Acrolein Inhibits Cytokine Gene Expression by Alkylation Cysteine and Arginine Residues in the NF-κB1 DNA Binding Domain

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Cigarette smoke is a potent inhibitor of pulmonary T cell responses, resulting in decreased immune surveillance and an increased incidence of respiratory tract infections. The αβ-un saturated aldehydes in cigarette smoke (acrolein and crotonaldehyde) inhibited production of interleukin-2 (IL-2), IL-10, granulocyte-macrophage colony-stimulating factor, interferon-γ, and tumor necrosis factor-α by human T cells but did not inhibit production of IL-8. The saturated aldehydes (acetaldelyde, propionaldehyde, and butyraldehyde) in cigarette smoke were inactive. Acrolein inhibited induction of NF-κB DNA binding activity after mitogenic stimulation of T cells but had no effect on induction of NFAT or AP-1. Acrolein inhibited NF-κB1 (p50) binding to the IL-2 promoter in a chromatin immunoprecipitation assay by >99%. Using purified recombinant p50 in an electrophoretic mobility shift assay, we demonstrated that acrolein was 2000-fold more potent than crotonaldehyde in blocking DNA binding to an NF-κB consensus sequence. Matrix-assisted laser desorption/ionization time-of-flight and tandem mass spectrometry demonstrated that acrolein alkylated two amino acids (Cys-61 and Arg-307) in the DNA binding domain. Crotonaldehyde reacted with Cys-61, but not Arg-307, whereas the saturated aldehydes in cigarette smoke did not react with p50. These experiments demonstrate that aldehydes in cigarette smoke can regulate gene expression by direct modification of a transcription factor.

Cigarette smoke produces profound suppression of pulmonary immunity, resulting in an increased incidence and severity of respiratory tract infections. A recent Institute of Medicine study concluded that smoking increased the incidence of influenza and bacterial pneumonia and accounted for 19,000 smoking-related deaths per year (1). Children infected with Mycobacterium tuberculosis are five times more likely to develop pulmonary tuberculosis if exposed to cigarette smoke (2), and smoking doubles the risk of developing Pneumocystis carinii pneumonia in human immunodeficiency virus-infected individuals (3). Several studies have demonstrated that smoking suppresses T and B cell responses in the lungs without affecting cells in the peripheral blood (4–7), but little research has been done to elucidate the underlying mechanism behind this phenomenon.

We have recently identified two classes of immunosuppressive compounds in cigarette smoke. The dihydropyrenols (hydroquinone and catechol) in the particulate phase inhibit T cell proliferation by blocking cell cycle progression in late G1 and S phase (8–12). In addition, the αβ-un saturated aldehydes, acrolein (CH₂=CHCHO) and crotonaldehyde (CH₃CH=CHCHO) in the gas phase of cigarette smoke inhibit the production of several proinflammatory cytokines including IL-2.

Recent studies have indicated that acrolein has the potential to regulate transcription of a variety of genes primarily though its effects on NF-κB, AP-1, and Nrf2 pathways (14–20). Members of the NF-κB transcription family include NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB, and c-Rel (21, 22). Each subunit includes a Rel homology domain containing a DNA binding domain and protein dimerization domains. RelA, c-Rel, and RelB contain an acidic transactivation domain at the C terminal end of the Rel homology domain. This work was supported by NIEHS, National Institutes of Health Grant ES05673 and by a grant from Philip Morris USA Inc. and Philip Morris International. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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4 The abbreviations used are: IL, interleukin; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; IC₅₀, inhibitory concentration of 50%; PMA, phorbol 12-myristate 13-acetate; ChIP, chromatin immunoprecipitation; 15d-PGJ₂, 15-deoxy-D₁₂,1₄-prostaglandin J₂, EMSA, electrophoretic mobility shift assay; LC, liquid chromatography; MS/MS, tandem mass spectroscopy.
nus. The predominant form of NF-κB consists of a heterodimer between p50 and p65 (23). NF-κB proteins are mainly localized in the cytoplasm and are associated with a family of inhibitory proteins known as IκB. IκB sequesters NF-κB in the cytoplasm by masking the nuclear localization sequence of p65. Upon stimulation, IκB is phosphorylated by the IκB kinase complex and quickly degraded. Consequently, NF-κB is released and translocates into the nucleus to activate NF-κB dependent genes (22, 24). The precise mechanisms by which acrolein regulates transcription are still poorly understood, but effects on glutathione, thioredoxin, IκB kinase, mitogen-activated protein kinase, and Jun kinase have been reported (25–33). We report here that acrolein inhibits cytokine gene expression in human T lymphocytes primarily by alkylating cysteine and arginine residues on the p50 subunit.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Acrolein, crotonaldehyde, acetaldehyde, butyraldehyde, and propionaldehyde (Acros Organics) were prepared in phosphate-buffered saline. The anti-IκBα (sc-371), anti-p50 (sc-8414 and sc-7178), and anti-nucleolin (sc-8031) antibodies were purchased from Santa Cruz Biotechnology, Inc. Anti-p65 (610868) was purchased from BD Transduction Laboratories. Anti-phospho-IκBα (pSer511) antibodies were purchased from BioSource. Anti-β-actin (A5441) was purchased from Sigma.

