Involvement of histone acetyltransferase (HAT) in ethanol-induced acetylation of histone H3 in hepatocytes: potential mechanism for gene expression

Pil-Hoon Park, Robert W. Lim, and Shivendra D. Shukla

Department of Medical Pharmacology and Physiology, School of Medicine, University of Missouri-Columbia, Columbia, Missouri

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Involvement of histone acetyltransferase in ethanol-induced acetylation of histone H3 in hepatocytes: potential mechanism for gene expression. Am J Physiol Gastrointest Liver Physiol 289: G1124–G1136, 2006. First published August 4, 2005; doi:10.1152/ajpgi.00091.2005.—Ethanol treatment increases gene expression in the liver through mechanisms that are not clearly understood. Histone acetylation has been shown to induce transcriptional activation. We have investigated the characteristics and mechanisms of ethanol-induced histone H3 acetylation in rat hepatocytes. Immunocytological and immunoblot analysis revealed that ethanol treatment significantly increased H3 acetylation at Lys9 with negligible effects at Lys14, -18, and -23. Acute in vivo administration of alcohol in rats produced the same results as in vitro observations. Nuclear extracts from ethanol-treated hepatocytes increased acetylation in H3 peptide to a greater extent than extracts from untreated cells, suggesting that ethanol either increased the expression level or the specific activity of histone acetyltransferases (HAT). Use of different H3 peptides indicated that ethanol selectively modulated HAT(s) targeting H3-Lys9. Treatment with acetate, an ethanol metabolite, also increased acetylation of H3-Lys9 and modulated HAT(s) in the same manner as ethanol, suggesting that acetate mediates the ethanol-induced effect on HAT. Inhibitors of MEK (U0126) and JNK (SP600125), but not p38 MAPK inhibitor (SB203580), suppressed ethanol-induced acetylation of H3. However, U0126 and SP600125 did not significantly affect ethanol-induced effect on HAT, suggesting that ERK and JNK regulate histone acetylation through a separate pathway(s) that does not involve modulation of HAT. Chromatin immunoprecipitation assay demonstrated that ethanol treatment increased the association of class I alcohol dehydrogenase (ADH I) gene with acetylated H3-Lys9. These data provide first evidence that ethanol increases acetylation of H3-Lys9 through modulation of HAT(s) and that histone acetylation may underlie the mechanism for ethanol-induced ADH I gene expression.

ethanol metabolism; selective acetylation; extracellular signal-regulated kinase; c-Jun NH2-terminal kinase

CHROMATIN IS A HIGHLY ORGANIZED AND DYNAMIC PROTEIN-DNA COMPLEX. The nucleosome, the fundamental subunit of chromatin, is composed of four core histones (H2A, H2B, H3, and H4) surrounded by 146 bp of DNA (53). Transcriptionally inactive chromatin is tightly wrapped around histone proteins, and binding with transcription factor is inhibited (22). Posttranslational modifications of histone, including acetylation, phosphorylation, methylation, ubiquitination, and ADP ribosylation, influence DNA wrapping with histones and the exposure of DNA to transcription factors as well as interactions among different histone molecules (17, 45). Histone modifications are therefore widely considered as important factors in the regulation of gene transcription and chromatin remodeling. Of these modifications, histone acetylation strongly correlates with enhanced gene transcription (2, 17) and is the most extensively studied in relationship to the mechanisms of transcriptional activation (46).

Histone acetylation occurs at the ε-amino groups of evolutionarily conserved lysine residues located at the NH2 termini. All core histones are acetylated in vivo, but acetylations of H3 and H4 have been more extensively characterized than those of H2A and H2B. Histone acetylation is regulated by a balance of opposing histone acetyltransferase (HAT) and histone deacetylase (HDAC) activities. HATs transfer acetyl moieties from acetyl CoA to the lysine residues, which reduces affinity of histone with DNA and increases accessibility of transcriptional regulatory proteins to chromatin templates (46). In the deacetylation reaction, HDACs catalyze the removal of acetyl groups from histones and increased HDAC activities are usually associated with transcriptional repression (42). Individual HATs and HDACs display distinct specificities in terms of the individual lysine residues and the particular histones they affect. This enzymatic specificity might reflect different biological functions of the various enzymes (48), although the mechanisms responsible for the specificity are poorly understood.

Mitogen-activated protein kinase (ERK1/2, p38 MAPK, and JNK) cascades are activated by diverse stimuli and play key roles in the regulation of cellular processes, such as cell growth, proliferation, differentiation, and apoptosis (23). These MAPKs have been shown to phosphorylate several types of HATs (e.g., CBP, ATF-2, and SRC-1) and directly increase their enzymatic activities (1, 20). MAPKs may also regulate HAT activity indirectly by modifying signaling pathways affecting HAT activity (21, 38).

