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# Detrimental effects of post-treatment with fatty acids on brain injury in ischemic rats

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**Abstract**

Studies have illustrated that fatty acids, especially polyunsaturated fatty acids (PUFA), have a role in regulating oxidative stress via the enhancement of antioxidative defense capacity or the augmentation of oxidative burden. Elevated oxidative stress has been implicated in the pathogenesis of brain injury associated with cerebral ischemia/reperfusion (I/R). The objective of this study was to assess whether treatment with fatty acids after focal cerebral I/R induced by occlusion of the common carotid arteries and the middle cerebral artery has effects on brain injury in a rat model. PUFA, including arachidonic acid (AA) and docosahexaenoic acid (DHA), and the saturated fatty acid, stearic acid (SA), were administrated 60 min after reperfusion via intraperitoneal injection. AA and DHA aggravated cerebral ischemic injury, which manifested as enlargement of areas of cerebral infarction and increased impairment of motor activity, in a concentration-dependent manner. However, there were no remarkable differences in post-ischemic alterations between the SA and saline groups. The post-ischemic augmentation of injury in AA and DHA treatment groups was accompanied by increases in the permeability of the blood–brain barrier (BBB), brain edema, metalloproteinase (MMP) activity, inflammatory cell infiltration, cyclooxygenase 2 (COX-2) expression, caspase 3 activity, and malondialdehyde (MDA) production, and by a decrease in the brain glutathione (GSH) content. Furthermore, we found that either AA or DHA alone had little effect on free radical generation in neuroglia, but they greatly increased the hydrogen peroxide-induced oxidative burden. Taken together, these findings demonstrate the detrimental effect of PUFA such as AA and DHA in post-ischemic progression and brain injury after cerebral I/R is associated with augmentation of cerebral I/R-induced alterations, including oxidative changes.


*Keywords:* Cerebral ischemia/reperfusion; Inflammation; Polyunsaturated fatty acid; Reactive oxygen species

## 1. Introduction

Cerebral ischemia or stroke is a leading cause of death and long-term disability in aged individuals. Ischemic stroke is caused by obstruction of blood flow to the brain, resulting in a failure of energy production that initiates a complex series of metabolic events culminating in the transformation of ischemic cerebral tissue into areas of infarction. In the acute phase, cell death in the ischemic core is conventionally considered necrotic, whereas after a short period of cerebral ischemia followed by reperfusion, the neurons in the penumbral regions undergo another wave of delayed cell death (apoptosis) (Garcia et al., 1995; Linnik et al., 1993). Evidence suggests that post-ischemic progression and neuronal injury are mediated by multiple mechanisms, such as excitatory neurotransmitter accumulation, calcium overload, free radical generation,

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inflammation, and apoptosis (Garcia et al., 1995; Linnik et al., 1993; Phillips et al., 2000; Siesjö, 1992; Yamagami et al., 1999; Yrjanheikki et al., 1999). Among the different mechanisms involved in the pathogenesis of cerebral ischemia/reperfusion (I/R) injury, there is increasing evidence to show that reactive oxygen species (ROS) and oxidative stress are important detrimental factors in post-ischemic progression. Specifically, the brain is characterized by a high rate of oxidative metabolic activities and a low capacity for antioxidative activities (Mizuno and Ohta, 1986; Sinet et al., 1980). The brain is exceptionally vulnerable to ROS, which cause oxidative damage to brain lipids, proteins, and nucleic acids, leading to brain dysfunction and cell death (Choi-Kwon et al., 2004). Therefore, ROS are critical intervention targets and studies have already shown the neuroprotective effects of free radical scavengers after cerebral I/R injury (Cao et al., 2004; Nagel et al., 2007).

Fatty acids are important for the histological, anatomical, and biochemical integrity of the brain, and fatty acid composition influences various physiological and biochemical processes. Many studies have documented the beneficial effects of polyunsaturated fatty acids (PUFA) and the potential detrimental effects of saturated fatty acids in humans. Case–control studies have shown that, compared with controls, stroke patients have lower proportions of PUFA and higher proportions of saturated fatty acids in their blood (Iso et al., 2002; Ricci et al., 1997). A lower level of PUFA in patients increases the risk and mortality of stroke (Simon et al., 1995; Tilvis et al., 1987). There is now considerable literature describing the beneficial effects of PUFA in the prevention of cerebral I/R injury in both human and animal studies (Belayev et al., 2005; Blondeau et al., 2002; Cao et al., 2004, 2005, 2006; Choi-Kwon et al., 2004; de Wilde et al., 2002; Katsumata et al., 1999; Lauritzen et al., 2000). In contrast, saturated fatty acid intake is found to correlate positively with stroke mortality (Sasaki et al., 1995). Generally, PUFA possess modulatory effects on the homeostasis of redox potential, either by activating antioxidant enzyme activity or by enhancing oxidative burden due to lipid peroxidation. Most neuroprotective effects of PUFA are demonstrated in dietary supplementation and/or chronic administration prior to cerebral I/R via the enhancement of antioxidative capacity. However, the effects of fatty acid treatment after cerebral I/R-induced brain alterations are largely unclear. Therefore, the present investigation was designed to evaluate the effects of post-treatment with fatty acids on post-ischemic brain injury in focal cerebral I/R-injured animals, and to attempt to elucidate the role and contribution of the oxidative burden in the underlying mechanism. The utilized fatty acids included the ω6 PUFA arachidonic acid (AA), the ω3 PUFA docosahexaenoic acid (DHA), and the saturated fatty acid stearic acid (SA).

## 2. Materials and methods

### 2.1. Animals and cerebral I/R

The Animal Experimental Committee of Taichung Veterans General Hospital approved the protocol of the animal study. Male Sprague–Dawley rats (300–350 g) were anesthetized with chloral hydrate (400 mg/kg) administered intraperitoneally. Body temperature was maintained at $37.0 \pm 0.5$ °C with a heating pad. A midline cervical incision on the ventral side was made in order to isolate the bilateral common carotid arteries (CCA). 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 CCA for 90 min followed by 24 h reperfusion, as described previously (Kao et al., 2006). In animals receiving sham operations, all surgical procedures were the same as above, but no arterial occlusion was performed. Fatty acid or saline vehicle was injected intraperitoneally 60 min after reperfusion. The dosage of fatty acids was determined and modified according to the methods of Blondeau et al. (2002).

