Research Article

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Pharmacological promotion of autophagy alleviates steatosis and injury in alcoholic and non-alcoholic fatty liver conditions in mice

Chih-Wen Lin[1,2,3,†], Hao Zhang[4,†], Min Li[4], Xiwen Xiong[5], Xi Chen[4], Xiaoyun Chen[4], Xiaocheng C. Dong[5], Xiao-Ming Yin[1,4,6,*]

[1]*Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, PA, United States;* [2]*Division of Gastroenterology and Hepatology, Department of Medicine, E-Da Hospital/I-Shou University, Kaohsiung, Taiwan;* [3]*Graduate Institute of Medicine, School of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan;* [4]*Department of Pathology and Laboratory Medicine, Indiana University School of Medicine, Indianapolis, IN, United States;* [5]*Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, IN, United States;* [6]*Department of Medicine, Indiana University School of Medicine, Indianapolis, IN, United States*

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Background & Aims: Pharmacological approaches can potentially improve fatty liver condition in alcoholic and non-alcoholic fatty liver diseases. The salutary effects of reducing lipid synthesis or promoting lipid oxidation have been well reported, but the benefits of increasing lipid degradation have yet to be well explored. Macroautophagy is a cellular degradation process that can remove subcellular organelles including lipid droplets. We thus investigated whether pharmacological modulation of macroautophagy could be an effective approach to alleviate fatty liver condition and liver injury.

Methods: C57BL/6 mice were given ethanol via intraperitoneal injection (acute) or by a 4-week oral feeding regime (chronic), or high fat diet for 12 weeks. An autophagy enhancer, carbamazepine or rapamycin, or an autophagy inhibitor, chloroquine, was given before sacrifice. Activation of autophagy, level of hepatic steatosis, and blood levels of triglycerides, liver enzyme, glucose and insulin were measured.

Results: In both acute and chronic ethanol condition, macroautophagy was activated. Carbamazepine, as well as rapamycin, enhanced ethanol-induced macroautophagy in hepatocytes *in vitro* and *in vivo*. Hepatic steatosis and liver injury were exacerbated by chloroquine, but alleviated by carbamazepine. The protective effects of carbamazepine and rapamycin in reducing steatosis and in improving insulin sensitivity were also demonstrated in high fat diet-induced non-alcoholic fatty liver condition.

Conclusions: These findings indicate that pharmacological modulation of macroautophagy in the liver can be an effective strategy for reducing fatty liver condition and liver injury.

Keywords: Macroautophagy; Hepatic steatosis; Alcohol; High fat diet; Carbamazepine.

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* Corresponding author. Address: Department of Pathology and Laboratory Medicine, Indiana University School of Medicine, Indianapolis, IN 46202, United States. Tel.: +1 317 491 6096; fax: +1 317 274 1782.

E-mail address: xmyin@iupui.edu (X.-M. Yin).

† These authors contributed equally to this work.

Abbreviations: AFLD, alcoholic fatty liver disease; ALT, alanine aminotransferase; CBZ, carbamazepine; CQ, chloroquine; EBSS, Earle's Balanced Salt Solution; HFD, high fat diet; LD, lipid droplets; NAFLD, non-alcoholic fatty liver disease; RP, rapamycin; TG, triglycerides.

Introduction

Fatty liver is the most common early response of the liver to heavy alcohol consumption, which can render the liver more susceptible to inflammatory mediators or other toxic agents, leading to alcoholic fatty liver disease (AFLD) with the presentation of steatohepatitis, fibrosis, and cirrhosis [1–4]. Non-alcoholic fatty liver disease (NAFLD) is a condition ranging from benign lipid accumulation to steatohepatitis and cirrhosis. NAFLD may be considered the hepatic event in an overall disturbed metabolic status and is therefore linked to common metabolic syndrome risk factors such as obesity, insulin resistance, dyslipidemia, and hypertension [5–8]. Fatty liver diseases, whether caused by alcoholic or non-alcoholic factors, seem to share similar mechanisms and pathologic features [4,9]. Alcoholic and non-alcoholic steatosis can be a reversible presentation of the liver pathology and reduction of steatosis could halt or slow the progression to inflammation and fibrosis.

Although life-style changes could be most beneficial in handling AFLD or NAFLD, this may not be always practical or sufficient. Hence, it would be important to develop specific pharmacological approaches to control hepatic steatosis. Consequently, pharmacological interventions have been attempted for NAFLD [10–12]. While the mechanisms could be multiple, the main results seem to be related to an increased utilization of fatty acids and/or their efflux out of the liver. Alternative

approaches, such as that aiming at promoting lipid degradation, have not been well explored.

Macroautophagy (here referred to as autophagy) is an essential cellular degradation process with important pathophysiological significance [13]. Autophagy involves the formation of double-membraned autophagosomes, which envelop the substrates and fuse with lysosomes for degradation. Autophagy is now known to be widely involved in the pathogenesis of many human diseases and is activated under a variety of stress conditions. Autophagy actively participates in liver physiology and pathogenesis [14,15]. Autophagy can constitute an effective defense mechanism against multiple pathological insults, including alcohol and non-alcoholic fatty liver conditions [13,16–19].

Autophagy can regulate the intracellular level of lipids through its function in removing lipid droplets (lipophagy) [16]. Inhibition of autophagy in cells cultured with lipids or ethanol increases intracellular lipid content [16,19]. Loss of the autophagy capacity *in vivo* also alters fatty liver condition and liver injury induced by high fat diet or acute alcohol exposure [16,18]. Conversely, genetically enhancing autophagy by overexpressing an autophagy gene, *Atg7*, could improve hepatic steatosis and insulin resistance in *ob/ob* mice and in high fat diet (HFD)-fed mice [17]. These observations led us to postulate that autophagy modulation via pharmacological agents may offer a new strategy for treating fatty liver conditions.