Cell Culture—Peripheral blood was drawn from healthy, non-smoking adult volunteers after informed consent. Peripheral blood mononuclear cells were prepared by density gradient centrifugation using Ficoll-Paque as described previously (11). T cells were negatively selected using a Pan T Cell Isolation Kit II (Miltenyi Biotec) and were consistently >90% CD3+ as analyzed by flow cytometry (FACScan, BD Biosciences). Cells (107) from individual donors were cultured in tissue culture flasks at 37 °C in 5% CO2 overnight. The cells were then pretreated with acrolein, crotonaldehyde, or acetaldehyde at various concentrations for 3 h. Cultures were stimulated with 10 ng/ml anti-CD3 and 10 nM phorbol 12-myristate 13-acetate (PMA, Sigma). Cells were collected at different time points depending upon the application. Each experiment was replicated three to six times with at least three different individuals. Data are presented as the mean ± S.E. To establish statistically significant p values for cytokine measurements, the values (pg/ml) obtained were converted to percent control (stimulated group) and evaluated by one-way analysis of variance and the Dunnett multiple comparison test. This method allowed us to account for significant variances among individuals.

Cytokine Measurement—T lymphocytes were pretreated for 3 h with various aldehydes as indicated and stimulated with 10 ng/ml anti-CD3 and 10 nM PMA, and culture supernatants were harvested 24 h later. Cytokine levels were measured by Luminex bead human cytokine multiplex kit (BioSource) according to the manufacturer’s instructions and as described (13).

Western Blotting—T lymphocytes were pretreated with acrolein, crotonaldehyde, or acetaldehyde for 3 h and harvested either 5 min after stimulation to detect IκB phosphorylation or 30 min after stimulation for detection of total IκBα. Whole cell lysates were prepared from purified T cells by lysing the cells in 70 μl of lysis buffer containing 50 mM Tris-HCl, pH 7.6, 300 mM NaCl, and 0.5% Triton X-100 at 4 °C for 10 min. Cellular debris was removed by centrifugation. Supernatants were stored at −70 °C. Protein concentrations were determined by the BCA assay. An equal amount (10 or 15 μg) of protein was loaded, separated on a 7.5–12% polyacrylamide gel, and electroblotted onto an Immobilon P (Millipore) membrane. The blots were incubated in 5% Blotto and then probed individually with the indicated antibodies. Detection was performed using secondary antibodies conjugated to horseradish peroxidase and the Western Lightning™ ECL chemiluminescence reagent. The intensity of protein bands was quantitated using LabWorks 4.0 software (UVP). Signals were normalized to either β-actin for whole cell lysates or nucleolin for nuclear extracts.