### 2.2. Quantification of ischemic infarction

After 24 h reperfusion, the brains were quickly removed and chilled in cold phosphate-buffered saline (PBS) for 5 min. Coronal sections 2 mm thick were cut using a tissue slicer. Sections were immersed in PBS containing 2% triphenyltertrazolium chloride (TTC) at 37 °C for 30 min, after which they were fixed in 10% phosphate-buffered formalin for 45 min (Kao et al., 2006). TTC is reduced by certain enzymes in normal tissues to a deep red, fat soluble, light-sensitive compound that turns normal tissue deep red and thereby clearly delineates ischemic areas. The areas of infarction were measured using a computer image analysis system (Alpha Innotech Corporation, IS1000).

### 2.3. Behavioral observations

An accelerating rotarod was used to measure rat motor function as previously reported (Kao et al., 2006). The rats were placed on the rotarod cylinder, and the time for which the animals remained on the rotarod was measured. The speed was slowly increased from 4 to 40 rpm within 5 min. The animals were trained 3 days before occlusion. The mean duration on the device was recorded with three rotarod measurements before surgery as baseline. Motor test data is presented as the mean duration (three trials) on the rotarod as a percentage of each baseline control measurement.

### 2.4. Measurement of blood–brain barrier (BBB) permeability

BBB permeability was assessed by measurement of Evans blue content in 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 trichloroacetic acid (TCA) (100%) overnight at 4 °C. After centrifugation at 12,000 rpm for 30 min, Evans blue was quantified in the

samples (by absorbance at 620 nm). A standard Evans blue curve was generated using a standard solution.

### 2.5. Gelatinase zymography

Protein extracts were harvested from cortical tissues with potassium phosphate buffer containing 0.5% hexadecyltrimethyl-ammonium bromide (Liao et al., 2001). The protein concentration in the supernatant was determined by Bradford assay. Protein extracts (100 μg) were assayed for gelatinase activity by zymography and underwent electrophoresis in polyacrylamide gels containing 0.5 mg/ml gelatin in the presence of sodium dodecyl sulfate (SDS) under nonreducing conditions. After electrophoresis, the gels were washed twice in 2.5% Triton X-100 for 1 h, rinsed briefly, and incubated at 37 °C for 24 h in 100 mM Tris–HCl, pH 7.4, and 10 mM $CaCl_2$. Thereafter, gels were stained with Coomassie Brilliant R-250 and destained in a solution of 7.5% acetic acid and 5% methanol. Zones of enzymatic activity appeared as clear bands against a blue background. The zone areas were measured using a computer image analysis system (Alpha Innotech Corporation, IS1000).

### 2.6. 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 calculated by the wet and dry weight method as follows: water content (%) = [wet weight − dry weight]/wet weight × 100 (Liao et al., 2001).

### 2.7. Myeloperoxidase activity (MPO) assay

In brief, protein extracts (100 μl) were mixed with 2.9 ml of the assay solution. The optical absorbance was determined at 470 nm for 1 min with a spectrophotometer (Liao et al., 2001). Arbitrary activity was expressed as the value of OD470 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; guaiacol, 0.048 ml.

### 2.8. Caspase activity assay (Kao et al., 2006)

Samples were homogenized on ice in a lysis buffer containing 20 mM HEPES, pH 7.4, 4 mM EDTA, 1 mM EGTA, 5 mM $MgCl_2$, 1 mM DTT, and a protease inhibitor cocktail. An aliquot of 50 μl of the supernatant 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 flourogenic peptide substrate Ac-DEVD-AMC. Enzymatic release of free AMC was measured at an excitation of 380 nm and an emission of 460 nm. Arbitrary activity was expressed as the fluorescence change per amount of protein.

### 2.9. Measurement of lipid peroxidation

A thiobarbituric acid reactive substances (TBARS) assay kit (ZeptoMetrix) was used to measure lipid peroxidation. 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 reaction buffer (provided in the kit) and heated at 95 °C for 60 min. After cooling, the absorbance of the supernatant was measured at 532 nm using a spectrophotometer. The TBARS are expressed in terms of malondialdehyde (MDA) equivalents.

### 2.10. Measurement of reduced glutathione (GSH)

GSH was determined using a commercially available glutathione 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% trichloroacetic acid to remove the proteins. An aliquot of 50 μl of homogenate was mixed with 150 μl reaction buffer (provided in the kit). The mixture was vortexed and the absorbance read at 405 nm within 30 min. The GSH content was calculated using a standard solution of GSH.

### 2.11. Isolation of RNA and reverse transcriptase-polymerase chain reaction (RT-PCR)

The isolation of RNA, synthesis of cDNA, and PCR were carried out as previously reported (Chen et al., 2004). DNA fragments of specific genes and internal controls were co-amplified in one tube. The PCR reaction was carried out under the following conditions: one cycle of 94 °C for 3 min, 28 cycles of (94 °C for 50 s, 58 °C for 40 s, and 72 °C for 45 s), and then 72 °C for 5 min. The amplified DNA fragments were resolved by 1.5% agarose gel electrophoresis and stained with ethidium bromide. The intensity of each signal was determined using a computer image analysis system (IS1000; Alpha Innotech Corporation). The primer sets used in this study were 5′-CTCACTTTGTTGAGTCATTC and 5′-TTTGATTAGTACTGTAGGGTTAATG for cyclooxygenase 2 (COX-2), and 5′-TCCTGTGGCATCCATGAAACT and 5′-GGAGCAATGATCTTGATCTTC for β-actin.

### 2.12. Cell cultures

Neuroglia were prepared from cerebral cortices of 1-day-old Sprague–Dawley rats as previously reported (Chen et al., 2004). The dissociated cells were resuspended in Dulbecco's modified Eagle's medium/10% bovine fetal serum. The medium was replenished 4 days after plating and changed every 3 days. The resultant neuroglia cultures were used 14–16 days after plating.

