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Synergistic Steatohepatitis by Moderate Obesity and Alcohol in Mice Despite Increased Adiponectin and p-AMPK

Jun Xu^{1,*}, Keane K.Y. Lai^{1,*}, Alla Verlinsky¹, Aurelia Lugea^{1,2}, Samuel W. French^{1,3}, Marcus P. Cooper⁴, Cheng Ji⁵, and Hidekazu Tsukamoto^{1,2}

¹Southern California Research Center for ALPD and Cirrhosis, Department of Pathology, Keck School of Medicine of the University of Southern California

²Veterans Affairs Greater Los Angeles Healthcare System, Los Angeles, California

³Harbor-UCLA Medical Center, Torrance, California

⁴Division of Cardiovascular Medicine, University of Massachusetts Medical School, Worcester, Massachusetts

⁵Department of Medicine, Keck School of Medicine of the University of Southern California

Abstract

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 (32g/kg/day) 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, accentuated ER and mitochondrial stress underlie potential mechanisms for synergistic steatohepatitis caused by moderate obesity and alcohol.

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Address correspondence to: Jun Xu, 1333 San Pablo Street, MMR-428, Los Angeles, CA 90089; Tel: 323-442-3121; Fax: 323-442-3126; junx@usc.edu.

*These two equally contributed to the project.

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Keywords

synergistic steatohepatitis; obesity and alcohol synergism; apoptosis; necrosis; macrophage activation; nitrosative stress

Introduction

Alcoholic liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD) are 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 are 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 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 underlying mechanisms. We found 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 immunofluorescence 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 not 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 micrograms/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 $\pm$ 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 $\sim 34.6 \pm 9.4\%$ 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/day) did not, but high alcohol dose (32 g/kg/day) 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 effect in overfed mice receiving high dose of alcohol, as compared to overfeeding or alcohol infusion alone (Fig. 1B). Histologically, mice receiving overfeeding plus high dose of alcohol exhibited severe steatohepatitis with multiple foci of inflammation and pericellular fibrosis as compared to steatosis, minor mononuclear cell infiltration, and less pericellular fibrosis in mice receiving high dose of alcohol only (Fig. 1C). The synergistic liver injury induced by overfeeding plus alcohol was confirmed by fibrosis scores and total pathological scores (Table 1). Together, these results demonstrate that moderate obesity and alcohol synergistically aggravate the severity of steatohepatitis in our synergism mouse model.

Effects of overfeeding and alcohol on adiponectin and lipogenic signaling

Plasma adiponectin was increased in our mice treated with overfeeding, high dose of alcohol, or both as compared to controls (Fig. 2A). Because more striking effects were induced by giving high dose of alcohol (referred to as alcohol hereafter) to the overfed mice (Fig. 1 and 2A, Table 1), we used these synergism mice for subsequent studies. As shown in Fig. 2B, alcohol induced hepatic *AdipoR1* expression, and this induction was abrogated in overfeeding plus alcohol mice. Expression of *AdipoR2*, the predominant form in the liver (7,8), was decreased in overfeeding plus alcohol mice when comparing to alcohol fed mice. However, AMPK, an effector of adiponectin signaling, is phosphor-activated (p-AMPK) equally in both alcohol groups, regardless of overfeeding status (Fig. 2C)

Activation of AMPK reduces hepatic lipogenesis through inhibition of SREBP-1 and ACC (7,8). Despite the increased p-AMPK, the nuclear (active) form of SREBP-1 (nSREBP-1) was increased and the phosphorylated (inactive) ACC (p-ACC) was decreased in both alcohol groups (Fig. 2C). Total ACC protein levels were also reduced in these two groups in spite of upregulated nSREBP-1c, a known transactivator of the *Acc* gene (29). The protein levels of total AMPK and PPAR δ , a known lipolytic transcription factor (30), were unchanged (Fig. 2C). Taken together, these results suggest that alcohol-associated activation of adiponectin signaling is defective downstream of AMPK, leading to the loss of hepatoprotective effect of adiponectin and subsequent liver injury.

