Docosahexaenoic Acid Complexed to Albumin Elicits High-Grade Ischemic Neuroprotection

Ludmila Belayev, MD; Victor L. Marcheselli, PhD; Larissa Khoutorova, BS; Elena B. Rodriguez de Turco, PhD; Raul Busto, BS; Myron D. Ginsberg, MD; Nicolas G. Bazan, MD, PhD

Background and Purpose—High-dose human albumin therapy is strongly neuroprotective in models of brain ischemia and trauma and is currently being studied in a pilot-phase clinical stroke trial. Among its actions in ischemia, albumin induces the systemic mobilization of n-3 polyunsaturated fatty acids and may help to replenish polyunsaturated fatty acids lost from neural membranes.

Methods—We complexed 25% human albumin to docosahexaenoic acid (DHA; 22:6n-3) and compared its neuroprotective efficacy with that of native albumin in rats with 2-hour focal ischemia produced by intraluminal suture-occlusion of the middle cerebral artery.

Results—In animals treated with DHA–albumin, 0.63 g/kg, the improvement in neurobehavioral scores at 72 hours significantly exceeded that of other treatment groups, and the extent of histological protection (86% reduction in cortical infarction) was highly significant and tended to surpass the degree of cortical protection produced by native albumin at 1.25 g/kg (65%). DHA–albumin 0.63 g/kg, but not native albumin, also significantly reduced subcortical infarction and markedly diminished brain swelling. Lipidomic analysis of DHA–albumin-treated postischemic brains revealed a large accumulation of the neuroprotective DHA metabolite, 10,17–docosatriene, in the ipsilateral hemisphere.

Conclusions—The high-grade neuroprotection afforded by the DHA–albumin complex at relatively low albumin doses is clinically advantageous in that it might reduce the likelihood of acute intravascular volume overload and congestive heart failure sometimes induced when patients with compromised cardiovascular function are treated with high-dose albumin. (Stroke. 2005;36:118-123.)

Key Words: albumins ■ fatty acids ■ ischemia ■ neuroprotection ■ rats

There is an unmet, compelling need for a safe and effective neuroprotective therapy for patients with acute ischemic stroke.1 Many potentially effective neuroprotective agents have emerged from experimental studies,2 but clinical trials to date have been disappointing.3,4

We have shown that moderate-dose to high-dose human albumin therapy affords consistent neuroprotection in animal models of temporary5–9 and permanent10 focal cerebral ischemia, as well as in global cerebral ischemia11 and traumatic brain injury.12 Among its multiple actions, albumin administration after middle cerebral artery occlusion (MCAo) induces a selective systemic mobilization of n-3 polyunsaturated fatty acids (PUFA) through the blood stream.13 Under these conditions, the n-3 PUFA docosahexaenoic acid (22:6n-3, DHA) and docosapentaenoic acid (22:5n-3) selectively increased within 30 minutes in systemic venous plasma, whereas lower free fatty acid (FFA) levels were present in jugular venous plasma, suggesting that these free PUFA were supplied to the postischemic brain from another site (eg, liver13) and might replenish PUFA lost from neural membranes during ischemia.14 We suspected that if albumin were complexed with DHA, it might be possible to achieve neuroprotection at lower albumin doses. This would be clinically desirable because high-dose albumin expands intravascular volume and may, on occasion, precipitate congestive heart failure.

Materials and Methods

Preparation of DHA–Albumin Complex

DHA was physically complexed to human albumin by incubating 20 mL of human serum albumin (25%; Baxter, Deerfield, Ill) with 4.0 mg DHA/g albumin (molar ratio=0.2) in a shaking incubator at 37°C for 30 minutes with vortex mixing every 5 minutes. Aliquots were extracted, and FFA were isolated by thin-layer chromatography, derivatized to fatty acid methyl esters, and analyzed by gas liquid chromatography. Each vial was aliquoted in 5-mL samples and kept under nitrogen in a cold room; vials were gassed with nitrogen every week. The DHA–albumin complex contained 2.1±0.1 μmol DHA per milliliter of albumin and was stable for at least 2 months.

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From the Cerebral Vascular Disease Research Center (L.B., L.K., R.B., M.D.G.), Department of Neurology, University of Miami School of Medicine, Miami, FL; and the Neuroscience Center of Excellence and Department of Ophthalmology (V.L.M., E.B.R.d.T., N.G.B.), Louisiana State University Health Sciences Center, New Orleans, La.

