ACUTE IN VIVO EFFECT OF ETHANOL (BINGE DRINKING) ON HISTONE H3 MODIFICATIONS IN RAT TISSUES

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(Received 4 August 2005; first review notified 22 September 2005; in revised form 13 October 2005; accepted 7 November 2005; advance access publication 28 November 2005)

Abstract — Aims: To investigate the effect of acute in vivo administration of ethanol on acetylation or methylation of histone H3 at lysine9 in different tissues in rat. Methods: Ethanol was injected into the stomach of Sprague–Dawley rats (8-weeks-old) using blunt tipped needle. The rats were divided into three groups based on ethanol exposure times (1, 3, and 12 h). Each group was compared with water-injected control group. The tissues from 14 different organs were removed. We essentially used similar type of protocol, tissue homogenization method, and sucrose density gradient centrifugation for isolation of nuclei with only minor modifications for some organs. Histone was isolated from the nuclei using acid extraction method. Acetylation of histone H3 at lysine9 (Ac-H3-lys9) and methylation of histone H3 at lysine9 (Me-H3-lys9) were analysed by western blotting. Results: Effect of ethanol on Ac-H3-lys9 was investigated in 11 out of 14 rat tissues. In liver, we observed an increase in Ac-H3-lys9 with maximal increase of ~6-fold after 12 h exposure. Lung also showed ~3-fold increase. In spleen, ethanol-induced Ac-H3-lys9 in all three ethanol-treated groups with similar increase (1.5–to 1.6-fold). Tests showed significant increase (3-fold increase) of Ac-H3-lys9 only at 1 h ethanol exposure. Ethanol had no affect on Ac-H3-lys9 in other tissues: kidney, brain, heart, stomach, colorectum, pancreas, and vessels. Ethanol had little effect on Me-H3-lys9 in all rat tissues examined. Conclusions: After in vivo administration of ethanol, analogous to binge drinking condition, the acetylations of H3-lys9 in rat tissues are not universal but tissue-specific events with different patterns of responses. Ac-H3-Lys9 in liver, lung, and spleen were significantly affected and it was demonstrated that ethanol causes this epigenetic alteration in rat tissues selectively.

INTRODUCTION

Ethanol causes diverse systemic effects on various organs/systems. A number of epidemiological studies have demonstrated a correlation between alcohol ingestion and the occurrence of cancer in mammalian tissues: digestive tract, liver, colorectum, and the breast (Pöschl and Seitz, 2004). The exact mechanism of ethanol-induced carcinogenesis, however, is not clear. Aberrant acetylation or deacetylation of histones plays an important regulatory role in developmental processes, proliferation, and differentiation leading to such diverse disorders as leukaemia, fragile X syndrome, and Rubinstein-Taybi syndrome (Timmermann et al., 2001). Expression of the histone H3 gene is elevated in benign, semi-malignant, and malignant lesions of the head and neck (Bosch et al., 1993). Recent studies implicate alterations in chromatin structure by histone hyperacetylation/deacetylation as playing an important role in either the genesis or the suppression of cancer (Archer and Hodin, 1999).

The accumulated literature on histone acetylation supports a general model in which histone acetylation contributes to the formation of a transcriptionally competent environment by ‘opening’ chromatin and allowing general transcription factors to gain access to the DNA template. Conversely, histone deacetylation mainly contributes to a ‘closed’ chromatin state and transcriptional repression (Cheung et al., 2000). Acetylation occurs at lysine residues on the N-terminal tails of the histones, neutralizing the positive charge of the histone tails and decreasing their affinity for DNA. As a consequence, histone acetylation alters nucleosomal conformation, which can increase the accessibility of transcriptional regulatory proteins to chromatin templates (Struhl, 2001). Ethanol causes selective, post-translational acetylation of histone H3 at lys9 (Ac-H3-lys9) in a dose- and time-dependent manner in cultured rat hepatocytes, with maximum response at 100 mM, 24 h (Park et al., 2003), and in cultured hepatic stellate cells with maximum response at 100 mM, 72 h (Kim and Shukla, 2005).

To examine the relationship between ethanol and histone acetylation in a variety of tissues, we have now tested the acute in vivo effect of alcohol (a condition similar to binge drinking) on different rat tissues by measuring the acetylation and also methylation of histone H3 at lysine9 (Me-H3-lys9).