### 2.13. Assessment of cellular redox potential

Changes in redox potential were measured by fluorescence signal after the oxidation of non-fluorescent 2′,7′-dichlorofluorescein by free radicals, as described previously (Chen and Liao, 2002). Cultures were loaded with 5 μM 2′,7′-dichlorofluorescein at 37 °C for 10 min, washed, and then subjected to the treatment. The fluorescence signal

of oxidized 2′,7′-dichlorofluorescein was measured using a fluorometer.

### 2.14. Statistical analysis

Data were expressed as the mean ± standard error of the mean (S.E.M.). The statistical significance of differences between groups was determined by one-way analysis of variance (ANOVA) followed by Dunnett's test. An alpha level of $p \leq 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Effects on cerebral I/R-induced infarction and behavioral impairment

Both CCA and MCA occlusion for 90 min followed by 24 h of reperfusion resulted in extensive and reproducible hemispheric swelling and focal infarction throughout the cortical and subcortical structures (Fig. 1A). PUFA such as AA and DHA administrated 60 min after reperfusion increased the infarct volume in a concentration-dependent manner. Under our experimental conditions, the administration of saturated fatty acid SA did not affect post-ischemic infarction (Fig. 1B). In behavioral testing, animals with cerebral I/R showed marked alterations in motor activity (Fig. 1C). Cerebral I/R animals demonstrated reduced mean duration on the rotarod. AA and DHA post-treatment aggravated the cerebral I/R-induced deficit in motor activity in a concentration-dependent manner. This behavioral alteration was not changed with SA administration. These results indicate that PUFA, but not saturated fatty acids, can exacerbate ischemic injury if administered post-reperfusion.

### 3.2. Effects on BBB permeability

Increased vascular permeability and disruption of the BBB could be initiating factors for the development of cerebral infarction. To elicit the effects of fatty acids on BBB permeability, we quantified the extravasation of Evans blue into 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 fatty acid administration (Fig. 2A). However, levels of Evans blue increased dramatically following cerebral I/R insult in the ipsilateral cortex. SA administration did not affect amount of Evans blue detected. In contrast, AA and DHA further increased the extravasation of Evans blue (Fig. 2A). Increased

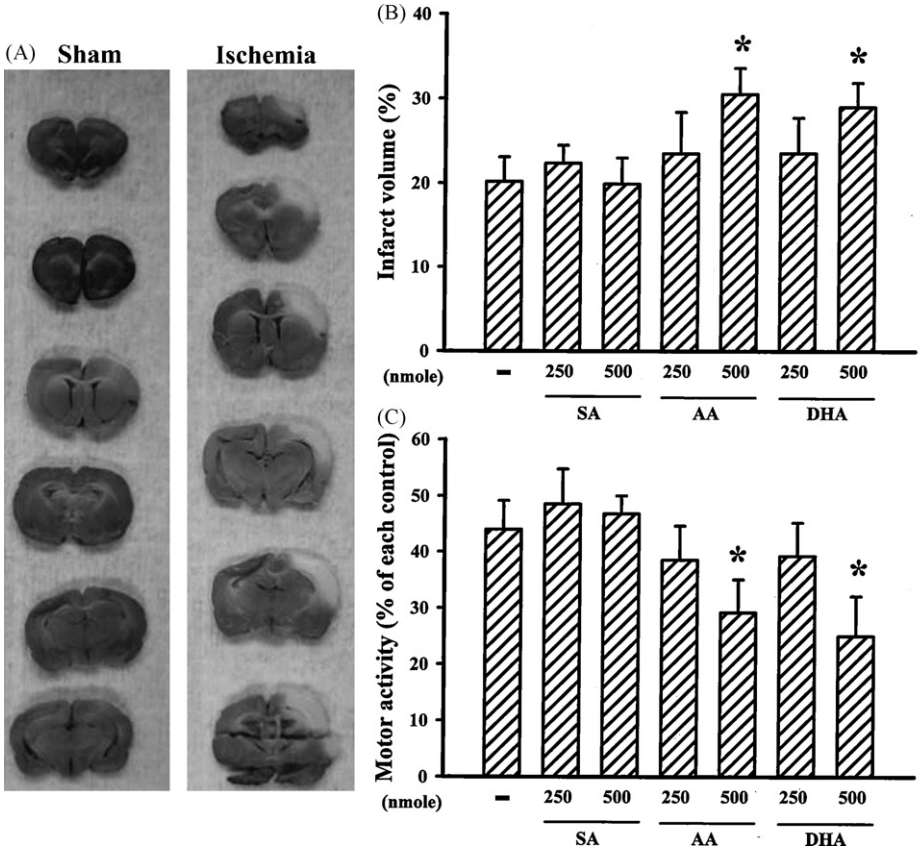


Fig. 1. Effects of post-treatment with fatty acids on cerebral I/R-induced brain infarction and behavioral deficits. Rats were subjected to sham operation (sham) or 90 min ischemia followed by 24 h reperfusion (ischemia). A representative photograph shows histological examination (A). Rats were subjected to 90 min ischemia followed by 24 h reperfusion, and received saline or various concentrations of fatty acids (SA, AA, and DHA) post-treatment (60 min after reperfusion). The average percentage of infarct volume (B) in each ipsilateral hemisphere was depicted. The rotarod test (C) was used to assess motor activity. The motor activity before occlusion in each group was defined as 100%. *$p < 0.05$ vs. saline control, $n = 10$.

