ALCOHOL INDUCED HISTONE ACETYLATION MEDIATED BY HISTONE ACETYL TRANSFERASE GCN5 IN LIVER

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LIST OF ABBREVIATIONS

ACTR   Activin receptor protein 1

ADH   Alcohol dehydrogenase

Ada   Adenosine deaminase

AIB1   Amplified in breast cancer-1

ALD   Alcoholic liver disease

ALDH   Aldehyde dehydrogenase

BRCA1   Breast cancer 1 early onset

BSO   Buthione-S-Sulfoximine

CARM1   Coactivator-associated arginine methyltransferase 1

CBP   CREB binding protein

CDK   Cyclin dependent kinase

cSKI   Cellular Sloan-Kettering Institute protein

CYP2E1   Cytochrome P4502E1

DMEM   Dulbecco's modified eagles's medium

DPI   Diphenylene iodonium

EZH2   Enhancer of Zeste homolog 2

GCN5   General Control Nonderepressible 5

GLDH   Glutamate Dehydrogenase

GPT   Glutamic-pyruvic transaminase

GRIP1   Glucocorticoid receptor interacting protein 1

GRP1	Glycine-rich RNA binding protein
GSH	Reduced glutathione
GTP	Guanosine triphosphate
HAT	Histone acetyltransferasedihydroethidium
HDAC	Histone deacetylase
HMTase	Histone methyltransferase
HRP	Horseradish peroxidase
IF2	Initiation factor 2
IKKα	Inhibitor of IκB kinase α
LDH	Lactate dehydrogenase
MAPK	Mitogen-activated protein kinas
MBD	Methyl CpG binding domain protein
MEF2	Myocyte enhancer factor-2
MEOS	Microsomal ethanol oxidizing system
MLTK	MLK-like mitogen-activated protein triple kinase
MOF	MOZ-realated factor
MOZ	Monocytic leukemia zinc-finger protein
MSK	Mitogen- and stress-activated protein kinase
MTA	Metastasis-associated protein
MYST	MOZ, Ybf, Sas and Tip60 together
NAC	N-acetyl cystein
NADH	Reduced form of nicotinamide-adenine dinucleotide
NADPH oxidase	Nicotinamide adenine dinucleotide phosphate-oxidase

NCoA	Nuclear receptor coactivator1
N-CoR	Nuclear receptor corepressor
NFκB	Nuclear factor-kappa B
NHK1	Nucleosomal histone kinase 1
NuRD	Nucleosome remodeling histone deacetylase
PCAF	P300/CBP-associated factor
PKA	Protein kinase A
PLA2	Phospholipase A2
RAC3	Receptor-associated coactivator 3
RAR	Retinoic acid receptor
RbAp	Retinoblastoma associated protein
ROS	Reactive oxygen species
RPD3	Reduced potassium dependency 3
RSK2	Ribosomal S6 kinase 2
RXR	Retinoid X receptor
SAHA	Suberoylanilide hydroxamic acid
SAP	Saposin
SAMe	S-adenosyl-L-methionine
Sas	Something about silencing
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SETDB1	SET domain, bifurcated 1
SIRT	Sirtuins family
SMYD3	SET- and MYND-containing protein 3

SOD	Superoxide dismutase
Spt	Serine palmitoyltransferase
Src-1	Steroid receptor coactivator-1
SUV39h	Suppressor of variegation 3-9 homolog 1
TBP	TATA binding protein
TIF2	Transcription intermediatory factor
Tip60	Tat-interactin protein of 60 kDa
TFIIB	Transcription factor IIB
TG2	Transglutaminase 2
TNF	Tumor necrosis factor

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ABSTRACT

Although several mechanisms and factors have been proposed to be responsible for alcoholic liver disease, at present there are no precise mechanisms for liver injury. Drinking ethanol as well as higher chain alcohols (called surrogate alcohol) causes severe liver problems and could be fatal. Emerging evidence highlight the importance of histone modifications, transcriptional regulators and gene expressions in liver disease. Histone acetylation plays an imperative role in transcription, and histone acetyl transferase (HAT) and histone deacetylase (HDAC) are important class of transcriptional regulators. This study describe the effects and the regulatory mechanism of surrogate alcohols on histone acetylation; the role of oxidative stress in histone acetylation and alcohol dehydrogenase 1 (ADH1) gene expression; and the identification of specific HAT in ethanol induced histone acetylation. We have observed that surrogate alcohols increase histone H3 acetylation selectively at Lys 9 (H3-Lys9) but not at Lys 14, 18, 23 or 27 in primary cultures of rat hepatocytes. The alcohol effect was inhibited by alcohol metabolizing enzyme inhibitors. Alcohol metabolites (various short chain fatty acids) showed a similar

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effect as the alcohols, suggesting the involvement of metabolism in histone acetylation. Both alcohols and their metabolites increased the HAT activity without inducing any significant change in HDAC activity in hepatocytes, but only the metabolites increased HAT activity in an in vitro assayand only propionate and butyrate HDAC activity when added to an in vitro assay. A series of experiments to manipulate oxidative stress were carried out and the results demonstrate for the first time that oxidative stress mediates ethanol induced histone acetylation and *ADH1* gene expression. It is likely that both NADPH oxidase and mitochondria derived ROS are involved. The study presented here also identifies for the first time the specific HAT, GCN5, responsible for ethanol induced histone H3 acetylation at lysine 9 in human hepatoma cell overexpressing ADH1 (VA-13 cells). siRNA knock down of GCN5 in VA-13 cells decreased both ethanol induced H3AcK9 and HAT activity. In summary, we conclude that ethanol increases histone H3 acetylation at lysine 9 via modulation of histone acetyl transferase GCN5 in the liver. These original findings may contribute to a better understanding of the mechanism underlying the pathogenesis of alcoholic liver disease and also contribute towards development of potential therapeutic target at the nucleosomal level.

Chapter I

Introduction

I.1 Epigenetics

For decades our vision of inheritance has been written in the language of DNA. Genetic mutations have driven most of the attention on the inheritance of phenotypic characteristics from one generation to the next. But recent discoveries in the field of epigenetics are changing the views about inheritance. Epigenetics means above genetics – something that affects a cell, organ or individual without affecting its DNA sequence. Epigenetic mechanism involves DNA, RNA and histone modifications. Untying the relationship among these components has led to rapidly evolving new concepts, showing how they interact with each other. Disruption of each of these epigenetic mechanisms resulted in aberrant transcription of genes involved in various diseases (Goodman and Smolik, 2000; Petrij et al., 1995; Kundu and Dasgupta, 2007). Epigenetics has an impact on various scientific enterprises including somatic gene therapy, cloning and transgenic technologies in plants and animals, aspects of cancer biology, genomic imprinting, and developmental abnormalities in plants and animals (Wolffe and Matzke, 1999; Egger et al., 2004). The excitement in this research area follows from the realization that diverse organisms have common molecular mechanisms that contribute to the epigenetic control of gene expression. Epigenetics represents a new frontier in genetics research. With the achievement of genome sequencing projects, a major challenge will be to understand

gene function and regulation. Researchers need to determine how epigenetic controls are imposed on genes.

I.1.1 Chromatin, nucleosome and histone

The most well characterized structural unit of the chromosome is the nucleosome (Kornberg, 1974; McGhee and Felsenfeld, 1980; Pondugula and Kladde, 2008). The nucleosomal complex formed by the histone octamer and associated DNA, is the fundamental unit of chromatin. There are two types of chromatin: heterochromatin and euchromatin. Heterochromatin is the condensed region with inactive genes and is transcriptionally silent. On the other hand, euchromatin contains transcriptionally active genes. Post-translational modifications of histone act as markers of these different chromatin regions (Jenuwein and Allis, 2001).

The nucleosome core particle consists of a histone octamer core around which 146 base pairs of DNA are wrapped (Fig. 1). There are four types of histones in the histone octamer (called core histone) and one linker histone (H1) is located outside the nucleosome. The histone octamer arranges as $(H3-H4)_2$ tetramer and two H2A-H2B dimers positioned on both sides of the tetramer. Each core histone is well conserved in eukaryotes and has a molecular weight from 11-15 kDa and 17~20 positive charges at pH 7.0. H1 is also a major component of nucleosome and links the bridge between adjacent nucleosomes. Nucleosomes play an important role in the formation of chromatin. X-ray crystallography has shown that the histone octamer has a disk like shape with the diameter of 6.5 nm and the thickness of 6 nm (Arents et al., 1991). The tails of H2A and

H4 stick out of the disk- shaped nucleosome and the tails of H3 and H2B extend between DNA (Suto et al., 2000).

The core histone has a basic N-terminal domain, a globular domain organized by a histone fold and a C-terminal tail. The histone fold domains mediate histone-histone or histone-DNA interactions. The N-terminal tail of H4 binds to the H2A-H2B dimer of a neighboring nucleosome which contributes to the folding of the chromatin fiber and may be involved in nucleosomal positioning (Luger and Richmond, 1998). The precise organization of DNA in chromatin has significant functional consequences in the processes such as transcription, replication, DNA repair, segregation, etc. These processes are influenced by the chromatin which is imposed at the most fundamental level by histones (Wolffe and Hayes, 1999). For example, HMG-14 and -17 proteins bind to the N-terminal tail of H3 and unfold the higher order chromatin fiber, thereby facilitating transcription (Trieschmann et al., 1998). Additionally, Lugar and Richmond showed that the H3 and H4 N-terminal tails bind to the yeast trans-acting repressors, Sir3 and Sir4, leading to the formation of transcriptionally repressed chromatin domain (Lugar and Richmond, 1998).
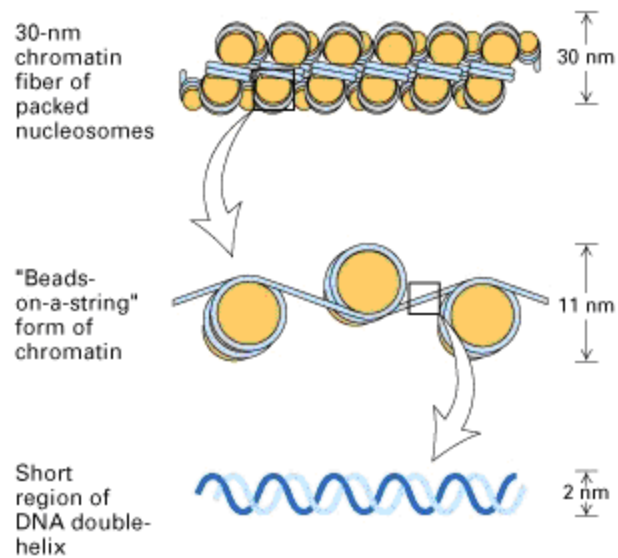
Fig. 1. Schematic diagram of chromatin formation.

Chromatin is composed of repeated subunit of nucleosome. Nucleosome consists of core histones which are wrapped around by DNA. (The figure is adapted from http://bioweb.wku.edu/courses/biol566/L5YeastSilencing.html).

I.2 Histone modifications

Core histones are subject to diverse post-translational modifications. Among them, acetylation, methylation and phosphorylation are extensively studied (Cheung et al**.,** 2000; Kouzarides, 2002; Peterson and Laniel, 2004). In addition, ubiquitination, glycosylation, ADP-ribosylation, sumoylation (Wong and Smulson, 1984; Jason et al., 2002; Shiio and Eisenman, 2003) are also on the list. The numbers are growing. Hence, one of the challenges is also to identify all of the histone modifications. Most modifications take place on the N-terminal basic tail domain with the exception of histone ubiquitination (Goldknopf and Busch, 1977; Weake and Workman, 2008). The large number of histone modifications and their potential role in physiological response has been postulated as "histone code" (Jenuwein and Allis, 2001; Strahl and Allis, 2000; Turner, 2000).

Histone Acetylation: One of the post-translational modifications described in the "histone code" is acetylation. Acetylation of histone occurs at lysine residues on the amino terminal tails which neutralizes their positive charges, weakening their interaction with DNA, thus resulting in an opened chromatin conformation. Such conformation facilitates access for transcriptional regulators which could result in increased transcriptional activity. Four decades ago, Allfrey et al. proposed that histone acetylation was associated with transcriptional activity (Allfrey et al., 1964). Later, acetylated core histones were shown to associate with transcriptionally active chromatin (Sealy and Chalkley, 1978; Hebbes et al., 1988) and active genes (Kimura et al., 2005). These observations suggest that acetylation of histones is closely linked to transcriptional

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activation. Although discovery of histone acetylation correlating transcriptional regulation occurred four decades ago, understanding the molecular mechanism of transcriptional regulation was hampered by a lack of knowledge about the enzymes that acetylate or deacetylate histones. Recently, with the identification of multiple histone acetyltransferases (HATs) and histone deacetylases (HDACs), it has become possible to analyze directly the effects of histone acetylation and deacetylation on transcriptional regulation.

Histone acetylation is a reversible process. HATs transfer the acetyl moiety from acetyl coenzyme A to the amino group of lysine residue and neutralize the positive charge. Acetyl coenzyme A is a high energy acetyl moiety donor for histone acetylation. Reversal of this reaction is catalyzed by HDACs. HDACs remove the acetyl group from the acetylated histone and re-establish the positive charge in the histone (Fig. 2). The discovery that several transcriptional coactivators contain HAT activity, while repressors possess HDAC activity, has strongly linked histone acetylation to transcriptional activation and deacetylation to repression (Kuo and Allis, 1998; Strahl and Allis, 2000). Histone acetylation can be a dynamic process; rates of acetylation/deacetylation vary throughout the genome. There are 1-6 lysine residues in each histone subunit that could be acetylated; Lys5 of H2A; Lys5, Lys12, Lys15, Lys20 of H2B; Lys4, Lys9, Lys14, Lys18, Lys23, Lys27, Lys36 of H3; and Lys5, Lys8, Lys12, Lys16 of H4. Though some enzymes are promiscuous in acetylating/deacetylating lysine residues, others show variety degree of site selectivity. For example, Gcn5 preferentially acetylates Lys8 and Lys16 of H4 (Kuo et al., 1996) and Rpd3 preferentially deacetylates Lys5 and Lys12 of histone H4 (Rundlett et al., 1996; Taunton et al., 1996). Most of the acetylation occurs in

euchromatin regions and some in heterochromatin regions. For example, the acetylation

of Lys12 of H4 causes telomeric silencing (Kelly et al., 2000). Lys 16 of H4 is acetylated

in the transcriptionally hyperactive X chromosome in the male larvae of *Drosophila*

(Turner et al., 1992). This dissertation is mainly focused on histone acetylation (Fig. 2).

Histone Acetylation
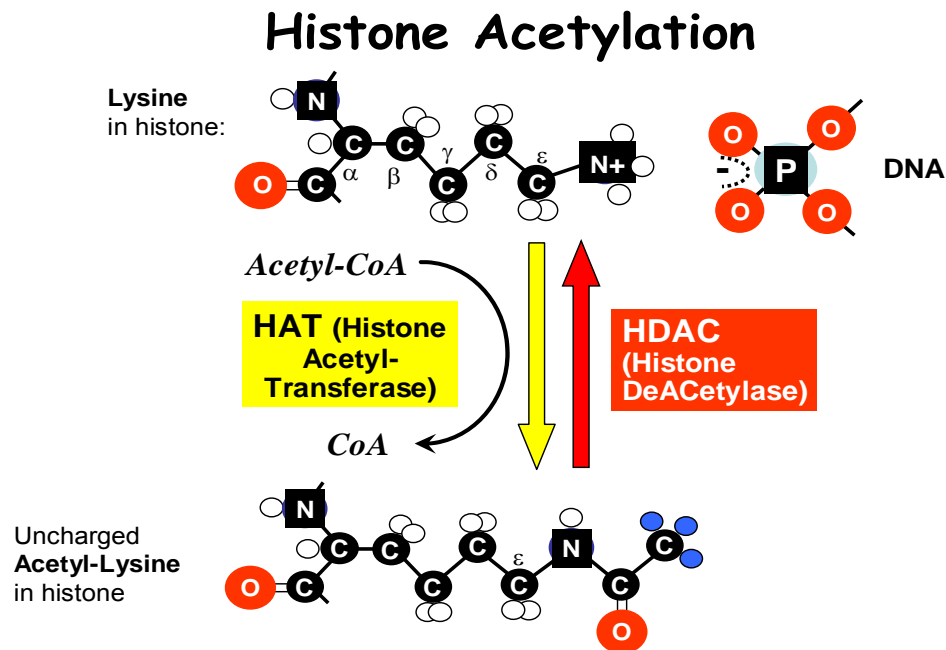
Fig. 2. Schematic Diagram of Histone Acetylation.

Acetylation of histone occurs at lysine residues on the amino terminal tails which neutralizes their positive charges, weakening their interaction with DNA, thus resulting in an opened chromatin conformation. HATs transfer the acetyl moiety from acetyl coenzyme A to the amino group of lysine residue and neutralize the positive charge. HDACs remove the acetyl group from the acetylated histone and re-establish the positive charge in the histone.

(The figure is adapted from http://sbs.umkc.edu/waterborg/chromat/chroma14.html)

Histone Phosphorylation: Although acetylation is a major pathway for histone mediated transcriptional regulation, phosphorylation also plays role in regulation. Phosphorylation has been correlated with mitotic condensation and transcriptional regulation in interphase (Nowak and Corces, 2004). Phosphorylation mainly occurs at serine10 and 28 (Gurley et al., 1978; Wei et al., 1998; Lee et al., 2007) and threonine 11, 119, 139 (Preuss et al., 2003; Kurose et al., 2005) residues of histone H3. Phosphorylation is catalyzed by the protein kinase and opposing reaction is carried out by protein phosphatase enzymes. In addition to the core histones, linker H1 histone can also be phosphorylated (Swank et al., 1997; Bhattacharjee et al., 2001). Interestingly, histone H1 phosphorylation regulates chromatin remodeling enzyme (Dou et al., 2002) and also activate oncogenes (Chadee et al., 2002). Histone H2AX was phosphorylated on Ser 139 (Kurose et al., 2005; McManus and Hendzel, 2005) in response to DNA double strand break which is a marker for cell death. In *Saccharomyces cerevisiae*, H4 was also shown to connect with DNA double strand breaks (Cheung et al., 2005). Beside these modifications, researchers also showed cross talk among all the modifications. H3 phosphorylation was shown to enhance acetylation of H3 at Lys14 (Cheung et al., 2000) and ameliorate acetylation of H3 at Lys9 (Edmondson et al., 2002). Rea et al. demonstrated that methylation of histone H3 at Lys9 interferes with H3 phosphorylation at Ser10 (Rea et al., 2000). Though scientists discovered various histone phosphorylation, function of these modifications remain to be explored in the near future.

Histone Methylation: Another important histone modification is histone methylation. It occurs at lysine and arginine residues. The amino group of the side chain is methylated. It

is a more stable modification than the others and is irreversible. There are few known histone demethylase enzymes that remove lysine or arginine methylations (Yamane et al., 2006; Whetstine et al., 2006). However, several histone methyltransferases (HMTase) have been characterized (Rea et al., 2000; Nishioka et al., 2002) and more yet to be recognized. HMTases regulate the site specific addition of methyl groups to histone. Lysine can be mono-, di-, or tri- methylated and arginine can be mono- or di-methylated on H3 and H4. Histone methylation can result in transcriptional activation or repression. For example, di- and tri-methylation of H3 Lys4 have been primarily linked to transcriptional activation (Santos-Rosa et al., 2002). In contrast, di-and tri-methylation of H3K9 have been associated with transcriptional repression (Noma et al., 2001). Another interesting topic is the cross-talk between methylation and other modifications. For example, phosphorylation on H3-Ser10 or methylation on H3 Lys4 enhances H3 Lys14 acetylation and these ordered activities lead to the transcription of specific genes (Lo et al., 2000; Wang et al., 2001). Another important issue is that the imbalance of epigenetic events is connected to human disease (Kundu and Dasgupta, 2007). For example, histone methyltransferase EZH2 methylates H3 Lys27 and interestingly, EZH2 is overexpressed in prostate cancer and breast cancer (Fujii et al., 2008; Wei et al., 2008). Hence, further understanding of the molecular mechanism of histone modification and their modifying enzymes will help to develop new therapeutic strategies.

I.2.1 Histone Modifying Enzymes

Histone Acetyltransferase (HAT): Histones are the targets for a variety of post-translational modifications where histone modifying enzymes are the key players.

Histone acetyltransferase (HAT) and histone deacetylase (HDAC) balance histone acetylation. There are two types of HATs (Brownell and Allis, 1996): Type A and Type B. Type A HATs are located in the nuclei and most likely acetylate histones closely tied to transcriptional activation. Type B HATs can be found in the cytoplasmic fraction and can acetylate newly synthesized histones before chromatin assembly during DNA replication. The first Type A HAT cloned and identified was 55 KDa polypeptide from the ciliated protozoan *Tetrahymena thermophila* (Brownell et al., 1996); which is highly similar to a known transcriptional co-activator, Gcn5p, in *Saccharomyces cerevisiae* (Georgakopoulos and Thireos, 1992). Researchers studied the HAT activity of these two proteins repeatedly and showed that they prefer H3 rather than other histones (shown in the Table 1). They also have site specificity at the amino acid level. Studies showed that yGcn5 functions in vivo in a multisubunit protein complex that contains Ada1p, Ada2p, Ada3p/Ngg1p, and Ada5p/Spt20p (Grant et al., 1997). In case of mammals, p/CAF (p300/CBP associated factor) and GCN5 together are assumed as one family and they are also a part of large multiprotein complex (Ogryzko et al., 1998; Brand et al., 1999; Martinez et al., 2001). The C terminal domain contains 160 amino acids and intrinsic HAT activity. The function of N-terminal domain is not known presently, but could be involved in substrate specificity (Yang et al., 1996).

Another important HAT family member is CREB binding protein (CBP) and related protein p300. They are present in many multicellular organisms, but not in yeast (Yuan and Giordano, 2002). Though the multidomain protein region varies from species to species, it at least contains three cysteine-histidine rich regions, the binding site for the CREB transcription factor, the bromodomain, the HAT domain and steroid receptor

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coactivator-1 (SRC-1) interaction domain (SID) (Arany et al., 1994). They interact with phosphorylated cAMP response element binding protein (CREB), p53, RXR, RAR, TBP, TFIIB and many more proteins (Kwok et al., 1994). As p300/CBP is associated with so many proteins, it acts as an integrator that mediates transcription of multiple signal transduction pathways (Kamei et al., 1996; Chan and Thangue, 2001). They also acetylate those proteins and are therefore called factor acetyltransferase (FAT) (Sterner and Berger, 2000). Fascinatingly, many of these protein-protein interactions can be regulated by the same post-translational modifications that chromatin is subject to, such as phosphorylation (Jankecht and Nordheim, 1996), methylation (Chevillard-Briet et al., 2002), and sumoylation (Girdwood et al., 2003). They acetylate specifically Lys12 and Lys15 in H2B; K14 and K18 in H3 and K5 and K8 in H4 (Schiltz et al., 1999).

Third family of histone acetyltransferases is the MYST family. This family contains MOZ, yeast YBF2 (renamed Sas3), yeast Sas2 and TIP60 (Borrow et al., 1996). Though they differ in the other structural part, they all contain a zinc finger domain and HAT domain (Yang, 2004). Drosophila MOF (Sas) may play a role in dosage compensation in male flies (Hilfiker et al., 1997). MOF is shown to acetylate histone H4 on male X chromosome at Lys16 and in case of loss of dosage compensation Lys16 acetylation was lost. In case of mammals, MOZ and MOZ related factor (MORF) both acetylate H3 and H4, only MOZ acetylates H2B (Champagne et al., 2001). In contrast to CBP/p300, there are no reports of non-histone substrates of MYST family HATs.

The last family of HAT in type A is composed of SRC1/NCoA1, F2/NCoA2/GRIP1 and ACTR/AIB1/RAC3/pCIP/NCoA3 (Leo and Chen, 2000). SRC1 is a steroid hormone receptor interacting co-activator that induces ligand inducible

transcription (Kamei et al., 1996; Yao et al., 1996). SRC1 interacts with PCAF (Spencer et al., 1997) and CBP/p300 (Yao et al., 1996; Onate et al., 1995; Sheppard et al., 2001) and also prefers H3 and H4 as substrates, similar to PCAF and CBP/p300 (Spencer et al., 1997). ACTR also has the similar structure like SRC1 and TIF2/GRIP1 and it forms multiprotein complex with PCAF and p300 which is essential for transcription of downstream genes (Chen et al., 1997).

The only known HAT in type B HAT family is Hat1p which was identified in yeast (Kleff et al., 1995; Benson et al., 2007). They appear to acetylate newly synthesized histone in the cytoplasm. After the acetylation process occurs, acetylated histone is transported to the nuclei and assembled into chromatin (Brownell & Allis, 1996; Grunstein, 1997).

Histone Deacetylase: In contrast to HATs, HDACs have roles in transcriptional repression. HDACs have been categorized into three classes. The first class consists of yeast Rpd3, Hos1 and Hos2 and mammalian HDAC1, HDAC2, HDAC3 AND HDAC8. However, HDAC8 stands at the border of class I and class II (Buggy et al., 2000; Hu et al., 2000). They are all located in the nucleus and associated with other proteins/corepressor complex (Sin3A, NuRD complex etc.) to direct transcriptional repression (Knoepfler and Eisemnman 1999; Ng and Bird, 2000). The Sin3 complex consist of mSin3, N-CoR or SMART (corepressors), Sap18, Sap30, RbAp48, RbAp46 and c-Ski (Nomura et al., 1999). Another complex NuRD consists of N-CoR, MTA2, Mi2, RbAP46/48, and MBD2 and has ATP dependent chromatin remodeling and HDAC activities (Zhang et al., 1999). BRCA1 was found to bind to RbAp46, RbAp48, HDAC1

and HDAC2, indicating that BRCA1 may be a component of HDAC1/2 multiprotein complex (Abbott et al., 1999). Sin3A complex is devoid of HDAC3 and classII HDAC (Grozinger et al., 1999). Class I HDACs are able to deacetylate four of the core histones depending on multiprotein complex compounds. For example, HDAC1 associated with nuclear matrix preferentially deacetylate histone H2B (Sun et al., 1999). In yeast, deletion of RPD3 causes hyperacetylation of histone H3 and histone H4 and specifically H4 Lys5 and Lys12 position (Rundlett et al., 1996). These classes of HDACs generally function by a Zn-dependent mechanism.

The second class consists of yeast HDAC1 and mammalian HDAC4, 5, 6, 7, 9 and 10 (Grozinger et al., 1999; Zhou et al., 2001; Guardiola and Yao, 2002). Similar to class I HDAC, they generally function by a Zn-dependent mechanism and form multiprotein complex (Fischle et al., 1999; Grozinger et al., 1999). In yeast, deletion of HDAC1 causes hyperacetylation of histone H3 and H4 and specifically at H4 Lys5 and Lys12 position (Rundlett et al., 1996). Mammalian class II HDACs were shown to acetylate all four core histones. HDAC 4, 5, and 7 bind with MEF2 family of transcription factor, repress specific genes in a calcium dependent manner (Miska et al., 1999; Lemercier et al., 2000; Lu et al., 2000). Recently HDAC6 is getting huge attention from the scientific community. Histone deacetylase 6 (HDAC6) is a unique enzyme with specific structural and functional features. It is localized exclusively in the cytoplasm and is the only member, within the histone deacetylase family, that harbors a full duplication of its deacetylase homology region followed by a specific ubiquitin-binding domain at the C-terminus end (Bertos at al., 2001). This deacetylase functions play critical role in the microtubule network. This enzyme was recently identified as a multisubstrate protein

deacetylase that can act on acetylated histone tails, alpha-tubulin and Hsp90 (Wang et al.,

2007; Dompierre et al., 2007). HDAC6-deficient mice showed hyperacetylated tubulin in

most tissues (Zhang et al., 2008). HDAC6 appears both as a sensor of stressful stimuli

and as an effector, mediates and coordinates appropriate cell responses (Matthias et al.,

2008). HDAC9 was shown to express in heart (Zhang et al., 2002) and in skeletal muscle

(Mejat et al., 2005) and is involved in chromatin acetylation.

