Histone H3 Phosphorylation (S10, S28) and Phosphoacetylation (K9/S10) are Differentially Associated With Gene Expression In Liver of Rats Treated In Vivo With Acute Ethanol

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Running title: In vivo histone H3 phosphorylation in liver by acute ethanol

Number of text pages:

Number of tables: 2

Number of figures: 11

Number of references: 39

Number of words in the abstract: 223

Number of words in the introduction: 520

Number of words in discussion: 1121

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List of nonstandard abbreviations: ADH-1: Alcohol dehydrogenase-1; ALD: alcoholic liver disease; ALT: alanine aminotransferase; BAC: blood alcohol concentration; ChIP: chromatin immunoprecipitation; H3-K9/S10: histone H3-lysine9/serine-10; H3-S10: histone H3-serine 10; H3-S28: histone H3-serine 28; IP: intraperitoneal; MKP-1: mitogen activated protein kinase phosphatase-1; PAI-1: plasminogen activator inhibitor-1; TSA: trichostatin A.
Abstract: The epigenetic histone modification by ethanol is emerging as one of the mechanisms for its deleterious effects in the liver. In this context, we have investigated the role of histone H3 phosphorylation at serine-10 (P-H3-S10), and serine 28 (P-H3-S28) in liver after acute ethanol treatment in vivo. Ethanol was administered intraperitoneally (IP) in male Sprague–Dawley rats. Ethanol dose response (1-5g/ Kg body weight) and time course (1-4 h) experiments were conducted and various parameters were monitored. Steatosis, and necrosis (serum ALT) of the liver increased in 4h suggesting liver injury. There were differences between P-H3-S10 and P-H3-S28 at 1h, the latter was more sensitive to lower ethanol doses. Interestingly, phosphorylation of both serines disappeared at the highest dose used (5g/Kg). We also examined phosphoacetylation of histone H3 at K9/S10 and observed a dramatic increase. The changes in histone H3 phosphorylation, and phosphoacetylation were also accompanied with expression of early response genes (c-Fos, c-Jun, MKP-1). Chromatin immunoprecipitation (ChIP) assays in samples from 1.5h and 4h of ethanol administration indicated that increased histone H3 phosphorylation at serine 28 was associated with the promoters of c-Jun and PAI-1. In conclusion, this study demonstrates for the first time that in vivo exposure of liver to acute ethanol induced phosphorylation, and phosphoacetylation of histone H3, and these modifications are differentially involved in the mRNA expression of genes.
Introduction

Alcohol addiction results in alcoholic liver disease (ALD) where the pathology is identified by steatosis (fatty liver), steatohepatitis, cirrhosis (fibrosis of the liver) and at times it progresses to hepatocellular carcinoma (Purohit et al., 2009). Repeated acute alcohol intake promotes phenotypic changes in the liver pathology; however the molecular and cellular events underlying acute ethanol induced liver injury are not clearly defined.

The epigenetic histone modifications by ethanol may be one of these mechanisms. We have previously shown that ethanol promotes the acetylation of histone H3 at lysine 9 leading to the upregulation of the ADH1 gene in liver (Park and Shukla, 2005). Moreover, we have reported ethanol induced histone H3 phosphorylation at serine 10 and serine 28 mediated by p38 MAPK in primary culture of rat hepatocytes (Lee and Shukla, 2007). Studies have shown that there may be a link between acetylation and phosphorylation of histone H3 (Grant, 2001), since some histone acetyltransferases (HATs) GCN5, PCAF, and p300 have preferences for phosphorylated histone H3 (Koch et al 2000; Merienne et al 2001; Cheung et al 2000; Lo, et al 2000; Clayton, et al 2000). It is believed that the phosphorylation moiety may serve as a docking site for HATs to bind and acetylate histone H3 (Nowak and Corces, 2004). It remains unknown if ethanol induced histone acetylation and phosphorylation are related.

Histone phosphorylation is mostly known to occur during mitosis and is involved in the expression of early genes such as c-Fos, c-Jun and c-Myc, but the role of histone
phosphorylation in other cell processes is not well defined. When c-Fos is upregulated it can form a heterodimer with c-Jun or Jun-D to become the AP-1 transcription factor and initiate gene expression. (Clayton et al, 2000). The activation of the MAP kinases ERK1/2 and JNK can increase the mRNA expression of c-Fos and c-Jun respectively, and hence AP-1 activation (Clayton and Mahadevan, 2003). MAP kinases are implicated in alcoholic liver disease as they regulate inflammation, steatosis, apoptosis and necrosis (Aroor and Shukla, 2004; Boutrous, 2008; Brown and Sacks, 2008). Ethanol is known to activate AP-1 in HepG2 cells (Roman et al, 1999), and chronic ethanol feeding also increases AP-1 in the liver (Wang et al, 1998). The activation of c-Fos is known to be associated with histone H3 S10 phosphorylation in neurons by drugs such as cocaine (Tsankova et al, 2007). However, in terms of ethanol and liver in vivo, the association of AP-1 and histone phosphorylation has not been determined. This may give insight into ALD because AP1 is a regulator of inflammatory genes such as PAI-1 (Stroschein, 1999; Mertens et al, 2006).

Recently, histone H3 phosphorylation has also been at the forefront of hepatocellular carcinoma where inhibition of histone H3 phosphorylation (with an Aurora kinase inhibitor) was correlated with apoptosis of cancer cells (Aihara et al, 2010). We have reported that in vivo intraperitoneal acute ethanol promotes histone acetylation at K9 and phosphorylation at serine 10 and 28 (Aroor et al, 2010). We have now investigated the characteristics of histone H3 phosphorylation (S10, S28) and phosphoacetylation (K9/S10) in vivo and examined their mechanistic relevance to gene expression.

