Linking Binge Alcohol-Induced Neurodamage to Brain Edema and Potential Aquaporin-4 Upregulation: Evidence in Rat Organotypic Brain Slice Cultures and *In Vivo*

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Abstract

Brain edema and derived oxidative stress potentially are critical events in the hippocampal-entorhinal cortical (HEC) neurodegeneration caused by binge alcohol (ethanol) intoxication and withdrawal in adult rats. Edema's role is based on findings that furosemide diuretic antagonizes binge alcohol-dependent brain overhydration and neurodamage *in vivo* and in rat organotypic HEC slice cultures. However, evidence that furosemide has significant antioxidant potential and knowledge that alcohol can cause oxidative stress through non-edemic pathways has placed edema's role in question. We therefore studied three other diuretics and a related non-diuretic that, according to our oxygen radical antioxidant capacity (ORAC) assays or the literature, possess minimal antioxidant potential. Acetazolamide (ATZ), a carbonic anhydrase inhibitor/diuretic with negligible ORAC effectiveness and, interestingly, an aquaporin-4 (AQP4) water channel inhibitor, prevented alcohol-dependent tissue edema and neurodegeneration in HEC slice cultures. Likewise, in binge alcohol-intoxicated rats, ATZ suppressed brain edema while inhibiting neurodegeneration. Torasemide, a loop diuretic lacking furosemide's ORAC capability, also prevented alcohol-induced neurodamage in HEC slice cultures. However, bumetanide (BUM), a diuretic blocker of Na⁺-K⁺-2Cl⁻ channels, and L-644, 711, a nondiuretic anion channel inhibitor—both lacking antioxidant capabilities as well as reportedly ineffective against alcohol-dependent brain damage *in vivo*—reduced neither alcohol-induced neurotoxicity nor (with BUM) edema in HEC slices. Because an AQP4 blocker (ATZ) was neuroprotective, AQP4 expression in the HEC slices was examined and found to be elevated by binge alcohol. The results further indicate that binge ethanol-induced brain edema/swelling, potentially associated with AQP4 upregulation, may be important in consequent neurodegeneration that could derive from neuroinflammatory processes, for example, membrane arachidonic acid mobilization and associated oxidative stress.

Key words: acetazolamide; brain damage; diuretic; ethanol; furosemide; neurotoxicity

Introduction

CHRONIC ALCOHOL (ETHANOL) ABUSE causes brain neurodegeneration, which is believed to result primarily from alcohol's neurocellular and neurovascular effects; in some cases, deficiencies of thiamine and other micronutrients may have important contributing roles. Despite considerable study with animal models, the specific mechanisms underlying malnutrition-independent brain damage due to alcohol remain unclear. One reason may be that, depending on the exposure model used and perhaps age and even gender, one mechanism might predominate over others and hence could involve different brain regions, cells, and signal transduction path-

ways. Two general *in vivo* paradigms have been used in adult rodents to simulate human alcoholic central nervous system (CNS) damage: (a) continuous alcohol intake, usually in liquid diets but sometimes in water, for periods of months or more to produce a low-to-moderate blood alcohol concentration (BAC) (Walker et al., 1980; Riikonen et al., 1999), and (b) binge alcohol treatment, typically via gastric intubation over a subchronic period (4–10 days, but sometimes longer), a trauma-like paradigm that generates episodically high BACs and repetitive withdrawal episodes (Collins et al., 1996; Crews et al., 2000). In addition, a variety of different *in vitro* (culture) models have been used to study neurodegeneration due to either sustained chronic or binge alcohol exposure.

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Perhaps the most widely disseminated hypothetical mechanism for alcohol-induced neurodegeneration is excitotoxicity based on synaptic excitatory glutamate receptors and elevated intraneuronal Ca^{2+} (Lovinger, 1993; Tsai and Coyle, 1998). However, although chronic alcohol exposure has been shown to increase expression of brain ionotropic glutamate receptors and Ca^{2+} channels (Hoffman, 2003), and pharmacological results in developing brain cultures indicate a role for the NMDA receptor (NMDAR) in alcohol withdrawal-dependent neurotoxicity (Prendergast et al., 2004), pharmacological attempts to demonstrate that excitotoxicity underlies alcohol-induced neurodegeneration *in vivo* have failed. Experiments with binge-intoxicated adult rats using NMDAR and Ca^{2+} channel antagonists have not supported a glutamatergic receptor-mediated mechanism (Zou et al., 1996; Collins et al., 1998; Corso et al., 1998), and the lack of effect of NMDAR inhibitors was recently confirmed by others (Hamelink et al., 2005). Spurred by indications that alcohol can induce cellular edema in astroglial and hypothalamic cultures (Sato et al., 1991; Snyder, 1996; Aschner et al., 2001), we considered the possible role of brain (particularly astroglial) edema in the binge alcohol models. Indeed, brain edema is implicated clinically in the neurodamaging sequelae of trauma, *status epilepticus*, stroke, and hepatic failure (Lassmann et al., 1984; Unterberg et al., 2004; Heo et al., 2005; Albrecht and Norenberg, 2006) and has been postulated to be important in alcohol abuse (Lambie, 1985). We found that brains of adult rats binge-intoxicated daily with alcohol for ~1 week were moderately but significantly edemic, and treatment with furosemide blocked the edema while significantly reducing entorhinal cortical and hippocampal dentate neurodegeneration. Furosemide also suppressed binge alcohol-induced cytotoxicity in organotypic hippocampal-entorhinal cortical (HEC) slice cultures (Collins et al., 1998).

