Robust Docosahexaenoic Acid–Mediated Neuroprotection in a Rat Model of Transient, Focal Cerebral Ischemia

Ludmila Belayev, MD; Larissa Khoutorova, BS; Kristal D. Atkins, MS; Nicolas G. Bazan, MD, PhD

Background and Purpose—Docosahexaenoic acid (DHA; 22:6n-3), an ω-3 essential fatty acid family member, is the precursor of neuroprotectin D1, which downregulates apoptosis and, in turn, promotes cell survival. This study was conducted to assess whether DHA would show neuroprotective efficacy when systemically administered in different doses after middle cerebral artery occlusion (MCAo) in rats.

Methods—Sprague-Dawley rats were anesthetized with isoflurane and subjected to 2 hour of MCAo. Animals were treated with either DHA (low doses=3.5 or 7 mg/kg; medium doses=16 or 35 mg/kg; and high dose=70 mg/kg) or an equivalent volume of saline intravenously 3 hours after MCAo onset. Neurologic status was evaluated during occlusion (60 minutes) and on days 1, 2, 3, and 7 after MCAo. Seven days after MCAo, brains were perfusion-fixed, and infarct areas and volumes were determined.

Results—Only the low and medium doses of DHA significantly improved the neurologic score compared with vehicle-treated rats at 24 hours, 48 hours, 72 hours, and 7 days. DHA markedly reduced total corrected infarct volume in all treated groups compared with vehicle-treated rats (3.5 mg/kg, 26±9 mm³; 7 mg/kg, 46±12 mm³; 16 mg/kg, 37±5 mm³; and 35 mg/kg, 34±15 mm³ vs vehicle, 94±12 mm³). Cortical and striatal infarct volumes were also significantly reduced by treatment with DHA. No neuroprotective effects were observed with 70 mg/kg DHA.

Conclusions—We conclude that DHA experimental therapy at low and medium doses improves neurologic and histologic outcomes after focal cerebral ischemia and might provide benefits in patients after ischemic stroke. (Stroke. 2009;40:3121-3126.)

Key Words: focal ischemia □ lipids □ neuroprotection □ animal models

Stroke is the third leading cause of death and the first cause of adult disability in industrial countries. Despite progress made in understanding the pathophysiology of stroke, today the only efficacious treatment approved for ischemic stroke is thrombolysis. Unfortunately, only 3% to 5% of patients can be selected to undergo this treatment. Therefore, the need for developing an effective treatment for stroke remains vital.

Cerebral ischemia results in the rapid accumulation of free fatty acids, including arachidonic and docosahexaenoic acids (DHA), which are released from membrane phospholipids. Both fatty acids are derived from dietary essential fatty acids; however, only DHA (22:6n-3), the ω-3 polyunsaturated fatty acyl chain, is concentrated in phospholipids of various cells of the central nervous system.1 After ingestion, DHA is processed in the liver and transported by the bloodstream to various tissues. The release of DHA from cell membranes occurs mainly under conditions of excessive oxidative stress.1 DHA, as an acyl chain of membrane phospholipids, is necessary for ion channels, receptors, and transporters to maintain their proper physical conformation1 and is involved in memory formation,2 excitable membrane function,3 and neuroprotection.4,5

DHA is the precursor of neuroprotectin D1 (NPD1) that, in turn, downregulates apoptosis and promotes cell survival.6–8 We have recently shown that NPD1 inhibits brain ischemia/reperfusion-mediated leukocyte infiltration and proinflammatory gene expression, promotes neurogenesis both in vitro and in vivo, and has been implicated in neuroprotection.5,9,10 This study was conducted to assess whether DHA would show neuroprotective efficacy when systemically administered in different doses after middle cerebral artery occlusion (MCAo) in rats.

Materials and Methods

Animal Preparation
All studies were approved by the institutional Animal Care and Use Committee of the Louisiana State University Health Sciences Center. Male Sprague-Dawley rats (Charles River Laboratory, Wilmington, Mass) weighing 279 to 329 g were fasted overnight but allowed free access to water. Rats were anesthetized and maintained with 1% to 3% isoflurane in 70% N₂O and 30% O₂, intubated, and mechanically ventilated. The right femoral artery and vein were catheterized for blood sampling for arterial gases, pH, plasma glucose, and drug infusion. Rectal (CMA/Microdialysis AB, Stockholm) and cranial (left temporalis muscle; Omega Engineering, Stamford, Conn) tem-
Fibrillary acidic protein (GFAP), as previously described.12 GFAP-positive groups were set aside for immunohistochemistry with antiglial swelling. Adjacent sections in saline- and DHA- (14 mg/kg) treated sectional area and the intersection distance and corrected for brain volumes of MCAo, rats were reanesthetized with the same anesthetic combination and intraluminal sutures were removed.

