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Binge Alcohol-Induced Microvesicular Liver Steatosis and Injury are Associated with Down-Regulation of Hepatic *Hdac 1, 7, 9, 10, 11* and Up-Regulation of *Hdac 3*

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Abstract

Background—Binge, as well as chronic, alcohol consumption affects global histone acetylation leading to changes in gene expression. It is becoming increasingly evident that these histone associated epigenetic modifications play an important role in the development of alcohol-mediated hepatic injury.

Methods—C57BL/6 mice were gavaged 3 times (12 h intervals) with ethanol (4.5 g/kg). Hepatic histone deacetylase (*Hdac*) mRNAs were assessed by qRT-PCR. Total HDAC activity was estimated by a colorimetric HDAC activity/inhibition assay. Histone acetylation levels were evaluated by Western blot. Liver steatosis and injury were evaluated by histopathology, plasma ALT activity, and liver triglyceride accumulation. Fatty acid synthase (*Fas*) and carnitine palmitoyl transferase 1a (*Cpt1a*) expression were also examined. HDAC 9 association with *Fas* promoter was analyzed.

Results—Binge alcohol exposure resulted in alterations of hepatic *Hdac* mRNA levels. Down-regulation of HDAC Class I (*Hdac 1*), Class II (*Hdac 7, 9, 10*), Class IV (*Hdac 11*), and up-regulation of HDAC Class I (*Hdac 3*) gene expression were observed. Correspondent to the decrease in HDAC activity an increase in hepatic histone acetylation was observed. These molecular events were associated with microvesicular hepatic steatosis and injury characterized by increased hepatic triglycerides (48.02±3.83 vs 19.90±3.48 mg/g liver, p<0.05) and elevated plasma ALT activity (51.98±6.91 vs 20.8±0.62 U/L, p<0.05). Hepatic steatosis was associated with an increase in FAS and a decrease in Cpt1a mRNA and protein expression. *Fas* promoter analysis

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revealed that binge ethanol treatment decreased HDAC 9 occupancy at the *Fas* promoter resulting in its transcriptional activation.

Conclusions—Deregulation of hepatic *Hdac* expression likely plays a major role in the binge alcohol-induced hepatic steatosis and liver injury by affecting lipogenesis and fatty acid β -oxidation.

Keywords

Binge ethanol exposure; liver steatosis and injury; HDACs

Binge drinking is an increasing public health issue which can have long-term consequences, such as alcohol-induced tissue/organ damage, including liver injury (Mathurin and Deltenre, 2009). Acute alcohol exposure due to binge drinking results in transient hepatic steatosis which is reversible and considered to be benign. However, it can also lead to the development of steatohepatitis, fibrosis, and cirrhosis associated with chronic alcoholic liver disease (Donohue, 2007).

In the past decade, it has been clearly demonstrated that alcohol administration results in epigenetic alterations in the liver including posttranslational histone modifications and DNA methylation (Shukla et al., 2008, Mandrekar, 2011, Moghe et al., 2011). Histone acetylation is a key component in the regulation of gene expression, and is associated with enhanced transcriptional activity, whereas deacetylation is typically associated with transcriptional repression (Khan and Khan, 2010, Kouzarides, 2007). Steady-state levels of acetylation of the core histones result from the balance between the opposing activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs). Currently, several different phylogenetic classes of HDACs are known, including Class I (HDAC 1, 2, 3, and 8) and Class II (HDAC 4, 5, 6, 7, 9 and 10) (de Ruijter et al., 2003). Class III HDACs (SIRT1-SIRT7) represent a distinct class of NAD-dependent enzymes, which are important in DNA repair (Kruszewski and Szumiel, 2005). Additionally, HDAC 11 has been recently identified as a new member of the HDAC family. HDAC11 possesses properties of HDACs Class I and Class II, and is classified as Class IV (Gao et al., 2002, Gregoret et al., 2004). A major function of HDACs is the regulation of transcription through deacetylation of histone and non-histone proteins (Shepard and Tuma, 2009, Yao and Yang, 2011).

