HISTONE H3 PHOSPHORYLATION AND PHOSPHOACETYLATION IN THE LIVER OF RATS TREATED IN VIVO WITH ACUTE ETHANOL

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The undersigned, appointed by the dean of the Graduate School,

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HISTONE H3 PHOSPHORYLATION AND PHOSPHOACETYLATION IN THE LIVER OF RATS TREATED IN VIVO WITH ACUTE ETHANOL

Presented by Taryn T James,

A candidate for the degree of

Doctor of philosophy

And hereby certify that, in their opinion, it is worthy of acceptance.

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Dr. Lene Holland

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LIST OF ABBREVIATIONS

ADH	Alcohol dehydrogenase
ALD	Alcoholic liver disease
ALDH	Aldehyde dehydrogenase
AMP	Adenosine monophosphate
AP1	Activator protein 1
CARM1	Coactivator-associated arginine methyltransferase 1
CBP	CREB binding protein
CDK	Cyclin dependent kinase
CHD1	Chromodomain 1
CYP2E1	Cytochrome P4502E1
DMEM	Dulbecco's modified eagles's medium
ERK	Extracellular related kinase
GCN5	General Control Nonderepressible 5
HAT	Histone acetyltransferase
H & E	Hematoxylin & Eosin
HDAC	Histone deacetylase
HMTase	Histone methyltransferase
HP	heterochromatin protein 1
HRP	Horseradish peroxidase

IF2	Initiation factor 2
IKKα	Inhibitor of IκB kinase α
MAPK	Mitogen-activated protein kinas
MBD	Methyl CpG binding domain protein
MCP-1	Monocyte Chemoattractant Protein 1
MEOS	Microsomal ethanol oxidizing system
MOF	MOZ-realated factor
MOZ	Monocytic leukemia zinc-finger protein
MKP	MAP Kinase Phosphatase
MSK	Mitogen- and stress-activated protein kinase
MTA	Metastasis-associated protein
MYST	MOZ, Ybf, Sas and Tip60 together
NFκB	Nuclear factor-kappa B
PC	Polycomb
PEPCK	Phosphoenolpyruvate carboxykinase
PCAF	P300/CBP-associated factor
PKA	Protein kinase A
PKCRK	Protein kinase C related kinase
qtRT-PCR	Quantitative Real Time PCR
RAR	Retinoic acid receptor
RbAp	Retinoblastoma associated protein

ROS	Reactive oxygen species
RSK2	Ribosomal S6 kinase 2
RXR	Retinoid X receptor
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIRT	Sirtuins family
Src-1	Steroid receptor coactivator-1
SRE	Sterol Regulatory Element
SREBP	Sterol regulatory element binding protein
TBP	TATA binding protein
TG2	Transglutaminase 2
TSA	Trichostatin A
TNF	Tumor necrosis factor

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ABSTRACT

Overindulgence of ethanol leads to alcoholic liver disease and in some cases death especially for the chronic abusers. Binge drinking is also on the rise and binge drinkers are more susceptible to alcohol induced injury over a shorter time. The relevance of histone modifications is gaining more light in the pathogenesis of alcoholic liver disease. Histone H3 phosphorylation which was previously only known to be involved in mitosis, has recently become known to be involved in the transcriptional regulation. Additionally, histone H3 phosphorylation is known to crosstalk with histone H3 acetylation and methylation. This study utilized an acute model where rats were administered ethanol intraperitoneally, to mimic the effects seen in binge drinking in humans. The model also helped to study rapid and transient responses of ethanol often encountered in binge intake. The effects of ethanol on histone H3 phosphorylation in rats in vivo; the role of this phosphorylation in gene regulation; the relationship between this phosphorylation and acetylation, and phosphoacetylation, and its implication in alcoholic liver disease are described. We have observed that ethanol

administration in vivo increased the phosphorylation of JNK, p38, and ERK MAP kinases in both the cytosol and nucleus of the liver. We also observed histone H3 phosphorylation specifically at serine-10 and serine-28. Serine 28 was more sensitive to ethanol at low dose (1.75 g/Kg). At a higher dose (5 g/Kg), the phosphorylation of both serines disappeared. Additionally, serine 10 phosphorylation was more rapid than serine 28 phosphorylation. Interestingly at the highest dose (5 g/Kg), phosphoacetylation significantly increased as compared to no change in the phosphorylation of both serine 10 and 28 alone. The differences in the profiles of these site specific different modifications in histone H3 suggest that these may reside in different domains in the nucleosome. The increase in histone phosphorylation and phosphoacetylation was followed by increases in the expression of a number of genes i.e. c-Fos, c-Jun and MKP-1, LDL-r, TNFα, PAI-1 all of whom play roles in alcoholic liver injury. Chromatin immunoprecipitation (ChIP) assays were performed with histone H3 antibodies that recognized 1) Phosphorylated serine-10, 2) phosphorylated serine-28, and 3) phosphoacetyl H3-lysine 9/serine-10, to determine their association with specific gene promoters. This thesis demonstrates for the first time that ethanol induction of histone H3 phosphorylation at serine 10 may be associated with immediate early genes c-Fos, and c-Jun, phosphorylation at serine 28 was associated with the promoters of early response genes c-Jun (AP-1 consensus sequence in the promoter region (-592 to -585). In contrast to serine 10, histone H3 serine 28 phosphorylation was associated with the promoter of plasminogen activator inhibitor 1 (PAI-1) which contains an SRE consensus

sequence (-701 to 693) for that could interact with AP1 transcription factors. Phosphoacetylation of histone H3 at lysine 9/serine 10 also specifically associated with PAI-1 promoter. The fact that histone H3 phosphorylation and phosphoacetylation are associating with genes that are changed after ethanol and that have roles in alcoholic liver injury suggests that these modifications may have a role in transcriptional events relevant to the liver injury. The study presented here also established for the first time that the ethanol metabolite, acetate may be involved in the phosphorylation of histone H3. In summary, we conclude that ethanol increases histone H3 phosphorylation at serine 10 and serine 28 in a distinctive manner. Since the phosphorylation of serine 28 and the phosphorylation of serine 10 in conjunction with lysine 9 acetylation was associated with PAI-1, these two modifications may be mechanistically important in the steatosis and fibrin accumulation after alcohol. The results from this in vivo study offer new insight into H3 phosphorylation, its transcriptional significance and possible involvement in the pathogenesis of alcoholic liver disease especially in the context of binge drinking.

CHAPTER I

INTRODUCTION

I.1 Alcoholic liver disease

Alcohol abuse is a very prevalent problem in the world today, as it leads to addiction and eventually socioeconomic and health problems that can have extreme negative consequences on society. The cost of alcohol abuse to society is estimated to be $180 billion per year. This is an important area for research because alcohol addiction often results in alcoholic liver disease; therefore it is necessary to understand the molecular and cellular events that lead to disease progression. The pathology for alcoholic liver is identified by steatosis (fatty liver), steatohepatitis (inflammation), cirrhosis (fibrosis of the liver) and at times hepatocellular carcinoma (Menon, et al 2001; Tilg and Diehl, 2000; Albano, 2008). Pathology results showed that alcohol abuse promotes phenotypic changes in the liver (Ray, 1987; Burt et al 1987; Harrison et al, 1990). The disease usually begins as fatty liver, and inflammation, then evolves to necrosis and apoptosis and leads to fibrosis (Lieber, 2000). Alcoholic hepatitis is an inflamed condition of the liver resulting from the metabolism of alcohol to toxic compounds such as acetaldehyde, and acetate (Menon, et al 2001; Miranda-Mendez et al, 2010). Additionally, ethanol metabolism in the liver triggers the production of tumor necrosis factor alpha (TNFα) by hepatic macrophages and hence the generation of

reactive oxygen species, another contributing factor to inflammation (Miranda-Mendez et al, 2010). With increased exposure to alcohol this inflammation can give way to liver cirrhosis, an irreversible condition where healthy tissue is replaced by scar tissue (Bataller & Brenner, 2005). Moreover, increased exposure of alcohol also results in fatty liver because the liver is not able to function normally in the oxidation of fats, as it is overwhelmed to metabolize alcohol (Menon et al, 2001).

I.1.1 Ethanol Metabolism in the liver

Ethanol is metabolized by three enzymatic (oxidative) pathways in the liver (Edenberg, 2007) [fig. 1). Most of the ethanol in the liver is metabolized by alcohol dehydrogenase leading to the formation of acetaldehyde (Lieber, 2000). The second major player for ethanol metabolism is the microsomal ethanol oxidizing system (MEOS) which utilizes the enzyme cytochrome P450E1 (CYP2E1) for ethanol degradation (Aroor & Shukla, 2003; Gemma et al, 2006). This pathway has been found mainly in chronic alcoholics and is believed to play a role in the tolerance to ethanol seen in these individuals (Lieber, 2000). The MEOS is also responsible for the production of reactive oxygen species (ROS), a contributing factor in the liver injury (Lu and Cedebaum, 2008). The third pathway for ethanol metabolism occurs in peroxisomes and employs the enzyme catalase and also requires hydrogen peroxide for metabolism. Ethanol can also be metabolized nonoxidatively. An example of this is the production of esterification products of ethanol and fatty acids known as fatty acid ethyl esters (FAEEs) a pathway

that utilizes the enzyme fatty acid ethyl ester (FAEE) synthase. FAEEs can collect in the plasma membrane as well as in the membranes of the organelles such as the mitochondria and lysosomes and prevent them from functioning properly (Best and Laposata, 2003). FAEEs can also serve as postmortem markers to differentiate chronic alcoholics from with a binge drinkers, the latter having more FAEEs in the blood and tissues (Sordeberg et al, 2003). Another nonoxidative product of ethanol is phosphatidylethanol. Therefore ethanol metabolism generates multiple products that have damaging consequences.

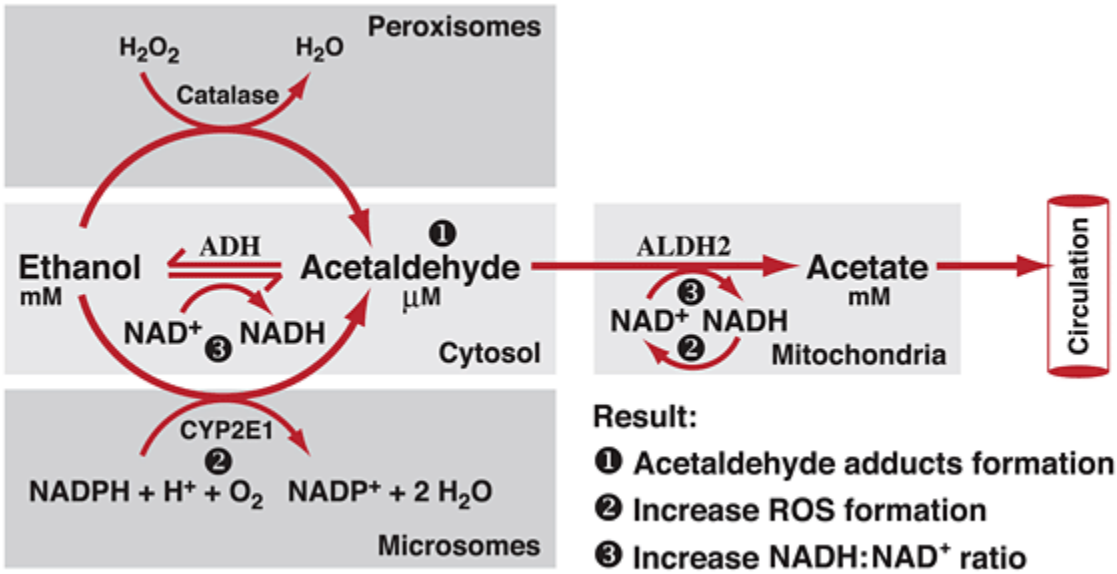

Fig 1. Pathways of Ethanol Metabolism

adapted from niaa.nih.gov

I.1.2 Effects of ethanol metabolism in the liver

Studies in animals have shown that the first major effect of ethanol metabolism in the liver is a reduction in the ratio of NAD/NADH (Geokas, 1981; Galli et al, 2001). This redox state increases fatty acid synthesis, whilst preventing gluconeogenesis and inhibiting the TCA cycle (Galli et al, 2001). However, continued exposure to ethanol normalizes the redox state of the liver. The production of acetaldehyde is also an important consequence of ethanol metabolism (Erickson, 2001). Acetaldehyde is a toxic compound that promotes changes in the liver and has further actions through its by-products: protein aldehyde adducts, acetate and adenosine monophosphate (AMP) (Nagy, 2004). Aldehyde adducts can lead to inflammation and fibrogenesis seen in alcoholic liver injury. When acetaldehyde is metabolized in the liver, it is converted to acetate and AMP. AMP is produced from the metabolism of acetate by ecto-5'-nucleotidase to acetylCoA which involves the catalism of ATP to AMP. AMP can be further metabolized to adenosine (Peng et al, 2009). Adenosine can bind to adenosine receptors on the surface of hepatocytes and increase cAMP production (Galli et al, 2001). Acetate can have effects in the central nervous system. The ROS produced by CYP2E1 may affect signal transduction pathways such as the mitogen activated protein kinase (MAPK) pathways and may also affect the transcription factors NFκB and AP-1 (Aroor & Shukla, 2003; Nagy, 2004).

I.1.3 Steatosis, Alcoholic Hepatitis and Cirrhosis

Steatosis (fatty liver) is the most common characteristic of alcoholic liver disease and it is marked by increased accumulation of fats inside liver cells (hepatocytes). As mentioned above, initial exposure of the liver to ethanol shifts the redox state to favor fatty acid synthesis over fatty acid oxidation (Nagy, 2004; Galli et al, 2001). This is mediated through alcohol dehydrogenase. Since the redox state of the liver returns to normal with increased exposure to ethanol, steatosis may be mediated by different mechanisms (Nagy, 2004). One likely explanation is that a change in gene expression could be responsible for steatosis. Ethanol is known to promote changes in gene expression. Our lab has shown that ethanol can induce epigenetic changes in histone H3 through modulation of the MAP kinase pathway (Aroor and Shukla 2004; Shukla et al, 2008). Epigenetic changes can alter gene expression (Grant, 2001). If these changes can affect genes involved in fatty acid regulation, then this may be the pathway for alcohol induced steatosis. A down regulation or up regulation of genes mediating fatty acid synthesis, oxidation and/or transport could be responsible for liver steatosis. Interestingly, it has been shown that PAI-1 may have a causal relationship. Recently PAI-1 has been implicated in this aspect of alcoholic liver disease (Arteel, 2008). Early stages of ALD involve chronic inflammation. Lipopolysaccharide is long known to play a role in ALD and since it can activate the coagulation cascade, LPS can lead to fibrin accumulation. Recently it was shown that ethanol induces activation of PAI-1, which causes fibrin accumulation in the liver by inhibiting fibrinolysis as well as sensitizes the

liver to LPS (Beier et al, 2009). Co-administration of the MEK inhibitor, U0126, and the thrombin inhibitor, hirudin (which prevents fibrin accumulation) decreases liver damage after ethanol exposure and also prevents extracellular accumulation of fibrin and the induction of PAI-1. Similar results were observed in TNF$\alpha^{-/-}$ mice and with PAI-inactivating antibody (Beier et al, 2009).

Acute and chronic alcohol use can lead to alcoholic hepatitis, however it usually develops in chronic alcohol users. Hepatitis can be mild (recognizable by liver function tests) or severe; recognizable by jaundice, ascites, hepatic encephalopathy, and microscopically by hepatocyte degeneration and inflammation with neutrophils); (McClain et al, 1997, Miranda-Mendez et al, 2010). As mentioned above, the metabolism of ethanol leads to the production of ROS which in turn can stimulate cytokine production, another contributuing factor to alcoholic hepatitis. The cytokines that are found at the forefront of alcoholic hepatitis are the interleukins [IL-1, IL-6, IL-8, and IL-10], tumor necrosis factor (TNF), and monocyte chemoattractant protein-1 [MCP-1] (McClain et al, 1997). Increased TNF in ALD patients means their prognosis is reduced (Hill et al, 1992). Consequently, IL-8 promotes neutrophilia and neutrophil infiltration of liver tissue in ALD (Sheron et al, 1993). These findings have also been found in laboratory animals. When there is an inflammatory process in the body the liver is one of the major organs affected. Inflammation causes the liver to focus on the inflammatory and immune system over its normal metabolic function (McClain et al, 1997). The secretion of acute-phase proteins, a marker of acute inflammation, is

increased whereas the production of albumin, the major protein in the soluble portion of the blood, is decreased. This reduction in metabolic activity is also seen in other inflammatory diseases (McClain et al, 1997). Therefore, understanding the change in the function of the liver during alcoholic hepatitis will also aid in understanding similar type of changes in other diseases. Alcoholic hepatitis can be reversed with alcohol abstinence. If it is not, it can lead to cirrhosis, a condition where nodules of regenerating heptocytes and fibrosis [caused by excess deposition of extracellular matrix (ECM) proteins, mainly collagen] develop in the liver (Bataller & Brenner, 2005). Hepatic fibrosis begins as a wound healing response of the liver to repeated injury (Bataller & Brenner, 2005). Acute liver injury causes parenchymal cells to regenerate and replace necrotic and apoptotic cells. During this process the inflammatory response occurs with limited ECM deposition. If the injury continues, the liver regeneration fails and abundant ECM replaces hepatocytes (Benyon and Iredale, 2000). The ECM proteins in advanced liver fibrosis include collagen (I, III, IV), fibronectin, undulin, elastin, laminin, hyaluronan, and proteoglycans (Bataller & Brenner, 2005). It was found that about 20 percent of patients with alcoholic hepatitis develop cirrhosis (McClain et al, 1997). Since cirrhosis is an irreversible condition, the cellular events that lead to its development need to be understood. Again, the post-translational modification of histone and chromatin remodeling may provide some answers. It is possible that histone phosphorylation may be linked to alcoholic hepatitis and cirrhosis. Perhaps the phosphorylation of H3 at serine 28 may have a role in these conditions, since phosphorylation at this residue is higher with acetaldehyde than with ethanol (Lee and Shukla, 2007). Since acetaldehyde

is a metabolic by-product of ethanol that leads to the generation of ROS, it is conceivable that ROS may be involved in histone H3 phosphorylation.

I.2 Ethanol and gene expression

In a microarray analysis where rats were fed ethanol for 14-15 weeks, genes that were affected by ethanol had a two-fold difference over control (down-regulation or up-regulation). Stearoyl Coenzyme A (CoA) desaturase, and acyl CoA desaturase, genes that are involved in fatty acid metabolism, were decreased by ethanol (Deacuic et al, 2004). These two desaturase enzymes are involved in the oxidation of fats, therefore ethanol clearly affects this normal process, which is a function of the liver. In the same study, glutathione S-transferase gene was found to be upregulated by ethanol. In another gene expression study (Deacuic et al, 2004), rats were fed ethanol for 4 weeks (chronically) by intragastric infusion. Again the effect of ethanol on genes was assessed via microarray analysis. Genes from many cellular processes such as gluconeogenesis, and cell proliferation were found to be affected by ethanol. The alcohol dehydrogenase gene, and two genes of the cytochrome P450 family were upregulated by ethanol while phosphoenolpyruvate carboxykinase (PEPCK), phosphatidylinositol 3-kinase, and Bcl-2 were downregulated by ethanol (Deacuic et al, 2004). The down regulation of PEPCK is an important finding because it is known that alcohol causes hypoglycemia by causing the NAD/NADH ratio to be reduced which promotes low levels of oxaloacetic acid, the precursor for PEP(Krebs, 1968). But it also suggests that hypoglycemia (by less gluconeogenesis) may develop due to reduced expression of PEPCK enzyme. The Bcl-2 down regulation may give insights into why alcohol causes apoptosis of liver cells in

experimental animals and humans as previously shown by Benedetti et al 1988, and by Kawahshara et al 1994, respectively.

I.2.1 Ethanol and its Effect on Transcription Factors

PPARS

Transcription factors that are affected by ethanol include peroxisome proliferator- activated receptors (PPARs). PPARs have diverse roles in cellular functions such as lipid metabolism, glucose metabolism, cell proliferation, differentiation, adipogenesis, and inflammatory signaling (Galli et al, 2001 & Lefebvre et al, 2006). PPARs are ligand (i.e. fatty acid) activated transcription factors that bind DNA as heterodimers with the retinoid X receptor (RXR). As a heterodimer, (PPAR/RXR) binds to PPAR response elements (PPRE) located in the enhancer regions of PPAR regulated genes to increase gene transcription. One member of this receptor family, PPAR α functions in fatty acid oxidation and studies in rats have shown that ethanol metabolism prevents the transcriptional activation of PPAR α by inhibiting it from binding DNA (Galli et al, 2001). Acetaldehyde was found to be important in this process. Previous studies in PPAR α null mice demonstrated that the oxidation of long chain fatty acid and palmitic acid were reduced; and that the levels of enzymes such as long chain acyl-CoA dehydrogenase were decreased (Aoyama et al, 1998). These studies demonstrate that PPARα may mediate alcoholic steatosis.

Sterol regulatory binding proteins (SREBPs)

SREBPs are transcription factors that aid in the regulation of cholesterol and fatty acid synthesis by directly activating over 30 genes in the liver (You et al, 2004). As suggested by their name, SREBPs have important roles in sterol regulation. SREBPs are basic-helix-loop-helix-leucine zipper (bHLH-LZ) transcription factors synthesized as membrane bound inactive precursors that reside on the rough endoplasmic reticulum (rER). When SREBPS are activated they undergo a two-step proteolytic cleavage to release their active form that can bind importins to localize to the nucleus. Once in the nucleus, they can activate their target genes by binding to E-boxes, and sterol regulatory element (SRE) sequences. There are three mammalian SREBPs, SREBP-1a, SREBP-1c, and SREBP-2. SREBP1a and 1c have important roles in the regulation of genes responsible for hepatic triglyceride synthesis, such as fatty acid synthase, stearoyl-CoA desaturase, acetyl CoA carboxylase, ATP citrate lyase, and malic enzyme (You et al, 2004). SREBP2 has a central role in lipid homeostasis since it can stimulate expression of genes in the cholesterol bisosynthetic pathway (Kim et al, 2008). Liver-specific overexpression of these transcription factors increases hepatic triglyceride content (Shimano et al, 1996). Interestingly, another study illustrated that ethanol metabolism (i.e. acetaldehyde) induces fatty acid synthesis by the activation of SREBP-1 in the rat hepatoma cell lines H411EC3 and McARH7777 (You et al, 2002).

NFκB

Nuclear factor kappa beta (NFκB) is a family of dimeric transcription factors that are able to activate multiple promoters (Lu and Stark, 2004). The members of this family that are present in mammalian cells are most notably RelA (p65), RelB, c-Rel, NFκB1 (p105/50), NFκB2 (p100/p52) (Li & Verma, 2002). These transcription factors are characterized by their Rel homology domain (RHD) which is involved in DNA binding, dimerization between family members, and for association with the inhibitors of kappa B [IκB] (Trachootham et al, 2008). The NFκB family of transcription factors are dimeric; many different combinations can exist. For instance NFκB exists most commonly as a p65/p50 heterodimer. In the cytoplasm of mammalian cells NFκB is inactive because it is bound to its repressor IκB (Lu and Stark, 2004). However, when the cell is stimulated, IκB becomes phosphorylated by an activated IκB kinase (IKK) complex, which targets it for degradation by the 26S proteasome, allowing NFκB to translocate into the nucleus, and bind to the promoters of NFκB regulated genes to activate transcription (Trachootham et al, 2008). NFκB also has an important role in the effects of alcohol on liver injury. It has been reported that ethanol can increase DNA binding of NFκB in rats fed ethanol via gastric infusion (Nanji et al, 1999, Tsukomoto et al, 1999). This is believed to mostly occur in the Kuppfer cells and so the role of NFκB is believed to be involved in the inflammatory response in ALD (Nagy, 2004). However it is possible that this transcription factor may also have important roles in hepatocytes and that this may be mediated in part by acetaldehyde. It has already been shown in HepG2 cells that activation of NFκB was due to acetaldehyde. It is important to note that cytochrome

P450 2E1 was overexpressed in these HepG2 cells (Roman et al, 1999). NFκB is known to regulate cellular responses to oxidative stress (Denk et al, 2000; Ha et al, 2010). The fact that ethanol promotes CYP2E1 mediated oxidative stress and this did not affect NFκB activation, suggests that acetaldehyde may have an important role in the activation of NFκB. Therefore acetaldehyde may have a similar role in the activation and recruitment of NFκB to gene promoters in primary hepatocytes or liver which may also be linked to histone H3 phosphorylation. It was demonstrated in dendritic cells that p38-dependent histone phosphorylation mediated by LPS tagged (inflammatory) genes for increased NFκB recruitment to promoters undergoing histone H3 serine 10 phosphorylation (Saccani et al, 2001). Thus, the fact that p38 MAPK was found to be the kinase mediating histone phosphorlation in primary cultures of hepatocytes (Lee and Shukla, 2007) lends support to this idea.

AP-1

The AP-1 family of transcription factors are interesting in that they have roles in both cell growth and apoptosis (Trachootham et al, 2008). The members of this family are basic leucine zipper protein domain proteins (bZIP) , namely, Jun (cJun, JunB, JunD), Fos (cFos, FosB, Fra-1, and Fra-2), Maf (cMaf, MafB, MafA, MafG/F/K, and Nrl), and ATF (ATF2, LRF1/ATF3, B-ATF, JDP1, JDP2) subfamilies (Trachootham et al, 2008). Like the NFκB family, AP-1 proteins form dimers to bind DNA (Angel & Karin, 1991). The activation of AP-1 is regulated at the transcript and protein levels (Trachootham et al,

2008). The amount of AP-1 in the cell is controlled by transcriptional rate which is regulated by a number of stimuli. Additionally AP-1 is regulated at the protein level: for example MAPK can phosphorylate and activate AP-1 proteins (Trachootam et al, 2008). Once activated AP-1 can target genes involved in cell survival or apoptosis. The question arises, what is the role of AP-1 in ALD? AP-1 is a transcription factor that exists as a c-Jun homodimer or heterodimer with c-fos (Angel & Karin, 1991). The activation of the MAP kinases (ERK1/2 and JNK) can increase the expression of c-Jun and c-fos and hence AP-1 (Thomson et al, 1999). Histone H3 phosphorylation at serine 10 and phosphoacetylation of histone H3 at serine 10/lysine 14 have been shown to be present on c-Fos nucleosomes (Clayton et al, 2000); Thomson et al, 2001). 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). Since the activation of c-fos may be associated with histone H3 phosphorylation at S10 after ethanol, it is possible that AP-1 is affected by histone phosphorylation. This may give insight into ALD because AP1 is a regulator of inflammatory genes such as TNFα (alcoholic hepatitis), PAI-1 (steatosis), as well as matrix metalloprotease, and collagen type I (fibrosis) (Yao et al, 1997; Wisdom, 1999; Benkossa et al, 2002; Ghosh, 2002; McDonald et al, 2008).

It will be interesting to see if the above changes in gene expression by ethanol are linked to phosphorylated histone H3. Since the changes in alcoholic liver disease are not solely specific for alcohol, this can provide information on other diseases with similar consequences. Additionally, since alcohol is a toxin, discovering how the

pathology of alcoholic liver develops may aid our understanding of how other toxins affect the body, since a major role of the liver is detoxification.

I.3 Epigenetics

Epigenetics, which means above the genetics, is a term coined by Waddington that is used to describe heritable changes (meiotic and mitotic) in gene expression that are independent of the DNA sequence itself (Waddington, 1942). There are three major processes involved, DNA methylation, RNA-associated silencing, and histone modifications (Egger et al., 2004). These components interact and stabilize each other and a disruption of their interaction can promote inappropriate expression or silencing of genes resulting in 'epigenetic diseases' (Goodman and Smolik, 2000; Petrij et al., 1995; Kundu and Dasgupta, 2007). Epigenetics is an exciting and promising area for research because epigenetic pathways are common in various organisms. The role of epigenetic modifications in gene function and regulation is thus important as a manipulation of these modifiers will be important in treating epigenetic diseases. Uncovering mechanisms of epigenetics as applied to gene states will be important for therapy across many applications such as somatic gene therapy, cancer biology; and cloning (Wolffe and Matzke, 1999; Egger et al., 2004). Epigenetics differs from genetics in that epigenetic changes are acquired gradually as opposed to be abruptly. This makes these modifications applicable to prevention strategies. Therefore for a certain disease it is possible to have genetic as well as epigenetic markers to which therapy can be targeted.

I.3.1 Chromatin, nucleosome and histone

The nucleosome is the structural unit of chromatin and it is formed by an octamer of histone, and the DNA to which it is associated (Kornberg, 1974; Kudu and Daguspta, 2007). There are two main types of chromatin: heterochromatin and euchromatin. Heterochromatin is tightly coiled and (transcriptionally) inactive while euchromatin is active in gene expression (Grewal and Jia, 2007).

The histone octamer of the nucleosome is comprised of two H2A-H2B dimers, and an H3- H4 tetramer. Furthermore, 1.65 turns of 146 base pairs (bp) of DNA wraps around this octamer to compact eukaryotic DNA to form the nucleosome (Kundu & Dasgupta, 2007). Nucleosomes connect to each other by 10-90 bp of DNA which is stabilized by the linker histone (H1) to help package the nucleosomes into 30nm fibers that contain 6 nucleosomes per turn in a spiral arrangement (Khan and Krishnamurthy, 2005) [Fig 2]. When these 30nm fibers unfold, a transcriptional template is created. The core histones are made up of a structured domain and an unstructured amino-acid tail that varies in length from 16 amino acid residues in H2A, 26 amino acids for H4, 32 for H2B, and 44 amino acids for H3 (Sng, 2004). These amino-acid tails can undergo a number of covalent modifications such as acetylation, methylation, phosphorylation, ADP-ribosylation, sumoylation, and ubiquitination. These modifications help to identify the different chromatin states (Jenuwein and Allis, 2001). For example, histones H3 and H4 are highly hypoacetylated in heterochromatic chromosomal regions in various organisms such as yeast, flies, and mammals. (Braunstein et al, 1993; Grunstein et al

1998; Jepperson and Turner, 1993; Nakayama et al, 2000). Additionally, histone modifications affect the chromatin structure by modifying histone-DNA contacts, or by acting as recognition motifs for protein interactions that may be enzymatic or act to recruit regulatory proteins as defined by the histone code hypothesis (Strahl and Allis, 2000, Jenewein and Allis, 2001).

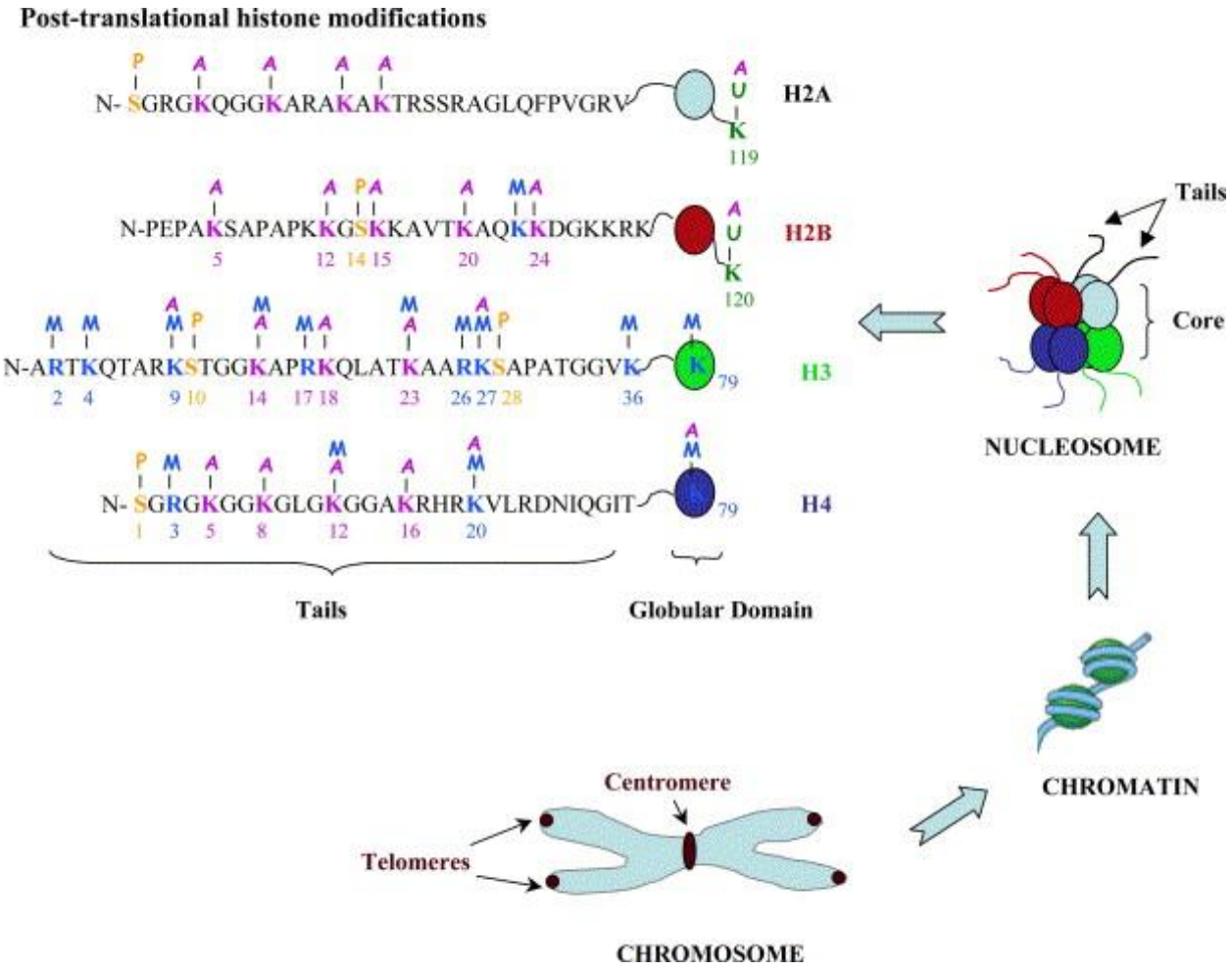

Fig 2. Post translational Histone Modifications

Adapted from Santos-Rosa, H and Caldas, C (2005) Chromatin Modifier enzymes, the histone code and cancer. Eur J Cancer. 41, 2381-2402.