MATERIALS AND METHODS

Reagent

Anti-acetyl-histone H3 antibodies (lys9) ChIP Grade (rabbit antisera: Anti-H3 Ac9, Anti-H3 Ac18 and Anti-H3 Ac23) and Anti-dimethyl-histone H3 (lys9) ChIP Grade (rabbit antiserum: Anti-H3 Me9) were obtained from Upstate Biotechnology (Lake Placid, NY). The goat anti-rabbit immunoglobulin G (IgG) conjugated horseradish peroxidase (HRP) was bought from Bio-Rad Laboratories (Richmond, CA).

Ethanol oral injection

Sprague–Dawley male rats (8-weeks-old, average weight: 263.5 ± 7.1 g) were starved 6 h before ethanol injection. We used the protocol of a previous study with some modifications (Carson and Pruett, 1996). Ethanol was diluted to 32% (v/v) in sterile water and injected 6 g/kg through the oral cavity to the stomach using an 18 gauge stainless steel blunt tipped needle. The average amount of injected alcohol was 6.3 ml (5.6–6.6 ml). The condition we used was analogous to binge drinking. Three ethanol treatment groups, 1, 3, and 12 h,
and colorectum were thoroughly washed with TKM solution to remove intraluminal contents and mucus. 1.6/2.3 M sucrose cushion was centrifuged at 37,000 g for 30 min whereas 1.0/1.6 M was centrifuged at 27,000 g for 20 min.

Tissue isolation

Rats were anesthetized with diethyl ether. The liver was perfused briefly with ice-cold TKM buffer (50 mM Tris–HCl, 25 mM KCl, and 5 mM MgCl₂) to eliminate blood and removed. The tissues of brain (cerebral hemispheres), testes, kidney, abdominal wall muscle, spleen, pancreas, lung, heart, esophagus, and great vessels (aorta and vena cava) were removed in sequences. The mucosal surface of the stomach, oesophagus, and great vessels (aorta and vena cava) were skipped for pancreas, oesophagus, and vessels due to insufficient amount of tissue. In 1.6/2.3 M sucrose pad centrifugation, the homogenate in 0.25 M sucrose/TKM was mixed with two volumes of 2.3 M sucrose/TKM to establish a 1.6 M sucrose/TKM cell-containing buffer. This buffer, underlayered by 2.3 M sucrose/TKM pad, was centrifuged at 37,000 g f 30 min (Beckman, L8-70 ultracentrifuge, SW 28 rotor). In 1.0/1.6 M method, the sucrose pad was centrifuged at 27,000 g for 20 min (Brown et al., 1987). The nuclei pellets, after washing with TKM buffer and centrifugation at 700 g for 5 min, were stored in –80°C.

Histone extraction

Acid extraction of histone from the nuclei was achieved as follows. The nuclei stored in –80°C were thawed rapidly at 37°C and centrifuged at 12,000 g for 5 min at 4°C. The pellet was mixed with 0.5 ml of 0.4 N HCl/10% glycerol, incubated for 30 min at 4°C, and centrifuged at 12,000 g for 10 min. The supernatant was mixed with trichloroacetic acid (TCA) in 4:1 ratio making a 20% TCA solution, incubated for 1 h at 4°C, and centrifuged at 12,000 g for 10 min. The pellets were washed with 0.5 ml of acetone/0.02 N HCl, centrifuged at 12,000 g for 5 min, and dried under the hood for 30 min. The dried pellets were resuspended in water and sonicated. After centrifugation at 12,000 g for 10 min, the supernatant was collected and stored in a –80°C freezer. Protein concentration was measured using the Bio-Rad DC protein assay kit.

Western blot

Equal amounts (5–20 μg) of proteins were subjected to 15% SDS–PAGE and transferred onto nitrocellulose membrane. After blocking with 5% non-fat dried milk for 1.5 h at room temperature, membranes were incubated overnight at 4°C with primary antibody with the dilution of 1:1000 for anti-H3, 1:2000 for anti-H3 acetyl lys9, 1:10,000 for anti-H3 dimethyl lys9. These dilutions were optimized for respective antibodies in the laboratory. After washing with TBST solution three times, membranes were incubated with goat anti-rabbit IgG HRP conjugated secondary antibody with 1:3000 dilution for 1 h at room temperature. Western blots were developed by peroxidase reaction with the ECL reagents (Pierce, Supersignal West Pico Chemiluminescent Substrate).

Analysis of data and statistics

We measured and compared the average value of each control group with corresponding ethanol pairs for individual time.

