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The IFN γ -PKR Pathway in the Prefrontal Cortex Reactions to Chronic Excessive Alcohol Use

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Abstract

Background—Brain cell death is a major pathological consequence of alcohol neurotoxicity. However, the molecular cascades in alcohol-induced brain tissue injury are unclear.

Methods—Using Western blot and double immunofluorescence, we examined the expression of interferon (IFN)-induced protein kinase R (PKR), phosphorylated-PKR (p-PKR), and IFN gamma (IFN γ) in the prefrontal cortex (PFC) of postmortem brains from subjects with alcohol use disorders (AUD).

Results—The protein levels of PKR, p-PKR, and IFN γ were significantly increased in subjects with AUD compared with control subjects without AUD, and a younger age of onset of AUD was significantly correlated with higher protein levels of p-PKR. In addition, elevated PKR- and p-PKR-IR were observed in both neurons and astrocytes in the PFC of subjects with AUD compared to subjects without AUD.

Conclusions—The activation of the IFN γ -PKR pathway in PFC of humans is associated with chronic excessive ethanol use with an age of onset dependent manner, and activation of this pathway may play a pivotal role in AUD-related brain tissue injury. This study provides insight into neurodegenerative key factors related to AUD and identifies potential targets for the treatment of alcohol-induced neurotoxicity.

Keywords

Alcohol Use Disorders; Protein Kinase R; Interferon Gamma; Human Postmortem Brains

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AUTHORS' CONTRIBUTION

SJ, JL, X-MO, CAS, and JMW designed the study. SJ, JD, SAH, GC, CM, and X-MO acquired the data; SJ, JD, SAH, GC, WM, GR, X-MO, CAS, and JMW wrote the article; all authors reviewed and approved for publication.

Alcohol use disorders (AUD) result in persistent social, economic, and health consequences. Individuals with AUD drink repeatedly and excessively despite knowledge of major social, familial, and health hazards (Cantrell et al., 2008). This lack of impulse control and loss of inhibition, as well as related mental and cognitive abnormalities, are associated with the neurobiological effects of excessive alcohol use in the brain tissue (Lyvers, 2000). Deficits in tissue volume have been reported in multiple brain regions, including the prefrontal cortex (PFC), of humans diagnosed with AUD (Brooks et al., 2014; Fein et al., 2013; Paul et al., 2008). In addition to decreased brain tissue volume, AUD is associated with decreases in neuronal and glial cell densities (Duncan et al., 2012; Miguel-Hidalgo et al., 2002, 2006). Ethanol (EtOH) exposure is also linked to apoptotic cell death through several potential mechanisms including oxidative stress, receptor inhibition, cytokine expression, transcriptional disruption, electrolyte abnormalities, and/or thiamine deficiency (Luo, 2012; Olney, 2014). Ultimately, specific factors underlying the detrimental consequences of alcohol misuse warrant identification.

Recently, a microRNA-29b-mediated SP1/RAX/PKR pathway has been proposed in EtOH-induced neuronal apoptosis in mouse cerebellum development (Qi et al., 2014). Double-strand RNA-dependent protein kinase R (PKR), also an interferon (IFN)-induced PKR, has been implicated in the neurodegeneration observed in Alzheimer's, Parkinson's, and Huntington's diseases (Bando et al., 2005; Dumurgier et al., 2013). Previous studies have documented increased phosphorylation of PKR (p-PKR) in the developing cerebellum following EtOH exposure, thereby implicating PKR as a functional player in the manifestation of fetal alcohol syndrome (Chen et al., 2006a; Qi et al., 2014).

Ubiquitously expressed in mammalian cells, PKR is a component of signal transduction pathways mediating cell survival, proliferation, differentiation, and stress responses (Chen et al., 2006b). PKR can be activated by multiple cellular stresses, including dsRNA, IFN gamma (IFN γ), cytokines, growth factors, serum deprivation, disruption of intracellular Ca⁺⁺ homeostasis, bacterial products, or physiochemical stress (Balachandran et al., 1998; Li et al., 2010; Marchal et al., 2014). However, the response of the *pkR* gene to the cellular stress of alcohol-induced neurotoxicity in the adult human has not been characterized.