NFAT, AP-1, NF-kB DNA Binding Assay, and NF-κB Translocation—Nuclear extracts were prepared from aldehyde-treated T cells 2 h after stimulation. T cells were washed once in cold phosphate-buffered saline and resuspended in 50 μl of buffer A (10 mM Hepes, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 1 mM dithiothreitol, 0.5% IGEPAL, and protease inhibitors from Sigma (P-8340) on ice for 10 min. Samples were centrifuged, and the nuclear pellets were resuspended in 40 μl of buffer C (20 mM Hepes, pH 7.9, 1.5 mM MgCl2, 25% glycerol, 0.2 mM EDTA, 0.42 NaCl, 1 mM dithiothreitol, and protease inhibitors as described above) for 15 min on ice. Then 40 μl of buffer D (20 mM Hepes, pH 7.9, 0.2 mM EDTA, 1 mM dithiothreitol, and protease inhibitors) was added. Samples were centrifuged, and supernatants were stored at −70 °C. Protein concentration was determined by the Quant-iTTM protein assay (Molecular Probes). The DNA binding activities of all transcription factors were evaluated in nuclear extracts by the TransAM™ transcription factor DNA binding enzyme-linked immunosorbent assay kits according to the manufacturer’s instructions (Active Motif). Wild-type and mutated consensus oligonucleotides (20-fold molar excess) were used as controls to determine specificity of the transcription factor complex. Nuclear translocation of NF-κB was monitored by Western blotting (as described above) with antibodies specific for p50 and p65.

Recombinant NF-κB1 p50—The coding region of p50 was cloned into the expression plasmid pQE-30 (Qiagen) which incorporates a hexahistidine tag on the N terminus. Escherichia coli were grown and induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside. Cells were lysed with lysozyme (1 mg/ml) and sonicated in lysis buffer (20 mM Tris-HCl, pH 8.0, 100 mM KCl, 10% glycerol, 1 mM phenylmethylsulfonflyl fluoride, and protease inhibitors). Cell debris was removed by centrifugation. Nickel-nitrilotriacetic acid-agarose beads (Qiagen) were added to the supernatant and incubated at 4 °C for 1 h. The protein-bead slurry was added to a disposable column (Bio-Rad) and the beads were washed with 10 ml of cold wash buffer A (20 mM Tris-HCl, pH 8.0, 300 mM KCl, 10 mM imidazole, 10% glycerol, 1 mM phenylmethylsulfonflyl fluoride, and protease inhibitors) and 10 ml of cold wash buffer B (20 mM Tris-HCl, pH 8.0, 300 mM KCl, 20 mM imidazole, 10% glycerol, 1 mM PMSF, and protease inhibitors). Bound protein was eluted in 10 × 350-μl fractions in elution buffer (20 mM Tris-HCl, pH 8.0, 100 mM KCl, 250 mM imidazole, 10% glycerol, 1 mM phen-
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FIGURE 1. Cigarette smoke aldehydes inhibit cytokine production. Purified human T lymphocytes were pretreated with acrolein (●), crotonaldehyde (○), or acetaldehyde (■) for 3 h and then stimulated with anti-CD3 and PMA for 24 h. All cytokine levels were measured simultaneously by Lumienx multiplex bead array using culture supernatants. Each experiment was replicated three to six times with at least three different individuals. The absolute values differ for the stimulated group due to study participant variability. Data are presented as the mean ± S.E., p < 0.05, * p < 0.01 compared with stimulated. GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; TNF, tumor necrosis factor.

For example, the unstimulated input CT value was subtracted from the unstimulated anti-p50 immunoprecipitated DNA CT value. A ΔCT value was then calculated by subtracting the ΔCt value for the control (unstimulated immunoprecipitated DNA) from the ΔCt value from each treatment (i.e., stimulated, acrolein-treated immuno-precipitated DNA). To calculate -fold differences, the ΔCT values were converted by raising 2 to the −ΔCT power. This calculation sets the p50 DNA binding basal measurement or the unstimulated, non-treated sample to 1.

