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Binge Ethanol-Induced HDAC3 Down-Regulates *Cpt1a* Expression Leading to Hepatic Steatosis and Injury

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Abstract

Background—Recently, we have demonstrated that acute alcohol exposure due to binge drinking leads to hepatic steatosis with the deregulation of hepatic histone deacetylase (HDAC) expression. Various class I, II, and IV HDACs were down-regulated, whereas expression of HDAC3 was solely up-regulated. Hence, in the present work, we specifically examined the mechanistic role of HDAC3 in the development of hepatic steatosis occurring in response to binge alcohol administration.

Methods—C57BL/6 mice were gavaged 3 times with ethanol (EtOH) at a dose of 4.5 g/kg. HDAC inhibitor, Trichostatin A (TSA) was simultaneously injected intraperitoneally at a dose of 1 mg/kg. Hepatic steatosis, injury, expression of HDAC3 and carnitine palmitoyltransferase 1 α (CPT1 α) were evaluated. HDAC3 and histone H3 acetylation levels at the *Cpt1a* promoter were analyzed by chromatin immunoprecipitation (ChIP).

Results—The binge EtOH-mediated increase in HDAC3 was prevented by simultaneous administration of HDAC inhibitor, TSA, which markedly attenuated hepatic steatosis and injury. Importantly, HDAC3 inhibition was able to normalize the down-regulation of *Cpt1a* expression. Causal role of HDAC3 in the transcriptional repression of *Cpt1a* was demonstrated by increased HDAC3 binding at the thyroid receptor element site in the *Cpt1a* distal promoter region. Further, a resultant decrease in the transcriptionally permissive histone H3 lysine 9 acetylation in the proximal promoter region near the transcriptional start site was observed. Notably, TSA treatment reduced HDAC3 binding and increased H3K9 acetylation at *Cpt1a* promoter leading to increased *Cpt1a* expression. These molecular events resulted in attenuation of binge alcohol-induced hepatic steatosis.

Conclusions—These findings provide insights into potential epigenetic mechanisms underlying transcriptional regulation of *Cpt1a* in the hepatic steatosis occurring in response to binge EtOH administration.

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

Keywords

Binge Alcohol Exposure; Liver Steatosis; HDAC3; CPT1 α

Excessive Alcohol Consumption continues to be a major public health problem in the United States with binge drinking as a common dangerous drinking pattern in the adolescent and young adult populations (Courtney and Polich, 2009; Gruzca et al., 2009). Mounting evidence demonstrates that binge drinking causes liver damage (Mathurin and Deltenre, 2009). Acute alcohol exposure due to binge drinking leads to transient hepatic steatosis which is usually benign and is reversible but can also lead to the development of steatohepatitis, fibrosis, and cirrhosis associated with chronic alcoholic liver disease (ALD) (Donohue, 2007). The pathophysiology of chronic alcohol abuse mediated steatohepatitis is well defined; however, the mechanisms underpinning steatosis following acute alcohol exposure are only beginning to be understood.

It is becoming increasingly evident that histone associated epigenetic modifications play a significant role in the development of hepatic pathology induced by chronic as well as binge alcohol exposure (Shukla and Aroor, 2006; Shukla et al., 2008). Recent studies demonstrated that binge, as well as chronic, ethanol (EtOH) consumption causes site selective modifications in histones leading to changes in histone acetylation, methylation, and downstream gene expression (Bardag-Gorce et al., 2009; Kim and Shukla, 2006; Moghe et al., 2011; Pal-Bhadra et al., 2007; Shepard and Tuma, 2009). Among the various modifications documented at the tails of histone proteins, acetylation and methylation are the best characterized. Particularly, acetylation at the lysine residues of histones represents a transcriptionally permissive state, allowing opening up of the chromatin structure and access to transcriptional machinery (Berger, 2002). Initial work carried out by Shukla's group demonstrated that EtOH caused posttranslational 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., 2003, 2005).

Steady-state levels of acetylation result from the balance between the opposing activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs). Our recent work examining the contribution of individual HDACs showed that decreased class I, II, and IV HDACs contribute to the increase in the hepatic histone H3 acetylation (H3Ac) status in response to binge EtOH treatment. Moreover, this work also revealed that although various class I, II, and IV HDACs were down-regulated, expression of HDAC3 was solely up-regulated (Kirpich et al., 2012). The present study is the extension of the mentioned above work with the goal to further pursue the understanding of the potential pathogenic role of HDAC3 in the binge alcohol-induced liver steatosis. Our findings revealed that increased HDAC3 plays a critical role in the binge alcohol-induced suppression of the carnitine palmitoyltransferase 1 α (*Cpt1a*) gene expression and development of binge alcohol-mediated hepatic steatosis.

