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Pathogenic role of acrolein in alcoholic liver disease.

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PATHOGENIC ROLE OF ACROLEIN IN ALCOHOLIC LIVER DISEASE

By

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B.S. in Chemistry, Tamkang University, Taipei, 2003
M.S. in Biochemistry, University of North Carolina-Greensboro, 2009

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A Thesis Approved on

November 20, 2014

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ABSTRACT

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Wei-Yang (Jeremy) Chen

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Alcohol is the most socially accepted addictive drug, and it can cause alcoholic liver disease (ALD), which is a major cause of morbidity and mortality in the United States and worldwide. Animal and human studies demonstrate that chronic alcohol consumption causes a pro-oxidant environment in the liver and increases hepatic lipid peroxidation and the accumulation of by-products such as acrolein and 4-hydroxynonenal. Acrolein is the most reactive and toxic aldehyde generated through lipid peroxidation. Also, acrolein is a major component of cigarette smoke, and there is increasing evidence that smoking negatively impacts the incidence, severity, and clinical course of chronic liver disease. Acrolein is known to form protein adducts, and can trigger endoplasmic reticulum (ER) stress. Notably, alcohol-induced perturbations in the ER have emerged as an important etiologic factor in alcoholic liver disease. This study investigated the role of acrolein as a mediator of hepatic ER stress and injury during alcohol consumption. Acrolein accumulation, activation of pro-apoptotic stress kinase-

JNK (the mitogen activated protein kinase c-jun N-terminal kinase) , ER stress, and apoptotic cell death was examined in vitro in alcohol-exposed rat hepatic cells (H4IIEC), and in vivo in a mouse model of alcohol consumption. Exposure to alcohol led to substantial accumulation of acrolein adducts both in vitro and in vivo. This was accompanied by phospho-activation of JNK and upregulation of ER stress transcription factors ATF3 and ATF4, and the pro-apoptotic protein, GADD153/CHOP. This study demonstrates that acrolein is likely to be a major culprit in the ER stress and hepatotoxicity associated with alcohol consumption. Also, the data show that acrolein removal protects against alcohol-induced ER stress and injury, suggesting that acrolein scavengers may have therapeutic potential in alleviating the adverse effects of alcohol consumption.

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CHAPTER 1

INTRODUCTION

Alcohol liver disease (ALD)

Alcohol is the most socially accepted addictive drug, and alcohol abuse/dependence is known to cause chronic organ disorders [1]. Alcohol abuse is the 3rd leading lifestyle-related cause of death in the United States and there are approximately 80,000 deaths annually attributed to excessive alcohol use [2, 3]. Binge and underage drinking are common types of excessive alcohol use and both are also linked to a wide range of health and social problems. Excessive alcohol use also costs about $185 billion each year in health care and criminal justice expenses, as well as lost productivity in the United States.

Alcohol quickly distributes into the portal circulation and across biological membranes to organs throughout the body. Alcohol injuries are observed in many organs including brain, gastrointestinal tract, immune system, kidney, lung, heart, pancreas, and particularly, the liver. In the liver, alcohol consumption is known to cause alcoholic liver disease (ALD) which includes a spectrum of liver disorders (Figure 1), ranging from fatty liver (steatosis), steatohepatitis, and fibrosis, to cirrhosis, end stage liver disease and hepatocellular carcinoma (HCC) [4]. Steatosis is commonly found in approximately 90 percent of individuals who consume more than 60 g per day of alcohol or about five drinks per day [5]. Steatosis is reversible if the individuals stop or dramatically reduce the amount of

alcohol consumption. Histology evidence has shown fat accumulation of both small (i.e., microvesicular) and large (i.e., macrovesicular) droplets within liver cells [6]. Some individuals with steatosis progress to more serious liver disease such as alcoholic hepatitis, which is a steatosis accompanied by inflammation, neutrophil infiltration, hepatocyte necrosis, and Mallory bodies. Approximately 35 to 40 percent of alcoholic patients may progress from steatosis to fibrosis and eventually cirrhosis followed by end stage liver disease and hepatocellular carcinoma (HCC). Currently, there is no FDA approved therapy for any stage of ALD; hence, the study of mechanisms and mediators that contribute to ALD is critical to understand the development and progression of ALD and to promote therapeutic development.

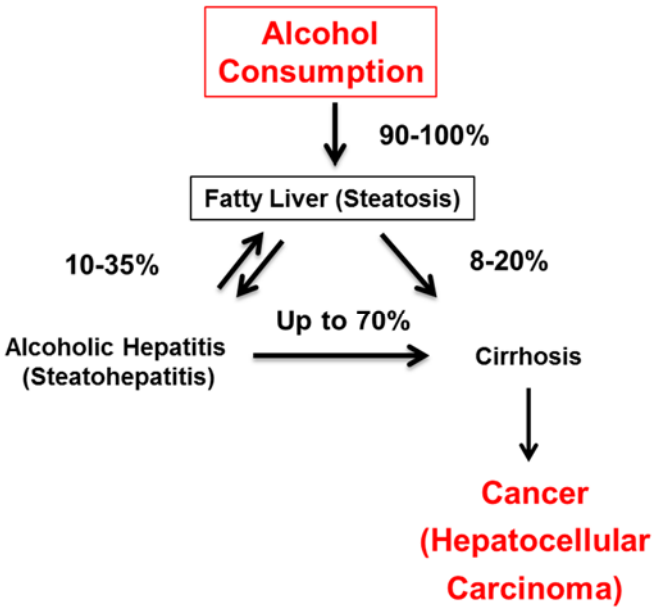


Figure 1: Progression of alcoholic liver disease.

Alcohol metabolism and oxidative stress

The portal circulation from the intestine passes first through the liver and ingested alcohol is primarily metabolized in the liver. The process of ethanol metabolism involves at least three distinct enzymatic pathways. Most of the alcohol in the human body is first oxidized in the liver to toxic acetaldehyde, catalyzed by the cytosolic alcohol dehydrogenase (ADH), an NAD^+-requiring enzyme expressed at high concentrations in hepatocytes. ADH oxidizes ethanol to acetaldehyde, which enters the mitochondria where it is oxidized to acetate by one of several aldehyde dehydrogenases (ALDH). Acetaldehyde is then converted by acetaldehyde dehydrogenase (ALDH) to acetic acid [7]. The second major pathway for ethanol metabolism is the microsomal ethanol oxidizing system (MEOS) which involves the cytochrome P450 enzyme 2E1 (CYP2E1) and requires NADPH instead of NAD^+ as for ADH. The MEOS pathway is highly induced in individuals who chronically consume alcohol. CYP2E1 also plays an important role in alcohol metabolism; alcohol can be oxidized by CYP2E1 to acetaldehyde, with generation of hydrogen peroxide which causes oxidative stress in the liver cells. Minor pathways for alcohol metabolism involve fatty acid ethyl ester (FAEE) synthase, or catalase in peroxisomes.

Studies have shown that consumption of ethanol disrupts antioxidants function, which leads to elevated oxidative stress and increased interactions between free radicals and cellular components including lipids, proteins, and DNA, which alter cellular structures and functions. Ethanol metabolism is responsible for the

production of reactive oxygen species (ROS) and a disturbance of cellular antioxidant capacity. These physiological conditions can be adapted by the antioxidant systems of the human body. However, when excessive ROS production goes over the capacity of physiological adaption to eliminate ROS, it could lead to health problems. The superoxide and hydrogen peroxide are the most dominant form of ROS and they can cause acetaldehyde accumulation due to imbalance of redox state. Aldehyde dehydrogenase (ADH) is responsible for the oxidation of ethanol by increasing the reduced form of nicotinamide adenine dinucleotide (NADH) which results in decreasing of NAD+/NADH ratio and triggers the conversion of cytosolic xanthine dehydrogenase to xanthine oxidase which is the enzyme responsible for the production of superoxide radicals. NADH is involved in the conversion of ferritin to ferrous (Fe) (II) ions and these ferrous ions play important roles in free radical reactions which lead to the generation of hydrogen peroxide, hydroxyl radicals, and increased lipid peroxidation [8, 9].

Alcohol-induced lipid peroxidation

Alcohol metabolism in the liver generates free radicals and these free radicals can lead to oxidative degradation of cellular polyunsaturated fatty acids (PUFAs), a process called lipid peroxidation [10]. Linoleic acid (LA) is the most common PUFA found in all foods in the western diet [11]. The consumption of LA has dramatically increased from 2% to over 7% in the last decade. Notably, studies by our group and others have shown that consumption of alcohol along with a diet rich in PUFA (particularly, LA) gives rise to worse alcohol-induced liver injury, compared to alcohol and saturated fat diet [12]. Moreover, LA is a precursor of

arachidonic acid converted to proinflammatory eicosanoids; these are thought to be involved in several chronic diseases including cancer and cardiovascular disease [13]. Linoleic acid enriched diet is known to elevate lipid peroxidation [14] which can generate higher acrolein in the body [15].

Free radicals produced by alcohol metabolism can damage the cells membrane by stealing electrons from the lipids in cell membranes in the process of free radical chain reaction. Alcohol-induced lipid peroxidation is well documented in animal models, as well as in patients with ALD [16]. The products of lipid peroxidation including malondialdehyde (MDA), 4-hydroxynonenal (4-HNE) and acrolein are detected in animal blood or tissues and in the humans consuming alcohol; these highly reactive aldehydes are likely to play a critical role in the pathology of ALD (Figure 2). Due to their chemical nature, these electrophiles can react with cellular protein nucleophiles and form aldehyde-modified protein adducts which generate a pro-oxidative environment in the liver, further promoting lipid peroxidation. These aldehyde protein adducts can serve as biomarkers of alcohol-induced oxidative stress in ALD [16].

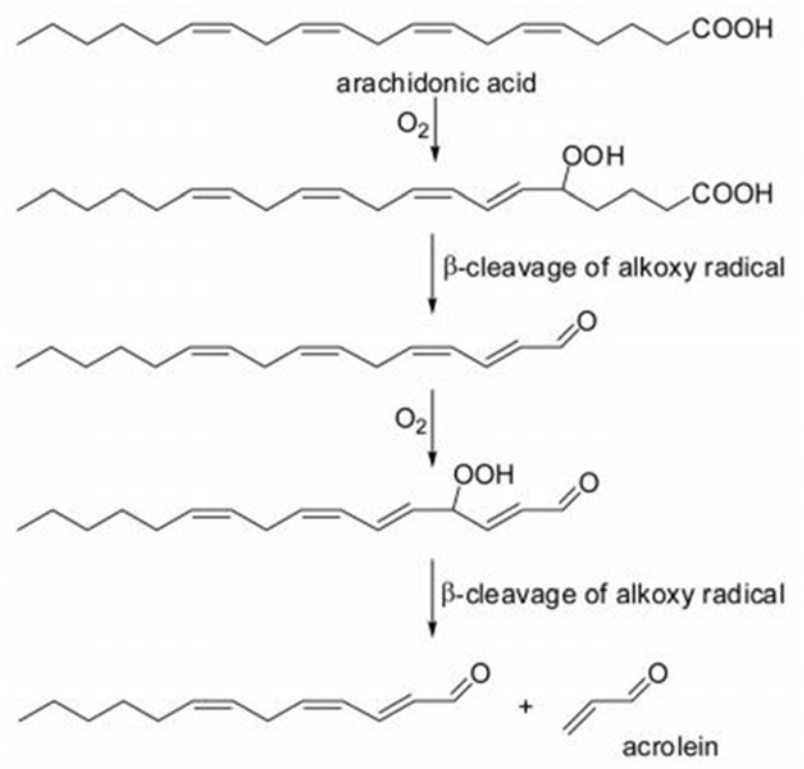


Figure 2: Proposed formation of acrolein from arachidonic acid (PUFA) [17].

