Airway Epithelial Epidermal Growth Factor Receptor Mediates Hogbarn Dust–Induced Cytokine Release but Not Ca^{2+} Response

Puttappa R. Dodmane[1], Nancy A. Schulte[1], Art J. Heires[2], Hamid Band[3], Debra J. Romberger[2,4], and Myron L. Toews[1]

[1]Department of Pharmacology and Experimental Neuroscience, University of Nebraska Medical Center, Omaha; [2]Pulmonary, Critical Care, Sleep and Allergy Section, Department of Internal Medicine, University of Nebraska Medical Center, Omaha; [3]Eppley Institute for Research in Cancer and Allied Diseases and Departments of Biochemistry & Molecular Biology; Genetics, Cell Biology & Anatomy; Pathology & Microbiology; and Pharmacology & Experimental Neuroscience; University of Nebraska Medical Center, Omaha; and [4]Veterans Administration Nebraska-Western Iowa Health Care System, Omaha, Nebraska

A subset of workers in swine confinement facilities develops chronic respiratory disease. An aqueous extract of dust from these facilities (hogbarn dust extract [HDE]) induces IL-6 and IL-8 release and several other responses in isolated airway epithelial cells. The cell membrane receptors by which HDE initiates these responses have not been identified. Because several other inhaled agents induce airway epithelial cell responses through epidermal growth factor receptor (EGFR) activation, we hypothesized that HDE would activate EGFRs and that EGFRs would be required for some of the responses to HDE. Exposure of Beas-2B cells to HDE caused EGFR phosphorylation and downstream ERK activation, and both responses were blocked by the EGFR-selective kinase inhibitor AG1478. AG1478 and EGFR-neutralizing antibody reduced HDE-stimulated IL-6 and IL-8 release by about half. Similar EGFR phosphorylation and requirement of EGFRs for maximal IL-6 and IL-8 release were found with primary isolates of human bronchial epithelial cells. Because HDE-stimulated IL-6 and IL-8 release involve the Ca^{2+}–dependent protein kinase Cα, we hypothesized that HDE would induce intracellular Ca^{2+} mobilization. HDE exposure induced intracellular Ca^{2+} mobilization in Beas-2B cells and in primary cell isolates, but this response was neither mimicked by EGF nor inhibited by AG1478. Thus, HDE activates EGFRs and their downstream signaling, and EGFR activation is required for some but not all airway epithelial cell responses to HDE.

Keywords: airway epithelial cells; EGF receptors; hogbarn dust; cytokines; calcium

Current approaches to commercial hog production in the United States and around the world involve feeding pigs in confinement facilities to maximize yields and profits. In the United States, over 40% of pigs are raised in facilities with more than 5,000 animals (1). The dusts present in these and other concentrated animal feeding operations are increasingly being associated with adverse health effects in the confinement facility workers and in people living in the surrounding communities. In particular, workers in swine confinement facilities have increased risk of lung disease, including chronic bronchitis and chronic obstructive pulmonary disease (2, 3). About 25% of swine confinement facility workers suffer from chronic respiratory diseases (4), characterized by increased bronchial inflammation along with elevations in proinflammatory cytokines IL-6 and IL-8 and increased numbers of neutrophils in bronchoalveolar lavage fluid (5, 6).

CLINICAL RELEVANCE

This work identifies epidermal growth factor receptors as important components in some but not all airway epithelial cell responses to components of hogbarn dust, a complex agricultural dust that causes chronic inflammatory disease in hogbarn workers. The studies better define the cellular targets and mechanisms for the responses to the dust, and they identify a new potential target for disease intervention.

These inflammatory changes and respiratory symptoms can be reproduced in experimental settings with exposure of naive human subjects to the swine confinement facilities (7, 8) or with exposure of laboratory animals to the dusts or dust components (9, 10). In addition, exposure of human airway epithelial cells in culture to components of the dust provides a convenient *in vitro* model system for assessing the molecular mechanisms that may contribute to the human disease. Our laboratories have shown that an aqueous extract of this dust (hogbarn dust extract [HDE]) induces multiple effects in cultured human airway epithelial cells that are similar to those seen in patients, including release of IL-6 and IL-8 (11, 12), recruitment of neutrophils (9), and increased lymphocyte adherence (13). Several components of the cellular signaling responses to HDE have been established, in particular the sequential roles of protein kinase (PK)C-α and tumor necrosis factor (TNF)-α to facilitate IL-6 release, followed by additional activation of PKC-ε to promote IL-8 release (12).