I.3.2 Histone modifications

Histones are highly conserved basic proteins whose main function is in the organization of chromatin as mentioned above and they can undergo diverse post-translational modifications: acetylation, methylation, phosphorylation, ubiquitination, glycosylation, ADP-ribosylation, and sumoylation. Of these, acetylation, methylation and phosphorylation are the most studied (Cheung et al**.,** 2000; Jason et al., 2002; Kouzarides, 2002; Peterson and Laniel, 2004; Wong and Smulson, 1984; and Shiio and Eisenman, 2003). Most of these modifications with the exception of ubiquitinization, occur on the N-terminal tail of the histones (Weake and Workman, 2008). Distinct patterns of post-translational modifications of histones, "the histone code," mark them for the recruitment of downstream chromatin modifiers (Strahl and Allis, 2000; Turner, 2000; Nakayama et al, 2001).

Histone Acetylation

Acetylation is the most extensively studied histone modification, and it is balanced by histone acetyltransferases or HATs (enzymes that catalyze the addition of acetyl groups to ε-lysine residues) and by histone deacetylases or HDACS (enzymes that catalyze the removal of these groups) (Rice and Allis, 2001). Acetylation of histone occurs at lysine (K) residues on the amino terminal tails (Sterner and Berger, 2000; Roth et al, 2001). The addition of an acetyl group to histones reduces their positive charges, and lessens their interaction with negatively charged DNA, leading to an open

(accessible) chromatin state (Norton et al, 1989, Hong et al, 1993; Lee et al, 1993). Histones are more acetylated in active chromatin regions than inactive regions (Grunstein, 1997; Kuo and Allis, 1998). It follows that most HATs are coactivators of transcription while most HDACs are corepressors (Kuo and Allis, 1998; Strahl and Allis, 2000). Inhibition of HDAC activity can turn on previously repressed genes (Roth et al, 2001).

Histone Methylation

The methylation of histones was first described in 1964 (Murray, 1964). Histone methylation occurs at lysine or arginine residues. The addition of methyl groups to histones are catalyzed by histone methyltransferases (HMTs) and their removal by histone demetylases (HDMs)**.** Lysine residues can be mono-, di-, or trimethylated whereas arginine residues are either mono- or dimethylated (Zhang & Rheinberg, 2001). This modification of histone took center stage with a paper in Science (Nakayama et al, 2001) where the relationship between histone methylation and deacytelation during gene silencing in fission yeast was analyzed. With the discovery of methyltransferases (Rea et al, 2000), they were able to develop a methylation antibody specific for lysine 9 of histone H3 which was used in ChIP assays to demonstrate that histone methylation at centromeres was dependent upon a methyltransferase (i.e Clr4) as well as histone deacetylase activity at lysine 14. This paper suggested that deacetylation at lysine 14 promotes methylation at lysine 9. Additionally methylation of histone H3 recruits heterochromatin protein 1 (HP1) [Swi6 in fission yeast] to heterochromatin (Bannister et

al, 2001; Nakayama et al, 2001). Histone methylation was first thought to be associated with gene repression but some active genes in euchromatin are methylated at lysine 4 and 36 in histone H3. In yeast, expressed genes undergo methylaton (normally trimethylation) at their 5' ends in specific forms and analysis of human euchromatin suggests that a similar pattern occurs in mammals (Margueron et al, 2005). Because a methyl group is small and does not neutralize the positive charge of lysines and arginines, it is logical that methylation provides binding sites for regulatory proteins (Bannister and Kouzarides, 2005). Methylation of histone H3 at K9 and K27 are known as repressive marks since they bind the repressive proteins HP1 and PC respectively; and CHD1 binds the H3K4, an activating methylation mark (Bannister and Kouzarides, 2005; Pray-Grant et al, 2005). Interestingly, consistent with the above observations, our lab has shown that methylation of H3K4 was associated with upregulated genes after ethanol treatment, and methylation of H3K9 was associated with downregulated genes in hepatocytes (Pal-Bhadra et al, 2007). Recently in brain (nucleus accumbens) another drug, cocaine, has been shown to affect histone methylation. It was found that histone lysine methylation may have an important role in regulating gene expression after repeated cocaine self-administration. Cocaine reduced the methlyation of histone H3 K9 which led to reward related changes in behavior in mice (Maze et al, 2010). Methylation of histone H3 at K9 has also been shown to be linked with DNA methylation. DNA methyltransferases and methyl-binding proteins can directly interact with histone-methylating enzymes (Freitag & Selker, 2005). For example, disruption of DNA methylation promoted a loss of histone H4K20 methylation in cancer cells (Fraga et al,

2005). RNAi is also linked to histone methylation. A disruption of the RNAi machinery affects heterochromatin formation, as well as the presence of H3K9 and HP1 in heterochromatin (Elgin and Grewal, 2003; Pal-Bhadra et al, 2004; White and Allshire, 2004; Bannister and Kouzarides, 2005).

Histone Phosphorylation

Histone phosphorylation is known to function in mitosis, cell death, repair, replication, and recombination (Kundu and Dasgupta, 2007). Histones, H1, H2A, and H2B, H3 and H4 as well as histone variants can be phosphorylated, and the phosphorylation of each is believed to have specific roles in mitosis (Ajiro, 2000). H1 phosphorylation is a marker for mitotic cells (Boulikos, 1995) and is highest during metaphase when the chromosomes are maximally condensed (Zhang and Dent). Cyclin dependent kinases 1 and 2 (CDK1 and CDK2) have been implicated in the phosphorylation of histone H1 (Kundu & Daguspa, 2007). H1 can be phosphorylated on its C and N-terminus. H2A is phosphorylated throughout the cell cycle. Histone H2B phosphorylation is low in quiescent and growth stimulated states in vitro but is increased in apoptotic cells (Ajiro, 2000). Phosphorylation of the histone variant H2A.X is involved in the DNA damage response (Hanosage and Ljungman, 2007). H4 phosphorylation is negligible depending on cell type. Histone H3 is highly phosphorylated during mitosis and is also associated with premature chromatin condensation (Strahl and Allis, 2000). Histone H3 is phoshorylated on serine residues (S10, S28) and threonine residues (T3, and T11). Phosphorylation of H3 on threonine residues is less studied than phosphorylation at

serine residues but has recently received more attention. Threonine 3 phosphorylation was found to concentrate at centromeric chromatin during metaphase (Tian et al, 2010). Protein kinase C related kinase 1 (PKCRK1) was shown to phosphorylate histone H3 at T11 resulting from ligand-dependent recruitment to androgen receptor (AR) target genes (Metger et al, 2008).

Rapid phosphorylation of histone H3 occurs after various stimuli i.e UV irradiation, 12-O-tetradecanoylphorbol-13-acetate, anisomycin, epidermal growth factor, and others (Zhong et al, 2001; Novak and Corces, 2004, Drobic et al 2010). Histone H3 phosphorylation by MAP kinases (MSK1 & MSK2) is known to be involved in the induction of immediate early (IE) genes such as the proto-oncogene c-myc and c-fos (Thomson et al, 2001). Recently it was reported that the remodeling of the promoters of immediate early genes is mediated through histone phosphorylation at serine 10 and serine 28 by an MSK-1 multi-protein complex (Drobic et al, 2010). H3 phosphorylation at serine 10 has also been implicated in a less condensed chromatin structure and aberrant gene expression in oncogene transformed cells (Chadee et al, 1999).

I.3.3 Crosstalk between phosphorylation and other modifications

It has previously been reported that increased HAT activity occurs on histone H3 phosphorylated at-ser 10, and that mutation of ser 10 decreases expression of GCN5-regulated genes (Grant, 2001). Several other HATs also have a preference for phosphorylated S10 including PCAF and p300 (Cheung et al, 2000; lo et al, 2000).

Additionally, it was found that phosphoacetylation of H3 was involved in gene activation in c-myc and c-fos nucleosomes (Clayton et al, 2000). Phosphorylation of histone H3 at S10 can increase acetylation of histone H3 at lysine K14, eliminate acetylation of histone H3 at K9, and methylation of histone H3 at K9 (Kundu & Dasgupta, 2007; Rea et al, 2000) [fig. 3]. The belief is that once histone is phosphorylated at the serine 10 position, GCN5 binds to this phospho-residue and acetylates lysine 14 (Nowak & Corces, 2004). However, analysis of c-Jun induction has shown that a prephosphorylated H3 tail isn't a tag for acetylation at lysine 14 but that the balance between HATs and HDACs may play more of a role (Nowak & Corces, 2004). In this case phosphorylation and acetylation will be occurring by two distinct pathways. Interestingly, it has been demonstrated that mutations in MSK1 and MSK2 did not lead to a loss of acetylation at IE genes (Nowak & Corces, 2004). Therefore, phosphoacetylation of histone H3 tail may occur via two ways. In the first (synergistic) pathway a preacetylated H3 at K9 (perhaps by GCN5) is phosphorylated at S10 by MAPK signaling; this increases binding affinity for GCN5 which then phosphorylates K14 and activates transcription of the associated genes (Nowak & Corces, 2004). In the second (parallel independent) pathway H3 is acetylated at K9 and K14 which is maintained by HATs and HDACs, while kinase activity via MAPK cascade, phosphorylates the same H3 at S10 leading to gene transcription (Nowak & Corces, 2004). Our lab has reported that ethanol stimulates acetylation of H3 at K9 and phosphorylation of H3 a S10 in primary hepatocyes, showing the greatest increase in both modifications at 24hrs. Interestingly, we haven't seen any changes in K14 acetylation (Lee and Shukla, 2007; Park et al, 2005). Additionally, we do not know if the

increases in acetylation and phosphorylation are occurring on the same nucleosomes, or if they are independent or synergistic. Therefore it is important to study the crosstalk between acetylation and phosphorylation as this may further our understanding of alcohol's effect on genes changes.

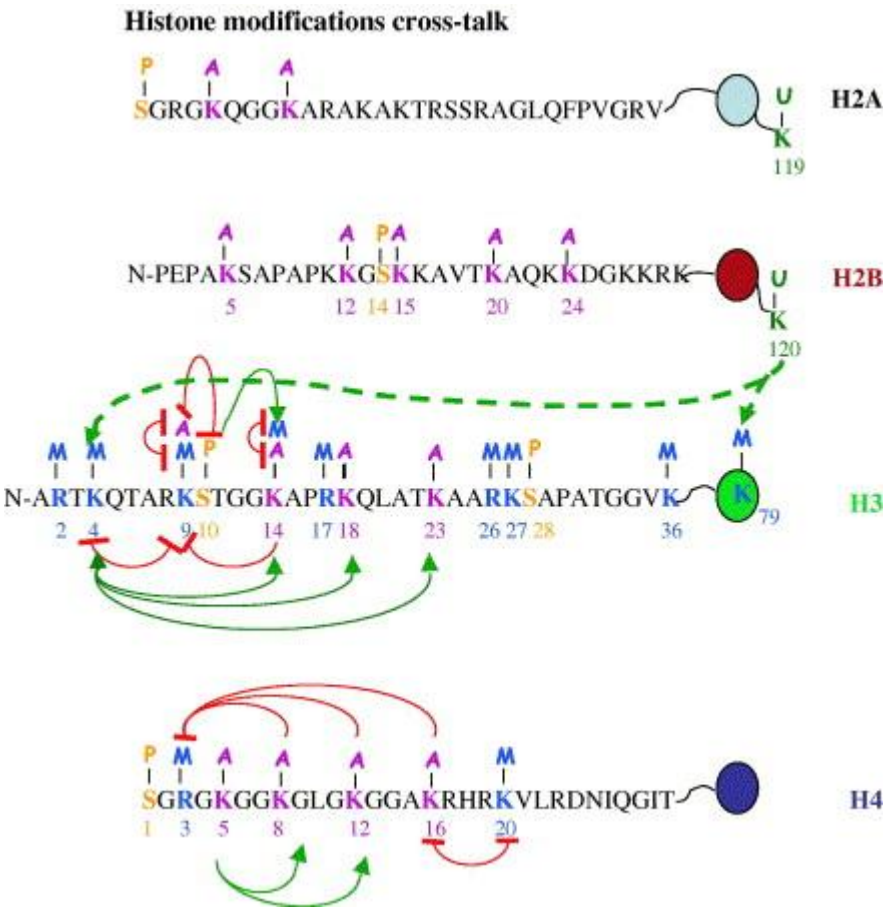

Fig 3. Histone Modification Crosstalks

Adapted from Santos-Rosa, H, Caldas, C (2005) Chromatin Modifier enzymes, the histone code and cancer. Eur J Cancer. 41, 2381-2402

I.4. Histone Modifying enzymes

Histone acetyltransferases (HATs)

HATs are known as transcriptional activators and are grouped based on sequence homology, and biological functions. They acetylate histones by using acetyl-CoA as a cofactor in the reaction and they are grouped into five different families i.e the GNAT, MYST, p160, p300/CBP, and TAFII250. In eukaryotic cells HATs are either in the nucleus or cytosol (Gallinari et al, 2007; Kundu and Dasgupta, 2007). The GNAT family which includes GCN5 and PCAF (p300/CBP associated factor), is the most studied family of HATs. This family has a C-terminal catalytic HAT domain, as well as a bromodomain (110 residues) for interaction with lysine-acetylated histone tails. GCN5L and PCAF are human HATs that are well studied members of this family. MYST family members are grouped based on a 370 residue MYST domain which has a different catalytic mechanism to other HATs. These HATs have many biological functions. For example the human MYST Tip60 has been reported to regulate DNA repair and apoptosis (Ikura et al, 2000). The p160 coactivators interact with nuclear hormone receptors and are involved in ligand dependent receptor-dependent gene transcription (Sterner and Berger, 2000; Roth et al, 2001). The HATs p300/CBP and TAFII250 regulate a wide range of genes.

Histone deactylases (HDACS)

HDACs are considered as transcriptional repressors and are grouped into three classes based on phylogenic and sequence homologies with the yeast proteins. They are Rpd3, Hos1 and Hos2 (class I), HDA1 and Hos3 (class II) and the sirtuins (class III) (de et al, 2003; Verdin et al, 2003; Blander et al, 2004). Therefore Class I HDACs are Rpd3, Hos1 and Hos2 and mammalian HDAC1, HDAC2, HDAC3 and HDAC8. However, HDAC8 can also be a class II HDAC (Buggy et al., 2000; Hu et al., 2000). Class II HDACs are yeast HDAC1 and mammalian HDACs 4, 5, 6, 7, 9 and 10 (Grozinger et al., 1999; Zhou et al., 2001; Guardiola and Yao, 2002; Richon and O'brien, 2002). Both class I and class II HDAC proteins are evolutionarily related and their enzymatic mechanisms involve Zn-catalyzed hydrolysis of the acetyl-lysine amide bond. There is a Zn-dependent HDAC in higher eukarytes (HDAC11 in mammals) which is phylogenetically different from both class I and class II enzymes, and is therefore classified as a different class (class IV) (Gallinari et al, 2007). Class I HDACs are found in the nucleus and form proteins/corepressor complexes (Sin3A, NuRD complex etc.). Depending on the makeup of the multiprotein complex they can differentially deacetylate the core histones. Functional diversity of HDACs is a result of their recruitment into different corepressor complexes and modulation of deacetylase activity by protein-protein interactions. However, post-translational modifications such as phosphorylation and sumoylation, increase the complexity of their regulation. All mammalian HDACs have potential phosphorylation sites and most of them have been shown to be phosphorylated *in vitro* and *in vivo* (Sengupta and Seto, 2004). Phosphorylation of class IIa HDACs on conserved serine residues in their N-terminal regions regulates their subcellular localization and thus their

biological functions (McKinsay et al, 2000, McKinsay et al, 2001; Verdin et al, 2003; Sengupta and Seto, 2004). The phospho-serine residues can bind 14-3-3 chaperone proteins, thereby removing phospho-HDACs from the nucleus to the cytoplasm, leading to activation of HDAC target genes. Protein kinase D and as well as various Ca^{2+}/calmodulin-dependent kinases can phosphorylate class II HDACs after activation by extracellular stimuli in a variety of cell types (Gallinari et al, 2007). Casein kinase 2 (CK2) which functions in cell growth and survival, phosphorylates class I HDACs with the exception of HDAC8, shown to be phosphorylated by protein kinase A (PKA). CK2 resides in both the cytoplasm and the nucleus of eukaryotic cells and is elevated in many tumors (Gallinari et al, 2007). Regulation of HDACs by phosphatases is becoming increasingly important. PP1 can associate specifically with HDAC1, 6 and 10 but it remains unknown if they are direct substrates. PP4 can directly dephosphorylate HDAC3 to negatively modulate its deacetylase function (Zhang et al, 2005).

Class III HDAC consists of yeast silent information regulator 2 (Sir2) (Buck et al., 2004) and mammalian SIRTs. Class III proteins are not linked evolutionarily to class I, II or IV and catalyze the transfer of the acetyl group onto the sugar moiety of NAD (Blander et al, 2004). NAD is the cofactor for this reaction, therefore class III HDACs are thought to link transcriptional regulation to energy metabolism where NAD levels are regulated. Class III enzymes are also believed to play a role in life span regulation by caloric restriction.

Histone Methyltransferases (HMTase)

Histone methyltransferase catalyze the transfer of methyl group from the donor SAMe to either lysine or arginine residues in the N-terminal histone tails of histones. Therefore they are grouped as lysine methyltransferase or HLMTase (Martin and Zhang, 2005) and arginine methyltransferase (Wysocka et al., 2006). Methylation of arginine and lysine residues are involved in the formation of active and inactive regions of the genome. Arginine methylation of histones H3 (Arg2, 17, 26) and H4 (Arg3) aids in transcriptional activation and is mediated by a family of protein arginine methyltransferases (PRMTs), such as PRMT1 and CARM1 (PRMT4) (Li and Zhao, 2008). Lysine methylation has roles in both transcriptional activation (H3 Lys4, 36, 79) and silencing (H3 Lys9, 27, H4 Lys20). Lysine methylation functions in transcription by coordinating the recruitment of chromatin modifying enzymes that contain chromodomains (HP1, PRC1), PHD fingers (BPTF, ING2), Tudor domains (53BP1), and WD-40 domains (WDR5). These domains are found in histone acetyltransferases, deacetylases, methylases and ATP-dependent chromatin remodeling enzymes. These enzymes bind methylated lysines to regulate chromatin condensation and nucleosome mobility to maintain local regions of active or inactive chromatin (Martin and Zhang, 2005).

HMTase can be further grouped into two categories, the first group methylates histones in the nucleosome (EZH2, pr-SET7/SET8, Suv4-20hl, h2), while the second group methylates histone octamers but has a low catalysis rate for nucleosome histones. H3K9 HMTase in mammals (Suv39h, G9a, GLP/Eu-HMTase1, and SETDBA/ESET) are all in this

second category (Kundu and Dasgupta, 2007). HMTase demonstrate substrate preferences and context- dependant enzymatic activities. For example EZH2, an H3K27 HMTase is only functional when it's in a complex with EED (Cao and Zhang, 2004; Pasini et al, 2004).

Histone Demethylase

Histone methylation is reversed by histone demetylase. Histone demethylase is one of the newer discovered group of histone modifying enzymes (Shi et al., 2004; Kubicek and Jenuwein, 2004). Methylation was thought to be irreversible until the discovery of demythlases such as LSD1/AOF2, JMJD1, JMJD2, AND JHDM1(Shi et al., 2004: Kubicek and Jenuwein, 2004; Wysocka, et al, 2006; Li and Zhao, 2008).

Histone H3 Kinases

The kinases that have been reported to phosphorylate histone H3 at S10 are MSK1/2, RSK2, aurora B kinase (ABK) and IkB kinase α (IKKα), and cAMP dependent protein kinase A (PKA) (Kundu &Dasgupta, 2007). Aurora B kinase has been reported to phosphorylate H3 S10 in many cancer cell lines (Rea et al, 2000). There are two major roles for phosphorylated H3 (S10); one is the initiation of chromosome condensation during mitosis and meiosis, and the other is transcriptional activation (Novak and Corces, 2004; Kundu & Dasgupta, 2007). Phosphorylation of histone H3 S28 has been reported to also occur by Aurora B kinase and occurs with chromosome condensation in

mitosis (Goto et al, 1999, Goto et al, 2002). As mentioned above our lab has reported that ethanol increases histone H3 phosphorylation at S10 and S28 in primary rat hepatocytes, and that this event was mediated by p38 MAPK (Lee and Shukla, 2007). JNK can phosphorylate histone H3 at serine 10 after stimulation with nickel (Ke et al, 2008). Tissue transglutaminase 2 (TG2), which has serine/threonine kinase activity, has also become known as a histone H3 kinase. TG2 phosphorylated H1, H2A, H2B, H3, and H4 histones in vitro. It phosphorylated histone H3 at serines 10 and 28 in nucleosomes (Mishra et al, 2006). Another serine/threonine kinase that has been shown to phosphorylate serine 10 is PIM1 which was shown to induce H3 serine 10 phosphorylation after growth factor stimulation in HUVECs (Zippo et al, 2007). In another paper by the same researcher serum stimulation of PIM1 kinase phosphorylated S10 of a preacetylated histone H3 on the FOSL1 enhancer. The binding of the adapator protein 14-3-3 to the phosphorylated S10 recruited the HAT MOF which then acetylated lysine 16 of histone H4, and resulted in a histone code for the elongation of the FOSL1 gene (Zippo, 2009).

Histone H3 Phosphatases

Rapid phosphorylation of MAP kinases leads to the induction and activation of MAP Kinase Phosphatase 1(MKP-1). Protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) have been postulated to dephosphorylate H3 (Murnion et al, 2001; Nowak et al, 2003). MKP-1 can dephosphorylate histone H3 at serine 10 in vitro after stimulation with VEGF or thrombin (Kinney et al, 2009; Pages, 2009).

Multimeric complexes with corepressor and coactivors

The modification of histones results from coordinated events in multisubunit complexes that may work together with nucleosome remodeling complexes (NuRCs). An example of this is the Swi/Snf RSC, NURF. Also the HMT CARM1 interacts with HAT (p160) coactivators to affect gene expreassion (Wu and Zhang, 2009). RSK kinase is also known to act in concert with CBP/300 (Strahl and Allis, 2000). These complexes are able to form because of binding domains in the interacting proteins such as bromodomains and chromodomains.

I.5. Disruption of Histone Modifications and Human Disease

A disruption of any of the aspects of histone modification can lead to human disease. A disruption of HATs or HDACs can lead to a change in acetylation and result in disease. For example, loss of recruitment of the histone H4-K16 HATs MOZ, MOF, and MORF to DNA sequences has been observed in cancer cells (Fraga et al, 2005). In leukemias, these HATs exist as fusion proteins with either CBP or p300, which are HATs that have various substrates (Esteller, 2006; Fraga and Esteller, 2005). CBP and p300 are suggested to have tumor suppressor activity. A germline mutation of CBP can cause Rubinstein-Tayi syndrome and patients with the disease tend to develop tumors at an early age (Gibbons, 2005). In transformed cells there is increased recruitment of Sirt1 to H4 K16. This has been the case in leukemia cells (Bradbury et al, 2005). Other HDACs such as HDAC1 and HDAC2 are dysregulated in many human neoplasia (Gibbons, 2005). Recently our lab has shown that GCN5 is one of the HAT mediating histone H3 lysine 9 acetylation in human hepatoma HEPGP2 cells that express a stable construct of the ethanol metabolizing enzyme ADH1 (VA-13) (Choudhury et al, 2011). Overexpression of the EZH2, a H3K27 methyltransferase occurs in metastatic prostate cancer and can cause widespread gene repression (Varambally et al, 2002; Feinberg and Tycko, 2004). In terms of histone phosphorylation, an increase of H1 phosphorylation is found in many cancer cell cultures and tumors. The aurora kinases are also found to be overexpressed in many aggressive human cancers which results in defective chromosome segregation and aneuploidy, early events in oncogenesis (Andrews et al, 2003; Katayama et al,

2003). Additionally a defect in RSK2 that inhibits phosphorylation of histone H3 at S10 can result in coffin-lowry syndrome (Trivier et al, 1996).

I.6 Intraperitoneal Administration of Ethanol Model

In this dissertation we used an in vivo rat model. We administered ethanol intraperitoneally to male Sprague Dawley rat. The rationale for the use of this model is listed below.

1. Binge drinking is on the rise and this is an acute binge model.
2. The blood alcohol levels mimics the levels seen in human binge patients.
3. The blood alcohol levels are less varied as compared to the intragastric route.
4. This model provides less stress to the animal as compared to other acute models (eg. Intragastric).
5. We are able to get injury in this model.
6. Rapid activation of MAP Kinases .
7. Kinase inhibitors can be injected to study their effects on phosphorylation.
8. Ethanol metabolites (acetate) can be injected to determine the role of ethanol metabolism on histone phosphorylation in vivo.
9. Crosstalk between histone H3 phosphorylation and acetylation can be examined in vivo.
10. Intraperitoneal administration of ethanol is widely used in other studies such as neurobehavioral responses.

I.7 Hypothesis, Specific Aims and the Significance of the Dissertation Project

The liver is the main site for the metabolism of alcohol and this laboratory has done extensive studies with primary hepatocytes isolated from the livers of male Sprague-Dawley rats. Most of these studies concerned the effects of alcohol on histone modifications. Ethanol affects the expression of hepatic genes that are involved in a wide array of liver functions such as ethanol metabolism, cell proliferation, and lipid metabolism gene changes that could possibly lead to alcoholic liver disease. The post-translational modification of histones may be important in such processes resulting in chromatin remodeling and altered gene expression. Histone phosphorylation is mostly known to occur during mitosis and is involved in the expression of early genes such as c-fos and c-myc (Kundu & Dasgupta, 2007). Recently, our lab has reported that ethanol also increases phosphorylation of histone H3 at serine 10 and 28 through a MAPK dependent pathway (Lee & Shukla, 2007). However, the characteristics of the phosphorylating kinases involved in the ethanol induced phosphorylation of histones and the consequences thereof remain to be identified especially in vivo. This thesis project was designed to addresss this issue using an in vivo rat model.

Hypothesis:

The hypothesis of this research project is that "ethanol induced histone phosphorylation at serine 10 and serine 28 in vivo initiate chromatin remodeling events that modulate transcription factors and promote gene changes leading to alcoholic liver

disease in vivo. Moreover phosphorylation at serine 10 may work in concert with other modifications, i.e. acetylation at lysine 9 and regulate a specific subset of genes." There are three specific aims in my dissertation.

Specific Aims:

1. Determine the effects of ethanol on MAP kinase activation, histone H3 phosphorylation, acetylation, in vivo. (See chapter 3)

i. Determine the effects of ethanol on liver injury in vivo.

ii. Determine the effects of ethanol on histone H3 phosphorylation, acetylation, and methylation at specific residues.

iii. Determine if MAP Kinases are activated in vivo by ethanol.

2. Determine the role of ethanol induced histone H3 phosphorylation and gene expression in vivo. (See chapter 4)

i. Determine the effect of different doses of ethanol on site specific histone H3 phosphorylation.

ii. Determine the effect of ethanol on gene expression.

iii. Determine the effect of ethanol on the association of site specific phosphorylated histone H3 with specific genes using ChIP assay.

3. Identification of the relationship between ethanol induced histone H3 phosphorylation, acetylation, methylation, and phosphoacetylation (S10/K9). The

effect of acetate on the same modifications above will be determined. (See Chapters 4 and 5)

i. Determine the effect of ethanol on histone H3 acetylation, phosphoacetylation, and methylation, in vivo in rat liver.

ii. Determine the effect of acetate on acetylation, phosphoacetylation, and methylation of histone H3.

Significance:

Binge drinking is on the rise worldwide and with it comes an increased chance of liver damage and death. Therefore investigations into molecular mechanisms involving binge ethanol is important. Evidence highlights that MAP Kinases are central to the injurious effects of alcohol (Aroor and Shukla, 2004). Histone H3 phosphorylation is downstream of MAP kinase activation and the role of epigenetics in the actions of ethanol is becoming increasingly appreciated. Additionally phosphorylation is known to affect other modifications of histone H3. This project aims to establish for the first time a role for histone phosphorylation in the actions of ethanol and gene expression in vivo. In addition, the effect of ethanol on other modifications and the relationship between histone H3 phosphoacetylation (K9/ S10) provides insight into the molecular effects of ethanol on liver injury. The focus onto the role of histone phosphorylation is innovative in that much less is known about histone phosphorylation over a wide scope as compared to other histone modifications, especially its role in transcriptional activation.

Since histone H3 phosphorylation is increased at serine 10 and 28 by ethanol and acetaldehyde, this may have significant role in disease (Lee & Shukla, 2007). Additionally, our lab has already shown that ethanol promotes the acetylation of lysine 9 leading to the upregulation of the ADH1 gene (Park et al, 2005) and that methylation of histone H3 K4 and K9 were involved in the upregulation and downregulation of selected genes respectively (Pal-Bhadra, 2007). It has also been postulated in the literature in a number of cell types that acetylation of lysine 9 and phosphorylation of serine 10 of H3 may be linked (Grant, 2001). Therefore it is timely to investigate if phosphorylation of H3 at serine 10 and 28 is of importance in the actions of ethanol. If so it may be plausible that ethanol induced histone phosphorylation may promote other modifications of histone that eventually result in alcoholic liver disease. These findings can give deeper insights into the nature of epigenetic changes caused by binge ethanol and may also help to develop tools for the treatment of alcoholic liver disease.

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Chapter II

Development and Evaluation of Methods of Histone Isolation

II.1 ABSTRACT

Traditionally, histones are isolated using high salt or acids. However these methods do not preserve all the post translational modifications of histones, particularly histone phosphorylation, the major form for my thesis project. Therefore the goal of this study is to determine a method that would preserve phosphorylated histone H3 during isolation from liver. In this study we compared five different methods: [1] high salt nuclear extraction, [2] high salt + 4M urea nuclear extraction, [3] 1% formaldehyde used in initial steps of extraction in a sucrose lysis buffer + 4M guanidine HCl (GdnHCl), [4] 1% formaldehyde total extraction, and [5] 2X sample buffer without bromophenol. The total extract methods were not very effective in recovering the amount of histones and 1% formaldehyde posed difficulty in getting an almost pure nuclear extract (method 4). The best results were obtained with our high salt + 4M urea and GdnHCl methods.

