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Docosahexaenoic acid (DHA) prevents binge ethanol-dependent aquaporin-4 elevations while inhibiting neurodegeneration: experiments in rat adult-age entorhino-hippocampal slice cultures

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Abstract

Repetitive binge intoxication with ethanol (alcohol) in adult rats, mimicking chronic ethanol abuse in alcoholics, causes trauma-like brain edema and relatively selective neurodegeneration of hippocampal dentate granule cells and pyramidal neurons in the temporal cortex (especially entorhinal cortex). We have now modeled aspects of this type of acquired brain damage *in vitro* with rat entorhino-hippocampal slice cultures of adult brain age (62 ± 3 days). When sequentially treated (four 16-hr overnight exposures) with 100 mM ethanol, the slices display elevated levels of aquaporin-4 (AQP4) water channels accompanied by significant neurodegeneration. Increased AQP4 has been associated with neuroinflammatory responses including edema, pro-inflammatory cytokine elevations, arachidonic acid release and oxidative stress. Co-treatment of ethanol-binged slice cultures with docosahexaenoic acid (DHA), an omega-3 fatty acid known to suppress brain damage from other insults, prevents both the AQP4 elevations and the neurodamage. Surmising that AQP4 augmentation is a causative neuroinflammatory component in this model, we are investigating several possibilities to explain the protective actions of the omega-3 fatty acid. Since the worldwide incidence of cognitive dysfunction and dementia from ethanol abuse and alcoholism is not inconsequential, DHA supplementation with chronic alcoholics could emerge to be a rational approach to potentially lessening brain disabilities.

INTRODUCTION

Despite four decades or more of experimental research on central nervous system pathologies related to chronic ethanol abuse, the neurochemical mechanisms causing alcoholic brain damage are not clearly understood. Although excitotoxic overstimulation of glutamatergic receptors is often central to neurodegeneration in other acquired brain damage conditions (e.g., trauma, stroke, status epilepticus), excitotoxicity as an essential toxic mechanism in ethanol abuse is not supported by pharmacological evidence *in vivo* in animal models (Collins and Neafsey 2012a). In contrast, recent studies from several laboratories indicate that neuroimmune activation, neuroinflammatory pathways, and associated oxidative stress are critical events (Zou and Crews 2010; Pascual et al. 2011; Haorah et al. 2008).

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With respect to alcoholic brain damage models, initial studies with adult rats repetitively binged with ethanol for several days replicated results in a seminal early abstract (Switzer et al. 1982); i.e., surprisingly focused neurodegeneration in the hippocampus (primarily dentate granule cells), temporal cortex (especially entorhinal cortical pyramidal neurons) and olfactory bulb (Collins et al. 1996). Subsequent experiments employing ethanol-binged adolescent- or juvenile-age slice cultures of rat entorhinal cortex and hippocampus (entorhino-hippocampal slices) reproduced the *in vivo* situation to some extent, although differences were apparent (Collins and Neafsey 2012b).

Since brain damage in chronic alcoholism primarily involves adults, it seemed more relevant to examine ethanol's neurotoxic mechanisms in an adult-age *in vitro* model. This decision is based on indications that ethanol's mechanisms may differ between adolescent/juvenile and adult brain. For example, excitotoxicity antagonists are ineffective in preventing neuronal damage in chronically intoxicated (binged) adult rats, but are neuroprotective in adolescent-age brain slice cultures (Collins and Neafsey 2012a). Also, such slice cultures incur regional neurodegeneration from chronic ethanol exposure (such as in CA1-CA3 hippocampal regions) that is different from that occurring *in vivo* in adult animals (i.e., minimal CA1-CA3 neurodamage, but extensive in the dentate gyrus).

With assistance from investigators who have pioneered in the preparation of the more difficult-to-prepare older hippocampal slice cultures for ischemic injury studies (Benardete and Bergold 2009; Bickler et al. 2010), we have generated rat adult-age entorhino-hippocampal slices in long-term culture [from 46–49 day-old male rats, cultured 2–2½ wks prior to the start of ethanol treatment]. The results to date from these slice cultures are consistent with those obtained with adolescent slice cultures in that binge ethanol treatment (100 mM or ~460 mg/dl, a concentration documented in chronic alcoholics (Allely et al. 2006; Jones 1999; Adachi et al. 1991), which we view as causing brain trauma, increases levels of aquaporin-4 (AQP4) and promotes neurodegeneration.