Materials and Methods

Animals and Treatment

C57BL/6 male mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were housed in a pathogen-free barrier facility with a 12-hour dark/light cycle and free access to food and water. Eight-week-old mice, weighing 20 to 25 g, were given EtOH by gavage using a steel blunt tipped needle. Animals were gavaged with EtOH at a dose of 4.5 g/kg, 3 times with 12-hour intervals (9 AM, 9 PM, 9 AM the next day, and sacrificed 4 hours later at 1 PM). The control animals were gavaged with the equal amount of saline (control for the

effects of gavage procedure) as well as maltodextrin (MD; pair-fed control for EtOH derived calories). Animals in all the groups were not fasted and were fed ad lib over the experimental period. This protocol was developed to reflect the potential human model of binge drinking without the influence of altered metabolic parameters caused by fasting. Trichostatin A (TSA), a reversible nonspecific inhibitor of HDACs was injected intraperitoneally simultaneously with EtOH gavage at a dose of 1 mg/kg. The TSA dose employed was established experimentally based on its protective effects on the binge EtOH-induced liver injury indicated by serum alanine aminotransferase (ALT). TSA was purchased from Sigma (St. Louis, MO). The protocol for the study was approved by the University of Louisville Institutional Animal Care and Use Committee.

Biochemical Analysis

Serum ALT levels were measured according to the manufacturer's instructions using Thermo Fisher Scientific Inc. reagents (Middletown, VA). Total liver triglycerides (TGs) were extracted from mouse liver tissue and quantified using TG reagents (Thermo Fisher Scientific Inc.) as previously described (Kirpich et al., 2011). Blood alcohol levels were measured using NAD-ADH Reagent Multiple Test (Sigma) according to manufacturer's instructions.

Liver Histopathological Examination

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

Oil-Red-O Staining

To examine the amount of fat accumulation, the liver sections were stained with Oil-Red-O. Frozen liver sections were washed in phosphate buffered saline twice for 5 minutes. Oil-Red-O and 85% propylene glycol were added with agitation for 15 minutes, followed by washing in tap water.

TdT-Mediated dUTP Nick-End Labeling (TUNEL)

Liver cell apoptosis was assessed by TUNEL assay using the ApopTag Peroxidase In Situ Apoptosis Detection commercially available kit (Chemicon, Temecula, CA), according to the manufacturer's instructions. TUNEL-positive cells were quantitated by counting in 5 randomly selected fields (×200 final magnification).

Immunohistochemistry

Commercially available antibody against HDAC3 (Santa Cruz Biotechnology, Hercules, CA) and CPT1α (Proteintech Group Inc., Chicago, IL) was used for immunohistochemical analysis. Assays were performed according to the manufacturers' protocols.

Liver Nuclear Protein Extraction

Liver nuclear proteins were extracted using Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific, Rockford, IL) according to the manufacturer's protocol.

Western Blot Analysis

To analyze HDAC3 protein expression, 60 µg of nuclear proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membrane. Commercially available primary antibody for HDAC3 was purchased from Santa Cruz Biotechnology. Detection of histone H3 (Abcam Inc., Cambridge, MA) served as a loading

control. Immunoreactive signals were visualized using enhanced chemiluminescence light detection reagents (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Band intensities were quantified using ImageJ software (<http://rsb.info.nih.gov/ij/>).