Acrolein, the reactive aldehyde from lipid peroxidation

Acrolein, a highly reactive α,β-unsaturated aldehyde with three carbon and a double bond structure (Figure 3), is formed endogenously during lipid peroxidation [18]. Acrolein is also a biotransformation metabolite formed from certain amino acids, allyl compounds and the anticancer drug cyclophosphamide [15]. Acrolein has the potential to rapidly deplete cellular glutathione, and reduces cellular antioxidant capacity [18]. Therefore acrolein may serve as an important oxidative stress biomarker for lipid peroxidation [19]. Acrolein is the most toxic and reactive product of lipid peroxidation; indeed, it is over 100 times

more reactive than HNE [15]. Due to its highly electrophilic nature, acrolein can form adducts with cellular nucleophilic groups in lipids, nucleic acids, and proteins; these acrolein adducts can cause cytotoxicity via irreversible adduction by disrupting cell signaling and mitochondrial dysfunction of the cells and produce oxidative stress. Acrolein is associated with several chronic diseases and elevated concentration of acrolein/acrolein-protein adducts are detected in the plasma of patients with chronic renal failure, Alzheimer's disease, Parkinson's disease, atherosclerosis, and chronic obstructive lung disease [18]. Notably, acrolein is also an environmental pollutant arising from combustion of wood, paper, fossil fuels, and plastics. It is a major component of cigarette smoke and up to 600µg of acrolein is generated per cigarette [20]. This is particularly relevant for ALD given the high prevalence of individuals that use/abuse both alcohol and cigarettes. Additionally, acrolein is produced by overheating fats and oils, and is now considered a significant dietary pollutant occurring in charred meats and fried foods [18, 20]. Thus, both exogenous and endogenous acrolein generation may contribute to the development/progression of liver diseases.

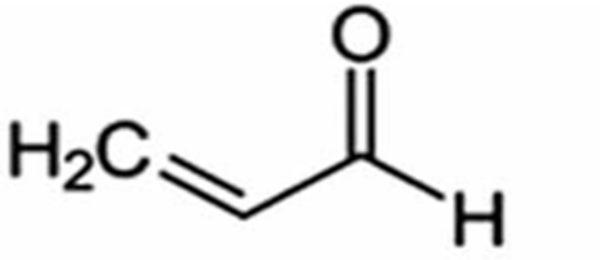


Figure 3: Chemical structure of acrolein.

The pathogenic mechanisms of alcohol liver disease

Some mechanisms postulated to contribute to ALD include inflammation, mitochondria dysfunction, ER stress, hepatocyte apoptosis and intestinal barrier dysfunction.

Mitochondria dysfunction

Mitochondria are the energy factory of the cells which produce ATP required to maintain normal metabolic and repair functions of the cells. Studies have shown that alcohol significantly decreases the viability of hepatocytes due to inability to maintain sufficient rate of ATP synthesis. The oxidation of alcohol by ALD and CYP2E1 and acetaldehyde by ALDH leads to significant increase in the hepatic $NADH/NAD^+$ ratio and in the production of ROS. The enzyme activities of NADH dehydrogenase, succinate dehydrogenase and ATP synthase are depressed when exposed to reactive species, including superoxide, hydrogen peroxide and peroxynitrite which result in the inactivation of mitochondria proteins and further decreases the function of mitochondria in the cells [16, 21]. Disruption of mitochondrial function can trigger signaling cascades involving the release of pro-apoptotic protein from mitochondria, and activation of initiator and effector caspases, leading to apoptotic cell death.

Inflammation

Inflammation is an early indicator of more serious liver disease and several inflammatory response pathways are linked to alcohol liver disease such as NFκB, JNK, ROS, interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α). TNF-α is a cytokine which responsible for the programmed cell death and apoptosis of hepatocytes in alcohol-induced liver injury. TNF-α promotes a series of inflammation responses in the cells and leads to the production of inflammatory cytokines such as IL-1β, IL-6, and IL-8 [22]. Persistent cytokine secretion causes chronic inflammation in the cells which leads to hepatitis, fibrosis, and cirrhosis [23].

Endoplasmic Reticulum (ER) stress and unfolded protein response (UPR)

The endoplasmic reticulum (ER) is an eukaryotic cell organelle composed of low concentrations of cholesterol and sphingolipids [24]. The ER plays a crucial role in cell homeostasis and survival, and is responsible for synthesis, folding, assembly, and trafficking of proteins, lipid biosynthesis, and regulation of intracellular calcium and redox homeostasis [25]. The molecular chaperones/folding proteins in the ER (e.g., Grp78/BiP, Grp94, protein disulfide isomerase (PDI), calnexin, and calreticulin) ensure that only properly folded proteins reach their destinations; inappropriately folded proteins are retained in the ER lumen for refolding or endoplasmic reticulum–associated degradation (ERAD) [25]. Certain stimuli (such as altered redox status, oxidative stress, unbalanced calcium, hypoxia, or energy deprivation) can disturb ER function and lead to the accumulation of unfolded proteins in the ER, thereby triggering ER

stress and the unfolded protein response (UPR). To restore ER homeostasis, the UPR adaptive mechanism upregulates the folding capacity of the ER through induction of chaperones and foldases, and downregulates the biosynthetic load through inhibition of protein synthesis. UPR adaptive responses can re-establish homeostasis and reduce ER stress via (i) increased folding capacity through upregulation of chaperones, and (ii) decreased protein burden through inhibition of protein synthesis and degradation of misfolded proteins [25].

ER stress/UPR signaling pathways involves three major ER resident transmembrane sensor proteins, inositol requiring protein 1 (IRE1), ds-RNA-activated protein kinase (PKR) like ER kinase (PERK), and activating transcription factor 6 (ATF6), which are activated from their inhibitory binding with chaperone GRP78/BiP. The first sensor IRE1, a type I transmembrane protein with both a serine/threonine kinase domain and an endoribonuclease (RNase) activities, is activated by transautophosphorylation. IRE1 transcriptionally form active spliced XBP1 (sXBP1) which activates UPR target genes and ERAD pathway genes. IRE1 also activates the stress-signaling kinase, JNK, and the transcription factor NFκB; both of which can lead to proinflammatory cytokine production. The second sensor PERK, a type I transmembrane protein with a cytosolic serine/threonine kinase domain, phosphorylates the eukaryotic initiation factor 2α-subunit (eIF2α), leading to inhibition of translation and reduction of the protein load during ER stress. Also, p-eIF2α triggers activating transcription factor 4 (ATF4), which regulates ER chaperone genes, ERAD pathway genes, amino acid metabolism genes, and the proapoptotic transcription factor C/EBP

homologous protein (CHOP, GADD153). The third sensor ATF 6, a type II ER transmembrane protein with a CREB/ATF bZIP transcription factor domain at the amino terminus, is activated by cleavage in the golgi and can translocate to the nucleus to activate chaperone gene expression [26, 27]. Additionally, ATF6 also leads to upregulation of CHOP, and affects SREBP activity and consequently, lipogenesis.

ER stress induces apoptosis

In order to counter ER stress in the cells, the UPR adaptive signaling pathway is triggered. However, excessive or prolonged ER stress can overcome the adaptive pathways, and cause apoptosis and necrotic cell death. The apoptosis pathway is triggered to protect the liver if the PERK, ATF6, and IRE1 adaptive pathways fail to mitigate ER stress [28]. Several mechanisms are involved in ER stress-induced apoptosis such as the transcription factor C/EBP homologous protein (CHOP), JNK, Bcl-2 family proteins, calcium and redox homeostasis, and caspase activation [15].

ER stress triggers JNK pathway via phosphorylation of IRE-1 with the adaptor protein TRAF2 (tumor necrosis factor receptor (TNFR)-associated factor-2) which phosphorylates and activates JNK [29]. Phosphorylated JNK activates proapoptotic Bim and inactivates antiapoptotic Bcl-2 proteins, and triggers release of cytochrome C through the mitochondrial death pathway. Calcium homeostasis is associated with apoptosis; excess calcium release from the ER results in calcium accumulation in the mitochondria and results in apoptosis

effectors release from mitochondria into the cytosol. Bcl-2 has the ability to reduce overflow of free calcium rest in the ER and prevent cell apoptosis.

The transcription factor CHOP/GADD153 is the best-known proapoptotic pathway associated with ER stress. CHOP is a bZIP-containing transcription factor that is a common point of convergence for all three arms of the UPR, with binding sites for ATF6, ATF4 and XBP1s present within its promoter. CHOP is primarily considered a pro-apoptotic transcription factor that mediates ER stress induced cell death through the regulation of Bcl-2 family members. Also, CHOP transcriptionally induces GADD34 and dephosphorylates p-eIF2α to enable protein synthesis, concomitantly generating ROS and leading to oxidative stress and inflammation [15]. The activation of ER stress, and consequent inflammation and apoptosis may be a significant contributor to many forms of liver injury, including ALD.

ER stress has been reported to contribute to alcohol-induced liver injury and the progression of alcohol live disease in both animal and cell models [30-33]. Upregulation of ER stress related genes such as Grp78, Grp94, and CHOP has been observed in acute and chronic alcohol feeding animal models and in patient biopsies [34, 35]. Some factors that are thought to contribute to alcohol-induced ER stress include acetaldehyde, oxidative stress, disrupted calcium homeostasis, impaired one carbon metabolism, and epigenetic modifications.

HYPOTHESIS

We hypothesize that the lipid metabolite acrolein is a major pathogenic contributor to ALD, based on the knowledge that alcohol metabolism via ADH and CYP2E1 increases lipid peroxidation leading to the generation/accumulation of lipid-derived acrolein, which forms protein adducts and triggers ER stress, ultimately leading to cell death and liver injury.

The study of cellular mechanisms and pathogenic mediators that contribute to ALD is critical to understand the development/progression of ALD and to promote therapeutic development. In this study, we investigated the pathological contribution of acrolein in a murine model of alcohol-induced liver disease, and we demonstrate that alcohol-induced generation of acrolein in the liver is a major pathogenic contributor to hepatic ER stress and liver injury in ALD.