Mammalian Class III HDAC is similar to yeast silent information regulator 2

(Sir2) (Buck et al., 2004). Sir2 related proteins were shown to involve in gene silencing

(Jin et al., 2008). This family of enzymes consists of NAD^+-dependent histone/protein

deacetylases that tightly couple the hydrolysis of NAD^+ and the deacetylation of an

acetylated substrate to form nicotinamide, the deacetylated product, and the novel

metabolite O-acetyl-ADP-ribose (OAADPR). It is named SIRT in mammals. It includes

7 types of SIRT. Among them, enzymatic analysis of recombinant SIRT2 in comparison

to a yeast homolog of Sir2 protein shows a striking preference of SIRT2 for acetylated

tubulin peptide as a substrate relative to acetylated histone H3 peptide (North et al.,

2003). Compared to other HDACs, structure and function of SIRT is relatively unknown.

This dissertation is mainly focused on HAT and HDAC regulation in histone H3

acetylation (Fig. 3).

Table 1. Summary of known HATs and HDACs (The table is taken from Adcock et

al., 2006).

HDAC families	Substrate
Class I (Rpd3 homologs)	
HDAC 1	H2A, 2B, 3, 4, AR, ER, SHP, YY1
HDAC 2	H2A, 2B, 3, 4, GR, YY1
HDAC 3	H2A, 2B, 3, 4, GR, SHP, GATA1, YY1
HDAC 8	H2A, 2B, 3, 4
Class II (Hda1 homologs)	
HDAC 4	H2A, 2B, 3, 4, GATA1
HDAC 5	H2A, 2B, 3, 4, GATA1
HDAC 6	H2A, 2B, 3, 4, tubulin, SHP
HDAC 7	H2A, 2B, 3, 4
HDAC 9	H2A, 2B, 3, 4
HDAC 10	H2A, 2B, 3, 4
Class III (Sir2 homologs)	
SIRT 1	
SIRT 2	
SIRT 3	
SIRT 4	Non-histone proteins
SIRT 5	e.g. tubulin, p65, p53
SIRT 6	
SIRT 7	
Class IV (Rpd3 homolog)	
HDAC 11	H2A/H2B/H3/H4
HAT families	
GNATs (Gcn5-related acetyltransferase)	
Hat1	H4/H2A
Gcn5 and Gcn5L	H3 K9/K14/H2B, c-Myc
Elp3	H3/H4
Hpa2	H3/H4
PCAF	H3/H4, c-Myc, GATA2
MYST (MOZ, Ybf2/Sas3, Sas2, Tip60-related)	
Esa1	H4/H2A
Tip60	H4/H2A, c-Myc, AR
MOF	H4 K16/H3/H2A
MOZ	
Sas3	H3/H4
Sas2	H4K16
P300/CBP	
P300/CBP	H2A/H2B/H3/H4, p53, p65, AR, ER
General transcription factor HATs	
TAF250	H3/H4
TFIIIC	H2A/H3/H4
Nuclear hormone related HATs	
SRC1	H3/H4
SRC3/ACTR	H3/H4

(a) Repressor-directed histone deacetylation

Rpd3
Sin3
Ume6
RD
DBD
URS1
TATA
Histone octomer
Deacetylation of histone N-terminal tails

(b) Activator-directed histone hyperacetylation

Gcn4
Gcn5
AD
DBD
UAS
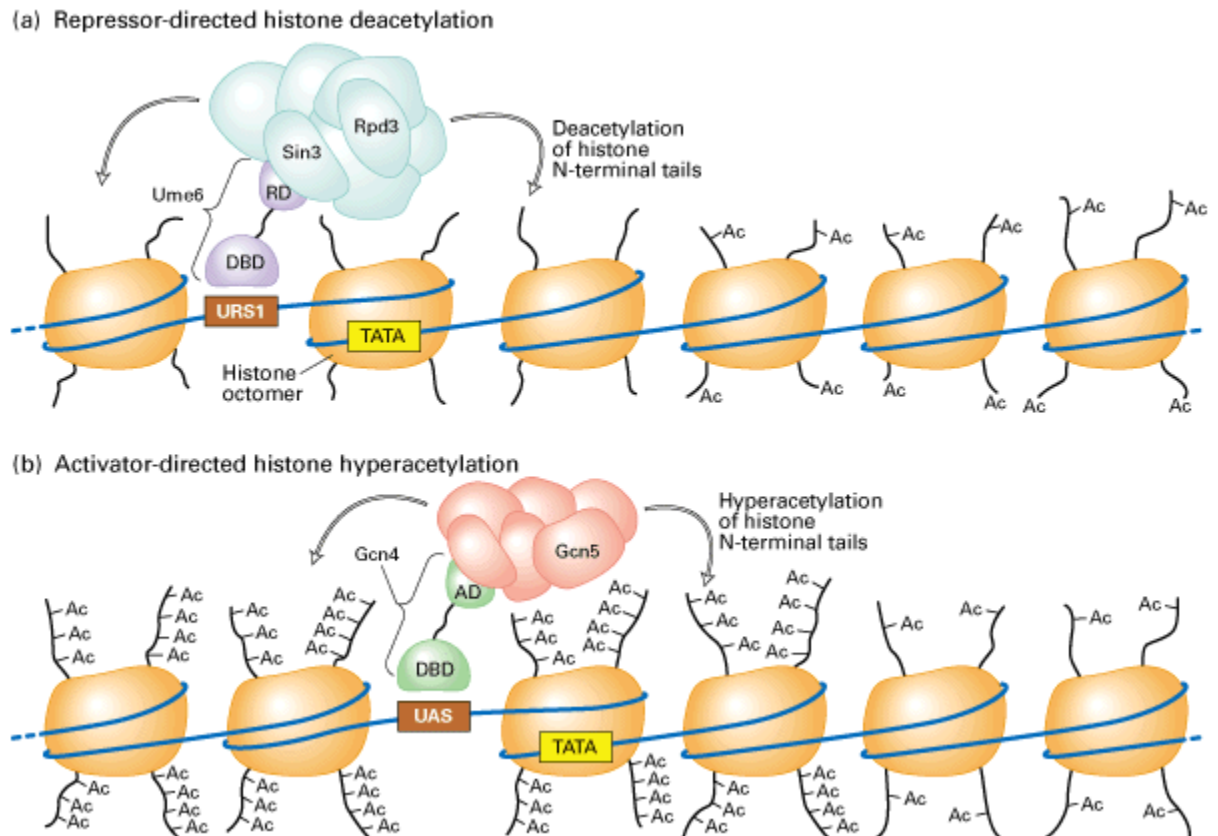TATA
Hyperacetylation of histone N-terminal tails

Fig. 3. Schematic diagram of HATs and HDACs on histone acetylation.

Histone acetylation is a balance between numerous HATs and HDACs. The figure shows

the regulation of histone acetylation/deacetylation by HATs and HDACs (taken from

http://departments.oxy.edu/biology/Stillman/bi221/110300/rna_polymerases.htm)

Histone Kinase: Histone kinases also play important role in chromatin remodeling (Strahl and Allis, 2000). Several kinases phosphorylate H3, H2A/B and even H1; for example: MAP kinase, RSK2, Fyn kinase, PKA, Aurora kinase, Nima kinase, IKKα (Prigent and Dimitrov, 2003). CDKs are involved in cell cycle dependent phosphorylation of histone H1 (Swank et al., 1997; Deterding et al., 2008). Upon exposure to DNA damaging agents, H2AX was phosphorylated at Ser139 (Thiriet and Hayes, 2005) and upon UV exposure, H3 was also phosphorylated at serine 28 position by MAP kinase, MSK1/2 and/or MLTK-α (Dong and Bode, 2006). Evidence showed that TG2 possessed an intrinsic serine/threonine kinase activity and H3 serine10 was phosphorylated (Mishra et al., 2006). In *Drosophila* embryo, NHK1 phosphorylates Thr 119 of H2A which is related to cell cycle progression and chromosome dynamics (Aihara et al., 2004). Though researchers discovered many histone kinases and connected histone phosphorylation with biological functions, the detailed mechanisms are poorly understood.

Histone Methyltransferase and Histone Demethylase: Recent studies have identified few histone modifying enzymes which are involved in histone methylation. Histone methyltransferase catalyze the transfer of methyl group from the donor SAMe to the lysine and arginine residues of the N-terminal histone tails. They are classified as lysine methyltransferase (Martin and Zhang, 2005) and arginine methyltransferase (Wysocka et al., 2006). All the histone lysine methyltransferase (HLMTase) and arginine methyltransferase have conserved catalytic domain. All of the known HLMTase (SUV39h, G9a, GLP/Eu-HMTase1, SETDB1/ESET) preferentially methylate histone

octamer and are involved in transcriptional repression (Wang et al., 2003; Schotta et al., 2004; Tachibana et al., 2005). On the other hand, arginine HMTase is involved in transcriptional activation. For example, arginine HMTase PRMT4/CARM1 has been shown to activate p53 and NF-κb responsive genes (Covic et al., 2005).

Another important histone modifying enzyme was recently discovered in the field of histone lysine demethylase (Shi et al., 2004; Kubicek and Jenuwein, 2004). It was KIAA0601 (BHC110) protein which showed the demethylase activity. The recombinant BHC110/LSD1 (lysine specific demethylase1) catalyzes the amine oxidation of methylated histone H3 K4 to generate unmodified lysine (Shi et al., 2004: Kubicek and Jenuwein, 2004).

I.3 Aberrant Histone Modification and Human Disease

Histone modification plays a central role in the epigenetic regulation of transcription process and because of that, dysfunction in epigenetic regulation causes human disease. CBP/ p300 and PCAF play key role in cell differentiation, transformation and apoptosis; and somatic mutation of these HATs are found in primary human tumors (Goodman and Smolik, 2000; Chan and La Thangue, 2001; Timmermann et al., 2001). A germline mutation of CBP was connected to Rubinstein Taybi syndrome (Petrij et al., 1995). Cells expressing mutant TIP60 showed a defect in the repair of double-strand DNA breaks and inefficient induction of apoptosis in response to such DNA damage (Ikura et al., 2000). Several studies strongly linked TIP60 to Alzheimer's disease (Baek et al., 2002; Jepsen and Rosenfeld, 2002; Kim et al., 2005). Most significantly, loss of H4 Lys16 acetylation resulted in cell cycle arrest and becomes a common hallmark for cancers (Fraga et al., 2005; Shogren-Knaak et al., 2006). Aberrant modifications are also connected to dysfunction in HDAC activities. Synthetic and natural HDAC inhibitors showed great potential utility for alleviating aberrant transcriptional repression (de Ruijter et al., 2003; Marks et al., 2001). A study with HDAC inhibitor SAHA showed a marked change in gene transcription which coincides with acetylated histones in tumor cells (Mitsiades et al., 2004).

In addition, misregulations of HMTs are also linked to human disease. The deregulation of SUV39H1 caused destabilization in the chromatin structure followed by increased risk of cancer (Peters et al., 2001). Studies showed that SMYD3 upregulated oncogene expression in hepatocellular and colorectal carcinoma (Hamamoto et al., 2004). Another important histone modification, i.e. phosphorylation, is indirectly implicated in

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many syndromes related to DNA damage as phosphorylation of H2AX is induced in the response to double-strand break (Medvedeva et al., 2007). As all the histone modifications are involved in nuclear processes, it is hardly surprising that aberrant histone modifications have been implicated in a number of human diseases. Even though continued effort in analyzing all the histone modifications and their function is going on, more work is needed to identify the molecular mechanism which assists to design therapeutic tools.

I.4 Alcohol (Ethanol) and Surrogate Alcohols

Alcohols: Alcohol, specifically ethanol, consumption is one of the leading causes of liver injury all over the world. Alcohol is a generic name for a group of organic hydrocarbon. One or more of the hydrogen atoms have been replaced by a hydroxyl (-OH) group in these hydrocarbons. Alcohols are named according to the radical to which the –OH group is attached. In case of ethanol, ethyl (C_2H_5) group is attached to –OH. Ethanol (molecular weight 46) is a colorless, flammable liquid at room temperature. Its boiling point is $78^{\circ}C$ at atmospheric pressure and the freezing point is -114°C. The pure ethanol is 200 proof. Most alcoholic beverages contain ethanol (C_2H_5OH) up to 50%, although some exceed this level. Ethanol mixes in all proportions with water. After consumption, ethanol is readily distributed throughout the body in the aqueous blood stream because of complete miscibility with water. It readily crosses the blood brain barrier and affects a large number of organs and biological processes in the body. Although the major route of entry of ethanol into the body is by drinking alcoholic beverages, absorption of ethyl alcohol into the blood can occur through the skin and via the lungs.

Surrogate Alcohols: Alcohols with more than two carbon atoms are commonly known as higher chain or fusel alcohols. In the 19th century, the predominant view was that higher chain alcohols were contaminants of alcoholic beverages (Huckenbeck and Bonte, 1988). Recently, concerns have been raised about these surrogate alcohols due to the increased incidence of liver disease. There are diverse definitions about these surrogate alcohols in the literature: a) As a synonym of all nonbevarage alcohols, i.e. not intended for drinking; b) Denoting only nonbevarage alcohols outside production data;

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c) Denoting both nonbevarage alcohols and illegally produced or homemade alcohols. Though wider definitions of the term 'surrogate alcohols' are available, the information about the health consequences and the effects of these alcohols are unknown.

Further research on various disease endpoints should be undertaken with priority. Most of the higher chain alcohols (1-propanol, isobutyl alcohol, isoamyl alcohol, and 2-methyl-1-butanol) denoted as "flavoring agent" and are mentioned as safe at current levels (JECFA, 1997). Though they are found in both legal and illegal alcohols (Table 2), there is concern about the illegal surrogate alcohols as higher alcoholic liver disease has been reported among the consumers of homemade country liquor (Narawane et al., 1998). An animal study on rats showed that Indian country liquor had an increased toxicity compared to pure ethanol (Lal et al., 2001). Rats treated with high dose of corn fusel oil containing aldehydes, esters and a large number of higher alcohols showed severe hepatic damage (Gibel et al., 1969) and those results were confirmed by Peneda et al. (1994) and concluded that hepatotoxicity of ethanol may be enhanced by interaction with its surrogate alcohols. Strubelt et al. (1999) showed that the capacity of the straight chain alcohols to release the hepatotoxic marker (GPT, LDH and GLDH) into the perfusate rat liver was strongly correlated with their carbon chain-length. Sinclair et al. (2000) suggested that short-term consumption of alcoholic beverages rich in isopentanol may be a risk for developing liver damage from acetaminophen. More interestingly, it is suggested that isopentanol can contribute to increase in cytochrome P450 following consumption of alcoholic beverages contaminated with isopentanol (Louis et al., 1993). In addition to the liver, other organ damage (central nervous system, retinal, renal

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damage, etc.) were also identified with the consumption of these surrogate alcohols. Unfortunately, the exact pathways underlying this link are far from clear and research is necessary to better understand the mechanism in order to develop preventive therapeutics.

Table 2. Composition of alcohols in the samples (This table is taken from Kang et al., 2006).

| Substance	Ethanol (mol/l)	1-Propanol (mmol/l)	Isobutanol (mmol/l)	Isoamyl alcohol (mmol/l)
Medicines				
Tinctura menthae	13	n. d.	n. d.	14.825
Valerianae tinctura	10.6	n. d.	n. d.	n. d.
Vilkabelu tinctura	11.2	n. d.	n. d.	n. d.
Nastoika Bojarõshnika 18%	10.9	n. d.	n. d.	n. d.
Materu tinctura	11.8	n. d.	n. d.	n. d.
Aftershaves etc.				
Leda perfume water (lemon)	10.7	n. d.	n. d.	0.794
eau de cologne 'Landõsh'	13.8	n. d.	n. d.	n. d.
Dushistaja voda 'Persik'	9.4	n. d.	n. d.	n. d.
Gigienitsheski fitoaromat	11.6	n. d.	n. d.	0.437
Leda perfume water (raspberry)	10.6	n. d.	n. d.	n. d.
Troinoi cologne	11	n. d.	n. d.	n. d.
Lemon lotion aftershave	11.5	n. d.	n. d.	n. d.
Lõhnavesi 60 K	9.5	n. d.	n. d.	n. d.
Illegal alcohols				
Rural 'brown' moonshine	8.3	0.153	5.107	15.914
Rural moonshine	9.2	7.502	8.506	15.512
Illegal spirits from soup kitchen	6.9	n. d.	n. d.	n. d.
Illegal spirits from soup kitchen	8.3	0.035	4.259	9.614
Illegal spirits from soup kitchen	6.4	n. d.	n. d.	n. d.
Illegal spirits from north-east	8.3	0.406	4.355	9.433
Illegal spirits from north-east	5.7	0.266	4.122	9.192
Illegal spirits from north-east	7.6	n. d.	2.949	6.974
Illegal spirits from north-east	5.5	0.606	3.187	8.054
Fire lighting fuels				
Fire lighting fuel 'hot'	13.5	0.526	n. d.	n. d.
Fire lighting fuel	11.4	n. d.	n. d.	n. d.

I.4.1 Alcohol Metabolism

Hepatotoxic effects of ethanol are tied to ethanol metabolism. There are three major pathways known for ethanol metabolism. They are cytosolic alcohol dehydrogenase (ADH), hepatic catalase, and the microsomal ethanol oxidizing system (MEOS). Hepatic catalase is specially located in peroxisome and the MEOS system is found in the endoplasmic reticulum. Each of these pathways can produce ROS and is thus involved in liver injury.

In the classical pathway, ADH converts ethanol to acetaldehyde in an oxidation reaction that is coupled to reduction of NAD+. In this reaction, a hydride ion is transferred from ethanol to NAD+ (Cunningham and Bailey, 2001).

$$CH_3CH_2OH \ + \ NAD^+ \xrightarrow{\text{ADH}} CH_3CHO + \ NADH + H^+$$
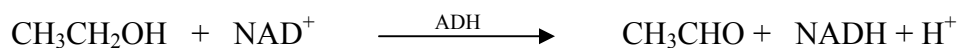
Majority of the conversion occurs by the cytosolic hepatic ADH. When large amounts of ethanol are consumed, an excessive amount of reduced NADH is formed which causes a variety of metabolic disorders (Lieber and Abittan, 1999).

Catalase can metabolize ethanol in the presence of hydrogen peroxide. Under in vivo condition, low hydrogen peroxide concentrations do not allow this reaction to proceed (Inatomi et al., 1989).

$$CH_3CH_2OH + H_2O_2 \xrightarrow{\text{Catalase}} CH_3CHO + 2H_2O$$
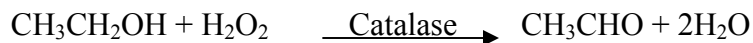
Another important system, the MEOS, is also involved in oxidation of ethanol. Ethanol can be oxidized to acetaldehyde via cytochrome p450 isoenzymes (Lieber et al., 1970). There are three isoforms- 2E1, IA2 and 3A4 with varying degree of ethanol oxidizing property (Salmela et al., 1998). Cyp450 enzyme is induced by ethanol

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consumption. Thus, the mechanism becomes more significant in the case of alcoholic liver disease (Lieber, 1997).

$$CH_3CH_2OH + NADP^+ \xrightarrow{\text{Cyp 450}} CH_3CHO + NADPH + H^+$$
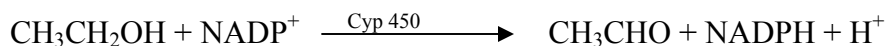
I.4.2 Alcoholic Liver Disease

Since the liver is the primary site of alcohol metabolism, it is most susceptible to alcohol-related injury. The injury to the liver from long-term drinking apparently comes not only from ethanol, but also from the products generated during ethanol metabolism. Alcohol induced liver injury progresses through different characterized stages (Diehl, 2005; Powell et al., 2005). These are characterized by fatty liver, inflammation, hepatocyte necrosis, fibrosis and ultimately cirrhosis.

Fatty liver indicates fat disposition in the liver. A single drinking session or chronic ethanol consumption can lead to fatty liver. This is reversible and may not lead to more serious liver problems (Eaton et al., 1997).

Hepatic fibrosis is a consequence of excessive deposition of extracellular matrix proteins by hepatic stellate cells in response to oxidants, cytokines and other paracrine factors. These are produced in hepatocytes, Kupffer cells and infiltrating blood cells (Friedman, 1998).

The next stage is cirrhosis. Alcoholic cirrhosis is the most advanced form of liver disease and is diagnosed in 15 to 30 % of heavy drinkers. This is the stage preceding liver carcinoma. Between 40 to 90% of the 26,000 annual deaths from cirrhosis are alcohol related (Dufour et al, 1993). Cirrhosis is described by extensive scar tissue (fibrosis) that

stiffens blood vessels and distorts the internal structure of the liver (Albanis and

Friedman, 2001). This stage is irreversible. Cirrhosis also causes failure of other organs

such as the brain and kidneys.

I.5 Alcohol, Oxidative Stress and Liver injury

The molecular mechanisms of alcohol induced liver injury are unknown. It is now well established that the progression of liver injury is a multifactorial event. Among these factors, the attention has focused on reactive oxygen species (ROS). The involvement of ROS in ethanol toxicity was first reported in 1963 by Di Luzio. It was shown that ethanol exposure enhanced lipid peroxidation which could be prevented by antioxidants. Subsequently, oxidative protein modification and enzyme inactivation (Rouach et al., 1997), mitochondrial damage (Cunningham et al., 1990; Albano, 2006), antioxidant depletion (Fernandez-Checa et al., 1987; Nordmann et al., 1992; Albano, 2006), and NADPH oxidase activation (Zhan et al., 2006) contributed to the development of alcoholic liver disease. ROS are small, highly unstable (because of the distribution of electrons within the molecule), oxygen containing species that can damage other cellular molecules in any organ, particularly in liver (Wu and Cederbaum, 2003). Overproduction of ROS in the body is called oxidative stress (Halliwell, 1999).

Oxidative stress is linked to ethanol metabolism. Alcohol dehydrogenase metabolizes ethanol and forms acetaldehyde and free radicals, and as a result, significantly increases the NADH/NAD+ redox ratio (Cederbaum, 1991) leading to the oxidative stress. The MEOS system also oxidizes ethanol with the help of cytochrome P450 isoenzymes (Lieber and DeCarli, 1970). Elevated isoenzymes were shown to generate higher amount of H_2O_2 (Nordsblom and Coon, 1977) as well as hydroxyl radicals in liver (Klein et al., 1983). Peroxisomal activity also oxidizes ethanol. Due to increased peroxisomal activity, accumulation of fatty acid in the liver was observed in case of heavy drinkers (Rubin et al., 1972; Donohue, 2007).

Distorted mitochondrion is one of the earliest effects of ethanol consumption

(Kiessling and Tobe, 1964). Rubin et al. demonstrated that these mitochondrial changes

were accompanied by the development of fatty liver in rat model (Rubin et al., 1972).

The increased mitochondrial production of O_2^- at complexes I and III, and consequently

H_2O_2 and other ROS , are the major causes of mitochondrial and cellular oxidative stress

and damage in acute and chronic alcoholism (Bailey et al., 1999; Bailey and

Cunningham, 2002). Evidence showed that alcoholism is associated with decreased level

of iron sulfur centers in complex I which leads to higher level of O_2^- production (Thayer

and Rubin, 1980; Bailey and Cunningham, 2002). Chronic ethanol feeding also leads to a

decrease in cytochrome c which increases the level of ubisemiquinone and causes higher

rates of O_2^- production (Bailey and Cunningham, 2002; Thayer and Rubin, 1981;

Coleman and Cunningham, 1990). In addition, a decrease in the heme content of

cytochrome c oxidase induced by chronic alcoholism may enhance mitochondrial O_2^-

production (Thayer and Rubin, 1981). Like chronic alcoholism, acute ethanol exposure

also stimulates mitochondrial ROS production (Bailey et al., 2001; Cahill et al., 2002).

During ethanol metabolism, NADH is generated and is shuttled to the mitochondria.

Inside the mitochondria, NADH serves as a substrate for the electron transport chain

(ETC). Hence, excess NADH causes increased flux of electrons and the ETC becomes

more reduced which facilitates the transfer of an electron to molecular oxygen to generate

the superoxide anion. Therefore mitochondrial ETC is the major intercellular source of

this ROS production (Bailey and Cunningham, 1999).

In relation to mitochondrial ROS, the endogenous glutathione–glutathione

peroxidase system and catalase are also important antioxidants and cytoprotective

machinery in the hepatocytes exposed to ethanol (Ishii et al., 1997). It is assumed that glutathione is synthesized in the cytosol and transported into the mitochondria to maintain a separate pool of glutathione (Mittur et al., 2002). Ethanol is known to deplete GSH levels via the generation of oxidants and inhibition of mitochondrial glutathione transporter (Colell et al., 1998; Wheeler et al., 2001). A carrier that transports GSH from the cytosol to the mitochondrial matrix becomes defective in chronic ethanol feeding which causes depletion in GSH level in the mitochondria (Colell et al., 1997) and this is mediated by decreased fluidity of the mitochondrial inner membrane (Fernandez-Checa et al., 1998a; Fernandez-Checa et al., 1998b). Alcohol also disrupts methionine and oxidative balances which may lead to alcoholic liver disease (Yalçinkaya et al., 2007). S-adenosyl-L-methionine (SAMe) acts as a methyl donor for methylation reactions and participates in the synthesis of glutathione. In experimental models, alcohol consumption results in a selective decrease in the mitochondrial GSH stores, which sensitize hepatocytes to TNF-α mediated cell death (Fernandez-Checa, 2003). Chronic ethanol consumption induces oxidative modification of liver mitochondrial proteins by altering glutathione system, leading to adverse effects on the liver (Bailey et al., 2001).

Another potential source of ROS is the NADPH system. NADPH oxidase (nicotinamide adenine dinucleotide phosphate oxidase) is a multi-subunit enzyme comprising of both membrane bound and cytosolic subunits. It is comprised of membrane bound subunits, including p22 phox and gp91 phox and cytosolic subunits, including p47 phox, p40 phox, p67 phox and Rac1/2, the small GTP-binding protein (Bedard & Krause 2007). Upon stimulation, the cytosolic subunits become phosphorylated and translocate to bind with the membrane subunits. This enzyme catalyzes the conversion of molecular

oxygen to superoxide anion by transferring electrons from NADPH (Quinn & Gauss 2004). Recent studies have identified several novel homologs of gp91 phox (Nox2) in animals that constitute the Nox family. The human genome contains 7 members: Nox1 through Nox5 and the dual oxidases Duox1 and Duox2 (Sumimoto et al., 2005). Studies have shown that differential expression of these homologs in different cell types and also different intracellular localization (Sumimoto et al., 2005). Scientists generally experiment with the mice that are genetically deficient for either gp91 phox (Pollock *et al.* 1995) or p47 phox (Jackson et al., 1995) or NADPH oxidase inhibitors DPI and apocynin (O'Donnell et al., 1993; Pearse & Dodd, 1999). However, there are several controversies about the specificity of these inhibitors. More studies are needed to find specific NADPH oxidase inhibitor.

There are two types of NADPH: phagocytic and nonphagocytic. In contrast to phagocytes, nonphagocytes constitutively produce low levels of ROS and also differ in the homologues as compared to phagocyte. Hepatocytes express both types of enzymes. Kupffer cells contain phagocytic form whereas hepatic stellete cell contain nonphagocytic form of NADPH oxidase. Alcohol metabolites activate NADPH oxidase to produce ROS which exert proinflammatory effects and hepatocytes undergo apoptosis. Emerging evidence support the fact that Kupffer cell derived ROS mediates hepatocellular damage and fibrosis in patients with alcoholic liver disease (Hasegawa et al., 2002; Wheeler et al., 2001). Beside Kupffer cell, there is overwhelming evidence suggesting the involvement of NADPH oxidase system of hepatocytes and hepatic stellete cells in alcoholic liver injury (De Minicis and Brenner, 2008).