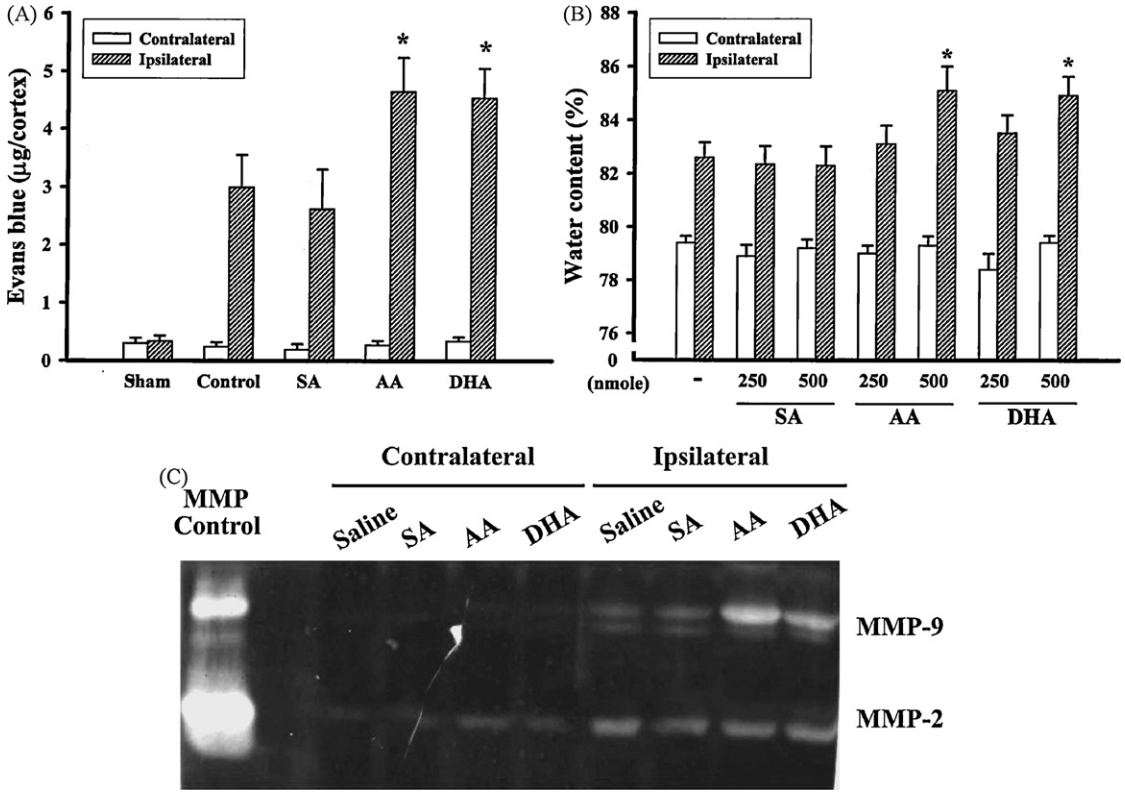


Fig. 2. Effects of post-treatment with fatty acids on cerebral I/R-induced BBB disruption. Rats were subjected to 90 min ischemia followed by 24 h reperfusion, and received saline or fatty acids (SA, AA, and DHA) post-treatment (60 min after reperfusion). The integrity of the BBB was determined by measuring the content of Evans blue in contralateral and ipsilateral cortical tissue (500 nmol of each fatty acid) (A). *$p < 0.05$ vs. saline control (ipsilateral), $n = 9$. The content of brain water was determined in contralateral and ipsilateral cortical tissue (B). *$p < 0.05$ vs. saline control (ipsilateral), $n = 10$. Total proteins were isolated from contralateral and ipsilateral cortical tissues and subjected to zymography for the measurement of MMP activity (C). One of six independent experiments is shown.

vascular permeability, which results in edema formation, is a major complication of stroke (Cao et al., 2005). The brain water content, used as an index of cerebral edema, was higher in the ipsilateral cortical tissues than on the contralateral side. AA and DHA, but not SA, post-treatment produced a significant increase in post-ischemic cerebral edema (Fig. 2B). It is proposed that destruction of vascular integrity is a result of the degradation of the basal lamina and extracellular matrix. MMP is thought to be the critical molecule in this process. To elicit the effects of fatty acids on MMP activity, gelatinase zymography was performed. Although the cerebral I/R insult significantly stimulated MMP-2 and MMP-9 gelatinase activities, fatty acids had little effect on MMP-2 activity (Fig. 2C). Significantly, cerebral I/R-stimulated MMP-9 activity was not affected by SA but was aggravated by AA (by a factor of $2.7 \pm 0.95$ compared with saline, $p < 0.05$) and DHA ($1.9 \pm 0.65$-fold, cf. saline, $p < 0.05$) (Fig. 2C). These results indicate that PUFA, but not saturated fatty acids, can exacerbate cerebral I/R-induced BBB disruption if administered post-reperfusion.

### 3.3. Effects on inflammation

Inflammation is characterized by the accumulation of inflammatory cells and mediators. The activation of resident microglia and the infiltration and accumulation of neutrophils and other leukocytes are postulated to be cytotoxic to brain tissues and the main cause of ischemic injury (Yrjanheikki et al., 1999; Liao et al., 2001). The assessment of neutrophils/leukocyte accumulation in the ischemic brain was based upon biochemical assay, MPO activity, and an index of neutrophils/leukocyte infiltration. Fatty acids alone had little effect on MPO activity in sham-operated animals (Fig. 3A). MPO activity was significantly increased in the ipsilateral cortex, and its activity was further elevated in AA- and DHA- but not SA-treated ischemic animals (Fig. 3B). In addition, we found that cerebral I/R stimulated pro-inflammatory cytokine COX-2 mRNA expression in the ipsilateral hemisphere, and its expression was elevated by AA ($2.1 \pm 0.65$-fold, cf. saline, $p < 0.05$) and DHA ($1.9 \pm 0.46$-fold, cf. saline, $p < 0.05$) but not post-treatment SA (Fig. 3C). These results indicate that PUFA, but not saturated fatty acids, can augment post-ischemic brain inflammation if administered post-reperfusion.

