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Cigarette smoke induces distinct chromatin histone modifications in lung cells: implication in pathogenesis of COPD and lung cancer

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Abstract

Cigarette smoke (CS)-mediated oxidative stress induces several signaling cascades, including kinases, which results in chromatin modifications (histone acetylation/deacetylation and histone methylation/demethylation). We have previously reported that CS induces chromatin remodeling in pro-inflammatory gene promoters; however, the underlying site-specific histone marks formed in histones H3 and H4 during CS exposure in lungs *in vivo* and in lung cells *in vitro*, which can either drive gene expression or repression are not known. We hypothesize that CS exposure in mouse and human bronchial epithelial cells (H292) can cause site-specific posttranslational histone modifications (PTMs) that may play an important role in the pathogenesis of CS-induced chronic lung diseases. We used a bottom-up mass spectrometry approach to identify some potentially novel histone marks, including acetylation, mono-methylation and di-methylation in specific lysine and arginine residues of histones H3 and H4 in mouse lungs and H292 cells. We found that CS-induced distinct posttranslational histone modification patterns in histone H3 and histone H4 in lung cells, which may be considered as usable biomarkers for CS-induced chronic lung diseases. These identified histone marks (histone H3 and histone H4) may play an important role in epigenetic state during the pathogenesis of smoking-induced chronic lung diseases, such as chronic obstructive pulmonary disease and lung cancer.

Keywords

Oxidants; chromatin; lung; acetylation; methylation; chronic obstructive pulmonary disease; mass spectrometry; lung cancer

INTRODUCTION

Cigarette smoke (CS) exposure causes oxidative stress and triggers inflammatory-immune response. This affects the host's ability to escalate appropriate immune and inflammatory responses leading to smoking-induced chronic lung diseases, including cancer^{1, 2}. Chronic

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AUTHOR CONTRIBUTIONS

IKS, IR: Conceived and designed the experiments; IKS, MZN, AEF: Performed the experiments; IKS, MZN, AEF: Analyzed the data; and IKS: Wrote the manuscript and IR, AEF Edited the manuscript.

inflammation, premature lung aging (cellular senescence), DNA damage/repair and steroid resistance are some of the key contributing factors in the pathogenesis of chronic obstructive pulmonary disease (COPD). CS is the most common etiological factor in the pathogenesis of COPD, which is characterized by irreversible airway obstruction with an increased resistance to breathing and by marked lung functional impairment (reduced lung function) associated with loss of lung tissue (emphysema), inflammatory small airway thickening (bronchiolitis), and excessive mucus production (bronchitis)^{2, 3}. Although several studies have shown the epigenetic events, such as histone acetylation and histone deacetylation, contributing to CS-induced lung inflammatory response using *in vitro* and *in vivo* models^{4–11}, the exact role of histone modifications in chromatin remodeling of chronic lung diseases remains unclear^{12, 13}. Posttranslational histone modification is one of the key processes that remain poorly understood, and it may have significant impact in the development of novel epigenetic-based therapeutic strategies for the treatment/management of smoking-induced chronic lung diseases including COPD and cancer.

In eukaryotes, DNA is tightly packed with histones known as chromatin. Nucleosomes form the basic structural unit of chromatin comprised of DNA wrapped around the octamer which is formed by two copies of each histone (H2A, H2B, H3 and H4)¹⁴. The amino acids that most commonly undergo posttranslational modifications are the basic lysine (K) and arginine (R) residues of histone tails, which either causes activation (active) or repression (inactive) of gene expression¹⁵. Core histones and their posttranslationally modified variants play a vital role in the nuclear scaffolding that controls the interaction of DNA and other transcription factors, including RNA polymerase, to modulate gene expression¹⁴. These changes in epigenetic marks of histone tails are regulated by histone modification enzymes, such as histone acetyltransferases (HATs)/histone deacetylases (HDACs) and histone methyltransferases (HMTs)/histone demethylases (HDMs)^{15–20}. We hypothesize that cigarette smoke causes distinct and differential posttranslational histone modifications both *in vivo* (mouse lung) and *in vitro* (H292: human bronchial epithelial cells) that can be identified using a bottom-up mass spectrometry approach. Increased acetylation of histones H3 and H4 has been directly correlated with regulation of proinflammatory gene expression both *in vitro* and *in vivo*^{21–23}. Earlier reports have demonstrated that cigarette smoke causes hyperacetylation of histone and decreased histone deacetylase activity in lungs of smokers²⁴, and lungs of patients with COPD²⁵, as well as in rodent lung exposed to CS²⁶ and human alveolar epithelial cells²⁷. This results in a heightened inflammatory response due to CS-mediated chromatin modifications. Recent studies have demonstrated the critical role of histone modifications in the development of fibroblast resistance to apoptosis using both mouse model and human patients with pulmonary fibrosis²⁸. Furthermore, chromatin forms the extracellular trap protein components in neutrophils, particularly histones, which cause host cell cytotoxicity leading to the destruction of lung tissue²⁹. It has been shown that circulating histones are mediators of trauma-associated lung injury³⁰ and are involved in apoptosis in lung cells³¹. Due to lack of evidence on the role of histone modifications in the pathogenesis of chronic lung diseases¹³, there is a need to study site-specific histone modifications using the rapidly growing mass spectrometry (MS) field. We report that CS induces distinct posttranslational histone modification patterns both *in vitro* and *in vivo* (histones H3 and H4). Identified posttranslational histone modifications in air versus CS-exposed C57BL/6J mouse lung (3 days), and control versus cigarette smoke extract (CSE)-treated human bronchial epithelial cells (H292) may be considered as potential epigenetic-based biomarkers for CS-induced chronic lung diseases and chronic CS exposure animal model studies. Our data reveals that identification of distinct histone marks (histones H3 and H4) plays an important role in understanding the epigenetic state during the pathogenesis of smoking-induced chronic lung diseases.

MATERIALS AND METHODS

Ethics statement

All experiments for animal studies were performed in accordance with the standards established by the United States Animal Welfare Act, as set forth by the National Institutes of Health guidelines. The research protocol for mouse studies was approved by the University Committee on Animal Research Committee of the University of Rochester.

Materials

Unless otherwise stated, all biochemical reagents used in this study were purchased from Sigma Chemicals (St. Louis, MO, USA). Penicillin-streptomycin, L-glutamine and RPMI-1640 were obtained from Gibco BRL (Grand Island, NY). Fetal bovine serum (FBS) was obtained from HyClone Laboratories (Logan, UT).

