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The Effects of Acetaldehyde Exposure on Histone Modifications and Chromatin Structure in Human Lung Bronchial Epithelial Cells

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Abstract

As the primary metabolite of alcohol and the most abundant carcinogen in tobacco smoke, acetaldehyde is linked to a number of human diseases associated with chronic alcohol consumption and smoking including cancers. In addition to direct DNA damage as a result of the formation of acetaldehyde-DNA adducts, acetaldehyde may also indirectly impact proper genome function through the formation of protein adducts. Histone proteins are the major component of the chromatin. Post-translational histone modifications are critically important for the maintenance of genetic and epigenetic stability. However, little is known about how acetaldehyde-histone adducts affect histone modifications and chromatin structure. The results of protein carbonyl assays suggest that acetaldehyde forms adducts with histone proteins in human bronchial epithelial BEAS-2B cells. The level of acetylation for N-terminal tails of cytosolic histones H3 and H4, an important modification for histone nuclear import and chromatin assembly, is significantly downregulated following acetaldehyde exposure in BEAS-2B cells, possibly due to the formation of histone adducts and/or the decrease in the expression of histone acetyltransferases. Notably, the level of nucleosomal histones in the chromatin fraction and at most of the genomic loci we tested are low in acetaldehyde-treated cells as compared with the control cells, which is suggestive of inhibition of chromatin assembly. Moreover, acetaldehyde exposure perturbs chromatin structure as evidenced by the increase in general chromatin accessibility and the decrease in nucleosome occupancy at genomic loci following acetaldehyde treatment. Our results indicate that regulation of histone modifications and chromatin accessibility may play important roles in acetaldehyde-induced pathogenesis.

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Statement of Author Contributions:

D.C. and C.J. designed the study. D.C. and L.F. performed experiments and prepared draft figures and tables. D.C., L.F., H.L., and C.J. analyzed the data. D.C. prepared the manuscript draft with important intellectual input from C.J. All authors approved the final manuscript.

Keywords

epigenetic alteration; histone acetylation; aldehyde; alcohol

Introduction

As a highly volatile, low molecular weight organic aldehyde, acetaldehyde is the primary and most toxic metabolite of ethanol metabolism. Small amounts of acetaldehyde are also found in some food products (Cheng 2010) and can be generated in the human body during threonine catabolism (Lin and Greenberg 1954). Moreover, acetaldehyde is the most abundant component in cigarette smoke (Counts et al. 2005). Acetaldehyde is defined as “carcinogenic to humans” (Group1) by the International Agency for Research on Cancer (IARC) (Pearce et al. 2015) based mainly on the elevated risk of esophageal cancer in alcohol abusers who lack the capacity to metabolize acetaldehyde into acetate as a result of a genetic variation in ALDH2 (Brooks et al. 2009). Kaphalia *et al.* showed that significant amounts of ingested alcohol reach the airways via the bronchial circulation where it is either metabolized or is excreted by exhalation. Although a majority is metabolized in the liver, the lung can also metabolize ingested alcohol through the action of alcohol dehydrogenase (ADH), which oxidizes ethanol into acetaldehyde (Kaphalia and Calhoun 2013). In fact, acetaldehyde is considered an important contributor to increased risks of developing cancers of the respiratory tract, upper digestive tract, liver, colon, and breast (Seitz and Becker 2007).

The formation of the different acetaldehyde-DNA adducts has been regarded as an important mechanism of acetaldehyde mutagenicity and carcinogenicity (Brooks and Theruvathu 2005). For example, acetaldehyde can directly react with the exocyclic amino group of deoxyguanosine to form DNA adducts (Brooks and Zakhari 2014). CrPdG adducts are another type of acetaldehyde adducts, which are formed as a result of binding between the DNA and two molecules of acetaldehyde (Chung and Hecht 1983; Wang et al. 2000). Replication of individual CrPdG adducts in nucleotide excision repair (NER) pathway-depleted cells led to a 5 to 10% mutation rate (Fernandes et al. 2005; Stein et al. 2006). The spectrum of mutations at the p53 gene in acetaldehyde-exposed cells was consistent with p53 mutational hotspots in esophageal cancer, suggesting that acetaldehyde-DNA adducts may play a critically important role in acetaldehyde carcinogenesis. Other mechanisms such as epigenetic changes may also contribute to acetaldehyde-related cancer development (Seitz and Stickel 2007). Epigenetic mechanisms including DNA methylation, histone modifications, non-coding RNAs, incorporation of histone variants, and nucleosome positioning/chromatin accessibility, are fundamental for almost all DNA-templated processes. A large number of studies have shown that epigenetic profiles such as DNA methylation and histone modifications could be affected by surrounding environmental factors (Jirtle and Skinner 2007; Dolinoy and Jirtle 2008). For example, exposure to metal toxicants causes global changes in post-translational histone modifications (PTMs) (Zhou et al. 2009). Worrall *et al.* (Worrall and Thiele 2001) demonstrated that a majority of histone modifications, which occur on the lysine residues of histone tails, is primarily targeted by acetaldehyde. The histone tails extend outside of the octamer core and are accessible to the

