Cigarette Smoke Alters Chromatin Remodeling and Induces Proinflammatory Genes in Rat Lungs

John A. Marwick, Paul A. Kirkham, Christopher S. Stevenson, Henry Danahay, June Giddings, Keith Butler, Kenneth Donaldson, William MacNee, and Irfan Rahman

Edinburgh Lung and the Environment Group Initiative Colt Laboratories, MRC Centre for Inflammation Research, University of Edinburgh, Edinburgh; Novartis Institute for Biomedical Research, Horsham, United Kingdom; Department of Environmental Medicine, Division of Lung Biology and Disease Program, University of Rochester Medical Center, Rochester, New York

Cigarette smoke–triggered inflammation is considered to play a central role in the development of chronic obstructive pulmonary disease by a mechanism that may involve enhanced proinflammatory gene transcription. Histone acetylation and deacetylation is a key regulator of the specificity and duration of gene transcription. Disruption in the nuclear histone acetylation:deacetylation balance (chromatin remodeling) may result in excessive transcription of specific proinflammatory genes in the lungs. In this study we show that cigarette smoke exposure results in an influx of inflammatory cells and chromatin modifications in rat lungs. This was associated with an increase in the active phosphorylated form of p38 mitogen-activated protein kinase concomitant with increased histone 3 phospho-acetylation, histone 4 acetylation, and increased DNA binding of the redox-sensitive transcription factor nuclear factor-κB, independent of inhibitory protein-κB degradation, and activator protein 1. We also observed decreased histone deacetylase 2 activity, which is due to protein modification by aldehydes and nitric oxide products present in cigarette smoke. Furthermore, we show that corticosteroid treatment has no effect on smoke-induced proinflammatory mediator release. These findings suggest a possible molecular mechanism by which cigarette smoke drives proinflammatory gene transcription and an inflammatory response in the lungs.

Enhanced inflammation in the lungs in response to inhaled particles or gases is considered to be a defining characteristic of chronic obstructive pulmonary disease (COPD) (1). This involves the recruitment and activation of inflammatory cells in the lungs by proinflammatory mediators. These activated inflammatory cells also produce mediators of inflammation, including oxidants and proinflammatory cytokines, perpetuating the cycle of inflammation. Cigarette smoke is the main etiological factor in the pathogenesis of COPD, causing oxidative stress, a key feature in smoking-induced lung inflammation (2–4). The mechanism of oxidative stress–induced inflammation is thought to involve activation of the redox-sensitive transcription factors nuclear factor (NF)-κB and activator protein (AP)-1 with subsequent nuclear translocation and DNA binding. Transcription factor binding to DNA occurs as part of a larger complex, incorporating coactivators, such as cyclic AMP response element binding protein–binding proteins/p300, which possess intrinsic histone acetyltransferase activity. This results in localized chromatin remodeling, and consequently enhanced gene transcription for proinflammatory mediators (4–7). Indeed, enhanced NF-κB activation has been shown in bronchial biopsies from smokers and in guinea pigs exposed to cigarette smoke, with subsequent increased interleukin (IL)-8 release (8, 9). However, the molecular mechanism of cigarette smoke–mediated enhanced expression of proinflammatory mediators is not yet understood.

The association of core histone proteins with DNA is critical to the regulation of gene transcription and expression by limiting the accessibility of the genome to the transcriptional machinery. The NH_2-terminal of the four core histones (H2A, H2B, H3, and H4) can undergo covalent modifications, including acetylation and phosphorylation (10). These directly affect the chromatin structure, thereby either indirectly or directly affecting transcription (11–13) by acetylation of the core histones, which uncoil DNA from around the core histones making genes more accessible for transcription. To regulate this process, acetylation is reversed by a group of enzymes called histone deacetylases (HDACs).

The HDAC family of enzymes consists of 17 isoforms that have been identified to date, each differentially expressed and regulated in different cell types (14, 15). HDACs remove acetyl moieties from the ε-acetamino groups of lysine residues of histones, restoring their positive charge, thereby causing rewinding/condensation of the DNA. This displaces transcription factors from their DNA binding sites, leading to silencing of gene transcription (16). It is through this process that steroids are thought to propagate some of their anti-inflammatory properties. Ito and coworkers (17) demonstrated that this is achieved by recruitment of HDAC2 into the activated complex, thereby closing down proinflammatory gene expression, a process referred to as transrepression.

Disruption of the acetylation:deacetylation balance may lead to sustained gene transcription of proinflammatory genes controlled by NF-κB and AP-1, resulting in a further influx of proinflammatory cells, creating a chronic cycle of inflammation.

Recent *in vitro* studies by Saccani and colleagues (12) showed that specific phosphorylation of H3 at serine 10, indirectly resulting from activation of p38 mitogen-activated protein kinase (MAPK), acts as a marker for transcription of specific inflammatory cytokine and chemokine genes. Moreover, this enhanced gene transcription positively correlated with p38 MAPK–dependent recruitment of NF-κB (12, 13). However, this concept was never tested *in vivo* in an animal model in response to cigarette smoke.

The field of COPD research currently lacks a clearly well defined *in vivo* model detailing, at the mechanistic level, the impact of cigarette smoke exposure on inflammation. We show here, for the first time, that *in vivo*, altered chromatin remodeling underlies the enhanced inflammatory responses in rat lungs exposed to cigarette smoke. Furthermore, we demonstrated that reduced HDAC activity and expression levels positively correlated with

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Address correspondence to: Dr. Irfan Rahman, Department of Environmental Medicine, University of Rochester Medical Center, 601 Elmwood Ave., Box 850, Rochester, NY 14642. E-mail: irfan_rahman@urmc.rochester.edu

Abbreviations: activator protein, AP; bronchoalveolar lavage fluid, BALF; chronic obstructive pulmonary disease, COPD; glyceraldehyde-3-phosphate dehydrogenase; GAPDH; histone deacetylase 2, HDAC2; 4-hydroxy-2-nonenal, 4-HNE; inhibitory protein-κB, IκB; interleukin, IL; mitogen-activated protein kinase, MAPK; macrophage inflammatory protein, MIP; mitogen- and stress-activated protein, MSK; nuclear factor, NF; phosphate-buffered saline, PBS; reverse transcriptase–polymerase chain reaction, RT-PCR; sodium dodecyl sulfate, SDS.

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increased posttranslational modification of HDAC itself. Finally, as HDAC activity is reported to be essential for the anti-inflammatory effect of corticosteroids, we investigated the ability of corticosteroid treatment to inhibit proinflammatory mediator release in response to cigarette smoke exposure.

Materials and Methods

Animals

Male, Sprague-Dawley rats (323 ± 2.5 g) (Charles River, Margate, UK) were divided into 4 groups: (*i*) 3 d sham-exposed ($n = 6$); (*ii*) 3 d smoke-exposed ($n = 6$); (*iii*) 8 wk sham-exposed ($n = 6$); and (*iv*) 8 wk smoke-exposed ($n = 6$). The rats were exposed to whole-body cigarette smoke, generated from 2R4F research cigarettes (University of Kentucky, Louisville, KY), in 7 liter smoking chambers at a rate of 4 cigarettes per day, Monday–Friday. The total particulate matter was 27.1 ± 0.8 mg total particulate matter per cigarette. To ensure a consistent exposure across exposed animals, cotinine levels were measured. There was 2.66 ± 0.12 μM cotinine in the plasma 1 h after exposure (none was detectable in the air-exposed animals) and 0.51 ± 0.07 μM cotinine after 24 h. There was no progressive increase in cotinine levels over 1 wk of exposures. Carboxyhemoglobin levels were measured immediately after the animals were removed from the chambers. A peak level of 42 ± 4.0 μM was reached after the fourth cigarette, which quickly decreased after exposure was stopped. Rats were killed 2 h after the last exposure by intraperitoneal injection of 200 mg sodium pentobarbital.