### 3.4. Effects on apoptosis

The occurrence of apoptosis is of paramount importance for ischemic progression and is a determining factor of ischemic severity. Biochemical features of apoptosis include internucleosomal DNA fragmentation, pro-apoptotic gene expression, and caspase activation (Cho et al., 2003; Linnik et al., 1993). Caspase 3 is the most abundant caspase, and serves as a downstream executioner for caspase. Fatty acids alone had little

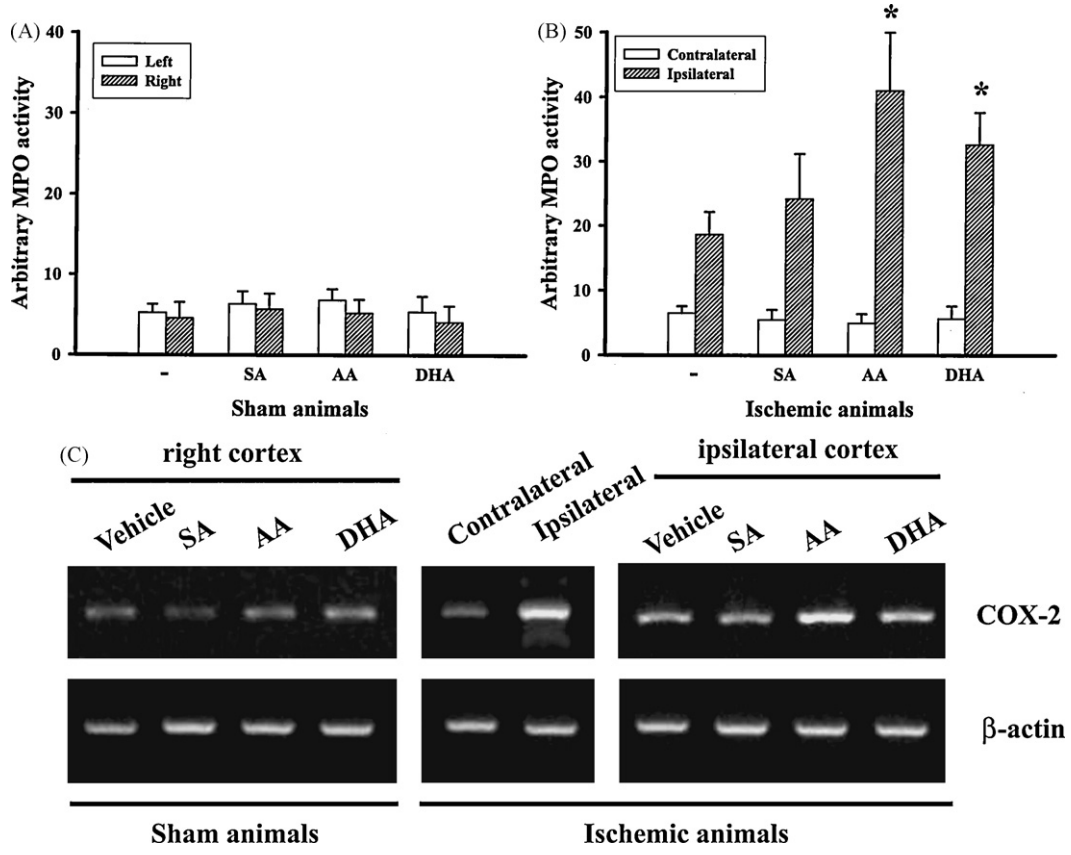


Fig. 3. Effects of post-treatment with fatty acids on cerebral I/R-induced inflammation. Rats were subjected to sham operation and received saline or 500 nmol fatty acid (SA, AA, or DHA) treatments for 24 h. Total proteins were isolated from the left and right cortical tissues and subjected to enzymatic assay for MPO activity (A), $n = 6$. Rats were subjected to 90 min ischemia followed by 24 h reperfusion receiving saline or 500 nmol fatty acid (SA, AA, or DHA) post-treatment (60 min after reperfusion). Total proteins were isolated from the contralateral and ipsilateral cortical tissues and subjected to enzymatic assay for MPO activity (B). $*p < 0.05$ vs. saline control (ipsilateral), $n = 6$. Rats were subjected to sham operation (sham) or 90 min ischemia followed by 24 h reperfusion (ischemic) and received saline or 500 nmol fatty acid (SA, AA, or DHA) post-treatment (60 min after reperfusion). Total RNA was isolated from contralateral and ipsilateral (right) cortical tissues and subjected to RT-PCR for the determination of COX-2 and β-actin. One of six independent experiments is shown (C).

effect on caspase 3 activity in sham-operated animals (Fig. 4A). Cerebral I/R caused a significant increase of caspase 3 activity in the ipsilateral cortex but not in the contralateral cortex. AA and DHA, but not SA, post-treatment further elevated cerebral I/R-induced caspase 3 activity in ischemic animals (Fig. 4B). These results indicate that PUFA, but not saturated fatty acids, can increase post-ischemic apoptosis in brain tissue if administered post-reperfusion.

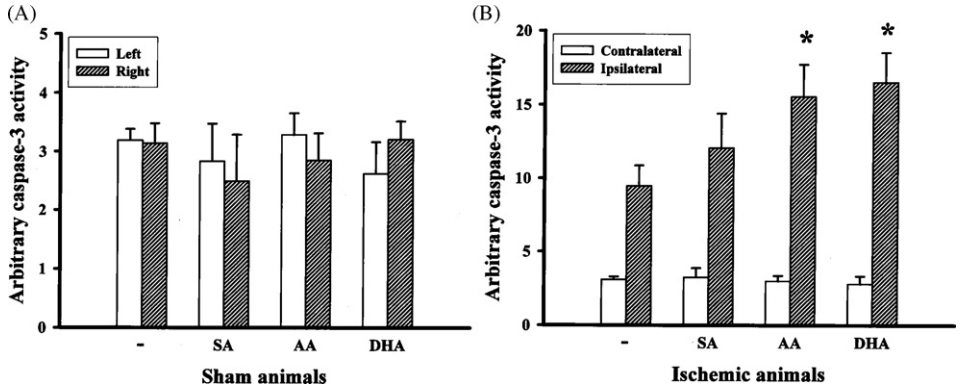


Fig. 4. Effects of post-treatment with fatty acids on cerebral I/R-induced caspase 3 activity. Rats were subjected to sham operation (A, sham) or 90 min ischemia followed by 24 h reperfusion (B, ischemic) and received post-treatment saline or 500 nmol fatty acid (SA, AA, or DHA) (60 min after reperfusion). Total proteins were isolated from the contralateral (left) and ipsilateral (right) cortical tissues and subjected to enzymatic assay for caspase 3 activity. $*p < 0.05$ vs. saline control (ipsilateral), $n = 6$.