Based on these results, we proposed that brain edema has a causative role in the brain neurotoxicity engendered by repetitive (binge) intoxication combined with withdrawal, via its promotion of neuroinflammatory-related oxidative stress, possibly accompanied *in vivo* by pressure necrosis. However, brain edema's essentiality as precursor for oxidative stress from binge alcohol exposure has been questioned (Hamelink et al., 2005), since—as antioxidants can neuroprotect in binge alcohol-intoxicated rats—furosemide was determined to be a potent antioxidant. To further study edema's role in alcohol-dependent brain damage, we first compared the antioxidant capabilities of furosemide and selected other diuretics to the vitamin E analog, Trolox, using the well-known oxygen radical absorbance capacity (ORAC) assay. We then examined the extent of neurodegeneration and brain tissue edema in binge alcohol-treated rat HEC slice cultures using those diuretics and a related compound that have negligible antioxidant capabilities. The compounds studied were acetazolamide (ATZ), a carbonic anhydrase inhibitor; torasemide, a pyridine-sulfonylurea loop diuretic resembling furosemide but with potassium-sparing ability; bumetanide (BUM), another loop-type diuretic; and L-644, 711, a nondiuretic anion channel inhibitor. Neurodegeneration in HEC slices was determined with propidium iodide (PI) staining and/or lactate dehydrogenase (LDH) release. Also, since ATZ potentially inhibits aquaporin-4 (AQP4) (Huber et al., 2007), the effect of binge alcohol on expression of this principal brain water channel in the HEC slices was examined. ATZ actions were

further tested in binge alcohol-intoxicated adult rats; similar to our published experiments with furosemide, edema was determined in fresh brain portions and entorhinal cortical/dentate gyrus neurodegeneration was assessed in fixed, cupric silver-stained brain sections (Collins et al., 1996; Corso et al., 1998). Overall, the findings were consistent with the development of binge alcohol-induced neurodegeneration being linked to brain edema, possibly involving AQP4 upregulation. As such, the neurodamage could ultimately result from oxidative stress arising in part from pro-inflammatory, edema-dependent processes such as phospholipase 2 (PLA_2) activation and arachidonic acid (AA) mobilization (Crews et al., 2004; Brown et al., 2008).

Methods

Chemicals

Furosemide, β -phycoerythrin (β -PE), and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical (St. Louis, MO). Torasemide was obtained from the Loyola University Hospital Pharmacy, 2,2'-Azobis (2-amidinopropane) dihydrochloride (AAPH) was purchased from Polyscience Co. (Warrington, PA), and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Modified Eagles' medium (MEM) media, Hanks' buffer, and horse serum were obtained from Gibco (Gaithersburg, MD). Tissue culture inserts and plasticware were from Fisher Scientific (Pittsburgh, PA).

Oxygen radical antioxidant capacity assay

Antioxidant activities of diuretics were determined with the oxygen radical antioxidant capacity (ORAC) assay (Cao et al., 1993), using β -PE as indicator protein, AAPH as a peroxy radical generator, and Trolox, a water-soluble vitamin E analog, as a standard. The reaction mixture, prepared fresh in triply-distilled deionized water, contained 1.67×10^{-8} M β -PE and 3×10^{-3} M AAPH in 7.5×10^{-2} M phosphate buffer, pH 7.0, in a final volume of 2 ml. Into each sample tube, 20 μ l of blank (1% dimethylsulfoxide [DMSO] or water) or the diuretics (1, 2, and 4 μ M) were added in 1% DMSO. After addition of AAPH to start peroxy radical generation and mixing well, the loss of fluorescence was measured every 5 min until zero fluorescence occurred, using a Perkin-Elmer fluorescence spectrophotometer at 565-nm emission and 540-nm excitation. Trolox was assayed during each run, and the ORAC value (U/ml) of the sample was calculated using the Trolox standard curve. One ORAC unit has been assigned the net protection area (S) provided by 1 μ M Trolox final concentration. The ORAC value was calculated as $(S_{\text{sample}} - S_{\text{blank}}) / (S_{\text{Trolox}} - S_{\text{blank}})$, where S is area under the quenching curve.

Organotypic hippocampal-entorhinal cortical slice cultures

Adult rats and rat pups were acquired and cared for in accordance with the guidelines published in the NIH *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health publication no. 85-23) and the principles presented in the "Guidelines for the Use of Animals in Neuroscience Research" by the Society for Neuroscience. All protocols have been approved by Loyola University Institutional Animal Care and Use Committee. Sprague-Dawley rat pups (7 days

old; maternal source, Zivic-Miller, Portersville, PA) were used to prepare organotypic HEC slice cultures (Stoppini et al., 1991; Collins et al., 1998). Pups were cold-anesthetized and then were decapitated. The HEC was removed and transverse slices (350 μ) were placed on Millipore 0.4- μ Millicel tissue culture inserts in six-well Falcon plates with covers; they were cultured in an atmosphere of 95% O₂/5% CO₂ at 37°C on MEM media containing 25% horse serum, 25% Hanks buffer, 20 mM HEPES, and 6.5 mg/ml glucose. Each treatment group contained six wells containing three to four slices per well. The media was changed every 3 days. Slices were periodically examined, and those appearing unhealthy under the phase contrast microscope (darkened appearance) were discarded.

After 14–16 days of culture and maturation, plates with HEC slices were treated with alcohol, and/or vehicle (0.1–1% DMSO) $\pm$ diuretic at the indicated concentrations over a period of 6 successive days, depending on the experiment. They were kept in the incubator in separate closed Tupperware brand containers; alcohol culture containers also had a small open dish of 1.8% v/v alcohol/water to maintain alcohol levels in culture wells, and controls had a similar dish with water only. One of two alcohol treatment modifications was used: slices were exposed daily to alcohol in culture media (initial concentration, 100 mM, but 150 mM for torasemide and L-644, 711 experiments) for 15 h (incubation), and then all transferred to alcohol-free media for 9 h (withdrawal), resulting in six withdrawal episodes; alternatively, slices were given one extended 3-day alcohol or control media incubation period, followed by the first 9-h withdrawal, and then three 15-h incubation days with three more withdrawals, for a total of four withdrawal episodes. Media alcohol concentrations, which dropped moderately (15–20%, unpublished data) during incubation periods, were re-established by addition of alcohol at each incubation period. In either modification, complete media changes were done after 3 days. When used, ATZ, torasemide, BUM, or L-644, 711, dissolved in dimethylsulfoxide and diluted appropriately into media, were present in both incubation and withdrawal media throughout the 6 days; control slices received changes with media only, $\pm$ relevant DMSO concentrations. Media aliquots pooled from the last three withdrawal periods were analyzed for LDH with a Sigma diagnostic kit. When required, tissue protein was determined in centrifuged extracts of slice homogenates with a bicinchoninic acid (BCA)-based method (Pierce Biotechnology, Rockford, IL).