active site of nuclear histone methyltransferase and acetyltransferase enzymes. On the other hand, as an aldehyde, acetaldehyde can react with nucleophilic side chains of residues such as lysine. Correspondingly, lysine residues are also readily accessible to small molecules like acetaldehyde. Furthermore, non-nucleosomal free histones such as cytosolic histones could also be targeted by acetaldehyde. Niemela *et al* (Niemela et al. 1990) reported that incubation of DNA-free histone H1 with acetaldehyde results in lysine modification and that these acetaldehyde modifications inhibit the binding of H1 to DNA. However, it has not been determined whether acetaldehyde forms adduct with other histones and in turn promote changes in the acetylation of the lysine residues.

Here we investigated the effects of acetaldehyde on post-translational histone modifications in human lung epithelial BEAS-2B cells. Our results show that exposure to acetaldehyde dramatically reduces the acetylation of N-terminal tails of cytosolic histones H3 and H4. The decrease in these modifications is expected to impact histone nuclear import and chromatin assembly (Burgess and Zhang 2013; Fang et al. 2014). To extend our findings, we monitored the amount of histones in the chromatin before and after acetaldehyde treatment. We find that the level of nucleosomal histones in the chromatin fraction and at several genomic loci is decreased after acetaldehyde treatment, suggesting that acetaldehyde exposure compromises chromatin assembly. Defective chromatin assembly is considered a direct cause of or significant contributor to cancer development (Burgess and Zhang 2013). These observations suggest a novel mechanism by which acetaldehyde increases the risk of developing cancers associated with alcohol consumption and cigarette smoking.

Materials and Methods

Cell Culture and treatment

The immortalized human lung bronchial epithelial BEAS-2B and the human lung adenocarcinoma A549 cell lines were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were incubated at 37 °C in a humidified atmosphere containing 5% carbon dioxide. In all experiments, cells (1×10^7) were seeded into 15 cm-diameter tissue-culture dishes with growth medium and allowed to reach 60% confluence before being exposed to acetaldehyde with doses ranging from 0 to 20 mM for 3 to 16 hours.

Antibodies

The antibodies were purchased as follows. Anti-histone H3 (1:1000 dilution, ab1791), anti-acetyl-histone H4 Lys-12 (1:2000 dilution, ab61238), and anti-gamma Tubulin (1:2000 dilution, ab11317) were from Abcam (Cambridge, MA); anti-dinitrophenyl (DNP; 1:1000 dilution, D9656), which reacts with DNP-BSA and DNP-rabbit serum albumin but not BSA or rabbit serum albumin, was from Sigma-Aldrich (St. Louis, MO); anti-histone H3 pan (1:1000 dilution, 07-690), anti-acetyl-histone H3 (1:1000 dilution, 06-599), anti-trimethyl-histone H3 Lys-4 (1:1000 dilution, 07-473), anti-dimethyl-histone H3 Lys-9 (1:1000 dilution, 07-441), anti-trimethyl-histone H3 Lys-27 (1:1000 dilution, 07-449), anti-acetyl-histone H3 Lys-18 (1:1000 dilution, 07-354), and anti-histone H4 (1:1000 dilution, 07-108) were from Millipore (Mahopac, NY); anti-GCN5 (1:400 dilution, sc20698), and anti-HAT1

(1:400 dilution, sc276200) were from Santa Cruz Biotechnology (Dallas, TX). According to the product information, the specificities of anti-trimethyl-histone H3 Lys-4 (Millipore, 07-473), anti-dimethyl-histone H3 Lys-9 (Millipore, 07-441), anti-trimethyl-histone H3 Lys-27 (Millipore, 07-449), and anti-acetyl-histone H3 Lys-18 (Millipore, 07-354) were validated by Dot blot or Western blot with antibody that was preincubated with histone peptides containing similar modifications, e.g., H3 peptides containing monomethyl-Lysine 9, dimethyl-Lysine 9, trimethyl-Lysine 9, or dimethyl-Lysine 27 were used to test the specificity of anti-dimethyl-histone H3 Lys-9. Moreover, the specificities of anti-acetyl-histone H4 Lys-12 (Abcam, ab61238) and anti-acetyl-histone H3 (Millipore, 06-599) were validated by the observation of one single band detected by Western blot analysis.

Trypan Blue Staining and Cell Viability Assay

After exposure to acetaldehyde for different time, cells were trypsinized, centrifuged, and resuspended in culture medium. The total number of viable cells was determined by trypan blue staining, which stains dead cells and shows a blue color under the microscope. The cell viability was assessed by calculating at least 3 times the percentage of live cells within five randomly selected fields.