Table 1. Tissue preparation and nuclei isolation protocols

<table>
<thead>
<tr>
<th>Luminal washing</th>
<th>Ten Broeck tissue homogenizera</th>
<th>Ultra-Turrax homogenizerb</th>
<th>1.6 M/2.3 M sucrose cushion</th>
<th>1.0 M/1.6 M sucrose cushion</th>
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Number of total homogenization and the concentration of sucrose pad used were varied according to the tissue type. Because of the small amount of tissue volume in oesophagus, vessels, and pancreas, we skipped density gradient centrifugation step and only used homogenization to isolate the nuclei. Stomach, small bowel, and colorectum were thoroughly washed with TKM solution to remove intraluminal contents and mucus. 1.6/2.3 M sucrose cushion was centrifuged at 37,000 g for 30 min whereas 1.0/1.6 M was centrifuged at 27,000 g for 20 min.

The number of up and down movement of Ten Broeck tissue homogenizer piston.

The number of 30 s bursts with Ultra-Turrax TP 18/10 S1.

were used; each contained four rats. As a control three rats for 1 h control and three rats for 12 h control were injected with comparable amount of sterile water instead of ethanol.
points: i.e. 1 h control group (C1) with 1 h ethanol (E1); 12 h control group (C12) with 12 h ethanol group (E12). For the 3 h control group (C3), we averaged the value of C1 and C12 rats and used it as the value for C3. There was no difference between C1 and C12 groups. The results are expressed as mean ± SD. The values are also expressed in graphs as fold increases over the control group. Paired or unpaired sample t-test was used to determine the significance among the three groups. Quantitative analysis was performed by densitometry analysis. We used Quantity-1 (version 4.1.1) software for the analysis of protein bands and the GraphPad Prism (version 3.03) software for plotting graphs and for statistical analysis.

RESULTS

Acetylation of Histone H3 at lysine9

We investigated the ethanol effect on Ac-H3-lys9 in 14 different tissues of rat: brain (cerebral hemispheres), lung, heart, liver, spleen, pancreas, kidney, oesophagus, stomach, small bowel, colorectum, testes, vessels (aorta and vena cava), and abdominal wall muscle. Compared with all other tissues, the amount of histone protein extracted from oesophagus, small bowel, and muscle was too small for the analysis. Therefore, any changes in the acetylation in these three tissues were hard to assess. We were able to isolate sufficient amount of histone H3 and detected Ac-H3-lys9 in other 11 tissues. Antibody against histone H3 protein (total H3) was used for loading control.

In liver (Fig. 1A), we observed a pattern of increase in the Ac-H3-lys9 in each three groups: 2.4-fold increase after 1 h exposure; 1.6-fold after 3 h exposure; 6.2-fold after 12 h exposure. There was significant increase of Ac-H3-lys9 both in 1 h (P = 0.0218) and 12 h (P = 0.0101) exposure groups. There was no difference noted between C1 and C12 groups indicating that the basal levels did not change. The loading amount of total histone protein was similar indicating that increase of total H3 protein expression. When the ratio of Ac-H3-lys9 to H3 proteins was determined (Fig. 1B) then also the pattern was similar to Fig. 1A.

Ethanol also increased Ac-H3-lys9 in lung tissue (Fig. 2) with statistically significant effects in all three groups. The folds increase value also accentuated with ethanol exposure time: 1.5 in EI group (P = 0.0243), 1.8 in E3 (P = 0.0029), and 2.3 in E12 group (P = 0.0256). Neither the levels of total H3 nor the values between C1 and C12 groups changed.

In spleen (Fig. 3), ethanol increased Ac-H3-lys9 in all three ethanol-treated groups somewhat to the same extent: 1.6 in EI group (P = 0.0051); 1.6 in E3 group (P = 0.0081); 1.5 in E12 group (P = 0.0159). The loaded amount of total H3 was similar in these groups.

Testes (Fig. 4) showed significant increase in Ac-H3-lys9 at 1 h ethanol exposure (3.0-fold increase, P = 0.0478) with no statistically significant effect in 3 and 12 h treated groups. Here also the total H3 levels did not change.

Ethanol had no significant affect on Ac-H3-lys9 in brain, heart, stomach, colorectum, kidney, pancreas, and vessels (data not shown).

