Research Article

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Aldehyde dedydrogenase-2 plays a beneficial role in ameliorating chronic alcohol-induced hepatic steatosis and inflammation through regulation of autophagy

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Background & Aims: Mitochondrial aldehyde dehydrogenase (ALDH2) plays a critical role in the detoxification of the ethanol metabolite acetaldehyde. This study was designed to examine the impact of global ALDH2 overexpression on alcohol-induced hepatic steatosis.

Methods: Wild type Friend virus B (FVB) and ALDH2 transgenic mice were placed on a 4% alcohol or control diet for 12 weeks. Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), bilirubin and cholesterol, hepatic triglyceride, steatosis, fat metabolism-related proteins, pro-inflammatory cytokines, glutathione (GSH), oxidized glutathione (GSSG), autophagy and autophagy signalling were examined. The role of autophagy was evaluated in alcohol dehydrogenase 1 (ADH1)-transfected human hepatocellular liver carcinoma cells (VA-13) treated with or without the autophagy inducer rapamycin and lysosomal inhibitors.

Results: Chronic alcohol intake led to elevated AST-, ALT-levels, bilirubin, AST/ALT ratio, cholesterol, hepatic triglycerides and hepatic fat deposition as evidenced by H&E and Oil Red O staining. Hepatic fat deposition was associated with disturbed levels of fat metabolism-related proteins (fatty acid synthase, *SCD1*), upregulated interleukin-6, TNF-α, cyclooxygenase, oxidative stress, and loss of autophagy, effects which were attenuated or ablated by the ALDH2 transgene. Moreover, ethanol (100 mM) and acetaldehyde (100 and 500 μM) increased levels of IL-6 and IFN-γ, and suppressed autophagy in VA-13 cells, effects which were markedly alleviated by rapamycin. In addition, lysosomal inhibitors mimicked ethanol-induced p62 accumulation with little additive effect with ethanol. Ethanol significantly suppressed LC3 conversion in the presence of lysosomal inhibitors.

Conclusions: In summary, our results revealed that ALDH2 plays a beneficial role in ameliorating chronic alcohol intake-induced hepatic steatosis and inflammation through regulation of autophagy.

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Introduction

Chronic alcoholism leads to the onset and progression of liver disease, including fatty liver (steatosis), hepatitis with hepatic fibrosis or cirrhosis, and hepatocellular carcinoma [1]. With chronic alcohol challenge, liver undergoes pathological events *en route* to alcoholic liver disease including steatosis characterized by excessive triglyceride deposition and steatohepatitis before alcoholic liver fibrosis is evident [2]. Earlier findings depicted a number of molecular mechanisms underlying alcohol liver injury including hepatotoxicity, oxidative stress, multiple cytokines, immune system and ethanol metabolites (e.g. acetaldehyde or lipid oxidation products) [2,3]. Acetaldehyde, the first metabolite of ethanol, is formed by oxidation of ethanol primarily through the action of alcohol dehydrogenase (ADH). Ample evidence has demonstrated that acetaldehyde serves as a major culprit, responsible for hepatic damage following chronic heavy alcohol intake, since the liver serves as the primary site of ethanol oxidation [4]. In fact, acetaldehyde has been considered as a hepatotoxin with an essential role in the onset and progression of alcoholic liver diseases through its direct cytotoxicity and pro-inflammatory responses [5,6]. Acetaldehyde is oxidized to acetic acid by aldehyde dehydrogenase (ALDH), among which mitochondrial ALDH2 is perhaps the most efficient isozyme [7–9]. Findings from our laboratory revealed that ALDH2 effectively rescued against myocardial ischemia/reperfusion injury, alcoholic cardiomyopathy and diabetic cardiomyopathy through regulation of oxidative stress, ER stress, apoptosis and autophagy [10–14]. Nonetheless, the role of ALDH2 in the aetiology of alcoholic liver disease remains largely elusive.

Keywords: Alcohol; ALDH2; Autophagy; Steatosis; Inflammation.

