The Tobacco Smoke Component, Acrolein, Suppresses Innate Macrophage Responses by Direct Alkylation of c-Jun N-Terminal Kinase

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The respiratory innate immune system is often compromised by tobacco smoke exposure, and previous studies have indicated that acrolein, a reactive electrophile in tobacco smoke, may contribute to the immunosuppressive effects of smoking. Exposure of mice to acrolein at concentrations similar to those in cigarette smoke (5 ppm, 4 h) significantly suppressed alveolar macrophage responses to bacterial LPS, indicated by reduced induction of nitric oxide synthase 2, TNF- α , and IL-12p40. Mechanistic studies with bone marrow-derived macrophages or MH-S macrophages demonstrated that acrolein (1–30 μ M) attenuated these LPS-mediated innate responses in association with depletion of cellular glutathione, although glutathione depletion itself was not fully responsible for these immunosuppressive effects. Inhibitory actions of acrolein were most prominent after acute exposure (<2 h), indicating the involvement of direct and reversible interactions of acrolein with critical signaling pathways. Among the key signaling pathways involved in innate macrophage responses, acrolein marginally affected LPS-mediated activation of nuclear factor (NF)- κ B, and significantly suppressed phosphorylation of c-Jun N-terminal kinase (JNK) and activation of c-Jun. Using biotin hydrazide labeling, NF- κ B RelA and p50, as well as JNK2, a critical mediator of innate macrophage responses, were revealed as direct targets for alkylation by acrolein. Mass spectrometry analysis of acrolein-modified recombinant JNK2 indicated adduction to Cys⁴¹ and Cys¹⁷⁷, putative important sites involved in mitogen-activated protein kinase (MAPK) kinase (MEK) binding and JNK2 phosphorylation. Our findings indicate that direct alkylation of JNK2 by electrophiles, such as acrolein, may be a prominent and hitherto unrecognized mechanism in their immunosuppressive effects, and may be a major factor in smoking-induced effects on the immune system.

Keywords: cigarette smoking; electrophile; inflammation; glutathione; NF-E2-related factor 2

The respiratory tract is equipped with a fine-tuned immune system to adequately respond to inhaled pathogens, and to tolerate

CLINICAL RELEVANCE

Our findings describe a previously unrecognized mechanism by which electrophiles, such as acrolein, a major component in tobacco smoke, can suppress innate macrophage responses, which involves direct alkylation of c-Jun N-terminal kinase and inhibition of its signaling. These findings offer new insights into the diverse health effects associated with cigarette smoking, and may also be relevant to reported anti-inflammatory actions of other electrophiles.

many harmless airborne animal or plant proteins to avoid chronic inflammatory responses to these agents. As such, immune responses to inhaled pathogens and allergens are orchestrated by complex interactions between various lung cell types, including lung epithelial cells and resident alveolar macrophages (AMs), which form a first-line general defense, and lung dendritic cells (DCs), which survey inhaled antigens and initiate adaptive immune responses (1). Cigarette smoking and exposure to environmental tobacco smoke remain major global health burdens, and are strongly associated with increased childhood respiratory infections and development of chronic lung diseases, such as chronic obstructive pulmonary disease, asthma, and lung cancer. The health effects of cigarette smoke (CS) are largely related to its adverse effects on innate or adaptive immune responses, leading to impaired innate immune responses, and reduced host defense or tumor surveillance, as well as predisposition to development of allergic diseases, such as asthma (2). Transcriptional profiling of AMs in smokers has indicated a marked macrophage reprogramming in smokers (3, 4), with suppression of inflammatory genes associated with classical M1-related inflammatory/immune genes and induction of genes associated with various M2-polarization programs relevant to tissue remodeling and immunoregulation, findings that are consistent with reduced innate immune responses and increased susceptibility to respiratory infections in smokers or CS-exposed individuals (2, 5). Actions of CS on airway epithelial cells affect their production of inflammatory or host defense mediators (6–9) and their interactions with DCs (10, 11). Furthermore, direct actions of CS on DCs may affect DC maturation (12) and polarize T helper (Th) 2 immune responses (13, 14).

Due to its complex nature, the mechanistic details by which CS affects these immune responses are incompletely understood, although oxidative mechanisms are typically implicated based on observations of altered pulmonary glutathione (GSH) status and protective effects of thiol-based antioxidants (14–16). Detailed analysis of CS-induced GSH modifications, however, revealed relatively minimal GSH oxidation, and the main fate of GSH is its alkylation by abundant α,β -unsaturated aldehydes within CS, most notably, acrolein (2-propenal) and

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crotonaldehyde (2-butenal) (17, 18). Mainstream CS contains over 90 ppm acrolein (19), and indoor levels of acrolein can be as high as 1 mg/m³ (0.2 ppm), especially in smoking areas (20, 21). Estimates of acrolein within airway secretions or tracheal aspirates from smokers indicate its presence at 1–10 μ M (22, 23), concentrations that are sufficient to induce major functional effects on several cell types. Acrolein is itself recognized as one of the greatest noncancer health risks of all organic air pollutants, and has been associated with decreased respiratory function in the United States (24, 25).

