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Docosahexaenoic acid complexed to albumin provides neuroprotection after experimental stroke in aged rats

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ABSTRACT

Recently we have shown that docosahexaenoic acid complexed to albumin (DHA–Alb) is neuroprotective after experimental stroke in young rats. The purpose of this study was to determine whether treatment with DHA–Alb would be protective in aged rats after focal cerebral ischemia. Isoflurane/nitrous oxide-anesthetized normothermic (brain temperature 36–36.5 °C) Sprague–Dawley aged rats (18-months old) received 2 h middle cerebral artery occlusion (MCAo) by poly-L-lysine-coated intraluminal suture. The neurological status was evaluated during occlusion (60 min) and on days 1, 2, 3 and 7 after MCAo; a grading scale of 0–12 was employed. DHA (5 mg/kg), Alb (0.63 g/kg), DHA–Alb (5 mg/kg + 0.63 g/kg) or saline was administered i.v. 3 h after onset of stroke (n = 8–10 per group). Ex vivo T2-weighted imaging (T2WI) of the brains was conducted on an 11.7T MRI on day 7 and 3D reconstructions were generated. Infarct volumes and number of GFAP (reactive astrocytes), ED-1 (activated microglia/microphages), NeuN (neurons)-positive cells and SMI-71 (positive vessels) were counted in the cortex and striatum at the level of the central lesion. Physiological variables were entirely comparable between groups. Animals treated with DHA–Alb showed significantly improved neurological scores compared to vehicle rats; 33% improvement on day 1; 39% on day 2; 41% on day 3; and 45% on day 7. Total and cortical lesion volumes computed from T2WI were significantly reduced by DHA–Alb treatment (62 and 69%, respectively). In addition, treatment with DHA–Alb reduced cortical and total brain infarction while promoting cell survival. We conclude that DHA–Alb therapy is highly neuroprotective in aged rats following focal cerebral ischemia and has potential for the effective treatment of ischemic stroke in aged individuals.

Introduction

Stroke is a major cause of death and disability in the elderly. However, experimental stroke research, including the evaluation of neuroprotective drugs, has almost universally relied on use of young animals despite the importance of aging on cerebrovascular disease in humans. This might reflect in part the considerable difficulties to establish a reproducible stroke model in aged animals. In addition, aging has been associated with a significant increase in cerebral infarct size and high mortality (Davis et al., 1995). The need to evaluate and develop an effective treatment for elderly stroke patients remains paramount. Thus animal studies which investigate the mechanisms and efficacy of novel treatments specifically in aged animal remain ideal.

High-dose human albumin (Alb) therapy is strongly neuroprotective in animal models of focal cerebral ischemia (Belayev et al., 2001), as well as in global cerebral ischemia (Belayev et al., 1999b) and traumatic brain injury (Belayev et al., 1999a). The neuroprotective efficacy of albumin is attributed to its multifunctional properties, which include antioxidant action, hemodilution and oncotic effects, binding of copper ions, fatty-acid transport, preservation of endothelial integrity, platelet anti-aggregatory effects and decreased red blood cell sedimentation under low-flow conditions (Belayev et al., 1997, 1998, 2002). Recently, Alb was studied in a phase III clinical trial for acute ischemic stroke (Ginsberg et al., 2011), but the trial was stopped, because it was associated with more adverse effects than saline control (administration of high-dose albumin in dose of 2 g/kg expanded intravascular volume, which leads to pulmonary edema in 13% of patients).

Recent studies have established that omega-3 fatty acids reduce inflammation and may help lower risk of chronic diseases such as heart disease, cancer, and arthritis. Docosahexaenoic acid (DHA; 22:6, n−3),

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a member of omega-3 fatty acid family, is highly concentrated in the brain and appears to be important for cognitive (brain memory and performance) and behavioral function. DHA therapy in low (3.5 mg/kg) and medium (5 mg/kg) doses improves neurological and histological outcome following focal cerebral ischemia in rats (Belayev et al., 2009). Recently, we complexed 25% human serum albumin to DHA and compared its neuroprotective efficacy with that of native albumin in young rats with 2 h of focal cerebral ischemia (Belayev et al., 2005). The DHA–Alb complex affords high-grade neurobehavioral neuroprotection in focal cerebral ischemia, equaling or exceeding that afforded by native albumin at considerably lower doses of Alb (0.63 g/kg). In addition, DHA–Alb leads to improved neurological outcomes and significant reductions of infarct volumes (especially in the salvageable penumbral region) even when treatment is initiated as late as 7 h after onset of middle cerebral artery occlusion (MCAo) (Eady et al., 2012b). The purpose of this study was to determine whether treatment with DHA–Alb would similarly protect aged rats from focal cerebral ischemia.

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, New Orleans. Twenty nine male Sprague–Dawley aged rats (18-months old, Harlan Laboratories, Inc., Indianapolis, IN) were fasted overnight but allowed free access to water. Atropine sulfate (0.5 mg/kg, i.p.) was injected 10 min before anesthesia. Anesthesia was induced with 3.5% isoflurane in a mixture of 70% nitrous oxide and 30% oxygen. All rats were orally intubated and mechanically ventilated. During ventilation, the animals were paralyzed with pancuronium bromide (0.6 mg/kg, i.p.). Temperature probes were inserted into the rectum and the left temporalis muscles. Heating lamps were used to maintain rectal and temporalis temperatures at 36° to 37 °C. Arterial blood gases, pH, hematocrit and plasma glucose were measured 15 min before, 15 min after stroke and 15 min after treatment (3 h 15 min after onset of MCAo). Body weight and rectal temperature were monitored daily during a one-week survival period.

