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Chronic Alcohol Consumption Causes Liver Injury in High-Fructose-Fed Male Mice Through Enhanced Hepatic Inflammatory Response

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Abstract

Background—Obesity and the metabolic syndrome occur in approximately one-third of patients with alcoholic liver disease (ALD). The increased consumption of fructose parallels the increased prevalence of obesity and the metabolic syndrome in the United States and worldwide. In this study, we investigated whether dietary high fructose potentiates chronic alcohol-induced liver injury, and explored potential mechanism(s).

Methods—Six-week-old male C57BL/6J mice were assigned to 4 groups: control, high fructose, chronic ethanol (EtOH), and high fructose plus chronic alcohol. The mice were fed either control diet or high-fructose diet (60%, w/w) for 18 weeks. Chronic alcohol-fed mice were given 20% (v/v) ethanol (Meadows-Cook model) ad libitum as the only available liquid from the 9th week through the 18th week. Liver injury, steatosis, hepatic inflammatory gene expression, and copper status were assessed.

Results—High-fructose diet and chronic alcohol consumption alone each induce hepatic fat accumulation and impair copper status. However, the combination of dietary high fructose plus chronic alcohol synergistically induced liver injury as evidenced by robustly increased plasma alanine aminotransferase and aspartate aminotransferase, but the combination did not exacerbate hepatic fat accumulation nor worsen copper status. Moreover, FE-fed mice were characterized by prominent microvesicular steatosis. High-fructose diet and chronic alcohol ingestion together led to a significant up-regulation of Kupffer cell (KC) M1 phenotype gene expression (e.g., tumor necrosis factor- α and monocyte chemoattractant protein-1), as well as Toll-like receptor 4 (TLR4)

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Diet components. Teklad Custom Research Diet Data Sheets TD.10781 and TD.10782.

signaling gene expression, which is also associated with the up-regulation of KCs and activation marker gene expression, including Emr1, CD68, and CD163.

Conclusions—Our data suggest that dietary high fructose may potentiate chronic alcohol consumption-induced liver injury. The underlying mechanism might be due to the synergistic effect of dietary high fructose and alcohol on the activation of the TLR4 signaling pathway, which in turn leads to KC activation and phenotype switch toward M1 polarization. This study suggests that alcohol–fructose combination contributes to ALD progression.

Keywords

Alcohol; Fructose; Kupffer Cell Activation; M1 Polarization; Nonalcoholic Fatty Liver Disease

Alcoholic liver disease (ALD) remains a major cause of chronic liver disease and liver failure. Although more than 90% of alcoholics have fatty liver, only 20 to 40% develop more severe forms of ALD, including alcoholic hepatitis, fibrosis, and cirrhosis. This suggests that other factors may contribute to the disease progression. The factors identified to date include gender, obesity, genetics, drinking pattern, smoking, dietary factors, hepatitis B/C virus infection, HIV, etc. (Gao and Bataller, 2011; Tsukamoto et al., 2009). In addition, the prevalence of obesity and the metabolic syndrome in patients with ALD is 44.5 and 32.4%, respectively, based on the NHANES III data (Stepanova et al., 2010). Moreover, a clinical study showed that alcoholism and obesity synergistically increased the risk of liver injury, and being overweight for at least 10 years in patients with ALD is a risk factor for more severe liver damage (Naveau et al., 1997). Recent studies on estimating the presence of liver fibrosis in the general population showed that nonalcoholic fatty liver disease (NAFLD) and ALD together account for more than 88% of cases (Roulot et al., 2011), and these 2 types of fatty liver disease may coexist in many patients. Recently, the term, “Syndrome of metabolic and alcoholic steatohepatitis,” was used to describe this combined ALD and NAFLD phenotype caused by the interactions between alcohol and overnutrition (Carr and Correnti, 2015).

The typical Western-style diet includes consumption of high-fat and high-fructose corn syrup (HFCS)-sweetened beverages (Odermatt, 2011). Diets high in fat have long been known to promote alcoholic liver injury (Naveau et al., 1997; Xu et al., 2011). HFCS consumption raises another important nutritional issue, because the increased consumption of fructose parallels the increased prevalence of obesity and the metabolic syndrome in the United States and worldwide (Hallfrisch, 1990; Spruss and Bergheim, 2009). Moreover, emerging evidence has shown that high-fructose intake may play an important role in the development of NAFLD (Assy et al., 2008; Ouyang et al., 2008; Spruss et al., 2009). The mechanism(s) by which dietary high fructose induces NAFLD may involve at least 2 mechanisms: fructose absorption from the duodenum may impair copper absorption leading to increased de novo lipogenesis (Song et al., 2012); and/or the limited absorptive capacity of the duodenum for fructose (Fujisawa et al., 1991; Riby et al., 1993) may allow extra fructose to pass to the distal intestine, thereby leading to bacterial overgrowth, intestinal barrier dysfunction, and subsequent Kupffer cell (KC) activation via Toll-like receptor 4 (TLR4) signaling pathway (Spruss and Bergheim, 2009; Spruss et al., 2009).

