Different Mechanisms for Histone Acetylation by Ethanol and Its Metabolite Acetate in Rat Primary Hepatocytes

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ABSTRACT

Ethanol and its major metabolite acetate both induced histone H3 acetylation in primary culture of rat hepatocytes. The acetylation by ethanol was dependent on the reactive oxygen species and mitogen-activated protein kinase pathway, whereas that by acetate was independent of both pathways. Ethanol increased CYP2E1 protein expression but acetate had negligible effect. The level of phospho-H2AX, an indicator of DNA breaks, was elevated by ethanol but not by acetate. Ethanol and acetate differentially activated mRNA expression for different genes, e.g., IL-6, PPARγ, c-Fos, Egr-1, and PNPLA3 in hepatocytes. The most striking increase (3-fold) was in PNPLA3 mRNA by ethanol with little change by acetate. It was further shown that acetate inhibited histone deacetylase activity. Taken together, these data establish for the first time that ethanol and acetate exhibit differences in their effects on hepatocytes in gene expression, P-H2AX levels, and the mechanism of histone H3 acetylation. The implications of these differences in the actions of ethanol in liver are discussed.

Introduction

The molecular mechanism of the action of ethanol in liver remains to be defined; a complicating factor is the generation of diverse metabolites of ethanol that include both oxidative (e.g., acetaldehyde and acetate) and non-oxidative (e.g., phosphatidylethanol and fatty acid ethyl ester) products. Ethanol is known to cause epigenetic changes in liver with implications in ethanol-induced steatosis and inflammation, leading to liver injury (Shukla and Lim, 2013). One of the epigenetic pathways involves post-translational modifications in histones, e.g., histone H3 acetylated at lysine 9 (H3AcK9). It has been demonstrated previously that ethanol causes an increase in liver H3AcK9 that was dependent in part on ethanol metabolism (Park et al., 2005) and involves both reactive oxygen species (ROS) and mitogen-activated protein kinase (MAPK) signaling components (Shukla et al., 2008; Choudhury et al., 2010). We have now investigated and compared the effects of ethanol and its major metabolite acetate on histone H3 acetylation in primary cultures of rat hepatocytes and also monitored differences in their effects on CYP2E1 protein, phospho-H2AX levels, histone deacetylase (HDAC) activity, and the expression of selected genes.

Materials and Methods

Hepatocyte Preparation and Treatment. Hepatocytes from male Sprague-Dawley rats (250–300 g) were isolated using an in situ collagenase perfusion method (Seglen, 1976; Weng and Shukla, 2000). The viability of the hepatocytes was monitored by trypan blue dye exclusion and was routinely 90 ± 5%. The cells were plated on collagen-coated culture dishes in Dulbecco’s modified Eagle medium (DMEM) (high glucose) containing 10% fetal bovine serum, 2 mM l-glutamine, and 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were allowed to attach to the dishes for 2 hours in the culture incubator equilibrated with air/CO2 (95%/5%). The medium was removed from the dishes and subsequently cells were treated with different agents, as required in DMEM medium containing 1% fetal bovine serum, for the desired length of times. Subsequently, the cells were processed for various analyses.

Collection and Storage of Cell Pellets after Treatment. Media from plates were aspirated and the plates washed by adding 2 ml of 1× sterile phosphate-buffered saline at room temperature. The phosphate-buffered saline was aspirated, and then the plates were kept on an ice block while 1 ml of ice-cold 1× phosphate-buffered saline was added. Cells were scraped and collected into a 1.5 ml centrifuge tube, kept on ice, and centrifuged at 1000g for 5 minutes at 4°C. The supernatant was aspirated and the cell pellets were stored at −80°C until processing for protein or RNA extraction. In some cases this pellet was resuspended in a lysis buffer (as needed) and then frozen.