Flow Cytometry for Cell Cycle Analysis

After different treatments, cells were harvested, washed with cold PBS, and processed for cell cycle analysis using flow cytometry. Briefly, the cells were fixed in pre-cooled 75% ethanol and stored at -20°C for later analysis. The fixed cells were centrifuged at 1,000 rpm for 10 min and washed with cold PBS twice. Propidium iodide (PI) staining solution (50 $\mu\text{g}/\text{ml}$ final concentration; Acros Organics, Fair Lawn, NJ) was added to the cells and incubated in the dark for 30 min at room temperature. Next, 100,000 cells were analyzed using a FACS instrument (Beckman Coulter Epics SL-MCL, Jersey City, NJ) equipped with a suitable laser and EXPO32 ADC software.

Cell Lysates, Acid Extraction, and Western Blot

After AA exposure, cells were washed with ice-cold PBS and collected by centrifugation. Whole cell lysates were prepared by using Triton X-100 lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM MgCl_2 , 10 mM sodium butyrate, 1 mM PMSF, 10 $\mu\text{g}/\text{ml}$ Aprotinin and 1 $\mu\text{g}/\text{ml}$ Leupeptin). For acid extraction, cells were resuspended in lysis buffer (10 mM HEPES, pH 7.5, 1.5 mM MgCl_2 , 10 mM KCl, 0.2 N HCl, 0.5 mM DTT, 10 mM sodium butyrate, 1 mM PMSF, 10 $\mu\text{g}/\text{ml}$ Aprotinin and 1 $\mu\text{g}/\text{ml}$ Leupeptin), incubated on ice for 60 min, and centrifuged at $11,000 \times g$ for 10 min. The supernatant was transferred to a new tube and neutralized with one-fifth the volume of 1.5 M Tris-HCl buffer (pH 8.8). Fifty micrograms of total protein were separated on a 14% polyacrylamide gel by electrophoresis and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA). The membrane was cut into different parts according to the predicted size of the target proteins. Non-specific binding sites were blocked by incubation in Tris-buffered saline (TBS) containing 0.1% Tween-20 and 5% dry non-fat milk. The membranes were immunoblotted with primary antibodies overnight at 4°C , followed by incubation with a peroxidase-conjugated

secondary antibody. The reagent for enhanced chemiluminescence (Bio-Rad, Hercules, CA) was used for detection and developed by X-ray film.

Cellular Fractionation

Cells (4×10^7) were suspended in 1.5 mL of hypotonic buffer (10 mM Tris-HCl, pH 7.4, 10 mM KCl, 1.5 mM MgCl₂, 1 mM DTT, 1 mM PMSF, 10 µg/ml Aprotinin, and 1 µg/ml Leupeptin) for 10 min on ice and centrifuged at $2,500 \times g$ for 10 min after passing four times through a 25-gauge needle. The supernatant was retained as the cytosolic fraction. The remaining pellet was resuspended in 0.5 pellet volume of low salt buffer (20 mM Tris-HCl, pH 7.4, 20 mM KCl, 1.5 mM MgCl₂, 1 mM DTT, 0.2 mM EDTA, 25% glycerol, 1 mM PMSF, 10 µg/ml Aprotinin, and 1 µg/ml Leupeptin) and homogenized with a 25-gauge needle. The sample volume was carefully measured and 0.5 volume of high salt buffer (20 mM Tris-HCl, pH 7.4, 1.2 M KCl, 1.5 mM MgCl₂, 1 mM DTT, 0.2 mM EDTA, 25% glycerol, 1 mM PMSF, 10 µg/ml Aprotinin, and 1 µg/ml Leupeptin) was subsequently added to obtain a final KCl concentration of 0.42 M. After rotating for 30 min at 4 °C, the suspension was centrifuged at $14,000 \times g$ for 15 min, and the supernatant was retained as nuclear extract. The insoluble pellet was resuspended in Tris buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, and protease inhibitors) plus 1.5 mM CaCl₂. After passing four times through a 20-gauge needle followed by four passes through a 25-gauge needle, the suspension was adjusted to $A_{260} = 100$ and digested with 12×10^{-2} units/µl of MNase for 12 min at 37 °C. The reaction was stopped by adding EDTA (10 mM, final concentration). After sitting on ice for 30 min, the supernatant was collected as S1. The remaining pellet was resuspended in Tris buffer plus 0.25 mM EDTA, incubated on ice for 15 min, and passed four times through a 25-gauge needle. After centrifugation at $14,000 \times g$ for 10 min, the supernatant was collected as S2 and combined with S1 as the chromatin fraction.

Histone Carbonyl Assay

Histone carbonyl assays were performed as described by Thompson *et al.* (Thompson and Burcham 2008) using 15 µg of acid-extracted total histones as substrates.

