IKK α Causes Chromatin Modification on Pro-Inflammatory Genes by Cigarette Smoke in Mouse Lung

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Cigarette smoke (CS) induces abnormal and sustained lung inflammation; however, the molecular mechanism underlying sustained inflammation is not known. It is well known that activation of I κ B kinase β (IKK β) leads to transient translocation of active NF- κ B (RelA/p65-p50) in the nucleus and transcription of pro-inflammatory genes, whereas the role of IKK α in perpetuation of sustained inflammatory response is not known. We hypothesized that CS activates IKK α and causes histone acetylation on the promoters of pro-inflammatory genes, leading to sustained transcription of pro-inflammatory mediators in mouse lung *in vivo* and in human monocyte/macrophage cell line (MonoMac6) *in vitro*. CS exposure to C57BL/6J mice resulted in activation of IKK α , leading to phosphorylation of ser10 and acetylation of lys9 on histone H3 on the promoters of IL-6 and MIP-2 genes in mouse lung. The increased level of IKK α was associated with increased acetylation of lys310 RelA/p65 on pro-inflammatory gene promoters. The role of IKK α in CS-induced chromatin modification was confirmed by gain and loss of IKK α in MonoMac6 cells. Overexpression of IKK α was associated with augmentation of CS-induced pro-inflammatory effects, and phosphorylation of ser10 and acetylation of lys9 on histone H3, whereas transfection of IKK α dominant-negative mutants reduced CS-induced chromatin modification and pro-inflammatory cytokine release. Moreover, phosphorylation of ser276 and acetylation of lys310 of RelA/p65 was augmented in response to CS extract in MonoMac6 cells transfected with IKK α . Taken together, these data suggest that IKK α plays a key role in CS-induced pro-inflammatory gene transcription through phospho-acetylation of both RelA/p65 and histone H3.

Keywords: histone acetylation; macrophages; NF- κ B; inflammation; chronic obstructive pulmonary disease

Cigarette smoke (CS) is a major etiologic factor in the pathogenesis of chronic obstructive pulmonary disease (COPD), which is characterized by chronic inflammatory response in the lungs with a progressive and irreversible airflow limitation (1). We and others have shown that CS exposure resulted in lung inflammation with an increased inflammatory cell influx such as macrophages, neutrophils, CD8⁺ lymphocytes, and increased release of pro-inflammatory mediators (2–8). Histone acetylation/deacetylation is an epigenetic event that

CLINICAL RELEVANCE

IKK α plays a key role in cigarette smoke-induced pro-inflammatory gene transcription by epigenetic/chromatin modifications, which explains the abnormal and sustained lung inflammatory response that occurs in smokers and in patients with chronic obstructive pulmonary disease.

plays an important role in perpetuation of CS-induced inflammatory response in the lung (3, 4, 6, 7). Acetylation of lysine residues on histones H3 and H4 has been suggested to be directly related to the regulation of pro-inflammatory gene transcription (9–11). Recently, it has been shown that increased acetylation of histones occurs in lungs of smokers (6), in bronchial biopsy/peripheral lungs obtained from patients with COPD (3), and in rat lung (7) leading to heightened inflammatory response. However, the molecular signaling mechanism underlying CS-mediated chromatin modifications (e.g., increased acetylation of histone proteins and nuclear factor- κ B [NF- κ B]-dependent gene transcription) is not known.

Pro-inflammatory genes are regulated by the transcription factor NF- κ B, which is activated in inflammatory cells, particularly in alveolar macrophages and in lungs of patients with COPD (2, 3, 5, 6, 12). It is well known that NF- κ B is activated via phosphorylation and degradation of inhibitor- κ B (I κ B) by I κ B kinases (IKKs), which in turn lead to nuclear translocation of NF- κ B and subsequent transcription of NF- κ B-dependent genes. Several studies have suggested that degradation of I κ B α and nuclear translocation of NF- κ B are not sufficient to promote a maximal NF- κ B transcriptional activity; rather, the NF- κ B complex must undergo additional post-translational modifications involving site-specific phosphorylation and acetylation (13–17). Previously, we have reported that CS extract (CSE)-mediated NF- κ B activation was associated with increased phosphorylation and acetylation of RelA/p65, implying that acetylation of RelA/p65 might be required for increased transcription of pro-inflammatory cytokine genes in response to CS exposure in macrophages (2, 13).

IKK is a heterotrimer which contains two functionally distinct kinases, IKK α and IKK β , and a regulatory subunit, IKK γ (NEMO). IKK β , in conjunction with NEMO/IKK γ , is directly involved in phosphorylation and degradation of inhibitory I κ B α in response to a variety of inflammatory stimuli, including CS (2, 7, 13, 18, 19). In contrast, IKK α is not essential for I κ B α phosphorylation and degradation, and nuclear translocation of RelA/p65 (20–22). However, it has been recently reported that transcription of NF- κ B-dependent genes is decreased in IKK α ^{-/-} cells, suggesting that IKK α might have an accessory role in the NF- κ B activation pathway (23). Furthermore, the pro-inflammatory cytokine-induced NF- κ B-dependent transcription and promoter activation was markedly decreased