Recent studies demonstrated that binge, as well as chronic, ethanol consumption causes site-selective modifications in histones leading to changes in histone acetylation, methylation and downstream gene expression (Bardag-Gorce et al., 2007, Pal-Bhadra et al., 2007, Bardag-Gorce et al., 2009). It was shown that ethanol caused post-translational acetylation of histone H3 at lys 9 in a tissue/organ specific manner in a rat binge drinking model (Kim and Shukla, 2006), and in a dose- and time-dependent manner in cultured rat hepatocytes (Park et al., 2005, Park et al., 2003). It has been demonstrated that epigenetic modifications of histones play a significant role in the development of alcohol-associated liver pathology (Shukla et al., 2008, Aroor et al., 2010). However, the molecular mechanisms involved in epigenetic histone modifications leading to alcoholic tissue/organ damage are only beginning to be understood. The aim of the present work was to systematically examine the

effects of binge alcohol exposure on the expression of liver Class I, II and IV HDACs, and to determine their relevant contributions to binge alcohol-induced liver steatosis and injury.

METHODS

Mouse Model of Binge Ethanol-Induced Liver Steatosis and Injury

C57BL/6 male mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were housed in a pathogen-free barrier facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Food and tap water were allowed ad libitum. Eight-week-old mice, weighing 20 to 25 g were given ethanol (EtOH) by gavage at a dose of 4.5 g/kg. Animals were gavaged with EtOH three times with 12 hours intervals (9 a.m., 9 p.m., 9 a.m. the next day, and sacrificed 4 hours later, at 1 p.m., Figure 1). Control animals were gavaged with saline. At the time of sacrifice, blood samples were collected from the inferior vena cava, and centrifuged at 300× g for 15 minutes at 4°C to obtain plasma. Part of the liver from the left lobe was harvested and fixed in 10% neutral-buffered formalin, while the remaining liver tissue was snap frozen in liquid N₂ and stored at –80°C. The protocol for this study was approved by the University of Louisville Institutional Animal Care and Use Committee.

Evaluation of Hepatic Steatosis and Liver Injury

For histological analysis, liver sections were fixed in 10% buffered formalin for 24 hours and embedded in paraffin. Tissue sections were deparaffinized, stained with hematoxylin-eosin (H&E) and examined by light microscopy (×200 final magnification).

Liver injury was assessed by measuring plasma aminotransferase (ALT) activity (Thermo Fisher Scientific Inc., Middletown, VA) according to the manufacturer's protocols.

Liver cell apoptosis was assessed by TdT-mediated dUTP nick-end labeling (TUNEL) assay using the ApopTag Peroxidase *In Situ* Apoptosis Detection kit (Chemicon, Temecula, CA), according to the manufacturer's instructions. TUNEL+ve cells were quantitated by counting five randomly selected fields (×200 final magnification).

Liver triglyceride (TG) measurement and Oil-Red-O staining were performed to evaluate fat accumulation in the liver. Hepatic TGs were measured as previously described (Kirpich et al., 2010) using TG reagents (Thermo Fisher Scientific Inc., Middletown, VA). For Oil-Red-O staining, frozen liver sections were washed in PBS twice for 5 minutes. Oil-Red-O and 85% propylene glycol were added with agitation for 15 minutes, followed by washing in tap water.

Analysis of Total Hepatic HDAC Activity

Liver HDAC activity was estimated using commercially available HDAC activity/inhibition assay kit (colorimetric) according to manufacturer's protocol (Epigentek, Farmingdale, NY).

