PHARMACOLOGY AND CELL METABOLISM

Gene-Selective Histone H3 Acetylation in the Absence of Increase in Global Histone Acetylation in Liver of Rats Chronically Fed Alcohol

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Abstract — Aims: The aim of this study was to determine the effect of chronic ethanol feeding on acetylation of histone H3 at lysine 9 (H3-Lys9) at promoter and coding regions of genes for class I alcohol dehydrogenase (ADH I), inducible nitric oxide synthase (iNOS), Bax, p21, c-met and hepatocyte growth factor in the rat liver. Methods: Rats were fed ethanol-containing liquid diet (5%, w/v) for 1–4 weeks. The global level of acetylation of H3-Lys9 in the liver was examined by western blot analysis. The levels of mRNA for various genes were measured by real-time reverse transcriptase-polymerase chain reaction. The association of acetylated histone H3-Lys9 with the different regions of genes was monitored by chromatin immunoprecipitation assay. Results: Chronic ethanol treatment increased mRNA expression of genes for iNOS, c-jun and ADH I. Chronic ethanol treatment did not cause increase in global acetylation of H3-Lys9, but significantly increased the association of acetylated histone H3-Lys9 in the ADH I gene, both in promoter and in coding regions. In contrast, chronic ethanol treatment did not significantly increase the association of acetylated histone H3-Lys9 with iNOS and c-jun genes. Conclusion: Chronic ethanol exposure increased the gene-selective association of acetylated H3-Lys9 in the absence of global histone acetylation. Thus, not all genes expressed by ethanol are linked to transcription via histone H3 acetylation at Lys9.

INTRODUCTION

Post-translational modifications of histone, including acetylation, phosphorylation, methylation, ubiquitination and ADP-ribosylation, play an important role in the regulation of gene expression and chromatin remodeling by influencing histone–DNA and histone–histone interactions in the chromatin (Strahl and Allis, 2000). Of these modifications, acetylation of core nucleosomal histones neutralizes the positive charge on lysine residues, disrupts electrostatic interaction between histone and DNA and increases the accessibility of transcriptional regulatory proteins to the DNA. Therefore, histone acetylation has been shown to correlate with enhanced gene transcription (Allfrey et al., 1964).

Chronic ethanol consumption increases expressions of various genes in the liver. Ethanol-induced gene expression covers a wide spectrum of cellular functions including ethanol metabolism (Mistilis and Birchall, 1969; Gong et al., 2003), cell signaling (Spitzer et al., 2002) and apoptosis (Rashid et al., 1999; Zhou et al., 2001). Recent studies have shown a profiling of altered hepatic gene expressions by alcohol consumption (Tadic et al., 2002; Deaciuc et al., 2004) and suggested that these are implicated in the development of alcoholic liver disease (ALD), a significant and growing global health problem. However, the molecular mechanisms underlying ethanol-induced gene expression are poorly understood and whether histone acetylation plays a wider role is unknown.

Ethanol is predominantly metabolized in the liver. In the ethanol metabolism, the alcohol dehydrogenase (ADH) system is responsible for the majority of ethanol oxidation and the contribution of non-ADH systems, such as cytochrome P450 2E1 in the endoplasmic reticulum and catalase in the peroxisome, is considered to be minor (Badger et al., 2000). Multiple isozymes of ADH have been identified through their kinetic properties, substrate specificities and tissue distribution. Among these, the class I alcohol dehydrogenase (ADH I), the most abundant in the liver, is the principal enzyme for ethanol oxidation (Bosron et al., 1983) and its gene expression is strongly induced by ethanol treatment (Mistilis and Birchall, 1969). However, the molecular mechanisms for ethanol-induced ADH I gene expression is poorly defined.

Previously, we have shown that ethanol induces an increase in the overall level of acetylation of histone H3 at Lys9 (Park et al., 2003), and it enhances acetylation of histone at the promoter region of ADH I gene (Park et al., 2005) in primary cultures of rat hepatocytes. However, in vivo effect of chronic ethanol in this process has not been demonstrated. In the present study, we have investigated the role of histone H3 acetylation in the expression of various genes induced by chronic ethanol treatment in the rat liver and provide here evidence that chronic ethanol exposure enhances the acetylation of histone H3 at the region of ADH I in a gene selective manner.

