Histone H3 Phosphorylation (Ser10, Ser28) and Phosphoacetylation (K9S10) Are Differentially Associated with Gene Expression in Liver of Rats Treated In Vivo with Acute Ethanol

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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 Ser10 (P-H3-Ser10), and Ser28 (P-H3-Ser28) in liver after acute ethanol treatment in vivo. Ethanol was administered intraperitoneally in male Sprague-Dawley rats. Ethanol dose-response (1–5 g/kg body weight) and time-course (1–4 h) experiments were conducted, and various parameters were monitored. Steatosis and necrosis (serum alanine aminotransferase) of the liver increased in 4 h, suggesting liver injury. There were differences between P-H3-Ser10 and P-H3-Ser28 at 1 h, with the latter being more sensitive to lower ethanol doses. It was noteworthy that phosphorylation of both serines disappeared at the highest dose used (5 g/kg). We also examined

phosphoacetylation of histone H3 at K9S10 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*, mitogen-activated protein kinase phosphatase-1). Chromatin immunoprecipitation assays in samples from 1.5 and 4 h of ethanol administration indicated that increased histone H3 phosphorylation at Ser28 was associated with the promoters of *c-jun* and plasminogen activator inhibitor-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 where the pathology is identified by steatosis (fatty liver), steatohepatitis, and 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 those mechanisms. We have shown previously that

ethanol promotes the acetylation of histone H3 at Lys9, leading to the up-regulation of the *ADH1* gene in liver (Park et al., 2005). Moreover, we have reported ethanol-induced histone H3 phosphorylation at Ser10 and Ser28 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), because some histone acetyltransferases (HATs), general control nonderepressible 5, P300/CBP-associated factor, and p300 have preferences for phosphorylated histone H3 (Cheung et al., 2000; Clayton et al., 2000; Merienne et al., 2001). 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 whether ethanol-induced histone acetylation and phosphorylation are related.

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ABBREVIATIONS: MAPK, mitogen-activated protein kinase; ALT, alanine aminotransferase; AP-1, activator protein-1; BAC, blood alcohol concentration; bp, base pairs; ChIP, chromatin immunoprecipitation; ERK1/2, extracellular signal-regulated kinase 1/2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HAT, histone acetyltransferase; JNK, *c-jun* NH₂-terminal kinase; LDL-r, low-density lipoprotein receptor; MKP-1, mitogen-activated protein kinase phosphatase-1; NaF, sodium fluoride; PAGE, polyacrylamide gel electrophoresis; PAI-1, plasminogen activator inhibitor-1; PBS, phosphate-buffered saline; qRT-PCR, quantitative real-time polymerase chain reaction; TBST, 20 mM Tris, pH 7.4, containing 0.1% Tween 20 and 150 mM NaCl; TNF, tumor necrosis factor; TSA, trichostatin A.

Histone phosphorylation is known to occur mostly during mitosis, and it 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 up-regulated 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 because they regulate inflammation, steatosis, apoptosis, and necrosis (Arora and Shukla, 2004; Boutros et al., 2008; Brown and Sacks, 2008). Ethanol is known to activate AP-1 in HepG2 cells (Román 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-Ser10 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 alcoholic liver disease because AP1 is a regulator of inflammatory genes such as *PAI-1* (Stroschein et al., 1999; Mertens et al., 2006).

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 Lys9 and phosphorylation at Ser10 and Ser28 (Arora et al., 2010). We have now investigated the characteristics of histone H3 phosphorylation (Ser10, Ser28) and phosphoacetylation (K9S10) in vivo and examined their mechanistic relevance to gene expression.

Materials and Methods

Reagents

Antibodies to P-H3-Ser10, P-H3-Ser28, Ac-H3-Lys9, and H3 protein were purchased from Millipore Bioscience Research Reagents (Temecula, CA). Antibody for phosphoacetyl K9S10 (i.e., anti-acetyl-Lys9/phospho-Ser10 histone H3) and antibody to cleaved caspase-3 were obtained from Cell Signaling Technology (Danvers, MA). Oligonucleotides were designed by using Primer 3 (Rozen and Skaltsky, 2000) and Primer blast (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and were obtained from Integrated DNA Technology Inc. (Coralville, IA). TRIzol reagent was purchased from Invitrogen (Carlsbad, CA), and QIAGEN RNeasy Midi column, RNase-free DNase, and Qiaquick PCR spin columns were obtained from QIAGEN (Valencia, CA). A high-capacity reverse transcription kit was purchased from Abcam Inc. (Cambridge, MA). Reagents for quantitative real-time polymerase chain reaction (qPCR) were obtained from Bio-Rad Laboratories (Hercules, CA). Protease inhibitor cocktail (P8340), phosphatase inhibitor cocktail (P2850), and anti- β -actin antibody were obtained from Sigma-Aldrich (St. Louis, MO).