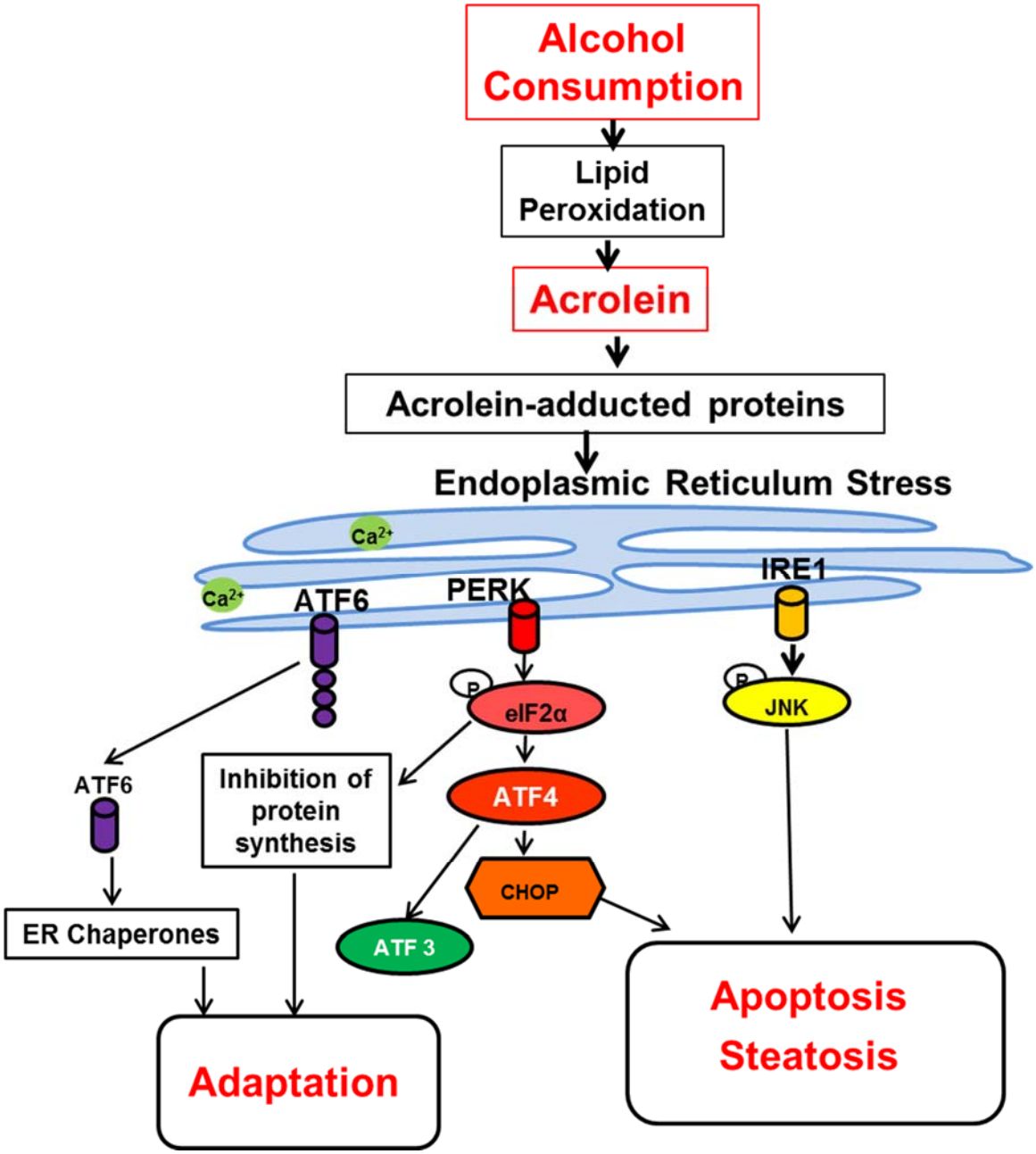


Figure 4: Alcohol-induced acrolein-mediated ER stress.

CHAPTER 2

MATERIALS AND METHODS

Animal studies

Male C57BL/6J mice (10 weeks of age) were obtained from Jackson laboratories (Bar Harbor, ME). They were maintained at 24°C with a 12h:12h light/dark cycle and had free access to normal chow diet and tap water for 5 days before the start of the experiment. The mice were fed (ad libitum) a Lieber-DeCarli liquid diet (Bio-Serv Inc., Frenchtown, NJ) containing 5% ethanol (w/v or 35% of calories) or maltose dextrin as control for 10 days, followed by a single oral gavage of ethanol (5g/kg body weight) on day 11. At the end of the experiment, the mice were anesthetized with Avertin. Plasma and tissue samples were collected for assays. Part of the liver from the left lobe was harvested and fixed in 10% neutral-buffered formalin, while the remaining liver tissue was snap frozen in liquid N2 and stored at −80°C. All mice were treated according to the protocols reviewed and approved by the Institutional Animal Care and Use Committee of the University of Louisville.

Reagents

General chemicals, N-acetyl cysteine (NAC), acrolein, and β-actin antibody were purchased from Sigma Aldrich (St. Louis, MO). All other antibodies were purchased from Cell Signaling (Beverly, MA). Cell culture supplies were obtained from Invitrogen (Carlsbad, CA).

Cell culture

H4IIEC, a rat hepatoma cell line obtained from American Type Culture Collection (Rockville, MD) was used according to company instructions. All treatments were performed on sub-confluent monolayers of cells. Cells were plated at the following densities: (i) 20,000 cells per well for 96-well plates; (ii) $0.25x10^6$ cells per well for 24-well plates; (iii) $0.5x10^6$ cells per well for 6-well plates; (iv) $2.5x10^6$ cells per 100mm plates. Cells were cultured in the DMEM media (10% horse serum, 5% .Cells were counted using a hemocytometer.

Isolation of RNA and RT-PCR

The messenger RNA (mRNA) levels of ER stress genes were analyzed by real time polymerase chain reaction (RT-PCR). 300ng of total RNA, isolated using Trizol (Invitrogen, Carlsbad, CA), was used in reverse transcription reactions as described by the manufacturer. The resulting complementary DNA was then subjected to PCR using specific primer pairs (Table 1), with 18s and TATA-binding protein as internal controls. SYBR Green I dye was used for Real Time PCR in an ABI prism 7500 sequence detection system.

Mouse	
mATF3_F1	GCGCTGGAGTCAGTTACCGTCA
mATF3_R1	TTCTTCAGGGGCCGCCTCAGAC
mATF4_F1	AAGCCATGGCGCTCTTCACGA
mATF4_R1	AGTCCCCCGCCAACACTTCG
mDDIT3_GADD_CHOP_F1	CCGGAACCTGAGGAGAGAGTGTT
mDDIT3_GADD_CHOP_R1	AGCTGCCATGACTGCACGTGG
mGRP78_F1	ACCACCTATTCCTGCGTCGGTGT
mGRP78_R1	AGGCCACATACGACGGCGTG
mGRP94_F1	AAAGGACTTGCGACTCGCCGG
mGRP94_R1	TCTGACGAACCCGAAGGTCAGC
Rat	
rATF3_F1	GGGCCACCTCAGACTTGGTGACT
rATF3_R1	CATCGGATGTCCTCTGCGCTGG
rATF4_F1	GACAAGGCGGGCTCCTCAGAA
rATF4_R1	AAAGGCATCCTCCTTGCCGGTGT
rDDIT3_GADD_CHOP_F1	TGTTGAAGATGAGCGGGTGGCAG
rDDIT3_GADD_CHOP_R1	TGGACCGGTTTCTCTCTCCTCAGGT
rGRP78_F1	GGACCACCTATTCCTGCGTCGGT
rGRP78_R1	TGGCCGCATCGCCAATCAGA
rGRP94_F1	CGTCCTGCTGACCTTCGGGTTT
rGRP94_R1	CCAGGTCCTCTTCTACCGTGCCA
Human/Rat/Mouse	
18Sreg3F	CTCAACACGGGAAACCTCAC
18Sreg3R	CGCTCCACCAACTAAGAACG
hrm_Tbp_F	CCTAAAGACCATTGCACTTCGT
hrm_Tbp_R	GCTCCTGTGCACACCATTTT

Table 1: Primers for quantitative Real Time-PCR.

MTT assay

Cell viability was assessed by the 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After treatments, cells were washed twice with PBS; then cell culture medium was replaced with medium containing 1 mg/ml MTT. After 1h, cells were lysed with100ul lysis buffer containing 20% SDS

and 50% N,N-dimethyformamide (DMF), and incubated at 37°C overnight. The OD values were read at 570 nm.

Western blot analysis

Western Blot Analysis: Cells were lysed in lysis buffer (50 mM Tris•HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 4 mM Na3VO4, 40 mM NaF, 1% Triton X-100, 1 mM PMSF, 1% protease inhibitor cocktail) and centrifuged at 14,000 g for 10 min. The supernatants were collected and 26μg of equivalent protein in total cell lysates was resolved by SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane ((Bio-Rad, Hercules, CA). Membranes were blocked for 1 h in blocking buffer (5% nonfat dry milk in 0.1%TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween 20)) and incubated overnight at 4°C with the primary antibodies diluted in blocking buffer. After washing with 0.1%TBST, the membranes were incubated with appropriate secondary antibodies for 1 h at room temperature. Proteins were visualized using an enhanced chemiluminescence system (ECL, GE Healthcare, Piscataway, NJ) and quantified by densitometry analysis using UNSCANIT (Silk Scientific, Inc, Orem, UT). The density ratio of each band compared to its corresponding β-actin band was determined. The density ratio was normalized to the untreated value which was set to 1.

Liver histology:

Liver sections were fixed in 10% buffered formalin for 24 hours and embedded in paraffin. Tissue sections were deparaffinized, stained with hematoxylin–eosin (H&E), and examined by light microscopy (×200 final magnification).

ALT & AST assay

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity was measured as a marker of liver injury using commercially available reagents from Thermo Fisher Scientific Inc. (Middletown, VA).

Oil Red O staining

Frozen liver sections were washed in phosphate buffered saline twice for 5 minutes. Oil-Red-O and 85% propylene glycol were added with agitation for 15 minutes, followed by washing in tap water.

Detection of apoptosis and acrolein adducts

TUNEL staining for apoptosis was performed using ApopTag® Peroxidase In Situ Apoptosis Detection Kit (EMD Millipore Corporation, Billerica, MA). Acrolein adducts were detected using an antibody specific for FDP-Lysine acrolein adducts obtained from Cell Sciences. (Canton, MA). Quantitation was done and calculated by MetaMorph microscope. Ten pictures from each liver were used for analyses.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, Inc., La Jolla, CA). One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used to evaluate significant differences between the compared groups. A p-value of <0.05 was considered statistically significant. Data were expressed as mean ± SEM.

CHAPTER 3

RESULTS

In this study, we have examined the contribution of the lipid-derived aldehyde, acrolein, to alcohol-induced liver injury in a “chronic+binge” murine model of ALD. This model reflects a common drinking pattern in humans, particularly in patients with ALD who are often chronic and binge drinkers. Moreover, the model has the advantage of shorter duration with increased liver inflammation and injury as compared to other alcohol feeding models [36, 37]. Figure 5 is a schematic representation of the mouse model of alcohol-induced liver injury in early ALD.

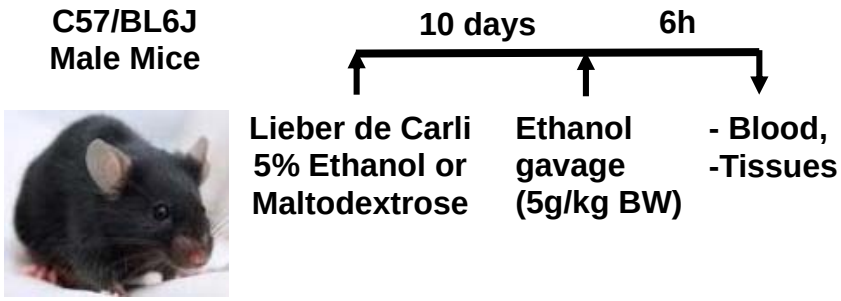


Figure 5: Schematic of murine model of alcohol consumption and ALD.

Alcohol consumption results in the accumulation of acrolein adducts in the liver

Alcohol consumption is known to upregulate CYP2E1 and increase oxidative stress and LPO, and hence is expected to generate higher levels of the lipid-derived aldehyde, acrolein. Free acrolein is extremely labile and reactive, and difficult to quantify; moreover, it is known to react with amino acid side chains, particularly lysine and cysteine, and can form adducts with cellular proteins. Accordingly, we examined (i) CYP2E1 gene expression and protein levels, and (ii) the levels of acrolein-protein adducts in the livers of control versus alcohol-fed mice. Alcohol feeding led to a robust increase in CYP2E1 protein levels (Figure 6A), but interestingly CYP2E1 mRNA was down-regulated in the alcohol-fed mice. Consistent with CYP2E1protein upregulation, a corresponding increase was observed in the levels of acrolein-protein adducts in the liver (Figure 6B). Some liver sections in the alcohol group had slightly higher accumulation of alcohol-induced acrolein adducts around the central veins, however, there was no zone-specific accumulation, and there was no particular increase in acrolein adducts at the sinusoids. In the majority of liver sections, elevated acrolein staining was seen in both cytoplasm and nuclei of hepatocytes. The acrolein adduct accumulation was quantified and there was a statistically significant difference in the adduct levels between control and alcohol-fed livers. Consistent with increased acrolein adducts, alcohol exposed mice also developed microvesicular and macrovesicular liver steatosis as compared to controls, as evaluated by

histological examination (Figure 6C) and confirmed by Oil-Red-O staining (Figure 6D).