The identity of any specific cell surface receptors that may detect active components of HDE and initiate the cellular responses to HDE has not been established. Epidermal growth factor receptors (EGFRs) are critical regulators of diverse functions of airway epithelial cells (14, 15). In addition to their well established role in lung cancer (16), EGFRs play important roles in mucus secretion (17), in IL-8 release (18), and in various other immune and inflammatory responses of these cells (19, 20). In particular, several inhaled agents have been shown to mediate effects on airway epithelial cells via EGFR transactivation, including bacterial lipopolysaccharide (21) and lipoteichoic acid (22), cigarette smoke (23), and various environmental and occupational dusts and particulates (24–27). Transactivation can occur by activation of cellular proteases

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Correspondence and requests for reprints should be addressed to Myron L. Toews, Ph.D., Professor, Department of Pharmacology and Experimental Neuroscience, University of Nebraska Medical Center, 985800 Nebraska Medical Center, Omaha, NE 68198-5800. E-mail: mtoews@unmc.edu

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that in turn release cell surface proligands to activate the EGFR extracellularly (18, 28) or by activation of cytosolic Src-family tyrosine kinases that directly phosphorylate the EGFR, leading to its ligand-independent intracellular activation (29, 30).

The current studies tested whether EGFRs are activated upon exposure of airway epithelial cells to HDE and assessed whether EGFR activation is required for downstream cellular responses. Because of the importance of the Ca^{2+}–dependent PKC-α isozyme for cytokine responses to HDE (12), the ability of HDE to stimulate Ca^{2+} mobilization was tested. The data show that HDE induces EGFR phosphorylation and that the tyrosine kinase activity of the activated EGFR is required for maximal IL-6 and IL-8 release in response to HDE. HDE also stimulates increases in intracellular Ca^{2+}, but this response is not mimicked by EGF and is independent of EGFR activation by HDE. Thus, EGFRs are essential for some but not all of the cellular responses initiated upon exposure of airway epithelial cells to HDE. Portions of this work have been presented previously in meeting abstracts (31, 32).

MATERIALS AND METHODS

Reagents

The sources of all reagents are presented in the online supplement.

Cell Culture

All assays were conducted with the Beas-2B cell line or with primary isolates of human bronchial epithelial cells (hBECs). Details of cell isolation and culture are presented in the online supplement.

Hogbarn Dust and Extract Preparation and Properties

The aqueous HDE used in these studies was prepared by dissolving 1 g of settled dust collected from regional swine confinement facilities in 10 ml of HEPES-buffered (pH 7.4) saline solution. This extract was centrifuged, and the supernate was filter sterilized and considered 100% HDE, as previously described (11, 33). This HDE was used at 5% (vol/vol) unless indicated otherwise. Additional details on the properties of the dust used for these studies are presented in the online supplement.

EGFR and ERK Phosphorylation Assays

Details of procedures are presented in the online supplement. Briefly, cells were grown to confluence and starved overnight before being treated with HDE or with EGF or lysophosphatidic acid (LPA) for comparison, both in the absence or presence of inhibitors in some experiments. For EGFR phosphorylation assays, EGFRs were immunoprecipitated, electrophoresed, and then blotted for quantification of total and phosphorylated EGFRs. For ERK phosphorylation assays, cell lysates were electrophoresed and blotted for quantification of phospho-ERK1/2 and total ERK1/2.

Cytokine Release Assays

Quantification of IL-6 and IL-8 release by ELISA was performed as previously described (12) and as detailed in the online supplement.

Ca^{2+} Mobilization Assays

Assays of Ca^{2+} mobilization were conducted with the FLIPR Calcium 4 Assay Kit reagents using a FlexStation and FlexStation accessories (Molecular Devices, Sunnyvale, CA) as described in detail in the online supplement.

Data Analyses

Data were analyzed using GraphPad Prism (GraphPad Software Inc., San Diego, CA). Statistical significance was determined using one-way ANOVA with the Newman-Keuls, Tukey's, or Dunnett's post-tests. Data are presented as means ± SE from at least three separate experiments unless otherwise indicated.

RESULTS

HDE-Induced Autophosphorylation of EGFRs

Exposure of Beas-2B cells to 5% HDE for 15 minutes induced a clear stimulation of EGFR phosphorylation (Figure 1). The magnitude of the stimulation by HDE (2.4 ± 0.3-fold) was similar to that for the G-protein–coupled receptor activator LPA (1.7 ± 0.2-fold), which is known to induce EGFR transactivation in these cells (18, 34), but was much lower than that seen with direct activation by 60 ng/ml EGF (28 ± 14-fold). Stimulation of EGFR phosphorylation by HDE was concentration dependent (*see* Figure E1 in the online supplement), with half-maximal stimulation at approximately 3% HDE, similar to the concentration of HDE required for various other cellular effects studied previously in these cells (11). The fold stimulation in this series of experiments was 3.6 ± 0.8-fold for 10% HDE, the highest concentration tested, and 22 ± 7-fold stimulation for 60 ng/ml EGF.