Correspondence to Dr Myron D. Ginsberg, Department of Neurology (D4–5), University of Miami School of Medicine, PO Box 016960, Miami, FL 33101. E-mail mdginsberg@stroke.med.miami.edu

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MCAo
Male Sprague–Dawley rats (Charles River Laboratories, Wilmington, Mass) weighing 260 to 357 grams were studied after an overnight fast; the University of Miami’s Animal Care and Use Committee approved all study protocols. After atropine sulfate (0.5 mg/kg, intraperitoneally), animals were anesthetized with halothane (3.5% for induction, 1% for maintenance), 70% nitrous oxide, and a balance of oxygen; immobilized with pancuronium bromide (0.6 mg/kg, intravenously); and mechanically ventilated. Rectal and cranial (left temporals muscle) temperatures were monitored with temperature probes and were regulated at 36°C to 37°C via separate heating lamps. Arterial blood pressure and blood gases, hematocrit, and plasma glucose were measured as previously described.15 The right MCA was occluded for 2 hours by our modification of the intraluminal suture occlusion method,16 in which a poly-L-lysine–coated nylon filament is introduced retrogradely into the MCA.15 Animals were allowed to awaken from anesthesia and, at 60 minutes of MCAo, were tested on a standardized neurobehavioral battery15 to confirm the presence of a high-grade neurological deficit. Rats that did not demonstrate an initial left upper extremity paresis were excluded from further study. Animals were then re-anesthetized for removal of the intraluminal filament after 2 hours of MCAo; treatment was administered immediately thereafter.

Animals were randomly assigned to 1 of 5 treatment groups: (1) albumin, 1.25 g/kg (n=10); (2) DHA–albumin, 1.25 g/kg (n=7); (3) albumin, 0.63 g/kg (n=7); (4) DHA–albumin, 0.63 g/kg (n=7); and (5) a comparable volume (5 mL/kg) of normal saline (n=8). These agents were infused intra-venously over 3 minutes. Rats were then transferred to a temperature-controlled incubator at 37°C for 24 hours, where they received supplemental oxygen and were observed carefully for signs of discomfort; no such signs were observed.

Neurobehavioral Testing
A standardized neurobehavioral test battery15 was used to quantify postural reflexes and forelimb placing responses in all 39 rats before MCAo, at 60 minutes of MCAo, and at 1, 24, 48, and 72 hours after treatment. A 12-point scale was used: normal=0, maximal=12.15 Tests were conducted by an observer blinded to the treatment group.

Quantitative Histopathology
Three days after MCAo, brains were perfusion-fixed, paraffin-embedded, sectioned coronally, and stained with hematoxylin and eosin as previously described.15 Sections were digitized at 9 standard coronal levels, and infarct areas and volumes were quantified by an investigator blinded to the experimental groups, using image analysis. Total infarct volume was corrected for the effects of brain swelling.17

Lipidomic Analysis
Separate groups of rats were subjected to 2 hours of MCAo, and then were treated at the onset of reperfusion with native albumin (0.63 g/kg), DHA–albumin (0.63 g/kg), or vehicle (saline) (n=6 in each group); animals were euthanized 20 hours later. Right- and left-hemisphere brain tissues were collected separately and kept frozen until lipid analysis. Samples were lipid-extracted,18 and lipids were purified by solid-phase extraction. The extracts were resuspended in methanol (EM Science), equilibrated in H2O at pH 3.0 at 10% methanol/H2O ratio, and then loaded onto C18-SPE columns (Varian). Lipids were eluted with 10 mL of ethyl acetate, then blown dry under N2 and resuspended in methanol. The DHA metabolite, 10,17S-docosatriene, was isolated on high-performance liquid chromatography (Surveyor; Thermo-Finnigan), equipped with a Biobasic-AX column (Thermo-Hipersyl-Keystone) (100 mm x 2.1 mm, 5-μm particle sizes) using a gradient, starting on solvent solution A (40:60:0.01 methanol/water/acetic acid, pH 4.5), at a flow rate of 300 μL/min, and reaching 100% solvent B (99.99:0.01 methanol/acetic acid) in 30 minutes, and then run isocratically for 5 minutes. Lipids were diverted to an electrospray-ionization probe (ESI) mounted on a TSQ Quantum (Thermo-Finnigan) triple quadrupole mass spectrometer running on negative ion-detection mode. Quantitative analysis of 10,17S-docosatriene was obtained by selected reaction monitoring (SRM). The selected pair parent/daughter ions for 10,17S-docosatriene were 359/205 m/z.

