PARP Inhibition Prevents Ethanol-Induced Neuroinflammatory Signaling and Neurodegeneration in Rat Adult-Age Brain Slice Cultures

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ABSTRACT

Using rat adult-age hippocampal-entorhinal cortical (HEC) slice cultures, we examined the role of poly [ADP-ribose] polymerase (PARP) in binge ethanol's brain inflammatory and neurodegenerative mechanisms. Activated by DNA strand breaks, PARP (principally PARP1 in the brain) promotes DNA repair via poly [ADP-ribose] (PAR) products, but PARP overactivation triggers regulated neuronal necrosis (e.g., parthanatos). Previously, we found that brain PARP1 levels were upregulated by neurotoxic ethanol binges in adult rats and HEC slices, and PARP inhibitor PJ34 abrogated slice neurodegeneration. Binged HEC slices also exhibited increased Ca^{+2} -dependent phospholipase A2 (PLA2) isoenzymes (cPLA2 IVA and sPLA2 IIA) that mobilize proinflammatory ω 6 arachidonic acid (ARA). We now find in 4-day-binged HEC slice cultures (100 mM ethanol) that PARP1 elevations after two overnight binges precede PAR, cPLA2, and sPLA2 enhancements by 1 day and high-mobility group box-1

(HMGB1), an ethanol-responsive alarmin that augments proinflammatory cytokines via toll-like receptor-4 (TLR4), by 2 days. After verifying that PJ34 effectively blocks PARP activity ($\uparrow$ PAR), we demonstrated that, like PJ34, three other PARP inhibitors—olaparib, veliparib, and 4-aminobenzamide—provided neuroprotection from ethanol. Importantly, PJ34 and olaparib also prevented ethanol's amplification of the PLA2 isoenzymes, and two PLA2 inhibitors were neuroprotective—thus coupling PARP to PLA2, with PLA2 activity promoting neurodegeneration. Also, PJ34 and olaparib blocked ethanol-induced HMGB1 elevations, linking brain PARP induction to TLR4 activation. The results provide evidence in adult brains that induction of PARP1 may mediate dual neuroinflammatory pathways (PLA2 $\rightarrow$ phospholipid $\rightarrow$ ARA and HMGB1 $\rightarrow$ TLR4 $\rightarrow$ proinflammatory cytokines) that are complicit in binge ethanol-induced neurodegeneration.

Introduction

Severe alcohol (ethanol) abuse and chronic binge alcoholism impair brain neuronal and glial pathways associated with memory and cognition. Consequently, ethanol abuse/addiction is a relatively widespread—yet surprisingly underacknowledged—reason for cognitive impairment or decline and dementia worldwide (Ilomaki et al., 2015; Sachdeva et al., 2016). We and others have linked neuroinflammatory mechanisms to brain damage in ethanol-binged adult rodent models. Our laboratory has focused on phospholipid-based pathways (Brown et al., 2009; Collins and Neafsey, 2012), specifically phospholipase A2 (PLA2) enzymes that govern the mobilization and availability of ω 6 arachidonic acid (ARA), an often proinflammatory precursor molecule and of anti-inflammatory ω 3 docosahexaenoic acid (DHA), both of which

are present in the mammalian brain in approximately equivalent amounts. Also of interest have been the possible role and association of aquaporin-4 (AQP4) water channels that stimulate brain edema (which also develops in ethanol-binged rats), increase PLA2 activities, and augment cytokines such as tumor necrosis factor when enhanced by ischemia or trauma. With respect to this latter proinflammatory cytokine and related “kines,” other laboratories have coupled ethanol's neuroinflammatory effects to increased cytokines, resulting via neuroimmune/inflammatory pathways involving toll-like receptors (especially TLR4) and brain danger signals or alarmins—notably, high mobility group box-1 (HMGB1).

We initially observed that comparatively moderate binge ethanol treatment ($\sim$ 3 g/kg per day) of male rats as juveniles and young adults promoted elevations of an important DNA-interacting enzyme, poly [ADP-ribose] polymerase-1 (PARP1), in brain regions particularly susceptible to ethanol-induced neurodegeneration (Tajuddin et al., 2013). Associated with the

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ABBREVIATIONS: AQP4, aquaporin-4; ARA, arachidonic acid; ATA, aristolochic acid; ATK, arachidonoyl trifluoromethyl ketone; cPLA2, Ca^{+2} -dependent cytosolic phospholipase A2; DHA, docosahexaenoic acid; HEC, hippocampal-entorhinal cortical complex; HMGB1, high-mobility group box 1; HS, horse serum; MEM, minimum Eagle's medium; OCM, one-carbon metabolism; PARP, poly [ADP-ribose] polymerase; PI, propidium iodide; RAGE, receptor for advanced glycation end products; ROS, reactive oxygen species; sPLA2, Ca^{+2} -dependent secreted PLA2; TLR4, toll-like receptor-4.