The EGFR-selective tyrosine kinase inhibitor AG1478 almost completely inhibited HDE- and EGF-induced EGFR phosphorylation (Figure 2), indicating that the increased phosphorylation is mediated by the tyrosine kinase activity of the EGFR itself.

Mechanisms of HDE Stimulation of EGFR Phosphorylation

Inhibitors of known pathways for transactivation of the EGFR failed to decrease HDE stimulation of EGFR phosphorylation (Figure 3). The broad-spectrum matrix metalloprotease inhibitor GM6001 did not inhibit HDE stimulation of EGFR phosphorylation, though it did inhibit stimulation by LPA by about half in side-by-side experiments (data not shown), in agreement with previous studies (18, 34) and confirming the effectiveness of the

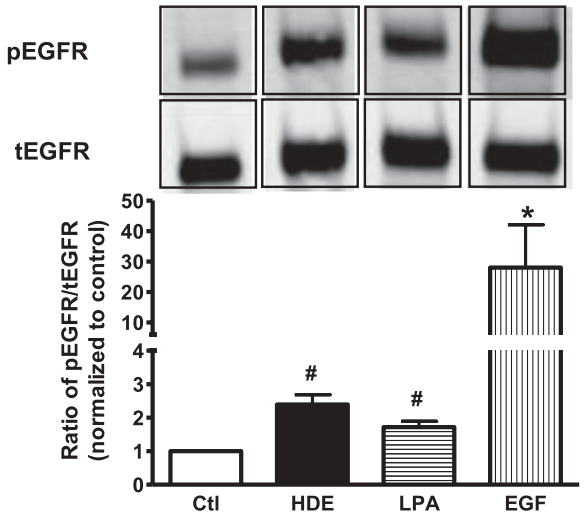

Figure 1. Hogbarn dust extract (HDE) stimulation of epidermal growth factor receptor (EGFR) phosphorylation. Beas-2B cells were incubated for 15 minutes in the absence (Ctl) or presence of 5% HDE, 10 μM lysophosphatidic acid (LPA), or 60 ng/ml EGF. Total EGF receptor (tEGFR) and phospho-EGFR (pEGFR) were assessed by immunoprecipitation and blotting. The *upper panel* shows a representative Western blot, with selected lanes from a gel that included other conditions not shown here. The *lower panel* shows the ratios of pEGFR to tEGFR expressed as the fold increase relative to untreated Ctl, and the data are means ± SE from three experiments. *Stimulation by EGF was significant ($P < 0.05$). #The stimulations by HDE and LPA were consistently observed but were significantly greater than control at $P < 0.05$ only in experiments or analyses where the much larger stimulation by EGF was not included.

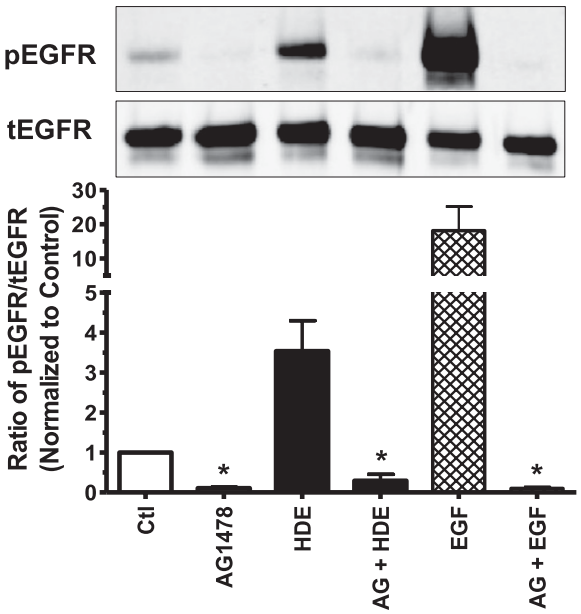

Figure 2. Effect of the EGFR-selective kinase inhibitor AG1478. Beas-2B cells were pretreated with 10 μM AG1478 or vehicle (Ctl, 0.1% DMSO) for 30 minutes and then stimulated with 5% HDE or 60 ng/ml EGF for 15 minutes. tEGFR and pEGFR were assessed by immunoprecipitation and blotting. The *top panel* is a representative blot; the *bottom panel* shows means ± SE from 3 to 11 experiments, with the ratios of pEGFR to tEGFR expressed as the fold increase relative to the untreated Ctl value. *Significant inhibition by AG1478 ($P < 0.05$).

