Research Article

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Synergistic steatohepatitis by moderate obesity and alcohol in mice despite increased adiponectin and p-AMPK

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Background & Aims: Mechanisms underlying synergistic liver injury caused by alcohol and obesity are not clear. We have produced a mouse model of synergistic steatohepatitis by recapitulating the natural history of the synergism seen in patients for mechanistic studies.

Methods: Moderate obesity was induced in mice by 170% overnutrition in calories using intragastric overfeeding of high fat diet. Alcohol (low or high dose) was then co-administrated to determine its effects.

Results: Moderate obesity plus alcohol intake causes synergistic steatohepatitis in an alcohol dose-dependent manner. A heightened synergism is observed when a high alcohol dose (32 g/kg/d) is used, resulting in plasma ALT reaching 392 ± 28 U/L, severe steatohepatitis with pericellular fibrosis, marked M1 macrophage activation, a 40-fold induction of *iNos*, and intensified nitrosative stress in the liver. Hepatic expression of genes for mitochondrial biogenesis and metabolism are significantly downregulated, and hepatic ATP level is decreased. Synergistic ER stress evident by elevated XBP-1, GRP78 and CHOP is accompanied by hyperhomocysteinemia. Despite increased caspase 3/7 cleavage, their activities are decreased in a redox-dependent manner. Neither increased PARP cleavage nor TUNEL positive hepatocytes are found, suggesting a shift of apoptosis to necrosis. Surprisingly, the synergism mice have increased plasma adiponectin and hepatic p-AMPK, but adiponectin resistance is shown downstream of p-AMPK.

Conclusions: Nitrosative stress mediated by M1 macrophage activation, adiponectin resistance, and accentuated ER and mitochondrial stress underlie potential mechanisms for synergistic steatohepatitis caused by moderate obesity and alcohol.

Keywords: Synergistic steatohepatitis; Obesity and alcohol synergism; Apoptosis; Necrosis; Macrophage activation; Nitrosative stress.

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Abbreviations: ALD, alcoholic liver disease; NAFLD, non-alcoholic fatty liver disease; ALT, alanine aminotransferase; AdipoR, adiponectin receptor; AMPK, AMP activated protein kinase; (n)SREBP-1, (nuclear) sterol regulatory element-binding protein-1; Acc, acetyl-CoA carboxylase; TNFα, tumor necrosis factor alpha; GRP78, glucose-regulated protein 78; NO, nitric oxide; JNK, c-Jun N-terminal kinase; DTT, dithiothreitol; eIF2α, eukaryotic initiation factor 2α; ERK, extracellular signal-regulated kinase; PARP, cleaved poly (ADP-ribose) polymerase; WAT, white adipose tissue; iNOS, inducible nitric oxide synthase; Arg1, arginase 1; NT, nitrotyrosine.

Introduction

Alcoholic liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD) are the most common life-style liver diseases caused by chronic alcohol consumption and obesity, respectively [1]. Epidemiological evidence shows that obese alcoholics have an accentuated rise in serum alanine aminotransferase (ALT) [2,3] and 2–3 times higher risk of developing steatohepatitis or cirrhosis [4,5] as compared to non-obese alcoholics or obese non-alcoholics. Alcohol consumption also synergistically increases the risk of hepatocellular carcinoma in obese diabetic patients [6]. These studies demonstrate synergistic effects of alcohol and obesity in liver injury and warrant further mechanistic studies.

In rodents, impaired adiponectin production and signaling are implicated in liver pathology of ALD and NAFLD [7,8]. Plasma adiponectin levels are inversely correlated with the disease stages of steatosis and steatohepatitis [7,8], and administration of adiponectin improves hepatic steatosis, necroinflammation, and fibrosis [9,10]. However, normal or increased plasma adiponectin has also been reported in NAFLD mouse models [11]. In humans, plasma adiponectin is reduced in NAFLD patients [8], but increased in ALD patients [12,13]. Thus, the role of adiponectin in the obesity-alcohol synergy in liver injury needs further elucidation.

Adiponectin, through its receptors (AdipoR1/2), activates AMP activated protein kinase (AMPK), which in turn inhibits lipogenesis through sterol regulatory element-binding protein-1 (SREBP-1) and acetyl-CoA carboxylase (ACC) [7,8]. In conditions of ALD and NAFLD, SREBP-1 can be induced by tumor necrosis factor-α (TNF-α) [14] and increased endoplasmic reticulum (ER) stress [15,16],

leading to enhanced hepatic lipogenesis. In intragastrically alcohol-fed mice, betaine supplement alleviates homocysteine-induced ER stress and ER stress-associated activation of SREBP-1, and reduces hepatic lipid contents [17]. In mice, overexpression of glucose-regulated protein 78 (GRP78) inhibits ER stress-induced SREBP-1c activation and reduces hepatic steatosis [15]. Collectively, these studies suggest SREBP-1 intersects multiple intracellular events associated with pathogenesis of ALD and NAFLD.

