, FST, CA) throughout the surgery. Mice were excluded if their rectal temperature was out of the range of the criterion (36.5–37.5°C) during ischemic surgery. The animal was placed on its back and an incision was made in the ventral neck skin. The thyroid and associated musculature and connective tissue were carefully dissected away from the trachea, and the common carotid arteries were separated from the vagus nerves and adjacent muscles using blunt forceps. A suture (5-0, silk) was looped around each common carotid artery and then the bilateral common carotid arteries were clamped with small vascular clips. Reperfusion was induced by removing the small vascular clips after 20 min occlusion. Shams were subjected to the same surgical procedure but without actual ligation. We measured regional cerebral blood flow from the start of anesthetic induction to 5 min after reperfusion with a Laser Doppler Blood Flow and Temperature Monitor System (moor-VMS-LDF™, Moor Instruments Ltd, UK). Animals with a mean cerebral blood flow successfully reduced to 10% baseline cerebral blood flow within the first minute of occlusion and recovered at least up to 75% after reperfusion were used in subsequent data analysis. Heart rate and blood oxygen saturation were continuously monitored via a pulse oximeter (MouseOx; Starr Life Sciences Corp., Oakmont, PA) clipped to the hind paw of the mouse. After surgery, all mice were allowed to recover for 24 h in a recovery cage (Harvard) and had free access to drinking water. Thereafter, mice were returned to general housing (22°C to 24°C). Overall, around 18% of all animals were excluded because of insufficient cerebral blood flow reduction and around 12% of animals died during or immediately after surgery. No further losses were observed after recovery from surgical anesthesia.

Morris Water Maze Test

Spatial learning and memory abilities were tested using the Morris Water Maze test on days 1, 3, 5, and 7 after the surgical procedures. A circular pool (90 cm diameter, 35 cm height) divided into four quadrants was filled with white opaque water by the addition of water-insoluble and nontoxic titanium dioxide (24 ± 1°C), and an 8 cm diameter × 25 cm platform was positioned 1 cm below the water surface in the center of one of the quadrants. The swimming trials were recorded using a video camera mounted above the center of the pool and analyzed using a video tracking and analysis system (Institute of Medica, Chinese Academy of Medical Sciences). One day prior to testing, animals were habituated to the water by swimming for 1 min, henceforth, the mice received four consecutive training trials during which the hidden platform was kept in a constant location. During the testing days, the mice started each trial facing the edge of the pool and were given 60s to complete a trial. The time to reach the platform was recorded. If a mouse could not find the platform within 60s, it was guided toward the platform and then permitted to stay on for 10s.

Space probe trial

On the 7th day, memory retention was evaluated during a 60s probe trial carried out 24 h after the last training session in the absence of the escape platform. The total number of crossings to the exact position where the platform was located was measured.

Tissue preparation

For histologic evaluation, on day 7 after 2VO and sham-operation, a total of 36 mice (n = 6 per subgroup) were perfused transcardially with 50ml of ice-cold saline followed by 200 ml of 4% (w/v) formaldehyde in PBS. Brains were then incubated overnight in 20°C. Brain tissue blocks were consecutively cryosectioned into 10 µm thick sections with a cryostat microtome (Leica Microsystems Inc., Jena, Germany). In order to ascertain that the hippocampus CA1 neurons were counted at the same rostrocaudal level, we first dissected the same regions from each brain using the Bregma as a reference point. Then we collected 10 µm thick coronal slices of the dorsal hippocampus (~3.3 mm to ~4.5 mm from the Bregma) at the same levels of sectioned specimens. A total of 30 tissue sections per brain were made and stored at ~4°C.
For protein analysis and QPCR analysis, on days 1, 3, and 7 after 2-VO, a total of 24 mice with a high ω-6 PUFA diet (fat−I: n = 4 per time point; WT: n = 4 per time point) were perfused transcardially with 50 ml of ice-cold saline. Brains from the remaining mice were rapidly removed from the skulls. These brains were placed in a brain blocker maintained on ice. The bilateral hippocampi were isolated and then further processed for protein extraction and total RNA isolation.