**Materials and methods**

**Reagents**
Antibodies to P-H3-S10, P-H3-S28, Ac-H3-K9, and H3 protein were purchased from Millipore (Temecula, CA). Antibody for phosphoacetyl K9/S10 (i.e. anti-acetyl-Lys 9/ phospho-ser-10 histone H3, Cat # 9711) and antibody to cleaved caspase-3 were obtained from Cell Signaling (Danvers, MA). Oligonucleotides were designed using Primer 3 and Primer blast and were obtained from Integrated DNA Technology Inc. (IDT, Coralville, IA). TriZol Reagent was purchased from Invitrogen (Carlsbad, CA) and Qiagen RNeasy Midi column, RNase free DNase, and Qiaquick PCR spin columns (cat # 28104) were obtained from Qiagen (Valencia, CA). High Capacity Reverse Transcription Kit was purchased from Abcam (Cambridge, MA). Reagents for quantitative real time PCR (qtPCR) were obtained from Bio-Rad (Hercules, CA). Protease inhibitor cocktail (P8340), phosphatase inhibitor cocktail (P2850), anti β-actin antibody were obtained from the Sigma-Aldrich (St. Louis, MO).

**Acute ethanol administration**

Twelve week old male Sprague–Dawley rats, with weights ranging from 300-550 g, were purchased from Harlan Laboratories (Indianapolis, IN) and were maintained on a 12-h/12-h light/dark cycle. All animals were allowed free access to water and standard laboratory rat chow. Rats were acclimated to surroundings for 1 week prior to experiments. For the dose response experiments ethanol (volume 7.5 ml or less) was administered intraperitoneally (32%, v/v in water in dosages of 1, 1.75, 2.5, 3.5, and 5 g/kg body weight) and the liver was removed after 1h. Control animals were administered equal volume of water. To assess the effects of ethanol on histone H3 phosphorylation at different time points, 3.5g/kg ethanol or water (control) was injected into rats and the liver was removed after 1.5h, 2h, 3h, and 4h. At the time of liver removal, blood samples were collected for blood alcohol and serum ALT analysis. A section of
liver was placed in formalin and the remaining sections of the liver were frozen in liquid nitrogen and stored at -80°C. This study was in accordance with the Guide for the Care and Use of Laboratory Animals published by National Institutes of Health and the protocol for their use was approved by the University of Missouri Animal Care & Use Committee.

**Serum Ethanol And ALT Analysis**

An alcohol dehydrogenase assay kit from Genzyme Diagnostics (Framingham, MA) was used to determine blood alcohol levels. Serum alanine aminotransferase (ALT) were measured by kinetic ALT assay in an automated analyzer.

**Histopathology**

The formalin fixed liver sections were sectioned and stained with hematoxylin and eosin (H&E) and analyzed by light microscopy.

**Preparation of Nuclear Extracts**

Nuclear protein extracts were obtained according to the methods detailed below and all steps were carried out at 4°C. One gram of frozen liver was homogenized in 0.25M sucrose lysis buffer containing 50 mM Tris-HCl pH 7.4, 25 mM KCl, 5 mM MgCl₂, 1 mM β-glycerophosphate, 1 mM EDTA, 1 mM Na-orthovanadate, 1 mM EGTA, 1 mM DTT, 100 nM trichostatin A (TSA), 5 mM sodium butyrate, 10 mM sodium fluoride (NaF), 2.5 mM sodium pyrophosphate, and 1X Sigma protease inhibitor cocktail (P8340). The homogenate was centrifuged at 1600 g for 10 min at 4°C. The supernatant was saved for cytoplasmic extract and stored at -80 °C until further analysis. The pellet was resuspended and washed in 0.25 M sucrose...
buffer. After a second centrifugation at 1600 g for 10 min at 4 °C, the pellet was resuspended in
1.35 M sucrose containing 0.3 % NP-40. After passage through a 22-gauge needle 3 times, the
nuclear suspension was subjected to another round of centrifugation at 1600 g for 10 min to
remove most of the cytosolic components. The nuclear pellet was then re-suspended in 1.35 M
sucrose, divided into four aliquots and collected by microcentrifugation at 16,000 g for 1 min.
The nuclear fractions were examined under light microscope for purity of nuclei (Lee and
Shukla, 2007). The nuclear pellets were flash frozen in liquid nitrogen and stored at -80 °C until
further analysis. For lysis of nuclei, an aliquot of each sample was thawed on ice and solubilized
in high salt detergent buffer containing urea (4 M urea, 0.45 M NaCl, 50 mM Tris pH 7.4, 2 mM
EDTA, 1 mM DTT, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 10 mM NaF, 2.5 mM Na
pyrophosphate, 1 mM Na-orthovanadate, 100 nM TSA, 5 mM Na-butyrate, 1 mM
β−glycerophosphate, 1X Sigma protease inhibitor cocktail P8340, and 1X Sigma phosphatase
inhibitor cocktail P2580). The nuclear preparations were sonicated three times for 5 sec. After
centrifugation at 16000 g for 10 min, the supernatant was used as nuclear fraction. Protein
concentrations in nuclear extracts were measured using the Bio-Rad Dc protein assay.

