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ORIGINAL RESEARCH

Acrolein Is a Pathogenic Mediator of Alcoholic Liver Disease and the Scavenger Hydralazine Is Protective in Mice

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SUMMARY

Acrolein is a pathogenic mediator of alcoholic liver disease; alcohol-induced acrolein accumulation triggers endoplasmic reticulum stress without endoplasmic reticulum adaptive/protective responses, leading to apoptosis and liver injury. Acrolein removal by scavengers (hydralazine) prevents alcohol-induced liver injury in mice, and shows therapeutic potential in alcoholic liver disease.

BACKGROUND & AIMS: Alcoholic liver disease (ALD) remains a major cause of morbidity and mortality, with no Food and Drug Administration–approved therapy. Chronic alcohol consumption causes a pro-oxidant environment and increases hepatic lipid peroxidation, with acrolein being the most reactive/toxic by-product. This study investigated the pathogenic role of acrolein in hepatic endoplasmic reticulum (ER) stress, steatosis, and injury in experimental ALD, and tested acrolein elimination/scavenging (using hydralazine) as a potential therapy in ALD.

METHODS: In vitro (rat hepatoma H4IIEC cells) and in vivo (chronic+binge alcohol feeding in C57Bl/6 mice) models were used to examine alcohol-induced acrolein accumulation and consequent hepatic ER stress, apoptosis, and injury. In addition, the potential protective effects of the acrolein scavenger, hydralazine, were examined both in vitro and in vivo.

RESULTS: Alcohol consumption/metabolism resulted in hepatic accumulation of acrolein-protein adducts, by up-regulation of cytochrome P4502E1 and alcohol dehydrogenase, and down-regulation of glutathione-s-transferase-P, which metabolizes/detoxifies acrolein. Alcohol-induced acrolein adduct accumulation led to hepatic ER stress, proapoptotic signaling, steatosis, apoptosis, and liver injury; however, ER-protective/adaptive responses were not induced. Notably, direct exposure to acrolein in vitro mimicked the in vivo effects of alcohol, indicating that acrolein mediates the adverse effects of alcohol. Importantly, hydralazine, a known acrolein scavenger, protected against alcohol-induced ER stress and liver injury, both in vitro and in mice.

CONCLUSIONS: Our study shows the following: (1) alcohol consumption triggers pathologic ER stress without ER adaptation/protection; (2) alcohol-induced acrolein is a potential therapeutic target and pathogenic mediator of hepatic ER stress, cell death, and injury; and (3) removal/clearance of acrolein by scavengers may have therapeutic potential in ALD. *(Cell Mol Gastroenterol Hepatol 2016;2:685–700; http://dx.doi.org/10.1016/j.jcmgh.2016.05.010)*

Keywords: Lipid Peroxidation; Apoptosis; Therapeutic; CHOP.

Alcohol consumption can lead to alcoholic liver disease (ALD), which remains a major cause of morbidity and mortality worldwide and in the United States. Despite the profound economic and health impact, there is no Food and Drug Administration–approved therapy for any stage of ALD,[1] emphasizing the need for research into therapeutic interventions during the early initiating stages of the disease. Furthermore, only approximately 20% of heavy alcohol drinkers develop liver disease, and diet and environment are considered potential determining factors. The pathogenesis of ALD is multifactorial, and although oxidative stress, lipid peroxidation, and endoplasmic reticulum (ER) stress are known etiologic factors,[2] the molecular mediators of hepatic injury remain poorly defined.

Hepatic ER stress and the unfolded protein response (UPR) are thought to play a critical role in the pathogenesis of ALD.[3] ER stress is mediated primarily by the ER protein BiP/glucose-regulated protein (GRP)78 via 3 ER sensors: activating transcription factor (ATF)6, inositol-requiring enzyme 1 (IRE1), and protein kinase RNA-like endoplasmic reticulum kinase (PERK). ER stress comprises both pathologic and adaptive responses; UPR adaptive responses reduce the protein burden by decreased synthesis and

Abbreviations used in this paper: **ADH, alcohol dehydrogenase; ALD, alcoholic liver disease; ALDH, aldehyde dehydrogenase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ATF, activating transcription factor; CHOP, CCAAT/enhancer-binding protein homologous protein; CYP2E1, cytochrome P4502E1; ER, endoplasmic reticulum; FDP-lysine, Nε-(3-formyl-3,4-dehydropiperidino)lysine; GRP, glucose regulated protein; GSTP, glutathione-s-transferase-Pi; IRE1, inositol-requiring enzyme 1; JNK, cJun N-terminal kinase; mRNA, messenger RNA; NIAAA, National Institute on Alcohol Abuse and Alcoholism; PERK, protein kinase RNA-like endoplasmic reticulum kinase; LPO, lipid peroxidation; PUFA, polyunsaturated fatty acids; siRNA, small interfering RNA; TRAF, TNF receptor-associated factor; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling; UPR, unfolded protein response; XBP1, X-box binding protein-1.**

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increased ER-associated degradation of proteins, and by inducing ER chaperones to enhance folding capacity. If the adaptive/protective reactions are inadequate or if the ER stress is prolonged or extreme, apoptotic cell death ensues via pathways involving activation of cJun N-terminal kinase (JNK) and caspases, and up-regulation of proapoptotic proteins such as CCAAT/enhancer-binding protein homologous protein (CHOP), growth arrest DNA damage (GADD) 153 and GADD 34.[4] Alcohol consumption is known to cause hepatic ER stress along with steatosis, inflammation, and apoptosis, and reducing ER stress decreases alcoholic liver injury.[5,6] Although oxidative stress is known to contribute to ER stress,[7] the exact cause and pathogenic mediators of alcohol-induced ER stress in ALD remain unclear.