Key Words: guanidine HCl, formaldehyde, histone isolation, histone phosphorylation, urea

II.2 INTRODUCTION

Histones are one of the most highly conserved proteins known (Smith et al, 1970; Bartova et al, 2008). These basic proteins have very important roles in chromatin organization and function. Chromatin is comprised of DNA and proteins (namely histones). The basic unit of chromatin is the nucleosome which is comprised of DNA wrapped around an octomer of histone dimers of four main types (H2A, H2B, H3 and H4 and at times may include their variant forms). Histone H1, or linker histone as it is commonly referred, links nucleosomes to create the larger chromatin fiber (Kornberg, 1974; Von Holt, 1989; Kudu and Daguspta, 2007). Histone proteins as well as their variants can be modified post-translationally by acetylation, methylation, phosphorylation, ADP-ribosylation, ubiquitination and sumoylation. These post translational modifications (PTMs) have important roles in chromatin remodeling and gene expression as they can allow or prevent access to the DNA (Khan and Krishnamurthy, 2005; Kouzarides, 2007). Combinations of these modifications on nucleosomes are highly involved in the regulation of gene expression (Shechter et al, 2007). As the role of histones in these two processes is expanding, it is necessary to have good methods for their isolation that retain these modifications in stable form. Since histones are ionically bound to DNA they are either isolated with high salt or in dilute acid. Interestingly, histones were first discovered as part of an acid-soluble protein extract of bird erythrocyte nucei (Van Holde, 1989; Shechter et al, 2007). The type and concentration of acid used can at times help to purify different subunits of

histones. For example, histone H1 is normally isolated with perchloric acid ($HClO_4$), and 0.25 M hydrochloric acid (HCl) has been effective in isolating histone H3 (Bolund and Johns, 1973; Paulston, 1980; Rodriguez-Collazo et al, 2009). In the same manner, different concentrations of salt can be used to differentially isolate subunits; H2A and H2B are extracted at salt concentrations greater than 1M while H3 and H4 are extracted at greater than 1.5M NaCl (Von Holt, 1989; Shechter et al, 2007). Thus, acid and salt extractions have become the two commonly used methods of histone isolation used today. Many different methods have been explored to isolate histones in the past. This is in part due to the fact that the chemistry of histones makes them very susceptible to the possibility of enzymatic and chemical degradation during isolation of nuclei, extraction of histones, and fractionization of said histone (Butler et al, 1954; Moore, 1959; Rasmussen et al, 1962). However, both salt and acid methods of isolation have drawbacks. While acid is a good inhibitor of enzyme activity (that degrade PTMs), some modifications such as phosphorylation and methylation are acid labile and are therefore not stable. In the case of high salt isolation, while the use of a neutral pH maintains acid-labile modifications some enzymes remain active (Shechter et al, 2007). Therefore other isolation methods are needed. When the interest in histones was renewed after the discovery of the structure of DNA, many techniques in their isolation were used utilizing acid, salt, ethanol, acetone, guanidine hydrochloride, urea, or combination of some of these (Bolund and Johns, 1973; Daskalov and Gavasova, 1977; Mende et al, 1983; Lu et al, 1995; Sweet el al, 1996; Pepinsky, 2004; Kizer et al, 2006). In this study we have tried a number of different methods of histone isolation based on some of the

results of above studies, to determine which one is more effective in the preservation of histone H3 phosphorylation.

II.3 MATERIALS AND METHODS

Reagents

Urea, guanidine HCl, anti-beta actin mouse monoclonal primary antibody, and protease inhibitor cocktail (P8340) were obtained from Sigma-Aldrich (St. Louis, MO). Formaldehyde (37%) was purchased from Fisher Scientific (Fair Lawn, NJ). Primary antibodies to histones were anti- phospho-Histone H3-S28 (cat #07-145) and anti-Histone-H3 (cat # 06-755) all purchased from Millipore (Temecula, CA). Goat anti-rabbit immunoglobulin G (IgG) (cat # 170-6515) and anti-mouse IgG (cat # 170-6516) both conjugated with horseradish peroxidase (HRP) and Bio-Rad D_c protein assay kit were purchased from Bio-Rad Laboratories (Hercules, CA).

For the methods discussed below it is worth mentioning that our primary concern was not to compare the different methods in terms of yield, therefore the same amount of tissue was not always used for each method of histone isolation. (This was also done to conserve the amount of tissues as these samples were important for future experiments). Rather, we wanted to compare the fold change in histone H3 phosphorylation at serine 28 between control and ethanol treated liver tissue samples to see if there's a difference among the different methods of histone isolation.

The samples used for method comparison were from rats treated intraperitoneally with either 3.5 g/Kg ethanol in a 32% solution or saline (control rat). We only studied the effects on serine 28, because the fold change was higher than at serine 10 as previously analyzed by salt extraction. Therefore to minimize the work we

studied only one modification of histone. In all methods, the proteins in the samples were quantified by Bradford Assay using the Bio-Rad D_c Assay kit and proteins were run on a 15% SDS-PAGE gel 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% nonfat dry milk for 1 h at room temperature. For primary antibodies, 1 to 1000 of both anti-phospho-histone H3-S28 and histone H3, and 1: 65 000 dilution of anti-β–actin were used overnight at 4°C. Phosphorylated histone H3-S28 and β–actin were probed on the same blot at the same time while histone H3 protein was probed on separate blot loaded in the same manner. For secondary antibody, 1: 2500 dilution of anti-rabbit HRP conjugated antibody was used for both phosphorylated histone H3-S28 and histone H3 protein whereas 1: 3000 dilution of anti-mouse HRP conjugated antibody was used to detect β–actin and membranes were incubated for 1.5 hrs. After washing with TBST, the membrane was incubated with secondary antibody conjugated to horseradish peroxidase for 1h 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 were quantified with Multi Gauge ^{TM} software and was analyzed within the linear range of detection. Histone H3 protein levels in nuclear extracts and β-actin levels in total extracts were used to determine equal loading of proteins. Levels of histone H3 and β-

actin were not altered after acute ethanol exposure. Histone H3 was used for data normalization to compare methods 2 and 3. We only did this for these two methods because we were unable to detect phosphorylated histone H3 S28 and histone H3 for the other methods.

Isolation of Nucleus for Methods 1 and 2

For methods 1 & 2 below, the nucleus was isolated as follows. Frozen liver (300 mg) of was homogenized on ice in hypotonic lysis buffer ((50 mM Tris- HCl, pH 7.4, 25 mM KCl, 5 mM $MgCl_2$, 1 mM β–glycerophosphate, 1 mM EDTA, 1 mM Na-orthovanadate, 1 mM EGTA, 1 mM DTT, 100 nM trichostatin A (TSA), 5mM sodium butyrate, 10 mM sodium fluoride (NaF), 2.5 mM sodium pyrophosphate, and 1X Sigma protease inhibitor cocktail (P8340)) containing 0.25M sucrose using a dounce homogenizer, 15 strokes with pestle A (large clearance pestle for tissue reduction), and 5 strokes with pestle B (small clearance pestle for the formation of the final homogenate). The homogenate was passed though a 0.2 μm filter before centrifugation at 800 g for 15 min at 4 °C. The pellet was resuspended and washed in the same lysis buffer. After a second centrifugation at 800 g for 15 min the pellet was resuspended in 1.35 M sucrose containing 0.3 % NP-40. After passage through a 22-gauge needle 3 times, the cellular 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 resuspended in 1.35M sucrose, divided equally into 2 aliquots and collected by microcentrifuge at 16

000 g for 1 min. The nuclear fractions were examined under light microscope for purity of nuclei. The nuclear pellets were flash frozen in liquid nitrogen and stored at -80 ^{o}C until further analysis.

Method 1: High Salt Extraction

The first nuclear aliquot was thawed on ice and lysed in high salt buffer (0.45M 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-orthovanodate, 100 nM TSA, 5 mM Na-butyrate, 1 mM β–glycerophosphate, and Sigma protease inhibitor cocktail 1X P8340) . The nuclear preparations were sonicated three times for 5 sec. After centrifugation at 16 000 g for 10 min, the supernatant was used as nuclear fraction. Protein concentrations in nuclear extracts were measured using 5 μl of extract. For analysis 20 μg of proteins were used. Samples were denatured in 2X sample buffer (see method 5) and heated at 100 ^{o}C for 10 min.

Method 2: High Salt + 4M Urea Extraction

For lysis of nuclei the 2nd aliquot was thawed on ice and was solubilized in high salt detergent lysis buffer as in method 1 above but with the addition of 4M urea. The nuclear preparations were sonicated three times for 5 sec. After centrifugation at 10 000 g for 10 min to remove insoluble material, the supernatant was used as nuclear fraction. Protein concentrations in nuclear extracts were measured using 5 μl of extract,

and 20 μg of protein was used for gel electrophoresis. Urea in solution can spontaneously rearrange to form ammonium cyanate which can give rise to isocyanic acid (Allen and Jones, 1964; Rodriguez-Collazo et al, 2009). Isocyanic acid in turn can cause carbamylation of proteins, in the presence of heat. Carbamylation affects the amino-terminus of proteins, and can prevent N-terminal sequencing of proteins (Lippincott and Apostle, 1999). The isocyanic acid can also react with side chains of lysine and arginine residues and form a protein that has altered mass and thus will migrate differentially during SDS-PAGE. Therefore to prevent this from occurring samples were only heated at 37 ^{o}C to aid in denaturation before electrophoresis.

Method 3: 1% Formaldehyde Lysis Buffer + 4M Guanidine HCl (GdnHCl)

This method was adapted from Lu et al, 1995 and Sweet el al, 1996. Frozen liver, (300 mg), was placed in hypotonic lysis buffer (see *Isolation of Nucleus for Methods 1 and 2* above) containing 1% formaldehyde. Samples were homogenized with a dounce homogenizer as described for methods 1 and 2 above. Samples were centrifuged at 800 g for 15 min to pellet cells. The pellet was resuspended and washed in the same buffer above. After a second centrifugation at 800g for 10 min at 4 ^{o}C, the pellet was resuspended in 1.35 M sucrose containing 0.3 % NP-40 and the same buffer components above. After passage through a 22-gauge needle 3 times, the suspension was subjected to another round of centrifugation at 1600 g for 10 min. Because of the formaldehyde crosslinks, the nuclei were unable to pellet because they did not separate from the cytosolic components. To overcome this, the sucrose was diluted back to

0.25M with lysis buffer and centrifuged at 1600g for 10min. The pellet containing nuclei was divided into two aliquots and collected by microcentrifuge at 16000 g for 1 min. It was then flash frozen in liquid nitrogen and stored at -80 ^{o}C until further analysis. To reverse formaldehyde crosslinks and isolate histones, samples were thawed on ice and resuspended in 4M guanidine HCl, 10mM EDTA, and 2mM DTT at a concentration of ~1-6 X 10^{8} nuclei/ml and boiled at 100 ^{o}C for 30min. GdnHCl has a high ionic strength and can interfere with the electrical field during SDS-PAGE . This will cause the gel to run too slowly and will cause distortion of bands. GdnHCl will also cause SDS to precipitate upon the addition of sample buffer. It follows that a primary method for SDS removal is by precipitation with guanidine HCl (Shively 1986). For these reasons, the GdnHCl was removed by the addition of 9 volumes of 100 % ethanol to 1 volume of protein sample and incubation overnight at -20 ^{o}C to precipitate proteins. Next samples were spun at 4 ^{o}C for 15 min at 16 000g in a microcentrifuge. The supernatant was carefully removed and samples were then washed in 90% ethanol and centrifuged at 16 000g for 5 min. The pellet was air dried to eliminate ethanol and resuspended in buffer containing 1 % SDS, 50 mM Tris pH 7.4, 1mM DTT, plus the same protease and phosphatase inhibitors in the hypotonic lysis buffer mentioned above (*Isolation of Nucleus for Methods 1 and 2*), sonicated two times for 5 sec, boiled for 10 min, centrifuged, then the supernatant was taken as protein extract and 20 μg of protein was run on the gel.

Method 4: 1% Formaldehyde Total Extract

This method was adapted from the protocol for Millipore catalogue # 17-371 (EZ ChIP Assay Kit**)** and changed for the use with tissue and for the isolation of protein. Approximately 130 mg of frozen liver was weighed, cut into 1-3mM pieces and incubated in 1% formaldehyde in 1X PBS for 20 min at room temperature. Next 130 mM of glycine was added to quench the unreacted formaldehyde. The liver was then washed twice in ice cold 1X PBS for 2 min at 4 ^{o}C and lysed in 1X PBS containing protease, phosphatase, and HDAC inhibitors (1X P8340, 10mM NaF, 2.5mM Na pyrophosphate, 1mM Na-orthovanodate, 100nM TSA, 5mM Na-butyrate, 1mM β–glycerophosphate) using a dounce homogenizer. The lysate was centrifuged at 1000 rpm for 10 min at 4 ^{o}C and the cellular pellet was resuspended in 800 μl of SDS lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris -HCl, pH8.1) containing the same phosphatase inhibitors as above. The cell lysate was divided equally (~400 μl) into two tubes. The cells were then sonicated 4 times for 10 secs at 50 % duty cycle set at microtip 4 on a Vibra Cell sonicator model VCX-600 on wet ice. Next the samples were centrifuged at 10 000 g for 10 min to pellet the insoluble material. The supernatant was saved and 1 tube was frozen at -80 ^{o}C. The other was used for protein analysis. First to remove the protein-DNA crosslinks created by formaldehyde, salt was added to a final concentration of 0.5 M and samples were boiled at ~ 100 ^{o}C for 20 mins. The samples (10 μl) were quantified and 55 μg of protein was used for gel electrophoresis.

Method 5: 2X Sample Buffer (without bromophenol blue) total extract

Approximately 60 mg of frozen liver was weighed and placed into a 1.5 ml tube containing 2X sample buffer without bromophenol blue (4% SDS, 100mM Tris pH 6.8, 20% glycerol, and 10mM DTT). The bromophenol was omitted for easy visualization of the tissue to determine when it was completely homogenized. A 1:1 ratio w/v of buffer was used, therefore 600 μl was added to 60 mg. The liver was boiled for 5mins at ~ 100 ^{o}C then sonicated 4 times as described in method 4. The lysates were then centrifuged for 10 mins at 16 000 g in a microcentrifuge to remove insoluble material. The extract (5 μl) was used for protein quantitation and 100 μg of protein was used for gel electrophoresis. Water was first added to a 100 μg aliquot to make it a 1X solution, a 200 μl pipet-tip was dipped in powdered bromophenol blue then briefly dipped in the sample to color the sample for visualization during gel electrophoresis. Samples were then boiled at ~ 100 ^{o}C for 5 mins before loading unto the gel.

II.4 RESULTS

In this study, histones were isolated from frozen liver by 5 different methods to compare them for fold change in phosphorylated histone H3-serine 28. First, histones were isolated from a nuclear extract either by lysis in a [1] high salt buffer or a [2] high salt buffer containing 4M urea. In method 3 the liver was homogenized in 1% formaldehyde and guanidine HCl was used to extract histones. The last two methods were total extracts utilizing [4] 1% formaldehyde or [5] 2X sample buffer. The results are presented below. Samples were analyzed by SDS-PAGE (15% gel) and western blotted with site specific antibodies to phosphorylated histone H3 at serine 28, histone H3 protein, and β-actin.

In method 1, salt extraction of nuclear histones, we did not detect any phosphorylated H3 band. Because we were able to see phosphorylated bands with the urea extracts (method 2), this indicated that the addition of urea increased the yield of nuclear proteins (Figure 1 & 2). Using salt and urea seem to be a very efficient way to isolate histones. The fold increase in phosphorylation histone H3 at S28 between the control and ethanol treated samples was 1.6.

In method 3, there was a 1.6 fold increase in phosphorylated histone H3 at S28 over control for the ethanol sample (Figure 1, and 2). While the same fold change was observed for the urea/salt extraction above (method 2), the amount of proteins seen is low compared with the urea extraction (Figure 1). This is most likely due to loss of nuclei during isolation. However, unlike in method 2, further purification was required to

remove the GdnHCl from the proteins for SDS-PAGE. This makes method 2 more attractive. A comparison of the fold change of methods 2 and 3 is presented in figure 2.

In method 4, (1% formaldehyde extract), it was difficult to see any bands corresponding to histone H3 in the gel. This could be overcome by using more proteins to analyze on the gel or by using less lysis buffer so that proteins can be concentrated in the extract. Perhaps, if we had concentrated the proteins by TCA precipitation or with an amicon, we would be able to visualize the histones on the gel and this method would have proved to be more fruitful.

As seen in figure 1 extraction of histones in 2X sample buffer (method 5) was not an ideal method for histone isolation. We loaded 100 μg of protein and phosphorylated histone H3 could not be detected in the gel. We then doubled the amount of protein loaded (200 μg) and were able to see a faint band of histone H3 and phosphorylated histone H3 (data not shown).

II.5 DISCUSSION

The need for the proper isolation of histones to preserve the different post translational modifications of interest is an important aspect in these studies. Phosphorylation of histones is one of the modifications that can be lost during histone isolation (Rodriguez-Collazo et al, 2009). An evaluation of 5 methods is presented here and was based upon much of the work done in the past when histones became of interests for roles besides DNA packaging.

The rationale for using method 1 was to compare a traditional histone isolation method (high salt extraction) with the other methods tested here. As compared to method 2, salt extraction was not very effective in isolating histones from such a small amount of tissue. The amount of urea (4 M) used in method 2 was based on the work published by Bolund and Johns in 1973. This concentration of urea worked well in combination with salt and ethanol to effectively isolate histone H3. We must also mention that the amount and combination of detergents used in this extract method were based on RIPA (radioimmune precipitation assay) buffer. To the best of our knowledge our exact urea buffer components are original, and have not been reported in the literature. Recently a methods paper published by Rodriguez-Collazo et al, 2009 details that they had issues isolating phosphorylated histone H3. In this context and to preserve histone phosphorylation as well as other PTMs of histones, they used 8M urea to bypass the usual nuclear isolation steps. With a combination of other methods including some salt extraction, they were able to purify different histone subunits,

omitting the need for HPLC which is normally used (Rodriguez-Collazo et al, 2009) to separate histones. The researchers hold a patent for their methods which was licensed to Actif Motif (cat # 40025) and is available as a histone isolation kit. We also noticed that another group of researchers who are studying phosphorylated histones were reconstituting histones from acid extraction and acetone precipitation in 4 M urea (Ke et al, 2008). Therefore having a denaturant such as urea in the procedure is becoming attractive for the isolation and preservation of phosphorylated histone H3.

Isolation steps to get to the nuclear fraction may lead to dephosphorylation of histone H3. Therefore in method 3 we first tried to denature enzymes that may degrade phosphorylation by adding 1% formaldehyde in the lysis buffer. To reverse the crosslinking and separate histones from DNA we used guanidine HCl. It must be mentioned that guanidine HCl is a denaturant that functions similar to urea. The addition of the formaldehyde prevented the pure nucleus from being isolated. Since a combination of guanidine HCl and SDS will affect the migration of protein bands during SDS PAGE as mentioned earlier in the methods section, the GdnHCl was removed by ethanol precipitation. This makes the isolation of histones a little more tedious as compared to the urea extraction method. Additionally the amount of GdnHCl used in this study was incompatible with quantitation of proteins via the Bio-Rad Dc protein assay.

The rationale for method 4 and 5 is that PTMs can be affected by the steps taken to isolate the nucleus, therefore if we bypass these steps, we should not alter phosphorylation. In method 4 we rationalized that first crosslinking the tissue in 1%

formaldehyde would prevent dephosphorylation. However, one of the major problems for this method is that it is harder to isolate nucleus, and so we did a total extract. It was difficult to detect phosphorylated histone H3 in this extract. Since SDS is a detergent that is capable of solubilizing the whole tissue to produce a total extract and it can prevent enzyme activity, we hypothesized that bypassing the nuclear isolation methods and putting the tissue directly in sample buffer, the last step before gel electrophoresis, would preserve histone phosphorylation. Another advantage is that this method takes less time and therefore more samples can be processed. However as shown by figure 1, a drawback is that a large amount of samples has to be loaded onto the gels to visualize the histones. Therefore as in method 4, we have to load a large amount of protein on the gel for visualization. Again it was difficult to detect histone H3. Also the antibodies may not work as well in a total extract.

Based on the results of this study, we chose a nuclear isolation of histones and further lysis of the nucleus in urea containing buffer as in method 2. This method of histone isolation was used throughout the rest of this thesis except in chapter 3. In this chapter, histones were isolated before our realization of a need to determine an optimal method of histone isolation for the study of phosphorylated histone H3.

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Fig.1. Phosphorylated histone H3 and its Protein Levels Observed Using Differerent Methods of Histone Isolation. 1A. Histones were isolated according to the methods described in the material and methods section. Proteins were quantified and equal amounts of control and ethanol treated samples were subjected to 15% SDS-PAGE. 20 μg was loaded for GdnHCl , salt, and salt+ urea method of extraction while 55 μg for 1% formaldehyde total extract, 100 μg for 2X sample buffer total extract. Proteins were transferred to nitrocellulose membrane. Phosphorylated histone H3 and histone H3 protein, and beta-actin were analyzed using anti-phospho-histone H3-Ser 28, anti-histone H3 antibodies, and anti-beta actin antibody respectively. m= marker, Salt = Salt Extract Method, GdnHCl = guanidine hydrochloride, HCHO = 1% formaldehyde extract, and 2X SB= 2X Sample Buffer extract. C = control and E = Ethanol.

1B. This is a longer exposure of figure 1 A so that the guanidine HCl bands can be more easily seen.

FIG.1

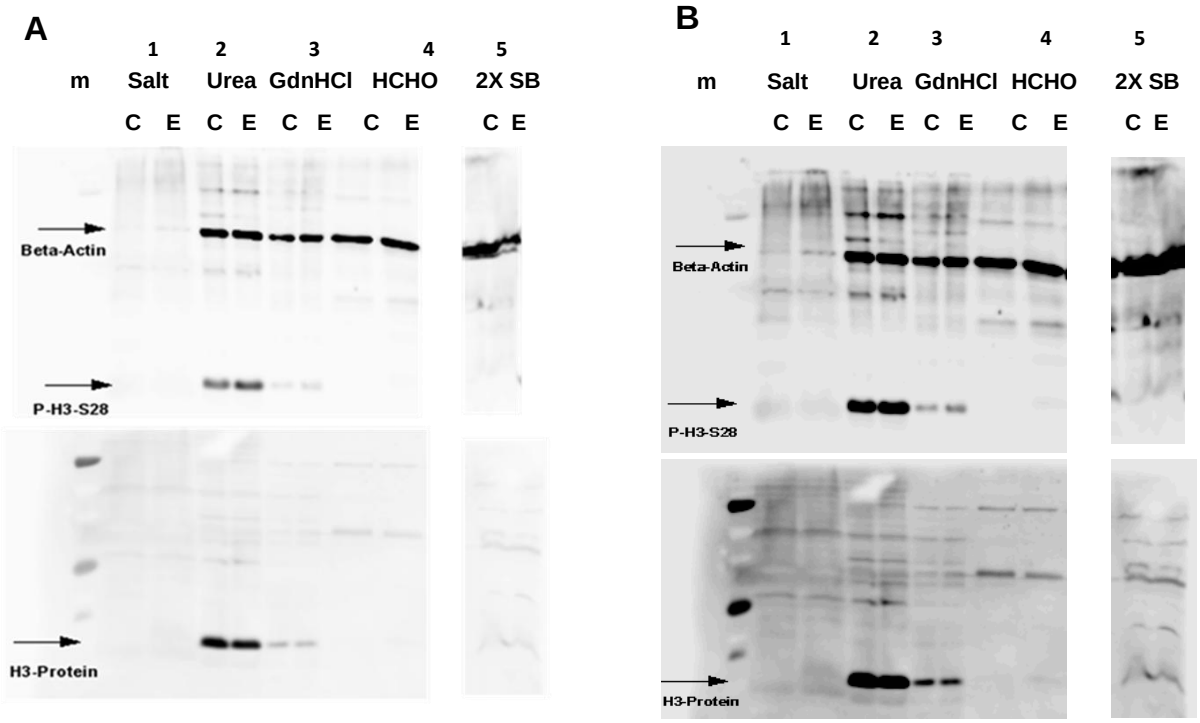
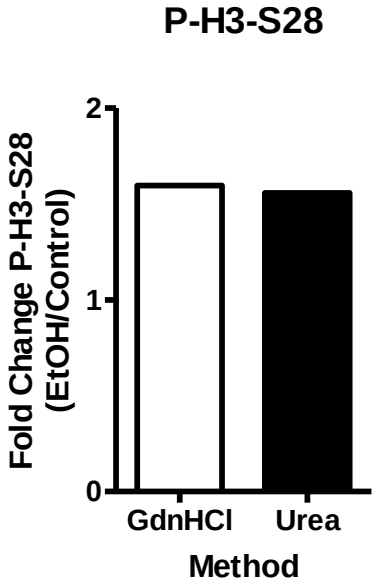

A
m
1
Salt
2
Urea
3
GdnHCl
4
HCHO
5
2X SB
C E C E C E C E C E
Beta-Actin
P-H3-S28
H3-Protein
B
m
1
Salt
2
Urea
3
GdnHCl
4
HCHO
5
2X SB
C E C E C E C E C E
Beta-Actin
P-H3-S28
H3-Protein

Fig.2. Histogram of the fold change for methods 2 and methods 3.

Blots from fig. 1 for methods 2 (urea/salt extraction) and method 3 (for GdnHCl) were quantified using Multigauge Software. The graph represents the fold change in ethanol treated samples over control. Phosphorylated histone H3 at serine 28 (P-H3-S28) was normalized with histone H3 protein.

FIG. 2


P-H3-S28
Fold Change P-H3-S28 (EtOH/Control)
2
1
0
GdnHCl
Urea
Method

CHAPTER III

DIFFERENTIAL CHANGES IN MAP KINASES, HISTONE MODIFICATIONS AND LIVER INJURY IN RATS ACUTELY TREATED WITH ETHANOL

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Differential Changes in MAP Kinases, Histone Modifications and Liver Injury in Rats Acutely Treated With Ethanol

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Contribution: In this manuscript I conducted all the histone modification experiments, phosphorylation, and acetylation (Fig.5). I also treated the animals IP with ethanol in these experiments and processed samples. I also analyzed and interpreted in the manuscript.

III.1 ABSTRACT

Background: Acute ethanol is known to affect cells and organs but the underlying molecular mechanisms are poorly explored. Recent developments highlight the potential importance of mitogen activated protein kinases, MAPKs (i.e. ERK1/2, p38 and JNK1/2) signaling, and histone modifications (i.e. acetylation, methylation and phosphorylation) in the actions of ethanol in hepatocytes. We have therefore investigated significance of these molecular steps *in vivo* using a model in which rats were acutely administered ethanol intraperitoneally (IP).

Methods: Ethanol was administered IP (3.5 g/ Kg body weight) to 12 weeks old male Sprague–Dawley rats. Liver was subsequently removed at 1 and 4 hr. Serum was used for alcohol and ALT assays. At the time of the removal of liver, small portions of each liver were formalin-fixed and stained with hematoxylin and eosin (H&E) and used for light microscopy. Western blot analysis was done with specific primary antibodies for various parameters.

Results: There were clear differences at 1 and 4 hr in blood ethanol, ALT, steatosis, and cleaved caspase 3. Apoptosis at 1 h was followed by necrosis at 4 hr. Acute alcohol elicited a marked increase in the phosphorylation of ERK1/2 and moderate increases in the phosphorylation of p38 MAPK and JNK. Temporally different phosphorylation of histone H3 at Ser-10 and Ser -28 occurred and acetylation of histone H3 at lys 9 increased progressively.

Conclusions: There were distinct differences in the behavior of the activation of the three MAP kinases and histone modifications after acute short exposure of liver to ethanol *in vivo*. Although all three MAPKs were rapidly activated at 1 h, the necrosis, occuring at 4 h, correlated to sustained activation of ERK1/2. Transient activation of p38 is associated with rapid phosphorylation of histone H3 whereas prolonged activation of ERK1/2 is correlated to persistent histone H3 acetylation.

Key words: Epigenetics, MAPK signaling, Alcoholic liver disease, Binge drinking, Steatosis,

III.2 INTRODUCTION

Alcoholic liver disease (ALD) is the most common hepatic disease in the western countries. More than 18 million adults in the United States abuse alcohol (Lucey et al, 2009, Purohit et al, 2009) and its chronic consumption causes liver damage leading to steatosis, alcoholic hepatitis, fibrosis, cirrhosis and leads to the development of hepatocellular carcinoma in susceptible individuals (Lucey et al, 2009; Purohit et al; 2009). Acute liver injury induced by ethanol is also drawing attention because the incidence of acute alcoholism or binge drinking is on the rise worldwide (Mathurin and Deltenre, 2009). Moreover, liver is sensitized to injury after acute ethanol in burn injury (Emanuele et al, 2009). Although mechanisms and mediators of chronic alcoholic liver injury have been extensively studied, little is known about the mechanisms underlying acute alcoholic liver injury.

The mitogen-activated protein kinase (MAPK) pathways represent a converging point for many signaling pathways, including tyrosine and serine/threonine kinases, G proteins, and calcium signaling (Roux et al, 2004). The most common MAPKs are the extracellular signal-regulated kinases ERK1 and ERK2 (also known as p44 and p42MAPK, respectively), p38MAPK, and c-Jun-N-terminal kinase/stress-activated protein kinases (JNK/SAPK). MAPKs regulate a variety of biologic processes, eg. cell growth and proliferation, chemotaxis, inflammation, steatosis, necrosis and apoptosis (Boutrous, 2008; Brown and Sacks, 2008). The activated MAP kinases translocate into nucleus and then phosphorylate transcription factors, and thereby regulate transcription. In addition

to phosphorylating specific transcriptional factors, MAP kinases and their downstream kinases are implicated in alterations in chromatin environment by modulating the phosphorylation and acetylation of nucleosomal and chromatin proteins (Bártová et al, 2008, Delcuve et al, 2009). Histone H3 at ser- 10 and ser-28 are rapidly and transiently phosphorylated during immediate-early response of mammalian cells to extracellular stimuli (Delcuve et al, 2009). There are a few kinases identified as histone H3 kinase, eg, mitogen-and stress-activated kinase-1 (MSK1), ribosomal S6 kinase 2 (RSK2), aurora B, and IkB kinase α (Bártová et al., 2008). RSK2, a downstream kinase of ERK1/2, is shown to be required for epidermal growth factor (EGF)-stimulated phosphorylation of histone H3 (Sassone-Corsi, et al, 1999). Histone H3 phosphorylation by p38 MAPK involves downstream kinase MSK-1 (Cheung, et al, 2000; Zhong et al, 2001). Acetylation of histone is also closely related to regulation of transcriptional activity (Delcuve et al, 2009). The regulation of histone acetylation may be indirect through phosphorylation of histone H3 by MAPKs (Sassone-Corsi,et al, 2000) or direct through phosphorylation of histone acetyltransfearses by MAPKs (Ait-Si-Ali S et al, 1999; Kawasaki et al, 2000; Merienne et al, 2001).

A role for MAPKs and epigenetic histone modifications in alcoholic organ damage is emerging (Aroor and Shukla, 2004; Shukla et al, 2008). However, the relationship between MAPK signaling and epigenetic histone modifications in acute alcoholic liver injury is not known. In primary culture of rat hepatocytes, ethanol caused moderate activation of ERK1/2 and marked activation of JNK (Lee et al, 2002). Although phospho-

p38 MAPK was not affected in the cytosol, nuclear p38 MAPK phosphorylation was increased by ethanol (Lee and Shukla, 2007). Previously we have reported histone H3 lys 9 acetylation and histone H3 ser-10 and ser-28 phosphorylation in primary cultures of hepatocytes (Park et al, 2005; Lee and Shukla, 2007). In hepatocytes, histone H3 lys 9 acetylation was decreased by inhibition of ERK1/2 and JNK whereas histone H3 ser-10 and ser-28 phosphorylations were reduced by inhibition of p38 MAPK (Park et al., 2005; Lee and Shukla, 2007). Although the activation of MAPKs and histone modifications by ethanol have been studied *in vitro*, very little is known about the activation of MAPKs and their relationship to histone acetylation and phosphorylation in liver acutely exposed to ethanol *in vivo*. This was investigated here.

III.3 MATERIALS AND METHODS

Reagents

Antibodies to phospho-ERK1/2, ERK1/2 protein, phospho-p38 MAPK, p38 MAPK protein, phospho-JNK1/2, JKN1/2 protein and calreticulin were purchased from Cell Signaling (Danvers, MA). Antibodies to phospho-H3 ser 10, Phospho-H3 ser 28, H3 protein and acetyl –H3 lys 9 were obtained from Millipore (Temecula, CA). Protease inhibitors cocktail (p8340), anti β-actin antibody and assay kit for triglycerides were obtained from the Sigma-Aldrich (St. Louis, MO).

Acute ethanol administration

Twelve week old male Sprague–Dawley rats, each weighing between 300-350 g, were purchased from Harlan Laboratories (Indianapolis, IN). They were housed under a 12-h/12-h light/dark cycle. All animals had free access to water and were permitted ad libitum consumption of standard laboratory rat chow. After one week, rats were divided into two groups: control and acute ethanol group. Ethanol was administered intraperitoneally (ip) (32%, v/v in saline 3.5 g/Kg body weight) and liver was subsequently removed at 1 and 4 hr. In the control group, ethanol was replaced by saline. Blood samples were collected at 1 hr and 4 hr after binge ethanol administration. A small portion of the liver was placed in formalin and remaining portion of the liver was frozen in liquid nitrogen and stored at -70^{0}C. This study conformed to 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 Committee.

Determination of serum ethanol and ALT

Blood alcohol was determined by alcohol dehydrogenase assay using kit from Genzyme Diagnostics Framingham, MA. Serum alanine aminotransferase (ALT) levels were determined by kinetic ALT assay in an automated analyzer.

Determination of hepatic triglyceride

For the determination of triglycerides, 100 mg of liver was homogenized in 8 volumes of hypotonic buffer containing 20 mM Tris, 50 mM EGTA and Sigma protease inhibitor cocktail (p8340) followed by addition of one volume of 5 M NaCl. 3.75 ml of chloroform –methanol (2:1) was added and the resulting homogenate was vortexed and left for 30 min at room temperature. After addition of 1.25 ml chloroform and 1.25 ml of water, the contents were mixed well and centrifuged at 1000 g for 5 min. The lower organic phase was removed and evaporated under nitrogen gas. The resulting lipid material was dissolved in 1.0 ml of phosphate buffered saline containing 2% Triton X -100. One hundred microliters of Triton X-100 fraction was used for triglyceride estimation using the assay kit as detailed by the supplier.