**Preparation of Cytoplasmic Extracts**

The cytoplasmic extracts from above were thawed on ice and 9 volumes were added to 1 volume
of 10% SDS to get a 1% SDS extract. Samples were boiled for 10 mins. After cooling at room
temperature, samples were sonicated 3 times for 5 secs then centrifuged at 12,000g for 10 mins.
The supernatant was kept as cytoplasmic extract. This extract (80μg) was used for the analysis of
cleaved caspase 3.
**Immunoblot analysis**

Nuclear extracts (30 µg of protein) were separated in a 15% SDS-PAGE and electrophoretically transferred onto nitrocellulose membrane (Bio-Rad, Hercules, CA) using Bio-Rad Trans-Blot apparatus. Membranes were washed with 1X TBST (20 mM Tris, pH 7.4, containing 0.1% Tween 20 and 150 mM NaCl) and incubated with 1X TBST containing 10% non-fat dry milk for 1 h at room temperature. The membrane was then incubated overnight at 4°C with antibody to phosphorylated, phosphoacetylated, acetylated, total histone H3, or cleaved caspase 3. After washing with TBST, the membrane was incubated with secondary antibody conjugated to horseradish peroxidase for 1 h at room temperature. The horseradish peroxidase was detected by enhanced chemiluminescence (ECL, Supersignal, Pierce Chemical, Rockford, IL). The membrane was scanned with a LAS-3000 imaging system (Fujifilm Life Science). The data was quantified with Multi Gauge™ software and was done within the linear range of detection. Total histone H3 protein levels in the nuclear extracts and β-actin levels in cytoplasmic extracts were used to monitor equal loading of proteins. Levels of histone H3 and β-actin were not altered after acute ethanol exposure and were used for data normalization.

**RNA Isolation and qRT-PCR**

Frozen liver was weighed and homogenized in TriZol Reagent according to manufacturer’s protocols. The RNA was extracted using chloroform, precipitated with 75% ethanol and cleaned up on Qiagen RNeasy Midi column (Valencia, CA). After on column DNase treatment, 2 µg of RNA was reversed transcribed using Abcam’s High Capacity cDNA kit in a 20µl reaction and the resulting cDNA was used for quantitative real time polymerase chain reaction (qRT –PCR) analysis in an iCycler 5 system (Bio-Rad, Hercules, CA). For analysis of
c-Fos, c-Jun, MKP-1, LDL-r, and TNF-α, the cDNA was diluted 10 fold. The cDNA was diluted 100 fold for the analysis of Gapdh and PAI-1 mRNA expression. The primers in Table 1 were used to amplify gene regions.

**Chromatin Immunoprecipitation Assays**

For ChIP assays, frozen liver was weighed then broken into 1-3mm fragments under liquid nitrogen and then fixed in formaldehyde (1%) for 15 min at room temperature to crosslink protein-DNA complexes. Next the liver pieces were washed with ice cold 1X PBS then resuspended in 1X PBS containing protease and phosphatase inhibitors [1X P8340, 10 mM NaF, 2.5 mM Na pyrophosphate, 0.1 mM Na-orthovanadate, 10 mM β-glycerophosphate, and 0.1 mM Na molybdate, and 5 mM sodium butyrate (for phosphoacetyl ChIP assay)] and disaggregated in a dounce homogenizer. After centrifugation at 2000 rpm for 5 mins the cell pellet was resuspended in cell lysis buffer (5 mM HEPES pH 7.9, 85 mM KCl, and 0.5 % NP-40 containing the same inhibitors as above) and incubated on ice for 15 min. Samples were vortexed briefly for 10 s every 5 min to aid in nuclei release. Samples were centrifuged at 4 °C at 2000 rpm for 5 min and resuspended in nuclei lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris-HCl, pH 8.1) containing the same protease and phosphatase inhibitors as above. Lysates were sonicated with 7-9 sets of 10 sec pulses in a VibraCell Sonicator model VCX-600 (Sonics and Materials, Newton, CT) at 90% DC, microtip 4 to obtain 200-1000 bp DNA fragments with average fragments at 350bp. Chromatin was centrifuged at 12, 000g for 10 min at 4°C to remove insoluble material, diluted ten fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.1, 16.7mM NaCl) supplemented with protease and phosphatase inhibitors as above and then was incubated for 1hr at 4°C with 60 μl of protein G beads (50% slurry-salmon sperm DNA) to preclear the lysates. After removal of the agarose
beads by centrifugation at 5000 g for 1 min, an aliquot (1%) of the supernatant was saved as input. The remaining supernatants were divided equally (corresponding to 40 mg of tissue each) and incubated with the desired antibody for immunoprecipitation as follows: 4 μg anti-phospho-H3 Ser 10 (Millipore cat # 17-685), 4 μg of normal mouse IgG (Millipore cat # 12-371B), 5 μg anti-phospho-H3 Ser 28 (Millipore cat # 07-145), 5 μg normal rabbit IgG (Cell Signal cat # 2729S), 1 μg anti-RNA-Polymerase (Millipore cat # 05-623B), or a no-antibody control, and incubated overnight at 4°C. In some experiments antibody for phosphoacetyl-H3 K9/S10 was also used. All antibodies were ChIP grade except anti-phospho-histone H3 Ser 28, but it has been previously used by Drobic et al, 2010 for ChIP assay. Antibody-DNA immunocomplexes were precipitated with 60 μl of protein G agarose beads for 1h and washed twice for 5 min each with the following wash buffers in the order, low-salt buffer (0.1% SDS, 1% Triton X-100, 1.2 mM EDTA, 20 mM Tris-HCl, 167 mM NaCl), high-salt buffer (0.1% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 20 mM Tris-HCl, 500 mM NaCl), and Li-Cl containing buffer (1% NP-40, 1% deoxycholic acid sodium salt, 1 mM EDTA, 10 mM Tris-HCl pH 8.1, and 0.25 M LiCl) and TE buffer (10 mM Tris-HCl pH 8.1, 1 mM EDTA). Immunocomplexes were eluted twice from the antibody in elution buffer in 100 μl of elution buffer (1% SDS, 0.1 M NaHCO3) at room temperature. DNA-protein crosslinks were reversed by the addition of 5M NaCl to a final concentration of 0.2 M for 4h-overnight at 65°C. DNA was purified by the use of Qiaquick PCR columns. Immunoprecipitated DNA was analyzed by qRT-PCR using primers listed in Table 2.