The central role of oxidative stress has been well established in the pathogenesis of ALD. Both an increase in reactive oxygen species and a decrease in antioxidant defenses will lead to oxidative stress (Arteel, 2003; Wu and Cederbaum, 2009). Previous research also showed that ethanol (EtOH) has an effect similar to fructose in exacerbating the clinical signs associated with copper deficiency (Fields et al., 1994). In addition, disruption of intestinal barrier function has been well documented in patients with ALD as well as in animal studies (Bjarnason et al., 1984; Parlesak et al., 2000; Zhong et al., 2010).

Both fructose and alcohol are metabolized in the liver, and both may lead to alterations of gut microbiota and permeability. We hypothesize that these 2 factors together may exacerbate liver injury and/or fat accumulation and promote disease progression in a mouse model of ALD.

MATERIALS AND METHODS

Animals

Six-week-old male C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). On arrival, the animals were group housed in a temperature- and humidity-controlled room with a 12:12-hour light–dark cycle and were acclimated for 1 week on a standard pelleted rodent chow diet. They were then randomly assigned to 4 groups: control (C), high-fructose diet (F), chronic EtOH (CE), and high-fructose diet plus chronic EtOH (FE). Mice in the high-fructose diet group or control group were fed (ad libitum) either high-fructose diet (60%) (TD.10781) or control diet (TD.10782, corn starch and maltodextrin) (both are pelleted diets from the Harlan Laboratories, Madison, WI) for 18 weeks. The diet components include protein 20.2% Kcal, carbohydrate 66.96% Kcal, and fat 12.9% Kcal. The detailed information for diet components is included in Supporting Information for review and online publication. For animals on the EtOH diet, deionized water containing EtOH was given ad libitum after 8 weeks of high-fructose diet feeding or control diet feeding. Otherwise, deionized water was the source of liquid for the animals. The Meadows–Cook model was used for chronic alcohol administration. Briefly, mice in the EtOH (CE and FE) groups were given 10% (v/v) EtOH for 2 days, followed by 5 days of 15% (v/v) EtOH. Then, mice were placed on 20% (v/v) EtOH and maintained at this concentration until the end of the experiment (Blank et al., 1991; Meyerholz et al., 2008; Song et al., 2002) (Fig. 1). An adequate amount of copper (6 ppm) was present in both the control and high-fructose diets. After fasting overnight, all the animals were sacrificed under anesthesia at the end of the experiment. Blood was collected from the inferior vena cava, and citrated plasma was stored at –80°C for further analysis. Portions of liver tissue were fixed with 10% formalin for sectioning, while others were snap-frozen with liquid nitrogen. Alcohol-enriched drinking water and regular drinking water were changed once a week. All studies were approved by the University of Louisville Institutional Animal Care and Use Committee, which is certified by the American Association of Accreditation of Laboratory Animal Care.

Assessment of Copper Status

Plasma ceruloplasmin was measured on the basis of its oxidase activity (Schosinsky et al., 1974). Plasma copper and liver copper were measured by Varian SpectrAA 880/GTA-100

graphite furnace atomic absorption spectrometer (Worcester Polytechnic Institute, Worcester, MA).

Plasma Biochemical Assays

The plasma biochemical assays were performed with commercially available kits: alanine aminotransferase (ALT), aspartate aminotransferase (AST), glucose, triglyceride, cholesterol (Infinity; Thermo Electron, Melbourne, Vic., Australia), nonesterified fatty acids (NEFAs) (Waco Chemicals, Richmond, VA), and insulin (Linco Research, St. Charles, MO).

Histology

Formalin-fixed, paraffin-embedded liver sections were cut at 5 μ m thickness and stained with hematoxylin and eosin (H&E). Cryostat sections of liver tissue were stained with Oil Red O (Sigma-Aldrich, St. Louis, MO).

Assessment of Micro- Versus Macrovesicular Steatosis

Microvesicular steatosis was defined as multiple lipid droplets smaller than the central nucleus. Macrovesicular steatosis was defined as a single lipid droplet that is larger than the nucleus and usually displaces the nucleus to the periphery of the cell. The number of hepatocytes with fat accumulation in the fat area was counted in 8 randomly selected high-power microscopic fields ($\times 400$) per mouse, and 5 mice in each group. The average percent of hepatocytes with micro- and macrovesicular steatosis were compared between groups (Lee et al., 2013; Oleszczuk et al., 2007).

Hepatic Triglyceride Assay

Liver tissues were homogenized in ice-cold phosphate-buffered saline. Hepatic total lipids were extracted with chloroform/methanol (2:1) according to the method described by Bligh and Dyer (1959). Hepatic triglyceride content was determined by commercially available kits (Infinity; Thermo Electron).

Isolation of RNA and Real-Time Reverse Transcription Polymerase Chain Reaction

Total RNA was extracted from liver tissues using TRIZOL (Invitrogen, Carlsbad, CA). Primers were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) (Table 1). Real-time polymerase chain reaction (PCR) was performed with an ABI prism 7500 sequence detection system and SYBR green master mix (Life Technologies, Grand Island, NY). The relative gene expression was analyzed using 2^{-C_t} method.