A number of studies demonstrate that inhalation of acrolein at relevant concentrations causes respiratory changes that are also characteristic of CS exposure, including epithelial alterations and compromised antibacterial and antiviral host defenses (21, 26–28). Acrolein is known to be capable of suppressing macrophage activation (21), and our previous studies indicate that *in vivo* exposure of mice to acrolein leads to reduced innate immune responses to LPS (29), similar to previously reported effects of CS. Although the biochemical mechanisms involved in these immunosuppressive effects are incompletely understood, they were associated with impaired NF- κ B signaling (29). Based on its chemical reactivity, the cellular effects of acrolein are mediated by depletion of cellular GSH and indirect dysregulation of redox signaling pathways, or by interference with cellular processes by direct alkylation of nucleophilic targets within critical proteins. Moreover, immunosuppressive effects of various electrophiles, including acrolein, are also strongly associated with activation of NF-E2-related factor 2 (Nrf2) and induction of anti-inflammatory genes (30–32). The present studies were designed to further detail the impact of acrolein exposure on AM responses and the mechanisms involved. Our findings demonstrate that acrolein exposure mimics the effects of cigarette smoking by selectively suppressing innate M1-polarized AM responses and favoring M2 polarization. These inhibitory actions are primarily mediated by acute actions related to GSH depletion and direct alkylation of critical proteins involved in NF- κ B and activator protein 1 (AP-1) activation. Most notably, our studies reveal alkylation of selective cysteines within c-Jun N-terminal kinase (JNK) 2 as a previously unrecognized mechanism involved in the immunosuppressive actions of acrolein.

MATERIALS AND METHODS

Mouse Exposure to Acrolein

Male C57BL/6J mice (6–8 wk old; Jackson Laboratories, Bar Harbor, ME) were placed in a small cylindrical glass chamber (part no. X02AI99C15A57H5; Specialty Glass, Houston, TX) and exposed to vaporized acrolein (Fluka BioChemika, Buchs, Switzerland) for 4 hours at a concentration of 5 ppm (11.5 mg/m³) (29). After exposure, AMs were obtained by bronchoalveolar lavage, involving four washes of 0.5 ml sterile PBS, collected by centrifugation (1,500 rpm; 5 min) and used for *in vitro* experiments and analysis.

In Vitro Macrophage Studies

Resuspended AMs in RPMI medium (1×10^5 cells/100 μ l), bone marrow-derived macrophages (BMDMs; 1×10^6 cells/ml), isolated and cultured as described previously (33), or MH-S macrophages (ATCC, Manassas, VA) were treated with acrolein (1–30 μ M) to achieve an exposure level of 1–30 nmol acrolein/10⁶ cells. After exposure to acrolein, cells were stimulated with LPS (0.1 μ g/ml), IFN- γ (1,000 U/ml), or IL-4 (10 U/ml), and cells and media were harvested for the various analyses outlined subsequently here. Pharmacological inhibitors were added 15 minutes before cell stimulation by LPS. Cellular GSH was depleted by preincubation with 100 μ M buthionine sulfoximine (Sigma, St. Louis, MO) for 18 hours, or supplemented by preincubation with 1 mM glutathione ethyl ester (GEE) (Sigma) for 18 hours, before treatment with acrolein.

Biochemical Analyses of Mediator Production and GSH

Conditioned media were collected after 24 hours for analysis of NO₂[−] using the Griess reagent (34), or IL-12p40, TNF- α , or IL-10 using ELISA (R&D Systems, Minneapolis, MN; BD Biosciences, San Diego, CA). RNA was extracted (Qiagen, Valencia, CA) for RT-PCR analysis of nitric oxide synthase (NOS) 2, IL-12 p40, TNF- α , and IL-10 mRNA. Cell lysates were prepared for analysis of NOS2 protein by Western blot, arginase activity (35), or cellular GSH (36). Cell lysates were also analyzed by SDS-PAGE and Western blot using polyclonal antibodies against (phosphorylated) inhibitor of κ B kinase β (I κ B β), inhibitor of NF- κ B α (I κ B α), extracellular signal-regulated kinase (ERK)1/2, or c-Jun-N-terminal kinase (JNK)1/2 (1:1,000; Cell Signaling, Danvers, MA), detected using horseradish peroxidase-conjugated secondary antibodies (Cell Signaling) and enhanced chemiluminescence (Pierce, Rockford, IL). Nuclear extracts were prepared using a Nuclear Extract Kit (Active Motif, Carlsbad, CA) for analysis of DNA binding activity of NF- κ B, or c-Jun using TransAM NF- κ B p65 and TransAM AP-1 c-Jun ELISA kits (Active Motif).

Identification of Protein–Acrolein Adducts

Michael addition of acrolein with proteins was determined by derivatization with biotin hydrazide (BH) and purification of BH-labeled proteins using avidin chromatography (37) for analysis by SDS-PAGE and Western blotting. Alternatively, proteins of interest were immunoprecipitated from BH-derivatized cell lysates and analyzed by SDS-PAGE and visualization of biotinylated proteins with streptavidin–horseradish peroxidase.

Mass Spectrometry Analysis of Acrolein Adduction of Recombinant JNK2

Human recombinant JNK2 α 2 (accession no. NP_002743; Invitrogen, Carlsbad, CA) was reacted with acrolein and after stabilization with NaBH₄ and removal of excess reagents, the protein was alkylated with iodoacetamide and digested using trypsin and/or Glu-C for peptide analysis by liquid chromatography–electrospray ionization–tandem mass spectrometry (LC–ESI–MS/MS) (Thermo Electron, Waltham, MA). Obtained MS spectra were searched against a single JNK2 α 2 protein sequence database using SEQUEST (Bioworks software, v3.3.1; Thermo Electron).