Detection of neurodegeneration in HEC slices with propidium iodide

Degenerating neurons were detected in HEC slices with PI (5 μ g/ml media), added during the final hour of the last alcohol withdrawal period. PI uptake in each slice was assessed by capturing 8–12-sec exposures with a DS-5M Nikon color camera attached to an Eclipse TS100 inverted microscope with an Epi-fluorescence attachment. Using Image J version 1.36 (NIH, Bethesda, MD), the entire slice was outlined, and the percentage of the entire slice area that fluoresced red was recorded and used for overall statistical analysis.

Western blot analysis of aquaporin 4

Following 4 days of binge alcohol treatment, HEC slices were rinsed twice with phosphate-buffered saline (PBS), lysed

by pipette action in radioimmunoprecipitation assay buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 2 mM NaF, 2 mM EDTA, 0.1% SDS, and protease inhibitor cocktail), centrifuged, and protein content determined by the BCA method. Samples of equal protein content (10–15 μ g) were resolved by 10% SDS-PAGE, transferred to Millipore Immobilon-P membranes, and blotted with AQP4 primary antibody (Genetex, San Antonio, TX) at a dilution of 1:1000. Peroxidase-conjugated secondary antibody was used at a dilution of 1:3000. Bound antibodies were visualized using an ECL detection kit (Pierce Biotechnology) and exposed to Kodak X-Omat film (Kodak, Rochester, NY).

Binge alcohol intoxication experiments

Adult male Sprague-Dawley rats, 280–320 g, obtained from Zivic-Miller Laboratories (Portersville, PA), were acclimatized in the animal facilities. Water and lab chow were provided *ad libitum*, and the animals were maintained in a temperature-controlled (25°C), light-controlled (12:12 h), and humidity-controlled (50–55%) room. Rats were divided into four experimental groups (8–10 rats/group): vehicle (50:50 water/Vanilla Ensure, 355 cal/8 fl oz; Ross Laboratories), alcohol, ATZ alone (10 mg/kg/day in 1% DMSO/H₂O), and alcohol + ATZ. They were intubated intragastrically with a single morning dose of 25–35% alcohol (w/v) in vehicle, or with vehicle only, for 8 days. Alcohol doses were 3.5–6.5 g/kg, depending on intoxication state and BAC, in a procedure extensively detailed previously (Collins et al., 1998). Importantly, the 8-day dose average, $\sim$ 5 g/kg, did not differ significantly between the alcohol-treated groups. The daily intraperitoneal dose of ATZ was based on our above-cited studies conducted with furosemide and on ATZ's estimated potency and plasma half-life.

Blood alcohol concentration assays

Tail blood samples were taken daily with 50- μ l heparinized micro-hematocrit capillary tubes 2 h after alcohol administration, placed in 2 ml of 3.5% HClO₄, and centrifuged for 5 min at 4°C. An 80- μ l aliquot of the supernatant was mixed with 2 ml of 0.5 M Tris buffer (pH 8.8) containing 5% NAD and 0.005% alcohol dehydrogenase (Boehringer Mannheim, Indianapolis, IN). After incubation for 15 min at 37°C, the absorbance for NADH was read at 340 nm, and BAC assays were calculated from a standard curve.

Brain water determination

Brain and brain slice edema measurements applied a basic wet weight/dry weight procedure (Olson et al., 1990). With *in vivo* studies, at 9 a.m. the morning following 8 days of alcohol or vehicle intubation with and without ATZ co-treatment, rats were lightly anesthetized with sodium pentobarbital (60 mg/kg i.p.) and decapitated. The brain cortices were rapidly removed, and wet weights were obtained using pre-weighed aluminum foil. The brain tissues in foil were heated at 102°C for 48 h, and the dry weights were obtained. The percent of brain slice water was calculated according to 100 $\times$ (wet weight minus dry weight)/(wet weight). For HEC slices, fresh slices were carefully severed from insert membranes, pooled on pre-weighed foil, and weighed before and after heating as above to obtain wet weight/dry weight ratios.

Detection and quantitation of *in vivo* neurodegeneration (De Olmos cupric silver staining)

At 24 h following the last alcohol/vehicle dose, rats were anesthetized with sodium pentobarbital (80 mg/kg ip) and perfused intracardially with 4% paraformaldehyde in 0.4 M phosphate (pH 7.4) buffer. Brains were removed and kept 2 days in a solution of buffered paraformaldehyde containing 30% sucrose (w/v). Frozen brain sections (40 μ) cut in the horizontal plane were taken at every 0.5-mm level, stained using the cupric silver technique for degenerating (argyrophilic) neurons (De Olmos et al., 1981), and mounted on glass slides. The mean number was obtained from counting and averaging the number of argyrophilic neurons in all brain sections/sides/regions, as described elsewhere (Collins et al., 1996).

Statistical analysis

Data were expressed as mean $\pm$ SEM, and the test for significance was achieved by first performing a one-way analysis of variance (ANOVA). Subsequent pair-wise comparisons were performed using Tukey LSD and Bonferroni post-hoc tests. Experiments were usually replicated at least three times. Mean BACs were analyzed with post-hoc Student *t*-test. The mean number of argyrophilic neurons counted in brain sections ($n = 8-9$ at each successive level chosen) from rats treated with or without alcohol and/or diuretic was compared by the non-parametric Wilcoxon Rank Sum test, because the distributions were not normal.

Results

The antioxidant capabilities of three diuretics—ATZ, torasemide, and furosemide—were compared with the ORAC assay to Trolox, a vitamin E analog and well-established antioxidant, in vehicle (1% DMSO/water, which had no effect on the assay). Figure 1A compares the effects of Trolox and increasing concentrations of furosemide over time on the relative fluorescence of β -PE indicator protein; the two other diuretics were similarly compared. Figure 1B summarizes ORAC values versus concentrations for the three diuretics. It confirms furosemide's potency and demonstrates similar dose-dependent increases for furosemide and Trolox with respect to antioxidant capabilities. However, ATZ and torasemide exhibited initially low ORAC values relative to Trolox that changed only minimally with increasing concentrations. The lack of increase with concentration indicates that ATZ and torasemide possess no discernible antioxidant activities. Although not examined here, BUM and L-644, 711 have been judged to have negligible "biological" antioxidant potencies using other assays (Hamelink et al., 2005).