We have shown previously that treatment of hepatocytes in vitro with ethanol increased the acetylation of histone H3 at Lys9 (39). Ethanol exposure is also known to activate various signal transduction processes, including G protein-coupled receptors and protein kinases (e.g., PKC, Src family kinases, and MAPK family) as well as transcription factors such as NF-κB, CREB, and AP-1 (10, 24, 35, 50). These complex responses to ethanol exposure ultimately translate into altered gene expression in the liver, leading presumably to the development of alcohol-induced liver injury. Recent evidences have shown a central role for MAPKs in mediating the diverse effects of ethanol (reviewed in Ref. 3). This prompted us to investigate the role of MAPK in ethanol-induced histone acetylation and whether HAT is modulated by ethanol.
Changes in the expression of genes covering a wide spectrum of cellular functions have been implicated in the development of alcoholic liver disease (ALD). Genes that are affected include those involved in ethanol metabolism (14, 30, 33), cell signaling (15, 44), and apoptosis (29, 40, 56). Several microarray studies have also shown a profiling of hepatic gene expressions induced by alcohol consumption (13, 47). However, the molecular mechanism for ethanol-induced gene expression in the hepatocytes is poorly understood and whether histone acetylations play a role is unknown.

One of the genes that is strongly induced in the liver by ethanol is the gene coding for class I alcohol dehydrogenase (ADH I) (30). Ethanol is metabolized predominantly in the liver, and ADH I, the most abundant isozyme in the liver (7), is believed to be the principal enzyme of ethanol oxidation. The contribution of non-ADH systems to ethanol metabolism, such as cytochrome P-450 in the endoplasmic reticulum and catalase in the peroxisomes, is considered to be minor (4). We report here characteristics of the ethanol-induced H3 acetylation in hepatocytes, and the relationship to changes in HAT activity and ADH I gene expression.

MATERIALS AND METHODS

Reagents

Polyclonal anti-histone H3, antiacetylated histone H3 antibodies targeting specific lysine residues, nonradioactive HAT activity assay kit, and chromatin immunoprecipitation (CHIP) assay kit were purchased from Upstate Biotechnology (Lake Placid, NY). The goat anti-rabbit immunoglobulin G (IgG) conjugated with horseradish peroxidase (HRP) was purchased from Bio-Rad Laboratories (Hercules, CA), and goat anti-rabbit IgG conjugated with FITC was purchased from Jackson Immunoresearch (West Grove, PA). Histone, trichostatin A (TSA), and protease inhibitors (aprotinin, pepstatin A, PMSF, and leupeptin) were obtained from Sigma (St. Louis, MO). Ethanol was purchased from Aldrich (Milwaukee, WI). SP600125 and SB203580 were purchased from Cell Signaling (Beverly, MA). Mounting medium with 4',6-diamidino-2-phenylindole (DAPI) was purchased from Vectashield (Burlingame, CA). TRIzol reagent for RNA isolation and PCR master mix for conventional PCR were purchased from Invitrogen (Carlsbad, CA). QPCR SYBR green I master mixes for real-time PCR was purchased from Abgene (Rochester, NY).

Isolation and Culture of Hepatocytes

Hepatocytes were isolated from male Sprague-Dawley rats (200–250 g) using a collagenase perfusion protocol as described previously (51). Viability of isolated hepatocytes was 90 ± 5%. Isolated hepatocytes were plated on collagen-coated dishes (7.5 × 10⁶ cells/100-mm dish) in DMEM containing 10% FBS.

Treatment of Cells

Hepatocytes were allowed to attach to culture dishes for 2 h and were then treated with different concentrations of ethanol or acetate in DMEM containing 0.1% FBS for indicated time periods. In experiments using inhibitors, hepatocytes were pretreated with inhibitors of MAPKs (U0126, SP600125, and SB203580) for 1 h and incubated with ethanol for 24 h. The selection of these inhibitors and the conditions in which they are used are based on published data in which these inhibitors have been shown to inhibit MAPKs (25, 52, 54). Reagents were dissolved in DMSO or distilled water, and an equivalent amount of DMSO was added to cells in mock-treated control samples.

Immunocytochemistry of Acetylated Histone H3

Hepatocytes were cultured in an eight-well chamber slide and treated with ethanol as above. After 24 h, cells were washed with PBS and fixed in ice-cold acetone-methanol [50:50 (vol/vol)] for 30 min at −20°C. After being blocked with 5% BSA for 1 h at room temperature, slides were incubated with site-specific anti-acetyl histone H3 antibody as the primary antibody for 2 h at room temperature and incubated with anti-rabbit IgG conjugated with FITC as the secondary antibody for 1 h at room temperature in a humidified slide box. Slides were washed with PBS three times to remove nonspecific bindings between each step. Finally, slides were stained with DAPI to show the nucleus. Cellular immunofluorescence was detected under the fluorescence microscope (Eclipse E600, Nikon).

Extraction of Nuclear Histones

Histones were extracted from nuclei based on the method of Bonner et al. (6) and Rogakou et al. (41). Hepatocytes were washed twice with cold PBS, scraped, and resuspended in hypotonic lysis buffer containing (in mM) 20 HEPES, 1 EDTA, 10 NaCl, 1 DTT, 1 sodium orthovanadate, 2 MgCl₂, and 1 PMSF with 10 μg/ml of leupeptin, aprotinin, and pepstatin A and 0.25% NP-40. Cells were then incubated on ice for 20 min and lysed by 10 passages through a 26-gauge syringe needle. Nuclei were pelleted by centrifugation at 12,000 g for 20 s, resuspended in 0.4 N HCl with 10% glycerol, and centrifuged at 12,000 g for 10 min. The supernatant fraction (acid-soluble) was carefully collected, precipitated with trichloroacetic acid (final concentration 20%), washed with acetone, dried under the vacuum, and dissolved in distilled water. Protein concentration was measured using the Bio-Rad DC protein assay kit.