I.6 Primary Rat Hepatocytes and HepG2 Cell line (VA-13)

Almost 70-80% of the liver consists of hepatocytes (parenchymal cells). These cells are involved in protein synthesis, protein storage and transformation of carbohydrates, synthesis of cholesterol, bile salts and phospholipids, detoxification, modification and excretion of exogenous and endogenous substances. They have a polyhedral shape and are arranged in layers, called hepatic laminae that typically are one-cell thick. Primary monolayer culture of collagenase-prepared rat hepatocytes represents a homogenous preparation. A successful preparation of isolated rat hepatocytes should retain all the functional properties of intact liver. Isolated rat hepatocytes showed similarities in various functions including gluconeogenesis, ketogenesis, oxygen uptake, maintainence of adenine nucleotides, cholesterol and fatty acid synthesis, ethanol oxidation when compared to the perfused or intact organ or liver in vivo (Cook et al., 1977; Mapes et al., 1975; Kulkosky and Cornell, 1979). However, isolated rat hepatocytes lack non-parenchymal cells, interstitial matrix and other elements of intact liver. It still serves as a useful model for examining the effects of different agents such as alcohol.

HepG2 (hepatocellular carcinoma cell line) cells are also considered an excellent model to study liver pharmacology. They also offer advantage in long-term studies in contrast to primary rat hepatocytes. After 3-4 days, primary hepatocytes loose their healthy polyhedral shape and most of the cells are detached from the collagen coated dish. Hepatocytes cultured in vitro for extended periods lose the ability to express many liver specific functions including alcohol metabolizing ability (Gregor et al., 2008). Although HepG2 cells are phenotypically similar to hepatocytes, they express less

alcohol dehydrogenase. Clemens et al. developed a hepatic cell line that stably and constitutively expresses alcohol dehydrogenase (Clemens et al., 1995; Clemens et al., 2002). In this case, the HepG2 cell line was stably transfected with a eukaryotic expression plasmid designed to express alcohol dehydrogenase; this cell line is called VA-13 (Donohue et al., 2006).

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

Alcohol is associated with diverse cellular effects in the body. However, the specific mechanisms or specific components that may play role in the actions of alcohols remain unknown. It has been shown that alcohol consumption changes the gene expression in the liver. Emerging evidence highlight the importance of histone modifications in this process. For example, histone acetylation is strongly correlated with transcriptional activation. Our laboratory has observed that ethanol increases histone H3 acetylation selectively at Lys 9 (H3-Lys9) but not at Lys 14, 18 or 23 in primary cultures of hepatocytes as well as in the liver of rats administered ethanol in vivo (Park et al., 2003; Kim and Shukla, 2006). In addition to ethanol, sometimes alcoholic beverages also contain other carbon chain-length alcohols often called surrogate alcohols. Though ethanol modulates histone H3 acetylation, the molecular mechanism of ethanol and other long chain alcohols in relation to histone modification are unknown.

Hypothesis:

The hypothesis of this research project is that "Ethanol induced histone acetylation is mediated by oxidative stress and involves a specific HAT in the liver". There are three specific aims in my dissertation.

Specific Aims:

1. Determine the effects of surrogate alcohols on H3 acetylation and its characteristics in primary rat hepatocytes.

i) Effects of methanol, ethanol, propanol, butanol, pentanol, hexanol and octanol on histone H3 acetylation at specific lysine residues will be examined.

ii) Determine if surrogate alcohols sensitize ethanol induced H3 acetylation.

iii) The effects of these alcohols on the hepatocyte toxicity will be determined.

iv) The effect of alcohol metabolites on histone H3 acetylation will be examined.

v) Determine if alcohol or metabolites modulate HATs and or HDACs.

2. Determine the role of oxidative stress in ethanol induced histone H3 acetylation and alcohol dehydrogenase 1 (ADH1) expression.

i) The effect of glutathione modulators on histone H3 acetylation will be examined.

ii) The effect of dietary antioxidants on histone H3 acetylation will be examined.

iii) The effect of glutathione modulators on ethanol induced ADH1 gene expression will be examined.

iv) The source of oxidative stress in ethanol induced histone H3 acetylation will be determined.

3. Identification of specific HAT which is responsible for ethanol induced H3 acetylation in ADH1 overexpressing human hepatoma cell (VA-13).

i) Effect of ethanol on histone H3 acetylation will be examined in VA-13 cells.

ii) Effect of ethanol on the expression of GCN5 will be determined.

iii) Knockdown of specific HAT (GCN5) will be carried out with siRNA methodology.

iv) The acetylation level in GCN5 knocked down cells with the treatment of ethanol will be examined.

Significance:

Alcohol consumption leads to liver damage. But drinking illegal and contaminated alcohol expedites the damage and also leads to death. Growing evidence indicate a relationship between ethanol and histone modifications involving gene expression. This project aims to establish a new concept related to the long carbon-chain alcohols and histone modifications and regulation of histone modifying enzymes. In addition, involvement of oxidative stress in ethanol induced histone modification will be valuable information in the case of alcoholic liver injury. Most importantly, molecular target(s) for the treatment of alcoholic liver disease are not clearly identified. So identification of specific HAT responsible for alcohol will be a novel finding in alcohol research. It will offer a new insight into molecular mechanisms of actions of alcohol and its relevance to the cellular response. Such molecular understanding is essential for the development of specific therapeutic tools to prevent and control alcohol induced cellular damage in liver. Identification of such novel regulator itself would constitute a significant advance in this field as they can be the subject of future studies on potential target for intervention.

Chapter II

Surrogate alcohols and their metabolites modify histone H3 acetylation: Involvement of histone acetyl transferase and histone deacetylase

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II.1 Abstract

Ethanol increases histone H3 acetylation in the rat liver. However, the effect of other carbon chain length alcohols, consumed as surrogate alcohols and used in industry, on H3 acetylation is unknown. Hence, we investigated the effect of these alcohols on histone H3 acetylation, cell toxicity and HAT and HDAC activity. Primary cultures of rat hepatocytes were incubated with selected concentration (40 mM) of different chain length alcohols with or without inhibitors of alcohol metabolizing enzymes. Cells were also treated with low concentration (2.5 mM) of 1-propanol or 1-butanol or isopentanol, with or without 40 mM ethanol for 24 hr. Effects of the metabolites of these alcohols were also studied. Cytotoxicity was determined by lactate dehydrogenase (LDH) release and mitochondrial activity (MTT assay). The degree of histone H3 acetylation at specific lysine residues were monitored by western bloting using site specific antibodies. Histone acetyltransferase (HAT) and histone deacetylase (HDAC) activities were measured by enzyme-linked immunosorbent assay (ELISA) and colorimetric assay respectively. Alcohols with increasing carbon chain length exhibited a variable effect on the ratio of acetylated lys9 histone H3 to β-actin. A graded increase (methanol< ethanol< 1-propanol< 1-butanol) followed by a gradual decrease (1-butanol> 1-pentanol> 1-hexanol> 1-octanol) in the ratio was observed. Other lysine sites were not affected. HAT activation also corresponded to the acetylation profile. These alcohols or their metabolites did not significantly alter HDAC activity in the hepatocytes. Low concentration (2.5 mM) of 1-propanol alone did not affect acetylation, but sensitized the ethanol induced H3 acetylation at lysine 9 (H3AcK9). 1-Butanol and isopentanol also increased the response of ethanol induced H3AcK9. Alcohol metabolizing inhibitors attenuated ethanol and

propanol induced increase in H3AcK9. Carboxylic acid metabolites of these alcohols also increased HAT activity and histone H3 acetylation at lysine 9. Propionate and butyrate modestly inhibited HDAC activity in an in vitro assay. Surrogate alcohols modulate H3AcK9 via increasing HAT activity and this is dependent on their metabolism. Furthermore, alcohol metabolites also increased H3AcK9, but in contrast, exhibit both HAT activation and HDAC inhibition.

II.2 Introduction

Alcohol, specifically ethanol, consumption is one of the leading causes of liver injury all over the world. In addition to ethanol, sometimes alcoholic beverages also contain, as contaminants, other carbon chain length alcohols such as propanol, isobutanol, isopentanol (Ribereau-Gayon and Peynaud, 1970; Greenshields, 1974; Lisle et al., 1978), often called surrogate alcohols. The concentration and the type of contaminant alcohols can be different. Surrogate alcohols may contain upto 7.5 mM of 1-propanol, 8.5 mM of isobutanol and 15.9 mM of isoamyl alcohol (Lang et al., 2006). Recent studies in Russia identified an unexpectedly high frequency of consumption of surrogate alcohols (McKee et al., 2005). Alcohols used in the rural areas of north-east Estonia in particular were found to contain long chain alcohols. Most of these contaminated alcohols are substantially more hepatotoxic than ethanol (Strubelt et al., 1999). Interestingly, many of these alcohols are also widely used as industrial solvents, components of automobile fluids, co-solvents and chemical intermediates (Nelson et al., 1990) and humans are exposed to them. Consumption of large quantities of the illicit alcohol has deleterious effects in many organs, particularly the liver. This is supported by the studies from Mexico City (Narro-Robles et al., 1992), Slovenia, Hungary, Romania, Moldova (McKee M et al., 2000, 2005; Pomerleau J, 2005), India (Narawane et al., 1998), all of them showing high death rates due to hepatic cirrhosis.

For several decades, it has been thought that the primary function of histone is to pack nuclear DNA and organize it into higher order chromatin structure (Kornberg and Lorch, 1999). However, recent studies have discovered that post-translational modifications of histone can alter the molecular activity of the cell and this raises the

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probability of an unparalleled degree of control and fine-tuning within the cell (Rice et al., 2001; Strahl et al., 2000; Jenuwein et al., 2001). Among the many histone modifications, acetylation has received most attention because site-specific acetylation of histones influences chromatin organization and is central to the switch between repressive and permissive chromatin structure. Irregular acetylation and deacetylation of histones have been linked to various developmental diseases, leukaemia, and fragile X-syndrome (Timmermann et al., 2001). The enzymes, histone acetyltransferase (HAT) and histone deacetylase (HDAC), add and remove acetyl groups to and from target lysine residues within histones, respectively (Kuo and Allis, 1998; Peterson and Laniel, 2004). Aberrant activities of several of these enzymes have been implicated in human disease, making them important drug targets. Recent evidence from this laboratory suggested that ethanol increases the acetylation of histone H3 at lysine 9 in primary culture of rat hepatocytes (Park et al., 2003) and that the acetylation event correlates with ADH1 expression (Park et al., 2005). Such histone modifications may underlie the mechanisms involved in ethanol induced cellular injury (Shukla and Aroor, 2006). This prompted us to investigate the effect of surrogate alcohols on histone acetylation in liver cells.

II.3 Materials and methods

Reagents

Polyclonal rabbit anti-acetylated histone H3 antibodies targeting specific lysine positions at 9, 14, 18, 23 or 27; sodium butyrate and nonradioactive HAT activity assay kit were purchased from Upstate Biotechnology (Lake Placid, NY). The 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). Protease inhibitors (aprotinin, pepstatin A, PMSF, and leupeptin) and monoclonal mouse anti-β-actin, 4-methylpyrazole (4-mp), cyanamide, sodium propionate, MTT were obtained from Sigma (St. Louis, MO). Ethanol, methanol, 1-propanol, 1-butanol, sodium acetate were purchased from Fisher Scientific (Fair Lawn, NJ); 1-pentanol and 1-hexanol from Acros Organics (NJ); 1-octanol and isopentanol (3-methyl-1-butanol) were obtained from Sigma -Aldrich (Milwaukee, WI). All alcohols were ≥98% pure. Cytotoxicity Detection Kit was purchased from Roche (Indianapolis, Indiana). HDAC colorimetric assay kit was purchased from Biomol (Plymouth Meeting, PA).

Isolation and Culture of hepatocytes

Rat hepatocytes were isolated from male Sprague-Dawley rats (200–250 g) using a collagenase perfusion protocol (Weng et al., 2000). Viability of isolated hepatocytes was ≥90% (trypan blue exclusion). Isolated hepatocytes were plated on collagen-coated dishes ($7\mathrm{x}\ 10^6$ cells/ 100 mm dish) in DMEM containing 10% FBS, 2 mM L-glutamine and 100 units/ml penicillin and 100 µg/ml streptomycin. The animal protocols were

approved by the University of Missouri Institutional Animal Care and Use Committee (IACUC).

Treatment of Cells

Isolated hepatocytes were allowed to attach to culture dishes for 2 hr and then treated with different concentrations of alcohols, inhibitors or other agents in DMEM containing 0.1% FBS, 2 mM L-glutamine and 100 units/ml penicillin and 100 µg/ml streptomycin for 24 hr. Duplicate sets of culture dishes were used for each treatment. The petridishes were covered with parafilm and clingwrap to avoid the evaporation of alcohols.

Preparation of nuclear extract

Hepatocytes were washed twice with cold 1X PBS, scraped, and resuspended in 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 and 0.25% NP-40. Lysates from the duplicate dishes were combined together and then lysed by 10 passages through 26-gauge syringe needle. Nuclei were pelleted by centrifugation at 14,000 g for 20 s. Proteins were extracted from nuclei by resuspending in the buffer containing 20 mM Hepes, 1 mM EDTA, 420 mM NaCl, 1 mM dithiothreitol (DTT), 20 mM glycerophosphate, 1 mM sodium orthovanadate, 2 mM $MgCl_2$, 1 mM PMSF, 10 µg/ml leupeptin, aprotinin, pepstatin A; 25% glycerol, and 0.25% NP-40 followed by sonication. Debris was pelleted by centrifugation at 14,000 g for 20 s and discarded. The supernatant (pure nuclear extract-investigated by Lee and Shukla, 2007) was used after determining its protein concentration by the Bio-Rad DC protein assay kit.

Western blot analysis

Identical amounts (40 μg) of nuclear extracts were run on 15% SDS–PAGE and transferred onto nitrocellulose membrane. After blocking with 5% nonfat dried milk in water for 1.5 hr, membrane was incubated separately with primary antibody with the dilution of 1:2000 for anti-H3 Lys9, 1:3000 for anti-H3 Lys14, 1:10000 for anti-H3 Lys18, 1:1000 for anti-H3 Lys23, 1:2000 for anti-H3 Lys27. When incubating with specific acetylated lysine antibody, the same membrane was also incubated at the same time with 0.25 μg/ml concentration of anti β-actin antibody for overnight at 4^0C. Membranes were incubated with horseradish-conjugated secondary antibody (both anti-mouse and anti-rabbit) with the dilution of 1:2000 (except in the case of Lys18, the dilution of secondary anti-rabbit was 1:5000) for 1 hr at room temperature. The horseradish peroxidase was detected by enhanced chemiluminescence (Supersignal, Pierce) and exposed to film. The bands were subjected to laser densitometry for quantitation using Bio-Rad Quantity One Software.

LDH release assay

The integrity of the cell membrane was measured using Cytotoxicity Detection Kit following manufacturer's protocol. Culture supernatant was collected (cell free) after 24 hr treatment with different alcohols and also from control dishes.The cell-free supernatant was incubated with the substrate mixture from the kit. LDH activity was determined in a coupled enzymatic reaction. During this reaction, the tetrazolium salt INT was reduced to formazan. In the first step of the enzymatic reaction, NAD+ is reduced to NADH/H+ by the LDH-catalyzed conversion of lactate to pyruvate. In the second step, the catalyst (diaphorase) transfers H/H+ from NADH/H+ to the tetrazolium

salt INT which is reduced to formazan. During the assay, LDH enzyme activity in the culture supernatant increases as the number of dead cells (or cells with damaged plasma membranes) increases. The increase in supernatant LDH activity directly correlates to the amount of formazan formed over time. The percentage of LDH release was expressed as the proportion of LDH released into the medium compared to the total amount of LDH released when the untreated (control) cells were lysed with 1% Triton X-100 (v/v). The activity was monitored at 530 nm with plate reader (Fusion ^{TM} α, Packard Bioscience Company).

MTT assay

Cell viability as well as mitochondrial activity was determined with a spectrophotometric assay using 3-[4, 5 Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide or thiazolyl blue (MTT) (Carmichael et al., 1987). After 24 hr treatment, MTT solution was added to the cells and incubated for 4 hr at 37°C. This water-soluble tetrazolium salt is cleaved by the mitochondria of living cells to an insoluble purple formazan. Formazan was next dissolved with DMSO and optical density was measured at 490 nm with plate reader (Fusion ^{TM} α, Packard Bioscience Company).

HAT assay

The effect of alcohols and their metabolites on HAT activity were measured by HAT activity assay kit according to the manufacturer's protocol. This was done by two experimental approaches. The nuclear extracts of hepatocytes treated with alcohols or metabolites for 24 hr were used in one assay. In the second assay, the nuclear extracts from control hepatocytes (cultured for 24 hr) were used. At first streptavidin-coated ELISA plate (96 well) was incubated with 100 μl of 1 μg/ml biotinylated histone H3

peptide (unmodified, not acetylated in lysine positions). The wells were washed with

Tris-buffered saline (TBS) and incubated with 3% BSA for 30 min. After washing the

wells with TBS, 50 μl of reaction cocktail (10 μl of 5x HAT assay buffer, 10 μl of 500

μM acetyl-CoA, 5 μl of 500 mM Na butyrate, 40 μg nuclear extracts and the rest was

adjusted with sterile water) was added to each well and incubated for 35 min at 30°C to

start acetylation of the H3 peptide. Since the sodium butyrate (known HDAC inhibitor) is

added in the HAT assay, this assay determines true HAT activity. In the second assay, we

added the alcohols or metabolites to the HAT reaction cocktails and then the control

nuclear extracts were added to the mixture. The concentration of all the alcohols was 40

mM. For the metabolites, 10 mM sodium acetate, 10 mM sodium propionate or 5 mM

sodium butyrate were added to the control nuclear extract. Rest of the assay was similar

for both the experimental approaches. After the incubation, the wells were again washed

with TBS and incubated with 100 μl of anti-acetyl-lysine antibody (1:250 diluted with

TBS) for 2 hr. The wells were again washed with TBS and 100 μl of anti-rabbit IgG

conjugated with HRP (1:5,000 diluted with TBS) were added and incubated for 1 hr.

Tetramethyl-benzidine (TMB) substrate mixture (100 μl) was added to each well and

incubated for 10 min. Sulfuric acid (50 μl) was added to stop the HRP reaction.

Colorimetric change was measured by plate reader at a wavelength of 450 nm with a

reference wavelength of 570 nm. All treatments were done in triplicate sets.

HDAC assay

This assay was performed using the colorimetric HDAC activity assay kit

according to manufacturer's instructions. Similar to HAT assays above, two experimental

approaches were taken to assay the HDAC activity. The nuclear extracts (source of

HDAC activity) from hepatocytes treated with alcohols or metabolites for 24 hr were used in one set of assays. In the second assay, the nuclear extracts from control hepatocytes cultured for 24 hr were used. In the first assay, 50 μg of nuclear extracts from the cells treated with alcohols or metabolites were incubated with 500 μM of colorimetric deacetylase lysyl substrate for 15 min at 37°C in a total volume of 50 μl. In case of direct in vitro assay, alcohols and metabolites were added to the substrate and then control nuclear extract was added to the mixture and incubated for 15 min at 37°C. Subsequently, 50 μl of 1x developer was added to the sample and again incubated for 15 min at 37°C. Colorimetric changes were measured at 405 nm using a plate reader. Treatments were conducted in triplicate sets.

To ensure that the direct addition of these alcohols and metabolites in the in vitro assay did not produce any artifactual effects in colorimetric assay, control experiments with HeLa extract were done. It was observed that the alcohols and metabolites, added after the substrate incubation or added after the color development or added before the substrate addition, did not artifactually affect the assay.

Statistical analysis

Data were combined from 3 separate experiments. Statistical analyses were performed using a standard one way ANOVA (Newman-Keuls Multiple Comparison Test). Differences with p< 0.05 considered significant. In all the figures, the bar without significance sign did not show any statistical significance.

II.4 Result

In the present study, we have investigated the effects of different carbon chain length alcohols on histone H3 acetylation and on cytotoxicity (mitochondrial dysfunction, LDH release) in cultured rat hepatocytes. The effects of ethanol alone and in combination with other alcohols on histone H3 acetylation were also analyzed. We further explored the effect of alcohol metabolites on HAT and HDAC activity. The in vivo peripheral blood concentration of ethanol in chronic alcoholics can reach ~ 50 mM (see Deitrich & Harris, 1996). Liver cells are exposed to higher concentration of ethanol than what might be expected in the peripheral blood (Elmer et al., 1982; Luca et al., 1997; Nuutinen et al., 1984, Orrego et al., 1988). We therefore selected 40 mM for each alcohol. The results are described below.

Effect of increasing carbon chain length alcohols on histone H3 acetylation at lysine 9 and other lysine residues

Previously it was shown that ethanol increases the acetylation of H3 lysine 9 in primary rat hepatocytes after 24 hr treatment with a maximal response at 100 mM (Park et al., 2003). In this study, we determined the effect of other carbon (C) chain length alcohols (40 mM for 24hr) on histone H3 acetylation. A variable effect on histone acetylation at lysine 9 (Fig. 1) was observed. The ratio of acetylated histone H3 to β-actin was determined in order to normalize the sample recovery and loading. A graded increase in the acetylation was observed from methanol (C1) to ethanol (C2), 1-propanol (C3) and 1-butanol (C4) i.e. methanol< ethanol< 1-propanol< 1-butanol. Increasing the carbon chain length further to 1-pentanol (C5), 1-hexanol (C6) or 1-octanol (C8) showed a

gradual decline in the ratio, i.e. 1-butanol> 1-pentanol> 1-hexanol> 1-octanol. The ratio

of acetylated histone H3 to total histone also exhibited similar pattern (data not shown).

1-Propanol and 1-butanol induced the highest, 3 fold, increase in H3 acetylation at lysine

9 compared with untreated control ($p<0.001$). Primary rat hepatocytes exposed to

methanol, ethanol, propanol, or butanol, all showed the typical morphology of mature

hepatocytes with mainly mono- and binuclear cells. The nuclei remained round shaped

and intact until the chain length was increased beyond that of butanol (data not shown).

Next, we investigated the effect of these alcohols on H3 acetylation at other lysine

residues (lys14, lys18, lys23 and lys27) using site-specific anti-acetyl histone H3

antibodies. Statistically insignificant changes in the acetylation at these lysine residues

were observed (Fig. 2).

Effect of increasing carbon chain length alcohols on LDH release from hepatocytes

Release of the cytoplasmic enzyme lactate dehydrogenase into the medium of the

cells is a marker for disruption of the cell membrane integrity and an index of cell death.

No marked increase in the LDH activity was observed in the culture supernatant of the

cells after 24 hr treatment with 40 mM of methanol, ethanol, 1-propanol, 1-butanol or 1-

pentanol. LDH release was substantially increased in 1-hexanol and 1-octanol treated

cells (Fig. 3).

Differential effect of various carbon chain length alcohols on hepatocyte

mitochondrial function

We next determined the mitochondrial activity by MTT assay. As shown in the

Fig. 4, no change in the mitochondrial function was observed when cells were treated

with 40 mM of methanol, ethanol, 1-propanol or 1-butanol for 24 hr**.** However, a large

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reduction in the mitochondrial activity compared to control was observed when rat hepatocytes were treated with 40 mM of 1-pentanol, 1-hexanol or 1-octanol for 24 hr. The reductions were about 54% for pentanol, 56% for 1-hexanol and 74% for 1-octanol.

Effect of treatment of hepatocytes with different alcohols on HAT activity

We evaluated the HAT activity using an ELISA HAT assay kit (Park et al., 2005; see Materials and Methods). For HAT assays, we selected three different alcohols representing three points in the Gaussian curve (Fig. 1). The nuclear extracts from the hepatocytes treated with 40 mM of ethanol, 1-propanol or 1-pentanol for 24 hr were studied for HAT activity. They all showed elevated HAT activities compared to control (Fig. 5). Similar to Fig. 1, a Gaussian curve for HAT activity was observed. For propanol, a 4.7 fold increased HAT activity was noted compared to control, and is consistent with the elevated H3 acetylation profile (Fig. 1).

HDAC activity in rat hepatocytes treated with different alcohols

The acetylation and deacetylation of histone proteins is influenced by HAT and HDAC enzymes, respectively. HDAC activity was therefore also measured using a HDAC colorimetric activity assay kit. The same samples as used above for HAT assays were utilized to determine the HDAC activity in the nuclear extract. We did not find any increase in HDAC activity by any of the alcohols tested (Fig. 6).

Sensitization of ethanol induced H3 acetylation by other alcohols

Surrogate alcohols contain long chain alcohols such as 1-propanol, 1-butanol and isopentanol. We investigated whether low concentrations of 1-propanol, 1-butanol or isopentanol would increase (or sensitize) ethanol- induced histone H3 acetylation in rat hepatocytes. Since 2.5 mM isopentanol in combination with ethanol increased P450 and

drug metabolism in rat hepatocytes (Louis et al., 1993, 1994), we used 2.5 mM of each of the long chain alcohol in our study. At 2.5 mM, 1-propanol by itself had little effect on acetylation (Fig. 7a). However, cells treated with 2.5 mM 1-propanol and 40 mM ethanol together for 24 hr, showed a dramatic increase in H3 lys9 acetylation. The increase in acetylation was statistically significant compared to control or ethanol or 1-propanol alone ($p < 0.001$) (Fig. 7a). Although 1-butanol alone at 2.5 mM showed an increase in acetylation compared to control ($p < 0.001$) or ethanol ($p < 0.05$); it also sensitized the ethanol-induced effect on acetylation when combined with ethanol (Fig. 7b). Isopentanol showed similar result as butanol (Fig. 7c). Similar results were also observed when 20 mM and 100 mM ethanol were used in combination with 2.5 mM isopentanol (data not shown). The cells treated with 5 mM 1-propanol or 1-butanol showed increased acetylation compared to 40 mM ethanol alone, but combination of 5 mM 1-propanol or 1-butanol with 40 mM ethanol showed no significant increase compared to 5 mM 1-propanol or 1-butanol induced acetylation (data not shown). Thus, sensitization of ethanol induced H3-acetylation was observed at lower concentration of surrogate alcohols. No significant changes were observed in the MTT and LDH assay in these combination experiments when compared to control (data not shown). However, treatment with 40 mM of 1-propanol, 1-butanol or 1-pentanol together with 40 mM ethanol, caused detachment of cells.

Involvement of alcohol metabolism in the H3 acetylation at lys9

To further characterize the mechanism of this acetylation, we investigated the role of alcohol metabolism. Hepatocytes were treated with ethanol or 1-propanol (40 mM) in the absence or presence of metabolizing enzyme inhibitors; 4-methylpyrazole (4-mp) for

alcohol dehydrogenase (ADH) and cyanamide for aldehyde dehydrogenase (ALDH). ADH and ALDH are known to be the important alcohol metabolizing enzymes present in liver and are inhibited by 4-mp and cyanamide respectively (Cheung et al., 2003). With 4-mp treatment, acetylation was reduced to almost basal level in ethanol or propanol-treated hepatocytes (Fig. 8a). Likewise, cyanamide also decreased the histone H3 acetylation in alcohol-treated hepatocytes (Fig. 8b). Thus, both inhibitors reduced the ethanol- or 1-propanol induced increase in histone acetylation at lys9. To further extend this observation, we treated hepatocytes with carboxylic acid products of these alcohols i.e. acetic acid (as sodium acetate), propionic acid (as sodium propionate) or sodium butyrate for 24 hr. Treatment with 10 mM acetate or propionate or 5 mM sodium butyrate caused 3.3, 6.8 and 4.8 fold increase, respectively, in histone H3 acetylation at lys9 (Fig. 8c).