Acute Ethanol Administration

Twelve-week-old male Sprague-Dawley rats, ranging from 300 to 550 g, were purchased from Harlan (Indianapolis, IN) and maintained on a 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 before 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 livers were removed after 1 h.

Control animals were administered an equal volume of water. To assess the effects of ethanol on histone H3 phosphorylation at different time points, 3.5 g/kg ethanol or water (control) was injected into rats, and the livers were removed after 1.5, 2, 3, or 4 h. At the time of liver removal, blood samples were collected for blood alcohol and serum alanine aminotransferase (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* (Institute of Laboratory Animal Resources, 1996), and the protocol for their use was approved by the University of Missouri Animal Care and 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 ALT were measured by kinetic ALT assay in an automated analyzer (University of Missouri Research Animal Diagnostic Laboratory, Columbia, MO).

Histopathology

The formalin-fixed liver sections were sectioned, stained with hematoxylin and eosin, 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 0.25 M sucrose lysis buffer containing 50 mM Tris-HCl, pH 7.4, 25 mM KCl, 5 mM MgCl_2 , 1 mM β -glycerophosphate, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM EGTA, 1 mM dithiothreitol, 100 nM trichostatin A (TSA), 5 mM sodium butyrate, 10 mM sodium fluoride (NaF), 2.5 mM sodium pyrophosphate, and $1\times$ Sigma protease inhibitor cocktail (P8340). The homogenate was centrifuged at 1600g 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 1600g for 10 min at 4°C , the pellet was resuspended in 1.35 M sucrose containing 0.3% Nonidet P-40. After passage through a 22-gauge needle three times, the nuclear suspension was subjected to another round of centrifugation at 1600g for 10 min to remove most of the cytosolic components. The nuclear pellet was then resuspended in 1.35 M sucrose, divided into four aliquots, and collected by microcentrifugation at 16,000g for 1 min. The nuclear fractions were examined under a 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 dithiothreitol, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 10 mM NaF, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 100 nM TSA, 5 mM sodium butyrate, 1 mM β -glycerophosphate, $1\times$ Sigma protease inhibitor cocktail P8340, and $1\times$ Sigma phosphatase inhibitor cocktail P2580). The nuclear preparations were sonicated three times for 5 s. After centrifugation at 16,000g for 10 min, the supernatant was used as nuclear fraction. Protein concentrations in nuclear extracts were measured by using the Bio-Rad D_c 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 min. After cooling at room temperature, samples were sonicated three times for 5 s, then centrifuged at 12,000g for 10 min. The supernatant was kept as cytoplasmic extract. This extract (80 μg) was used for the analysis of cleaved caspase 3.

Immunoblot Analysis

Nuclear extracts (30 µg of protein) were separated by 15% SDS-PAGE and electrophoretically transferred onto nitrocellulose membrane (Bio-Rad Laboratories) using Bio-Rad Trans-Blot apparatus. Membranes were washed with 1× TBST (20 mM Tris, pH 7.4, containing 0.1% Tween 20 and 150 mM NaCl) and incubated with 1× TBST containing 10% nonfat 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 (Supersignal; Thermo Fisher Scientific, Waltham, MA). The membrane was scanned with a LAS-3000 imaging system (Fujifilm Life Science, Tokyo, Japan). The data were quantified with Multi Gauge software (Fujifilm Life Science) and 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 Quantitative Real-Time Polymerase Chain Reaction

Frozen liver was weighed and homogenized in TRIzol reagent according to the manufacturer's protocols. The RNA was extracted by using chloroform, precipitated with 75% ethanol, and cleaned up on a QIAGEN RNeasy Midi column. After on-column DNase treatment, 2 µg of RNA was reversed-transcribed by using Abcam's high-capacity cDNA kit in a 20-µl reaction, and the resulting cDNA was used for qRT-PCR analysis in an iCycler 5 system (Bio-Rad Laboratories). For analysis of *c-fos*, *c-jun*, mitogen-activated protein kinase phosphatase-1 (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- to 3-mm fragments under liquid nitrogen and then fixed in formaldehyde (1%) for 15 min at room temperature to cross-link protein-DNA complexes. Next, the liver pieces were washed with ice-cold 1× PBS then resuspended in 1× PBS containing protease and phosphatase inhibitors [1× P8340, 10 mM NaF, 2.5 mM sodium pyrophosphate, 0.1 mM sodium orthovanadate, 10 mM β-glycerophosphate, and 0.1 mM sodium molybdate and 5 mM sodium butyrate (for phosphoacetyl ChIP assay)] and disaggregated in a dounce homogenizer.