IFN γ expression is up-regulated following exposure to EtOH in pancreatic cancer cells (Koido et al., 2013). Furthermore, the activation of PKR by IFN γ elicits the endoplasmic reticulum stress response in oligodendrocytes and demonstrates that IFN γ induces apoptosis in oligodendrocyte progenitor cells (Mecha et al., 2012). In addition, IFN γ -induced neurotoxicity has been demonstrated in microglia, astrocytes, and neuroblastoma cells in vitro (Hashioka et al., 2012). These studies suggest the possibility that EtOH exposure increases the protein expression of IFN γ , PKR, and activated p-PKR in the PFC of humans, followed by downstream effects of p-PKR, which can trigger apoptotic cell death (for our working hypothesis, see Fig. 1). Our study using postmortem brain samples from subjects with AUD suggests that IFN γ /PKR pathway may be a mediator of alcohol-induced neurotoxicity in adult PFC.

MATERIALS AND METHODS

Human Subjects

This study was conducted in accordance with the declaration of Helsinki and with approved Institutional Review Board policies at the University Hospitals of Cleveland (protocol # 11-88-233) and the University of Mississippi Medical Center (# 1999–1002) (Stockmeier et al., 2009). Brain samples (Brodmann area 8/9; right hemisphere) from all subjects were collected at autopsy at the Cuyahoga County Coroner's Office (Cleveland, Ohio). Next-of-kin identified in the Coroner's records were contacted by telephone using an approved script describing the overall purpose of the study. The legally defined next-of-kin were asked for permission to donate tissue, to participate in an interview about the deceased, and to give permission for the release of medical records regarding the deceased. Once verbal approval was given, written consent was obtained by a research assistant for all subjects in the study. The Structured Clinical Interview for DSM-IV Psychiatric Disorders was administered by a trained research assistant to family members or acquaintances that either lived with or had frequent contact with the deceased to determine current and lifetime psychopathology (First et al., 1996). Participants in the interviews were given the opportunity to receive \$25 as compensation for their time. Diagnosis of AUD, and confirmation of its absence (controls), was independently assessed by a clinical psychologist and psychiatrist, and consensus diagnosis was reached in conference.

Seventeen Control Subjects Without AUD—The average age of this control group was 50 ± 3.3 years, 10 subjects were male, and 7 were female (Table 1). The average postmortem interval (PMI; time between death and tissue processing) was 18.5 ± 2.1 hours. The average pH of the brain tissue was 6.6 ± 0.1 . The average freezer storage time was 11.7 ± 0.9 years. Assessment of postmortem blood and urine from normal control subjects did not reveal the presence of psychoactive drugs. These control subjects never met criteria for a psychiatric disorder and were closely matched to the 17 subjects with AUD regarding age, sex, race, PMI, pH, toxicology, and freezer storage time (Ou et al., 2010).

Seventeen Subjects With AUD—The average age for this group was 46 ± 2.2 , 11 male and 6 female. Fourteen subjects were diagnosed with AUD for the duration of at least 9 years and average AUD duration was 23.4 years, with a documented age of onset for alcohol use when they were 22.4 ± 1.7 years old. The age of onset for alcohol use of the other 3 subjects with AUD was not documented, and the duration of AUD was not available. Based on toxicological screening of postmortem blood and urine, phenothiazine metabolites were noted in 1 subject; no other psychoactive drugs were present in these subjects at the time of death, although 3 subjects were prescribed psychoactive medications within the last 30 days prior to death. EtOH was detected in the postmortem toxicology screening of 5 subjects with AUD. Three subjects with AUD were comorbid for schizophrenia, while another subject with AUD was comorbid for major depressive disorder. However, these 4 subjects did not show any difference in PKR/p-PKR levels compared with the remaining 13 subjects with AUD (data not shown).