Effect of treatments of hepatocytes with different alcohol metabolites on HAT and HDAC activity

The data in Fig. 8c showed an increase in H3 lys9 acetylation in hepatocytes treated with alcohol metabolites. We next questioned whether HAT is involved in metabolite induced histone acetylation. To test this, we treated hepatocytes with 10 mM sodium acetate, 10 mM sodium propionate or 5 mM sodium butyrate for 24 hr, and evaluated the HAT activity in the nuclear extracts using an ELISA HAT assay kit. Acetate, propionate and butyrate also significantly increased (*$p<0.001$) the HAT activity by 1.4, 2 and 1.3 fold respectively, compared to control nuclear extract (Fig. 9a). In contrast, treatment of hepatocytes with any of these alcohol metabolites had no detectable effect on HDAC activity (data not shown).

Effect of alcohols and metabolites when added directly to HAT and HDAC activity assay

We next examined whether alcohols or metabolites 'directly' increase HAT activity or inhibit HDAC activity. We added the alcohols (each 40 mM) or metabolites (10 mM sodium acetate, 10 mM sodium propionate or 5 mM sodium butyrate) to the nuclear extract from control hepatocytes. The results showed that alcohols had negligible direct effect on HAT activity (Fig. 9b). In contrast, the different metabolites activated HAT to similar extents as those seen with the nuclear extracts of hepatocytes treated with the metabolites for 24 hr (Fig. 9a). HDAC activity was measured by HDAC colorimetric activity assay kit. Alcohols and their metabolites were added at the same concentrations used in the HAT assay to control nuclear extracts. None of the alcohols tested (ethanol, propanol and butanol), inhibited HDAC activity. In contrast, propionate and butyrate both inhibited (*p<0.001) HDAC activity by 11.4 % and 15.4%, respectively. Acetate, which increased HAT activity at 10 mM (Fig. 9a and 9b), had no measurable effect on HDAC enzyme activity (Fig. 9c). HeLa extract used as a positive control, exhibited robust HDAC activity indicating that the assay is functioning normally (not shown).

To see if the various alcohols and their metabolites have effects on cell extract enriched in HDAC activity, we added these compounds to the HeLa extract provided as positive control in HDAC assay kit. HDAC activity was then determined. Acetate, propionate and butyrate showed a graded inhibition in HDAC activity (42.8%, 65.8%, and 79.5%). On the other hand, ethanol, propanol and butanol had negligible effect on HDAC activity in the HeLa extract (Fig. 9d). A summary of the effects of alcohols and

their metabolites on HAT and HDAC activities in intact cells and in in vitro, is provided

in the Table1.

II.5 Discussion

The pharmacological effect of various chain length alcohols, often termed as surrogate alcohols, on histone H3 acetylation in rat hepatocytes and its relationship to HAT and HDAC activities were examined in this study. Acetylation is one of the most studied post-translational modifications of histone. The sites of modification are the lysine residues of the positively charged amino terminal tail of histone. Our findings showed that exposure of primary rat hepatocyte to different carbon chain length alcohols resulted in selective acetylation of lys9 in histone H3 protein, but to a varying degree. 1-Propanol and 1-butanol were more effective than ethanol. In contrast, methanol, 1-pentanol, 1-hexanol or 1-octanol was not effective either at 40 mM or 20 mM (data not shown). We observed no significant changes in the H3 acetylation at lys14, lys18, lys23 and lys27 by any of the alcohols examined. Lower concentration of 1-propanol, 1-butanol or isopentanol amplified the ethanol induced H3 acetylation at lys9. Since these surrogate alcohols are the most abundant contaminants in illicit alcoholic beverage, their presence can increase the effectiveness of ethanol in inducing H3 acetylation.

Though there are several lysine residues on the histone H3, acetylation at lys9 is relevant for the transcriptional regulation (Jenuwein and Allis, 2001). Preferential acetylation of specific lysine residue may lie in the specificity of HAT and HDAC. The levels of histone acetylation are determined by the combined activities of these two enzymes. These enzymes are often responsible for setting patterns of acetylation. Several members of HAT and HDAC have been shown to acetylate or deacetylate preferentially specific histones and/or specific lysine residues (Sobel et al., 1994; Kuo et al., 1996).

This specificity also depends on the composition of the enzyme complex. Most HATs are found in vivo as multiprotein assemblies or associated with other proteins.

Since the balance between HATs and HDACs is crucial for the net histone acetylation, we therefore investigated the effect of common surrogate alcohols on these enzymes. Hepatocytes treated with different alcohols showed a pattern of increasing HAT activity that parallels the increase in H3AcK9, but none showed any detectable change in HDAC activity. It can be hence suggested that alcohol-induced increase in H3AcK9 is due to HAT activation. It was also apparent that the increased HAT enzyme activity following alcohol treatment of the hepatocytes persisted through subsequent nuclear extraction protocols.

The present study also showed that at 40 mM, only the higher chain alcohols (1-hexanol and above) induced LDH release, whereas mitochondrial function was decreased by 1-pentanol and higher chain alcohols. Presumably, MTT assay detects the 'early stage' whereas LDH release indicates 'late stage' of the cell death. A significant reduction in the mitochondrial activity by 1-pentanol, 1-hexanol or 1-octanol indicates decreased cell viability. Ethanol, 1-propanol or 1-butanol increases acetylation without affecting LDH release or mitochondrial function, but such post-translational modifications in histones may still be a prelude to the long term detrimental effect of these alcohols. To discern the damaging effect of the combined treatment of these surrogate alcohols with ethanol, in vivo studies have to be performed in the future.

Both ADH and ALDH enzyme inhibitors attenuated the H3K9 acetylation induced by different chain length alcohols, therefore their metabolism and the resulting metabolites are likely involved in the histone H3 acetylation. Since cyanamide inhibition

of ALDH is expected to result in aldehyde accumulation, the aldehydes themselves are unlikely to be inducing histone acetylation. Rather its further metabolism to the carboxylic acids may be needed. Indeed, the carboxylic acid metabolites of the respective alcohols did increase acetylation. Others have shown that 24 hr incubation with various short chain fatty acids also led to the selective induction of histone H4 hyperacetylation in the HT-29 cell line (Hinnebusch et al., 2002). Taken together, these data suggest that the carboxylic acid metabolites mediate, at least in part, the increase in H3 acetylation by the corresponding alcohols.

We next examined the effect of the various metabolites on HAT and HDAC activities, using two complementary experimental approaches. In the first approach, we treated hepatocytes with these compounds for 24 hr, prepared the nuclear extracts and then assayed the HAT and HDAC activities. Using this approach, we found a significant but variable increase in HAT activity by the different metabolites (control<butyrate< acetate<propionate), but could detect no change in HDAC activity. It is possible that the HDAC assay kit does not detect all the members of HDAC family (e.g. sirtuins) and might not be sensitive enough to reveal decreases by individual HDACs preferentially targeting particular histones/lysine residues. Hence, we can not completely rule out the inhibition of HDAC as a possible mechanism. In addition, we would also have not seen any inhibition in HDAC if it was rapidly reversible during the isolation procedure. The alcohols also did not block HDAC activity when added directly to the assay, thus providing further evidence that H3 hyperacetylation in hepatocytes is not due to a direct inhibition of HDAC caused by alcohols.

In the second approach, we added the various alcohols or their metabolites directly to HAT or HDAC assay mixture containing nuclear extracts from untreated (control) hepatocytes. Interestingly, none of the alcohols affected the HAT activity in this assay, thus ruling out any direct physical effect on HAT enzymes. Instead, the alcohols may affect HAT activity indirectly, for example, by sensitizing signaling pathways mediating the HAT activation or enhancing the interaction of HAT with co-activators in hepatocytes. Our results open up an avenue to investigate these provocative possibilities in future. The alcohols also did not block HDAC activity when added directly to the assay, thus providing further evidence that H3 hyperacetylation in hepatocytes is not due to a direct inhibition of HDAC by alcohols.

In contrast to the results with the alcohols, all the metabolites tested did significantly increase HAT activity in the in vitro assay. The results support our observation that alcohol metabolism is important for H3K9 acetylation and suggest that the carboxylic acids derived from these alcohols contribute to a direct increase in HAT activity. Propionate and butyrate but not acetate modestly inhibited HDAC activity when added to the hepatocyte extract. The inhibition is smaller than the propionate/butyrate effect on the HeLa extract in vitro (see Fig. 9d). The result is consistent with earlier observations that butyrate inhibits HDAC activity to varying degree in different types of cells (Kruh, 1982), and that butyrate and propionate were particularly weak inhibitors towards rat liver HDAC activity (Skarpidi et al., 2003). One explanation is that butyrate may not inhibit all types of HDAC equally and that the HDAC present in hepatocytes may be less sensitive to butyrate.

In our system, butyrate appears to increase HAT and decrease HDAC. Nakatani et al. have also shown that sodium butyrate dose dependently enhanced HAT activity in SK-N-MC cells (Nakatani et al., 2003). In contrast, propionate acts as a weak HDAC inhibitor and strong HAT activator in hepatocytes. Acetate only activates HAT with little effect on HDAC. It seems that HAT activation and HDAC inhibition varies depending on the compound used and the net effect is based on the contribution of both enzymes.

In conclusion, an interesting pattern of histone H3 acetylation by alcohols and their metabolites emerges from these studies. In intact cells, both alcohols and metabolites increased HAT suggesting that the increase in H3AcK9 is likely due to HAT activation. It can be argued that effects of surrogate alcohols on HAT, in hepatocytes, are due to its metabolites. This is supported by the fact that different alcohols had no direct effect on HAT in vitro activity but the metabolites did (Table 1). But, one can also speculate that alcohol may still indirectly increase HAT activity mediated via an unknown mechanism / signaling pathway yet to be identified. In addition, in vitro assays showed that metabolites, but not alcohols, had a modest direct inhibitory effect on HDAC and this may also represent part of the mechanism for increase in H3AcK9. This highlights differences in the mechanisms involved in the modulatory effects of surrogate alcohols on HAT and HDAC leading to histone H3 acetylation and indicates the role of both enzymes in this process.

Since multiple families of HATs and HDACs have been identified (Peterson and Laniel, 2004), the alcohols and their metabolites may not act identically on all types of HATs and HDACs. The study described here raises many interesting questions: What are the specific HAT and HDAC regulated by these metabolites? Are there binding sites of

these metabolites on the HAT or HDAC molecules? The detail mechanisms of HAT

activation or HDAC inhibition by the alcohols and their metabolites should be of

considerable interest for future investigation.

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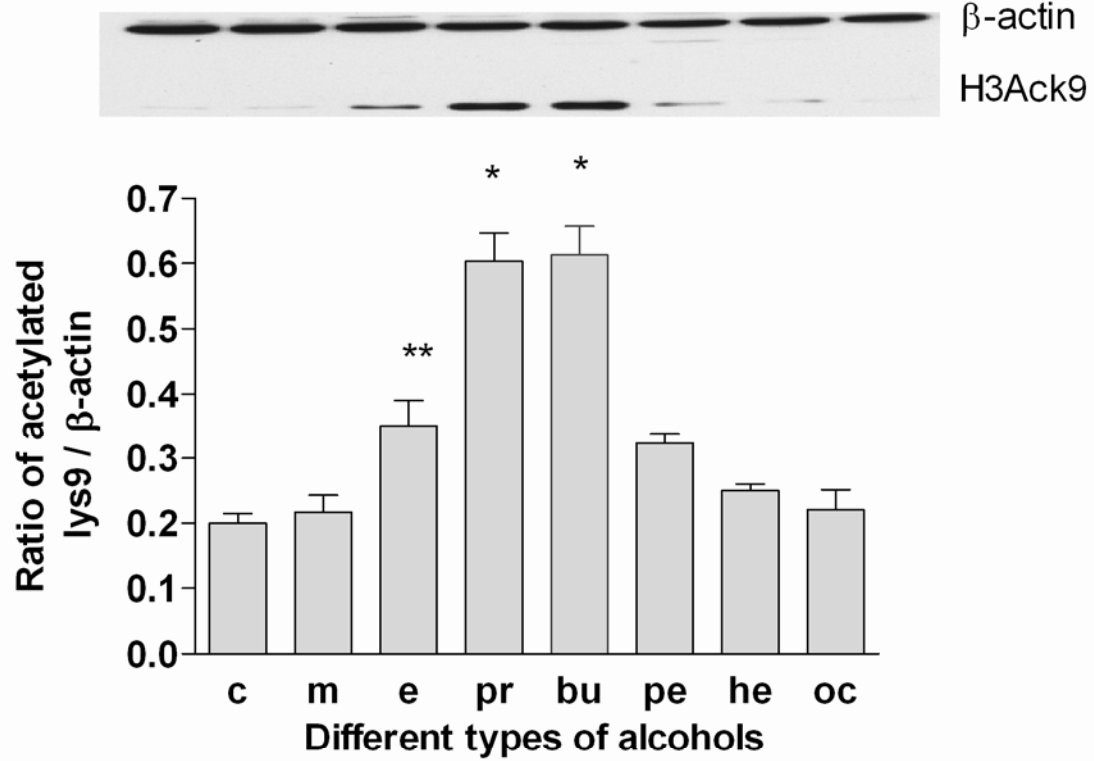
Fig. 1. *Effect of different carbon chain alcohols on acetylation of histone H3 at lys9.* Hepatocytes were treated with 40 mM of methanol (m), ethanol (e),1- propanol (pr), 1-butanol (bu), 1-pentanol (pe), 1-hexanol (he) or 1-octanol (oc) for 24 hr. Nuclear extracts were used for Western blot analysis to detect acetylated histone H3 at lys9. Equal amounts (40 μg) of extract proteins were subjected to 15% SDS–PAGE and transferred onto nitrocellulose membrane. Acetylated histone H3 lys 9 levels were monitored using anti-H3 Ac 9 antibody and ECL detection. Quantitative analysis of acetylated histone H3 was performed by densitometric analysis and is presented as mean ±SE (bar), n=3 experiments. Values represent ratio of acetylated H3 lys 9 and β-actin. *$p < 0.001$ (Control vs Propanol or Butanol), **$p < 0.05$ (Control vs Ethanol).

Fig. 1

Fig. 2. *Effect of different carbon chain alcohols on acetylation of histone H3 at lys 14, lys 18, lys 23, lys27.* Hepatocytes were treated with 40 mM of methanol, ethanol, 1-propanol, 1-butanol, 1-pentanol, 1-hexanol or 1-octanol for 24 hr. Nuclear extracts were used for Western blot analysis as in Fig. 2. Acetylated histone H3 lys14, lys18, lys23 or lys27 levels were monitored using site-specific antibodies and ECL detection. Quantitative analysis of acetylated histone H3 was performed by densiometric analysis and is presented as mean of ±SE (bar), n=3 experiments. Values represent ratio of acetylated H3 at lysine residues and β-actin.

Fig. 2a
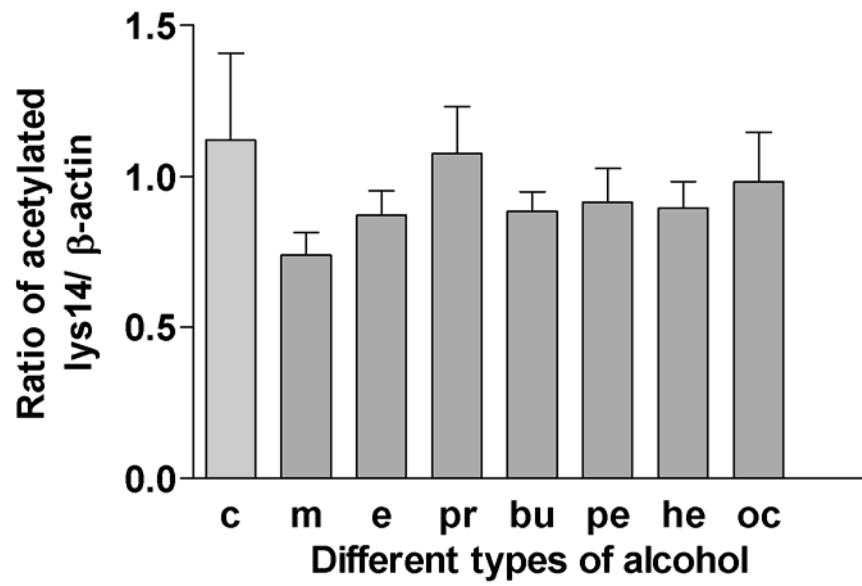
Fig. 2b
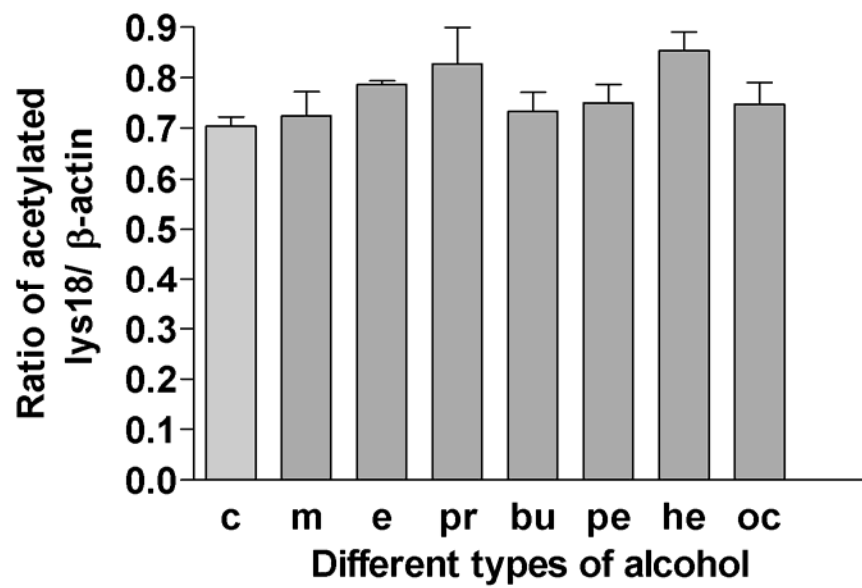
Fig. 2c

Fig. 2d

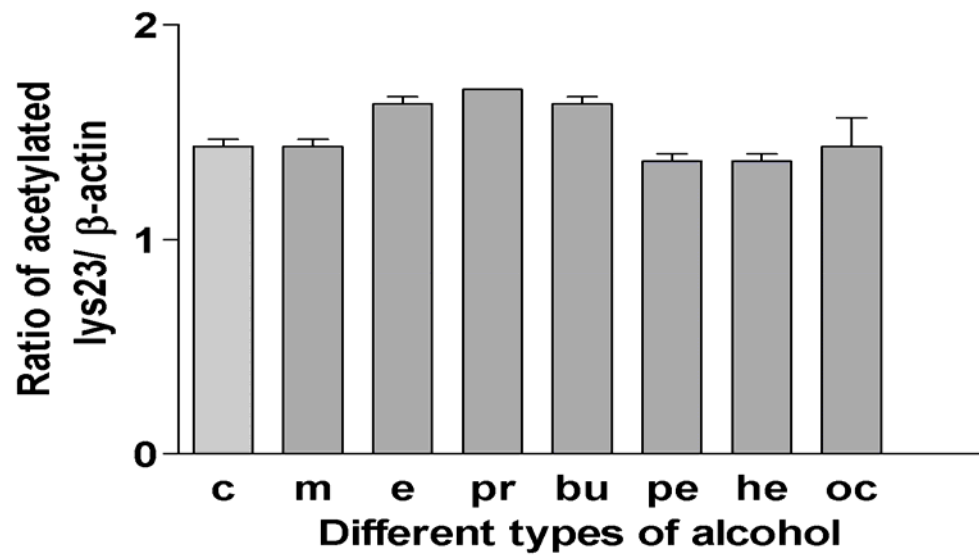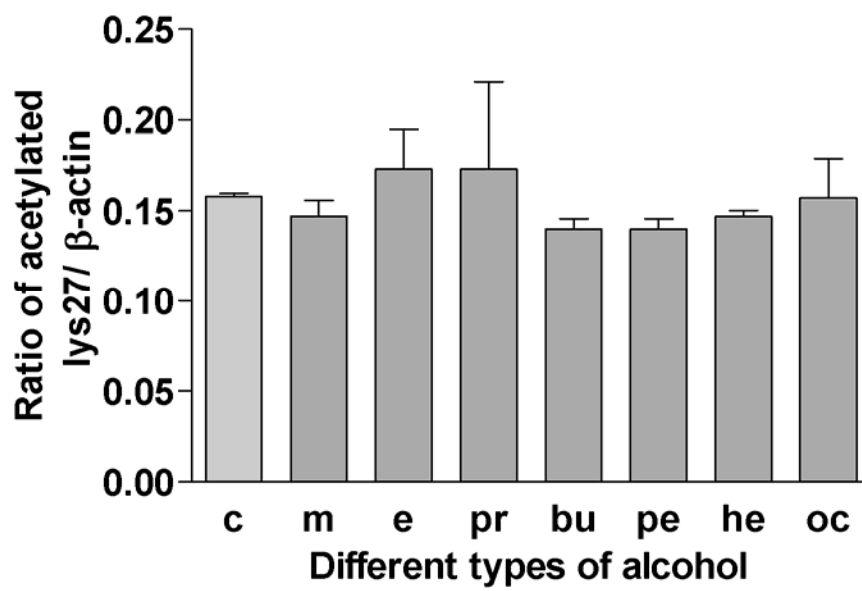**Fig. 3.** *Lactate dehydrogenase (LDH) release from primary rat hepatocytes treated with different alcohols*. Hepatocytes were treated with 40 mM of methanol (m), ethanol (e), 1-propanol (pr), 1-butanol (bu), 1-pentanol (pe), 1-hexanol (he) or 1-octanol (oc) for 24 hr and LDH activity was determined in the culture medium (see Materials and Methods). Percent LDH release was calculated based on a 100% value obtained by lysing hepatocytes with 1% Triton X-100. Each value represents the mean of three different experiments, ± SE, indicated by vertical line. c, control without any treatment; m, methanol; e, ethanol; pr, 1-propanol; bu, 1-butanol; pe, 1-pentanol; he, 1-hexanol; oc, 1-octanol.
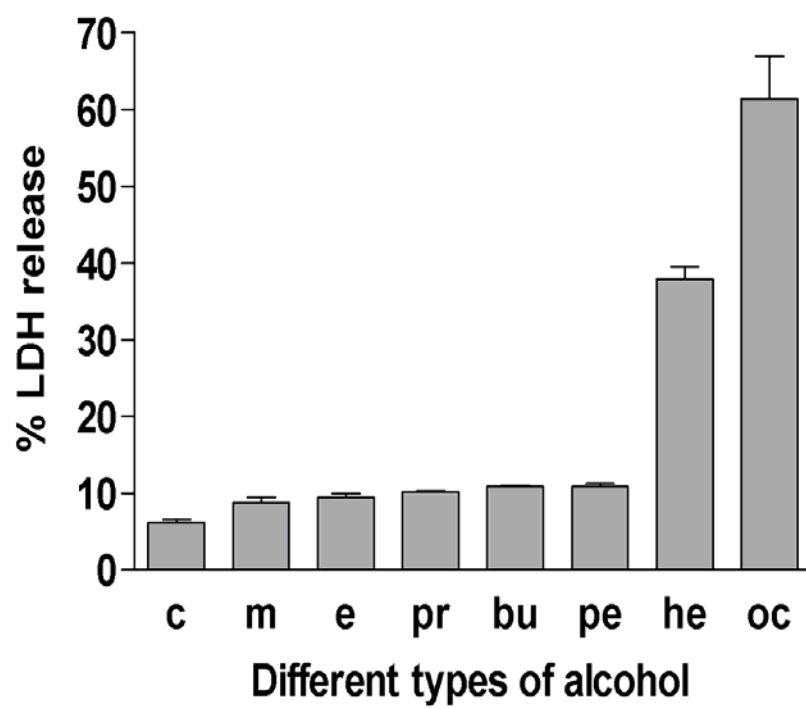
Fig. 3

Fig. 4. *Effect of different carbon chain alcohols on hepatocyte mitochondrial activity.* Hepatocytes were treated with 40 mM of methanol (m), ethanol (e), 1-propanol (pr), 1-butanol (bu), 1-pentanol (pe), 1-hexanol (he) or 1-octanol (oc) for 24 hr. After treatment, mitochondrial activity was assessed using the MTT assay (see Materials and Methods). Data represent mean ± SE (bar), n= 3 experiments.
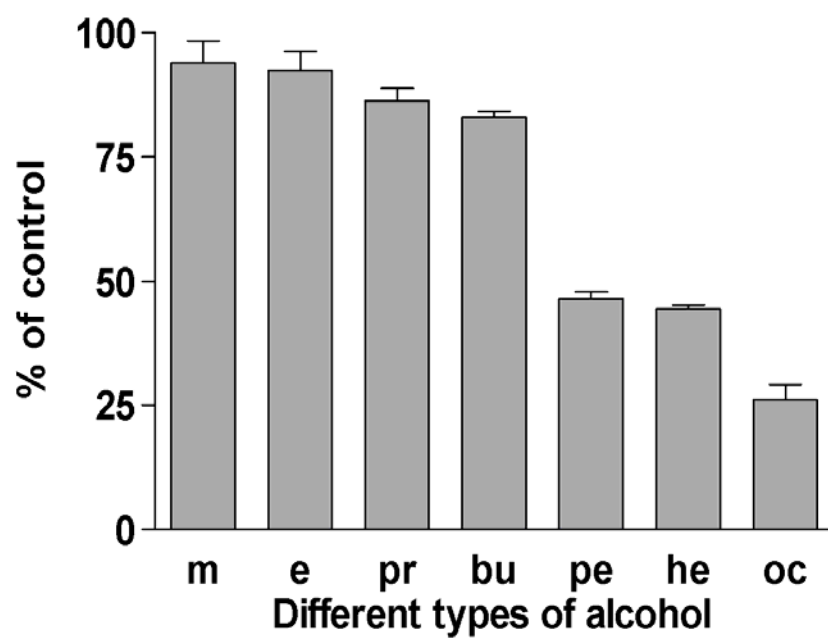
Fig. 4

Fig. 5. *Effect of different alcohols on histone acetyltransferase (HAT) activity*. Effects of different alcohols on HAT were examined by the ELISA method. Hepatocytes were cultured with 40 mM of ethanol, 1-propanol or 1-pentanol for 24 hr. Nuclear extracts were prepared and incubated with histone H3 peptide in the presence of HAT assay cocktail containing HAT assay buffer, Na-butyrate and acetyl-CoA. HAT activity was measured by ELISA plate reader (see Materials and Methods). Values are presented as mean ± SE (bar), n=3 experiments and represent fold increase over control group (control =1).
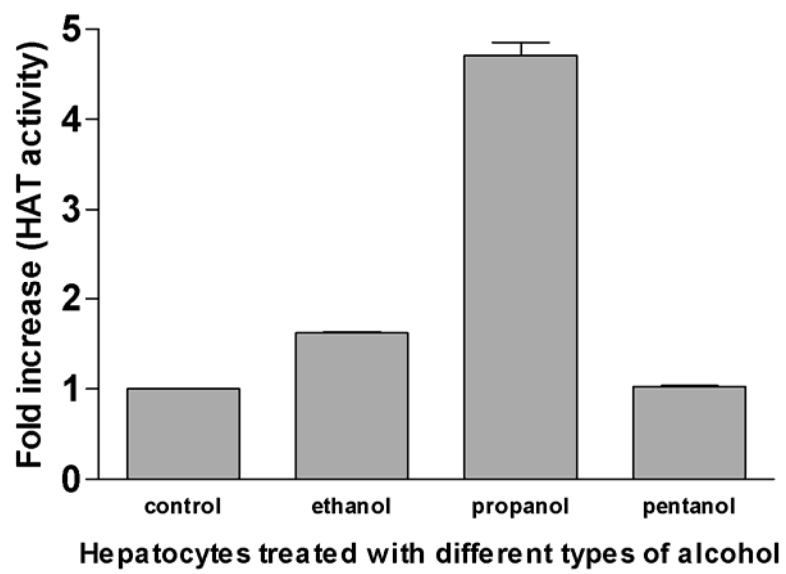
Fig. 5