Behavioral Tests
Behavioral tests were performed by an observer blinded to the treatment groups at 60 minutes (during MCAo) and then on days 1, 2, 3, and 7 after MCAo. The battery consisted of 2 tests that have been used previously to evaluate various aspects of neurologic function: (1) the postural reflex test, to examine upper-body posture while the animal is suspended by the tail, and (2) the forelimb placing test, to examine sensorimotor integration in forelimb-placing responses to visual, tactile, and proprioceptive stimuli. Neurologic function was graded on a scale of 0 to 12 (normal score = 0, maximal score = 12), as previously described.11

Treatment and Experimental Groups
At 3 hours after onset of stroke, the agent (DHA: 3.5, 7, 14, 35, or 70 mg/kg) or vehicle (saline, 5 mL/kg) was administered into the femoral vein over 3 minutes by an infusion pump. Rats were randomly assigned to the following groups: (1) vehicle=saline (n = 14), (2) 3.5 mg/kg DHA (n = 9), (3) 7 mg/kg DHA (n = 9), (4) 14 mg/kg DHA (n = 8), (5) 35 mg/kg DHA (n = 8), and (6) 70 mg/kg DHA (n = 5).

Histopathology
Animals were maintained for 7 days, reanesthetized with the same anesthetic combination, and transcardially perfused with saline followed by 4% paraformaldehyde. Brains were removed and embedded in paraffin. Histologic sections were cut and stained with thionine (Nissl) and digitized at 9 standardized coronal levels. The area of infarction was measured with an MCID image-analysis system (MCID Core imaging software; InterFocus Imaging Ltd, Linton, Cambridge, England).11 An investigator blinded to the experimental groups then outlined the zones of infarction as well as the outlines of the left and right hemisphere on each section. Infarct volume was calculated as the integrated product of the cross-sectional area and the intersection distance and corrected for brain swelling. Adjacent sections in saline- and DHA- (14 mg/kg) treated groups were set aside for immunohistochemistry with antiglial fibrillary acidic protein (GFAP), as previously described.12 GFAP- and Nissl-positive cell counts were conducted in the cortex and striatum at the level of the central lesion (bregma level -0.3 mm; Figure 1). Data were expressed as numbers of GFAP- and Nissl-positive cells per high-power microscopic field (magnification ×40).

Statistical Analysis
Data are presented as mean±SEM. Repeated-measures ANOVA, followed by Bonferroni procedures to correct for multiple comparisons, was used for intergroup comparisons of neurobehavioral scores over time and infarct areas across coronal levels. Two-tailed Student’s t tests were used for 2-group comparisons. Differences at P<.05 were considered statistically significant.

Results

Physiologic Variables
Rectal and cranial (temporalis muscle) temperatures, plasma glucose, and blood gases showed no significant differences among groups (the Table).

Figure 1. Schematic diagram of the rat brain, showing locations of regions for GFAP- and Nissl-positive cell counts in the cortex (A, B, and C) and striatum (S).

Neurobehavioral Assessment
Before MCAo, the neurologic score was normal (score=0) in all animals. High-grade behavioral deficits (score=10 or 11) were present in all animals when tested at 60 minutes of MCAo (Figure 2). Saline-treated animals continued to exhibit severe behavioral impairments throughout the 7-day survival period. Only the low and medium doses of DHA (3.5, 7, 14, and 35 mg/kg) significantly improved the neurologic score compared with vehicle-treated rats at 24 hours, 48 hours, 72 hours, and 7 days (Figure 2). Rats treated with 70 mg/kg DHA tended to have a slightly improved neurologic score, but this did not reach statistical significance (Figure 2).