Nucleosome Preparation and ChIP

Mono- and di-nucleosomes were isolated by micrococcal nuclease (MNase) digestion and sucrose gradient purification and subjected to ChIP analysis, as described previously (Jin and Felsenfeld 2006). The following primers were used: IAP (inhibitor of apoptosis), 5'-CCGCTGGAGTTCCTTAAG-3' (forward) and 5'-CGCACTCCTCCAGTGGTT-3' (reverse); MT1F (metallothionein-1F), 5'-AGCGCCGGCTGTTG-3' (forward) and 5'-ACTTTCCAAGAGAGAAGCGAAGAA-3' (reverse); S100A10, 5'-GCAGGGTCATCCAGCAAGTAA-3' (forward) and 5'-GCGCAGAACCAGAGAAGCGAAGAA-3' (reverse); GAPDH body, 5'-AGGCTGTGGGCAAGGTCAT-3' (forward) and 5'-CAGGTCCACCACTGACACGTT-3' (reverse); SAT2 (spermidine/spermine N1-acetyltransferase family member 2), 5'-TGAATGGAATCGTCATCGAA-3' (forward) and 5'-CCATTCGATAATCCGCTTG-3' (reverse); TM4SF1 (transmembrane 4 L six family member 1) TTS, 5'-AAGACAGGAAGCCGTTAGCA-3' (forward) and 5'-

GGCAGGAGGACCACGAGGAA-3' (reverse); GADD45, 5'-GCCCTTGTCTTTCCACCAATAG-3' (forward) and 5'-CGGCACCGACTTTTATAGACTTG-3' (reverse).

MNase Digestion Assay

Nuclei were isolated from acetaldehyde-treated and control BEAS-2B cells as described (Jin and Felsenfeld 2007). Briefly, the nucleus suspension was adjusted to an A_{260} of 100. MNase was added (2, 6, or 12×10^{-3} units/ μ l, final concentration) and the suspension was incubated for 8 min at 37 °C. The reaction was stopped by adding EDTA to a final concentration 10 mM. Proteinase K (1%, v/v) and SDS (1%, w/v) were added and the mixture was incubated overnight at 37 °C. The DNA was purified by phenol/chloroform extraction and ethanol precipitation. Three μ g of purified DNA was separated on a 2% agarose gel and visualized by ethidium bromide staining. The monomeric DNA band was excised and the DNA was extracted for quantitative real-time PCR (qPCR) analysis.

Determination of Nucleosome Occupancy

DNA fragments from MNase-digested nucleosomes (see above) were purified by phenol/chloroform extraction and ethanol precipitation, and quantified using the PicoGreen fluorescence kit (Molecular Probes, Eugene, OR). The same amount of DNA in each sample was used in qPCR to determine the differences in the abundance of specific target DNA sequences obtained from cells treated with or without acetaldehyde. If a genomic locus is occupied by nucleosomes in untreated cells, the corresponding DNA fragment will be protected from the MNase treatment and can easily be amplified by qPCR. However, if nucleosome occupancy of the region is reduced following acetaldehyde exposure, the DNA fragment is largely removed by MNase digestion and cannot be properly amplified. Accordingly, the ratio of the concentration of a target sequence protected from the MNase digestion in acetaldehyde-treated cells versus that in untreated cells will be smaller than 1.

Quantitative Real-Time PCR

Quantitative real-time PCR analysis was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on the ABI PRISM 7900HT system. All PCRs were performed in triplicates. Relative gene expression levels were normalized to Tubulin expression. The results were presented as –fold change to the level expressed in control cells. The following primers were used: EGR-2 (early growth response-2) pro, 5'-CAGCGACGTCACGGGTATT-3' (forward) and 5'-CGCCGAGCTATTAATCAATTGC-3' (reverse); EGR2-dEnh, 5'-CATTTGCATAATTCGGCTTTCC-3' (forward) and 5'-CCCCGCGCTGACCAT-3' (reverse); Chr1, 5'-TCATTCCCACAACTGCGTTG-3' (forward) and 5'-TCCAACGAAGGCCACAAGA-3' (reverse); Chr4, 5'-CTGCACTACCTGAAGAGGAC-3' (forward) and 5'-GATGGTTCAACACTCTTACA-3' (reverse); EGR2-pEnh, 5'-TCCGTGAATGCATGTAGCTTTT-3' (forward) and 5'-CTCACTTTCTTGGCAGCTCAAA-3' (reverse); H3.3B, 5'-ACGAAAGCCGCCAGGAA-3' (forward) and 5'-CTGTAGCGATGAGGCTTCTTCA-3' (reverse); D4Z4, 5'-CTCAGCGAGGAAGAATACCG-3' (forward) and 5'-ACCGGGCCTAGACCTAGAAG-3' (reverse), NBL2, 5'-

TCCCACAGCAGTTGGTGTTA-3' (forward) and 5'-TTGGCAGAAACCTCTTTGCT-3' (reverse). The other primers used are the same as listed for the ChIP assay.