To limit the contribution from other neurological disorders which had been reported or had the potential to affect the expression of PKR/p-PKR, the following subjects were excluded from this study: subjects with evidence of Alzheimer's disease, Parkinson disease, brain infarcts, demyelinating diseases, atrophy, heterotopia, head trauma, Korsakoff's psychosis, Wernicke's encephalopathy, and Creutzfeldt–Jakob disease; subjects with a psychoactive substance abuse disorder other than alcohol or nicotine; subjects experiencing prolonged hospitalization or life support measures prior to death; subjects with HIV, hepatitis A, B, or C.

Western Blot Analysis

Brain tissue from each subject was homogenized in a solution containing 1 mM EDTA, 10 mM Tris-HCL, and fresh protease inhibitor (Sigma, St. Louis, MO) and centrifuged at 4°C (550 *g*) for 10 minutes. The supernatants were isolated and stored at –80°C. Forty micrograms of total protein were separated by 10.5% SDS–polyacrylamide gel electrophoresis (Johnson et al., 2011). After transfer, the membranes were incubated with mouse anti-PKR antibody (1:500; BD Transduction Laboratories™, San Jose, CA; cat # 610764). For the analysis of p-PKR, the membranes were incubated with rabbit anti-p-PKR (phosphor Thr451) antibody (1:500; Cell Signaling Technology, Danvers, MA; cat # 3072) overnight at 4°C. Rabbit anti-IFN γ antibody was utilized for the assessment of IFN γ (1:500, Abcam, Cambridge, MA; cat # ab9657). Membranes were then incubated with their respective secondary antibodies prior to exposure.

An equal number of samples from each group (control and alcohol exposed subjects) were immunoblotted on separate membranes as duplicates. Protein bands were visualized by the ChemiDoc XRS+ Imaging System (Bio-Rad, Hercules, CA). The optical density of each band for PKR and p-PKR was calculated and normalized to that of β -actin using Bio-Rad Image Lab Software analysis software (Johnson et al., 2011). Additionally, a standard curve was established for PKR and p-PKR using increasing concentrations of total protein from a human control subject. The optical densities for PKR and p-PKR exhibited a linear correlation with the total protein concentration (Johnson et al., 2011; Ou et al., 2010).

Immunohistochemistry

To assess the cellular distribution of PKR and p-PKR, immunofluorescence double-staining was conducted. Duplicate brain sections (20 μ m) were obtained with a cryostat from human PFC tissue (Brodmann area 8/9; right hemisphere) of control and an AUD subjects. The sections (3 pairs of each condition) were fixed in 4% paraformaldehyde for 30 minutes followed by phosphate buffered saline washes and Tris-buffered saline (TBS; 0.1M, pH 7.6) washes. A pre-incubation solution with bovine albumin serum and TBS was used as blocking buffer.

The brain sections were then incubated with rabbit anti-p-PKR antibody (Aviva Systems Biology, San Diego, CA; 1:150), or rabbit monoclonal to PKR (clone Y117, 1:500; Abcam) and either mouse anti-NeuN antibody (a specific marker for neuronal cells; Millipore (Chemicon), Temecula, CA; 1:50) or chick anti-GFAP antibody (a specific marker of astrocytes; Abcam; 1:1,000) overnight at 4°C followed by TBS washing. Secondary

antibody incubation was performed with a Cy2-conjugated anti-rabbit secondary antibody (for PKR or p-PKR, green) and either Cy3-conjugated anti-mouse (red, for NeuN), or anti-chicken (red, for GFAP) secondary antibody at room temperature for 90 minutes. All secondary antibodies (1:2,000) were purchased from Jackson ImmunoResearch (West Grove, PA). Prior to visualization, the sections were incubated with Autofluorescence Eliminator Reagent (Chemicon, Billerica, MA) for 3 minutes and subsequently washed with 75% EtOH. Nuclei were stained by 4',6-diamino-2-phenylindole (DAPI) (blue; Vectashield mounting medium with DAPI; Vector Labs, Burlingame, CA) (Ou et al., 2010). The cell and nuclear size was estimated by a fully automated Zeiss fluorescent microscope (Carl Zeiss Microscopy GmbH, Goettingen, Germany) equipped with a 3D reconstruction and volume measurement module in Slidebook 6.0 (Intelligent Imaging Innovations, Inc., Denver, CO). The area of NeuN-IR neurons and DAPI positive nuclei were masked, and the number of masked cells was counted, and the area volumes were assessed. For each section, data were averaged from 3 counting frames, which were randomly and automatically selected by the software in the PFC region.

