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Kupffer cells activation promoted binge drinking-induced fatty liver by activating lipolysis in white adipose tissues

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ABSTRACT

Kupffer cells (KCs) have been suggested to play critical roles in chronic ethanol induced early liver injury, but the role of KCs in binge drinking-induced hepatic steatosis remains unclear. This study was designed to investigate the roles of KCs inhibitor ($GdCl_3$) and TNF-α antagonist (etanercept) on binge drinking-induced liver steatosis and to explore the underlying mechanisms. C57BL/6 mice were exposed to three doses of ethanol (6 g/kg body weight) to mimic binge drinking-induced fatty liver. The results showed that both $GdCl_3$ and etanercept partially but significantly alleviated binge drinking-induced increase of hepatic triglyceride (TG) level, and reduced fat droplets accumulation in mice liver. $GdCl_3$ but not etanercept significantly blocked binge drinking-induced activation of KCs. However, neither $GdCl_3$ nor etanercept could affect binge drinking-induced decrease of PPAR-α, ACOX, FAS, ACC and SCD protein levels, or increase of the LC3 II/LC3 I ratio and p62 protein level. Interestingly, both $GdCl_3$ and etanercept significantly suppressed binge drinking-induced phosphorylation of HSL in epididymal adipose tissues. Results of *in vitro* studies with cultured epididymal adipose tissues showed that TNF-α could increase the phosphorylation of HSL in adipose tissues and upgrade the secretion of free fatty acid (FFA) in the culture medium. Taken together, KCs inhibitor and TNF-α antagonist could partially attenuate binge drinking-induced liver steatosis, which might be attributed to the suppression of mobilization of white adipose tissues. These results suggest that KCs activation may promote binge drinking-induced fatty liver by TNF-α mediated activation of lipolysis in white adipose tissues.

1. Introduction

Alcoholic fatty liver (AFL) is the earliest and most common histological manifestation of alcoholic liver disease (ALD), and has been reported to occur in more than 95% of heavy drinkers (O'Shea et al., 2010). Although being considered as a benign status previously, accumulating evidences have demonstrated that AFL is a pathologic condition which can progress to more severe injuries such as hepatic necroinflammation and cirrhosis (Purohit et al., 2009). Importantly, AFL is recognized as the optimal stage for ALD intervention, as the subsequently advanced ALD are usually irreversible. Thus, it has long been the research focus to elucidate the underlying mechanisms and to explore the potential therapeutic agents for ethanol-induced steatosis.

Binge drinking is a major public health issue which can no longer be considered simply as a momentary risk factor of behavioral concerns, but must be viewed in light of its deleterious effects on liver (Mathurin and Deltenre, 2009). A binge drinking is defined as consumption of 5 and 4 drinks for men and women, respectively, in 2 h to produce a blood ethanol level over 80 mg/dl (Shukla et al., 2013). In humans, binge drinking is more common than chronic alcoholism, particularly among males in their late teens and early 20 s (Waszkiewicz et al., 2009; Waszkiewicz and Szulc, 2009). In the past several decades, considerable studies have revealed that chronic ethanol-induced liver steatosis may be attributed to many factors including the impaired activity of peroxisomal proliferator activation receptor α (PPAR-α), the induction of sterol regulatory element binding protein 1c (SREBP-1c), reactive oxygen species (ROS) and oxidative stress, cytochrome P4502E1 (CYP2E1), and the activation of Kupffer cells (KCs) (Liu, 2014; Livero and Acco, 2016; Zeng and Xie, 2009). Although acute and chronic AFL may share similar mechanisms, the deleterious effects of

