HISTONE H3 MODIFICATIONS IN RAT HEPATIC STELLATE CELLS BY ETHANOL

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Abstract — Aims: Hepatic stellate cells (HSCs) play critical roles in the development of hepatic fibrosis caused by various agents including alcohol. Ethanol causes post-translational modification in histone. The goal of this study is to investigate whether ethanol affected acetylation and methylation of histone H3 in rat HSCs. Methods: We isolated and separated HSCs using collagenase perfusion of liver followed by Nycodenz density gradient centrifugation. HSCs were divided and treated with different concentrations of ethanol for various times. Histone was isolated using acid extraction method. Acetylation and methylation of histone H3 at Lys9 (Ac-H3-lys9), Lys14 (Ac-H3-lys14), Lys18 (Ac-H3-lys18), or Lys23 (Ac-H3-lys23) was checked by western blotting. Results: At lysine 9, ethanol caused dose-dependent increase of Ac-H3 up to 200 mM. Ac-H3-lys9 increased with a maximum of 86-fold at 72 h and 200 mM ethanol treatment, and decreased thereafter. This increase was confirmed by both western blotting and FITC stain. At high dose, ethanol increased acetylation of histone H3 at Lys23 (Ac-H3-lys23), but it had no effect on Ac-H3-lys14 or Ac-H3-lys18. The intensity of the FITC-labelled dimethyl-histone H3 at Lys9 (Me-H3-lys9) antibody appeared to decrease slightly with increasing dose of ethanol. But this did not appear to change when monitored by western blotting. Conclusions: Ethanol caused dose and time-dependent increase in acetylation of histone H3 at Lys9, but not at Lys14 or Lys18. Compared with hepatocytes the Ac-H3-lys9 in HSCs required longer ethanol exposure. Levels of Me-H3-lys9 seemed to remain unaltered. Thus increase in Ac-H3-lys9 represents a nuclear-chromatin modification event in HSCs exposed to ethanol.

INTRODUCTION

Hepatic Stellate cells (HSCs) are also known as Ito cells or lipocytes. Stereological analysis reveals that hepatocytes constitute 92.5% of the total volume of liver cells, whereas HSCs constitute 1.7%. The relative numbers of the each liver cell are also quite different: hepatocytes, 65%; HSCs, 5.5% (Pertoff and Smedsrod, 1987). But in a more recent study, HSCs were estimated to constitute up to 10–15% of the total number of cells in normal liver (Ramm, 1998). Although HSCs constitute a relatively small portion of the liver, they have unique roles in hepatic physiology and patho-physiology. In normal liver, HSCs are responsible for the transport and storage of retinoid and for the production of basement membrane component. However, when activated by viral infection, various drugs and alcohol, HSCs are transformed into myofibroblast-like cells that produce increased levels of extracellular matrix components, particularly fibrillar collagen, matrix remodelling metalloproteinase and their inhibitors. HSCs, therefore, are known to play central roles in fibrosis of the liver (Friedman and Roll, 1987; Friedman, 1996; Kawada et al., 1998).

In eukaryotic chromatin, the nucleosomal complexes formed by the histone octamer and associated DNA are the fundamental organizational units. The precise organization of DNA in chromatin has important functional consequences in processes such as transcription, replication, repair, recombination, and segregation (Cheung et al., 2000). The N-terminal and C-terminal tails of histone, which protrude from the surface of the chromatin polymer, undergo post-translational modifications including acetylation, phosphorylation, methylation, ubiquitination, and ADP-ribosylation. Histone modifications may alter chromatin structure by influencing histone–DNA and histone–histone contacts. Of the modifications listed above, histone acetylation has been the most studied. In H3 from most species, the main acetylation sites include lysine 9, 14, 18 and 23 (Strahl and Allis, 2001; Timmermann et al., 2001). In rat hepatocytes, we reported ethanol-induced selective, post-translational Ac-H3-lys9 in a dose-dependent and time-dependent manner with maximum response at 100 mM, 24 h (Park et al., 2003). However, whether ethanol affects HSCs is not known. Therefore, we have investigated the ethanol effect on acetylation and methylation of histone H3 in these cells.