Liver Chromatin Immunoprecipitation Assay

The liver chromatin immunoprecipitation (ChIP) assay was performed following the Enzymatic ChIP protocol (Cell Signaling, Danvers, MA) with slight modifications using minced tissue for formaldehyde cross-linking. Briefly, 50 mg of tissue from each mouse was minced and cross-linked in 1% formaldehyde for 15 minutes. After 2 washes, cells were subjected to nuclear lysis and sonication. The lysates were incubated overnight with respective antibody followed by immunoprecipitation of antibody/protein complexes with magnetic beads and 1 wash each with low salt, high salt, LiCl (lithium chloride), and Tris EDTA buffers. After reverse cross-linking and purification, pure DNA was eluted in 1% SDS, 0.1 M NaHCO₃. Chromatin was immunoprecipitated with anti-HDAC3 (Santa Cruz Biotechnology), anti-NCoR (Thermo Scientific/Pierce Biotechnology, Rockford, IL), and anti-H3Ac (Cell Signaling). Two primer pairs were used for ChIP PCR: *Cpt1a* TRE and *Cpt1a* (-1). The *Cpt1a* TRE ChIP primer is located at the thyroid receptor element (TRE) site in the *Cpt1a* upstream promoter region and was previously described and extensively studied (Alenghat et al., 2008). The *Cpt1a* TRE ChIP primer was used for quantification of HDAC3 and N-CoR binding at the TRE site. A commercially available *Cpt1a* (-1) ChIP primer (SA Biosciences, Frederick, MD) was used for quantification of H3Ac in the proximal promoter region. Semiquantitative ChIP PCR was performed using DNA Thermal Cycler 480 System, with input DNA as a reference control. Twenty-nine PCR cycles were used for input, HDAC3, N-CoR, and H3Ac.

Cell Culture and Treatments

Human hepatoma HepG2 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, penicillin 100 IU/ml, and streptomycin 100 µg/ml. Transient transfections in these cells were performed 1 day after plating using FuGENE HD transfection reagent (Promega, Madison, WI). The plasmids, human pcDNA3-Flag-HDAC1, and human pcDNA3-Flag-HDAC3 were purchased from Addgene (Cambridge, MA). These plasmids have been used and described previously (Emiliani et al., 1998). Transfection mixes were prepared according to manufacturer's recommendations. Briefly, 2 µg plasmid DNA and 4 µl FuGENE HD transfection reagent were combined in 200 µl of DMEM and incubated for 30 min at room temperature. The mix was used to transfect HepG2 cells in 6 well plates (1 × 10⁶ cells in 2 ml culture medium). Total RNA was extracted 24 hours after transfection and used for *Cpt1a* reverse transcription quantitative polymerase chain reaction (RT-qPCR) assay.

RNA Isolation and Real-Time PCR Analysis

Total RNAs were isolated from liver tissue and cell cultures using TRIzol reagent (Invitrogen, Carlsbad, CA) and treated with DNase I to remove any contaminating genomic DNA (RQ1 RNase-Free DNase; Promega). For RT-qPCR, 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). Primers specific for *Hdac1*, *Hdac3*, *Hdac4*, *Hdac6*, *Hdac10*, *Cpt1a*, and peroxisomal proliferator-activated receptor gamma co-activator 1 alpha (*Pgc-1a*) mRNA, and 18s rRNA were purchased from SA Biosciences.

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).

Statistical Analysis

Data are expressed as mean $\pm$ standard error of the mean (SEM). Statistically significant differences were determined by 1-way analysis of variance (ANOVA) followed by Tukey's honest significance test, which compares all possible pairs of means and is based on a studentized range distribution with control of the experimental error rate at 5% (Ravishanker and Dey, 2002). $P < 0.05$ was considered statistically significant. Statistical analysis was performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, Inc., La Jolla, CA).

Results

Increased Hepatic HDAC3 Expression in Response to Binge EtOH Administration

Recent studies demonstrated that alcohol induces epigenetic modifications leading to changes in histone acetylation, methylation, and downstream gene expression (Bardag-Gorce et al., 2009; Moghe et al., 2011; Pal-Bhadra et al., 2007; Shepard and Tuma, 2009). It has been demonstrated that acute EtOH exposure causes histone H3 hyperacetylation through modulation of activity of HATs (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. Our recent work screening hepatic class I, II, and IV *Hdac* mRNA in the binge EtOH animal model showed that *Hdacs 1, 7, 9, 10, and 11* were significantly down-regulated and only *Hdac3* was up-regulated upon binge EtOH exposure (Kirpich et al., 2012). Recent work has shown that HDAC3 plays a significant role in the regulation of hepatic lipid metabolism (Feng et al., 2011). Hence, in the present work, we specifically examined the pathogenic role of increased HDAC3 expression in the development of hepatic steatosis and injury in response to binge EtOH exposure. Because only *Hdac3* expression was increased in response to binge EtOH administration, we hypothesized that its inhibition may prevent/attenuate liver steatosis and injury. Inhibition of HDAC3 was achieved by treating animals with TSA, a known HDAC-specific inhibitor.