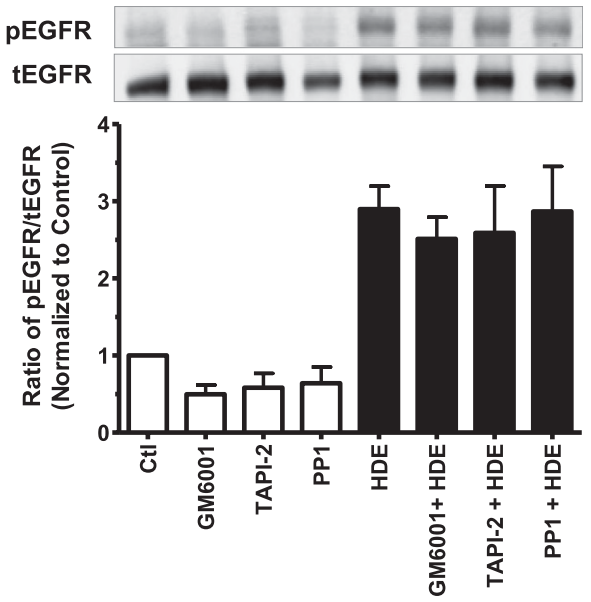

Figure 3. Lack of inhibition of HDE stimulation by transactivation inhibitors. Beas-2B cells were pretreated with vehicle (Ctl, 0.1% DMSO), 25 μM GM6001, 10 μM TAPI-2, or 1 μM PP1 for 30 minutes and then stimulated with 5% HDE for 15 minutes. tEGFR and pEGFR were assessed by immunoprecipitation and blotting. The *top panel* is a representative blot; the *bottom panel* shows means ± SE from three experiments for GM6001 and TAPI-2 and two experiments for PP1, with the ratios of pEGFR to tEGFR expressed as the fold increase relative to the untreated Ctl value. None of the values in the presence of the inhibitors was significantly lower than those in the absence of inhibitor at $P < 0.05$.

inhibitor. The TNF-α–converting enzyme inhibitor TAPI-2 and the Src family tyrosine kinase inhibitor PP1 also failed to block HDE stimulation of EGFR phosphorylation. These data suggest that neither the extracellular nor the intracellular pathways of EGFR transactivation are responsible for the EGFR phosphorylation induced by HDE.

HDE Activates Intracellular Signaling Downstream of EGFRs

To confirm that the HDE-induced phosphorylation of EGFRs leads to activation of downstream EGFR signaling, HDE effects on ERK1/2 phosphorylation were assessed (Figure 4). Exposure of cells to 5% HDE for 5 minutes induced a 6- to 7-fold increase in the phosphorylation of ERK1/2, comparable to the 9- to 10-fold stimulation by EGF itself. The increases in ERK phosphorylation by HDE and EGF were completely inhibited by the EGFR kinase inhibitor AG1478, confirming that the HDE stimulation of ERK1/2 phosphorylation requires the tyrosine kinase activity of the EGFR. Furthermore, EGF and HDE stimulations of ERK1/2 phosphorylation were prevented by an antibody that blocks ligand-dependent EGFR activation, indicating that HDE activation of the EGFR is via an extracellular ligand.

EGFR Involvement in HDE-Stimulated Release of IL-6 and IL-8

To test whether the EGFR phosphorylation and activation documented above are important for inflammation-related downstream responses to HDE, the effects of AG1478 on HDE stimulation of IL-6 and IL-8 release from Beas-2B cells were assessed (Figure 5). Exposure of cells to 5% HDE for 18 to 24 hours led to a 7.4-fold stimulation of IL-6 release and a 7.0-fold stimulation of IL-8 release, similar to data from previous studies (11, 12). Treatment of cells with 10 μM AG1478 before and during the exposure to HDE significantly inhibited these effects of HDE by 36% for IL-6 release and by 48% for IL-8 release (Figure 5). Treatment of cells with 10 μg/ml of anti-EGFR antibody before and during the exposure to HDE similarly inhibited these HDE effects by 58% for IL-6 release and by 69% for IL-8 release. GM6001 failed to inhibit IL-6 or IL-8 responses (Figure 5), consistent with its lack of effect on EGFR phosphorylation (*see* Figure 3).