Liver Histone Extraction

An acid extraction of histones from liver tissue was performed according to the Abcam (Cambridge, MA) protocol with modifications. Briefly, 80–100 mg of liver tissue was homogenized in a Dounce homogenizer on ice in Triton Extraction Buffer (TEB, containing 0.5% Triton X-100, 2 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM sodium butyrate). Homogenates were incubated on ice for 10 min, and centrifuged at 2500 rpm for 10 min at 4°C. The pellet was washed in TEB, resuspended in 0.2N HCl and incubated overnight at 4°C. The extraction was centrifuged at 10,000 rpm for 10 min at 4°C, and the acid-insoluble pellets were discarded. The supernatant fraction, which contains the acid soluble proteins (histones) was neutralized by adding NaOH to a final concentration of 200 mM. Protein was measured by using Bio-Rad reagents (Hercules, CA).

Western Blot Analysis

To analyze histone acetylation status equal amounts of histones (40 µg) were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a nitrocellulose membrane. The membrane was probed with antibody against acetyl-histone H3 (Cell Signaling Technology, Danvers, MA). Total histone H3 (Abcam, Cambridge, MA) was used as a loading control.

To analyze fatty acid synthase (FAS) protein expression, total protein was extracted from liver tissue using RIPA buffer (50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 2mM EDTA, 4 mM Na₃VO₄, 40 mM NaF, 1% Triton X-100, 1 mM PMSF, 1% protease inhibitor cocktail). Equal amounts of protein (60 µg) were separated by SDS-PAGE, and transferred to polyvinylidene fluoride membrane. Commercially available primary antibody for FAS was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Detection of GAPDH (Cell Signaling Technology, Danvers, MA) was served as a loading control.

Protein signals were visualized using the enhanced chemiluminescence system (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Band intensities were quantified using ImageJ software (NIH, Bethesda, MD, USA).

Immunohistochemical Evaluation of Liver CPT1a, FAS, and HDAC 9 Protein Levels

Hepatic carnitine palmitoyl transferase 1a (CPT1a), fatty acid synthase (FAS), and HDAC 9 protein levels were evaluated by immunohistochemical analysis using commercially available antibodies against CPT1a (Proteintech Group Inc., Chicago, IL), FAS (Santa Cruz Biotechnology, Santa Cruz, CA), and HDAC 9 (Abcam, Cambridge, MA). Analysis was performed according to the manufacturer's protocols.

RNA Isolation and Real-Time PCR Analysis

Total RNAs were isolated from liver tissue using TRIzol reagent (Invitrogen, Carlsbad, CA) and treated with DNase I to remove any contaminating genomic DNA (RQ1 RNase-Free DNase; Promega, Madison, WI). For qRT-PCR, the first-strand cDNA was synthesized using qScript cDNA SuperMix (Quanta Biosciences, Inc., Gaithersburg, MD). qRT-PCR was performed in triplicate with an ABI Prism 7500 sequence detection system and PerfeCTa SYBR Green FastMix, Low ROX reagents (Quanta Biosciences, Inc., Gaithersburg, MD).

The specific primers for *Hdac(s) 1 to 11*, carnitine palmitoyltransferase (*Cpt1a*), fatty acid synthase (*Fas*), and *18s* were purchased from SA Biosciences (Frederick, MD). *18s* mRNA expression was used to normalize the obtained data.

Liver Chromatin Immunoprecipitation (ChIP) Assay

ChIP assay was performed in order to detect the status of HDAC9 in the mouse FAS promoter region. Briefly, 100 mg of liver tissue from control and binge ethanol treated mice were minced and cross-linked in 1% formaldehyde followed by 0.125M glycine neutralization. Tissue was then homogenized and resuspended in lysis buffer containing protease inhibitors (Magnify ChIP system, Invitrogen, Carlsbad, CA). Chromatin fragments were prepared using a Covaris S2 as recommended for the Magnify ChIP system. Chromatin was immunoprecipitated with anti-HDAC9 (Abcam, Cambridge, MA), and non-specific control rabbit IgG (Cell Signaling Technology Inc., Beverly, MA) ChIP antibodies. The lysates were incubated overnight with respective antibody followed by capture of antibody/protein complexes with protein A/G magnetic beads (Thermo scientific, Rockford, IL) and one wash each with low salt, high salt, LiCl and Tris EDTA buffers (Millipore, Temecula, CA). After reverse crosslinking and purification, pure DNA was eluted in 10mM Tris buffer. Semi quantitative ChIP-PCR was performed using the primers for the *Fas* promoter, 5'-CAGCCCCGACGCTCATTGG-3' and 5'-CTCTCTGGCTCCCTCTAGGC as described (Wong et al., 2009). Product was ran on 1.4% agarose gels and stained with ethidium bromide.