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Abbreviations: ACC, acetyl-CoA carboxylase; ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; ALT, alanine aminotransferase; AMPK, AMP-activated protein kinase; AST, aspartate aminotransferase; Cox, cyclooxygenase; FAS, fatty acid synthase; FVB, Friend virus B-type; IL-6, interleukin-6; IFN-γ, interferon-γ; DMEM, Dulbecco's modified Eagle's medium; SCD1, stearoyl-CoA desaturase 1; PPAR-γ, peroxisome proliferator-activated receptor-γ.

Autophagy, a vital dynamic process of degradation, participates in the lysosomal turnover of damaged, dysfunctional or harmful intracellular products and components, and serves as a catabolic energy source under nutrient deficiency. Recent findings depicted a new concept named macrolipophagy, a process through which lipid droplets are engulfed by double-membrane-bound autolipophagosome vesicles to be transported to lysosomes, where they are degraded into fatty acids [15]. This is consistent with the pivotal role of autophagy in the regulation of lipid transport, storage and metabolism. With a key role of LC3 in autolipophagosome formation and lipolysis, impaired autophagy may facilitate abnormal deposition of lipid droplets contributing to the pathogenesis of metabolic diseases [15–18]. Although ethanol and acetaldehyde have been shown to affect hepatic autophagy and lysosomal proteolysis [19], the precise role of autophagy in alcoholic injury in particular alcoholic liver diseases remain elusive. To this end, this study was designed to examine the impact of facilitated acetaldehyde metabolism through elevated ALDH2 level on chronic alcohol ingestion-induced hepatic steatosis, inflammation and lipid metabolic perturbations. To examine the role of autophagy in ethanol- and ALDH2-induced responses in hepatic lipid accumulation, the ALDH2 activator Alda-1 and the autophagy inducer rapamycin were applied in VA-13 cells (HepG2 cells that stably express murine class 1 ADH) exposed to ethanol.

Materials and methods

Generation of ALDH2 mice and chronic alcohol feeding

The animal procedures described were approved by the Institutional Animal Care and Use Committee at the University of Wyoming. ALDH2 transgenic mice were produced as described [14]. All mice were housed in a temperature-controlled room under a 12 h/12 h-light/dark cycle and were allowed access to tap water *ad libitum*. Three month-old adult female Friend virus B (FVB) and ALDH2 mice were placed on a nutritionally complete liquid diet (Shake & Pour Bio-Serv Inc., Frenchtown, NJ) for a one-week acclimation period. The use of a liquid diet is based on the scenario that ethanol self-administration results in less nutritional deficiencies and less stress to the animals in comparison to forced-feeding regimens, intravenous administration, or aerosolized inhalation. Upon completion of acclimation, half of the FVB and ALDH2 mice were maintained on the regular liquid diet (without alcohol), and the remaining half began a 16-week period of isocaloric 4% (vol/vol) alcohol diet feeding, with around 24% total calories originated from ethanol. An isocaloric pair-feeding regimen was employed to eliminate the possible nutritional deficits. Control mice were offered the same quantity of diet, which the alcohol-consuming mice drank the previous day. Body weight was monitored weekly.

Serum cholesterol, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and total bilirubin

Please refer to the online Supplementary Materials and methods section for full method description.

Measurement of blood or hepatic ethanol and acetaldehyde levels

In the morning of the last day of alcohol or control diet feeding, mice were sacrificed under anaesthesia (ketamine/xylazine: 3:1, 1.32 mg/kg, i.p.). Blood was collected and centrifuged prior to the collection of plasma in sealed 5 ml vials. Liver samples were homogenized and deproteinized with 3 M perchloric acid prior to the collection in sealed 5 ml vials. All samples were stored at −80 °C. Immediately before analysis, all vials were incubated in an oven for 15 min at 60 °C. A 2 ml aliquot of the headspace gas from each vial was removed through the septum on the cap with a gas tight syringe and transferred to a 200-μl loop injection system on an Agilent 6890 gas chromatograph (Agilent Technologies, Inc, Wilmington, DE) equipped with a flame ionization detector. Ethanol, acetaldehyde and other components were separated using a 9-meter VOCOL capillary column (Supelco Inc., Bellefonte, PA) with a film of 1.8 μm in thickness and an inner diameter of 320 μm. The temperature was held isothermally at 30 °C, and the carrier gas was helium at a flow rate of 1.8 ml/min. Under such condition, separation of acetaldehyde from ethanol and other compounds was complete within one min. Quantitation was achieved by calibrating the gas chromatographic peak areas against those from headspace samples of known ethanol and acetaldehyde standards [20,21].