Lack of EGFR Involvement in HDE Stimulation of Calcium Mobilization

Because HDE has been shown to activate the Ca^{2+}–sensitive PKC-α isozyme, an effect that is also important for HDE stimulation of IL-6 and IL-8 release (11), the effects of HDE on Ca^{2+} mobilization were assessed (Figure 6). HDE stimulated a concentration-dependent increase in Ca^{2+} mobilization that was about half as large as that induced by LPA, which was used as a positive control and an internal standard for normalization of responses (Figure 6A). Half-maximal Ca^{2+} mobilization occurred with 0.6% HDE, a lower concentration than that required for EGFR phosphorylation (*see* Figure 1) and several other responses to HDE. In contrast to the other HDE responses studied here, the HDE stimulation of Ca^{2+} mobilization was not inhibited by AG1478, consistent with the lack of a Ca^{2+} mobilization response to EGF itself in the same experiments (Figure 6B). Thus, EGFR activation is important for several downstream responses to HDE but is not the only mechanism for initiating HDE responses.

HDE Induces EGFR Phosphorylation and Ca^{2+} Mobilization in Primary Human Bronchial Epithelial Cells

Beas-2B cells are a convenient airway epithelial cell model system but are a virally transformed cell line. Key findings from

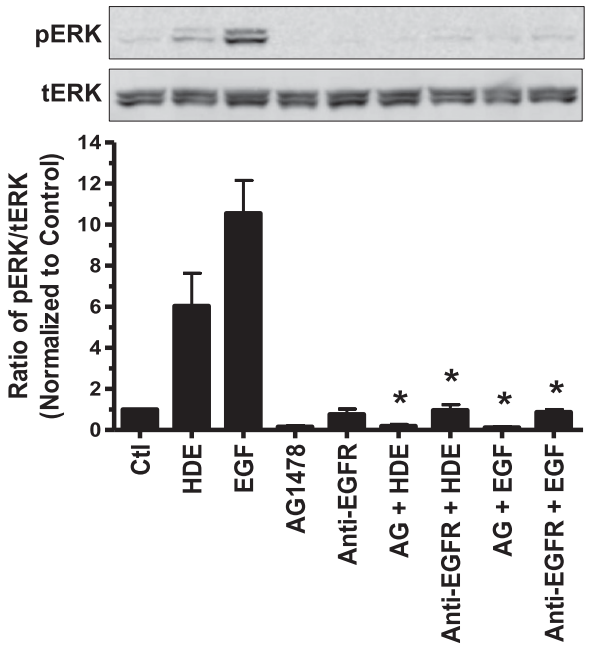

Figure 4. HDE phosphorylates ERK1/2 in an EGFR kinase–dependent manner. Beas-2B cells were pretreated with vehicle (Ctl, 0.1% DMSO), 10 μM AG1478, or 1 μg/ml anti-EGFR neutralizing antibody for 30 minutes and then stimulated with 5% HDE or 60 ng/ml EGF for 5 minutes. Total ERK1/2 (tERK) and phospho-ERK1/2 (pERK) were assessed by Western blotting. The *top panel* is a representative blot; the *bottom panel* shows the means ± SE from at least three experiments for AG1478 and two experiments for anti-EGFR antibody, with the ratios of pERK to tERK expressed as the fold increase relative to untreated Ctl. *Significant inhibition of the responses to HDE or EGF ($P < 0.05$).