Statistical Analysis

Data are expressed as Mean $\pm$ standard error of the mean (SEM). Statistically significant differences were determined by Student's *t*-test. $P < 0.05$ was considered statistically significant. Statistical analysis was performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, Inc, La Jolla, CA)

RESULTS

Effects of Binge-Ethanol Exposure on Hepatic Class I, II and IV Hdac mRNA Expression

It has been demonstrated that alcohol induces histone modifications leading to histone H3 hyperacetylation through modulation of HAT activity (Kim and Shukla, 2006, Park et al., 2005). However, the histone acetylation status results from the balance between the opposing activities of HATs and HDACs. We therefore examined hepatic HDAC Class I, II and IV gene expression in response to binge ethanol exposure. Hepatic mRNA levels of HDAC family members were assessed by qRT-PCR in binge ethanol gavaged animals compared to control group (saline gavaged, age-matched littermates). In our binge-ethanol animal model, ethanol (4.5 g/kg body weight) was administered to C57BL/6 mice by oral gavage, 3 times, 12 hours apart (Figure 1). Most of the hepatic *Hdacs* analyzed were expressed at low levels in binge ethanol treated mice compared to control group. *Hdac 1*, 7, 9, 10, and 11 were significantly ($p < 0.05$) down-regulated upon binge ethanol exposure. In contrast, the only HDAC that was found to be up-regulated in response to binge ethanol treatment was the Class I HDAC – *Hdac 3* ($p < 0.05$) (Figure 2A, 2B, 2C). These results

strongly suggest that binge ethanol treatment differentially affects the normal regulatory mechanisms of hepatic *Hdac* expression.

Effects of Binge-Ethanol Administration on Hepatic HDAC Activity and Histone Acetylation

To evaluate the consequence of the general down-regulation of the Class I, II and IV *Hdacs* (with the exception of *Hdac 3*), global hepatic HDAC activity and histone H3 acetylation (H3Ac) were examined. Consistent with the gene expression pattern, hepatic HDAC activity was significantly decreased ($p<0.05$) in response to binge ethanol exposure (Figure 3A). Moreover, in agreement with previously published reports (Kim and Shukla, 2006, Park et al., 2005), significant elevation in the acetylation of total histone H3 in ethanol treated mice compared to control animals was observed in our study (Figure 3B and 3C). These results demonstrate that binge ethanol-induced deregulation of liver Class I, II and IV *Hdac* expression correlates with decreased hepatic HDAC activity leading to increased hepatic histone acetylation.

Binge Ethanol-Induced Liver Injury and Steatosis were Associated with Decreased CPT1a and increased FAS mRNA and Protein Levels

Liver injury, defined by elevated plasma ALT activity (51.98 ± 6.91 vs 20.8 ± 0.62 U/L, $p<0.05$) was found in binge ethanol treated mice compared to control, saline gavaged animals (Figure 4A). Consistent with elevated ALT activity, presence of apoptotic hepatocytes as indicated by TUNEL-positivity was considerably higher in ethanol-fed mice compared to control animals (Figure 4B). These data demonstrate that binge ethanol administration protocol used in our study can induce hepatocyte apoptosis leading to liver injury.