Mouse cigarette smoke exposure

C57BL/6J (Jackson Laboratory, Bar Harbor, ME, USA) were bred and maintained under pathogen-free conditions with a 12 h light/dark cycle in the vivarium facility of the University of Rochester. Adult C57BL/6J mice were exposed to CS using research grade cigarettes (3R4F) according to the Federal Trade Commission protocol (1 puff/min of 2 sec duration and 35 mL volume) using a Baumgartner-Jaeger CSM2072i automatic CS generating machine (CH Technologies, Westwood, NJ)^{4, 8, 9, 32}. Mainstream CS was diluted with filtered air and directed into the exposure chamber. The smoke exposure [total particulate matter (TPM) in per cubic meter of air] was monitored in real-time with a MicroDust Pro-aerosol monitor (Casella CEL, Bedford, UK) and verified daily by gravimetric sampling. The smoke concentration was set at a value of ~300 mg/m³ TPM by adjusting the flow rate of the diluted medical air and the level of carbon monoxide in the chamber was 350 ppm^{4, 9}. Mice (n = 4 per group) received two one h exposures (one h apart) daily for three consecutive days, and were sacrificed at 24 h post-final exposure. Control mice were exposed to filtered air in an identical chamber according to the same protocol as described for CS exposure. Mice were anesthetized by an intraperitoneal injection of pentobarbital sodium (100 mg/kg; Abbott Laboratories, Abbott Park, IL), and then sacrificed by exsanguination 24 h after last exposure. The lungs were removed *en bloc* and frozen in -80°C for nuclear extraction followed by acid extraction of histones.

Cell culture

Human bronchial epithelial cells (H292) derived from human lung mucoepidermoid carcinoma were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). H292 cells were grown in RPMI-1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. The cells were cultured at 37°C in a humidified atmosphere containing 7.5% CO₂.

Preparation of cigarette smoke extract

Research grade cigarettes 3R4F were obtained from the Kentucky Tobacco Research and Development Center at the University of Kentucky (Lexington, KY). Ten percent CSE was prepared by bubbling smoke from one 3R4F research-grade cigarette into 10 mL culture medium at a rate of one cigarette per min, as described previously^{9, 33}, using a modification of the method as described³⁴. The CSE was adjusted to pH 7.4 and then sterile-filtered through a 0.45 µm filter (25-mm Acrodisc; Pall Corporation, Ann Arbor, MI, USA). CSE preparation was standardized by measuring the absorbance at 320 nm (OD=1.00 ± 0.05). The spectral variations observed between different CSE preparations at 320 nm were minimal.

Cell treatments

Human bronchial epithelial cells (H292) (4×10^6) were grown in 100 mm dishes to ~80–90% confluency in respective cell culture medium with 0.5% FBS. The cells were treated with CSE (1%) for 1 h at 37°C with 7.5% CO₂. At the end of treatment, the cells were washed with cold sterile Ca²⁺/Mg²⁺-free PBS and harvested cell pellets were stored in –80°C for nuclear extraction followed by acid extraction of histones.

Nuclear protein isolation and acid extraction of histone proteins

For nuclear extracts, H292 cells/lung tissues were lysed/homogenized in buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF). After 15 min, Nonidet P-40 was added to a final concentration of 0.6% and vortexed for 15 sec. Samples were centrifuged for collection of the supernatants containing cytosolic proteins. The nuclear pellets were re-suspended in buffer B (20 mM HEPES [pH 7.9], 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF). After 30 min at 4°C, lysates were centrifuged and supernatants containing the nuclear proteins were stored at –80°C. The final pellet obtained after nuclear extraction was used for preparation of histones by acid extraction. To the nuclear pellet, 150 µl acid extraction buffer containing 0.2 N HCl and 0.36 N H₂SO₄ was added. The mixture was rotated on a rocker at 4°C for 6–18 hrs. The pellet was sonicated for 2 sec on ice and centrifuged at 14,000 g for 10 min at 4°C. The supernatant containing acid extracted histones was then transferred to a fresh tube and mixed with 1.1 mL ice-cold acetone to precipitate histones. The tubes were incubated at –20°C overnight, and then centrifuged at 14,000 g for 10 min at 4°C. The pellet was then washed with ice-cold acetone and centrifuged to remove the acid. The pellet containing the isolate was dried in ambient air and dissolved in sterile distilled water for protein quantification using a bicinchoninic acid (BCA) kit as per the manufacturer's protocol (Pierce, Rockford, IL, USA). Isolated histones were stored at –80°C until SDS-PAGE analysis.

Trypsin digestion

Samples analyzed by SDS-PAGE, followed by band excision, were digested with trypsin overnight³⁵. Peptides were twice extracted with 50% acetonitrile containing 5% trifluoroacetic acid.

LC/MS/MS analysis

For LTQ analysis, 2 µL of each sample was loaded onto a home-pulled, home-packed C₁₈ analytical column. The tip was pulled to ~10 µm with a Sutter Laser puller. Columns were packed to 10 cm with C₁₈ AQ 5 µm 200 Å media (Michrom) using a pressure bomb. The internal diameter of the columns used was 75 µm. Prior to loading samples, the columns were equilibrated to initial run conditions. Peptides were eluted with the following chromatographic profile: 5% B for 6 min, ramping to 20% B over one minute then to 60% B over 113 min, washing at 95% B for 3 min, and finally returning to initial run conditions, with Solvent A as LC/MS grade water (Burdick & Jackson) + 0.1% formic acid (Pierce) and Solvent B as LC/MS grade methanol (Burdick & Jackson) + 0.1% formic acid. The flow rate was 350 nL/min. Instrument specific parameters included analysis by data-dependent MS/MS mode, where a survey scan was performed followed by MS/MS analysis of the top 7 analytes in each survey scan. Once fragmented, each analyte was placed on an exclusion list for 45 sec to avoid repetitive identifications. Helium was used as collision gas, with an activation Q of 0.25, activation time of 30 ms and normalized collision energy of 35%. Data were collected as raw files.

Data processing

LTQ files were converted from .raw files to .mgf files using BioWorks Browser (Thermo). The processed data were exported as an xml file. Resultant .mgf and .xml files were imported into ProteinScape (Bruker Daltonics) and searched via MASCOT (MatrixScience)³⁶. Search parameters included: trypsin as an enzyme; 9 missed cleavages; MS tolerance of 1.5 Da; MS/MS tolerances of 0.8 Da for LCQ data and 0.5 Da for MS and MS/MS data from the MicrO-TOF QII; one for #13C, +2; +3 for charge state; instrument set to ESI-TRAP or ESI-QUAD TOF appropriately; decoy search and acceptance criteria of minimum one peptide greater than identity score; minimum score of 15; and False Discovery Rate less than 5%. The high number of missed cleavages was chosen under the assumption that trypsin would not cut at a modified residue, as modified residues would not fit in the trypsin active site. Data were searched for variable modifications of oxidized methionine, carbamidomethyl cysteine, acetyl lysine, acetyl protein n-terminus, methyl- and dimethyllysine and arginine. The ProteinExtractor function of ProteinScape combined search results and compiled a non-redundant list of identifications. Matched spectra were manually validated using BioTools (Bruker Daltonics), with poor matches being excluded from the final results.