Histopathology
The brains of saline-treated animals with MCAo exhibited a consistent pan necrotic lesion involving both cortical and subcortical regions of the right hemisphere, characterized microscopically by the destruction of neuronal, glial, and vascular elements. By contrast, infarct size was dramatically reduced by DHA therapy in the low- and medium-dose treatment groups but not in the high-dose group (Figure 3).

The dose-response study revealed that the extent of neuroprotection was profound in the neocortex (mean tissue salvage, 83% and 74%, respectively, in the 3.5 and 35 mg/kg DHA-treated groups) and extended across multiple coronal levels (Figures 4A and 4B). The subcortex was also significantly protected by DHA in 3.5, 7, and 35 mg/kg DHA-treated groups (by 57%, 44%, and 61%, respectively; Figures 4C and 4D). Total infarct volume, corrected for brain swelling, was reduced by a mean of 72%, 51%, 59%, and 63%, respectively, by treatment with 3.5, 7, 14, and 35 mg/kg DHA (Figures 4E and 4F). No protection was observed with 70 mg/kg DHA compared with saline treatment (cortex, 82±40 vs 55±11 mm³; subcortex, 37±11 vs 38±19 mm³; total infarct volume, 119±52 vs 93±12 mm³, respectively; repeated-measures ANOVA followed by Bonferroni tests). Figure 5A presents Nissl- and GFAP-stained brain sections from saline- and DHA- (14 mg/kg) treated rats. Saline-treated rats showed large cortical and subcortical infarcts. In contrast, rats treated with DHA showed less extensive damage, mostly in the subcortical area. GFAP- and Nissl-positive cell counts are shown in Figure 5B. Treatment with DHA (14 mg/kg)

Peratures were maintained at 36°C to 37°C during surgical procedures.

Middle Cerebral Artery Occlusion
The right MCA was temporarily occluded for 2 hours by an intraluminal filament coated with poly-L-lysine.11 A 4-cm length of 3-0 monofilament nylon suture was inserted via the proximal external carotid artery into the internal carotid artery and MCA, a distance of 20 to 22 mm from the common carotid artery bifurcation. Animals were allowed to awaken from anesthesia and, at 60 minutes of MCAo, were tested on a standardized neurobehavioral battery to confirm the presence of a high-grade neurologic deficit. After 2 hours of MCAo, rats were reanesthetized with the same anesthetic combination and intraluminal sutures were removed.
Twelve animals died during the experiment: 5 rats in the saline group (died on days 2, 3, 4, 6, and 7), 1 rat in the 3.5-mg DHA group (died on day 7), 3 rats in the 7-mg DHA group (died on days 2, 5, and 7), 1 rat in the 35-mg DHA group (died on day 7), and 2 rats in 70-mg DHA group (both died during drug administration). Three animals in the 70-mg DHA group developed transient hematuria, which disappeared after 24 hours.

### Discussion

The goal of our study was to determine whether DHA was efficacious in protecting the brain when systemically administered after transient, focal cerebral ischemia. Our results clearly demonstrate that this treatment, when applied at 3 hours after MCAo onset, improves the outcome as measured by neurologic score and by pathologic estimation of the size of infarction.

DHA (22:6, n-3), an ω-3 fatty acid and the main polyunsaturated fatty acid in the mammalian brain, plays a crucial role in the development and function of brain neurons. Although every cell contains DHA, its highest concentrations

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**Table. Physiologic Variables**

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Values are mean±SEM.
are found in the brain and retina. Once in the nervous system, it is incorporated into phospholipids in neuronal and photoreceptor membranes, where it supports neural activity and vision. Synaptic membranes and photoreceptors share the highest content of DHA of all cell membranes. In vivo, the active DHA supply to the brain is necessary for normal brain function. It also may play a critical role in conditions where, due to enhanced oxidative stress, the polyunsaturated fatty acyl chains of membrane phospholipids are decreased as a consequence of lipid peroxidation, as occurs in aging, retinal degenerations, and neurodegenerations such as Alzheimer’s disease.