Statistical Analysis

Gel intensities were quantified using ImageJ image processing software (National Institutes of Health, Rockville, MD). Relevant results are represented as mean $\pm$ S.D. (error bars). Significance was assessed by Student's *t* test. $p < 0.05$ was considered statistically significant. The number of times each experiment was repeated is indicated in the figure legends.

Results

Cytotoxicity of Acetaldehyde to human lung bronchial epithelial BEAS-2B cells

In an effort to establish an appropriate experimental condition for acetaldehyde exposure in our study, immortalized human bronchial epithelial BEAS-2B cells were treated with 10 mM of acetaldehyde at different time intervals for up to 12 hours. Subsequently, the cytotoxicity of acetaldehyde was determined by conventional trypan blue dye exclusion assays. About 50% of cells died 12 hours after the treatment, while the majority of cells (>90%) were still alive 6 hours after acetaldehyde exposure (Fig. 1A). Furthermore, we carried out cell death analysis by flow cytometry. Propidium iodide (PI) staining shows no DNA fragmentation, a key feature of cell apoptosis, when cells were treated with 10 mM acetaldehyde for 6 hours (Fig. 1B). In *in vitro* carcinogenesis studies, sub-cytotoxic doses (>80% cell viability) are considered suitable for yielding meaningful outcomes (Mathijs et al. 2010; van Kesteren et al. 2011). Based on these results, we determined that exposure to 10 mM of acetaldehyde for 6 hours would be the appropriate condition used in this study.

Acetaldehyde forms adducts with histone proteins in BEAS-2B cells

Acetaldehyde has been shown to form adducts with free histone H1 *in vitro* (Niemela et al. 1990). However, it is not known whether acetaldehyde reacts with histone proteins in cells. We used protein carbonyl assays to investigate whether acetaldehyde forms adduct with histone proteins in BEAS-2B cells. The reaction between acetaldehyde and protein nucleophiles forms carbonyl-retaining adducts, which can be detected using Western blot analysis. Total histones were prepared by acid extraction from acetaldehyde-treated and untreated BEAS-2B cells. We next incubated the total histones with dinitrophenyl hydrazine solution (0.5% 2, 4-dinitrophenyl hydrazine (w/v) in 10% trifluoroacetic acid (v/v)). The carbonylated proteins were then detected by Western blot analysis using antibodies against dinitrophenyl. Acetaldehyde reacts with proteins with molecular weight of approximately 17 kDa, which roughly coincides with the size of core histones H3, H2B, H2A, and H4 (Fig. 1C, top). The protein band overlaps with the H3 band when the same membrane was re-probed with antibodies against histone H3 (Fig. 1C, bottom). These results suggest that acetaldehyde forms adducts with histone H3 and probably other histones in BEAS-2B cells as well.

Acetaldehyde Downregulates Levels of N-terminal Tail Acetylations of Cytosolic Histones H3 and H4

It has been shown that acrolein- or formaldehyde-modified histones are resistant to PTMs *in vitro* (Lu et al. 2008), suggesting that reaction of histone residues with other types of aldehyde might have similar impacts on PTMs. Thus, we hypothesized that the formation of acetaldehyde-histone adducts could inhibit proper PTMs of histone proteins. In order to test this hypothesis, we isolated total histones from acetaldehyde-treated and untreated BEAS-2B cells and measured global levels of a number of different types of histone PTMs by Western blot analysis. Interestingly, while the levels of histone lysine methylation, such as H3K4me3, H3K9me2, and H3K27me3, are upregulated after acetaldehyde exposure, the amount of histone lysine acetylation, such as H3K9&K14ac, H3K18ac, and H4K12ac, are decreased following exposure to 10 mM of acetaldehyde (Fig. 2).

We previously reported that acrolein, an unsaturated aldehyde, preferentially reacts with unmodified newly synthesized free histones and dramatically decreases the acetylation of N-terminal tail lysine residues of cytosolic histones (Chen et al. 2013). In light of these findings, we focused our study on how acetaldehyde regulates histone lysine acetylation in different cell fractions. We isolated cytosolic, nuclear extract, and soluble chromatin fractions from BEAS-2B cells treated with or without acetaldehyde and subjected them to Western blot analysis. As shown in Figure 3A, a drastic decrease in H3K9&14ac and H4K12ac levels is observed in the cytosolic fraction of cells treated with 10 mM of acetaldehyde. Exposure to acetaldehyde also results in the downregulation of H3K9&14ac and H4K12ac in the chromatin fraction (nucleosomal histones).