Model structure for identified histone H3 and H4

This model structure was constructed using the core histone crystal structure available in the protein data bank (<http://www.rcsb.org/>). The entire structure pdb file of core histones (1KX5) reported previously³⁷ was downloaded and chain B (histone H4) and chain C (histone H3) were extracted separately. The model was generated from 1KX5 using Pymol³⁸ for both human and mouse histone H3 and H4, as they have high sequence similarity. We have defined specific PTMs within the lateral chain of the amino acid residue (lysine and arginine) of histone H3 and H4 in CS/CSE treated samples compared against those identified in air/control with a particular color code, as described: Ac = acetylation (yellow spheres); me1 = mono-methylation (cyan spheres); me2 = di-methylation (olive spheres); ac + me2 = acetylations and di-methylations (red spheres); me1 + me2 = mono- and di-methylation (orange spheres); and ac + me1 + me2 = acetylation, mono- and di-methylation (grey spheres) (Fig 3).

Human histone database and PubMed search

The identified posttranslational histone modifications in this study from both mouse lung and human bronchial epithelial cells were searched using a database of human histones, their posttranslational modifications and modifying enzymes available online (<http://www.histome.net>)³⁹. In brief, the HIstome database consists of information gathered from PubMed-listed literature and the publicly available UniprotKB/Swiss-Prot database⁴⁰ on marked sites for human histone modifications and histone modifying enzymes. We performed a database search analysis using the HIstome database combined with a PubMed search for the posttranslational histone modifications identified in this study.

RESULTS

CS induced distinct posttranslational histone modifications in mouse lung *in vivo*

We have previously reported that cigarette smoke caused posttranslational histone modifications both *in vitro* using human bronchial epithelial cells and *in vivo* mouse lung exposed to CS^{4-9, 41}. Posttranslational histone modifications have been implicated in the pathogenesis of COPD that is associated with chronic lung inflammation, imbalance in antioxidant defense, DNA damage/repair, epigenomic stability, and cellular senescence¹². To date, there has not been a study that has identified the specific histone marks and their

role in CS-mediated lung diseases, particularly in response to environmental stimuli. We hypothesize that cigarette smoke induces chromatin modifications as a result of posttranslational histone modifications leading to chromatin remodeling in the lungs. C57BL/6J mice were exposed to CS for 3 days (acute), and the lung tissues were harvested 24 hrs after the last exposure. Large left lobe of the lungs was used for nuclear extract preparation followed by acid extraction of histones for SDS-PAGE analysis. Gels were staining with Coomassie Brilliant Blue to identify the histone bands and destained with standard destaining solution. Histone H3 and H4 bands identified from stained gels were excised for in gel trypsin digestion as described earlier⁴². Peptides were extracted twice with 50% acetonitrile containing 5% trifluoroacetic acid for LC/MS/MS analysis. The brief schematic used for identification and detection of posttranslational histone modifications by LC/MS/MS analysis is described in Scheme 1.

The sequence coverage observed for histone H3 and histone H4 in acute air and CS-exposed mouse lungs were 64% (air) and 63.9% (CS) and 81.6% (air) and 92% (CS), respectively (Fig. 1A–B). The majority of the posttranslational histone modifications identified in air and CS-exposed mouse lungs were found in the first 56 amino acids of histone H3 and in the first 77 amino acids of histone H4 N-terminal tails as summarized (Table 1). MS analysis detected acetylation of histone H3 (H3K23ac, H3K36ac, and H3K56ac) in air and CS-exposed mouse lungs. In addition, CS-exposed mouse lungs also showed H3K79ac along with the other histone modifications detected in the air exposed mouse lung. Furthermore, methylation (mono- and di-) of histone H3 were detected in air (H3K23me2, H3K36me2, H3R72me2, and H3K79me1/2) and CS-exposed (H3K27me2, H3K36me1/2, H3K56me2, and H3K79me1/2) mouse lungs. MS analysis for acetylation of histone H4 detected specific residues in air (H4K16ac and H4K31ac) and CS-exposed (H4K12ac and H4K31ac) mouse lungs. We found more number of mono-methylation of histone H4 (H4K20me1, H4R23me1, H4R35me1, H4R55me1 and H4R77me1) residues in air-exposed mouse lungs. CS-exposed mouse lung showed increase in both mono-and di-methylation of histone H4 residues (H4K20me1/2, H4R23me1, H4K31me2, H4R35me1/2, H4R36me1, H4R55me1 and H4K77me1).

CS-induced distinct posttranslational histone modifications in human bronchial epithelial cells *in vitro*

H292 cells were treated with and without CSE (1% and 2% CSE) for one hr, and the cells were harvested, washed with sterile ice-cold 1X PBS, and nuclear extracts were prepared followed by acid extraction of histone for SDS-PAGE analysis. The gels were stained with Coomassie Brilliant Blue in order to identify the histone bands and destained with standard destaining solution. Histone H3 and H4 bands identified from stained gels were excised for in-gel trypsin digestion, as described previously⁴². Peptides were then extracted twice with 50% acetonitrile containing 5% trifluoroacetic acid for LC/MS/MS analysis.

The sequence coverage observed for histone H3 and histone H4 in control and one hr CSE-treated (1% or 2%) H292 cells were 63% (control), 67.4% (CSE), 90.3% (control) and 85.4% (CSE), respectively (Fig. 2A–B). The majority of the posttranslational histone modifications identified in control and CSE-treated H292 cells were found in the first 79 amino acids of histone H3 and in the first 35 amino acids of histone H4 N-terminal tails as summarized (Table 2). MS analysis detected acetylation of histone H3 in control (H3K14ac, H3K27ac, H3K56ac and H3K122ac) and in CSE-treated (H3K23ac, H3K56ac and H3K79ac) H292 cells. Furthermore, methylation (mono- and di-) of histone H3 were detected in control (H3K36me1/2, H3K37me2, H3K56me2, H3K79me1/2, H3K122me2 and H3R128me1) and CSE-treated (H3K27me1/2, H3K36me1/2, H3K37me2, H3K56me2, H3K79me1 and H3K122me2) H292 cells. MS analysis for acetylation of histone H4 detected specific residues in control (H4K12ac, H4K16ac and H4K31ac) and CSE-treated

(H4K8ac, H4K12ac, H4K16ac and H4K31ac) H292 cells. We found mono- and di-methylation of specific histone H4 residues in control (H4K31me2, H4R35me1/2) and CSE-treated (H4K16me2, H4K31me2, H4R35me1) H292 cells. These findings confirm that CS induces histone modifications in mouse lung *in vivo* and H292 cells *in vitro*.