AQP4, the major water channel protein in mammalian brain, is constitutively expressed largely in astroglia (Sato et al. 2007), and is upregulated in reactive astroglia as well as possibly reactive microglia (Tomas-Camardiel et al. 2004). Such upregulation underlies central nervous system (CNS) cytotoxic (cellular) edema associated with ischemia, infectious insults and trauma (Zador et al. 2009). Brain cytotoxic edema and neurologic dysfunction after ischemia are reduced in AQP4 knockout (KO) mice (Verkman 2005). A key finding is AQP4's regulatory role in CNS neuroinflammation: results with AQP4 KO mice show that the channels, concomitant with glial edema, are required for significant brain proinflammatory cytokine expression and neurodamage due to experimental autoimmune encephalomyelitis or endotoxin treatment (Li et al. 2011). We have hypothesized that AQP4 upregulation has a critical role in binge ethanol-induced brain edema, neuroinflammation and neurodegeneration (Collins and Neafsey 2012b).

We report here that DHA supplementation not only inhibits the neurodamage due to binge ethanol in the adult-age brain slices, as in adolescent-age slices, but it also prevents induction of elevations in AQP4 levels. Several possible mechanisms for this effect exist and require experimental examination. This first evidence that DHA can antagonize neuroinflammatory increases in AQP4 levels suggests that other brain insults where AQP4 involvement has been proven, such as brain trauma (Higashida et al. 2011), could benefit from DHA treatment.

MATERIALS AND METHODS

Antibodies for AQP4 (sc-20812) and GAPDH (sc-166545) were from Santa Cruz Biotechnology (Santa Cruz CA), and secondary antibodies were from Jackson ImmunoResearch (West Grove PA). Luminol reagent for immunoblot detection was from Pierce Chemical Co. (Rockford IL). Other chemicals and reagents were from Sigma Chemical Co. (St. Louis MO). Following a Loyola University IACUC-approved protocol, male Sprague-Dawley rats, 42–45 days old, were anesthetized with isoflurane and thoroughly perfused *via* an intracardiac route with 150 ml of ice-cold lactate-Ringer's solution. Rat brains were placed in ice-cold Gey's buffer solution; the entorhinal cortical-hippocampal complex was removed and 250 micron slices were prepared using a McIlwain tissue chopper. The slices were placed on Millipore 0.4 μ m membrane inserts (2–3 slices/insert), and inserts were placed in 6-well plates with 1.2 ml of MEM media with 25% heat-inactivated horse serum (HS). Slices were incubated at 32°C and 5% CO₂ for the first 48 hr. Media was then changed to 20% heat-inactivated HS and slices were transferred to a 37°C incubator (5% CO₂). Cultures were then maintained 15–17 days, changing the media every 2–3 days; and slices were monitored visually and those appearing unusually dark were discarded.

At brain ages of 62 $\pm$ 3 days, slices in the experimental groups were treated over the subsequent 4 days with 100 mM ethanol in MEM/20% HS overnight (~16 hrs), and media without ethanol during daylight hours. Control slices were subjected to similar media changes throughout the 4 days with HS-containing MEM media lacking ethanol. Approximately half of the experimental and control slices were treated with DHA beginning 4 hrs prior to the start of ethanol treatment and throughout both overnight ethanol/media and daytime media only exposures for 4 days. The DHA concentrations of 25 and 50 μ M were chosen because they can enhance neuronal viability in primary cultures (Cao et al. 2005). Slices then were stained with propidium iodide (PI), or pooled for western blot analyses; selected slices were taken for cresyl violet staining.

PI staining

Media were removed from the wells and slices were incubated with 1.2 ml fresh serum-free media containing 10 μ M PI for 20–30 min. After removal of PI-containing media, slices were washed twice with fresh serum-free media, followed by addition of complete media with 20% HS for 1 hr before observing results of PI staining.

Cresyl Violet staining

Slices were washed twice with 1X phosphate buffered saline (PBS), and were fixed with 10% formalin for 30 min at room temperature. After washing the slices with PBS twice, slices were subjected to a series of ethanol washes (100%, 95, and 70%), stained in cresyl violet for 15 min, and then again treated with the above ethanol washes before mounting on coverslips.