PARP1 elevations were regionally selective increases of two Ca^{+2} -dependent PLA2 enzymes responsible for ARA liberation from membrane phospholipids—cytosolic PLA2 (cPLA2 IVA; also termed cPLA2 α), phosphorylated (activated) cPLA2 IVA, and secreted PLA2 (sPLA2 IIA). Reductions in Ca^{+2} -independent PLA2 (iPLA2 VIA), a regulator of mitochondrial function (Kingham et al., 2015) and brain ω 3 docosahexaenoic acid dynamics (Cheon et al., 2012), also occurred, along with regional upregulation of AQP4 water channels. More recently, similar alterations of PARP1, the preceding PLA2 isozymes, and AQP4 selectively occurred in the hippocampus and entorhinal cortex of severely binged adult male rats (Tajuddin et al., 2014)—that is, the Majchrowicz binge model, which undergoes neurodegeneration chiefly in these two regions (Collins et al., 1996) and displays cognitive impairments (Obernier et al., 2002b). In parallel with the preceding *in vivo* experiments, repeated ethanol treatment of adult rat organotypic hippocampal-entorhinal cortical (HEC) slice cultures produced corresponding PARP1, PLA2, and AQP4 changes.

This article reports pharmacologic studies using adult HEC slice cultures to examine the possibility that excessive PARP1 activation engendered by binge ethanol exposures mediates dual neuroinflammatory phospholipid-ARA and neuroimmune HMGB1-cytokine pathways leading to neuronal damage—fundamentally, a pleiotropic master inflammatory regulator in brain. A growing literature confirms that, upon hyperinduction from DNA strand breaks arising from pro-oxidative brain insults (i.e., stroke or trauma), PARP1 and its PAR products elicit neuroinflammation and neurodegeneration (Narne et al., 2017). Mechanisms proposed for PARP1-triggered neuronal impairments include neuronal energy/ATP deficits from PARP-dependent depletion of NAD^{+} and/or PAR inhibition of hexokinase (glycolysis) and PAR-initiated mitochondrial release of apoptosis-inducing factor—which, diverging from its name, promotes nuclear chromatin damage and a regulated necrosis termed *parthanatos* (David et al., 2009).

Current experimental studies of ethanol and brain PARP have used primarily fetal and developmental rat models (Cherian et al., 2008; Gavin et al., 2016). With respect to neuroinflammation, brain PARP has been reported to stimulate cyclooxygenase-2 (Koh et al., 2004), an important enzyme in ARA metabolism that is responsive to ethanol (Knapp and Crews, 1999; Valles et al., 2004), but to our knowledge no connection has been reported with neurodegeneration models between PARP and key isoforms of PLA2, the rate-limiting process liberating ARA for cyclooxygenases. Thus, in addition to results verifying that PARP (and, most likely, the predominant brain form, PARP1) is important in binge ethanol-related neurotoxicity, we provide evidence that PARP amplifies PLA2 isozymes that mobilize ARA, which would generate a plethora of neuroinflammatory lipid mediators and reactive oxygen species (ROS) that can cause neurodamage, and our results with two PLA2 inhibitors support this possibility. Furthermore, consistent with evidence using non-neuronal cells that couples PARP1 activation to the endogenous TLR4 agonist, HMGB1 (Ditsworth et al., 2007; Yang et al., 2014), our results suggest that the overall levels of this ethanol-responsive danger signal for proinflammatory cytokine stimulation (Crews et al., 2017) are regulated by PARP in the adult brain.

Materials and Methods

Inhibitors and Antibodies. Olaparib and veliparib were obtained from Selleckchem (Houston, TX), and PJ34 was from Enzo Life Sciences (Ann Arbor, MI). Arachidonoyl trifluoromethyl ketone (ATK) and aristolochic acid (ATA) were from Sigma-Aldrich (St. Louis, MO). Santa Cruz Biotechnology (Santa Cruz, CA) provided antibodies for cPLA2 IVA (1:1000, sc-454), AQP4 (1:1000, sc-20812), and GAPDH (1:2000, sc-34391); Millipore (Temecula, CA) was the source of iPLA2 VIA antibody (1:1000, 07-169-1). Antibodies for sPLA2 IIA (1:500, 1811055100) was obtained from BioVendor (Candler, NC), and PAR antibody (1:500, BML-SA216.0100) was from Enzo Life Sciences; all were rabbit except PAR (mouse) and cPLA2 (mouse). Secondary antibodies (1:10,000) were from Jackson ImmunoResearch (West Grove, PA), and luminol reagent for immunoblot detection was from Pierce Chemicals (Rockford, IL). Other chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of Rat Adult-Age HEC Slice Cultures. Based on a Loyola University Medical Center Institutional Animal Care and Use Committee–approved protocol, 40- to 42-day old male Sprague-Dawley rats were anesthetized with isoflurane and thoroughly perfused via an intracardiac catheter with 150 ml ice-cold lactate-Ringer's solution. Translucent brains were removed and placed in ice-cold Gey's buffer solution. The HEC was isolated and 250- to 300- μm slices were prepared with a McIlwain tissue chopper. Slices were transferred carefully to sterile Millicell-CM membrane inserts (0.4- μm pore size, 30-mm diameter, ~3 slices/insert; Millipore Corp) in six-well plastic plates. Each well contained 1.2 ml of minimum Eagle's medium (MEM)/25% heat-inactivated horse serum (HS) containing 6.5 mg/ml glucose, a volume that saturates insert membranes but does not cover slices. Plates with inserts + slices were then incubated in 5% CO_2 at 32°C; after 48 hours, plates were transferred to a 37°C incubator at 5% CO_2 , with a complete media change (but with 20% HS), and then cultured as such for 16–18 days with media changes every 3 days. Slices were visually monitored throughout, and those appearing abnormally dark or with darkened regions were discarded.