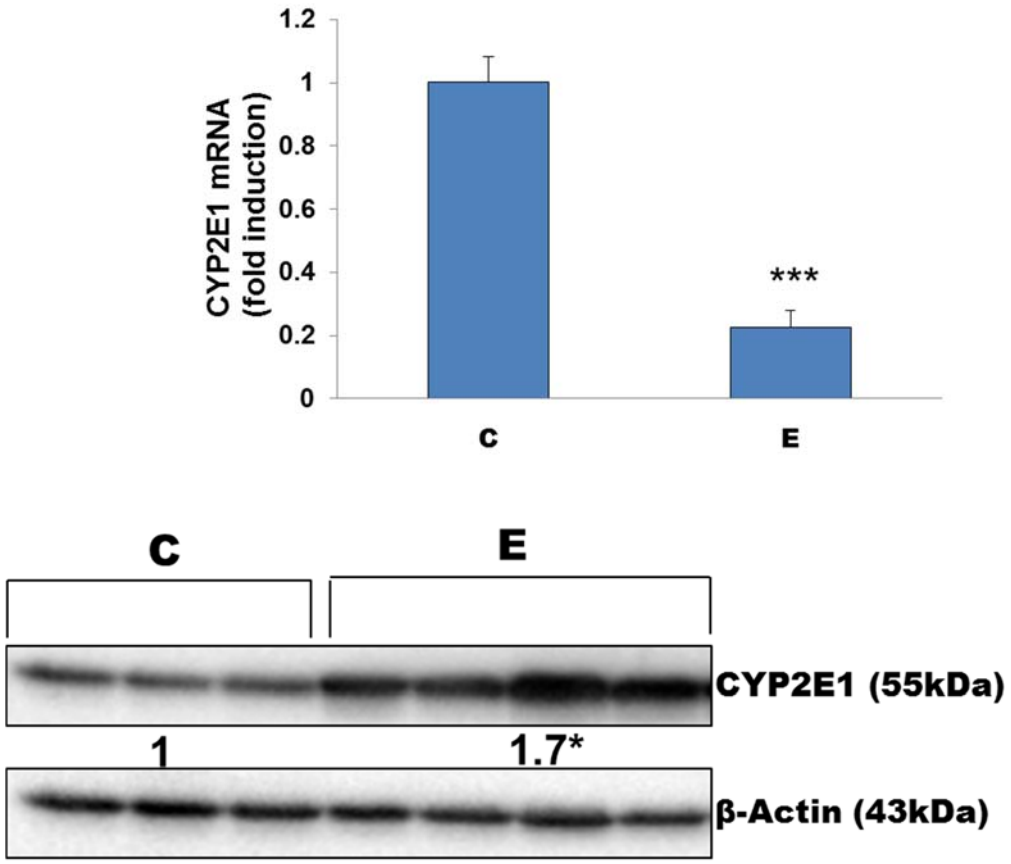


Figure 6A. Alcohol consumption decreases mRNA and increases CYP2E1 protein levels in mouse livers.

Top: CYP2E1 mRNA by real time-PCR. Data are presented as the mean ± SD. Statistical analysis was done by student's t-test. *p,0.05, ***p<0.001compared to control (n=5). **Bottom:** CYP2E1 protein by Western blot analysis of total lysates. Blots were reprobed with antibody to β-actin to ensure equivalent loading. Densitometry analysis was performed using Imagelab software. Density ratio was calculated using β-actin as control. Numbers represent the mean of density ratio for each group of Control (C) and alcohol-fed (E) mice.

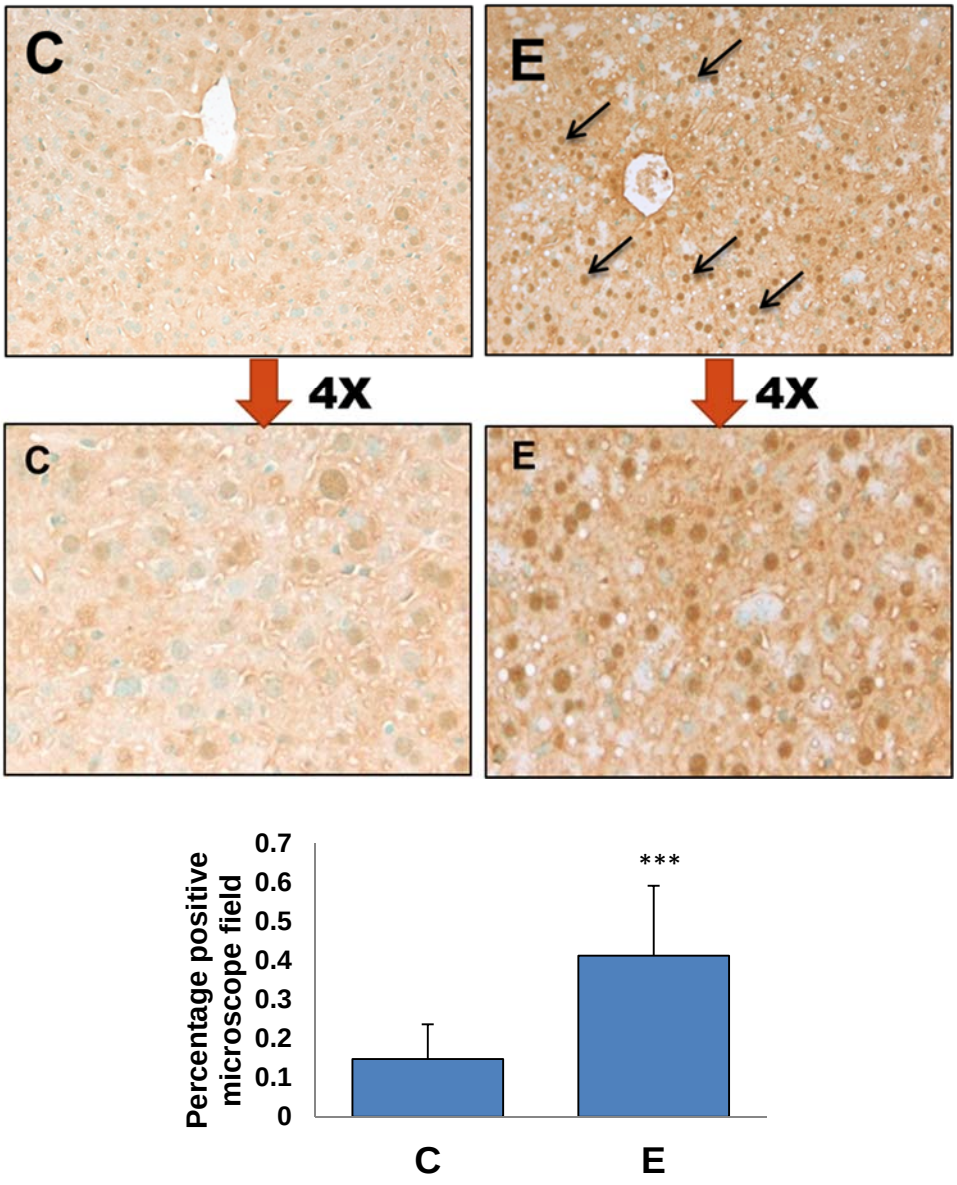


Figure 6B. Alcohol consumption leads to accumulation of acrolein adducts in mouse livers.

Top: Immunostaining for Acrolein FDP-lysine adducts in liver of control (C) and alcohol-fed (E) mice from 10+1 chronic alcohol binge model. 5μM liver sections were stained with antibodies for acrolein FDP-lysine adducts. Brown color indicates presence of acrolein adducts (denoted by arrows).

Bottom: Quantification of acrolein adducts by percentage positive microscope fields was calculated by MetaMorph microscopy in control (C) and alcohol-fed (E) mice. Data are presented as the mean ± SD. Statistical analysis was done by student's t-test. ***$p<0.001$ compared to untreated control (C) (n=10).

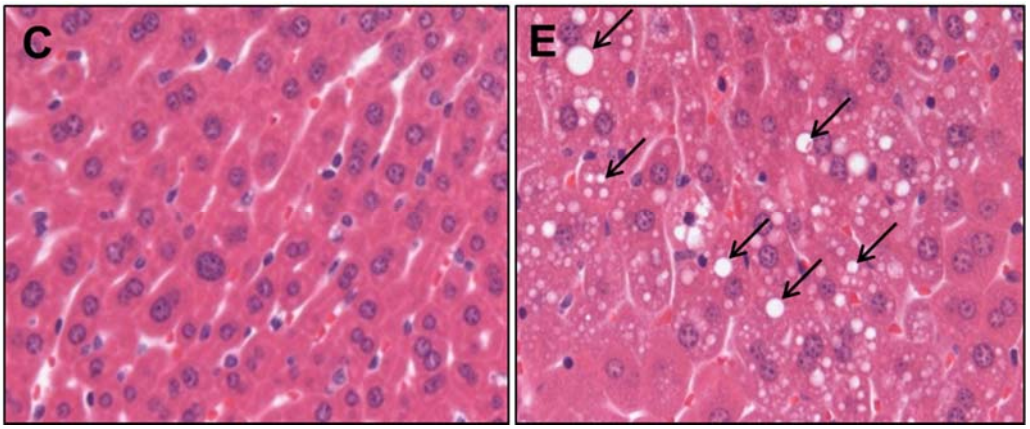


Figure 6C. Alcohol consumption results in hepatic steatosis in mouse livers.

Liver were stained with Hematoxylin and Eosin staining (H&E, ×200 final magnification). Alcohol exposure resulted in microvesicular and macrovesicular fat accumulation. Liver sections from mice fed control diet (C) or alcohol (E). Arrows indicate the fat droplets.

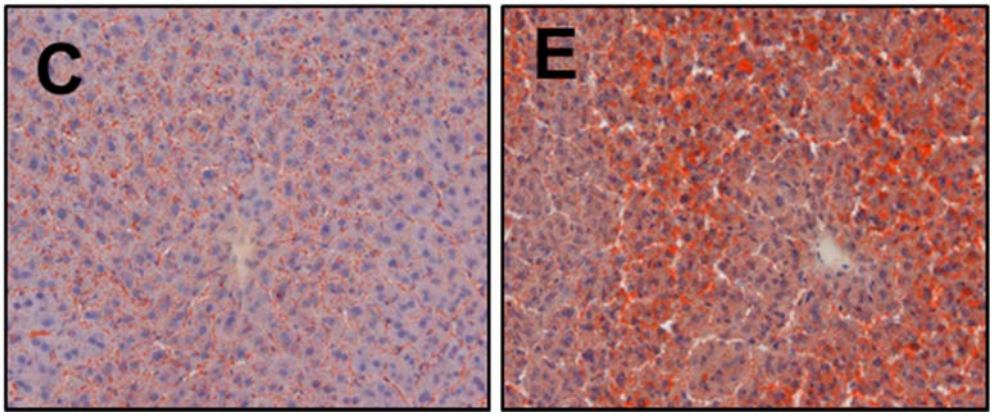


Figure 6D. Alcohol consumption results in hepatic steatosis in mouse livers.

Liver sections from mice fed control diet (C) or alcohol (E) were stained with Oil-Red-O (×20 final magnification). Alcohol exposure resulted in microvesicular and macrovesicular fat accumulation (red color).