Overfeeding and alcohol induce synergistic ER stress and JNK1 activation, but not hepatocellular apoptosis

The concurrent activation of AMPK and SREBP-1 in both alcohol-fed groups led us to evaluate whether ER stress was an inducer of SREBP-1 activation (15–17) in our model. To test this, we did immunoblot analysis on ER stress marker proteins - phosphorylated eukaryotic initiation factor 2 α (p-eIF2 α), GRP78, and XBP-1. As shown in Fig. 3A, overfeeding increased XBP-1 levels; while alcohol caused moderate increases in p-eIF2 α and GRP78. However, overfeeding plus alcohol significantly induced all three ER stress marker proteins, with enhanced induction of p-eIF2 α and XBP-1, and synergistic induction of GRP78 as compared to either overfeeding or alcohol mice (Fig. 3A). Moreover, plasma homocysteine levels were significantly elevated in the overfeeding plus alcohol mice ($34.4 \pm 4.56 \mu\text{M}$) as compared to alcohol fed ($24.7 \pm 6.85 \mu\text{M}$) or overfed mice ($2.97 \pm 0.47 \mu\text{M}$). These results suggest the role of hyperhomocysteinemia in heightened ER stress and ER stress-associated SREBP-1 activation in our model.

Sustained ER stress is known to promote apoptosis through CHOP and JNK1 activation (16). Activated CHOP suppresses pro-survival gene *Bcl-2* (31) and induces pro-apoptotic gene *Bim* (32). Our qRT-PCR analysis showed that hepatic *Chop* mRNA was significantly increased in alcohol but not in alcohol plus overfeeding mice (Fig. 3D). Hepatic anti-apoptotic *Bcl-2* was induced and *Bcl-xL* remained unchanged in both alcohol groups. On the other hand, pro-apoptotic genes *Bax* and *Bim* were significantly induced by alcohol, and these effects were abrogated in alcohol plus overfeeding mice (Fig. 3D). At protein level, phosphor-activated JNK1 (p-JNK1) was induced by alcohol and synergistically by overfeeding plus alcohol (Fig. 3B). In contrast, the phosphor-activation of pro-survival protein, the extracellular signal-regulated kinase (p-ERK) (33), was significantly decreased in both alcohol groups. Moreover, the heightened ER stress and JNK1 activation were associated with increased cleavage of caspases 3/7 and induction of CHOP in overfeeding plus alcohol mice (Fig. 3C), suggesting activation of apoptotic pathway by p-JNK1 (34,35). However, the cleaved poly (ADP-ribose) polymerase (PARP), a product of activated caspases 3/7 and a marker of cells undergoing apoptosis (36), was not induced by overfeeding plus alcohol (Fig. 3C). Our repeated TUNEL staining also failed to detect significantly increased apoptotic hepatocytes in these mice compared with controls (Supplementary Fig. 1).

We next measured hepatic caspase 3/7 activities in the absence or presence of DTT. Preincubation of protein lysates with DTT restores the cysteine residue in the catalytic site of caspases to its initial reduced (active) state and thereby reverse posttranslational inhibition of caspase activity such as by S-nitrosylation (27). As shown in Fig. 3E, intraperitoneal injection of Jo2 anti-Fas antibody in mice resulted in a > 3-fold induction of hepatic caspase 3/7 activities, and this induction was not significantly changed by DTT. The caspase activities were significantly decreased in alcohol mice and were further decreased by 26% in overfeeding plus alcohol mice. DTT preincubation did not affect caspase activities in control, overfeeding, and alcohol fed mice, but it caused a significant 40%

increase in overfeeding plus alcohol mice (Fig. 3E). Taken together, these results suggest an incomplete execution of apoptosis and a potential shift of apoptotic to necrotic cell death in the synergistic steatohepatitis of our mouse model.

Overfeeding and alcohol synergistically suppress genes of mitochondrial biogenesis and metabolism