Statistical Analysis

Quantitative data from at least three separate experiments are presented as mean values ($\pm$ SE), and analyzed using two-tailed Student's *t* test. Differences were considered statistically significant at *P* less than 0.05.

RESULTS

Acrolein Inhalation *In Vivo* Alters AM Responsiveness to LPS and IFN- γ

To determine the effect of *in vivo* acrolein inhalation on innate macrophage responses to common stimuli, we exposed C57BL/6J mice to acrolein (5 ppm, 4 h) and collected AMs for *in vitro* stimulation with LPS or a combination of LPS plus IFN- γ . As shown in Figure 1, stimulation of AMs with LPS and, especially LPS/IFN, led to robust induction of innate immune responses illustrated by induction of NOS2 and production of NO₂[−] (a measure of NO production), as well as the inflammatory cytokines, TNF- α and IL-12p40. Each of these responses was significantly reduced in AMs obtained from acrolein-exposed animals (Figures 1A–1D). Because these mediators are indicative of classical M1 macrophage activation, and cigarette smokers display features of alternative M2-favored macrophage polarization, we also evaluated AMs for two markers of M2 macrophage activation, IL-10 and arginase. As shown, AMs from acrolein-exposed mice displayed slight increases in basal or LPS/IFN-

Figure 1. *In vivo* acrolein exposure alters alveolar macrophage (AM) responsiveness to LPS/IFN- γ . C57BL/6J mice were exposed to acrolein *in vivo* (5 ppm, 4 h) and AMs were collected and stimulated *in vitro* with LPS (0.1 μ g/ml) and/or IFN- γ (1,000 U/ml) for 24 hours. Cell lysates were analyzed for nitric oxide synthase (NOS) 2 protein expression (A), and media were analyzed for accumulation of NO₂⁻ (B), IL-12p40 (C), TNF- α (D), or IL-10 (E) by Griess assay or ELISA. Cell lysates were also analyzed for arginase activity using the urea method (F). * P < 0.05 compared with untreated control groups; # P < 0.05 compared with corresponding air control. ACR, acrolein.

elicited IL-10 production, but did not induce significant changes in arginase activity (Figures 1E and 1F). Thus, acrolein exposure primarily results in suppression of innate M1 macrophage responses, whereas M2 responses are largely unaffected.

Acrolein Exerts Direct Immunosuppressive Effects on Murine Macrophages

To determine whether these immunosuppressive effects are due to direct actions of acrolein on macrophages, isolated AMs or BMDMs were exposed to acrolein *in vitro* before stimulation with LPS or IFN- γ . As expected, exposure of BMDMs to acrolein caused dose-dependent inhibition of LPS-mediated induction of NOS2 (Figure 2A) and production of NO₂⁻ (Figure 2B) as well as IL-12p40 and TNF- α (Figure 2C). In apparent contrast to Figure 1E, acrolein suppressed LPS-induced IL-10 production *in vitro*, possibly reflecting differences between AMs and BMDMs in their responses to LPS or differences in effects of acrolein exposure *in vivo* compared with *in vitro* treatment. Qualitatively similar findings were obtained using AMs, with marked suppression of LPS-induced NO₂⁻, IL-12p40, and TNF- α by acrolein, although production of IL-10 was not significantly altered (data not shown). It should be noted that acrolein up to 30 μ M (the highest concentration tested) did not significantly reduce cell viability, measured by analysis of lactate dehydrogenase (LDH) release or CellTiter-Blue cell viability assay (data not shown). Analysis of mRNA levels of NOS2, IL12p40 (the inducible component of IL-12 and IL-23 [38]), and TNF- α indicated that acrolein exposure inhibited these innate immune responses primarily at the transcriptional level (*see* Figure E1 in the online supplement).

The most marked reduction was seen with respect to LPS-induced production of NO₂⁻ and IL-12p40, both characteristic features of classical M1 macrophage responses. To further explore potentially selective effects of acrolein on M1 or M2

macrophage responses, we investigated the effects of acrolein on BMDM stimulation by the Th1 cytokine, IFN- γ , or the Th2 cytokine, IL-4, which leads to induction of NOS2 or arginase as indicators of M1 and M2 responses, respectively (35). BMDM pre-exposure to acrolein (10 μ M) inhibited IFN-induced increases in NO₂⁻ production, but had no effect on IL-4-mediated induction of arginase activity (Figure E2). Higher concentrations of acrolein (>30 μ M) also suppressed the induction of arginase (data not shown). Thus, these findings indicate that acrolein, at intermediate doses, selectively suppresses classical M1 macrophage responses, and may thereby contribute to the development of altered macrophage polarization in favor of M2 phenotype.