**Nissl staining**

The frozen sections were selected at intervals of 6 in consecutive frozen section (10 μm thickness, 50 μm apart), dried at room temperature, rehydrated, and immersed in 0.1% cresyl violet (5 min) for Nissl staining. The sections were rinsed in distilled water, dehydrated in a graded series of ethanol (70%, 80%, 90%, and 100%), cleared in xylene, and coverslipped with xylene using Permount. The number of cells that presented a well-delimited, spherical form with a distinct nucleus and nucleolus was counted in the hippocampus CA1 region per section at 40× magnification; we counted the number of neurons in five random sections from each animal. The final average number of intact neurons in the hippocampus CA1 region for each animal (total of six animals in each group).

**TUNEL staining**

Apoptosis was determined by using the in situ cell death detection, Fluorescein isothiocyanate (FITC)–dUTP (Roche Applied Science), according to the manufacturer’s instructions. Briefly, sections were treated with 0.3% Triton and 10% anti-donkey serum for 1 h at room temperature. Subsequently, the sections were incubated with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) reaction mixture for 1 h at 37°C in the dark. After washing with PBS, the sections were incubated with the polyclonal antibody NeuN (1:500, Millipore) overnight at 4°C. Then the sections were incubated with the secondary antibody Alexa Fluor® 555 donkey anti-rabbit IgG (1:500, Invitrogen) for 1 h at room temperature in the dark and washed with PBS again. Cells expressing NeuN with red fluorescence were considered to be surviving neurons and cells with green fluorescence in the nucleus were considered to be the apoptotic cells under the microscope. Total TUNEL-positive cells and NeuN-positive cells in the hippocampus (CA1) were calculated in five random fields under the light microscope with 20× magnification. The final average number of TUNEL-positive cells and NeuN-positive cells of three sections from each animal was used for analysis and the severity of brain damage was evaluated by apoptotic index defined as the percentage of TUNEL-positive cells over NeuN-positive cells.

**Immunofluorescence**

To assess reactive astrogliosis and microgliosis of hippocampus CA1 region after 2-VO, frozen sections were selected at intervals of six in consecutive frozen sections. The frozen sections were treated with 0.3% Triton and 10% anti-donkey serum for 1 h at room temperature. Subsequently, the sections were incubated with goat polyclonal antibody Iba-1 (1:400, abCAM) and monoclonal antibody glial fibrillary acidic protein (GFAP) (1:500, abCAM) overnight at 4°C in dark, and then incubated with secondary antibodies at 37°C for 1 h in the dark. The secondary antibodies were Alexa Fluor® 488 donkey anti-mouse IgG (1:500, Invitrogen) and Alexa Fluor® 555 donkey anti-goat IgG (1:500, Invitrogen) respectively. The expression of Iba-1 and GFAP was examined using the Olympus fluorescence microscope (20× magnification). Quantification of Iba1 labeled macrophages/microglia and GFAP labeled astrocytes was performed using the image analysis program ImageJ (ImageJ 1.90u, the National Institutes of Health). For evaluating the average density of fluorescence, three measuring frames of 1360 × 1024 pixels in the hippocampus (CA1) region per sample was used for analysis. In the ImageJ program, these images were converted to a binary image using a threshold that was set manually at a level to detect most of the finest microglial or astrocytes processes and then counted automatically using the “analyze particles” program. The level of immunoreactivity was expressed as the percentage of the area of the measuring frame that contained immunoreactivity (field detected area).

**Real-time PCR**

The total hippocampus RNA was isolated using the E.Z.N.A.® Total RNA Kit II (Omega Bio-Tech, Doraville, GA). cDNA was produced using Taqman reverse transcription kit (TaKaRa Biotechnology, Dalian). Real-time PCR was performed on an ABI-Prism 7700 using SYBR Green II as a double stranded DNA specific dye according to the manufacturer’s instructions (TaKaRa Biotechnology, Dalian). Each sample was tested in triplicate and the average values were used for calculations. The primers used were 5′-CTGCC-CCTCTGATCATTTGTT-3′ and 5′-AGATTTTCTCCATGCGGT-TGG-3′ for GPR120, 5′-CATTGCTAAGACCTCTATGGAACAG-3′ and 5′-ATGGAGCCACCGATCCACA-3′ for β-actin.