Alcohol-induced hepatic accumulation of acrolein adducted proteins causes ER stress

The alcohol-induced accumulation of acrolein adducted proteins in the liver is a likely burden on the ER protein folding machinery, which if overwhelmed can result in the induction of ER stress and activation of UPR. To evaluate the consequence of alcohol-induced acrolein adduct accumulation, we therefore examined hepatic ER stress following alcohol feeding. Indeed, alcohol feeding and resultant accumulation of acrolein adducts led to ER stress and extensive upregulation of the prototypical ER stress markers, activating transcription factors ATF3 and ATF4, at the mRNA (Figure 7A). In agreement with gene expression, western blot analysis also demonstrated increased protein levels in the livers of alcohol-fed animals (Figure 7B). The increase in ATF3 and ATF4 could be attributed to phospho-activation of the upstream signaling proteins, PKR-like endoplasmic reticulum kinase (PERK) and eukaryotic translation initiation factor 2A, (eIF2α) (Figure 7B). The effect of alcohol consumption was also examined on gene expression of ER chaperone proteins GRP-78 and GRP94, which are upregulated as part of the adaptation responses to ER stress. Alcohol feeding increased the mRNAs of both GRP78 and GRP94 to a small but statistically significant extent (Figure 7A); however, at the protein level, only GRP78 was elevated (Figure 7B).

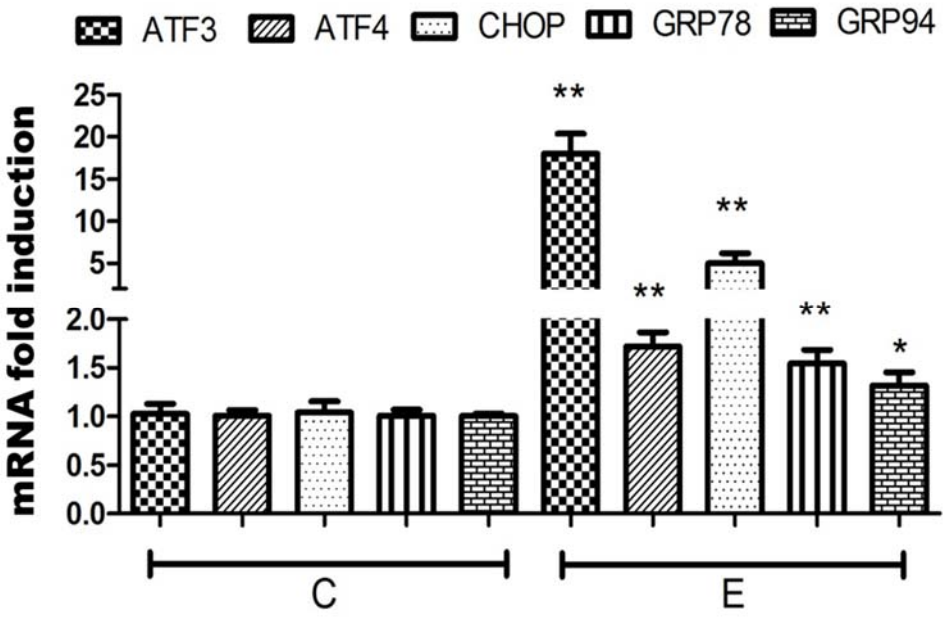


Figure 7A. ER stress gene expression is up-regulated in mouse livers. mRNA expression was examined by real-time PCR using primers specific for ATF3, ATF4, GADD/CHOP, GRP78, and GRP94 genes. Data are presented as the mean ± SD (n=6). Statistical analysis was performed using GraphPad Prism Software using one-way ANOVA followed by Bonferroni posttest. *P<0.05, **P<0.01.

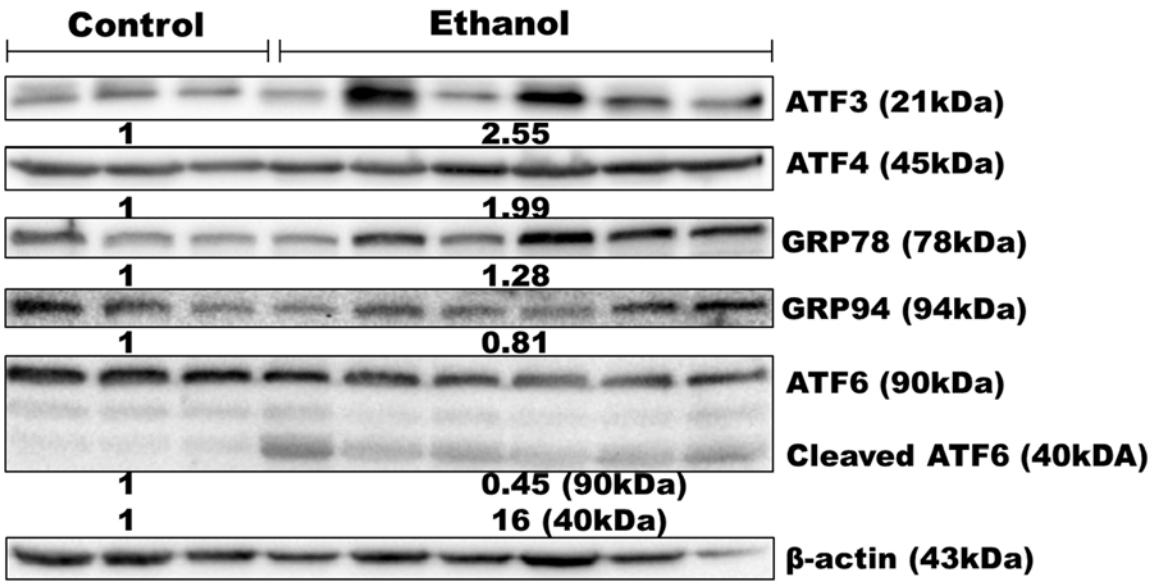


Figure 7B. Alcohol consumption induces ER stress in mouse livers.

Western blot analysis of ATF3, ATF4, GADD/CHOP, GRP78, GRP94, and ATF6 was performed using total cell lysates. Blots were reprobed with antibody to β-actin to ensure equivalent loading. Statistical analysis was performed using GraphPad Prism Software using one-way ANOVA followed by Bonferroni posttest. Densitometry analysis was performed using Imagelab software. Density ratio was calculated using β-actin as control. Numbers represent the mean of density ratios for each group of mice pair-fed control or ethanol diet.

Alcohol-induced hepatic acrolein build-up and consequent ER stress lead to apoptotic signaling, hepatocyte death and liver injury

ER stress-induced apoptosis is thought to occur via various apoptotic pathways. The first is activation by phosphorylation of the stress kinase JNK (cJUN NH2-terminal kinase), which is primarily involved in proinflammatory signaling and gene expression, but is also linked to hepatocyte apoptosis. Another arm of ER stress-associated apoptosis is postulated to involve activation of caspase-12 (caspase-4 in humans), a phylogenetic family member of the inflammatory caspases which is proteolytically activated during ER stress-induced cell death [38], rather than other apoptotic pathways. ER stress increases cytosolic calcium and activates m-calpain, which cleaves Bcl-XL and proteolytically activates caspase12; active caspase12 then activates caspase-9 and caspase-3 [39], resulting in apoptosis. The third and possibly the most important pathway of ER-stress induced apoptosis is upregulation of C/EBP homologous protein (CHOP, GADD153), the pro-apoptotic protein primarily responsible for ER stress-induced cell death. Accordingly, we examined these apoptotic signals following alcohol feeding in mice.

In hepatocytes, JNK1 (46kDa) and JNK2 (54kDa) are expressed and sustained JNK activation has been implicated in several forms of liver injury. In particular, it is thought that JNK1 may be responsible for inducing cell death, and JNK2 may primarily promote proliferation. In our model system, alcohol caused a significant phospho-activation of JNK1 which was undetectable in control mice, while phospho-JNK2 was detected in both control and alcohol fed mice at the same

level (Figure 8). Also, alcohol feeding led to proteolytic activation of caspase12, with a decrease in pro-caspase12 (apparent ~55kDa) and a concurrent increase in the cleaved form (38kDa) in mice fed alcohol compared to controls (Figure 8). Notably, alcohol consumption also led to the induction of C/EBP homologous protein (CHOP, GADD153). CHOP is a pro-apoptotic basic-leucine zipper (bZIP)-containing transcription factor which is a point of convergence for all three arms of UPR; CHOP has binding sites within its promoter for ATF6, ATF4 and XBP1. CHOP mediated upregulation of Bim and downregulation of Bcl-2 has been reported as the primary mechanism by which CHOP favors pro-apoptotic Bcl-2 proteins and mediates ER stress induced cell death. CHOP mRNA was increased approximately 5-fold (Figure 7A) and CHOP protein was dramatically upregulated in the alcohol-fed group compared to control (Figure 8).

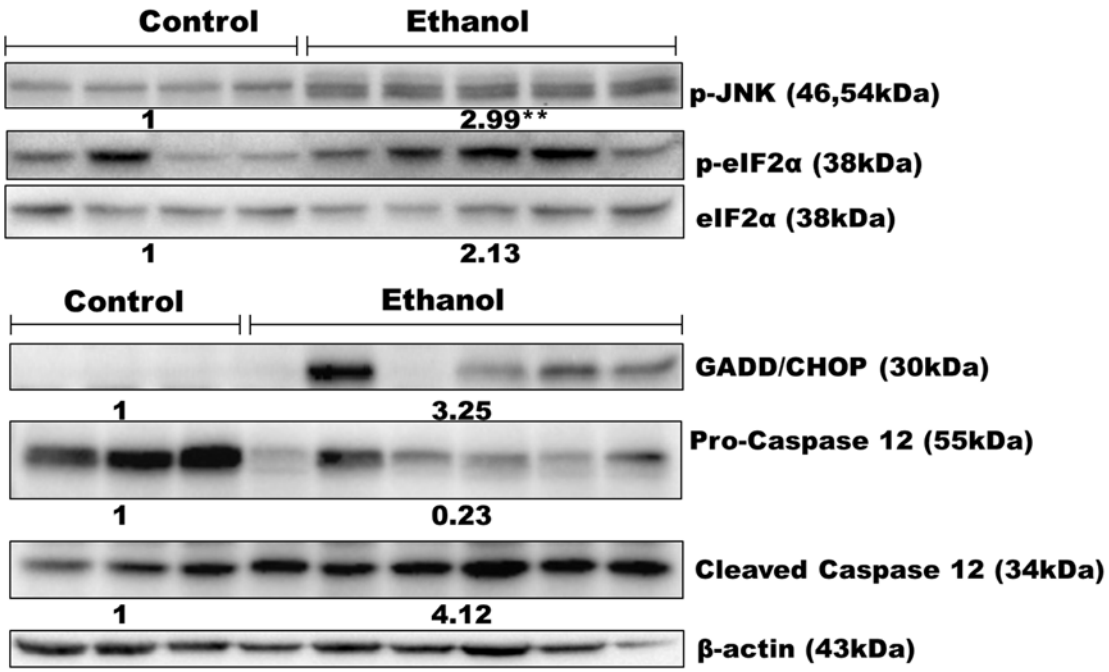


Figure 8. Hepatic activation of JNK, induction of ER stress proteins, and ER stress induced apoptotic proteins in mouse livers.

Western blot analysis of p-JNK, p-eIF2α, GADD/CHOP, and Caspase 12 was performed using total cell lysates. Blots were reprobed with antibody to β-actin to ensure equivalent loading. Statistical analysis was performed using GraphPad Prism Software using one-way ANOVA followed by Bonferroni posttest. **P<0.01. Densitometry analysis was performed using Imagelab software. Density ratio was calculated using β-actin as control. Numbers represent the mean of density ratio for each group of mice pair-fed control or ethanol diet.