Excessive production of reactive oxygen species (ROS) and nitric oxide (NO) is also implicated in the pathogenesis of ALD and NAFLD [18]. ROS and lipid peroxidation products cause mitochondrial DNA damage, impaired mitochondrial function, ATP depletion, and necrosis. ROS signals to induce TNF-α and activate c-Jun N-terminal kinase 1 (JNK1), both of which are implicated in hepatocellular apoptosis and development of steatohepatitis [19,20]. NO causes mitochondrial disruption, calcium efflux, and upregulation of Grp78, suggesting coupling of NO-induced mitochondrial dysfunction to ER stress response [21]. In adipocytes, impaired mitochondrial function leads to increased ER stress, JNK activation, and decreased adiponectin expression [22]. These results support an emerging concept that mitochondria-ER interaction is not only an integral component of cellular homeostatic response to stress but may also be a participant in pathological processes [16,18].

Previously, we created NAFLD [23] and ALD [24] mouse models, which exhibited histological resemblance of liver injury in human, using intragastric feeding of high fat diet and alcohol, respectively. In the present study, we used intragastric co-feeding of high fat diet and alcohol in mice to study obesity-alcohol synergism in liver injury and explored the underlying mechanisms. We found that synergistic steatohepatitis caused by moderate obesity and alcohol was associated with increased plasma adiponectin and hepatic AMPK activation. However, defective signaling downstream of AMPK was identified. Furthermore, heightened ER stress and suppression of genes involved in mitochondrial functions were associated with conspicuous M1 *iNos* induction and nitrosative stress in the liver.

Materials and methods

Animals, overfeeding, and alcohol infusion

Age matched (8 weeks of age) male C57BL/6j mice were purchased from Jackson Laboratories (Bar Harbor, ME). All animals were treated in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. The surgical procedure for intragastric catheter placement was performed as previously described [23,24]. Moderate obesity was induced by intragastric overfeeding of high fat diet, and synergistic liver injury in these mice was induced by co-administration of alcohol. Detailed methods for generation of this mouse model are described in Supplementary materials.

Blood biochemistry

Plasma ALT and adiponectin concentrations were measured by ELISA kits (Sigma Diagnostic, MO; Alpoco Diagnostics, NH), and blood alcohol levels were measured by an ANALOX GM7 metabolite analyzer (Analox Instruments USA, MA) following manufacturers' protocols.

Morphological evaluation

Paraffin-embedded sections of liver or WAT were stained with hematoxylin and eosin to determine the histological score for hepatic steatosis, inflammation, and fibrosis (based on reticulin staining), and individually scored to derive the total pathological score [25]. Macrophages in the liver and WAT were evaluated by immunostaining with anti-CD68 antibody (Dako, Carpinteria, CA). Hepatic nitrosative stress was evaluated by immunofluoresence using anti-nitrotyrosine antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and fluorescein isothiocyanate labeled secondary antibody.

Hepatic caspase activity assay

Liver protein extraction was performed as previously described [26]. Hepatic caspase 3/7 activities were determined using a commercial Caspase-Glo 3/7 Assay kit (Promega). To assess caspase inactivation by nitrosative stress, hepatic protein lysates were preincubated with or without 5 mM dithiothreitol (DTT) [27] and the activity of caspases 3/7 was then determined. For negative and positive controls of hepatic apoptosis, liver proteins from mice injected i.p. with PBS and 10 μg/mouse anti-Fas Jo2 antibody were used, respectively.

Hepatic ATP, triglyceride, and malondialdehyde measurements

Hepatic ATP was determined as previously described [28] using a commercial kit (Molecular Probes). Hepatic triglyceride was measured using commercial reagents (Wako Chemical, Richmond VA), and hepatic malondialdehyde was determined as described [25].

Immunoblotting and qRT-PCR

Liver protein extracts in RIPA buffer were resolved on a 10% SDS–PAGE for immunoblot analysis with the primary antibodies listed in Supplementary materials. RNA extracted from liver and WAT were subjected to SYBR Green (Applied Biosystems, CA) qRT-PCR analysis. The mRNA levels are relative to *Gapdh* and normalized to Control group. The primer sequences are listed in Supplementary Table 1.

Data analysis

Numerical data were expressed as mean ± SD. Student's *t* test was performed and *p* values less than 0.05 were considered statistically significant.

Results

Synergistic steatohepatitis induced by alcohol and moderate obesity

Feeding regimen for the synergistic mouse model is shown in Fig. 1A and detailed in Supplementary materials. On average, overfed mice reached 28% heavier body weight (moderate obesity) than regular-fed controls before starting alcohol infusion. At the end of the experiments, the overfed mice with or without alcohol feeding remained moderately obese, being ~34.6 ± 9.4% heavier than the controls (Table 1). Overfeeding also caused an increase in liver weight, but not in liver/body weight ratio and hepatic triglyceride contents. Low alcohol dose (23 g/kg/d) did not, but high alcohol dose [32 g/kg/d) did cause significant increases in these three parameters as compared to controls. However, when either dose of alcohol was given to the overfed obese mice, it caused further and/or synergistic increases in liver weight, liver/body weight ratio, and hepatic triglyceride contents as compared to overfeeding or alcohol infusion (Table 1). These results, along with liver histology, (Fig. 1C, H&E staining) clearly showed that overfeeding plus alcohol synergistically aggravate the mild to moderate steatosis induced by overfeeding or alcohol feeding alone. Importantly, the blood alcohol levels in mice receiving either dose of alcohol were comparable between the respective alcohol *vs.* overfeeding plus alcohol groups (Table 1), which validated the experimental protocol for the obesity and alcohol synergism in our model.

Giving alcohol at either dose to overfed obese mice caused a synergistic increase in plasma ALT levels, with a more intensified