In response to binge alcohol exposure mice developed microvesicular liver steatosis, evaluated by histological examination (Figure 5A). Oil-Red-O staining further demonstrated significantly increased hepatic fat accumulation (Figure 5B) in the livers of ethanol treated mice compared to control group, which was confirmed by biochemical assessment of hepatic triglyceride accumulation (48.02 ± 3.83 vs 19.90 ± 3.48 mg/g liver, $p<0.05$, Figure 5C).

Numerous lines of evidence have shown that hepatocyte lipid accumulation depends on the balance of de-novo lipogenesis and fatty acid oxidation. Thus, we evaluated the effect of binge ethanol exposure on gene expression of *Fas* and *Cpt1a*, which play critical roles in the regulation of hepatic lipid metabolism. *Cpt1a*, the critical rate-determining regulator of fatty acid β -oxidation in hepatocytes, was significantly ($p<0.05$) down-regulated at the gene expression levels in response to binge ethanol administration (Figure 6A). Down-regulation of *Cpt1a* mRNA also led to a decline in hepatic CPT1a protein expression as documented by immunohistochemical staining (Figure 6B). Also, in contrast, the expression of the *Fas* gene which encodes the key lipogenic enzyme was significantly ($p<0.05$) up-regulated in the livers of ethanol treated mice compared to control animals (Figure 7A). In agreement with *Fas* gene expression, immunohistochemical staining and Western blot analysis also demonstrated increased FAS protein levels in the livers of binge ethanol treated animals (Figure 7B, 7C and 7D).

Evaluation of the Role of HDAC9 in the Transcriptional Regulation of Fas Gene Expression

To determine the contribution of deregulated *Hdac* expression in the development of hepatic steatosis we primarily investigated the role of HDAC 9 since (i) amongst all the HDACs, HDAC 9 was maximally down-regulated and (ii) recent studies have shown that HDAC 9 interacts with the *Fas* promoter and plays a significant role in repressing the transcriptional activation of *Fas* gene expression (Wong et al., 2009). Interaction of HDAC 9 with the hepatic *Fas* promoter region in saline treated and binge ethanol treated conditions was examined by ChIP analysis. The *Fas* promoter region that was interrogated is involved in HDAC 9 binding leading to the deacetylation of the transcription factor USF-1 and consequent down-regulation of *Fas* mRNA expression (Wong et al., 2009). PCR analysis of chromatin fragments immunoprecipitated with anti-HDAC9 specific antibody revealed that ethanol treatment leads to decreased HDAC 9 binding to the hepatic *Fas* promoter (Figure 8A–B). Indeed, the decrease in HDAC 9 binding correlated with the decrease observed in HDAC 9 mRNA and protein levels (Figure 2B, 8C) and a correspondent increase in FAS expression (Figure 7A–D). These data strongly suggest that binge ethanol mediated decrease in HDAC 9 expression plays a contributory role in the up-regulation of FAS expression and development of hepatic steatosis. Moreover, we have also observed the contributory role of HDAC 3, which was the only HDAC found to be increased in response to the binge ethanol treatment, in the down-regulation of Cpt1a expression; the detailed findings are being presented as a separate manuscript. Overall, these results strongly support the notion that binge ethanol-induced microvesicular hepatic steatosis likely occurs due to the deregulation of hepatic *Hdac* expression affecting lipogenesis and fatty acid β -oxidation.