Fig. 6. *Effect of different alcohols on histone deacetylase (HDAC) activity.* Effect of different alcohols on HDAC was examined by the cleavage of acetylated histone H3 substrate as described in the Materials and Methods. Hepatocytes were cultured with 40 mM of ethanol, 1-propanol or 1-pentanol for 24 hr. Nuclear extracts were prepared and incubated with the substrate. Deacetylated substrate level was measured by colorimetric change using ELISA plate reader (see Materials and Methods). Values are presented as mean ± SE (bar), n=3. no, empty well; hela, positive hela cell control; c, control (nothing added); e, ethanol; pr, 1-propanol ; pe, 1-pentanol.
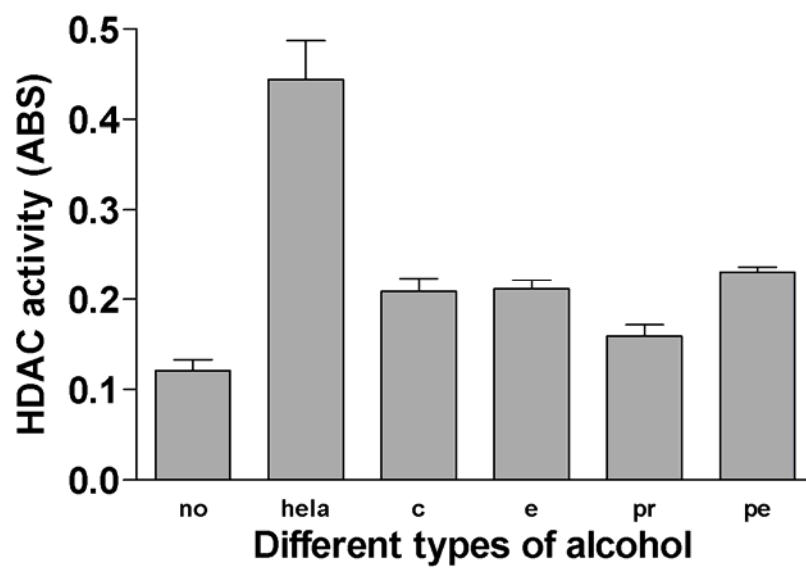
Fig. 6

Fig. 7. *Effect of higher concentration of ethanol alone and in combination with surrogate alcohols on acetylation of histone H3 at lys9.* Acetylated histone H3 at lys9 was measured as described in the Materials and Methods. Each value represents the mean of three different experiments, with the ±SE indicated by vertical lines. a) Control, no alcohol; e, 40 mM ethanol; pr, 2.5 mM 1-propanol; e + pr, 40 mM ethanol in combination with 2.5 mM 1-propanol; b) Control, no alcohol; e, 40 mM ethanol; bu, 2.5 mM 1-butanol; e + bu, 40 mM ethanol in combination with 2.5mM 1-butanol; c) Control, no alcohol; e, 40 mM ethanol; iso, 2.5 mM isopentanol; e + iso, 40 mM ethanol in combination with 2.5 mM isopentanol. Treatments were for 24 hr. * $p < 0.001$, ***$p < 0.05$, * *$p < 0.01$(compared with the control, ethanol compared to ethanol combined with propanol or butanol or pentanol).
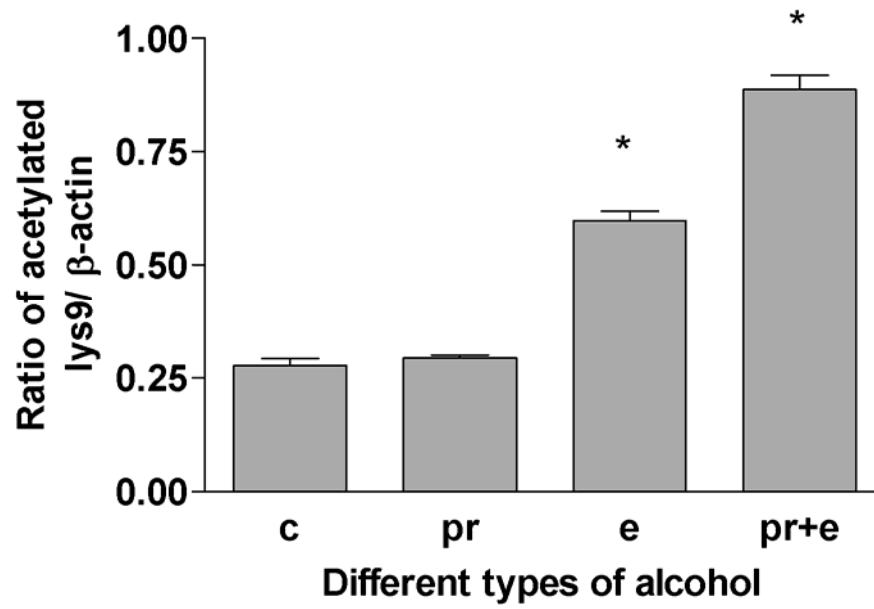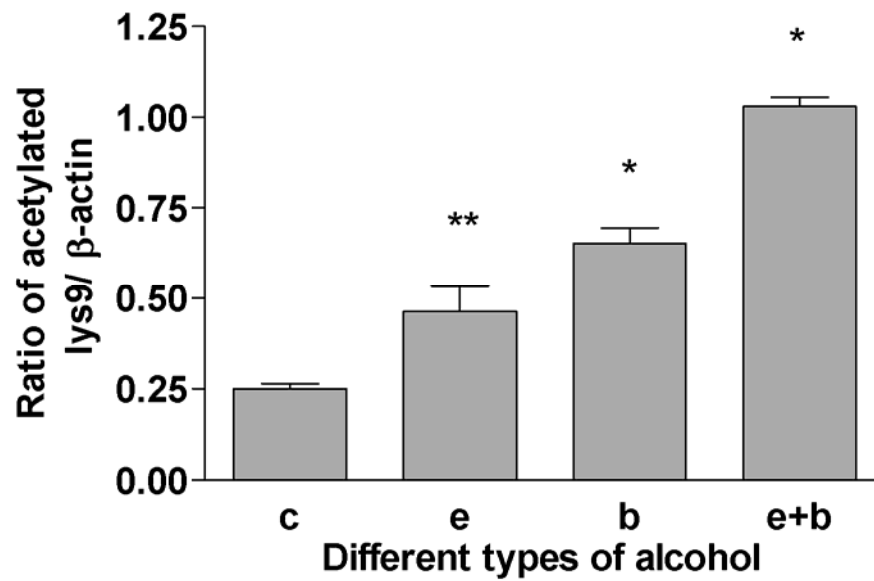
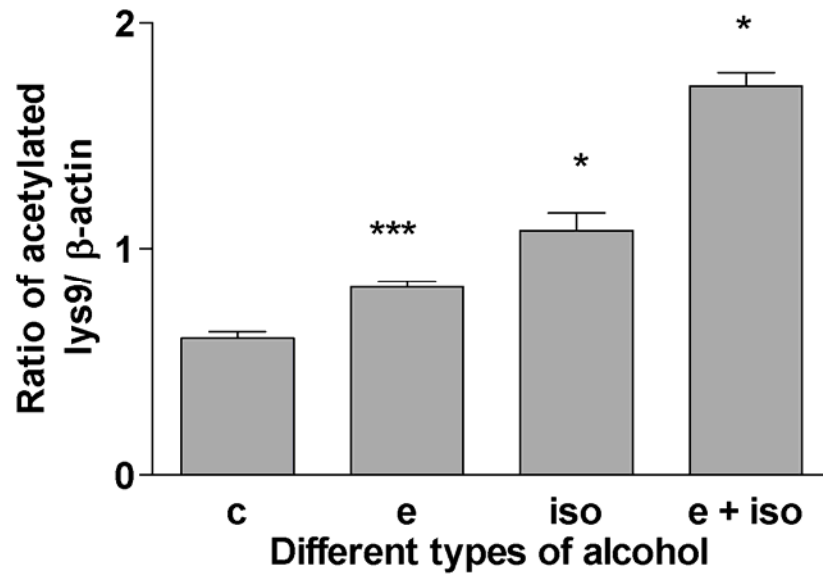**Fig. 7a**

Fig. 7b

Fig. 7c

Fig. 8a. *Effect of 4-mp on ethanol or 1-propanol-induced histone H3 acetylation at lys9.* Hepatocytes were treated with ethanol (40mM) for 24 h in the absence or presence of 4-mp (0.5 mM). Nuclear extracts were prepared and acetylated histone H3 was detected by Western blot analysis using anti-H3 Ac9 antibody. Quantitative analysis of acetylated histone H3 was performed by densitometric analysis and values are presented as mean ± SE (bar), n=3 experiments. c, control; e, ethanol; pr, 1-propanol e; e+mp, ethanol and 4-mp; pr+mp, 1-propanol and 4-mp; mp, 4-mp.

Fig. 8a
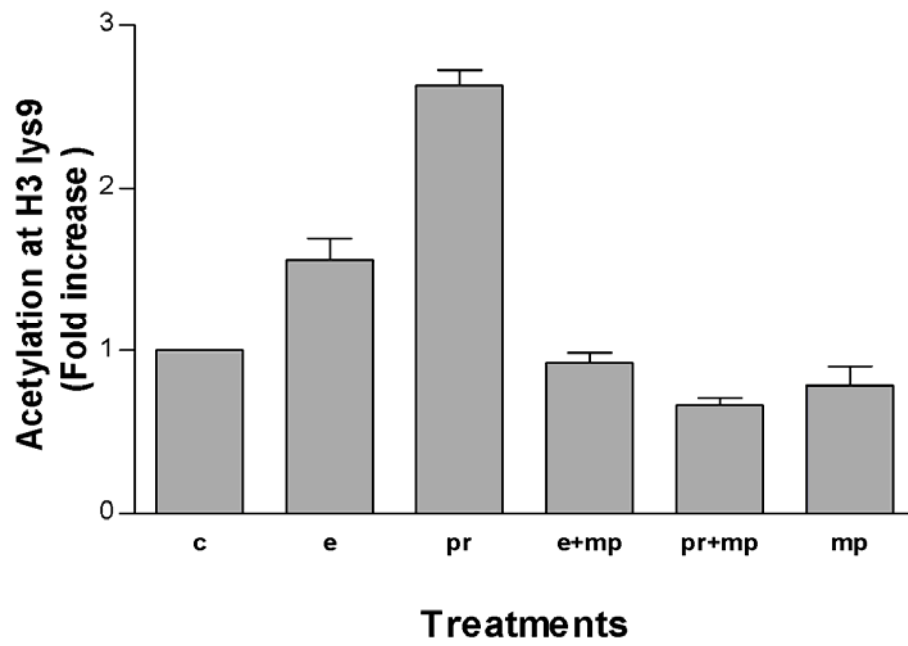
Fig. 8b. *Effect of cyanamide on ethanol or 1-propanol induced histone H3 acetylation at Lys9*. Hepatocytes were treated with ethanol (40 mM) for 24 h in the absence or presence of cyanamide (1 mM). Nuclear extracts were prepared and acetylated histone H3 was detected by Western blot analysis using anti-H3 Ac9 antibody. Quantitative analysis of acetylated histone H3 was performed by densitometric analysis and is presented as mean ± SE (bar), n=3 experiments. c, control; e, ethanol; pr, 1-propanol; e+cy, ethanol and cyanamide; pr+cy, 1-propanol and cyanamide; cy, cyanamide.

Fig. 8b
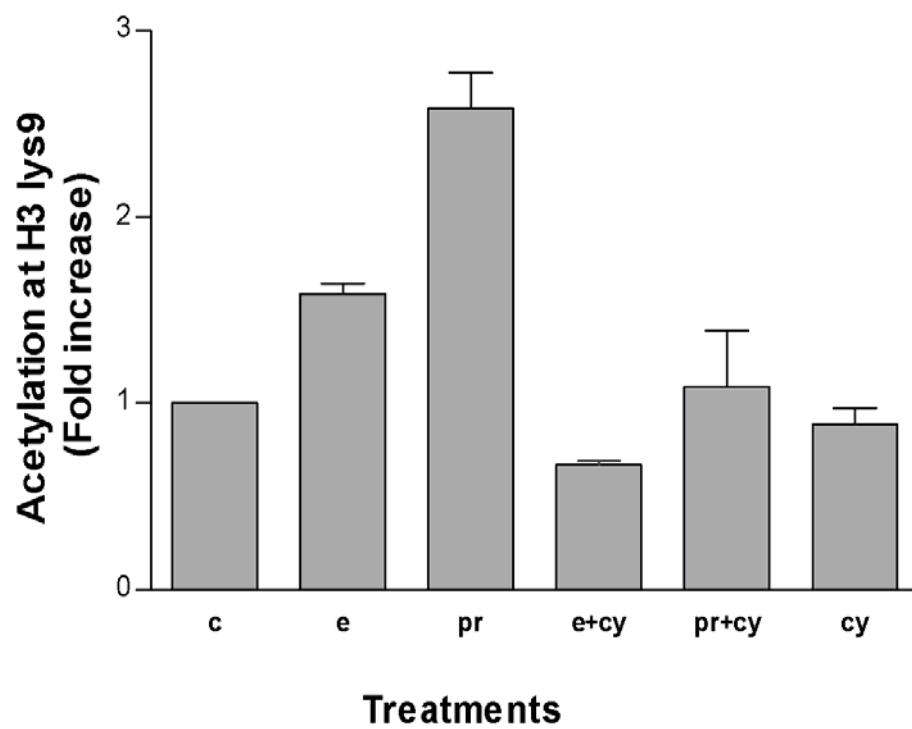
Fig. 8c. *Effect of acetate and propionate on acetylation of histone H3 at lys9.* Hepatocytes were treated with sodium acetate (10 mM), sodium propionate (10 mM) and sodium butyrate (5 mM) for 24 h. Nuclear extracts were prepared and acetylated histone H3 was detected by Western blot analysis using anti-H3 Ac9 antibody. Quantitative analysis of acetylated histone H3 was performed by densitometric analysis and is presented as mean ± SE (bar), n=3 experiments.

Fig. 8c
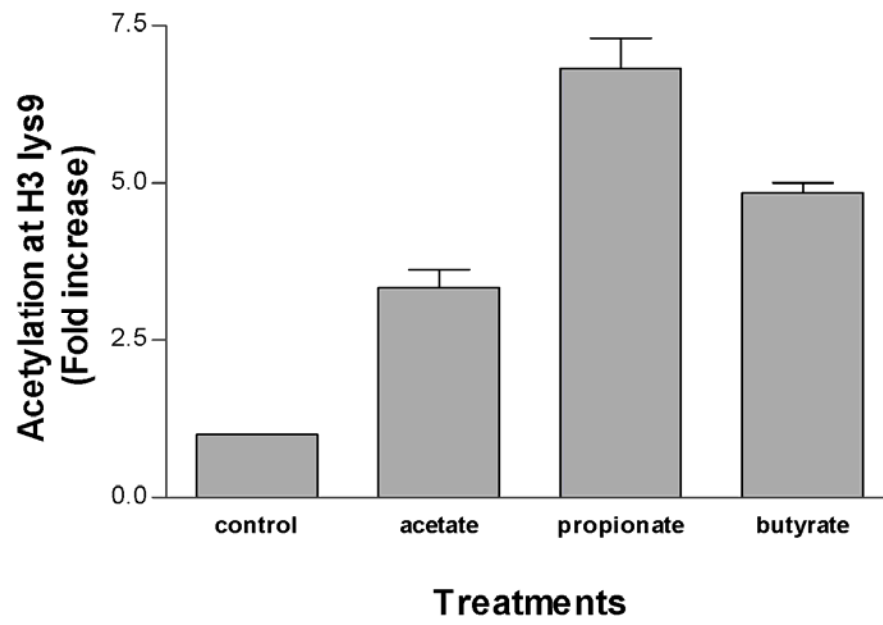
Fig. 9a. *Effect of different alcohol metabolites on histone acetyltransferase (HAT) activity.* Effects of various metabolites on HAT were examined by the ELISA method. Hepatocytes were cultured with sodium acetate (10 mM), sodium propionate (10 mM) or sodium butyrate (5 mM) for 24 hr. Nuclear extracts were prepared and incubated with histone H3 peptide in the presence of HAT assay cocktail containing HAT assay buffer, Na-butyrate and acetyl-CoA. HAT activity was measured by ELISA plate reader (see Materials and Methods). Values are presented as mean ± SE (bar), n=3. * $p < 0.001$ (compared with the control). c, control; ac, sodium acetate; pro, sodium propionate; buty, sodium butyrate.

Fig. 9a

Fig. 9b. *Effect of different alcohols and metabolites added directly in HAT assay.* Control nuclear extract was prepared from untreated rat hepatocytes. Ethanol (40 mM), propanol (40 mM), butanol (40 mM), acetate (10mM), propionate (10mM) or butyrate (5 mM) was added directly to the HAT assay mixture followed by the control nuclear extract. HAT activity was measured as described in Materials and Methods. Values are presented as mean ± SE (bar), n=3. * $p < 0.001$ (compared with the control). c, control; e, ethanol; pr, 1-propanol; bu, 1-butanol; ac, sodium acetate; pro, sodium propionate; buty, sodium butyrate.
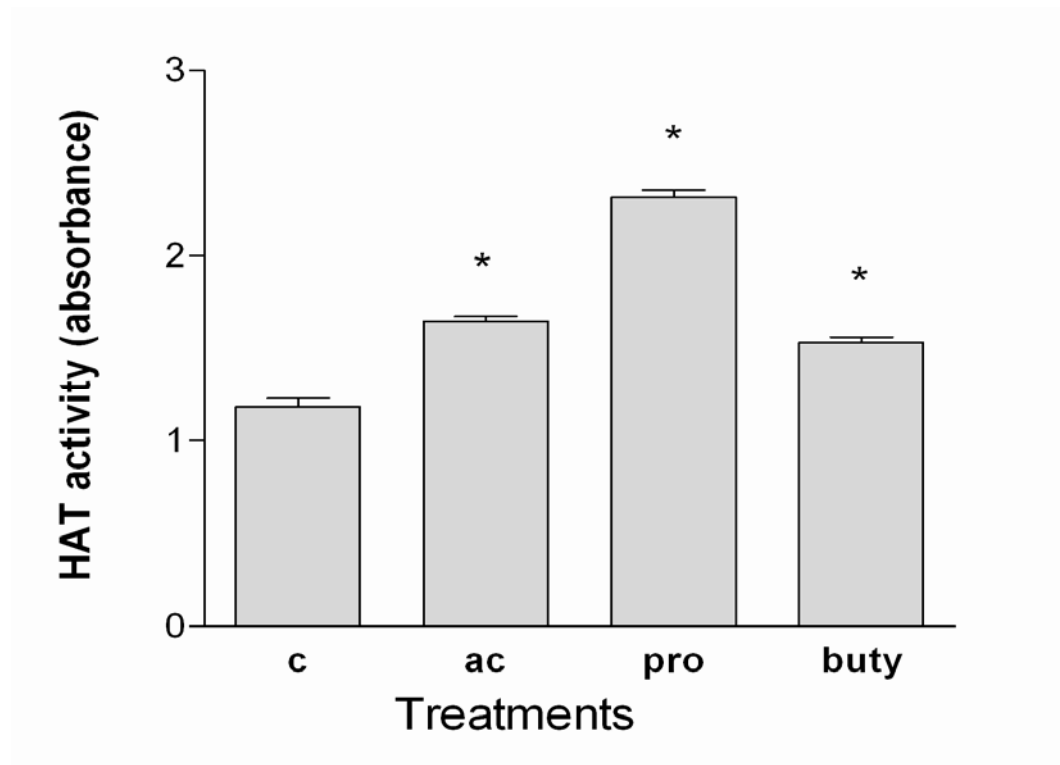
Fig. 9b

Fig. 9c. *Effect of different alcohols and metabolites added directly in HDAC assay.* Control nuclear extract was prepared from untreated rat hepatocytes. Ethanol (40 mM), propanol (40 mM), butanol (40 mM), acetate (10mM), propionate (10mM) or butyrate (5 mM) was added directly to the substrate followed by the control nuclear extract. HDAC activity was measured as described in Materials and Methods. Values are presented as mean ± SE (bar), n=3. * $p < 0.001$ (compared with the control). c, control; e, ethanol; pr, 1-propanol; bu, 1-butanol; ac, sodium acetate; pro, sodium propionate; buty, sodium butyrate.
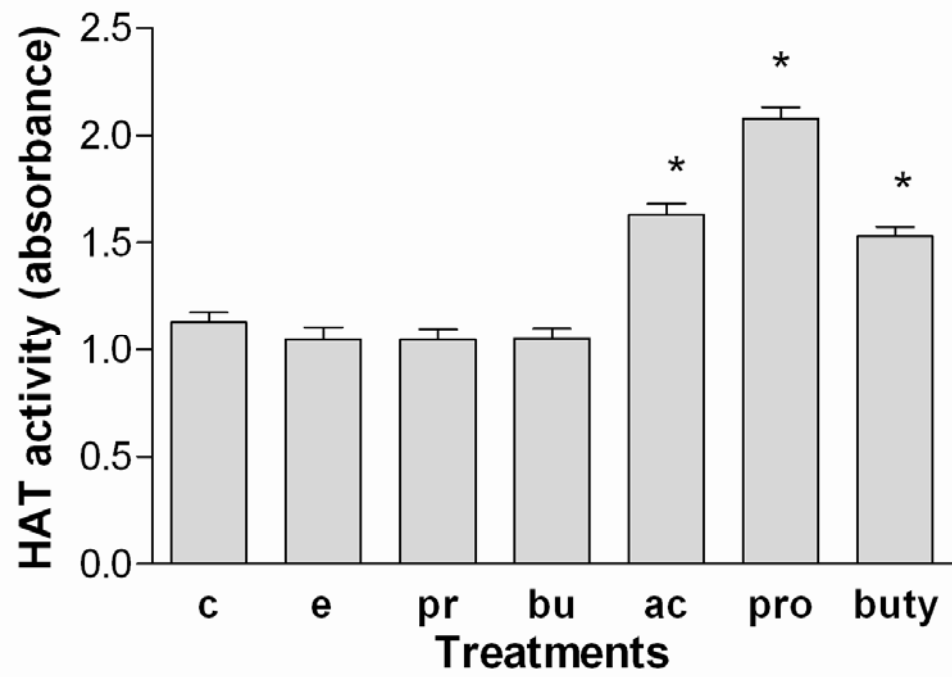
Fig. 9c
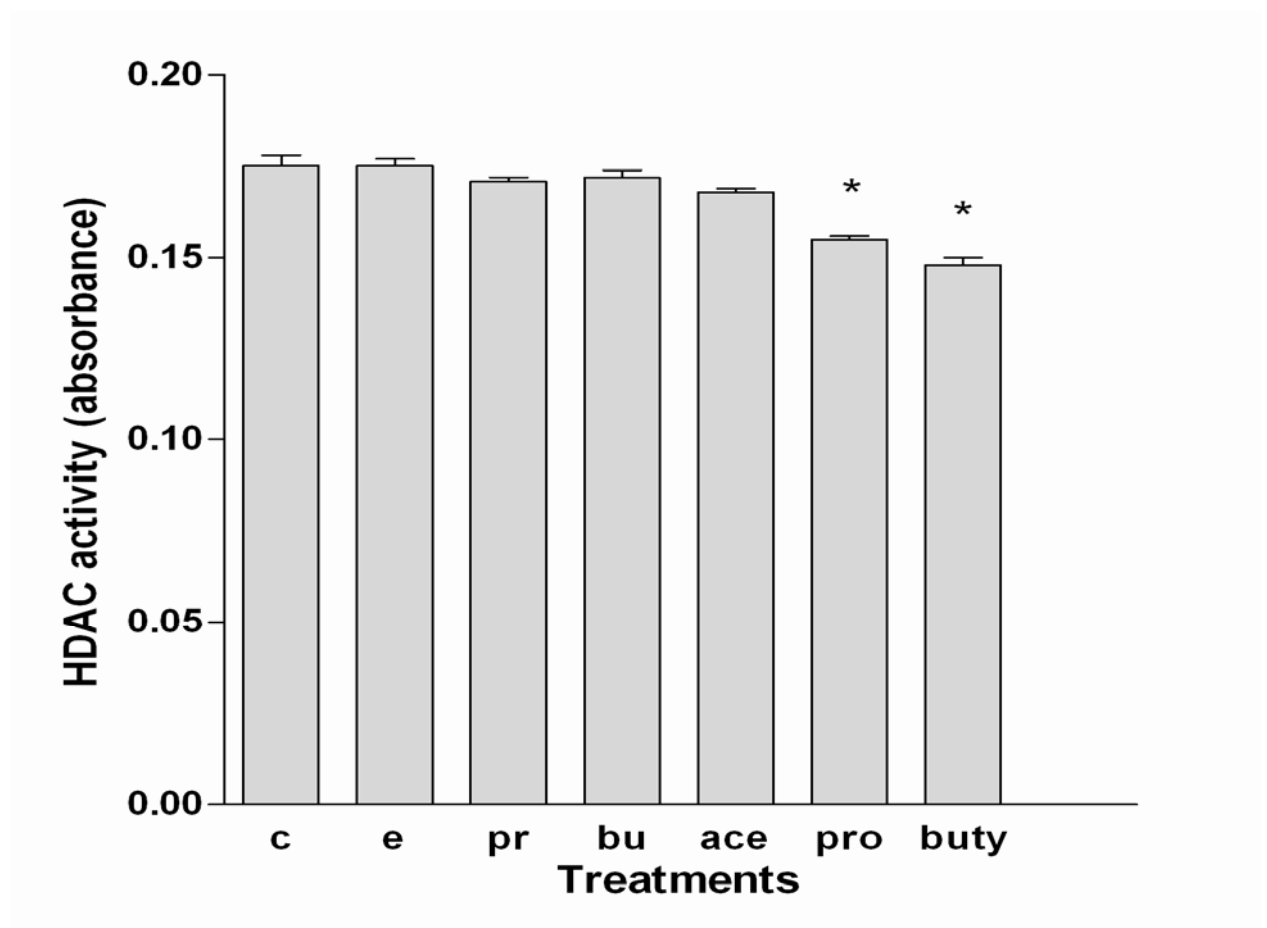
Fig. 9d. *Effect of different alcohols and alcohol metabolites on HDAC activity in HeLa cell extract.* Ethanol (40 mM), propanol (40 mM), butanol (40 mM), acetate (10mM), propionate (10mM) or butyrate (5 mM) was added directly to the substrate followed by the control nuclear extract. HDAC activity was measured as described in Materials and Methods.Values are presented as mean ± SE (bar), n=3. * $p<0.001$ (control versus acetate or butyrate, ** $p<0.01$ (control versus propionate). e, ethanol; pr, 1-propanol; bu, 1-butanol; ac, sodium acetate; pro, sodium propionate; buty, sodium butyrate.