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in IKK $\alpha^{-/-}$ fibroblasts even though I κ B α degradation and NF- κ B *in vitro* DNA binding activity were normal in these cells in response to tumor necrosis factor- α (TNF- α) or interleukin-1 (IL-1) (24). These studies implied that IKK α is required for full activation of NF- κ B-dependent gene expression (10, 25). Further evidence for the involvement of IKK α in pro-inflammatory gene transcription comes from our own observation that IKK β inhibitors were not effective in controlling CS-induced pro-inflammatory cytokine release from macrophages *in vitro* (2). Interestingly, it has been shown that IKK α is an essential regulator of NF- κ B-dependent gene expression through promoter-associated histone H3 phosphorylation (H3) in response to TNF- α (10, 25–27). The phosphorylation of histone H3 in turn facilitates its interaction with CREB-binding protein (CBP) through histone acetyltransferase activity, leading to acetylation of histone H3 on lysines 9 and 14 (28). In light of the regulatory role of IKK α in chromatin modifications on pro-inflammatory gene promoters, we hypothesized that CS exposure leads to increased activation of IKK α and that the activated IKK α induces phosphorylation/acetylation of histones (H3 and H4) on promoters of NF- κ B-driven pro-inflammatory genes, leading to sustained transcription of pro-inflammatory genes. To test this hypothesis, we investigated the role of IKK α in CS-induced histone acetylation on promoters of key pro-inflammatory genes in mouse lung. Furthermore, the role of IKK α in regulation of CS-mediated chromatin remodeling and pro-inflammatory cytokine release was confirmed using molecular approaches in a human monocyte-macrophage cell line (MonoMac6).

MATERIALS AND METHODS

Reagents

Unless otherwise stated, all biochemical reagents used in this study were purchased from Sigma Aldrich, Inc. (St. Louis, MO). Antibodies used in this study include the following: β -actin (CP-01; Oncogene, San Diego, CA), histone H3, acetylated (Ac)/phosphorylated (P) histone H3 (lys9/ser10), histone H4, Ac histone H4 (Lys12), IKK β , and phospho-RelA/p65 (ser276) (antibody catalog nos., 9715, 9711, 2592, 2591, 2370, and 3037, respectively; Cell Signaling Technology Inc., Danvers, MA), IKK α and RelA/p65 (SC-7182 and SC-372, respectively; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-acetylated histone H3 and histone H4, and IKK α (06-599, 06-598 and 05-536; Upstate, Charlottesville, VA), IKK α pS176/180 (ab17943; Abcam, Inc., Cambridge, MA). The anti-acetylated RelA/p65 antibody specific for AcLys310 or AcK310 (16) was provided by Dr. Leonard Buckbinder at Pfizer Global R&D (Groton, CT).

Animals

Adult male C57BL/6J mice (8–10 wk, 37 ± 1.5 g; Jackson Laboratory, Bar Harbor, ME) were housed in the Inhalation Core Facility of the University of Rochester. The Animal Research Committee of the University of Rochester approved all animal experimental procedures described in this study.

CS Exposure

Mice (six to eight per group) were used for acute (3 d) and sub-chronic (8 wk) CS exposure. The mice were placed in individual compartments of a wire cage that was placed inside an aerated plastic box connected to the smoke source. The CS was generated from 2R4F research cigarettes (total particulate matter [TPM] concentration 11.7 mg/cigarette, tar 9.7 mg/cigarette, nicotine 0.85 mg/cigarette; University of Kentucky, Lexington, KY). CS exposure was performed according to the Federal Trade Commission protocol (1 puff/min of 2-s duration and 35 ml volume) in an automatic Baumgartner-Jaeger CSM2082i CS machine (CH Technologies, Westwood, NJ). Mainstream CS was diluted with filtered air and directed into the exposure chamber. The smoke exposure (TPM per cubic meter of air, mg/m³) 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 nominal value of approximately 300 mg/m³ TPM by adjusting the flow rate of the dilution air (13, 29–31). Sham control animals were exposed only to filtered air in the same manner for the same duration. Mice received two 1-hour exposures (1 h apart) per day for 3 days and 8 weeks (5 d/wk), and were killed 2 and 24 hours after the final exposure. Concentration of carbon monoxide in the CS-filled chamber was approximately 350 ppm. The dosimetry of carbon monoxide in CS was estimated by measuring the blood carboxyhemoglobin levels. Mice tolerated CS without the evidence of toxicity (carboxyhemoglobin, COHb levels $\sim 17\%$ and no body weight loss).

Tissue Harvest and Bronchoalveolar Lavage

Mice were injected with 100 mg/kg (body weight) of pentobarbiturate (Abbott laboratories, Abbott Park, IL) intraperitoneally and killed by exsanguinations. The heart and lung were removed *en bloc*, and the lungs were lavaged three times with 0.5 ml of 0.9% sodium chloride. The lavage fluid was centrifuged, and the cell-free supernatants were frozen at -80°C for later analysis. The bronchoalveolar lavage (BAL) fluid cell pellet was resuspended in saline, and the total cell number was determined with a hemocytometer. Differential cell count (500 cells/slide) was performed on cytopspin-prepared slides (Thermo Shandon, Pittsburgh, PA) stained with Diff-Quik (Dade Bering, Newark, DE).

The detailed procedures for hematoxylin and eosin (H&E) staining, macrophage immunohistochemistry, and analysis of pro-inflammatory mediators in BAL fluid are provided in the online supplement.

Immunohistochemical Localization of IKK α

The levels of IKK α were measured in the fixed lung sections (4 μm thick) by immunohistochemical staining using IKK α rabbit polyclonal antibody (1:100 dilution) with avidin-biotin-peroxidase complex (ABC) method followed by hematoxylin counterstaining. Appearance of dark brown color represents the presence of IKK α in the lung tissue. The numbers of IKK α -positive cells in the lung sections (five random microscopic fields per lung section in three different sections) were counted manually in a blinded manner under a magnification of $\times 200$, and the numbers were averaged.

Cell Culture

The human monocyte-macrophage cell line (mature monocytes-macrophages) MonoMac6, which was established from peripheral blood of a patient with monoclastic leukemia (32, 33), were grown in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 $\mu\text{g}/\text{ml}$ penicillin, 100 U/ml streptomycin, 1% nonessential amino acids, 1 mM sodium pyruvate, 1 $\mu\text{g}/\text{ml}$ human holo-transferrin, and 1 mM oxaloacetic acid. These cells do not require PMA to differentiate into the macrophages, thus avoiding any stress to the cells. The cells were cultured at 37°C in a humidified atmosphere containing 7.5% CO₂.