Role of GSH Alterations in Immunosuppressive Effects of Acrolein

Based on the chemical reactivity of acrolein, its cellular effects are most likely due to reactions with cellular nucleophiles, such as GSH. Depletion of cellular GSH may have indirect consequences for immune responses, by impairing GSH-dependent regulation of redox-dependent signaling pathways or by reducing antioxidant mechanisms and increasing cellular oxidant production. Acrolein exposure resulted in dose-dependent depletion of cellular GSH (Figure 3A) at doses similar to those necessary to suppress immune responses (Figure 2). To determine whether GSH depletion *per se* could be responsible for the immunosuppressive effects of acrolein, we depleted BMDM GSH by preincubation with 100 μ M buthionine sulfoximine (an inhibitor of GSH synthesis [37]) for 24 hours before LPS stimulation. Depletion of GSH to less than 10% of initial levels (Figure 3B) slightly reduced LPS-induced production of NO₂⁻ and IL-12p40, but did not significantly affect TNF- α production (Figure 3C). Conversely, elevation of BMDM GSH by cell loading with the cell-permeable GEE (1 mM) slightly enhanced LPS-induced IL-

Figure 2. Acrolein dose-dependently inhibits innate macrophage responses *in vitro*. Bone marrow-derived macrophages (BMDMs) were exposed to acrolein (0–30 μM) for 10 minutes and then stimulated with LPS (0.1 mg/ml) or IFN-γ (1,000 U/ml) for 24 hours. Induction of NOS2 protein (A) and accumulation of NO₂⁻ (B) or inflammatory cytokines (C) were analyzed as in Figure 1. **P* < 0.05 compared with untreated control groups; #*P* < 0.05 compared with corresponding control (CTL).

IL-12p40 production by LPS, but did not significantly affect production of NO₂⁻ or TNF-α (Figure 4C). Thus, although GSH depletion may account for some of the actions of acrolein, the dramatic immunosuppressive effects of acrolein are largely due to mechanisms independent of GSH depletion and indirect alteration of GSH-regulated processes, and likely involve direct actions on other susceptible cellular pathways.

Immunosuppressive Effects of Acrolein Are Primarily due to Acute Actions Independent of Nrf2-Mediated Gene Expression

A number of studies have indicated that immunosuppressive effects of electrophiles are closely associated with activation of Nrf2 and induction of anti-inflammatory genes, such as HO-1 or NQO1 (39, 40). Indeed, acrolein was found to readily induce the Nrf2-responsive protein, HO-1, in BMDMs, which was detectable as early as 4 hours after acrolein treatment (Figure 4A). To address the potential importance of Nrf2 activation and induction of anti-inflammatory genes in the immunosuppressive actions of acrolein, we performed time-dependent studies in which BMDMs

Figure 3. Importance of glutathione (GSH) on macrophage responses. (A) Analysis of cellular GSH levels after BMDM exposure to indicated doses of acrolein for 15 minutes. (B) Effect of preincubation with buthionine sulfoxide (BSO; 100 μM; 18 h) or glutathione ethyl ester (GEE; 1 mM, 18 h) on cellular GSH levels. (C) Effect of BSO or GEE pretreatment on BMDM production of NO₂⁻, IL-12p40, or TNF-α in response to LPS. **P* < 0.05 compared with corresponding controls.

were pretreated for various periods of time (30 min to 8 h) with acrolein before cell stimulation with LPS, to distinguish between acute and more delayed mechanisms. As shown in Figure 4B, the ability of acrolein to suppress LPS-mediated induction of NOS2 and IL-12p40 was most pronounced after acute treatment (30 min to 2 h), preceding the induction of HO-1, and was less pronounced after more prolonged acrolein treatment (<4 h). This finding indicates that acrolein suppresses immune responses primarily by acute mechanisms independent of Nrf2-mediated induction of anti-inflammatory genes, although Nrf2-dependent mechanisms are likely to be involved in the more prolonged immunosuppressive effects of acrolein.

Sulforaphane (SFN), a cruciferous-derived isothiocyanate, is a potent anti-inflammatory and chemopreventative agent, and is thought to act primarily by activating Nrf2 (41, 42). We investigated the immunosuppressive actions of SFN by preincubating BMDMs with SFN for 30 minutes or 8 hours before stimulation with LPS. As shown in Figure E3, suppression of LPS-induced production of NO₂⁻ or IL12p40 by SFN was more pronounced at 30 minutes than at 8 hours, at which time HO-1 induction was apparent. Thus, similar to acrolein, SFN has acute immunosuppressive actions that appear to be largely unrelated to Nrf2-mediated anti-inflammatory gene induction.

Acrolein Inhibits LPS-Induced Activation of NF-κB and Mitogen-Activated Protein Kinase Signaling Pathways

LPS-induced production of NOS-2, as well as IL-12p40 and TNF-α, involves activation of NF-κB (43), a redox-sensitive

Figure 4. Time-dependent effects of acrolein on BMDM immune responses. (A) BMDMs were pre-exposed to acrolein for various time points (30 min to 8 h) before stimulation with LPS, and production of NO₂⁻ and IL-12p40 were measured after 24 hours. Data represent mean values (±SE) from two experiments in duplicate. (B) BMDMs were treated as in (A), and induction of NOS2 or HO-1 (NF-E2-related factor 2 [Nrf2]-responsive gene) was determined in cell lysates by Western blot. **P* < 0.05 compared with untreated control; #*P* < 0.05 compared with corresponding LPS control.

transcription factor that is susceptible to inhibition by electrophiles, such as acrolein (44, 45). Therefore, we explored the effects of acrolein on NF-κB signaling by monitoring phosphorylation and degradation of IκBα and by analysis of NF-κB activity in nuclear extracts. Acrolein treatment of BMDMs was

found to slightly inhibit LPS-induced IκBα phosphorylation, although it did not acutely prevent IκBα degradation (Figure 5A). More prolonged exposure to acrolein (>4 h) slightly prevented LPS-induced IκBα degradation (Figure 5B), which might be associated with increased Nrf2-dependent induction of anti-inflammatory genes, such as HO-1. Both acute and chronic acrolein treatment tended to inhibit LPS-induced activation of NF-κB binding activity (Figure 5C), but this was not statistically significant.