Western Blot Analysis

Equal amounts (5–10 μg) of proteins were fractionated by 15% SDS-PAGE and transferred onto a nitrocellulose membrane. After being blocked with 5% nonfat dried milk for 2 h, the membrane was incubated with primary antibody (anti-histone H3 or site-specific anti-acetyl histone H3 antibody) overnight at 4°C. The membrane was incubated with HRP-conjugated secondary antibody for 1 h at room temperature. The HRP was detected by enhanced chemiluminescence (ECL; Pierce) according to the manufacturer’s protocol. The bands were subjected to laser densitometry for quantitation.

Measurement of HAT Activity

The effect of ethanol on HAT activity was examined by two methods: ELISA and immunoblot analysis. In the ELISA method, HAT activity was measured by nonradioactive ELISA HAT assay kit (Upstate Biotechnology) according to the manufacturer’s instruction using different types of histone H3 peptide. The histone H3 peptides used in this assay were purchased from Upstate Biotechnology and correspond to the first 21 amino acids of the NH₂-terminal of histone H3 followed by a GG linker and biotinylated lysine (ARTKQTARK-GGK-biotin). The peptides used were either unmodified, acetylated on K9 (H3-AcK9), or acetylated on K14 (H3-AcK14). In this assay, a streptavidin-coated ELISA plate (96 well) was incubated with 100 μl of 1 μg/ml biotinylated histone H3 peptides (unmodified H3 peptide, H3-AcK9, or H3-AcK14). The wells were washed with Tris-buffered saline (TBS) and incubated with 3% BSA for 30 min. After the wells were washed with TBS, 50 μl of reaction cocktail (10 μl of 5× HAT assay buffer, 10 μl of 500 μM acetyl-CoA, 5 μl of 500 mM Na butyrate, 40 μg of nuclear extracts from cells cultured in the absence or presence of ethanol, adjusted to 50 μl with sterile water) were added to each well and incubated for 15 min at 30°C to induce acetylation of H3 peptide. Preliminary experiments measuring the activity response (incorporation of acetyl-CoA to H3 peptide) using different amounts of nuclear extract (0–80 μg) and a constant amount of acetyl-CoA (100 μM)
showed that HAT activity increased with increasing the amount of nuclear extracts, suggesting that the acetyl-CoA level being used is not rate limiting. 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 h. After the wells were washed with TBS, 100 μl of anti-rabbit IgG conjugated with HRP (1:5,000 diluted with TBS) were added and incubated for 1 h. Tetramethyl-benzidine (TMB) substrate mixture (100 μl) was added to each well and incubated for 10 min before the addition of 50 μl of sulfuric acid to stop the HRP reaction. Colorimetric change was measured by plate reader on a wavelength of 450 nm with a reference wavelength of 570 nm.

RT-PCR

For RT-PCR, hepatocytes were treated with indicated concentrations of ethanol for 24 h and RNA was isolated using TRIzol reagent (Invitrogen). Two micrograms of total RNA was reverse transcribed with 100 μg of total histone in the presence of acetyl-CoA and Na butyrate (50 mM) for 20 min at 37°C. The reaction mixtures were then mixed with Lammeli buffer and used for immunoblot analysis using site-specific anti-acetyl H3 antibodies.

Analysis of CHIP Assay

The immunoprecipitated DNA was analyzed by conventional PCR and real-time PCR. For conventional PCR, aliquots of immunoprecipitated and input DNA were separately amplified for 35 cycles at 94°C for 45 s, 58°C for 45 s, and 72°C for 1 min using two primer pairs spanning different regions of the ADH I gene; the sequences of the primer pairs are listed in Table 1. The PCR products were run in 1.5% agarose-ethidium bromide gel electrophoresis.

CHIP Assay

CHIP assay was carried out according to the manufacturer’s instructions (Upstate Biotechnology). Hepatocytes were cultured with 100 units of Moloney murine leukemia virus reverse transcriptase at 42°C for 60 min and 92°C for 10 min. Aliquots from each cDNA preparation were amplified by 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 24 cycles. GAPDH was used as the internal control. The PCR conditions for GAPDH were 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C for 23 cycles. The sequences for the different primer pairs are listed in Table 1. The PCR products were visualized by 1.5% agarose-ethidium bromide gel electrophoresis.

RESULTS

In the present study, we have investigated the characteristic features of ethanol-induced histone H3 acetylation in cultured rat hepatocytes and in the rat liver in vivo and its mechanisms involving modulation of HAT and MAPK cascades. The effect of ethanol on the association of acetylated histones with the ADH I gene in the chromatin was also analyzed. The results are summarized below.
Fig. 1. Analysis of ethanol (EtOH)-induced histone acetylation by immunocytochemical and immunoblot analysis in the hepatocytes. A: hepatocytes were cultured in a chamber slide in the presence of ethanol (100 mM) or trichostatin A (TSA; 2 μg/ml) as a positive control for 24 h. Histone acetylation was examined by immunocytochemical staining. Cells were incubated with site-specific anti-acetyl histone H3 (AC-H3) antibody, secondary antibody conjugated with FITC, and finally stained with 4′,6-diamidino-2-phenylindole (DAPI). Columns 1, 3, and 5 (blue) show the images stained with DAPI to show nuclei, and columns 2, 4, and 6 (green) show the same field stained with site-specific anti-acetyl histone H3 antibody. Immunocytochemistry pictures shown are representative of 3 separate experiments.