**Western blot assay**

The nuclear and cytoplasmic proteins of the hippocampus were extracted by Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Institute of Biotechnology, China), respectively, according to the manufacturer’s instructions. SDS-PAGE (7.5% gel) was carried out on each sample (30 μg of protein). Separated proteins were subsequently electrotransferred to 0.2 μm nitrocellulose membranes at 16 V for 30 min. The membranes were blocked with 5% fat-free milk in TBS containing 0.05% Tween 20 (TBST; 2 h at room temperature) and washed in TBST. The blots were probed with primary antibodies: NF-κB P65 (Sigma) diluted 1: 1000, α-tubulin (Sigma) diluted 1: 2000, and histone 3 (Sigma) diluted 1: 2000 at 4°C overnight, washed for 30 min in TBST, incubated with horseradish peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG (both at 1:5000) for 2 h at room temperature, and visualized using a DAB kit. Densitometric quantification of blots was carried out using Alphapart 11 Ease version 5.0, and a ratio relative to control was calculated.

**ELISA**

The supernatant of the hippocampus homogenate (10%) was collected after 4,000 × g centrifugation for 15 min. ELISA measurements were performed with TNF-α ELISA kit (BD), IL-1β, IL-6, MCP-1 ELISA kits (R and D), and RvD1 EIA kits (Cyman Chemical) following the recommendations of the manufacturers.

**Statistical analysis**

All values are expressed as mean ± SEM. ANOVA followed by post hoc Newman-Keuls multiple range tests was used for multiple groups. Significance was defined as P < 0.05 in all statistical analyses.

**RESULTS**

**Fatty acid profiles of the hippocampus in fat-1 mice and WT mice**

The profiles of PUFAs in the hippocampus from fat-1 mice and their WT littermates fed with a diet enriched in ω-6

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PUFAs were analyzed (Table 1). A significantly higher level of ω-3 docosapentaenoic acid (DPA) and DHA was found in the hippocampus of fat-1 mice compared with their WT littermates. Meanwhile, a concomitant decrease in ω-6 docosatetraenoic acid (DTA), EPA, and arachidonic acid (AA) was found in fat-1 mice with the marked effects found for ω-6 DTA and EPA. The ratio of omega-6/omega-3 PUFAs was significantly lower in fat-1 mice compared with WT mice.

**Improved spatial learning and memory in fat-1 mice after 2-VO**

To investigate whether the increase of endogenous ω-PUFAs affects the ability of hippocampus-dependent deficits in spatial learning and memory after 2-VO, we analyzed the performance of the mice in the Morris Water Maze task. Compared with their WT counterparts, fat-1 mice spent less time finding the hidden platform underneath the water (Fig. 1A, P < 0.05) on days 5 and 7 after 2-VO, indicating that the spatial learning ability of fat-1 mice was significantly increased as compared with the WT mice on either the high ω-6 PUFA diet or the normal diet. In the space probe trial, the number of platform crossings was significantly increased in the fat-1 mice as compared with the two WT controls at 7 days after 2-VO (Fig. 1B, P < 0.05). Taken together, these findings suggest that the increase of endogenous ω-PUFAs improved impairment of spatial learning and memory induced by transient global cerebral ischemia.

**Improved neuronal survival and attenuated neuronal apoptosis in the hippocampus CA1 region of fat-1 mice after 2-VO**

Next, we evaluated the neuroprotective effect of the increase of endogenous ω-PUFAs on ischemia-induced delayed neuronal death with histological analysis (by Nissl staining and TUNEL staining) in the hippocampus CA1 regions on day 7 after 2-VO surgery. The neuronal survival rate of the sham group was defined as 100% at the end of the experiment (day 7) (Fig. 2A). Significantly more surviving neurons were found in the hippocampus CA1 region of fat-1 mice compared with their WT counterparts on day 7 after 2-VO (68.2 ± 8.7 vs. 22.3 ± 5.8, P < 0.05) (Fig. 2A, B).