Statistical Analysis

Continuous data are reported as mean $\pm$ SD, and statistical significance was evaluated using Student's *t*-test for 2 group comparison for continuous data that met the normality requirement. For PKR, the normality assumption was not met and medians are reported along with the results of a nonparametric Mann-Whitney *U*-test comparing the PKR distributions between the 2 groups. A value of $p < 0.05$ was considered statistically significant for all comparisons.

RESULTS

PKR Protein Expression is Increased in the PFC of Human Subjects with AUD

To better categorize its functional influence, PKR protein expression was assessed in the postmortem PFC of 17 subjects with AUD and 17 matched control subjects without AUD. Figure 2A depicts a representative immunoblot of PKR using individual PFC samples from 4 human control subjects and 4 subjects with AUD with β -actin shown as well. Western blot analysis revealed that PKR levels were significantly increased by ~2.3-fold in the PFC of subjects with AUD compared with controls (Fig. 2B). The potential influences of age, sex, race, smoking habit, PMI, tissue pH, tissue storage time, age of onset, and duration of AUD on levels of PKR were examined by linear regression. PKR levels were not significantly correlated with any of these demographic variables between the AUD and control groups. In the statistical analysis using the Student's *t*-test, there is a significant difference in the PKR/actin ratio ($t_{32} = 11.28$, p -value = 0.0008) and p-PKR/actin ratio ($t_{32} = 2.8$, p -value = 0.0093) also differs between AUD and subjects without AUD (Table 1).

To further identify the specific cell type expression, double-immunofluorescence images are presented. Figure 2C shows PKR-IR (green) signal in GFAP-positive glial cells (red) in both cohorts and more pronounced in subjects with AUD. Interestingly, the GFAP-positive cells in subjects with AUD appear with enlarged processes and pronounced GFAP signals. Images in Fig. 2D depict the cellular localization of PKR-IR (green) primarily in the cytosol of

neurons (red) in controls subjects. While in the brains of subjects with AUD, PKR-IR signal is more pronounced and located in the area near the nucleus. Interestingly, the nuclear size (blue, DAPI stained) is increased and the cytosol is reduced in neurons of the subjects with AUD. These results in combination with the Western blot data demonstrate a significant increase of PKR expression in both astrocytes and neurons in the PFC of subjects with AUD in comparison with controls without AUD. Moreover, AUD subjects displayed an increase of expression of p-PKR in processes of astrocytes and a reduction of soma of neuronal cells [(AUD, 706 ± 54 ($n = 86$) vs. control $1,147 \pm 134$ ($n = 89$), $t = 10.82$, $p < 0.001$)] and increase of nuclei [(AUD, 525 ± 29 ($n = 161$) vs. control 349 ± 23 ($n = 182$), $t = 17.85$, $p < 0.00001$)].

Phosphorylated-PKR Protein Expression is Increased and Negatively Correlated with Age of Onset in Human Subjects with AUD

We next analyzed the distribution of p-PKR-IR in the adjacent sections of those used for PKR-IR labeling. Images in Fig. 3A show the cellular localization of p-PKR (green) in neurons labeled by NeuN-IR (red) in the PFC of both cohorts. There appeared to be more p-PKR-IR cells and greater p-PKR colocalization with DAPI in AUD subjects compared with controls, suggesting a novel role for the presence and nuclear translocation of p-PKR in neurons of subjects with AUD. As demonstrated in Fig. 3B, the p-PKR-IR was also observed in GFAP-expressing cells with stronger signal in those with AUD.