Mass Spectrometry—Purified recombinant p50 (27.7 pmol) was incubated with various concentrations of acrolein, crotonaldehyde, acetaldehyde, propionaldehyde, or butyraldehyde diluted in phosphate-buffered saline (50 μl total volume) for 1 h at room temperature. Sequencing grade trypsin (2.5 μg) from Promega (Madison, WI) was added, and samples were digested at 37 °C overnight. For MALDI-TOF analysis, 1 μl of sample was deposited on a plate then co-crystallized with 1 μl of α-cyano-4-hydroxycinnamic acid matrix in acetonitrile-water (1:1) containing 1% trifluoroacetic acid. The samples were analyzed on a PE-Biosystems Voyager-DE STR MALDI operating in reflectron and positive ion mode; 256 scans were accumulated for each sample. Approximately 50% of the digested sample was analyzed by reverse phase nanospray LC-MS/MS (Agilent 1100 high performance liquid chromatography, 75 μm inner diameter × 15-cm column, Zorbax C18). Samples were loaded onto the column using 3% acetonitrile in 0.1% formic acid and washed for 2 min. Spray was induced using a capillary voltage of 1750 V applied to a fused silica emitter (PicoTip, New Objective, Inc., Woburn, MA) with an 8-μm aperture. Peptides were eluted into the mass spectrometer using a gradient of increasing buffer B (90% acetonitrile, 0.1% formic acid) at a flow rate of 300 nl/min. The gradient was ramped from 3 to 8% B in 3 min, then from 8 to 40% B over 40 min. Finally, buffer B was increased to 90% for 5 min, held for 5 min, and returned to initial conditions. Spectra were collected over a m/z range of 350–18,000 Da (Agilent LC/MSD Ultra Trap). Three MS/MS
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FIGURE 2. Acrolein inhibits NF-κB DNA binding but not AP-1 or NFAT. T lymphocytes were pretreated with acrolein (1 or 5 μM) or crotonaldehyde (10 μM) for 3 h then stimulated with anti-CD3 and PMA. Cells were harvested 2 h later, and nuclear extracts were analyzed for AP-1 and NFAT DNA binding (A) and NF-κB (B) using a DNA-based enzyme-linked immunosorbent assay. Nuclear extracts from stimulated T cells were incubated with a 20-fold molar excess of a wild-type consensus oligonucleotide showed binding comparable with unstimulated cells (10% c-Fos, 39% JunB, 43% NFATc1, 32% p50, and 8% p65). Competition with a 20-fold molar excess of a mutated consensus oligonucleotide showed binding comparable with stimulated cells (103% c-Fos, 92% JunB, 103% NFATc1, 94% p50, and 80% p65). Cells from one individual were used per experiment, and each experiment was replicated a minimum of three times. Data are presented as the mean ± S.E. *, p < 0.01 compared with stimulated. Unstim, unstimulated; croton, crotonaldehyde.

FIGURE 3. Acrolein inhibits p50 DNA binding to the IL-2 promoter in vivo. Purified T cells were pretreated with either 1 or 5 μM acrolein or 10 μM crotonaldehyde for 1 h and then stimulated with anti-CD3 and PMA for 2 h. p50 DNA binding to the −233 to −133 region of the IL-2 promoter was assessed using chromatin immunoprecipitation. Recovered DNA was quantitated by real-time quantitative PCR and is expressed as -fold change compared with the amount of DNA recovered in unstimulated cells immunoprecipitated with anti-p50. Error bars represent the S.E. of triplicate PCR reactions. The amount of DNA recovered in the negative control (no antibody) was less than 0.3 and is not shown. Croton, crotonaldehyde; Exp., experiment; Unstim, unstimulated.

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Quantitative Mass Spectrometry Analysis—Because of the global approach taken to identify modified peptides using LC-MS/MS with dynamically excluded precursor ions, a traditional quantitative approach with a labeled internal standard and integration of extracted chromatographic peaks was not possible. Instead, peak areas were calculated by multiplying signal intensity by peak width (full weight half maximum) for ions above a signal-to-noise level of 5. The peak area for the precursor of interest was divided by the sum of all peak areas to provide a ratio. The ratio was calculated for every observance of the precursor ion, which ranged from 7 times for crotonaldehyde-modified Cys-61 to 12 times for acrolein-modified Cys-61. A scale factor was determined by dividing the acrolein peak area (either averaged or the area providing the maximum ratio) by the crotonaldehyde peak area. The acrolein ratio (either averaged or maximum) was normalized by multiplying with this

spectra were collected for the three most abundant m/z values, after which those masses were excluded from analysis for 1 min, and the next three most abundant m/z values were selected for fragmentation. This maximized the dynamic range of peptides sequenced in the mixture. More than 4000 MS/MS spectra were obtained during each run. Compound lists of the resulting spectra were generated using an intensity threshold of 10,000 and a minimum of 0.2% relative abundance with grouping within 5 scans. The compound lists were exported as Mascot generic format (.mgf) files and searched against a database consisting of the His-tag p50 sequence and nine other contaminating proteins including E. coli proteins and human keratin. Parameters used in the data base search were: monoisotopic mass, peptide mass tolerance of 2 Da, fragment ion mass tolerance of 0.8 Da, and allowance of up to 2 missed tryptic cleavages. These were searched for aldehyde modifications using MASCOT (Matrix Science, Ltd.). Spectral assignments were manually confirmed, and fragment ions were required to have a minimum signal-to-noise ratio of five to be considered as observed.