The neuroprotective effect of DHA was also evaluated in animal models of cerebral ischemia. Long-term fish oil treatment was able to improve learning/memory function in rats subjected to global cerebral ischemia. Pretreatment with DHA (100 and 500 nmol/kg IP at 1 hour and 3 days or daily for 6 weeks before ischemia) was accompanied by decreases in blood/brain barrier disruption, brain edema, malondialdehyde production, inflammatory cell infiltration, interleukin-6 expression, and caspase-3 activity in rats subjected to 90 minutes of MCAo. However, acute posttreatment with DHA (500 nmol per rat [0.16 mg] IP 1 hour after reperfusion) after 90 minutes of MCAo remarkably exacerbated cerebral ischemia/reperfusion injury. Recently, we have demonstrated that administration of DHA complexed to human serum albumin (DHA-Alb; 2 mmol DHA per mL of Alb) provides an improvement of behavioral function and a reduction of brain infarction and edema (1.25 or 0.63 g/kg IV 2 hours after onset of ischemia) after 120 minutes of MCAo.

Lipidomic analysis of DHA-Alb–treated brains revealed the formation of the neuroprotective DHA-derived mediator NPD1 in the ipsilateral hemisphere. In the present study, we found that much lower concentrations of DHA were required to elicit protection (3.5 to 35 mg/kg). These relatively lower DHA concentrations may be carried by endogenous proteins (eg, Alb, lipoproteins). The reason higher concentrations of DHA were used in the Alb/DHA studies was because DHA may be tightly retained by added human serum Alb, and only a fraction of DHA may be delivered to the brain.

Recently, we discovered that brain ischemia/reperfusion generates stereospecific DHA pathways that lead to the formation of novel messengers. One of them is NPD1, a stereospecific derivative of DHA formed through a lipoxigenase enzyme that acts on free DHA. The synthesis of NPD1 after 1 hour of MCAo in the mouse coincides with free DHA availability that results from phospholipase A2 activation. NPD1 is formed in the ipsilateral hippocampus and peaks at 8 hours of reperfusion, and it was still elevated 25 hours later. In that study, NPD1 was formed from endogenous DHA. NPD1 was found to serve an endogenous neuroprotective role by inhibiting apoptotic DNA damage, upregulating antiapoptotic and downregulating proapoptotic proteins, and also binding toxic peroxides, particularly in hemorrhagic stroke. The relatively large ischemic insult may overcome the ability of endogenously generated doco-

Figure 3. Computer-generated MosaiX processed images (Zeiss Axio Imager.M1; AxioVision Release 4.6.3) of Nissl-stained, paraffin-embedded brain sections from rats treated with saline or 3.5, 7, 14, 35, or 70 mg/kg DHA. Saline-treated rat shows large cortical and subcortical infarction. In contrast, rats treated with low and medium doses of DHA show less extensive damage, mostly in the subcortical area. Rat treated with a high dose of DHA (70 mg/kg) shows a large infarct involving cortical and subcortical regions.

Figure 4. Cortical, subcortical, and total infarct areas measured at 9 coronal levels and integrated infarct volumes in rats with 2-hour MCAo and on day 7 of survival. Shown are the rostrocaudal distribution of cortical infarct area and cortical infarct volume (A and B), subcortical infarct area and subcortical infarct volume (C and D), and total infarct area and total corrected infarct volume (E and F) in all groups. Data are presented as mean ± SEM. *Significantly different from the corresponding vehicle group at P < 0.05 by repeated-measures ANOVA followed by Bonferroni tests.
of the rat brain cortex. Our study shows that 70 mg/kg DHA caused a toxic effect as well. All animals developed hematuria, and 40% of the animals died during drug injection. Three animals survived but had very extensive damage, mostly in the cortical area. Thus, we would like to emphasize that the low to medium doses of DHA will be considered in the future as therapeutic doses for the treatment of ischemic stroke.

Our data show that after 7 days of MCAo, GFAP expression was found in the boundary zone of the infarct or in the areas of selective, incomplete ischemic necrosis. GFAP expression was localized to the same areas where neurons are destined to survive the ischemic insult (detected by Nissl-positive cell counts). Glial cells are activated after brain ischemia. It has been demonstrated that microglia secrete neurotoxic agents and astrocytes that produce neurotrophic factors. Astrocytes can release a number of growth factors for neurons. Some of these, such as nerve growth factor, may stimulate the neuron as a whole as well as promote neurite growth. Thus, GFAP expression may be related to a possible “protective” effect on adjacent neurons within regions of the brain that remain viable after focal cerebral ischemia.