Western blot analyses were then used to quantitatively estimate the p-PKR expression in PFC of these subjects. The data indicated a significant increase in protein expression of p-PKR, by 2-fold ($t_{32} = 2.7$, $p < 0.01$), in the PFC of subjects with AUD compared with the control subjects (Fig. 3C). A correlation analysis was performed between p-PKR and demographic and clinical characteristics associated with subjects with or without AUD. There is not a significant correlation relating PKR or p-PKR with age, PMI, pH, storage time, gender, race, or smoking history. There is also no significant correlation between toxicology and duration of AUD with PKR or p-PKR in subjects with AUD. There is, however, a significant negative correlation between p-PKR/actin ratio and age of onset ($r = -0.57$, $p\text{-value} = 0.0336$) in 14 subjects with AUD (Fig. 3D and Table 1). Three subjects with AUD were not included in this correlation assay because information regarding their age of onset was not available. Our results demonstrate that a younger age of onset of AUD is associated with higher expression levels of p-PKR protein.

Chronic Excessive Alcohol Use Affects the Protein Expression of IFN γ in the PFC of Humans

The protein expression of IFN γ was assessed by Western blot analysis in the PFC of humans compared with controls without AUD. IFN γ expression levels in subjects with AUD exhibited a greater than 2-fold ($t_{32} = 2.8$, $p < 0.01$) increase in the PFC protein extracts as compared to control subjects (Fig. 4A). As expected, the expression of p-PKR was highly correlated with the expression of IFN γ as analyzed with a Pearson correlation ($r = 0.817$, $p < 0.0001$) (Fig. 4B).

DISCUSSION

The current study proposes a novel mechanism that the neuropathological detriment in PFC of subjects with AUD may be mediated by the $\text{INF}\gamma$ -PKR pathway. This is the first study that examines the relationship between chronic alcohol use in humans and protein levels of PKR, p-PKR, and $\text{INF}\gamma$ in the postmortem PFC of subjects with AUD. Our results reveal that AUD is associated with a significant increase in both PKR and p-PKR protein expression in the PFC and these increases may be regulated by EtOH-induced $\text{INF}\gamma$. Interestingly, none of the subjects included in this study had thiamine deficiency or Wernicke–Korsakoff syndrome, which is associated with thiamine deficiency, suggesting a thiamine-independent mechanism in EtOH-induced activation of PKR in the PFC of AUD.

Activation of PKR, a cell stress factor, is associated with neural cell apoptosis in various neurodegenerative diseases (Bando et al., 2005; Dumurgier et al., 2013) and also in manifestation of fetal alcohol syndrome (Chen et al., 2006b; Qi et al., 2014). The role of PKR in neuronal cell apoptosis is further supported by use of a PKR inhibitor, which has been shown to protect against cell death in cerebellar granule neurons exposed to EtOH (Chen et al., 2006b; Qi et al., 2014). Our findings about activation of the $\text{INF}\gamma$ -PKR pathway in PFC of subjects with AUD, combined with previous studies, suggest that activation of PKR may contribute to alcohol-induced brain injury in adult humans.

In addition, this study not only characterizes activation of PKR in AUD, but further associates markedly increased activated p-PKR with younger ages of onset for AUD; the younger the onset for AUD, the higher p-PKR expression in the PFC of subjects with AUD. It is now known that the adolescent brain undergoes substantial and protracted development in terms of both structure and function throughout the teenage years and into the 20s and even 30s. Because of this ongoing development, young brains are more vulnerable to neurotoxicity of heavy alcohol use (Feldstein Ewing et al., 2014; Hanson et al., 2010; Jacobus et al., 2013; Medina et al., 2008). This elevated p-PKR expression in subjects with younger age of onset for AUD likely leads to significant neurobiological changes that take place during this developmental period. Although numerous studies have been reported on the prevalence, psychosocial correlates, and potential consequences, as well as the region-related disruptions in white matter microstructure and gray matter volumes of adolescent alcohol use (Feldstein Ewing et al., 2014; Jacobus et al., 2013), surprisingly little is known about how drinking alcohol affects the developing human brain. This negative correlation of age of onset of AUD and p-PKR expression in PFC of postmortem brain of subjects with AUD suggests that the particular susceptibility of adolescent brain to alcohol use may be mediated by the activation of PKR.