MATERIALS AND METHODS

Reagents

Chromatin immunoprecipitation (CHIP) assay kit and polyclonal anti-acetyl histone H3 targeting specific lysine residues were purchased from Upstate Biotechnology (Lake Placid, NY, USA). Ethanol was purchased from Aldrich (Milwaukee, WI, USA). Trizol reagent for RNA isolation was purchased from Invitrogen (Carlsbad, CA, USA) and QPCR SYBR Green I Master Mixes for real-time polymerase chain reaction (PCR) was purchased from Applied Biosystems (Foster City, CA, USA).

Chronic ethanol feeding

Male Sprague–Dawley rats (200 g) were fed a nutritionally adequate ethanol-containing liquid diet for 4 weeks, as
described previously (De Carli and Lieber, 1967). Ethanol introduction was gradually increased starting with 1.25% (w/v) for the first day, 1.67% for the second day, 2.5% for third and fourth days and then increased to 5% for 4 weeks. The control rats were fed on the same liquid diet, except that ethanol was replaced by dextrin–maltose and strictly pair-fed isocalorically by administration of the same amount of liquid diet as taken by ethanol-fed rats on the previous day. After 1–4 weeks treatment, livers were removed, weighed, immediately immersed in liquid nitrogen and subsequently stored at −80°C. The animal study was in accordance with the guidelines of the National Institutes of Health (USA) and the protocol for their use was approved by the University of Missouri Animal Care & Use Committee (protocol approval # 7178).

**Western blot analysis**

At indicated time periods after ethanol administration, the liver was removed. Nuclei were collected by sucrose-density gradient centrifugation as described previously (Ho and Guenthner, 1997) and nuclear acid extracts were prepared as described previously (Park et al., 2003). Equal amounts (5–10 µg) of proteins were fractionated on 15% sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane. After blocking with 5% non-fat dried milk for 2 h, membrane was incubated with site-specific acetyl histone H3 antibody overnight at 4°C. Membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature. The HRP was detected by enhanced chemiluminescence (Pierce) according to the manufacturer’s instructions. Two to three hundred nanograms of total RNA were reverse transcribed using the RETROscript RT–PCR Kit (Ambion, Austin, TX, USA) with random decamers as primers. RT–PCR amplification was performed using QPCR SYBR Green Master Mix (Abgene) at 95°C for 15 s and 60°C for 1 min. The relative amount of target mRNA was determined using the CT method by normalizing target mRNA to that derived from the input (starting) supernatants. The rest of the samples were incubated with anti-acetylated H3-Lys9 antibody or normal rabbit immunoglobulin G overnight at 4°C and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature. The HRP was detected by enhanced chemiluminescence (Pierce) according to the manufacturer’s protocol.

**RNA isolation and quantitative reverse transcriptase-polymerase chain reaction**

The mRNA level of various genes in chronic ethanol-treated liver was measured by real-time reverse transcriptase-polymerase chain reaction (RT–PCR). Total RNA was isolated from the liver using the RNeasy Micro Kit (Qiagen, Valencia, CA, USA), with on-column DNA digestion using the RNase-free DNase set (Qiagen) according to the manufacturer’s instructions. Two to three hundred nanograms of total RNA were reverse transcribed using the RETROscript Kit (Ambion, Austin, TX, USA) with random decamers as primers. Primers sequence used for real-time RT–PCR were designed previously (De Carli and Lieber, 1967). Ethanol introduction was gradually increased starting with 1.25% (w/v) for the first day, 1.67% for the second day, 2.5% for third and fourth days and then increased to 5% for 4 weeks. The control rats were fed on the same liquid diet, except that ethanol was replaced by dextrin–maltose and strictly pair-fed isocalorically by administration of the same amount of liquid diet as taken by ethanol-fed rats on the previous day. After 1–4 weeks treatment, livers were removed, weighed, immediately immersed in liquid nitrogen and subsequently stored at −80°C. The animal study was in accordance with the guidelines of the National Institutes of Health (USA) and the protocol for their use was approved by the University of Missouri Animal Care & Use Committee (protocol approval # 7178).

**CHIP assay**

CHIP assay was carried out essentially as described previously (Park et al., 2005). Briefly, liver tissue was fixed with formaldehyde and homogenized in phosphate-buffered saline. Liver cells were lysed in SDS lysis buffer and cellular lysates were sonicated with four sets of 10-s pulses to shear DNA. After centrifugation, an aliquot (2%) of the supernatants was kept and designated as ‘input (starting)’ fraction supernatants. The rest of the samples were incubated with anti-acetylated H3-Lys9 antibody or normal rabbit immunoglobulin G overnight at 4°C and incubated with protein A agarose/salmon sperm DNA slurry for 1 h at 4°C. After washing, DNA was extracted from the precipitates by phenol–chloroform extraction and ethanol precipitation.