After centrifugation at 2000 rpm for 5 min the cell pellet was resuspended in cell lysis buffer (5 mM HEPES, pH 7.9, 85 mM KCl, and 0.5% Nonidet P-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 seven to nine sets of 10-s pulses in a VibraCell Sonicator model VCX-600 (Sonics and Materials, Newton, CT) at 90% duty cycle, microtip 4 to obtain 200- to 1000-bp DNA fragments with average fragments at 350 bp. Chromatin was centrifuged at 12,000g for 10 min at 4°C to remove insoluble material, diluted 10-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, and 16.7 mM NaCl) supplemented with protease and phosphatase inhibitors as above, and then incubated for 1 h 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 5000g 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 of anti-phospho-H3-Ser10 (Millipore Bioscience Research Reagents), 4 µg of normal mouse IgG (Millipore Bioscience Research Reagents), 5 µg of anti-phospho-H3-Ser 28 (Millipore Bioscience Research Reagents), 5 µg of normal rabbit IgG (Cell Signaling Technology), 1 µg of anti-RNA-polymerase (Millipore Bioscience Research Reagents), or a no-antibody control and incubated overnight at 4°C. In some experiments antibody for phosphoacetyl-H3 K9S10 was also used. All antibodies were ChIP grade. Antibody-DNA immunocomplexes were precipitated with 60 µl of protein G agarose beads for 1 h 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, and 167 mM NaCl), high-salt buffer (0.1% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 20 mM Tris-HCl, and 500 mM NaCl), lithium chloride-containing buffer (1% Nonidet P-40, 1% deoxycholic acid sodium salt, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1, and 0.25 M lithium chloride), and Tris-EDTA buffer (10 mM Tris-HCl, pH 8.1, and 1 mM EDTA). Immunocomplexes were eluted twice from the antibody in elution buffer in 100 µl of elution buffer (1% SDS and 0.1 M NaHCO₃) at room temperature. DNA-protein cross-links were reversed by the addition of 5 M NaCl to a final concentration of 0.2 M for 4 h overnight at 65°C. DNA was purified by the use of Qiaquick PCR columns. Immunoprecipitated DNA was analyzed by qRT-PCR using the 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 that were isolated and frozen at the time of

TABLE 1

Primer sequences used for qRT-PCR for mRNA expression

Gene	Forward	Reverse
<i>GAPDH</i>	5'-AGACAGCCGCATCTTCTTGT-3'	5'-CTTGCCGTGGGTAGAGTCAT-3'
<i>TNFα</i>	5'-AAATGGGCTCCCTCTCATCAG-3'	5'-TTCTCTGCTTGGTGGTTTGCTACGAC-3'
<i>PAI-1</i>	5'-AACCCAGGCCGACTTCA-3'	5'-CATGCGGGCTGAGACTAGAAAT-3'
<i>c-fos</i>	5'-GCGGACTACGAGGCGTCAT-3'	5'-GGAGGAGACCAAGAT-3'
<i>c-jun</i>	5'-CAGGTGGCACAGCTTAAACA-3'	5'-CGCAACCAGTCAAGTTCTCA-3'
<i>MKP-1</i>	5'-GCTGTCCTTGACCGGGGCAC-3'	5'-CCACACCTCCCTGGCCCTT-3'
<i>LDL-r</i>	5'-TTCTTCAGGTTGGGGATCAG-3'	5'-CAGCTCTGTGTGAACCTGGA-3'

TABLE 2

Primer sequences used for qRT-PCR for promoter regions

Gene	Forward	Reverse
<i>GAPDH</i>	5'-CGTAGCTCAGGCCTCTGCGCCCTT-3'	5'-CTGGCACTGCACAAGAAGATGCGGCT-3'
<i>PAI-1</i>	5'-CCACCCAGTACACCTCAAA-3'	5'-GGTGAAGACCTCTGACTCC-3'
<i>c-fos</i>	5'-TTCTCTGTTCCGCTCATGACGT-3'	5'-CTTCTCAGTTGCTAGCTGAATCG-3'
<i>c-jun</i>	5'-GGGGTGACATCATGGGCTAT-3'	5'-GCCACACTCAGTGCAACTCT-3'