MATERIALS AND METHODS

Reagents

Nycodenz® (NycoPrep™ Universal, Oslo, Norway) was purchased from Greiner-Bio-One, Inc. (Longwood, FL). Anti-acetyl-histone H3 antibodies (Lys9, Lys18, or Lys23) ChIP Grade (rabbit anti serum; Anti-H3 Ac9, Anti-H3 Ac18 and Anti-H3 Ac23), Anti-acetyl-Histone H3(Lys14) (rabbit polyclonal IgG; Anti-H3 Ac14), and Anti-dimethyl-histone H3 (Lys9) ChIP Grade (rabbit anti serum; Anti-H3 Me9) were obtained from Upstate Biotechnology (Lake Placid, NY). The goat anti-rabbit immunoglobulin G (IgG) conjugated horseradish peroxidase was bought from Bio-Rad Laboratories (Richmond, CA). Trichostatin A (TSA) was attained from Sigma (St Louis, MO). The culture slides (BD Falcon™) came from BD Biosciences Discovery Labware (Bedford, MA). Fluorescein isothiocyanate [Fluorescecin (FITC)-Conjugated AffiniPure Fab Fragment Goat Anti-rabbit IgG(H+L)] was obtained from Jackson ImmunoResearch Lab Inc (West Grove, PA), 4′,6′-diamidino-2-phenylindole, dihydrochloride (DAPI; VECTASHIELD® mounting medium with DAPI H-1200) was supplied by Vector laboratories Inc (Burlingame, CA).
HSC isolation and culture

Male Sprague-Dawley rats (300–400 g) were obtained from Charles River Laboratories (Wilmington, MA) and were fed standard regular diet for rodent (LabDiet®). All animals received care in compliance with the guidelines of Animal Care Quality Assurance, University of Missouri-Columbia (ACQA). The rats were anesthetized with ethyl ether. The liver was perfused using collagenase-perfusion protocol (Weng and Shukla, 2000), and HSCs were isolated using Nycodenz density gradient centrifugation as described previously (Vyas et al., 1995; Lu et al., 1998; Ramm, 1998) with some modifications. Briefly, the rat liver was perfused with Krebs-Ringer bicarbonate buffer containing Ca++/Mg++ free KRB with 0.5% bovine serum albumin (BSA). After gentle dispersion, the hepatic non-parenchymal cells (NPC) were separated from the hepatocytes by centrifugation at 50 g for 2 min at 4°C twice. The supernatant, composed of mainly NPC but also hepatocytes, was centrifuged at 450 g for 10 min at 4°C. The pellet was resuspended in 30 ml of 17.2% Nycodenz and centrifuged at 1400 g for 20 min at 4°C. The white, diffuse top layer of Nycodenz cushion, which is enriched with HSCs, was collected and diluted with phosphate-buffered saline (PBS) with 0.3% BSA and centrifuged at 450 g for 10 min at 4°C. The cells were plated on Petri dish (100 mm) in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), 4 mM L-glutamine, 100 IU/ml penicillin, 100 g/ml streptomycin and 2.5 g/ml amphotericin B (antifungal). The culture medium was changed 24 h later and thereafter every 2–3 days. The HSCs were identified by typical stellate shaped morphology (Friedman and Roll, 1987; Kawada et al., 1998).

Ethanol treatment and histone isolation

After HSCs achieved a 90% confluence (usually 2–3 weeks), the culture medium was changed to DMEM containing 0.1% FBS with or without alcohol and sealed with Parafilm® (Pechiney plastic packaging, Chicago, IL). HSCs were divided and treated with ethanol (0, 50, 100, and 200 mM) for various times (24, 48, 72, 96, and 120 h). TSA (2 µg/ml) was used as a positive control. The ethanol-containing medium was changed every 24 h during incubation period. Histone was isolated from HSCs as follows. After the desired time, HSCs were rinsed twice with ice-cold PBS, scraped into 800 µl ice-cold lysis buffer containing 20 mM HEPES (pH 7.9), 1 mM EDTA, 10 mM NaCl, 2 mM MgCl2, 20 mM glycerophosphate, 0.25% NP-40, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml pepstatin A, 10 µg/ml leupeptin, and 1 mM diithiothreitol and transferred into Eppendorf tube at 4°C. The cells were incubated on ice for 20 min and passed through a 26-gauge syringe needle 10 times. After centrifugation at 12 000 g for 10 min at 4°C, the pellet was washed again with ice-cold lysis buffer. 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 the ratio 4:1 making 20% TCA solution, incubated for 1 h at 4°C and centrifuged at 12 000 g for 10 min. The pellet was washed by 0.5 ml of acetone/0.02 N HCl, centrifuged at 12 000 g for 5 min and the pellets were 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, supernatant was collected and stored in −70°C freezer. Protein concentration was measured using the Bio-Rad DC protein assay kit.