Preparation of Aqueous CSE

Research-grade cigarettes (1R3F) were obtained from the Kentucky Tobacco Research and Development Center at the University of Kentucky (Lexington, KY). Tar and nicotine contents of 1R3F were 15 mg/cigarette and 1.16 mg/cigarette, respectively. CSE (10%) was prepared by bubbling smoke from one cigarette into 10 ml of culture medium supplemented with 1% FBS at a rate of one cigarette per 2 minutes as described previously (34), with modifications (2, 13, 35, 36). The pH of the CSE was adjusted to 7.4 and was sterile filtered through a 0.45- μm filter (25-mm Acrodisc; Pall, Ann Arbor, MI). The CSE preparation was standardized by monitoring the absorbance at λ_{320} (optical density of 0.74 ± 0.05). The absorption spectrum observed at λ_{320} showed very little variation between different preparations of CSE. Freshly prepared CSE was diluted with culture medium containing 1% FBS immediately before use for each experiment. Control medium was prepared by bubbling air through 10 ml of culture medium supplemented with 1% FBS, adjusting pH to 7.4, and sterile filtered as described for CSE preparation.

Treatments

MonoMac6 cells were seeded at a density of 1×10^6 cells/well (total final volume = 2 ml), grown to approximately 80 to 90% confluence in 6-well plates containing RPMI 1640 medium with 10% FBS, washed

in Ca²⁺- and Mg²⁺-free PBS, and then exposed to various treatments in media containing 1% serum. All treatments were performed in duplicate. The cells were treated with CSE (0.5%, 1.0%, and 2.5%) for 1 hour at 37°C with 7.5% CO₂. At the end of treatment, the cells were washed with cold, sterile Ca²⁺- and Mg²⁺-free PBS and were lysed in RIPA buffer and stored at -80°C. Culture media from these cells were collected and stored at -80°C until analyzed for IL-8 release.

Transfection

The plasmids for overexpression and dominant-negative IKK α were obtained as described previously (37). Transient transfection was performed with 1 μ g of plasmids in the presence of Lipofectamine 2000 transfection reagent (product no. 11668-027; Invitrogen, Carlsbad, CA) in MonoMac6 cells. Transfection efficiency in case of both plasmids transfection was more than 80%. One day after transfection, MonoMac6 cells were treated with CSE (0.5%, 1.0%, and 2.5%). Supernatant of transfected cells was assayed for IL-8 release and cytospin slides were prepared for immunocytochemistry. Whole cell lysate was used in Western blotting analysis.

Analysis of Pro-Inflammatory Mediators in BAL Fluid, Lung Homogenates, and Cell Culture Media

Mice BAL fluid (150 μ l) was analyzed for the cytokine levels using the sensitive mice Multi-Analyte Profile (version 1.6) screening by Luminex (Rules Based Medicine, co-marketed with Charles River Laboratories, Austin, TX). The assays permit simultaneous cytometric quantification of multiple chemokines/cytokines with minimal sample volume.

The levels of keratinocyte chemoattractant (KC), IL-6, monocyte chemotactic protein (MCP)-1, and granulocyte macrophage colony-stimulating factor (GM-CSF) in lung homogenates (100 μ l) were measured by the Luminex 100 using the beadlyte mouse multi-cytokine beadmaster kit (Upstate) according to the manufacturer's instructions. The assays use microspheres as the solid support for immunoassays. The levels are expressed as pg/mg protein.

In MonoMac6 cells, the culture medium was collected after treatment and centrifuged at 2,500 rpm for 5 minutes to pellet the cells. The supernatant was then removed and stored at -80 °C for later analysis. IL-8 level in the supernatant was determined by enzyme-linked immunosorbent assay from the respective human IL-8 Cytoset (catalog no. CHC1303; BioSource International, Inc., Camarillo, CA) according to the manufacturer's instructions.

Nuclear Protein Isolation and Acid Extraction of Histone Proteins

One hundred milligrams of lung tissue was mechanically homogenized in 0.5 ml buffer A (10 mM HEPES [pH 7.8], 10 mM KCl, 2 mM MgCl₂, 1 mM DTT, 0.1 M EDTA, 0.2 mM NaF, 0.2 mM Na orthovanadate, 1% [vol/vol] NP-40, 0.4 mM phenylmethylsulfonyl fluoride, and 1 μ g/ml leupeptin) on ice. The homogenate was centrifuged at 2,000 rpm in a benchtop centrifuge for 30 seconds at 4°C to remove cellular debris. The supernatant was then transferred to a 1.7-ml ice-cold microtube and further centrifuged for 30 seconds at 13,000 rpm at 4°C. The supernatant was collected as a cytoplasmic extract. The pellet was resuspended in 200 μ l of buffer C (50 mM HEPES [pH 7.8], 50 mM KCl, 300 mM NaCl, 0.1 M EDTA, 1 mM DTT, 10% [vol/vol] glycerol, 0.2 mM NaF, 0.2 mM Na orthovanadate, and 0.6 mM phenylmethylsulfonyl fluoride) and placed on the rotator in the cold room for 30 minutes. After centrifugation at 13,000 rpm in a micro Eppendorf tube for 5 minutes, the supernatant was collected as the nuclear extract and kept frozen at -80°C for Western blotting. For extraction of histone protein, pellets from the nuclear extraction were resuspended in 150 μ l deionized water, 0.2 N HCl, and 0.36 N H₂SO₄. The histone proteins were precipitated from the supernatant, agitated overnight at 4°C, and then centrifuged at 13,000 rpm for 10 minutes, and the supernatant decanted into a fresh tube. Ice-cold acetone precipitation samples were incubated overnight at -80°C, centrifuged, and the air-dried pellets were resuspended in 50 μ l deionized water.