Tissue Processing

The lungs were excised from the rats and the right lobe tied off and then snap-frozen in liquid nitrogen. The left lobe was inflated with 5 ml of 10% neutral buffered formalin and then immersed in neutral buffered formalin to complete fixation for 24 h. The left lobe was then sliced tangentially into six slices, which were processed as two tissue blocks. Sections (3 μm) of the four central lung slices were cut using a Leica rotary microtome (Leica, Bannockburn, IL). The sections were mounted on to Polysine slides (Surgipath Europe Ltd., Cambridge, UK) and dried overnight at 37°C.

Immunohistochemistry

Briefly, lung sections were dewaxed in xylene, rehydrated, and endogenous peroxidase inhibited with 0.5% hydrogen peroxide in methanol for 10 min. A controlled digestion was performed in warmed trypsin solution (ICN Pharmaceuticals, Basingstoke, UK). To detect macrophages, slides were incubated in mouse anti-rat macrophage specific antigen ED1 (Serotec, Oxford, UK) (diluted 1:1,000) overnight at 4°C. Immunodetection was performed using biotinylated rabbit anti-mouse immunoglobulin reagent (Dako Cytomation, Cambridgeshire, UK), SABC reagent (Dako Cytomation), and 3,3′-diaminobenzidine (Sigma, Dorset, UK). The nuclei were counterstained with Cole's hematoxylin solution. In rat lungs, the bronchus-associated lymphoid tissue acted as an internal positive control. For negative controls, the primary antibody was omitted from one section of each of the samples. Rat neutrophils were identified with the substituted naphthol method for AS-D chloroacetate esterase. Briefly, solution Y (1.5% pararosaniline, 1.5% $NaNO_2$, pH 6.3) was mixed with 1 ml of solution Z (Naphthol AS-D chloroacetate 20 mg/ml in NN dimethyl formamide [Sigma]) and added to the sections for 2 h at room temperature. Nuclei were counterstained with Mayer's hemalum. For a negative-control solution, Z was prepared without the substrate. Both neutrophils and macrophages were counted using the Axiohome Microscope (Carl Zeiss, Hertfordshire, UK). Cells were identified by both positive staining and standard morphologic criteria. Two fields to the right of the large airway in two pieces of the left lobe were counted (i.e., four fields, total area ~ 6.5 mm^2). When there was a difference of more than 5 cells/mm^2 between the average counts of two and four fields, an extra field was counted in piece three (total area of ~ 8.3 mm^2).

Whole-Cell Lung Tissue Homogenate

Fresh snap-frozen rat lung tissue (0.1 g) was homogenized in 1 ml of ice-cold lysis buffer containing 1% Nonident 40, 0.1% sodium dodecyl sulfate (SDS), 0.01 M deoxycholic acid, and a complete protease cocktail inhibitor tablet with ethylenediaminetetraacetic acid (Roche, East Sussex, UK) and incubated on ice for 45 min. The samples were then centrifuged at 13,000 rpm for 25 min at 4°C and the supernatant aliquoted and stored at −80°C.

Nuclear Protein Isolation and Acid Extraction of Histone Proteins

Fresh snap-frozen rat lung tissue (0.1 g) was homogenized in Buffer A containing 0.4 mM phenylmethylsulfonyl fluoride and 0.3 mg/ml leupeptin (6, 18). The extraction was followed as described previously (18). The supernatant obtained from nuclear lysis was aliquoted and stored at −80°C until used.

Pellets from the nuclear extraction were resuspended in 150 μl of deionized water, 2.6 μl of 11.6 M HCl (final concentration, 0.2 N), and 1.5 μl of 18 M H_2SO_4 (final concentration, 0.36 N). The samples were then agitated overnight at 4°C, and then centrifuged at 13,000 rpm at 4°C for 10 min and the supernatant decanted into fresh eppendorf tubes. Ice-cold acetone (1.1 ml) was added and left over night at −80°C to precipitate out the protein. The samples were then centrifuged at 13,000 rpm for 10 min, the supernatant removed, and 1 ml of ice-cold acetone added, mixed, and left at −80°C for 1 h. The samples were then centrifuged at 13,000 rpm at 4°C for 10 min, the supernatant decanted, and the pellet air-dried and then resuspended in 50 μl deionized water.

Western Blotting

Whole-cell lysate was subjected to electrophoresis using SDS–polyacrylamide gel electrophoresis and transferred to a Proban BA 85 nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). Western blots were visualized using enhanced chemiluminiscence fluid (Amersham, Buckinghamshire, UK). Bands were analyzed using ImageQuant software (Molecular Dynamics Ltd., Buckinghamshire, UK). The blots were then stripped using Chemicon Re-Blot Plus Western recycling kit (Chemicon International, Temecula, CA), blocked, and reprobed. The antibodies used were: HDAC1 (#PC544; Oncogene, San Diego, CA), HDAC2 (#382153; Calbiochem, San Diego, CA), phosphorylated p38 MAPK (#44–684; BioSource, Nivelles, Belgium), p38 MAPK (#9212; Cell Signaling, Beverly, MA), inhibitory protein-κB (IκB) (# sc-371-G; Santa Cruz Biotechnology, Santa Cruz, CA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (# ab9485; Abcam, Cambridge, UK), H4 (#2592; Cell Signaling), acetyl-H4 (Upstate, Charlottesville, VA), H3 (#9715; Cell Signaling) and phosphoacetylated-H3 (#9711; Cell Signaling).

RNA Isolation and Reverse Transcriptase–Polymerase Chain Reaction

Lung tissue (0.1 g) was homogenized in 1 ml of Trizol (InVitrogen Life Technologies, Carlsbad, CA) and left at room temperature for 15 min. RNA was extracted according to the manufacturer's instructions.

RNA (2 μg) was reverse-transcribed into cDNA as described previously (6, 18). Reverse transcriptase–polymerase chain reaction (RT-PCR) amplification was performed for macrophage inflammatory protein (MIP)-1α and MIP-2 for 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min (19) and for GAPDH, 25 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 1 min, and extension at 68°C for 2 min (20). MIP-1α sense = 5′-GATGTATTCTTGGACCCAGGT-3′, anti-sense = 5′-TATGGAGCTGACACCCC-GAC-3, MIP-2 sense = 5′-GGCACATCAGGTACGATCCAG-5′, anti-sense = 5′-ACCCTGCCAAGGGTTGACTTC-3′, GAPDH sense = 5′-ACCACCATGG-AGAAGGCTGC-3′, anti-sense = 5′-CTCAGTGTAGCCCAGGATGC-3′, resulting in PCR products of 189, 287, and 520, respectively. RT-PCR reactions were performed using a thermo cycler (MJ Research, Genetic Research Instrumentation, Essex, UK) and PCR products (base pairs, bp) were electrophoresed on a 1.5% agarose gel containing ethidium bromide. The bands were visualized and quantified by densitometry using ultraviolet radiation and the Grab-IT software package (Ultra-Violet Products, Cambridge, UK).

Electrophoretic Motility Shift Assay

Binding reactions were established in 20 μl of binding buffer as recommended by Promega (Southampton, UK), using 10 μg of nuclear extract protein per reaction for the consensus probe as described previously

(18). Samples were then loaded and electrophoresed through a 6% polyacrylamide gel. The gels were dried, scanned, and analyzed using a phosphorImager system (STORM; Molecular Dynamics).

HDAC2 Immunoprecipitation and Activity Assay

A 200-μg aliquot of protein in a final volume of 100 μl was incubated for 1 h with anti-HDAC2 (#7029; Abcam), then immunoprecipitated out with Protein-A-agarose beads (Calbiochem) at 4°C overnight with constant agitation. HDAC2 activity was measured using an HDAC activity assay kit (BioVision, Mountain View, CA) according to the manufacturer's instructions. For Western blots, sample buffer was added to immunoprecipitated HDAC2 agarose bead suspension, boiled, and run using SDS–polyacrylamide gel electrophoresis as described previously (5, 21). Blots were probed with anti-HDAC2 (#7029; Abcam) as a loading control and stripped as described elsewhere (5, 21). To determine the posttranslational modification of HDAC2, blots were reprobed with antinitrotyrosine, stripped, reprobed with anti–4-hydroxy-2-nonenal (4-HNE), and finally stripped and reprobed with anti-acrolein. Mouse monoclonal anti–4-HNE was purchased from the Japan Institute for the Control of Aging (Fukusoi, Shizoaka Prefecture, Japan). Mouse monoclonal anti-nitrotyrosine was purchased from HyCult Biotechnology (#HM5001) and polyclonal rabbit anti-acrolein was prepared as described previously (22, 23).