Western Blot

Equal amounts of protein extracted from the liver homogenate were loaded on 4 to 15% SDS-polyacrylamide gels and transferred to PVDF membrane (Millipore, Bedford, MA). Membranes were probed with primary antibody for cytochrome P450 2E1 (CYP2E1; catalog number: ab28146; 1:1,000 dilution; Abcam, Cambridge, MA), and fructokinase/ketohexokinase (KHK; catalog number: sc-366024; 1:500 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), F4/80 (catalog number: sc-25830; 1:500 dilution;

Santa Cruz Biotechnology, Inc.), interferon regulatory factor 3 (IRF3; catalog number: #4302; 1:1,000 dilution; Cell Signaling Technology, Inc., Danvers, MA), interferon regulatory factor 7 (IRF7; catalog number: sc-9083; 1:500 dilution; Santa Cruz Biotechnology, Inc.), tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6; catalog number: sc-7221; 1:500 dilution; Santa Cruz Biotechnology, Inc.), or GAPDH (catalog number: sc-25778; 1:1,000 dilution; Santa Cruz Biotechnology, Inc.) and then incubated with the corresponding horseradish peroxidase-conjugated secondary antibody. Protein bands were visualized using the enhanced chemiluminescence system (Amersham Biosciences, Little Chalfont, UK). Band intensities were quantified using NIH ImageJ software (<http://rsb.info.nih.gov/ij/>).

Plasma Endotoxin and Lipopolysaccharide-Binding Protein Assays

Plasma samples were diluted 5-fold in pyrogen-free water and heated at 75°C for 10 minutes and cooled down to room temperature. Endotoxin was measured using the limulus amoebocyte lysate kit (Lonza, Walkersville, MD) according to the manufacturer's instructions. Plasma lipopolysaccharide-binding protein (LBP) was measured using ELISA kit (Cell Sciences, Canton, MA).

Statistical Analysis

All data were expressed as mean $\pm$ SD (standard deviation) and analyzed by analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison test. The interactions between EtOH and fructose were examined by 2-way ANOVA. Differences at $p < 0.05$ were considered to be statistically significant.

RESULTS

Effects of High-Fructose Diet and Chronic Alcohol Consumption on Body Weight, Liver Weight, Epididymal Fat Weight, and Blood Metabolites

As shown in Table 2, neither high-fructose diet nor chronic alcohol consumption alone led to significant differences on body weight (BW) or liver weight. While liver weight and liver/BW ratio were significantly higher in response to combined high-fructose diet and chronic alcohol consumption, the BW was not changed significantly compared to controls (Table 2). The food consumption was significantly higher in high-fructose-diet-fed mice as compared to controls. However, food consumption was significantly lower in both EtOH- and EtOH-plus-high-fructose-diet-fed animals compared to their controls. The high-fructose diet caused a significantly higher epididymal fat weight (EFW) as well as EFW/BW ratio compared to controls, and these changes were negated by chronic alcohol consumption. Chronic alcohol intake alone did not lead to a significant change in EFW compared to controls. Plasma triglycerides were significantly elevated in the chronic alcohol-consuming mice compared to those without alcohol intake. However, there were no significant differences between control and high-fructose-diet-fed mice with or without alcohol. Plasma cholesterol levels were lower in the mice with chronic alcohol intake compared to controls. Chronic alcohol consumption alone led to markedly elevated plasma NEFAs compared to mice fed a high-fructose diet or those fed chronic alcohol plus high fructose. Plasma glucose levels were markedly elevated in the mice fed a high-fructose diet compared to controls.

Importantly, plasma glucose levels were significantly lower in both EtOH- and EtOH-plus-high-fructose-diet-fed animals compared to their controls. Plasma insulin levels were markedly elevated in high-fructose-diet-fed mice and the chronic alcohol mice alone compared to controls. However, high-fructose diet and chronic alcohol intake together did not increase the plasma insulin level. HOMA-IR value was significantly higher in high-fructose-diet-fed mice compared to controls, whereas it was markedly lower in both alcohol- and high-fructose-diet-plus-alcohol-fed mice.

High-Fructose Diet and Chronic Alcohol Consumption Synergistically Induce Liver Injury

Liver injury was assessed by plasma levels of liver enzymes (ALT and AST). Neither plasma ALT nor plasma AST levels were significantly changed by high-fructose diet or chronic alcohol consumption alone. However, plasma ALT and AST levels were significantly higher in mice fed chronic alcohol plus high fructose compared to controls (Fig. 2).

Effects of High-Fructose Diet and Chronic Alcohol Consumption on Hepatic Lipid Accumulation

Hepatic triglyceride levels were significantly higher than controls in both chronic alcohol consumption and high-fructose-diet-fed mice. However, there was no further increase in the hepatic fat accumulation with the combination of high-fructose-diet plus chronic alcohol intake (Fig. 3B). Those changes are shown by H&E and Oil Red O staining (Fig. 3A). Quantification of the percentage of hepatocytes with micro- versus macrovesicular steatosis showed that chronic alcohol-fed mice had a higher percentage of microvesicular steatosis and less macrovesicular steatosis compared to controls and to high-fructose-fed mice (Fig. 3C). In particular, the hepatic phenotype of alcohol plus fructose mice was characterized by predominantly microvesicular steatosis and minimal macrovesicular steatosis with scattered foci of inflammatory cell infiltration.