We further analyzed the distribution of PKR and p-PKR in PFC of subjects with AUD and compared with control subjects. Although PKR signaling has been assessed in human brain tissue associated with neurodegenerative diseases (Alirezai et al., 2007; Hashioka et al., 2012; Ill-Raga et al., 2011), the distribution of p-PKR in either neurons or astrocytes in brain tissue related to an adult AUD has not been investigated. An obvious elevation of PKR-IR and p-PKR-IR in PFC of subjects with AUD was observed, which is consistent with and further support our Western blot data. Double immunofluorescence also demonstrated that

the majority of PKR-IR and p-PKR-IR signals are located in the cytosol of both neurons and astrocytes in the PFC of subjects without AUD, while nuclear expression of PKR and p-PKR is evident in neurons in the PFC of subjects with AUD. It had been reported that elevated p-PKR in the nucleus of neuronal cells was associated with neuronal cell death in cultured cerebellar granule neurons (Ke et al., 2009) and brain tissue from subjects with Parkinson's or Huntington's disease (Bando et al., 2005; Paquet et al., 2012). Therefore, the nuclear translocation of p-PKR in PFC of subjects with AUD may be a pivotal factor in the neurodegenerative pathology of PKR-induced brain cell death (Bando et al., 2005; Ke et al., 2009).

In our morphometric analysis, it appeared that there was no significant difference in neuronal cell numbers between controls without AUD (average $n = 89$ /counting frame) and subjects with AUD ($n = 86$ /counting frame) in PFC. Furthermore, smaller neuronal cell bodies and larger nuclei were observed in the PFC of subjects with AUD compared to controls. This morphological abnormality of neurons in the PFC of subjects with AUD suggests a brain region-specific neuronal response to alcohol. The smaller neuronal cell bodies observed support previous discoveries that the loss of gray matter associated with AUD can be due, in part, to decreases in the size of cells in the brain parenchyma (Boutte et al., 2012; Crews et al., 2004; Fein et al., 2013; Spear and Swartzwelder, 2014). Smaller size of the soma of neurons in the PFC of subjects with AUD may be a major reason for the reduction of PFC volume.

Activation of PKR could contribute to a common neuronal machinery leading to cell apoptosis and cell death (Hugon et al., 2009). The double-strand RNA-dependent PKR is a serine–threonine kinase composed by a kinase domain and 2 double-strand RNA binding domains. The activation of PKR initiates a dimerization of the protein, and followed by autophosphorylation at the Thr446 and Thr 451 sites. PKR activation can be initiated by viral infection and also by different types of stresses including cytokines, such as tumor necrosis factor alpha and IFN γ . Therefore, it is also named as an IFN γ -induced PKR. Interestingly, our results indicated that IFN γ was increased and highly correlated the expression of p-PKR in the brains of subjects with AUD. Suzuki and colleagues (2005) characterized astrocytes as the major producer of IFN γ in the brain following cellular stress, which coincides with our immunohistochemical observations related to activation (enlarged cell body and processes with high GFAP immunoreactive signals) of astrocytes in response to EtOH-induced neurotoxicity. In fact, glia cells express cytokine receptors, including IFN γ , which serves a functional role in astrocytes (Lee et al., 2006). Therefore, it is possible that excessive and chronic EtOH exposure may activate astrocytes and increase the expression of IFN γ , which then activates PKR, and this process is more vulnerable in youth. Although, it cannot be excluded that chronic alcohol use may trigger viral infection, leading to double-stranded viral RNA expression and subsequent PKR activation. Our observed up-regulation of IFN γ expression, which is associated with activation of astrocytes (reactive astrocytosis) and oxidative stress, is also capable of activating PKR as supported by the high correlation between IFN γ and p-PKR expression.

