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Moderate alcohol consumption aggravates high-fat diet induced steatohepatitis in rats

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Abstract

Background—Nonalcoholic steatohepatitis (NASH) develops in the absence of chronic and excessive alcohol consumption. However, it remains unknown whether moderate alcohol consumption aggravates liver inflammation in pre-existing NASH condition.

Methods—Sprague-Dawley rats were first fed *ad libitum* with Lieber-DeCarli high-fat diet (71% energy from fat) for 6 weeks to induce NASH, as demonstrated previously. Afterwards, these rats were continuously fed with high-fat diet (HFD, 55% total energy from fat) or high fat plus alcohol diet (HFA, 55% energy from fat and 16% energy from alcohol) for an additional 4 weeks. Pathological lesions including fat accumulation and inflammatory foci in liver were examined and graded. Lipid peroxidation and apoptotic hepatocytes in the liver were assessed. The mRNA expressions of tumor necrosis factor- α (TNF α) and TNF receptor 1 (TNFR1), Fas death receptor (Fas) and Fas ligand (FasL), IL-1 β and IL-12 were determined by real time PCR. Protein levels of total and cleaved caspase-3, CYP2E1, Bax and Bcl-2 were measured by western blotting.

Results—The number of hepatic inflammatory foci and apoptotic hepatocytes were significantly increased in rats fed HFA as compared with those in HFD-fed rats. The aggravated inflammatory response and cellular apoptosis mediated by HFA were associated with elevated mRNA expression of Fas/FasL and cleaved caspase-3 protein. Although no significant differences were observed between HFD and HFA groups, the levels of lipid peroxidation, Bax and Bcl-2 protein concentration and mRNA levels of other inflammatory cytokines were significantly higher in these two groups than those in the control group.

Conclusions—This data suggests that even moderate alcohol consumption can cause more hepatic inflammation and cellular apoptosis in a pre-existing NASH condition.

Keywords

nonalcoholic steatohepatitis; high-fat diet; moderate alcohol; rats

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The detrimental effects of chronic and excessive alcohol consumption, e.g., prolonged alcohol intake in excess of approximately 100 g/day, on human health have been well documented (Bloss, 2005; Dawson et al., 2005; Ellison et al., 2001; Hoek and Pastorino, 2002; Seitz and Stickel, 2007; Wang, 2005). Recently, a growing number of evidence shows that “moderate” alcohol consumption without heavy drinking episodes is associated with multiple health benefits and lower risks for some chronic diseases (Koppes et al., 2005; Rimm et al., 1991; Stampfer et al., 2005). The term “moderate alcohol” has been established as 1~2 drinks/day for men (Dawson, 2003), which is equivalent to 14–28 g/day based on the energy value of pure ethanol (Meister et al., 2000). Data from the Framingham Heart Study revealed that the age-adjusted mean alcohol consumption was 21.0~30.6 g/day among adult men and 10.4~14.2 g/day among adult women (Zhang et al., 2008). The inferior limit for the moderate alcohol consumption category is established on 4 drinks/day (Dawson et al., 1995), which accounts for 19.6% of a total 2000 kcal intake.

Nonalcoholic steatohepatitis (NASH) has been recognized as a severe stage of nonalcoholic fatty liver disease, the most common form of chronic liver disease in the United States (Ong and Younossi, 2007). Diagnosis of NASH is defined by the concurrence of fat accumulation and infiltration of inflammatory cells in the liver, in the absence of chronic & excessive alcohol consumption history (Brunt, 2005). Certain risk factors for NASH development have been suggested to potentiate the capacity of alcohol-mediated liver damage (Diehl, 2004). Although patients with NASH have been advised against drinking alcohol, experimental evidence is lacking regarding whether alcohol consumption, especially at moderate levels, can increase their susceptibility to more liver injuries.

Previously, a high-fat diet induced NASH rat model was developed by feeding rats a 71% liquid high-fat diet enriched with corn oil for three weeks (Lieber et al., 2004). Recently, we demonstrated that a higher rate of cellular apoptosis was induced in this rat model, which was associated with increased hepatic inflammation and oxidative stress (Wang et al., 2008). Since excessive alcohol feeding to rats increased apoptosis (Baroni et al., 1994; Mi et al., 2000; Slomiany et al., 1999), we investigated whether moderate alcohol consumption (16% of total calories intake) with the pre-existing NASH condition, aggravates apoptosis and hepatic inflammation in rats.