Neurologic deficits are also an important indicator of stroke development in humans as well as in experimental cerebral ischemia models. Stroke in patients is commonly associated with impaired sensorimotor and cognitive function, and ≈80% of all patients experience hemiparesis after the insult. Rodents that experience focal ischemia also exhibit a neurologic deficit characterized by sensorimotor dysfunction. In the present study, animals treated with low and medium doses of DHA showed neurologic improvements within the first day after the insult that continued for 7 days. Rats treated with higher doses of DHA tended to have a slightly improved neurologic score, but this did not reach statistical significance.

The timing of DHA administration is another important factor. In several previous studies, DHA was given before or shortly after experimental cerebral ischemia. During that time, it is logistically difficult to institute therapy in most patients with acute stroke. Other focal ischemia models have shown posttreatment successes even when treatment is delayed >1 hour after the onset of ischemia. However, in most models, benefit is lost if treatment is delayed by >2 or 3 hour. It is therefore important that a significant neuroprotective effect of DHA in our study was achieved when therapy was initiated 3 hours after the onset of transient MCAo. We demonstrated that DHA did not have direct effects on brain temperature or arterial blood gases because these variables were carefully controlled and did not differ among groups.

The beneficial effect of DHA has been shown in a well-controlled animal model of MCAo. In the present study and in recently published observations, we used a poly-L-lysine–coated suture and found that this technique produces substantial, consistent cortical plus subcortical infarction. This infarction closely mimics, in extent and severity, the large hemispheric infarcts resulting from proximal MCA and internal carotid artery occlusions in patients.

**Figure 5.** A, Representative Nissl- and GFAP-stained brain sections of rats treated with vehicle and DHA (14 mg/kg). Saline-treated rats show large cortical and subcortical infarcts. In contrast, rats treated with DHA show less extensive damage and increased GFAP staining. B, GFAP- and Nissl-positive cell counts in the cortex (A, B, and C) and striatum (S; see Figure 1 for details). Treatment with DHA (14 mg/kg) significantly increased the numbers of GFAP- and Nissl-positive cells compared with saline-treated rats. Values shown are mean±SEM. *Significantly different from the corresponding vehicle-treated group at **P<0.05 by repeated-measures ANOVA followed by Bonferroni tests.

The results of this study established efficacy and the dose–response relation for the neuroprotective effects of DHA in a rat model of focal cerebral ischemia. The most remarkable effect was found in rats treated with the lowest (3.5 and 7 mg/kg) and medium (14 and 35 mg/kg) doses of DHA. Vehicle-treated rats showed a large cortical and subcortical infarct at multiple coronal levels. By contrast, DHA-treated animals had significantly reduced cortical (by 74% to 83%), subcortical (by 38% to 61%), and total (by 51% to 72%) infarct volumes compared with those in the vehicle-treated group. No significant neuroprotective effects were observed at the highest dose (70 mg/kg) of DHA. A previous study had shown that high doses of DHA induced brain edema in slices

sanoids to elicit neuroprotection. Thus, when NPD1 (400 ng) was infused into the third ventricle during 48 hours after 60 minutes of MCAo, a reduced infarct size (by 50%) and 80% inhibition of leukocyte infiltration in the mouse hippocampus and neocortex were detected. It is interesting that ischemia/reperfusion-induced nuclear factor κB activation and proinflammatory cyclooxygenase-2 expression, but not cyclooxygenase-1, were downregulated by this docosanoid. 

In addition to its neuroprotective effects, DHA also inhibited leukocyte infiltration in the mouse hippocampus shortly after experimental cerebral ischemia. During that time, it is logistically difficult to institute therapy in most patients with acute stroke. Other focal ischemia models have shown posttreatment successes even when treatment is delayed >1 hour after the onset of ischemia. However, in most models, benefit is lost if treatment is delayed by >2 or 3 hour. It is therefore important that a significant neuroprotective effect of DHA in our study was achieved when therapy was initiated 3 hours after the onset of transient MCAo. We demonstrated that DHA did not have direct effects on brain temperature or arterial blood gases because these variables were carefully controlled and did not differ among groups.

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Our results show that DHA is markedly neuroprotective in acute focal cerebral ischemia in rats, improving neurologic function and reducing the extent of histologic damage with a therapeutic window of at least 3 hours after MCAo onset. We therefore suggest that this agent offers great promise in developing therapies for cerebral ischemia in patients with acute ischemic stroke.

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References


Disclosures
None.

Summary
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