Abbreviations: ACC, acyl-CoA carboxylase; ACOX, acetyl-CoA oxidase; AFL, alcoholic fatty liver; ALD, alcoholic liver disease; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AMPK, AMP-activated protein kinase; CPT-1, carnitine palmitoyltransferase 1; CYP2E1, cytochrome P4502E1; FAS, fatty acid synthase; FFA, free fatty acid; HSL, hormone-sensitive lipase; KCs, Kupffer cell; LFABP, liver fatty acid binding protein; LPS, lipopolysaccharide; MDA, malondialdehyde; NO, nitric oxide; PPAR-α, peroxisomal proliferator activation receptor α; ROS, reactive oxygen species; SREBP-1c, sterol regulatory element binding protein 1c; TG, triglyceride; TNF-α, tumor necrosis factor α

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alcohol can be affected by the doses, duration, and patterns of drinking. In contrast to the well documented deleterious effects of chronic ethanol drinking on liver, the mechanisms underpinning steatosis following acute ethanol exposure are only beginning to be understood (Kirpich et al., 2013). Considering the extraordinary rapid growth of the binge drinking worldwide, much more works are urgently needed.

KCs are the resident macrophages in liver which play key roles in host defense by removing foreign, toxic and infective substances from the portal blood, and have been demonstrated to be involved in the pathogenesis of many kinds of liver diseases (Dixon et al., 2013). Considerable evidences have demonstrated that KCs play critical roles in chronic ethanol-induced early liver injury (Smith 2013; Zeng et al., 2016). It has been demonstrated that activated KCs can provide a large amount of ROS by NADPH oxidase (Kono et al., 2001, 2000). Furthermore, M1 polarized KCs stimulated by gut-sourced lipopolysaccharide (LPS) can secrete a series of proinflammatory cytokines including tumor necrosis factor α (TNF-α) (Zeng et al., 2016). Previous studies have revealed that TNF-α could directly induce hepatic steatosis, which might be related with the upregulation of SREBP-1c and deregulation of PPAR-α (Beier et al., 1997; Endo et al., 2007; Lawler et al., 1998). However, the role of KCs in the pathogenesis of binge drinking-induced fatty liver remains to be investigated.

The current study was designed to evaluate the effects of KCs inhibitor ($GdCl_3$) and TNF-α antagonist (etanercept, a genetically engineered, soluble, systemic TNF-α receptor fusion protein) on binge drinking-induced liver steatosis and to explore the underlying mechanisms.

2. Materials and methods

2.1. Materials

Ethanol was purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). $GdCl_3$, Oil Red O, and primary antibodies against LC3, p62, and β-actin were bought from Sigma (St. Louis, MO, USA). Etanercept was purchased from Shanghai CP Guojian Pharmaceutical Co.Ltd (Shanghai, China). Primary antibodies against PPAR-α, SREBP-1c, and fatty acid synthase (FAS) were purchased from Santa Cruz (CA, USA). Primary antibodies against AMP-activated protein kinase α (AMPK-α), *p*-AMPKα, acyl-CoA carboxylase (ACC), hormone-sensitive lipase (HSL), *p*-HSL^{ser660} and *p*-HSL^{ser563} were provided by Cell Signaling Technology Inc. (Beverly, MA, USA). Rabbit acyl-CoA oxidase (ACOX) antibody and liver fatty acid-binding protein (LFABP) antibody were bought from Abcam (Cambridge, UK). Rabbit F4/80 polyclonal antibody and carnitine palmitoyltransferase 1 (CPT-1) antibody were provided by Proteintech Group (Chicago, USA). Tissue triglyceride (TG) assay kit was purchased from Applygen Technologies Inc. (Beijing, China). Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) assay kits were bought from Ningbo Medical System Biotechnology Co., Ltd (Ningbo, China). Malondialdehyde (MDA), nitric oxide (NO) and free fatty acid (FFA) assay kits were supplied by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Recombinant human TNF-a protein was bought from R & D systems (Minneapolis, MN, USA). All other reagents were purchased from Sigma, unless indicated otherwise.