Interestingly, the levels of H3K9&14ac and H4K12ac remain unchanged or slightly increased in the nuclear extract, which contains free nuclear histones (Fig. 3A). Downregulation of cytosolic H3K9&14ac and H4K12ac is also observed in the human lung adenocarcinoma cell line A549, suggesting a common effect across different cell types (Fig. 3B). Since acetaldehyde is the primary by-product of ADH-mediated alcohol metabolism in liver, we next measured the level of H4K12 acetylation in hepatocellular carcinoma HepG2 cells. As shown in Figure 3C, H4K12 acetylation is decreased in HepG2 cells after exposure to 15 mM of acetaldehyde for 6 hours, implicating a possible role of deregulation of cytosolic histone PTMs in alcohol-related liver diseases.

Acetaldehyde Downregulates Expression of Histone Acetyltransferases (HATs)

In addition to the formation of acetaldehyde-histone lysine adducts, changes in expression and/or activity of histone modifying enzymes may contribute to acetaldehyde-induced reduction in histone modifications. It is known that GCN5, a HAT, can acetylate histone H3 at K9 and K14 (Nagy and Tora 2007; Jin et al. 2011), whereas type-B HATs such as HAT1 transfer acetyl groups specifically to free histone H4 at K5 and K12 (Verreault et al. 1998). Therefore, we examined how protein levels of GCN5 and HAT1 are altered following acetaldehyde exposure. We isolated cell lysate from acetaldehyde-treated and untreated BEAS-2B cells and measured the level of GCN5 and HAT1 using Western blot analysis. Figure 4 shows that the level of both GCN5 and HAT1 is reduced following 10 mM acetaldehyde exposure in BEAS-2B cells. These results suggest that the observed reduction

in histone acetylation following acetaldehyde exposure is probably due to the direct reaction between acetaldehyde and lysine residues in histones H3 and H4 and/or the downregulation of the expression of histone modifying enzyme(s).

Acetaldehyde Exposure May Compromise Chromatin Assembly

Newly synthesized histone H4 is acetylated at K5 and K12. H4K5&12ac regulates nucleosome assembly at least in part by modulating histone nuclear import, which is influenced by the interaction between histone and translocator proteins. The acetylation of N-terminal tail of H3 is also considered to play a role in nucleosome assembly by regulating the interactions between histones and histone chaperones (Ejlassi-Lassalette et al. 2011; Ejlassi-Lassalette and Thiriet 2012; Zhang et al. 2012). Thus, the reduction in cytosolic H3K9ac, H3K14ac, and H4K12ac by acetaldehyde treatment may have an impact on histone nuclear import and assembly into the chromatin. If histone nucleosome assembly were inhibited by acetaldehyde exposure, we would expect to see a reduction in the amount of nucleosomal histones following acetaldehyde treatment. In agreement with this hypothesis, the level of histones H3 and H4 in the chromatin fractions was downregulated in BEAS-2B cells exposed to acetaldehyde (compare lanes 1 and 3, bottom panel in Fig. 3A; Fig. 5A). To further test this hypothesis, we next measured the level of histone H4 marked with lysine 12 acetylation at several genomic loci before and after acetaldehyde treatment. Results from ChIP assays show that the level of histone H4 marked with lysine 12 acetylation is significantly reduced following acetaldehyde exposure at the majority of loci tested, especially in the promoters of *MT1F*, *S100A10*, *GAPDH*, *SAT2*, and *TM4SF1* genes (Fig. 5B). These data suggest that nucleosome assembly may be compromised by acetaldehyde exposure.

Chromatin accessibility and Nucleosome Occupancy Are Changed by Exposure to Acetaldehyde

Defective nucleosome assembly could result in changes in chromatin structure. We used MNase digestion assays to examine the influence of acetaldehyde exposure on chromatin accessibility. Nuclei isolated from BEAS-2B cells treated with or without 10 mM acetaldehyde were digested with MNase. We then extracted DNAs and monitored changes of protective nucleosome ladders by agarose gel electrophoresis. Acetaldehyde-treated cells showed a loss of bands indicative of polynucleosomes (larger than 5-mers), suggesting that acetaldehyde exposure can lead to overall increases in MNase sensitivity (compare lanes 1 and 4, Fig. 6A). Next, we utilized MNase-qPCR to examine how nucleosome occupancy at genomic loci changes after acetaldehyde treatment. We determined the changes in nucleosome occupancy by measuring the ratio of the abundance of target DNA sequences in MNase-digested mononucleosomes isolated from acetaldehyde-treated versus untreated cells (Jin and Felsenfeld 2006). As shown in Figure 6B, nucleosome occupancy is diminished in the majority of the sites tested in response to acetaldehyde exposure. The reduction is most apparent at *S100A10*, *MT1F*, *GAPDH*, *Chr 4*, and *NBL2*. Together, these data indicate that acetaldehyde exposure can increase chromatin accessibility.