Western Blotting

After washing twice in 1X PBS, slices were pooled (6–8 slices per sample) in lysis buffer (0.1% SDS, 1% sodium deoxycholate, 1% triton X-100, 150 mM NaCl, and 25 mM Tris HCl) containing protease and phosphatase inhibitors. Pooled slices in buffer were then sonicated for 15 sec, and centrifuged for 10 minutes at 13,000 rpm. The supernatant was removed for western blotting by standard procedures, and for the determination of protein concentrations using the bicinchoninic acid method. The intensity of immunostaining was analyzed by scanning the images with LABwork 4.5 image acquisition and analysis software from Ultra Violet Products (Upland CA).

Statistical Analyses

Results in Figures 2 and 3 were reproduced thrice with 12–15 brain slices per group in each experiment. Quantitation of PI fluorescence employed 1.99 NIH Image software. AQP4 levels were determined relative to levels of GAPDH as housekeeping protein and expressed as percent of control (mean $\pm$ standard error). All results were analyzed for statistically significant differences ($p < 0.05$ or $p < 0.01$) by Tukey's t-test and one-way analysis of variance (ANOVA) with completely randomized design.

RESULTS

Figure 1 shows representative images of the rat adult-age (~60 days of age) entorhino-hippocampal slices following culturing and the described treatments. The brain slice in Figure 1A after fixation and cresyl violet staining, prior to initiation of binge ethanol treatment, demonstrates the integrity of the hippocampal region and the entorhinal cortex. Fig. 1B is a representative brain slice of ~63 days of age treated for 24 hr with a neurotoxic concentration of N-methyl-D-aspartate (NMDA; 50 μ M). Widespread neurodegeneration as indicated by PI staining is evident, being particularly intense in the hippocampal CA regions; a control slice treated with PI shows little or no PI staining (Fig. 1C). A representative brain slice after 4 days of 100 mM ethanol treatment and PI-treated shows considerable neurodamage (Fig. 1D), whereas a brain slice similarly binged with ethanol and co-treated with DHA (50 μ M) demonstrates negligible PI fluorescence, indicating neuroprotection (Fig. 1E).

Quantitation of neurodegeneration in the adult-age brain slice cultures binge-exposed for 4 days to 100 mM ethanol alone or with DHA supplementation (25 or 50 μ M) is in Figure 2. As indicated in Figure 1D, binge ethanol exposure caused a significant increase in PI-stained neurons over control. DHA exposure alone at two different concentrations did not significantly alter PI staining in the slices, indicating that the concentrations used are not cytotoxic. Supplementation of binge ethanol-treated slice cultures with DHA at either 25 μ M or 50 μ M completely reduced PI staining to control levels, confirming potent neuroprotection by the omega-3 fatty acid.

Immunoblot results for levels of AQP4 and GAPDH (housekeeping control) in adult-age entorhino-hippocampal slices after binge ethanol treatment alone (100 mM) and supplemented with the 50 μ M concentration of DHA are in Fig. 3. Robust elevations in AQP4 (~225% of control levels; $p < 0.05$) were induced by the binge ethanol exposure. DHA alone did not increase control AQP4 levels and even tended to non-significantly lower levels of the water channel. As shown, co-treatment with 50 μ M DHA completely inhibited ($p < 0.05$) the binge ethanol-induced increase in AQP4 in the adult-age entorhino-hippocampal slices.

DISCUSSION

Four key points from these results can be emphasized. The data show that adult-age hippocampal slice cultures mimic the adult rat brain *in vivo* with respect to binge ethanol-induced neuroinflammation and damage —i.e., 4 days of exposure to fluctuating blood levels of ethanol approaching 100 mM (Collins et al. 1996; Knapp and Crews 1999). The results also provide evidence that AQP4 levels are significantly potentiated in response to binge ethanol treatment in an adult-age organotypic model, consistent with findings *in vivo* in adult rats (Sripathirathan et al. 2009). Furthermore, DHA supplementation is as effective a neuroprotectant against binge ethanol in the adult-age cultures as it is in the previously-used adolescent slice cultures (Brown et al. 2009). Finally, and perhaps most unexpected, the omega-3 fatty acid prevents binge ethanol-dependent elevations in AQP4 in the adult-

age EH slice cultures, which is in harmony with a mechanism-based relationship between the water channel, tissue edema, and neurodegeneration due to ethanol binging.