B: effects of ethanol on H3 acetylation were also examined by immunoblot analysis. Hepatocytes were treated with indicated concentrations of ethanol or TSA (2 μg/ml) for 24 h. Nuclear acid extracts were prepared and used for Western blot analysis to detect acetylated histone H3 (see MATERIALS AND METHODS). Equal amounts (10 μg) of proteins were subjected to 15% SDS-PAGE and transferred onto a nitrocellulose membrane. Acetylated histone H3 levels were monitored using site-specific (at Lys9, -14, -18, and -23) anti-acetyl H3 antibody and enhanced chemiluminescence detection. Data shown are the representative of 5 separate experiments. Quantitative analysis of acetylated histone H3 at each lysine residue by 100 mM ethanol was performed by densitometric analysis and is represented by the bar graph. Data are presented as means ± SD; n = 5. Values represent the fold increase over the control group (control = 1). **P < 0.01 compared with the control group.
Characteristics of Ethanol-Induced H3 Acetylation

Analysis of ethanol-induced histone H3 acetylation at specific lysine residues by immunocytochemical and immunoblot analysis. Previously, we have shown that ethanol increases acetylation of H3 at Lys9 in primary culture of rat hepatocytes in dose- and time-dependent manners with a maximal response at 100 mM and 24 h of treatment (39) without affecting the acetylation state of Lys14. In the present study, we have extended our examination of the effect of ethanol on H3 acetylation at two additional lysine residues (Lys18 and -23) using site-specific anti-acetyl histone H3 antibodies. As expected, immunocytochemical analysis indicated that acetylation of histone H3 at Lys9 was increased by ethanol exposure (H3-AcK9; Fig. 1A). However, ethanol had a negligible effect on the acetylation of H3 at Lys14, -18, or -23 (Fig. 1A). This ethanol-induced selective effect on H3 acetylation at Lys9 was confirmed by immunoblot analysis. Ethanol (100 mM) treatment of hepatocytes caused a ninefold increase in H3 acetylation at Lys9, whereas ethanol-induced H3 acetylations at Lys14, -18, and -23 were not statistically significant (Fig. 1B), indicating that ethanol treatment preferentially increased acetylation of H3 at Lys9. In both studies, TSA, a HDAC inhibitor, was used as a positive control, and, as expected, it increased acetylation of H3 at each of the lysine residues.

Effect of glucose and serum levels on ethanol-induced histone H3 acetylation. The hepatocyte culture medium used in this experiment contains glucose and serum, both of which can affect histone acetylation. Glucose can be converted to acetyl-CoA, a substrate for the histone acetylation reaction, and serum may also contain miscellaneous factors that could affect histone acetylation. Therefore, we asked whether ethanol-induced

Fig. 2. Ethanol-induced histone H3 acetylation is reversible. Hepatocytes were cultured for 24 or 48 h in the absence (C) or presence (E) of 100 mM ethanol. In another group, hepatocytes were cultured with ethanol (100 mM) for 24 h, and the medium was then replaced and the cells were cultured without ethanol for another 24 h (W, withdrawal). H3 acetylation level at Lys9 was determined by Western blot analysis. Data shown are the representative of 3 separate experiments. Densitometric quantitations of acetylated histone H3 at Lys9 in Western blot analysis are shown in the bar graph. Values are presented as means ± SD (n = 3) and represent the fold increase over the control group (control = 1). **P < 0.01 compared with the control group; ###P < 0.01 compared with the 48-h ethanol-treated group.

Fig. 3. In vivo effects of acute ethanol treatment on the histone H3 acetylation. A: effects of acute ethanol treatment on histone acetylation in vivo were investigated. Rats were administered alcohol (6 g/kg) intragastrically. After the indicated time periods (1, 3, and 12 h), liver nuclear acid extracts were prepared and used for Western blot analysis to determine the level of acetylated H3 at Lys9. Four rats were used for each group. Quantitative analysis of acetylated histone H3 at Lys9 shown in the Western blot analysis was performed by densitometric analysis and is represented by the bar graph. Values are presented as means ± SD; n = 4. Band intensity from 1 and 3 h was normalized to the signal from the 12-h ethanol-treated sample. **P < 0.01 compared with the control group. B: the same nuclear extracts as A were used for Western blot analysis to determine the level of acetylation of H3 at Lys14, -18, and -23. C, control; E, ethanol-treated group (6 g/kg). H3 acetylation is affected by changes in glucose and serum levels. Cells were incubated with or without ethanol in media containing different concentrations of glucose (high concentration, 25 mM; or a physiologically relevant concentration, 6 mM) and serum levels (0.1–20%), and the degree of ethanol-induced H3 acetylation was investigated. Ethanol (50 and 100 mM) increased H3 acetylation at Lys9 to a similar extent, without significantly affecting Lys14 acetylation at either glucose levels (data not shown). Ethanol also increased H3 acetylation at Lys9 to a similar extent independent of the serum
levels (0.1–20%; data not shown). Thus ethanol-induced H3 acetylation is not affected by glucose and serum levels.