Effects of Dietary High Fructose and Chronic Alcohol Consumption on Copper Status

Plasma ceruloplasmin activity and plasma copper were significantly lower in the mice fed with either high-fructose diet or alcohol. High-fructose diet plus alcohol together also markedly inhibited plasma ceruloplasmin activity. However, no additive or synergistic effect between fructose and EtOH was observed. While liver copper levels were significantly lower in both chronic alcohol consumption and alcoholplus-high-fructose-diet-fed mice, liver copper levels as well as plasma copper levels were significantly higher in alcoholplus-high-fructose-diet-fed mice compared to chronic alcohol intake alone mice (Fig. 4).

Liver Injury Induced by High-Fructose Diet and Chronic Alcohol Consumption Is Independent of Either Alcohol or Fructose Metabolism in the Liver

Hepatic expression of CYP2E1, a major alcohol-metabolizing enzyme during chronic consumption, was significantly up-regulated by chronic alcohol feeding. However, the combination of fructose and alcohol did not further up-regulate the hepatic CYP2E1 expression compared to alcohol alone (Fig. 5A). Similarly, hepatic expression of KHK, the first enzyme in fructose metabolism, was markedly higher in high-fructose-diet-fed mice, whereas its expression was not further enhanced by high-fructose-plus-chronic-alcohol-fed

mice compared to only high-fructose-diet-fed mice (Fig. 5B). Taken together, our data suggest that liver injury induced by high-fructose diet plus chronic alcohol consumption is unlikely due to accelerated metabolism of either fructose or alcohol.

High-Fructose Diet and Chronic Alcohol Consumption Lead to KC Activation and Phenotype Switch Toward M1 Polarization

The expression of hepatic KC marker gene, *Emr1* (F4/80) mRNA (Louvet et al., 2011; Wan et al., 2014), was significantly higher in the fructose-plus-alcohol-fed mice than in the controls, suggesting expansion of the total KC number (Fig. 6A). Hepatic F4/80 protein expression was also significantly higher in alcohol- or fructose-fed mice compared to controls and was the highest in alcohol-plus-fructose-fed mice (Fig. 6B). Moreover, hepatic expression of KC activation markers, CD68 (M1 phenotype) and CD163 (M2 phenotype) mRNAs (Louvet et al., 2011; Wan et al., 2014), was significantly up-regulated by chronic alcohol consumption. While CD68 mRNA expression was further significantly up-regulated by the combination of fructose and alcohol, CD163 mRNA expression was only slightly up-regulated compared to the mice fed alcohol alone, and the difference did not reach the statistical significance, suggesting M1 polarization is the predominant phenotype of KC activation (Fig. 6A). Hepatic mRNA expression of TNF- α and monocyte chemoattractant protein-1 (MCP-1) was significantly up-regulated in high-fructose diet plus alcohol-fed animals compared with mice fed alcohol alone, whereas the expression of interleukin-10 (IL-10) and arginase 1 mRNAs was not increased. On the contrary, they tended to be down-regulated although the differences did not reach the statistical significance or were borderline significant ($p = 0.0557$, arginase 1) (Fig. 6C). IL-1 β mRNA expression was significantly up-regulated in both EtOH- and high-fructose-plus-EtOH-fed animals compared to the controls; however, there was no statistical difference between EtOH and alcohol plus high fructose-fed mice. IL-6 mRNA expression tended to be up-regulated in EtOH- and fructose-plus-EtOH-fed animals, but the difference did not reach statistical significance (Fig. 6C). These data further support the concept that M1, a pro-inflammatory phenotype of KC, is predominant in the high-fructose-plus-alcohol-induced KC activation (Louvet et al., 2011; Wan et al., 2014).

Liver Injury Induced by High-Fructose Diet Plus Chronic Alcohol Consumption Might Involve Both MyD88-Dependent and MyD88-Independent TLR4 Signaling Pathways

Although the TLR4 signaling pathway is critical in KC activation and liver injury in both ALD and NAFLD (Wan et al., 2014), the downstream pathways initiated by TLR4 are different, that is, MyD88-dependent and MyD88-independent pathways (Hritz et al., 2008; Spruss et al., 2009). To further explore the molecular mechanisms underlying KC activation, we examined mRNA expression of molecular markers in these 2 signaling pathways. The hepatic expression of TLR4 was significantly up-regulated by high-fructose diet plus chronic alcohol feeding compared to controls. Moreover, hepatic expression of MyD88 as well as IRF3, a marker of the Myd88-independent pathway (Hritz et al., 2008; Petrasek et al., 2011), was significantly up-regulated by chronic alcohol feeding and further increased by the combination of high-fructose diet plus chronic alcohol consumption (Fig. 7A). IRF3 protein was also significantly up-regulated in the livers of alcohol plus fructose mice compared to controls (Fig. 7B). The mRNA expression of IRF7, an IRF3-inducible gene (Petrasek et al.,