Cell apoptosis is one of the major causes for ischemia-induced delayed neuronal death in the hippocampus CA1 region (18). The severity of apoptosis in hippocampus CA1 neurons was evaluated by apoptotic index, defined as the percentage of TUNEL-positive cells in neurons. TUNEL-positive cells were indicated by apoptotic bodies and green staining. Apoptotic cells were not detectable in either sham group (Fig. 2C). Significantly fewer TUNEL-positive cells were found in the CA1 region of the fat-1 mice compared with their WT counterparts on day 7 after 2-VO (27.1 ± 4.8 vs. 49.5 ± 7.2, P < 0.05) (Fig. 2C, D).

**Inhibited NF-κB activation, reduced production of pro-inflammatory factors, and increased generation of Resolvin D1 in the hippocampus CA1 region of fat-1 mice after 2-VO**

Accumulating evidence demonstrates that programmed neuronal death is greatly enhanced in response to global ischemia (18, 19) and inflammatory signaling plays important roles in ischemia-induced programmed neuronal death (10, 20). It was reported that the G protein-coupled receptor 120 (GPR120) is a molecular target of ω-3 PUFAs for anti-inflammatory effects (21, 22). Therefore, we first investigated the expression of GPR120 in the hippocampus of fat-1 mice and their counterparts after global ischemia. Quantitative PCR analyses showed that the expression level of GPR120 did not change between fat-1 mice and their WT counterparts after global ischemia (Fig. 3A).

It is believed that NF-κB activation contributes to the infarction and neuronal death in middle cerebral artery occlusion models of rodents (23–25) as well as stroke patients (26). We then investigated the NF-κB activation as proved by nuclear translocation of the NF-κB subunit p65. Western blot assay revealed that the level of nuclear p65 was greatly increased on days 3 and 7 after transient global ischemia in WT mice (Fig. 3B, C), demonstrating that global ischemia significantly induces NF-κB activation. In contrast, the level of nuclear p65 was pronouncedly decreased on days 3 and 7 after transient global ischemia in fat-1 mice (Fig. 3B, C), indicating that enriched endogenous ω-3 PUFAs successfully inhibit NF-κB activation. Because NF-κB activation is known to stimulate pro-inflammatory gene transcription (20, 27), we then further measured the protein levels of TNF-α, IL-1β, IL-6, and MCP-1 in the hippocampus using ELISA analyses. As expected, the protein levels of TNF-α, IL-1β, IL-6, and MCP-1 in fat-1 mice were significantly decreased compared with those in WT mice at 7 days after global ischemia (Fig. 3D).

Resolvin D1, a metabolite derived from DHA, could function as an endogenous anti-inflammatory molecule that suppresses inflammation and helps in the resolution of inflammatory events including leukocyte infiltration.

**Table 1.** Profiles of polyunsaturated fatty acid in hippocampus from fat-1 transgenic mice and their WT littermates fed with a diet enriched in ω-6 PUFAs (n = 3)

| Fatty acid | WT | Fat-1 |
|-----------|----|-------|
| LA (C18:2 ω-6) | 0.73 ± 0.07 | 0.94 ± 0.06<sup>a</sup> |
| EDA (C20:2 ω-6) | 0.15 ± 0.02 | 0.14 ± 0.02 |
| DGLA (C22:3 ω-6) | 0.42 ± 0.02 | 0.67 ± 0.08<sup>b</sup> |
| AA (C20:4 ω-6) | 14.08 ± 0.49 | 13.08 ± 0.45<sup>c</sup> |
| DTA (C22:4 ω-6) | 3.86 ± 0.04 | 2.82 ± 0.12 |
| ω-6 DPA (C22:5 ω-6) | 2.82 ± 0.21 | 0.57 ± 0.05<sup>a</sup> |
| Total | 22.06 ± 0.21 | 18.23 ± 0.49<sup>b</sup> |
| ω-3 DPA (C22:5 ω-3) | 0.19 ± 0.00 | 0.41 ± 0.05 |
| DHA (C22:6 ω-3) | 15.87 ± 0.63 | 18.73 ± 0.87<sup>c</sup> |
| Total DHA | 16.06 ± 0.63 | 19.14 ± 0.84<sup>b</sup> |
| Total PUFA | 38.12 ± 0.42 | 37.37 ± 1.18 |
| SFA | 46.48 ± 1.43 | 46.85 ± 0.82 |
| MUFA | 15.40 ± 1.00 | 15.78 ± 1.05 |
| ω-6/ω-3 | 1.37 ± 0.07 | 0.95 ± 0.03<sup>a</sup> |