MATERIALS AND METHODS

Animals and diets

The Institutional Animal Care and Use Committee at the Jean Mayer USDA Human Nutrition Research Center on Aging approved the animal experiment protocol. As shown in Figure 1, eight-week old male Sprague-Dawley rats (Charles River Co., Wilmington, MA) were initially divided into two groups and fed *ad libitum* with either a Lieber-DeCarli control diet (CD, 35% energy from fat, n=10) or a high-fat diet, (71% energy from fat, n=20) (Dyets Inc., Bethlehem, PA) for 6 weeks as described previously (Wang et al., 2008). Afterwards, all of the rats in the high-fat diet group were equally divided into two sub-groups and fed either a modified high-fat diet (HFD, 55% energy from fat, n=10) or a modified high-fat alcoholic diet (HFA, 55% energy from fat and 16% energy from ethanol, n=10) (Dyets Inc., Bethlehem, PA). The composition for each modified diet is listed in Table-1. All three groups (CD, HFD and HFA) were then fed their respective diets for another 4 weeks. Rats were housed individually in temperature and humidity controlled rooms and kept on a 12-hour light: dark cycle. The body weight of the rats was recorded weekly. After killing, the liver was promptly excised and removed. Two small portions of the liver at the right lobe were fixed in a 10% neutral-buffered formalin for histological examination, and the remaining liver was snap frozen in liquid nitrogen and stored at –80°C for subsequent analysis.

Histological examination

Formalin-fixed and paraffin-embedded liver tissues were routinely processed for hematoxylin and eosin (H&E) staining. Liver histology was examined and graded by two independent investigators that were blind to the samples according to published criteria for magnitude analysis of steatosis and inflammation (Brunt et al., 1999; Matteoni et al., 1999). Briefly, the degree of steatosis was graded 0–4 based on the average percent of fat-accumulated hepatocytes per field at $\times 200$ magnification under H&E staining (Grading 0 = $<5\%$, 1 = 5–25%, 2 = 26–50%, 3 = 51–75%, 4 = $>75\%$). The inflammation was graded 0–3 based on the average number of inflammatory foci (clusters of infiltrating mononuclear cells) at $\times 200$ magnification per area (cm^2) (Grading 0 = 0; 1 = 1–2/20 $\times$; 2 = 2–4/20 $\times$; 3 = $>4/20\times$).

Hepatic lipid peroxidation

A lipid peroxidation colorimetric microplate assay (Oxford Biochemical Research Inc, Oxford MI) was used to assay lipid peroxidation end products malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) in the liver.

Quantification of apoptotic hepatocytes

Apoptotic hepatocytes were evaluated in the rat liver by using terminal deoxynucleotide transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay. In Situ Cell Death Detection Kit, TMR red (Roche Diagnostics, Indianapolis, IN) was used for detection of DNA strand breaks in apoptotic cells by fluorescence microscope, as previously described (Wang, 2008). The number of TUNEL-positive cells was counted from 10 randomly selected fields at $\times 100$ magnification per liver sample. Results were expressed as the mean number of TUNEL-positive apoptotic hepatocytes per microscopic field.

Gene expression by Real-time PCR

Total RNA was isolated from the liver by TriPure Isolation Reagent (Roche Diagnostics, Indianapolis, IN) according to the instructions. cDNA was then prepared from the RNA samples using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA) and an automated thermal cycler (Bio-Rad Laboratories, Hercules, CA). After quantification and qualification, the PCR reaction for mRNA detection was carried out in each well using 20 μL reaction mixture containing 10 μL 2 $\times$ SYBR Green Supermix, 0.4 μL of 10 $\mu\text{mol/L}$ primer mix (including forward and reverse primers) and 2.5 μL cDNA diluted in RNase-free water. Cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. Gene-specific primer sequences were designed using the Primer Express version 2.0 software (Applied Biosystems, Foster City, CA). PCR results were then normalized to the levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and calculated by reference to the average value for the control group using the comparative Ct method. For each sample and each gene, PCR reactions were carried out in duplicate and repeated twice. Gene expression was analyzed using the following pairs of primers: TNF α (forward, CCAGACCCTCACACTCAGATCA; reverse, TCCGCTTGGTGGTTTGCTA), TNF receptor 1 (forward, GCACCAAGTGCCACAAAGGAA; reverse, TGCGAAGCTGTAAAGGTGCCT), Fas (forward, TTTGGAGTTGAAGAGGAGCGTT; reverse, TTTCGTTCAACAGGCTGACAC), FasL (forward, ACTGGCAGAAGTCCGTGAGTT; reverse, CACACTCCTCGGCTCTTTTTT), IL-1 β (forward, CTCTCCAGTCAGGCTTCCTTGT; reverse, CAGGTCATTCTCCTCACTGTCTG), IL-12 (forward, ACATCATCAAACCGGACCCA; reverse, TGCGGACGAAGAAGTGTGAGG), Bax (forward, AACAAACATGGAGC; reverse, GAAGTTGCCGTCT), Bcl-2 (forward, CATGTGTGTGGAGA; reverse,