In Vitro Modified HDAC2 Activity Assay

HDAC2 was immunoprecipitated from a sham exposed animal lung homogenate as previously described (5). The immunoprecipitated HDAC2 was then split five ways and left either untreated or treated with 2% cigarette-smoke condensate as described previously (21), 100 μM 1,2,3,4-Oxatriazolium-5-amino-3-(3,4-dichlorophenyl)-chloride (Alexis, Nottingham, UK), 100 μM 4-HNE (Alexis), and 100 μM Acrolein (Sigma-Aldrich, Dorset, UK) in triplicate for 1 h at 37°C. HDAC activity was measured by HDAC activity kit (BioVision).

Bronchoalveolar Lavage Fluid

The lungs were lavaged by inserting a cannula into the trachea and instilling the lungs with 3 × 4 ml aliquots of sterile phosphate-buffered saline (PBS). All aliquots were combined for individual rats and brought up to 12 ml with PBS. Bronchoalveolar lavage fluid (BALF) was taken by instilling three 4 ml aliquots of sterile PBS into the lungs. Total cell counts were measured using an automatic cell counter (Sysmex UK Ltd., Milton Keynes, UK). Differential cell counts were performed using standard morphologic criteria on Hema-Gurr–stained cytospins (Merck, Poole, UK).

The Effect of Budesonide on Cigarette Smoke–Induced Proinflammatory Cytokine Production

Male Sprague-Dawley rats were anesthetized using halothane (Merial Animal Health, London, UK, http://uk.merial.com/) and N_2O. A microspray (Penn-Century, Philadelphia, PA), inserted into the trachea, delivered either budesonide (as a 2, 6, or 20% lactose dry-powder blend in 5 mg) or 5 mg lactose (vehicle control groups) ($n = 9$ per treatment group). The dose of budesonide equated to 0, 1, or 3 mg/kg/animal. One hour after dosing, the animals were exposed to whole-body cigarette smoke, as described previously, for a total of three consecutive days. Twenty-four hours after the final exposure, the animals were killed with an overdose of sodium pentobarbitone (200 mg given intraperitoneally) followed by exsanguination. For cytokine analysis, lung tissue was snap-frozen in liquid nitrogen and stored at −80°C until the lung was homogenized in 2 ml of ice-cold saline. Homogenates were spun at 2,000 rpm and supernatant IL-1β levels measured by enzyme-linked immunosorbent assay using an IL-1β Duo-Set, following the manufacturer's protocol (R&D Systems, Abingdon, UK).

Protein Assay

Protein concentrations were determined by bicinchoninic acid (BCA) protein assay kit according to the manufacturer's instructions (Bio-Rad Laboratories Inc., Hercules, CA).

TABLE 1. Inflammatory cell profile in the bronchoalveolar lavage fluid from rat lungs after cigarette smoke exposure

	Total cells ($\times 10^6$)	Macrophages ($\times 10^6$)	Neutrophils ($\times 10^6$)
Sham-exposed rats, 3 d	1.47 ± 0.20	1.46 ± 0.20	0.01 ± 0.01
Cigarette smoke–exposed rats, 3 d	1.55 ± 0.11	0.79 ± 0.06*	0.84 ± 0.11*
Sham-exposed rats, 8 wk	1.62 ± 0.12	1.37 ± 0.17	0.14 ± 0.09
Cigarette smoke–exposed rats, 8 wk	1.82 ± 0.19	0.79 ± 0.12*	0.82 ± 0.17*

Total cell counts were measured using an automatic cell counter (Sysmex UK, Ltd.). Differential cell counts were performed using standard morphological criteria on Hema-Gurr–stained cytospins.

*$P < 0.05$ compared to sham animals ($n = 6$).

Statistical Analysis

All data are expressed as means ± SEM. Statistical analysis of significance was preformed using Minitab software (Minitab, Coventry, UK). The data were normally distributed and values obtained in the different groups of rats were compared using one-way analysis of variance using Tukey's *post hoc* test. Statistical significance was accepted at $P < 0.05$.

Results

Cigarette Smoke Induces Inflammatory Cell Influx in the Lungs

To assess the inflammatory response to cigarette smoke exposure in rat lungs, inflammatory cell counts were performed on the BALF and lung sections. Significant increases in BALF neutrophils were observed after both 3 d and 8 wk of cigarette smoke exposure compared with sham-exposed animals (Table 1). Immunohistologic studies also revealed a significant elevation of neutrophils in the rat lungs after 3 d of cigarette smoke exposure (Figure 1b) compared with sham-exposed animals. There was also a significant increase in the number of macrophages after 8 wk of smoke exposure (Figure 1c) compared with that in sham-exposed animals.

Cigarette Smoke Alters Histone Acetylation and Phosphoacetylation

We first sought to confirm that cigarette smoke exposure would result in elevated histone acetylation. To test this hypothesis, western blot analysis was performed using acid-extracted histones. H4 acetylation was unaltered in the lungs of rats after 3 d of smoke exposure; however, after 8 wk of exposure, H4 acetylation was significantly increased compared with that observed in sham-exposed animals (Figure 2a). Recently, specific phosphorylation of serine 10 on histone 3 has been shown in response to inflammatory stimuli *in vitro* (12). To establish whether this was the case *in vivo* in the rat smoking model, Western blot analysis was performed for acetylated H3 that was specifically phosphorylated at serine 10. Similarly to H4, we observed no increase in acetylated H3 phosphorylation after 3 d of smoke exposure, but significant increases after 8 wk of smoke exposure (Figure 2b).

Cigarette Smoke Causes p38 MAPK Phosphorylation

It has recently been shown that inflammatory stimuli result in the activation of p38 MAPK, which may be responsible for the subsequent specific phosphorylation of serine 10 on H3 seen *in vitro* (12). To determine if this was the case *in vivo* in the rat smoking model, Western blot analysis was performed to assess p38 MAPK phosphorylation (activation). p38 MAPK phosphorylation remained unchanged in the lungs after 3 d of cigarette smoke exposure, but was increased significantly after 8 wk of smoke exposure (Figure 3). The increased p38 MAPK phosphorylation was positively correlated with the observed elevation in H3 phosphorylation. Therefore, the data suggest that the mecha-

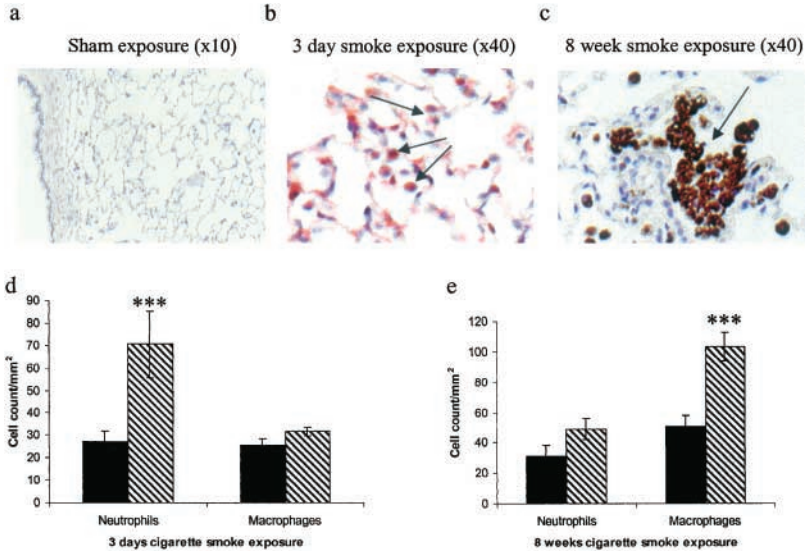

Figure 1. The effect of cigarette smoke exposure in rat lungs. Histologic sections of rat lungs in (*a*) sham-exposed rats at ×10; (*b*) 3-d smoke-exposed rats stained for neutrophils at ×40 (*arrows* show neutrophils in the alveolar space); (*c*) 8-wk smoke-exposed rats stained for macrophages at ×40 (*arrow* shows alveolar macrophages); (*d*) histogram representing neutrophil and macrophage cell counts in 3-d smoke-exposed rat lungs ($n = 6$); (*e*) histogram representing neutrophil and macrophage cell counts in 8-wk smoke-exposed rat lungs ($n = 6$). *Histograms* represent means and *bars* the SEM of inflammatory cells counted per mm^2. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with sham-exposed animals. *Black bars*, sham; *hatched bars*, exposed.