Preparation of Whole Cell Extract and Nuclear Extracts from Primary Hepatocyte Cultures. Cell pellets were resuspended in 150 μl of buffer-1 (lysis buffer with 0.25 M sucrose as described by Aroor et al., 2012) and homogenized by 10 strokes using...
a 1 ml syringe with a 25 gauge needle. Cell suspensions in 1.5 ml microfuge tubes were centrifuged at 1000g for 10 minutes at 4°C. Supernatant (140 μl) was collected into tubes and glycerol was added to a final concentration of 10% (v/v), vortexed, and stored at −80°C as whole cell extract (WCE). The pellet was resuspended in 150 μl of buffer-2 (lysis buffer with 0.25 M sucrose and 0.25% Nonidet P-40), homogenized 8–10 times as previously described, and centrifuged. The supernatant was discarded. The remaining pellet was resuspended in 150 μl of buffer-3 (lysis buffer with 2.0 M sucrose) and homogenized 3–5 times as previously described. The resulting suspensions were centrifuged at 4000g for 15 minutes at 4°C. The supernatant was discarded and the remaining nuclear pellet was resuspended using 75 μl of urea/salt/radioimmunoprecipitation assay buffer, with vortexing 5 times every 5 minutes at 4°C. The nuclear suspensions were next sonicated 3 times for 10 seconds and centrifuged at 16,000g for 10 minutes at 4°C. The supernatant was transferred to a new tube and stored at −80°C as nuclear extract and used for quantification of modified histones. Protein concentrations in the extracts were determined using the Bio-Rad DC Protein Analysis Kit (Hercules, CA). About 10–15 μg of protein were used for Western blotting.

**RNA Extraction.** Cell pellets were resuspended in 400 μl of TRI REAGENT T9424 (Sigma-Aldrich, St. Louis, MO), transferred into a 2 ml dounce homogenizer, and homogenized 5 times with pestle B. Homogenized samples were collected into 1.5 ml centrifuge tubes and RNA was isolated according to the manufacturer’s instructions.

**Western Blotting.** WCEs were used for Western blotting of CYP2E1 and 20μg of protein per lane was loaded. Western blotting for histones and proteins smaller than 50 kDa was performed in 12.5% mini-gels and for all other proteins in 10% mini-gels. The immuno-bLOTS were quantified by imaging (Aroor et al., 2014).

**HDAC Assay.** A HDAC Fluorometric Assay Kit, (Millipore, Temecula, CA) was used. A Spectramax M2 (Molecular Devices, Sunnyvale, CA) fluorescence microplate reader was used.

**Determination of ROS.** For the assessment of ROS produced by ethanol, cells were grown in 6-well plates for 23 hours in phenol-free DMEM media and a dichlorofluorescein-diacetate assay was used. The method was slightly modified from a previously described technique (Sheehan et al., 1997); instead of Hepes-Krebs-Ringer buffer, phenol-free media was used. Dichlorofluorescein-diacetate was added to the wells and incubated for 1 hour. During this time they were shielded from light. After 1 hour the media containing excess dichlorofluorescein was removed from the cells and replaced with fresh DMEM media. The fluorescent intensity of the cells was measured by excitation at 485 nm and emission at 520 nm using a Fuji LAS-3000 imager (FujiFilm, Tokyo, Japan). The imager measured fluorescence in the linear range before saturation occurred. The background values were appropriately subtracted using Fuji multigauge software before the data were calculated and analyzed.

**Quantitative Polymerase Chain Reaction.** cDNA was made using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Primers for probing various genes (see Supplemental Table 1 in the Supplemental Material) were supplied by IDT (Coralville, IA). A CFX96 (Bio-Rad) real-time polymerase chain reaction detection system was used.
Data are mean ± S.E.M.; n = 2–3 experiments. D, dimethylsulfoxide vehicle; asterisks show significant change over control.