TTCCACAAAGGCAT) and GAPDH (forward, AGTGCCAGCCTCGTCTCATAG; reverse, CCTTGACTGTGCCGTTGAACT).

Protein concentrations by Western Blotting

Whole liver homogenate was prepared from liver samples as previously described (Chung et al., 2002). Liver protein extracts (40 µg each) were resolved on sodium dodecyl sulfate--polyacrylamide (SDS-PAGE) gel electrophoresis. After blocking membrane, immunoblotting was performed based on the manufacturer's instruction for each primary antibody against total and phosphorylated cleaved caspase-3 (Cell Signaling Technology, Inc. MA), CYP2E1 (Chemicon International, Inc. MA), Bax and Bcl-2 (Santa Cruz Biotechnology, Inc. CA). Membranes were then incubated with the secondary antibodies against rabbit or mouse (Bio-Rad Laboratory, Hercules, CA). Anti-GAPDH antibody (Chemicon International, Inc, CA) was used as internal control for equal loading of proteins.

Statistical analysis

SAS (version 9.12) software was used for all data analysis. The histological grading in liver among different groups was compared using non-parametric Wilcoxon's test. One-way analysis of variance (ANOVA) with *post hoc* analysis using Tukey's multiple comparison test was used for the rest data. All group values are presented as Mean $\pm$ Standard error of the mean (SEM). A *P* value of < 0.05 was regarded as significant difference.

RESULTS

General appearance

There were no significant differences on body weight and liver weight among all groups at the end of this experiment (Table-2). However, liver index, a ratio of liver weight to body weight, was significantly higher in the HFA group than the other two groups.

Histological examinations

A mild steatosis was observed in the livers of rats fed with the CD for 10 weeks (Figure 2A), whereas a significant accumulation of fat droplets in the liver was observed from either HFD or HFA groups (Figure 2B and C). The semi-quantitatively histological grading for steatosis showed that feeding rats with HFD or HFA led to a more than five fold increase of steatosis as compared to the rats fed with the CD (Table 2). Although there was a tendency for steatosis to be higher in the HFA as compared with the HFD, it did not reach a statistically significant level. A higher number of inflammatory foci was found in HFD (Figure 2E) than in the CD (Figure 2D), whereas even a greater amount of inflammatory foci was found in the HFA group (Figure 2F). As compared with rats fed with the CD, the semi-quantitatively examination for inflammatory foci showed that feeding rats with HFD led to a significantly higher degree (e.g. two fold) of inflammation, which was further induced in the HFA group (e.g. three fold) (Table 2).

Cellular apoptosis and its regulation pathways

The number of TUNEL-positive apoptotic hepatocytes was significantly higher in rats fed with the HFD as compared with that in the CD group; while the incorporation of moderate alcohol into the HFD caused a further increased rate (25%) of cellular apoptosis ($P < 0.05$, Figure 3A). The protein concentration of cleaved (activated) caspase-3, the major executor of intracellular apoptotic pathway, was also significantly higher in the HFA than that in the HFD (Figure 3B). No differences were found for its total form of Caspase 3. Since intracellular apoptotic event can be regulated by either intrinsic pathway, which is triggered by intracellular stresses (e.g. oxidative stress) and its related imbalanced Bcl-2 family or