Innate immune responses by LPS are also mediated by AP-1 through stimulation of mitogen-activated protein kinase pathways, such as JNK and ERK. Although acrolein is capable of stimulating JNK and ERK (46, 47), acrolein pretreatment of BMDMs was found to dose-dependently inhibit LPS-mediated phosphorylation of JNK and ERK (Figure 5A). Again, the inhibitory actions of acrolein were more pronounced after acute treatments (<4 h), and less prominent after prolonged exposure (8 h) (Figure 5B). Similarly, acute acrolein also significantly attenuated LPS-mediated nuclear accumulation of active c-Jun, which was not seen after more delayed treatment (>4 h) (Figure 5C).

The importance of ERK and JNK activation in LPS-induced production of NO₂⁻, IL-12p40, or TNF-α was explored using pharmacological inhibitors. Inhibition of ERK phosphorylation using U0126 did not affect LPS-induced NOS2 induction and NO₂⁻ production (Figures 6A and 6B), but slightly increased IL-12p40 production (Figure 6C), and inhibited LPS-mediated production of TNF-α (Figure 6D). Thus, the immunosuppressive actions of acrolein cannot be fully explained by its inhibitory effects on ERK signaling. In contrast, LPS-induced production of NOS2 and NO₂⁻ (Figures 6A and 6B) and TNF-α (Figure 6D) were significantly inhibited in the presence of the JNK inhibitor, SP600125. This inhibitor slightly reduced IL-12p40 production as well, although this didn't reach statistical significance (Figure 6C). These findings implicate inhibition of JNK activation as a potential common mechanism involved in its immunosuppressive effects by acrolein. Neither inhibitor significantly affected acrolein- or LPS-mediated induction of HO-1 (Figure 6A). In addition, the Nrf2 activator, SFN, was also found to acutely inhibit LPS-induced IκB degradation

Figure 5 Effects of acrolein on inflammatory signaling pathways. (A) Dose-dependent effect of acrolein pretreatment on LPS-mediated phosphorylation and degradation of inhibitor of NF-κB α (IκBα) (top) and extracellular signal-regulated kinase (ERK)1/2 and c-Jun N-terminal kinase (JNK)1/2 (bottom), evaluated by Western blot. (B) Time-dependent effect of acrolein pretreatment (30 min to 8 h) on LPS-mediated phosphorylation and degradation of IκBα and phosphorylation of JNK. (C) Time-dependent effect of acrolein pretreatment (30 min to 8 h) on DNA binding activity of RelA or c-Jun, evaluated using an ELISA-based DNA binding assay (Activ Motif). #*P* < 0.05 compared with corresponding LPS control.

Figure 6. Involvement of ERK and JNK in macrophage LPS responses. BMDMs were preincubated with the mitogen-activated protein kinase (MAPK) kinase (MEK) inhibitor, UO126 (1 μM), or the JNK inhibitor, SP600125 (10 μM), for 15 minutes before exposure to acrolein (10 μM) and/or LPS (0.1 μg/ml). Protein expression of NOS2 and HO-1 (A), and accumulation of NO₂⁻ (B), IL-12p40 (C), or TNF-α (D), were analyzed after 24 hours by Western blot or ELISA, respectively. #*P* < 0.05 compared with corresponding LPS control.

and JNK phosphorylation (Figure E4), indicating that this isothiocyanate is capable of inhibiting inflammation independent of Nrf2-dependent mechanisms.

Acrolein Causes Direct and Transient Alkylation of NF-κB and JNK

Because the immunosuppressive effects of acrolein involved acute inhibitory actions on NF-κB and JNK signaling, we wanted to determine whether these inhibitory effects are related to direct modification of proteins in these signaling pathways using BH labeling to detect protein-bound aldehydes formed by Michael addition. As illustrated in Figure 7B, BH labeling of cell lysates from acrolein-exposed MH-S macrophages revealed increased abundance of biotinylated proteins, especially after acute acrolein exposure. BH labeling after more prolonged acrolein treatment indicated that the abundance of biotinylated proteins gradually reversed to basal levels after 8–24 hours. This indicates that acrolein exposure results in rapid carbonylation of a range of proteins, which gradually disappear by reversal of protein carbonylation or by enhanced protein turnover.