Model structure for identified posttranslational histone modifications in H3 and H4

A cartoon summary of all the PTMs observed in this study for posttranslational histone H3 and H4 modifications is shown (Fig. 3A–B). Additionally, all of the modified peptides identified in acute 3 days of air and CS-exposed mouse lung, control and CSE-treated H292 cells are summarized (Tables 1 and 2). Fragmentation spectra of the identified peptides modified in air/CS and control/CSE-treated groups are summarized in Tables 3 and 4, and in online supplemental data files (Supplemental Figs. 1 and 2). Key and distinct histone H3 and H4 PTMs in mouse lungs and epithelial cells exposed to CSE/CS are tabulated in Table 5.

Histone modifications database and PubMed search

Based on the available resources from human histone database³⁹ and PubMed, we performed searches to identify some “Writer(s)” and “Eraser(s)” that may be responsible for causing the PTMs identified in this study. Histone PTMs were classified into eight different types depending on the type of amino acid and its modification (e.g. lysine acetylation and methylation, arginine methylation, serine and threonine phosphorylation, ADP ribosylation, proline isomerization, arginine deamination, ubiquitination and sumoylation)^{15, 43, 44}. Histone acetylation and methylation plays a crucial role during DNA damage response after double-strand break induction at the DNA damage foci^{45–49}. Histone H3K9 and H3K56 acetylation and H3K4 and H3K36 trimethylation have been linked with active gene transcription (euchromatin), whereas trimethylation of histone H3K9 and H3K27 are associated with silenced gene loci (heterochromatin) by closing the active chromatin^{45, 50–52}. In the present study, we focused mainly on histone H3 and H4 acetylation (Lysine: K) and mono- and di-methylation (Lysine: K and Arginine: R) caused by cigarette smoke exposure in cells and mouse lung to link CS-induced DNA damage response (impaired repair) that may contribute to the changes in regulation of gene expression due to site-specific histone modifications. The enzymes responsible for creating histone PTMs were termed as histone modifying enzymes⁵³. These histone modifying enzymes are broadly categorized into “Writer(s)” and “Eraser(s)”, based on the ability to catalyze either addition or removal of specific PTMs, respectively^{16, 20, 53}. A search in the database revealed some of the known histone marks reported earlier, along with their writers (histone acetyltransferases/ histone methyltransferases) and erasers (histone deacetylases/ histone demethylases). These results may be responsible for the changes caused in epigenetic histone marks identified in this study (Supplemental Tables 1 and 2).

Interestingly, we found histone acetylation at histone H3K14ac, may be caused by known histone acetyltransferases (HATs: MGEA5, CLOCK, GTF3C4, KAT2A, and MYST3) and for histone H3K27ac and H3K56ac, (HATs: CREBBP, and EP300). Based on the database search and recent literature from PubMed, we found that only a few specific known histone deacetylases (HDACs) were reported for deacetylation of histone H3K56ac (HDACs: HDAC1, SIRT2, SIRT3 and SIRT6). For the identified methylation mark at H3K27me1, potential histone methyltransferases (HMTs) reported includes: EZH1, EZH2, DOTL1, EHMT1 and EHMT2. Methylation at H3K27me2 was reported by EZH1 and WHSC1L1, and histone demethylation of the same residue by PHF8 and KDM6B. We found H3K27 methylation (me1/2) to be very unique in both CS-exposed mouse lung and CSE-treated H292 cells compared to air/control groups (Fig. 4; Table 5). Other histone modifications and their spectra in various groups are depicted in Supplemental Figs. 1 and 2. Methylation of H3K36me1, possibly due to ASH1L, and methylation of H3K36me2 were reported to be

modified by several HMTs (SETMAR, NSD1, SMYD2 and ASH1L) and demethylation by HDMs (KDM2A, KDM2B and JMJD5). However, DOT1L is the only histone methyltransferase reported to cause histone H3K79me1/2 in humans (Supplemental Table 1).

Similarly, we also performed searches from databases and recent literature from PubMed for histone H4 modifications to identify the known writer(s) and eraser(s) leading to specific PTMs. We found acetylation at H4K8 and H4K12 may be caused due to HATs (MGEA5, CREBBP, GTF3C4, KAT2A, KAT5, MYST2 and EP300) and (CREBBP, KAT2A, KAT5, MYST2, EP300 and HAT1), respectively. Histone H4K16ac was reported to be caused by HATs CREBBP, KAT2A, EP300, and MYST1, and deacetylation of the same residue was reportedly mediated by SIRT1 and SIRT2. Overall, there were only a few histone H4 methylations reported previously, including H4K20me1 and H4K20me2 mediated by HMTs [(SETD8, NSD2, and WHSC1) and (SETD8, SUV420H1, SUV420H2 and NSD1), respectively]. PHF8 is the only known histone demethylase that can demethylate histone H4K20me1 (Supplemental Table 2). Overall, it remains clear that only a few of the specific writer(s) and eraser(s) creating specific PTMs in histone H3 and H4 were known. Hence, future studies will be conducted to explore the dose response effects of CS exposure to identify if these posttranslational histone modifications are dose-dependent that may have greater impact in serving as biomarkers of CS-induced chronic lung diseases. We are currently using gene expression profiling analysis combined with proteomics approach to determine a possible causal relationship between site-specific histone modifications and associated histone modifying enzymes on the epigenetic regulation during CS exposure in lung cells *in vitro* and mouse model studies *in vivo*.