The reversibility of ethanol-induced H3 acetylation. The reversibility of this acetylation event was examined by ethanol withdrawal experiments. Hepatocytes were exposed to 100 mM of ethanol for 24 h to increase acetylation of H3 at Lys9. The medium was replaced after 24 h, and the cells were cultured with or without ethanol for another 24 h. In the continuous presence of ethanol, the level of H3 acetylation at Lys9 reached maximal at 24 h and decreased slightly at 48 h of treatment [Fig. 2, bottom; compare E (24 h) and E (48 h)]. However, withdrawal of ethanol for the last 24 h accelerated the decrease of H3 acetylation [Fig. 2, bottom; compare E (48 h) and W] with acetylation level returning to almost the control level, suggesting that ethanol-induced histone acetylation is reversible.

In vivo effects of acute ethanol treatment on H3 acetylation in rat liver. To investigate whether ethanol can also increase histone acetylation in vivo, rats were administered alcohol (6 g/kg body wt) intragastrically and were killed at 1, 3, and 12 h after ethanol treatment. Nuclear acid extracts were prepared from the liver and used for immunoblot analysis. Acetylation of histone H3 at Lys9 was slightly (~23%) increased at 1 h and by twofold at 3 or 12 h (Fig. 3A). However, ethanol had a negligible effect on the H3 acetylation at Lys14, -18, and -23 at 12 h (Fig. 3B), showing that acute in vivo ethanol treatment causes selective acetylation of H3 at Lys9 consistent with in vitro observations.

![Fig. 4. Ethanol modulates histone acetyltransferase (HAT) responsible for H3 acetylation at Lys9. A: HAT activity assay was performed by ELISA using various H3 peptides. Hepatocytes were cultured with ethanol (50 and 100 mM) for 24 h. Nuclear extracts were prepared and incubated with either histone H3 peptide (unmodified) or peptide acetylated on Lys9 (H3-AcK9) or on Lys14 (H3-AcK14) in the presence of HAT assay cocktail containing HAT assay buffer, Na butyrate, and acetyl-CoA. The acetylation level of each peptide was measured by ELISA (see MATERIALS AND METHODS). Values are presented as means ± SD (n = 4 or 5) and represent the fold increase over the control group (control = 1). *P < 0.05 compared with the control group. B: hepatocytes were cultured with ethanol (50 and 100 mM) for 24 h. Nuclear extracts were prepared and incubated with total histone, HAT assay buffer, Na butyrate, and acetyl-CoA (see MATERIALS AND METHODS). Reaction mixtures were taken and used for Western blot analysis using anti-H3 AcK9 antibody (top), and the same samples were used for Western blot analysis using anti-H3 AcK14 antibody (bottom). Each experiment was performed in duplicate, and the blot shown is the representative of 3 separate experiments. After incubation of nuclear extracts with histone, acetylated H3 at Lys9 shown at top was quantitated by densitometric analysis and is represented by the bar graph. Values are presented as means ± SD (n = 3) and represents the fold increase over control group (control = 1). *P < 0.05 and **P < 0.01 compared with the control group.
Mechanisms Involved in Ethanol-Induced Histone Acetylation

Effect of ethanol on HAT. We next examined the effect of ethanol on HAT by an ELISA method using three different types of H3 peptides: unmodified H3 peptide or modified H3 peptides already acetylated on Lys9 (H3-AcK9) or Lys14 (H3-AcK14). In this experiment, nuclear extracts were prepared as sources of HATs from hepatocytes treated with ethanol and incubated with the H3 peptide. The acetylation level of H3 peptides was measured by colorimetric change (see MATERIALS AND METHODS). Acetylation of unmodified H3 peptide containing the first 21 amino acids of NH2 terminal of histone H3 was increased by incubation with nuclear extracts from ethanol-treated cells, by 1.5- and 1.7-fold at 50 and 100 mM ethanol, respectively (Fig. 4A, unmodified H3 peptide). When we used the modified H3 peptide already acetylated on Lys9 as the substrate, the acetylation level was not significantly affected by incubation with nuclear extracts from ethanol-treated cells (Fig. 4A; H3-AcK9 peptide). In contrast, when the modified H3 peptide already acetylated on Lys14 was incubated with nuclear extracts from 100 mM ethanol-treated hepatocytes, the acetylation level of this peptide was significantly increased by twofold (Fig. 4A; H3-AcK14 peptide). These results imply that ethanol selectively modulates HAT(s) targeting the acetylation of H3 at Lys9.

We also examined ethanol effect on HAT by immunoblot analysis. Nuclear extracts from control and ethanol-treated hepatocytes were incubated with histone in the presence of acetyl-CoA and Na butyrate (HDAC inhibitor; see MATERIALS AND METHODS). The reaction mixtures were then analyzed by immunoblot analysis using site-specific anti-acetylated H3 antibodies to determine the levels of H3 acetylation at Lys9 and Lys14. H3 acetylation at Lys9 was increased when purified histone was incubated with nuclear extracts prepared from ethanol-treated hepatocytes (Fig. 4B, top) in a dose-dependent manner, by 2.3-fold at 50 mM of ethanol and 3.9-fold at 100 mM. However, H3 acetylation at Lys14 was not affected by ethanol treatment (Fig. 4B, bottom). These data further support that ethanol selectively modulates HAT(s) targeting acetylation of H3 at Lys9 without affecting Lys14.