Ethanol Treatments and Neurodegeneration and Immunoblot Assays. At brain ages of 62 ± 2 days (18–20 days in culture), slices in experimental groups were treated over four successive overnight intervals (~16 hours) with 100 mM ethanol in MEM/20% HS and to the complete media without ethanol during subsequent daylight hours (~8 hours) for 3 of the 4 days. The binge exposure, designed to mirror repetitive binge intoxication in severe, chronic alcoholism, has been shown to promote HEC neuroinflammation and neuronal damage in this *in vitro* model (Collins et al., 2013; Tajuddin et al., 2014). Control slices were treated similarly throughout to changes of ethanol-free complete media. At concentrations stated, PARP inhibitors or PLA2 inhibitors were added 2 hours before ethanol + media or media only and were present throughout the 4 nights and interior 3 days. The morning of day 4, all slices were allowed ~4 hours in freshly added media, less inhibitors and ethanol, before propidium iodide (PI) analysis or preparation for immunoblot assays. In the time-course studies (Fig. 1) that did not include inhibitors, respective immunoblot analyses were done after ~4 hours on the specific day indicated.

For PI staining, media were discarded, and slices were incubated with 1.2 ml of fresh serum-free media containing 10 μM PI, a widely used fluorescent stain used with neurotoxin-treated brain slice cultures to quantify dead or dying neurons (Collins et al., 2013). After 25–30 minutes, media were removed, and slices were rinsed twice with fresh serum-free media, followed by the addition of MEM/20% HS for 1 hour before observation of slice fluorescence results of PI-stained cells. Fluorescence was quantitated with 1.99 National Institutes of Health Image J software using a Nikon DS-5 camera + Nikon TS100 inverted microscope. The percentage of slice area with PI fluorescence above background was calculated in relation to the total area for each slice; the same threshold level was used for all slices in an experiment.

Fig. 1. Time course for PARP1, PAR, cPLA2, sPLA2, and HMGB1 levels in adult rat organotypic HEC slice cultures throughout 4 days of binge ethanol (E) exposure reveal temporal priority of PARP1. Slice levels of PARP1 were increased significantly above control levels beginning after the second overnight E exposure. Slice levels of PAR, cPLA2 IVA, and sPLA2 IIA were augmented significantly above respective control levels beginning after the third overnight E exposure. Slice levels of HMGB1 were increased significantly above control levels beginning after the fourth overnight E exposure (** $P \leq 0.001$; * $P \leq 0.05$; $n = 3-5$).

For immunoblot analyses, slices were rinsed twice with $1\times$ phosphate-buffered saline, pooled (six to eight slices) in lysis buffer (0.1% SDS, 1% sodium doxycholate, 1% Triton X-100, 150 mM NaCl, and 25 mM Tris HCl) containing protease and phosphatase inhibitors, sonicated for 15 seconds, and centrifuged 10 minutes at 13,000 rpm. Supernatants were removed, and aliquots were taken for protein assays (BCA method; Pierce Biotech., Rockford, IL). Additional aliquots (30–40 μ g protein/aliquot) were then separated by 4%–20% SDS-PAGE, transferred to nitrocellulose membranes, and treated with respective protein or PAR antibodies. Intensities of immunoblots were normalized to GAPDH, obtained on the same gels after stripping and reprobing, and images were scanned with LABwork 4.5 image acquisition and analysis software (UltraViolet Products, Upland, CA). Immunoblot levels were determined relative to levels of GAPDH as housekeeping protein and expressed as percent of control levels (mean $\pm$ S.E.M.).

Statistical Analyses. For immunoblots, one n contains 8–10 pooled slices (two wells). For PI analyses, the number of slices is stated. Results were analyzed for statistically significant differences ($P < 0.01$ or $P < 0.05$) by Tukey's test and one-way analysis of variance with completely randomized design.

Results

Ethanol Significantly Increases PARP1 before Causing Elevations of Neuroinflammation-Associated Proteins cPLA2, sPLA2, and HMGB1. In our previous binge ethanol experiments with adult HEC slice cultures, PARP1 was significantly increased after four sequential overnight binge exposures, in concert with PAR, cPLA2, and sPLA2. To obtain insight into the possible temporal signaling involving these species as well as the alarmin protein, HMGB1, we ascertained with immunoblots the daily time courses of significant changes in HEC slice levels resulting from ethanol binges. As shown in Fig. 1, PARP1 preceded the other species in being significantly elevated after the second overnight ethanol exposure (day 2; $F(1:5) = 64.1$, $P = 0.001$), and it continued to escalate on days 3 ($P = 0.05$) and 4 ($P = 0.05$). Levels of its PAR product, although apparently higher than control on day 2, were slightly outside statistical significance ($P = \sim 0.06$) at this juncture; however, after the third and fourth ethanol exposures, PAR levels were significantly elevated (day 3: $F(1:5) = 10.97$, $P = 0.03$; day

4: $F(1:5) = 9.58$, $P = 0.036$). Also significantly above their respective control levels by days 3 and 4 were both cPLA2 IVA (day 3: $F(1:7) = 5.985$, $P = 0.05$; day 4: $F(1:7) = 15.45$, $P = 0.008$) and sPLA2 IIA (day 3: $F(1:9) = 10.794$, $P = 0.011$; day 4: $F(1:9) = 6.63$, $P = 0.033$). Not until the 4th day of ethanol exposure, however, two binge exposure periods after PARP1 elevations and one ethanol exposure after increases of PAR and the Ca^{+2} -dependent PLA2 enzymes, were HMGB1 levels significantly elevated above control values ($F(1:7) = 6.53$, $P = 0.043$).