Western Blotting

Lung tissue homogenate samples (cytoplasmic and nuclear proteins) were separated on a 7.5%-12% SDS-PAGE. MonoMac6 cells were

harvested (24 h after transfection), lysed, and the nuclear fraction was separated with 10% Igepal CA-630 lysis buffer supplemented with a protease inhibitor cocktail (leupeptin, aprotinin, pepstatin, and PMSF). Equal amount of protein was subjected to electrophoresis on 7.5%-12% PAGE gels, electroblotted onto nitrocellulose membranes (Amersham Bioscience, Piscataway, NJ), and then incubated overnight with primary antibodies at 4°C. The next day, membranes were washed and incubated for 1 hour at room temperature with the appropriate secondary antibody linked to horseradish peroxidase (Dako, Santa Barbara, CA); bound complexes were detected with the use of the enhanced chemiluminescence method (Jackson Immunology Research, West Grove, PA).

Immunocytochemistry

MonoMac6 cells (1 $\times$ 10⁴ cells/slide) were used to prepare cytospin slides (1,500 rpm for 5 min), and fixed in 4% paraformaldehyde for 10 minutes. The cells were then permeabilized for 10 minutes in 0.3% Triton X-100 in PBS, and blocked for 1 hour using 10% normal goat serum in TBS. Samples were incubated with antibodies specific for acetylated (Ac)/phosphorylated (P) histone H3 (lys9/ser10) and IKK α using 1% BSA in TBS in a humidified chamber overnight. The primary antibodies were detected with FITC-labeled anti-mouse (MP Biomedicals, Solon, OH), or Alexa Flour 594 goat anti-rabbit secondary antibody (catalog no. A-11037; Invitrogen). Nuclei were stained with 1 μ g/ml Hoechst 33342 for 1 minute. Samples without primary antibodies were used as negative controls. The coverslips were mounted onto the slides (Product no. H-1000; Vector Laboratories, Burlingame, CA) and viewed under a fluorescence microscope.

Chromatin Immunoprecipitation

One hundred milligrams of lung tissue was homogenized in 1 mg/ml BSA with protease inhibitors (one tablet) in PBS, and cross-linked with 1% formaldehyde for 10 minutes, rinsed three times with PBS, and then 0.5 ml 2.5 M glycine was added. After a brief centrifugation, cell pellets were resuspended in SDS-lysis buffer (50 mM Tris-HCl, 1% SDS, 5 mM EDTA, 5 mM Na-butyrate, protease inhibitors). Sonication of nuclear pellet containing chromatin was performed four times for 30 seconds and one time for 15 seconds at a maximum speed using Misonix-3000 Sonicator (Misonix Inc, Farmingdale, NY). Supernatants were collected and diluted (1:10 dilution) with buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl pH 8.0, 5 mM NaButyrate, protease inhibitor) followed by preclearing the extract with 60 μ l of protein A agarose/salmon sperm DNA (Cat no. 16-157; Upstate) for 3 hours at 4°C (38). Immunoprecipitation was carried out overnight at 4°C with 1 μ g of specific antibodies as mentioned above. After immunoprecipitation, 40 μ l of protein A agarose/salmon sperm DNA was added and incubated for 2 hours, followed by brief centrifugation. Precipitates were washed with Paro buffer I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl), Paro buffer II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 500 mM NaCl), and Paro buffer III (0.25 M LiCl, 1% Igepal CA-630, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8.1) for 5 minutes at 4°C. Precipitates were then washed again with Tris-buffer twice for 5 minutes each. The antigen-antibody complexes were extracted two times with 50 μ l elution buffer (0.6 μ g/ μ l proteinase K, 1% SDS, 0.1 M NaHCO₃). The eluted samples were heated at 65°C overnight to reverse formaldehyde cross-linking. The recovered DNA was purified with a QIAquick PCR purification kit (Product no. 28106; Qiagen, Valencia, CA) (38). Samples of input DNA were also prepared in the same way as described above. PCR amplification was performed using a PTC-200 DNA engine (M. J. Research, Waltham, MA) under the following conditions: 94°C for 180 seconds; 30 to 38 cycles at 94°C for

TABLE 1. PRIMER SEQUENCES USED IN CHROMATIN IMMUNOPRECIPITATION ASSAY

Gene	Primer	Sequence
MIP-2	Sense	5'-CAA CAG TGT ACT TAC GCA GAC G-3'
	Antisense	5'-CTA GCT GCC TGC CTC ATT CTA C-'
IL-6	Sense	5'-GAC ATG CTC AAG TGC TGA GTC AC-3'
	Antisense	5'-AGA TTG CAC AAT GTG ACG TCG-3'

Figure 1. Cigarette smoke (CS) exposure increased the levels of RelA/p65 NF- κ B-dependent pro-inflammatory mediators in mouse lung. (A) Levels of RelA/p65 NF- κ B-dependent pro-inflammatory cytokines were analyzed by Luminex assay in bronchoalveolar lavage fluid (150 μ l) of mice exposed to CS for 3 days. The animals were killed 24 hours after the last CS exposure. Open bars indicate air-exposed mice, and solid bars indicate CS-exposed mice. (A) MIP-2 (macrophage-inflammatory protein-2) and IL-6. Acute and sub-chronic CS exposure significantly increased the levels of keratinocyte chemoattractant, MCP-1, IL-6, and granulocyte macrophage colony-stimulating factor in the lungs of mice exposed for (B) 3 days and (C) 8 weeks. The levels of pro-inflammatory mediators in lung homogenates were measured by the Luminex 100 using a beadlyte mouse multi-cytokine beadmaster kit. Data are shown as mean $\pm$ SEM ($n = 5-6$ /group). ** $P < 0.01$, *** $P < 0.001$, significant compared with air-exposed group.