Data expressed as mol% of total fatty acids ± SD (<sup>a</sup>P < 0.05; <sup>b</sup>P < 0.01; <sup>c</sup>P < 0.001). AA, arachidonic acid; DGLA, dihomo-γ-linolenic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; DTA, docosatetraenoic acid; EDA, eicosadienoic acid; LA, linoleic acid; MUFA, monounsaturated fatty acids (the value is given as follows: C16:1 Δ9 + C18:1 Δ9 + C20:1 Δ9 + C22:1 Δ9); SFA, saturated fatty acids (the value is given as follows: C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C20:0 + C22:0 + C24:0); PUFA, polyunsaturated fatty acids.
and clearance of the cellular debris from the site of inflammation. A significant difference in the expression of Resolvin D1 was detected between fat-1 mice and WT controls after global ischemia injury in that the level of Resolvin D1 in the hippocampus of fat-1 mice was greatly increased on days 1 and 3 after transient global ischemia compared with the expression level in WT mice (Fig. 3E).

**Attenuated microglia activation and astrocytes reaction in the hippocampus CA1 region of fat-1 mice after 2-VO**

Under normal conditions, nonactivated microglia perform a surveillance function in the CNS. If ischemic/reperfusion develops, microglia become activated with morphological changes, secretion of chemokines and cytokines, and increased phagocytic activity, subsequently leading to inflammatory injury (12). We thus investigated whether the increase of endogenous ω-3 PUFAs had the ability to suppress microglia activation in the hippocampus CA1 region on 7 days after 2-VO. Microglia were stained with the antibody against Iba-1, which is known to be specifically expressed in macrophages/microglia and up-regulated during the activation of these cells. In the Sham group, only a few Iba-1-positive cells were observed as an inactivated form (smaller cell bodies; ramified microglia) in the hippocampus CA1 region (Fig. 4A). In the 2VO group, Iba1-positive cells were present in an activated form (larger cell bodies and short ramifications; amoeboid microglia) (Fig. 4A) and the level of Iba1 immunoreactivity was significantly increased compared with their counterparts sham group (0.86 ± 0.18 vs. 7.38 ± 0.86, P < 0.05; 1.0 ± 0.21 vs. 5.33 ± 0.53, P < 0.05) (Fig. 4A, C). However, in the fat-1 mice group, Iba1 immunoreactivity in the hippocampus CA1 region was significantly suppressed compared with the WT groups (7.38 ± 0.86 vs. 5.33 ± 0.53, P < 0.05) 7 days after 2-VO (Fig. 4A, C).

Aside from microglia, astrocytes are known to participate in inflammation in the ischemic brain injury by expressing major histocompatibility complex and secreting inflammatory factors (28). Following ischemia/reperfusion, brain astrocytes are activated, resulting in increased GFAP expression (29). Therefore, we investigated the effect of the increase of endogenous ω-3 PUFAs on ischemia-induced reactive astrocytosis. On day 7 following global ischemia/reperfusion, high levels of GFAP immunoreactivity were found in the hippocampus CA1 region of the WT mice (Fig. 4A). In the fat-1 mice group, the level of GFAP immunoreactivity was significantly lower than that of the counterpart 7 days after 2-VO (Fig. 4A, B; 11.0 ± 1.21 vs. 8.4 ± 1.25, P < 0.05).