protein extraction (see *Preparation of Nuclear Extracts*). For ChIP assay the nuclei were processed as described below. First, the nuclei were thawed on ice, then resuspended in 1 ml of cross-linking buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 7.5, 3 mM MgCl₂, and phosphatase and protease inhibitors mentioned above). Proteins were cross-linked to DNA by the addition of 27 μ l of 37% formaldehyde for 7.5 min at room temperature. Samples were then centrifuged for 6000g at room temperature to remove formaldehyde then washed in 1 $\times$ PBS containing the same phosphatase and protease inhibitors as above. After centrifugation at 6000g, nuclei was resuspended in nuclei lysis buffer and sonicated as above. Next, protein was quantified and 40 μ g of protein was used per ChIP assay. The chromatin immunoprecipitation steps were carried out as described above. The background C_t 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 $\pm$ S.E.M. The values on the graphs (Figs. 4–7) represent fold change from control values that were normalized to the levels of histone H3 protein for each sample. Prism (version 4) software (GraphPad Software, Inc., San Diego, CA) was used for statistical analysis. Changes in proteins were analyzed by using the Student's *t* test (two-tailed, paired). Differences with a *P* value < 0.05 were determined to be statistically significant. It should be mentioned that experiments reported here involved in vivo acute/short-term treatments, 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 the mean values did not reach statistical significance.

RNA Analysis. The qRT-PCR data for changes in mRNA expression between ethanol and control were analyzed by using the comparative $\Delta\Delta C_t$ method, and the levels of GAPDH were used as a normalizer. Statistical analysis was performed by using the Student's *t* test (two-tailed, paired).

ChIP Assay/Analysis. To determine fold enrichment of histone phosphorylation and phosphoacetylation at promoter regions, the comparative $\Delta\Delta C_t$ method was used, and differences between immunoprecipitated samples between ethanol and control were normalized to the normal IgG antibody (anti-mouse IgG for P-H3-Ser10, anti-rabbit IgG for both P-H3-Ser28, and phosphoacetyl-H3-K9S10). Input samples were diluted (1/100), and ChIP samples (no dilution) was used for real-time PCR on iCycler 5 (Bio-Rad Laboratories) using primers for the promoter regions specified in Table 2. The average C_t 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 was 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 Ser10, and Ser28), phosphoacetylation at H3-K9S10, acetylation at H3-Lys9, 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-Ser10, P-H3-Ser28, and phosphoacetyl H3-K9S10 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 Immunoprecipitation as a Model for Acute Effects In Vivo

There are several laboratory models available for studying alcohol-induced organ damage by using acute or chronic treatment conditions (D'Souza El-Guindy et al., 2010). Intragastric and intraperitoneal administration are two methods used widely to examine acute effects of ethanol. In comparison to the intragastric ethanol administration model, blood ethanol levels after intraperitoneal administration are more controlled and exhibit less variation between animals. In addition, intraperitoneal 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 intraperitoneal model. Intraperitoneal administration of ethanol has been used widely, among others, in neurobehavioral 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

Intraperitoneal administration of different doses of alcohol (1, 1.75, 2.5, 3.5, and 5 g/kg body weight) to rats for 1 h resulted in blood ethanol levels ranging from 27 to 108 mM (Fig. 1A). The control rats with no ethanol administered had an average basal (background) value of 6 mM. In time-course experiments, a single intraperitoneal dose of ethanol (3.5 g/kg body weight) to rats increased BAC up to 2 h (152 mM) and declined thereafter at 3 h (98 mM) and 4 h (65 mM; Fig. 1B). At 4 h, the blood alcohol was approximately one third of the 2-h peak value (Fig. 1B). For comparison, in humans BACs ranging from 20 to 170 mM can be seen in moderate to heavy alcohol users and binge drinkers (Rivara et al., 1993; Haycock, 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 doses of 3.5 and 5 g/kg the level of cleaved caspase 3 increased slightly but did not reach a level of statistical significance because of high variability among individual rats (Fig. 2A). The serum ALT levels in control and ethanol-treated rats were not different (Fig. 2B). A time-course study using 3.5 g/kg ethanol for 1 to 4 h 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 4 h were all similar (47–56 U/liter), and no difference between control and ethanol-treated rats was observed at 1 h after intraperitoneal injection. The ALT levels seemed elevated at later time points compared with controls, but because of variability among animals, values only at the 2- and 3-h time points reached levels of statistical significance (*p* < 0.05) (Fig. 3B). Histological examination by hematoxylin and eosin staining revealed no apparent steatosis at 1 h (Fig. 3C). However, from 1.5 to 4 h, steatosis increased over time as fatty deposits were evident in the liver sections (Fig. 3C, see arrows). Thus, in this intraperitoneal model mild steatosis and necrosis (increased serum ALT) were observed. It was also apparent that although BAC was maximal at 2 h, liver injury by acute ethanol may continue to increase gradually at time points later than the peak in BAC.