The observed alcohol-induced activation of apoptotic signals culminates in apoptotic cell death of hepatocyte in the livers of alcohol-fed mice, as seen by an increase in TUNEL positive staining in the livers of alcohol-fed mice compared to control (Figure 9A, apoptotic cells denoted by arrows). Consistent with elevated apoptosis in the liver, alcohol-fed mice exhibited higher liver injury as compared to control; liver injury was defined by elevated serum ALT **(53.89 ± 9.93 vs. 7.21 ± 1.05 U/l, $p < 0.05$),** and AST **(66.35 ± 25.87 vs. 11.06± 2.18 U/l, $p < 0.05$)** activities (Figure 9B). Thus, alcohol-induced accumulation of acrolein and consequent ER stress resulted in hepatocyte apoptosis leading to liver injury in mice fed alcohol.

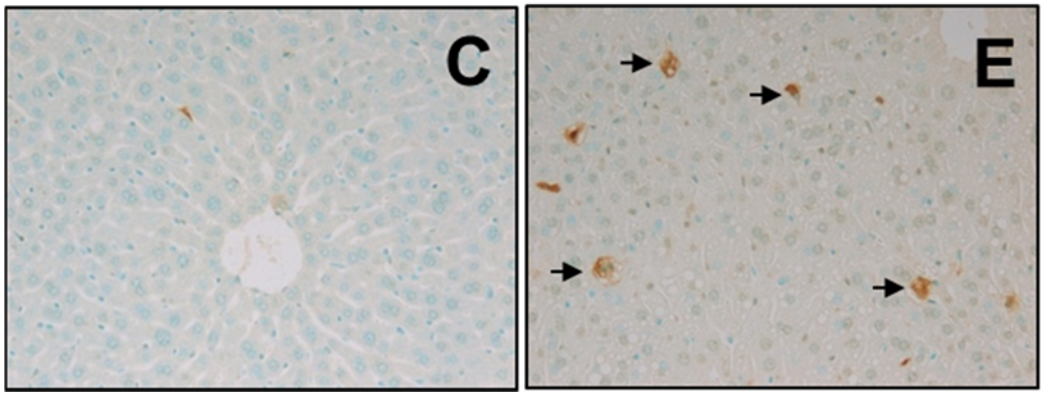


Figure 9A. Alcohol consumption results in hepatic apoptosis by terminal transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) staining assay.

TUNEL staining of liver sections from Control (C) and alcohol (E) fed mice. Arrows indicate TUNEL-positive cells.

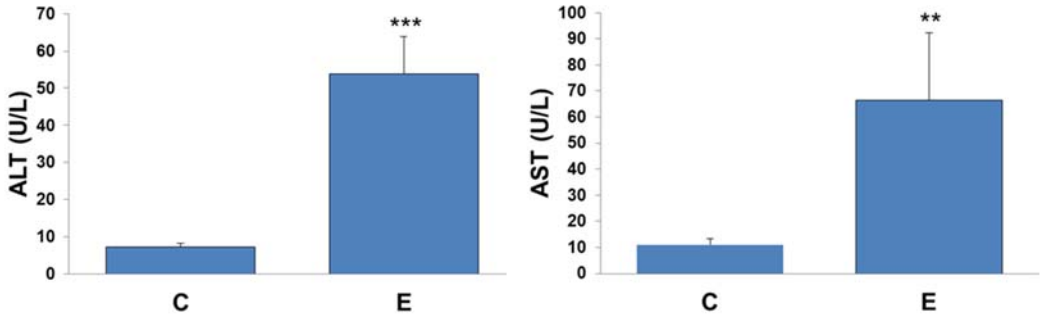


Figure 9B. Alcohol consumption leads to liver injury (serum ALT and AST).

Control (C) and alcohol (E). AST and ALT serum levels were measured by commercial kits as described in methods. Data are presented as the mean ± SD. Statistical analysis was done by t-test. **p<0.01 compared to Control and ***p<0.001 compared to Control (n=6).

Acrolein mimics the in vivo effects of alcohol in cultured hepatic cells.

Alcohol consumption and metabolism in the liver is capable of giving rise to many toxic metabolites including acetaldehyde, HNE and acrolein. To isolate and determine the sole contribution of acrolein in alcohol-induced hepatic injury, we used cultured rat hepatic H4IIEC cells to examine the direct in vitro effects of acrolein exposure in comparison to alcohol exposure. These cells are well characterized model system for hepatocytes and importantly, are known to metabolize alcohol. We first assessed whether in vitro exposure to alcohol resulted in the accumulation of acrolein adducts, similar to adducts seen in alcohol-fed mouse livers. H4IIEC cells were treated for 24h with relevant concentrations of alcohol (200mM) and acrolein (20μM). Considerable acrolein adduct accumulation was seen within 6h of alcohol exposure and acrolein adduct levels continued to be elevated at 24h compared to untreated cells (Figure 10A). Interestingly, the in vitro acrolein adduct accumulation appeared to be greater in the cell cytoplasm, rather than the nucleus; whereas in vivo, acrolein adducts were equally distributed throughout the cells.

To further characterize the acrolein-protein adducts, we subjected total protein extracts from treated cells to Western blot analysis (Figure 10B). Compared to untreated cells, we observed a significant increase in the overall levels of acrolein-FDP lysine adducts in the cells treated for 24h with acrolein or alcohol. In particular, three proteins with an estimated molecular mass of 46kDa, 56kDa and 64kDa were considerably elevated in both alcohol and acrolein exposed cells. The identity of these proteins is currently unclear and under investigation.

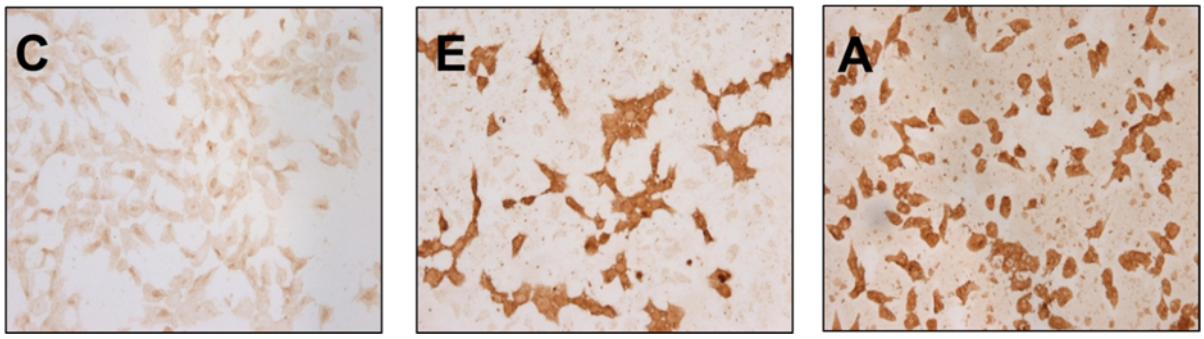


Figure 10A. Acrolein adduct accumulation in H4IIEC cells (Immunostaining with acrolein FDP-lysine antibody).

Control (C); 200mM Alcohol (E); 20μM Acrolein (A) for 24 hours. Brown color indicates presence of acrolein adducts.

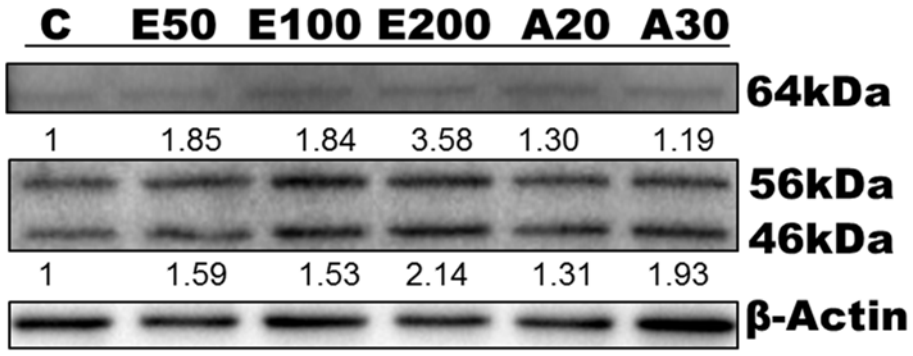


Figure 10B. Acrolein adduct accumulation in H4IIEC cells (Western Blot).

H4IIEC cells were treated with varying concentrations of alcohol (50mM, 100mM, and 200mM, denoted by E50, E100, and E200) and acrolein (20μM and 30μM, denoted by A20 and A30) 24 hours. Western blot analysis was performed using total lysates. Blots were reprobed with antibody to β-actin to ensure equivalent loading. Densitometry analysis was performed using Imagelab software. Density ratio was calculated using β-actin as control. Numbers represent the means of density ratio for each treatment.

Next, we determined the effects of alcohol and elevated acrolein on induction of ER stress and consequent apoptotic cell death in hepatocytes. We exposed H4IIEC cells to different concentrations of either ethanol (50mM, 100mM or 200mM) or acrolein (20μM or 30μM). Similar to in vivo alcohol exposure, alcohol triggered ER stress and caused increased gene expression of ATF3, ATF4 mRNA (Figure 11A) and protein (Figure 11B). A parallel result was seen with direct treatment of cells with acrolein (Figure 11A and 11B). As seen with in vivo alcohol exposure, the adaptive responses to ER stress, namely, upregulation of GRP78 and GRP94, were not robust (Figure 11A and 11B). Notably, proapoptotic CHOP was significantly upregulated at both mRNA and protein levels (Figure 11A and 11B). The up-regulation of proapoptotic signal and CHOP expression resulted in apoptotic cell death and decreased cell survival by MTT assay (Figure 11C).Thus, our data demonstrate that acrolein mimics the effects of alcohol in cultured hepatic cells, suggesting that the adverse effects of alcohol may indeed be attributed to elevated acrolein in the liver, occurring as a result of alcohol consumption.

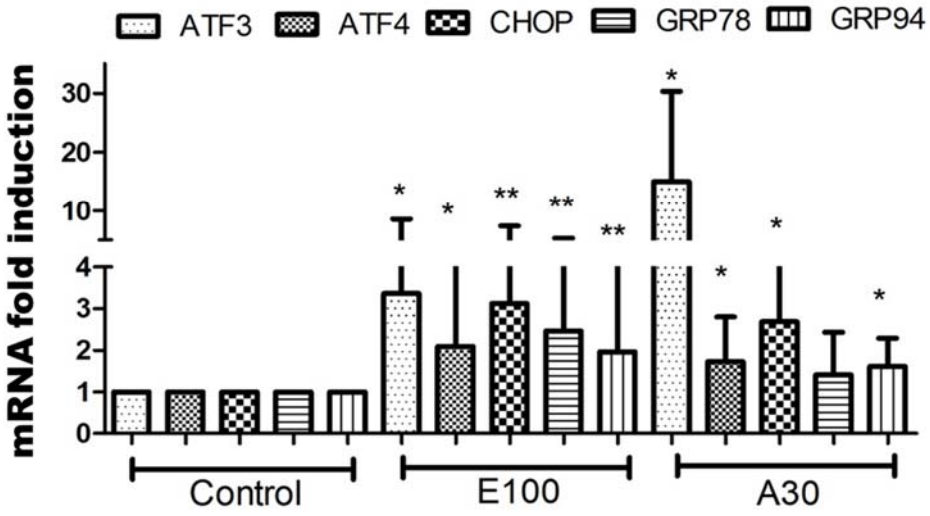


Figure 11A. Exposure of H4IIEC cells to alcohol or acrolein causes upregulation of ER stress genes by Real time qPCR.

C=Control; E=alcohol (mM); A=Acrolein (μM). Data are presented as the mean ± SD. Statistical analysis was performed using GraphPad Prism Software using one-way ANOVA followed by Bonferroni posttest. *p<0.05, **p<0.01, n=3.