Structure and Amino Acid Sequence of p50—The NF-κB1 structure (1LE5) used for modeling purposes was downloaded from the Research Collaboratory for Structural Bioinformatics (35). The mouse p50 protein sequence was used for these crystallization studies and, thus, did not align perfectly with the human protein. In this article the amino acid numbers refer to the homologous human p50 sequence and not the mouse sequence. Also, there were discrepancies in the literature regarding the numbering of p50 amino acids, in particular Cys-62. In 1993, the original p50 protein sequence (NCBI GI:189179) had an additional alanine inserted at position 40. A more recent annotated p50 sequence (NCBI GI:21542418) noted a conflict at this position, stating that the alanine had been omitted. This omission changed the p50 amino acid numbering, resulting in amino acid Cys-62 being redesignated as Cys-61.

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## Immune Suppression by Cigarette Smoke

### A. IκB phosphorylation

| Acrolein μM | NS | Stim | 0.1 | 0.5 | 1 | 2.5 | 5 | 10 |
|-------------|----|------|-----|-----|---|-----|---|----|
| p-IκB       |    |      | 4   | 100 | 107| 94  | 87 | 57 |
| Band        |    |      | 6   | 100 | 106| 99  | 77 | 49 |
| Intensity   |    |      | 120 | 120 | 118| 98  | 77 | 49 |

| Crotonaldehyde μM | NS | Stim | 0.1 | 0.5 | 1 | 2.5 | 5 | 10 |
|-------------------|----|------|-----|-----|---|-----|---|----|
| p-IκB             |    |      | 4   | 16  | 01 | 77  | 57 | 33 |
| Band              |    |      | 16  | 16  | 12 | 12  | 12 | 12 |
| Intensity         |    |      | 33  | 33  | 33 | 33  | 33 | 33 |

| Acetaldehyde μM   | NS | Stim | 0.1 | 0.5 | 1 | 2.5 | 5 | 10 |
|-------------------|----|------|-----|-----|---|-----|---|----|
| p-IκB             |    |      | 0   | 16  | 10 | 87  | 77 | 66 |
| Band              |    |      | 16  | 16  | 16 | 16  | 16 | 16 |
| Intensity         |    |      | 16  | 16  | 16 | 16  | 16 | 16 |

### B. IκB degradation

| Acrolein μM | NS | Stim | 0.1 | 0.5 | 1 | 2.5 | 5 | 10 |
|-------------|----|------|-----|-----|---|-----|---|----|
| IκB         |    |      | 100 | 100 | 88 | 77  | 66 | 55 |
| Band        |    |      | 100 | 100 | 88 | 77  | 66 | 55 |
| Intensity   |    |      | 100 | 100 | 88 | 77  | 66 | 55 |

### C. Nuclear localization

| Acrolein μM | NS | Stim | 1 | 2.5 | 5 | 10 |
|-------------|----|------|---|-----|---|----|
| p50         |    |      | 53 | 100 | 89 | 69 |
| Band        |    |      | 53 | 100 | 89 | 69 |
| Intensity   |    |      | 53 | 100 | 89 | 69 |

|      |    |      | 50 | 100 | 99 | 112| 124| 116|

|      |    |      | 65 | 100 | 99 | 112| 124| 116|

|      |    |      | 65 | 100 | 99 | 112| 124| 116|

### FIGURE 4. Acrolein inhibits IκBα signaling.** T cells were pretreated with acrolein, crotonaldehyde, or acetaldehyde for 3 h and stimulated with anti-CD3 and PMA for 5 min (IκB phosphorylation), 30 min (IκB degradation), or 2 h (NF-κB nuclear localization). A, IκB phosphorylation (p). Whole cell lysates were Western-blotted and probed with anti-phospho-IκBα. The (upper band; arrow) indicates phospho-IκBα; the lower band is non-phosphorylated IκBα. B, IκB degradation. Whole cell lysates were Western-blotted and probed with anti-IκBα to assess total cellular IκB levels. C, nuclear localization of NF-κB subunits. Extracts were Western-blotted and probed with anti-p50 or anti-p65. The quantitated band values represent the average value normalized to the loading control, either actin or nucleolin, from three experiments. NS, non-stimulated; Stim, stimulated.
RESULTS