With regard to binge treatment of HEC slices with alcohol (100 mM), Figure 2A shows that such treatment causes statistically significant brain tissue edema, consistent with findings in intact binge alcohol-intoxicated rats (Collins et al., 1998). Slice edema was apparent at 4 days, prior to evident neurotoxicity. Furthermore, whereas daily treatment with ATZ, a diuretic with minimal antioxidant capability, did not significantly change control slice water content, it blocked binge alcohol-dependent brain tissue edema. At the 4-day duration of alcohol treatment and withdrawals, expression levels of AQP4 water channel in the HEC slices were exam-

FIG. 1. Oxygen radical antioxidant capacity (ORAC) assays. (A) Comparison of the effects of Trolox and increasing concentrations of furosemide on the relative fluorescence of β -phycoerythrin (β -PE). (B) Relative ORAC values demonstrating dose-related increases for the established antioxidant, Trolox, and furosemide, while acetazolamide and torasemide showed minimal basal activities with no significant dose-dependent increases. ORAC values are means $\pm$ standard error of the mean (SEM); $F(3, 8) = 5.20$. Standard errors for acetazolamide and torasemide are too small to be seen in the figure.

ined by Western blot analysis. Quantitation of AQP4 blots (representative images in Fig. 2B) revealed that binge alcohol treatment demonstrably increased AQP4 about 2.5-fold over its expression levels in control slices.

Neuronal death in HEC slices that were binge-exposed to alcohol and the effect of ATZ co-treatment were assessed with the vital dye, PI. As shown in Figure 3A, representative control and ATZ-treated slices showed little PI labeling, but binge alcohol-treated slices showed relatively intense PI fluorescence due to neurodegeneration that was prominent in the entorhinal cortex. It was also clearly evident in the hippocampal dentate gyrus, but less so in CA1 and CA3 regions. ATZ co-treatment largely prevented the binge alcohol-induced PI labeling in the dentate and greatly suppressed labeling in the entorhinal cortical and CA regions. Figure 3B shows quantitation of total slice PI labeling in control and

FIG. 2. (A) Increase in water content in rat organotypic hippocampal-entorhinal cortical (HEC) slices induced by repetitive binge alcohol exposure (100 mM) and withdrawal for 4 days, and prevention of the increased edema by co-treatment with acetazolamide (ATZ, 1.25 mM). Vehicle (0.1% dimethyl sulfoxide [DMSO] final concentration) had no effect on basal tissue water content. $N = 10\text{--}15$ slices/sample. $F(3, 54) = 4.92$, $p = 0.004$; $*p < 0.01$ compared to control slices. (B) Representative Western blot analysis of aquaporin-4 (AQP4) in protein extracts of control (left lane) and binge-exposed (right lane) HEC slices. Bottom: Induction of AQP4 expression levels in HEC slices by binge alcohol (100 mM) for 4 days. $N = 3$ /group; $t = -2.3$; $*p < 0.05$ compared to control.

binge-exposed HEC slices $\pm$ ATZ co-treatment. Neuronal PI fluorescence in slices treated with ATZ alone did not differ significantly from that in control slices. The density of PI labeling in slices episodically exposed to 100 mM alcohol for 6 days was significantly greater than in control slices. ATZ co-treatment during binge alcohol exposure completely abrogated the increased PI density of degenerating neurons. Also consistent with this PI data, Figure 3C shows that 6 days of binge alcohol treatment of HEC slice cultures increased LDH leakage (180% of control), which is a measure of neurotoxic damage in organotypic slices (Bruce et al., 1996). ATZ, having no effect on LDH release/leakage itself, significantly

FIG. 3. Effect of acetazolamide (ATZ) on neurodegeneration in hippocampal-entorhinal cortical (HEC) slice cultures binge-exposed to alcohol (100 mM, 6 days). (A) Representative photomicrographs of propidium iodide (PI) labeling in HEC slices: Cont (0.1% dimethyl sulfoxide [DMSO] vehicle-treated) slices with normal background fluorescence and minimal cellular labeling; ATZ treatment (1.25 mM) only, with no degenerating neurons above control; binge alcohol (Alc)-treated slices showing bright cellular fluorescence due to PI uptake by degenerating neurons in the hippocampal areas and entorhinal cortex; Alc + ATZ co-treatment demonstrating evident suppression of PI-labeled neurons compared to alcohol. (B) Quantitation of PI labeling density in HEC slices from cultures represented in photomicrographs of A. $N = 6\text{--}12$ wells/group. $F(3, 40) = 20.01$; $*p < 0.01$ compared to control. (C) Increase in withdrawal media lactate dehydrogenase (LDH) activity in HEC slice cultures binge-exposed to alcohol as in A, and its prevention by co-treatment with acetazolamide (ATZ, 1.25 mM). Vehicle (0.1% DMSO) had no effect on LDH activity. $N = 6\text{--}9$ wells/group. $F(3, 16) = 14.46$, $p = 0.0008$; $*p < 0.05$ compared to control.

FIG. 4. Effects of torasemide or L-644, 711 on lactate dehydrogenase (LDH) release induced by binge alcohol treatment of hippocampal-entorhinal cortical (HEC) slice cultures. **(A)** Prevention by co-treatment with torasemide (1 mM) of increases in LDH in withdrawal media during binge alcohol treatment (150 mM, 6 days) and withdrawal in HEC slice cultures. $N=6-9$ wells/group. $F(3, 25)=39.58$; $*p < 0.05$ compared to control. **(B)** Failure of co-treatment with L-644, 711 (1 mM) to prevent increases in LDH in withdrawal media due to binge alcohol treatment (150 mM, 6 days) and withdrawal in HEC slice cultures. $N=6-9$ wells/group. $F(3, 24)=9.83$; $*p < 0.05$ compared to control.

prevented the elevation in media LDH when co-administered throughout the binge alcohol treatment.

Figure 4 shows the results of two further compounds, torasemide and L-644, 711, which were screened for neuroprotection using LDH leakage from binge alcohol-treated HEC slice cultures. Torasemide, the second diuretic in Figure 1B with negligible ORAC antioxidant potential, had no effect on basal LDH release; however, torasemide co-treatment completely suppressed binge alcohol-induced LDH leakage (Fig. 4A), indicating effective neuroprotection. On the other hand, L-644, 711, an agent without antioxidant potential that did not affect neurotoxicity in binge-intoxicated rats (Hamelink et al., 2005), also had no effect on LDH release in alcohol-exposed HEC slice cultures, confirming that it lacks neuroprotective effects in these binge alcohol models. Neither compound was examined further with PI labeling.