Results

Dose- and Time-Dependent Effects of Acetate on Histone Acetylation. Ethanol treatment of hepatocytes has been shown to cause an increase in H3AcK9 in a dose-dependent manner, with increases noted at as low as 5 mM and with a peak at 100 mM (Park et al., 2003). This was also time dependent with a peak at 24 hours after ethanol treatment. It should be mentioned that 100 mM ethanol is a concentration that has been observed in chronic alcoholics and binge drinkers (Shukla et al., 2013). In a rat model that was treated with chronic ethanol followed by three binge doses of ethanol, the serum levels of ethanol reached 117 mM (Aroor et al., 2011). Therefore, the levels of 100 mM ethanol are also observed in vivo and are clinically significant. It should also be emphasized that only 15–20% of chronic alcoholics develop alcoholic liver disease and most of them are binge drinkers. This also indicates that higher ethanol concentrations are experimentally useful in order to understand the pathologic effects of alcohol in liver. Acetate is a major product of ethanol metabolism. Because the liver is the primary organ for ethanol metabolism, hepatocytes are exposed to rather high acetate concentrations in vivo following ethanol administration, with the rate of acetate production from ethanol in the rat liver estimated to be about 1.4 μmol/min per 10^8 cells, or approximately 3.5–7 μmol/min per milliliters of cell volume (assuming a typical per cell volume of 2 to 4 × 10^-9 cm^3 per cell) (Buckley and Williamson, 1977). Perfusion of liver in live rat with ethanol has been shown to result in acetate concentrations as high as 10 mM in the perfusate (Topping et al., 1984), and blood levels of acetate can reach 2.5–3 mM in rats and rabbits following acute administration of ethanol (Suokas et al., 1984; Fujimiya et al., 2003). In human chronic alcoholics, blood acetate levels close to 2 mM have been reported (Nuutinen et al., 1985). Intraperitoneal administration of acetate in mice elevated acetate levels in plasma up to 10 mM (Ishiguro et al., 2007). It is likely that the local concentration of acetate in and around hepatocytes where ethanol metabolism occurs may be much higher than the serum levels. To generate a dose-response profile we used the range of 1–10 mM acetate (sodium salt), which covers both the physiological and pathological/toxicological levels. Acetate caused an increase in H3AcK9 at concentrations above 1.0 mM, and this increase continued to a maximum at 10 mM with an EC50 value of 5 mM (Fig. 1). At concentrations of acetate 15 mM or higher, a decrease in the degree of induction of H3AcK9 was observed. We next examined the time dependency of acetate-induced changes in H3AcK9 and used 10 mM acetate to increase the detection sensitivity. In contrast to ethanol’s peak effect at 24 hours, the acetate-induced increase in H3AcK9 was highest at 4 hours and remained at that level at 10 hours with a small decrease at 24 hours (Fig. 2). We chose to use 10 mM acetate for this and other experiments to make it easier to follow the changes. We have shown previously that treatment of hepatocytes with acetaldehyde causes increases in H3AcK9 in hepatocytes (Shukla et al., 2007). However, due to toxic vapors and the volatility of acetaldehyde it is not easy to manipulate the acetate levels in hepatocytes by exposing them to acetaldehyde. In this regard, it has been shown that ethanol-induced increases in H3AcK9 are reduced by the presence of 4-methyl pyrazole, an inhibitor of alcohol dehydrogenase, or cyanamide, an inhibitor of aldehyde dehydrogenase (Park et al., 2003). This further suggests a role for ethanol metabolic processes including acetate in histone H3.
Acetylation. It may be noted that ethanol up to 200 mM does not alter the osmolality or pH of the medium (Reddy and Shukla, 2000) during the 24 hour treatment of hepatocytes. Furthermore, glucose up to 25 mM in the medium had no effect on the ethanol-induced increases in H3AcK9 (Park et al., 2005).

It has been reported that hepatocytes placed into an incubation medium, where most of the chloride anion was replaced by high concentrations of sodium or potassium acetate (142 or 150 mM, respectively), resulted in a rapid and transient decrease in intracellular pH, which was followed by cell swelling as the intracellular pH equilibrated with the extracellular pH (Stewart, 1989). In the present study, incubation of primary hepatocytes in culture medium with acetate (0–20 mM range) for 24 hours did not alter the pH of the medium. It was shown by Stewart (1989) that at a more physiologic pH of 7.4, the volume increase was only about 10% in a 20–40 minute time frame. Stewart (1989) also showed that hepatocytes incubated with ethanol and the resulting acetic acid formation did not cause Na:H exchanger–mediated cellular swelling. Because our experiments were performed in a bicarbonate-buffered cultured medium at a physiologic pH containing normal concentrations of chloride and relatively low acetate concentrations (≤10 mM), we consider it unlikely that either a rapid/transient acidification or a small increase in cell volume, if they occur at all, would be responsible for the change in histone acetylation that occurs several hours later. Increases in histone acetylation were not apparent at 1 hour but were noticeable at 2 hours, with further increases at 4 and 10 hours (Fig. 2). Thus, histone acetylation is observed at much lower acetate concentrations and at longer times, as opposed to the reported cell volume changes that occur at very high acetate concentrations, and is rapid and transient.

**Effect of Acetate on ROS Production.** Ethanol is known to induce oxidative stress via increasing ROS (Cederbaum, 2012). To determine the involvement of ROS, we monitored the production of ROS by ethanol and acetate in hepatocytes using the dichlorofluorescein assay. Ethanol significantly increased ROS at 24 hours, an observation consistent with other reports (Cabrales-Romero et al., 2006). In contrast to ethanol, acetate at 1, 5, or 10 mM was unable to elicit any increase in ROS levels at 24 hours (or at earlier time points, data not shown) (Fig. 3).