A**Ethanol Dose and Time Course of Histone H3 Phosphorylation**

We had reported previously that after an acute administration of ethanol histone H3 phosphorylation at Ser10 and Ser28 increased (Aroor et al., 2010). We have now determined the effect of different doses of ethanol on H3 phosphorylation at the 1-h time point. As shown in Fig. 4, increased phosphorylation of H3-Ser10 and H3-Ser28 was apparent with ethanol (1.75 and 3.5 g/kg), reaching a level of statistical significance ($p < 0.05$) for doses of 1.75 and 3.5 g for H3-Ser10 and doses of 1.75, 2.5, and 3.5 g for H3-Ser28. It is noteworthy that no significant increase occurred at either site with the 5 g/kg dose. At lower doses (1 g/kg) of ethanol, a smaller increase in phosphorylation at Ser28 was observed (1.3-fold increase) although it did not reach statistical significance. In contrast, at 1 g/kg the phosphorylation at Ser10 did not change. This may suggest that phosphorylation of Ser28 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 Ser10 and Ser28 (Fig. 5). The time course of Ser10 phosphorylation was biphasic with apparent peaks at 1.5 and 4 h (Fig. 5C). No significant difference between ethanol-treated and control samples was observed at the 2-h time point. Ser28 phosphorylation remained elevated from 1 to 4 h, although a small reduction was noted at the 2-h point. Taken together, these data indicate that histone H3 phosphorylation was altered with dose of ethanol and time of treatment.

B

Fig. 1. A, serum ethanol levels after acute ethanol administration at 1 h. Rats were given different doses of ethanol intraperitoneally (1, 1.75, 2.5, 3.5, and 5 g/kg) for 1 h, and the levels of serum ethanol were measured as described under *Materials and Methods*. Control animals given water showed an average serum ethanol value of 6 mM (not shown). Values are mean $\pm$ S.E. ($n = 3$ rats). B, serum ethanol levels after acute ethanol administration of 3.5 g/kg at different time points. Rats were given a single intraperitoneal dose of ethanol (3.5 g/kg), and the levels of serum ethanol were measured after different times. Values are mean $\pm$ S.E. ($n = 3-4$ rats). *, statistically significant from control ($p < 0.05$).

Ethanol Dose Response and Time Course of Changes in Histone H3 Phosphoacetylation

Ethanol also increased phosphoacetylation of histone H3 at K9S10, but seemed to do so at higher ethanol doses than Ser10 and Ser28 phosphorylation (Fig. 6C). Phosphoacetylation levels remained elevated from 1 to 4 h, reaching statistical significance at the 1.5-, 2-, and 4-h time points (Fig. 6D). It may be noted that an increase in phosphoacetylation was noticeable with ethanol at 5 g/kg, whereas increases in the phosphorylation at individual Ser10 or Ser28 sites were negligible at this dose (Fig. 4 versus Fig. 6). Thus, phosphoacetylation seems to occur with a different time-course and dose-response profile to H3-Ser10 and H3-Ser28 phosphorylation.

Fig. 2. Serum ALT and cleaved caspase 3 levels after administration of different doses of ethanol. Rats were given different doses of ethanol intraperitoneally for 1 h. A, cleaved caspase 3 was analyzed by Western blot. B, serum ALT was measured by using an automated analyzer as described under *Materials and Methods*. Values are mean $\pm$ S.E. ($n = 3-5$ rats).

Fig. 3. Serum ALT, cleaved caspase 3, and steatosis at different time points after ethanol administration. Rats were given a single intraperitoneal dose of ethanol (3.5 g/kg) for different times. A, cleaved caspase 3 levels were determined by Western immunoblot analysis in cytoplasmic extracts at the time points indicated and normalized to control for each time point. B, levels of serum ALT were determined at different times as described under *Materials and Methods*. Values are mean $\pm$ S.E. ($n = 3-5$ rats). *, significant values compared with control group ($p < 0.05$). C, steatosis was monitored at the above time points by hematoxylin and eosin staining of liver sections. Control represents animals given water.

Fig. 4. Ethanol dose-dependent changes in the levels of phosphorylated histone H3. A and B, representative bands from individual rats for H3-Ser10 (A) and H3-Ser28 (B). C and D, rats were given different intraperitoneal doses of ethanol for 1 h, and levels of phosphorylated H3 Ser10 (C) and Ser28 (D) in nuclear extracts were determined at 1 h by SDS-PAGE and Western blotting with site-specific antibodies. Data were normalized to histone H3 protein. Histone H3 protein levels did not change. Values are mean $\pm$ S.E. ($n = 3-6$ rats). *, significant differences compared with control (C; $p < 0.05$).