**Analysis of CHIP assay**

Aliquots of the immunoprecipitated DNA from each sample were amplified by real-time PCR. The amplification was performed using QPR SYBR Green Master Mix (Abgene) at the following conditions: 50°C for 2 min, 95°C for 15 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The relative amount of target mRNA was determined using the comparative threshold (Ct) method by normalizing target mRNA Ct values to those for glyceraldehyde-3-phosphatedehydrogenase (GAPDH;ΔCt). The sequences for the different primer pairs are listed in Table 1.

**Statistical analysis**

Data are expressed as mean ± SD and obtained by combining data from separate experiments. Statistical significance was determined by one-way analysis of variance for comparison of multiple samples or t-test (two-tailed, unpaired).

### Table 1. Primer sequence for real-time PCR used for CHIP assay

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>ADH I (P1)</td>
<td>5'-TTGCGACCCAGCAGTGA-3' (F)</td>
</tr>
<tr>
<td>iNOS (P1)</td>
<td>5'-AACAGGAGCTAGCTGTAA-3' (F)</td>
</tr>
<tr>
<td>c-Jun (P1)</td>
<td>5'-CTTCAATCGCCCGCATTC-3' (F)</td>
</tr>
<tr>
<td>c-met (P1)</td>
<td>5'-GTACGGTGTCTCCAGCATTTT-3' (F)</td>
</tr>
<tr>
<td>p21 (P1)</td>
<td>5'-AGAGCAACCACCTGCATGAAG-3' (F)</td>
</tr>
<tr>
<td>HGF (P1)</td>
<td>5'-AGAGCGGAGGCTGAGCTGAAT-3' (F)</td>
</tr>
<tr>
<td>GAPDH (P1)</td>
<td>5'-TATGATGACATCAAGTGTTG-3' (F)</td>
</tr>
</tbody>
</table>

F, forward primer; R, reverse primer.
Differences with a \( P < 0.05 \) were considered statistically significant.

**RESULTS**

**Effect of chronic ethanol treatment on mRNA expression of target genes in the rat liver**

Prior to investigating the role of histone acetylation in gene expression by chronic ethanol treatment in the rat liver, we first identified target genes whose mRNA expressions are increased by chronic ethanol treatment in the liver. In this study, rats were fed with ethanol-containing liquid diet (5% w/v) for 4 weeks. After 4 weeks ethanol treatment, the plasma alanine aminotransferase level was significantly increased to 108.14 \( \pm \) 13.13 mU/ml when compared with pair-fed control rats (51.66 \( \pm \) 4.71 mU/ml, \( P < 0.01 \)). Blood alcohol levels in 4-week ethanol-fed rats were 22 \( \pm \) 9 mM. There was no significant difference in body weights between the two groups. However, the liver weight was significantly increased in ethanol-treated rats, consequently the ratio of liver weight to body weight was significantly increased in ethanol-fed rats (18%, \( P < 0.01 \)), indicating fatty liver in alcohol-fed animals.

Ethanol treatment has been shown to affect expression of various genes. The gene expression pattern by ethanol exposure largely depends on the experimental conditions. We first examined the effects of chronic ethanol exposure on the mRNA expression of various possible target genes. As shown in Fig. 1, treatment of rats with ethanol (5% w/v) for 4 weeks significantly increased the mRNA levels of ADH I, c-jun and inducible nitric oxide synthase (iNOS) in the liver consistent with previous studies (Wang et al., 1998; He et al., 2002; Yuan et al., 2006). The mRNA levels of other target genes, including Bax, p21, hepatocyte growth factor (HGF) and HGF receptor (c-met), were not significantly affected under these experimental conditions. It may be noted that the ethanol exposure has been shown by many studies to increase protein levels of above components (Buris et al., 1985; Wang et al., 1998; He et al., 2004; Yuan et al., 2006).

**Effect of chronic ethanol treatment on the acetylation of histone H3 at Lys9 at the different regions of target genes in the rat liver**

We have previously shown that ethanol treatment selectively increases acetylation of histone H3 at Lys9 in rat hepatocytes (Park et al., 2003). We next examined, using CHIP assay, whether chronic ethanol-induced expression of ADH I, c-jun and iNOS are related to increase in histone acetylation. Liver lysates from ethanol-treated and control pair-fed rats were immunoprecipitated with anti-acetyl H3-Lys9. DNA was isolated from immunoprecipitates and amplified by PCR using primer pairs in the ADH I promoter region (P1) and the ADH I coding region (P2). As shown in Fig. 2A, chronic ethanol treatment increased accumulation of acetylated H3-Lys9 with promoter (P1) and coding (P2) region of ADH I gene by 2.4- and 2.1-fold, respectively, consistent with in vitro study. However, as shown in Fig. 2B and C, no significant changes in the levels of acetylated H3-Lys9 in iNOS and c-Jun genes were detected in the liver after chronic ethanol treatment. This was established by CHIP assay using four primer pairs encompassing different regions (promoter and coding regions) of iNOS and c-jun gene (Table 1).