nism of specific phosphorylation at serine 10 on H3 by activated p38 MAPK in response to inflammatory stimuli seen *in vitro* (12) may also occur *in vivo* in rat lungs exposed to cigarette smoke, as shown by our data.

Cigarette Smoke Activates NF-κB, AP-1, and cAMP Response Element Binding Protein DNA Binding without Affecting IκB Levels

An increase in redox-sensitive transcription factor DNA binding is known to occur in response to oxidative stress. To further investigate the inflammatory response to cigarette smoke exposure seen in rat lungs, we determined the levels of transcription factor DNA binding by electrophoretic mobility shift assay. Nuclear binding of NF-κB and AP-1 was significantly elevated after both 3 d and 8 wk of cigarette smoke exposure, compared with the that in the lungs of sham-exposed animals (Figure 4). However, the increase in NF-κB activation was not associated with degradation of IκB at either 3 d or 8 wk of smoke exposure (Figure 5). This suggests that another pathway, independent of IκB, is involved in NF-κB activation.

Cigarette Smoke Alters HDAC Expression and Activity

Previous studies have reported decreased HDAC2 expression in smokers (5). To assess HDAC1 and HDAC2 expression in the rat-smoking model, Western blot analysis was performed. HDAC2 protein expression was significantly decreased in the rat lungs after 3 d of smoke exposure. However, after 8 wk of exposure, there was no difference between the smoke-exposed and sham-exposed animals (Figure 6a). HDAC1 protein expression remained unchanged at both 3 d and 8 wk smoke exposure (data not shown); this is an agreement with previous studies, in which HDAC2 but not HDAC1 was affected by cigarette smoke (5). To investigate further the apparent decrease in HDAC2 protein expression, we examined HDAC2 activity in the rat lung tissue as an index of deacetylation. In correlation with HDAC2 protein expression, we observed substantially reduced HDAC2 activity after 3 d exposure but no difference after 8 wk exposure between smoke-exposed and sham-exposed animals (Figure 6b).

Cigarette Smoke Causes HDAC Modifications

Cigarette smoke–imposed oxidative stress results in lipid peroxidation. End products of lipid peroxidation, such as 4-HNE and

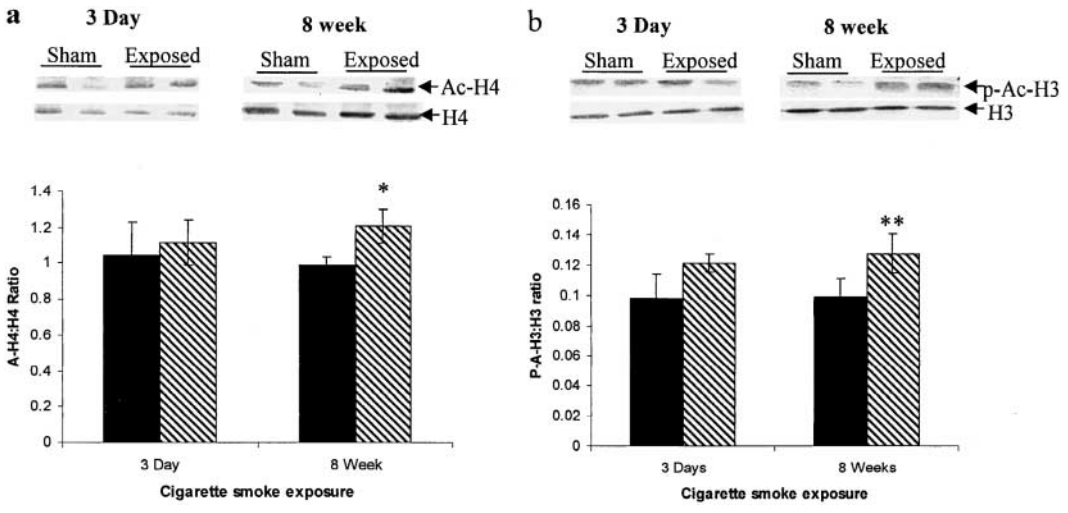

Figure 2. Cigarette smoke increases H4 acetylation and H3 phospho-acetylation. (*a*) Acetylation of histone 4 was increased after 8 wk of cigarette smoke exposure but not after 3 d of exposure in rat lungs compared with sham-exposed animals, as assessed by immunoblotting with anti-acetylated H4 and anti-H4 antibodies ($n = 6$). (*b*) Phosphorylation of serine 10 on acetylated H3 was significantly increased after 8 wk of cigarette smoke exposure but not after 3 d in rat lungs compared with sham-exposed animals, as assessed by immunoblotting with anti-serine 10 phosphoacetylated H3 and anti-H3 antibodies. *Histograms* represent mean and the *bars* the SEM of the ratio of unmodified H4 (*H4*) to acetylated H4 (*Ac-H4*) and unmodified H3 (*H3*) to phospho-acetylated H3 (*p-Ac-H3*). $^*P < 0.05$, $^{**}P < 0.01$ compared with sham-exposed animals. *Black bars*, sham; *hatched bars*, exposed.

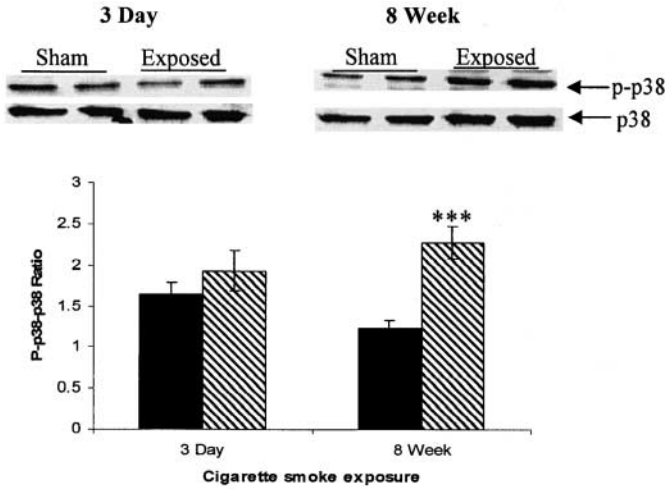

Figure 3. Cigarette smoke increases p38 MAPK activation. Phosphorylated p38 MAPK is increased after 8 wk of cigarette smoke exposure but not after 3 d of exposure in rat lungs compared with sham-exposed animals, as assessed by immunoblotting using anti-phophorylated p38 MAPK and p38 MAPK antibodies ($n = 6$). *Histograms* represent mean and the *bars* the SEM of the ratio of native p38 MAPK (*p-38*) to phosphorylated p38 MAPK (*p-p38*). ***$P < 0.001$ compared with sham-exposed animals. *Black bars*, sham; *hatched bars*, exposed.