In Figure 5A, representative PI uptake results in HEC slices during binge alcohol treatment are shown with BUM, a loop diuretic that, unlike furosemide, was neither an effective "biological" antioxidant nor a neuroprotectant in binge-

intoxicated rats (Hamelink et al., 2005). In our HEC slice experiments, BUM appeared to cause a modest degree of neurotoxicity in the slices at 10 μ M, and higher concentrations (20–50 μ M) evoked considerable neurodegeneration (J. Brown, unpublished data). BUM at 10 μ M also failed to noticeably reduce binge alcohol-induced PI labeling in any region of the slice. In Figure 5B, quantitation of the PI fluorescence in regions of binge-exposed HEC slices co-treated with 10 μ M BUM confirms the interpretation of representative PI images in Figure 5A, since there was a tendency for 10 μ M BUM alone to potentiate PI staining, and a 10-fold increase in PI fluorescence over control due to binge alcohol that was not reduced or changed by 10 μ M BUM co-treatment. We speculated that the inability of BUM to reduce neurodegeneration in this model might indicate failure to counter alcohol-induced brain slice edema. In support of this speculation, Figure 5C unmistakably shows that binge alcohol-dependent edema in HEC slices was not significantly suppressed by co-treatment with BUM.

ATZ was examined *in vivo* in the once-daily binge intoxication and neurodegeneration protocol with adult male rats. Figure 6A shows that, as with furosemide and alcohol (Collins et al., 1998), neither the daily mean 2-h BAC values from a single alcohol intubation for 8 days nor the averages of these means differed significantly between the alcohol $\pm$ ATZ groups. In Figure 6B, the daily binge alcohol treatment provoked brain edema, significantly increasing brain water content above control rat brain values. Daily ATZ co-treatment in control rats did not significantly affect brain water content; however, as evident in Figure 6B, ATZ completely inhibited the alcohol-induced brain edema.

With regard to *in vivo* neurodegeneration, De Olmos cupric silver staining of fixed brain sections from either the control or ATZ-treated rats revealed little or no argyrophilia, in agreement with our published studies (Collins et al., 1998), and are therefore omitted from Figure 6B. Cupric silver-stained sections from binge-exposed rats (not shown) revealed argyrophilic degenerating neurons in the entorhinal cortex, particularly layer III, and to a lesser extent in the dentate gyrus, consistent with our results and those of others using either binge intoxication procedure (Collins et al., 1998; Crews et al., 2000; Hamelink et al., 2005). Scattered argyrophilic pyramidal neurons were also apparent in other regions of the temporal cortex of alcohol-intoxicated rats, as expected from previous studies in our laboratory and in others (Collins et al., 1996). Quantification of mean argyrophilic cell counts in the entorhinal cortex (EC) and dentate gyrus (DG) of binge alcohol-intoxicated rats (Fig. 6C) showed that binge alcohol-induced neurodegeneration in the EC and DG was significantly inhibited (>85%) by ATZ co-treatment. Thus ATZ, a diuretic lacking in antioxidant capability, significantly prevented brain edema concomitant with neurodegeneration in both binge alcohol-treated HEC slices as well as in binge alcohol-intoxicated rats.

Discussion

We have proposed that alcohol-induced degeneration of entorhinal cortical pyramidal neurons and dentate granule cells in adult rats that occurs due to repetitive bouts of exposure and withdrawal is linked to a significant extent to nonsynaptic cellular (especially glial) swelling phenomena.

This proposal was based on the suppression of both brain edema and neurodegeneration *in vivo* and *in vitro* was achieved with the diuretic furosemide, a potent K^+Cl^- co-transport inhibitor (Collins et al., 1998; Corso et al., 1998); the present study is concerned mainly with ATZ and, to a lesser extent, torasemide and BUM. Among the key results herein are that AQP4 water channels appear to be upregulated by the neurotoxic binge alcohol exposure *in vitro*, and that ATZ, a diuretic lacking antioxidant potency—unlike furosemide—and also a potent inhibitor of AQP4 activity, suppresses binge alcohol-dependent brain edema and neurodegeneration *in vitro* and *in vivo*.

To verify furosemide's antioxidant potential and to determine whether or not ATZ and torasemide possessed similar capabilities, we utilized the ORAC assay, a widely accepted standard tool for antioxidant activity measurements in the pharmaceutical and food industries (Huang et al., 2002). Like all antioxidant estimations, the ORAC assay has its short-

comings, but its use of peroxy (or hydroxyl) radicals as pro-oxidants makes it different from assays that involve oxidants that are not necessarily pro-oxidants (Prior et al., 2005). Nevertheless, as emphasized elsewhere (Hamelink et al., 2005), for a variety of reasons a single *in vitro* parameter of antioxidant activity does not necessarily predict biological activity *in vivo*.

For *in vitro* assessments of binge alcohol neurotoxicity, the organotypic HEC slice culture tends to replicate the regional brain degeneration pattern observed *in vivo*, with some differences such as sometimes evident CA1 damage and possibly NMDAR involvement. We suspect that these differences are related to the adolescent age of the HEC slices in culture (~ 4 weeks of age overall) relative to adult brain, but this requires further study. Nevertheless, in retaining much of the neuron-glia relationships and neuronal connectivity of intact maturing brain, brain slice cultures have distinct advantages for neurotoxicity experiments over dispersed (usually fetal) hippocampal or cortical cultures (Diekmann et al., 1994; Holopainen, 2005). Alcohol-induced neurodegeneration in brain slice cultures has generally required subchronic exposure to concentrations approaching ~ 100 mM, combined with withdrawals (Collins et al., 1998; Prendergast et al., 2004). However, such concentrations are not uncommon in bingeing chronic alcoholics (Lindblad and Olsson, 1976; Urso et al., 1981; Minion et al., 1989). Determination of neurodegeneration in the slice cultures used PI, a vital stain that labels dying neurons (Vornov et al., 1991), and LDH release, a general measure of neurotoxicity in brain slice cultures that correlates well with PI labeling (Bruce et al., 1996; Norberg et al., 1999). With regard to media conditions in our brain slice experiments, we acknowledge they are likely to be hyper-osmolar to varying degrees during the alcohol exposure (consistent with the plasma of alcoholics during intoxication (Snyder et al., 1992; Pursell et al., 2001)), but iso-osmolar during withdrawal periods.