The ChIP assay on the 4 h samples was performed on frozen nuclei as an attempt to reduce the background signal. To do this we used frozen isolated nuclei which were isolated and frozen at the time of protein extraction (see “preparation of nuclear extracts above”). For ChIP
assay the nuclei were processed as described below. First the nuclei were thawed on ice, then resuspended in 1ml of crosslinking buffer (0.25 M sucrose, 10 mM Tris-HCl pH 7.5, 3 mM MgCl₂, and phosphatase and protease inhibitors mentioned above). Proteins were crosslinked to DNA by the addition of 27 μl of 37% formaldehyde for 7.5 min at room temperature. Samples were then centrifuged for 6000 g at room temperature to remove formaldehyde then washed in 1X PBS containing the same phosphatase and protease inhibitors as above. After centrifugation at 6000 g, nuclei was resuspended in nuclei lysis buffer and sonicated as above. Next protein was quantified and 40 μg of protein was used per ChIP assay. The chromatin immunoprecipitation steps were carried out as described above. The background Ct values were similar to that obtained when the ChIP assay was done using the whole liver.

Data analysis

Protein analysis: All results are expressed as mean ± S.E.M. The values on the graph represent fold change from control values that were normalized to the levels of histone H3 protein for each sample. Graph Pad PRISM (version 4) software was used for statistical analysis. Changes in proteins were analyzed using the Student t test (two-tailed, paired). Differences with a P value of <0.05 were determined to be statistically significant. It may be mentioned that experiments reported here involved in vivo acute/short term treatments and hence in a few cases variations in values among different rats were noted. In these few cases although noticeable changes in various parameters were observed but the mean values did not reach statistical significance, and these are explained in the text.
RNA analysis: The qRT-PCR data for changes in mRNA expression between ethanol and control were analyzed using the ΔΔ comparative Ct method and the levels of Gapdh were used as a normalizer. Statistical analysis was performed using the Student t test (two-tailed, paired).

ChIP assay/analysis: To determine fold enrichment of histone phosphorylation and phosphoacetylation at promoter regions, the ΔΔ comparative Ct method was used and differences between immunoprecipitated samples between ethanol and control was normalized to the normal IgG antibody (anti-mouse IgG for P-H3 S10, anti-rabbit IgG for both P-H3 S28, and phosphoacetyl-H3-K9/S10). Input samples were diluted (1/100) and ChIP samples (no dilution) was used for real time PCR on iCycler 5 (Bio-Rad) using primers for the promoter regions specified in Table 2. The average Ct of three replicates was taken for analysis. The differences in site occupancy between control and ethanol samples were analyzed by first normalizing ChIP samples to the input. For comparison of a gene that is not altered by ethanol we monitored GAPDH and no change in the association of modified histone H3 with the GAPDH promoter was seen between control and ethanol (data not shown).

Results

In this study, we have measured the effect of dose and time of acute ethanol administration in vivo in rats on various parameters, i.e blood alcohol concentration (BAC), ALT, cleaved caspase 3, histone H3 phosphorylation (at Ser 10, and Ser 28), phosphoacetylation at H3-K9/S10, acetylation at H3-K9, expression of early response genes (c-Fos, c-Jun, MKP-1) and genes involved in alcoholic liver injury (LDL-r, and PAI-1). Next we performed ChIP assays with site
specific antibodies to P-H3-S10, P-H3-S28, and phosphoacetyl H3- K9/S10 and analyzed the association between promoter regions of different genes with the phosphorylated, and phosphoacetylated histone H3. The results are presented below.

**Rationale for the use of IP as a model for acute effects in vivo**

There are several laboratory models available to study alcohol-induced organ damage using acute or chronic treatment conditions (D’Souza El-Guindy et al, 2010). Intragastric and intraperitoneal (IP) are two methods used widely to examine acute effects of ethanol. In comparison to intragastric ethanol administration model, blood ethanol levels after IP administration are more controlled, and exhibits less variation between animals. Additionally, IP administration of ethanol itself is less stressful to the animals. Furthermore, the phosphorylation responses, mediated mostly via rapid kinase activation, can be monitored in the IP model. IP administration of ethanol has been used widely, among others, in neuro-behavioral studies to assess acute effects of ethanol. This in vivo model is therefore useful for acute studies and was chosen for our phosphorylation experiments.

**Blood ethanol levels after acute ethanol administration**

IP administration of different doses of alcohol (1, 1.75, 2.5, 3.5, 5 g/Kg body weight) to rats for 1 h resulted in blood ethanol levels ranging from 27-108 mmol/L (Fig 1A). The control rats with no ethanol administered had an average basal (background) value of 6 mmol/L. In time course experiments, a single IP dose of ethanol (3.5 g/Kg body weight) to rats increased BAC up to 2h (152 mmol/L) and declined thereafter at 3h (98 mmol/L) and 4h (65 mmol/L, Figure 1B). At 4h, the blood alcohol was about one third of the 2h peak value (Fig 1B). For comparison, it
may be mentioned that in humans BACs ranging from 20-170 mM can be seen in moderate to heavy alcohol users and binge drinkers (Rivara et al, 1993; Haycock, 2009; Mathurin and Deltrenre, 2009).

**Serum ALT, cleaved caspase 3 and liver injury**

Rats were treated with ethanol for 1 h at different doses, and cleaved caspase 3 and serum ALT levels were examined. At the 3.5g/kg and 5g/kg doses the level of cleaved caspase 3 increased slightly but did not reach level of statistical significance due to high variability among individual rats (Fig. 2A). The serum ALT levels in control and ethanol-treated rats were not different (Figure 2B). A time course study using a 3.5g/Kg ethanol for 1-4h showed variable increase in the levels of cleaved caspase 3 which did not reach statistical significance (Fig. 3A). The serum ALT values in the control rats from 1.5 to 4h were all similar (47-56 U/L) and no difference between control and ethanol treated rats was observed at 1h after IP injection. The ALT levels appeared elevated at later time points compared to controls, but because of variability among animals, values only at the 2 and 3h time points reached levels of statistical significance (p<0.05) (Fig. 3B). Histological examination by H&E staining revealed no apparent steatosis at 1h (Fig. 3C). However, from 1.5 to 4h, steatosis increased over time as fatty deposits were evident in the liver sections (Fig. 3C, see arrows). Thus, in this IP model mild steatosis, and necrosis (increased serum ALT) were observed. It was also apparent that although BAC is maximum at 2 h, liver injury by acute ethanol may continue to increase gradually at time points later than the peak in BAC.
Ethanol dose and time course of histone H3 phosphorylation