Necrosis occurs when ATP is depleted secondary to mitochondrial dysfunction (37). Based on aforementioned results, we hypothesized that hepatic mitochondria were deleteriously targeted in our synergism model. To test this, we first screened a panel of 8 genes involved in mitochondrial biogenesis and metabolism, including *Pgc-1 α* , *Prc*, *Nrf-1* (nuclear regulatory factors); *Ppara* (mitochondrial fatty acid β -oxidation); *Tfb1m* and *Tfb2m* (mitochondrial transcription specificity factors); *CoxII* (mitochondrion-encoded respiratory subunit); and *Cyto c* (nucleus-encoded respiratory subunit) (38,39), as well as *Acox1*, a *Ppara* target gene controlling peroxisomal fatty-acid β -oxidation and therefore ATP production. As shown in Fig. 4A, overfeeding alone caused suppression of *Ppara* and *CoxII* genes, while alcohol alone had essentially no effect on all genes examined. Overfeeding plus alcohol caused consistent suppression of all genes examined except for *Cyto c* (Fig. 4A). We next measured hepatic ATP content as an index of mitochondrial function. As shown in Fig. 4B, overfeeding ($p=0.051$) or alcohol alone caused a decrease in hepatic ATP level, which was further decreased by 26% in alcohol plus overfeeding mice. These results suggest that impairments in mitochondrial biogenesis, fatty acid oxidation, and ATP production contribute to the necrosis in synergistic steatohepatitis.

Synergistic and differential macrophage activation in liver and adipose tissue induced by overfeeding and alcohol

A growing body of evidence suggests that macrophage infiltration in WAT and liver plays a critical role in the pathogenesis of insulin resistance and fatty liver disease due to imbalanced activation of the classical, pro-inflammatory (M1) vs. the alternative, anti-inflammatory (M2) macrophages (40). In our mice, overfeeding or alcohol increased macrophage marker *Cd68* expression in the liver, but not in WAT; and overfeeding plus alcohol caused a synergistic induction of *Cd68* expression in both tissues (Fig. 5A and 5B). Histologically, macrophage infiltration in the WAT was most prominent in overfeeding plus alcohol mice (Fig. 5C and 5D), similar to histological findings in the liver (Fig. 1C).

To characterize macrophage polarization in the liver and WAT, we examined gene markers of M1 activation (*iNos* and *Tnf- α*) and M2 activation (*Arg1* and *Il-10*) (40). As shown in Fig. 5A, in the liver of overfeeding plus alcohol mice, the M1 gene *iNos* was conspicuously induced (~ 40-fold) and *Tnf- α* was moderately upregulated, while the M2 genes *Arg1* and *Il-10* were suppressed. Since iNOS competes metabolically with ARG1 for the same substrate arginine to produce NO, these results suggest significant increase in hepatic NO production in these mice. In the presence of oxidative stress, NO reacts rapidly with superoxide (O_2^-) to produce strong oxidant peroxynitrites ($ONOO^-$), which can subsequently produce a variety of reactive nitrogen species and cause nitrosative stress (27). Indeed, in overfeeding plus alcohol mice, hepatic malondialdehyde (MDA), a biomarker for oxidative stress and lipid peroxidation (25,41), was synergistically elevated, especially when high alcohol dose was given (Table 1). Consistent with these findings, immunostaining for hepatic nitrotyrosine (NT) demonstrated a striking increase in fluorescence intensity in the liver of overfeeding plus alcohol mice (Fig. 5E), suggesting a synergistic nitrosative stress (42).

In contrast to the liver, the WAT of overfeeding plus alcohol mice had synergistic increase in expression of M2 *Arg1* (~ 45 fold) and *Il-10* genes. Interestingly, the M1 genes *iNos* and

Tnf- α were also upregulated in WAT (Fig. 5B). Despite the increased *Tnf- α* expression in WAT by overfeeding or alcohol, and its synergistic increase by both, adiponectin expression was still elevated in WAT (Fig. 5B), correlating with the increased plasma adiponectin (Fig. 2A). Taken together, these results demonstrate a synergistic induction of macrophage infiltration in both liver and adipose tissue by overfeeding plus alcohol, with a predominant M1 phenotype and increased nitrosative stress in the liver. On the other hand, the infiltrating macrophages in WAT appear composed both M1 and M2 activated cells.

DISCUSSION

The present study has utilized the intragastric feeding mouse model to co-introduce two common life-style factors for fatty liver disease in humans (overnutrition and alcohol) and to demonstrate synergistic steatohepatitis caused by the two. Previously, we utilized the same mouse model to induce severe obesity (a 75% increase in body weight), hyperglycemia, insulin resistance, and steatohepatitis by excessive overfeeding (23). This time, we overfed mice using a moderate feeding regimen to induce “moderate obesity” (a 28~35% increase in body weight) which simulates the degree of obesity commonly observed in more than 30% of the US adult population (43). Liver damage induced by this moderate obesity is minimal; however, when alcohol is co-infused in these mice, severe liver injuries are observed in an alcohol dose-dependent manner. A conspicuous synergism is demonstrated when high alcohol dose is used, as manifested by severe steatosis, necroinflammation, and pericellular fibrosis.