Western blot analysis

Equal amounts (5–10 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 (RT), membranes were incubated overnight at 4°C with primary antibody with the dilution of 1:1000 for anti-H3 acetyl Lys23, 1:2000 for anti-H3 acetyl Lys9 or anti-H3 acetyl Lys14, 1:10 000 for anti-H3 acetyl Lys18 or anti-H3 dimethyl Lys9. 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 RT. Western blots were developed with peroxidase reaction with the ECL reagents (Pierce, Supersignal West Pico Chemiluminescent Substrate). Quantitative analysis was performed by densitometry analysis. We used Quantity-1 (version 4.1.1) software for the analysis of protein bands and GraphPad Prism (version 3.03) software for graph formation and statistical analysis (one-way ANOVA).

Immunofluorescence stain

HSCs were placed and cultured on culture slides (BD Falcon™). After 24 h, HSCs were treated with ethanol-containing medium and were incubated; ethanol-containing medium was changed every 24 h. After 72 h ethanol treatment, the slides were immersed in pre-cooled (−20°C) acetone/methanol (1:1) mixture for 30 min at −20°C. The slides were next incubated in PBS containing 0.5% Triton X-100 for 10 min at RT. For blocking, the slides were incubated in 5% BSA–PBS for 1 h at RT. Primary antibody for acetyl-H3 (lys9) or dimethyl-H3 (lys9), diluted 1:50 in 1% BSA–PBS, was applied and incubated overnight at 4°C in a humidified chamber with light shield. On the next day, the slides were incubated with 500 µl of 1% BSA–PBS containing secondary antibody conjugated with FITC (1:200 diluted) for 1 h at RT in the humidified chamber. After washing, the slides were incubated with DAPI for 5 min. The slides were washed with PBS for 10 min three times between each step. Specimens stained with DAPI and FITC were examined using Epi-fluorescence microscopy (Nikon, Japan) equipped with filters for FITC and ultra violet (×400).

RESULTS

The dose-dependent increases of Ac-H3-lys9 were observed at each 24, 48, 72, 96, and 120 h ethanol exposure (Fig. 1A and B). The values expressed are fold increases over control group and represent 2–3 separate experiments. At lysine 9, ethanol caused dose-dependent increase in acetylation up to 200 mM at each exposure time period (Fig. 1A and B). Ac-H3-lys9 increased gradually up to 72 h and decreased thereafter. Treatment of cells with ethanol for 72 h showed largest increase in Ac-H3-lys9 in a dose-dependent manner: 5.0 ± 5.9 folds at 50 mM, 16.5 ± 8.0 folds at 100 mM, and maximum 86.2 ± 18.1 folds at 200 mM. Based on this observation HSCs
were treated with ethanol for 72 h in subsequent experiments. TSA also showed the increase of Ac-H3-lys9 at 24, 48, and 72 h. However, at 96 and 120 h, TSA caused detachment of HSCs and therefore data for these time points are not shown (Fig. 1A).

Modifications of histone-H3 at various other lysine residues by ethanol, were also evaluated by western blotting (Fig. 2). HSCs were incubated with ethanol for 72 h followed by monitoring of Ac-H3 at three different lysine residues: lysine 14, lysine 18, and lysine 23 and also methylation of histone H3 at lysine 9 (Me-H3-lys9). No influence on Ac-H3-lys14 or Ac-H3-lys18 was noticed. Acetylation on H3-lys23 increased ~10-folds after 200 mM ethanol treatment. The levels of Me-H3-lys9 did not appear to change (Fig. 2A and B). Noticeably, TSA caused decrease in Me-H3-lys9. Antibody against histone H3 protein (total H3) was used for loading control and showed no change in the protein levels indicating that ethanol did not affect the histone H3 protein expression under these conditions. It is of interest to mention that the expression of histone H3 mRNA, used as a marker for cellular regeneration, was increased in colonocytes (rectal crypts) in chronic alcoholics (Simanowski et al., 2001).

In order to confirm the Ac-H3-lys9 and Me-H3-lys9, we used immunofluorescent stain for these antibodies. FITC-labelled Ac-H3-lys9 antibody displayed dose dependent increase in fluorescence intensity from 50 to 200 mM in 72 h-ethanol treated HSCs (Fig. 3A). On the contrary, the intensity of the FITC-labelled dimethyl histone H3 at Lys9 antibody appeared to decrease slightly with increasing dose of ethanol (Fig. 3B). DAPI was used as nuclear stain to confirm the location of the nucleus (Fig. 3A and B).