We had previously reported that after an acute administration of ethanol histone H3 phosphorylation at serine-10 and serine-28 increased (Aroor et al, 2010). We have now determined the effect of different doses of ethanol on H3 phosphorylation at 1h time point. As shown in Figure 4, increased phosphorylation of H3-S10 and H3-S28 was apparent at ethanol 1.75 and 3.5 g/Kg, reaching a level of statistical significance (p<0.05) for the 1.75 and 3.5 g dose for H3-S10 and for 1.75, 2.5 and 3.5 g doses for H3-S28. Interestingly, no significant increase occurred at either site with the 5 g/Kg dose. At lower dose (1 g/Kg) of ethanol, a smaller increase in phosphorylation at S28 was observed (1.3 fold increase) although not reaching statistical significance. In contrast at 1g/Kg the phosphorylation at S10 did not change. This may suggest that phosphorylation of Ser 28 is more sensitive to lower blood alcohol levels. A time course profile of ethanol (3.5 g/Kg) induced histone phosphorylation was generated and differences were noted between the phosphorylation of serine-10 and serine-28 (Fig. 5). The time course of serine-10 phosphorylation was biphasic with apparent peaks at 1.5 and 4h (Fig. 5C). No significant difference between-ethanol treated and control samples was observed at the 2h time point. S-28 phosphorylation remained elevated from 1h to 4h, although a small reduction might be noted at the 2h point. Taken together, these data indicate that histone H3 phosphorylation was altered with dose of ethanol and time of treatment.

Ethanol dose response and time course of changes in histone H3 phosphoacetylation

Ethanol also increased phosphoacetylation of histone H3 at K9/S10, but appeared to do so at higher ethanol doses than S-10 and S-28 phosphorylation (Fig. 6C). Phosphoacetylation level
remained elevated from 1h-4h, reaching statistical significance level at 1.5h, 2h and 4h time points (Fig. 6D). It may be noted that increase in phosphoacetylation was noticeable at ethanol 5g/Kg whereas increase in the phosphorylation at individual serine 10 or serine 28 sites were negligible at this dose (Fig. 4 vs Fig. 6). Thus, phosphoacetylation appears to occur with a different time course and dose response profile to H3-S10 and H3-S28 phosphorylation.

**Ethanol dose and time related acetylation at K9 of histone H3**

For comparison, we next examined acetylation of H3 at K9 in this model. Statistical significant increases in the acetylation of histone H3 at K9 were observed at ethanol doses from 1-3.5 g/Kg (Fig. 7C), similar to the phosphorylation of histone H3 at serine 28. Significant increase in K9 acetylation was observed at 1 h and remain elevated up to 4h with an apparent peak at 1.5h (Fig. 7D). It may be noted that the time and dose response pattern of Ac-H3-K9 (Fig. 7) and phosphoacetylation at K9/S10 (Fig. 6) were different.

**The effect of acute ethanol on the gene expression**

**Dose Response**

Ethanol is known to increase gene expression. In studies (not related to ethanol) phosphoacetylation of histone H3 at serine 10 and lysine-14 has been shown to correlate with induction of c-Fos and c-Jun expression. Furthermore, histone H3 phosphorylation is known to affect these genes during mitosis (Clayton et al, 2000). In this study we analyzed the acute effects of ethanol on immediate early gene mRNA expression for c-Fos, c-Jun, and MKP-1. There was induction of c-Fos and c-Jun by ethanol (1.75 and 3.5g/kg) after 1 h of ethanol
treatment (Figure 8 A & B). For MKP-1, 1.75g and 3.5g ethanol caused about 2 fold increase although the increase did not reach level of statistical significance because of large variability among the different animals. There was no induction of c-Fos, c-Jun and MKP-1 at 5 g/kg.

In this series of experiments we also determined the effects of ethanol on LDL-r, PAI-1, and TNFα, since these genes are known to play role in alcoholic liver injury. LDL-r is long known to be involved in alcohol induced steatosis (Wang et al, 2010). PAI-1 has been implicated in alcoholic liver disease (Arteel, 2008), and TNFα is a well known marker for alcoholic liver injury (McClain and Cohen 1989). We detected some increases in transcript levels of all three genes but the difference reach statistical significance only for LDL-r at the 1.75g and 5g dose and for PAI-1 at the 1.75g dose. Interestingly, the 5g dose caused a statistically significant decrease of TNFα transcript (Fig. 8).

Time course

We next examined the time course of gene expression response to 3.5g/Kg ethanol (Fig. 9). c-Fos was induced up to 12 fold at 1.5h followed by gradual decrease. By 4h following ethanol injection, only a 1.9 fold change was observed which was not statistically different from the control (Fig. 9A). c-Jun also increased at 1.5 h then decreased rapidly thereafter (Fig. 9B). MKP-1 mRNA showed somewhat a variable pattern of increase and these differences did not reach statistical significance (Fig. 9C). In the case of LDL-r mRNA expression (Fig. 9D), there was no change at 1 h, gradually peaked at 2 h, and then decreased. There was no change in TNFα mRNA expression at early time points but showed a trend towards increase at 3 and 4h.
after ethanol administration (Fig. 9E). In contrast, PAI-1 showed gradual increase with increasing time after ethanol administration (Fig. 9F).