45 seconds, 60°C for 60 seconds, and 72°C for 60 seconds; and final elongation at 72°C for 10 minutes. PCR for the input reaction was performed using 100 ng of genomic DNA. Mouse primer sequences are given in Table 1, and PCR products were analyzed on a 1.5 to 2.0% agarose gel.

Protein Assay

Protein level was measured with a BCA kit (Pierce, Rockford, IL). Protein standards were obtained by diluting a stock solution of BSA. Linear regression was used to determine the actual protein concentration of the samples.

Figure 2. CS exposure caused histone H3 phospho-acetylation and H4 acetylation on pro-inflammatory gene promoters in mouse lung. CS caused acetylation of histone H3 and H4 on promoter sites of MIP-2 and IL-6 genes after 3 days of CS exposure and killed 24 hours after the last exposure (A). Phosphorylation on ser10 and acetylation on lys9 of histone H3, and acetylation on lys12 of histone H4 were increased in response to CS exposure after 3 days. Lung homogenates were immunoprecipitated with anti-acetylated histone H3 and H4 antibodies, and chromatin modification on the promoter region of pro-inflammatory cytokine genes was detected by PCR using primers for IL-6 and MIP-2. The bands were measured by densitometry (fold induction versus control). Chromatin modification on various promoters was compared with input DNA against IL-6 and MIP-2 primers. PCR products were separated on agarose gel and stained with SYBR green. Immunoglobulin G was used as a negative control in ChIP assay. (B) Acid-extracted protein was used for immunoblotting against anti-acetylated and phosphorylated histone H3 (lys9/ser10) and acetylated histone H4 (lys12). The levels of phosphorylation (histone H3 on ser10) and acetylation (histone H3 on lys9 and histone H4 on lys12) of histones were increased in response to CS exposure after 3 days. Data are shown as mean $\pm$ SEM, ** $P < 0.01$, *** $P < 0.001$, $n = 4$ /group significantly different from respective air-exposed mice. Ac-P-H3, acetylated- and phosphorylated histone H3; Ac-H3, acetylated histone H3; Ac-H4, acetylated histone H4. Open bars, air; solid bars, CS.

isolated-histone H3 (lys9/ser10) and acetylated histone H4 (lys12). The levels of phosphorylation (histone H3 on ser10) and acetylation (histone H3 on lys9 and histone H4 on lys12) of histones were increased in response to CS exposure after 3 days. Data are shown as mean $\pm$ SEM, ** $P < 0.01$, *** $P < 0.001$, $n = 4$ /group significantly different from respective air-exposed mice. Ac-P-H3, acetylated- and phosphorylated histone H3; Ac-H3, acetylated histone H3; Ac-H4, acetylated histone H4. Open bars, air; solid bars, CS.

Statistical Analysis

Results are shown as means $\pm$ SEM. Statistical analysis of significance was calculated by one-way ANOVA followed by Fisher's PLSD *post hoc* test for multigroup comparisons (StatView 5.0; SAS Institute, Cary, NC). Statistical significance is as indicated in figure legends.

RESULTS

CS Increased the Levels of NF- κ B-Dependent Pro-Inflammatory Mediators in Mouse Lung

In order to determine whether CS induces inflammatory lung response, C57BL/6J mice were exposed to CS for 3 days and 8 weeks. Inflammatory response in mouse lung tissue was evaluated by differential cell counts, H&E staining, and Mac-3 staining. CS exposure resulted in increased accumulation of inflammatory cells in mouse lung tissue (Figures E1 and E2 in the online supplement). We next determined the NF- κ B-dependent pro-inflammatory cytokines in response to CS in mouse lung by Luminex-based multiplex assay in BAL fluid of mice exposed to CS for 3 days or 8 weeks after the mice were killed at 24 hours after the last exposure. As shown in Figure 1, the levels of MIP-2 and IL-6 were significantly increased in BAL fluid of mice exposed to CS for 3 days, and the levels of KC, MCP-1, IL-6, and GM-CSF were significantly increased in mouse lung homogenate at both 3 days and 8 weeks of CS exposure. The levels of NF- κ B-dependent pro-inflammatory mediators, such as IL-2, IL-10, IP-10, LIF, MCP-1, MCP-3, MCP-5, MIP-3 β , and MMP-9, were significantly increased at 3 days in CS-exposed mice (Figure E3). Similar data were obtained for these cytokines at 8 weeks of CS exposure in mouse lung (data not shown). These data confirmed the pro-inflammatory effect of CS in mouse lung.

Chromatin Modification Occurred on Pro-Inflammatory Gene Promoters in Response to CS in Mouse Lung

Chromatin immunoprecipitation (ChIP) assay is the most powerful tool for identifying proteins/nuclear factors associated with specific promoter regions. We studied the CS-induced acetylation of histones H3 and H4 on pro-inflammatory promoters (IL-6 and MIP-2) by the ChIP assay in lungs of mice. The levels of acetylated histone H3 and H4 on pro-inflammatory promoter sites of MIP-2 and IL-6 were increased (Figure 2A). However, the regions that were non-coding for IL-6 and MIP-2 showed no change in gene expression, attesting the specificity of the ChIP assays (data not shown).

It has been reported that phosphorylation of histone H3 on ser10 is known to be an early step in chromatin remodeling. Moreover, histone H4 lys12 residue is mainly known as the typical acetylation site in chromatin remodeling (39). We therefore next determined the levels of histone H3 on ser10/lys9 and H4 on lys12 using acid-extracted histone proteins by Western blot analysis in lungs of mice exposed to CS. Phosphorylation on ser10 and acetylation on lys9 of histone H3 was significantly increased in response to CS exposure (Figure 2B). We also observed increased acetylation on lys12 of histone H4 as well as histone H3 acetylation (Figure 2B). These results suggested that the pro-inflammatory effect of CS is associated with chromatin modifications on various pro-inflammatory gene promoters.