**Effect of MAPK Inhibitors on Acetate- and Ethanol-Induced Histone Acetylation.** Hepatocytes were treated with acetate in the presence or absence of ERK1/2 inhibitor U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene; 10 μM) or c-Jun N-terminal kinase inhibitor SP600125 (anthra[1-9cd]pyrazole-6(1H)-one; 30 μM) for 24 hours. Levels of H3AcK9 were similar for acetate with or without U0126 or SP600125 (Fig. 4, left). Thus, the MAPK inhibitors for ERK1/2 and c-Jun N-terminal kinase had no effect on acetate-induced H3AcK9 increase. However, increases in H3AcK9 by ethanol were attenuated by U0126 and SP600125 by about 30 and 60%, respectively (Fig. 4, right). This reflected differences in the involvement of MAPKs between ethanol and acetate.

**Differences in Dual Modification (Phospho-Acetylation) of Histone H3 by Acetate and Ethanol.** In primary hepatocytes ethanol increased histone H3 phosphorylation at serine 10 and serine 28 residues (Lee and Shukla, 2007); this has also been shown to occur in vivo (James et al., 2012). Furthermore, ethanol in vivo caused increases in the dually modified histone H3, i.e., H3AcK9 and H3AcK9 phosphorylated at serine 10 (James et al., 2012; Aroor et al., 2014). Whether this unique dual modification also occurs in primary hepatocytes remained unknown. We have, therefore,
to ethanol causes induction of CYP2E1, resulting in the shown). They also inhibited HDAC about 90% (data not propionate (metabolite of propanol) and butyrate (metabolite ison. In this experiment we also examined the influence of HDAC inhibitor trichostatin A was also included for compar-

indicating that acetate inhibited HDAC (Fig. 6). The known source of HDAC. The in vitro addition of 5 and 10 mM acetate acetate on HDAC activity using HeLa cell extract, a rich HDAC activity (Park et al., 2005). We tested the effect of HDAC inhibition using HeLa cell extract, a rich source of HDAC. The in vitro addition of 5 and 10 mM acetate decreased HDAC activity by 50 and 60%, respectively, indicating that acetate inhibited HDAC (Fig. 6). The known HDAC inhibitor trichostatin A was also included for comparison. In this experiment we also examined the influence of propionate (metabolite of propanol) and butyrate (metabolite of butanol). They also inhibited HDAC about 90% (data not shown).

Effect of Ethanol and Acetate on the Levels of CYP2E1 and P-H2AX in Hepatocytes. Chronic exposure to ethanol causes induction of CYP2E1, resulting in the production of ROS. Increased ROS can initiate lipid peroxidation, leading to cytotoxic effects (Lieber, 2004; Cederbaum, 2012). In the next series of experiments we monitored the protein and mRNA levels of CYP2E1 after ethanol (100 mM) and acetate (10 mM) treatments of hepatocytes up to 24 hours (Figs. 7 and 8). Ethanol significantly increased CYP2E1 protein levels in WCEs in a time-dependent manner with the highest effect (about 4-fold over control) after 24 hours of treatment (Fig. 7). Interestingly, the ethanol-induced increase in CYP2E1 was statistically significant for the protein levels but not for the mRNA (Fig. 8). In contrast, acetate had no effect on the mRNA level of CYP2E1 and caused only a statistically significant, but very small, increase in the protein level that reached a plateau (~2-fold) after 10 hours (Figs. 7 and 8).

H2AX is one of core histone proteins in the nucleosome. The phosphorylated form of H2AX (at ser-139) is produced as a result of DNA double-strand breaks caused by external/toxic agents (Rogakou et al., 1998). Phospho-H2AX (abbreviated as P-H2AX or γ-H2AX) is a major hallmark of double-strand break recognition. Increases in the levels of P-H2AX are therefore reflective of double DNA breaks (Smeenk and Attikum, 2013). In the context of our histone modification studies, we therefore addressed this issue and monitored the levels of P-H2AX in hepatocytes treated with ethanol and acetate. Ethanol caused a statistically significant rise in the P-H2AX levels, whereas acetate was without effect (Fig. 9), indicating clear differences between ethanol and its metabolite.