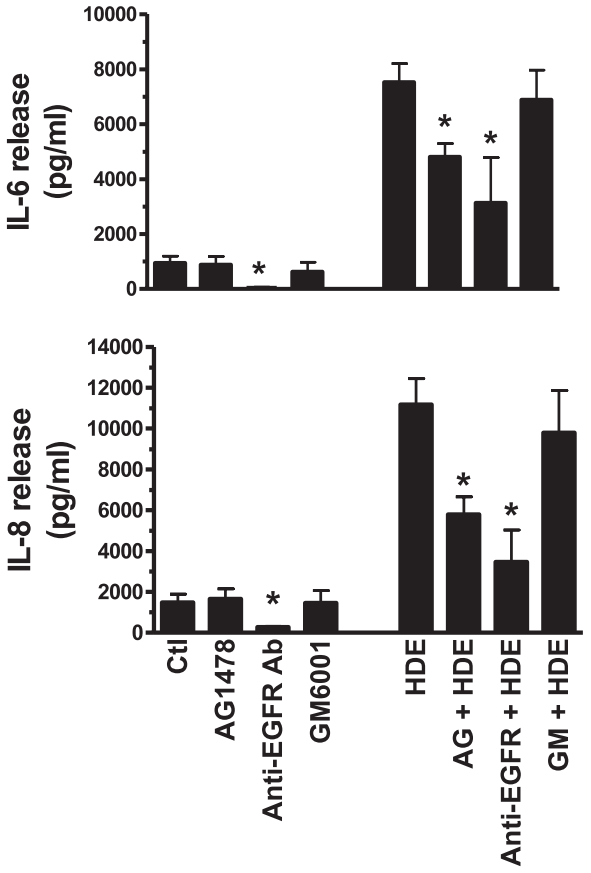

Figure 5. Inhibition of IL-6 and IL-8 release by AG1478 and anti-EGFR antibody but not by GM6001. Beas-2B cells were pretreated with vehicle (Ctl, 0.1% DMSO), 10 μM AG1478, 10 μg/ml anti-EGFR neutralizing antibody or 25 μM GM6001 for 30 minutes and then stimulated with 5% HDE for 18 or 24 hours. The supernates were collected and assayed for IL-6 and IL-8 by ELISA. The data are the means ± SE from 15 experiments for AG1478, three experiments for anti-EGFR antibody, and five experiments for GM6001. *Significantly lower value in the presence of the inhibitor ($P < 0.05$).

Beas-2B cell studies were therefore confirmed with primary isolates of hBECs. Stimulation of hBECs with 5% HDE for 15 minutes clearly induced EGFR phosphorylation (Figure 7A); the magnitude of the stimulations by HDE (1.4 ± 0.1-fold), LPA (1.4 ± 0.1-fold), and EGF (7.5 ± 2.3-fold) were smaller in hBECs than in Beas-2B cells, perhaps in part because of the higher baseline phosphorylation. Exposure of hBECs to 5% HDE for 18 hours induced a 6.4-fold stimulation of IL-6 release and a 10.0-fold stimulation of IL-8 release (Figure 7B). AG1478 significantly reduced these HDE effects by 45% for IL-6 release and by 47% for IL-8 release (Figure 7B). Finally, HDE stimulated Ca^{2+} mobilization in hBECs, and this response was not inhibited by AG1478 (*see* Figure E2). Thus, the key HDE responses identified in Beas-2B cells are similar in primary isolates of hBECs.

DISCUSSION

The current studies show that exposure of human airway epithelial cells to aqueous HDE leads to rapid auto-phosphorylation and activation of EGFRs and that EGFR phosphorylation and activation are critical contributors to HDE stimulation of IL-6 and IL-8 release from these cells. These are the first studies to implicate an EGFR-dependent signaling pathway in the response to HDE, a complex environmental dust, and they place HDE among a subset of inhaled agents that cause airway injury or inflammation through EGFR activation and signaling (27, 35–38). Our studies also show that HDE exposure leads to a rapid increase in intracellular Ca^{2+} but that this Ca^{2+} mobilization response is not dependent on EGF receptor activation. The EGFR is thus critical for the initiation of some but not all of the airway epithelial cell responses to HDE. Studies in primary hBECs confirmed the key results obtained using the immortalized Beas-2B cell line, including HDE stimulation of EGFR phosphorylation, the requirement of EGFR signaling for maximal stimulation of IL-6 and IL-8 release, and the EGFR-independent stimulation of Ca^{2+} mobilization.

The magnitude of the stimulation of EGFR phosphorylation by 10% HDE was lower than that with maximal activation by EGF itself but was similar to that induced by LPA in the same experiments and to that seen with several other agents that mediate their effects at least in part via EGFRs, including allergen and cigarette smoke (38–42). The inhibition of downstream HDE responses by EGFR inhibitors, in particular IL-6 and IL-8 release, provides the most compelling evidence that this level of phosphorylation is not only sufficient but is also critical for HDE to mediate its effects.