Histopathology

After formalin fixation, specimens were sectioned and stained with hematoxylin and eosin (H&E) and used for light microscopy.

Preparation of cytosolic and nuclear extracts

The cytosolic and nuclear protein extracts were obtained following our previously published protocols with some modifications (Park et al., 2005, Aroor et al, 2009). All steps were conducted at 4^0C. One gram of frozen liver was homogenized in lysis buffer containing (50 mM Tris. HCl. pH 7.4, 25 mM KCl, 5 mM $MgCl_2$, 5 mM glycerophosphate, 1 mM EDTA, 1 mM Na-orthovanadate, 1 mM EGTA, 1 mM DTT, and Sigma protease inhibitor cocktail (p8340). The homogenate was layered over 1.0 M sucrose cushion and centrifuged at 1600 g for 10 min at 4 °C. The supernanatant containing cytosolic proteins was transferred to a precooled microcentrifuge tube, frozen in liquid nitrogen and stored at -70C. The nuclear pellet was resuspended in 1.0 M sucrose containing 0.25 % NP-40. The nuclear suspension was passed through a 26-gauge needle 6 times, followed by centrifugation at 1600 g for 10 min. This step successfully removed contaminated endoplasmic reticulum and plasma membrane. The nuclear pellet was re-suspended again in 1.0 M sucrose. After centrifugation at 1600 g for 10 min, the pellet containing nuclei was washed once with homogenization buffer. The nuclear fractions were examined under light microscope for purity of nuclei and were free of membrane contamination and other subcellular organelles. The isolated nuclei preparations were solubilized in high salt detergent buffer (0.5M NaCl, 1% Triton X-100, 1% deoxycholate and 0.1% SDS). The nuclear preparations were sonicated for 5

sec. After centrifugation at 14000 g for 10 min, the supernatant was used as nuclear fraction. The purity of cytosolic and nuclear fractions were verified by the presence of β tubulin and absence of histone H3 in the cytosolic fraction and absence of β tubulin and presence of histone H3 in the nuclear fraction as reported earlier (Lee and Shukla 2007). The nuclear preparations were also free from contamination with endoplasmic reticulum (ER) as evaluated by western blot with calreticulin, a marker for ER. For histone extraction, nuclear pellets were resuspended in 0.4 N HCl with 10 % glycerol and centrifuged at 12,000 *g* for 10 min. The supernatant fraction (acid-soluble) is carefully collected, precipitated with trichloroacetic acid (final concentration 20%, w/v), washed with acetone, dried under the vacuum and dissolved in distilled water. Protein concentrations in cytosolic and nuclear extracts were measured using the Bio-Rad DC protein assay.

Immunoblot analysis

The cytosolic extract (80 μg) and nuclear extracts (40 μg for MAPKs and 10 μg for histones) were subjected to 10% SDS-PAGE and electrophoretically transferred onto nitrocellulose membrane (Bio-Rad) using Bio-Rad Trans-Blot apparatus. The membrane was washed with 20 mM Tris, pH 7.5, containing 0.1% Tween 20 and 150 mM NaCl (TBST) and incubated with TBST containing 5% nonfat dry milk for 2 h at room temperature. The membrane was next incubated with antibody to phospho- or total p42/p44 ERK1/2, p38 MAPK, JNK 1/2 overnight at 4°C. For western blot of cleaved caspase 3, membrane was incubated with antibody to cleaved caspase 3 (1:1000

dilution). After washing with TBST, the membrane was incubated with secondary antibody conjugated horseradish peroxidase for 1 h at room temperature. The horseradish peroxidase was detected by enhanced chemiluminescence (ECL) (Supersignal, Pierce Chemical, Rockford, IL). The membrane treated with ECL reagent was exposed to x-ray film or scanned with a LAS-3000 imaging system (Fujifilm life science). Quantitation of the data was done using Multi Gauge ^{TM} software. The intensity of the chemiluminescence was always determined within the linear range of detection. For repeat immunoblotting, membrane was stripped using Restore Western blot stripping buffer (Pierce). Equal loading of protein was confirmed by determining β-actin levels for cytosolic extracts and histone H3 protein levels for nuclear extracts. Levels of β-actin or histone H3 did not change after acute ethanol exposure.

Data analysis

All results are expressed as mean ± S.E and were obtained by combining data from individual experiments. Graph Pad PRISM (version 4) software was used for statistical analysis using the Student *t* test (two-tailed, unpaired). Differences with a *P* value of <0.05 were considered statistically significant.

III.4 RESULTS

Effect of acute ethanol binge on liver injury in rats

Intraperitoneal administration of 3.5 g of ethanol/Kg body weight to rats resulted in marked elevation of ethanol levels in the blood. It ranged 60-90 mmol/L at 1 hr and 36-66 mmol/L at 4 hr (Fig 1). The magnitude of increase seen in these experiments was similar to levels seen after heavy consumption of alcohol in humans (Dietrich and Harris, 1996). In one series of study, 7.2 % of patients with alcoholic intoxication (190 out of 1250 patients with alcohol detected in the blood) had blood levels exceeding 60 mmol/L with alcohol levels reaching up to 100 mmol/L in the blood in some patients (Rivara et al., 1993). Alcohol caused mild apoptosis at early time points (1 hr) as evaluated by the increased levels of cleaved caspase 3 (Fig 2A). There was no significant necrosis at 1 h (Fig 2A). However, at 4 h ethanol significantly increased hepatic necrosis as indicated by the release of ALT from hepatocytes; a 4.2 fold increase in serum ALT levels (Fig 2B). Histochemical examination of liver sections revealed mild steatosis in acute ethanol treated rat liver at 4 (Fig 2.C) but not at 1 h (data not shown). The presence of stetaosis is further supported by 1.7 fold increase in liver triglycerides at 4 hr after ethanol treatment (Fig. 2D).

Activation of mitogen activated protein kinases after ethanol binge

We have determined the activation of MAPK by evaluating western immunoblots of phosphorylated MAPKs**.** The sample loading were similar for individual samples as

evaluated by western blot for β-actin (data not shown). Moreover, the protein levels of MAPK did not change between the control and ethanol treated groups (data not shown). We have previously shown a relatively modest increase in the phosphorylation of ERK1/2 and marked increase in phosphorylation of JNK in the cytosolic fractions at 1 hr after addition of ethanol to cultured primary hepatocytes (Lee et al., 2005; Lee and Shukla, 2007). In contrast to vitro findings, acute administration of ethanol in vivo produced a large increase in the phosphorylation of ERK1/2 levels and a smaller increase in phospho-JNK1/2 after 1 hr of ethanol administration (Fig. 3). The mean increases in phospho-ERK1 and phospho-ERK2 levels were 6.0 fold & 9.2 fold, respectively. The magnitude of increase in phospho-JNK 1 and JNK 2 levels (2.1 and 2.4 fold, respectively) were less pronounced than increase in phospho-ERK1/2. Ethanol activated p38 MAPK with a 1.5 fold increase in the levels of cytosolic phosphorylated p38 MAPK at 1hr. When MAPKs were monitored at 4 hr, activation of ERK1/2 was still significantly elevated (2.3 fold for ERK1 and 5.1 fold for ERK2) compared to control although the magnitude of increase was blunted at 4 hr as compared to 1 hr. In contrast to ERK1/2, the activation of JNK and p38 MAPK were transient and the levels of phosphorylated JNK and p38 MAPK at 4 hr were not significantly different from control group. These results suggest marked and prolonged activation of ERK1/2 by acute ethanol treatment in liver *in vivo*.

The effects of ethanol on changes in the levels of phosphorylated-MAPKs in the nuclear extracts of liver after acute ethanol administration are shown in Fig. 4. Loading

of samples were similar for control and ethanol treated samples as assessed by western immunoblot of histone H3 or β- actin protein levels. The increase in the levels of phosphorylated ERK1/2 in the nuclear extracts was significantly higher (4.7 for ERK1 and 4.9 fold for ERK2) at 1 hr. Moreover, the increases in the levels of phosphorylated ERK 1/2 were also significant (2.6 fold and 2.4 fold) at 4 hr. Increases in the levels of phosphorylated JNK1/2 (1.45 and 1.9 fold respectively) and p38 MAPK (1.5 fold) after 1 hr of ethanol treatment in the nuclear compartment was also moderate. At 4 hr, the levels of phosphorylated JNK1/2 and p38 returned to near normal values.

Acetylation and phosphorylation of histone H3

We next determined the phosphorylation of histone H3 at Ser-10 and Ser-28 at 1hr and 4r hr after IP ethanol administration. As shown in Fig. 5 the phosphorylation of histone H3 at Ser-10 and Ser -28 increased significantly at 1 hr. Phosphorylation of histone H3 Ser-28 (1.9 fold increase) was slightly higher compared to phosphorylation of histone H3 Ser-10 (1.6 fold increase). The levels of these phosphorylations returned to near control levels at 4 hr. In contrast to transient nature of histone phosphorylation, alcohol caused sustained increase in histone acetylation. Acetylation of histone H3-lys 9 at 1hr was marginally increased but was significant at 4 hr (1.4 fold, Fig. 5). A comparative assessment of the changes in the levels of phosphorylated MAPKs in the cytosolic and nuclear fractions and changes in the levels of modified histones is given in Table 1.

III.5 DISCUSSION

To the best of our knowledge, no study has been reported on the activation pattern of liver cell MAPK during acute ethanol binge *in vivo*. This study has assessed the relationship among MAPK activation, histone modifications and liver injury induced by acute intraperitoneal ethanol administration. We noted some contrasting effects of acute ethanol on MAPK signaling *in vivo* compared to the effects of ethanol on MAPK activation in primary cultured rat hepatocytes (Lee et al, 2002; Lee and Shukla, 2007). While ERK1/2 activation was modest *in vitro*, increase in the phosphorylation of ERK1/2 by ethanol *in vivo* was more dramatic and marked. In contrast, phosphorylation of JNK1/2 was more marked in cultured hepatocytes whereas its activation in vivo after acute ethanol was moderate. In cultured hepatocytes, we have demonstrated increased phosphorylation of JNK by ethanol when ERK1/2 phosphorylation was inhibited by U-0126, a MEK1/2 inhibitor (Lee and Shukla, 2005). Recently, administration of resistin to endotoxin treated mice resulted in increased phosphorylation of ERK1/2 with concomitant decrease in phosphorylation in JNK1/2 (Beier et al, 2008). Therefore, marked activation of ERK1/2 with modest activation of JNK suggests the possibility of cross talk between these two signaling pathways after acute ethanol treatment *in vivo*. With regard to the phosphorylation of p38 MAPK, acute ethanol administration *in vivo* caused 1.6 fold increase in p38 MAPK in cytosolic extracts (Fig. 2) whereas no such increase in phosphorylation of p38 MAPK in the cytosolic fraction was observed in cultured hepatocytes (Lee and Shukla, 2007). However, when hepatocytes were

cultured in the presence of serum, in contrast to our studies on serum free medium, increased phosphorylation of p38 MAPK was seen in cytosolic extracts (Zhang et al, 2007). These results suggest further modulation of ethanol induced MAPK activation by serum derived factors or cytokines *in vitro* and *in vivo*. In this regard, acute ethanol has been shown to cause hypoxia in liver (French, 2004) and hypoxia is associated with activation of three MAPKS in the cytoplasmic fraction in liver (Kaizu et al, 2008).

Pattern of caspase 3 cleavage and ALT at 1 and 4 h lead us to suggest that apoptosis precedes necrosis in acute binge. In hepatocyte cultures, transient activation of JNK1/2 was associated with transient apoptosis. The findings in vitro are also seen *in vivo* after acute ethanol treatment. Moreover, marked activation of JNK1/2 correlated with increase in apoptosis. In contrast to marked apoptosis of hepatocytes, necrotic damage to hepatocytes was minimal in vitro as evaluated by LDH release (Weng and Shukla, 2000; Lee and Shukla 2005). On the other hand, acute ethanol in vivo caused significant increase in necrosis. This correlated with activation of ERK1/2 *in vivo*. Although ERK1/2 has been reported to mediate suppression of necrotic response in vitro and *in vivo*, ERK1/2 activation under hypoxic conditions has been shown to promote necrotic cellular injury *in vivo* and *in vitro* (Schattenberg et al, 2004, Sabbatini et al, 2006). Ethanol has been shown to induce hypoxic damage at high blood ethanol levels under both acute and chronic conditions in vivo (French, 2004).

Ethanol induced phosphorylation of histone *in vivo*. Acetylation of histone H3 lys 9 also increased after IP administration of ethanol. Although the type of MAPKs involved

in ethanol induced histone phosphorylation and acetylation *in vivo* remain to be determined, our studies with hepatocytes suggested regulation of histone phosphorylation by p38 MAPK, and histone acetylation by ERK1/2 and JNK1/2 (Park et al, 2005; Lee and Shukla, 2007). The pattern of histone acetylation and phosphorylation by ethanol also demonstrated distinct temporal changes. Although the time course of histone acetylation induced by ethanol at 1 hr and 4 hr *in vitro* and *in vivo* are comparable, the magnitude of histone acetylation in vitro was more marked compared to *in vivo*. The increased acetylation *in vitro* may be due to marked activation of JNK1/2 *in vitro* since histone acetylation was dependent on both ERK1/2 and JNK1/2 activation in hepatocytes (Park et al, 2005). Interestingly, histone H3 ser-10 and ser-28 phosphorylations were evident at 1 hr and declined at 4 hr where as histone aectylation increased progressively with further increase at 4 hr both *in vitro* (Park et al, 2005; Lee and Shukla, 2007) and *in vivo* (the present study). The persistent acetylation observed at 4 hr may be due to persistent accumulation of phosphorylated ERK/2 in hepatocyte nucleus or due to MAPK independent effects of ethanol metabolite acetate as observed in *in vitro* hepatocyte cultures (Park et al, 2005). In contrast, the transient nature of histone H3 Ser-10 and Ser-28 phosphorylation may be linked to transient activation of p38 MAPK in the nuclear compartment after ethanol treatment. Although we have consistently observed ethanol induced H3 lys 9 acetylation, significant increase in H3K9 acetylation was not demonstrated after acute bolus administration of ethanol orally in another study (Bardag-Gorce et al, 2009). The discrepancy may be attributed to their administration of dextrose as control for ethanol binge. We have observed that histone

is acetylated after administration of dextrose alone orally as bolus (unpublished observations).

The issue of how MAP kinase cascades can transduce the acetylation of nucleosomal and chromatin proteins in response to extracellular signals, remains to be known. MAPKs may cause increased phosphorylation of histone, and phosphorylated histone has been shown to be a better substrate for histone acetylation (Merienne et al., 2001). The HAT GCn5 displays a strong preference for phosphorylated histone H3 over non-phosphorylated histone H3 as substrate *in vitro,* suggesting that histone H3 phosphorylation can affect the efficiency of subsequent acetylation reactions (Cheung et al., 2000). Interestingly, GCn5 has been implicated in ethanol induced histone acetylation (Choudhury et al, 2011). Other human HATs such as PCAF and p300 also prefer phosphorylated histone H3 as substrate (Clayton et al, 2003). Taken together it is apparent that histone H3 is phosphorylated via MAP kinase cascades. It can be speculated that histone kinases downstream of MAP kinase, such as RSK, are activated by ERK1/2 and that the resulting histone phosphorylation triggers further histone acetylation. However, ethanol induced phosphorylation of histone H3 was not inhibited by ERK1/2 (Lee and Shukla, 2007). Moreover, inhibition of p38 MAPK pathway in hepatocytes inhibits ethanol induced phosphorylation without affecting histone acetylation (Lee and Shukla, 2007). MAPKs phosphorylate several types of HATs (e.g., CBP, p300, ATF-2, and SRC-1) and directly increase their enzymatic activities (Ait-Si-Ali et al., 1999; Kawasaki et al., 2000; Li et al., 2001). p300, a substrate of ERK1/2 *in vivo* and *in vitro* (Li et al., 2003) has been shown to acetylate histone H3.

Epigenetic histone modifications contribute to inflammatory response, necrosis, and apoptosis under diverse types of tissue injury (Waring, 1997; Tikoo et al, 2001, Bártová et al., 2008, Delcuve et al., 2009). The role of epigenetic histone modifications in alcoholic organ damage is poorly understood (Shukla et al, 2008). Histone H3 phosphorylation by ethanol in hepatocytes had no influence on apoptosis (Lee and Shukla, 2007). On the other hand, modifications of histones have been shown to be relevant to transcriptional activation induced by ethanol. Histone H3 acetylation is linked to ethanol induced regulation of ADH1 gene expression in vitro (Park et al, 2005; Pal-Bhadra et al, 2007) and expression of lipogenic genes after chronic ethanol treatment in vivo (You et al., 2008). We believe that differential behaviors of histone acetylation and transient increase in histone H3 phosphorylation may determine activation of different sets of genes, perhaps in a sequential manner.

In summary, acute administration of ethanol caused a marked increase in the phosphorylation of ERK1/2 and modest increase in the phosphorylation of p38 MAPK and JNK. The acetylation of histone H3 K9 may be attributed to increased phosphorylation of ERK1/2 and JNK1/2. Likewise, increased phosphorylation of histone H3 Ser-10 and Ser-28 may be attributed to the phosphorylation of p38 MAPK. Taken together, these results suggest the existence of molecular axis between MAPK and epigenetic histone modifications in ethanol induced acute alcoholic liver injury *in vivo.*

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Fig. 1. Serum ethanol levels after acute ethanol administration Rats were given IP a single ethanol binge dose (3.5 g/Kg) and the levels of serum ethanol were determined at 1 hr and 4 hr as described under materials and methods. Control represents animals given saline for binge control. Values are mean ± SE (n=5 rats).

FIG. 1

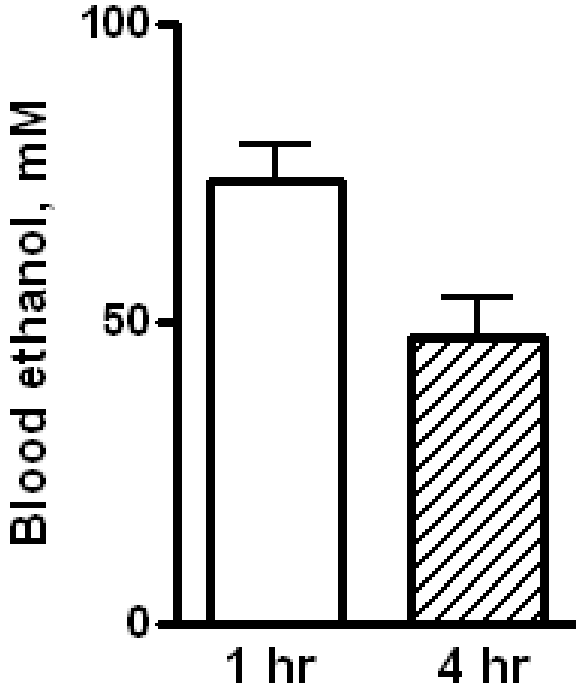

Fig. 2. Serum ALT, cleaved caspase 3, and steatosis after acute ethanol administration

Rats were given IP a single ethanol binge dose (3.5 g/Kg) and the levels of serum ALT were determined at 1 hr and 4 hr as described under materials and methods. Cleaved caspase 3 levels were determined by western immunoblot at 1 hr and 4 hr after ethanol administration. Steatosis was monitored at 1hr and 4 hr after ethanol administration. Sections of liver samples were stained with hematoxylin and eosin. Liver triglycerides were determined at 4 hr by colorimetric method. Control represents animals given saline for binge control. Values are mean ± SE (n=4 to 5 rats) * significant compared to control group ($p<0.05$).

FIG. 2

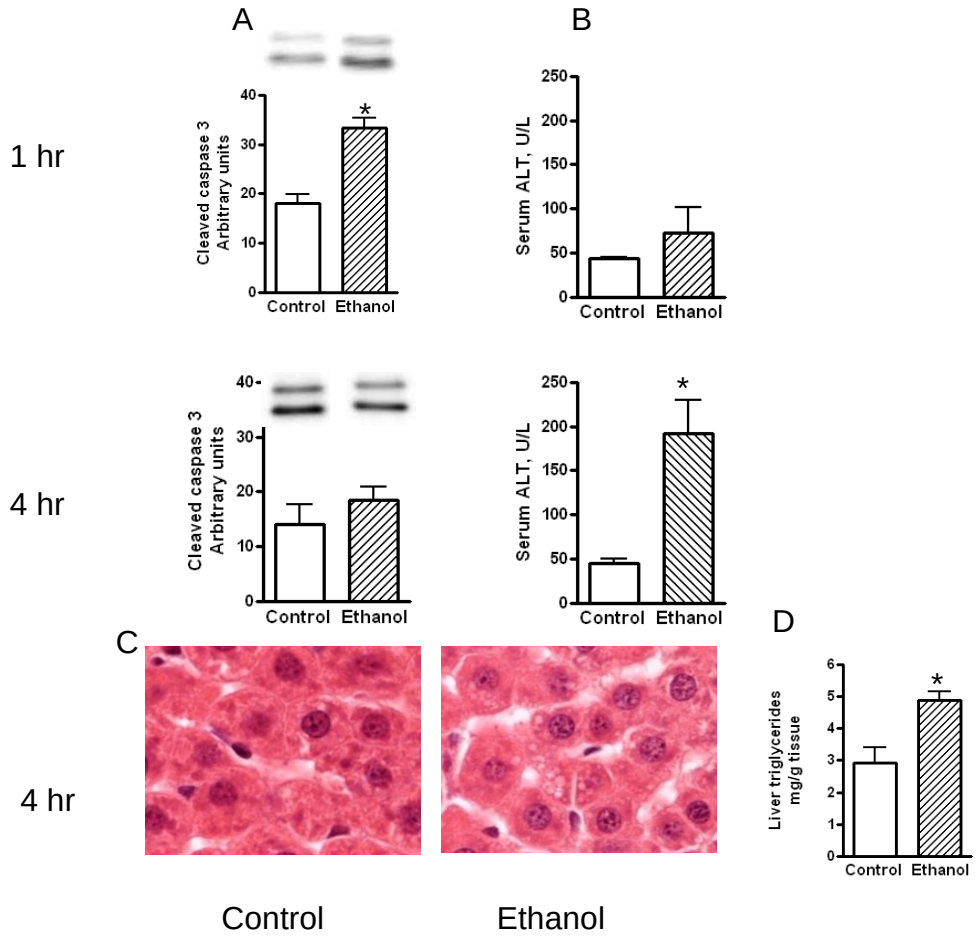
A
B
1 hr
Cleaved caspase 3
Arbitrary units
Control
Ethanol
Serum ALT, U/L
4 hr
C
D
Liver triglycerides
mg/g tissue
4 hr
Control
Ethanol

Fig. 3. Levels of phosphorylated ERK1/2, p38 MAPK and JNK1/2 in cytosolic extracts

Ethanol binge was administered as described in Fig .1 The levels of phosphorylated ERK1/2 , p38 MAPK and JNK1/2 in cytosolic cell extracts were determined at 1 hr and 4 hr. Control represents animals given saline for binge control. Values are mean ± SE (n= 3 to 5 rats) * significant compared to control group ($p<0.05$)

FIG. 3

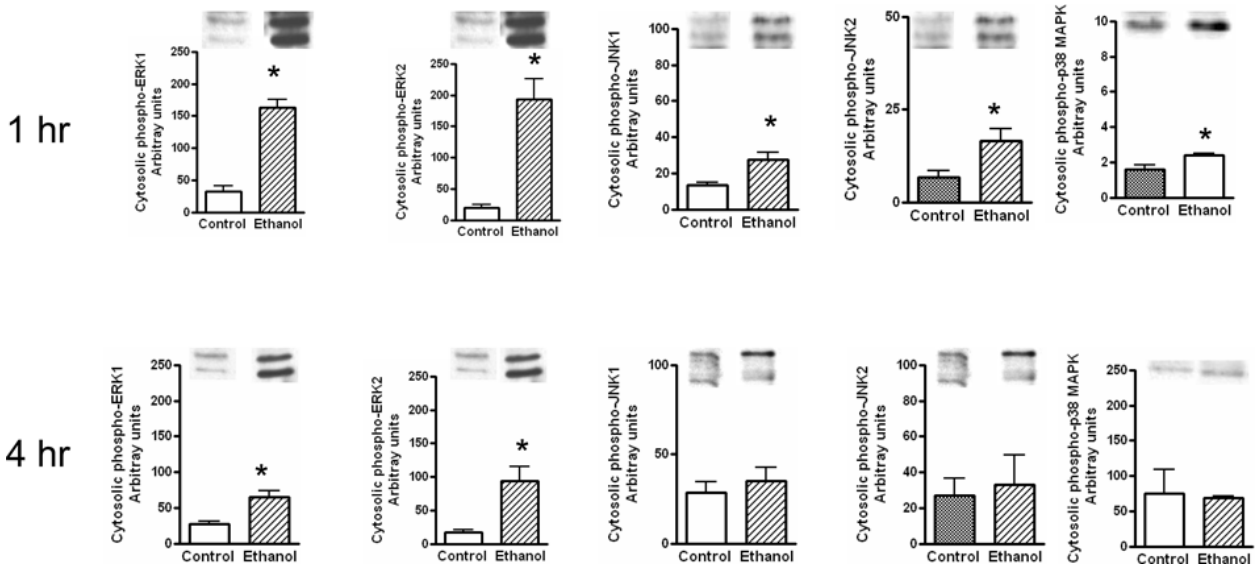
1 hr
4 hr
Cytosolic phospho-ERK1 Arbitray units
Cytosolic phospho-ERK2 Arbitray units
Cytosolic phospho-JNK1 Arbitray units
Cytosolic phospho-JNK2 Arbitray units
Cytosolic phospho-p38 MAPK Arbitray units
Control
Ethanol
*

Fig. 4. Levels of phosphorylated ERK1/2, p38 MAPK and JNK1/2 in nuclear extracts. Ethanol binge was administered as described in Fig.1. The levels of phosphorylated ERK1/2, p38 MAPK and JNK1/2 in nuclear extracts were determined at 1 hr and 4 hr as described under materials and methods. Control represents animals given saline for binge control. Values are mean ± SE (n=3 to 4 rats) * significant compared to control group ($p<0.05$).

FIG. 4

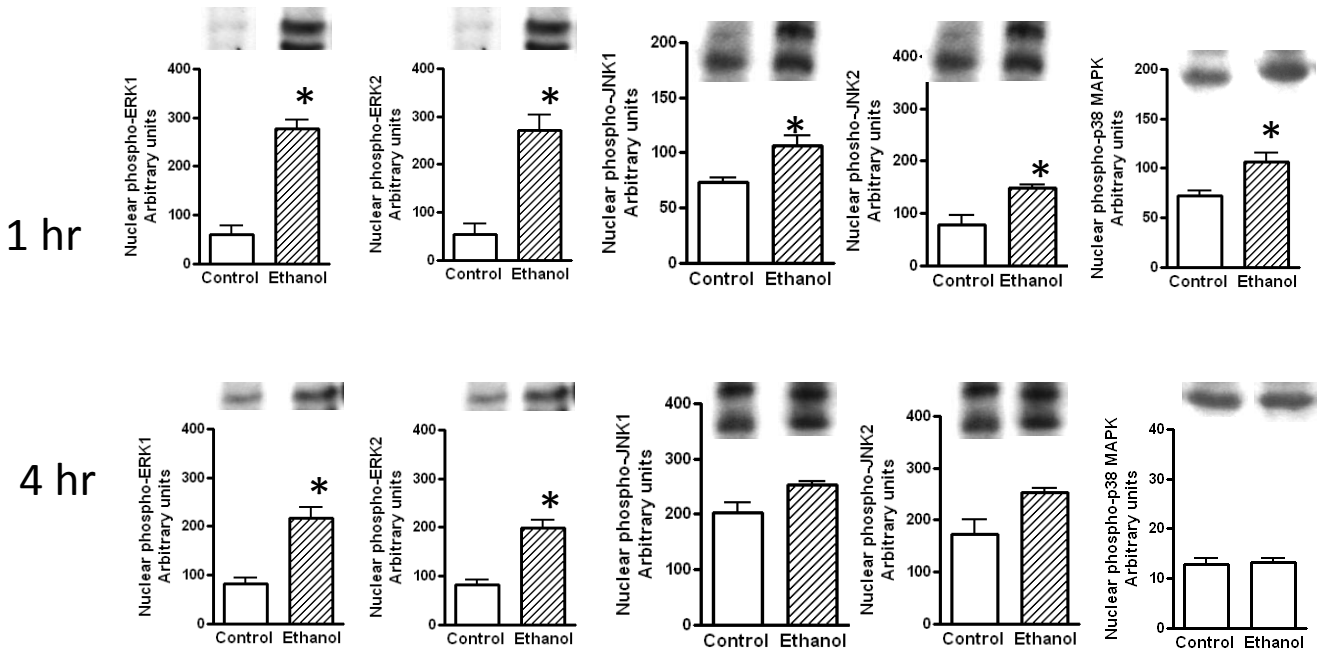
1 hr
4 hr
Nuclear phospho-ERK1 Arbitrary units
Nuclear phospho-ERK2 Arbitrary units
Nuclear phospho-JNK1 Arbitrary units
Nuclear phosho-JNK2 Arbitrary units
Nuclear phospho-p38 MAPK Arbitrary units
Control
Ethanol
*

Fig. 5. Levels of phosphorylated histone and acetylated histone after acute ethanol administration Rats were given IP a single ethanol binge dose (3.5 g/Kg) and levels of phosphorylated H3 ser-10 & ser-28 and acetylated H3 Lys 9 in nuclear extracts were determined at 1 hr and 4 hr as described under materials and methods. Values are mean ± SE (n=4 to 5 rats) *significant compared to control group ($p<0.05$).

FIG. 5

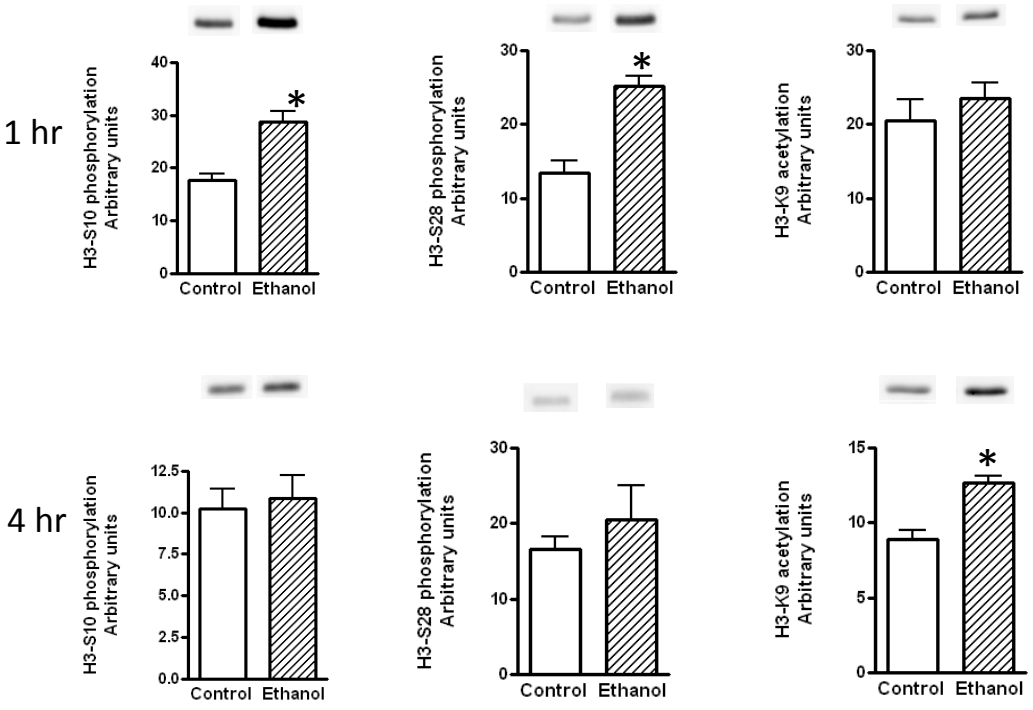
1 hr
4 hr
H3-S10 phosphorylation Arbitrary units
H3-S28 phosphorylation Arbitrary units
H3-K9 acetylation Arbitrary units
Control
Ethanol
*

Table 1. MAPK activation and histone modifications after acute alcoholic intoxication

	1 hr	**4 hr**
Injury		
Apoptosis	++	±
Steatosis	±	+
Necrosis	±	++
MAPK phosphorylation: Cytosolic		
ERK1	++++	++
ERK2	++++	++
JNK1	++	+
JNK2	++	+
p38 MAPK	++	+
MAPK phosphorylation: Nuclear		
ERK1	++++	++
ERK2	++++	++
JNK1	++	+
JNK2	++	+
p38 MAPK	+	±
Epigenetic histone modifications		
H3 Ser 10 phosphorylation	++	±
H3 Ser 28 phosphorylation	++	±
H3 K9 acetylation	+	++

CHAPTER IV

Histone H3 Phosphorylation (Ser-10, Ser-28) and Phosphoacetylation (Lys-9/Ser-10) are Differentially Associated With Gene Expression In Liver of Rats Treated In Vivo With Acute Ethanol

IV.1 ABSTRACT

The epigenetic histone modifications by ethanol are emerging as one of mechanisms for the deleterious effects in the liver. In this context, the role of histone H3 phosphorylation at serine-10 (P-H3-S10) and serine 28 (P-H3-S28) in acute ethanol liver injury in vivo, was investigated here. Ethanol was administered intraperitoneally (IP) (1-5g/ Kg body weight) in 12 weeks old male Sprague–Dawley rats. Serum ALT and steatosis of the liver increased from 1.5 to 4h suggesting liver injury. Based on the profile of histone phosphorylation as determined by western blot analysis, 3.5g/kg ethanol was selected to determine histone H3 phosphorylation at different times (1-4h). There were obvious differences between P-H3-S10 and P-H3-S28 at 1h, the latter was more sensitive at lower ethanol doses. Interestingly, phosphorylation of both serines disappeared at the highest dose used (5g/Kg). We also examined phosphoacetylation of H3 at K9/S10. The change in histone H3 phosphorylation and phosphoacetylation correlated with early response gene expression (c-Fos, c-Jun, MKP-1). We performed chromatin immunoprecipitation (ChIP) assays at 1.5h and 4h after ethanol administration. Histone H3 phosphorylation at serines 10 and 28 associated with the promoters of c-Jun. However, only S28 associated with PAI-1 at 1.5h. Interestingly, phosphoacetylation at K9/S10 was associated to PAI-1 at 4h only. In conclusion, after acute exposure of liver to ethanol in vivo, histone phosphorylation and phospoacetylation occur, and play a differential role in the expression of early response genes.