Effect of acetate on HAT. Ethanol is metabolized into acetaldehyde and further oxidized into acetate. We have previously shown that inhibitors of enzymes responsible for ethanol metabolism decreased ethanol-induced H3 acetylation and acetate treatment increased H3 acetylation in the hepatocytes, suggesting that acetate plays a role in ethanol-induced H3 acetylation (39). We therefore examined the effect of acetate on HAT to investigate whether the acetate-induced histone acetylation might also involve HAT. Acetylation of unmodified H3 peptide was increased by 2.3-fold following incubation with nuclear extracts from acetate (5 mM)-treated (24 h) hepatocytes (Fig. 5; H3). Acetate treatment also increased the acetylation level of H3 peptide already acetylated on Lys14 (Fig. 5; H3-AcK14) but not the acetylation level of H3 peptide already acetylated on Lys9 (Fig. 5; H3-AcK9). Thus acetate treatment also modulated HAT selectively required for H3 acetylation at Lys9, suggesting that the conversion to acetate may mediate the effect of ethanol on HAT.

Effects of MAPK inhibitors on ethanol- and acetate-induced histone H3 acetylation. The MAPK cascade has been shown to increase histone acetylation in other systems (1, 20, 21, 38). Ethanol modulates MAPK pathways (3). The question, therefore, arises as to whether MAPK is involved in the increase of histone acetylation by ethanol. To examine this possibility, we used selective inhibitors of MAPK. Blockade of the MAPK (ERK1/2) pathway with a MEK inhibitor (U0126; 10 μM) decreased H3 acetylation by 52 and 57% in the 50 and 100 mM ethanol-treated cells, respectively (Fig. 6A). Likewise, treatment with SP600125 (JNK inhibitor; 30 μM) reduced H3 acetylation by 52 and 65% in the 50 and 100 mM ethanol-treated hepatocytes, respectively (Fig. 6B). However, p38 MAPK inhibitor (SB203580) at 10 or 20 μM, concentrations that have been shown to inhibit p38 MAPK activity (8, 54), were without effect on ethanol-induced H3 acetylation (Fig. 6C). Actually, SB203580 treatment slightly increased both basal (Fig. 6C, compare lanes 1 and 8) and ethanol-induced H3 acetylation (Fig. 6C, compare lanes 2 and 4, 5, and 7). These data indicate that ethanol-induced H3 acetylation at Lys9 is dependent, at least in part, on ERK1/2 and JNK signaling but not on p38 MAPK.

Metabolism of ethanol to acetate plays a role in H3 acetylation (39), and acetate treatment results in an increase in total HAT activity assayed in vitro (Fig. 5). We investigated whether ERK and JNK are involved in acetate-induced H3 acetylation. Treatment of hepatocytes with acetate (5 mM, 24 h) caused an increase of H3 acetylation at Lys9, but MEK and JNK inhibitors did not affect H3 acetylation induced by acetate (Fig. 7), showing that ERK and JNK pathways are not involved in acetate-induced histone acetylation. This result suggests that ERK and JNK pathways regulate ethanol-induced histone acetylation through separate pathway(s) distinct from those activated by acetate.

Effects of MEK and JNK inhibitors on ethanol-modulated HAT. We next examined the role of ERK and JNK in ethanol-modulation of HAT. Nuclear extracts were prepared from hepatocytes treated with ethanol (100 mM) in the absence or presence of MEK or JNK inhibitor. Extracts were incubated with modified H3 peptide acetylated on Lys14 (H3-AcK14).
Ethanol (100 mM, 24 h) treatment increased the acetylation level of this peptide consistent with previous experiments; however, the presence of MEK and JNK inhibitor did not significantly affect the increase in acetylation of H3-AcK14 peptide caused by ethanol treatment (Fig. 8). Taken together, these experiments (Figs. 7 and 8) suggest that the ERK/JNK signaling cascades are implicated in ethanol-induced histone acetylation independent of a direct modulation of HAT.

**Effect of ethanol on the association of acetylated histone with the ADH I gene.** Ethanol treatment has been shown to induce ADH I gene expression (30). Ethanol treatment increases the overall acetylation level of histone H3 at Lys9. To investigate whether ethanol induction of ADH expression is related to this increase in histone acetylation, we examined the effect of ethanol on the association of acetylated H3-Lys9 with the ADH I gene using the CHIP assay. Formaldehyde cross-linking was performed, and acetylated H3 was detected by Western blot analysis using anti-H3 AcK9 antibody. Data shown are the representative of 4 separate experiments. Quantitative analysis of acetylated histone H3 at Lys9 shown in A was performed by densitometric analysis and is represented by the bar graph. Data are presented as means ± SD; n = 4. Values represent the fold increase over the control group (control = 1). *P < 0.05 and **P < 0.01 compared with the control group; #P < 0.05 compared with the ethanol-treated group. B: hepatocytes were cultured with ethanol (50 or 100 mM) for 24 h in the absence or presence of the JNK inhibitor SP600125 (SP). Nuclear acid extracts were prepared, and acetylated H3 was detected by Western blot analysis using anti-H3 AcK9 antibody. Data shown are the representative of 4 separate experiments. Quantitative analysis of acetylated histone H3 at Lys9 shown in B was performed by densitometric analysis and is represented by the bar graph. Data are presented as means ± SD; n = 4. Values represent the fold increase over the control group (control = 1). *P < 0.05 and **P < 0.01 compared with the control group; ###P < 0.001 compared with the ethanol-treated group. C: hepatocytes were cultured with ethanol (50 or 100 mM) for 24 h in the absence or presence of the p38 MAPK inhibitor SB203580. Nuclear acid extracts were prepared, and acetylated H3 was detected by Western blot analysis using anti-H3 AcK9 antibody. Data shown are the representative of 3 separate experiments that showed similar results.
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linked chromatin fragments from ethanol-treated hepatocytes were immunoprecipitated with anti-acetyl H3-Lys9 antibody. DNA from the immunoprecipitates was amplified by PCR using primer pairs in the ADH I promoter region (P1) and the ADH I coding region (P2). As shown in Fig. 9B, ethanol treatment increased association of DNA from both the ADH promoter region (P1) and the coding region (P2) with Lys9-acetylated histone H3 (Fig. 9B; H3-AcK9). PCR amplification using either primer pairs with the input DNA (i.e., chromatin fragments before the immunoprecipitation) from ethanol-treated and control cells yielded similar amounts of PCR product (Fig. 9B; input), whereas no amplified product was detected when DNA was immunoprecipitated with rabbit IgG as a negative control (Fig. 9B; IgG). Increased association of acetylated histone with the ADH I gene by ethanol treatment was confirmed by real-time PCR analysis. Immunoprecipitated DNA was used for real-time PCR reaction using SYBR green I dye to quantify CHIP assay. As expected from the data shown in Fig. 9B, ethanol (100 mM) treatment increased the accumulation of acetylated histone H3-Lys9 with the promoter (P1) and coding (P2) region of the ADH I gene by 3.7- and 3.9-fold, respectively (Fig. 9C). Finally, total RNA was isolated from hepatocytes treated with ethanol under the exact same conditions as for CHIP assay and used for RT-PCR to analyze ADH I mRNA expression. As shown in Fig. 9D, ethanol (100 mM) treatment increased the level of ADH I mRNA by twofold.