Neuroprotective PJ34 Dose Inhibits Ethanol-Dependent PARP Activity (PAR Levels) and Three Other PARP Inhibitors Neuroprotect from Ethanol-Like PJ34. To verify that by increasing PARP1 ethanol does enhance PARP activity and that the neuroprotective effectiveness of PJ34 involves curbing elevated activity, we assayed slice PAR levels by immunoblot after 4 days of binge ethanol. For immunoblot quantitation, integrated densities of the entire lanes, which encompass 50- and 125-kDa parylated species and other faint PAR-associated macromolecular species as expected, were taken. Figure 2 shows that ethanol augmented the PAR species ($F(3:15) = 7.785$, $P = 0.004$) and PJ34 effectively blocked the ethanol-induced elevations of PAR ($F(3:15) = 7.785$, $P = 0.001$). To establish definitively the connection between PARP and ethanol neurotoxicity in the HEC slice cultures, we examined the effects on neurodegeneration from 4 days of ethanol binges of three other PARP inhibitors, as evidenced by PI staining. In Fig. 3A, ethanol induced significant neurodegeneration ($F(5:119) = 3.79$, $P = 0.003$) and olaparib, a selective PARP inhibitor approved by the Food and Drug Administration for cancer cotherapy treatment, significantly counteracted the ethanol-elicited neurodegeneration at both 0.1 ($P = 0.004$) and 1 μ M concentrations ($P = 0.008$). Similarly, as shown in Fig. 3B, the neurodegeneration caused by 4 days of ethanol ($F(5:100) = 9.85$, $P = 0.01$) was completely prevented by veliparib, a another selective chemotherapy-used PARP inhibitor, at 0.1 ($P = 0.05$) and 1 μ M ($P = 0.001$). Furthermore, Fig. 3C shows that the ethanol-induced neurodegeneration [$F(3:29) = 7.76$, $P = 0.01$] was reduced by $\sim 60\%$ by an older and less specific PARP inhibitor, 4-aminobenzamide (4-AB) ($P = 0.05$), at

Fig. 2. PARP inhibition by PJ34 suppresses binge ethanol (E)-induced PAR elevations in adult rat HEC slice cultures. Overnight exposure of HEC slice cultures to E (100 mM) for 4 days significantly increased slice levels of PAR products, and cotreatment throughout with PJ34 (10 μ M) abolished the increased PAR content in ethanol-binged HEC slices. * $P \leq 0.01$, E vs. control (C) or E vs. E + PJ34 ($n = 4$).

500 μ M. In Fig. 3D, representative images of PI-stained slices display the fluorescent density in a binge ethanol-exposed slice, and diminished densities in images for E cotreated with the above three PARP inhibitors—olaparib (1 μ M), veliparib (1 μ M), and 4-AB (500 μ M). Not shown for convenience are slices containing only the PARP inhibitors, which show negligible PI staining that is indistinguishable from control slices.

PARP Inhibitors Counteract Ethanol-Induced Elevations of cPLA2 and sPLA2 but Not Reductions of iPLA2. In previous immunoblot assays of extracts of adult HEC slice cultures undergoing neurotoxic ethanol binges, we found significantly elevated cPLA2 IVA and sPLA2 IIA, two Ca^{+2} -dependent enzymes that have vital roles in mobilization of ARA, which is often neuroinflammatory and pro-oxidative, and significant reductions in a major brain Ca^{+2} -independent PLA2 (iPLA2 VIA). As connections between brain PARP and PLA2 proteins have not been reported, we explored whether PARP inhibition interfered with binge ethanol's effects on these key isoenzymes. The results, shown in Fig. 4A, confirmed that ethanol promoted significant cPLA2 IVA elevations ($F(3:53) = 4.7$, $P = 0.05$) and show for the first time that PJ34 cotreatment (10 μ M) effectively prevented the increases ($P = 0.04$). Furthermore, Fig. 4B shows that augmentation of ethanol-induced cPLA2 IVA ($F(3:24) = 2.97$, $P = 0.04$) was also blocked by the more selective olaparib at 1 μ M ($P = 0.043$). (Although olaparib at the 10-fold lower, neuroprotective 0.1- μ M dose reduced cPLA2 IVA to about control values, statistical significance versus ethanol-induced levels was not obtained ($P = 0.08$)). With sPLA2 IIA, Fig. 4C shows that, as with cPLA2, ethanol-induced increases in this sPLA2 isoform ($F(3:31) = 11.91$, $P = 0.001$) that we reported earlier were