Key words: Alcoholic liver disease, binge drinking, epigenetics, histone H3 phosphorylation, immediate early genes

IV.2 INTRODUCTION

Alcohol addiction often results in alcoholic liver disease (ALD) where the pathology is identified by steatosis (fatty liver), steatohepatitis, cirrhosis (fibrosis of the liver) and at times progresses to hepatocellular carcinoma (Lucy et al., 2009; Purohit et al., 2009). Repeated alcohol abuse (binge) promotes phenotypic changes in the liver leading to pathology; however the molecular and cellular events that lead to the progression of ALD 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 et al, 2005). Moreover, we have reported ethanol induced histone H3 phosphorylation at serine 10 and serine 28 mediated by p38 MAPK in primary 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 (Clayton et al, 2000; Cheung et al, 2000; Lo 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). Therefore ethanol induced histone acetylation and phosphorylation may be 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 expression c-Fos and c-Jun respectively, and hence AP-1 (Clayton and Mahadevan, 2003). MAP kinases are implicated in alcoholic liver disease as they regulate inflammation, steatosis, apoptosis and necrosis (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 the 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 (S10/K9) in vivo and examined their mechanistic relevance to the gene expression.

IV.3 MATERIALS AND METHODS

Reagents

Antibodies to P-H3- S10, P-H3-S28, and H3 protein were purchased from Millipore (Temecula, CA). Cleaved caspase-3, and anti acetyl- and phospho-Histone H3 (Lys9/Ser10) antibody (Cat # 9711) was 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 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 water. To assess the effects of ethanol on histone H3 phosphorylation at different time points, 3.5g/kg ethanol or water (control) was injected to rats and the liver was removed at 1h, 1.5h, 2h, 3h, and 4h. At the time of liver removal, blood samples were collected for blood alcohol and serum ALT analysis. Additionally, a section of liver was placed in formalin and the remaining sections of the liver were frozen in liquid nitrogen and stored at -80^{0}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^{o}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 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 ^{o}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 ^{o}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 ^{o}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 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 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 with antibody to phosphorylated, phosphoacetylated, acetylated, total histone H3, or cleaved caspase 3 overnight at 4°C. After washing with TBST, the membrane was incubated with secondary antibody conjugated to horseradish peroxidase for 1h 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 ^{TM} 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 20 μl 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 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 ^{o}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 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^{o}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) and then incubated for 1hr at 4^{o}C with 60 μl of protein G beads (50% slurry-salmon sperm DNA) to preclear the lysates. After removal of agarose 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) and incubated with the immunoprecipitating antibody, 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) and 1 μg anti-RNA-Polymerase (Millipore cat # 05-623B) or a no-antibody control, and incubated overnight at 4^{o}C. 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, 1mM 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 ^{o}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 4h 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 $MgCl_2$, and the same concentrations of phosphatase and protease inhibitors mentioned above). Proteins were then crosslinked

to DNA by the addition of 27 μl of 37% formaldehyde for 7.5 mins 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 C_t values were similar to that obtained when the ChIP assay was done from 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. 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.

RNA Analyis: The qRT-PCR data for changes in mRNA expression between ethanol and control were analyzed using the the average C_t of three replicates and the ΔΔ comparative C_t 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 C_t 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-S10/K9). Input samples were diluted (1/100) and ChIP samples (no dilution) was used for real time PCR in a iCycler 5 (Bio-Rad) 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 should not be altered by ethanol induced histone phosphorylation, Gapdh promoter was analyzed (data not shown).

IV.4 RESULTS

In this study, we have assessed the effect of dose and time of acute binge 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-S10/K9, acetylation at H3-K9, early response gene expression in liver (c-Fos, c-Jun, MKP-1) and genes involved in alcoholic liver injury (LDL-r, TNFα and PAI-1). Next we performed ChIP assays with site specific antibodies to P-H3-S10, P-H3-S28 and phosphoacetyl H3-S10/K9 and analyzed the association between promoter regions of those genes with phosphorylated, and phosphoacetylated histone H3. The results are presented below.

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

Because of the rapid response in phosphorylation of H3, we felt that IP administration will be a good model to study the relationship between phosphorylation and acetylation i.e. phosphoacetylation and their relevance to gene expression. In comparison to intragastric ethanol administration model, blood ethanol levels after IP administration are more controlled, and exhibits less variation between animals. The administration of ethanol itself is less stressful to the animals by IP route. The technique also offers the ability in the future to inject metabolites of ethanol such as acetate, or various inhibitors/drugs, to study their effects in vivo. Additionally, IP administration of ethanol is widely used, among others, in neuro-behavioral studies assessing acute binge effects.

Blood ethanol levels after acute ethanol binge

IP administration of different doses of alcohol (1, 1.75, 2.5, 3.5, 5 g/Kg body weight) to rats resulted in blood ethanol levels ranging from 27-108 mmol/L after 1h (Fig 1A). The 2.5g, 3.5g and 5g doses were able to achieve blood ethanol levels that mimic human binge where blood alcohol levels can reach 60 mmol/l and can reach up to 100 mmol/L (Rivara et al, 1993). The National Institute on Alcohol Abuse and Alcoholism (NIAAA) defines binge drinking as 5 or more drinks for men, or 4 or more drinks for women within a 2 h period (Mathurin and Deltrenre, 2009). Three to five drinks in humans give a BAC of ~ 33 mM (Gohlke et al, 2005). However, it is important to consider the actual BAC rather than the number of drinks. Realistically, pharmacologically relevant doses in humans, rarely exceeds 200 mM. In a casualty ward in a German hospital, BACs of 109-170 mM were reported in 3/1000 patients in a period of 3 months (Haycock, 2009). BACs ranging from 20- 170 mM can be seen in moderate to heavy users (Pantazis et al, 1992). In our study the BAC at 1h at all doses was in this range (Figure 1A). Additionally after a single IP dose of ethanol (3.5 g/Kg body weight) to rats, BAC increased up to 2hrs (152 mmol/L) but declined at 3h (98 mmol/L) and 4hrs (65 mmol/L, Figure 1B). At 4h, the blood alcohol was less than half that of the peak at 2hrs (Fig 1B).

Serum ALT, cleaved caspase 3 and liver injury

There were no statistically significant changes in serum ALT between control and ethanol treated animals at all doses (Figure 2) treated for 1h. A time course experiment using 3.5g/Kg ethanol showed that after 1h there were significant differences between ALT from control and ethanol treated animals (Fig. 3). From 1.5 -4h all control values are similar (47-56 U/L), but increased ($p < 0.05$) in ethanol samples. The levels of ALT were 134.3 at 1.5h and increased about 40% to 231.5 at 4h. Interestingly, the levels of ALT were similar in the ethanol treated animals up to 3h and dramatically increased to its highest at 4h.

Cleaved caspase 3 increased when 3.5 g/Kg and 5 g/Kg ethanol was administered. Interestingly histone phosphorylation at both serines 10 and 28 increased at this dose. A time course profile of 3.5 g/Kg showed that cleaved caspase 3 decreased at 1.5h then peaked again at 2h and decreased thereafter at 3 and 4 hours.

There was no apparent steatosis at 1h as determined by H&E staining. In Fig.3 (see arrows) it is evident that steatosis increased over time as fatty deposits are evident in the liver. Therefore in this model both necrosis and steatosis is observed which shows that acute alcohol is causing injury to the liver.

Ethanol Dose Related Phosphorylation of histone H3

Previously we have reported that after an acute administration of ethanol histone H3 phosphorylation at serine-10 and serine-28 significantly increased (Aroor et al, 2010). In this study we have determined the ethanol dose effects on H3

phosphorylation at 1h. As shown in Figure 4, the fold change in phosphorylation of P-H3-S10 and P-H3-S28 increased at 1.75, 2.5, and 3.5 g ($p < 0.05$) whereas no significant increase occurred with the 5 g/Kg dose. It may be noted that at lower dose of 1 g/Kg the phosphorylation at S28 is more sensitive (1.3 fold increase) than at S10 which did not change. Likewise, 1.75g caused a peak increase in phosphorylated histone H3-Ser 28, as opposed to S10 that peaked at 3.5g. This indicated that phosphorylation of Ser 28 is more sensitive at lower blood alcohol levels. At 5 g/kg there was no change in phosphorylation at either site.

Time course of change in histone H3 phosphorylation by ethanol

A time course profile of ethanol (3.5 g/Kg) induced histone phosphorylation was generated. There were clear differences between the phosphorylation of serine-10 and serine-28 (Fig. 5). There was a biphasic response in the phosphorylation of serine 10. Treating the rats for extra 30 min (1h versus 1.5h) with ethanol almost doubled the fold change in histone phosphorylation at Ser 10 (2.4 fold to 4.4 fold increase). At 2h the fold change came down to 1.7 (lowest) and then increased up to 4h (4.7). The phosphorylation of serine 28 increased only 20% from 1h to 1.5 h (1.88 to 2.44 fold) compared to 100% for serine 10. While the decrease in Ser 10 phosphorylation at 2h was more pronounced, there was only a slight decrease at 2h in Ser 28 phosphorylation. At 3 and 4h phosphorylation was similar at Ser 28 compared to a moderate increase for Ser 10. The data also show that there was a higher degree of change in the phosphorylation of H3-Ser 10 as compared to Ser 28.

Thus, as seen in (Fig 4), histone H3 phosphorylation increased from 27.6 mmol/L to 90.37 mmol/L of ethanol (Fig 1A), suggesting that at low to medium levels of ethanol binge, histone phosphorylation can occur rapidly after 1h. Histone H3 phosphorylation also increased at all time points. Therefore, histone H3 phosphorylation was altered with dose of ethanol and time of treatment.

Time and ethanol dose related changes in histone H3 phosphoacetylation (K9/S10)

Phosphoacetylation of histone H3 at K9/S10 did not change at 1 g, peaked at 2.5g, and decreased at 5 g/Kg (Fig. 6). Phosphoacetylation increased at 1h, remained at that level until 3h, and increased again to its highest value at 4h. It may be noted that the fold increase in phosphoacetylation at 5g/Kg, was significantly higher than the negligible fold increase in the phosphorylation at serine 10 or serine 28 alone (Fig. 4 vs Fig.6) indicating differences in profile.

Ethanol dose and time related acetylation at K9 of histone H3

For comparison, we next tested the acetylation of H3 at K9. Acetylation of histone H3 at K9 was similar to the phosphorylation of histone H3 at serine 28 in that it was more sensitive to lower doses of ethanol. Acetylation increased further at 2.5g/Kg and declined thereafter (Fig.7). It must be noted that the time and dose response pattern of Ac-H3-K9 and phosphoacetylation at K9/S10 (fig. 6) were different.

The effect of ethanol on gene expression

Dose Response

Ethanol is known to increase gene expression. Phosphoacetylation of histone H3 at serine 10 and lysine-14 have previously been shown to induce c-Fos and c-Jun expression and histone H3 phosphorylation is known to affect these genes during mitosis (Clayton et al, 2000). Induction of c-Fos has been implicated in ALD as described in the Introduction. In this study we analyzed the effects of ethanol on immediate early gene (IEG) expression of c-Fos, c-Jun, and MKP-1. After a 1h administration of ethanol there was a significant ($p < 0.05$) induction of c-Fos and c-Jun by 1.75 and 3.5g ethanol per kg body weight (Figure 8). For MKP-1, 1.75g caused a 4.5 fold change in MKP-1 as compared to 2 fold by 3.5g. The induction of these genes coincided with the increase in histone H3 phosphorylation at 1.75 and 3.5g (Fig.4). There was no induction of IEG expression with 5 g/kg ethanol which suggests that their expression may be related to histone phosphorylation.

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 important roles in alcoholic liver injury. LDL-r is long known to be involved in alcohol induced steatosis. Recently PAI-1 has been implicated in this aspect of alcoholic liver disease (Arteel, 2008) and TNFα is a well known marker for alcoholic liver injury (McClain and Cohen 1989; McClain et al. 1997). Because PAI-1 and TNFα can be activated by AP1 they were important genes to analyze after acute ethanol injury.

At 1.75 g/Kg, LdL-r increased about 2 fold. There was also an increased seen at 3.5 and 5g/Kg, 1.2 and 1.5 fold respectively. TNFα increased at 1.75 g/Kg then decreased at 3.5 and 5 g/Kg. PAI-1 increased at 1.75 and 3.5 g/Kg then decreased at 5 g/Kg (Fig. 5).

Time course

c-Fos was induced up to 13.4 fold ($p < 0.05$) at 1.5h, 8 fold at 2h ($p < 0.05$), and 5 fold ($p < 0.01$) at 3h and there was a small change at 4h (1.89 fold). c-Jun followed a somewhat similar pattern of induction albeit the fold changes were less robust; 1.5h (12 fold), 2h (1.9 fold), and 3h (2.6 fold). MKP-1 mRNA increased to 1.85 fold at both 1 and 1.5h, decreased at 2h (0.6 fold), increased again at 3h (2.1 fold), then decreased at 4h (0.87 fold).

At 1h there was no change in LDL-r mRNA expression however it increased at the other time points and was highest at 1.5h. Its pattern of change was similar to the change in the blood alcohol levels. In contrast, PAI-1showed gradual increase with increasing time points and was highest at 4h. There was no change in TNFα mRNA expression at early time points but it increased at 3 and 4h after ethanol administration (Fig. 9).

ChIP Assay to determine association between phosphorylated Histone H3 and the gene promoter

To determine the association of histone H3 phosphorylation with gene expression, we chose to compare our 1.5h and our 4h time points (Figure 5) where phosphorylation of S10 was highest. This also offers the ability to compare an early response gene and a gene that is more dramatically affected at a later time point. We chose the 4h time point to determine the phosphoacetylation and gene association since it was highest at 4h (Fig. 6). Analysis of gene association by ChIP assay (Fig. 10) revealed that serine 10 showed a trend of association to the AP1-CRE site of the rat c-Fos with primers spanning a 140 bp region upstream of the transcription start site (TSS). Phosphorylated serine 28 does not appear to associate with this region of the c-fos promoter indicating a degree of selectivity. In contrast, both serines showed association to the AP1 site (-592 to -585) of the c-Jun promoter. We used primers spanning a region -632 to -537 upstream of the TSS. The association of serine 28 was significant as compared to serine 10 at the c-Jun promoter. RNA Polymerase II (RNA Pol II) antibody showed higher association at the promoter of the early response genes in the ethanol treated samples, further suggested that transcription is occurring. It is known that RNA Pol II is found at the promoters of early response genes under basal activity (Sandoval et al, 2004).

We found an association between phosphorylated H3-S28 and not phosphorylated H3-S10 at the PAI-1 promoter at region -724 to -613 upstream of the TSS which contains an SRE binding domain at region -701 to -693. Phosphorylated H3-S28 was associated with this region of PAI-1 after both 1.5 and 4 hours of ethanol

treatment. Additionally we found an association of phosphoacetylated histone H3-K9/S10 at this same region of PAI-1 but only at 4h.

IV.5 DISCUSSION

In this study, we have observed that acute IP administration of ethanol can promote liver injury i.e apoptosis and necrosis, as measured by cleaved caspase 3 and ALT, respectively (Fig. 2 and Fig. 3). In addition, after the administration of ethanol, mild steatosis occurs in the liver (Fig 3). Therefore this model is relevant in studying acute ethanol binge in terms of liver injury. 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. It has been previously shown that histone phosphorylation at serine 10 was correlated with caspase 10 activation. Also in the same study, ChIP assay revealed that histone H3 phosphorylation was also associated with the promoter of caspase 10 (Li et al, 2002). Since cleaved caspase 3 can result from caspase 10 activation (Kumar, 2007), it is feasible that histone phosphorylation may have a role in inducing cleaved caspase 3 and thus apoptosis after ethanol. However, histone phosphorylation cannot be the only factor since cleaved caspase 3 increased at 5 g/Kg ethanol where phosphorylation did not alter. Interestingly, Li et al also observed that phosphoacetylation of histone H3 at serine 10 and lysine 14 was associated with the promoter of caspase 10 as well. Phosphoacetylation at these two residues have also been implicated in the induction of MKP-1 mRNA. Phosphoacetylation of H3 at S10 and K14 is induced after ethanol (data not shown) and can therefore be another mechanism for effects on caspase 3

activation. In this paper we focused on the effects of ethanol on the phosphorylation of histone H3 at serine 10 and 28 and phosphoacetylation at lysine 9/ serine 10.

An important finding observed in this study is that acute IP administration of ethanol can promote site specific histone H3 phosphorylation at serine 10 and serine 28 in rat liver in vivo after acute ethanol administration. Phosphorylation of H3 at S28 was more sensitive at lower dosages (1g/Kg) of ethanol than P-H3-S10. At the higher 5g dosage, phosphorylation was not altered (Figure 4). It has been reported that rapid activation of MAP Kinases can lead to induction of MAP Kinase Phosphatase-1 (MKP-1). MKP-1 is a negative regulator MAP Kinases and hence a decrease in MAP Kinases 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). Interestingly there was a remarkable increase in the phosphoacetylation (S10/K9) status of histone H3 at 5 g/kg dose of ethanol (Fig. 6). Oxidative stress has been reported to cause dephosphorylation and deacetylation of histone H3 (Kabra et al 2008) and may be an explanation for 5 g/Kg not affecting phosphorylation or acetylation of H3 (Fig. 4 and Fig.7, respectively). The differences in phosphorylation of serine 10 and phosphoacetylation at K9/S10 after 5g/Kg ethanol suggests that phosphorylation and phosphoacetylation resides in different domains in the chromatin after ethanol. The phosphorylated serine 10 and the acetylated antibodies only bind to H3 when it's mono-

modified, and the phosphoacetyl antibody only binds when H3when it is di-modified. This also suggests that different nucleosomal domains exist for these modifications.

The phosphorylation of serine 10 and serine 28 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. From the 1-3h time points MKP-1 mRNA induction was similar in pattern to that of phosphorylated histone H3 at S10 (Fig. 9 vs Fig. 5). Therefore MKP-1 could be responsible for the down regulation of Ser 10 phosphorylation either by dephosphorylating MAP kinases directly or histone H3 S10 itself (Kinney et al, 2009). It remains to be tested in the future.

To the best of our knowledge this is the first report of increase in phosphoacetyl-histone H3- K9/S10 after ethanol. We predicted that the dual modification would be involved in transcriptional activation of genes. There are published studies indicating that phosphorylation at serine 10 of histone H3 oppose acetylation at lysine 9, but enhances acetylation at lysine 14 (Edmondson et al, 2002). However this is unlikely to be the case in our model. We have also observed that HDAC inhibition by trichostatin A induced increase in P-H3-S10 and P-H3-S28 in rat primary hepatocytes (see miscellaneous data). Histone H3 phosphorylation at serine 28 after TSA treatment has also been observed in JB6 cells (Zhong et al, 2003). Earlier we

reported that 100 mM ethanol in primary hepatocytes 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 HATs are known to exhibit increased activity on histone H3 with phosphorylated S10 (Merienne et al 2001, Clayton, et al 2000), we hypothesized that ethanol induced in vivo histone phosphorylation may increase acetylation. In the present study phosphorylation is high at 4h, and the acetylation is high at 1.5h. Thus both modifications appear to be occurring independently as opposed to regulating each other.

Because serine 28 was more responsive at 1.75g and serine 10 at 3.5g, we conclude that serine 10 and serine 28 may have differential roles in gene expression after ethanol. Their differential roles were seen at the promoter of PAI-1 since phosphorylated histone H3-S28 associated with PAI-1, whereas phosphorylated serine 10 did not. However, we cannot rule out that phosphorylated serine 10 does not associate with PAI-1 at all, because we have not tested all regions of the promoter. Additionally, phosphoacetyl H3-K9/S10 was associated with the same region of the promoter of PAI-1 as serine 28. Therefore in terms of this region of the PAI-1 promoter, phosphorylated serine 10 in conjunction with acetylated lysine 9, and not phosphorylated serine 10 alone, is associated with the promoter. This is an important finding because ethanol induces activation of PAI-1, which causes fibrin accumulation in the liver by inhibiting fibrinolysis (Beier et al, 2009). PAI-1 was also implicated in liver steatosis (Arteel, 2008). Taken together these data suggest that the phosphorylation of

histone H3 at serine 10 and serine 28 and phosphoacetylation of histone H3-K9/S10 are affected during acute ethanol binge administration in vivo, and have relationship to gene expression.

It will also be interesting in the future to determine if the histone phosphorylation is associated with LDL-r or TNF-α. After chronic alcohol consumption, hypercholesterolemia develops because LDLr levels decrease whilst VLDL increases in liver (Wang et al, 2010). Since LDL-r was previously found to be associated with histone phosphorylation in HepG2 cells (Huang et al, 2004, Huang et al, 2006), we were interested in studying the effects of acute binge alcohol on LDLr.

In summary we have demonstrated that ethanol induced histone H3 phoshorylation plays a role in acute ethanol induced gene expression in vivo. An increase in histone phosphorylation correlated with an increase in its association with the promoter of the early response genes, c-Fos and c-Jun, thus histone phosphorylation may have roles in the induction of the AP1 responsive genes. An important finding in this work is that histone H3 phosphorylation at serine 28 as well as phosphoacetylation was associated with the PAI-1 promoter. Since histone phosphorylation was found at the promoter of c-Fos and c-Jun, which forms the transcription factor AP1, and since AP1 can bind to the SRE site of PAI-1 promoter, then histone H3 phosphorylation and phosphoacetylation may have direct and indirect roles in regulating the expression of PAI-1 after ethanol, since PAI-1 has an AP- 1 binding site in its promoter. This suggests that the role of ethanol induced histone H3 phosphorylation is in transcriptional

activation and is further supported by the fact that RNA Pol II was increased at the promoter of c-Fos and c-Jun genes tested in this study. To the best of our knowledge this is the first study establishing a relationship between histone H3 phosphorylation at serine-10 and serine-28 and gene expression after ethanol, showing the effects of ethanol on phosphoacetylation of H3 at K9/S10, and lastly showing that this phosphoacetylation is involved in the expression of genes that have roles in alcoholic liver injury.

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Table 1. Primer Sequences Used for qRT-PCR for mRNA expression

Gene	Forward	Reverse
GAPDH	5'-AGACAGCCGCATCTTCTTGT-3'	5'-CTTGCCGTGGGTTAGAGTCAT-3'
TNF α	5'-AAATGGGCTCCCTCTCATCAG-3'	5'TTCTCTGCTTGGTGGTTTGCTACGAC-3'
PAI-1	5'-AACCCA GGC CGA CTT CA-3'	5'-CATGCGGGCTGAGACTAGAAT-3'
c-Fos	5' GCGGACTACGAGGCGTCAT-3'	5'- GGAGGAGACCAGAGT -3'
c-Jun	5'-CAGGTGGCACAGCTTAAACA-3'	5'- CGCAACCAGTCAAGTTCTCA-3'
MKP-1	5'- GCTGTCCTTGACCGGGGCAC-3'	5'-CCACACCTCCCCTGGCCCTT-3'
LDL-r	5'- TTCTTCAGGTTGGGGATCAG-3'	5'-CAGCTCTGTGTGAACCTGGA-3'

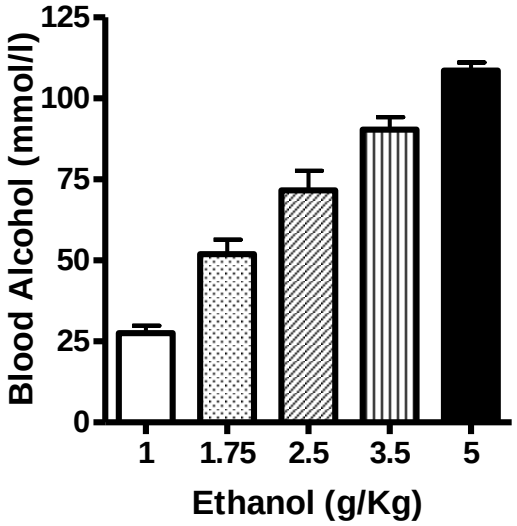
Table 2. Primer Sequences Used for qRT-PCR for Promoter Regions

Gene	Forward	Reverse
GAPDH	5'- CGTAGCTCAGGCCTCTGCGCCCTT-3'	5'CTGGCACTGCACAAGAAGATGCGGCT-3'
PAI-1	5'- CCCACCCAGTACACCTCAAA -3'	5'- GGTGAAGCACCTCTGACTCC -3'
c-Fos	5' TTCTCTGTTCCGCTCATGACGT -3'	5'- CTTCTCAGTTGCTAGCTGCAATCG -3'
c-Jun	5'- GCCACACTCAGTGCAACTCT -3'	5'- CGGAGGAGGGGACAGTTG -3'

Fig. 1. Serum ethanol levels after acute IP in vivo: dose response and timecourse. 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 1h and the levels of serum ethanol were measured as described in materials and methods. Control represents animals given water. Values are mean ± SE (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 IP dose of ethanol (3.5g/Kg) and the levels of serum ethanol were measured after 1, 1.5, 2, 3, and 4h as described under materials and methods. Values are mean ± SEM (n= 3 to 4 rats). The serum ethanol levels were measured from control animals [5 g/Kg] (n = 3). The average value was 6 mmol/l (data not shown).

FIG. 1

A


Blood Alcohol (mmol/l)
125
100
75
50
25
0
1
1.75
2.5
3.5
5
Ethanol (g/Kg)

B

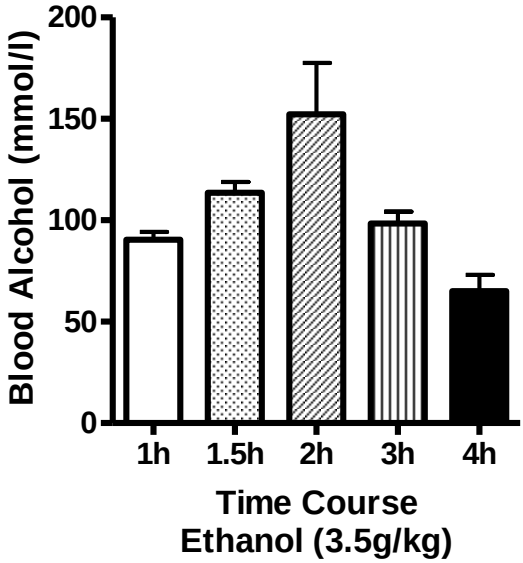

Blood Alcohol (mmol/l)
200
150
100
50
0
1h
1.5h
2h
3h
4h
Time Course
Ethanol (3.5g/kg)

Fig. 2. Serum ALT and cleaved caspase 3 after ethanol administration at different doses. Rats were given IP, different doses of ethanol (1, 1.75, 2.5, 3.5, 5 g/Kg). Cleaved caspase 3 was analyzed by western blot and serum ALT was measured using an automated analyzer as described under materials and methods. Values represent mean ± SEM (n= 3 to 5 rats). The asterisk (*) represent significant values at $p < 0.05$ compared to control as analyzed by the student t test.

FIG. 2

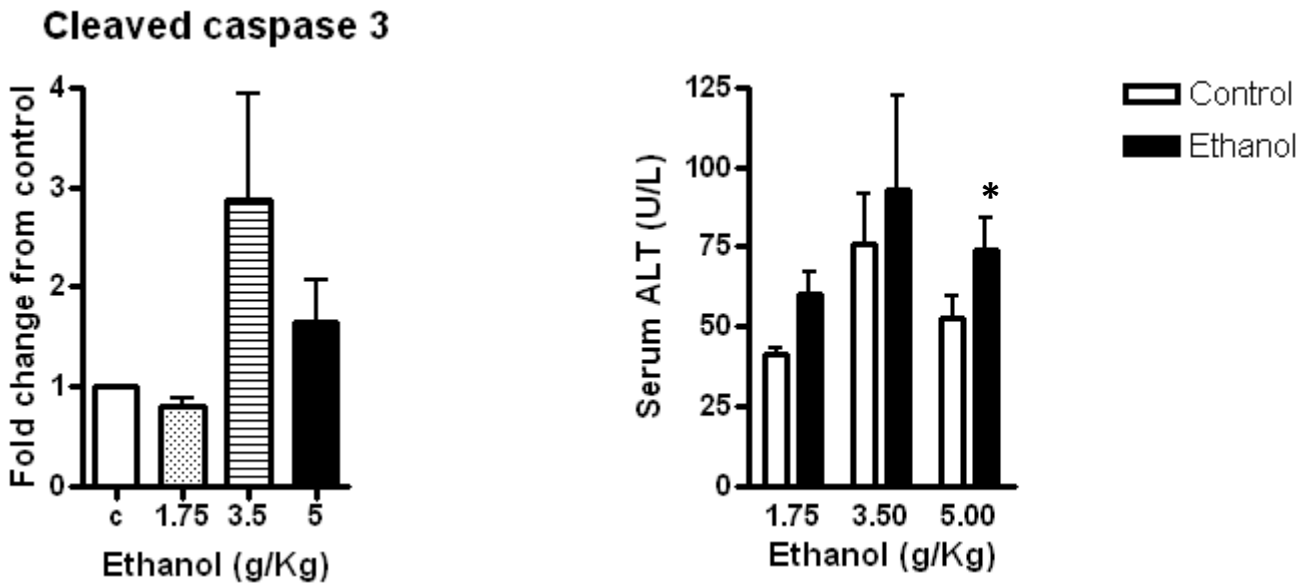
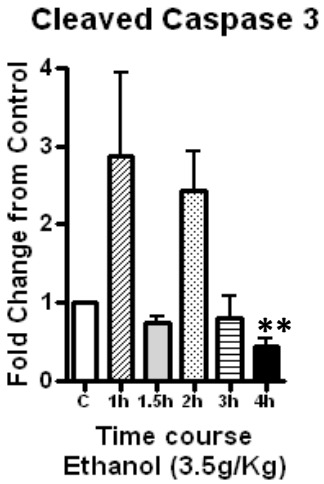
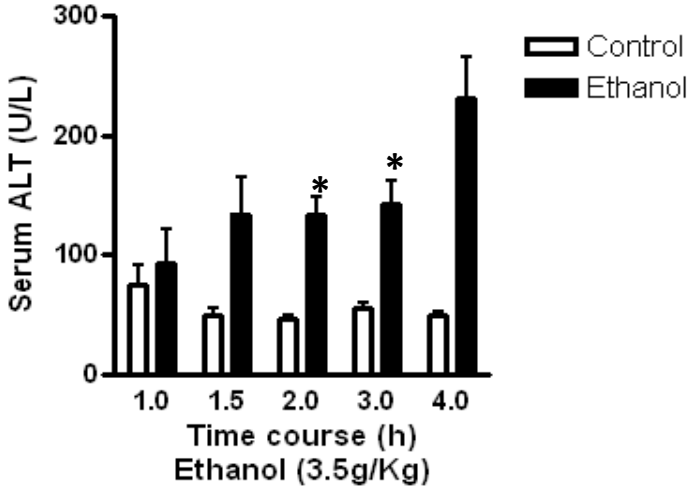

Cleaved caspase 3
Fold change from control
0
1
2
3
4
c
1.75
3.5
5
Ethanol (g/Kg)
Serum ALT (U/L)
0
25
50
75
100
125
1.75
3.50
5.00
Ethanol (g/Kg)
*
Control
Ethanol

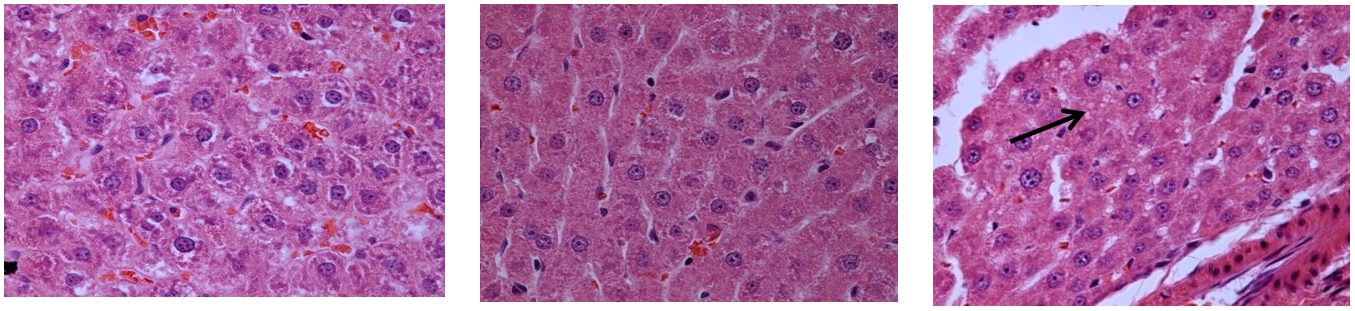
Fig. 3. Serum ALT, cleaved caspase 3, and steatosis at different time points after ethanol administration. Rats were given IP a single ethanol binge dose (3.5 g/Kg) and the levels of serum ALT were determined at different times i.e. at 1 h, 1.5 h, 2 h, 3 h and 4h as described in the materials and methods section. Cleaved caspase 3 levels were determined by western immunoblot analysis in cytoplasmic extracts. Steatosis was monitored by hematoxylin and eosin staining of liver sections. Control represents animals given water. Values are mean ± SEM (n= 3 to 4 rats). The asterisk (*) represent significant values at $p < 0.05$ compared to control as analyzed by the student t test.