Discussion

In the present study, we demonstrate that acetaldehyde exposure reduces acetylation of lysine residues at the N-terminus of cytosolic histones H3 and H4 and may inhibit chromatin assembly. Defective chromatin assembly is associated with dysregulation of transcription, DNA replication and repair, and causes genomic instability (Ye and Adams 2003; Ye et al. 2003; Ransom et al. 2010; Corpet et al. 2011; Burgess and Zhang 2013; Gurard-Levin et al. 2014). Thus, the ability of acetaldehyde to compromise chromatin assembly by modulating histone acetylation may represent a novel mechanism for toxic effects of acetaldehyde.

It has been reported that acetaldehyde forms adducts with lysine residues of free histone H1 (Niemela et al. 1990). Note that the ϵ -amino group of lysine residues in the histone tails is the primary target of acetaldehyde (Worrall and Thiele 2001). Protein carbonylation assays demonstrate the formation of acetaldehyde-histone adducts in cells as well (Fig. 1C). Consequences of the formation of acetaldehyde-histone adducts have remained elusive. Our previous studies showed that acrolein, an aldehyde, forms adducts with lysine residues and in turn downregulates acetylation at the N-terminal tail of newly-synthesized cytosolic histones (Chen et al. 2013). Lu *et al.* demonstrated that formaldehyde, the simplest of aldehydes, reacts with lysine residues of unmodified histone H4 and prevents those sites from being properly modified *in vitro* (Lu et al. 2008). In agreement with these observations, the acetylation of H3K9, H3K14, and H4K12 were decreased in BEAS-2B cells following acetaldehyde exposure (Fig. 2). The most obvious effect of acetaldehyde on histone acetylation was found in cytosolic fractions (Fig. 3). The illustrated decrease in histone acetylation level could result from aldehyde-histone adduct formation or reduced expression and/or activity of histone acetyltransferases following aldehyde exposure. The activity and expression of HAT1, a histone acetyltransferase specific for H4K12 acetylation, were not changed by acrolein treatment (Chen et al. 2013; Fang et al. 2016). Thus, it seems fair to assume that acrolein-induced reduction of histone acetylation is triggered by the formation of acrolein histone lysine adducts. Unlike acrolein, acetaldehyde exposure downregulated both GCN5 and HAT1 expression (Fig. 4), suggesting that reduction of histone modifying enzymes may have directly contributed to the observed decrease in histone acetylations by acetaldehyde. This could also explain the apparent decrease in acetylation of total histones by acetaldehyde, whereas no such alterations were detected following acrolein exposure (Chen et al. 2013). In future studies, it would be important to characterize the extent in which histone acetylation is downregulated in response to the formation of acetaldehyde-histone adducts or the reduction in expression of histone acetyltransferases. Moreover, the global levels of histone lysine methylations, such as H3K4me3, H3K9me2, and H3K27me3, were all increased in BEAS-2B cells treated with acetaldehyde. Of note, exposure to acrolein did not yield similar results. It would be interesting to elucidate mechanisms that underlie acetaldehyde-mediated upregulation of histone methylations.

Appropriate acetylation of N-terminal tails of newly synthesized histones H3 and H4 is considered critical for histone nuclear import and nucleosome assembly (Burgess and Zhang 2013; Fang et al. 2014). Histone modifications affect chromatin assembly in various ways, including the regulation of histone folding and processing, histone nuclear import, and the interaction between histones and histone chaperones (Adkins et al. 2004; Groth et al. 2005;

Jin et al. 2006; Zhang et al. 2012). H4K5&K12Ac, a di-acetylation catalyzed by HAT1, is detected in newly synthesized histone H4 from yeast to human as an early modification occurring in H3-H4 (Ejlassi-Lassalette et al. 2011; Nagarajan et al. 2013). The H4K5&K12 double-mutants are imported less efficiently into the nucleus compared to wild-type histones (Ejlassi-Lassalette et al. 2011). These results indicate that H4K5&K12 might regulate chromatin assembly pathways through regulating H3-H4 nuclear import. Other histone acetylations such as H4K79ac and H4K91ac (Yang et al. 2011), H3K9ac and H3K27ac (Burgess et al. 2010), as well as H3K56ac (Driscoll et al. 2007; Das et al. 2009), are also reported to play important roles in chromatin assembly, probably through regulating the interaction between histones and histone chaperones. We have shown that acrolein can inhibit acetylation of N-terminal tails of cytosolic histones H3 and H4 and compromise chromatin assembly pathways. Reduction in cytosolic H3K9ac, H3K14ac, and H4K12ac levels suggests that acetaldehyde may also have an impact on chromatin assembly. In fact, cell fractionation followed by Western blot analysis as well as ChIP assays demonstrated that the general level of nucleosomal H4 and that of H4 marked with H4K12ac at several genomic loci we tested are both decreased by acetaldehyde exposure, which is suggestive of inhibition of chromatin assembly. Our findings are likely to be applicable to other aldehydes and electrophiles in consideration to similarities in chemical properties. Thus, reaction with nucleophilic side chains of lysines in histone proteins and subsequent disruption of proper chromatin assembly may represent a general mechanism by which electrophiles influence the biological functions of the genome. In addition to chromatin assembly regulation, acetaldehyde exposure may have broader and more complicated impact than acrolein on the chromatin landscape. In contrast to acrolein, which cannot change the expression and activity of histone acetyltransferase, acetaldehyde downregulates the expression of histone acetyltransferases. Thus, it is very likely that acetaldehyde exposure perturbs the epigenetic profiles of chromatin by directly targeting histone-modifying enzymes through mechanisms not yet characterized.