DISCUSSION

All histones are reported to undergo posttranslational modifications including acetylation, methylation, phosphorylation, ubiquitination, SUMOylation and ADP ribosylation, which occur mainly in the histone tails^{15, 43}. Histone modifications play an essential role in transcriptional regulation, DNA damage/repair, replication⁵⁴, alternative splicing⁵⁵, chromosome condensation¹⁵ and cellular senescence/aging^{56–58}. It is critical to understand the transcriptional state (active – euchromatin and inactive – heterochromatin) within the genome that regulates gene expression. For example, active gene expression is characterized by increased acetylation and tri-methylation of histones H3K4, H3K36 and H3K79 that were commonly referred to as euchromatic modifications. Similarly, gene repression is characterized by decrease in acetylation and increase in methylation of histones H3K9, H3K27 and H4K20, regarded as heterochromatic marks^{45, 50–52, 59, 60}. In this study, we determined the novel histone acetylation and methylation marks in histones H3 and H4 caused by CS exposure *in vitro* and *in vivo* due to its involvement in DNA damage/repair, pro-inflammatory gene activation, genomic instability and cellular senescence in a site-specific manner^{45–49, 61, 62}. A recent study showed that extracellular, degradation-resistant, hyperacetylated histone H3.3 was elevated in lung of patients with COPD³¹. We and others have shown that human lung epithelial cells and mouse lung exposed to cigarette smoke causes histones H3 and H4 modifications limited to specific residues (e.g. H3K9ac and H4K12ac)^{3–8, 37, 63}. Therefore, in this study our aim was to identify novel site-specific histone H3 and H4 acetylation and methylation marks induced by CS/CSE in H292 cells and mouse lungs using a bottom-up mass spectrometry approach.

COPD proteomics remains one of the less explored and emerging areas in mass spectrometry. Previous observations using lung tissues and induced sputum from patients with COPD by proteomic and mass spectrometry approaches revealed an increase in surfactant protein A (SP-A) as a potential biomarker and sputum polymeric immunoglobulin

receptor (PIGR) in smokers with mild to moderate COPD linked to the pathogenesis of smoking-induced chronic lung disease, including COPD^{64, 65}. Another study has demonstrated the role of CS-induced unfolded protein response (UPR) in human lung by comparative proteomic approach. Proteomes of lungs from chronic smokers showed up-regulation of several UPR proteins (chaperones, glucose-regulated protein 78, calreticulin a foldase, protein disulfide isomerase and enzymes involved in antioxidant defense) compared to nonsmokers and ex-smokers, suggesting the activation of UPR may protect the lung from CS-mediated oxidant injury and development of COPD⁶⁶. Thus, proteomic analysis highlights strategies to identify novel biomarkers for the diagnosis, treatment and prognosis in chronic lung diseases such as COPD^{67, 68}. We tested our hypothesis in lung tissue of mice exposed to acute CS for 3 days and H292 cells treated with CSE, so as to identify CS-mediated posttranslational histone modifications by mass spectrometry approach.

The combinations of PTMs on particular histone comprise histone codes⁶⁹, which specify distinct regulatory states. Traditional biochemical or immunochemical approaches have been widely employed to identify histone codes, but these have shown it to be extremely difficult in identifying multiple PTMs. Mass spectrometry-based approaches (bottom-up proteomics) are now being used to catalog histone PTMs⁷⁰. Our characterization of posttranslational histone modifications showed majorly acetylations (K) and mono- and di-methylations (K and R). In this study, we clearly noted that histones H3 and H4 have distinct epigenetic states during control/air-exposed and CSE-treated/CS-exposed conditions. The dynamic nature of histone modifications that occur in the N-terminal histone tail plays a vital role in the opening and closing of the chromatin, allowing access for DNA repair enzymes and chromatin complexes, which mediate the regulation of gene transcription¹⁵. Histones that are differentially methylated form unique association patterns with specific protein complexes, which recognize the PTM marks to convey their activating or silencing effects⁷¹.

Alterations in histone biosynthesis and redistribution of epigenetic marks occur as a result of chronic damage and cellular aging (replicative senescence)⁵⁶. Some of the key markers, such as H3K56ac, H3K79me2 and H4K20me2, which have been previously implicated to be involved in DNA damage and replicative stress were significantly altered in response to chronic bleomycin treatment in IMR90 cells in a dose-dependent manner⁵⁶. Our data also suggest that CS exposure in mouse and CSE treatment in H292 cells significantly alters some of these known histone marks involved in DNA damage and cellular senescence, suggesting these epigenetic signatures might play an important role in CS-mediated chronic lung diseases. In both mice exposed to CS and H292 cells treated with CSE, we observed the site-specific histone PTMs at H3K27me1 and H3K27me2 only in the CS/CSE exposure group. This suggests that polycomb and associated H3K27 methylation via its HP1 chromodomain⁷² may have some role in DNA replication⁷³ process during which CS-mediated damage alters the transcriptome and replication dynamics⁷⁴. In mouse lung and H292 cells from control/air and CSE/CS exposure, we observed acetylation of H3K56, H4K8, H4K12 and H4K16. These findings suggest a strong link among chromatin replication, compaction and transcriptional control and DNA damage sensing that occurs during normal states and in response to CS-mediated oxidative stress.

The histone modification status in lung tissue or cells was previously investigated by determining the protein levels of modified histones H3 and H4^{24–27}. It has been clearly demonstrated that levels of H4 acetylation was significantly increased in smokers with and without COPD suggesting the effect of smoking on chromatin modification and thus, enhancing proinflammatory cytokine gene transcription²⁴. Increased acetylation of histone H4 at the IL-8 promoter in peripheral lung tissues samples also showed direct correlation with COPD disease severity (GOLD Stage 4)²⁵. Studies in the past have demonstrated global posttranslational histone modification profile in normal lung tissues and primary lung

tumors implicating the role of site-specific histone modification in lung cancer progression^{51, 75}. Non-small cell lung cancer (NSCLC) cells exhibited increase in H4K5ac and H4K8ac but decreased H4K12ac, H4K16ac and H4K20me3⁷⁶. Loss of H4K20me3 correlates reduced survival in patients (stage I adenocarcinoma), along with decreased expression of the histone methyltransferase SUV4-20H2⁷⁶. Hence, changes in global levels of histone modifications directly influence disease prognosis. Site-specific histone modification at H3K4me2, H2AK5ac and H3K9ac influences the clinical outcome of NSCLC patients during early-stage tumors⁷⁷. Based on the published reports, the novel CS-induced site-specific histone acetylation and methylation marks identified in this study will have greater translational impact on understanding the pathogenetic process involved in smoking-mediated chronic lung diseases, such as COPD and lung cancer.