The synergistic steatohepatitis in the alcohol-fed obese mice is associated with synergism in M1 macrophage activation, *iNos* induction, and elevated MDA levels. These results, along with the enhanced hepatic NT-immunostaining strongly suggest the presence of profound hepatic oxidative stress and NO-induced nitrosative stress in these mice. Nitrosative stress is known to cause: 1) S-nitrosylation and inactivation of caspases (27); and 2) tyrosine nitration of mitochondrial proteins leading to mitochondrial dysfunction (42,44). Although we do not directly assess S-nitrosylation of caspases, our finding of DTT-mediated increase in hepatic caspase 3/7 activities in alcohol-fed obese mice suggests the involvement of redox-mediated inhibition of these caspases (27). Interestingly, the immunoblot results show increased cleavage of caspase 3/7 in the overfeeding plus alcohol mice, indicating activation of apoptotic pathway. However, the cleavage of PARP by caspase 3/7 is not increased in these mice. Previously, *Kim et al.* showed that fully activated (cleaved) recombinant human caspase 3 could be inhibited by NO through posttranslational modification, i.e. S-nitrosylation *in vivo* and *in vitro* (45). Thus, we believe that apoptotic pathway is activated in the overfeeding plus alcohol mice, as evident by the cleaved caspases 3/7, but failed to be executed because of inactivation of caspases due to profound nitrosative stress. We propose that this condition plus enhanced ER stress and ATP depletion has resulted in necrotic cell death. This notion agrees with our TUNEL study, which shows no significant apoptotic hepatocytes in the synergism mice compared with other groups.

Our results demonstrate heightened hepatic ER stress in the overfeeding plus alcohol mice. This is also attributed to hepatic nitrosative stress, as well as hyperhomocysteinemia (17) in these mice. Nitrosative stress-induced mitochondrial dysfunction (42,44) leads to impaired ATP production and potentiates ER stress through increasing cytosolic free Ca^{2+} (21,46). The synergistic ER stress in the overfeeding plus alcohol mice is associated with increased p-JNK1, suppression of genes for mitochondrial biogenesis and oxidative function, and decreased hepatic ATP levels, suggesting an ER-mitochondrial stress crosstalk. This crosstalk has previously been observed in the setting of hepatic insulin resistance (46), apoptosis (47), and NO-mediated inhibition of the mitochondrial function (21). The coexistence of ER stress, mitochondrial dysfunction, and ATP depletion is likely to cause

hepatic necrotic cell death. Moreover, Kruman *et al.* have reported that caspase inhibitor zVAD-fmk decreases the percentage of neurons undergoing apoptosis, accelerates the loss of mitochondrial potential, and shifts the mode of cell death to necrosis (48). Thus, the synergistic M1 macrophage activation in the liver of overfeeding plus alcohol mice provides a mechanism that potentiates aforementioned NO-induced adverse effects on hepatocytes, leading to shift apoptosis to necrosis and synergistic aggravation of steatohepatitis.

The impaired ATP production due to mitochondrial perturbation in the synergism mice will result in increased AMP/ATP ratio and subsequent AMPK activation. Although growth factor signaling can also activate AMPK (49), this is less likely because p-ERK1/2, the downstream event of growth factor receptor activation, is reduced. The increased nSREBP-1 and decreased p-ACC protein levels in alcohol-fed obese mice, in spite of AMPK activation, indicate defective adiponectin-AMPK signaling and account for the hepatic steatosis. At least for SREBP-1c, the hyperhomocysteinemia and heightened ER stress are likely responsible for induction of this lipogenic transcription factor (16,17). Thus, in our mouse model, the increase plasma adiponectin and inadequate ATP production likely contribute to induce and/or maintain high p-AMPK levels.