**ChIP assay to determine association between phosphorylated histone H3 and the gene promoter**

To determine the association between histone H3 phosphorylation and gene expression, we selected samples from 1.5h and 4h time points and processed for the ChIP assay. Analysis of the ChIP assay data revealed that there was a statistically significant increased association of P-H3 S28 with the c-Jun promoter at the 1.5h time point (Fig. 10B) and the PAI-1 promoter at the 4h time point (Fig. 10D) consistent with the different time courses of expression for the two genes. The PCR primers used for the chip assays span a region of approximately 500-700 bp upstream of the transcription start site (TSS) for both promoters (-632 to -537 for C-Jun that includes an AP1 site and -724 to -613 for PAI-1 which contains an SRE, serum response element, binding site). Slight increases in the association of P-H3 S10, P-H3 S28 and phosphoacetylated H3 K9/S10 were also seen, respectively, with the c-Jun promoter at 1.5h (Fig. 10B), the PAI-promoter at 1.5h (Fig. 10C) and the PAI-promoter at 4h (Fig. 10D) after ethanol administration, but the differences did not rise to the level of statistical significance. No statistically significant change was observed for the association of P-H3 S10 or P-H3 S28 with the c-fos promoter (Fig. 10A) or the association of phosphoacetylated K9/S10 with the PAI-promoter at 1.5h (Fig. 10C). Taken together the data indicate that differentially modified histones might be preferentially found at specific gene promoters following IP administration of ethanol in a manner that is consistent with a role in gene activation.
Discussion

Acute IP administration of ethanol can promote mild steatosis, apoptosis and necrosis in liver. Therefore this model was considered useful to study the effects of acute ethanol in liver. An important finding in this study is that acute & rapid IP administration of ethanol promoted site-specific histone H3 phosphorylation at serine 10 and serine 28 in rat liver in vivo. Phosphorylation of H3 at S28 was more sensitive to lower dosages (1g and 1.75g) of ethanol than P-H3-S10. At the highest dosage (5g/kg), phosphorylations at these two sites were not altered. It has been reported that rapid activation of MAP kinases can lead to induction of MAP Kinase phosphatase-1 (MKP-1; Clark, 2003). MKP-1 is a negative regulator of MAP Kinase and hence a decrease in MAP Kinase activity will result from an induction of MKP-1 (Clark, 2003; Kuwano et al, 2008). Additionally, MKP-1 can directly dephosphorylate P-H3-S10 in vitro (Kinney et al, 2009). However, in this model there was no induction of MKP-1 mRNA at the 5g dose (Fig. 8) suggesting that MKP-1 induction may not be responsible for the decrease in the histone phosphorylation seen at this dose of ethanol. Instead, oxidative stress which has been reported to cause dephosphorylation of histone H3 (Kabra et al 2008) may be an explanation for the lack of phosphorylation of H3 at higher dose (Fig. 4). Interestingly there was a remarkable increase in the phosphoacetylation (K9/S10) status of histone H3 at 5 g of ethanol. The differences in the dose response relationship for the phosphorylation of serine 10, and the phosphoacetylation at K9/S10 suggest that the two modes of histone modifications are independently regulated. It may be noted that the phosphorylated S10 and the acetylated K9 antibodies only bind to H3 when it is mono-modified (K9 or S10), and the phosphoacetyl antibody only binds when H3 is di-modified (K9/S10). It is therefore possible that these
modifications may occur on different sites of histone H3 located on same or on different nucleosome domains in chromatin as shown schematically in Fig. 11.

The phosphorylation of serine 10 appeared to be biphasic. The first phase could be due to p38 MAPK activation and the second phase could be due to sustained activation of ERK ½. In this context, we have reported that both p38 MAPK as well as ERK ½ MAPK are activated in both the cytosol and nucleus in liver after acute ethanol (Aroor et al, 2010). While p38 phosphorylation came down at 4h the phosphorylation of ERK was sustained at 4h. Since the time course of MKP-1 mRNA induction from 1-3 h was similar in pattern to that of phosphorylated histone H3 at S10 (Fig. 9C vs Fig. 5C), increased MKP-1 activity could be responsible for the subsequent decrease in S10 phosphorylation either by dephosphorylating MAP kinases or histone H3 S10 itself (Kinney et al, 2009). Alternatively, the change in S10 phosphorylation may be regulating the transcription of MKP-1. However, this remains to be established in the future.

It is known that cleaved caspase 3 can result from JNK activation and JNK was previously shown to be activated after ethanol (Aroor and Shukla, 2004; Aroor et al, 2010) in vivo. Cleaved caspase 3 may also be affected from the changes in histone H3 phosphorylation and MKP-1 induction. Modified histones have also been shown to associate with the caspase 10 promoter and linked to its activation (Li et al, 2001). Increase in caspase 10 activity might be responsible in part, to the observed modest increase in caspase 3 cleavage in the present study.

It was also demonstrated here for the first time that ethanol elevated phosphoacetyl-histone H3- K9/S10 in vivo. It has been reported that phosphorylation at serine
10 of histone H3 oppose acetylation at lysine 9, but enhances acetylation at lysine 14 (Edmondson et al, 2002). However, our data suggest that modification of K9 and S10 could also be co-ordinately upregulated. We have also observed that HDAC inhibition by trichostatin A induced increase in P-H3-S10 and P-H3-S28 in rat primary hepatocytes (unpublished data), and increased histone H3 phosphorylation at serine 28 after TSA treatment has also been observed in JB6 cells (Zhong et al, 2003). In addition, exposure of primary hepatocytes to 100 mM ethanol caused maximal phosphorylation of both P-H3-S10 and Ac-H3-K9 at 24 h (Lee and Shukla, 2007; Park and Shukla, 2003). Because several histone acetyltransferases (HATs) are known to exhibit increased activity on histone H3 containing phosphorylated S10 (Merienne et al 2001, Clayton, et al 2000), ethanol induced in vivo histone H3 S10 phosphorylation may enhance acetylation by these HATs. The biphasic increase in H3S10 phosphorylation (with an early peak at 1.5h) and the delayed peak of dual-modified H3 (at 4 h) provides support to this possibility. However, the early peak of H3K9 acetylation alone at 1.5h suggests that the two modifications could be occurring independently of each other.