Fig. 9d
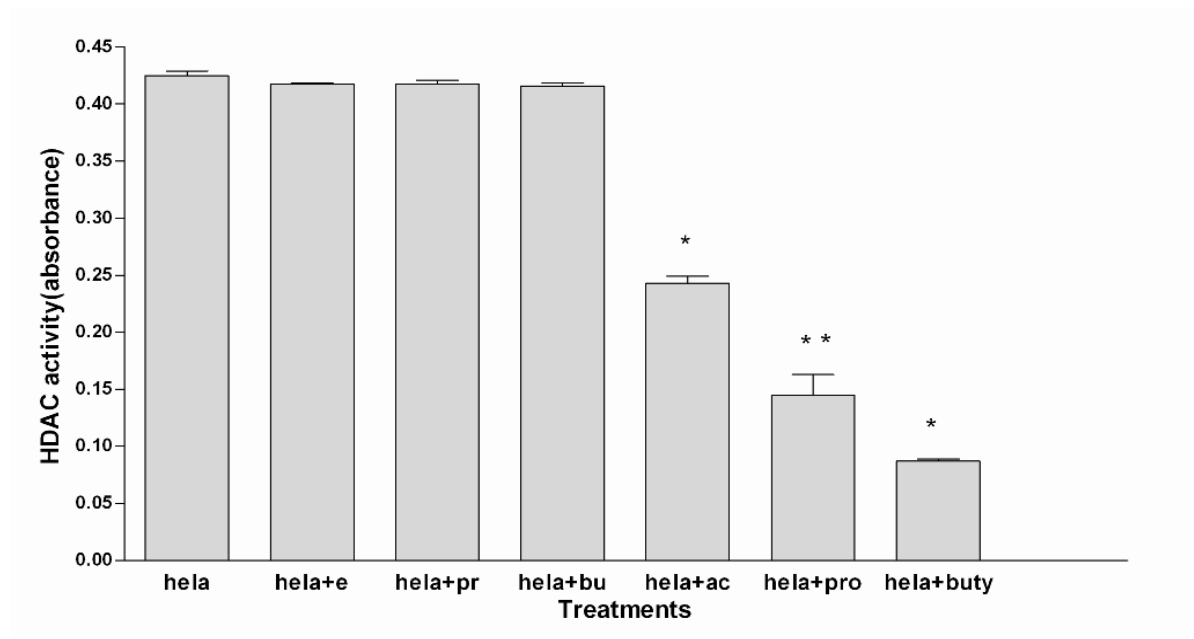
Table. 1. Effects of alcohols and its metabolites on HAT and HDAC activity in isolated nuclear extract or in intact hepatocytes.

Hepatocytes treated with alcohols and metabolites followed by assay of nuclear extract			
TYPES	COMPOUNDS	HAT activity	HDAC activity
Alcohols	Ethanol	↑	No change
	Propanol	↑	No change
	Butanol	↑	No change
Metabolites	Acetate	↑	No change
	Propionate	↑	No change
	Butyrate	↑	No change
Addition of compounds in the assay using control hepatocytes nuclear extract			
Alcohols	Ethanol	No increase	No change
	Propanol	No increase	No change
	Butanol	No increase	No change
Metabolites	Acetate	↑	No change
	Propionate	↑	↓ small
	Butyrate	↑	↓ small

Chapter III

Oxidative stress mediates ethanol induced histone H3 acetylation in rat hepatocytes

Oxidative stress mediates ethanol induced histone H3 acetylation in rat hepatocytes

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III.1 Abstract

Ethanol induced oxidative stress has been implicated in the development of alcoholic liver disease but its mechanism remains to be understood. We have shown earlier that ethanol and surrogate alcohols increased histone H3 acetylation at lysine 9 (H3AcK9) in rat hepatocytes. Acetylation of histone plays a role in transcriptional activation. Whether oxidative stress plays any role in the ethanol induced H3AcK9 in liver, is not known and therefore has been investigated. Ethanol (50 or 100 mM, 24 hr) increased reactive oxygen species (ROS) production ($p < 0.001$). Interestingly, when hepatocytes were exposed to ethanol (50 mM, 24 hr) in the presence of N-acetyl cystein (NAC; ROS reducer) or dietary antioxidants (quercetin, resveratrol), or NADPH oxidase inhibitor apocynin, ethanol induced H3AcK9 was significantly reduced. On the other hand, l-buthionine-sulfoximine (BSO; ROS inducer) and inhibitor of mitochondrial complex I (rotenone) and III (antimycin) increased ethanol induced H3AcK9 ($p < 0.01$). Oxidative stress was also involved in ethanol induced alcohol dehydrogenase 1 (ADH1) mRNA expression. Taken together, these results demonstrate for the first time that oxidative stress mediates ethanol induced histone acetylation and ADH1 gene expression.

III.2 Introduction

Epigenetics is defined as the changes in gene expression without affecting DNA sequence. Histone acetylation is one of the important epigenetic events with a pivotal role in the control of eukaryotic gene transcription (Jenuwein & Allis, 2001). Transcriptionally active genes are correlated with hyperacetylated histone, while their paucity (hypoacetylated histone) is associated with transcriptionally repressed genes (Smith, 2008). Especially, acetylation of histone H3 at lys9 is considered to be a specific marker of various active genes (Morinobu et al., 2004). Irregular acetylation and deacetylation of histones have been linked to various developmental diseases, eg. leukaemia, fragile X-syndrome and Rubinstein-Taybi syndrome (Kundu & Dasgupta, 2007).

Alcohol consumption has long been associated with liver damage (Reuben, 2008). But, the mechanisms underlying deleterious effects of alcohol are not completely understood. They could result from the production of the toxic derivatives from alcohol metabolism or from a direct action of alcohol on cellular components. Recent evidence from this laboratory indicated that ethanol as well as other higher chain alcohols increased the acetylation of histone H3 at lysine 9 in primary culture of rat hepatocytes (Park et al., 2003; Choudhury & Shukla, 2008) and that this acetylation correlated with ADH1 gene expression (Park et al., 2005). Such histone modifications are proposed to underlie the mechanisms involved in ethanol induced cellular injury (Shukla et al., 2008).

On the other hand, acute and chronic alcohol have been shown to increase the production of reactive oxygen species (ROS), lower cellular antioxidant levels, and

enhance oxidative stress in many tissues, especially in the liver. The formation of reactive oxygen species (ROS) such as the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) represent an important cause of oxidative injury. Several enzymatic systems, including the CYP2E1-dependent microsomal monoxygenase system, NADPH oxidase, the mitochondrial respiratory chain have been implicated as the sources of ROS during ethanol intoxication (Albano, 2006; Dey & Cederbaum, 2006). It is notable that alcohol is metabolized predominantly by ADH1 enzyme in the liver which leads to the production of ROS. Although ethanol induced oxidative stress in the liver is known, the molecular mechanism by which this contributes to the pathogenesis of alcoholic liver disease (ALD) is still incompletely understood.

Recently, it has been shown that oxidative stress and redox status of the cells can also regulate nuclear chromatin remodeling (histone acetylation/deacetylation) leading to gene expression (Ito et al., 2004). It was also demonstrated that oxidative stress altered histone acetylation/deacetylation and increased the activation of NF-κB and AP-1, leading to the release of the pro-inflammatory cytokine IL-8 in human alveolar epithelial cells (Rahman et al., 2002). Interestingly, ROS generation has been reported to regulate histone acetylation differentially (increase/decrease) in different cell types (Kang et al., 2003; Ito et al., 2004). However, the relationship between ethanol induced oxidative stress and histone acetylation in the liver remains unknown. To address this issue, we designed a series of experiments to manipulate oxidative stress using glutathione modulators, dietary antioxidants and exogenous H_2O_2 and investigated their effects on ethanol induced H3AcK9 in rat hepatocyte. Furthermore, we investigated if mitochondria

and/or NADPH oxidase system play any role in this histone acetylation. The relationship

of oxidative stress to ethanol induced ADH1 gene expression was also examined.

III.3 Materials and Methods

Polyclonal anti-acetylated histone H3 lysine9 antibody was purchased from Upstate Biotechnology (Lake Placid, NY). The 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). RNeasy Mini Kit was bought from Qiagen (Valencia, CA). DCF-DA was bought from Invitrogen (Eugene, Oregon). RETROscript was purchased from Ambion (Austin, TX). Ethanol (≥98% pure) was purchased from Fisher Scientific (Fair Lawn, NJ). All other chemicals were obtained from Sigma (St. Louis, MO).

Isolation and Culture of Rat Hepatocytes

Rat hepatocytes were isolated from male Sprague-Dawley rats (200–250 g) using an in situ liver perfusion protocol with collagenase (Choudhury and Shukla, 2008). Trypan blue exclusion showed 90%-95% hepatocyte viability. Hepatocytes were plated on collagen-coated dishes (7×10^6 cells∕100 mm dish) in DMEM containing 10% FBS, 2 mM l-glutamine and 100 units∕ml penicillin and 100 µg∕ml streptomycin. The animal protocols were approved by the University of Missouri Institutional Animal Care and Use Committee (IACUC).

Treatment of Cells

Hepatocytes were allowed to attach to culture dishes for 2 hr and then treated with different concentrations of modulators, inhibitors or other agents in DMEM containing 0.1% FBS, 2 mM l-glutamine and 100 units∕ml penicillin and 100 µg∕ml streptomycin for 1 hour. Ethanol was next added as needed for different experiments (see result

section). Duplicate sets of culture dishes were used for each treatment. The petridishes were sealed with parafilm and clingwrap to avoid the evaporation of ethanol.

Preparation of Nuclear Extract

Primary culture of hepatocytes were washed twice with cold 1X PBS, scraped, and resuspended in hypotonic lysis buffer containing 20 mM HEPES, 1 mM 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 and 0.25% NP-40. Lysates from the duplicate dishes were combined and then subjected to 8 passages through 26-gauge syringe needle. Nuclei were pelleted by centrifugation at 14,000·g for 20 seconds. They were resuspended in the buffer containing 20 mM Hepes, 1 mM EDTA, 420 mM NaCl, 1 mM dithiothreitol (DTT), 20 mM glycerophosphate, 1 mM sodium orthovanadate, 2 mM $MgCl_2$, 1 mM PMSF, 10 µg∕ml leupeptin, aprotinin, pepstatin A; 25% glycerol, and 0.25% NP-40, sonicated and re-centrifuged at 14,000g for 20 seconds (4°C). Protein concentration of the supernatant corresponding to the nuclear extract was measured by the Bio-Rad DC protein assay kits.

Western Blot Analysis

Equal amounts (40 µg) of nuclear extracts were run on 15% SDS–PAGE and transferred onto nitrocellulose membrane and then blocked with 5% nonfat dried milk for 1.5 hours. After washing the membrane with 1X TBST (Tris-Buffered Saline Tween-20), membrane was incubated with anti-H3AcK9 primary antibody (1:2,000 dilution) together with 0.25 µg∕ml concentration of anti-β-actin, overnight at 4°C. After washing, membranes were incubated with horseradish-conjugated secondary antibody for 1 hour at room temperature. The horseradish peroxidase was detected by enhanced

chemiluminescence (Supersignal, Pierce) and x-ray film exposure. The band density was measured using Bio-Rad Quantity One Software. This band quantification was always done within the linear range of X-ray film sensitivity by selecting appropriate exposure time. Linearity was tested and confirmed using standard amount of protein and various exposure times.

Measurement of reactive oxygen species accumulation

For the assessment of ROS produced by ethanol, cells were grown in 6 well plates for 23 hr in phenol-free DMEM media and the DCF-DA (dichlorofluorescein diacetate) assay was used. The method was slightly modified (instead of HKRB buffer, phenol free media was used) from previous described techniques (Sheehan et al, 1997). DCF-DA (10 μM) was added to the wells and incubated for 1hr. During this time they were shielded from light. After 1 hr the medium containing excess DCF was removed from the cells and replaced with fresh DMEM media. Fluorescent intensity of the cells was measured at 485 nm excitation and 520 nm emission using a Packard Fusion plate reader.

RNA isolation and RT-PCR

Hepatocytes were treated with various modulators in the presence or absence of ethanol for 24 h and RNA was isolated using Qiagen RNAeasy kit. Two micrograms of total RNA were reverse transcribed with 100 units of Moloney murine leukemia virus reverse transcriptase at 55°C for 60 min, and 92°C for 10 min. Each cDNA preparation was amplified by polymerase chain reaction (PCR). The PCR conditions for ADH I were 45 s at 94°C, 2 min at 55°C, and 2 min at 70°C for 35 cycles. The sequences for the primers were 5'-ACCATCGAGGACATAGAA-3' (forward) and 5'-

GTGGAGCCTGGGGTCAC-3' (reverse). For consistency, GAPDH mRNA was also used as the internal control. The PCR products were visualized by 1.5% agarose-ethidium bromide gel electrophoresis.

Statistical Analysis

Each experiment was performed 3-5 times. Each figure was created from 3 separate experiments. Statistical analyses were performed using a standard one-way ANOVA (Newman-Keuls Multiple Comparison Test). Values with $p < 0.05$ considered significant**.** In all the figures, the bar without significance sign did not show any statistical significance.

III.4 Results

In order to investigate the role of oxidative stress in ethanol induced histone acetylation, we have employed various experimental approaches. This includes pharmacological agents to increase (eg. BSO, H_2O_2) or decrease (eg. resveratrol, quercetin, NAC) oxidative stress and study their effects on H3AcK9. We also studied the source of the oxidative stress and its relationship to ADH1 gene expression. The in vivo peripheral blood concentration of ethanol in chronic alcoholics can reach 50 mM (Deitrich & Harris, 1996) and was therefore the selected concentration in this study. The concentration ranges of the modulators were chosen from the literature. Ethanol induced H3 acetylation at lys9 was analyzed by western blotting using anti-acetyl histone H3 lysine 9 antibody. For consistency, it was also probed with anti-β-actin antibody (an endogenous housekeeping protein).

ROS production in ethanol treated hepatocytes

Ethanol, treatment of hepatocytes at 50 mM and 100 mM for 24 hr increased ROS production by 1.3 and 1.5 fold, respectively, over control (p<0.001) (Fig. 1). These results are in agreement with other reports demonstrating increased ROS by ethanol in hepatocytes (Lee and Shukla, 2005; Cabrales-Romero et al., 2006).

Role of glutathione modulators in ethanol induced H3AcK9 and ADH1 gene expression

Cellular oxidative state is largely determined by glutathione (GSH), which is important in protecting the cell from ROS produced during coupled mitochondrial electron transport and oxidative phosphorylation. We, therefore, investigated the role of oxidative stress in ethanol induced histone acetylation using glutathione precursor NAC

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or depletor BSO (an inhibitor of glutamate-cystein ligase). NAC (10 mM) itself did not affect the histone acetylation, but when hepatocytes were pretreated with NAC for 1 hr followed by 23 hr with 50 mM ethanol (in the same medium), it ameliorated ethanol induced acetylation by 46% (p<0.01) (Fig. 2a). On the other hand, 1 mM BSO significantly increased H3AcK9 on its own by 1.9 fold (p<0.05) and it amplified ethanol induced acetylation by 1.8 fold (ethanol=2.9 fold and ethanol +BSO=4.7 fold) (p<0.001) (Fig. 2b).

In a similar approach as above, we also examined the relationship between ethanol induced oxidative stress and ADH1 gene expression. We have previously shown that ethanol induced ADH1 gene expression correlates with histone H3 acetylation at lys9 (Park et al., 2005). We pretreated hepatocytes with 10 mM NAC or 1 mM BSO for 1 hr followed by 50 mM ethanol for 23 hr. Cells were also treated with only 10 mM NAC or 1 mM BSO. Subsequently, we examined the ADH1 mRNA expression with RT-PCR. Ethanol (50 mM) increased the ADH1 gene expression by 1.3 fold and when combined with BSO, ADH1 gene expression went up to 1.8 fold. NAC decreased ethanol induced ADH1 gene expression by 15% (Fig. 3).

Effects of dietary antioxidants on ethanol induced H3AcK9

Resveratrol and quercetin, used at different concentrations did not show any effect on their own on histone acetylation. When hepatocytes were pretreated (1 hr) with resveratrol (0.5 mM and 5 mM) followed by 50 mM ethanol for 23 hr, histone acetylation was decreased to near basal level (p<0.01) (Fig. 4a). A similar effect was seen when hepatocytes were preincubated (1hr) with 5 mM or 20 mM quercetin followed by 50 mM

ethanol treatment for 23 hr. Quercetin decreased ethanol induced H3AcK9 by 56% (p<0.001) (Fig. 4b).

Effect of exogenous H_2O_2 on ethanol induced H3AcK9

ROS include oxygen ions, free radicals, and peroxides, both inorganic and organic. Hydrogen peroxide is damaging to the cell because it can easily transform into a hydroxyl radical (via reaction with Fe^{2+}: Fenton reaction), one of the most destructive free radicals. Hepatocytes treated with 50 µM H_2O_2 for 24 hr showed 2.7 fold increase in acetylation of H3K9 (Fig. 5). Hepatocytes preincubated with 50 µM H_2O_2 (1hr) followed by 50 mM ethanol for 23 hr showed 4.3 fold increase in H3AcK9 (p<0.001, Fig. 5).

Role of mitochondrial and NADPH oxidase derived ROS species in histone H3 acetylation at lys9

The mitochondrial electron transport chain is the major intracellular source of ROS. Ethanol stimulates the production of reactive oxygen species at mitochondrial complexes I and III (Dawson et al., 1993). Generation of this ROS is augmented by selective inhibitors of mitochondrial complex. We used these inhibitors as tools in our study. Hepatocytes were pretreated with inhibitors of mitochondrial complex I (rotenone) and III (antimycin) for 1 hr and then incubated in the presence or absence of 50 mM ethanol for 23 hr. Hepatocytes incubated with 0.5 µM and 1 µM rotenone increased H3AcK9 by 1.5 fold and 2.9 fold, respectively (Fig. 6a). Though rotenone (0.5 µM) increased the ethanol induced acetylation, it is not statistically significant compared to ethanol induced H3AcK9. But 1 µM rotenone increased ethanol induced acetylation up to 5.2 fold (p<0.01). Similarly, 0.025 mM antimycin also significantly increased ethanol

induced H3AcK9 (p<0.01). Antimycin itself showed 2 fold increase in H3AcK9 compared to control (p<0.01) (Fig. 6b).

Another potential candidate for the ROS generation is the NADPH oxidase system in hepatocytes. It is composed of two essential membrane bound components, $gp91^{phox}$/Nox2 and $p22^{phox,}$ which comprise flavocytochrome $b_{558,}$ and four cytosolic componenets, $p47^{phox}$, $p40^{phox}$, $p67^{phox}$, and the small G protein Rac1/2. Following stimulation (e.g. ethanol), translocation of cytosolic subunits to the membrane occurs and NADPH oxidase generates superoxide anion (Babior, 1999; Kono et al., 2000). To explore if NADPH oxidase is involved in the histone acetylation, we used apocynin. Apocynin has been shown to prevent the translocation of the cytosolic subunits to the membrane bound catalytic subunit, thereby inhibits the formation of an active NADPH oxidase complex (Reinehr et al., 2005). Hepatocytes incubated with increasing concentration of apocynin decreased ethanol induced H3AcK9. Apocynin at 0.1 mM reduced the ethanol induced acetylation by 31% (p<0.01). Interestingly, at higher concentration, 1 mM apocynin abolished the ethanol induced histone acetylation by 72% (p< 0.001) (Fig. 7). Apocynin itself did not show any significant effect on histone acetylation.

III.5 Discussion

Generation of ROS during alcohol exposure is suggested as one of the important mechanisms of alcohol-induced liver injury. ROS are considered hepatotoxic because of their potential to react with macromolecules, inactivate enzymes, cause DNA damage, modify proteins, and induce lipid peroxidation. In the present study, we have established that ethanol generated ROS play important role in the histone acetylation and that both mitochondria and NADPH dependent pathways could be involved.

Hepatocytes exposed to ethanol, show robust increase in ROS production within 30 min (Cabrales-Romero et al., 2006). As ROS production occurs in cells, a variety of endogenous enzymatic (superoxide dismutase, catalase, glutathione peroxide, etc.) and nonenzymatic (vitamin C, vitamin E, etc.) mechanisms have evolved against ROS to maintain the homeostasis. Some of the mechanisms are reported to be impaired after long-term alcohol consumption and may therefore contribute to the liver and other organ damage. In order to investigate the relationship between ROS and histone acetylation we chose 24 hr time point for ethanol treatments, because histone acetylation is highest in hepatocytes at this time (Park et al., 2003). We observed a significant ROS accumulation after 24 hr ethanol treatment in hepatocytes (Fig. 1). It is possible that ethanol causes an early robust ROS production (Cabrales-Romero et al., 2006) which triggers the process of increasing histone acetylation monitored at 24 hr.

Studies using confocal laser scanning microscopy suggest that active oxidants produced during ethanol metabolism modulate mitochondrial energy synthesis in isolated and cultured hepatocytes (Kurose et al., 1996). In addition, endogenous glutathione-glutathione peroxidase system serve as important antioxidants/ cytoprotective machinery

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in the hepatocyte exposed to ethanol. Due to its high concentration and its central role in maintaining the cells redox state, glutathione is one of the most important cellular antioxidants. In our study, hepatocytes treated with glutathione precursor NAC decreased ethanol induced histone acetylation and the glutathione depletor BSO showed the opposite result. This clearly implicates oxidative stress in ethanol induced histone H3 acetylation at lysine 9.

To pursue further the role of the oxidative stress in this epigenetic modification, we examined the effect of dietary antioxidants on ethanol induced H3AcK9. Resveratrol and quercetin, polyphenol compounds contained in several dietary products, reduced ethanol induced histone acetylation. This further supports a role for ethanol induced oxidative stress in histone acetylation. Recently, it was demonstrated that resveratrol protects against oxidative stress in ethanol induced lipid peroxidation in rats (Kasdallah-Grissa et al., 2006). Notably, resveratrol activates sirtuin family of NAD-dependent histone deacetylases involved in regulation of various cellular processes including gene transcription, DNA repair and apoptosis (Howitz et al., 2003). Quercetin also showed protective effect in case of iron overload liver injury (Zhang et al, 2006) or dimethylnitrosamine induced liver damage (Lee et al., 2003).

Superoxide anion is primarily converted to H_2O_2 by superoxide dismuatse. Our results indicated that H_2O_2 also increased ethanol induced acetylation. The effect persisted up to 24 hr treatment with 50 µM exogenous $H_2O_2.$ It is relevant to note that H_2O_2 (200 µM) has been shown to induce prolonged histone acetylation in bronchial epithelial cells (Tomita et al., 2003).

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Though the mechanism by which ethanol triggers an increase in reactive oxygen species in the liver is complex, mitochondria may play major role (Bailey & Cunningham, 2002; Dey and Cederbaum, 2006). It is recognized that mitochondrial oxidative stress contributes to the injury observed in several liver diseases including alcoholic liver disease (Cahill et al., 1997; Bailey et al., 1999; Bailey & Cunningham, 2002). Liver pathologies are associated with significant alterations in the functional state of the mitochondrial respiratory chain (Krahenbuhl and Reichen, 1992). Though these electron transporters are composed of four complexes: complex I-IV, data in the literature concerning mitochondrial respiratory chain function in hepatitis and cirrhosis showed that complex I and III are the key sources of ROS in the cell (Barja and Herrero 1998; Barja 1999; Bailey & Cunningham, 2002). It is assumed that complex II is the most resistant complex which is not damaged even in liver cirrhosis (Cederbaum et al., 1974). If the electron flow along the respiratory chain is interrupted (by inhibitors like complex I inhibitor rotenone and complex III inhibitor antimycin) then ROS formation increases as a result of autooxidation of the reduced electron transport components. It has been shown by others that both rotenone and antimycin increased hepatocyte mitochondrial ROS production, and it was further enhanced by the addition of ethanol (Bailey & Cunningham, 1998; Bailey et al., 1999). In an analogous profile, rotenone and antimycin also increased histone acetylation and it was enhanced by ethanol (Fig. 6). This argues that of mitochondrial ROS could be a source of ethanol induced histone acetylation.

Though hepatocytes express components of phagocytic and nonphagocytic forms of NADPH oxidase, the inhibitory action of apocynin on ethanol induced histone acetylation in rat hepatocytes earn some comment. Previous studies have shown that

inhibition of NADPH oxidase by apocynin is not mediated by apocynin itself but by a compound derived from apocynin in a peroxidase-dependent manner (Stolk et al., 1994). Due to this mechanism, apocynin was shown to inhibit NADPH oxidase in phagocytic cells, whereas in some (peroxidase-deficient) nonphagocytic cells, such as fibroblasts, apocynin stimulates, rather than inhibits, ROS formation (Vejrazka et al., 2003). Hepatocytes contain peroxidases and exhibit endogenous H_2O_2 formation, which are prerequisites for the inhibitory action of apocynin on NADPH oxidase. This may explain why in ethanol treated hepatocytes (preincubated with apocynin), histone acetylation was inhibited. Since apocynin abolished the ethanol induced histone acetylation, it suggests that NADPH oxidase system is also involved in the oxidative stress induced histone H3 acetylation.

Park et al. (2005) showed that ethanol increased the association of H3AcK9 with the promoter region of ADH1 gene in rat hepatocytes. The fact that pharmacological manipulations to increase or decrease the ethanol induced oxidative stress showed a concomitant change in both H3AcK9 level and ADH1 gene expression implies that oxidative stress, H3 acetylation and ADH1 expression may be related.

Further understanding of the effects and roles of ROS in ethanol induced chromatin modeling (histone acetylation / phosphorylation/ methylation) and gene expression, will provide important information regarding basic pathological processes contributing to alcoholic liver disease. The histone acetylation–deacetylation is maintained through a balance of histone acetyltransferase (HAT) and histone deacetylase (HDAC) enzyme activities in normal cells. As we mentioned above, there is a growing body of evidence suggesting antioxidant as one of the effective therapy strategies to treat

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alcoholic liver disease (Kasdallah-Grissa et al., 2006). So the detail mechanism of the modulation of HAT or HDAC or both (directly and indirectly) or signaling pathways leading to the modulation of ethanol induced histone acetylation should be of considerable interest for future investigation. Then probably, combination of antioxidants and HAT/ HDAC inhibitor or activator will be a more effective remedy to halt the progression of alcoholic liver disease. Furthermore, as our understanding of gene expression/epigenetics/genomics increases, additional clinical targets and therapeutic strategies are likely to emerge.