CS Increased the Levels of IKK α in Alveolar Macrophages and Alveolar/Airway Epithelial Cells in Mouse Lung

We determined the localization of IKK α and its activation in lungs of mice exposed to CS. To assess the level and distribution of IKK α in lung sections, immunostaining for IKK α was performed in mid-sagittal sections. Immunostaining of IKK α

Figure 3. CS exposure increased the expression of IKK α in alveolar macrophages and alveolar/airway epithelial cells in mouse lung. Immunohistochemistry data revealed that IKK α is not only localized, but also increased, in alveolar macrophages, alveolar type II cells, and airway epithelial cells in response to CS both after 3 days and 8 weeks. (A) Representative photographs ($\times 200$) from immunostaining for IKK α in lung tissues from air- and CS-exposed mice. The insets in boxes are magnified alveolar macrophages and epithelial cells showing increased IKK α staining in response to CS compared with air-exposure. Appearance of dark brown color represents the presence of IKK α , which was increased in CS exposed mouse lung. E, epithelial cells; M, macrophage; Alv, alveoli; Aw, airway. (B) Immunostaining scores for IKK α per cell type in alveolar and airway regions of the lung. The assessment of immunostaining intensity was performed semi-quantitatively and in a blinded fashion. Black bars, intense staining; grey bars, moderate/weak staining; open bars, no staining. Results are mean of four experiments $\pm$ SEM. ** $P < 0.01$ and *** $P < 0.001$, significant compared with air-exposed mice.

Figure 4. Nuclear levels of IKKα and RelA/p65 (total and acetylated) were increased in lung tissue of mouse exposed to CS. (A) Mice were exposed to CS for 3 days and 8 weeks, and were killed 24 hours after the last CS exposure. CS increased the nuclear levels of IKKα and RelA/p65, and induced hyper-acetylation of RelA/p65 in mouse lung tissue. The levels of IKKα, RelA/p65, and acetylated RelA/p65 were determined by Western blotting. Histone H3 and β-actin were used for nuclear protein loading controls. β-tubulin was used for cytoplasmic protein loading control, which was not expressed in the nuclear extracts of mouse lung tissue (data not shown). (B) The bands were measured by densitometry (fold induction versus control). Data are shown as mean ± SEM; **P* < 0.05, ***P* < 0.01, and ****P* < 0.001, significantly different from respective air-exposed mice (*n* = 4/group).

showed a significantly increased levels of IKKα in macrophages and airway/alveolar epithelial cells in lung tissue of CS-exposed mice compared with air-exposed mice (Figures 3A and 3B). The levels of IKKβ (an isoform of IKKα) were not changed in response to CS exposure. These data suggest that CS exposure increased the level of IKKα, particularly in macrophages and epithelial cells of CS-exposed mouse lungs.

CS Caused the Recruitment of IKKα and RelA/p65 on Pro-Inflammatory Gene Promoters in Mouse Lung

Given the importance of altered chromatin remodeling in CS-induced pro-inflammatory gene transcription (Figures 2A and 2B), we hypothesized that CS causes IKKα-mediated chromatin modification, thereby binding of RelA/p65 NF-κB on pro-inflammatory gene promoters in mouse lung. To test this hypothesis, Western blotting was performed to determine whether CS induces the nuclear levels of IKKα and RelA/p65 in mouse lung. IKKα nuclear accumulation and nuclear translocation of RelA/p65 were increased at both 3 days and 8 weeks of CS exposure (Figures 4A and 4B). Interestingly, increased levels of RelA/p65 were associated with increased hyper-acetylation of RelA/p65 on lys310/K310 residue in response to CS exposure in mouse lungs (Figures 4A and 4B). We also determined whether IKKα and acetylated RelA/p65 were recruited on pro-inflammatory genes, and are induced by CS. The ChIP assay was performed in lung homogenates of air- and CS-exposed mice using the antibodies against IKKα, RelA/p65, and its acetylated form so as to assess the chromatin remodeling on the promoters of pro-inflammatory genes. As expected, IKKα, RelA/p65, and acetylated RelA/p65 were recruited on the promoters of MIP-2 and IL-6 after 3 days of CS exposure (Figures 5A and 5B). These results suggest that CS caused recruitment of IKKα and RelA/p65 on pro-inflammatory promoters associated

with hyper-acetylation of RelA/p65 in response to CS in mouse lung.

IKKα Regulates IL-8 Release and Phospho-Acetylation of Histone H3 and RelA/p65 in Response to CSE in Macrophages (MonoMac6 Cells)

CS increased the nuclear level of IKKα, and this protein was recruited on the pro-inflammatory gene promoters. We also showed the increased localization of IKKα in alveolar macrophages of lungs in response to CS exposure. In view of this, it is possible that IKKα regulates phosphorylation/acetylation of histone H3 and pro-inflammatory mediators release in response to CS exposure. Hence, to assess the role of IKKα in CSE-induced chromatin modification, MonoMac6 cells were transfected with IKKα plasmids (overexpression and mutant) and treated with CSE (0.5%, 1.0%, and 2.5%), and IL-8 level was measured. Our data showed that the level of IL-8 was increased in MonoMac6 cells treated with CSE, which was consistent with our previous data (2). Furthermore, the level of IL-8 was not changed in MonoMac6 cells transfected with plasmid lacking IKKα, whereas overexpression of IKKα exhibited augmented level of IL-8 compared with MonoMac6 cells without the transfection of IKKα plasmid in response to CSE treatment (Figure 6A). Transfection of IKKα significantly increased, whereas IKKα mutant had no effect on, basal levels of IL-8 compared with nontransfected control (Figure 6A).