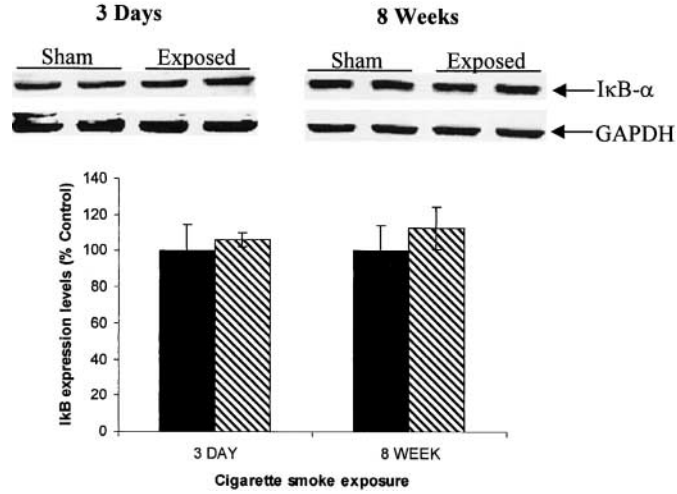

Figure 5. Cigarette smoke has no effect on IκB degradation. (*a*) IκB expression levels remain unaltered after both 3 d and 8 wk of cigarette smoke exposure in rat lungs compared with sham-exposed animals, as assessed by immunoblotting using anti-IκB and anti-GAPDH antibodies ($n = 6$). *Histogram* represents means and *bars* the SEM of IκB expression, expressed as percentage of control (sham-exposed animals). *Black bars*, sham; *hatched bars*, exposed.

acrolein, may react with proteins, leading to protein modification, thereby altering protein function. To investigate whether protein modification is involved in the observed altered HDAC2 expression and activity, we assessed covalent modification of the HDAC2 protein by immunoprecipitation, followed by Western blot analysis using monoclonal antibodies for 4-HNE nitrated tyrosine, and a polyclonal anti-acrolein antibody. There was a significant increase in the tyrosine nitration, 4-HNE and acrolein modification of HDAC2 after 3 d cigarette smoke exposure compared with sham-exposed animals (Figure 7a). However, there was no significant difference in either tyrosine nitration, 4-HNE, or acrolein modification of HDAC2 after 8 wk exposure (Figure 7b). To directly assess the impact of these modifications on HDAC activity, immunoprecipitated HDAC2 was incubated with cigarette-smoke condensate, the lipid peroxidation products

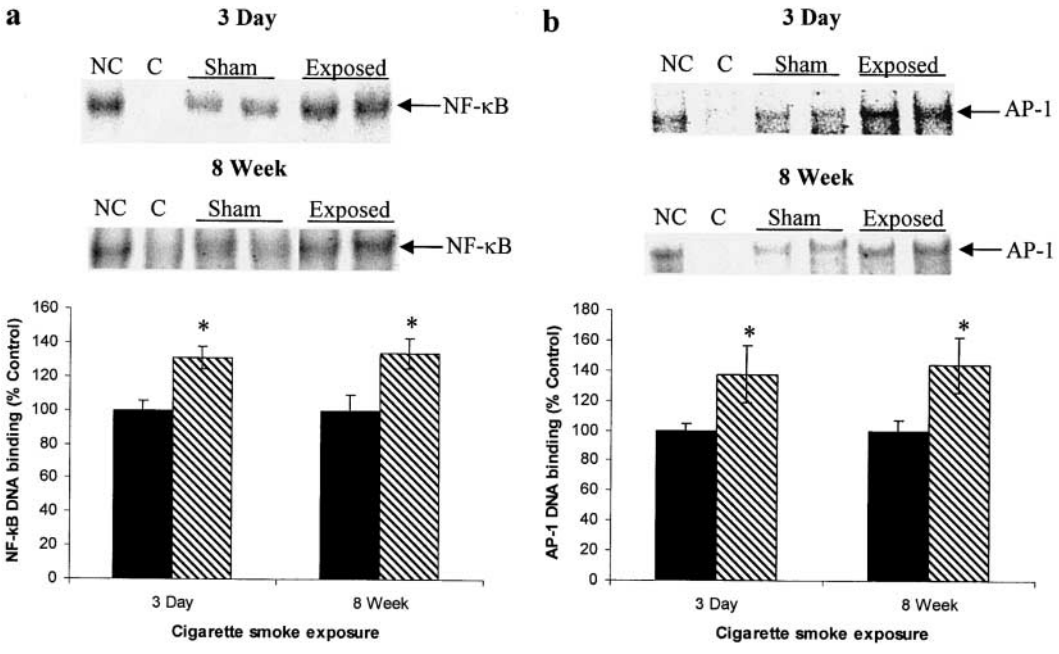

Figure 4. Cigarette smoke increases NF-κB and AP-1 DNA binding. (*a*) Cigarette smoke increases NF-κB DNA binding after both 3 d and 8 wk of cigarette smoke exposure in rat lungs compared with sham-exposed animals, as assessed by electrophoretic mobility shift assay ($n = 6$). (*b*) Cigarette smoke increases AP-1 DNA binding after both 3 d and 8 wk of cigarette smoke exposure in rat lungs compared with sham-exposed animals, as assessed by electrophoretic mobility shift assay ($n = 6$). *Histograms* represent means and *bars* the SEM of the percentage change in transcription factor DNA binding. For controls, the NF-κB– or AP-1–labeled probes were coincubated with a nonspecific cold probe (NC, non-competitor) or coincubated with cold NF-κB or AP-1 probe (C, competitor). *$P < 0.05$ compared with sham-exposed animals. *Black bars*, sham; *hatched bars*, exposed.

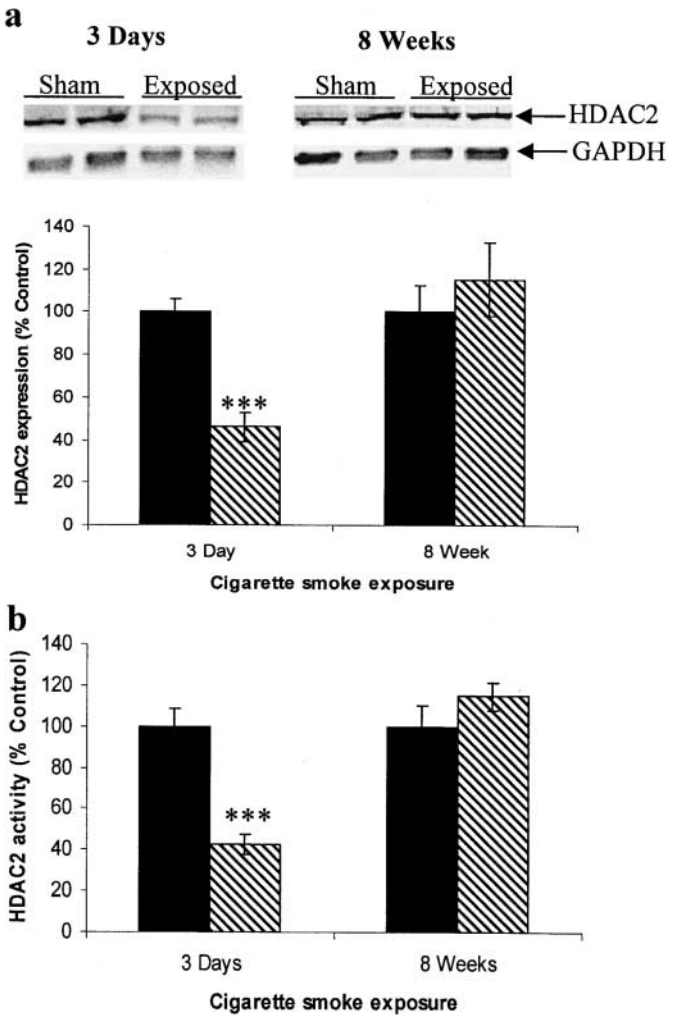

Figure 6. Cigarette smoke decreases HDAC2 expression and activity. (*a*) HDAC2 protein expression is decreased after 3 d but not after 8 wk of cigarette smoke exposure in rat lungs compared with sham-exposed animals, as assessed by immunoblotting using anti-HDAC2 and anti-GAPDH antibodies ($n = 6$). (*b*) HDAC2 activity is also decreased after 3 d but not after 8 wk of smoke exposure in rat lungs compared with sham-exposed animals, as assessed by HDAC activity assay ($n = 6$). *Histograms* represent means and *bars* the SEM of the percentage of HDAC2 expression and activity respectively. ***$P < 0.001$ compared with sham-exposed animals. *Black bars*, sham; *hatched bars*, exposed.