In addition, the *pkrr* gene is regulated under the TATA-less promoter, which harbors the IFN-stimulated response element, kinase conserved sequence, and IFN-gamma-activated

sequence elements (GAS), and thus responds to IFN γ transcriptional activation (Kuhlen and Samuel, 1997). IFN γ is particularly sensitive to reactive oxygen species (ROS), which can result from several factors, including viral infections, cytokines, or even EtOH. In a recent study, administration of ROS alone induced an increase in IFN γ levels, which targeted the transcriptional activation of the PKR gene. Furthermore, IFN γ siRNA abrogated the ROS-mediated PKR induction, indicating that oxidative stress induces PKR expression via the IFN γ activation cascade, which is completely independent of viral infection (Pyo et al., 2008). Although it is quite possible that chronic alcoholics are more susceptible to viral infection, be it either weakened immune response or through engaging in riskier behaviors or lack of hygiene. However, none of our subjects were diagnosed with viral infections at the time of autopsy.

Therefore, we propose a mechanism by which alcohol use leads to cell death as presented in Fig. 1. Alcohol use leads to the activation of astrocytes, elevated expression of IFN γ , and activation of the IFN γ receptor at the cellular membrane. This interaction results in the phosphorylation of PKR. The activated PKRs translocate into the nuclei of neural cells and trigger the p-PKR-mediated apoptosis. Our previous study of chronic EtOH-treated rats demonstrated that the apoptotic cell death pathway was activated following EtOH exposure as levels of the anti-apoptotic protein, Bcl-2, were reduced and active caspase 3 (pro-apoptotic) was increased in the PFC of rats following exposure to EtOH compared with controls (Ou et al., 2011). These findings further explicate alcohol-induced cell death and collectively elucidate possible pathways and particular proteins that may be involved in the neurotoxicity associated with AUD.

Furthermore, a significant negative correlation was observed between activated p-PKR and age at onset of AUD. Abnormally elevated p-PKR may also be a biological marker for determining earlier versus later age at onset of AUD and possibly subsequent progression of neuropathology in AUD. In an effort to define the signaling pathways linked with alcohol-induced neuropathology, this study suggests that PKR is a prominent element underlying alcohol-induced neurotoxicity and implicates IFN γ , an upstream activator of PKR, as an EtOH-responsive constituent in this pathway as well.

CONCLUSION AND LIMITATIONS

In summary, the results of this study identified a strong association between PKR levels and chronic and excessive alcohol use in the PFC of subjects with AUD. Our findings for PKR, p-PKR, and IFN γ further suggest a role for these proteins in the mechanisms underlying EtOH-induced neurotoxicity. Therefore, PKR, p-PKR, and IFN γ may be potential drug targets for the development of new pharmacological therapeutics for the treatment of this neurotoxicity and associated pathology. Despite the limitations of this study, our findings warrant interpretation. Overall, our study was limited by the small sample sizes for both the Western blot and immunohistochemistry assessments. While our results may have been affected by demographic and tissue characteristics of postmortem human samples, we correlated our results with factors such as age, race, gender, nicotine exposure, tissue pH, PMI, and freezer storage time in the data analysis. Immunohistochemical studies were limited by factors such as exposure to light and the selection and analysis of comparable

tissue sections. We minimized these limitations by analyzing sections with good histological quality and conducting the immunohistochemical techniques in the dark or with minimal light. This study was also limited by *only* examining the PFC. Future studies of other brain regions related to alcohol-induced neurotoxicity may help further elucidate underlying mechanisms.

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Alcohol use disorder is mediated by IFN γ /p-PKR activation

Fig. 1.

Schematic illustration of proposed mechanisms underlying alcohol-induced apoptosis. Excessive and chronic ethanol exposure increases the expression of interferon (IFN) γ which binds to its receptor at the cellular membrane, resulting in the activation (phosphorylation) of protein kinase R (p-PKR). This process is more vulnerable in youth as the younger the onset for alcohol use disorders (AUD), the higher p-PKR expression in the prefrontal cortex of subjects with AUD. Activated PKR in turn phosphorylates eukaryotic initiation factor-2 (eif2 α), which inhibits translation and negatively impacts cell growth. p-PKR nuclear translocation also triggers several apoptotic pathways, such as the p53 and p38 pathways, which ultimately result in cell death.

Fig. 2.