Effect of Ethanol and Acetate on Hepatocyte Gene Expression in Hepatocytes. In this study, we next examined the effect of ethanol and acetate on the levels of selected genes implicated in alcoholic liver disease. The mRNA levels of inducible nitric oxide synthase (iNOS), tumor necrosis factor α (TNFα), interleukin-6 (IL-6), peroxisome proliferator-activated receptor γ (PPARγ), c-Fos, early growth response 1 (Egr-1), and patatin-like phospholipase domain-containing 3 (PNPLA3) were determined in hepatocytes treated for 24 hours with ethanol (100 mM) or acetate (10 mM). Ethanol increased mRNA of IL-6, PPARγ, c-Fos, Egr-1, and PNPLA3. Noticeable changes by acetate were observed in c-Fos and Egr-1 but were not statistically significant. Acetate had no effect on iNOS, TNFα, IL-6, and PPARγ. The most pronounced and statistically significant increase by ethanol was in the mRNA levels of PNPLA3 (by about 3.0-fold), whereas acetate had no effect (Fig. 10). Changes in iNOS and TNFα were not discernable between ethanol and acetate. These data demonstrated differences in the behavior of ethanol and acetate in inducing mRNA expression of different genes.

Discussion

Both acetate and ethanol increased H3AcK9 levels in the primary cultures of rat hepatocytes. Inhibitors of alcohol dehydrogenase and aldehyde dehydrogenase have been shown to diminish ethanol-induced increases in H3AcK9 (Park et al., 2003). Although there is metabolic dependence of ethanol-induced changes in H3AcK9, the differences between ethanol and its major metabolite have not been investigated. The fact that ethanol but not acetate increased ROS suggests that ethanol and acetate induced increases in H3AcK9 by both ROS-dependent and ROS-independent pathways, respectively. Oxidative stress has been demonstrated to be involved in the ethanol-induced
increases in H3AcK9 (Choudhury et al., 2010). It is well recognized that oxidative stress plays a role in the damaging effects of ethanol. Therefore, it is plausible that the ROS-independent acetate-induced increase in H3AcK9 may not be involved in the damage caused by ethanol. As described in Results, the acetate effect on H3AcK9 is unlikely due to a change in pH or cell volume under the conditions examined in the present study. It is noteworthy that ethanol elicited increases in P-H2AX and also in CYP2E1 protein, whereas acetate had negligible effect on both. The differences in the ethanol- and acetate-induced changes reported here may be relevant to ethanol-induced liver injury. It should be mentioned that in recent studies unrelated to ethanol and liver, it has been shown that acetate imparts protective effects. For example, acetate attenuated lipopolysaccharide-induced neuro-inflammation (Reisenauer et al., 2011) and inhibited the nuclear transcription factor of activated T cells (Ishiguro et al., 2007, 2011). It is also of interest to note that acetate supplementation inhibited HDAC activity in brain (Soliman and Rosenberger, 2011). Acetate is also a modulator of inflammatory responses (Kendrick et al., 2010). The literature evidence and results presented here support the view that ethanol itself activates histone acetyltransferase, whereas acetate inhibits HDAC. The fact that acetate and metabolites of other similar short-chain alcohols decreased HDAC activity further favors this conclusion. An important dimension to the effects of ethanol and acetate is the difference in gene expression. For example, a remarkable increase in PNPLA3 mRNA was evident after ethanol treatment but not with acetate. This is similar to mRNA levels reported in vivo in nonalcoholic fatty liver disease (Romeo et al., 2008) and alcoholic liver disease (Tian et al., 2010), and these results offer primary hepatocytes as a useful model for additional studies. Because histone modification is often linked to changes in gene expression, it would be important to determine if and how ethanol- and acetate-induced histone modifications are causally linked to changes in mRNA levels.

Based on our observations, a diagram illustrating the mechanistic differences in histone H3 acetylation by ethanol and acetate is shown in Fig. 11. This study also highlights the point that manipulation of ethanol metabolism and its metabolites (e.g., acetate) may be a promising avenue to modulate histone modifications. Increase in the levels of acetate will inhibit HDAC, and the resulting increase in H3AcK9 will likely represent a different pool of histones than those increased by ethanol. These different histone pools and their modifications may be associated with different genes and may result in different consequences downstream. The type of HDAC affected by ethanol and acetate and their role(s) in hepatic damage are still present unknown. The present investigation sets the stage to address these questions in future studies.

Authorship contributions

**Participated in research design:** Shukla, Restrepo, Lim.

**Conducted experiments:** Shukla, Restrepo, Fish.

**Performed data analysis:** Shukla, Restrepo, Fish, Lim, Ibdah.

**Wrote or contributed to the writing of the manuscript:** Shukla, Restrepo, Lim, Ibdah.

References