Statistical Analysis
Data are expressed as means±SEM. Neurobehavioral scores, infarct sizes, and brain DHA metabolite levels were compared among groups by analysis of variance (ANOVA) or repeated-measures ANOVA, as appropriate, with post-hoc Dunnett and Bonferroni tests to correct for multiple comparisons. Statistical significance was accepted at the P<0.05 level.

Results
Physiological Variables
Cranial and rectal temperatures, blood pressure, and arterial blood gases were in the normal range throughout the study. In the subset of animals in which hematocrit was measured before and after treatment, corresponding doses of albumin and DHA–albumin led to equivalent degrees of hemodilution (21.3±2.5% and 11.3±2.0% hematocrit reductions at the 1.25 g/kg and 0.63 g/kg doses, respectively [mean±SEM]).

Neurobehavior
All rats had a total neurological score of 0 before ischemia and a high-grade neurological deficit (11 of a maximum possible 12) when examined at 60 minutes of MCA occlusion. At all post-treatment evaluation times, saline-treated rats showed a more severe neurological deficit than the other 4 groups [F(4,34)=15.93, P<0.0001, repeated-measures ANOVA], and animals that received 0.63 g/kg of DHA–albumin exhibited neurological improvement similar to that produced by higher-dose albumin or DHA–albumin (1.25 g/kg) (Figure 1). At 72 hours after treatment, animals treated with DHA–albumin at the 0.63 g/kg dose exhibited significantly improved total neurological scores compared with the
groups treated with either albumin, 0.63 g/kg, or DHA–albumin, 1.25 g/kg (P < 0.05; Bonferroni tests) (Figure 1).

**Histopathology**

A large, consistent zone of cortical and striatal infarction was present in saline-treated rats with MCA occlusion and 3-day survival (Figure 2). When 0.63 g/kg of DHA–albumin was administered, the mean volume of cortical infarction was reduced by 86% compared with saline-treated animals (P < 0.05). The extent of neuroprotection achieved with 0.63 g/kg DHA–albumin was statistically comparable to (but tended to exceed) the 65% cortical neuroprotection achieved at the higher dose of albumin (1.25 g/kg). Similarly, mean total corrected infarct volumes of the DHA–albumin 0.63 g/kg and albumin 1.25 g/kg groups were reduced by 70% and 50%, respectively, compared with saline-treated animals (P < 0.05) (Figure 2). The striatal component of the infarct was not significantly protected by native albumin but was reduced significantly, by 49%, with 0.63 g/kg of DHA–albumin (P < 0.05) (Figure 2).

Saline-treated ischemic rats showed a marked degree of brain swelling (mean, 12.5%), computed as the percentage increase in right (ipsilateral) hemisphere volume relative to left (Figure 3). Compared with this group, mean brain swelling in DHA–albumin 0.63 g/kg group was markedly reduced, by 58% (P < 0.05), whereas other treatment groups did not differ from saline-treated animals.

**Lipidomic Analysis**

Quantitative analysis of rat brain extracts by liquid chromatography–tandem mass spectrometry at 20 hours after MCAo revealed a large accumulation of 10,17S-docosatriene in the ipsilateral hemisphere of rats treated with DHA–albumin, 0.63 g/kg (P < 0.003), but not in animals treated with native albumin or with saline (Figure 4).
Discussion

We have shown that by complexing the essential fatty acid DHA to human albumin, high-grade neurobehavioral and histological neuroprotection is achieved at a low albumin dose (0.63 g/kg) that, in the absence of DHA, is not robustly neuroprotective (Figures 2 and 3). The DHA–albumin complex also increases the production of a neuroprotective, endogenous DHA-derived mediator in ipsilateral brain tissue.

The 2-hour MCA suture-occlusion model used in this study gives rise to a sizeable infarct that resembles large hemispheric ischemic strokes caused by MCA/ICA occlusion in patients. This model is routinely used in our laboratory5–8,17,19–21 and by other investigators throughout the world to facilitate DHA delivery to the brain.13 DHA, in turn, may gain access to the brain through the astrocytic foot processes of the microvasculature and then be used to rebuild critical phospholipid pools in astrocytes and/or neurons that had been degraded during ischemia-reperfusion. DHA–albumin may also exchange peroxidized PUFA from the brain parenchyma with intact DHA. It has been suggested that DHA is necessary for ion channels, receptors, and transporters to maintain their proper physical conformation.26 The relatively high DHA content in phosphatidylserine, and the direct relationship between DHA viability and DHA–phosphatidylserine content, point to its central role in the regulation of cell signaling and apoptosis.27,28 DHA-containing phospholipids confer a highly fluid-dynamic state to membranes and are actively involved in synaptic and dendritic plasticity. In fact, there is a very active flow of receptor-containing vesicles in dendrites.26 DHA is involved in memory formation,29 excitable membrane function,30 and neuroprotection.13,27,31,32 PUFA may confer neuroprotection by opening background K+ channels or by inhibiting apoptosis.31,32