4-HNE and acrolein, and the nitric oxide/peroxynitrite donor (tyrosine nitration) compound Oxatriazolium-5-amino-3-(3,4-dichlorophenyl)-chloride, *in vitro*. There was a substantial decrease in HDAC2 activity with all the treatments compared with the control, nontreated HDAC2 immunoprecipitate (Figure 7c). This observation supports the concept that decreased HDAC2 activity after 3 d smoke exposure was in part a result of covalent modification of HDAC2 protein by components of cigarette smoke.

Cigarette Smoke Induces MIP1 and MIP2 mRNA Expression in Rat Lungs

To directly examine proinflammatory gene expression in the rat lungs in response to smoke exposure, the proinflammatory cytokines MIP-1α and MIP-2 mRNA expression were assessed by RT-PCR. MIP-1α and MIP-2 mRNA expression increased significantly in the lungs of cigarette smoke–exposed animals after both 3 d and 8 wk compared with that of sham-exposed animals (Figure 8).

Budesonide Does Not Inhibit Cigarette Smoke–Induced Proinflammatory Cytokine Production in Rat Lungs

To investigate whether pretreatment with corticosteroid would attenuate the inflammatory response in the rat smoking model, rats were dosed with budesonide and then exposed to cigarette smoke, as previously described (*see* MATERIALS AND METHODS/Animals). The levels of the proinflammatory cytokine IL-1β in lung homogenate were used as an index of the inflammatory response. None of the corticosteroid doses reduced the IL-1β levels significantly in smoke-exposed animals (Figure 9). This suggests that cigarette smoking interferes with the anti-inflammatory effect of corticosteroids.

Discussion

Chronic inflammation in the lungs of smokers involves a sustained elevation of proinflammatory mediator transcription. We hypothesize that altered chromatin remodeling may be involved in the increased transcription of proinflammatory mediator genes in response to cigarette smoke. We therefore investigated the effect of cigarette smoke on chromatin remodeling in rat lungs. In this study, we show, for the first time, exposing rats to cigarette smoke results in altered chromatin associated with a resultant increased transcription of proinflammatory genes.

As expected, an inflammatory response was observed in the rat lungs after both 3 d and 8 wk of cigarette smoke exposure consistent with the results of previous studies (10, 24) and similar to that seen in smokers (25). The macrophage counts in the BALF are reduced but are paradoxically increased in the alveolar regions as assessed by immunohistochemistry. This may be explained by the large "plug" like foci of activated macrophages in the alveolar spaces, which may be difficult to wash out in the BALF. Moreover, cigarette smoke can also modify matrix proteins, resulting in macrophage activation and adherence, which again may be difficult to wash out (23).

Regulation of gene transcription is vital for an orchestrated inflammatory response. In an inflammatory setting, histone acetylation and deacetylation play a critical role in the control of proinflammatory gene transcription by regulating the access of transcription factors to proinflammatory genes. Our finding that H4 acetylation was elevated after 8 wk of cigarette smoke exposure indicated that the histone acetylation:deacetylation balance was indeed disrupted, leaving genes open to sustained transcription, thereby conceivably leading to excessive expression of inflammatory mediators. As oxidative stress has been shown to cause increased histone acetylation *in vitro* (6, 26), we speculate that the oxidative stress imposed by cigarette smoke may be responsible for the alteration in the histone acetylation:deacetylation balance *in vivo*.

In addition to acetylation, histones can undergo further modifications, such as phosphorylation (10). Our observation of elevated acetylated H3 phosphorylation on serine 10, concomitant with elevated p38 MAPK activation in rat lungs after 8 wk of smoke exposure, is of considerable interest. Indeed, Saccani and colleagues showed increased phosphorylation of H3 at serine 10 with concomitant p38 MAPK activation in response to inflammatory stimuli *in vitro* (12). These data suggest that *in vivo,* oxidative stress imposed by cigarette smoke results in the upregulation of specific proinflammatory genes by the same mechanism described by Saccani and colleagues *in vitro*. However, significant increases in H4 acetylation and H3 phosphoacetylation were not seen until 8 wk of exposure, reflecting the fact that the response may be due to a small subpopulation of cells.

The mitogen- and stress-activated protein (MSK) 1 is thought to be the histone kinase activated by p38 MAPK, which directly phosphorylates H3 at serine 10 (12, 27). In support of this, So-

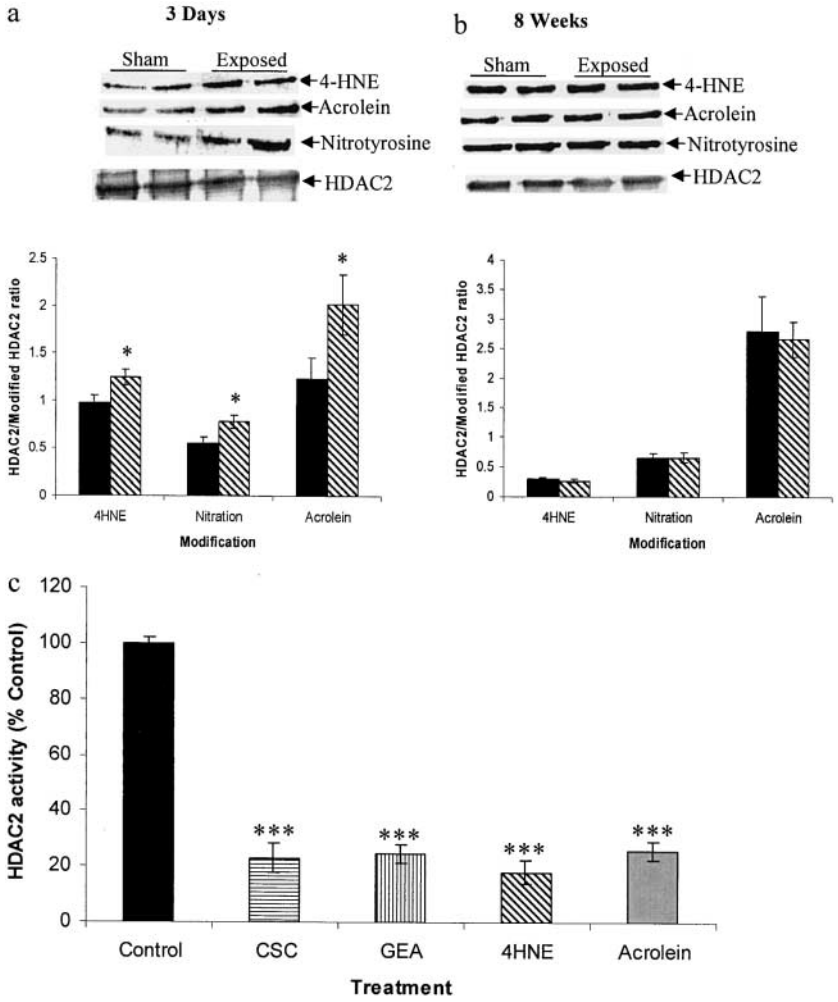

Figure 7. Cigarette smoke causes HDAC2 protein modification. (*a*) 4-HNE, tyrosine nitration and acrolein modification of HDAC2 protein is increased after 3 d of cigarette smoke exposure in rat lungs compared with sham-exposed animal, as assessed by immunoprecipitation of HDAC2 with an anti-HDAC2 antibody followed by immunoblotting using anti–4-HNE, anti-nitrotyrosine, anti-acrolein, and anti-HDAC2 antibodies (n = 6). (*b*) 4-HNE, tyrosine nitration, and acrolein modification of HDAC2 protein showed no change after 8 wk of cigarette smoke exposure in rat lungs compared with sham-exposed animals, as assessed by immunoprecipitation of HDAC2 with an anti-HDAC2 antibody followed by immunoblotting using anti–4-HNE, anti-nitrotyrosine, anti-acrolein, and anti-HDAC2 antibodies (n = 6). (*c*) immunoprecipitated HDAC2 activity was decreased after treatment with cigarette smoke condensate (2%), Oxatriazolium-5-amino-3-(3,4-dichlorophenyl)-chloride (100 μM), 4-HNE (100 μM), and acrolein (100 μM) (n = 6). *Histograms* represent means and *bars* the SEM of the ratio of HDAC2 modified protein to total HDAC2 protein. $^{*}P < 0.05$ compared with sham-exposed animals. *Black bars*, sham; *hatched bars*, exposed.

loaga and colleagues (28) reported that H3 phosphorylation was almost abolished in MSK1 and MSK2 mouse knockouts. The molecular mechanisms of the p38 MAPK downstream signaling with respect to H3 phosphorylation requires further studies.