2011), was not changed by either high-fructose diet or chronic alcohol feeding alone. However, it was significantly up-regulated by the combination of high-fructose diet plus chronic alcohol feeding (Fig. 7A). IRF7 expression was significantly up-regulated by high-fructose diet feeding, alcohol feeding, or alcohol plus high-fructose diet feeding. However, there was no significant difference between these 3 groups (Fig. 7B). Hepatic gene expression of TRAF6, a marker of the MyD88-dependent pathway (Seki and Brenner, 2008), was significantly up-regulated by chronic alcohol feeding and further enhanced by the combination of high-fructose diet plus alcohol (Fig. 7A). However, TRAF6 protein was not elevated in alcohol plus fructose mice, but it was significantly up-regulated in mice fed alcohol alone (Fig. 7B). Taken together, our data indicate that both MyD88-dependent and MyD88-independent TLR4 signaling pathways may be involved the inflammatory response induced by the combination of high-fructose diet plus chronic alcohol feeding.

Effects of High-Fructose Diet and Chronic Alcohol Consumption on Plasma Endotoxin and LBP

Plasma endotoxin level was slightly elevated in high-fructose-diet-plus-chronic alcohol-fed mice compared to controls. However, the difference did not reach statistical significance. Both fructose and alcohol lowered plasma LBP, and there was no apparent synergism between the 2 agents in regulating LBP levels (Fig. 8).

DISCUSSION

In the current study, we found a synergistic effect of dietary fructose and alcohol on the induction of liver injury, but not on steatosis, and this effect is characterized by enhanced hepatic TLR4 signaling pathway, KC M1 activation, and prominent microvesicular steatosis in the alcohol-plus-high-fructose-diet-fed mice. The liver injury induced by the fructose–alcohol combination seems to be caused by neither accelerated fructose nor alcohol metabolism in the liver. Our data showed that copper levels were lower in mice fed either high fructose or alcohol alone. However, it was not further worsened by the alcohol and fructose together. On the contrary, it was slightly improved compared to the chronic alcohol-alone-fed mice. Because 80% of the copper leaving the liver is excreted via the bile, biliary excretion represents the major pathway of copper elimination (Luza and Speisky, 1996). The copper level is decreased in the early stages of some liver diseases, such as alcoholic steatosis (Uhlíkova et al., 2008) and NAFLD (Aigner et al., 2008, 2010), probably due to impaired intestinal absorption (Song et al., 2012). Copper levels tend to be elevated in the later stages of liver disease or cholestatic liver disease (Deering et al., 1977; Rodriguez-Moreno et al., 1997) as well as in experimental hepatic fibrosis (Song et al., 2011), probably due to impaired excretion. In the current study, the modestly increased copper levels observed in fructose plus alcohol combination may be a reflection of fatty liver progression from an early stage to a later stage.

Both hepatic CYP2E1 and KHK expressions were not further enhanced in mice fed high-fructose diet plus chronic alcohol compared to mice fed either high fructose or alcohol alone. Moreover, blood alcohol concentration did not show a significant difference between alcohol-alone- and alcohol-plus-fructose-fed mice (data not shown). Taken together, our data

do not support the concept that either accelerated fructose or alcohol metabolism is the major cause of liver injury in this model. In addition, we also observed that the hepatic phenotype of the alcoholplus-high-fructose-fed mice was characterized by predominantly microvesicular steatosis, which is the histological hallmark of impairment of mitochondrial function (Jaeschke et al., 2002). This pathological change may contribute to liver injury. However, the underlying mechanisms remain to be determined.

A growing body of evidence has indicated that obesity is an independent risk factor for alcohol-induced liver damage (Carr and Correnti, 2015; Chiang and McCullough, 2014; Naveau et al., 1997; Stepanova et al., 2010). In our current study, high-fructose diet alone did not lead to obesity, although food consumption was higher in the high-fructose-diet-fed mice than in the control mice. Similar observations have been reported by other investigators (Bergheim et al., 2008), suggesting that dietary fructose-associated obesity might involve multiple factors, possibly including the interaction between nutrition and the environment. Although the BW was not increased, the EFW and EFW/BW ratio were significantly increased by high-fructose diet. Moreover, fasting plasma glucose and insulin levels as well as HOMA-IR were significantly elevated by high-fructose diet, suggesting the existence of insulin resistance. However, this effect was attenuated by chronic alcohol feeding. A recent study showed that either dietary high fructose or the combination of high fructose plus alcohol significantly decreased the hepatic insulin receptor substrate (Irs) 1 and 2 mRNA expression in the Lieber–DeCarli rat model, which includes high fat in the diet (Alwahsh et al., 2014). In the current study, we did not observe exacerbated insulin resistance by the high-fructose diet plus chronic alcohol feeding, suggesting that impaired insulin signaling in Alwahsh and colleagues' (2014) study may be due to the effect of dietary high fat in combination with fructose and/or alcohol.