DISCUSSION

In the present study, we investigated the biochemical effects of ethanol on histone H3 acetylation and its underlying mechanisms with particular focus on HAT. Histone acetylation occurs on various lysine residues. It has been shown that specific lysine residues on histone tails can be modified and hypothesized that a specific pattern of histone modification could determine the transcriptional activity of the gene (19). In the case of H3, acetylation is known to occur at Lys9, -14, -18, and -27. Among these, acetylations at Lys9 and Lys14 have been known as important positions for transcriptional activation (5, 42). Data shown here clearly demonstrated that ethanol treatment increases acetylation of histone H3 in the liver, selectively, at Lys9 both in vivo and in vitro. This acetylation reaction was reversible and unaffected by the changes in glucose or serum levels.

HATs play a major role in histone acetylation and preferentially acetylate specific lysine residues (22, 48). We, therefore, investigated the effect of ethanol on HAT to identify the mechanisms for ethanol-induced selective histone acetylation. In the HAT ELISA assay using different types of H3 peptides (Fig. 4), nuclear extracts (source of HATs) from ethanol-treated cells increased acetylation of unmodified H3 peptide and H3 peptides already modified at Lys14 (H3-AcK14) but did not increase acetylation of H3 peptide already modified on Lys9 (H3-AcK9). In this assay, H3 peptides were incubated with nuclear extracts in the presence of Na butyrate (a HDAC inhibitor, see MATERIAL AND METHODS) to remove the possibility of involvement of HDAC in the reaction. These data, therefore, suggest that ethanol affects HAT(s) selectively targeting H3 acetylation at Lys9. Whether this increase in total HAT activity is due to an increased expression level or the activation of specific HAT(s) remains to be determined.

Interestingly, this ethanol effect may be mediated by a metabolite of ethanol, acetate. Acetate is converted to acetyl-CoA (49), which is a substrate used by HAT to acetylate histone. However, the effect of acetate on histone acetylation in the hepatocytes cannot be explained simply by the production of acetyl-CoA, because acetate is barely metabolized to acetyl-CoA in the liver due to low activity of the enzyme (acetyl-CoA synthetase) responsible for the conversion of acetate to acetyl-CoA (55). The level of acetyl CoA does not change significantly after ethanol exposure in the liver (55). Moreover, increases in acetyl-CoA levels might be expected to cause a generalized increase in H3 acetylation rather than a selective increase in acetylation at Lys9 observed with ethanol treatment. Acetyl-CoA production is thus unlikely to be the mechanism responsible for ethanol-induced histone acetylation.

Our immunoblot analysis (Fig. 4B) using total histone also showed that ethanol modulation of HAT is required for H3 acetylation at Lys9. Because nuclear extracts were used as sources of HATs, it can be questioned whether the acetylation status of endogenous histones in the nuclear extracts might have contributed to the immunological signal. However, the amount of added “exogenous” histones was much higher than the endogenous histone level in the nuclear extracts. In addition, when nuclear extracts were used alone in the immunoblot analysis without added histone, acetylated H3 at Lys9 was not detected (data not shown). This precludes the effect of endogenous histones in this assay. Hence, two independent HAT activity assays indicate that ethanol increases activity of HAT for H3 acetylation at Lys9. However, a HAT that specifically acetylates H3 at Lys9 has not been reported to date. The identity of the HAT whose activity is regulated by ethanol is thus at present unknown.

The degree of histone acetylation is controlled by the balance between HDAC and HAT. Therefore, it is also possible that ethanol increases histone acetylation by inhibition of HDAC. We investigated the effect of ethanol on HDAC using a colorimetric assay kit (BioMol). Incubation of acetylated substrates (provided with the kit) with nuclear extracts prepared from cells treated with ethanol (or acetate) did not significantly change the release of the acetyl group from the substrates compared with extracts from untreated cells. The data suggest that ethanol treatment did not significantly affect...
However, because individual HDACs preferentially target particular histones and lysine residues in histone deacetylation, we cannot completely rule out the possibility that a specific type of HDAC may be inhibited by ethanol and contributes to the increase in histone H3 acetylation at Lys9.