The α,β-Unsaturated Aldehydes in Cigarette Smoke Inhibit Cytokine Production—Of all the cigarette smoke aldehydes tested, acrolein was clearly the most potent inhibitor of T cell activation. Acrolein at concentrations as low as 2.5 μM suppressed production of IL-2, IL-10, granulocyte-macrophage colony-stimulating factor, interferon-γ, and tumor necrosis factor-α by 75–99% but had no effect on production of IL-8 (Fig. 1). The other α,β-unsaturated aldehyde in cigarette smoke, crotonaldehyde, had similar effects on T cell responses but was significantly less potent. Acetaldehyde, the most abundant saturated aldehyde in cigarette smoke, did not suppress production of any cytokine tested. Other saturated aldehydes present in cigarette smoke (propionaldehyde and butyraldehyde) were also tested and found to be inactive at doses up to 1 mM (data not shown). None of the aldehydes affected cell viability as measured by 7-amino-actinomycin D at doses <10 μM.

Acrolein Inhibits Induction of NF-κB DNA Binding but Not NFAT or AP-1—The cytokines inhibited by acrolein in T cells are regulated primarily by three transcription factors, NFAT, AP-1, and NF-κB. Induction of NFAT, c-Fos, and JunB DNA binding activities by anti-CD3 and PMA were not affected by acrolein as assessed by an enzyme-linked immunosorbent assay-based oligonucleotide assay (Fig. 2A). In contrast, DNA binding of NF-κB1 (p50) in nuclear extracts from acrolein-treated cells were decreased to levels seen in unstimulated cells, and DNA binding of RelA (p65) decreased 55% (Fig. 2B). The overall levels of NF-κB or AP-1 proteins in T cells, as assessed by Western blotting, did not change in response to acrolein (data not shown), suggesting that acrolein inhibited either nuclear translocation or the ability of NF-κB to bind DNA or both. Surprisingly, crotonaldehyde had no effect on p50 or p65 DNA binding (Fig. 2B). Because both acrolein and crotonaldehyde were most effective at inhibiting IL-2 production, we performed a ChIP to assess the effects of acrolein and crotonaldehyde on in vivo p50 DNA binding activity to the IL-2 promoter (Fig. 3). Acrolein inhibited p50 binding to levels even below the basal levels seen in unstimulated T cells. Crotonaldehyde also inhibited p50 binding in vivo, and its effect was consistent with inhibition of IL-2 cytokine production.

Acrolein, Unlike Crotonaldehyde, Inhibits IkBα Activity and Nuclear Translocation of NF-κB—Activation and nuclear translocation of NF-κB is initiated by phosphorylation and subsequent degradation of its cytosolic inhibitor, IkB. Acrolein inhibited IkBα phosphorylation by 50% at 5 μM, but neither crotonaldehyde nor acetaldehyde had an effect (Fig. 4A). IkBα degradation, as assessed by measuring total IkBα protein, was also inhibited by 5 μM acrolein (Fig. 4B). Nuclear localization of p50 and p65 was also decreased at 5 μM acrolein (Fig. 4C).
Crotonaldehyde had no effect on NF-κB translocation. However, the effect of acrolein on NF-κB translocation was significantly less dramatic than its effect on cytokine production. For example, 2.5 mM acrolein inhibited production of IL-2 and interferon-γ by 90%, but nuclear localization of p50 and p65 was inhibited by 15%. These observations coupled with the observed inhibition of p50 binding in the ChIP suggested that acrolein might directly inhibit p50 DNA binding activity.