**Effect of chronic ethanol exposure on global histone H3 acetylation in the rat liver**

We have previously shown that acute ethanol treatment causes a selective acetylation of histone H3 at Lys9 without effect at Lys14, 18 and 23 in rat liver (Park et al., 2005). In the present study, we examined the effect of chronic ethanol treatment on the overall level of acetylation of histone H3 in rat liver. As shown in Fig. 3, treatment of rat with ethanol for 1, 2 or 4 weeks produced negligible change in total acetylation level of histone H3 at both Lys9 and Lys14 in the liver, showing an effect distinct from acute ethanol treatment (Park et al., 2003, 2005).

**DISCUSSION**

Acetylation of core histones play important role in transcriptional activation by altering chromatin structure (Grunstein, 1997). Especially, acetylation of histone H3 at Lys9 is considered to be a marker of active genes (Thiagalingam et al., 2003; Jayani et al., 2010). Ethanol treatment has been shown to increase activity of ADH I, most likely due to increased enzyme expression levels (Mistilis and Birchall, 1969; He et al., 2002). We have previously shown that ethanol increases the global acetylation of histone H3 at Lys9 in rat hepatocytes through modulating the activity of histone acetyltransferases (HATs) and increases the association of acetylated H3-Lys9 with ADH I gene in cultured rat hepatocytes (Park et al., 2005). In the present study, we have investigated the effect of chronic ethanol treatment on the acetylation of histone in the region of iNOS, c-fos and ADH I gene in vivo.

Ethanol exposure has been shown to increase expression of various genes in the liver. To investigate the role of histone acetylation in ethanol-induced gene expressions, we first examined the effects of chronic ethanol treatment on mRNA expression of selected target genes in the rat liver. Four-week treatment with ethanol increased expressions of ADH I, iNOS and c-jun consistent with previous reports, whereas it did not significantly affect expressions of other candidate genes, i.e. p21, Bax, c-met and HGF, in the rat liver. Even though previous reports have shown that ethanol treatment increased expressions of these genes (Rashid et al., 1999; Koteish et al., 2002; Lalani et al., 2005), the gene expression profile by ethanol treatment depends on experimental condition, such as acute or chronic exposure, feeding methods and species and tissues. The difference between our results and previous studies may be reflective of different experimental paradigms.

Chronic ethanol treatment enhances acetylation of H3-Lys9 at the region of ADH I gene in the rat liver determined by CHIP assay (Fig. 2A). In this study, DNA from the immunoprecipitates was isolated and amplified by real-time PCR using primer pairs encompassing ADH I promoter region (P1) and the ADH I coding region (P2). As shown in Fig. 2A, the association of acetylated histone H3-Lys9 with both promoter and coding regions of ADH I was...
significantly increased by chronic ethanol treatment consistent with in vitro observations, suggesting a potential role of histone acetylation in increased ADH I gene expression in the liver by chronic ethanol exposure. These results also indicate that histone acetylation may occur throughout the entire ADH I gene rather than localized to the promoter-associated chromatin.

We have also examined acetylation of histone H3-Lys9 at the region of iNOS and c-Jun gene after chronic ethanol treatment. Same as in ADH I gene, DNA isolated from chromatin immunoprecipitated with acetyl H3-Lys9 antibody was amplified using four primer pairs encompassing iNOS and c-Jun gene; P1, P2, P3 are covering promoter region and P4 is covering coding region of each gene. Contrary to our expectations, no significant changes in the levels of acetylated histone H3-Lys9 in the iNOS and c-Jun gene were detected in the liver after chronic ethanol treatment, suggesting that chronic ethanol-induced increases in histone acetylation in the rat liver are localized to specific areas of the chromatin and that histone acetylation might be involved in the regulation of ethanol-induced gene expression only in a selected subset of expressed genes.