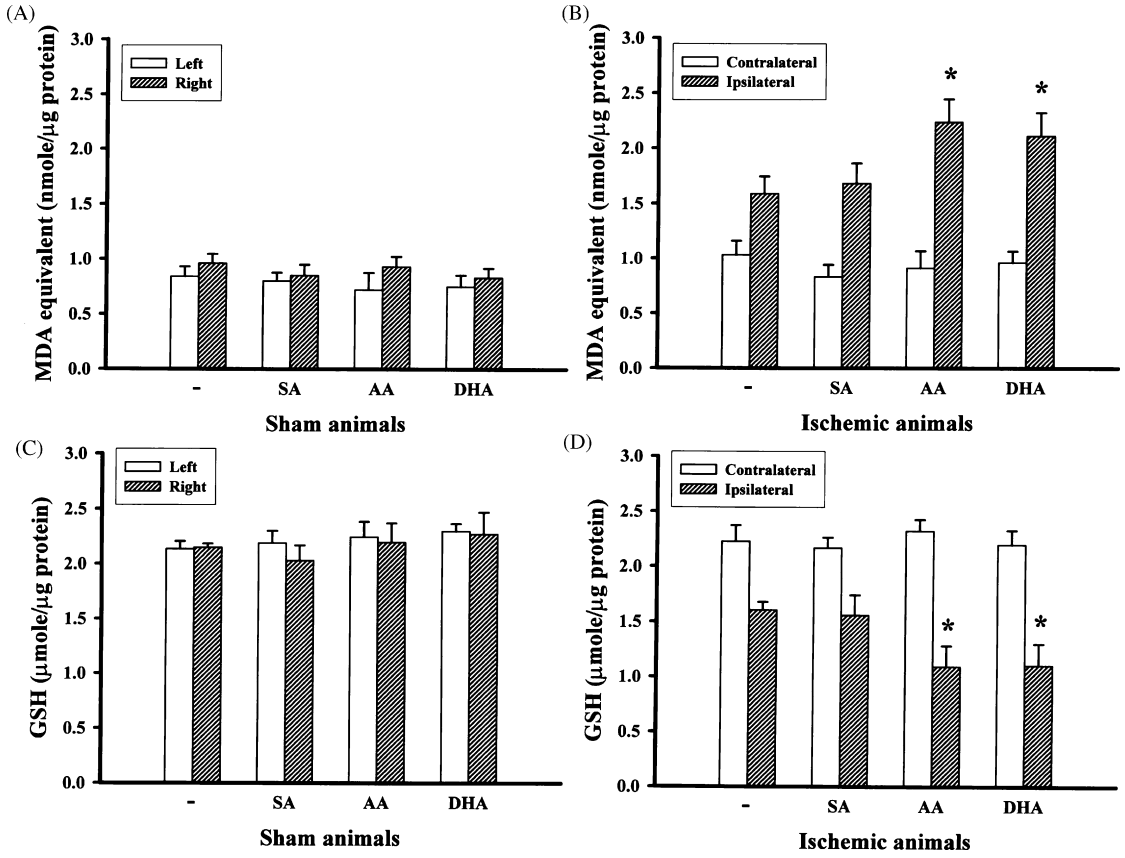


Fig. 5. Effects of post-treatment with fatty acids on cerebral I/R-induced oxidative stress. Rats were subjected to sham operation (sham) or 90 min ischemia followed by 24 h reperfusion (ischemic) and received post-treatment saline or 500 nmol fatty acid (SA, AA, or DHA) (60 min after reperfusion). The content of brain MDA (A and B) and GSH (C and D) was determined in contralateral (left) and ipsilateral (right) cortical tissues. *$p < 0.05$ vs. saline control (ipsilateral), $n = 9$.

### 3.5. Effects on oxidative stress

Oxidative stress has been implicated in the pathogenesis of cerebral injury after I/R (Cao et al., 2004). The increased 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 differences in brain MDA levels were found between vehicle and fatty acid treatment in sham operated animals (Fig. 5A). The level of post-ischemic brain MDA significantly increased in the ipsilateral cortex compared with the contralateral side in ischemic animals. Post-ischemic MDA

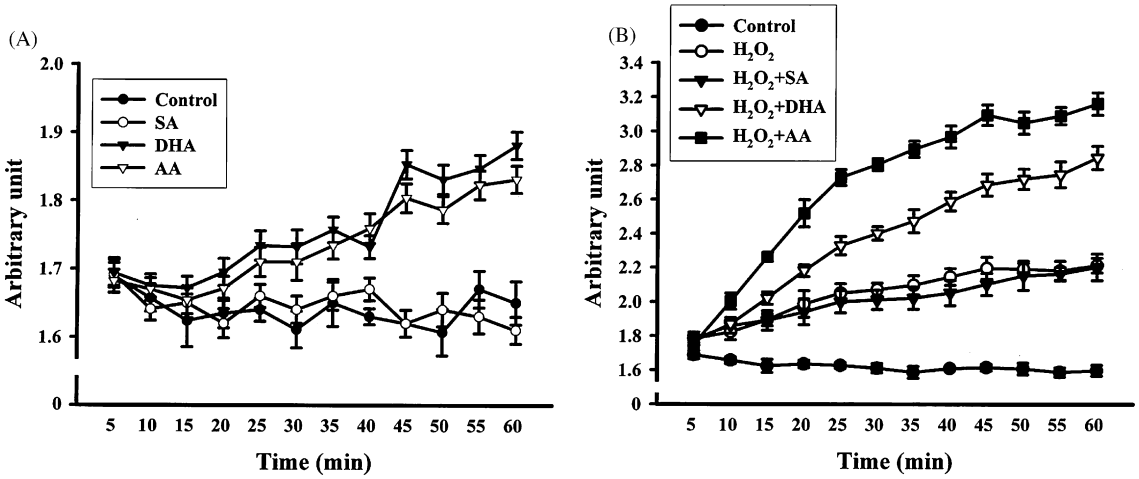


Fig. 6. Effect of fatty acids on $H_2O_2$-induced oxidative stress in neuroglia. Neuroglia were loaded with 5 μM 2′,7′-dichlorofluorescein for 10 min. The resultant cells were treated with medium (control) or 50 μM fatty acids (SA, DHA, AA) alone (A), or 100 μM $H_2O_2$ alone or 100 μM $H_2O_2$ plus 50 μM fatty acids (B) for 60 min. The fluorescent signals were recorded with an interval of 5 min and the kinetics depicted, $n = 6$.