Ethanol Dose- and Time-Related Acetylation at Lys9 of Histone H3

For comparison, we next examined acetylation of H3 at Lys9 in this model. Statistically significant increases in the acetylation of histone H3 at Lys9 were observed at ethanol doses from 1 to 3.5 g/kg (Fig. 7C), similar to the phosphorylation of histone H3 at Ser28. A significant increase in Lys9 acetylation was observed at 1 h and remain elevated up to 4 h with an apparent peak at 1.5 h (Fig. 7D). It may be noted that the time and dose-response pattern of Ac-H3-Lys9 (Fig. 7) and phosphoacetylation at K9S10 (Fig. 6) were different.

The Effect of Acute Ethanol on Gene Expression

Dose Response. Ethanol is known to increase gene expression. In studies (not related to ethanol) phosphoacetyla-

tion of histone H3 at Ser10 and Lys14 has been shown to correlate with the 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 3.5 g/kg) after 1 h of ethanol treatment (Fig. 8, A and B). For *MKP-1*, 1.75 and 3.5 g ethanol caused an approximately 2-fold increase although the increase did not reach levels 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 *TNF α* , because these genes

Fig. 5. Time-dependent alterations in levels of phosphorylated histone H3 after acute ethanol administration. A and B, for simplicity only one set of bands (4 h) representing different rats (five control and four ethanol) is shown for P-H3-Ser10 (A) and P-H3-Ser28 (B). C and D, rats were given a single intraperitoneal dose of ethanol (3.5 g/kg), and the levels of phosphorylated H3 Ser10 (C) and Ser28 (D) in nuclear extracts were determined at different times by SDS-PAGE and Western blotting with site-specific antibodies. Values are mean $\pm$ S.E. ($n = 3-5$ rats). *, significant values compared with control (C; $p < 0.05$).

Fig. 6. Dose- and time-dependent alterations in the levels of phosphoacetylated histone H3-K9S10 after acute ethanol administration. A and B, only the bands for one dose (5 g/kg) (A; four control and six ethanol rats) and one time point (4 h) (B; six control and five ethanol rats) are shown where each band represents a different animal. C, rats were given different doses of ethanol intraperitoneally, and the levels of phosphoacetylated-H3-K9S10 in nuclear extracts were determined after 1 h by SDS-PAGE and Western blotting with site-specific antibody. D, for the time-course experiments rats were given intraperitoneal a single dose of ethanol (3.5 g/kg), and the levels were determined at different time periods. Values are mean $\pm$ S.E. ($n = 3-5$ rats). *, significant values compared with control (C; $p < 0.05$). Values without * were also higher than control but did not show a p value < 0.05 because of animal variations in these samples.

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 *TNF α* 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 differences reached statistical significance only for *LDL-r* at doses of 1.75 and 5 g and for *PAI-1* at the 1.75-g dose. It is noteworthy that the 5-g dose caused a statistically significant decrease of *TNF α* transcript (Fig. 8).

Time Course. We next examined the time course of gene expression response to 3.5 g/kg ethanol (Fig. 9). *c-fos* was induced up to 12-fold at 1.5 h followed by a gradual decrease. By 4 h after 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 some-

what 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, it gradually peaked at 2 h, and then it decreased. There was no change in *TNF α* mRNA expression at early time points but showed a trend toward an increase at 3 and 4 h after ethanol administration (Fig. 9E). In contrast, *PAI-1* showed a 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 1.5- and 4-h time points and processed them for the ChIP assay. Analysis of the ChIP assay data revealed that there was a statistically significant increased association of P-H3-

Fig. 7. Dose- and time-dependent alterations in the levels of acetylated histone H3-Lys9 after acute ethanol administration. A and B, rats were given different doses of ethanol intraperitoneally for 1 h. The levels of acetylated-H3-Lys9 in the nuclear extracts were determined by SDS-PAGE and Western blotting with site-specific antibodies. For clarity only the bands of one dose (2.5 g/kg) (A, four control and four ethanol) and one time point (1.5 h) (B, three control and three ethanol) are shown for different animals. C and D, for the time-course experiments rats were given a single intraperitoneal dose of ethanol (3.5 g/kg) for different time periods, and the levels acetylated H3-Lys9 in the nuclear extracts were determined. Values are mean $\pm$ S.E. ($n = 3-5$ rats). *, significant values compared with control (C; $p < 0.05$).