Using mass spectrometry, we were able to map histone modifications between air versus CS-exposed mouse lung and control versus CSE-treated human bronchial epithelial cells. We found site-specific lysine acetylations and mono- and di-methylations. Arginine mono- and di-methylations were identified in both histones H3 and H4, along with multiple modifications at both the N- and C-terminals, including the histone-fold domain. Some unique PTMs, such as lysine acetylation, lysine methylation, and arginine methylation, were identified in the same peptide sequence (e.g. H4K31me2 and H4R35me2), suggesting the dynamic nature of histone PTMs between control/air and CSE/CS-exposed mouse lung and H292 cells. Studies in the recent past have shown considerable understanding of the role of specific histone modifications in organismal aging. Trimethylation of histone H4 at lysine 20, a hallmark of constitutive heterochromatin, increases in rat liver age-dependently, and the same mark was found upregulated in a cellular progeria model^{78, 79}. When we compared the histone modification data obtained from mouse lung exposed to acute CS with that of *in vitro* H292 cells treated with CSE they are very distinct. The difference in site-specific histone modifications identified from *in vivo* and *in vitro* may be due to the following: 1) *in vitro* experiment was performed using transformed human bronchial epithelial cells which showed epithelial cell specific effect of CSE and the treatment duration was short for 1 hr, 2) *in vivo* experiment was conducted in mice for 3 days (acute CS exposure) which involves complex interaction of CS-induced inflammatory signaling pathways including DNA damage/repair and cross-talk between different cell types in the lung, and 3) Overall, CS-induced site-specific histone modifications observed in lungs *in vivo* is from the effect of CS on mixed lung cell types, whereas the site-specific changes identified *in vitro* in H292 cells is specific to human bronchial epithelium. Hence, this variability that we see in the site-specific histone modifications *in vivo* and *in vitro* is scientifically justified. Although we have identified several novel histone marks between the air/control versus CS/CSE exposure groups, we are now performing quantitative analyses to prove these changes in specific histone PTMs were significant between the treatment groups.

There are reports that demonstrate genetic inactivation of histone methyltransferase Suv4-20 results in proliferation defects due to increased sensitivity to DNA damage, but their exact role in cellular senescence still remains elusive⁸⁰. EZH2, a histone methyltransferase, is implicated to have a direct role in cellular senescence. INK4A locus is a major player in inducing cellular senescence in mammalian cells. During replicative or oncogenic stress, the products of INK4A locus, such as p19^{ARF} and p16^{INK4A}, were accumulated in the cells, resulting in growth arrest. Reports have clearly shown that the EZH2-containing complex, also known as PRC2 complex, mediates transcriptional repression of the INK4A locus in proliferating cells. When senescence was triggered, EZH2 levels were reduced concomitant with the loss of the H3K27me3 mark at the INK4A locus⁸¹. Histone demethylases KDM2A and KDM2B, which directly target methylated H3K36, prevent cellular senescence via modulation of p53 and pRB pathways^{82, 83}. It has been observed that levels of HDAC1 were decreased after serial passaging of primary human fibroblasts⁸⁴. Another study

demonstrated a senescence-like state induced in primary human fibroblasts treated with HDAC inhibitors, such as trichostatin A (TSA) or sodium butyrate, suggesting that modulation of histone acetylation through class I and II HDACs is important in mediating cellular senescence⁸⁵. A recent study shows that concurrent to proteomic analysis for histone posttranslational modifications, transcriptomic analysis on histone modifying enzymes correlates with predicted histone posttranslational modification abundances along with enzyme abundances in different human cancer cells⁸⁶. Indeed, our preliminary data support this notion that alterations in the expression levels of histone modifying enzymes may directly affect changes in site-specific posttranslational histone modifications *in vitro* in cells and *in vivo* in mouse lungs. We found H292 cells treated with 1% CSE for 24 hrs show increased (> 1.5 fold) mRNA expression of specific chromatin modifying enzymes that are involved in acetylation (HAT1, MYST3, and NCOA3) and methylation (PRMT5, SETD4, and SETDB2) of histones H3 and H4 (Sundar *et al.*, unpublished observations), suggesting that these enzymes may play an important role in modulating chromatin modifications that may have implications in CS-induced chronic lung diseases including COPD and lung cancer. Overall, these studies have suggested that histone modifying enzymes, such as HATs/HDACs and HMTs/HDMs, play a crucial role in the induction or prevention of inflammatory response, premature lung aging/cellular senescence, DNA damage/repair, and steroid resistance.

Our earlier studies have demonstrated significant increases in global acetylations, as well as specific histone H3 and H4 acetylations, including H3K9, H4K8 and H4K12, between control/CSE-treated human bronchial epithelial cells and air/CS-exposed mouse lungs, using ChIP assay and immunoblot analysis³⁻⁸. Methylations were not shown in the same study groups. Here, we report several CS/CSE-specific posttranslational histone modifications in the lung of acute CS-exposed mouse and H292 cells treated with CSE. We have identified several novel histone marks, acetylations and mono- and dimethylations, at specific lysine and arginine residues that were distinct between air/control and CS/CSE-exposed groups. Understanding the role of these identified specific histone marks and their associated histone modifying enzymes will provide insights into the pathogenesis of smoking-induced chronic lung diseases, including COPD. Additional studies to quantitate these histone marks both *in vivo* and *in vitro* using specific gene knockout mouse models *in vivo* and *in vitro* cells along with different dose and time of CS exposure will help us understand the functional aspects of site-specific histone modifications in inflammatory gene expression and broaden their utility as novel biomarkers in CS-induced chronic lung diseases. Furthermore, the role of identified PTMs and their associated writer(s) and eraser(s) using different dose of CS exposure may be useful in developing novel biomarker, which can be used as specific drug targets, thereby regulating the transcriptional state during the progression of CS-induced chronic lung diseases, such as COPD and lung cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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A**Histone H3 - Air** **Histone H3 - CS**

Figure 1. Protein sequence coverage of histone H3 and H4 from air and CS-exposed mouse lungs
 Red peptides were identified using the LCQ. (A) Representative sequence coverage for amino acids in histone H3 (air 87/136 vs. CS 85/136), and (B) Representative sequence coverage for amino acids in histone H4 (air 84/103 vs. CS 92/103). Both A and B were identified in air and CS-exposed mouse lung.

A**Histone H3 - Control** **Histone H3 - CSE**

Figure 2. Protein sequence coverage of histone H3 and H4 from control and CSE-treated H292 epithelial cells

Red peptides were identified using the LCQ. (A) Representative sequence coverage for amino acids in histone H3 (control 86/136 vs. CSE treated 92/136), and (B) Representative sequence coverage for amino acids in histone H4 (control 93/103 vs. CSE treated 88/103). Both A and B were identified in control and CSE-treated H292 cells.

Figure 3. Ribbon model of histone H3 and H4 showing a comparison of differentially modified residues identified by mass spectrometry

(A) Histone H3 and H4 modifications identified in air and CS-exposed mouse lung. Several amino acids showed multiple posttranslational histone modifications, though not all of them were observed simultaneously on the same peptide. Table 1 indicates the number of modified peptides observed for each histone modifications. (B) Histone H3 and H4 modifications identified in control and CSE-treated H292 cells. Again, several amino acids show multiple posttranslational histone modifications, though not all of them were observed simultaneously on the same peptide. Tables 1 and 2 indicate the number of modified peptides observed for each histone modifications. The identified posttranslational histone modifications shown in ribbon model are indicated based on the following color code: Ac (acetylation) = yellow spheres; me1 (mono-methylation) = cyan spheres; me2 (di-

methylation) = olive spheres; ac + me2 (acetylations and di-methylations) = red spheres; me1 + me2 (mono- and di-methylation) = orange spheres; and ac + me1 + me2 (acetylation, mono- and di-methylation) = grey spheres.