FIG. 5. Effect of bumetanide (BUM, $10 \mu M$) on propidium iodide (PI) labeling of degenerating neurons and tissue edema in hippocampal-entorhinal cortical (HEC) slice cultures binge-exposed to alcohol (100 mM). **(A)** Representative photomicrographs of propidium iodide (PI) labeling in HEC slices: Cont (vehicle-treated) slices with normal background fluorescence and no cellular labeling; BUM treatment only, with indications of a detectable modest increase in degenerating neurons above control; slices treated with alcohol (Alc) for 6 days showing increased cellular fluorescence due to PI uptake by degenerating neurons in the hippocampus and entorhinal cortex; slices treated with Alc + BUM for 6 days showing no apparent suppression of PI-labeled neurons compared to alcohol alone. **(B)** Quantitation of PI labeling densities in A, showing failure of BUM co-treatment to reduce neurodegeneration in HEC slice cultures due to binge alcohol exposure (100 mM, 6 days) and withdrawals. $N = 6-12$ slices/group. $F(3, 48) = 50.21$; $*p < 0.01$ compared to control slices. **(C)** Increase in water content induced in organotypic HEC slices by repetitive binge alcohol exposure and withdrawal for 4 days, as in Figure 2, and the lack of significant suppression by co-treatment with BUM. Vehicle (0.1% DMSO final concentration) had no effect on brain water content. $N = 10-15$ slices/sample. $F(3, 60) = 9.82$; $*p < 0.01$ compared to control slices.

FIG. 6. Effect of acetazolamide (ATZ) co-treatment (10 mg/kg/days i.p.) on 2-h BAC, brain edema, and regional neurodegeneration in adult rats binge-intoxicated once daily with alcohol (3.5–6.5 g/kg/days) for 8 successive days. **(A)** No significant differences in the daily 2-h BAC means ($\pm$ SEM) between alcohol, and alcohol + ATZ rats. Parentheses contain means $\pm$ SEM averaged from the 8 days, $F(7, 8) = 4.53$. **(B)** Suppression of brain edema induced by once-daily binge alcohol intoxication for 8 days by ATZ co-treatment. $N = 6$ –8 rats/group. $F(3, 25) = 23.88$; $*p < 0.05$ compared to control. **(C)** Significant reduction by ATZ co-treatment of argyrophilia (mean degenerating neuronal cell counts) in the entorhinal cortex and dentate gyrus of adult rats binge alcohol-intoxicated once daily for 8 days. $N = 6$ rats/group. $*p < 0.05$ compared to alcohol by individual nonparametric Wilcoxon Rank Sum tests (for entorhinal cortex, $W = 32$, $*p = 0.015$; for dentate gyrus, $W = 30.5$, $*p = 0.027$).

Turning to the *in vivo* methodology in this report, our earlier binge alcohol-induced brain neurodegeneration studies (Corso et al., 1990; Collins et al., 1996) utilized the original approach to induce alcohol withdrawal seizures (Majchrowicz, 1975) that was first noted to promote degeneration of pyramidal neurons in limbic (especially entorhinal) cortical regions and granule cells of the dentate gyrus (Switzer et al., 1982). This entailed alcohol intubations three to four times daily (9–12 g/kg/day) for a 4-day duration to generate episodically high average BAC values (360–450 mg/dl), but with a mortality rate sometimes approaching 40%. We modified the model to a less severe treatment (Collins et al., 1998) of a single daily alcohol intubation (~ 5 g/kg) for 7–10 days, yielding average 2-h BAC values of ~ 250 mg/dl—still considered clinically severe intoxication (Lowenstein et al., 1990)—and lower ($\sim 20\%$) mortality rates; this modification was used here with ATZ. The fact that in this study the daily and aggregate 2-h BAC values did not differ between alcohol- and alcohol + ATZ-treated rats (Fig. 6A) obviates the possibility that lower brain alcohol concentrations might explain the diuretic's anti-edemic and neuroprotective actions.

The less severe single daily subchronic binge generates regional distributions of degenerating (argyrophilic) neurons that are indistinguishable from—but less intense than—those in the original Majchrowicz (1975) procedure. No significant neuroprotection is achieved by NMDAR inhibition in either intoxication protocol. The degree of induced brain edema is similar in both binge models; in that regard, the brain water increase in Figure 6B, although a seemingly low percentage ($\sim 0.6\%$), represents nearly 2.5% brain swelling (Elliott and Jasper, 1949). In brief, there is no indication that the original Majchrowicz intoxication procedure and its once-daily modification differ in the cellular mechanisms responsible for alcohol-induced brain edema and neurodegeneration.

Concomitant with inhibiting neurodamage, furosemide suppressed the brain edema in adult rats due to repetitive once-daily intoxication/withdrawal—an effect consistent with the diuretic's blockade of Ca^{2+} -independent astroglial swelling in epileptogenic hippocampal slices (Hochman et al., 1995). Lack of neuroprotection in the binge intoxication models by MK-801, 6,7-dinitroquinoxaline-2,3-dione (non-NMDAR glutamate [ionotropic] receptor antagonist), nimodipine, or nitric oxide synthase inhibitors provides no support for a central role for glutamate receptor-dependent excitotoxicity, extracellular Ca^{2+} uptake, or nitric oxide generation (Zou et al., 1996; Collins et al., 1998; Corso et al., 1998). Also, the facts that binge alcohol-induced neurodegeneration in rats was not reduced by the noncompetitive NMDAR antagonist, memantine (Hamelink et al., 2005), nor was it accompanied by increased brain NMDAR as ascertained with [^3H]MK-801 binding (Rudolph et al., 1997), further argue against a prominent excitotoxic mechanism. It is still possible, as suggested by studies of ionotropic glutamate receptors in alcoholics (Preuss et al., 2006) and reviewed by others (Tsai and Coyle, 1998), that excessive glutamatergic transmission could be involved in alcohol withdrawal seizures and disturbed autonomic activation. However, in binge alcohol-intoxicated adult rats, the density of neurodegeneration reaches a maximum considerably earlier than the time of greatest seizure activity (Majchrowicz, 1975) and does not increase throughout a 36-h withdrawal period (Collins et al.,