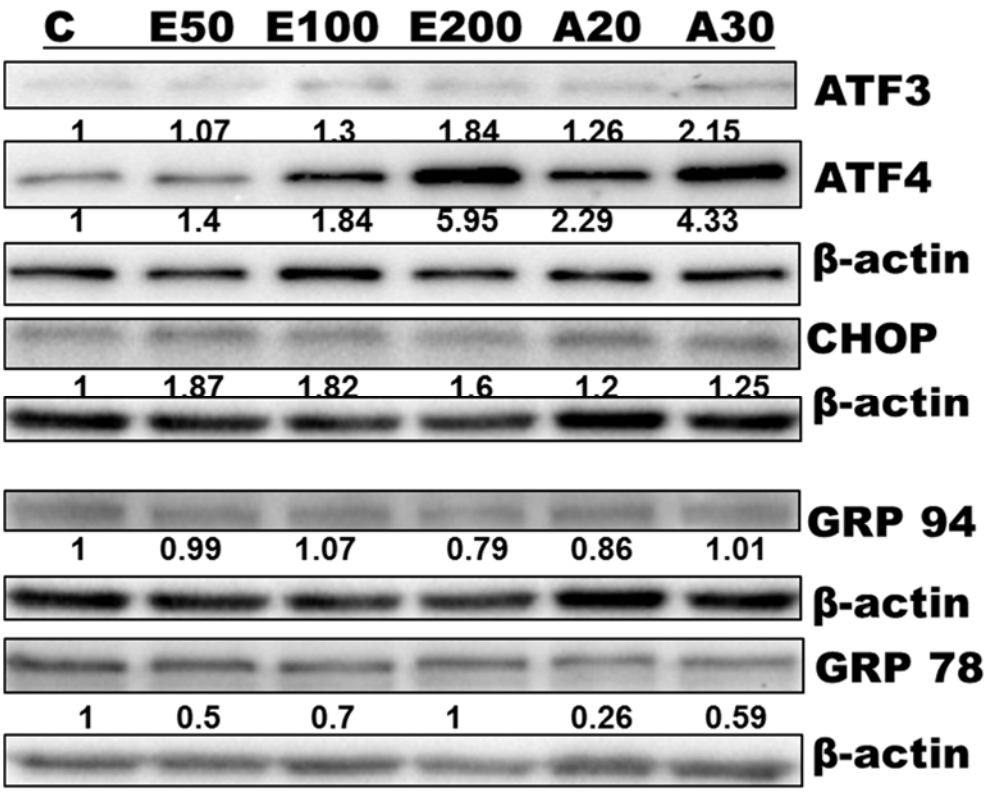


Figure 11B. ER stress gene expression ATF3, ATF4, GADD/CHOP, GRP78, and GRP94 by alcohol (E) or acrolein (A).

H4IIEC cells were treated with varying concentrations of ethanol (50mM, 100mM, and 200mM) and acrolein (20μM and 30μM) 24 hours. Western blot analysis was performed using total cell lysates. Blots were probed with ATF3, ATF4, GADD/CHOP, GRP78, and GRP94 antibody, then stripped and reprobed with antibody to β-actin to ensure equivalent loading. Densitometry analysis was performed using Imagelab software. Density ratio was calculated using β-actin as control. Numbers represent density ratio for each treatment.

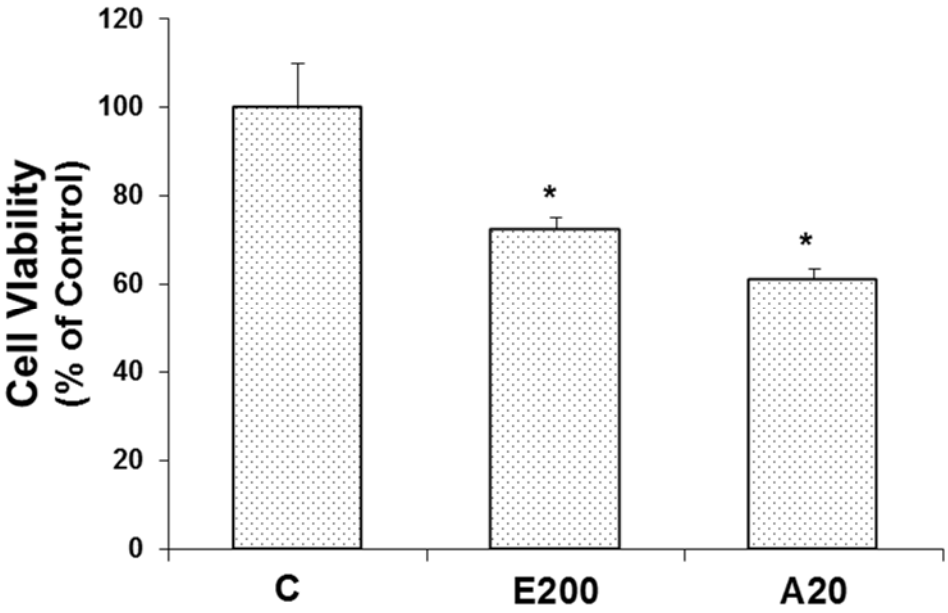


Figure 11(C). Cell viability of hepatocytes from alcohol (E) or acrolein (A) toxicity.

Cell viability was measured by MTT assay as described in methods. Alcohol 200mM (E200) and acrolein 20μM (A20). Data are presented as the mean ± SD. Statistical analysis was performed using GraphPad Prism Software using one-way ANOVA followed by Bonferroni posttest. *$p<0.05$ compared to Control (n=3).

Acrolein scavengers were effective in attenuating the adverse effects of both acrolein and alcohol exposure in vitro

In order to confirm the contribution and establish the pathogenic role of acrolein in alcohol-induced ER stress and liver injury, we used known acrolein scavengers (N-acetyl cysteine, carnosine and hydralazine) to try to mitigate the injurious effects of acrolein build-up in hepatocytes resulting from alcohol and acrolein exposure. Acrolein is known to rapidly deplete cellular GSH and cause oxidative stress in hepatocytes [1]. Hence, we tested the N-acetylcysteine (NAC), a glutathione precursor and antioxidant that was shown to build up intracellular GSH and protect against acrolein-induced apoptosis [1, 40]. Carnosine (beta-alanyl histidine) is an endogenous protein building block that is known to scavenge aldehydes, such as acrolein, and to inhibit harmful oxidation and glycation reactions [41]. Carnosine is thought to help against aging, autism, cataracts, brain and kidney disorders, and diabetic complications. H4IIEC cells were pretreated with NAC or carnosine for 45m prior to exposure to alcohol or acrolein, and the effects were studied on cell viability. Cell death was monitored by the MTT assay. The data show that both NAC and carnosine partially attenuated the cytotoxic effects of alcohol and acrolein (Figure 12).

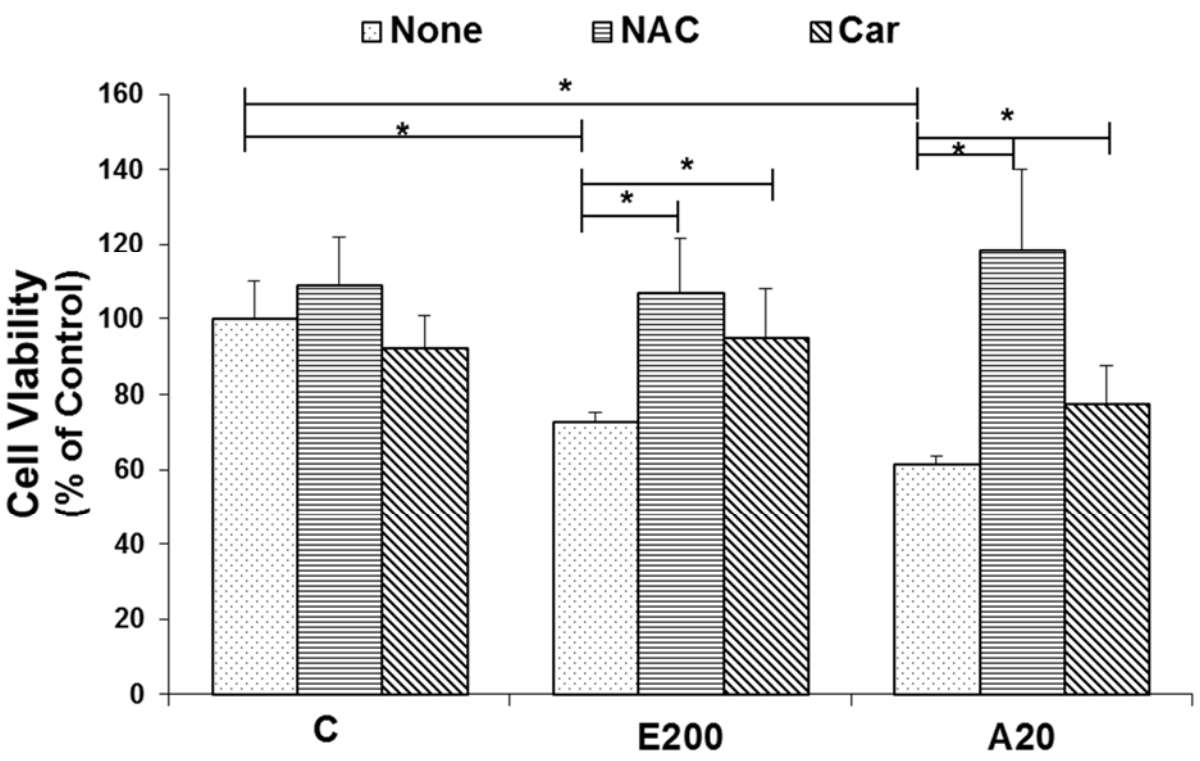
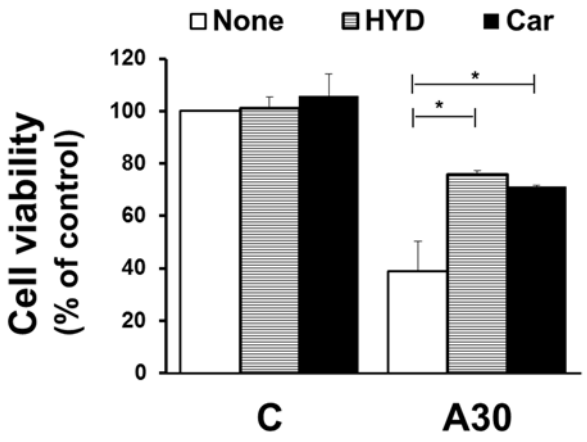


Figure 12. Acrolein scavengers protect hepatocytes from alcohol (E) or acrolein (A) toxicity.

Cell viability was measured by MTT assay as described in methods. Control (C); 20μM Acrolein (A); 200mM Alcohol (E). Data are presented as the mean ± SD (n=3). Statistical analysis was performed using GraphPad Prism Software using one-way ANOVA followed by Bonferroni posttest. *p<0.05.

Another acrolein scavenger, hydralazine, which is a vasodilator and hypertension drug, is highly selective and effective in preventing acrolein-mediated oxidative stress and cell death in PC12 cells, and tissue injury in spinal cord injury models [42]. Notably, the cytoprotective effects of hydralazine are shown to occur not merely by acrolein scavenging but also by effective trapping of acrolein-protein adducts [43]. We tested the protective effects of hydralazine against acrolein cytotoxicity in vitro (Figure 13A) and against alcohol-induced liver injury in vivo in the NIAAA mouse model (Figure 13B).

A.



B.

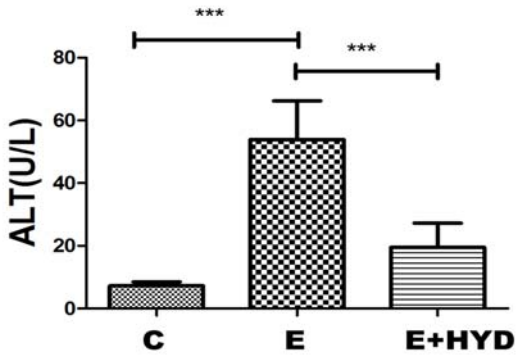


Figure 13. Acrolein scavengers protect from acrolein cytotoxicity in vitro (A), and alcohol-induced liver injury in vivo (B).