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Figure 1: Ethanol induced ROS generation in hepatocytes. Primary culture of rat hepatocytes were treated with 50 mM or 100 mM ethanol in 6 well plates for 23 hr. DCF-DA (10 µM) was added to the wells. After 1 hr, the media was removed from the wells and replaced with fresh phenol free DMEM media. Fluorescent intensity of the cell was measured at 485 nm excitation and 520 nm emission in a Packard Fusion plate reader. Data represent mean ± SE (bar), n=3 experiments. Values correspond to fold increase compared to control. *p<0.001 (Ethanol compared to Control) (C=Control, E50=50 mM Ethanol, E100=100 mM Ethanol).
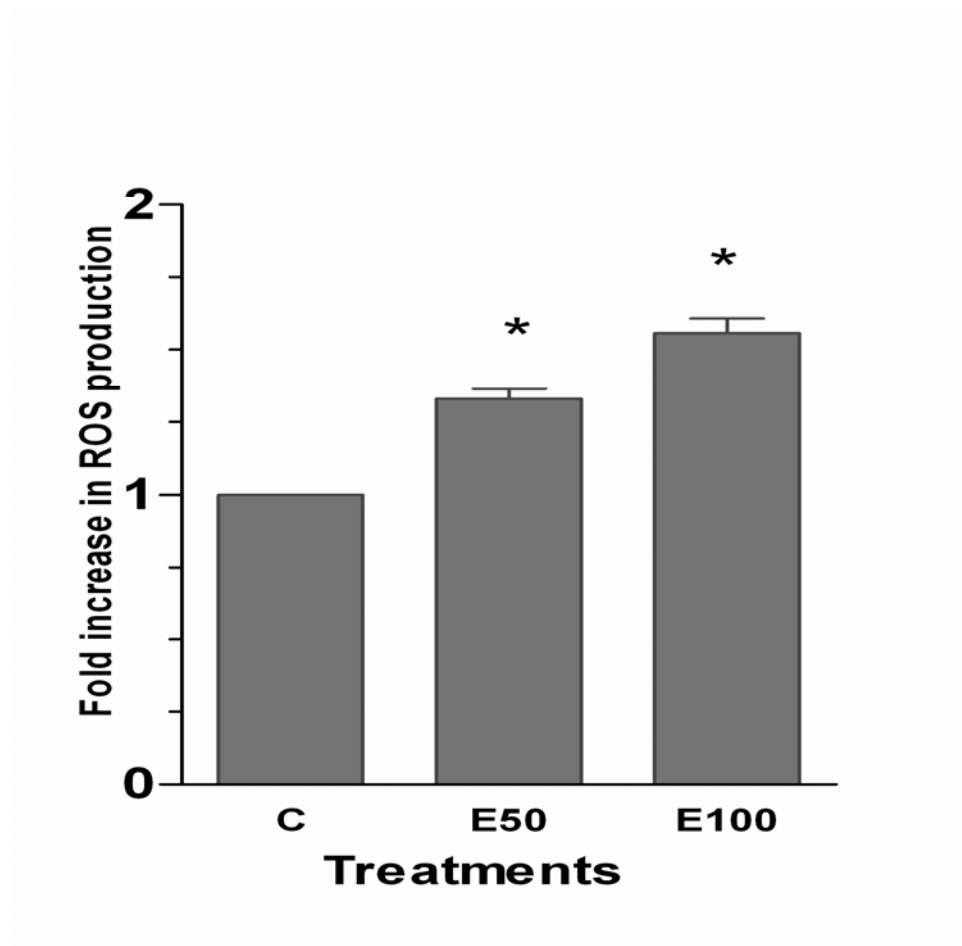
Fig. 1

Figure 2: Effect of glutathione modulators on ethanol induced histone H3K9 acetylation in hepatocytes. Hepatocytes were pretreated with a) 10 mM NAC or b) 1 mM BSO for 1 hr followed by with or without 50 mM ethanol for 23 hr. Nuclear extracts were prepared for immunoblot analysis to detect acetylated histone H3 at lys9. Identical amounts (40 µg) of extract were subjected to 15% SDS–PAGE and transferred onto nitrocellulose membrane. H3AcK9 levels were monitored using anti-H3 AcK9 antibody and ECL detection. Quantitative analysis of acetylated histone H3 was performed by densitometric analysis and is presented as mean ± SE (bar), n = 3 experiments. Values represent fold increase compared to control (C=Control, E50=50 mM Ethanol, N=10mM N-acetyl cystein, E50+N=50 mM Ethanol+10 mM N-acetyl cystein, B=1 mM Buthionine -S-sulfoxamine, E50+B=50 mM Ethanol+1 mM Buthionine -S-sulfoxamine). a) *p<0.01(E compared to C) and **p<0.01 (E50 + N compared to E50). b) *p<0.001 (E compared to C), **p<0.001 (E compared to E + B), *p<0.05 (C compared to B).
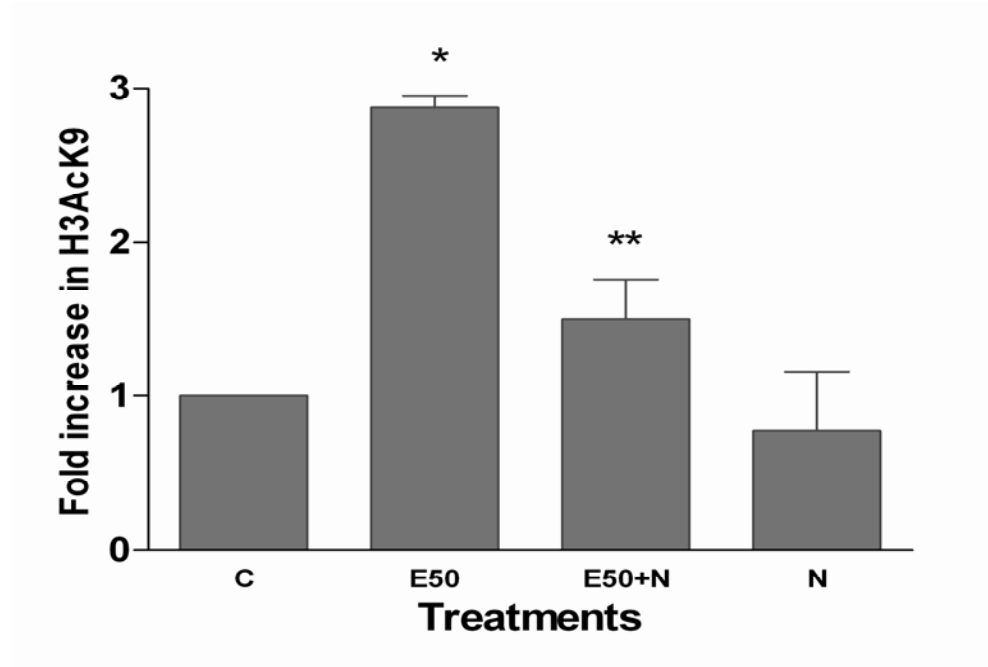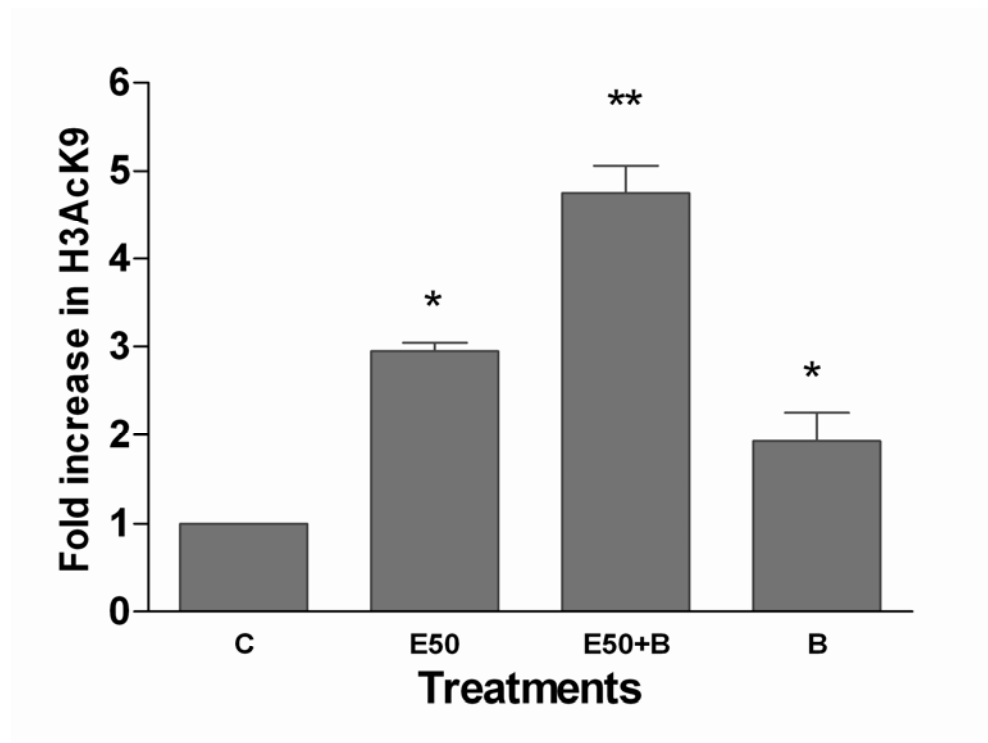
Fig. 2a

Fig. 2b

Figure 3: Effect of NAC and BSO in ADH1 gene expression. Hepatocytes were pretreated with 10 mM NAC or 1 mM BSO for 1 hr. Then cells were stimulated with 50 mM ethanol for 23 hr. Total RNA was isolated and reverse transcribed to cDNA. cDNA were amplified by PCR with ADH1 primers and PCR products were visualized by 1.5% agarose gel elctrophoresis (C=Control, E50=50 mM Ethanol, N=10 mM N-acetyl cystein, B=1 mM Buthionine -S-sulfoxamine, E50+N=50 mM Ethanol+ 10 mM N-acetyl cystein , E50+B=50 mM Ethanol+1 mM Buthionine -S-sulfoxamine). *p<0.001 (E50 compared to C), **p<0.001 (E50 compared to E50 + B or E50 + N).
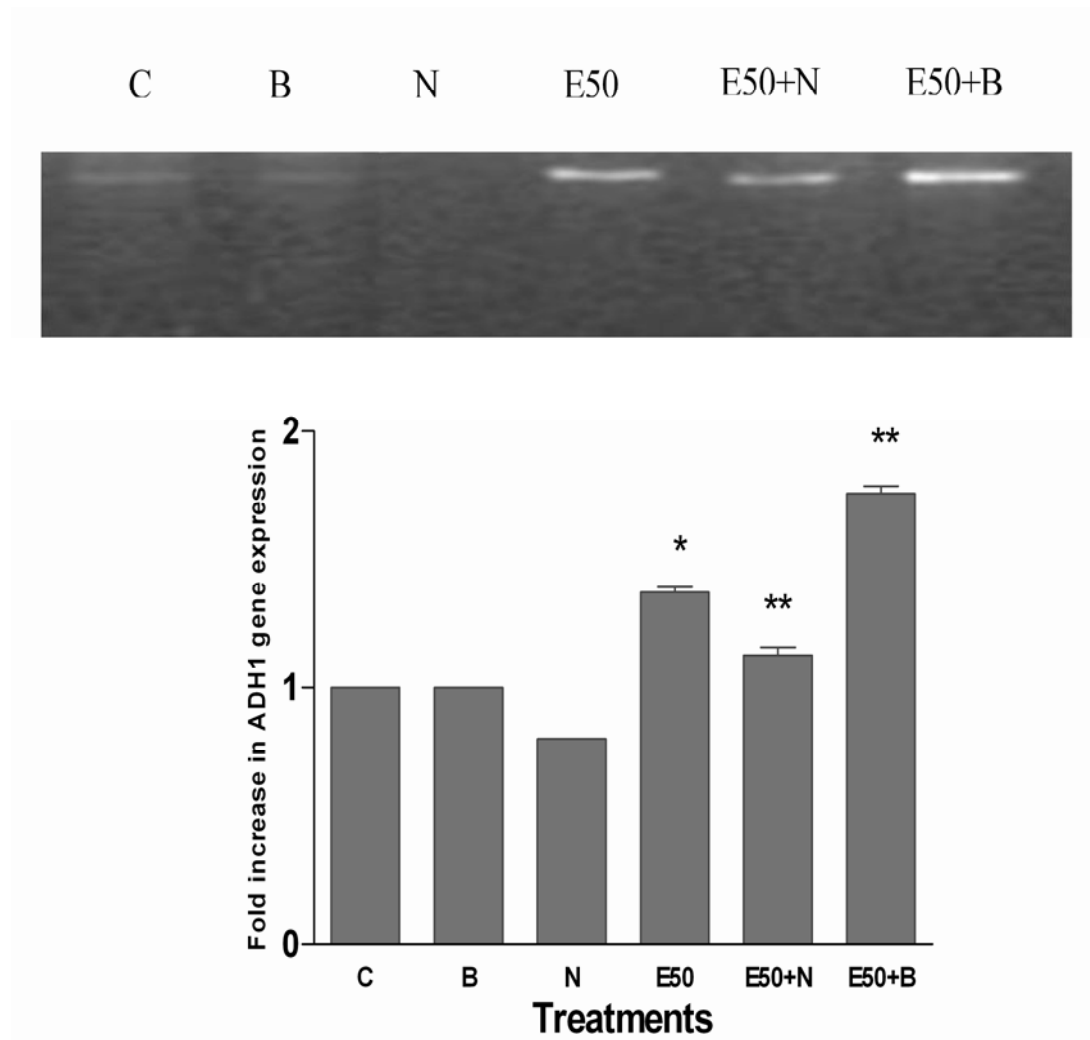
Fig. 3

Figure 4: Effect of dietary antioxidants on ethanol induced histone H3 acetylation in hepatocytes. Hepatocytes were pretreated with a) resveratrol (0.5 mM or 5 mM) and b) quercetin (5 or 20 mM) for 1 hr. Then cells were stimulated with 50 mM ethanol for 23 hr. Nuclear extracts were used for Western blot analysis as in Fig. 2. Quantitative analysis of acetylated histone H3 was performed by densitometric analysis and is presented as mean ± SE (bar), n = 3 experiments. Values represent fold increase compared to control. (C=Control, E50=50 mM Ethanol, R0.5=0.5 mM Resveratrol, R1=1 mM Resveratrol, E50+R0.5=50 mM Ethanol+0.5 mM Resveratrol, E50+R1=50 mM Ethanol+1 mM Resveratrol, Q5=5 mM Quercetin, Q20=20 mM Quercetin, E50+ Q5=50 mM Ethanol+5 mM Quercetin, E50+ Q20=50 mM Ethanol+20 mM Quercetin). a) *$p<0.01$ (E50 compared to C), **$p<0.01$ (E50 compared to E50 + R0.5/R5) b) *$p<0.01$ (E50 compared to C), **$p<0.01$ (E50 compared to E50 + Q5/Q20).
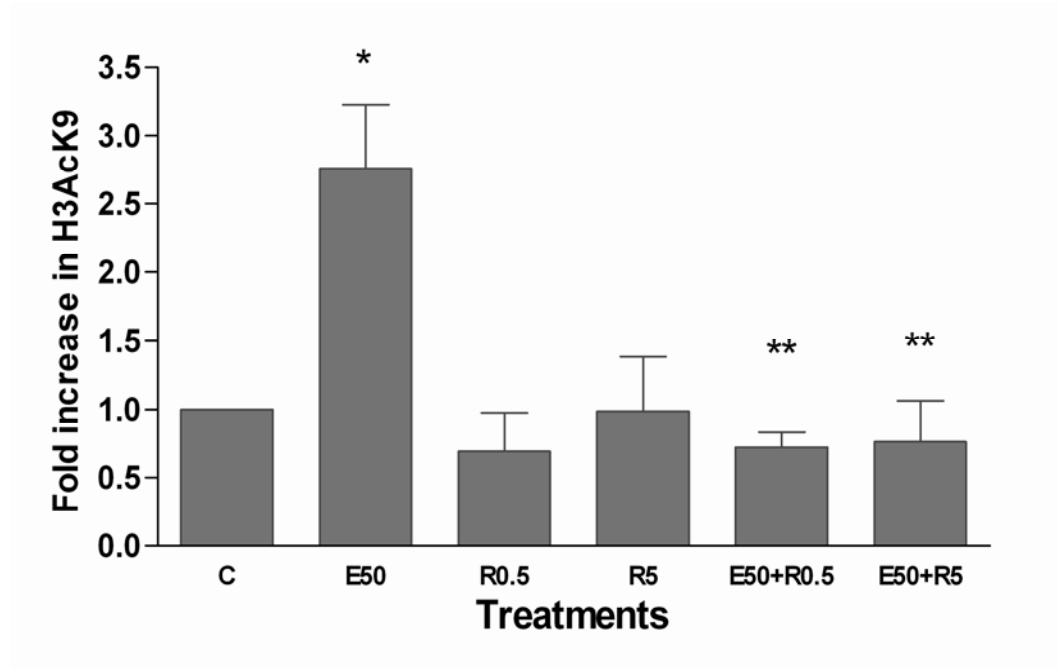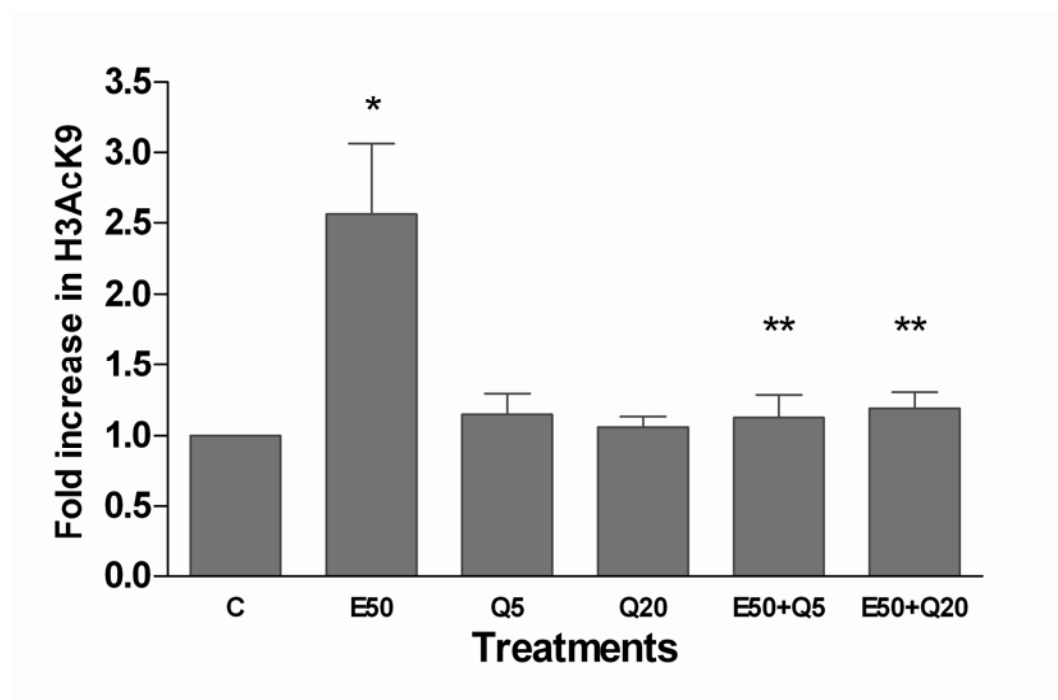
Fig. 4a

Fig. 4b

Figure 5: Effect of exogenous hydrogen peroxide (H₂O₂) treatment on ethanol induced histone H3 acetylation in hepatocytes. Hepatocytes were pretreated with H_2O_2 (10 or 50 µM H_2O_2) for 1 hr. Subsequently, cells were stimulated with 50 mM ethanol for 23 hr. Acetylated histone H3 at lys9 was measured as described in the Materials and Methods and Fig. 2. Values represent the mean of 3 separate experiments, with the ±SE indicated by vertical lines. Values represent as fold increase compared to control (C=Control, E50=50 mM Ethanol, H10=10 µM Hydrogen peroxide, H50=50 µM Hydrogen peroxide, E50+ H10=50 mM Ethanol+10 µM Hydrogen peroxide, E50+ H50=50 mM Ethanol+50 µM Hydrogen peroxide). *$p<0.001$ (C compared to E50 and H50), **$p<0.001$ (E compared to E50+ H50).
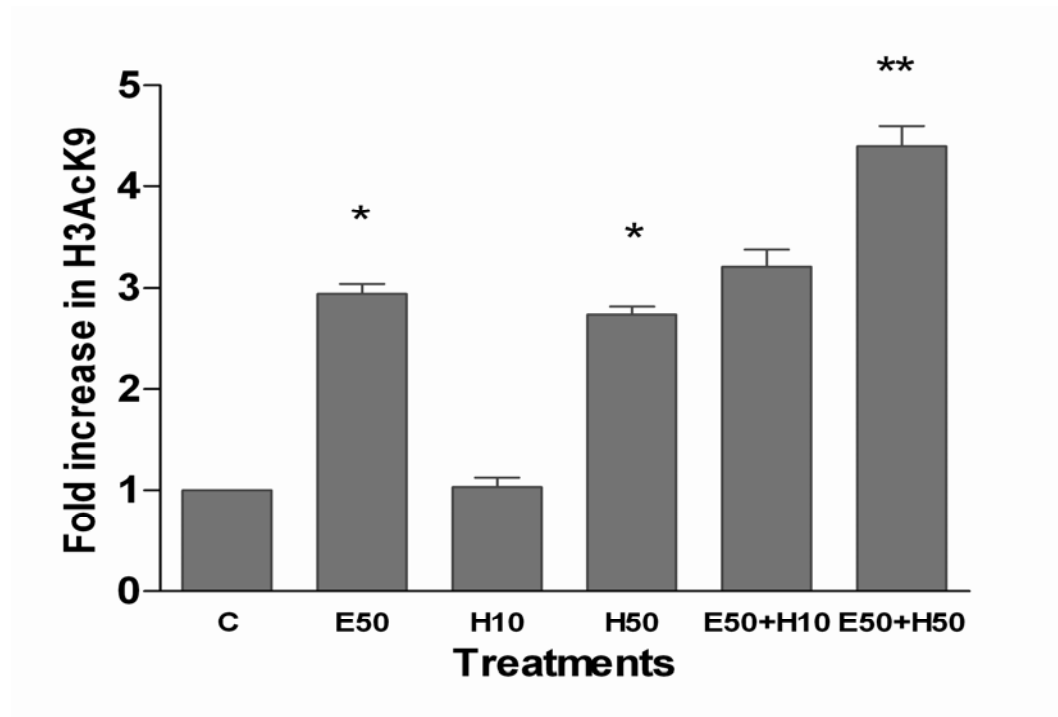
Fig. 5

Figure 6: Effect of mitochondrial respiratory chain inhibitors on ethanol induced histone H3 acetylation in hepatocytes. Hepatocytes were pretreated with a) rotenone (0.50 μM or 1 μM) and b) 0.025 mM antimycin for 1 hr. Then cells were treated with 50 mM ethanol for 23 hr. Equal amounts (40 μg) of nuclear extract were used for Western blot analysis to detect acetylated histone H3 at lys9. Quantitative analysis of acetylated histone H3 was performed by densitometric analysis and is presented as mean ± SE (bar), n = 3 experiments. Values represent as fold increase compared to control (C=Control, E50=50 mM Ethanol, Rt0.5=0.5 μM Rotenone, Rt1=1 μM Rotenone, E50+ Rt0.5=50 mM Ethanol+0.5 μM Rotenone, E50+ Rt1=50 mM Ethanol+1 μM Rotenone, An=0.025 mM Antimycin, E50+An=50 mM Ethanol+0.025 mM Antimycin) a) *$p<0.05$ (C compared to E and Rt1), **$p<0.01$ (E compared to E+ Rt1)

Fig. 6a
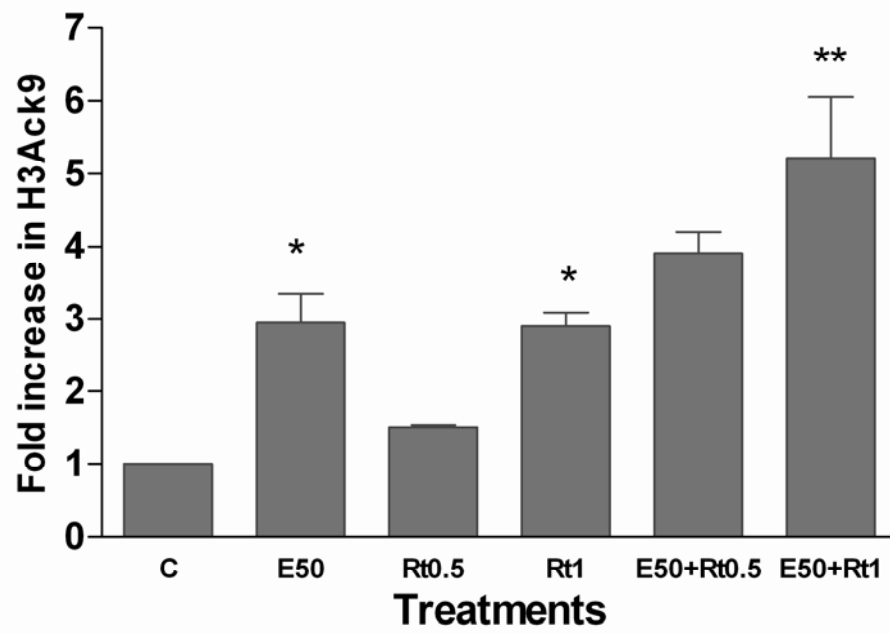
Fig. 6b
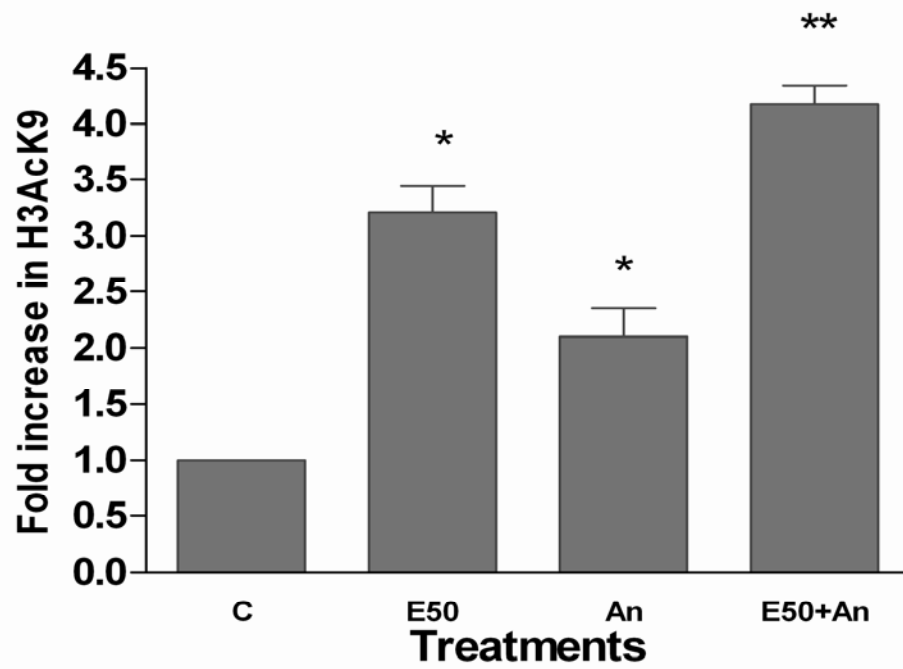
Figure 7: Effect of NADPH oxidase inhibitor apocynin on ethanol induced histone H3 acetylation in hepatocytes. Hepatocytes were pretreated with apocynin (0.1 mM or 1 mM) for 1 hr. Then cells were stimulated with 50 mM ethanol for 23 hr. Nuclear extracts were used for Western blot analysis as in Fig. 2. Quantitative analysis of acetylated histone H3 was performed by densitometric analysis and is presented as mean ± SE (bar), n = 3 experiments. Values represent here as fold increase compared to control (C= control, E50=50 mM Ethanol, E50+A0.1=50 mM Ethanol +0.1mM Apocynin, E50+A1=50 mM Ethanol +1mM Apocynin, 0.1A=0.1mM Apocynin, 1A=1mM Apocynin). *p<0.001 (C compared to E), **p<0.01 (E compared to E + A0.1), **p<0.001 (E compared to E + A1).