We evaluated the effect of TSA treatment on hepatic HDAC3 gene as well as protein expression levels in animals exposed to the binge EtOH regimen. In our binge EtOH model, EtOH (4.5 g/kg body weight) was administered by oral gavage, 3 times, 12 hours apart. The animals were sacrificed at 4 hours after the third EtOH gavage, and typically yielded blood alcohol levels at $0.24 \pm 0.02\%$. TSA was administered intraperitoneally at a concentration of 1 mg/kg immediately after each binge challenge. Control baseline observations were obtained from animals gavaged with saline as well as MD providing equivalent EtOH calories. No statistically significant difference was observed between control saline and control MD gavaged animals in terms of major outcomes of this study (liver histology, *Hdac3* and *Cpt1a* expression) (Fig. S1). Accordingly, the baseline data from the saline gavaged animals have been used for comparative analysis.

Binge EtOH exposure up-regulated *Hdac3* gene expression (2-fold, $p < 0.05$), and this increase was prevented by TSA treatment (Fig. 1A). Correspondent to *Hdac3* mRNA expression levels, HDAC3 protein levels were also increased upon EtOH treatment, as shown by Western blot analysis (Fig. 1B,C). Immunohistochemical analysis demonstrated that HDAC3 protein levels were elevated in hepatocyte nuclei in response to binge EtOH exposure, and this effect was markedly decreased by TSA treatment (Fig. 1D).

Because TSA inhibits the activity of both class I and II HDACs, its effect on other representative class I and II HDACs (HDACs 1, 4, 6, and 10) was also evaluated. The HDAC mRNA expression observed in binge EtOH-treated animals as well as saline control animals was not affected by TSA treatment (Fig. S2); further, commensurate with its HDAC inhibitory function TSA treatment was observed to decrease total hepatic HDAC activity (Fig. S3).

Increased Hepatic HDAC3 Expression is Associated with Microvesicular Liver Steatosis and Liver Injury which are Significantly Attenuated by HDAC Inhibition

Next, to determine whether increased HDAC3 expression plays a pathogenic role in alcohol-mediated liver pathology, we examined the effect of its inhibition on binge EtOH-induced liver steatosis. As expected, liver histological examination revealed microvesicular liver steatosis in response to binge EtOH exposure in comparison to control (Fig. 2A). Further, Oil-Red-O staining demonstrated significantly increased hepatic fat accumulation in the livers of EtOH-treated mice (Fig. 2B). Liver steatosis was strikingly attenuated by TSA treatment which was confirmed by biochemical assessment of TG accumulation (48.02 ± 3.83 mg/g liver in EtOH vs. 19.9 ± 3.48 mg/g liver in control, and vs. 24.4 ± 2.09 mg/g liver in EtOH+TSA-treated groups, $p < 0.05$; Fig. 2C).

To detect liver injury in response to binge EtOH exposure, we measured serum ALT activity. As shown in Fig. 3A, elevated ALT activity was found in alcohol-treated mice compared to control animals, and attenuated by TSA treatment (51.98 ± 6.19 U/l in EtOH vs. 20.80 ± 0.62 U/l in control, and vs. 25.46 ± 2.95 U/l in EtOH+TSA-treated animals, $p < 0.05$). In correspondence to elevated ALT activity, the number of apoptotic TUNEL-positive hepatocytes was observed to be greater in EtOH exposed mice compared to control and EtOH+TSA-treated animals ($4.36 \pm 0.55\%$ vs. $0.41 \pm 0.13\%$ and $1.67 \pm 0.19\%$, respectively, $p < 0.05$; Fig. 3B). Prevention of hepatic steatosis and injury by TSA-mediated HDAC inhibition strongly suggest that increased HDAC3 expression plays a significant pathogenic role in our model.

Microvesicular Liver Steatosis in Response to Binge EtOH Exposure is Associated with Decreased CPT1 α mRNA and Protein Levels

Because we obtained compelling evidence for the involvement of HDAC3 in binge EtOH-induced hepatic steatosis, we then examined the potential targets of HDAC3 regulation. In this regard, it has been shown that HDAC3 plays a critical role in influencing circadian cellular metabolism by regulating CPT1 α expression, a key enzyme in free fatty acid β -oxidation (Alenghat et al., 2008). Additionally, earlier work has shown that the EtOH-mediated decrease of *Cpt1a* expression plays a major role in the development of microvesicular steatosis (Ajmo et al., 2008; Jeong et al., 2008; Kang et al., 2009; Zhang et al., 2010). Hence, the effects of binge EtOH administration and TSA treatment on CPT1 α expression were evaluated.