DISCUSSION

The pathophysiology of chronic alcohol-mediated steatohepatitis is largely defined. On the other hand, the mechanisms underlying hepatic steatosis following acute ethanol exposure are only beginning to be understood. In the current study we report, for the first time, that binge ethanol exposure resulted in significantly altered mRNA expression of liver Class I, II and IV *Hdacs*, which correlated with the development of hepatic steatosis and injury. Deregulation of *Hdac* expression also correlated with an increase in total histone H3 acetylation. The Increased histone acetylation observed in our study is in agreement with previously published observations demonstrating that binge ethanol administration, analogous to binge drinking, caused histone hyperacetylation in a tissue/organ specific manner (Kim and Shukla, 2006, Park et al., 2003). The mechanisms responsible for ethanol-induced histone hyperacetylation are not fully understood. An increase in the acetylation of histones in response to ethanol may be due to modulation of the activities of enzymes that control histone acetylation (HATs and HDACs), and/or an increase in the substrate for the acetylation reaction, acetyl-coA, or both. In our study, we showed that hepatic histone hyperacetylation in response to binge alcohol exposure is associated with the down-regulation of HDAC Class I (*Hdac 1*), Class II (*Hdac 7, 9, 10*) and Class IV (*Hdac 11*) gene expression and decreased HDAC activity. In agreement with our observations, binge drinking-mediated down-regulation of HDAC 2 and HDAC 11 was recently reported (Li J et al., 2010). A significant decrease in total nuclear HDAC activity and increased histone H3 acetylation have also been demonstrated in ethanol-treated hepatic WIF-B cells (Shepard et

al., 2008). On the other hand, recent studies have reported that acute alcohol exposure *in vivo* as well as *in vitro* led to an increase in histone H3 at Lys9 acetylation, possibly due to an increase in either the expression levels or the specific activity of HATs with no effect on overall HDAC activity (Park et al., 2005, Choudhury and Shukla, 2008). Nevertheless, the authors reporting these observations have acknowledged that the suppressive effects of ethanol on specific types of HDACs may also contribute to the increase in histone H3 acetylation at Lys9.

Our data strongly suggest that binge ethanol induced inhibition of HDAC Class I (*Hdac 1*), Class II (*Hdac 7, 9, 10*) and Class IV (*Hdac 11*) leads to increased histone acetylation. Recently it has been demonstrated that the formation of acetate from alcohol is critically involved in alcohol-induced inflammatory gene expression by promoter histone acetylation in acute alcoholic hepatitis (Kendrick et al., 2010). Hence, in addition to down-regulating HDACs, it is possible that binge alcohol exposure may increase cellular acetate levels, leading to enhanced HAT activity due to increased substrate availability. Additionally, since acetate is also the product of deacetylation, free acetate may cause feedback inhibition of HDACs (Kendrick et al., 2010).

In our study, binge alcohol-mediated changes in *Hdac* gene expression, decrease in HDAC activity, and consequent increase in histone acetylation were associated with hepatic steatosis and injury. In the context of liver specific HDAC functions, it has been demonstrated that several HDACs are involved in the regulation of liver metabolic functions. Accordingly, Class I HDACs have been demonstrated to play an essential role in liver gluconeogenesis (Oiso et al., 2011), and conditional HDAC3 deletion in mice induces severe disruption of carbohydrate and lipid metabolism, resulting in organ hypertrophy and hepatocellular damage (Alenghat et al., 2008, Feng et al., 2011)

To date, the involvement of HDACs in binge alcohol-induced liver steatosis and injury has not been fully investigated. We observed that down-regulation of *Hdac 1, 7, 9, 10, 11*, and over expression of *Hdac 3* were associated with hepatic steatosis and liver injury. Hepatic triglyceride accumulation and steatosis can occur as a consequence of increased fatty acid synthesis as well as decreased β -oxidation of fatty acids. Recent studies have pointed to possible defects in both of these pathways in liver steatosis (Browning and Horton, 2004, Caldwell et al., 1999, Diraison et al., 2002). Indeed in the present work, *Cpt1a* which is involved in intracellular regulation of fatty acid metabolism by transporting long chain fatty acids into mitochondria for β -oxidation was found to be down-regulated in alcohol treated animals. Further, hepatic expression of *Fas* was significantly up-regulated in response to binge-ethanol treatment. Hence, up-regulation of lipogenesis as well as decreased free fatty acid β -oxidation may play a significant role in the development of hepatic steatosis in our model. HDACs mainly function as transcriptional repressors by influencing promoter activation. Hence, binge-ethanol mediated alterations in HDAC expression can influence the extent of their interaction with the *Fas* and *Cpt1a* promoter regions and could be envisaged to play a contributory role in their expression including other genes involved in hepatic lipid metabolism. With regards to the *Fas* gene expression, the data obtained showed that the binge ethanol treatment decreased the association of HDAC 9 with the *Fas* promoter (Figure 8A–B) and correlated with its transcriptional activation. Notably, the decrease in the level of