KC activation is a key event contributing to the alcoholic liver injury as well as NAFLD (Miura et al., 2012; Spruss et al., 2009; Thurman, 1998). KCs, as the resident macrophages in the liver, play a critical role in innate and adaptive immune responses. The phenotype of KCs may change in response to different environmental stimuli: the classically activated (M1) phenotype, which is pro-inflammatory; and alternatively activated (M2) phenotype, which is anti-inflammatory. Our data showed that KC activation induced by dietary high fructose plus alcohol favored the M1 phenotype, as supported by the significantly increased expression of M1 markers, such as CD68, TNF- α , and MCP-1. The expression of M2 markers, such as IL-10 and arginase 1, was not up-regulated compared to alcohol feeding alone. On the contrary, they tended to be down-regulated (Fig. 6). The up-regulated expression of KC markers was associated with the inflammatory cytokine mRNA expression. However, the contribution of cytokine expression from other inflammatory cells (besides KCs) in the liver cannot be ruled out. In agreement with this, a recent study showed that alternatively activated M2 KCs may protect against ALD and NAFLD by promoting M1 KC apoptosis via secreting IL-10 (Wan et al., 2014), lending credence to the concept that an M1/M2 imbalance toward M1 polarization likely contributes to the liver damage.

It has been well documented that TLR4 is a critical signaling pathway leading to KC activation. While it is implicated in both fructose- and alcohol-induced hepatic steatosis and liver injury, the downstream pathways are different. It has been shown that TLR4-mediated

liver injury in ALD is MyD88 independent and involves the role of IRF-3-mediated type I interferon production (Hritz et al., 2008; Petrasek et al., 2011), whereas fructose-induced NAFLD is likely MyD88 dependent (Spruss et al., 2009). IRF-3 is considered to be a marker of MyD88-independent signaling. IRF-7 is an IRF3-inducible gene. The up-regulated expression of IRF-3 and IRF-7 supports the concept that liver injury induced by high-fructose diet plus alcohol consumption may be due to the enhanced MyD88-independent pathway. However, the role of the MyD88-dependent pathway cannot be ruled out. Recent studies also indicated cross talk between the MyD88-dependent and MyD88-independent pathways (Bagchi et al., 2007). How these 2 pathways interact and contribute to the liver injury remains to be defined.

To further determine whether increased gut permeability and/or gut bacteria contribute to the enhanced TLR4 signaling and hepatic inflammatory response, we examined the plasma endotoxin and LBP levels. The decreased plasma LBP levels were observed in both high-fructose- and chronic alcohol-alone-fed mice, and mice fed the combination of the 2 factors, suggesting an augmented cell response to LPS (Minter et al., 2009). Although we did not detect a significant difference in the plasma endotoxin levels among the groups, it does not rule out differences in levels of endotoxin delivered into the liver. LPS level is not only determined by the gut bacteria and gut permeability, but is also related to the detoxification by the liver and sequestration by plasma lipoprotein (Munford, 2005). Our data suggested that enhanced TLR4 signaling and KC M1 polarization induced by fructose–alcohol interaction may occur at either the gut or liver level and the underlying mechanisms remain to be defined.

In summary, our data provide evidence that chronic alcohol consumption acts with dietary fructose to induce liver injury, suggesting that the dietary fructose–alcohol combination contributes to the disease progression, and fructose is a dietary “second hit” in the transition of steatosis to steatohepatitis in ALD (Tsukamoto et al., 2009). The underlying mechanism might be enhanced hepatic TLR4 signaling pathways, which lead to KC activation and a phenotype switch toward M1 polarization, pro-inflammatory response, and liver injury. However, how fructose causes inflammation in ALD is not known and requires further study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Timeline of Experiment

Fig. 1.

Timeline of the experiment. Mice in high-fructose diet group were fed (ad libitum) high-fructose diet (60%, pelleted diet) (TD.10781; Harlan Laboratories) for 18 weeks. In the chronic ethanol (EtOH) and high-fructose diet plus chronic EtOH groups, deionized water containing EtOH as the only water source was given ad libitum after 8 weeks of high-fructose diet feeding until the end of the experiment. Dashed red line and arrow represent ramp-up phase of alcohol feeding (1 week) and solid red line and arrow represent 20% EtOH feeding period (9 weeks).

A **B** **Fig. 2.**

Effect of chronic alcohol drinking and high-fructose diet on liver enzymes. (A) Alanine aminotransferase (ALT) and (B) aspartate aminotransferase (AST). Data represent means $\pm$ SD ($n = 7$ to 9). Statistical significance was set to $p < 0.05$. * versus C; # versus F; \$ versus CE. † Interaction between ethanol (EtOH) and fructose is significant ($p < 0.05$, 2-way ANOVA). C, control; F, high-fructose diet; CE, control + EtOH; FE, high-fructose diet + EtOH.