Assuming for the present that the AQP4 protein elevations mainly are the outcome of increased protein expression, how does binge ethanol treatment induce the water channel? A number of cellular molecules have been reported to increase astrocytic AQP4 levels or activity; they include free radicals (Esposito et al. 2008), glutamate (Gunnarson et al. 2008), lactate (Morishima et al. 2008), and proinflammatory cytokines—notably, interleukin-1 β (Ito et al. 2006) and tumor necrosis factor- α (Huang et al. 2012). Ethanol at various concentrations and modes of exposure is known to cause cellular and/or extracellular increases in all of these players. Also, from research with astrocyte cultures, upstream signaling that stimulates AQP4 increases also involves NF-kappaB, mitogen-activated protein kinases, mitochondrial permeability transition, and the Na(+),K(+),Cl(-) co-transporter (Rao et al. 2011). In-depth research is needed to determine which of these signaling events is being triggered by binge ethanol exposure.

Associated with the above question and critical to our hypothesis is the mechanism responsible for the inhibitory action of DHA on AQP4 elevations. Studies have shown that DHA is extensively incorporated into phosphatidylserine (PS) in neuronal membranes (Guo et al. 2007). In the face of neuronal challenges such as high ethanol levels, omega-3 enrichment of PS and other phospholipids can suppress apoptotic signaling, sustain pro-survival pathways, and inhibit neuronal death (Kim et al. 2010). Furthermore, DHA and PS co-treatment is reported to protect developing rat brain from oxidative stress caused by induced seizures (Liu et al. 2012). How potential phospholipid (esp. PS) enrichment with DHA in these DHA-treated cultures possibly relates to (prevention of) induced AQP4 expression requires continued study.

There is an additional route of action for DHA that may also be associated with a PS mechanism of neuroprotection in response to the repetitive binge ethanol exposure. DHA is reported to upregulate as well as activate nuclear peroxisome proliferator-activated receptor (PPAR) transcription factors (Dyall et al. 2010). Notably, PPAR (and esp. PPARgamma) activation is neuroprotective against traumatic brain injury, among others (Semple and Noble-Haesslein 2011). Also, it should be noted that lipoxygenase-generated oxo-forms of DHA such as neuroprotectin-1 are neuroprotective ligands for PPARgamma (Zhao et al. 2011). Interestingly, PS liposomes apparently have anti-inflammatory effects that are dependent in part on PPAR activation (Ramos et al. 2007). It is possible that DHA-modulated PPARgamma activation in some manner, potentially *via* PS, is linked to the observed neuroprotection *via* suppression of AQP4 expression.

Concluding, it is likely that throughout most of human history, chronic abuse of/addiction to alcoholic beverages has been a widespread cause of acquired brain damage (neurodegeneration), cognitive decline—often subtle—and evident dementia (Gupta and Warner 2008). Omega-3 fatty acids might be an under-recognized countermeasure to inhibit this process of neurodamage, which could depend significantly on AQP4-mediated and activated neuroinflammatory processes.

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Figure 1.

Photomicrographs of representative rat organotypic adult-age entorhino-hippocampal slices following long-term culture and treatments as detailed in Methods. A: Cresyl violet-stained slice showing hippocampal CA1, CA3, dentate gyrus (DG) and entorhinal cortex (EC) subregions (Bar=1000 μ). B. Slice exposed to 50 μ M NMDA for 1 day and treated with PI (10 μ M), demonstrating fluorescence-labeled degenerating neurons. C. E-C slice exposed to media only and treated with propidium iodide (PI) reveals no apparent neurodegeneration. D. Experimental slice binge-treated with ethanol (100 mM) over 4 days as described in Methods, followed by PI staining, showing hippocampal and entorhinal cortical neurodegeneration. E. Entorhino-hippocampal slice binge-exposed to 100 mM ethanol for 4 days as with D, and co-treated with DHA (50 μ M), shows minimal fluorescence, indicating neuroprotection by DHA.

Figure 2.

Neurodegeneration (% PI staining) in adult-age entorhino-hippocampal slice cultures following binge treatment for 4 days with 100 mM ethanol (E100), and prevention of the neurodegeneration with docosahexaenoic acid (DHA) at 25 μ M (DHA25) or 50 μ M (DHA50) concentrations. N = 12–15 slices/group. * p <0.05 vs. Control (C); # p <0.05 vs. Ethanol (E100).

Figure 3.

Elevated levels of AQP4 protein relative to GAPDH protein in organotypic adult-age entorhino-hippocampal slices following 4 days binge exposure to 100 mM ethanol (E100) and prevention of the induced elevations by co-treatment with 50 μ M docosahexaenoic acid (DHA50), as described in Methods. Results based on GAPDH expression were replicated in three experiments with pooled slices. * p <0.05 vs. Control (C); # p <0.05 vs. Ethanol (E100).