Our data also showed that peak phosphorylation of serine 28 and serine 10 occurred at different ethanol doses and therefore may have differential roles in gene expression after ethanol treatment. This was supported by the observation that the promoter of PAI-1 showed increased association with phosphorylated histone H3-S28, but not with phosphorylated H3-S10 following ethanol treatment. However, we cannot completely rule out the possibility that increased association of phosphorylated H3-S10 might occur at regions of the PAI-1 promoter that were not tested in this study. Ethanol induced activation of PAI-1 is responsible, atleast in part, in causing fibrin accumulation in the liver by inhibiting fibrinolysis (Beier et al, 2009) and has also
been implicated in alcoholic liver steatosis (Arteel, 2008). Taken together these data demonstrate that the phosphorylation of histone H3 at serine 10 and serine 28 and phosphoacetylation of histone H3-K9/S10 are affected during acute ethanol administration in vivo. It will be interesting in the future to determine if changes in the association of modified histone with LDL-r or TNF-α promoter also occur following acute ethanol. LDL-r promoter was previously found to be associated with S-10 histone phosphorylation in HepG2 cells (Huang et al, 2004, Huang et al, 2006) and TNFα promoter was associated with H3K9 acetylation (Miao et al, 2004).

In summary, we have demonstrated that ethanol induced histone H3 phosphorylation plays a role in acute ethanol induced gene expression in vivo. An increase in histone phosphorylation was accompanied by an increase in its association with the promoter of the early response gene c-Jun, and correlated with increases in the levels of their transcripts, suggesting that histone phosphorylation underlies the mechanism for the induction of the AP1 transcription factors and AP1 responsive genes. Furthermore, histone H3 phosphorylation at serine 28 showed association with the PAI-1 promoter. In conclusion, ethanol induced histone H3 phosphorylation in liver, in vivo, is involved in transcriptional activation.

Acknowledgements: The authors thank Dr. Ricardo Restrepo and Mr. Daniel Jackson for their help in this study.
Authorship contribution

Participated in research design: Taryn James, A. Aroor, S.D. Shukla

Conducted experiments: Taryn James, A. Aroor, S.D. Shukla,

Performed data analysis: T James, R.W. Lim, S.D. Shukla

Contributed to manuscript writing: Taryn James, R.W. Lim, S.D.Shukla
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Kabra, DG; Gupta, J; Tikoo, K (2009) Insulin induced alteration in post-translational modifications of histone H3 under a hyperglycemic condition in L6 skeletal muscle myoblasts. *Biochemica et Biophysica Acta.* *Molecular Basis of Disease* **1792:** 574-583.


Footnotes:

This work was supported in part by the National Institute of Alcohol Abuse & Alcoholism [grant # AA16347].
Legends for Figures

Figure 1 [A]. Serum ethanol levels after acute ethanol administration at 1h. Rats were given IP, different doses of ethanol (1, 1.75, 2.5, 3.5, 5 g/Kg) for 1hr and the levels of serum ethanol were measured as described in materials and methods. Control animals given water showed an average serum ethanol value of 6 mmol/l (not shown). Values are mean ± SE (n= 3 rats).

Serum ethanol levels after acute ethanol administration of 3.5 g/Kg at different time points.
Rats were given a single IP dose of ethanol (3.5g/Kg) and the levels of serum ethanol were measured after different times. Values are mean ± SE (n= 3 to 4 rats) and were statistically significant (*) from control (p<0.05).

Figure 2. Serum ALT and cleaved caspase 3 levels after administration of different doses of ethanol. Rats were given different doses of ethanol IP for 1 h. Cleaved caspase 3 (A) was analyzed by western blot. Serum ALT (B) was measured using an automated analyzer as described under materials and methods. Values are mean ± SE (n= 3 to 5 rats).

Figure 3. Serum ALT, cleaved caspase 3, and steatosis at different time points after ethanol administration. Rats were given IP a single ethanol dose (3.5 g/Kg) for different times. Cleaved caspase 3 levels (A) were determined by western immunoblot analysis in cytoplasmic extracts at the time points indicated and were normalized to control for each time point. Levels of serum ALT (B) were determined at different times as described under materials and methods. Steatosis was monitored at the above time points by hematoxylin and eosin staining of liver sections (C). Control represents animals given water. Values are mean ± SE (n= 3 to 5 rats). Asterisk (*) represent significant values compared to control group (p<0.05).
Figure 4. **Ethanol dose dependent changes in the levels of phosphorylated histone H3.** Rats were given IP different doses of ethanol for 1h and levels of phosphorylated H3 ser-10 (C) and ser-28 (D) in nuclear extracts were determined at 1 h by SDS-PAGE & western blotting with site-specific antibodies. Data were normalized to histone H3 protein. Histone H3 protein levels did not change. The upper panels show representative bands from individual rats for H3 ser-10 (A) and H3-ser-28 (B). Values are mean ± SE (n=3-6 rats) and the asterisk (*) represents significant differences compared to control (p<0.05).

Figure 5. **Time dependent alterations in levels of phosphorylated histone H3 after acute ethanol administration.** Rats were given IP a single dose of ethanol 3.5 g/Kg and the levels of phosphorylated H3 ser-10 (C) and ser-28 (D) in nuclear extracts were determined at different times by SDS-PAGE & western blotting with site-specific antibodies. For simplicity only one set of bands (4h) representing different rats (5 control and 4 ethanol) is shown in the upper panel for P-H3-S10 (A) and P-H3-S28 (B). Values are mean ± SE (n=3-5 rats). Asterisk (*) represent significant values compared to control (p<0.05).