TABLE 1. EFFECTS OF DIURETICS, ANTIOXIDANTS, AND L-644-711 ON BINGE ALCOHOL-INDUCED BRAIN EDEMA AND/OR NEUROTOXICITY IN HIPPOCAMPAL-ENTORHINOCORTICAL (HEC) SLICE CULTURES AND *IN VIVO*: COMPARISON WITH *IN VITRO* ANTIOXIDANT CAPABILITY

Compound or agent	Edema inhibition in HEC slices	Prevention of neurotoxicity in HEC slices	Edema inhibition <i>in vivo</i>	Prevention of neurotoxicity <i>in vivo</i>	Antioxidant capability <i>in vitro</i>
Furosemide	n.d.	Yes	Yes	Yes	High
Acetazolamide	Yes	Yes	Yes	Yes	Negligible
Toraseamide	n.d.	Yes	n.d.	n.d.	Negligible
Bumetanide	No	No	n.d.	No ^a	Negligible ^a
L-644, 711	n.d.	No	n.d.	No ^a	Negligible ^a
Cannabidiol	n.d.	n.d.	n.d.	Yes ^a	High ^a
Vitamin E	n.d.	n.d.	n.d.	Yes ^{a,c}	High ^a
BHT	n.d.	n.d.	n.d.	Yes ^{a,b}	High ^a

^aHamelink et al. (2005).

^bCrews et al. (2006).

^cYes in Alkamuls solubilizer (Hamelink et al., 2005); no in corn oil (Crews et al., 2006).

n.d., not determined; BHT, butylated hydroxytoluene.

1996)—suggesting that seizure propensity and neurodamage are not directly related.

Table 1 summarizes the effects of the three diuretics on binge alcohol effects *in vitro* and/or *in vivo*, and contrasts these findings with their antioxidant potentials. Our HEC slice culture results as well as the reported *in vivo* studies with BUM and L-644, 711 are included. It also includes reported results with several anti-oxidants already alluded to, which will be discussed below. The ORAC assays verified that furosemide is an effective antioxidant, being at least equipotent with the vitamin E-related Trolox. Based on furosemide's activity, as well as positive results with several established antioxidants and negative results with L-644, 711 and BUM, Hamelink et al. (2005) suggested that furosemide's protection could be more closely associated with its antioxidant properties than with edema reduction. However, we emphasize that no confirmatory brain edema assessments were done in the abovementioned study. For example, if binge alcohol-induced brain edema had been found to be unaffected *in vivo* by L-644, 711 or BUM for blood-brain barrier, metabolic, or other reasons, the Hamelink et al. (2005) conclusions would, in our opinion, require revision. And to that point, BUM failed to prevent edema in HEC slices binge-exposed to alcohol (Fig. 5C). In addition, if a mechanism other than edema deterrence (such as free radical trapping) was the principal neuroprotective one employed by furosemide, the diuretic would have been expected to significantly reduce binge alcohol-induced neurodamage in all affected regions, but it failed to do so in the olfactory bulb glomeruli (Collins et al., 1998), a region not examined by Hamelink et al. (2005).

Table 1 also summarizes that, despite lacking antioxidant capability, ATZ diuretic, the primary focus of these current experiments, prevented binge alcohol-induced tissue water accumulation and neurodegeneration both in HEC slice cultures and *in vivo*. A carbonic anhydrase inhibitor and cerebrovascular dilator stimulus (Settakias et al., 2003), ATZ has been reported to reduce ischemic brain edema in rats (Czernicki et al., 1994), but there apparently is no further information linking the diuretic to possible neuroprotection. Furthermore, and particularly germane to our alcohol exper-

iments, ATZ and several arylsulfonamide isomers have been shown to potently inhibit the AQP4 water channel (Huber et al., 2007); the possible importance of this effect is discussed further below.

Toraseamide, a sulfonyleurea-based compound that is considered a more potent loop diuretic than furosemide but, according to the ORAC results, possesses no antioxidant ability, has had some success as a neuroprotective agent in stroke/edema models (Plangger, 1992; Staub et al., 1994). It also selectively inhibits Cl^- transport-related glial swelling, attenuating acidosis-dependent but not extracellular glutamate-evoked cell volume increases (Staub et al., 1993). In our HEC slice cultures, toraseamide largely blocked the LDH release evoked by binge alcohol exposure/withdrawal. Although toraseamide might protect through other molecular mechanisms such as blocking of angiotensin/angiotensin receptor pathways (Fortuno et al., 1999; Muniz et al., 2001), the result is consistent with the view that brain tissue overhydration and its downstream effects are potentially important factors in alcohol's neurotoxic mechanism.

The failure of L-644, 711 to protect against binge alcohol-induced neurotoxicity in the HEC slice cultures (Fig. 6) and *in vivo* (Hamelink et al., 2005) indicates that the compound, which primarily inhibits $\text{Cl}^-/\text{HCO}_3^-$ exchange, may not effectively counter the binge alcohol-induced brain water increases. Although lacking sufficient sample for *in vivo* binge alcohol/edema studies, we note that in a stroke model in rats, L-644, 711 was ineffective in reducing brain edema (Cole et al., 1991). With respect to BUM, in harmony with the *in vivo* results of Hamelink et al. (2005), this diuretic did not protect against binge alcohol neurodamage in the HEC slice culture model. We further showed that, unlike ATZ and furosemide, HEC slice edema induced by binge alcohol was not prevented by this diuretic, possibly explaining its lack of neuroprotection. Some considerations that might underlie this are that BUM is considerably less potent than furosemide in terms of inhibition of the Cl^- -extruding KCC2 transporter, but is a 500-fold stronger inhibitor of the electroneutral $\text{Na}^+-\text{K}^+-2\text{Cl}^-$ (NKCC1) co-transporter (Payne et al., 2003). This difference in potencies is thought to underlie BUM's reported absence of

anti-epileptic effects as compared to furosemide's inhibition of K^+ -induced epileptiform activity in hippocampal slices (Margineanu and Klitgaard, 2006). It is therefore possible that this divergence between BUM and furosemide is also important in the context of inhibition/prevention of alcohol-induced brain edema and neurodegeneration.