**DISCUSSION**

Several HSC isolation methods have been developed, which can be classified into three groups: simple washing and centrifugation (Riccalton-Banks et al., 2003), density gradient centrifugation, and enzymatic digestion. We tested and used the density gradient centrifugation method because it had several advantages: greater yield, less likelihood of contamination, availability of functionally intact cells, and appropriateness for combining with hepatocyte isolation. Compounds employed in density gradient centrifugation are arabinogalactan (Stractan).
Exposure of HSCs to ethanol resulted in time-dependent and dose-dependent increases in selective acetylation of Lys9 in histone H3 protein. A maximum increase of ~80-fold was observed at 200 mM ethanol treatment for 72 h. The in vivo peripheral blood concentration of ethanol in chronic alcoholics varies from 8.2 to 18.0% (Kawada et al., 1993, 1998; Lu et al., 1998; Ramm, 1998; Benyon et al., 1999). We adopted relatively high Nycodenz concentration (Ramm, 1998), 17.2%, to increase the number of HSCs isolated.

Exposure of HSCs to ethanol resulted in time-dependent and dose-dependent increases in selective acetylation of Lys9 in histone H3 protein. A maximum increase of ~80-fold was observed at 200 mM ethanol treatment for 72 h. The in vivo peripheral blood concentration of ethanol in chronic alcoholics is normally <50 mM. Therefore, the concentrations used in the present study for HSCs may appear higher. However, the exact concentration of ethanol to which HSCs are exposed in situ in the liver remain poorly understood. It may be noted that in pigs orally administered with ethanol, portal venous blood ethanol was 2-fold higher than in peripheral blood (Elmer et al., 1982; Luca et al., 1997). First pass hepatic metabolism can explain this difference. Furthermore, the hepatic venous blood is diluted 4–5 times in the caval vein (Nuutinen et al., 1984). The liver is also well known for its high blood supply, 70% of blood supplied from mesenteric portal venous system. In addition, acute administration of ethanol increases portal blood flow by 40–60% (Orrego et al., 1988). Taken together, liver cells are exposed to higher concentration and higher amounts of ethanol than expected by the peripheral blood level. It is also worth mentioning that the upper limit of ethanol in chronic alcoholics can be 100 or 245 mM or in one report as high as 300 mM (Deitrich and Harris, 1996). Thus use of ethanol in the range of 50–200 mM provides a reasonable range for experimental studies. We have consistently observed effects of ethanol on HSCs at 50 mM. However, to increase the sensitivity of our assay we have also used 100 and 200 mM concentrations.

The time required for the largest increment of Ac-H3-lys9 in HSCs, 72 h, was longer compared with hepatocytes where maximal acetylation occurred at 24 h ethanol exposure (Park et al., 2003). This finding suggested that HSCs are relatively...

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**Fig. 2. Dose-dependent modification of H3 at various lysine residues by ethanol.** (A) Acetylation of histone H3 at lysine 14 (Ac-H3-lys14), lysine 18 (Ac-H3-lys18) and lysine 23 (Ac-H3-lys23) and methylation of histone H3 at lysine 9 (Me-H3-lys9) were evaluated after 72 h ethanol treatment of HSC. Antibody against total amount of histone H3 (total H3) was used for loading control. (B) Densitometry analysis. Values for modification at various lysine residue and total H3 are expressed as fold increases compared with control value. Data are from one of the two separate but similar experiments.
slow reacting cells that might require longer ethanol exposure for H3 acetylation. The role of histone modifications by ethanol in vitro or in vivo, for example, in cell proliferation, growth, or gene expression, remains unknown at present. Lys9 in the H3 tail can be targeted for both acetylation and methylation and these modifications have opposite effects on the affinity with DNA and on transcriptional activity (Rice and Allis, 2001). Because ethanol caused dose-dependent increase of Ac-H3-lys9, we expected methylation to decrease with increasing ethanol concentration. We monitored this by western blot and immunofluorescence stain. Although the intensity of the FITC-labelled dimethyl histone H3 at Lys9 antibody appeared to decrease slightly with increasing dose of ethanol, the levels of Me-H3-Lys9 did not appear to change when monitored by western blotting. Thus there is no significant change in the Me-H3-lys9 by ethanol in HSCs.

In conclusion, ethanol caused dose-dependent-and time-dependent increase of Ac-H3-lys9 monitored by both western blot and FITC stain in HSC cells. This is not owing to increased H3 protein expression. Levels of Ac-H3-lys14 or Ac-H3-lys18 were unaffected. Compared with hepatocytes the Ac-H3-lys9 in HSCs required longer ethanol exposure (24 vs 72 h). Levels of Me-H3-lys9 seemed to remain unaltered. Thus increase of Ac-H3-lys9 represents a nuclear-chromatin modification event in HSC exposed to ethanol.

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