Figure 6. **Dose and Time dependent alterations in the levels of phosphoacetylated histone H3-K9/S10 after acute ethanol administration.** Rats were given different doses of ethanol IP and the levels of phosphoacetylated-H- K9/S10 (C) in nuclear extracts were determined after 1 h, by SDS-PAGE & western blotting with site-specific antibody. For the time course experiments rats were given IP a single dose of ethanol (3.5g/kg) and the levels were determined at different time periods (D). In the upper panel only the bands for one dose 5 g/Kg (A: left panel; 4 control and 6 ethanol rats) and one time point 4h (B: right panel; 6 control and 5 ethanol rats) are shown where each band represents different animal. Values are mean ± SE (n=3-5 rats). Asterisk (*)
represent significant values compared to control (p<0.05). Values without asterisk were also higher than control but did not show a p value below 0.05 due to animal variations in these samples.

**Figure 7. Dose and Time dependent alterations in levels of acetylated histone H3-K9 after acute ethanol administration.** Rats were given different doses of ethanol IP for 1 h (A). The levels of acetylated-H- K9 in the nuclear extracts were determined by SDS-PAGE & western blotting with site-specific antibodies. For the time course experiments (D) rats were given IP a single dose of ethanol 3.5g/Kg for different time periods and the levels acetylated H3-K9 in the nuclear extracts were determined. For clarity only the bands of one dose 2.5 g/Kg (A: left panel; 4 control and 4 ethanol) and one time point 1.5h (B: right panel; 3 control and 3 ethanol) are shown for different animals. Values are mean ± SE (n=3-5 rats). Asterisk (*) represent significant values compared to control (p<0.05).

**Figure 8. The effect of different doses of ethanol on mRNA expression 1h after acute ethanol administration** Based on the results on phosphorylated H3 ser-10 & ser-28 we analyzed gene expression in liver samples from animals that received 1.75g, 3.5g, and 5g/Kg ethanol for 1h. RNA was isolated and reverse transcribed as described in the materials and methods. qRT-PCR was performed using specific primers listed in Table 1 on a Bio-Rad iCycler 5 and were done in triplicate and were repeated for confirmation. The average C_{t} values were used to calculate fold changes in gene expression which was determined using the \Delta\DeltaComparative C_{t} method and were normalized to the Gapdh values. Values are mean ± SE (n=3-8 rats). Significance was analyzed using paired student’s t test and asterisk (*) represent significant values compared to control (p<0.05).
Figure 9. Changes in mRNA expression for genes at different time points after acute ethanol administration. Rats were given IP a single dose of ethanol 3.5g/Kg and the livers were extracted at times indicated. RNA was isolated and reverse transcribed as described in the materials and methods. qRT-PCR was performed on a Bio-Rad iCycler 5 using specific primers listed in Table 1 and were done in triplicate. The average Ct values were used to calculate fold changes in gene expression was determined using the $\Delta\Delta$Ct method and were normalized to the Gapdh values. Values are mean ± SE (n=3-5 rats). Asterisk (*) represent significant values compared to control (p<0.05).

Figure 10. ChIP assay with different antibodies. Frozen liver from the 1.5h and 4h time course samples (Fig. 5), where maximum fold change in histone phosphorylation at ser-10 and ser-28 occurred, were used for ChIP assays with antibodies to phosphorylated -histone- H3-S10, phosphorylated –histone H3-S28, phosphoacetylated histone H3-K9/S10. The ChIP protocol is described in the materials & methods section. The association of modified histone with gene promoters was determined by quantitative RT-PCR using specific primers in Table 2. The data represent fold increase in PCR products relative to samples from control rats not exposed to ethanol. Values are mean ± SE (n=3 rats). Asterisk (*) represent significant values compared to control (p<0.05).

Figure 11. A schematic diagram depicting different doamains in the chromatin nucleosome for differential gene expression. The diagram shows the possible domains in the nucleosome of the chromatin where site-specific histone H3 modifications may be associated with expression of different genes. Different domains may be located on the same or different nucleosomes.
Table 1. Primer Sequences Used for qRT-PCR for mRNA expression

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Table 2. Primer Sequences Used for qRT-PCR for Promoter Regions

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<td>c-Jun</td>
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<td>5’- CCGAGGAGGGGACAGTTG -3’</td>
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</table>
Figure 2

[A] Cleaved caspase 3

[B] Serum ALT (U/L)

- Control
- Ethanol

Ethanol (g/Kg):
- 1.75
- 3.50
- 5.00

Fold change from control:
- c
- 1.75
- 3.5
- 5
Figure 3

[A] Cleaved Caspase 3

[B] Serum ALT (U/L)

[C] Control

Ethanol (3.5g/Kg)

Time course

1.0 1.5 2.0 3.0 4.0

Time course (h)

Control

Ethanol

*
Figure 7

[A] 2.5g/Kg

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Ac-H3-K9

H3 protein

[B] 1.5h

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[C] Ac-H3-K9

Fold change from control

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[D] Ac-H3-K9

Fold Change from Control

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</table>
Figure 8

[A] c-Fos

[B] c-Jun

[C] MKP-1

[D] LDL-\( r \)

[E] TNF\( \alpha \)

[F] PAI-1

mRNA Expression

Fold change from control

Ethanol (g/Kg)

C 1.75 3.5 5
Figure 9

[A] c-Fos

[B] c-Jun

[C] MKP-1

[D] LDL-\(\text{r}\)

[E] TNF\(\alpha\)

[F] PAI-1
Figure 10

[A] 1.5h  
c-Fos

[B] 1.5h  
c-Jun

[C] 1.5h  
PAI-1

[D] 4h  
PAI-1
Figure 11

P-H3-S10: genes?
P-H3-S28: genes?
P/Ac-H3-K9/S10: genes?
Ac-H3-K9: genes?