To determine whether specific proteins within NF-κB or JNK and signaling pathways were directly alkylated by acrolein, BH-labeled proteins were purified by avidin chromatography and analyzed by Western blotting for proteins of interest. As shown in Figure 7C, such analysis revealed the alkylation of NF-κB RelA and p50, primarily in response to acute acrolein exposure, consistent with previous studies demonstrating these proteins as direct targets for acrolein (44). In addition to NF-κB RelA and p50, we also identified JNK, and primarily JNK2, as a target for carbonylation by acute acrolein exposure (Figure 7C). The fact that these biotinylated proteins were not detected after more prolonged acrolein exposure indicates that alkylation of these proteins by acrolein is transient, and is primarily related to the acute immunosuppressive effects of acrolein. To confirm that NF-κB or JNK were not merely copurified with other BH-labeled proteins and, indeed, directly alkylated and biotinylated, we purified RelA or JNK from BH-labeled cell lysates by immunoprecipitation, followed by SDS-PAGE and detection of BH labeling by streptavidin blotting. Again, this

revealed the presence of both RelA and JNK as carbonylated proteins in response to acrolein exposure (Figure 7D).

We next determined whether such alterations in NF-κB or JNK signaling and alkylation of RelA or JNK were also detectable in AMs from mice after *in vivo* acrolein exposure. AMs were collected from acrolein- or air-exposed mice (5 ppm, 4 h), and stimulated with LPS or LPS/IFN-γ *in vitro* to monitor IκB degradation and JNK phosphorylation. As shown in Figure 8A, AMs from acrolein-exposed mice showed similar IκB degradation, but markedly reduced JNK phosphorylation in response to LPS or LPS/IFN-γ, compared with AMs from air-exposed mice. AMs from air- or acrolein-exposed mice were also subjected to BH labeling for analysis of protein-acrolein adducts, which revealed increased levels of carbonylated proteins in AMs from acrolein-exposed mice (Figure 8B). Furthermore, Western blot analysis of avidin-purified, BH-labeled proteins demonstrated increased alkylation of RelA and JNK2 (Figure 8C), consistent with *in vitro* findings with MH-S macrophages. Collectively, these findings demonstrate that both NF-κB RelA and, especially, JNK2 are direct AM targets for alkylation by acrolein, which may be responsible for reduced enzymatic activity or DNA binding and impaired innate macrophage responses to LPS.

Identification of Site of Alkylation within JNK2 by MS

To identify the sites of acrolein adduction within JNK2, recombinant JNK2α2 was reacted with acrolein, and protein modifications were identified by LC-MS/MS after trypsin digestion. As shown in Table 1, a large number of peptides could be identified upon protein digestion with trypsin and Glu-c, representing 48% coverage of the total protein. The amino acid sequence of JNK2α2 and detected peptide coverage is illustrated in Figure E5A. Of the various available cysteines within JNK2, two cysteine residues were found to be adducted by acrolein—Cys⁴¹ and Cys¹⁷⁷—based on a molecular weight increase of 58 (representing adduction of acrolein and reduction to the corresponding alcohol) (Table 1). Further confirmation of acrolein adduction to these cysteine residues was obtained by evaluation of b and y ions in MS2 spectra of these two Cys-containing peptides (Figures E5B and E5C). Nearly identical peptide coverage was obtained using unreacted

Figure 7. Identification of protein adduction by acrolein in MH-S macrophages. MH-S cells were exposed to 30 μ M acrolein for the indicated times, and biotin hydrazide (BH) labeling was used to derivatize carbonylated proteins in cell lysates. (A) Schematic illustration of BH labeling of protein carbonyls formed by Michael addition of acrolein with protein cysteine residues. (B) Visualization of biotin-labeled proteins in lysates from acrolein-treated MH-S cells by SDS-PAGE and blotting with streptavidin–horseradish peroxidase (HRP). (C) Identification of RelA, p50, and JNK as targets for acrolein adduction by avidin purification of biotin-labeled proteins and Western blot analysis. (D) RelA or JNK were immunoprecipitated from BH-derivatized cell lysates, and their degree of biotin labeling was determined by blotting with streptavidin–HRP. IP, immunoprecipitation.

JNK2 protein, except that no acrolein modifications were detected.

DISCUSSION

Chronic lung diseases associated with cigarette smoking are often associated with alterations in innate and adaptive immune responses, with altered macrophage or DC phenotypes and increased susceptibility to respiratory infections, which may contribute to chronic lung diseases, such as chronic obstructive pulmonary disease (2). One aspect of such altered immune response is an altered ability of AMs or DCs to respond appropriately to common infectious stimuli due to attenuated M1 or Th1 activation and increased M2 or Th2 polarization (3, 48). Such alterations in macrophage or DC responses have been associated with immunoregulatory actions of nicotine (49, 50), but also are noted to involve redox-dependent mechanisms, demonstrated by findings that CS-mediated alterations in AM phenotype and function are linked to changes in GSH, and can be prevented by approaches to restore GSH status (14–16). Our results point to a prominent role for the reactive electrophile, acrolein, as exposure to acrolein readily suppresses innate AM responses, primarily M1-polarized responses characterized by production of NOS2, IL-12, and TNF- α (29). Moreover, similar to reported effects of smoking (3, 4), acrolein exposure was found to primarily attenuate macrophage M1 responses, with relatively

little effect or even stimulatory actions on anti-inflammatory M2 responses. Therefore, acrolein or related electrophiles present within tobacco smoke may be among the most prominent factors contributing to disrupted innate immune responses associated with smoking.