FIG. 3





Control **1 h** **1.5 h**



2 h **3 h** **4h**

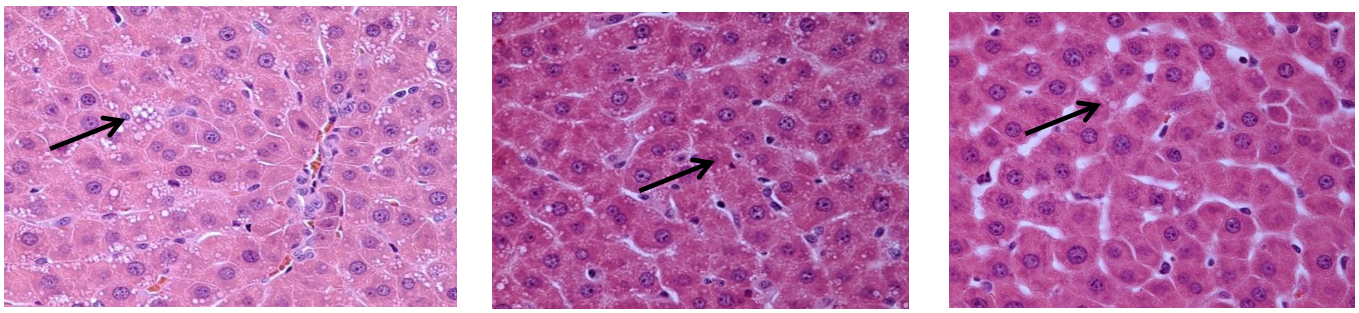


Fig. 4. Ethanol dose dependent changes in phosphorylated histone H3 after acute ethanol administration at 1h. Rats were given IP different doses of ethanol (1, 1.75, 2.5, 3.5, 5g/Kg) for 1h and levels of phosphorylated H3-ser-10 & ser-28 in nuclear extracts were determined at 1 h by SDS-PAGE & western blotting with site specific antibodies. Data were normalized to histone H3 protein. Values are mean ± SEM (n=4-7 rats). The bands above the graphs represent phosphorylated histone H3 and H3 protein from the 1.75 g/Kg samples. (The first set of bands represents 3 control, and 3 ethanol treated rats, the second set represent 2 contol and 2 ethanol treated rats). Asterisk (*) represent significant values compared to control ($p<0.05$) as analyzed by the student t test. Double asterisk (**) represent significant changes compared to control ($p<0.01$).

FIG.4

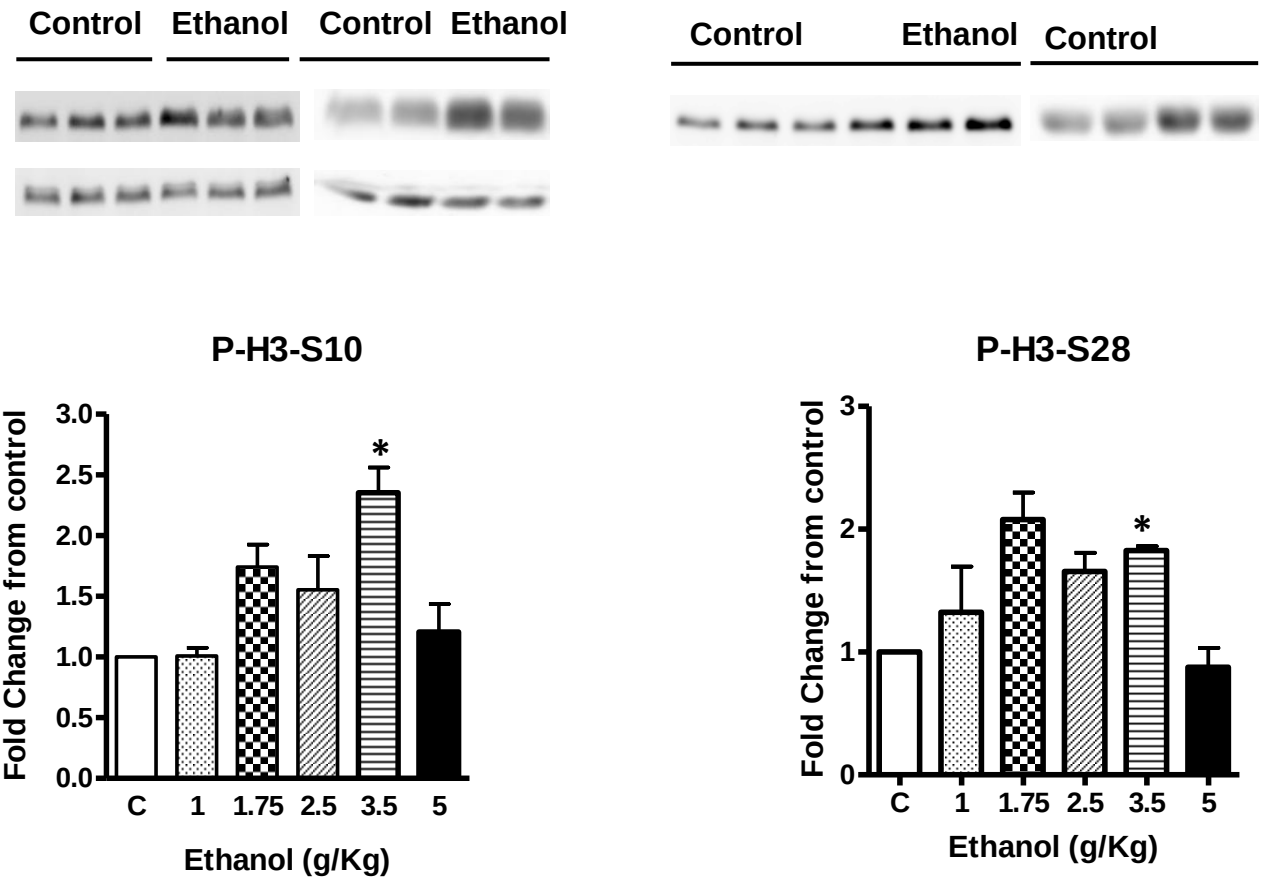

Control
Ethanol
Control
Ethanol
Control
Ethanol
Control
P-H3-S10
Fold Change from control
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
C
1
1.75
2.5
3.5
5
Ethanol (g/Kg)
P-H3-S28
Fold Change from control
3
2
1
0
*
C
1
1.75
2.5
3.5
5
Ethanol (g/Kg)

Fig. 5. Time dependent alterations in 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 & Ser-28 in nuclear extracts were determined at 1 h, 1.5h, 2h, 3h and 4h by SDS-PAGE & western blotting with site specific antibodies. For simplicity only two sets of bands representative of 4h phosphorylated histone H3 and H3 protein is shown (representing 5 control, and 4 ethanol treated rats). Values on the graphs represent mean ± SEM (n=3-5 rats). Asterisk (*) represent significant values compared to control ($p<0.05$), and double asterisk (**) represent significant values compared to control ($p<0.01$) as analyzed by the student t test.

FIG. 5

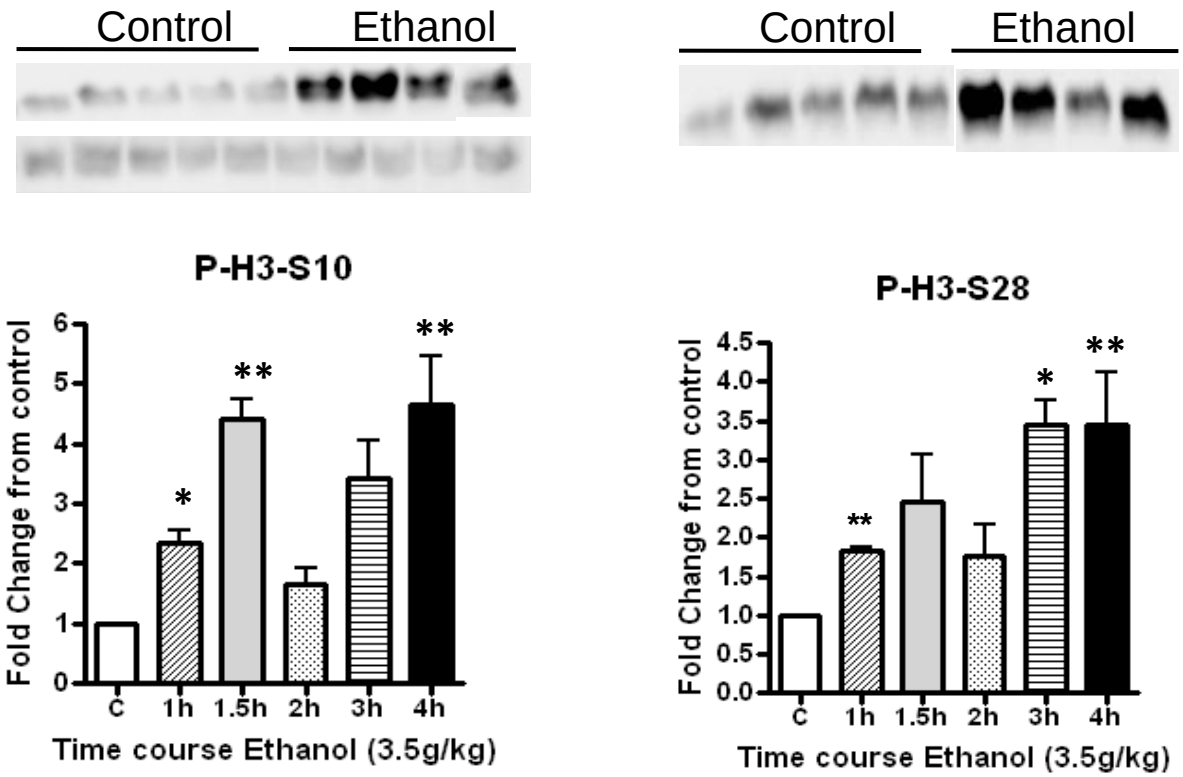
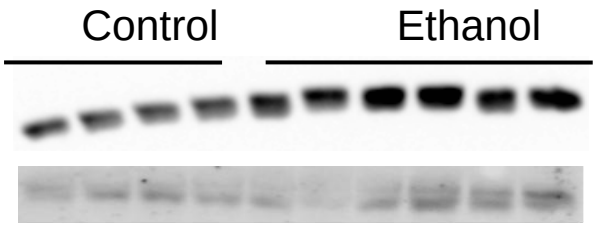
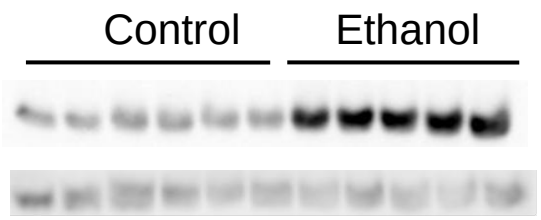
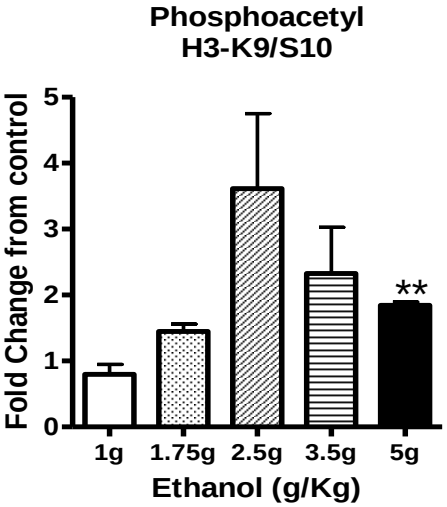

Control
Ethanol
Control
Ethanol
P-H3-S10
Fold Change from control
Time course Ethanol (3.5g/kg)
C
1h
1.5h
2h
3h
4h
P-H3-S28
Fold Change from control
Time course Ethanol (3.5g/kg)

Fig. 6. Dose and time dependent alterations in the levels of phosphoacetylated histone H3-K9/S10 after acute ethanol administration. Rats were given IP different doses (1, 1.75, 2.5, 3.5, 5 g/Kg) of ethanol. The levels of phosphoacetylated-H- K9/S10 in nuclear extracts were determined after 1 h by SDS-PAGE & western blotting with site specific antibodies. For the time course experiments rats were given IP a single dose of ethanol (3.5g/kg) and the levels phosphoacetylated H3 ser-10/lys-9 in nuclear extracts were determined at 1h, 1.5h, 2h, 3h and 4h. For simplicity only the bands (phosphoacetyl and histone H3 protein) representing 4 control and 6 ethanol treated rats of one dose (5 g/Kg, left panel) and those (6 control and 5 ethanol treated rats) of one time point (4h, right panel) are shown. Values are mean ± SEM (n=3-5 rats). Asterisk (*) represent significant values compared to control ($p < 0.05$). Double asterisk (**) represent significant values compared to control ($p < 0.01$).

FIG. 6


Control
Ethanol


Control
Ethanol


Phosphoacetyl
H3-K9/S10
Fold Change from control
0
1
2
3
4
5
**
1g
1.75g
2.5g
3.5g
5g
Ethanol (g/Kg)

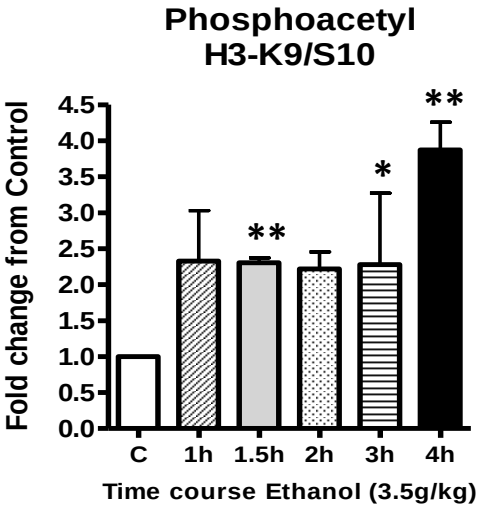

Phosphoacetyl
H3-K9/S10
Fold change from Control
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
**
*
**
C
1h
1.5h
2h
3h
4h
Time course Ethanol (3.5g/kg)

Fig.7. Dose and time dependent alterations in levels of acetylated histone H3-K9 after acute ethanol administration. Rats were given IP different doses (1, 1.75, 2.5, 3.5, 5 g/Kg) of ethanol. The levels of acetylated-H-K9 in nuclear extracts were determined after 1 h by SDS-PAGE & western blotting with site specific antibodies. For the time course experiments rats were given IP a single dose of ethanol 3.5g/Kg and the levels acetylated H3-K9 in nuclear extracts were determined at 1h,1.5h, 2h, 3h and 4h. For clarity only the bands of one dose representing 4 control and 4 ethanol treated rats (2.5 g/Kg, left panel) and those of one time point representing 3 control and 3 ethanol treated rats (1.5h, right panel) are shown. Values are mean ± SEM (n=3-5 rats). Asterisk (*) represent significant values compared to control ($p<0.05$). Double asterisk (**) represent significant values compared to control ($p<0.01$).

FIG. 7

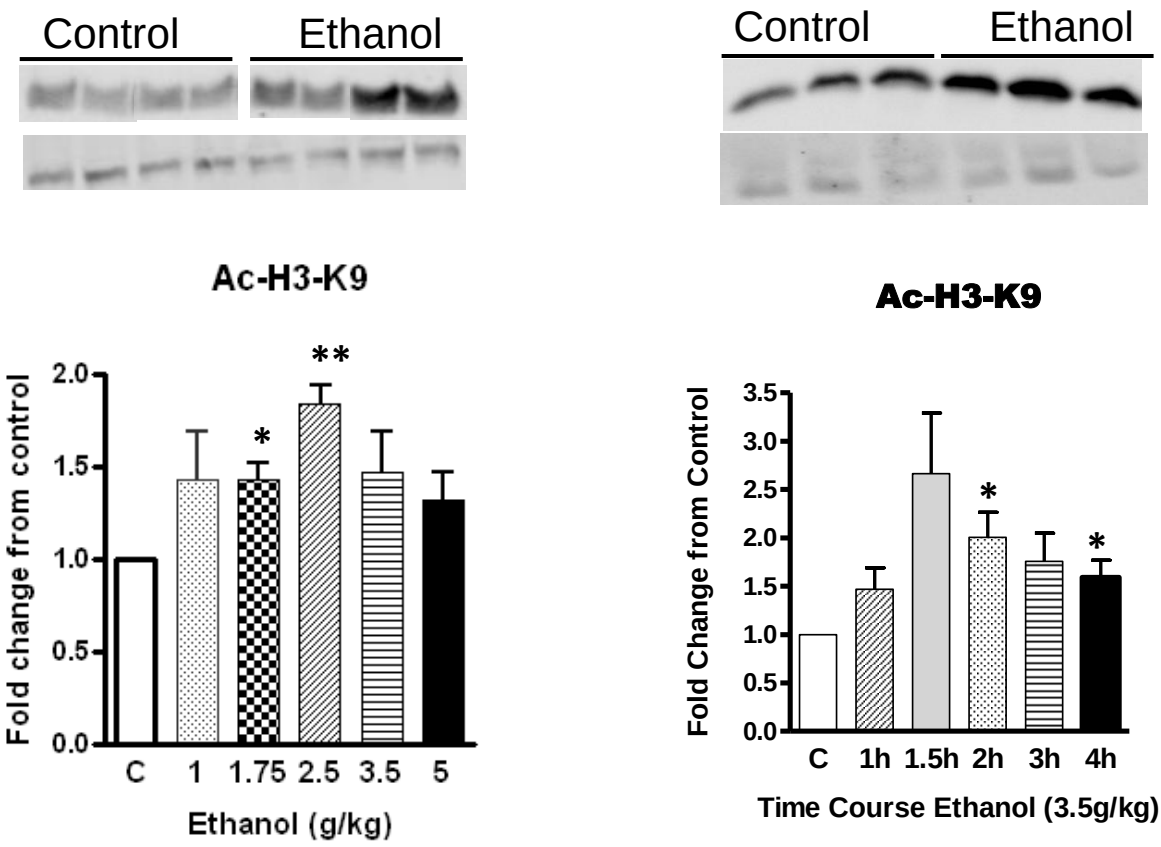

Control
Ethanol
Control
Ethanol
Ac-H3-K9
Fold change from control
2.0
1.5
1.0
0.5
0.0
**
*
C
1
1.75
2.5
3.5
5
Ethanol (g/kg)
Ac-H3-K9
Fold Change from Control
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
C
1h
1.5h
2h
3h
4h
Time Course Ethanol (3.5g/kg)

Fig. 8. The effect of different doses of ethanol on mRNA expression after 1h of acute ethanol administration. Before assaying for gene expression, we first determined the levels of phosphorylated histone H3 Ser-10 and Ser-28. Based on the results, we analyzed gene expression in liver samples from animals that received 1.75g, 3.5g, and 5g/Kg ethanol. RNA was isolated and reverse transcribed as described in materials and methods. qRT-PCR was performed using specific primers listed in Table 1 in 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 were determined using the ΔΔComparative C_t method and were normalized to the Gapdh values**.** Values are mean ± SEM (n=6-7 rats). Significance was analyzed using paired student's t test and asterisk (*) represent significant values compared to control ($p<0.05$) and the triple asterisk (***) represents significance ($p < 0.001$).

FIG. 8

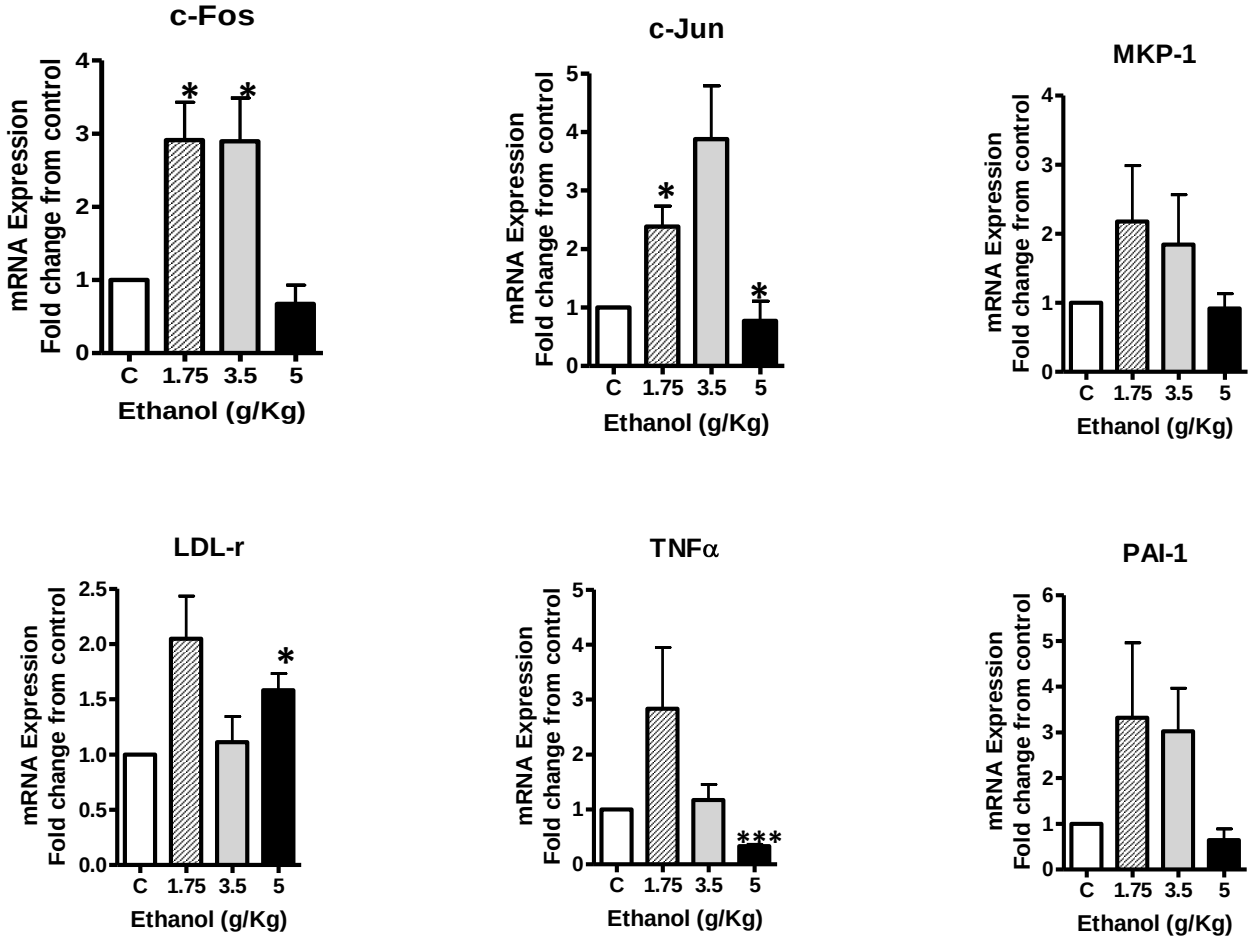

c-Fos
mRNA Expression
Fold change from control
0
1
2
3
4
*
*
C
1.75
3.5
5
Ethanol (g/Kg)
c-Jun
mRNA Expression
Fold change from control
0
1
2
3
4
5
*
*
C
1.75
3.5
5
Ethanol (g/Kg)
MKP-1
mRNA Expression
Fold change from control
0
1
2
3
4
C
1.75
3.5
5
Ethanol (g/Kg)
LDL-r
mRNA Expression
Fold change from control
0.0
0.5
1.0
1.5
2.0
2.5
*
C
1.75
3.5
5
Ethanol (g/Kg)
TNFα
mRNA Expression
Fold change from control
0
1
2
3
4
5
***
C
1.75
3.5
5
Ethanol (g/Kg)
PAI-1
mRNA Expression
Fold change from control
0
1
2
3
4
5
6
C
1.75
3.5
5
Ethanol (g/Kg)

Fig. 9. Changes in mRNA expression 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 1.5h, 2h, 3h and 4h. RNA was isolated and reverse transcribed as described in materials and methods. qRT-PCR was performed in a Bio-Rad iCycler 5 using specific primers listed in Table 1 and were done in triplicate. The average C_t values were used to calculate fold changes in gene expression were determined using the ΔΔCt method and were normalized to the Gapdh values**.** Values are mean ± SEM (n=3-5 rats) Asterisk (*) represent significant values compared to control ($p<0.05$).

FIG. 9

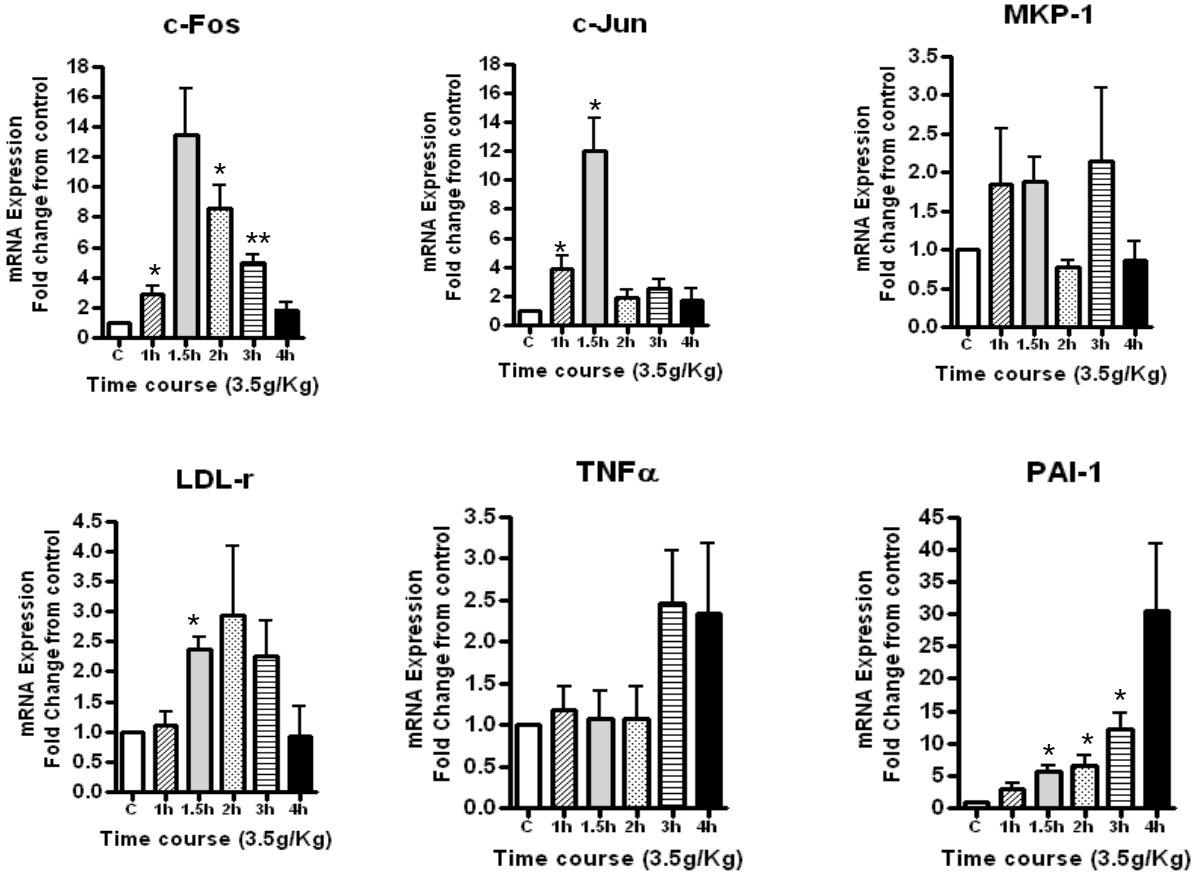

c-Fos
mRNA Expression
Fold change from control
Time course (3.5g/Kg)
c-Jun
mRNA Expression
Fold change from control
Time course (3.5g/Kg)
MKP-1
mRNA Expression
Fold change from control
Time course (3.5g/Kg)
LDL-r
mRNA Expression
Fold Change from control
Time course (3.5g/Kg)
TNFα
mRNA Expression
Fold Change from control
Time course (3.5g/Kg)
PAI-1
mRNA Expression
Fold Change from control
Time course (3.5g/Kg)
C 1h 1.5h 2h 3h 4h

Fig. 10. ChIP Assay with Phospho-specific antibodies for P-H3-S10 and P-H3-S28, and phosphoacetyl antibody for phospacetyl-H3-K9/S10 and analysis of gene expression. Frozen liver from the 1.5h, and frozen nuclei from 4h time course samples, where maximum fold change in histone H3 phosphorylation at Ser-10 and Ser-28 occurred, were used for ChIP assays. Antibodies to phosphorylated histone H3-Ser-10, phosphorylated histone H3-Ser-28, phosphoacetylated histone H3-K9/S10, or RNA polymerase II (RNA Pol II), were used for the ChIP. Normal mouse IgG and normal rabbit IgG were used as controls for the ChIP procedure. The association of histone phosphorylation with gene promoters was determined using specific primers in Table 2. Values are mean ± SEM (n=3 rats). Asterisk (*) represent significant values compared to control ($p<0.05$).