Fig. 7
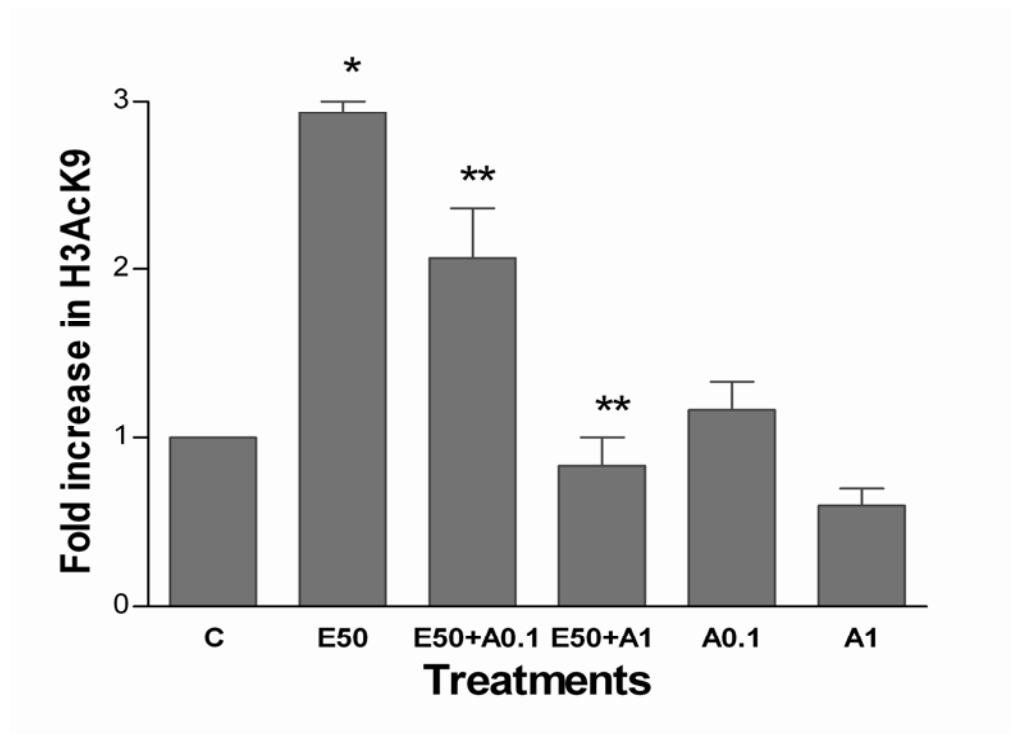
CHAPTER IV

Histone acetyltransferase GCN5 is involved in ethanol induced histone acetylation in Hepatoma cells

Histone acetyltransferase GCN5 is involved in ethanol induced histone acetylation in Hepatoma cells

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IV.1 Abstract

Ethanol causes cellular injury, but its molecular mechanisms of action are poorly understood. We have shown earlier that ethanol induces histone H3 lysine9 acetylation in liver with an increase in transcription of genes. However, the mechanism of ethanol induced histone acetylation and the identity of HAT that might be involved remain unknown. We demonstrate here that histone acetyltransferase GCN5 is responsible for ethanol induced histone H3AcK9 in hepatoma cells. Knock down of GCN5 by siRNA caused decrease in GCN5 protein levels, GCN5 HAT activity and also ethanol induced acetylation of H3K9. This is the first report of a specific HAT involved in the action of ethanol in cells.

IV.2 Introduction

Alcoholic liver disease is the second leading cause of death among all the liver diseases (Lieber, 2004). However, its biochemical action remains poorly understood. We have shown that ethanol and surrogate alcohols caused selective acetylation of histone H3 at lys9 in primary rat hepatocytes (Choudhury and Shukla, 2008). This was also demonstrated in rats acutely treated with ethanol (Kim and Shukla, 2006). Ethanol metabolism (Choudhury and Shukla, 2008), oxidative stress (Choudhury et al, submitted) and MAPKinase signaling pathway, are involved in this process (Shukla et al., 2008).

Histone acetylation has been linked to the transcriptional activity of cellular chromatin (Turner, 1998). The steady state level of histone acetylation is a balance between the action of histone acetyltransferases and histone deacetylases. Given the central role of these enzymes in transcriptional regulation, it is not surprising that aberrant regulation of these enzymes are linked to human disease (Kundu and Dasgupta, 2007). These enzymes are often found to be associated with large multisubunit protein complexes and contain known regulators of transcription (Struhl, 1998).

In the yeast, one of the best known HATs is Gcn5 which is the catalytic subunit of the SAGA complex that acetylates primarily histones H3 and H2B (Zhang et al., 1998; Suka et al., 2001). The transcriptional adaptor GCN5 was originally demonstrated to be a histone acetyltransferase predominantly modifying H3 at K14 *in vitro* (Kuo et al., 1996). Adkins et al. showed that GCN5 is responsible for S-phase –specific peak of H3AcK9 in yeast (Adkins et al., 2007). Carre et al. found that dGCN5 mutations abolished the K9 and K14 acetylation of H3 but had no effect on H4K8 acetylation in Drosophila (Carre et

al., 2005). In addition, Imoberdorf et al. showed a global acetylation by GCN5 (Imoberdorf et al., 2006) in yeast. Even though extensive biochemical and structural analysis of GCN5 complexes exist (Marmorstein and Roth, 2001), the role of GCN5 is not well understood in mammalian cells. One major limitation is the lack of GCN5 knockout mice. Deletion of GCN5 in mice leads to embryonic death (Xu et al., 1998; Yamauchi et al., 2000). In addition, histone H3 Lys9 is highly disordered within the crystal structure, so its location reactive to specific active site residues in GCN5 can not be discerned from the structures in hand (Rojas et al., 1999). In the context of ethanol induced histone acetylation, the mechanism and identity of specific HAT targeted by ethanol remain unknown. We have therefore investigated this in relation to GCN5 in human hepatoma cells.

IV.3 Experimental Procedures

Materials

Polyclonal anti-acetylated histone H3 lysine9 antibody and HAT assay kit were obtained from Upstate Biotechnology (Lake Placid, NY). GCN5 antibody and donkey anti-goat IgG were from Santa Cruz Biotechnology (Santa Cruz, CA). The 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). Ethanol (≥98% pure) was purchased from Fisher Scientific (Fair Lawn, NJ). All other chemicals were obtained from Sigma (St. Louis, MO). Small interfering RNA (for hGCN5, non-targeting RNA) and the transfection reagent were purchased from Dharmacon (Lafayette, CO).

Cell culture

VA-13 is a clonal derivative of Human hepatoma HepG2 cells stably transfected with a eucaryotic expression plasmid containing a cDNA copy of murine alcohol dehydrogenase 1 (*ADH1*) (Clemens et al., 2002). These cells were cultured in DMEM medium with 400 μg/ ml zeocin. The cells were subcultured at appropriate intervals according to experimental protocol. During ethanol treatment, 25 mM HEPES (pH 7.3) was added to the growth media and flasks were tightly sealed to minimize the evaporation of ethanol.

siRNA silencing studies

Cells were seeded at 2 X 10^5 per well in 6 well culture plates in an antibiotic free normal growth medium DMEM with 10% fetal bovine serum. After 14 hr of plating, the cells were washed and the growth medium was replaced with 0.5 ml low serum Opti-

MEM medium (GIBCO)**.** GCN5 target (from Dharmacon SMARTpool- combination of four siRNAs) or non-target siRNA oligonucleotides (each 40 nM); and transfection reagent were diluted with Opti-MEM medium separately for 5 min under sterile conditions. Target and non-target siRNA were then mixed with the diluted transfection reagent and incubated for 30 min inside the sterile hood before adding to the culture wells. After 10 hr, 1 ml of growth medium was added to each well. The media was replaced with the serum free media 42 hr after siRNA addition,. The cells were then treated with 100 mM ethanol according to the experimental design. Nuclear extraction was performed after 10 hr of ethanol incubation. The nuclear extraction protocol was described in our earlier publication (Choudhury and Shukla, 2008).

Western blotting

Equal amounts of nuclear extracts were run on various percentage of SDS–PAGE depending on the molecular weight of the protein of interest. The detailed protocol was described in our earlier publication (Choudhury and Shukla, 2008).

HAT assay

HAT activity was measured using an assay kit according to the manufacturer's protocol (Upstate) and reported earlier (Choudhury and Shukla, 2008).

Statistical Analysis

Each experiment was performed in triplicates and at least 3-5 times. Each figure was created from 3 separate experiments. The statistical significance was calculated using a standard one-way ANOVA (Newman-Keuls Multiple Comparison Test). Values with $p < 0.05$ were considered significant**.**

IV.4 Results

We have investigated the role of GCN5 in ethanol induced histone H3 acetylation at lysine 9 in VA-13 cells. In primary hepatocytes, 100 mM ethanol showed the highest histone acetylation and was the selected concentration for these experiments. However, we were unable to use primary hepatocytes for the GCN5 downregulation experiments because in the absence of antibiotics (necessary for siRNA transfection), cells were easily contaminated and could not be cultured for longer periods. In addition, primary hepatocytes cultured in vitro for extended period lose the ability to express many liver-specific functions including alcohol metabolism and ethanol metabolism is required for histone acetylation (Park et al., 2003; Choudhury and Shukla, 2008). Since normal HepG2 cells, which contain low ADH1 activity, also did not show any appreciable change in histone acetylation by ethanol (unpublished data), we decided to use for this study a clonal derivative of HepG2 VA-13, which constitutively expresses ethanol-metabolizing enzyme ADH1. VA-13 cells are well characterized and have been shown to possess primary hepatocytes-like characteristics (Clemens et al., 2002).

Effects of ethanol on histone H3 acetylation at lysine 9 and GCN5 in VA-13 cells

First we confirmed that ethanol can induced histone acetylation in VA-13 cells. At 10 hr, ethanol increased the amount of acetylated H3K9 by 4.5 fold compared to control (Fig. 1a). Increases in H3AcK9 compared to control were also seen at other time points (data not shown). For designing siRNA experiment, we chose the 10 hr time point. Importantly, VA-13 cells expressed GCN5 (Fig.1b) although the GCN5 protein expression level did not change significantly following ethanol exposure.

Silencing of GCN5 and ethanol effect on histone H3 acetylation at lys9 in GCN5 knocked down VA-13 cells

To examine GCN5 involvement in ethanol induced H3AcK9, we performed siRNA mediated GCN5- silencing experiments. VA-13 cells were transfected with GCN5 siRNA (target) or scrambled siRNA (non –target) oligoneucleotides as described in the 'Experimental Procedures'. The target siRNA transfection casused > 80% inhibition of GCN5 protein expression (Fig. 2a). β-actin levels (a house keeping protein) were unchanged in these samples. As expected, nontarget siRNA transfected cells did not show any change in GCN5 expression. To assess whether the knockdown of GCN5 had any influence on the ethanol induced H3AcK9, we incubated the transfected or non-transfected VA-13 cells with 100 mM ethanol for 10 hr and used western blot to examine the nuclear extracts for H3AcK9. Ethanol increased H3AcK9 by 4.3 and 4.2 fold, respectively in ethanol treated normal cells and cells transfected with scrambled siRNA (Fig. 2b). This ethanol induced increase in H3AcK9 was reduced in GCN5 siRNA-transfected cells by 67% ($p<0.001$) (Fig. 2b).

Effects of ethanol on HAT activity in the absence and presence of GCN5

In a similar approach as above, we conducted HAT activity assay in various nuclear extracts from cells transfected with target and nontargeting siRNA. A 2.9 and 3 fold increased HAT activity were noted in 100 mM ethanol treated normal cells and cells transfected with scrambled siRNA, respectively, were noted. On the other hand, VA-13 cells transfected with GCN5 siRNA showed 63% decease in ethanol induced HAT activity (Fig. 3).

IV.5 Discussion

The data presented in this study demonstrate a role for a specific HAT, GCN5, in the action of ethanol. Knockdown of GCN5 with siRNA and the concomitant decreases in GCN5 protein expression, HAT activity and ethanol induced acetylation of H3K9, provide evidence that GCN5 is involved in ethanol induced increase in H3AcK9. Though our data showed that GCN5 downregulation alone almost eliminated the increase in H3AcK9, we can not completely exclude the role of other HATs and co-activators. It may be quite possible that the other HATs or co-activators' functions were also suppressed by the knockdown of GCN5. Hence, it will be interesting to examine the involvement of others coactivators on histone acetylation.

There are controversies about the specificity of lysine residue acetylation by GCN5 in vivo or in vitro (Kuo et al., 1996; Adkins et al., 2007). Although there are several lysine residues on the histone H3, acetylation at lys9 is particularly relevant for the transcriptional regulation (Jenuwein and Allis, 2001). Preferential acetylation of specific lysine residue may lie in the specificity of HAT or the complex it is associated with. Grant et al. showed that the GCN5-dependent complex Ada preferentially acetylates Lys-14 > Lys-18, while SAGA acetylates Lys-14 > Lys-18 > Lys-9 = Lys-23 on synthetic H3 peptide substrates and on nucleosomal H3 in yeast (Grant et al., 1999). Therefore, it will be interesting to investigate the effect of GCN5 on other positions.

The data obtained on the mechanisms of histone acetylation by ethanol will be important in the construction of the 'molecular map' of ethanol induced liver injury and

will help develop therapeutic tools targeting specific molecules/steps. In order to develop new therapeutic tools for the diagnosis, prevention and control of alcohol induced liver injury; GCN5 may function as a new target in the action of alcohol.

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Fig. 1 Effect of ethanol on histone H3 acetylation at lys9 and GCN5 in VA-13 cells.
Cells were treated with 100 mM ethanol for 10 hours. Nuclear extracts were used for
Western blot analysis to detect a) acetylated histone H3 at lys9 and b) GCN5. Equal
amounts (40 µg) of extract were subjected SDS–PAGE and transferred onto
nitrocellulose membrane and monitored with ECL detection. Quantitative analysis was
performed by densitometric analysis and is presented as mean ± SE (bar), n = 3
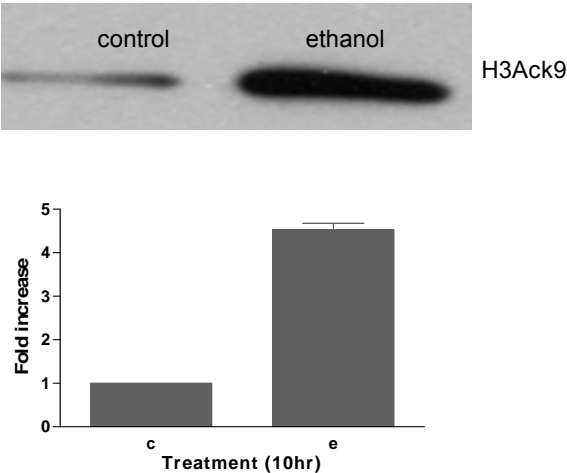experiments (c= control, e= 100 mM ethanol).
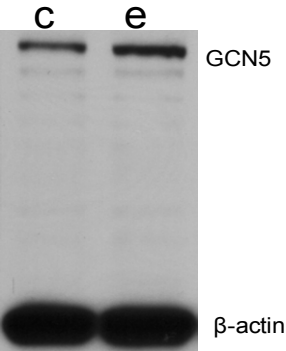
Fig.1a

Fig. 1b

Fig. 2 Effect of GCN5siRNA oligonucleotides on ethanol induced histone H3 acetylation in VA-13 cells. Cells were transfected with scrambled (non targeted siRNA) or GCN5 siRNA oligonucleotides as described in "Experimental Procedures". Expression of GCN5 and H3AcK9 were analyzed by Western blots. a) Equal amount of nuclear extracts were run on the blots for GCN5 or β-actin. b) The siRNA transfected cells were next treated with 100 mM ethanol for 10 hr, the nuclear proteins were extracted and western blot for H3Ack9 were performed. Quantitative analysis of the bands was performed by densitometry and is presented as mean ± SE (bar), n = 3 experiments. Values represent as fold increase compared to control (c- untransfetecd cells, no ethanol; e- ethanol, untransfected cells; t- GCN5 siRNA transfected cells, no ethanol; n - non-target siRNA transfected cells, no ethanol; t+e- GCN5 siRNA transfected cells, with 100 mM ethanol; n+e- non-target siRNA transfected cells, with 100 mM ethanol).#p<0.001 (t+e compared to n+e), *p<0.001 (c compared to e or n+e). In this figure, the bar without significance sign did not show any statistical significance.
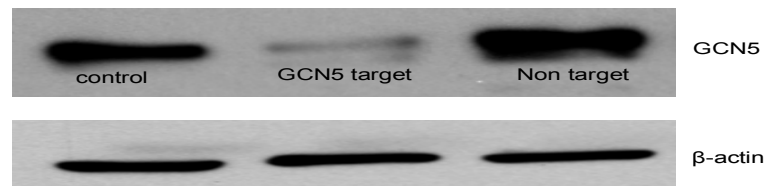
Fig. 2a

Fig. 2b

Fig. 3 Effect of GCN5siRNA oligonucleotides on ethanol induced HAT activity in VA-13 cells. Effects of GCN5siRNA ologonucleotides on ethanol induced HAT activity was examined by the ELISA method. Cells were transfected with scrambled (non targeted siRNA) or GCN5 siRNA oligonucleotides as described in "Experimental Procedures" followed by ethanol treatment. Nuclear extracts were prepared and incubated with histone H3 peptide in the presence of HAT assay cocktail containing HAT assay buffer, Na-butyrate and acetyl-CoA. HAT activity was measured by ELISA plate reader (see Experimental Procedures). Values are presented as mean ± SE (bar), n = 3 experiments and represent fold increase over control group (control = 1) (c- untransfetecd cells, no ethanol; e- ethanol, untransfected cells; t- GCN5 siRNA transfected cells, no ethanol; n - non-target siRNA transfected cells, no ethanol; t+e- GCN5 siRNA transfected cells, with 100 mM ethanol; n+e- non-target siRNA transfected cells, with 100 mM ethanol). *$p<0.001$ (c compared to e or n+e), #$p<0.001$ (t+e compared to n+e). In this figure, the bar without significance sign did not show any statistical significance.
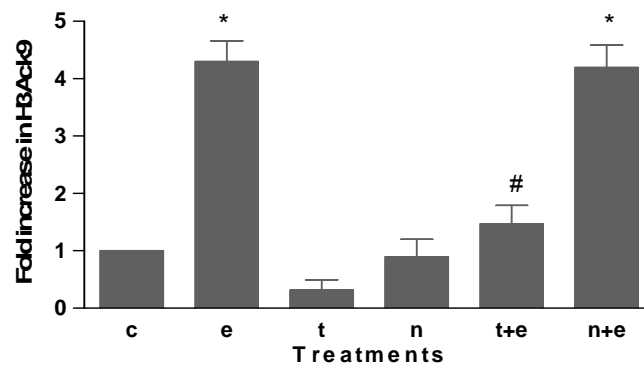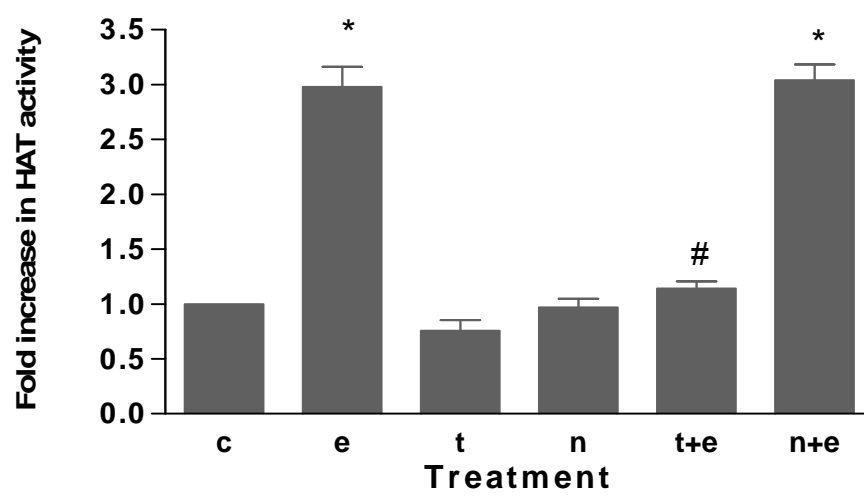
Fig. 3

CHAPTER V

Conclusions and Future Directions

Alcohol affects all the major organs in human body especially liver which is the major site of alcohol metabolism. Exposure to alcohol causes oxidative and metabolic stress and most importantly liver damage. Actions of alcohol in the liver are complex. In order to develop new therapeutic tools for the diagnosis, prevention and control of alcohol induced liver injury, it is necessary to identify new targets which are specific for alcohol. Therefore, research on both acute and chronic effects of alcohol on liver is beneficial.

Ethanol induced liver injury is associated with a global change in gene expression but its mechanisms are not known. Our laboratory has shown that ethanol altered histone acetylation, methylation, and phosphorylation in hepatocytes and these changes are correlated with various gene expression (Park et al., 2005; Bhadra et al., 2007). The research described in this dissertation was intended to determine the mechanism and regulation of histone acetylation by ethanol and surrogate alcohols.

An interesting pattern of histone H3 acetylation by alcohols and their metabolites emerges from these studies. Preferential acetylation of specific lysine residues may be indicative of transcriptional regulation. Surrogate alcohols showed variable effect on histone acetylation and metabolites play key role in this process. It can be argued that individual alcohol and metabolites mediate their action by different pathways. Metabolites can exert their action via modulation of the HAT or HDAC

activity, though the exact mechanism of action is still unknown. Previous study has shown the involvement of MAPK signaling pathway in ethanol induced H3AcK9 (Park et al., 2005). In addition, in the present study, we have shown that oxidative stress plays a role in ethanol induced histone acetylation. Oxidative stress was also shown to be partially involved in ADH1 gene expression which is correlated with ethanol induced histone acetylation. However, it is still unknown if oxidative stress modulates HAT or HDAC activity or MAPK or other signaling pathways and subsequently induces histone acetylation. Further investigation is needed to understand this complex mechanism.

An appreciation for this complex network of histone acetylation comes from the fact that there are several HATs and HDACs present in the liver. These enzymes are shown to acetylate or deacetylate preferentially specific histone or specific lysine residue. In this dissertation, I identified a specific HAT, GCN5, which is responsible for ethanol induced histone H3 acetylation at lysine 9. However, GCN5 is found in association with other multiprotein complex. So we can not rule out the other associated HATs or coactivators' role in ethanol induced histone acetylation. In that scenario, it is speculated that GCN5 inactivation leads to shut down of the others' activities. Hence, if there is any link between ethanol induced histone acetylation and liver injury, GCN5 will be a powerful therapeutic target to prevent the injury. In conclusion, these original findings may contribute to a better understanding of the mechanism underlying the pathogenesis of alcoholic liver disease.

Based on these conclusions, I propose a model illustrating the mechanism and regulation of alcohol induced histone acetylation (Fig. 1)

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

The model shows the complex network of alcohol induced histone H3 acetylation at lysine 9. Alcohols increase the histone acetylation via metabolites. Alcohols and metabolites both increased the HAT activity in hepatocytes. However, only the fatty acid metabolites increased HAT activity in vitro and only propionate and butyrate reduced HDAC activity when added directly to an in vitro assay. Ethanol specifically activates GCN5 and leads to histone H3 acetylation at lysine 9. More complexity is added to the network since oxidative stress also plays a role in ethanol induced acetylation and ADH1 gene expression.
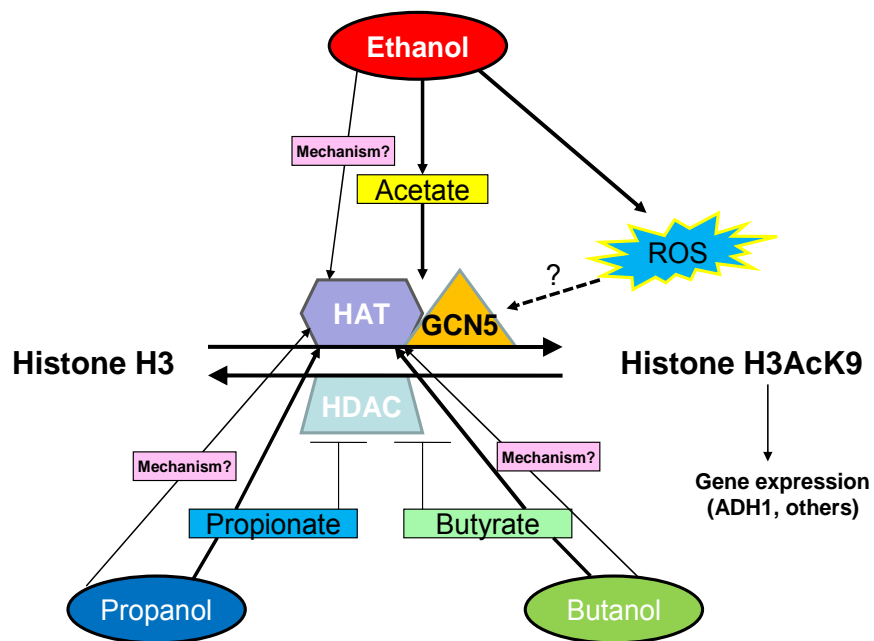
Future Direction

The goal of this dissertation was to determine if surrogate alcohols modulate histone acetylation, to elucidate the mechanism involved in alcohol induced histone acetylation, the role of reactive oxygen species and involvement of any specific HAT in ethanol induced histone acetylation. Based on the acquired data, many additional questions were raised for the future. It is important to have more knowledge on the regulation of alcohol induced histone modifications in relation to liver pathophysiology.

1. **Investigation of the effects of the surrogate alcohols on histone methylation, histone phosphorylation and histone ubiquitination.**

In addition to histone acetylation, other post translational modifications are also associated with transcription, regulation of gene expression and several biological processes (Lee and Peter, 2003; Zhang, 2003). They can occur on all four histones. Our lab has recently shown that ethanol altered the methylation of H3 at Lys4 and Lys9 (Bhadra et al., 2007) and phosphorylation of H3 at Ser10 and Ser28 in rat hepatocytes (Lee and Shukla, 2007). Hence, it would be interesting to look at the effects of surrogate alcohols on these post translational modifications on all the four types of histone.

Other emerging research shows that different histone modifications could affect each other (Zhang and Reinberg, 2001). Several studies showed that H3 phosphorylation at Ser10 or H3 methylation at K4 promoted histone acetylation (Lo et al., 2000; Wang et al., 2001). It is assumed that various transcriptional regulators utilize a distinct combination of enzymes and cofactors to induce various histone

modifications in different signaling pathways and also activating the gene transcription. Therefore, it is necessary to investigate the detailed mechanism and the regulation of alcohol induced post-translational modifications.

2. **Identification of specific genes which are upregulated or downregulated with the ethanol treatment in the presence or absence of GCN5 or other associated co-activators.**

   Histone acetylation is important in the regulation of gene expression. Although histone acetylation is generally associated with actively transcribed genes, there are also some exceptions. With the help of human gene array platform (Illumina, Affymetrix or Nimblegene) system, one should be able to discover genes which could be affected by this specific HAT. In addition, it will be interesting to investigate if GCN5 regulate any protein expression. Role of some selected proteins (HNF4α, GATA4, ApoCIII, PGC1α etc) which are connected to liver pathophysiology and histone acetylation will be of interest.

3. **Investigation of mechanism of GCN5 activity regulation in response to ethanol.**

   Ethanol increases histone H3 acetylation at lysine 9 via increasing HAT activity and GCN5 is involved. However, it may be possible that other HATs or coactivators associated with GCN5 take part in ethanol induced acetylation. So immunoprecipitation studies with various HAT antibodies will be a good addition to this project. In addition, it is still unknown how this reaction occurs. It would be

interesting to study the interaction of ethanol and HAT. More importantly, the GCN5 study should be conducted in in vivo binge drinking model.

3. **Relevance of the ethanol induced histone acetylation to the liver pathophysiology.**

Histone acetylation has been extensively studied to show roles in biological processes. It also varies from species to species, cells to cells. Histone acetylation was shown to be involved in apoptosis, cell differentiation, cell growth arrest and ethanol been shown to induce apoptosis in liver (Zhou et al., 2001; Mei et al., 2004). In addition, with the gene array (depending on the gene class) we can connect more biological responses to ethanol induced histone acetylation. Therefore, investigation of the ethanol induced histone acetylation's role in pathophysiological responses (apoptosis, necrosis, steatosis, etc.) would be valuable information in the treatment of alcoholic liver disease.

5. **Investigation of ethanol's effect on metal ion overload disease.**

The abnormal accumulation of the metal $Cu2^+$ is closely correlated with the incidence of various diseases, such as Alzheimer's disease and Wilson disease (the liver does not release copper into bile). In addition, carcinogenesis was caused by $Ni2^+$ ion exposure. Both cations were shown to induce histone hypoacetylation in human hepatoma cells (Kang et al., 2004; Kang et al., 2003). So, it will be interesting to investigate the role of ethanol in these diseases. Ethanol may be found as a preventive tool in these metal ion overload diseases. Some of my preliminary results showed inhibition of hypoacetylation (caused by copper ion) with the ethanol treatment.

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