The increased adiponectin expression in WAT and in circulation in our mouse model differs from findings in other rodent models of ALD and NAFLD (7–9). This discrepancy could be firstly a result of different etiological backgrounds used in the studies. Genetic models of NAFLD tend to accentuate phenotypes caused by gene-specific manipulations, which differentially affect metabolic parameters as exemplified by increased levels of adiponectin in liver-specific PTEN knockout mice and normal adiponectin in MCD mice (11). Secondly, in the present study, we purposely induce “moderate obesity” to examine its synergistic effect with alcohol in liver injury. These moderate-obese mice exhibit only minimal liver damage on histology and do not show overt ER stress, mitochondrial dysfunction, and oxidative/nitrosative stress in the liver. The increased plasma adiponectin in these mice could be a compensatory response of WAT to the early liver damage. In contrast, our previous study (23) of severe-obese mice with overt NASH induced by excessive intragastric overfeeding showed decreased adiponectin expression in WAT. Lastly, the increased plasma adiponectin in the alcohol and overfeeding plus alcohol mice is similar to the findings in ALD patients (12,13). Although the underlying mechanism is not fully understood, the increased adiponectin expression in WAT and in circulation in our synergism mice results probably from the effect of alcohol.

The concomitant induction of TNF- α and adiponectin in WAT in the overfeeding plus alcohol mice is unexpected as they are mutually antagonistic (7). In addition, the infiltrating macrophages in WAT of these mice appear composed of both M1 and M2 activated cells, as evident by increased expression of both M1 (*iNos* and *Tnfa*) and M2 (*Arg1* and *Il10*) genes. It has been shown that defective M2 activation due to macrophage-specific PPAR δ deficiency results in decreased adiponectin expression in WAT (50). Moreover, adiponectin has been shown both *in vitro* and *in vivo* to promote macrophage M2 polarization (51) and this might have maintained M2 cells in our model. At this point, it is not clear to us whether the increased adiponectin and M2 gene expression represent compensatory, hepatoprotective mechanisms or are somehow causally related. Physiological significance of the mixed macrophage activation in WAT of our model is a question of our future investigation.

In conclusion, our study clearly demonstrates the synergistic effect of the common life-style “two-hits”, alcohol and moderate obesity, in the genesis of severe steatohepatitis. Our findings pinpoint the potential importance of M1 activation of hepatic macrophages with intensified nitrosative stress, defective p-AMPK signaling, and mitochondrial-ER stress crosstalk in inducing synergistic hepatic necroinflammation. We believe that our

intra-gastric feeding model represents an ideal system in which precise manipulation of "hits" can be achieved, and thus a broad spectrum of investigation to recapitulate and better understand human fatty liver disease can be conducted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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 2 α
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

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Figure 1. Overfeeding plus alcohol induces synergistic steatohepatitis

(A) Schematic presentation of concomitant OF and alcohol infusion. The caloric intake in the overfed mice were gradually increased from initial 580 Cal/kg/day to 986 Cal/kg/day on day 17 and kept to the end. On day 17, alcohol was added and gradually increased to a final alcohol dose of 23g/kg/day or 32g/kg/day. Shaded area represents the amount of calories derived from alcohol in daily caloric intake. (B) Plasma ALT levels, n=5 for each group. (C) Liver H&E, arrowhead indicates mononuclear cell infiltration; and reticulin staining of fibrosis. ALT, alanine aminotransferase; OF, overfeeding; Alc, alcohol; Reti, reticulin.

Figure 2. Adiponectin signaling in overfeeding plus alcohol mice
(A) Plasma adiponectin levels, n=5 for each group. **(B)** Hepatic mRNA levels of AdipoR1 & 2 by qRT-PCR, n=5 for each group. **(C)** Immunoblot of adiponectin effectors involved in lipid metabolism. * p < 0.05 compared to control; † p < 0.05 compared to alcohol only group. OF, overfeeding; Alc, alcohol; AdipoR, adiponectin receptor.

Figure 3. Overfeeding and alcohol enhance ER stress and activate apoptotic pathway
 Immunoblot for (A) ER stress markers eIF2 α , GRP78, and XBP-1; (B) cell death signaling molecules JNK 1&2 (arrow, p-JNK1) and survival signaling molecules ERK 1&2; and (C) apoptotic executioner caspase-3 & -7; CHOP, a mediator of ER-induced apoptosis; and PARP, a downstream target of caspase-3 & -7. * $p < 0.05$ compared to control; † $p < 0.05$ compared to respective alcohol groups. (D) qRT-PCR analyses of anti-apoptotic genes Bcl-2, Bcl-xL, pro-apoptotic genes Bad, Bax and Bim, as well as Chop. $n = 5$ for each group. * $p < 0.05$ compared to control; † $p < 0.05$ compared to respective alcohol group. (E) Hepatic caspase 3 & 7 activities were expressed as percent values relative to the Control's, which was not preincubated with DTT (the 3rd open bar from left). DTT preincubation is shown in black bar. Livers from mice injected with PBS ($n = 2$) and Jo2 antibody ($n = 2$) serve as negative and positive controls of apoptosis, respectively. $n = 5-8$ for each experimental groups. * $p < 0.05$ compared to their respective Control groups.