Ser28 with the *c-jun* promoter at the 1.5-h time point (Fig. 10B) and the *PAI-1* promoter at the 4-h 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 to 700 bp upstream of the transcription start site for both promoters (-632 to -537 for *c-jun* that includes an AP-1 site and -724 to -613 for *PAI-1*, which contains an serum response element binding site). Slight increases in the association of P-H3-Ser10, P-H3-Ser28, and phosphoacetylated H3-K9S10 were also seen, respectively, with the *c-jun* promoter at 1.5 h (Fig. 10B), the *PAI* promoter at 1.5 h (Fig. 10C), and the *PAI* promoter at 4 h (Fig. 10D) after ethanol administration, but the differences did not rise to the level of statistical significance. No statistically significant changes were observed for the association of P-H3-Ser10 or P-H3-Ser28 with the *c-fos* promoter (Fig. 10A) or the association of phosphoacetylated K9S10 with the *PAI* promoter at 1.5 h (Fig. 10C). Taken together, the data indicate that differentially modified histones might be preferentially found at specific gene promoters after intraperitoneal administration of ethanol in a manner that is consistent with a role in gene activation.

Discussion

Acute intraperitoneal administration of ethanol can promote mild steatosis, apoptosis, and necrosis in liver. Therefore, this model was considered useful for studying the effects of acute ethanol in liver. An important finding in this study is that acute and rapid intraperitoneal administration of ethanol promoted site-specific histone H3 phosphorylation at Ser10 and Ser28 in rat liver in vivo. Phosphorylation of H3 at Ser28 was more sensitive to lower dosages (1 and 1.75 g) of

Fig. 8. The effect of different doses of ethanol on mRNA expression 1 h after acute ethanol administration. A, *c-fos*. B, *c-jun*. C, MKP-1. D, LDL-r. E, TNF α . F, PAI-1. Based on the results on phosphorylated H3-Ser10 and H3-Ser28 we analyzed gene expression in liver samples from animals that received 1.75, 3.5, and 5 g/kg ethanol for 1 h. RNA was isolated and reverse-transcribed as described under *Materials and Methods*. qRT-PCR was performed by using specific primers listed in Table 1 on a Bio-Rad iCycler 5 and was done in triplicate and repeated for confirmation. The average C_t values were used to calculate fold changes in gene expression, which were determined by using the comparative $\Delta\Delta C_t$ method and normalized to the GAPDH values. Values are mean $\pm$ S.E. ($n = 3-8$ rats). Significance was analyzed by using paired Student's t test. *, significant values compared with control (C; $p < 0.05$).

Fig. 9. Changes in mRNA expression for genes at different time points after acute ethanol administration. A, *c-fos*. B, *c-jun*. C, MKP-1. D, LDL-r. E, TNF α . F, PAI-1. Rats were given a single intraperitoneal dose of ethanol (3.5 g/kg), and the livers were extracted at the times indicated. RNA was isolated and reverse-transcribed as described under *Materials and Methods*. qRT-PCR was performed on a Bio-Rad iCycler 5 using the specific primers listed in Table 1 and was done in triplicate. The average C_t values were used to calculate fold changes in gene expression and were determined by using the $\Delta\Delta C_t$ method and normalized to the GAPDH values. Values are mean $\pm$ S.E. ($n = 3-5$ rats). *, significant values compared with control (C; $p < 0.05$).

ethanol than P-H3-Ser10. At the highest dosage (5 g/kg), phosphorylations at these two sites were not altered. It has been reported that the rapid activation of MAP kinases can lead to the induction of MKP-1 (Clark, 2003). MKP-1 is a negative regulator of MAP kinase, hence a decrease in MAP kinase activity will result from induction of MKP-1 (Clark, 2003; Kuwano et al., 2008). In addition, MKP-1 can directly dephosphorylate P-H3-Ser10 in vitro (Kinney et al., 2009). However, in this model there was no induction of MKP-1 mRNA at the 5-g 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 that has been reported to cause dephosphorylation of histone H3 (Kabra et al., 2009) may be an explanation for the lack of phosphorylation of H3 at higher doses (Fig. 4). It is noteworthy that there was a remarkable increase in the phosphoacetylation (K9S10) status of histone H3 at 5 g of ethanol. The differences in the dose-response relationship for the phosphorylation of Ser10 and the phosphoacetylation at K9S10 suggest that the two modes of histone modifications are independently regulated. It should be noted that the phosphorylated Ser10 and the acetylated Lys9 antibodies only bind to H3 when it is monomodified (Lys9 or Ser10), and the phosphoacetyl antibody only binds when H3 is dimodified (K9S10). It is therefore possible that these modifications may

occur on different sites of histone H3 located on the same or different nucleosome domains in chromatin as shown schematically in Fig. 11.