In agreement with earlier observations, binge EtOH administration significantly suppressed hepatic *Cpt1a* mRNA expression and also led to a decline in CPT1 α protein expression (Fig. 4A,D). Notably, TSA treatment reversed EtOH-induced inhibition of *Cpt1a* expression, strongly indicating the involvement of HDAC3 in binge EtOH-mediated deregulation of *Cpt1a*. Moreover, because *Cpt1a* is the rate limiting enzyme in mitochondrial free fatty acid β -oxidation, its repression by increased HDAC3 could play a significant role in the development of binge alcohol-induced liver steatosis. It is important to note that transcriptional regulation of *Cpt1a* involves several transcriptional factors, including peroxisomal proliferator-activated receptor alpha (PPAR α), and *Pgc-1 α* (Napal et

al., 2005; Song et al., 2004). Examination of *Pgc-1 α* expression demonstrated that TSA significantly increased *Pgc-1 α* mRNA levels in the livers of EtOH binged mice (Fig. 4B).

To further evaluate the causal role of HDAC3 in the transcriptional repression of *Cpt1 α* , we examined the effect of HDAC3 overexpression on *Cpt1 α* mRNA expression in a human hepatoma cell line (HepG2 cells). Transient transfections in HepG2 cells were performed with pcDNA3-Flag-HDAC3 plasmid. Total RNAs were extracted 24 hours after transfection and used for RT-qPCR. The data obtained showed that the basal levels of *Cpt1 α* mRNA were significantly decreased by the overexpression of HDAC3 (Fig. 4C). These data further support the mechanistic role of HDAC3 in the down-regulation of *Cpt1 α* gene expression.

Evaluation of the Role of HDAC3 in the Transcriptional Regulation of *Cpt1 α* Gene Expression

HDAC3 is known to partner with other HDACs and nuclear repressors like N-CoR to coordinate transcriptional repression of target gene expression (Karagianni and Wong, 2007; Li et al., 2000). Particularly, in the context of *Cpt1 α* gene expression, the N-CoR/HDAC3 complex has been shown to interact with the TRE in the *Cpt1 α* promoter region (Fig. 5A) (Alenghat et al., 2008). Further, HDAC3-mediated transcriptional repression involves the deacetylation of promoter-associated histones. Hence, in the livers of animals exposed to binge EtOH challenge with and without TSA treatment, we examined the *Cpt1 α* promoter region for N-CoR and HDAC3 binding as well as *Cpt1 α* -promoter-associated H3Ac status by ChIP analysis. The data obtained showed that binge EtOH treatment led to an increase in HDAC3 binding at the TRE site in the *Cpt1 α* distal promoter region (Fig. 5B). In comparison, EtOH+TSA treatment prevented the increase in HDAC3 binding in response to EtOH administration (Fig. 5B). Interestingly, the levels of N-CoR binding were found to be unaffected by either EtOH or TSA treatment (Fig. 5C). Further, evaluation of the *Cpt1 α* -promoter-associated H3Ac showed that the pattern of histone acetylation was consistent with the observed HDAC3 binding. It was decreased in binge EtOH-treated animals and increased in response to TSA treatment (Fig. 5D).

Discussion

Currently, very little is known about the role of individual HDACs in alcohol-induced liver steatosis and injury. In this regard, our recent work demonstrated that binge alcohol consumption solely increased HDAC3 expression (Kirpich et al., 2012). Hence, in the present work, we pursued the potential pathogenic role of HDAC3 in the binge EtOH-mediated hepatic steatosis and injury. In the context of liver specific HDAC functions, HDAC3 has been recently shown to play a pivotal role in liver homeostasis and development. Specifically, lack of HDAC3 in zebrafish leads to abnormalities in liver development, 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; Farooq et al., 2008; Feng et al., 2011). To date, however, no studies have examined the role of HDAC3 in the binge alcohol-induced hepatic steatosis and injury.

Recent work has shown that transcriptional repression mediated by the N-CoR-HDAC3 corepressor complex binding at the TRE underlies inhibition of *Cpt1 α* gene expression which plays a critical role in regulating hepatic β -oxidation of fatty acids impacting hepatic TG accumulation (Alenghat et al., 2008). Hence, the essential role of increased HDAC3 in the binge alcohol-mediated down-regulation of hepatic *Cpt1 α* gene expression was investigated in our study by examining the effect of the HDAC-specific inhibitor TSA. The increased levels of hepatic *Hdac3* mRNA expression caused by binge EtOH treatment correlated with the increased association of HDAC3 at the *Cpt1 α* N-CoR/HDAC3 TRE

element (-3045 to -2985) and a correspondent decrease in H3Ac at the *Cpt1a* promoter region closer to the transcriptional start site.