HDAC 9 (Figure 8C) targeted to the *Fas* promoter correlated with the significant suppression of *Hdac 9* mRNA levels. Recently, HDAC 9 has been shown to play a major role in the suppression of the *Fas* gene expression by interacting with the *Fas* promoter and deacetylating the key transcription factor USF-1 (Wong et al., 2009). Under metabolic states (e.g. fasting) where *Fas* gene expression is inhibited, significant nuclear protein levels of HDAC 9 are observed; whereas, under conditions leading to *Fas* gene expression (e.g. feeding) the nuclear protein levels of HDAC 9 are not detectable (Wong et al., 2009). Hence it is likely that binge ethanol induced decline in *Hdac 9* mRNA expression leads to decreased HDAC 9 nuclear protein levels and consequent interaction with the *Fas* promoter. These events then result in relieving the HDAC 9 mediated suppression and inducing the transcriptional activation of the *Fas* promoter resulting in an increase in the *Fas* mRNA expression. Additionally, our recent studies have also demonstrated that binge ethanol-induced increase in HDAC 3 expression is directly responsible for the suppression of the *Cpt1a* promoter activity and the consequent down-regulation of the *Cpt1a* gene expression (manuscript under review).

In summary, our data strongly suggest that HDACs play a major role in regulating the expression of genes that are relevant for hepatic fat metabolism and identify their deregulation occurring in response to binge ethanol consumption as a significant pathogenic mechanism leading to hepatic steatosis.

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Ethanol Gavage- 4.5 g/kg

Figure 1. Schematic representation of the animal model of binge ethanol induced liver steatosis and injury

C57BL/6 male mice were gavaged 3 times (12 hours interval) with ethanol at a dose of 4.5 g/kg. Control animals were gavaged with saline at the same time. Animals were sacrificed 4 hours after last gavage; blood and liver tissue were collected for analysis.

Figure 2. Expression of hepatic Class I (A), Class II (B), and Class IV (C) HDAC mRNA in response to binge alcohol exposure

Significant ($*p < 0.05$) down-regulation of HDAC Class I (*Hdac 1*), Class II (*Hdac 7, 9, 10*), Class IV (*Hdac 11*), and up-regulation of HDAC Class I (*Hdac 3*) gene expression were observed in binge ethanol treated mice compared to control animals. Results are presented as fold changes relative to control group denoted by the dashed line set to 1.0. Values are mean $\pm$ SEM, $n=6$ animals/group. Student's *t*-test was used to evaluate significant differences between binge ethanol treated and control animals.

Figure 3. Effects of binge ethanol administration on total liver HDAC activity and hepatic histone acetylation

(A) Total liver HDAC activity was significantly ($*p < 0.05$) decreased in mice binged with ethanol compared to control animals. HDAC activity was evaluated with colorimetric HDAC activity/inhibition assay kit. Data are presented as arbitrary units. Values are mean $\pm$ SEM, $n = 5$ animals/group. Student's t -test was used to evaluate significant differences between ethanol treated and control animals. (B) Western blot analysis demonstrated increase in histone H3 acetylation (H3Ac) in the livers of ethanol treated mice compared to control animals. (C) Quantitative analysis of histone H3Ac levels. Expression of total histone H3 was used as a loading control. The intensity of protein bands was quantified by densitometry using the NIH ImageJ software.