levels were elevated significantly in AA- and DHA- but not SA-treated animals (Fig. 5B). The content of brain GSH was relatively constant with or without the administration of fatty acids in sham operated animals (Fig. 5C). The ipsilateral cortex of ischemic animals showed a significant reduction in brain GSH content compared with the contralateral side. The reduction in post-ischemic GSH level was highly significant in AA- and DHA- but not SA-treated animals (Fig. 5D). These results indicate that PUFA, but not saturated fatty acids, can augment the oxidative burden in the ischemic brain if administered post-reperfusion.

### 3.6. Effects on free radical generation

To further determine whether fatty acids induce free radical generation, changes in intracellular redox potential were assessed in the *in vitro* cell model because of the feasibility of direct measurement of free radical generation. Intracellular ROS levels were assayed by changes in fluorescent signal after the addition of a nonfluorescent dye, 2′,7′-dichlorofluorescein (Chen and Liao, 2002). In control cells, the addition of SA was unable to cause significant changes in fluorescent signal. However, AA and DHA slightly increased the fluorescent signal from arbitrary unit 1.69–1.83 (Fig. 6A). Hydrogen peroxide exposure caused a change from 1.69 to 2.03. Remarkably, AA (1.69–3.1) and DHA (1.69–2.78), but not SA, increased the hydrogen peroxide-induced elevation of the fluorescent signal (Fig. 6B). These findings indicate that fatty acids alone have little effect on free radical generation in neuroglia, and that PUFA, but not saturated fatty acids, can augment insult-induced oxidative alterations.

## 4. Discussion

In this study, occlusion of the CCA and MCA was used to produce focal ischemic lesions in the rat. The ischemic lesion begins with a small core at the center of the region perfused by the MCA, and this becomes enlarged throughout the duration of reperfusion. Progressive expansion of brain infarction is often observed during the acute phase of cerebral ischemia in clinical practice, and is considered to involve various factors. Oxidative stress plays a pivotal role in the pathophysiology of ischemia, and can enlarge the initial ischemic damage when it appears at toxicologically relevant or unphysiologically high levels (Choi-Kwon et al., 2004). In the present study, we demonstrated that PUFA such as AA and DHA, if administered 60 min after reperfusion, aggravated cerebral ischemic injury as manifest in the enlargement of cerebral infarction and impairment of motor activity. However, there was no remarkable difference in these post-ischemic alterations between the SA treatment group and the vehicle treatment group (Fig. 1). The post-ischemic augmentation in the AA and DHA treatment groups was accompanied by increases in the permeability of the BBB (Fig. 2A), brain edema (Fig. 2B), MMP activity (Fig. 2C), inflammatory cell infiltration (Fig. 3B), COX-2 expression (Fig. 3C), caspase 3 activity (Fig. 4), and MDA production (Fig. 5), and by a decrease in the brain content of GSH (Fig. 5). The in vitro cell study further demonstrated that AA and DHA alone were relatively inert in inducing free radical generation in neuroglia, but they augmented hydrogen peroxide-induced oxidative change (Fig. 6). These findings demonstrate the detrimental effect of PUFA such as AA and DHA in post-ischemic progression and brain injury after cerebral I/R is strongly associated with augmentation of cerebral I/R-induced alterations, including oxidative changes.

Focal ischemia is characterized by an ischemic core surrounded by a penumbra region that retains relatively greater circulation of blood. The infarct can progressively propagate into the penumbra. Generally, the ischemic core is considered unsalvageable, whereas the penumbra may be rescued with proper intervention and is theoretically recognized as a critical target for the judgment of the severity of ischemic damage. Evidence suggests that reperfusion-associated alterations play an important role in the enlargement of infarction. The induction of oxidative stress is one of these alterations. Possible consequences of elevated oxidative stress are overproduction of lipid peroxidation products such as MDA and depletion of reduced GSH (Droge, 2002). Using a redox-sensitive fluorescent dye, there was little change in fluorescent signal between control and SA-, AA-, and DHA-treated neuroglia (Fig. 6). No significant difference in the brain MDA level was found between vehicle and fatty acid treatment in sham-operated animals (Fig. 5). Interestingly, AA and DHA, but not SA, increased hydrogen peroxide-induced oxidative change in neuroglia and augmented cerebral I/R-induced MDA production and GSH depletion (Figs. 5 and 6). These experimental findings suggest that PUFA such as AA and DHA may not primarily serve as initiators of free radical generation under normal conditions, but instead could augment the oxidative burden in oxidized environments. Generally, an oxidative state can be produced by dysfunctional cells with impaired metabolic activity and/or immune cell infiltration. The cessation of cerebral blood flow during ischemic periods leads to an energy crisis and necrotic neuronal death, which could initially induce ROS generation and trigger inflammatory responses leading to oxidative stress. Reperfusion of the occluded vessels leads to further elevation of oxidative burden, either by reperfusion with oxygenated blood or induction of activated immune cells. In the present study, an intraperitoneal injection of fatty acids was given 60 min after reperfusion. Thus, the augmented oxidative burden after reperfusion was probably a determining step in the detrimental effects of AA and DHA in cerebral I/R injury.