Gene expression is dynamically regulated by chromatin modifications on histone tails, and histone acetylation promotes transcription, whereas histone deacetylation negatively regulates transcription (Khan and Khan, 2010). Accordingly, increased hepatic levels of *Hdac3* and HDAC3 binding and concomitant decrease in the promoter-associated H3Ac in the binge EtOH-treated animals correlated with a decrease in the transcriptional competence of the hepatic *Cpt1a* expression. Notably, inhibition of HDAC3 activity by TSA decreased HDAC3 binding at the *Cpt1a* TRE site, increased promoter-associated H3Ac and rescued and normalized *Cpt1a* expression (Figs 4 and 5). These data strongly suggest a critical role for HDAC3 in the binge EtOH-induced transcriptional repression of *Cpt1a*. Because *Cpt1a* catalyzes a rate limiting step in mitochondrial free fatty acid β -oxidation, repression by increased HDAC3 could play a significant role in the development of binge alcohol-induced liver steatosis. As has been observed earlier, a global decrease in HDAC activity (Kirpich et al., 2012) and an increase in histone acetylation occurs in binge EtOH-treated animals (Choudhury and Shukla, 2008; Kendrick et al., 2010). Hence, the present data in relation to HDAC3 and regulation of *Cpt1a* promoter-associated histone acetylation underscores the relevance of examining localized genespecific histone modifications in order to identify specific pathogenic mechanisms in binge alcohol-induced hepatic steatosis and injury.

In relation to alcohol-induced hepatic steatosis contribution of Sirt1, which is a class III atypical HDAC and is distinct from class I and II HDACs, has been reported (You et al., 2008a,b). It should be noted that perturbations in Sirt1 expression have been observed only in response to chronic EtOH feeding (Bardag-Gorce et al., 2009; You et al., 2008a). In contrast, binge EtOH treatment does not have any effect on Sirt1 expression (Bardag-Gorce et al., 2009). Further, TSA treatment which markedly attenuated HDAC3 expression and hepatic steatosis and injury is a HDAC class I and class II inhibitor and has no effect on Sirt1 (Imai et al., 2000; Pagans et al., 2005). Taken together, it is highly likely that deregulation of HDAC3 and *Cpt1a* expression in response to binge EtOH treatment plays a major role in the development of hepatic steatosis and injury. Hepatic TG accumulation and steatosis can occur not only as a consequence of decreased β -oxidation of fatty acids but also increased fatty acid synthesis. 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). Hence, up-regulation of lipogenesis could also play a significant role in the development of hepatic steatosis in our model. Indeed, hepatic expression of fatty acid synthase (*Fas*) was significantly up-regulated in response to binge EtOH treatment (Kirpich et al., 2012); however, there was no statistically significant decrease in *Fas* gene expression in response to TSA treatment (data not shown). These observations strongly suggest that normalizing HDAC3 repressed *Cpt1a* expression was sufficient to markedly attenuate binge EtOH-induced hepatic TG accumulation. These findings are also supported by the recent studies examining the direct role of *Cpt1a*-mediated increased fatty acid oxidation (Stefanovic-Racic et al., 2008). Specifically, these studies showed that a moderate increase in *Cpt1a* expression and activity is able to substantially reduce the hepatic TG burden on either a standard or high-fat diet.

Besides relieving the transcriptional repression possibly caused by increased HDAC3 expression and decreased H3Ac, TSA-mediated HDAC3 inhibition was also observed to increase the expression of *Pgc-1 α* . PGC-1 α is the critical transcription factor that has been shown to regulate hepatic *Cpt1a* gene expression (Zhang et al., 2004). Importantly, recent work has demonstrated that the transcriptional activation of PGC-1 α expression in hepatocytes is also suppressed by the N-CoR-HDAC3 corepressor complex (Wu et al., 2009). Hence, the increase in hepatic *Pgc-1 α* mRNA expression observed in EtOH+TSA-

treated animals could be due to HDAC3 inhibition. The increase in *Pgc-1α* observed in response to TSA is highly relevant because it correlates with the concomitant increase in *Cpt1α* gene expression and attenuation of hepatic steatosis.