The redox-sensitive transcription factors NF-κB and AP-1 are closely linked to the transcription of specific inflammatory cytokines and chemokines (4–6). We found increased binding of NF-κB and AP-1 to the DNA after 3 d and 8 wk of smoke exposure, with parallel increases in the expression of the inflammatory chemokines MIP-1α and MIP-2. Although this DNA binding does not directly link NF-κB and AP-1 to gene transcription of MIP-1α and MIP-2, both have NF-κB and AP-1 binding sites in their promoter region and are expressed concomitantly with the increase in DNA binding for both these transcription

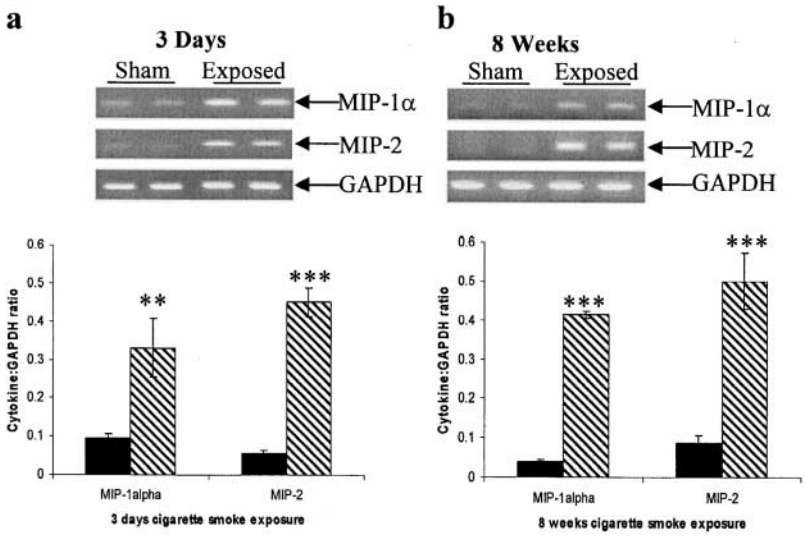

Figure 8. Cigarette smoke increases proinflammatory mediator gene expression. (*a*) MIP-1α gene expression is increased after both 3 d and 8 wk of cigarette smoke exposure in rat lungs compared with sham-exposed animals, as assessed by RT-PCR using GAPDH as a housekeeping gene (n = 6). (*b*) MIP-2 gene expression is increased after both 3 d and 8 wk cigarette smoke exposure in rat lungs compared with sham-exposed animals, as assessed by RT-PCR using GAPDH as a housekeeping gene (n = 6). *Histogram* represents means and *bars* the SEM of the ratio of cytokine to GAPDH housekeeping gene. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with sham-exposed animals. *Black bars*, sham; *hatched bars*, exposed.

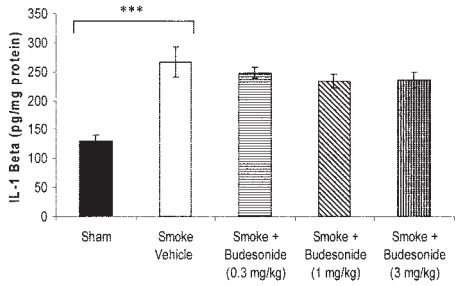

Figure 9. Corticosteroid pretreatment has no effect on 3-d cigarette smoke–induced inflammation. *Histogram* represents means and *bars* the SEM of IL-1β levels in rat lung homogenate; measured in pg/mg of protein, as a measure of the inflammatory response after 3 d of cigarette smoke exposure. The *x axis* shows treatments and doses where sham-exposed animals represent basal IL-1β levels ($n = 9$). $^{***}P < 0.001$ compared with sham-exposed animals.

factors (19). This observation supports our hypothesis that cigarette smoke results in an enhanced inflammatory response in the lung *in vivo*. In support of our data, an increase in NF-κB DNA binding has been shown in bronchial biopsies from smokers, and elevated NF-κB and AP-1 DNA binding has been shown in response to oxidative stress *in vitro* (6, 8). This suggests that cigarette smoke may induce similar proinflammatory pathways in both human and rat lungs in response to oxidative stress imposed by cigarette smoke.

The classical mechanism for NF-κB activation involves the phosphorylation of IκB by IκB kinase, ubiquitination, and then degradation. Interestingly, we found that NF-κB activation was not associated with IκB degradation. Our data suggests that another, IκB kinase–independent mechanism may be initiated by cigarette smoke, allowing for NF-κB nuclear binding. One possible mechanism may be through tyrosine phosphorylation of IκB. Tyrosine phosphorylation of IκB renders IκB unrecognizable for ubiquitination and degradation (29). Another possible explanation involves the p38 MAPK pathway (30). Activation of p38 MAPK by oxidative stress leads to NF-κB activation *in vitro*. This results in phosphorylation of NF-κB at serine 276, leading to p65 transactivation (12, 31). Therefore the mechanism of increased NF-κB DNA binding observed *in vivo* in rat lungs after smoke exposure in the absence of IκB degradation may be due to increased tyrosine phosphorylation of IκB and direct activation of NF-κB by p38 MAPK. However, it must be noted that this may not necessarily reflect an IκBα-independent mechanism of NF-κB activation, as this is a reflection of expression across the whole tissue at a single snapshot in time. Local or temporal changes in IκBα expression may indeed be occurring in distinct cell types.

Deacetylation of histones involves a group of enzymes known as the histone deacetylases (HDACs). Any alteration in the expression or activity of the HDACs may lead to altered acetylation of histones. Furthermore, HDAC2 is recruited by glucocorticoids in the transcriptional initiation complex to inhibit the transcription of proinflammatory mediators (32). Interestingly, we found that HDAC2 expression and activity was decreased after 3 d but not after 8 wk of smoke exposure, whereas HDAC1 expression remained unaltered at both time points. This is consistent with previous *in vitro* studies in alveolar macrophages obtained from smokers, in whom HDAC2 is decreased without change in HDAC1 (5). Intriguingly, the alteration in HDAC2 expression and activity after 3 d of exposure is the reverse of the histone acetylation, and therefore cannot directly explain the increased proinflammatory gene expression at this time point. However, NF-κB is known to form a complex with some members of the class I deacetylases (HDAC1, 2, 3, and 8) (33–35). This complex prevents the DNA binding, and thereby gene transcription, by NF-κB. Therefore, a decrease in HDAC2 may lead to a decrease in complex formation, increasing the free NF-κB in the nucleus to bind to the DNA. This could be seen as an early response to cigarette smoke in the rat lungs with direct access of NF-κB to the opened promoters, irrespective of histone acetylation (35, 36). However, we found that HDAC2 expression and activity had returned to control levels in rat lungs after 8 wk of exposure, possibly as an adaptive response, although histone acetylation and phosphorylation were increased. As both NF-κB and AP-1 activation were still elevated, the genes were opened for transcription of proinflammatory mediators. This could be seen as a late response and the onset of conditions sufficient for a sustained chronic inflammatory response seen here, the level of acetylation exceeds that of deacetyation.