Methylation of histone H3 at lysine9

We also analysed the acute in vivo effect of ethanol on Me-H3-lys9. Under these conditions ethanol showed no discernable effect on liver Me-H3-lys9. The fold increases were similar.
Fig. 2. Ac-H3-lys9 in lung. Statistically significant increase of Ac-H3-lys9 was shown in each three groups. The graph shows that the values increased with ethanol exposure time. Figures on the top represent immunoblotting against Ac-H3-lys9 and total H3. Other details are as described in Fig. 1. The values in graph are expressed as folds increase over the control group. Antibody against total H3 was used for loading control. Value of C3 group is the average value of C1 and C12 groups. *Statistically significant; P-values for 1, 3, and 12 h are 0.024, 0.002, and 0.025, respectively.

Fig. 3. Ac-H3-lys9 in spleen. Spleen showed statistically significant increase of Ac-H3-lys9 (paired sample t-test: $P = 0.0050$). Statistical significance was also shown in each three groups. Figures on the top represent immunoblotting against Ac-H3-lys9 and total histone. The values in graph are expressed as fold increase over the control group. Antibody against total H3 was used for loading control. Value of C3 group is the average value of C1 and C12 groups. Other details are similar to that in Fig. 1. *Statistically significant; P-values for 1, 3, and 12 h were 0.005, 0.008, and 0.015, respectively.
The amount of ethanol injected in this study, 6 g/kg, was selected to mimic acute consumption of ethanol (binge drinking) based on previous studies. Blood ethanol levels peak at \( \sim 0.4\% \) (w/v) 0.5–1 h after ethanol injection. The level decreases thereafter and is cleared by 8 h after injection (Carson and Pruett, 1996). We used three time points to compare the effects of ethanol on Ac-H3-lys9 in different tissues: at peak blood ethanol level, 1 h; in the middle of ethanol clearing phase, 3 h; and after clearance of ethanol from blood, 12 h.

In muscle, small bowel and oesophagus, we detected very weak signals for Ac-H3-lys9, although the amount of protein isolated after histone acid extraction was not small. We investigated whether these low levels of expressions reflect either tissue-specific resistance to Ac-H3-lys9 or lack of total H3 isolated. With immunoblotting using antibody against Ac-H3-lys9 and total H3 protein, we confirmed that the weak signal in muscle, small bowel, and oesophagus was not due to lack of Ac-H3-lys9 but due to a low level of total H3 in the samples. This means that non-histone protein may be present among the protein samples of these tissues isolated with histone acid extraction.

Ethanol effects on different tissues or organs are not similar. In a study which investigated the level of phosphatidylethanol (PEth) in organs of rats after \textit{in vivo} alcohol exposure, acute injection gave highest levels in intestines, stomach, and lung. No PEth was detected in skeletal muscle, pancreas, or testis. Variations in the level and rates of formation and degradation of PEth are organ specific (Aradottir \textit{et al.}, 2002). In another study, ethanol exerted oxidative stress on antioxidant systems of liver, lung, and kidney in proportion to the amount of ethanol ingestion, showing dose-dependant and tissue-specific changes in the antioxidant system after ethanol ingestion (Scott \textit{et al.}, 2000). We demonstrated here that acute ethanol increased Ac-H3-lys9 in liver, lung, spleen, and testes but...
had little effect on Ac-H3-lys9 in brain, heart, pancreas, stomach, colorectum, kidney, and vascular tissue. The mechanism for the differences in H3 acetylation among tissues is at present unknown. Whether this is a reflection of differences in the ethanol metabolism in different organs or in the capacity to acetylate histone remains to be investigated. However, the increases in acetylation are not due to increase in the expression of H3 protein by ethanol.

The gastrointestinal system is exposed to higher concentration of alcohol than any other tissue in the body. Alcohol is absorbed by passive diffusion throughout the entire length of the gastrointestinal tract. Tissue damage may, therefore, occur anywhere along the length of the gastrointestinal tract. Alcohol can be a potent cause of oesophagitis with acute administration. The relationship between oesophageal cancer and alcohol abuse has been demonstrated especially with strong alcohol consumption (Homann, 2001). Exposure of the gastric mucosa to alcohol results in the rapid development of structural changes such as patchy haemorrhages within 1 h of exposure. It is generally accepted that the direct effect of alcohol on gastric mucosa is to stimulate gastric acid secretion. The small intestinal lumen, like the stomach, is also exposed to high concentrations of alcohol. Alcohol can cause structural and functional changes such as malabsorption of amino-acid, vitamin, water and electrolytes in the small intestine. Diarrhoea occurs in about one-third of ‘binge’ drinkers. Alcohol thus inflicts severe morbidity on the gastrointestinal tract (Lake-Bakar, 1984). Chronic alcohol abuse leads to increased expression of histone H3 with rectal mucosal hyperproliferation in humans (Simonowski et al., 2001). Contrary to our expectations, both stomach and colorectum did not show any change in Ac-H3-lys9 after acute alcohol injection.