The observed effect of inhibition of HDAC activity in binge alcohol-treated animals by TSA, in agreement with other recently published reports, supports the critical regulatory role of HDAC3 in the coordinated expression of *Pgc-1α* and *Cpt1α* and consequent development of hepatic steatosis and injury. Interestingly, inhibition of HDAC activity by TSA also decreased the binge EtOH-induced as well as the baseline hepatic *Hdac3* mRNA expression (Fig. 1). Hence, the observed effects of HDAC activity inhibition by TSA could occur due to a lack of HDAC3 function caused by TSA or a net decrease in its expression caused by TSA or both. This effect of TSA on *Hdac3* gene expression could possibly occur through an as yet unknown negative feedback mechanism. This feedback mechanism could be speculated to occur via a negative regulatory protein (NRP) whose expression is affected by HDAC3. Accordingly, EtOH could decrease the expression of NRP which would then lead to an increase HDAC3 expression. TSA treatment which blocks HDAC3 activity would result in up-regulation of NRP that then interact with HDAC3 promoter leading to reduction/normalization of HDAC3 mRNA and protein expression.

Overall, the findings from this study suggest that increased HDAC3 expression and resultant decrease in *Cpt1α* promoter-associated histone acetylation, and CPT1α expression play a major role in the binge EtOH-induced hepatic steatosis (Fig. 6). Thus, derepression and normalization of *Cpt1α* and hepatic fatty acid oxidation capacity by targeting HDAC activity represents a potential therapeutic approach in the treatment of binge alcohol-induced hepatic steatosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Overexpression of liver histone deacetylase 3 (HDAC3) in response to binge ethanol (EtOH) treatment. **(A)** *Hdac3* mRNA was up-regulated in the livers of EtOH-treated animals compared to control and Trichostatin A (TSA)-treated mice. *Hdac3* mRNA was analyzed by RT-qPCR and normalized to *18s* rRNA. Data are expressed as mean $\pm$ SEM, $n = 6$ to 8 animals/per group. $*p < 0.05$ was considered statistically significant (1-way ANOVA with Tukey's honest significance test). **(B)** Western blot analysis demonstrating increased HDAC3 protein levels in the livers of binge EtOH-treated mice. Expression of H3 total was used as a loading control. **(C)** Quantitative analysis of HDAC3 protein expression. The intensity of protein bands (from **B**) was quantified by densitometry using the ImageJ software (NIH, Bethesda, MD). **(D)** Immunohistochemical staining with anti-HDAC3 antibody demonstrating HDAC3 elevation in the hepatocyte nuclei in response to binge EtOH treatment, which was attenuated by TSA ($\times 200$ final magnification).

Fig. 2. Effects of histone deacetylase (HDAC) inhibitor, Trichostatin A (TSA), on binge alcohol-induced liver steatosis and injury. **(A)** Liver hematoxylin and eosin staining (H&E, $\times 200$ final magnification). Binge ethanol (EtOH) exposure resulted in microvesicular fat accumulation in the livers of C57BL/6 mice. HDAC inhibition (TSA treatment) attenuated EtOH-induced liver steatosis. Arrows indicate the fat droplets. **(B)** Oil-Red-O staining demonstrated significant fat accumulation in the liver of mice exposed to binge EtOH versus control and EtOH+TSA-treated animals ($\times 200$ final magnification). **(C)** Liver triglycerides (TGs), assessed by biochemical assay, were significantly elevated in binge EtOH-treated mice and attenuated by TSA treatment (48.02 ± 3.83 vs. 24.47 ± 2.09 mg/g liver). Data are expressed as mean $\pm$ SEM, $n = 6$ to 8 animals/per group. $*p < 0.05$ was considered statistically significant (1-way ANOVA with Tukey's honest significance test).

Fig. 3.

Effects of histone deacetylase inhibitor, Trichostatin A (TSA), on binge alcohol-induced liver injury. **(A)** ALT activity was significantly elevated in alcohol-treated mice compared to control animals and markedly attenuated by TSA treatment (51.98 ± 6.19 vs. 20.80 ± 0.62 and 25.46 ± 2.95 U/l, respectively). Data are expressed as mean $\pm$ SEM, $n = 6$ to 8 animals/ per group. $*p < 0.05$ was considered statistically significant (1-way ANOVA with Tukey's honest significance test). **(B)** Mice gavaged with ethanol (EtOH) had significantly increased liver apoptosis compared to control and EtOH+TSA animals (4.36 ± 0.55 vs. 0.41 ± 0.13 and vs. $1.67 \pm 0.19\%$, respectively). Data are expressed as mean $\pm$ SEM, $n = 5$ to 6 animals/ per group. $*p < 0.05$ was considered statistically significant (1-way ANOVA with Tukey's honest significance test).