In order to investigate the involvement of IKKα in phosphorylation/acetylation of histone H3, immunocytochemistry was performed in the transfected MonoMac6 cells for IKKα and acetyl-phospho-histone H3 (lys9/ser10) using specific antibodies. CSE dose-dependently induced the translocation of IKKα from cytoplasm to nucleus, and the increased nuclear IKKα level and its phosphorylation at ser176/180 residue were

Figure 5. CS exposure led to recruitment of IKK α and RelA/p65 (total and acetylated) on promoters of IL-6 and MIP-2 genes. Mice were exposed to CS for 3 days and killed 24 hours after the last CS exposure. (A) CS exposure caused recruitment of IKK α , RelA/p65 (total and acetylated) on MIP-2 and IL-6 gene promoters. The nuclear extracts were immunoprecipitated with specific antibodies, and binding to the promoter of pro-inflammatory mediator genes was detected by PCR-primers for IL-6 or MIP-2 ($n = 3/\text{group}$). Binding to the promoters is compared with PCR of the input DNA. (B) The bands were measured by densitometry (fold induction versus control). Data are shown as mean $\pm$ SEM; ** $P < 0.01$, and *** $P < 0.001$, significantly different from respective air-exposed mice. Open bars, air; solid bars, CS.

associated with increased acetylation and phosphorylation of histone H3 (lys9/ser10) in the nucleus (Figures 6B and 6C). Furthermore, overexpression of IKK α led to increased acetylation and phosphorylation of histone H3 (lys9/ser10) in response to CSE treatments in MonoMac6 cells, whereas transfection of MonoMac6 cells with plasmid lacking IKK α led to disappearance of histone H3 acetylation and IKK α levels (Figure 6B). We assessed the effects of overexpression and knock-down of IKK α on basal protein level of IKK α in transiently transfected MonoMac6 cells. We found that the level of IKK α was substantially increased in IKK α transfected cells, whereas the level of IKK α was abolished in dominant-negative IKK α -transfected cells (Figure 6C).

We next performed Western blotting to determine the modification of RelA/p65 in response to CSE in MonoMac6 cells transfected with overexpression or dominant-negative IKK α plasmid. We found that the levels of phosphorylated RelA/p65 (ser276) and acetylated RelA/p65 (lys310/K310) were increased in response to CSE, and that phosphorylation of RelA/p65 on ser276 and acetylation of RelA/p65 on lys310/K310 were further increased in IKK α -transfected cells (Figure 6C). These data suggest that IKK α regulates IL-8 release

associated with phospho-acetylation of both histone H3 and RelA/p65 on specific lysine/serine residues in response to CSE in MonoMac6 cells.

DISCUSSION

It is currently thought that abnormal inflammatory responses to smoking in patients with COPD are due to chronic pro-inflammatory effects of CS. We and others have recently shown that CS induces pro-inflammatory genes by chromatin remodeling in the lung (6, 7). However, the molecular mechanism whereby CS alters chromatin remodeling on promoters of pro-inflammatory genes was not studied. It has been shown that IKK α induces NF- κ B-dependent gene expression by causing phosphorylation of histone H3 on the pro-inflammatory gene promoters in response to TNF- α treatment (10, 25–27). It is also known that IKK α , but not IKK β , constitutively shuttles between the cytoplasm and the nucleus, suggesting a nuclear function of IKK α (26). In light of the above-mentioned observations, we hypothesized that CS triggers the activation of IKK α , leading to acetylation of histones (H3 and H4) and RelA/p65 subunit of NF- κ B on the promoters of pro-inflammatory genes in mouse

Figure 6. IKKα regulated the release of IL-8 associated with histone H3 phospho-acetylation (lys9/ser10) and phosphorylation/acetylation of RelA/p65 in response to cigarette smoke extract (CSE) in macrophages (MonoMac6 cells). (A) MonoMac6 cells were transfected with IKKα lacking/expressing plasmids, and treated with CSE (0.5%, 1.0%, and 2.5%) for 1 hour; then the level of IL-8 in supernatant was measured by enzyme-linked immunosorbent assay. CSE significantly increased the level of IL-8 release, and cells lacking IKKα showed decreased level of IL-8 in MonoMac6 cells in response to CSE. Data are shown as mean ± SEM; ****P* < 0.001, ###*P* < 0.001, significant compared with control groups (*n* = 4). (B) CSE increased acetylated and phosphorylated histone H3, and the cells lacking IKKα showed decreased levels of acetylated and phosphorylated histone H3. However, the cells overexpressing IKKα increased the levels of acetylated and phosphorylated histone H3 in response to CSE treatments. The results are presented as an average of three separate experiments performed in triplicate. For immunocytochemistry, cells were fixed, and the expression of acetylated and phosphorylated histone H3 and IKKα were determined by immunofluorescence. Acetylated and phosphorylated histone H3 is shown in red, IKKα in green, and DNA (Hoechst nuclear staining) in blue. The images were taken by magnification (×400), and selected a representative cellular morphology from three separate experiments. The group without primary antibodies was used for negative control. (C) CSE increased the levels of total IKKα and phosphorylation of IKKα, and overexpression of IKKα augmented the phosphorylation of RelA/p65 on ser276 and acetylation of RelA/p65 on lys310/K310 in MonoMac6 cells. MonoMac6 cells were transfected with overexpression or dominant-negative IKKα plasmid and treated with CSE (0.5%, 1.0%, and 2.5%) for 1 hour; and then whole cell lysate was used to determine the protein levels of phosphorylated (ser176/180 residue) and total IKKα, IKKβ, total RelA/p65, phosphorylation and acetylation of RelA/p65. β-actin was used as a loading control (*n* = 4).

lung *in vivo* and macrophages *in vitro* which in turn results in sustained transcription of pro-inflammatory genes. Our data show that CS increased the nuclear levels of IKKα in lungs of mouse exposed to CS and macrophages treated with CSE. This increased level of IKKα was associated with phosphorylation of

IKKα at ser176/180 residue in response to CS. We further show that CS exposure induced lung inflammation *in vivo* which is reflected by inflammatory cell influx and NF-κB-dependent pro-inflammatory mediators release. We also show that this was associated with increased phosphorylation/acetylation of specific

histone H3 (lys9/ser10) and histone H4 (lys12) on pro-inflammatory gene promoters using the ChIP assay in CS exposed mouse lung. Furthermore, these modifications of histones, particularly H3 (lys9/ser10), had direct interaction with IKK α on pro-inflammatory gene promoters in macrophages (MonoMac6 cells) in response to CS. These findings are in agreement with previous studies showing that IKK α directly phosphorylates histone H3 (independent of IKK β) in mouse embryonic fibroblasts in response to TNF- α treatment (25).