During postnatal brain development and synaptogenesis, n-3 PUFA must be supplied for the synthesis of excitable membranes, which are highly enriched in DHA phospholipids.33 At the same time, photoreceptor biogenesis is also very active.33 The building blocks of excitable-membrane DHA are actively supplied by the liver, where PUFA elongation, desaturation, and phospholipid synthesis and assembly into plasma lipoproteins occur.14,33 Because albumin is a major carrier of hydrophobic molecules in the blood stream, it is well-suited to deliver DHA. In an in vitro retinal preparation—a model of the central nervous system—bovine serum albumin added to the incubation medium was an avid acceptor of DHA.34–36 At that time, it was suggested that an albumin-like molecule, by removing excessive amounts of free PUFA that could become toxic to neurons, might be a valuable therapeutic approach to brain ischemia or trauma.34

In brain ischemia-reperfusion, DHA is a target of oxidative stress, and nonenzymatic DHA derivatives, including the neuroprostanes, have been identified in brain injury and neurodegeneration.37 Very recently, a stereospecific DHA-oxygenation pathway was identified in mouse brain ischemia-reperfusion.18 One of the DHA derivatives, 10,17-docosatriene, is a potent inhibitor of polymorphonuclear leukocyte infiltration and pro-inflammatory gene expression, and an overall mediator of neuroprotection.18

Because DHA is the precursor of 10,17-docosatriene, we tested the prediction that DHA–albumin may give rise to this neuroprotective brain docosanoid during ischemia-reperfusion by liquid chromatography photodiode array-electrospray ionization tandem mass spectrometry-based lipidomic analysis.18 After DHA–albumin administration, there was an increase in 10,17-docosatriene in the ipsilateral brain at 20 hours of reperfusion after 2 hours of ischemia (Figure 4). In contrast, in vehicle-injected as well as albumin-injected animals, the docosanoid did not change in either ipsilateral or contralateral hemispheres; 10,17-docosatriene is a stereospecific derivative of DHA, formed through a lipoxygenase enzyme that acts on free DHA.38 The free DHA pool in the brain is extremely small. Brain ischemia-reperfusion, by activating phospholipase A2, results in the release of free DHA and arachidonic acid.
from phospholipids. In mice, 10,17S-docosatriene was formed in the ipsilateral hippocampus after 1 hour of MCAo; the docosanoid content peaked at 8 hours, and after 25 hours of reperfusion it was still elevated. In that study, no DHA–albumin was administered; thus, all 10,17S-docosatriene was formed from endogenous DHA.

When the docosanoid was infused into the third ventricle of the mouse brain during the first 48 hours of reperfusion after 1 hour of MCAo, neuroprotection occurred, as evidenced by reduced infarct size and diminished polymorphonuclear leukocyte infiltration. It is of interest that ischemia-reperfusion–induced NFκB activation and pro-inflammatory COX-2 expression (but not COX-1) were downregulated by the docosanoid.

A very recent study has shown that DHA–albumin can also prevent injury to cultured human retinal pigment epithelial cells triggered by oxidative stress, and that this effect is mediated by 10,17S-docosatriene. That study also demonstrates unambiguously that the effect of this neuroprotective lipid is not produced by other essential fatty acids (arachidonic acid) or by eicosanoids.

The lower DHA–albumin dose used in this study, 0.63 g/kg, was highly neuroprotective, whereas the higher DHA–albumin dose (1.25 g/kg) was not. Although the present study does not elucidate the basis of this dose-dependent effect, several possible explanations must be considered. At higher concentrations, DHA may exceed the equilibrium distribution between the microvascular endothelium and albumin and either not desorb efficiently and/or undergo impaired cellular utilization. In addition, findings in other tissues suggest the possibility that at higher DHA–albumin doses, DHA may become a substrate for lipid peroxidation or may induce uncoupling of mitochondrial respiration. In the clinical context, caution in applying higher DHA–albumin doses is therefore appropriate until future studies have clarified the mechanism of this dose-dependent effect.

To summarize, the administration of low-dose DHA–albumin confers high-grade neuroprotection in a model of focal cerebral ischemia and delivers the precursor of the neuroprotective metabolite, 10,17S-docosatriene, to the ischemic brain. These findings have important potential therapeutic implications.

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