Control – H3K27 **CSE – H3K27me1** **CSE – H3K27me2**

Figure 4. Identification of novel sites of methylations on histone H3 by LC/MS/MS analysis that were differentially modified between control and CSE treated H292 epithelial cells

MS spectrum from control H292 cells showing unmodified H3K27 compared to CSE-treated H292 cells, which showed both H3K27me1 and H3K27me2 modifications in the same residue. Methylated lysine (K) residues were marked in red color (annotated peptide sequence) shown in the spectra.

Scheme 1. Schematic for identified posttranslational histone modifications

Wild-type (C57BL6/J) mice were exposed to acute CS for 3 days. Human bronchial epithelial (H292) cells were treated with 1% or 2% CSE for one hr. Harvested lung tissues and cells were processed for nuclear extraction followed by acid extract of histones. Histone samples were analyzed by SDS-PAGE and stained to identify specific histones. Histone H3 and H4 bands were excised from the gel, followed by trypsin digestion and LC/MS/MS analysis.

Table 1
Identified residues and PTMs in air and CS groups in mouse lungs

Posttranslational modifications of histone H3 and H4 in acute air and CS-exposed C57BL/6J mouse lungs. Identified residues showing evidence of histone H3 and H4 modifications are listed in the table based on the PTM type detected.

PTMs	Identified residue	Air group	CS group
Histone H3			
Acetylation	Lys23	1	1
Dimethylation	Lys23	1	0
Dimethylation	Lys27	0	1
Acetylation	Lys36	1	1
Methylation	Lys36	0	1
Dimethylation	Lys36	1	1
Acetylation	Lys56	1	1
Dimethylation	Lys56	0	1
Dimethylation	Arg72	1	0
Acetylation	Lys79	0	1
Methylation	Lys79	1	2
Dimethylation	Lys79	1	2
Histone H4			
Acetylation	Lys12	0	2
Acetylation	Lys16	1	0
Methylation	Lys20	3	1
Dimethylation	Lys20	0	1
Methylation	Arg23	3	1
Acetylation	Lys31	4	4
Dimethylation	Lys31	0	2
Methylation	Arg35	2	2
Dimethylation	Arg35	0	1
Methylation	Arg36	0	1
Methylation	Arg55	1	1
Methylation	Lys 77	1	1

Table 2
Identified residues and PTMs in control and CSE groups in H292 epithelial cells

Posttranslational modifications of histone H3 and H4 in control and 1% CSE-treated H292 cells. Identified residues showing evidence of histone H3 and H4 modifications are listed in the table based on the PTM type detected.

PTMs	Identified residue	Control	CSE
Histone H3			
Acetylation	Lys14	1	0
Acetylation	Lys23	3	1
Acetylation	Lys27	1	0
Methylation	Lys27	0	1
Dimethylation	Lys27	0	1
Methylation	Lys36	2	4
Dimethylation	Lys36	1	2
Dimethylation	Lys37	1	2
Acetylation	Lys56	2	2
Dimethylation	Lys56	2	1
Acetylation	Lys79	2	1
Methylation	Lys79	4	4
Dimethylation	Lys79	3	1
Acetylation	Lys122	3	0
Dimethylation	Lys122	1	1
Methylation	Arg128	2	0
Histone H4			
Acetylation	Lys8	0	1
Acetylation	Lys12	1	1
Acetylation	Lys16	1	1
Dimethylation	Lys16	0	1
Acetylation	Lys31	2	6
Dimethylation	Lys31	2	1
Methylation	Arg35	1	1
Dimethylation	Arg35	1	0

Table 3

Summary of peptides identified for histone H3 and H4 from acute air and CS-exposed mouse lungs. Identity thresholds for MASCOT scoring are not noted because all peptides in the table were manually verified. A MASCOT score is presented as a means of identifying that a peptide was present, and peptide sequence provided with residue numbers in the beginning and the end indicates the cleavage sites.

Histone H3 - Air vs. CS Group in mouse lungs						
Peptide Sequence	Detected PTM	m/z (Air)	m/z (CS)	Mascot Score (Air)	Mascot Score (CS)	
¹⁹ QLATKAAR ²⁶	Lys ² 3ac	452.05	451.19	32.6	38	
¹⁹ QLATKAAR ²⁶	Lys ² 3me2	444.31	0.0000	21.8	0	
²⁷ KSAPATGGVK ³⁶	Lys ² 7me2	0.0000	472.53	0	38.8	
²⁸ SAPSTGGVKKPHR ⁴⁰	Lys ³ 6ac	682.74	674.73	23.9	17.5	
²⁸ SAPATGGVKKPHR ⁴⁰	Lys ³ 6me	0.0000	660.79	0	58.2	
²⁸ SAPSTGGVKKPHR ⁴⁰	Lys ³ 6me2	675.4	667.65	50.4	60.4	
⁵⁴ YQKSTELLIR ⁶³	Lys ⁵ 6ac	646.75	431.37	20	30.8	
⁵⁴ YQKSTELLIR ⁶³	Lys ⁵ 6me2	0.0000	640.12	0	44.5	
⁷⁰ L VREIAQDFK ⁷⁹	Arg ⁷ 2me2	417.04	0.0000	21.6	0	
⁷³ EIAQDFKTDLR ⁸³	Lys ⁷ 9ac	0.0000	689.89	0	39.7	
⁷³ EIAQDFKTDLR ⁸³	Lys ⁷ 9me	451.27	674.88	20.4	59	
⁷³ EIAQDFKTDLR ⁸³	Lys ⁷ 9me2	683.78	683.29	32.8	40.6	
Histone H4 - Air vs. CS Group in mouse lungs						
Peptide Sequence	Detected PTM	m/z (Air)	m/z (CS)	Mascot Score (Air)	Mascot Score (CS)	
⁹ GLGKGGAK ¹⁶	Lys ¹ 2ac	0.0000	729.58	0	27.5	
¹³ GGAKR ¹⁷	Lys ¹ 6ac	530.57	0.0000	29.3	0	
²⁰ KVLRDNIQGITKPAIR ³⁵	Lys ² 0me, Arg ²³ me	617.34	617.78	54.9	41.8	
²⁰ KVLRDNIQGITKPAIR ³⁵	Lys ² 0me2	0.0000	617.64	0	46.7	
²⁴ DNIQGITKPAIR ³⁵	Lys ³ 1ac	684.28	684.77	37.9	33	
²⁴ DNIQGITKPAIR ³⁵	Lys ³ 1me2	0.0000	677.5	0	40.5	
²⁴ DNIQGITKPAIR ³⁵	Lys ³ 1ac, Arg ³⁵ me	691.9	692.12	26.1	23.4	
²⁴ DNIQGITKPAIR ³⁵	Lys ³ 1me2, Arg ³⁵ me2	0.0000	770.24	0	18	
²⁴ DNIQGITKPAIRRLARR ⁴⁰	Arg ³ 6me	0.0000	664.42	0	25.6	