A number of studies have demonstrated that acetaldehyde is linked to inhalation toxicity, self-administration, addiction of tobacco and alcohol, among others (Oyama et al. 2007; Talhout et al. 2007; Cacace et al. 2012). Thus, in addition to its role in cancer pathogenesis, it would also be interesting to examine the function of defective chromatin assembly in the above-mentioned processes in relation to acetaldehyde exposure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Cytotoxicity in BEAS-2B cells after acetaldehyde treatment

(A) Cells were exposed to acetaldehyde (10 mM) for 3, 6, 9 and 12 h. Cell viability was determined by trypan blue dye exclusion assays. Viability is represented as the percentage of live cells at each time point. Data represent the mean $\pm$ S.D. (error bars) of triplicate tests. (B) BEAS-2B cells were treated with or without acetaldehyde (10 mM) for 3, 6, 9 and 12 h. Treated cells were trypsinized and collected for flow cytometry analysis to monitor apoptosis. Apoptotic cells appeared only in 12-h acetaldehyde-treated cells. (C) Acid-extracted histones were subjected to carbonyl assays. Carbonylated proteins were detected by Western blot analysis with anti-DNP antibodies (top). The same membrane was re-probed with anti-H3 antibody (bottom). AA, acetaldehyde.

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Fig. 2. Acetaldehyde changes histone modification levels
BEAS-2B cells were exposed to various concentrations of acetaldehyde for 6 h. Total histones were prepared by acid extraction and subjected to Western blot analysis with antibodies against indicated histone modifications. Full Western blot images are presented in Supplemental Figure 1.

Fig. 3. Acetaldehyde inhibits N-terminal tail acetylation of newly synthesized histones
(A) Cytosolic cell fractions, nuclear extracts, and soluble chromatin fractions were isolated from BEAS-2B cells treated with or without acetaldehyde for 6 h and then subjected to Western blot analysis with indicated antibodies. (B and C) Cytosolic cell fractions were prepared from A549 (B) or HepG2 (C) cells after acetaldehyde exposure and then subjected to Western blot analysis with antibody for H4K12ac.

Fig. 4. Acetaldehyde downregulates expression of histone acetyltransferases (HATs)

Whole cell lysates were prepared from acetaldehyde-treated and control cells and subjected to Western blot analysis with antibodies against GCN5 and HAT1.

Fig. 5. Acetaldehyde exposure may compromise chromatin assembly

(A) Reduced H4 levels in chromatin fraction after acetaldehyde exposure. The quantification of Western blot (Fig. 3A) by ImageJ software is shown. The data are shown as mean $\pm$ S.D. (error bars) of three independent experiments. (*, $p < 0.05$; **, $p < 0.01$ when compared with the untreated control). (B) BEAS-2B cells were treated with or without 10 mM acetaldehyde for 6 h. Mono- and dinucleosomes were prepared by MNase digestion and subjected to ChIP assays with antibodies specific for H4K12ac. Representative data from two independent experiments are shown. The data shown are the mean $\pm$ S.D. (error bars) from qPCRs performed in duplicates or triplicates. *, $p < 0.05$; **, $p < 0.01$.

Fig. 6. Acetaldehyde exposure changes chromatin accessibility and nucleosome occupancy
 (A) Acetaldehyde exposure results in the increase in chromatin accessibility. Nuclei were isolated from acetaldehyde-treated and -untreated BEAS-2B cells and digested with different concentrations of MNase. Digested DNA was extracted and separated in a 2% agarose gel and visualized by ethidium bromide staining. Representative results from two independent experiments are shown. (B) Acetaldehyde exposure reduces nucleosome occupancy. MNase-digested monomeric DNA bands from acetaldehyde-treated and -untreated cells (Fig. 6A, lanes 3 and 6) were excised, and DNA was extracted. The abundance of a sequence in protected nucleosomes from acetaldehyde-treated cells relative to that from the control cells was determined by qPCR analysis. Representative data from three independent experiments are shown. The data shown are the mean $\pm$ S.D. (error bars) from qPCRs performed in duplicates or triplicates. *, $p < 0.05$; **, $p < 0.01$. The raw Ct values are shown in Supplemental Table 1.