**DISCUSSION**

Transient global ischemia caused by cardiac arrest and cardiovascular surgery leads to selective delayed death of hippocampal CA1 neurons and cognitive deficits (1–4, 18). The results of our study showed that the hippocampal CA1 neuronal loss and cognitive deficits induced by global ischemia insult were significantly less severe in fat-1 mice than in WT mice controls. Meanwhile, NF-κB activation was markedly suppressed in the hippocampus of fat-1 mice, which was accompanied with an increased level of Resolvin D1, a decreased level of pro-inflammatory mediators, and a reduction of astrocytic and microglial activation. Analysis of the fatty acid profiles showed that the expression of fat-1 gene significantly increased the concentration of ω-6 DPA and DHA, with a concomitant decrease in the concentration of ω-6 DTA, DPA, and AA in the hippocampus of fat-1 mice. Consistently, the ratio of ω-6/ω-3 PUFA was significantly lower in fat-1 mice compared with WT mice. Our study demonstrated that fat-1 mice with high endogenous ω-3 PUFAs exhibit protective effects on hippocampal CA1 neurons and cognitive functions in a global ischemia injury model.

The ω-3 PUFAs cannot be synthesized de novo in mammals and have to be supplied by the diet (14, 15). During
The last several decades, the ratio of ω-6/ω-3 PUFAs in the diet has changed dramatically from 1–2:1 previously to 15–20:1 presently, which leads to an increase in the intake of ω-6 PUFAs and a concomitant decrease in the intake of ω-3 PUFAs (30). The deficiency in ω-3 PUFAs is considered to be a risk factor for human mental and neurological disorders (13, 31, 32). Exogenous application of ω-3 PUFAs slowed the progression of Alzheimer’s disease in mice (33, 34), prevented Huntington’s disease (35), showed therapeutic potential for spinal cord injury (36–39), and improved neurological and histological outcomes in the focal cerebral ischemia model (5, 40–42). However, these beneficial effects of ω-3 PUFAs are not consistently detected (15, 43). One of the reasons is that feeding animals with different experimental diets may impose confounding effects on the results because of many variables arising from the diets and feeding procedures. Another reason is that some studies focused only on the therapeutic effects of ω-3 PUFAs and neglected the relevance of the ratio of ω-3/ω-6 PUFAs (44–46).

The fat-1 mouse provides a desirable model to study the effects of ω-3 PUFAs because it allows well-controlled studies to be performed without the interference of potential confounding factors (47). Many studies have used this model to interpret the beneficial effects of ω-3 PUFAs in various experimental paradigms (15, 16, 48–51). For example, fat-1 mice exhibited increased hippocampal neurogenesis evidenced by elevated density of dendritic spines of CA1 neurons, leading to a better spatial learning performance in the Morris Water Maze compared with control
activation (9, 58) are reported to be possible mechanisms underlying ischemia-induced programmed neuronal death. In the present study, we found a significant difference in the expression of Resolvin D1 between fat-1 mice and WT controls after global ischemia injury in that the level of Resolvin D1 in fat-1 mice was greatly increased on days 1 and 3 after transient global ischemia compared with the expression level in WT mice. Resolvin D1, a metabolite derived from DHA, could function as an endogenous anti-inflammatory molecule that suppresses inflammation and helps in the resolution of inflammatory events including leukocyte infiltration and clearance of the cellular debris from the site of inflammation. We therefore propose that the increased production of Resolvin D1 could be one of mechanisms underlying beneficial effects of fat-1 mice against ischemic injury.

WT littermates (16); fat-1 mice conferred robust protection against peripheral nerve injury and spinal cord injury (15, 51). In our present study, we reported that fat-1 mice protected CA1 neurons and cognitive functions against global ischemia.