completely prevented by 10 μ M PJ34 ($P = 0.004$). Similarly, in Fig. 4D, ethanol-induced enhancement of sPLA2 levels over control ($F(3:15) = 4.1$, $P = 0.032$) were blocked by 1 μ M olaparib ($P = 0.044$). In contrast to the counteracting effect of PARP inhibition on cPLA2 and sPLA2 elevations, the reduction in levels of iPLA2 VIA caused by 4 days of ethanol binges that we have observed in vivo and shown in Fig. 4E ($F(3:11) = 5.9$, $P = 0.05$) was not significantly reversed by 10 μ M PJ34 ($P = 0.72$). Likewise, Fig. 4F shows that the significantly reduced brain slice iPLA2 VIA owing to binge ethanol ($F(1,12) = 6.35$, $P = 0.028$) was not prevented by 1 μ M olaparib ($P = 0.152$).

Inhibitors of cPLA2 and sPLA2 Are Highly Neuroprotective against Binge Ethanol. Pharmacologic examination of the importance of PARP-upregulated cPLA2 and sPLA2 in binge ethanol-induced neurodegeneration was done with adult HEC slice cultures using the relatively specific inhibitors of these two phospholipase classes, ATK and ATA, respectively. In Fig. 5A, ethanol's promotion of neurodegeneration ($F(5:129) = 16.77$, $P = 0.001$) was significantly reduced ~45% by coexposure with 5 μ M of cPLA2 inhibitor, ATK ($P = 0.03$), whereas at 25 μ M, ATK provided essentially complete neuroprotection ($P = 0.001$). In Fig. 5B, the significant increase in ethanol-elicited neurodegeneration ($F(5:139) = 33.4$, $P = 0.001$) was completely blocked by cotreatment with the sPLA2 inhibitor ATA at both 50 ($P = 0.001$) and 500 μ M ($P = 0.001$).

PARP Inhibition Does Not Obstruct Ethanol-Induced Upregulation of AQP4. In our previously reported HEC slice and in vivo experiments, ethanol binges over 4 or more days promoted significant enhancements of AQP4 levels, concomitant with brain edema and neurodegeneration

Fig. 3. Multiple PARP inhibitors prevent binge ethanol (E)-induced neurodegeneration (PI staining) in adult rat HEC slice cultures. (A) Overnight exposure of HEC slice cultures to E (100 mM) for 4 days significantly increased neurodegeneration above control PI staining, and olaparib at 0.1 or 1 μ M throughout prevented neurodegeneration from E (** $P \leq 0.01$; $n = 13$ –20 slices). (B) Overnight exposure of HEC slice cultures to E (100 mM) for 4 days significantly increased neurodegeneration above control PI staining, and veliparib at 0.1 and 1 μ M throughout suppressed neurodegeneration from E (* $P \leq 0.05$; ** $P \leq 0.01$; $n = 13$ –25 slices). (C) Overnight exposure of HEC slice cultures to E (100 mM) for 4 days significantly increased neurodegeneration above control PI staining, and 4-aminobenzamide (4-AB) at 500 μ M throughout significantly reduced neurodegeneration from E (* $P \leq 0.05$; ** $P \leq 0.01$; $n = 10$ –20 slices). (D) Representative photomicrographs of PI-stained HEC slices for control; E, 100 mM for 4 days; E + olaparib; E + veliparib; and E + 4-aminobenzamide (4-AB).

(Sripathirathan et al., 2009; Tajuddin et al., 2014). As the brain's foremost water channel that is highly enriched in astroglia, AQP4 elevations have been linked to neuroinflammatory stimulation (Fukuda and Badaut, 2012). Positing that PARP activity might also promote AQP4 increases, we examined the effects of PARP inhibition on the elevated water channel levels caused by 4 days of ethanol binges. As shown in Fig. 6A, ethanol-stimulated elevations of AQP4 levels ($F(3:21) = 3.62$, $P = 0.033$) were not significantly affected by cotreatment with 10 μ M PJ34 ($P = 0.82$). Similarly, Fig. 6B shows that the significant increases in AQP4 due to ethanol ($F(5:23) = 4.66$, $P = 0.01$) also were not prevented by olaparib coexposure at either 0.1 μ M ($P = 1.0$) or at 1 μ M ($P = 0.99$).

PARP Inhibitors Prevent Ethanol-Induced Elevations of HMGB1. Since studies of ethanol's in vivo neuroinflammatory mechanisms by other laboratories have implicated HMGB1, a key brain neuroimmune agonist of TLR4 and other receptors (see *Discussion*), we examined whether the binge ethanol-induced significant increases of

this brain alarmin in the adult HEC slice cultures shown in Fig. 1 were counteracted by PARP inhibition. Accordingly, the results shown in Fig. 7 confirm that HMGB1 levels were significantly elevated by 4 days of ethanol binges (Fig. 7A: $F(3:19) = 5.13$, $P = 0.011$ and Fig. 7B: $F(3:16) = 7.57$, $P = 0.04$), consistent with in vivo reports by others (see *Discussion*). Furthermore, Fig. 7A shows that PJ34 coexposure (10 μ M) significantly abrogated ethanol's amplification of this brain neuroimmune proinflammatory agonist ($P = 0.025$). In addition, 1 μ M olaparib cotreatment completely suppressed the induction of HMGB1 by ethanol binges in the HEC slice cultures (Fig. 7B; $P = 0.01$).