(A) Cell viability was measured by MTT assay as described in methods. Control (C); 30μM Acrolein (A30). 50μM Hydralazine (HYD). 100μM Carnosine (CAR) (B) Serum ALT was measured by commercial activity assay kit. Control (C), Alcohol (E), (5mg/kg) body weight Hydralazine (HYD). Statistical analysis was done by (A) ANOVA. *$p<0.05$ compared to Control. (B) ANOVA. ***$p<0.001$ compared to Control (n=5).

CHAPTER 4

DISCUSSION

In this study, we examined using in vitro (rat hepatic H4IIEC cells) and in vivo (C57Bl/6 mice - chronic+binge (NIAAA) alcohol feeding) models how acrolein plays a key role on hepatic effects in alcoholic liver disease. We investigated the effects of alcohol or acrolein on hepatic ER stress and cell death, and studied how acrolein contributed to liver injury in the alcoholic liver disease. Finally, acrolein scavengers were tested as potential therapeutic agents in ALD by examining their protective effects in the prevention of alcohol-induced ER stress and injury cell death.

Oxidative stress is a salient feature of ALD, and oxidative stress has been demonstrated in the absence of ER stress in alcohol fed mice [44]. In the present study our experimental results demonstrate elevated protein level of CYP2E1 in the livers of alcohol-fed mice following alcohol exposure. Also, the accumulation of acrolein adducts were detected in alcohol fed mice liver indicating that alcohol consumption results in considerable generation of lipid peroxidation product, acrolein, leading to the buildup of acrolein adducts. These results were also supported by in vitro acrolein adducts staining in rat hepatocyte H4IIEC treated with alcohol or acrolein which indicated the formation of acrolein adducts from alcohol treatment in vitro. Compared to untreated cells, we observed a significant increase in the overall levels of acrolein-FDP lysine adducts in the cells treated for 24h with acrolein or alcohol. Increased acrolein adducts were detected in vivo and in vitro, both in the cytoplasm as well as the nuclei. The accumulation of

cytoplasmic adducts is likely to directly trigger ER stress and alter calcium homeostasis, and may also lead to the mitochondrial permeability changes that are associated with ALD. The nuclear association of acrolein adducted proteins is especially interesting and may indicate changes in transcription factors or chromatin-modifying proteins such as histone deacetylases and DNA/histone methylases, which are likely to have significant effects on gene expression. Interestingly, in vitro exposure to either alcohol or acrolein for 24h resulted predominantly in cytoplasmic acrolein adducts; the significance of this finding is not clear and it is possible that longer exposures may be needed for nuclear acrolein buildup. By western blot analysis, we observed an increase in several proteins; however, three proteins with an estimated molecular mass of 46kDa, 56kDa and 64kDa were considerably elevated. The identity of these proteins is currently under investigation.

Our results demonstrate hepatic ER stress and injury in the NIAAA model of chronic + binge alcohol consumption. Notably, all three ER stress/UPR pathways were stimulated with phosphorylation of PERK, eIF2α, and JNK, proteolytic activation of ATF6α, and upregulation of ATF3 and ATF4 mRNA and proteins. The mechanisms by which acrolein affects ER stress proteins may be direct via adduction of ER stress molecules, or indirect via adduction and alteration of function of other proteins that regulate the transcription, translation, stability or localization of the ER response proteins. A direct adduction of the ER chaperone GRP78 by the lipid aldehyde HNE was shown by Galligan et al, 2014 [45]; notably, although HNE adduction led to a decrease in ATP binding, no change

was seen in the chaperone activity of GRP78 [45]. Notably, among the ER chaperone proteins, GRP78 mRNA and protein were upregulated but GRP94 was only minimally increased (~1.3 fold) at the mRNA level in this in vivo model of ALD. Thus, the adaptive responses were not robust and appeared inadequate. This is in keeping with the in vitro alcohol and acrolein exposure in H4IIEC cells showing modest increase in mRNA but no increases in protein level of GRP78 or GRP94; these data are also similar to our published work in HepG2 cells that do not express alcohol dehydrogenase or CYP2E1 and are unable to metabolize alcohol [1]. The alcohol-induced acrolein accumulation and ER stress were associated with significant hepatic steatosis, hepatocyte apoptosis and liver injury with a highly significant increase in AST and AST in the alcohol fed mice. Thus, these data suggest that alcohol consumption leads to acrolein adducts accumulation in the liver, with ER stress and apoptosis, which correlate with liver injury.

Overall, our results suggest that the effects of alcohol on hepatic ER stress and hepatocyte cell death and liver injury are mediated, at least in part, by acrolein, which is substantially generated with alcohol consumption/metabolism. This result is further supported by the in vitro data in H4IIEC cells treated with acrolein alone showing that acrolein is able to fully mimic the in vivo effects of alcohol. Finally, our data demonstrating that acrolein removal by acrolein scavengers protected cells against alcohol induced apoptosis confirmed that acrolein is indeed a major mediator of alcohol-induced injury. The mechanisms underlying the alcohol-induced acrolein-mediated ER stress and apoptotic injury requires

further investigation. Additionally, our data indicate that acrolein clearance may be an effective means of curtailing alcohol-induced acrolein-mediated hepatic injury, and may be effective clinically protective agents for alcoholic liver disease.

SIGNIFICANCE AND CLINICAL RELEVANCE

Alcohol consumption causes alcoholic liver disease (ALD) which remains a major cause of morbidity and mortality worldwide. Currently, there is no FDA approved therapy for ALD. Of the people who drink heavily, only a subset develop clinically important ALD and dietary and environmental factors may be critical determinants. Dietary polyunsaturated fatty acids (PUFAs), particularly linoleic acid, promote alcohol-induced liver damage; linoleic acid consumption has dramatically increased and is now a major fat source in the Western diet. Alcohol consumption increases lipid peroxidation of PUFAs leading to the generation and accumulation of lipid-derived oxidized metabolites, such as acrolein.

In this study, we investigated the pathological contribution of acrolein in alcohol-induced liver disease using in vitro (rat hepatic H4IIEC cells) and in vivo (C57Bl/6 mice - chronic+binge (NIAAA) alcohol feeding) model systems. Our data demonstrate that alcohol-induced generation of acrolein in the liver is a major pathogenic contributor to hepatic ER stress and liver injury in ALD. Additionally, our data indicate that acrolein clearance may be an effective means of curtailing alcohol-induced acrolein-mediated hepatic injury, and acrolein scavengers may be effective clinical therapeutic agents for Alcoholic Liver Disease.

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Education

2010-Present	Graduate Student, Department of Pharmacology & Toxicology, **University of Louisville, KY**
2007-2009	Master of Science in Biochemistry, **University of North Carolina-Greensboro, NC** *Thesis: The inhibition of human cytochrome P4502E1 by essential oil of Lemongrass and the primary aldehyde constituent of lemongrass, citral*
1999-2003	Bachelor of Science in Chemistry, **Tamkang University, Taipei, Taiwan**

Positions Held

2003-2003	Infantry, Army of R.O.C, Taiwan
2004-2007	Environmental Technician, SGS Group – Société Générale de Surveillance, Taipei, Taiwan
2007-2009	Teaching Assistant, University of North Carolina-Greensboro, Greensboro, NC
2010-2010	Research Assistant, Kaohsiung Medical University, Kaohsiung, Taiwan
2010-2015	Graduate Student (PhD), University of Louisville, Louisville, KY

Awards and Honors

2010-2012	IPIBS Integrated Programs in Biomedical Sciences (IPIBS) fellowship award, University of Louisville
2012-2013	Sponsored Research Tuition Award, University of Louisville
2013	Graduate School Council Travel Award for AASLD 2013, University of Louisville

2014	UofL School of Medicine Travel Award for SOT 2014, University of Louisville
2014-2015	Sponsored Research Tuition Award, University of Louisville
2014	Graduate School Council Travel Award for DDW 2014, University of Louisville
2014	**1st Place Student Poster Award**, NIEHS Tamburro Environmental Liver Symposium, Louisville, KY

Professional Society Memberships

The American Society for Pharmacology and Experimental Therapeutics
Society of Toxicology
Ohio Valley Society of Toxicology

Abstracts

2008	**SERMACS 2008** (Southeastern Regional Meeting of the American Chemical Society), **Nashville, TN** Essential oils with high Aldehyde Content as inhibitors of Human Cytochrome P4502E1
2011	**Research! Louisville 2011** **Louisville, KY** Induction of the neutrophil chemotactic cytokine - interleukin 8 (IL-8) by HAART (Highly Active Anti-Retroviral Therapy): Relevance to HAART induced hepatotoxicity
2012	**Research! Louisville 2012** **Louisville, KY** The role of neutrophil chemotactic cytokine - interleukin 8 in HAART-induced hepatotoxicity
2012	**OVSOT (Ohio Valley Society of Toxicology) 2012 Annual Meeting** **Columbus, OH** The role of neutrophil chemotactic cytokine - interleukin 8 in HAART-induced hepatotoxicity
2013	**Research Society for Alcoholism (RSA) 2013 Annual Meeting** **Orlando, FL** Binge alcohol impairs IFNα antiviral gene expression in mice
2013	**OVSOT (Ohio Valley Society of Toxicology) 2013 Annual Meeting, Louisville, KY** Acrolein, a reactive aldehyde metabolite, is a major mediator of alcohol-induced endoplasmic reticulum stress and liver injury
2013	**Research! Louisville 2013** **Louisville, KY** Acrolein, a reactive aldehyde metabolite, is a major mediator of alcohol-induced endoplasmic reticulum stress and liver injury

2013 **AASLD 2013 The Liver meeting**
Washington, D.C.
Acrolein, a reactive aldehyde metabolite, is a major mediator of alcohol-induced endoplasmic reticulum stress and liver injury

2014 **Society of Toxicology (SOT) 2014**
Phoenix, AZ
Acrolein, a Lipid-Derived Aldehyde Metabolite, Is a Critical Mediator of Alcohol-Induced Endoplasmic Reticulum Stress and Liver Injury

2014 **American Society for Pharmacology and Experimental Therapeutics (ASPET) 2014**
San Diego, CA
Lipid-derived aldehyde, acrolein, is a critical mediator of alcohol-induced gut-liver injury

2014 **NIEHS Tamburro Environmental Liver Symposium**
Louisville, KY
Lipid-derived aldehyde, acrolein, is a critical mediator of Alcohol- induced gut-liver injury in alcoholic liver disease

2014 **Research! Louisville 2014**
Louisville, KY
Lipid-derived aldehyde, acrolein, is a critical mediator of alcohol-induced gut-liver injury in alcoholic liver disease

2014 **OVSOT (Ohio Valley Society of Toxicology) 2014 Annual Meeting**
Dayton, OH
Lipid-derived aldehyde, acrolein, is a critical mediator of alcohol-induced gut-liver injury in alcoholic liver disease

Lecture presentation

2014 **Digestive Disease Week (DDW) 2014**
Chicago, IL
Lipid-derived aldehyde, acrolein, is a critical mediator of alcohol-induced gut-liver injury

2014 **OVSOT (Ohio Valley Society of Toxicology) Summer Student Meeting**
Louisville, KY
Lipid-derived aldehyde, acrolein, is a critical mediator of alcohol-induced gut-liver injury

Publication

2014 **Lipid-derived aldehyde, acrolein, is a critical mediator of alcohol-induced ER stress and liver injury.**
Wei-Yang (Jeremy) Chen, Jingwen Zhang, Craig McClain, Shirish Barve, and Swati Joshi-Barve (Manuscript in Preparation)