FIGURE 10

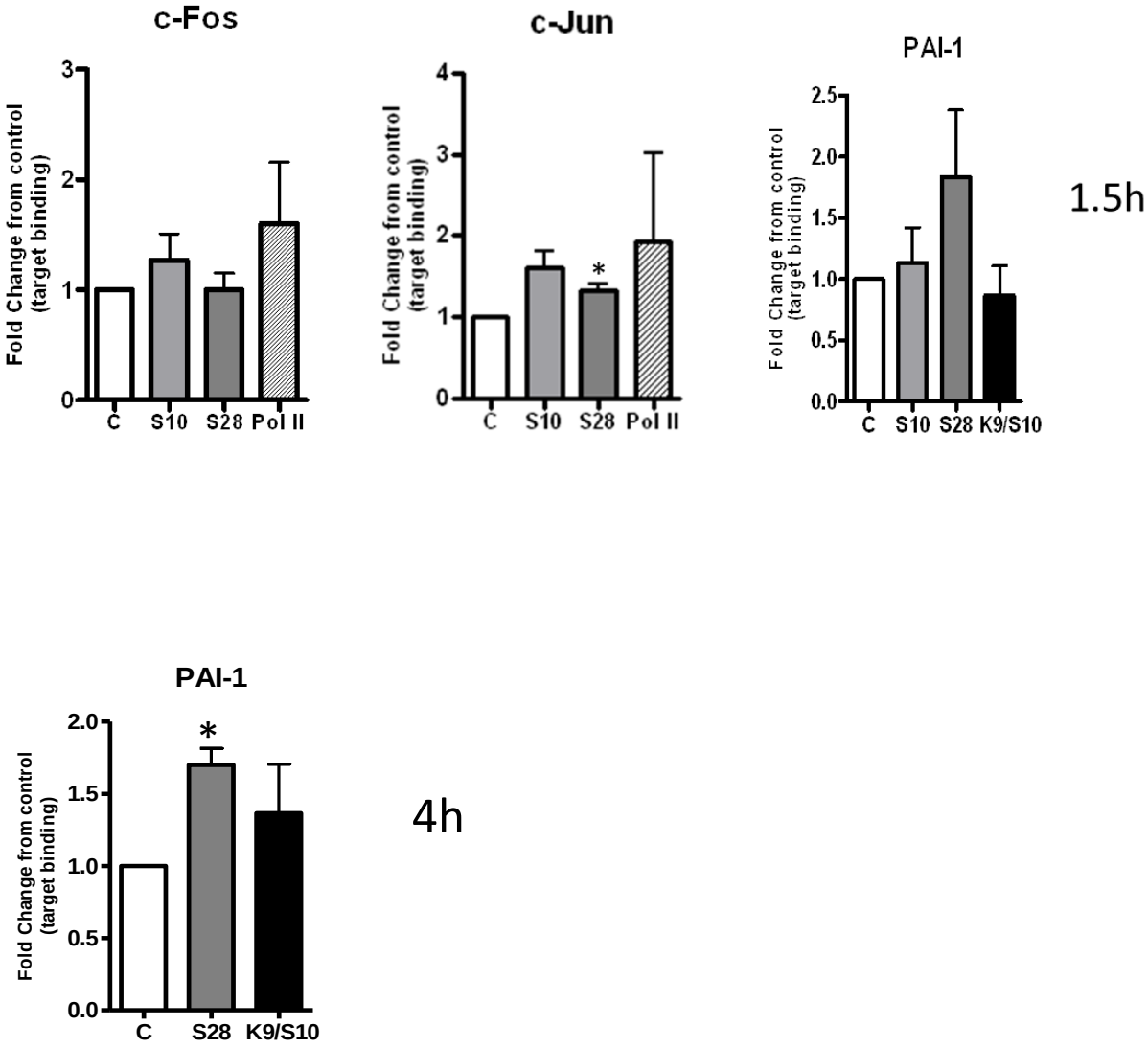
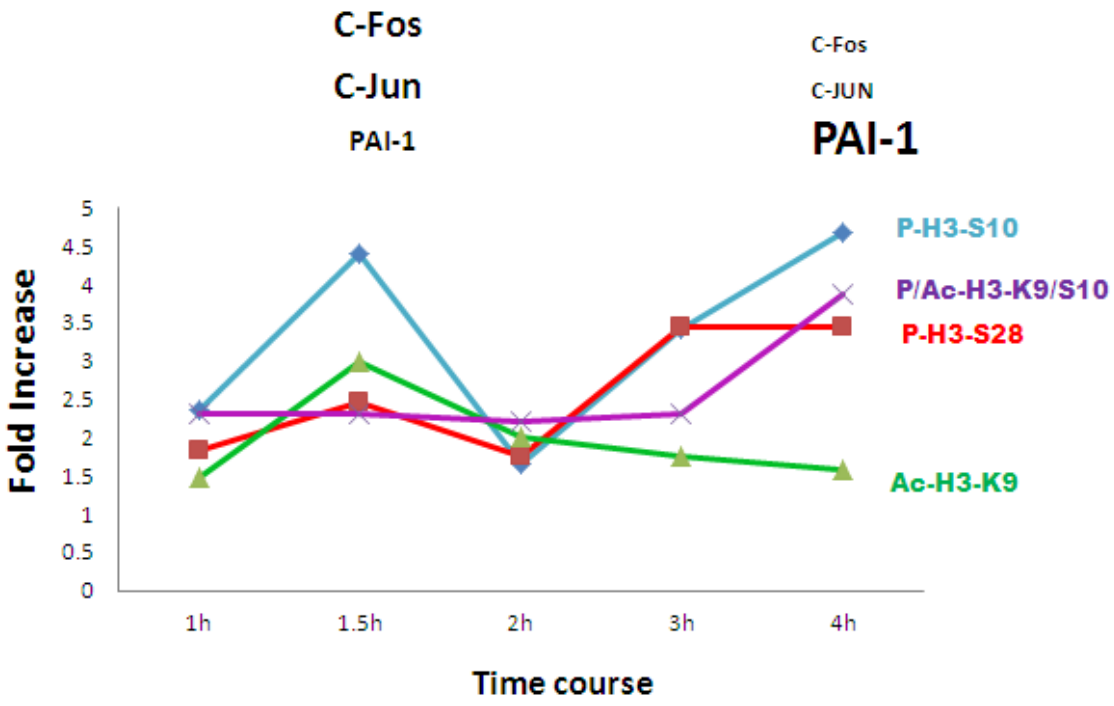

c-Fos
Fold Change from control (target binding)
3
2
1
0
C
S10
S28
Pol II
c-Jun
Fold Change from control (target binding)
4
3
2
1
0
*
C
S10
S28
Pol II
PAI-1
Fold Change from control (target binding)
2.5
2.0
1.5
1.0
0.5
0.0
C
S10
S28
K9/S10
1.5h
PAI-1
Fold Change from control (target binding)
2.0
1.5
1.0
0.5
0.0
*
C
S28
K9/S10
4h

Fig. 11. Summary of modified histone profile and gene expression after acute binge administration of ethanol in vivo. Using the values of average fold changes we created a schematic diagram to compare the modifications of histone H3 highlighting the key findings in this chapter. The size of the fonts are meant to indicate the levels of gene expression.

FIG. 11


C-Fos
C-Jun
PAI-1
C-Fos
C-JUN
PAI-1
Fold Increase
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
1h
1.5h
2h
3h
4h
Time course
P-H3-S10
P/Ac-H3-K9/S10
P-H3-S28
Ac-H3-K9

Chapter V

Miscellaneous Data

The effect of acute in vivo IP administration of ethanol on histone H3 acetylation at lysine 14 and phosphoacetylation at serine 10/lysine 14

Objective: Although we have never observed acetylation at lysine 14 in vitro (Park et al, 2005), we do not know if the same will be true in vivo. Therefore we wanted to first determine if acetylation of lysine 14 occurs in vivo after acute IP ethanol.

We previously observed that ethanol can induce phosphoacetylation of histone H3 at lysine 9 and serine 10 (see chapter 4). When phosphoacetylation is investigated in the literature it is mostly phosphoacetylation at S10/K14. Therefore we wanted to determine acetylation of lysine 14 in vivo after acute IP. Phosphoacetylation at S10/K14 has been shown to regulate the expression of early response genes c-Fos and c-Jun (Clayton et al, 2000). After acute IP administration of ethanol there was an induction of c-Fos, c-Jun as well as another IE gene, MKP-1 (see chapter 4). Therefore phosphoacetylation at S10/K14 may play a role. In this context, we present here an investigation of histone acetylation at lysine 14, and phosphoacetylation in an acute in vivo model of ethanol injury.

Methods: See chapter 4. Lysine 14 acetylation was analyzed at 1.5h. Phosphoacetylation was analyzed at 1.5 and 4h.

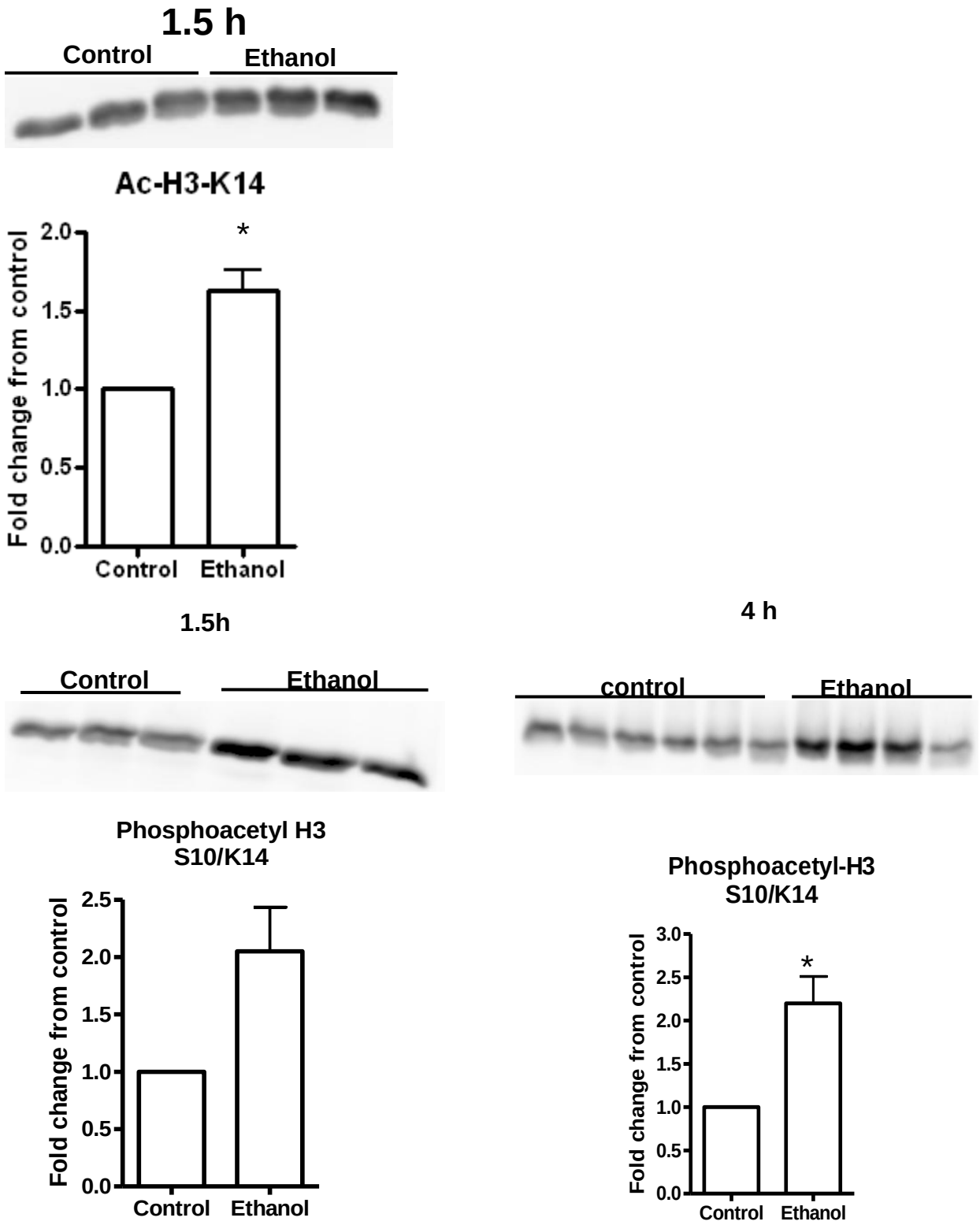
Results: Unlike in primary cultures of hepatocytes, ethanol significantly increased lysine 14 acetylation in vivo after acute IP. Ethanol also significantly increased phosphoacetylation of serine 10/lysine 14 after acute IP at 1.5 and 4h.

Conclusions: The increase in histone H3 ysine 14 acetylation may be involved in gene expression after ethanol. Because phosphoacetylation of S10/K14 has been previously shown to be involved in increased expression of c-Fos (Clayton et al, 2000), the role of phosphoacetylation of serine 10/lysine 14 on gene expression should be investigated in the future. Perhaps the robust change in c-Fos expression at 1.5h could be due to many different modifications of histone H3.

Fig 1. Acetylation of lysine 14 and Phosphoacetylation of Histone H3 at serine 10/lysine 14 after ethanol IP administration of ethanol.

Rats were injected IP with a fixed dose of (3.5g/Kg) ethanol. The liver was extracted at different timepoints as described in Chapter IV. We analyzed phosphoacetyl histone H3-S10/K14 at 1.5h and 4h since these were the timepoints that we chose for our chromatin immunoprecipitation assay experiments. The bands for the 1.5 h samples represent 3 control rats, and 3 ethanol rats. The bands for the 4h samples represent 6 control, and 5 ethanol treated rats.

FIG. 1


1.5 h
Control
Ethanol
Ac-H3-K14
Fold change from control
2.0
1.5
1.0
0.5
0.0
*
Control
Ethanol
1.5h
Control
Ethanol
4 h
control
Ethanol
Phosphoacetyl H3
S10/K14
Fold change from control
2.5
2.0
1.5
1.0
0.5
0.0
Control
Ethanol
Phosphoacetyl-H3
S10/K14
Fold change from control
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Control
Ethanol

The effect of Acute IP Ethanol Administration on histone H3 Phosphorylation at threonine 3 and threonine 11

Objective: We have shown in Chapter 3 and 4 that ethanol induces phosphorylation at serine 10 and serine 28 of H3. Does phosphorylation occur at other residues of H3? Threonine 3 and threonine 11 are the other two well known phosphorylated sites on histone H3 and are known to function in mitosis (Takashi, 2007). It was recently shown that phosphorylation of threonine 11 was a novel mark for transcriptional regulation (Metzger et al, 2007).

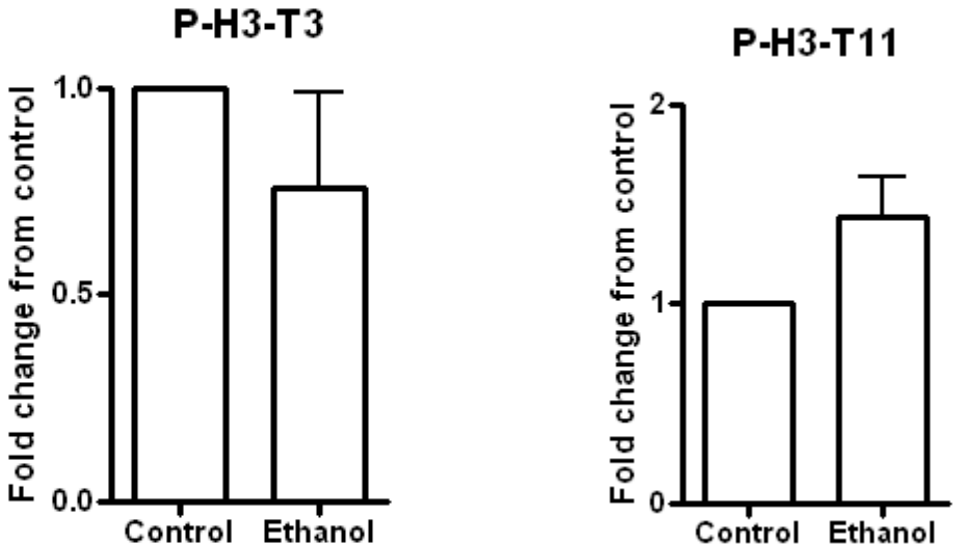
Methods: See Chapter 4. Phosphorylation of threonine 3 and 11 was analyzed in 3.5 g/kg 1h samples.

Results: Ethanol did not significantly alter the phosphorylation of threonine 3 and 11after a 1h treatment of 3.5g/Kg. The phosphorylation of threonine 3 was marginally increased while the phosphorylation of threonine 11 marginally increased.

Conclusion: Although phosphorylation did not significantly alter in the dose and time selected it is possible that ethanol may still alter threonine 3 and 11 phosphorylation. Other doses and times should be analyzed. This was only a preliminary study.

Figure. 2. Phosphorylation of histone H3 at threonine 3 and 11 in 3.5 g/Kg samples. Rats were given IP 3.5 g/Kg ethanol and the liver was extracted after 1h. Levels of phosphorylated histone H3 at threonine 3 and threonine 11 in nuclear extracts were determined by SDS-PAGE & western blotting with a site specific antibody. Values are mean ± SEM (n=4-7 rats) and the asterisk represents significant as compared to control ($p<0.05$).

FIG. 2


P-H3-T3
Fold change from control
1.0
0.5
0.0
Control
Ethanol
P-H3-T11
Fold change from control
2
1
0
Control
Ethanol

The effect of Acute IP Ethanol Administration on histone H3 Methylation

Objective: We have determined histone phosphorylation and acetylation after acute IP. Since methylation is known to crosstalk with phosphorylation. We next determine histone methylation after acute IP.

Methods: See Chapter 4. Dimethlylation of histone H3 at lysine, and trimethylation of histone H3 at lysine 9 was determined.

Results

Dose Response

There was little effect of dose on dimethylation of histone H3 at K4 or at K9. However at 2.5g/Kg there was an obvious increase in dimethylation at K4.

Time Course

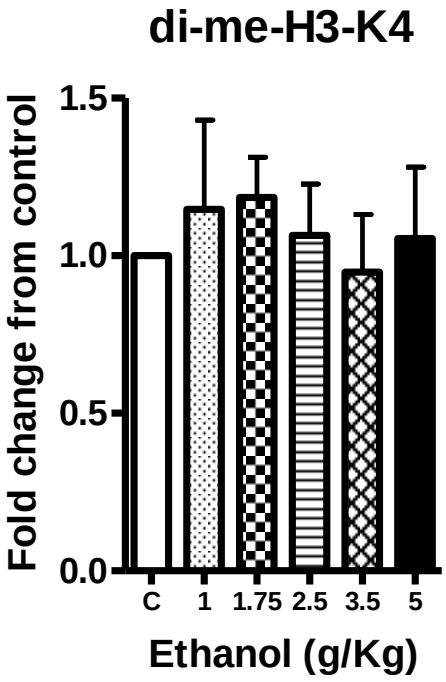
Dimethylation at K4 and K9 did not change with time. However at 4h the trimethylaion of histone H3 at K9 was increased as compared to control.

Conclusion:

Figure. 3. Dimethylation of histone H3 at K4 after ethanol administration in vivo. A. Dose dependent levels of dimethylated Histone H3 K4 after acute ethanol administration at 1h Rats were given IP different doses of ethanol (1, 1.75, 2.5, 3.5, 5g/kg) and the liver was extracted after 1h. Levels of dimethylated H3 lysine 9 in nuclear extracts were determined by SDS-PAGE & western blotting with a site specific antibody. Values are mean ± SEM (n=4-7 rats). **B. Time dependent alterations in levels of dimethylated Histone H3 K4 after acute ethanol administration.** Rats were given IP a single dose of ethanol 3.5g/kg and the levels of dimethylated H3 lysine 4 in nuclear extracts were determined at 1 hr, 1.5h, 2h, 3h and 4hrs by SDS-PAGE & western blotting with site specific antibodies. Values are mean ± SEM (n=3-5 rats).

FIG. 3

A


di-me-H3-K4
Fold change from control
1.5
1.0
0.5
0.0
C
1
1.75
2.5
3.5
5
Ethanol (g/Kg)

B

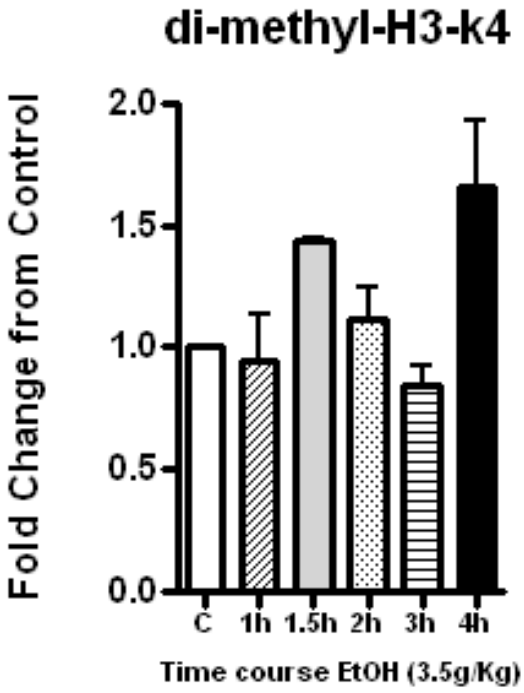
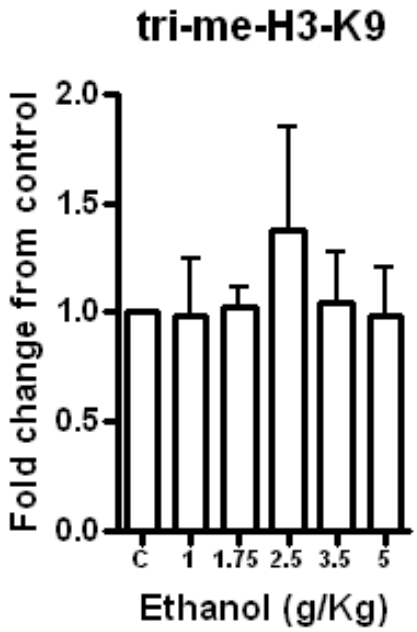
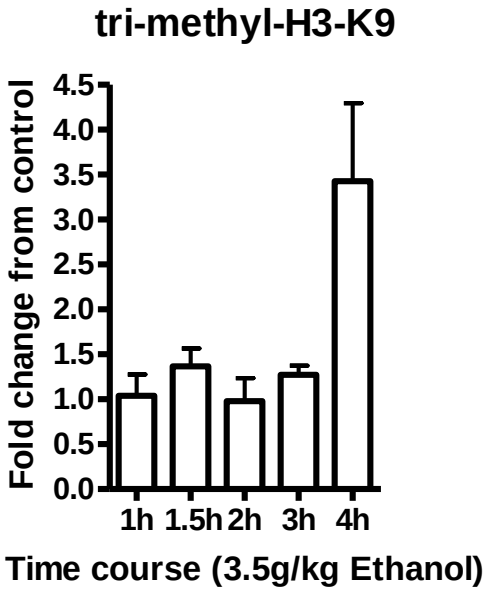

di-methyl-H3-k4
Fold Change from Control
2.0
1.5
1.0
0.5
0.0
C
1h
1.5h
2h
3h
4h
Time course EtOH (3.5g/Kg)

Figure. 4. Trimethylation of histone H3 at K9 after ethanol administration in vivo. A. Dose dependent levels of trimethylated Histone H3 K9 after acute ethanol administration at 1h Rats were given IP different doses of ethanol (1, 1.75, 2.5, 3.5, 5g/kg) and the liver was extracted after 1h. Levels of trimethylated H3 lysine 9 in nuclear extracts were determined by SDS-PAGE & western blotting with a site specific antibody. Values are mean ± SEM (n=4-7 rats). **B. Time dependent alterations in levels of trimethylated Histone H3 K9 after acute ethanol administration.** Rats were given IP a single dose of ethanol 3.5g/kg and the levels of trimethylated H3 lysine 9 in nuclear extracts were determined at 1 hr, 1.5h, 2h, 3h and 4h by SDS-PAGE & western blotting with site specific antibodies. Values are mean ± SE (n=3-5 rats).

FIG. 4


tri-me-H3-K9
Fold change from control
2.0
1.5
1.0
0.5
0.0
C
1
1.75
2.5
3.5
5
Ethanol (g/Kg)


tri-methyl-H3-K9
Fold change from control
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1h
1.5h
2h
3h
4h
Time course (3.5g/kg Ethanol)

Histone Modifications after Acetate IP Administration in Vivo in Rats

Objective: To determine if acetate can cause any epigenetic changes on histone H3 in vivo which would suggest a role for metabolism in the effects of ethanol on histone H3 modifications in vivo.

Methods

Acetate Administration

Based on a study done by Carmichael et al, 1991, an administration of 1g/Kg or 2g/Kg acetate gave a serum acetate concentration that is equivalent to the addition of 1 to 4g/kg ethanol in rats after 30 minutes. Therefore to determine if a metabolite of ethanol can lead to epigenetic modifications of histones, we injected 12 week old rats intraperitoneally with water, 1g or 2g of acetate (trihydate) as a 20% solution. The liver was extracted after 1.5h. We chose 1.5h to extract the liver because phosphorylation was highest at this timepoint (see chapter IV). Liver was then snapped frozen in liquid nitrogen and stored at -80 ^{o}C until further analysis.

Preparation of Nuclear Extracts and Immunoblot Analysis

Nuclear protein extracts and inmmunoblot analysis were obtained according to the methods detailed in chapter IV.

Data analysis

All results are expressed as mean ± SEM. Graph Pad PRISM (version 4) software was used for statistical analysis using the Student *t* test (two-tailed, unpaired). Differences with a *P* value of <0.05 were determined to be statistically significant.

Results: Acetate did not increase histone acetylation. However 2g/Kg acetate increased phosphorylation of histone H3 at serine 10 and serine 28. There was also no change in phosphoacetylation after acetate. We also observed no change in dimethylated H3 at K4, dimethylated H3 at K9, and trimethylated histone H3 at K9.

Conclusions: At the dosage used in this study acetate increased phosphorylation of histone H3 at serine 10 and 28 suggesting that ethanol metabolism may be implicated for its effects in vivo. Interestingly, we saw no change in acetylation. This study is preliminary and a dose response and timecourse (starting with 30 mins) should be done to determine the effect of acetate on histone H3 modifications in vivo.

Figure. 5. Phosphorylation, phosphoacetylation, and acetylation of histone H3 in vivo after Acetate Administration. A. Representative western blots for epigenetic modifications after acetate administration at 1.5h Rats were given IP water (control) or different doses of acetate (1 or 2g/kg) and the liver was extracted after 1.5h. Levels of histone H3 protein, acetylation at K9, phosphoacetylation at K9/S10 and phosphorylation at serine 10 and 28 in nuclear extracts were determined by SDS-PAGE & western blotting with a site specific antibody. Values are mean ± SEM (n=3 or 4 rats i.e at 1g/Kg). **A.** The bands represent 3 control rats, 4 1 g/Kg acetate treated rats, and 3 2 g/Kg acetate treated rats. **B-E. Graphs representing the Fold change in acetylation at K9, phosphoacetylation at K9/S10 and phosphorylation at serine 10 and 28 after acute acetate administration.** Rats were given water or acetate as described above. Fold increase in the modifications described above were calculated over control and was normalized to H3 protein. Values are mean ± SEM (n=3-4 rats).

FIG. 5

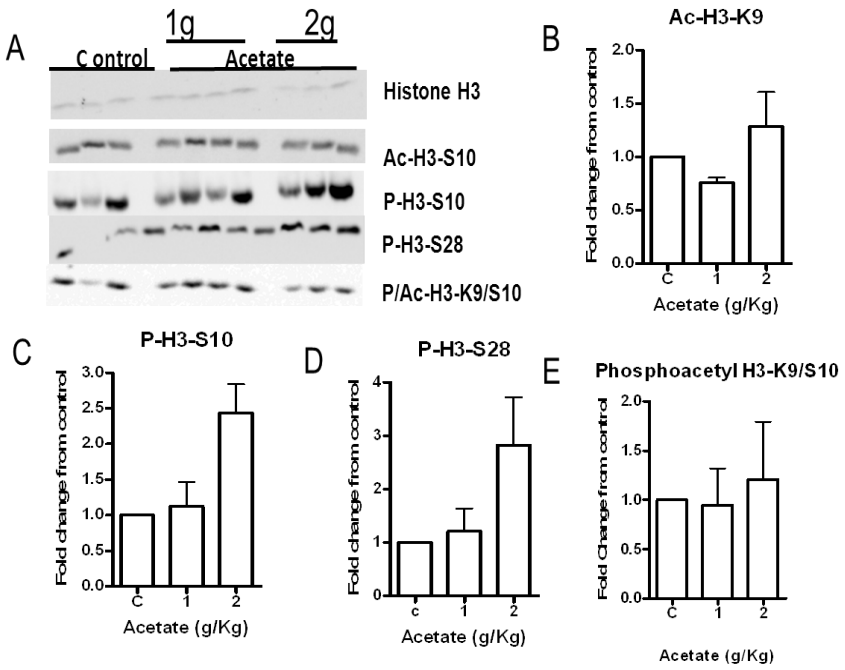
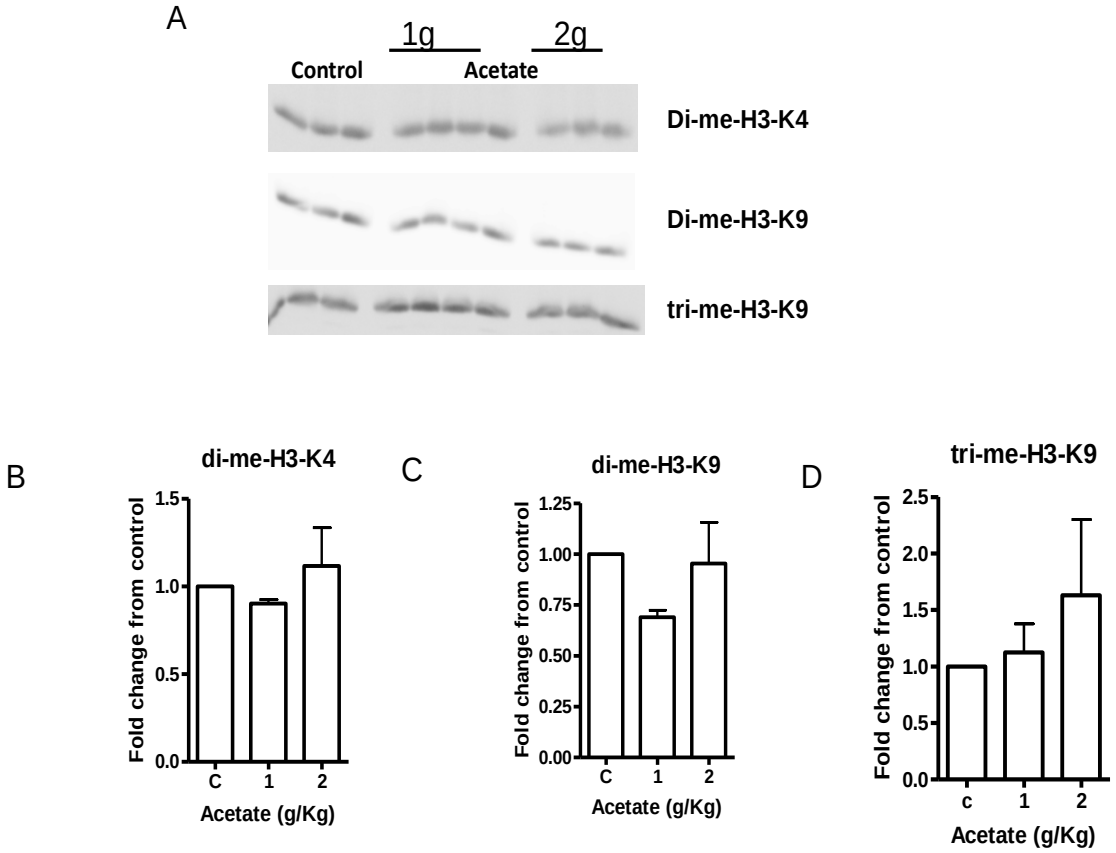

A
1g
2g
Control
Acetate
Histone H3
Ac-H3-S10
P-H3-S10
P-H3-S28
P/Ac-H3-K9/S10
B
Ac-H3-K9
Fold change from control
Acetate (g/Kg)
C
P-H3-S10
D
P-H3-S28
E
Phosphoacetyl H3-K9/S10

Figure. 6. Methylation in vivo after acetate administration. A.Representative western blots for epigenetic modifications after acetate administration at 1.5 h. Rats were given IP water (control) or different doses of acetate (1 or 2 g/kg) and the liver was extracted after 1.5h. Levels of dimethylation at K4, dimethylation at K9 and trimethylation at K9 in nuclear extracts were determined by SDS-PAGE & western blotting with a site specific antibody. Values are mean ± SEM (n = 3-4 rats). The bands represent 3 control rats, 4 (1 g/Kg) acetate treated rats, and 3 (2 g/Kg) acetate treated rats. **B-D. Graphs representing the Fold change in dimethylation at K4, dimethylation at K9 and trimethylation at K9 after acute acetate administration.** Rats were given water or acetate as described in part A above. Fold increase in the modifications described above were calculated over control. Values are mean ± SEM (n = 3-4 rats).

FIG. 6


A
1g
2g
Control
Acetate
Di-me-H3-K4
Di-me-H3-K9
tri-me-H3-K9
B
di-me-H3-K4
Fold change from control
1.5
1.0
0.5
0.0
C
1
2
Acetate (g/Kg)
C
di-me-H3-K9
Fold change from control
1.25
1.00
0.75
0.50
0.25
0.00
C
1
2
Acetate (g/Kg)
D
tri-me-H3-K9
Fold change from control
2.5
2.0
1.5
1.0
0.5
0.0
c
1
2
Acetate (g/Kg)

The effect of 200 mM Ethanol on Histone H3 phosphorylation in vitro

Objective: To determine if a high dose of ethanol can increase histone H3 phosphorylation at serine 10 and serine 28 in vitro in primary hepatocytes. We have previously shown that at 100mM can increase histone H3 phosphorylation in primary hepatocytes in vitro. Therefore we wanted to determine if histone H3 phosphorylation at serine 10 and serine 28 increases at higher ethanol levels in vitro.

Methods

Phosphorylated histone H3 at serine (06-570), phosphorylated histone H3- at serine 28 (07-145), and histone H3 antibodies (06-755) were purchased from Millipore (Temecula, CA).). Goat anti-rabbit and anti-mouse immunoglobulin G (IgG) conjugated with horseradish peroxidase (HRP) and Bio-Rad DC protein assay kit were purchased from Bio-Rad Laboratories (Hercules, CA). The ethanol used in this study was purchased from Fisher and was ≥99% pure.