Multiple signaling pathways are known to converge on the chromatin by targeting the NH2-terminal tails of histones (11). The MAPK cascades in particular have been shown to elicit both histone phosphorylation and acetylation (11, 38). In the present study, we have shown that ethanol-induced H3 acetylation is reduced by MEK inhibitor U0126 and JNK inhibitor SP600125 but not by p38 MAPK inhibitor SB203580, suggesting a role of ERK and JNK in histone H3 acetylation induced by ethanol. Several previous studies (20, 31, 38) have implicated MAPKs in the upregulation of HAT activity. However,
in the present study, MEK or JNK inhibitors did not affect the ethanol-induced increase in total HAT activity (Fig. 8), although these inhibitors decreased ethanol-induced H3-Lys9 acetylation (Fig. 6). In addition, MEK and JNK inhibitors did not affect acetate-induced histone acetylation (Fig. 7), indicating that these MAPK cascades are not downstream targets of acetate for histone acetylation. Thus ethanol treatment may modulate HAT, at least in part, through its metabolism to acetate, but the MAPK cascades might influence H3 acetylation independent of the HAT modulation pathway (Fig. 10).

How else might the ethanol-induced MAPK cascades contribute to increase acetylation of H3 without directly affecting HAT activity? There are several possibilities. For example, many HATs exist as multiprotein complexes inside the cell (37), and the formation of such complexes is necessary for the efficient acetylation of histones. Also, several HATs have been shown to preferentially increase acetylation of histone H3 previously phosphorylated at Ser10 (12, 28). Therefore, it is possible that MAPKs may indirectly affect histone acetylation following ethanol treatment by acting on other components of the HAT complexes to modulate their formation or by regulating the process of histone phosphorylation. In addition, phosphorylation of several types of HDACs has been shown to result in their export from the nucleus to the cytosol (42). Although we had not detected a decrease in total nuclear HDAC activity, it is also possible that ethanol-activated MAPKs may selectively modulate specific types of HDAC to increase H3-Lys9 acetylation. Details of the mechanisms involving MAPKs in the ethanol-induced histone acetylation have yet to be investigated.

Many evidences suggest that acetylation of core histones plays an important role in transcriptional activation by altering chromatin structure (16). Especially, acetylation of histone H3 at Lys9 is considered to be a specific marker of active genes (34). We have shown that ethanol increased the overall level of acetylation of H3-Lys9 in the nucleus and the increased accumulation of acetylated histone H3-Lys9 with the ADH I gene (Fig. 9). Ethanol also increased the mRNA level of this gene. These results suggest a role of histone acetylation in the ethanol induction of ADH I gene expression. In this study, the increases in association of acetylated histones occurred in the coding region as well as the promoter of ADH I, suggesting that histone acetylation occurs throughout the entire ADH I gene rather than localized to the promoter-associated chromatin.

This is the first report of an involvement of ethanol-induced histone acetylation on a target gene. Histone acetylation has been shown to induce various biological responses including apoptosis and cell growth arrest (26, 27, 43), most of which are likely to involve selective gene expression. It will be interesting to investigate the role of histone acetylation and other histone modifications in the many different ethanol-induced biological responses.

Combinations of different histone modifications induced by ethanol and their reciprocal interaction would function differently from singular modification and may generate distinct biological responses (19). Our preliminary data showed that ethanol treatment indeed affected histone methylation and phosphorylation in hepatocytes (unpublished observations). Thus an intriguing interplay among the different histone modifications induced by ethanol may have important consequences on ethanol-induced biological responses, a detailed understanding of which awaits future study.

In summary, the data reported here establish for the first time that, in the liver, ethanol increases acetylation of H3 at Lys9 through modulation of HAT, and this acetylation can be regulated by ERK and JNK signaling pathways. Furthermore, this ethanol-induced increase in acetylated histone H3-Lys9 may underlie the mechanism for ADH I gene expression by ethanol in hepatocytes.

Fig. 10. Proposed pathways for ethanol-induced histone acetylation in the hepatocytes. Ethanol induces H3 acetylation by dual pathways. Ethanol modulates HAT targeting H3-Lys9, and this leads to the selective acetylation of H3 at Lys9. Ethanol metabolism is involved in acetylation of H3-Lys9. Acetate may mediate the effect of ethanol on histone acetylation by modulation of HAT. MAPK cascades (ERK and JNK) also regulate ethanol-induced H3 acetylation. MAPK cascades are not implicated in direct modulation of HAT induced by ethanol nor H3 acetylation induced by acetate, suggesting that MAPK signaling cascades and HAT modulation pathway regulate H3 acetylation in different ways. Detailed mechanisms of how acetate affects HAT and how MAPKs contribute to H3 acetylation remain to be determined. Ethanol does not affect total nuclear histone deacetylase (HDAC) activity; however, further studies are required to test for the possibility that ethanol regulates a specific type of HDAC to modulate H3 acetylation. CHIP assay reveals that ethanol increased accumulation of acetylated histones with the ADH I gene, suggesting that the increase H3-Lys9 acetylation might be involved in the ethanol-induced ADH I gene expression.
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