Recently, a role of NF- κ B-inducing kinase (NIK) has been shown in histone H3 phosphorylation by IKK α , and the phosphorylation of H3 in turn facilitates its interaction with CBP, leading to acetylation of histone H3 on lysines 9 and 14 (26). Our data showing increased nuclear IKK α and modifications of histone H3 in response to CS may involve NIK as an intermediary of IKK α -induced histone modification. Indeed, our preliminary data support this notion that NIK is activated in response to CS in mouse lung (S.-R. Yang and coworkers, unpublished observations). In addition, our data showed that recruitment of IKK α and acetylation of RelA/p65 on pro-inflammatory gene promoters in response to CS in mouse lung is associated with acetylation of specific histone proteins on these promoters. CBP is a master co-activator and known to be recruited on promoters of NF- κ B-dependent pro-inflammatory genes, thereby leading to acetylation of histone proteins. Furthermore, CBP was recruited onto the pro-inflammatory gene promoters in response to CS exposure in mouse lung (S.-R. Yang and coworkers, unpublished observations), suggesting that once IKK α is activated, it forms a complex with CBP and RelA/p65 which may then lead to acetylation of histone H3 and RelA/p65. Indeed, increased acetylation of RelA/p65 at lys310/K310 residue was observed on the pro-inflammatory gene promoters as detected by the ChIP assay. Therefore, increased levels of acetylated RelA/p65 would interact with IKK α and transcriptional co-activators on the promoters of pro-inflammatory genes, and hence will lead to sustained gene transcription of pro-inflammatory mediators in response to CS exposure.

Macrophages are the main orchestrators and amplifiers of the chronic inflammatory responses seen in smokers and patients with COPD, and have the ability to cause lung damage when they are recruited and activated by CS (40, 41). We have previously shown that CS induced the release of pro-inflammatory mediators by activating NF- κ B, and with the reduction of HDAC levels/activity in monocyte-macrophage cell line (MonoMac6 cells) (2). We also show the increased levels of nuclear IKK α in lung interstitial macrophages in response to CS exposure. Using an approach of gain and loss of IKK α in MonoMac6 cells, we demonstrated that the IKK α is activated by CS and is involved in phosphorylation of ser10 and acetylation of lys9 on histone H3, and phosphorylation of ser276 and acetylation of lys310/K310 on RelA/p65. It is known that IKK β regulates activation of RelA/p65 by I κ B degradation in response to diverse stimuli (14, 15, 42). However, recent studies showed that RelA/p65 can also be phosphorylated on ser536 residue by IKK α and on ser276 residue by MSK1 (mitogen- and stress-activated protein kinase), leading to acetylation of RelA/p65 (43–45). Our finding is consistent with the phenomenon that phosphorylation of RelA/p65 at ser276 promotes the acetylation of RelA/p65 at lys310/K310, and this acetylation is required for the full transcriptional activity of RelA/p65 (16). Recent studies showed that IKK α can interact and/or phosphorylate CBP, which enhances the interaction between CBP and RelA/p65 on pro-inflammatory gene promoters in response to TNF- α (10, 46). In the present study, we showed that IKK α and RelA/p65 were recruited on the promoters of MIP-2 and IL-6 genes in response to CS in mouse lung. In addition, knockdown of IKK α

decreased the phosphorylation/acetylation of histone H3 and IL-8 release in MonoMac6 cells in response to CSE treatment, suggesting that IKK α might directly interact with acetylated histones by co-activating CBP. However, this effect is not global, since acetylation of RelA/p65 on lys310 is not observed on IL-6 promoter, suggesting that acetylation of RelA/p65 on promoters of pro-inflammatory genes are specific in response to CS. These findings are corroborated by a recent finding showing a promoter-specific NF- κ B recruitment and histone acetylation in LPS-mediated pro-inflammatory and anti-microbial genes (11).

MSK1 is a major kinase that phosphorylates CBP in mouse fibroblast in response to cellular stress (47). This leads to phosphorylation of histone H3 at ser10 (44, 45). However, the role of MSK1 in IKK α -mediated chromatin modification and NF- κ B activation in response to CS is not known. We found that the level of MSK1 was increased in MonoMac6 cells in response to CSE treatment, but the level was decreased in IKK α -knockdown MonoMac6 cells (S.-R. Yang and coworkers, unpublished observations), suggesting that MSK1 might play an important role in IKK α -mediated chromatin modifications in response to CS on pro-inflammatory gene promoters. However, further studies are required to confirm this contention using knockdown or overexpression of MSK1. Overall, our data suggest that CS-induced IKK α is directly associated with histone modifications (phosphorylation and acetylation of histone H3) on pro-inflammatory gene promoters, and activation of RelA/p65 led to recruitment of acetylated RelA/p65 on pro-inflammatory gene promoters culminating in sustained inflammatory cytokine release.

In conclusion, we have demonstrated a novel role of IKK α in CS-mediated chromatin modifications and acetylation of RelA/p65 in mouse lung *in vivo* and in macrophages *in vitro*. Increased activation of IKK α and acetylation of histones and RelA/p65 were associated with sustained pro-inflammatory gene transcription. These findings not only provide new insight into the molecular mechanisms that underlie COPD pathogenesis, but also validate the relevance of chromatin remodeling in CS-induced abnormal and sustained lung inflammation.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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