The BBB is composed of a continuous layer of brain capillary endothelial cells together with pericytes, a basal lamina, and astroglial cells. In general, the BBB is responsible for the limited and regulated movement of blood-borne constituents into the brain parenchyma. BBB disruption is found to precede, and may be the initiating event in, focal ischemic lesions, and can further contribute to secondary brain injury. Secondary brain damage can develop as a consequence of brain edema, oxidative stress, immune cell infiltration, and inflammation (Hamann et al., 1996). In accordance with these theories, we hypothesized that AA and DHA treatment might

lead to augmented, or at least comparable, destruction of the basal lamina of the cerebral microvessels caused by cerebral I/R. The damaged basal lamina might then suffer increased microvascular permeability, thereby eliciting cerebral edema and peripheral inflammatory cell infiltration, and result in elevation of intracranial pressure and aggravation of ischemic progression. This assumption was confirmed and supported by our experimental findings that AA and DHA increased extravasation of Evans blue into brain parenchyma (Fig. 2A), brain water retention (Fig. 2B), MPO activity (Fig. 3B), COX-2 mRNA expression (Fig. 3C), and caspase 3 activity (Fig. 4), and eventually exacerbated brain infarction (Fig. 1B) and neurobehavioral deficits (Fig. 1C).

Increasing evidence suggests that oxidative stress can increase the occurrence of edema, cytokine expression, MMP activation, and apoptosis (Choi-Kwon et al., 2004; Gursoy-Ozdemir et al., 2004; Kondo et al., 1997; Nagel et al., 2007). It is well known that a loss of vascular integrity results from the degradation of the basal lamina and extracellular matrix. During this process, MMPs are thought to be the critical molecules. Previously, we demonstrated that cerebral I/R induces both MMP-2 and MMP-9 activation in the ipsilateral but not the contralateral hemisphere (Liao et al., 2001). In experimental stroke models, MMP inhibition reduces brain infarction, edema, cytokine expression, and hemorrhage (Pfefferkorn and Rosenberg, 2003). In this study, we found that AA and DHA increased cerebral I/R-induced MMP activity (Fig. 2C). MPO, an enzyme produced by leukocytes such as neutrophils and monocytes, is linked to inflammation, and MPO activity is used as a marker of polymorphonuclear neutrophil infiltration (Liao et al., 2001). An enhancement of immune cell infiltration in ischemic brain after AA and DHA treatments was demonstrated by increased MPO activity (Fig. 3B). Recent evidence suggests that glia-mediated inflammation and the subsequent release of pro-inflammatory mediators are involved in the induction of cell apoptosis (Kreutzberg, 1996). The subsequent activation of immune cells by AA and DHA was shown by the increased expression of COX-2 mRNA (Fig. 3C). Evidence has indicated that the prostaglandin E2 EP1 receptor is a downstream effector responsible for ischemic neurotoxicity, and other studies have shown that treatment with COX-2 inhibitors improves neurological outcome after stroke (Kawano et al., 2006; Liao et al., 2004; Sugimoto and Iadecola, 2003). Apoptosis, the genetically and biochemically controlled process of cell death, plays an important role in ischemia-induced neurological deficits (Lee et al., 2000). Caspases, a family of cysteine proteases, play a central role in the control of apoptosis progression. In this study, we demonstrated activation of caspase-3 after the induction of cerebral I/R, and the results showed that its activation was augmented by AA and DHA treatment (Fig. 4). These observations indicate that augmented production of damaging factors is likely to contribute to the increased post-ischemic brain injury after treatment with AA and DHA.

A variety of neural actions of PUFA have been demonstrated, including protection of neurons from apoptotic death, control of gene expression, and maintenance of the structural integrity of neural membranes (Black et al., 1984; Cao et al., 2004; Choi-Kwon et al., 2004). Numerous studies have shown that dietary supplementation with PUFA, or chronic administration shortly prior to a severe ischemic insult, may ameliorate some of the symptoms associated with cerebral I/R via increased antioxidative capacity, reduced lipid peroxidation, induction of chaperon molecules, or stabilization of membrane integrity (Blondeau et al., 2002; Cao et al., 2004, 2005, 2006; Choi-Kwon et al., 2004; de Wilde et al., 2002). Regarding oxidative stress, it is possible that chronic administration of PUFA may render the brain more vulnerable to lipid peroxidation, thus inducing the antioxidative defense capacity and leading to elevated tolerance and protection against free radical-induced injury. Post-treatment, an elevated level of free PUFA could reach a critical threshold for free radical reaction. Free radicals attack and react instantaneously with unsaturated fatty acids. Once begun, this reaction continues and propagates an amplification cycle of free radical generation, leading to augmented oxidative stress. Generally, AA (20:4n-6)-derived eicosanoids are associated with increased inflammation, whereas those derived from DHA (22:6n-3) are anti-inflammatory (Calder and Grimble, 2002). In this study, AA and DHA shared similar deleterious effects, including inflammatory reactions, which were not found with SA (18:0). Katsumata et al. (1999) reported that daily oral administration of ethyl eicosapentate for 4 weeks after reperfusion improved local blood flow and metabolism without affecting brain infarction, using an embolization model of stroke. The difference in molecular structure, especially the unsaturated bonds that make them vulnerable to lipid peroxidation, is the likely explanation of the neural actions of PUFA observed in our experiments.

Induction of oxidative stress, activation of inflammatory cells and the subsequent release of cytotoxic factors leading to apoptosis have been demonstrated in ischemic brain injury. Accumulating evidence suggests that antioxidants, anti-inflammatory agents, or anti-apoptotic agent treatment can reduce the occurrence of apoptosis and ischemic brain injury (Chen et al., 2001; Liao et al., 2001; Phillips et al., 2000; Satoh et al., 1999; Yrjanheikki et al., 1999). The main findings of this study are that treatment with AA and DHA via intraperitoneal injection after arterial occlusion aggravated cerebral I/R-induced neurological deficits and brain injury in rats. AA and DHA significantly augmented the cerebral I/R-induced oxidative burden. Moreover, post-treatment with AA and DHA significantly increased the cerebral I/R-induced increase in BBB dysfunction, edema, MMP activity, recruitment and activation of inflammatory cells, COX-2 expression, and caspase 3 activity in post-ischemic brain tissue. Taken together, these findings demonstrate the detrimental effect of PUFA such as AA and DHA in post-ischemic progression and brain injury after cerebral I/R is associated with augmentation of cerebral I/R-induced alterations, including oxidative changes. However, current data of this study is not enough to identify and conclude the specific target, which is causal to the initiation of post-injury effects of PUFA.

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