The phosphorylation of Ser10 seemed to be biphasic. The first phase could be caused by p38 MAPK activation, and the second phase could be caused by the sustained activation of ERK1/2. In this context, we have reported that both p38 MAPK and ERK1/2 MAPK are activated in both the cytosol and nucleus in liver after acute ethanol (Aroor et al., 2010). Although p38 phosphorylation came down at 4 h, the phosphorylation of ERK was sustained at 4 h. Because the time course of MKP-1 mRNA induction from 1 to 3 h was similar in pattern to that of phosphorylated histone H3 at Ser10 (Fig. 9C versus Fig. 5C), increased MKP-1 activity could be responsible for the subsequent decrease in Ser10 phosphorylation either by dephosphorylating MAP kinases or histone H3-Ser10 itself (Kinney et al., 2009). Alternatively, the change in Ser10 phosphorylation may be regulating the transcription of MKP-1 (Li et al., 2001). However, this remains to be established in the context of ethanol.

It is known that cleaved caspase 3 can result from JNK activation, and JNK was shown previously 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 induc-

Fig. 10. ChIP assay with different antibodies. A, *c-fos*. B, *c-jun*. C and D, PAI-1. Frozen liver from the 1.5- and 4-h time-course samples (Fig. 5), where maximum fold change in histone phosphorylation at Ser10 and Ser28 occurred, were used for ChIP assays with antibodies to phosphorylated histone-H3-Ser10, phosphorylated histone H3-Ser28, and phosphoacetylated histone H3-K9S10. The ChIP protocol is described under *Materials and Methods*. The association of modified histone with gene promoters was determined by quantitative RT-PCR using the 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 $\pm$ S.E. ($n = 3$ rats). *, significant values compared with control ($p < 0.05$).

tion. Modified histones have also been shown to associate with the caspase 10 promoter and linked to its activation (Li et al., 2002). Increase in caspase 10 activity might be responsible in part for 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-K9S10 in vivo. It has been reported that phosphorylation at Ser10 of histone H3 opposes acetylation at Lys9, but enhances acetylation at Lys14 (Edmondson et al., 2002). However, our data suggest that modification of Lys9 and Ser10 could also be coordinately up-regulated. We have also observed that histone deacetylase inhibition by trichostatin A induced increases in

P-H3-Ser10 and P-H3-Ser28 in rat primary hepatocytes (unpublished data), and increased histone H3 phosphorylation at Ser28 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-Ser10 and Ac-H3-Lys9 at 24 h (Park and Shukla, 2003; Lee and Shukla, 2007). Because several HATs are known to exhibit increased activity on histone H3 containing phosphorylated Ser10 (Clayton et al., 2000; Merienne et al., 2001), ethanol induced in vivo histone H3-Ser10 phosphorylation may enhance acetylation by these HATs. The biphasic increase in H3-Ser10 phosphorylation (with an early peak at 1.5 h) and the delayed peak of dual-modified H3 (at 4 h) provide support to this possibility. However, the early peak of H3Lys9 acetylation alone at 1.5 h suggests that the two modifications could be occurring independently of each other.

Our data also showed that peak phosphorylation of Ser28 and Ser10 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-Ser28, but not with phosphorylated H3-Ser10 after ethanol treatment. However, we cannot completely rule out the possibility that increased association of phosphorylated H3-Ser10 might occur at regions of the PAI-1 promoter that were not tested in this study. Ethanol-induced activation of PAI-1 is responsible, at least in part, for causing fibrin accumulation in the liver by inhibiting fibrinolysis (Beier et al., 2009), and it has also been implicated in alcoholic liver steatosis (Arteel, 2008). Taken together, these data demonstrate that the phosphorylation of histone H3 at Ser10 and Ser28 and phosphoacetylation of histone H3-K9S10 are affected during acute ethanol administration in vivo. It will be interesting to determine whether changes in the association of modified histone with LDL-r or TNF- α promoter also occur after acute ethanol administration. LDL-r promoter was found previously to be associated with Ser10 histone phosphorylation in HepG2 cells (Huang et al., 2004, 2006), and TNF α promoter was associated with H3-Lys9 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 AP-1 transcription factors and AP-1-responsive genes. Furthermore, histone H3 phosphorylation at Ser28 showed association with the PAI-1 promoter. In conclusion, ethanol-

Fig. 11. A schematic diagram depicting different domains 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 the expression of different genes. Different domains may be located on the same or different nucleosomes.

induced histone H3 phosphorylation in liver, in vivo, is involved in transcriptional activation.

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Authorship Contributions

Participated in research design: James, Aroor, and Shukla.

Conducted experiments: James, Aroor, and Shukla.

Performed data analysis: James, Lim, and Shukla.

Wrote or contributed to the writing of the manuscript: James, Lim, and Shukla.

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