While many agents could stimulate autophagy *in vitro*, few have been tested *in vivo*, particularly in mammals. Rapamycin is a well-established autophagy inducer, by inhibiting mTORC1, which controls the activation of the core autophagy machinery [20]. Rapamycin could reduce the level of Mallory-Denk bodies, one of the hallmarks of chronic ethanol intoxication, in genetically susceptible mice through autophagy degradation [21], and the level of steatosis in mice given binge alcohol drinking [18]. Another FDA-approved drug, carbamazepine (CBZ), has been used as an anti-epileptic drug, but can induce autophagy by disturbing inositol metabolism [20]. Although direct evidence lacks that CBZ can induce autophagy in the liver, its use in mice was found to promote degradation of α1-antitrypsin mutant proteins in a transgenic model [22].

We thus investigated whether pharmacological modulation of autophagy in the liver with CBZ and rapamycin could be effective for alleviating fatty liver conditions caused by an alcoholic or non-alcoholic etiology. Indeed, we found that autophagy-enhancing drugs alleviated liver steatosis, liver injury, and dyslipidemia in both alcohol-fed and HFD-fed mice. These findings indicate that autophagy-promoting drugs have important implications as a new genre of therapeutic agents for fatty livers.

Materials and methods

Reagents

Ethanol was purchased from Pharmaco, Inc. (Shelbyville, KY). Other chemicals were from Sigma, Invitrogen, or Calbiochem. Antibodies used were anti-LC3 [23], anti-β-actin (Sigma, St. Louis, MO), and horseradish peroxidase-labeled secondary antibodies (Jackson ImmunoResearch Lab., West Grove, PA).

Animal models

Wild type C57BL/6 mice and GFP-LC3 transgenic mice [24] were used in this study. All procedures were approved by the Institutional Animal Care and Use Committee. For acute ethanol treatment, mice were given ethanol intraperitoneally (33%, v/v in normal saline) at a dose of 1.2 g/kg body weight [25]. Control mice received the same volume of normal saline. For chronic ethanol treatment, mice were given the Lieber-DeCarli low fat liquid diet for 4 weeks as previously described [26]. Ethanol-containing diet was prepared with ethanol added to the liquid diet base (DYETS, #710261, Bethlehem, Philadelphia, PA), accounting for 29% or 36% of the total calorie intake (1 kcal/ml). Control diet substitutes ethanol with maltose dextrin to account for the same level of calorie requirement. In the HFD model, mice were fed with a regular chow diet (Fat 14%, Lab Diet, #5P76) or an HFD (60% kcal in fat; Research Diets, #D12492) for 12 weeks. Pharmacological modulation of autophagy is achieved by intraperitoneal administration of chloroquine (60 mg/kg), rapamycin (2 mg/kg) or CBZ (25 mg/kg) unless otherwise indicated in figure legends.

Cell culture

Murine hepatocytes were isolated and cultured in William's medium with 10% FCS as previously described [18]. For modulation of autophagy, hepatocytes were cultured in Earle's Balanced Salt Solution (EBSS) or treated with ethanol (80 mM), CBZ (50 μM), rapamycin (5 μM) or 3-methyladenine (10 mM). Autophagy was assessed as previously described [18,27] by long-lived protein degradation assay and GFP-LC3 quantification, in which cells were pre-infected with adenoviral GFP-LC3 the night before the indicated treatment.

Biochemical analyses

Hepatic and plasma triglycerides (TG) were determined using a commercial kit (Pointe Scientific, MI) [18]. Levels of blood ALT, glucose and insulin were measured using a kit from Biotron Diagnostics (Hemet, CA), an Ascensia Contour glucose meter (Bayer HealthCare, IN), and a commercial kit from Millipore (Billerica, MA), respectively. Levels of insulin resistance were determined by Homeostatic Model Assessment (HOMA = [fasting glucose (mmol/L) × fasting insulin (μU/ml)]/22.5) with a conversion factor of 1 μU/ml = 6 pmol/L for insulin measurement [28].

Immunoblotting and fluorescence microscopic analyses

Immunoblot assays with liver lysates were performed as previously described [23]. Cryosections of livers were stained with Bodipy 493/503 (0.1 μM) for 15 min for quantification of lipid droplets [18]. GFP-LC3 puncta were quantified for autophagy status. All fluorescence images were digitally acquired with a Nikon epifluorescence microscope (Nikon TE200). At least 150 cells from different fields were examined for each mouse and the number of mice used per treatment group was indicated in the figure legend.

Quantitative RT-PCR

Total RNA was extracted from livers and quantitative RT-PCR was performed as previously described [29] using cyclophilin A as a housekeeping gene control, which remains stable in the liver under different conditions.

Statistical analysis

Quantitative data (mean ± SEM) are subjected to Student's *t* test or one way ANOVA with Holm-Sidak post-hoc analysis, using SigmaStat 3.5 (Systat Software, Inc., San Jose, CA).

Results

Carbamazepine promoted autophagy in hepatocytes

CBZ has been shown to possess a pro-autophagy activity in several cell lines [20]. It also promotes an effective clearance of aggregated α1-antitrypsin mutant proteins in mouse livers [22]. In order to determine whether CBZ could be used to alleviate fatty liver condition through autophagy induction, we first examined whether CBZ could directly induce autophagy in hepatocytes.