Discussion

The results indicate that PARP is an important early initiator in ethanol-binged adult brain of neuroinflammatory and neurodegenerative pathways, particularly PLA2-dependent ARA cascades, but also HMGB1, which promotes

Fig. 4. PARP inhibition normalizes elevations in cPLA2 and sPLA2 levels, but not reductions of iPLA2 levels, in binge ethanol (E)-exposed adult rat HEC slice cultures. (A) Overnight exposure of HEC slice cultures to E (100 mM) for 4 days significantly elevated slice levels of cPLA2 IVA, and PJ34 (10 μM) copresent throughout blocked cPLA2 increases due to E (* $P \leq 0.05$; $n = 5$ to 6). (B) Overnight exposure of HEC slice cultures to E (100 mM) for 4 days significantly elevated slice levels of cPLA2 IVA, and olaparib at 1 μM copresent throughout blocked cPLA2 increases from E (* $P \leq 0.05$; $n = 3$ –4). (C) Overnight exposure of HEC slice cultures to E (100 mM) for 4 days significantly elevated slice levels of sPLA2 IIA, and PJ34 (10 μM) copresent throughout prevented sPLA2 increases from E (** $P \leq 0.01$; $n = 5$). (D) Overnight exposure of HEC slice cultures to E (100 mM) for 4 days significantly augmented slice levels of sPLA2 IIA, and olaparib (1 μM) copresent throughout blocked sPLA2 increases because of E (* $P \leq 0.05$; $n = 5$). (E) Overnight

Fig. 5. Inhibition of cPLA2 with ATK or of sPLA2 with ATA prevents binge ethanol (E)-induced neurodegeneration in adult rat HEC slice cultures. (A) Overnight exposure of HEC slice cultures to E (100 mM) for 4 days significantly increased neurodegeneration above control PI staining, and ATK (5 or 25 μ M) addition throughout prevented neurodegeneration resulting from E ($*P \leq 0.05$ or $**P \leq 0.01$; $n = 30$ –40 slices). (B) Overnight E treatment of HEC slice cultures for 4 days significantly increased neurodegeneration above control PI staining, and addition of ATA (50 or 500 μ M) throughout completely protected against neurodegeneration resulting from E ($**P \leq 0.01$; $n = 20$ –30 slices).

proinflammatory cytokines. Our model, long-term cultures of rat HEC slices of ~60 “brain age” days upon binge ethanol treatment, is uncommon. Brain slice cultures typically are hippocampal and early adolescent at time of treatment with ethanol or toxins (Sarnowska, 2002; Reynolds et al., 2015). Instead, we have developed slices incorporating both hippocampus and entorhinal cortex, two regions extensively neurodamaged during binge ethanol intoxication and withdrawal in adult rats (Collins et al., 1996; Obner et al., 2002b; Kelso et al., 2011). Furthermore, although we first introduced conventional (neonatal) rat HEC slice cultures for in vitro studies of binge ethanol neurotoxicity (Collins et al., 1998), we recently perfected HEC slice cultures of young adult brain age at ethanol exposure (Collins et al., 2013) since these cultures are the more appropriate in vitro model for study of neuromechanisms in repetitively intoxicated adult rats.

In adult HEC slice cultures, immunoblots revealed binge ethanol-related PARP1 increases that are in accord with PARP1 elevations in the HEC of adult male rats subjected to binge intoxication (Tajuddin et al., 2014). PARP1, which constitutes >90% of brain PARP, is primarily nuclear, but it is also mitochondrial (Brunyanszki et al., 2016). DNA strand breaks induce or activate the polymerase, producing PAR that recruits DNA repair molecules directly or when protein-linked. Indeed, PARP1 “parylates” a wide array of cellular proteins, including itself (autoPARylation) (Basello and Scovassi, 2015); however, PARP hyperactivation from brain insults can precipitate regulated neuronal necrosis via PAR—that is, parthanatos (Andrabi et al., 2014; Narne et al., 2017). In experimental ischemia, brain PARP1 is elevated, and PARP inhibitors suppress necrotic neurodamage (Moroni et al., 2001; McCullough et al., 2005; Kauppinen et al., 2009). In brain trauma (TBI) models, PARP inhibition significantly attenuates neurodegeneration (Stoica et al., 2014; Tao et al., 2015). Parenthetically, binge ethanol neurodamage mechanistically mirrors TBI (Sripathirathan et al., 2009). Neuroprotection by PARP inhibitors has been reported in status epilepticus (Kim et al., 2014) and with neurotoxins—for example, soman (Meier et al., 1999), MPTP

(Yokoyama et al., 2010), and kainic acid (Mazzone and Nistri, 2011).