2.2. Animals and treatment

Male C57BL/6 mice (8 weeks) were provided by Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China). The animals received standard chow and tap water, and were maintained in a temperature-controlled environment (20–22 °C) with a 12-h light: 12-h dark cycle and 50%-55% humidity. After acclimation for 7 days, the mice were randomly divided into 5 groups, i.e. Control group, Ethanol group, $GdCl_3$/ethanol group, and Etanercept/ethanol group. All mice in ethanol-exposed groups were treated with three doses of ethanol (6 g/kg bw, by gavage) with a 12-h intervals, while control group mice received isocaloric/isovolumetric maltodextrin (Abdelmegeed et al., 2013). Mice in $GdCl_3$/ethanol group were pretreated with $GdCl_3$ (10 mg/kg bw, i.p.) at 24 h before the first dose of ethanol and after the first and third doses of ethanol, while the etanercept (1 mg/kg bw, i.p.) was administered to mice immediately after each of the three ethanol doses. The animals were sacrificed at 6 h after the last dose of ethanol or maltodextrin. The blood, liver tissues, and epididymal adipose tissues were collected. The use of animals was approved by the Ethics Committee of Shandong University Institute of Preventive Medicine.

2.3. Liver histopathological examination

Liver fat accumulation was evaluated by using H & E staining and Oil Red O staining as we previously reported (Chen et al., 2014; Zeng et al., 2012). Briefly, paraffin sections (5 μm) were prepared from 10% neutral formalin-fixed liver tissues, and subjected to hematoxylin and eosin (H & E) staining. For the Oil Red O staining, frozen sections (10 μm) were stained in Oil Red O dye for 10 min, and then counterstained with hematoxylin for 30 s. The sections were viewed and the representative photographs were captured using a Nikon microscope (Nikon, Melville, NY, USA).

2.4. Determination of hepatic TG, MDA and NO contents

Hepatic TG content was detected based on the optimized GPO Trinder reaction by using a commercial kit bought from Applygen Technologies Inc. (Beijing, China). Hepatic MDA was measured based on the reaction of thiobarbituric acid (TBA) with MDA, which could form pink product. The content of hepatic NO was detected using commercial assay kit from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.5. Real-time PCR analysis

Total RNA was isolated from frozen mice liver using RNAiso Plus (Takara, Japan). The concentration of RNA samples was measured by NanoDrop 2000c. 1 μg of total RNA was firstly incubated with gDNA eraser to eliminate genomic DNA, and then reverse-transcribed into complementary DNA (cDNA) at 37 °C for 15 min and then 85 °C for 5 s according to the manufacturer's protocol (PrimeScript™RT reagent kit, TakaRa). Real-time quantitative PCR was performed using TaKaRa SYBR Premix EX Taq™ to quantify the mRNA levels of PPAR-α, ACOX, LFABP, SREBP-1c, FAS, ACC and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primers were synthesized by Sangon Biotech Co., Ltd (Shanghai, China), and sequences of the primers were listed in Table 1. The PCR amplification reactions were performed using Roche LightCycler 480 Instrument (Roche, Germany).

2.6. Western blot analysis

Total protein was extracted from mouse liver and adipose tissues using RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 1 mM

Table 1
Gene-specific primers used in quantitative real-time PCR.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
PPAR-α	ATGCCAGTACTGCCGTTTTC	GGCCTTGACCTTGTTCATGT
ACOX-1	TCCAGACTTCCAACATGAGGA	CTGGGCGTAGGTGCCAATTA
CPT-1	TGAGTGGCGTCCTCTTTGG	CAGCGAGTAGCGCATAGTCATG
SREBP-1c	CTTTGGCCTGCCTTTTCGG	TGGGTCCAATTAGAGCCATCTC
FAS	TCCTGGAACGAGAACACGATCT	GAGACGTGTCACTCCTGGACTTG
ACC	GCCATTGGTATTGGGGCTTAC	CCCGACCAAGGACTTTGTTG
GADPH	GCATGGCCTTCCGTGTTCC	GGGTGGTCCAGGGTTTCTTACTC