Inhibition of Recombinant p50 DNA Binding by Cigarette Smoke Aldehydes—To assess the effects of acrolein on p50 DNA binding, recombinant p50 was purified, and its DNA binding activity was analyzed by an EMSA. As can be seen in Fig. 5, acrolein was 2000-fold more potent than crotonaldehyde in blocking p50 DNA binding activity. Treatment of 27 pmol of p50 with 5 μM acrolein reduced DNA binding >99%, whereas 10 mM crotonaldehyde was required to achieve an equivalent level of inhibition. Acrolein had no effect on p50 DNA binding activity even at a dose of 10 mM. Other saturated aldehydes in cigarette smoke, including formaldehyde, propionaldehyde, and butyraldehyde, also had no effect on p50 DNA binding activity (data not shown). These data suggested that the potency of acrolein in suppressing cytokine production was related in part to its ability to bind p50 directly.

Acrolein Covalently Modifies Cys-61 and Arg-307 of p50—A combination of MALDI-TOF and LC-MS/MS mass spectrometry analysis was used to identify the acrolein-modified p50 residues. The unsaturated β-carbon of acrolein readily forms thioether adducts with cysteine residues. In addition, the carbonyl carbon could potentially react with the imidazole group of histidine, the primary amine of lysine and the guanidino group of arginine (36–41). Acrolein-modified lysines are poorly cleaved by trypsin, and lysine adducts could not be detected after trypsin digestion. Digestion of p50 with chymotrypsin or Glu-C, which are commonly used in mass spectrometry, resulted in detection of less than 14 peptides and <25% sequence coverage and no detection of any residue modified by acrolein. Improved methodologies will, therefore, be needed to detect acrolein-modified lysines. Nevertheless, we used MALDI-TOF to detect mass changes (56.06 Da for acrolein) in peptides containing modified cysteine, histidine, and arginine residues after trypsin digestion of acrolein-treated p50. Fig. 6A shows the amino acid sequence of the peptide 59–76, which contains a cysteine residue at position 61 that is known to mediate DNA binding (42). The observed mass of the singly charged ion, either acrolein addition (916.8, as indicated by a diamond) or crotonaldehyde addition (923.2, as indicated by a diamond), of p5059–76 was further fragmented, and the resulting spectra are shown. The masses of the theoretical fragment ions are listed with the observed ions underlined, and diagnostic ions used to detect modifications are shown in bold. All fragment ions diagnostic for the aldehyde modification were required to have a signal-to-noise ratio of at least 5.

A similar analysis was then performed on p50 treated with 5 mM crotonaldehyde, 100 μM acetaldehyde, or 100 μM propionaldehyde. In each treatment the unmodified [M+H]⁺ peptide of p5059–76 was further fragmented, and the resulting spectra are shown. The observed masses of the theoretical fragment ions are listed with the observed ions underlined, and diagnostic ions used to detect modifications are shown in bold. All fragment ions diagnostic for the aldehyde modification were required to have a signal-to-noise ratio of at least 5.
Cys-61 peptide, 1774.81, was detected by MALDI-TOF. Neither acetaldehyde nor propionaldehyde reacted with p50 (data not shown). However, crotonaldehyde was also detected on Cys-61 (Fig. 7B). The relative amounts of acrolein and crotonaldehyde that reacted with Cys-61 were assessed by comparing the peak areas of the [M+2H]^{2+} ions from p50 treated with equimolar amounts of the two aldehydes. Approximately twice as much acrolein reacted with Cys-61 compared with crotonaldehyde (Fig. 8). However, this difference did not account for the 2000-fold difference in their effects on p50 DNA binding activity. Analysis of all p50 peptide fragments revealed that acrolein reacted with Cys-87, Cys-118, Cys-123, Cys-261, Cys-272, Arg-230, His-306, and Arg-307, whereas crotonaldehyde reacted with Cys-87, Arg-186, Arg-213, and Arg-230 (Fig. 9). Arg-307 is also involved in p50 DNA binding (35), and alkylation of this residue by acrolein probably contributes to its increased effects on DNA binding compared with crotonaldehyde. In addition, Cys-272 and His-306 are important for interactions with p65 (35), and alkylation of these residues by acrolein may contribute to the overall chromosomal instability of the NF-κB complex. The additional cysteine and arginine residues adducted by crotonaldehyde are not known to contribute to DNA binding or p65 interactions. However, it is possible that adduction of these residues could affect the ability of NF-κB to interact with the transcriptional machinery, thereby explaining the discrepancy between the effect of crotonaldehyde on p50 DNA binding in the EMSA and ChIP.