The HDE-induced release of IL-6 and IL-8 was significantly reduced but not completely eliminated by the EGFR-selective tyrosine kinase inhibitor AG1478, even though this inhibitor essentially completely blocked EGFR phosphorylation. Anti-EGFR antibody caused a similar partial inhibition of cytokine release, suggesting that HDE leads to EGFR activation by an extracellular mechanism. The partial inhibition of IL-6 and IL-8

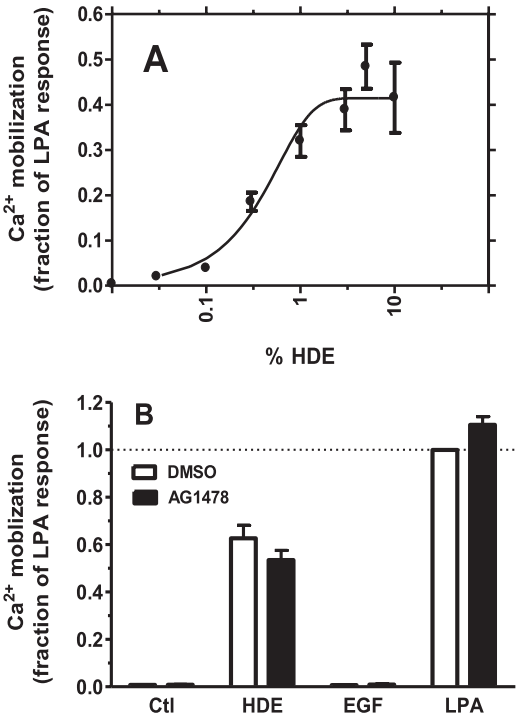

Figure 6. HDE stimulation of intracellular Ca^{2+} mobilization independent of EGFR activation. Beas-2B cells were assayed for calcium mobilization for 120 seconds using a FlexStation, and responses to test agents were normalized to that induced by 10 μM LPA included as an internal standard. (*A*) Data for cells exposed to increasing concentrations of HDE expressed as the means ± SE from at least three experiments. (*B*) Data for cells incubated with vehicle (0.1% DMSO) or 10 μM AG1478 for 1 hour and then stimulated with 5% HDE, 60 ng/ml EGF, or 10 μM LPA expressed as the means ± SE from four experiments. None of the values in the presence of AG1478 was significantly lower than those in the absence of inhibitor at $P < 0.05$.

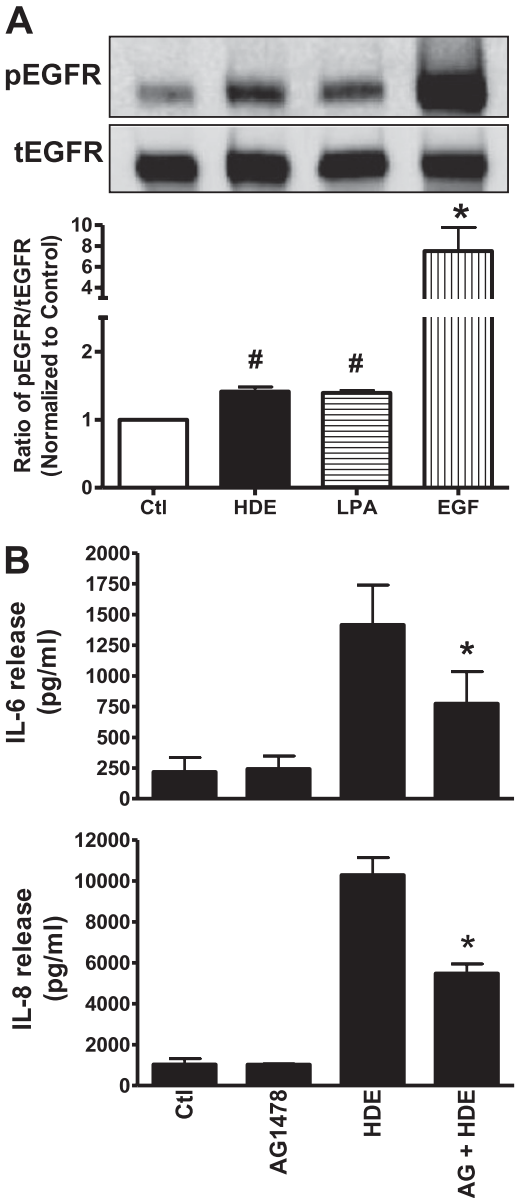

Figure 7. HDE effects on EGFR phosphorylation and IL-6 and IL-8 release in hBECs. (*A*) Primary hBECs were incubated in the absence (Ctl) or presence of 5% HDE, 10 μM LPA, or 60 ng/ml EGF for 15 minutes, and then tEGFR and pEGFR were assessed by immunoprecipitation and blotting. A representative Western blot is shown along with a bar graph of the means ± SEM from three experiments. *Stimulation by EGF was significant ($P < 0.05$). #The stimulations by HDE and LPA were consistently observed but were significantly greater than control at $P < 0.05$ only in experiments or analyses where the much larger stimulation by EGF was not included. (*B*) Primary hBECs were pretreated with vehicle (Ctl) or 10 μM AG1478 for 30 minutes and then stimulated with 5% HDE for 18 hours. The supernates were collected and assayed for IL-6 and IL-8 by ELISA, and the data are means ± SE from three experiments. *Significantly lower value in the presence of AG1478 ($P < 0.05$).