extrinsic pathway which is mediated by interaction between death ligands and their receptors (e.g. TNF α /TNF receptor or Fas ligand /Fas), we carried out further analysis to examine their potential alteration by HFD or HFA treatment. Lipid peroxidation end products MDA plus 4-HNE were increased in HFD as compared with CD, and no further change was observed when alcohol was added (Figure 4A). Similarly, hepatic CYP2E1 protein concentrations did not show a significant difference between the HFD and HFA groups, although both of them were higher than that in the CD (data not shown). The mRNA expressions of pro-apoptotic Bax and anti-apoptotic Bcl-2 did not show any significant changes among all three groups in this study (data not shown). However, the protein concentration of Bax was significantly higher in both the HFD and the HFA group than in the CD (Figure 4B). The anti-apoptotic Bcl-2 protein, on the other hand, was significantly lower in either HFD or HFA groups (Figure 4C). We did not detect any significant differences on these markers between the HFD and HFA groups. Hepatic mRNA expression of TNF α and its receptor 1 (TNFR1), which were upregulated by HFD vs. CD, did not show a significant increase after the addition of alcohol. (Figure 5A and B). The inflammatory cytokines, including IL-1 β and IL-12, did not display any significant changes between the HFD and HFA groups (data not shown). Interestingly, although the mRNA expression of Fas death receptor did not exhibit significant changes between the CD and HFD in rat liver, it was significantly induced by HFA (Figure 5C). Fas ligand mRNA level, on the other hand, was found to be significantly higher in HFA group as compared with CD while no changes was found between HFD and CD groups (Figure 5D).

DISCUSSION

Previous studies have reported that rats fed the liquid high-fat diet (71% energy from fat) for 3 to 6 weeks developed NASH (Lieber et al., 2004; Wang et al., 2008). In the present study we demonstrated, for the first time, that moderate consumption of alcohol in those with the pre-existing NASH condition led to more hepatic inflammation and cellular apoptosis. Our results indicate that patients diagnosed with NASH should use great caution in consuming alcohol, even at a moderate level. This study may also help to understand why a certain proportion NASH cases evolve into advanced liver diseases.

In the present study, we substituted ethanol for a small portion of carbohydrates while maintaining equal total fat and total calorie intake (isocaloric) between the HFD and HFE-fed rats, which enabled us to better observe the effects of moderate alcohol consumption on the pre-existing HFD-induced NASH condition. Increased cellular apoptosis has been indicated to be a prominent feature of NASH (Feldstein and Gores, 2004; Ribeiro et al., 2004). Its correlation with hepatic inflammation and fibrosis, instead of steatosis, suggested that increased apoptosis could be a key component involved in NASH pathogenesis, development and progression (Feldstein et al., 2003; Ribeiro et al., 2004). In our study, we found a significant increase of apoptotic hepatocytes in the HFA group vs. HFD group, which is in agreement with our observation that the protein concentration of cleaved caspase-3 is also significantly higher in HFA than in HFD. In addition, the number of hepatic inflammatory foci was further increased in the HFA group as compared with the HFD alone. Since the apoptotic body itself has been demonstrated to be able to act as a direct stimulus for Kupffer activation and the following production and secretion of multiple proinflammatory cytokines (Canbay et al., 2003), it is reasonable to postulate that the enhanced cellular apoptosis by alcohol might, at least partially, contribute to its further induced hepatic inflammation in this model. It should be pointed out that dietary carbohydrate/fat ratio change was reported to be able to affect alcohol-induced liver damage. However, the decrease of carbohydrate/fat ratio (e.g. from 2.36 to 0.84) only induced more steatosis without any other signs of hepatic pathology, such as inflammation, in a rat model (Korourian et al., 1999). In addition, the increased hepatic CYP2E1 and focal necrosis was

only observed when the dietary carbohydrate/fat ratio was extremely lower than control (0.022) (Korourian et al., 1999). Since the dietary carbohydrate/fat ratio was only modestly adjusted (e.g. 1.0 in HFD vs. 0.4 in HFA) in our model, and we did not detect any focal necrosis or any difference on CYP2E1 expression between the HFD and HFA groups, therefore, this mild change of the carbohydrate/fat ratio may not make a major contribution to the liver injury in the HFA group.

Chronic oxidative stress, implicated as the primary “2nd hit” in NASH pathogenesis, not only poses a constant threat to cells living through their direct damage to DNA, protein and lipids, but also can trigger cell death or apoptosis via its interference with Bcl-2 family member balance and mitochondria function (Martindale and Holbrook, 2002). In the present study, acute treatment of moderate ethanol did not induce a further increase of lipid peroxidation in HFD-induced NASH condition. One of the essential contributions to increased oxidative stress by chronic and excessive alcohol is *via* upregulation of CYP2E1 (Lieber, 1997). Since hepatic CYP2E1 protein had already been induced at a significant level in our HFD-induced NASH model (Wang et al., 2008) it is possible that the moderate alcohol under current dosage is insufficient to cause a further upregulation of this enzyme, and thus oxidative stress in this model. Similarly, the main downstream regulators involved in oxidative stress-mediated intrinsic pathway, such as Bax and Bcl-2, failed to show any significant difference between the HFD and HFA groups. Overall, this data may suggest that the oxidative stress may not contribute to the further upregulation of cellular apoptosis by moderate alcohol intake.