Several mechanisms are considered to be involved in selective neuronal death and cognitive deficits in global ischemia injury (52). Accumulating evidence demonstrates that programmed neuronal death plays a pivotal role in global ischemia (18), although clear mechanisms underlying global ischemia-induced programmed death are not fully understood. The mitochondrial pathway of apoptosis (53), the death receptor such as the Fas receptor and the TNF receptor pathway of apoptosis (54, 55), oxidative stress induced cell death (56), cascades underlying lysosomal stabilization (20, 57), and cascades regulating NF-κB activation (9, 58) are reported to be possible mechanisms underlying ischemia-induced programmed neuronal death. In the present study, we found a significant difference in the expression of Resolvin D1 between fat-1 mice and WT controls after global ischemia injury in that the level of Resolvin D1 in fat-1 mice was greatly increased on days 1 and 3 after transient global ischemia compared with the expression level in WT mice. Resolvin D1, a metabolite derived from DHA, could function as an endogenous anti-inflammatory molecule that suppresses inflammation and helps in the resolution of inflammatory events including leukocyte infiltration and clearance of the cellular debris from the site of inflammation. We therefore propose that the increased production of Resolvin D1 could be one of mechanisms underlying beneficial effects of fat-1 mice against ischemic injury.
The results of our study showed that NF-κB activation was significantly suppressed in the hippocampus of fat-1 mice. NF-κB is a key regulator of hundreds of genes encoding proteins that play a determining role in the process of cell death and its activation stimulates pro-inflammatory gene transcription (27). It has been reported that ω-3 PUFAs suppress NF-κB activation via several pathways: ω-3 PUFAs could inhibit NF-κB activation via the peroxisome proliferator-activated receptor γ pathway (59), the toll-like receptors pathway (60), or GPR120 pathway (22). The results of the present study showed that the expression level of GPR120 did not change before and after global ischemia. However, GPR120 may play an important role without any change in its expression, because GPR120 is a receptor for ω-3 PUFAs, especially DHA. In the presence of high levels of DHA in fat-1 mice, GPR120 may become an important mechanism of action. Further work is needed to investigate whether GPR120 is activated due to high levels of DHA in fat-1 mice and is inactive due to relatively low levels of DHA in WT mice. Future work also needs to use GPR120 knockout mice to further clarify whether this receptor is involved in protective effects of ω-3 PUFAs against ischemic injury.

We did not monitor the brain temperature in the present study. During ischemic surgery, we measured rectal temperature, which was maintained at 37 ± 0.5°C with a regulated heating pad throughout the surgery. Mice were excluded if their rectal temperature was out of the range of the criterion (36.5–37.5°C). However, rectal temperature does not always accurately predict brain temperature. Our future study should monitor brain temperature during and after ischemia. Regarding brain temperature in global ischemia, it was reported that global ischemia in rodents could result in a prolonged period of mild hypothermia (61). This suggests that temperature measurement and control during and after ischemia are important. We
did not do rectal temperature measurement after the recovery period (1–7 days) because we have concerns that rectal probe measurements after the recovery period would cause a stress injury and confound the study. However, given that delayed hypothermia is cytotoxic and delayed fever is detrimental, temperature measurement for at least 2 postoperative days is strongly recommended (62). Therefore, a safe and convenient temperature monitor system should be used to monitor temperature postischemia in our future ischemic study.

Our study also shows that the protein levels of some pro-inflammatory factors (TNF-α, IL-1β, IL-6, and MCP-1) and the astroglial and microglial activation were reduced in the hippocampus of fat-1 mice after ischemia injury. The reduced production of pro-inflammatory factors could result from the suppression of NF-kB activation in fat-1 mice because NF-kB is closely related to pro-inflammatory gene transcription (27). It is also noted that the reduced production of pro-inflammatory factors in fat-1 mice could be achieved by direct suppression of pro-inflammatory factors by ω-3 PUFAs metabolites such as prostaglandin E3, resolvins, and protectins (63). The glial activation is closely related to inflammation in ischemic brain injury (12, 28). Taken together, our data suggest that the improved CA1 neuronal survival and cognitive function in fat-1 mice after ischemia injury are attributable to the inhibition of NF-kB activation, the reduced production of pro-inflammatory factors, and the suppression of glial activation in the hippocampus. However, there may exist other mechanisms by which endogenous ω-3 PUFAs protect ischemia-injured neurons. For example, ω-3 PUFAs and their metabolites prevent cell apoptosis by upregulating the expression of the Bcl-2 family of anti-apoptotic proteins (34, 64) and ω-3 PUFAs enhance expression of neurotrophins, including brain-derived neurotrophic factor (65). Whether these mechanisms are involved in neuronal protection of fat-1 mice against global ischemia needs to be explored in the future.

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