Figure 4. Effects of binge ethanol exposure on liver injury

(A) Plasma ALT activity was significantly elevated in alcohol treated mice compared to control animals (51.98 ± 6.91 vs 20.8 ± 0.62 U/L, $*p < 0.05$). Values are mean $\pm$ SEM, $n = 6$ animals/group. Student's t -test was used to evaluate significant differences between ethanol treated and control animals. (B) Mice gavaged with ethanol had significantly increased liver apoptosis compared to control animals. Apoptosis was evaluated by Terminal dUTP nick end labeling (TUNEL) staining. Arrows indicate TUNEL+ve hepatocyte nuclei ($\times 200$ final magnification).

Figure 5. Effects of binge alcohol administration on the development of microvesicular liver steatosis

(**A**) Liver hematoxylin and eosin staining ($\times 200$ final magnification). Microvesicular liver steatosis was observed in mice binged with ethanol compared to control animals. Arrows indicate the fat droplets. (**B**) Liver Oil-Red O staining demonstrated significant fat accumulation in the livers of mice exposed to binge ethanol vs control group ($\times 200$ final magnification). (**C**) Binge ethanol exposure resulted in significant hepatic triglyceride accumulation compared to control animals (48.02 ± 3.83 vs 19.90 ± 3.48 mg/g liver, $*p < 0.05$). Values are mean $\pm$ SEM, $n = 6$ animals/group. Student's t -test was used to evaluate significant differences between ethanol treated and control animals.

A **B**

Figure 6. Binge alcohol-mediated down-regulation of hepatic CPT1a expression
(A) Hepatic carnitine palmitoyltransferase 1a (*Cpt1a*) mRNA was markedly ($*p<0.05$) down-regulated in alcohol treated mice compared to control animals. Results are given as fold changes relative to control. Values are mean $\pm$ SEM, n=6 animals/group. Student's *t*-test was used to evaluate significant differences between ethanol treated and control animals. (B) Representative images of immunohistochemical staining with the anti-CPT1a antibody ($\times 200$ final magnification). The images demonstrated that hepatic CPT1a protein levels (red staining) were decreased in the livers of binge alcohol treated mice compared to control animals.

Figure 7. Binge alcohol-mediated up-regulation of hepatic FAS expression

(A) Hepatic fatty acid synthase (*Fas*) mRNA was significantly ($*p<0.05$) up-regulated in response to binge EtOH exposure. Results are given as fold changes relative to control. Values are mean $\pm$ SEM, n=6 animals/group. Student's *t*-test was used to evaluate significant differences between EtOH treated and control animals. (B) Representative images of immunohistochemical staining with the anti-FAS antibody ($\times 200$ final magnification). The images demonstrated that hepatic FAS protein levels (dark brown staining) were increased in the livers of binge EtOH treated mice compared to control animals. (C) Representative Western blot image of FAS protein expression. GAPDH was used as a loading control. (D) Quantitative analysis (n=4) demonstrated significant increase ($p<0.05$) in FAS protein levels in the livers of binge EtOH treated mice compared to control animals. The intensity of protein bands was quantified by densitometry using the NIH ImageJ software.

Figure 8. Evaluation of the role of HDAC9 in transcriptional regulation of *Fas* gene expression (A) Representative ChIP analysis images demonstrated decreased binding of HDAC9 to the *Fas* promoter region in the livers of binge ethanol treated mice compared to control animals. Chromatin was immunoprecipitated with anti-HDAC 9 antibody; rabbit IgG (RIgG) was used as a non-specific control. Diluted fraction of non-immunoprecipitated chromatin was used for input. Semi-quantitative PCR was performed to confirm the protein interaction with the target promoters. (B) Quantitative analysis of the images (n=4) revealed significant ($p < 0.05$) decrease in recruitment of HDAC 9 to the *Fas* promoter region in the livers of binge ethanol treated mice compared to control animals. The band intensity was quantified by densitometry using the NIH ImageJ software. (C) Representative image of immunohistochemical staining with the anti-HDAC 9 antibody ($\times 400$ final magnification). The images demonstrated decreased HDAC 9 protein levels in the hepatocyte nuclei in response to binge ethanol treatment compared to control animals. Arrows indicate nuclei HDAC 9 levels.