Fig. 3. Effect of chronic alcohol drinking and high-fructose diet on hepatic lipid accumulation. **(A)** Representative photomicrographs of the hematoxylin and eosin (H&E) (9400) and Oil Red O staining of liver section ($\times 200$). **(B)** Hepatic triglyceride. **(C)** Percentage of micro- and macrosteatosis. The number of hepatocytes with fat accumulation in the fat area was counted in 8 randomly selected high-power microscopic fields ($\times 400$) per mouse, and 5 mice in each group. The average percentage of hepatocytes with micro- and macrovesicular steatosis were compared between groups. Data represent means $\pm$ SD ($n = 5$ to 10). Statistical significance was set to $p < 0.05$. * versus C; # versus F; \$ versus CE. † Interaction between ethanol (EtOH) and fructose is significant ($p < 0.05$, 2-way ANOVA). C, control; F, high-fructose diet; CE, control + EtOH; FE, high-fructose diet + EtOH.

Fig. 4.

Effect of chronic alcohol drinking and high-fructose diet on copper status. (A) Plasma ceruloplasmin. (B) Liver copper. (C) Plasma copper. Data represent means $\pm$ SD ($n = 6$ to 10). * versus C; # versus F; \$ versus CE (statistical significance was set to $p = 0.05$, 2-way ANOVA). C, control; F, high-fructose diet; CE, control + ethanol (EtOH); FE, high-fructose diet + EtOH.

A **B** **Fig. 5.**

Effect of chronic alcohol drinking and high-fructose diet on hepatic cytochrome P450 2E1 (CYP2E1) and fructokinase/ketohexokinase (KHK) protein expression. **(A)** CYP2E1. **(B)** KHK. Hepatic CYP2E1 and KHK expression were determined by Western blots. Optical density of the band was quantified by ImageJ software. The ratio to GAPDH was calculated by assigning the value from controls as 1. Data represent means ± SD ($n = 5$). * versus C; # versus F (statistical significance was set to $p < 0.05$, 2-way ANOVA). C, control; F, high-fructose diet; CE, control + ethanol (EtOH); FE, high-fructose diet + EtOH.

A **B** **C** **Fig. 6.**

Effect of chronic alcohol drinking and high-fructose diet on hepatic expression of (A) Emr1, Cd68, and Cd163 mRNA. (B) F4/80 protein (C) M1 and M2 phenotype markers. Hepatic F4/80 expression was determined by Western blots. Optical density of the band was quantified by ImageJ software. The ratio to GAPDH was calculated by assigning the value from controls as 1. Data represent means $\pm$ SD (polymerase chain reaction data, $n = 5$ to 9 ; Western blot data, $n = 5$). Statistical significance was set to $p < 0.05$. * versus C; # versus F; \$ versus CE. † Interaction between ethanol (EtOH) and fructose is significant ($p < 0.05$, 2-

way ANOVA). C, control; F, high-fructose diet; CE, control + EtOH; FE, high-fructose diet + EtOH.

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Fig. 7. Effect of chronic alcohol drinking and high-fructose diet on hepatic expression of Toll-like receptor 4 (Tlr4) signaling molecules. (A) Tlr4, Myd88, interferon regulatory factor 3 (Irf3), Irf7, and Traf6 mRNA. (B) IRF-3, IRF-7, and TRAF6 protein. Hepatic IRF-3, IRF-7, and TRAF6 expressions were determined by Western blots. Optical density of the band was quantified by ImageJ software. The ratio to GAPDH was calculated by assigning the value from controls as 1. Data represent means ± SD (polymerase chain reaction data, $n = 5$ to 9 ; Western blot data, $n = 5$). * versus C; # versus F; \$ versus CE (statistical significance was set

to $p < 0.05$, 2-way ANOVA). †Interaction between ethanol (EtOH) and fructose is significant ($p < 0.05$, 2-way ANOVA). C, control; F, high-fructose diet; CE, control + EtOH; FE, high-fructose diet + EtOH.

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Fig. 8. Plasma endotoxin and lipopolysaccharide-binding protein (LBP). Data represent means $\pm$ SD ($n = 5$ to 9). Statistical significance was set to $*p < 0.05$. C, control; F, high-fructose diet; CE, control + ethanol (EtOH); FE, high-fructose diet + EtOH.