In this study, global acetylation levels of histone H3 at Lys9 and Lys14 were not significantly altered by chronic ethanol exposure (1–4 weeks) in the rat liver. Interestingly, these results are in contrast to previous studies showing that acute ethanol (binge drinking) treatment (3–12 h) moderately but significantly increases global acetylation of histone H3 selectively at Lys9 in the rat liver (Park et al., 2005), implying that ‘acute’ and ‘chronic’ ethanol exposures generate different patterns of global histone acetylation. In fact, a recent study using microarray analysis revealed that, in addition to histone acetylation, changes in gene expression by acute ethanol treatment are also substantially different from chronic ethanol feeding (Bardag-Gorce et al., 2009).

With regard to global effect of chronic ethanol consumption on histone acetylation, it was reported that ethanol infusion by intragastric tube caused a significant increase in the global level of histone acetylation in the rat liver (Bardag-Gorce et al., 2007). Both intragastric alcohol infusion and liquid-diet ethanol feeding (De Carli and Lieber, 1967) have been extensively utilized to study various aspects of ALD pathogenesis. However, in many cases, alcohol infusion via intragastric tube and liquid-diet feeding generate different biological responses in the rat liver. Inconsistent result from intragastric infusion model with the current study may be due to different protocol for ethanol feeding and illustrates that care must be taken in the interpretation and comparison of data from different animal models.

Histone acetylation is regulated by a balance between HATs, which is implicated in transcriptional activation, and histone deacetylases (HDACs), which mediates transcriptional repression. Ethanol has been demonstrated to increase total histone acetylation with either increased HAT activity (Park et al., 2005) or reduced HDAC activity (Lieber et al., 2008). Further, increase in expression of specific genes may also be associated with either increased HAT activity (Choudhury et al., 2011) or reduced HDAC activity (Kendrick et al., 2010). It would be interesting to investigate if the effects of chronic ethanol on histone acetylation at the region of ADH I gene is mediated by changes in HATs and HDACs. The mechanism for gene-selective association remains to be known. In this context, alterations in the recruitment of HAT, HDAC and co-activators, co-repressors in HAT/HDAC complexes, may underlie the mechanism of ethanol-induced gene expression. Since increase in H3-Lys9 acetylation was not observed for other ethanol-induced genes such as iNOS and c-jun, it might be particularly illuminating to see whether acetylation of other histone lysine residues occurs in the chromatin domains of these other genes.
Taken together, the present study for the first time demonstrated that chronic ethanol exposure increases acetylation of histone H3 at Lys9 in the ADH I gene in the rat liver in vivo, suggesting that histone acetylation may underlie the mechanism for ethanol-induced ADH I expression in vivo. Another important finding of this study is that, in vivo, the acetylated H3-Lys9-related ADH-1 gene expression occurred in the absence of any detectable increase in the global

Fig. 2. The effect of chronic ethanol treatment on the association between acetylated histone H3-lys9 and region of ADH I, iNOS and c-jun genes in the rat liver. The effects of long-term ethanol treatment on acetylation of histone H3 associated with ADH I, iNOS and c-Jun gene were examined by CHIP assay. A piece of liver tissue (0.1 g) was prepared from the same liver shown in Fig. 1. After cross-linking, chromatin was immunoprecipitated with anti-acetyl H3-Lys9 antibody. DNA was isolated from each sample and amplified by real-time PCR. Sequences of the primers used in PCR are listed in Table 1. Two primer sets were prepared for ADH I amplification. P1 and P2 pair is located in the promoter and coding region, respectively (A). Four different primer sets were designed for amplification of iNOS (B) and c-Jun (C) genes. P1, P2 and P3 pairs target promoter region and P4 pair targets the coding region of each gene. The relative quantification of DNA immunoprecipitated by anti-acetyl H3-Lys9 antibody between pair-fed and ethanol-fed samples was determined after normalization of differences in the level of input samples. Values are presented as mean±SD, n = 6 (control value = 1). *P < 0.05 (compared with control group). C, control (pair-fed); E, ethanol-fed.
Histone H3 acetylation at Lys9. The lack of such changes at other ethanol inducible genes indicates that ethanol may induce expression of different genes by distinct mechanisms. Histone acetylation has been shown to induce various biological responses including inflammation (Villagra et al., 2010), fibrosis (Ghosh and Varga, 2007), apoptosis (Marks et al., 2001) and cell growth arrest (Sambucetti et al., 1999; Marks et al., 2001), most of which are likely to involve selective gene expression. It is possible that genes whose expressions are not affected by acetylation may be affected by other types of histone modifications (e.g. phosphorylation, methylation). This would also provide a rationale as to why ethanol elicits different types of site-specific histone modifications (Shukla et al., 2008).

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**REFERENCES**