release observed for HDE stimulation is similar to the partial inhibition of cytokine release and other airway epithelial responses to other agents that have been reported to require EGFR activation (e.g., IL-8 release stimulated by LPA [18] or LL-37 [43]). However, there are other agents whose responses are nearly completely blocked by AG1478, including IL-8 release by neutrophil elastase (20) and cigarette smoke (23). EGFR activation may be the primary or only key step for initiating responses to these latter stimuli, whereas for HDE and some other agents, EGFR signaling is one but not the only required mediator of their responses.

The mechanisms involved in HDE-induced phosphorylation of EGFRs appear to be different from previously described pathways for other agents that induce EGFR-dependent responses in airway epithelial cells. The inhibition by AG1478 indicates that it is an autophosphorylation mediated by the tyrosine kinase activity of the EGFR itself. The broad-spectrum matrix metalloproteinase inhibitor GM6001 that blocks EGFR phosphorylation induced by several other agents in airway epithelial cells (22, 39, 44) did not block EGFR phosphorylation or downstream responses to HDE. We confirmed the ability of GM6001 to inhibit EGFR phosphorylation induced by LPA in side-by-side experiments (data not shown), consistent with our previous studies and those of others (18, 34). Similarly, the TNF-α–converting enzyme inhibitor TAPI-2 and the Src inhibitor PP1 failed to inhibit EGFR phosphorylation, though they have been shown to inhibit this response for several other agents, such as allergen and cigarette smoke (17, 27, 38, 44).

The inhibition of HDE-induced signaling by an extracellular EGFR-blocking antibody but not by inhibitors of cellular proteases that release surface-bound EGFR ligands suggests at least two intriguing mechanisms for EGFR activation by

HDE. First, HDE could contain a direct-acting EGFR ligand, including perhaps EGF itself. Previous studies have shown the HDE factor(s) responsible for IL-6 and IL-8 release to be stable to boiling (45), a property long known for EGF (46). Alternatively, HDE itself could contain proteases capable of activating EGFRs. Several allergens have protease activity and at least some signal via EGFRs, consistent with this idea (38, 47). A variety of gut and bacterial proteases could be excreted in hog manure, a presumed contributor to the hogbarn dust. Our recent preliminary data are consistent with contributions of hog manure components and of active proteases to some but not all of the responses to HDE (48, 49).

Previous studies have documented a role for the Ca^{2+}–dependent PKC-α enzyme in IL-6 and IL-8 release by HDE (12). Our data provide the first demonstration that HDE can promote the increase in intracellular Ca^{2+} that is required for PKC-α activation. This Ca^{2+} mobilization may also be important for other steps in cytokine release or for other responses to HDE. However, the lack of inhibition of HDE-stimulated Ca^{2+} mobilization by AG1478 and the lack of a Ca^{2+} response to EGF itself clearly indicate that HDE-induced Ca^{2+} mobilization does not occur through EGFR activation. The ways in which EGFR signals interact with PKC-α activation and the other HDE signaling pathway components identified previously, including the Ca^{2+}–independent PKC-ε and TNF-α release, is an important area for future research.

Multiple mechanisms are involved in the various downstream responses induced by HDE, and our preliminary studies indicate that multiple active components are present in this dust extract, each of which may signal via its own unique mechanism. Studies are in progress to identify specific mediators of the multiple cellular responses to HDE, including the EGFR activation and the Ca^{2+} mobilization documented for the first time here. However, it may be the combined effects of multiple components of the hogbarn dust that mediate the human lung disease that underlies these studies. Thus, the current studies with the complete though complex dust extract make it likely that EGFRs do in fact play an important role in the context of hogbarn dust–induced lung disease. Identifying specific individual components will facilitate further mechanistic studies for each of those components alone and of the perhaps interactive mechanisms operative in the context of the complete dust present in the animal facilities.

In summary, HDE can be added to a growing list of diverse mediators of airway inflammation whose effects on airway epithelial cells are mediated at least in part through EGFR activation. Given that EGFR-targeted therapeutic agents are already in clinical use (16), the current findings provide a rationale for exploring the possible use of EGFR inhibitors for the treatment of respiratory illnesses caused by hogbarn dust exposure.

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