Alcohol consumption and metabolism via alcohol dehydrogenase (ADH), cytochrome P4502E1 (CYP2E1), and catalase pathways generates free radicals, leading to increased lipid peroxidation (LPO) of polyunsaturated fatty acids (PUFAs).[6–8] The dietary consumption of PUFAs, particularly linoleic acid, has increased dramatically in the past 2 decades, thereby increasing the substrate availability for LPO. LPO gives rise to α, β-unsaturated aldehydes, such as 4-hydroxynonenal, malondialdehyde, and acrolein. Acrolein is the most reactive and toxic electrophile produced by LPO[9,10]; moreover, it is found at approximately 40 times greater concentration and has a significantly longer half-life than other reactive oxidative species (days as compared with less than a second).[11,12] Acrolein is metabolized and cleared primarily by conjugation to glutathione catalyzed by glutathione-s-transferase-Pi (GSTP). If not removed, acrolein can form covalent protein adducts, generally leading to impaired protein structure and function. Accumulation of such adducted/misfolded proteins can trigger ER stress, and acrolein-induced ER stress is shown in vitro in various cell types.[13] Increased acrolein adduct levels are linked pathologically with several diseases that are associated with oxidative stress, including cancer,[14] Alzheimer's,[15] Sjogren's syndrome autoimmune disorder,[16] and cerebral stroke/infarction.[17] However, the role of acrolein in ALD has not been investigated. In this study, we investigated the pathologic contribution of acrolein in alcohol-induced hepatic ER stress, steatosis, and liver injury, and we tested, both in vitro and in vivo, whether removal of acrolein by using scavengers is effective in preventing liver injury in ALD.

Materials and Methods

Reagents

General chemicals, hydralazine, carnosine, acrolein, and β-actin antibody were purchased from Sigma Aldrich (St. Louis, MO). Acrolein Nε-(3-formyl-3,4-dehydropiperidino) lysine (FDP-lysine) antibodies were purchased from Abcam (Cambridge, MA). 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, was obtained from American Type Culture Collection (Rockville, MD) and used according to the manufacturer's instructions. All treatments were performed on subconfluent monolayers of cells.[18] Each experiment was replicated independently at least 3 times.

Animal Studies

We used an established model of ALD (National Institute on Alcohol Abuse and Alcoholism [NIAAA] model[19]). Male C57BL/6J mice (12 weeks of age) were obtained from Jackson Laboratories (Bar Harbor, ME), and maintained at 25°C with a 12:12-hour light/dark cycle. The mice were fed (ad libitum) a Lieber–DeCarli liquid diet (corn oil/LA enriched; Research Diet, New Brunswick, NJ.) containing 5% ethanol (wt/vol or 35% of calories) or an isocaloric maltose dextrin Lieber–DeCarli liquid diet as (pair-fed) control for 10 days. This was followed by a single oral gavage of ethanol (5 g/kg body weight, or maltose dextrin as control) on day 11. Blood and liver samples were collected 9 hours later. Hydralazine (5 mg/kg body weight) was administered by daily intraperitoneal injection along with alcohol feeding. We used 6 mice in each group based on our earlier observations and published literature in this mouse model. All experimental protocols were conducted under a protocol approved by the University of Louisville Institutional Animal Care and Use Committee in accordance with the National Institutes of Health Office of Laboratory Animal Welfare Guidelines (available: http://grants.nih.gov/grants/olaw/olaw.htm).

Acrolein Adducts Assay

Cryostat mouse liver sections (5 μmol/L) and H4IIEC cells grown on coverslips were fixed, stained using polyclonal rabbit antibodies specific for acrolein FDP-lysine adducts, and examined by light microscopy. Quantification was performed using ImageJ Microscopy Image Analysis Software (National Institutes of Health, Bethesda, MD) by calculating the average intensity of the field-of-view from microscope fields (at least 4 images per sample from cells and 10 images/mouse from each group).

Small Interfering RNA Transfection

Before alcohol or acrolein treatment, small interfering RNAs (siRNAs) specific for rat GSTP or scrambled RNA (Thermo Fisher, Grand Island, NY) as a negative control were transfected into H4IIEC cells with Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. The final concentration of each transfected RNA was 25 nmol/L. Inhibition of GSTP was confirmed by examining GSTP messenger RNA (mRNA) and protein levels. Cells were treated 72 hours after transfection with alcohol or acrolein for 24 hours.

Cell Viability–3, (4, 5-Dimethylthiazol-2-Yl) 2, 5-Diphenyltetrazolium Bromide Assay

Cell survival/cell death was measured in treated cells by the 3, (4, 5-dimethylthiazol-2-yl) 2, 5-diphenyltetrazolium bromide assay as described.[18]