Fig. 4.

Binge ethanol (EtOH)-induced down-regulation of liver *Cpt1a* and *Pgc-1a* levels. **(A)** Liver *Cpt1a* mRNA was significantly down-regulated in alcohol-treated animals, which was restored by Trichostatin A (TSA). **(B)** Liver *Pgc-1a* mRNA levels were elevated in EtOH-treated animals compared to control mice. *Cpt1a* and *Pgc-1a* mRNA were analyzed by RT-qPCR and normalized to *18s* rRNA. Data are expressed as mean $\pm$ SEM, $n = 6$ animals/per group. $*p < 0.05$ was considered statistically significant (1-way ANOVA with Tukey's honest significance test). **(C)** Histone deacetylase 3 (HDAC3) overexpression in a human hepatoma cell line (HepG2 cells) resulted in down-regulation of *Cpt1a* mRNA expression. HepG2 cells were transiently trans-fected with human pcDNA3-Flag-HDAC3 for 24 hours. Total RNA from cell cultures was isolated using TRIzol reagent, *Cpt1a* mRNA expression was analyzed by RT-qPCR. Data are presented as mean $\pm$ SEM. Results are from 3 independent experiments. $*p < 0.05$ was considered statistically significant. **(D)** Immunohistochemical staining with anti-CPT1 α antibody (9200 final magnification). The images demonstrated that hepatic CPT1 α protein levels (staining) were decreased in the livers of binge alcohol-treated mice and restored by TSA treatment.

Fig. 5. Evaluation of the role of histone deacetylase 3 (HDAC3) in the transcriptional regulation of *Cpt1a* gene expression. **(A)** Schematic representation of a mouse *Cpt1a* promoter. Locations of chromatin immunoprecipitation (ChIP) quantitative polymerase chain reaction (qPCR) primer pairs for analysis of HDAC3, N-CoR binding, and histone acetylation in the vicinity of the mouse *Cpt1a* promoter. The coordinate locations shown are with respect to the transcription start site (TSS) in REFSEQ NM_013495.2. **(B)** Increased HDAC3 recruitment to the *Cpt1a*-TRE promoter region in the livers of binge ethanol (EtOH)-treated mice compared to control and Trichostatin A (TSA)-treated animals. **(C)** N-CoR binding to the *Cpt1a*-TRE promoter region was unaffected by either EtOH or TSA treatment. **(D)** Histone acetylation at the *Cpt1a* (-1) region was decreased in the livers of binge EtOH-treated animals and increased in response to TSA treatment. Chromatin was immunoprecipitated with anti-HDAC3, anti-N-CoR, anti-histone H3 acetylation (H3Ac), and ChIP qPCR was performed to confirm the protein interaction with the target promoters. The "input" lanes represent the results of PCR using diluted fractions of nonimmunoprecipitated chromatin as templates. Densitometry analysis was performed to evaluate specific binding of each protein relative to input. HDAC3 and N-CoR recruitment to the *Cpt1a* promoter was analyzed using *Cpt1a*-TRE site. Histone acetylation was analyzed at the *Cpt1a* (-1) site. For semi-qPCR of ChIP assay 29, PCR cycles were used for input, HDAC3, N-CoR, and H3Ac.

Fig. 6.

Model of the pathogenic role of Histone deacetylase 3 (HDAC3) in the development of binge ethanol (EtOH)-induced hepatic steatosis. **(A)** Physiologic expression of HDAC3 in the liver leads to the normal constitutive levels of acetylation of the promoter-associated histones and *Cpt1a* mRNA expression. **(B)** Binge EtOH-induced increase in hepatic HDAC3 expression leads to decreased acetylation of promoter-associated histones, repression of *Cpt1a* mRNA expression, and steatosis. **(C)** Inhibition of binge EtOH-induced HDAC3 activity restores acetylation levels of the promoter-associated histones and derepresses *Cpt1a* mRNA expression and markedly attenuates hepatic steatosis.