Figure 4. Effect of overfeeding and alcohol on hepatic mitochondrial biogenesis and ATP production

(A) qRT-PCR analysis of hepatic genes involved in mitochondrial biogenesis (PGC-1 α , Nrf-1, TFB1M, TFB2M) and metabolism (PPAR α , CoxII, Cyto C), as well as peroxisomal fatty acid oxidation (Acox1). * $P < 0.05$ and ** $P < 0.01$ compared to Control; † $P < 0.05$ compared to respective alcohol group, $n = 5$ for each group. (B) Hepatic ATP contents, $n = 5-8$ for each groups.

Figure 5. Over feeding and alcohol result in synergistic macrophage infiltration in the liver and white adipose tissue (WAT), with contrasting M1 activation in the liver and M2 activation in WAT
(A) and (B) qRT-PCR analyses of macrophage marker (CD68), M1 markers (iNOS, TNF α), M2 marker (Arg1, Il-10), and adiponectin. **(C)** H&E staining of WAT. **(D)** immunostaining of macrophage marker CD68. Arrowheads in C and D indicate macrophage infiltration. **(E)** Immunostaining of nitrotyrosine in the liver. The green fluorescence intensity correlates with the degree of tyrosine nitration. * $p < 0.05$ and ** $p < 0.01$ compared to control; † $p < 0.05$ compared to respective alcohol group, $n = 5$ for each group.

TABLE 1

Pathophysiological and biochemical characterization of synergism mouse model.

	Control	OF	Alc (23g/kg/d)	Alc (32g/kg/d)	OF + Alc (23g/kg/d)	OF + Alc (32g/kg/d)
Final BW (g)	26 ± 1.14	36 ± 0.73 *	21 ± 0.63 *	24 ± 2.17	36 ± 1.35 *,†	32 ± 1.43 *,†
Liver Wt (g)	1.4 ± 0.26	1.8 ± 0.26 *	1.2 ± 0.06	2.4 ± 0.43 *	2.9 ± 0.24 *,†	3.6 ± 0.56 *,†
Liver Wt/BW (%)	5.5 ± 1.13	4.9 ± 0.48	5.9 ± 0.12	10 ± 1.01 *	7.9 ± 0.40 *,†	11 ± 1.54 *
Liver Tg (mg/g liver)	18 ± 3	20 ± 4	25 ± 4	51 ± 11 *	37 ± 6 *,†	79 ± 12 *,†
BAL (mg/dL)	4.62 ± 3.27	3.08 ± 1.27	63.4 ± 66.9	309 ± 50.9 *	53.9 ± 85.5	285 ± 58.5 *
Fibrosis Scores	0	0	0	0.6 ± 0.55 *	0.2 ± 0.45	1.7 ± 0.58 *,†
Total Path Scores	2.0 ± 2.00	1.0 ± 0.43	2.6 ± 1.87	6.4 ± 0.89 *	8.0 ± 1.55 *,†	11 ± 1.58 *,†
Liver MDA (nmol/mg proteins)	0.4 ± 0.10	0.5 ± 0.08	0.5 ± 0.09	1.3 ± 0.13 *	0.9 ± 0.11 *,†	1.9 ± 0.21 *,†

Data are expressed as mean ± SD (n = 5 to 6).

* p < 0.05 for all groups compared to the Control;

† p < 0.05 for OF + Alc groups compared to pair-fed Alc groups, e.g. OF + Alc (23g) vs. Alc (23g), or OF + Alc (32g) vs. Alc (32g).

OF, overfeeding; Alc, alcohol feeding; BW, body weight; Wt, weight; Tg, triglyceride; BAL, blood alcohol level; MDA, malondialdehyde.