The immunosuppressive actions of acrolein are shared by other electrophiles, such as curcumin or SFN, which are being promoted as potent food-derived anti-inflammatory agents. One common property of these electrophiles is the ability to activate Nrf2 and to induce anti-inflammatory genes, such as HO-1 and NQO1. Although activation of Nrf2 has indeed been demonstrated to be of importance in the immunosuppressive actions of electrophiles, our present results point to more acute mechanisms related to depletion of GSH and resulting effects on redox-dependent signaling mechanisms. Alterations in cellular GSH status by inhibiting its synthesis or GSH supplementation did, indeed, impact on macrophage production of NOS2 and IL-12p40 in response to LPS, although production of TNF- α was not affected. Thus, depletion of GSH may account for some, but not all, of the immunosuppressive actions of acrolein. Our results indicate that acrolein also acutely inhibits LPS-induced activation of NF- κ B and AP-1, which was associated with modification of proteins involved in these inflammatory signaling pathways. Indeed, our present data reveal that both RelA and JNK2 are directly and transiently alkylated by acrolein, which may account for much of its immunosuppressive effects. The

Figure 8. Identification of acrolein adduction of macrophage RelA and JNK after *in vivo* acrolein exposure. Mice were exposed to 5 ppm acrolein or air for 4 hours, and AMs were collected for *in vitro* LPS stimulation (0.1 μ g/ml; 10 min) and analysis of phosphorylation of I κ B α or JNK (A). AMs from acrolein-exposed mice were lysed and derivatized with BH for analysis of total carbonylated proteins by streptavidin blotting (B) or Western blot analysis of purified biotinylated proteins or input lysates for RelA or JNK (C).

more prolonged effects of acrolein on immune responses (>4 h) are likely to be independent of these direct, reversible modifications, and are probably associated with induction of anti-inflammatory genes (e.g., HO-1) by activation of Nrf2.

The importance of NF- κ B in LPS-mediated inflammatory mediator production is well established, and the NF- κ B pathway has previously been shown to be subject to inhibition by various electrophiles, including acrolein (44, 51–54). Moreover, these inhibitory actions have been associated with direct alkylation of IKK β , an upstream kinase that mediates NF- κ B activation, or of the NF- κ B subunits, RelA and p50, which interferes with its transcriptional activity. In agreement with recent findings by Lambert and colleagues (44), we observed direct alkylation of NF- κ B RelA as well as p50. However, although IKK β , the enzyme responsible for phosphorylation and subsequent degradation of I κ B α , is subject to inhibition by various electrophiles and thiol-reactive agents (53–55), we did not observe detectable alkylation of IKK β in acrolein-exposed macrophages (data not shown), consistent with the relatively modest effects of acrolein on I κ B α phosphorylation.

In contrast to the relatively modest effects of acrolein on NF- κ B activation, acrolein exposure had more profound effects on LPS-mediated activation of ERK and JNK signaling, which are also important in regulating innate immune responses (14, 56). Most notably, pharmacologic inhibition of JNK almost completely prevented LPS-mediated production of NOS2/NO and

TABLE 1. MASS SPECTROMETRY ANALYSIS OF ACROLEIN-MODIFIED C-JUN N-TERMINAL KINASE 2

Peptide Sequence	MH ⁺	Δ M	z	XC
K.LSRPFQNTQTHAK.R	1,426.75497	0.00281	3	4.65
K.TSQARDLLSK.M	1,118.61641	0.00217	2	2.78
K.MLVDPDKR.I	1,086.59759	0.00011	2	3.21
K.EVMDWEER.S	1,093.46189	0.00240	2	2.51
R.TAC#TNFMMTPYVTR.Y	1,791.82270	0.00633	2	4.46
R.TAC@TNFMMTPYVTR.Y	1,792.80120	0.04724	2	4.41
K.NIISLLNVFTPK.T	1,486.86279	0.00751	3	4.73
R.YQQLKPIGSGAQGIVC#AAFDTVLGINAVVK.L	3,245.77152	0.03258	4	4.95
K.ENVDIWSVGC#IMGELVK.G	1,948.95073	0.01367	2	2.96
R.YQQLKPIGSGAQGIVC@AAFDTVLGINAVVK.K	3,118.65505	0.04524	3	3.07
E.HAIEEWKE.L	1,041.49999	0.00079	2	2.75
E.NRPKYPGKFEEL	1,477.77979	0.00052	2	3.09
E.FMKKLQPTVRNYVE.N	1,752.94654	0.00517	2	3.13
E.VILGMGYKE.N	1,009.53868	0.00122	2	3.38
E.LVKGK#VIFQGTDHIDQWNKVE.Q	2,599.33376	0.00696	4	3.73
E.FQDYLVLME.L	1,143.53907	0.00365	1	2.37
E.NVDIWSVGC#IMGEL	1,479.66070	0.00622	2	3.14
E.LVLLKC#VNHKNIISLLNVFTPKTLE.E	3,034.74860	0.01717	4	2.20
E.LVLLKC#VNHKNIISLLNVFTPKTLEE.F	3,163.79119	0.01633	4	2.39
E.LFPDWIFPSESE.R	1,466.68382	0.00575	1	3.39

Definition of abbreviations: Δ M, difference between theoretical and actual peptide mass; MH⁺, protonated peptide mass; XC, cross-correlation score; z, peptide charge.