Table 1

Primers Used for Real-Time PCR Analysis

Gene	Sequence(5'–3')	Refseq no.
Tnf- α	Forward: GTGATCGGTCCCCAAAGG	NM_013693.3
	Reverse: GGTGGTTTGCTACGACGTG	
IL-1 β	Forward: TTCATCTTTGAAGAAGAGCC CAT	NM_008361.3
	Reverse: TCGGAGCCTGTAGTGCAGTT	
IL-6	Forward: TGGAAATGAGAAAAGAGTTG TGC	NM_031168.1
	Reverse: CCAGTTTGGTAGCATCCATCA	
IL-10	Forward: CCTTAATGCAGGACTTTAAGG GTTA	NM_010548.2
	Reverse: ACCCAGGGAATTCAAATGCT	
Ccl2 (MCP-1)	Forward: GGCTCAGCCAGATGCAGT	NM_011333.3
	Reverse: TGAGCTTGGTGACAAAACTA CAG	
Tlr4	Forward: GATCTGAGCTTCAACCCCTTG	NM_021297.2
	Reverse: TGCCATGCCTTGCTCTCAAT	
Traf6	Forward: TCGGAGTGCCGTGTATGTAG	NM_009424.2
	Reverse: ACACACACAACCCGATCGTA	
Irf3	Forward: GAACCGGAAAGAAGTGTTC	NM_016849.4
	Reverse: GGCACCCAGATGTACGAAGT	
Irf7	Forward: AAGCCCTCTGCTTTCTAGTGAT	NM_016850.3
	Reverse: TAGGGTTCCTCGTAAACACG	
Myd88	Forward: TGTCTCCAGGTGTCCAACAG	NM_010851.2
	Reverse: CTTGGTGCAAGGGTTGGTAT	
Arg1	Forward: GACATCAACACTCCGCTGAC	NM_007482.3
	Reverse: CACGATGTCCTTGGCAGATA	
Emr1 (F4/80)	Forward: TTTTCAGATCCTTGGCCATC	NM_010130.4
	Reverse: GGGTGGCAAGTGCAGAAGTA	
Cd68	Forward: GGACCCACAACCTGCTACTCA	NM_009853.1
	Reverse: GCCAACAGTGGAGGATCTTG	
Cd163	Forward: TGAAGCGACGACAGATTCAG	NM_053094.2
	Reverse: AATGACCCCCGAGGATTT	
Actb	Forward: CAGCTGAGAGGGAAATCGTG	NM_007393.3
	Reverse: CTCCAGGGAGGAAGAGGATG	

Tnf, tumor necrosis factor; IL-1 β , interleukin-1 beta; Ccl2, chemokine (CC motif) ligand 2; Tlr4, Toll-like receptor 4; Traf6, TNF receptor-associated factor 6; Irf, interferon regulatory factor; Myd88, myeloid differentiation primary response gene 88; Arg1, arginase 1, liver; Emr1, EGF-like module containing, mucin-like, hormone receptor-like sequence 1; Actb, actin, beta.

Table 2

Effects of Chronic Alcohol Drinking and High-Fructose Diet on Body Weight (BW), Liver/BW ratio, EFW, and Plasma Indices

Variable	EtOH (-)		EtOH (+)	
	Control	High fructose	Control	High fructose
BW (g)	26.37 ± 1.58	26.97 ± 3.52	26.32 ± 1.83	25.87 ± 1.75
Food consumption (g/mouse/wk)	18.97 ± 0.82	24.78 ± 3.46 [*]	16.78 ± 1.50 ^{*,**}	21.69 ± 2.27 ^{*,**,***,†}
Liver weight (g)	1.063 ± 0.066	1.057 ± 0.232	1.185 ± 0.149	1.327 ± 0.121 ^{*,**}
Liver/BW ratio (%)	4.031 ± 0.089	4.051 ± 0.127	4.511 ± 0.351 ^{*,**}	5.136 ± 0.402 ^{*,**,***,†}
EFW (g)	0.422 ± 0.062	0.845 ± 0.276 [*]	0.425 ± 0.092 ^{**}	0.470 ± 0.102 ^{**}
EFW/BW ratio (%)	1.617 ± 0.293	2.984 ± 0.736 [*]	1.605 ± 0.274 ^{**}	1.739 ± 0.271 ^{**}
Plasma indices				
Triglyceride (mg/dl)	19.0 ± 3.8	14.9 ± 2.77	27.4 ± 4.8 ^{*,**}	26.4 ± 5.8 ^{*,**}
Cholesterol (mg/dl)	111.4 ± 19.6	110.1 ± 20.8	93.0 ± 20.4 [*]	97.2 ± 9.4
NEFA (μM)	343.0 ± 77.0	246.9 ± 85.6	437.6 ± 121.9 ^{**}	274.1 ± 62.1 ^{***}
Glucose (mg/dl)	175.4 ± 35.1	249.8 ± 49.1 [*]	109.9 ± 24.0 ^{*,**}	179.9 ± 41.8 ^{*,**,***}
Insulin (ng/ml)	0.231 ± 0.01	0.258 ± 0.02 [*]	0.254 ± 0.02 [*]	0.249 ± 0.02
HOMA-IR	2.48 ± 0.48	3.68 ± 1.03 [*]	1.71 ± 0.42 ^{*,**}	2.69 ± 0.68 ^{*,***}

EFW, epididymal fat weight; NEFA, nonesterified fatty acids; HOMA-IR, homeostasis model assessment of insulin resistance. International formula for HOMA-IR calculator: [fasting glucose (mmol/l × fasting Insulin (mU/l) /22.5].

^{*} Significantly different from control group;

^{**} significantly different from high-fructose group;

^{***} significantly different from control + ethanol (EtOH) group.

[†] Interaction between EtOH and fructose is significant ($p < 0.05$, 2-wayANOVA).

Six-week-old male C57BL/6J mice were fed a 60% high-fructose diet for 18 weeks. Deionized water or deionized water containing 20% EtOH (v/v) was given ad lib after 8 weeks until the end of the experiment. Results are shown as means ± SD ($n = 7$ to 10).