To further investigate the observed alteration in HDAC2 expression and activity, we investigated the possibility of protein modification due to oxidative stress of HDAC2. HDAC2 is recruited into transcriptional complex to inhibit proinflammatory

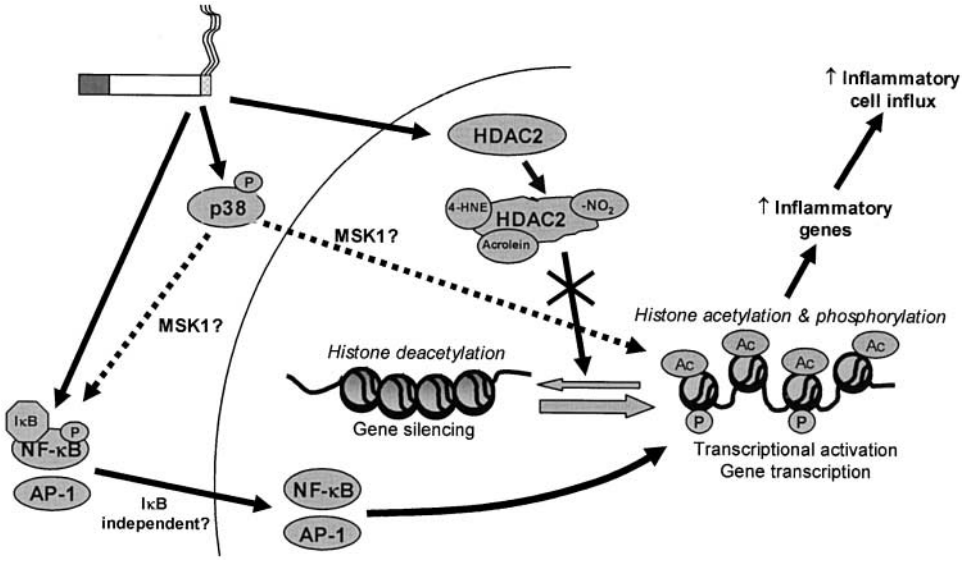

Figure 10. The hypothesized mechanism of cigarette smoke–induced chromatin remodeling through a decrease in HDAC2 and increase in p38 MAPK phosphorylation resulting in an increase in histone 4 acetylation and histone 3 phosphoacetylation. The increased DNA binding of redox-sensitive transcription factors resulting in an increased transcription of specific proinflammatory genes marked out by phosphorylation of serine 10 on histone 3.

gene transcription; therefore, modification of HDAC2 may render it unable to function in the regulation of gene transcription. Our findings showed increased tyrosine nitration, 4-HNE, and acrolein covalent modification of HDAC2 after 3 d of exposure, but interestingly, no change after 8 wk of exposure. Acrolein, a reactive aldehyde, is a major component of cigarette smoke and reacts with proteins to form protein carbonyls. Both acrolein and 4-HNE, also a reactive aldehyde, can be ubiquitously generated in biological systems under oxidative stress during lipid peroxidation reactions, and are known markers of oxidative stress (22, 23, 37). The reactive aldehyde/carbonyls can react with cysteine, histidine and lysine residues and may therefore be considered an important mediator of cell damage by disruption of protein function through covalent modification (38, 39). As the active site of HDAC2 contains histidine groups, the increased acrolein and 4-HNE modification seen after 3 d of smoke exposure may be part of the mechanism responsible for the decrease in HDAC2 activity.

Tyrosine nitration of proteins may also significantly disrupt protein function and turnover (40). Posttranslationally modified proteins are often the targets of proteolytic degradation and removal. This may be important for removal of dysfunctional proteins after by *de novo* synthesis to re-establish protein levels before a pathogenic outcome ensues. The increased HDAC2 tyrosine nitration seen at 3 d of exposure may result in the increased proteolytic degradation of HDAC2, resulting in decreased HDAC2 expression. Indeed, it has recently been shown that inhibited or modified HDAC2 undergoes proteolysis through the ubiquitination pathway (41). Increased *de novo* synthesis of HDAC2 as part of the adaptation to the insult of the cigarette smoke may explain the unaltered HDAC2 expression after 8 wk of exposure. However, despite this reversal of HDAC2 modification, expression, and activity, this ultimately fails to suppress the elevated histone acetylation and proinflammatory gene transcription. Further studies are needed to assess the direct structural changes that are imposed by these covalent modifications on HDAC2 and the relevant contributions of each of the modifications to both the activity and expression of HDAC2.

These results do, however, bring up a new paradox. Global HDAC2 expression and activity is decreased at 3 d, but global changes in histone acetylation are not seen until 8 wk; therefore, it is difficult to invoke a cause and effect mechanism at present. It is, however, more likely that global changes are limited in the key information that this observation provides, and that marked changes in MIP-1α and MIP2 promoter histone H4 acetylation and histone H3 phosphorylation may be present. Global effects on histone H4 acetylation and histone H3 phosphorylation may by confounded by the induction of acute anti-inflammatory genes, which is lost when a chronic inflammatory status is achieved or when only a subset of lung cells are affected. This is more than likely in an acute inflammatory process. Further investigation is also needed into the effect of cigarette smoke exposure on both the other members of the HDAC family, such as HDAC3, which plays a role in shuttling NF-κB out of the nucleus, and specific cell types *in vivo* (34).

Interestingly, HDAC2 has been implicated in corticosteroid-mediated inhibition of proinflammatory mediators by acting as chaperone for corticosteroids to bind to the DNA (17). Patients with COPD are largely unresponsive to the anti-inflammatory effects of corticosteroid treatment (1). Our findings that pretreatment with corticosteroid failed to diminish the elevated inflammatory response seen in rat lungs *in vivo* after 3 d of smoke exposure, may be due to the decreased HDAC2 expression also seen after 3 d of smoke exposure. Interestingly, this steroid unresponsiveness in the cigarette smoke–exposed animals also compliments studies of smoking subjects with asthma, in which smoking subjects with asthma are steroid-unresponsive compared with non-smoking subjects with asthma, which are largely steroid-responsive (42). Furthermore, *in vitro* studies have also shown that cigarette smoke–induced IL-1β expression was not inhibited by steroids (43).

In conclusion, this study provides novel data on an important molecular mechanism by which cigarette smoke affects gene transcription and promotes an inflammatory response *in vivo* in the lungs (Figure 10). Furthermore, regulation of histone acetylation may provide a target for therapeutic intervention in cigarette smoke–induced lung inflammation/COPD.

Conflict of Interest Statement: J.A.M. has no declared conflicts of interest; P.A.K., C.S.C., H.D., J.G., and K.B. are employees of Novartis Pharmaceuticals PLC; K.D. has no declared conflicts of interest; W.M. has been reimbursed by GlaxoSmithKline, Zambon, AstraZeneca, Boehringer Ingelheim, and Pfizer for attending several conferences, received £3,000 from Zambon for a lecture tour, and also participated as a speaker in scientific meetings or courses organized and financed by various pharmaceutical companies (GlaxoSmithKline, AstraZeneca, Boehringer Ingelheim, Zambon, Allen & Hanburys, Pfizer), receiving £4,500 in 2002 and £10,843 in 2003 for serving on advisory boards for GlaxoSmithKline, Almirall, and Amgen, and the sum of £1,700 in 2004 from Micromet for participating in an Expert Panel Meeting, and serves as a consultant for Pfizer and SMB Pharmaceuticals, receiving £6,500 in 2002 for speaking at meetings sponsored by GlaxoSmithKline and AstraZeneca, and £3,600 in 2003 for speaking at meetings sponsored by GlaxoSmithKline, AstraZeneca, and Boerhinger Ingelheim; and I.R. has no declared conflicts of interest.

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