Revisiting the issue of furosemide and its effectiveness, the diuretic's neuroprotective effect against binge alcohol-induced damage thus might arise from a combination of actions not available to BUM or L-644, 711. Its prevention of alcohol-induced brain edema (Collins et al., 1998) could first derive from the diuretic's potent inhibition of the brain-specific KCC2 co-transporter as mentioned, with resulting restoration of cellular ionic strength and volume. Of interest is that apoptotic events induced by etoposide in fibroblasts—the translocation of cytosolic BAX protein to the mitochondria and the consequent release of mitochondrial cytochrome c—were suppressed by furosemide (Karpnich et al., 2002). The explanation was that BAX translocation is the outcome of a conformational alteration in BAX resulting from ionic and pH changes in the cytosolic milieu, changes that furosemide might counter via inhibition of Cl^- extrusion. Parenthetically, whether classically apoptotic events such as cytochrome c release contribute significantly to alcohol-induced neurodegeneration is uncertain, as a study in binge alcohol-intoxicated rats using a terminal indicator of apoptosis, TUNEL staining, was largely negative (Obernier et al., 2002). Nevertheless, BAX translocation and cytochrome c release should be examined in the binge alcohol models, since these events can arise independent of classical apoptosis mechanisms.

We do not question, however, that additional mode of furosemide action that might contribute to neuroprotection (and possibly the reduction of brain edema) could well be anti-oxidative. Oxidative stress has been postulated by us and several others as fundamental to alcohol's neurotoxic mechanism; indeed, as shown in Table 1, administration of selected anti-oxidants (notably cannabidiol, vitamin E, and butylated hydroxytoluene) provided significant neuroprotection in binge alcohol-intoxicated rats (Hamelink et al., 2005; Crews et al., 2006), but the sources of reactive oxygen species (ROS) are imprecisely understood. Other potential actions of furosemide appear to be less relevant to the alcohol models. The diuretic is reported to be a GABA-A receptor antagonist at concentrations similar to those used in HEC slice cultures, but antagonism is manifested primarily in the cerebellum and not in the hippocampus and cortex (Korpi and Luddens, 1997).

A key associated point is that our preliminary finding of increased AQP4 during binge alcohol-induced edema and neurodegeneration in HEC slices could be of emerging significance in terms of alcohol's edema-based mechanism. The aquaporin water channel family consists of several gene products, with AQP4 the primary form in brain (Gunnarsson et al., 2004). While expressed primarily in astroglia (Amiry-Moghaddam et al., 2003), it is also expressed by microglia activated by inflammatory stimuli (Tomas-Camardiel et al., 2004). Increasing evidence indicates that AQP4 activity plays an instigating part in cellular (cytotoxic) glial edema in animal models of trauma, stroke and ischemia (Taniguchi et al., 2000; Badaut et al., 2007; Neal et al., 2007). Although the precise nature (i.e., cytotoxic versus vasogenic) of the binge alcohol-dependent edema is still uncertain, we suspect that glial edema is an important component, and AQP4 could thus

have an early neuropathological role. However, it is still possible that AQP4 elevation is a cellular survival response to the edema and associated neuroinflammatory responses rather than (or in addition to) a causative step. For example, upregulation of a number of cell death/survival genes, including AQP4, was associated with ischemic neuroprotection due to inflammatory (endotoxin) brain preconditioning (Mallard and Hagberg, 2007). Studies with knockdown or knockout models are needed to answer this question.

Our current view is that, by whatever molecular process it is initiated, brain edema (in part cytotoxic) and associated cell stress deformation due to repetitive high alcohol exposure and withdrawal promotes pro-inflammatory processes encompassing activation of PLA2 and excessive mobilization of AA, with increased oxidative stress as one downstream outcome (Lehtonen and Kinnunen, 1995; Basavappa et al., 1998). The work of Crews et al. (2004) suggests that increased pro-inflammatory cytokines (e.g., TNF α), may also be involved. While intracellular ROS elevations due to the alcohol/alcohol withdrawal stress and cellular edema can result from a number of routes in addition to PLA2 (e.g., cytochromes P450, xanthine oxidase, ribonucleotide reductase, NADPH oxidase, and mitochondrial leakage), AA is sometimes the major contributor in a neurodegenerative ROS process (Bobba et al., 2008). AA could produce oxidative stress enzymatically and nonenzymatically (Chan, 2001; Farooqui et al., 2004) as well as indirectly via NADPH oxidase induction (Dana et al., 1998); it also could aggravate the edema (Chan et al., 1983; Winkler et al., 2000). In addition, ROS could be positive feedback signals, further activating PLA2 isoforms (Martinez and Moreno, 2001). Possibly distinct from ROS generation, AA could fuel cell death processes by stimulating mitochondrial permeability transition (Scorrano et al., 2001). Glutamate, released by astrocytic swelling as well as by AA (Freeman et al., 1990; Kimelberg and Mongin, 1998), can exacerbate oxidative stress via a non-excitotoxic pathway involving inhibition of glutathione biosynthesis (oxidative glutamate toxicity (Tan et al., 2001). Of note, our current inhibitor studies with HEC slice cultures indicate that blockade of PLA2 activity is neuroprotective against binge alcohol treatment (Brown et al., 2008). However, a further mechanistic possibility is the potential integrative role in alcohol-dependent neurodamage for nuclear factor kappa B (NF- κ B), a neuroinflammatory-associated transcription factor which is reported to be upregulated by polyunsaturated fatty acids like AA (Maziere et al., 1999) as well as by alcohol in cultures and binge alcohol intoxication *in vivo* (Zima and Kalousova, 2005; Crews et al., 2006; Zou and Crews, 2006).

In summary, since two diuretics that lack antioxidant potency, ATZ and torasemide, prevent both brain edema and binge alcohol-induced neurodegeneration, we argue that brain edema is likely to be a critical factor leading to neurodegeneration, with its suppression by these diuretics, as well as by furosemide (but not by BUM or possibly L-644, 711) providing significant neuroprotection. Nevertheless, additional protective mechanisms could exist for each of the effective agents. Also, AQP4 water channels, evidently increased by binge alcohol, could be important in the generation or maintenance of the observed edema. Further studies are needed to understand the mechanisms by which repetitive alcohol intoxication and withdrawal promote brain edema, and how/whether AQP4 is centrally involved.

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No competing financial interests exist.

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