Protein kinase R (PKR) expression was increased in the prefrontal cortex (PFC) of humans with alcohol use disorders (AUD). Protein expression levels of PKR was examined by Western blotting in the postmortem PFC of 17 control subjects without AUD and 17 subjects with AUD. **(A)** Western blots showing the immunolabeling of PKR and β -actin in individual postmortem PFC specimens of 4 control subjects and 4 subjects with AUD. **(B)** PKR expression is plotted for individual subjects with the mean $\pm$ SEM represented by the long and 2 short horizontal lines, respectively. Two independent preparations were conducted to evaluate PKR expression by measuring its relative optical density (ROD) and normalizing that value to the ROD of β -actin. **(C)** Images depict the localization of PKR (green) with astrocytes (red, GFAP positive), and **(D)** images show the expression of PKR in neurons (red, NeuN positive) in PFC of control subjects and of subjects with AUD. DAPI stands for nuclear (blue).

Fig. 3.

The expression of phosphorylated protein kinase R (p-PKR) was increased in human prefrontal cortex (PFC) of subjects with alcohol use disorders (AUD). **(A)** Distribution of p-PKR (red) in neurons (red, NeuN positive) and **(B)** in astrocytes (red, GFAP positive) is shown in prefrontal cortex (PFC) of controls and subjects with AUD. Quantitative analysis of p-PKR protein expression was examined by Western blotting. **(C)** Protein expression levels of p-PKR in the postmortem PFC of 17 control subjects without AUD and 17 subjects with AUD. p-PKR expression is plotted for individual subjects with the mean $\pm$ SEM represented by the long and 2 short horizontal lines, respectively. Each p-PKR protein band was evaluated from 2 independent preparations by measuring its relative optical density (ROD) and normalizing that value to the ROD of β -actin. **(D)** Graphic representation of the negative correlation between p-PKR and the age of onset for 14 subjects with AUD.

Fig. 4.

Ethanol increases interferon (IFN) γ protein expression and phosphorylated protein kinase R (p-PKR) expression positively correlated with IFN γ expression in the prefrontal cortex (PFC) subjects with alcohol use disorders (AUD). **(A)** Upper panels are the Western blots of IFN γ and β -actin. Lower panels are the quantitative average relative optical density (ROD) of IFN γ bands normalized to β -actin. **(B)** Pearson's correlation analysis of the expression of IFN γ and p-PKR in PFC protein extracts of 17 subjects with AUD.

Table 1

Statistical Analysis of Human Subjects Included in this Study

	Non-AUD (<i>n</i> = 17)	AUD (<i>n</i> = 17 ^a)	Statistic ^b	<i>p</i> -Value
Age (years)	50.12 ± 13.54	46.18 ± 8.99	$t_{32} = -0.99$	0.3251
Age of onset	N/A	22.36 ± 1.65		
Duration of AUD	N/A	23.43 ± 1.94		
Gender	7F/10M	6F/11M	Equation	0.6357
Race	8C/9AA	11C/6AA	Equation	0.3136
PMI (hours)	18.51 ± 8.45	21.64 ± 8.25	$t_{32} = 1.09$	0.2825
Brain pH	6.61 ± 0.33	6.52 ± 0.31	$t_{32} = -0.79$	0.4342
Storage Time (years)	11.35 ± 3.69	11.35 ± 4.81	$t_{32} = -0.24$	0.9999
PKR/actin (median)	1.29 ± 1.20 (0.80)	2.62 ± 1.68 (1.90)	$t_{32} = 11.28$	0.0008
p-PKR/actin	1.19 ± 0.80	2.12 ± 1.12	$t_{32} = 2.77$	0.009

^aThe age of onset of 3 subjects was not available. Therefore, the age of onset and duration of AUD are *n* = 14.

^bContinuous data compared with *t*-statistic with 32 degrees of freedom; Categorical data compared with chi-square test with 1 degree of freedom.

AA, African American; AUD, alcohol use disorders; C, Caucasian; F, female; m, Male; PMI, postmortem interval; PKR, protein kinase R; p-PKR, phosphorylated-PKR.

Summary of statistical analysis of PKR and p-PKR protein levels between subjects without AUD (non-AUD) and subjects with AUD (AUD) comparing demographic and clinical characteristics.