The detected mass shift of the acrolein addition to arginine was only 54 Da, suggesting the formation of a cyclized adduct and the loss of two protons (Fig. 9C). Using NMR, we verified the structure for the reaction between N-tert-butoxycarbonyl-L-arginine methyl ester and acrolein (supplemental Figs. 1 and 2). The crotonaldehyde adduct of arginine resulted in a 72-Da shift, suggesting a non-cyclic adduct in which the carbonyl group is subsequently reduced (supplemental Fig. 3).

**DISCUSSION**

Aldehydes fall into two distinct categories based on reactivity. Acrolein and crotonaldehyde are α,β-unsaturated aldehydes with strong reactivity toward cysteine residues. Thiol reactivity is due to the presence of a positive dipole on the olefin carbon, rendering it highly susceptible to nucleophilic attack by thiolate anions. However, α,β-unsaturated aldehydes are also more strongly reactive with basic amino acids due to the dipole effect of the olefin on the carbonyl carbon. The increased reactivity of α,β-unsaturated aldehydes was clearly manifest in their ability to react with p50 and inhibit gene expression. Acrolein and crotonaldehyde reacted with cysteine, arginine, and histidine residues on the p50 subunit of NF-κB and inhibited p50 DNA binding, whereas the saturated aldehydes did not react with these residues and had no effect on DNA-binding activity.

Cys-61 is known to play a critical role in p50 DNA binding activity (42, 43). Replacement of this cysteine residue with a serine increases the dissociation rate with DNA by >500-fold (42). Both acrolein and crotonaldehyde alkylated Cys-61, but a comparison of precursor ion peak areas in the ion trap MS scan suggested that acrolein reacted with twice as much Cys-61 on a molar ratio. We hypothesize that the increased potency of acrolein is due to its additional reactivity with other amino acids in the DNA-binding domain. We detected one such
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Studies demonstrated that both the basal and activated NF-κB human alveolar macrophage cytokine production (14). Further inflammation, limiting the process by suppressing production after an immune response. Further work is needed to assess the mediator of inflammation, down-regulating T cell responses acid, threonine (53). Thus, acrolein may also be an endogenous action is the inability to detect every tryptic peptide of p50. In our analyses the sequence coverage was only 60%. There remains the possibility that other amino acids were modified that cause a biological effect but were not detected.

Other studies, which utilize different cell types, have also shown that acrolein affects cytokine release and NF-κB DNA binding activity. Treatment of 5–25 μM acrolein suppressed human alveolar macrophage cytokine production (14). Further studies demonstrated that both the basal and activated NF-κB DNA binding activity was inhibited by acrolein due to a decrease in IκBα phosphorylation and degradation (14). In contrast, inhibition of NF-κB DNA binding activity in human lung adenocarcinoma cells was independent of IκBα (15). In primary or immortalized human bronchial epithelial cells, acrolein (25 μM) suppressed both IL-8 protein and mRNA levels reportedly through inactivation of NF-κB and reduced IκBα degradation (16).

The Cys-61 residue in p50 appears to be a target of pharmaceutical and endogenous regulators of NF-κB (44–49). 15-Deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2) is a metabolite of prostaglandin D2 that reacts with cysteines via a Michael addition. 15d-PGJ2 is thought to be an important feedback regulator of inflammation, limiting the process by suppressing production of tumor necrosis factor-α and IL-1β. Like acrolein, 15d-PGJ2 may inactivate NF-κB by alkylating Cys-61 (45, 50). 15d-PGJ2 also inhibits NF-κB DNA binding at concentrations that did not inhibit nuclear localization. Both acrolein and 15d-PGJ2 induce IL-8 production, thereby conferring both proinflammatory and anti-inflammatory properties (51, 52). Thus, acrolein may exert its effects on pulmonary immunity by mimicking a natural inflammatory mediator. In fact, acrolein itself is produced by neutrophils in high concentration as a result of the effects of myeloperoxidase, H₂O₂, and Cl⁻ on the free amino acid, threonine (53). Thus, acrolein may also be an endogenous mediator of inflammation, down-regulating T cell responses after an immune response. Further work is needed to assess the regulatory roles of these α,β-unsaturated keto compounds in pulmonary immunity.

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