Several neurodamaging mechanisms may be involved in brain PARP1 hyperactivation (Conrad et al., 2016). Perhaps earliest is ATP loss and energy failure secondary to NAD^+ depletion from excessive PAR formation (Ha and Snyder, 1999). Evidence also indicates that energy failure can be NAD^+ -independent, stemming from PAR’s impairment of hexokinase, gatekeeper for glycolysis (Andrabi et al., 2014; Fouquerel et al., 2014). The mechanism for parthanatos relies on nuclear PAR translocation to mitochondria, stimulating apoptosis-initiating factor release, which promotes chromatinolytic neurodegeneration with assistance from a nuclease, macrophage-inhibiting factor (Fatokun et al., 2014; Wang et al., 2016). Cleaved PARP1 can also denote a caspase-dependent apoptotic mechanism via ~85-kD fragments, but apoptosis in the Majchrowicz model is negligible (Obner et al., 2002a).

Regardless of PARP’s mechanism, our inhibitor results indicate that PARP fuels amplification of cPLA2 IVA and sPLA2 IIA, previously reported in ethanol-binged HEC slice cultures and brains of intoxicated rats (Tajuddin et al., 2014), along with consequent ARA mobilization. Published inhibitor studies of brain ischemia with aminobenzamide revealed that PARP activity also may increase inducible cyclooxygenase-2 (Koh et al., 2004), which metabolizes ARA into pro-oxidative eicosanoids. Furthermore, brains of postischemic mice lacking PAR-degrading enzyme (enhancing PAR levels above those in wild-type ischemic mice) have elevations in cyclooxygenase-2 (Cozzi et al., 2006). Our results are the first using a brain damage model showing that PARP (up)regulates the rate-controlling steps (PLA2 isoenzymes), rendering ARA available for cyclooxygenases, as well as lipoxygenases forming leukotrienes and nonenzymatic oxidations to isoprostanes, isofurans, and isoketals, concurrent with increased ROS.

Aware that chronic ethanol in vivo can increase cPLA2 activity (Hudspeth et al., 1985), we were interested in cPLA2 IVA (~85 kDa) because of neuroinflammatory roles via ARA (Sun et al., 2014; Thomas et al., 2016). Furthermore, cellular

exposure of HEC slice cultures to E (100 mM) for 4 days significantly reduced slice levels of iPLA2 VIA, and PJ34 (10 μ M) copresent throughout failed to reverse significantly depletion of iPLA2 resulting from E ($*P \leq 0.05$; ns, not significant; $n = 4$). (F) Overnight exposure of HEC slice cultures to E (100 mM) for 4 days significantly reduced slice levels of iPLA2 VIA, and olaparib (1 μ M) copresent throughout (E + Ola) failed to prevent significantly iPLA2 depletion resulting from E ($*P \leq 0.05$; ns, not significant; $n = 6$ to 7).

Fig. 6. PARP inhibition with PJ34 or olaparib does not block the significant augmentation of aquaporin-4 (AQP4) levels by binge ethanol (E) in adult rat HEC slice cultures. (A) Overnight exposure of HEC slice cultures to E (100 mM) for 4 days increased total slice levels of AQP4, and PJ34 (10 μM) copresent throughout did not significantly antagonize AQP4 elevations resulting from E (* $P \leq 0.05$; ns, not significant; $n = 5-7$). (B) Overnight exposure of HEC slice cultures to E (100 mM) for 4 days amplified the total slice levels of AQP4, and olaparib (0.1 or 1 μM) throughout did not significantly suppress AQP4 elevations resulting from E (* $P \leq 0.05$; ns, not significant; $n = 4$).

swelling and deformation activate cPLA2 family members (Lambert et al., 2006) that respond as mechanosensitive transducers to cellular osmotic stress and membrane stretching (Lehtonen and Kinnunen, 1995; Alexander et al., 2004)—and hence a relationship to binge alcohol-induced cytotoxic edema. The sPLA2 family members (~15 kDa)—notably sPLA2 IIA—are known to be neuroinflammatory through extracellular receptor-mediated mechanisms (Moses et al., 2006), and sPLA2 inhibitors have confirmed that sPLA2 is involved in stroke and brain ischemia (Hoda et al., 2009; Chen et al., 2012). Consequently, we find that two widely used cPLA2 and sPLA2 inhibitors—ATK and ATA—neuroprotect against binge ethanol (Fig. 5), thus furnishing support for the possible mechanism of $\uparrow$ PARP $\rightarrow$ $\uparrow$ PLA2 $\rightarrow$ $\uparrow$ ARA $\rightarrow$ $\uparrow$ ROS $\rightarrow$ neurodegeneration from binge alcohol.

Whether upregulation of cPLA2 and sPLA2 by PARP is direct (e.g., PAR transcriptional role) or indirect is an unanswered issue. An indirect prospect in brain cells might be via HMGB1 since in immune cells, cPLA2 IVA activity and ARA mobilization have been connected to TLR4 activation and proinflammatory cytokines (Ruiperez et al., 2009). Also, sPLA2 IIA regulation in brain ischemia is reported to trail proinflammatory cytokine stimulation (Adibhatla and Hatcher, 2007); however, the observed time course induced by binge ethanol, with HMGB1 upregulation after cPLA2 and sPLA2 (Fig. 1), rules against the above mechanistic scenarios.