The bulk of alcohol ingested has to be metabolized by alcohol dehydrogenase (ADH) in the hepatic cytosol, and only 2–10% of ethanol consumed is excreted by the lungs and kidneys. Coupled with the conversion of NAD to NADH there is an accumulation of H+ and disturbance of the redox potential of the hepatocyte during the alcohol metabolism, which can explain many metabolic abnormalities seen in alcoholics (Jenkin, 1984). In the present study a time-dependent increase of acetylation was observed in liver after acute ethanol administration with a peak 6-fold increase at 12 h. Although we only checked in vivo effect of ethanol up to 12 h, this result is comparable to in vitro study with cultured hepatocytes (Park et al., 2003), which showed peak response at 100 mM, 24 h exposure. Thus, in liver, acute ethanol effect on Ac-H3-lys9 may peak at between 12–24 h both in vivo and in vitro situations.

A history of alcohol abuse is an independent outcome variable in the development of acute respiratory distress syndrome. In a rat model, chronic ingestion of ethanol decreased pulmonary glutathione concentration, increased alveolar barrier permeability, and increased the risk of acute lung injury (Brown et al., 2004). Although acute effects of ethanol on lung are not well studied, we observed a dose-dependent H3-lys9-acetylation after acute ethanol administration. It is of interest that patients with chronic obstructive pulmonary disease were recently shown to have a progressive reduction in total histone deacetylase (HDAC) activity (Ito et al., 2005).

Major ethanol effect on pancreas is related to chronic not acute alcohol consumption. Alcoholic pancreatitis is mostly observed in chronic alcoholics (Guy and Sarles, 1984). In our study, ethanol showed no affect on pancreas tissue in this binge drinking setting.

Ethanol evoked a decrease in both volume density of follicle and the mean follicle diameter of spleen (Budec et al., 2000). A single dose of ethanol decreased splenic NK cell activity. This decrease was maximal 12 h after dosing and was no longer evident at 60 h. Splenic T cells were not depleted, but B cells were significantly decreased at the highest ethanol dose (Wu et al., 1994). Spleen showed increase of Ac-H3-lys9 after ethanol injection which began 1 h after exposure lasting for 12 h. Because spleen is related to immunity, this rapid and sustained response may be relevant to self protection against injuries.

Ethanol, in general, causes decrease of total plasma testosterone concentration below the reference range in 12–50% of subjects. Testicular biopsy reveals severe reduction in germinal elements and also peritubular fibrosis. This is due to the direct toxic effect of alcohol and its metabolite, acetaldehyde. Whatever the mechanism, the deleterious effect of alcohol on testosterone synthesis is rapid (Fink, 1984). HDAC inhibitors, such as Trichostatin-A (TSA), are able to induce cell cycle arrest by stimulating transcription of genes that negatively regulate cell growth and survival. TSA treatment of cultured murine germ cells from whole testes resulted in an increase of histone H4 acetylation in round spermatids, suggesting that hypoacetylated states of these cells are important for their normal differentiation (Fenic et al., 2004). In this study, testes showed an increase in Ac-H3-lys9 at 1 h after ethanol injection and it normalized rapidly. Whether increase in Ac-H3-lys9 influences testosterone synthesis or spermatogenesis is not obvious, but the possibility of a close relationship can be speculated.

In summary, we have investigated the acute effect of ethanol on Ac-H3-lys9 and Me-H3-lys9 in various rat tissues. Ethanol treatment had no discernable effect on Me-H3-lys9 in all the tissues examined. The ethanol effect on Ac-H3-lys9 was increased in liver, lung, spleen, and testes but not in other tissues. In conclusion, after acute ethanol administration, analogous to binge drinking, the H3-lys9 in rat tissues are not universal but tissue-specific events with different patterns of responses.

Acknowledgements — Authors are thankful to Drs Qun Wang and Pil-Hoon Park for help in tissue isolation and to Mr Daniel Jackson for laboratory technical help. This work was supported by grant no. AA14852 from NIAAA of the National Institutes of Health, USA.

REFERENCES