Isolation and Culture of hepatocytes

Rat hepatocytes were isolated from male Sprague-Dawley rats weighing 200–250 g using an in situ collagenase perfusion protocol (Weng et al., 2000). Viability of hepatocytes was measured by trypan blue exclusion and cells used in all experiments were ≥90% viable. Isolated hepatocytes were plated on collagen-coated dishes (7.5×10^6 cells/ 100 mm dish) in high glucose DMEM containing 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. The protocols were approved by the University of Missouri Institutional Animal Care and Use Committee (IACUC).

Treatment of Cells

After plating, isolated hepatocytes were allowed to attach to culture dishes for 2 hr followed by two washes in 1X PBS, and then treated with 200mM ethanol. For each treatment, plates were done in duplicates. All culture dishes were covered with parafilm to prevent the evaporation of ethanol and cells were harvested after 24hrs.

Preparation of nuclear extract

Hepatocytes were washed three times with cold 1X PBS. Hypotonic lysis buffer containing 20 mM HEPES, 1mM EDTA, 10 mM NaCl, 1 mM DTT, 1 mM sodium orthovanadate, 2 mM $MgCl_2$, 20 mM glycerophosphate and 1 mM PMSF with 10 μg/ml of leupeptin, aprotinin, and pepstatin A was added to culture dishes and cells were scraped and then transferred to a 1.5 ml eppendorf tube. Cells from the duplicate dishes were combined into 1 tube. Next 0.25% NP-40 was added and samples were vortexed for 10 s. Nuclei were pelleted by centrifugation at 14,000 g for 20 s and then washed in the same buffer above. Proteins were extracted from nuclei by resuspended in 500 μl of 0.4N HCL/ containing 10 % glycerol and the pellet was dissolved with a pipet, vortexed and incubated with shaking at 30min in a 4 ^{o}C refrigerator. The samples were then centrifuged at 12, 000 g for 10min. Trichloroacetic Acid (TCA) was added to a final concentration of 24% and samples were incubated on ice for 1hr. After centrifugation at 12, 000 g for 10 min, the pellet was washed with acetone containing 0.02N HCl. Samples were pelleted by centrifugation at 12, 000g for 5 min at 4^{o}C. The supernatant was removed and the pellet was washed with pure acetone, centrifuged at

12, 000 g for 5 min at 4^{o}C, and dried under the hood for 30 min. The pellet was then resuspended in water. Proteins were estimated and 20 μg of protein was used for gel electrophoresis.

Western blot analysis

Equal amounts (20 μg) of nuclear extracts were run on 15% SDS–PAGE and transferred onto nitrocellulose membrane. Membranes were blocked with 5% nonfat dried milk in water for 1.5 hr, membranes were then incubated overnight with primary antibody with the following dilutions 1: 2000 for anti-H3-S10, and 1: 1000 for anti-H3 Ser 28 overnight at 4 ^{o}C, and 1: 65 000 for beta-actin. Membranes were incubated with horseradish-conjugated secondary antibody (both anti-mouse and anti-rabbit) with the dilution of 1: 3000 for 1 hr at room temperature. The horseradish peroxidase was detected by enhanced chemiluminescence (Supersignal, Pierce) and exposed to film. The bands were analyzed laser densitometry using Bio-Rad Quantity One Software.

Statistical analysis

Data were combined from 3 separate experiments. Statistical analyses was performed using a paired two-tailed t-test. Differences with $p< 0.05$ considered significant and is denoted by an asterisk (*).

Results

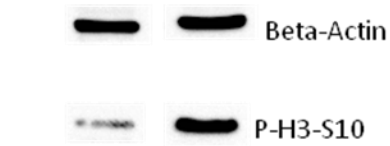
At a high dose of 200mM ethanol increased histone H3 phosphorylation at serine 10 and serine 28 in vitro. However, phosphorylation at serine 10 was more sensitive to 200mM ethanol than phosphorylation at serine 28. This is consistent with our vivo data demonstrating that serine 28 phosphorylation is more responsive at low doses of ethanol. This is also lends support to our finding in vivo that serine 10 phosphorylation is more rapid than serine 28 phosphorylation.

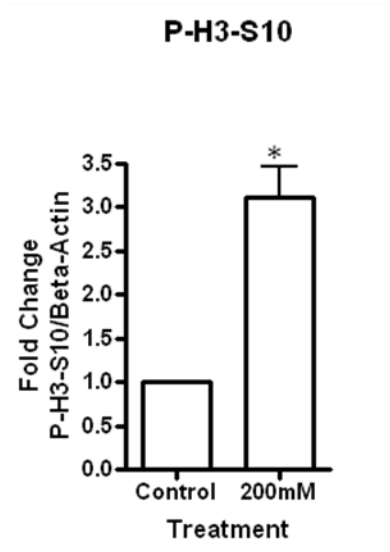
Conclusions

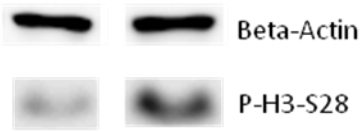
Our ultimate goal is to determine the effects of histone H3 phosphorylation on gene expression after ethanol. While we were able to see a significant change in phosphorylation with a 200mM dosage of ethanol, we notice that a lot of cells also detaches from the plate during washing. Therefore we decided that it was a good idea to study the role of histone H3 phosphoryltion on gene expression in vivo in an acute binge model (see previous chapters).

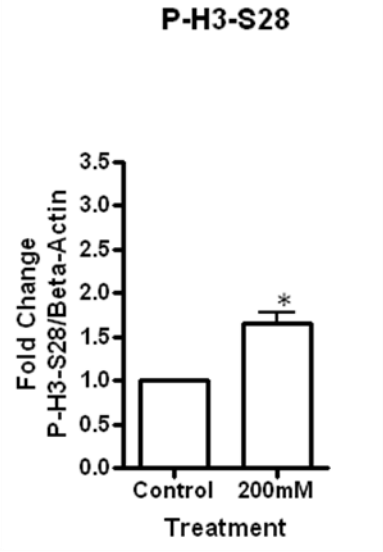
Fig 7. Effect of 200 mM Ethanol on Histone H3 phosphorylation in vitro at ser-10 and ser-28 in rat primary hepatocytes. Hepatocytes were cultured with or without 200mM ethanol for 24hrs. Nuclear extracts were prepared and used for western blot analysis using anti-phospho-histone H3-S10 and anti-phospho-histone H3-S28. Data shown are representative of 3 separate experiments. Quantitative analysis of phosphorylated histone H3 at serine 10 and serine 28 were performed by densometric analysis and is represented as a bar graph. The phosphorylation was normalized to the levels of beta-actin. Data are presented as means ± SEM. n = 3. Values represent fold increase over control group (control =1). Asterisk (*) represent P-values compared with the control group and was determined using the student t test, paired (two-tailed).

FIG . 7.


Beta-Actin
P-H3-S10


P-H3-S10
Fold Change
P-H3-S10/Beta-Actin
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Control
200mM
Treatment


Beta-Actin
P-H3-S28


P-H3-S28
Fold Change
P-H3-S28/Beta-Actin
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Control
200mM
Treatment

The effect of TSA on histone H3 phosphorylation

Objective: TSA treatment was done as a positive control for acetylation in antioxidant experiments. However when we ran the blots for posphorylated histone H3 we included this sample.

Methods: isolation, plating, culture, and isolation of hepatocytes are the same as described for figure 7 above.

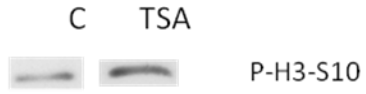
Results: TSA increases both histone H3 phosphorylation at serine 10 and serine 28. The response to TSA was higher for serine 10 as compared to serine 28.

Conclusion: The fact that TSA induced histone H3 phosphrylation at serine 10 and serine 28 suggests that HDAC inhibition can increase histone H3 phosphorylation. Could this be a mechanism for ethanol induced histone H3 phosphorylation? Since HDAC inhibition increases histone acetylation, do an increase in acetylation lead to an increase histone H3 phosphorylation?

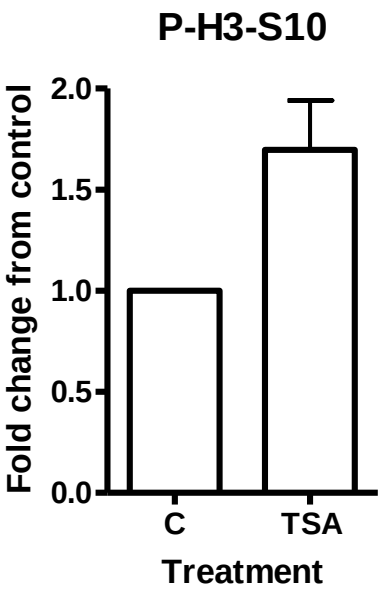
Fig. 8. Effect of TSA on histone H3 phosphorylation in rat primary hepatocytes.

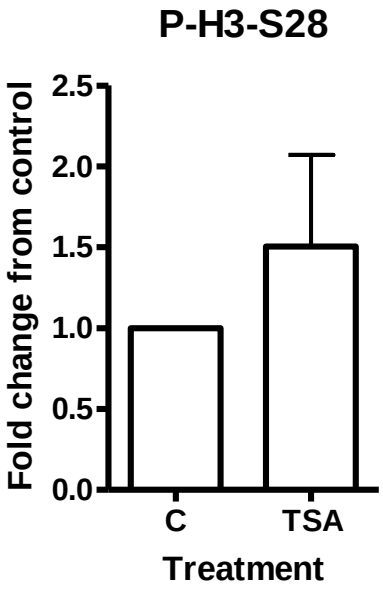
Hepatocytes were cultured with or without 2 μg/ml TSA for 24hrs. Nuclear extracts were prepared and used for western blot analysis using anti-phospho-histone H3-S10 and anti-phospho-histone H3-S28. Data shown are representative of 3 separate experiments. Quantitative analysis of phosphorylated histone H3 at serine 10 and serine 28 were performed by densometric analysis and is represented as a bar graph. The phosphorylation was normalized to the levels of beta-actin. Data are presented as means ± SEM (n = 3). Values represent fold increase over control group (control =1). Significance was analyzed using the paired two-tailed student t test.

FIG .8.


C
TSA
P-H3-S10


C
TSA
P-H3-S28


P-H3-S10
Fold change from control
2.0
1.5
1.0
0.5
0.0
C
TSA
Treatment


P-H3-S28
Fold change from control
2.5
2.0
1.5
1.0
0.5
0.0
C
TSA
Treatment

The Effect of Ethanol on Histone H3 Phosphorylation in VA-13 Cells

Objective: We have previously shown in primary hepatocytes that ethanol can increase histone H3 phosphorylation at serine 10 and serine 28. We also previously showed that a metabolite of ethanol, acetaldehyde is also implicated in histone H3 phosphorylation by ethanol (Lee and Shukla, 2007). In chronic alcoholics there is a loss of function of ADH. VA-13 is a clonal derivative of human hepatoma HepG2 cells that were stably transfected with a eucaryotic expression plasmid containing a cDNA copy of murine alcohol dehydrogenase 1 (*ADH1*) (Clemens et al, 1995; Clemens et al., 2002; Donohue et al, 2006). Therefore these cells can aid in determining if ethanol metabolism is a factor in its actions on histones.

Methods: Cells were grown and cultured according to our previous methods (Choudhury et al, 2011). For experiments, cells were plated at a density of 10×10^6 cells/ 100 mm dish in high glucose DMEM supplemented with 10% FBS, 1 mM sodium pyruvate, 4 mM L-glutamine, 50 units/ml penicillin and 50 μg/ml streptomycin and 400 μg/ml zeocin. Cells were allowed to grow for 2 days before treatment. Cells were treated with 100 mM ethanol for 24 hours. After treatment, cells were washed three times in 1X PBS and then scraped in 1X PBS containing protease inhibitors **(**0.1M Na_3VO_4, 20 mM glycerophosphate and 1 mM PMSF with 10 μg/ml of leupeptin, aprotinin, pepstatin A and 1mM DTT**)** and transferred to 1.5ml eppendorf tubes.

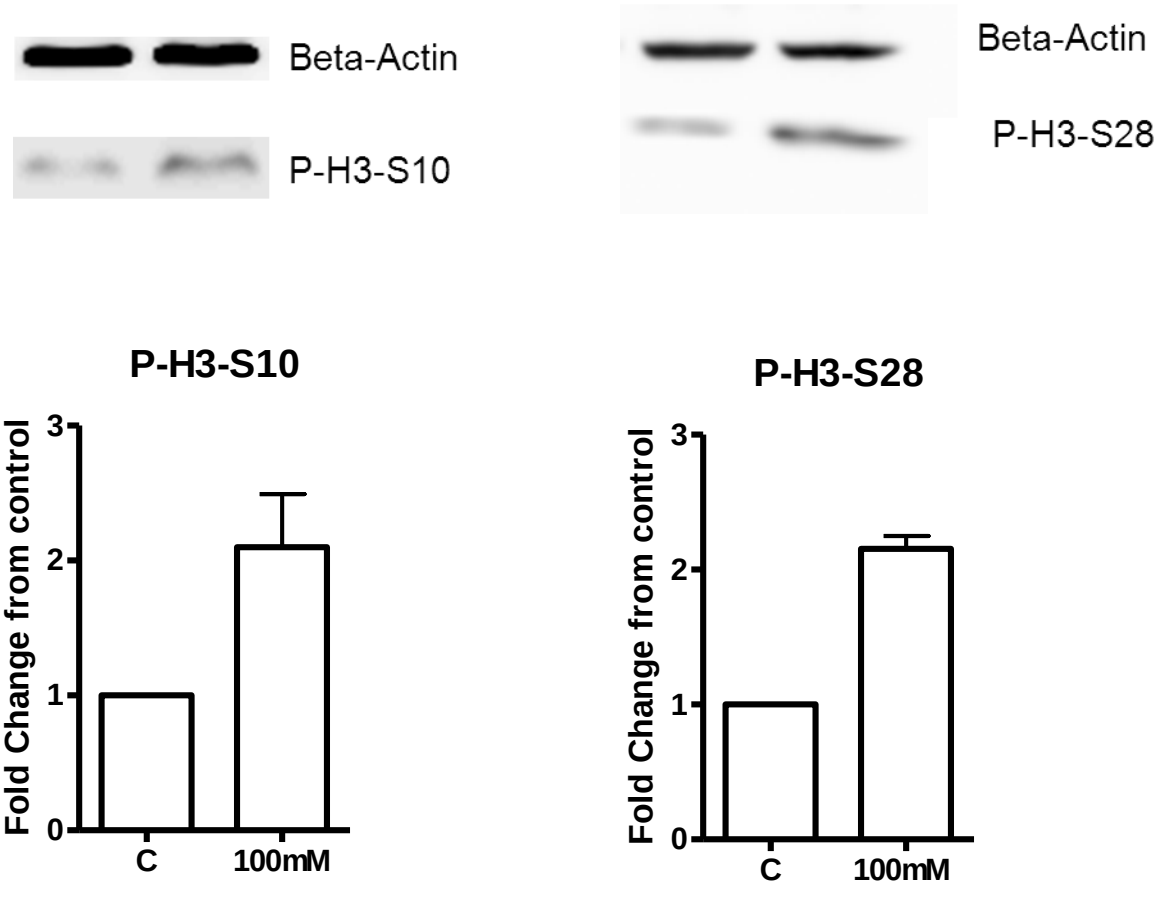
Samples were then flash frozen in liquid nitrogen and stored at -80 ^{o}C until the next day. A total extract was performed from the cells using lysis buffer (0.5M NaCl, 25% glycerol, 1% NP-40, 2mM HEPES, 1mM EDTA, 2mM $MgCl_2$) containing the same protease inhibitors as above.

Results: Histone phosphorylation was increased after the addition of 100 mM ethanol to VA-13 cells. The fold change in phosphorylation at serine 10 and 28 was similar, 2.1 and 2.2 respectively.

Conclusions: Ethanol increases histone phosphorylation in vitro in VA-13 cells at both serine 10 and serine. This is a preliminary and more experiments need to be done to find out the effect of time and dose.

Fig.9. The effect of ethanol on histone H3 phosphorylation in VA-13 cells. VA-13 cells were treated with or without 100mM ethanol for 24hrs. Whole cell extracts were prepared and used for western blot analysis using anti-phospho-histoneH3-S10 and anti-phospho-histone H3-S28. Data shown are representative of 3 separate experiments for P-H3-S10 and 2 experiments for P-H3-S28. Quantitative analysis of phosphorylated histone H3 at serine 10 and serine 28 were performed by densometric analysis and is represented as a bar graph. The phosphorylation was normalized to the levels of beta-actin. Data are presented as means ± SEM. Values represent fold increase over control group (control =1).

FIG. 9


Beta-Actin
P-H3-S10
Beta-Actin
P-H3-S28
P-H3-S10
Fold Change from control
0
1
2
3
C
100mM
P-H3-S28
Fold Change from control
0
1
2
3
C
100mM

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Clemens DL, Halgard CM, Miles RR, Sorrell MF, Tuma DJ (1995) Establishment of a recombinant hepatic cell line stably expressing alcohol dehydrogenase. Arch Biochem Biophys 321, 311-318.

Donohue TM, Osna NA, Clemens DL (2006) Recombinant Hep G2 cells that express alcohol dehydrogenase and cytochrome P450 2E1 as a model of ethanol-elicited cytotoxicity. Int J Biochem Cell Biol 38, 92-101.

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CHAPTER VI

CONCLUSIONS AND FUTURE DIRECTION

The liver is the main site of ethanol metabolism in the human body. It follows that ethanol causes deleterious effects on the liver. Over exposure of the liver to ethanol prevents the liver from performing its normal functions properly. Ethanol also affects many signaling pathways in the liver, the mechanisms of which are complex. Since binge drinking is on the rise, it is important to analyze the mechanisms related to binge and how it amplifies alcoholic liver disease. In recent years epigenetic modifications of histones have been shown to play roles in liver injury by ethanol. Therefore, an analysis of the effects of ethanol on histones, and the effect on gene expression will give insight into alcohol induced liver injury as well as may lead to the identification of new targets for the development of new therapeutic tools for the prevention and control of alcohol induced liver injury.

It has long been known that ethanol affects gene expression. This laboratory has shown that ethanol modification of histone acetylation and methylation in hepatocytes was associated with various gene expression (Park et al, 2005; Pal-Bhadra et al, 2007). While we have shown that ethanol affects histone phosphorylation in hepatocytes (Lee and Shukla, 2007), we have not shown its relationship to gene expression either in hepatocytes or in vivo. The research described in this dissertation aimed to determine the pharmacological mechanism of histone H3 phosphorylation by ethanol and acetate

in vivo mimicking acute binge conditions. The important conclusions and future studies from this original work are summarized below.

After binge administration of ethanol intraperitoneally (IP) in vivo, there were changes in the markers of alcoholic liver disease i.e ALT (necrosis), and cleaved caspase 3 (apoptosis). Apoptosis occurred before necrosis and steatosis, both of which increased with time. Therefore this model was considered suitable for studying mechanisms involved in binge effects in vivo. The rapid responses of kinases also occur in this model which makes it suitable for studying histone phosphorylation.

Ethanol induced the phosphorylation (activation) of the three major MAPKs i.e ERK1/2, JNK, and p38, in both the cytosol and the nucleus. Therefore MAPK may have a role in gene expression after ethanol in vivo.

IP administration of varying doses of ethanol for 1h, and a fixed dose (3.5g/Kg) over a time course showed elevated blood ethanol levels dose dependently and interestingly, was in the range, 20 to 170 mmol/L, observed in binge drinking in humans. This further supports this as a useful model to study ethanol binge effects in vivo in a rapid and controlled manner.

Phosphorylation of histone H3 at serine 10 and 28 altered after short exposure of the liver to different doses of ethanol in vivo for 1h. Phosphorylation of serine 28 was more

sensitive to lower doses of ethanol as compared to serine 10. There was no change in phosphorylation or acetylation when a higher dose, 5g/Kg was given. The phosphorylation of H3 serine 10 and 28 was reduced when the blood ethanol level was the highest. Therefore, phosphorylation of serine 10 and 28 in vivo occurs at lower blood ethanol levels. Phosphorylation of serine 10 and serine 28 was biphasic, suggesting the possible role of phosphatases. Since, MAP Kinase Phosphatase I (MKP-1) mRNA expression at the early time points resembled the pattern of histone phosphorylation, it is possible that it may play a role.

In this IP model ethanol induced the expression of the early response genes, c-Fos, C-Jun, MKP-1, and PAI-1. There was minor change in LDL-r expression. Therefore MAPK activation acting though ldl-r may not be implicated in steatosis in this model. There were major changes in PAI-1 mRNA levels. PAI-1 has been correlated to the induction of steatosis and in fibrin accumulation after ethanol in an LPS model.

The pattern of phosphorylation of histone H3 at serine 10 and 28 had distinctive similarities with the expression of c-Fos, c-Jun, MKP-1, and PAI-1 mRNA expression. Phosphorylated histone H3 at serine 10 and serine 28 associated with the promoter regions of c-Fos, c-Jun. However, only the phosphorylation of serine 28 was found to be associated with the promoter of PAI-1 containing an SRE binding domain.

Therefore serine 28 phosphorylation may be implicated in steatosis and fibrin accumulation after ethanol.

A unique aspect of this thesis work is the finding that ethanol induced phosphoacetylation at histone H3 lysine 9/ serine 10. Unlike phosphorylation at serine 10, the phosphoacetylation was increased at all doses of ethanol used in this study. Phosphoacetylation increased to at all time points and was marked at 4h. Furthermore, phoshoacetylation of histone H3 at K9/S10 also found to have increased association with the promoter of PAI-1 after ethanol.

Dimethylation of histone H3 was marginally affected by dosage of ethanol used in this study. Like phosphoacetylation, trimethylation of histone H3 at K9 was marked at 4h, as was the reduced expression of early response genes.

Preliminary observations using this model suggest that acetate induced the phosphorylation of histone H3 at serine 10 and 28 after 1.5h IP administration. Therefore, the effects of ethanol on histone phosphorylation may involve its metabolism in vivo. However, its details and mechanism remain to be investigated in the future.

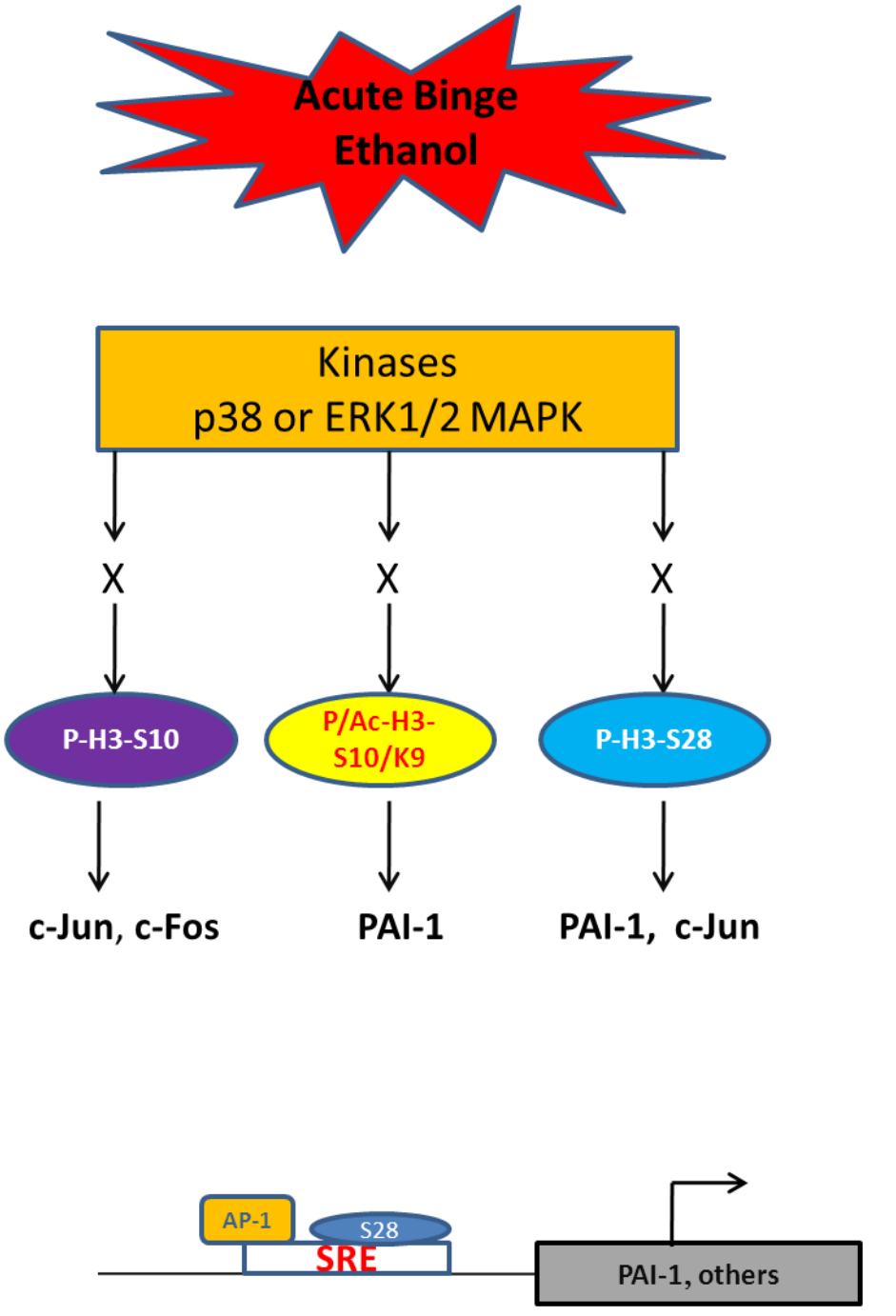
Taken together, phosphorylation of histone H3 by ethanol may have a role in transcriptional regulation. An appreciation for the role of histone phosphorylation

in the actions of ethanol comes from the fact that there is crosstalk between phosphorylation and acetylation/methylation. We demonstrated that two MAP kinases (p38 and ERK1/2) may be involved in histone phosphorylation in this in vivo binge model. Ethanol also promoted phosphoacetylation of histone H3 at K9/S10. Previous data from this laboratory showed that the MAPK signaling pathway is involved in ethanol induced increase in Ac-H3-K9 (Park et al, 2005). However, more studies are needed to understand this complex mechanism. In this dissertation, I demonstrated for the first time that histone phosphorylation and phosphoacetylation after acute binge ethanol is associated with gene expression in the liver at specific promoters. I may mention that this is also the first evidence of H3 phosphorylation and phosphoacetylation effect in vivo in a condition that mimics human binge drinking. In conclusion, these original findings may contribute to a newer understanding of the mechanism underlying liver injury during acute ethanol binge. Based on these conclusions, I propose a model illustrating the mechanism(s) of alcohol induced histone phosphorylation in vivo and its downstream consequences (Fig. 1).

Fig. 1. Proposed model of alcohol induced histone phosphorylation based on the dissertation project.

The model shows the effect of acute alcohol on histone H3 phosphorylation at serine 10 and 28 and phosphoacetylation at serine 10 and lysine 9 in vivo. Ethanol modulates p38 and ERK ½ MAPK Kinases which is speculated to activate a downstream kinase (denoted as X). The figure highlights different effects of phosphorylation at serine 10 and serine 28 and phosphoacetylation on gene expression. Ethanol induced histone H3 phosphorylation which at serine 10 was associated with the promoters c-Fos and c-Jun. Serine 28 phosphorylation was found at the promoters of c-Jun and PAI-1 and phosphoacetylation was associated with PAI-1 promoter. AP-1 can bind to the SRE site in PAI-1 promoter as well. Therefore c-Fos, and c-Jun may be inducing PAI-1. This highlights a role for MAPK and histone phosphorylation and phosphoacetylation in binge induced liver injury.

FIG.1


Acute Binge Ethanol
Kinases p38 or ERK1/2 MAPK
X
X
X
P-H3-S10
P/Ac-H3-S10/K9
P-H3-S28
c-Jun, c-Fos
PAI-1
PAI-1, c-Jun
AP-1
S28
SRE
PAI-1, others

Future Direction

The goal of this dissertation was to determine if acute ethanol modulates MAPK kinases, and increases histone phosphorylation in vivo. Furthermore, we addressed whether a crosstalks exist for histone phosphorylation and other modifications i.e. acetylation and methylation, and if acetate has any role. The data in this dissertation have provided some insight and raised some important and additional questions that would help to elucidate in the future the mechanism and importance of histone phosphorylation in vivo.

Inhibition of upstream (ERK1/2 and p38MAPK) and downstream kinases (MSK-1 and RSK) to determine the effect on ethanol induced histone H3 phosphorylation and acetylation. This lab has previously shown that ethanol increased phosphorylation of H3 at Ser10 and Ser28 in rat hepatocytes (Lee and Shukla, 2007). This phosphorylation was mediated by p38 MAPK. In chapter 3 I have demonstrated that ethanol increases ERK1/2 and p38 MAPK in vivo. Therefore, in order to understand the importance of ethanol induced histone phosphorylation we need to know how it is mediated. An inhibition of histone H3 phosphorylation by different strategies will determine how it affects other modifications namely acetylation and methylation. To determine which upstream MAPK is involved we can inject antisense oligonucleotides (ASO) into the animal prior to the addition of ethanol then inject ethanol and extract liver. Histone phosphorylation is known to be affected by many kinases. Our data only preliminarily

demonstrated the upstream kinases which are affected by ethanol. Therefore we need to determine the downstream kinases (eg MSK-1, RSK, histone kinase) which are affected.

Crosstalk of histone phosphorylation with acetylation and methylation. Histone modifications are known to crosstalk with each other (Zhang and Reinberg, 2001; Santos-Rosa and Caldas, 2004). These modifications act via transcriptional regulators and cofactors to affect gene transcription. Our results showed that there are different modifications of modified histone H3 after acute binge ethanol in vivo, i.e phosphorylated histone H3-S10, phosphorylated histone-H3 S28, phosphoacetylated histone -H3-K9/S10, and trimethylated histone H3-K9. Therefore, it will be exciting to investigate the mechanism and the regulation of ethanol induced subpopulations that are variably or dually modified at specific sites on histone H3.

Immunoprecipitation studies using antibodies to the differently modified histones. Immunoprecipitation of modified histones as well as regulatory enzymes i.e HATs (GCN5, p300), PCAF, etc), HDACs, kinases (MSK-1, RSK), phosphatases(MKP-1) and cofactors (c-Fos, c-Jun) will give some insight into this project. In addition, after the experiment mentioned above is performed the effect of the inhibitors on acetylation and methylation should be determined. Additionally, to determine if histone phosphorylation is involved in transcription one can also analyze for 14-3-3 proteins which are known to bind to phosphorylated histone H3 at serine 10 and serine 28. It

remains unknown if crosstalk occurs between phosphorylated histone H3-S28 and other modifications of histone H3 after ethanol. The same consensus sequence that exists around the S10 residue i.e. ARKS (alanine, arginine, lysine, and serine) of histone H3 is also evident for S28 (Prigent & Dimitrov, 2003). TSA treatment in cells have been shown to increase histone H3 phosphorylation (Zhong et al, 2003). We have also seen this effect in primary hepatocytes (see figure 8 of miscellaneous chapter). While we have not observed histone acetylation by ethanol in vitro in hepatocytes at any other residues but lysine 9, like phosphoacetyl histone H3-S10/K9, phosphoacetyl histone H3-S28/K27 may exist.

Do the genes changes relate to changes in respective gene expression? In this dissertation we have first observed that acute binge ethanol can modulate gene expression. We next showed that histone phosphorylation and phosphoacetylation is associated with the promoter of specific genes and are thus implicated in their modulation. Since these genes have roles in liver injury it is important to assess if the change in gene expression also lead to changes in protein expression of these respective genes.

ChIP assay with c-Fos antibody to determine if c-Fos is at the promoter of genes involved in liver injury in this model such as PAI-1. PAI-1 is increased after ethanol binge. PAI-1 has an AP-1 binding site in its promoter. Therefore performing a ChIP assay

with c-Fos will help to determine if c-Fos is involved in ethanol induced gene expression. Additionally, it will give insight into other genes that are affected by c-Fos.

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VITA

Taryn Typhaine James was born in the island of St. Christopher commonly known as St.Kitts in the two-island nation of St.Kitts and Nevis in the West Indies. She lived in St.Kitts until age 14 at which time she migrated to Miami, Florida. She finished high school in Miami and went on to pursue an AA degree in pharmacy at Miami Dade College. She graduated with a Bachelor's of Science degree in Biology with a minor in chemistry from Florida A&M University in 2003. She first discovered research as an undergraduate in the Life Sciences Summer Research Program at MU and decided to gain more experience in research in the MU PREP Scholar's Program to decide if she wants a research career. Because of her long standing interest in herbal medicine, she decided that pharmacology is a good area to study and has been working in this area for the past six years.