Although iPLA2 responds differently than do the Ca^{+2} -dependent PLA2s we are studying (i.e., not significantly normalized by PARP inhibition; Fig. 4, E and F), it could nonetheless be a component in ethanol's neurodegenerative mechanism(s) owing to its depletion by ethanol (Tajuddin et al., 2014). Evidence indicates that iPLA2 activity stabilizes brain synapses and mitochondria (Allyson et al., 2012; Nordmann et al., 2014), and during neurotoxic insults that evoke elevated cPLA2, iPLA2 is decreased (Cheon et al., 2012). Also, iPLA2 regulates brain $\omega 3$ fatty acid turnover—for example, DHA (Green et al., 2008), the loss of which is neurodeleterious, indeed, DHA is reduced by ethanol in our slices (Tajuddin et al., 2014). With respect to AQP4, the lack of the effect of PARP inhibition on ethanol's induction of the levels of this water channel is not readily explained since we previously found that pharmacologic blockade of AQP4 blockade in vivo neuroprotected against ethanol binges (Sripathirathan et al., 2009), consistent with AQP4's role in proinflammatory cytokine upregulation (Li et al., 2011).

Classic cytokines have been implicated in neuroinflammatory sequelae of ethanol in animal and cell culture models (Valles et al., 2004). An ethanol-induced route stimulating proinflammatory cytokines encompasses TLR4, inflammasomes (Alfonso-Loeches et al., 2010), and endogenous HMGB1 (Crews et al., 2013; Montesinos et al., 2016). Indeed, TLR4 activation is a known neurodegenerative trigger (Lehnardt et al., 2003). A neuronal alarmin source is suggested by

Fig. 7. PARP inhibition with PJ34 or olaparib prevents the elevations of HMGB1 levels by binge ethanol (E) in adult rat HEC slice cultures. (A) Overnight exposure of HEC slice cultures to E (100 mM) for 4 days significantly increased slice levels of total HMGB1, and cotreatment with PJ34 (10 μM) throughout completely prevented HMGB1 elevation as a result of E (* $P \leq 0.05$; $n = 6$). (B) Overnight exposure of HEC slice cultures to E (100 mM) for 4 days significantly increased slice levels of HMGB1, and cotreatment with olaparib (1 μM) throughout significantly prevented the increase in brain slice HMGB1 resulting from E (** $P \leq 0.01$ or * $P \leq 0.05$; $n = 3$ or 4).

evidence in neuroblastoma culture that ethanol increases or releases HMGB1 (Wang et al., 2015). Our results with two inhibitors (Fig. 7) implicate positive regulation of this neuro-inflammatory alarmin by PARP, possibly driven by ethanol-dependent, oxidative stress-provoked DNA strand breaks (Chiu et al., 2011). Another HMGB1 target is the receptor for advanced glycation end products (RAGE), increased in binge-intoxicated rat brains (Vetreno et al., 2013; Byun et al., 2014). This neuroinflammatory receptor is related to PARP via evidence in a stroke model that PJ34 modulates ischemia-induced rat brain RAGE (Greco et al., 2014) and that PARylation of HMGB1 in macrophages amplifies its activation of RAGE (Davis et al., 2012).

Our inhibitor results make sense if PARP activation is temporally upstream of cPLA2, sPLA2, and HMGB1, and the results shown in Fig. 1 suggest that this is so; PARP1 becomes significantly elevated after the second binge ethanol, and PAR values achieve significance by day 3, continuing to be high on day 4. Ca^{+2} -dependent PLA2s are elevated on day 3 and day 4, followed on day 4 by HMGB1. One speculation is that HMGB1 is dependent on a level of PARP1/PAR activity exceeding that needed to promote PLA2 isoforms, but the results also might indicate that HMGB1 requires PLA2 activity in the model.

Unlike stroke, TBI, and other acquired brain disorders, no studies have directly coupled PARP to ethanol's neurotoxicity in the adult nervous system; however, in a chronic ingestion model with adult mice (ethanol diet for 1+ month), ethanol-generated oxidative stress caused cerebrocortical DNA damage that was derived from one-carbon metabolism (OCM) impairment (Fowler et al., 2012). OCM is a well known metabolic process essential for DNA stability and also brain neuronal preservation. Coinciding with OCM impairment, immunocytochemical evidence was presented for increased PAR + PARylation, implicating PARP1 activity. Although differences exist between chronic liquid diet models and binge intoxication/withdrawal models that we use, it would not be remarkable if brain OCM dysfunction was involved in binge models.

As recently suggested (Berger et al., 2018), repurposing of PARP inhibitors developed for chemotherapy purposes could provide neuroprotective possibilities in acquired brain insults and chronic neurodegenerative diseases. Our in vitro results, as well as our initial in vivo results in the Majchrowicz binge model, that showed significant neuroprotection by veliparib against HEC neurodamage (Kouzoukas et al., 2017), indicate that PARP inhibition could provide brain neuroprotection in chronic alcohol abuse situations. In this context, it is noted that, although brain results were unreported, liver steatohepatitis and metabolic dysfunction were prevented by PARP inhibitors in chronic ethanol-exposed adult mice (Zhang et al., 2016; Mukhopadhyay et al., 2017).

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Authorship Contributions

Participated in research design: Tajuddin, Kim, Collins.

Conducted experiments: Tajuddin, Collins.

Performed data analysis: Tajuddin, Collins.

Wrote or Contributed to the writing of the manuscript: Tajuddin, Kim, Collins.

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