Stroke model

Focal cerebral ischemia was induced by the intraluminal-filament method as we previously described (Belayev et al., 1996). The right middle cerebral artery (MCA) was temporarily occluded for 2 h by a filament coated with poly-L-lysine (Belayev et al., 1996). In brief, the right common carotid artery (CCA) was exposed through a midline neck incision and dissected free of the surrounding nerves. The occipital branches of the external carotid artery (ECA) were coagulated, and the pterygopalatine artery was ligated. A 4-cm length of 3–0 monofilament nylon suture was inserted via the proximal ECA into the internal carotid artery (ICA) and MCA. Prior to use, the tip of the suture was heat-blunted and a 20-mm distal segment of the suture was coated with poly-L-lysine solution (0.1% [wt/vol]) and dried at 60 °C for 1 h (Belayev et al., 1996). Following suture placement, the neck incision was closed, animals were allowed to awaken from anesthesia, and they were tested using a standardized neurobehavioral battery. After 2 h of MCAo, rats were reanesthetized with the same anesthetic combination. Temperature probes were reinserted, and intraluminal sutures were carefully removed. The neck incisions were closed with silk sutures and animals were allowed to survive with free access to food and water. Because of severe neurological deficit and high mortality after MCAo in the aged rats, we limited our study up to 7 days after stroke.

Neurobehavioral testing

Behavioral tests were performed by an observer blinded to the treatment groups at 60 min during MCAo and then on days 1, 2, 3 and 7 after MCAo. The battery consisted of two tests 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 (Belayev et al., 1996). Neurological function was graded on a scale of 0–12 (normal score = 0, maximal score = 12), as previously described (Belayev et al., 1996). Only those animals with a high-grade neurological deficit (10 or greater) were used.

Treatment

Docosahexaenoic acid (DHA) in acid form (Cayman Chemical, Ann Arbor, MI) was physically complexed to human albumin (Alb) by incubating 20 ml of human serum albumin (25%; Baxter, Westlake Village, CA) with 5 mg DHA/g albumin (molar ratio = 0.2) as we previously described (Belayev et al., 2005). Animals were randomly assigned to four treatment groups: DHA (5 mg/kg; $n = 6$), Alb (0.63/kg; $n = 6$), DHA–Alb (5 mg/kg + 0.63 g/kg; $n = 8$) or saline ($n = 9$). Treatment was administered 3 h after onset of stroke into the femoral vein over the course of 3 min using an infusion pump.

Ex vivo MRI

High resolution ex vivo MRI was performed on 4% paraformaldehyde fixed brains on day 7 using an 11.7T Bruker Advance 8.9 cm horizontal bore instrument equipped with an 89 mm (ID) receiver coil (Bruker Biospin, Billerica, MA). MRI-derived lesion volumes were extracted from high resolution T2 weighted images (T2WI) and encompassed cortical and subcortical regions of the entire brain from which 3D reconstructions were generated to illustrate the extent of the lesions. Cheshire image processing software (Hayden Image Processing Group, Waltham, MA) and Amira (Mercury Computer Systems, Visage Imaging, Inc., San Diego, CA) were used for MRI analysis. We have previously reported no volumetric differences between in vivo and ex vivo MRI lesion assessment (Obenaus et al., 2011).

Histopathology and immunostaining

After completion of ex vivo MRI studies, brains were embedded in a gelatin matrix using MultiBrain™ Technology (NeuroScience Associates, Knoxville, TN). Coronal sections were stained with thionine (Nissl), digitized at nine standardized coronal levels and analyzed (MCID™ Core imaging software, Linton, Cambridge, UK) as we previously described (Belayev et al., 1996). An investigator blinded to the experimental groups then outlined the zones of infarction (which were clearly demarcated) as well as the left and right hemispheres of each section. Infarct volume was calculated as the integrated product of cross-sectional area and inter-section distance and corrected for brain swelling.

Immunohistochemical procedures were performed on the adjacent sections to identify specific vascular and neuronal elements in the infarct area. The following antibodies were used: glial fibrillary acid protein (GFAP, Santa Cruz, SDS Biosciences, Sweden) to label reactive astrocytes, Cd68/ED-1 (Serotec, Raleigh, NC) for activated microglia/microphages, Neuron-Specific Nuclear Protein (NeuN, Chemicon/Millipore, Billerica, MA) and rat blood–brain barrier (SMI-71, Sternberger Monoclonals, Inc., Baltimore, MD) as a vascular marker. Numbers of GFAP, ED-1, NeuN positive cells and SMI-71 immunopositive vessels were counted in the three cortical and one subcortical area (ipsi- and contralateral sides) at the level of the central lesion (bregma level −0.3 mm). Data were expressed as numbers of positive cells and vessels per high-power microscopic field (magnification ×40).