Hepatic triglyceride and ATP determination

Hepatic triglyceride (TG) level was measured using a kit from Biovision (Mountain View, CA). In brief, triglycerides were dissolved by heating the samples in 5% NP-40 solution to 90 °C for 5 min followed by vortexing. This was repeated before the lysate was cleared by centrifugation. The supernatant was used for triglyceride assay according to the manufacturer's instructions [22]. Triglyceride levels were measured in triplicates using the Spectra Max 190 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). Hepatic ATP levels were measured using HPLC as described in our lab [23].

Hepatic glutathione and glutathione disulfide assay

The ratio of glutathione (GSH) to oxidized glutathione (GSSG) was used as an indicator of oxidative stress [24]. Hepatic tissues were homogenized in 4 volumes (w/v) of 1% picric acid. Acid homogenates were centrifuged at 16,000 g (30 min) and supernatant fractions were collected for the measurement of GSH and GSSG by the standard recycling method. Half of each sample was used for GSH. Samples for GSSG determination were incubated at room temperature with 2 μl 4-vinyl pyridine (4-vp) per 100 μl sample for 1 h after vigorous vortexing. Incubation with 4-vp conjugates any GSH present in the sample, so that only GSSG is recycled to GSH without potential interference by GSH. GSSG (as GSH × 2) was subtracted from total GSH to determine the actual GSH and GSH/GSSG levels [24].

Histological analysis for lipid droplet determination

The accumulation of lipid droplets was observed by H&E and Oil Red O staining. Following anaesthesia (ketamine 80 mg/kg and xylazine 12 mg/kg, i.p), mouse livers were excised and immediately placed in 10% neutral-buffered formalin at room temperature for 24 h after a brief rinse with PBS. The tissues were dehydrated through serial alcohols and cleared in xylenes. The specimen were embedded in paraffin, cut in 5 μm sections and stained with haematoxylin and eosin (H&E) [25]. For Oil Red O staining, livers were sliced and snap-frozen in isopentane-cooled liquid nitrogen prior to cutting into 10 μm sections with a cryostat. Sections were fixed with 4% paraformaldehyde and placed in absolute propylene glycol for 5 min, then stained in pre-warmed Oil Red O solution for 15 min at 60 °C followed by the differentiation with 85% propylene glycol and brief counterstaining. Sections were then mounted with aqueous VECTASHIELD mountant (Vector Laboratories Ltd, Burlingame, CA) [25]. A digital Olympus BX-51 microscope (400×) (Olympus America Inc., Melville, NY) was used to digitalize sections. Quantification of lipid droplets (average size and fraction) measured by H&E and Oil Red O staining in each group were calculated using the colour-based threshold plugin of the ImageJ (version1.43u, NIH) software [25].

Cell culture

VA-13 cells (HepG2 cells transfected with mouse ADH1) were generously provided by Dr. Dahn L. Clemens from the University of Nebraska (Omaha, NE) and were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS and 1% P/S. Cells were subcultured at appropriate intervals and maintained at subconfluent densities. During alcohol studies, cells were pretreated with or without the autophagy inducer rapamycin (5 μM, 1 h) or the mixture of lysosomal inhibitors (bafilomycin A1 [50 nM], E64D [2.5 μg/ml] and pepstatin A methyl ester [2.5 μg/ml], 1 h) prior to ethanol exposure (100 mM, 4 days) [26]. Culture medium with ethanol was replaced daily. Four days later, cells were collected for protein extraction.

Data analysis

Data are mean ± SD. Statistical significance ($p < 0.05$) for each variable was estimated by analysis of variance (ANOVA) followed by a Tukey's *post hoc* analysis.

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