Recombinant c-Jun N-terminal kinase 2 α 2 was treated with acrolein, digested with trypsin and Glu-C and analyzed by liquid chromatography–tandem mass spectrometry. Cysteines detected either contain a carboxyamidomethyl (#, +57) or reduced acrolein (@, +58) modification.

TNF- α , indicating the critical importance of JNK in these immune responses. Although JNK1 and JNK2 may have largely overlapping and redundant functions, studies using transgenic approaches or genetic deficiency of specific JNK isoforms indicate that it is primarily JNK2 that is responsible for these innate responses (56–58). In this regard, our discovery of JNK2 as the primary target for direct alkylation implicates JNK2 as a direct site for inhibition of JNK signaling and innate immune responses by acrolein.

The activation of JNK signaling and AP-1 has been found to be subject to inhibition by various electrophiles (59–62), although some reports also claim activation of JNK by acrolein or 4-hydroxynonenal (46, 63). Suppression of JNK/AP-1 signaling has been reported to occur at the level of DNA binding by direct alkylation of c-Jun (59, 61), or by more upstream mechanisms (60, 62), although the direct molecular mechanisms are still elusive. JNK1 has also been identified as a direct target for alkylation by 4-hydroxynonenal (63), and is subject to oxidation or S-nitrosylation at Cys¹¹⁶, which inhibits JNK signaling by disrupting its interaction with c-Jun (64, 65). In our studies, we observed JNK (and primarily JNK2) as a direct target for alkylation by acrolein. Because of the importance of JNK2 in macrophage immune responses (56), we further characterized JNK2 modifications by LC-MS/MS. Although this did not reveal direct targeting of Cys¹¹⁶, because tryptic peptides containing Cys¹¹⁶ could not be identified using our MS analysis, we did observe acrolein alkylation of two other Cys residues, Cys⁴¹ and Cys¹⁷⁷. Other Cys-containing peptides were not found to be adducted by acrolein, indicating specificity in Cys alkylation. Cys¹⁷⁷ is located within the activation loop of JNK2, and is highly exposed. In fact, in attempts to crystallize JNK2, this Cys residue was mutated to avoid protein dimerization or other interactions with external factors (66). The fact that Cys¹⁷⁷ is in close proximity to Thr¹⁸³ and Tyr¹⁸⁵, phosphorylation targets for MKK4/7, would suggest that alkylation of Cys¹⁷⁷ could potentially interfere with interactions of MKK4/7 with JNK,

thereby reducing its phosphorylation and activation. Also, because the activation loop of JNK2 is thought to have the ability to fluctuate between multiple conformations, as is the case for many other protein kinases (66), alkylation of Cys¹⁷⁷ within this activation loop could alter such flexibility, and negatively impact on JNK2 activation. Both Cys¹⁷⁷ and Cys⁴¹ were also recently demonstrated as targets for oxidation within JNK (67). Oxidation of Cys³⁸ of ERK2, which is homologous to Cys⁴¹ in JNK2, was recently reported to impact on mitogen-activated protein kinase (MAPK) kinase (MEK)1/2-ERK2 interaction (67). By analogy, alkylation of these corresponding Cys residues within JNK or ERK might interfere with their interaction with upstream MEK proteins, and thereby contribute to the observed inhibition of their phosphorylation. The precise mechanistic consequences of such alkylations, and their impact on interactions with MKK isozymes, will need to be addressed in future studies.

It would be naïve to presume that cellular effects of acrolein can be attributed to a single molecular modification. However, our studies demonstrate that the immunosuppressive actions of acrolein and of other electrophiles extend beyond the well recognized activation of Nrf2 and induction of anti-inflammatory and antioxidant genes, and rely importantly on acute immunosuppressive actions related to direct alkylations of critical redox-sensitive proteins, such as NF- κ B and JNK2. As addressed in our present study, the immunosuppressive actions of acrolein can also partly be accounted for by acute depletion of cellular GSH and consequent interference with redox-dependent signaling mechanisms. In addition, recent studies have also suggested that electrophilic compounds, such as acrolein, can also inhibit LPS-mediated signaling by inhibiting homodimerization of Toll-like receptor 4 (68), although such a proximal mechanism would not fully explain the relatively modest inhibitory actions on NF- κ B compared with the more robust inhibition of JNK-c-Jun activation.

In summary, we conclude that acrolein contributes importantly to the immunosuppressive actions of CS, similar to previous findings by others (69), and that these anti-inflammatory actions of acrolein, as well as those of other dietary or endogenously generated electrophiles, involve a combination of diverse mechanisms. Our studies highlight the potential importance of JNK2 as a previously unrecognized direct target for alkylation by these electrophiles as an immunosuppressive mechanism. A recent report demonstrated decreased macrophage immune responses by CS in relation to carbonylation of various proteins, and claimed that oxidative stress was responsible for this protein carbonylation (70). Instead, our results would argue that such carbonylation most likely results from alkylation by acrolein or related electrophiles present within CS. Identification of the cellular targets for such alkylations will offer new insights into the biological effects of these electrophiles or of CS-related diseases in general. Moreover, establishing the importance of CS-derived electrophiles rather than oxidative stress in smoking-related pathologies will redirect attention to the potential importance of enzyme systems that detoxify these electrophiles, such as GSH *S*-transferase P1 (71) or alkenal/one oxidoreductase (72), as genetic determinants of such diseases.

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