Up-regulation of death receptors and their ligands, such as TNF α /TNF-R1 and Fas/FasL has been associated with hepatocyte apoptosis in steatohepatitis (Malhi and Gores, 2008). The importance of TNF α and its receptor I in the onset of hepatotoxicity and cellular apoptosis has been indicated in both patients (McClain et al., 1999) and animal models (Thurman et al., 1999; Tilg and Diehl, 2000) with NASH and alcohol-related liver injury. However, the little difference of TNF α and TNFR1 expressions between the HFA and HFD groups seems to exclude their potential contribution to moderate alcohol-induced further increase of cellular apoptosis. Previous experiments using TNF α or TNF-R1 knockout mice indicate that signaling through TNF-R1 may not be essential for the pathogenesis of steatohepatitis (Memon et al., 2001; Dela et al., 2005).

Interestingly, despite the little changes between HFD and CD; the Fas/FasL expression was significantly induced by moderate alcohol consumption in the HFA group, which is in agreement with previous findings in which ethanol and its metabolite acetaldehyde can induce the Fas/FasL expression from both *in vitro* and *in vivo* (Minana, et al., 2002; McVicker et al., 2006; Zhou et al., 2001). Moreover, it has been demonstrated that the hepatic steatosis sensitizes the liver to more injury and inflammation via up-regulation of Fas expression (Feldstein et al., 2003). Thus, we believe that the increased inflammatory foci (as detected by pathological examination) caused by the moderate alcohol consumption could be due to its induction of Fas/FasL signaling and its mediated apoptosis. In addition to its independent role in mediating cellular apoptosis or inflammation, recent evidence also suggests that Fas and TNF α may act cooperatively in the induction of alcohol-mediated cell death in the liver (Minagawa et al., 2004). For example, treatment of hepatocytes isolated from alcohol-fed mice with TNF α alone did not induce cell death, while the addition of TNF α to cells incubated with anti-Fas antibody significantly induced cell death in a TNF α -concentration dependent manner.

In summary, the present study demonstrated that the consumption of moderate alcohol in those with a pre-existing NASH condition can lead to more hepatic inflammation and cell death in this rat model. This further increased rate of cell apoptosis could be related to the

upregulated Fas/FasL signaling. Considering the significant variability of hepatic lesions and progression among NASH patients, additional investigation may be needed to identify whether this variation could correlate with the extent of alcohol ingestion.

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FIGURE 1.
Experimental design for NASH induction and incorporation of moderate alcohol consumption in rats.

FIGURE 2.

Histological examination of liver in rats (hematoxylin and eosin staining, $\times 200$). (A) Mild steatosis in CD (B) and (C) Remarkable fat accumulation from HFD and HFA, respectively (D) Scattered inflammatory cells in CD (E) Increased inflammatory foci formation from HFD (F) Significant presence of multiple inflammatory foci from HFA.

FIGURE 3.

Assessment of cellular apoptosis. (A) Apoptotic hepatocytes in rat liver were identified by TUNEL assay. TUNEL (+) cells were quantified from 10 randomly selected fields at $\times 100$. (B) Total and cleaved caspase-3 proteins measured by western blotting. Bars represent means $\pm$ standard error of the means (SEM). Bars sharing different letters are statistically significant at a level of $p < 0.05$.

FIGURE 4.

Lipid peroxidation and Bcl-2 family members. (A) End products of lipid peroxidation MDA plus 4-HNE assayed by LPO 586 kit (B) and (C) Hepatic protein concentration of Bax and Bcl-2 as measured by western blotting, respectively. Bars represent means $\pm$ standard error of the means (SEM). Bars sharing different letters are statistically significant at a level of $p < 0.05$.

FIGURE 5. Hepatic mRNA expressions of death ligand and receptors as measured by real-time PCR. (A) TNF α ; (B) TNFR1 (C) Fas; and (D) FasL. Bars represent means $\pm$ standard error of the means (SEM). Bars sharing different letters are statistically significant at a level of $p < 0.05$.