Histone H3 - Air vs. CS Group in mouse lungs						
Peptide Sequence	Detected PTM	m/z (Air)	m/z (CS)	Mascot Score (Air)	Mascot Score (CS)	
⁴⁶ SGLIYEE ^{TR} ⁵⁵	Arg ⁵⁵ me	598.02	598.19	29.9	43.9	
⁶⁸ DAVTY ^{TEHAK} ⁷⁷	Lys ⁷⁷ me	575.16	575.19	25.3	19.8	

Summary of peptides identified for histone H3 and H4 modifications in control and 1% CSE-treated H292 cells. Identity thresholds for Mascot scoring are not noted because all peptides in the table were manually verified. A Mascot score is presented as a means of identifying that a peptide was present, and peptide sequence provided with residue numbers in the beginning and the end indicates the cleavage sites.

Table 4

Histone H3 - Control vs. CSE treated H292 epithelial cells						
Peptide Sequence	Detected PTM	m/z (Control)	m/z (CSE)	Mascot Score (Control)	Mascot Score (CSE)	
¹⁰ STGGKAPR ¹⁷	Lys ^{14ac}	408.1708	0.0000	22.3	0	
¹⁹ QLATKAAR ²⁶	Lys ^{23ac}	451.0259	451.0329	48.1	29.7	
²⁷ KSAPATGGVK ³⁶	Lys ^{27ac}	478.7184	0.0000	38.2	0	
²⁷ KSAPATGGVK ³⁷	Lys ^{27me}	0.0000	465.5419	0	44.7	
²⁷ KSAPATGGVK ³⁷	Lys ^{27me2}	0.0000	943.6948	0	42.5	
²⁸ SAPATGGVKKPHR ⁴⁰	Lys ^{36me}	660.622	660.7842	63.9	65.3	
²⁸ SAPATGGVKKPHR ⁴⁰	Lys ^{36me2}	667.5405	667.7484	62.8	65	
²⁸ SAPATGGVKKPHR ⁴⁰	Lys ^{36me} , Lys ^{37me2}	674.8908	674.7555	28.1	22	
⁵⁴ YQKSTELLIR ⁶³	Lys ^{56ac}	646.8881	646.8543	50.1	56.9	
⁵⁴ YQKSTELLIR ⁶³	Lys ^{56me2}	640.1702	640.177	36.4	43.8	
⁷³ EIAQDFKTDLR ⁸³	Lys ^{79ac}	690.2346	690.321	37.3	21.7	
⁷³ EIAQDFKTDLR ⁸³	Lys ^{79me}	675.7364	674.8785	52.4	55.9	
⁷³ EIAQDFKTDLR ⁸³	Lys ^{79me2}	682.6239	682.9022	48	59.4	
¹¹⁷ VTMPKDIQLAR ¹²⁸	Lys ^{122ac}	713.863	0.0000	24.5	0	
¹¹⁷ VTMPKDIQLAR ¹²⁸	Lys ^{122ac} , Arg ^{128me}	722.1547	0.0000	29.9	0	
¹¹⁷ VTMPKDIQLAR ¹²⁸	Lys ^{122me2}	715.6589	715.6348	30.2	27.4	
Histone H4 - Control vs. CSE treated H292 epithelial cells						
Peptide Sequence	Detected PTM	m/z (Control)	m/z (CSE)	Mascot Score (Control)	Mascot Score (CSE)	
⁶ GGKGLGK ¹²	Lys ^{8ac}	0.0000	658.5278	0	23.7	
⁹ GLGKGGAK ¹⁶	Lys ^{12ac}	729.48	729.5429	29.1	19.7	
⁹ GLGKGGAK ¹⁶	Lys ^{16me2}	0.0000	715.4609	0	15.8	
¹³ GGAKR ¹⁷	Lys ^{16ac}	530.6036	530.6256	24.4	20.9	
²¹ VLRDNIQITKPAIR ³⁵	Lys ^{31ac}	0.0000	579.4067	0	33.5	

Histone H3 - Control vs. CSE treated H292 epithelial cells					
Peptide Sequence	Detected PTM	m/z (Control)	m/z (CSE)	Mascot Score (Control)	Mascot Score (CSE)
²⁴ DNIQGITKPAIR ³⁵	Lys ³¹ ac	684.667	684.42	36.6	35.1
²⁴ DNIQGITKPAIR ³⁵	Lys ³¹ me2	677.7654	677.6439	38.5	38.1
²⁴ DNIQGITKPAIR ³⁵	Lys ³¹ ac, Arg ³⁵ me	692.1958	692.251	28	22.8
²⁴ DNIQGITKPAIR ³⁵	Lys ³¹ me2, Arg ³⁵ me2	461.9516	0.0000	29.2	0

Table 5
Distinct Histone H3 and H4 PTMs in mouse lungs and H292 epithelial cells treated with cigarette smoke

Summary of differentially identified histone H3 and H4 modification residues from both air/CS-exposed mouse lung and control/CSE-treated H292 cells identified in this study.

Sl. No.	Air	CS	Control	CSE
1	-	-	H3K14ac	-
2	-	-	H3K27ac	-
3	H3K23me2	-	-	-
4	-	-	-	H3K27me1
5	-	H3K27me2	-	H3K27me2
6	-	H3K36me	-	-
7	-	H3K56me2 [*]	-	-
8	H3R72me2	-	-	-
9	-	H3K79ac [*]	-	-
10	-	-	H3K122ac [*]	-
11	-	-	H3K122ac + H3R128me [*]	-
12	-	H4K12ac	-	-
13	H4K16ac	-	-	-
14	-	H4K20me2	-	-
15	-	H4K31me2 + H4R35me2 [*]	H4K31me2 + H4R35me2 [*]	-
16	-	H4R35me2 [*]	-	-
17	-	H4R36me1 [*]	-	-

^{*} Spectra for the above mentioned differentially modified peptides are included in the support information section online (see Supplemental Figures 1 and 2).