Feedlot dust stimulation of interleukin-6 and -8 requires protein kinase Cε in human bronchial epithelial cells

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[1]*Pulmonary and Critical Care Medicine Section, Department of Internal Medicine, Omaha;* [2]*Research Service, Department of Veterans Affairs Medical Center, Omaha;* [3]*Texas A & M Agricultural Research & Extension Center, Amarillo, Texas; and* [4]*Department of Ophthalmology and Visual Sciences, Nebraska Medical Center, Omaha, Nebraska*

Wyatt TA, Slager RE, DeVasure J, Auvermann BW, Mulhern ML, Von Essen S, Mathisen T, Floreani AA, Romberger DJ. Feedlot dust stimulation of interleukin-6 and -8 requires protein kinase Cε in human bronchial epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 293: L1163–L1170, 2007. First published August 24, 2007; doi:10.1152/ajplung.00103.2007.—Individuals exposed to dusts from concentrated animal feeding operations report increased numbers of respiratory tract symptoms, and bronchoalveolar lavage samples from such individuals demonstrate elevated lung inflammatory mediators, including interleukin (IL)-8 and IL-6. We previously found that exposure of bronchial epithelial cells to hog barn dusts resulted in a protein kinase C (PKC)-dependent increase in IL-6 and IL-8 release. We hypothesized that cattle feedlot dusts would also generate bronchial epithelial interleukin release in vitro. To test this, we used interleukin ELISAs and direct PKC isoform assays. We found that a dust extract from cattle feedlots [feedlot dust extract (FLDE)] augments PKC activity of human bronchial epithelial cells in vitro. A 5–10% dilution of FLDE stimulated a significant release of IL-6 and IL-8 at 6–24 h in a PKC-dependent manner vs. control medium-treated cells. An increase in PKCα activity was observed with 1 h of FLDE treatment, and PKCε activity was elevated at 6 h of FLDE exposure. The PKCα inhibitor, Gö-6976, did not inhibit FLDE-stimulated IL-8 and IL-6 release. However, the PKCε inhibitor, Ro 31-8220, effectively inhibited FLDE-stimulated IL-8 and IL-6 release. Inhibition of FLDE-stimulated IL-6 and IL-8 was confirmed in a dominant-negative PKCε-expressing BEAS-2B cell line but not observed in a PKCα dominant negative BEAS-2B cell line. These data support the hypothesis that FLDE exposure stimulates bronchial epithelial IL-8 and IL-6 release via a PKCε-dependent pathway.

feedlot dust extract

CONCENTRATED ANIMAL FEEDING OPERATIONS (CAFOs) generate inhalable dusts that are the focus of numerous health concerns (6, 10, 23, 26). Such airborne dusts include endotoxin and other microbial products as well as ammonia, hydrogen sulfide, and a variety of volatile organic compounds (6). Increased medical complaints are more common in populations living near some CAFOs (30). Symptoms of respiratory disease and lung function test abnormalities have been described in workers employed in CAFOs, but the impact of CAFO dusts on ambient public exposure is not defined. Furthermore, the type of animal involved in the feeding operation may likely be important in the airborne agents generated and the effect on public health. CAFOs where the animals are located inside buildings such as hogs, poultry, and dairy cattle have been investigated more thoroughly than CAFOs where animals are located outdoors.

Clinical treatment of CAFO dust exposure is of importance to the general population. Simulation studies suggest that when CAFO workers comprise 15–45% of the community, human influenza cases are increased by 42–86% (22). Overall, in the United States, an increased rate of asthma among school children has occurred, and a recent study suggests that children in schools near CAFOs have a significantly increased prevalence of physician-diagnosed asthma (24). Some studies have suggested that dairy farmers' exposure to cattle dust leads to increased airborne particulate matter exposure and the potential for long-term development of chronic lung disease (4, 5).

Occupational exposures are responsible for ~15% of chronic obstructive pulmonary disease (COPD) (2). COPD is a disease syndrome characterized by chronic bronchitis, chronic asthma, and emphysema. COPD involves airway remodeling in response to chronic lung inflammatory events. While the mechanisms are complex and poorly defined, one hallmark of COPD is the chronic recruitment of inflammatory cells through the production of cytokines such as IL-6 and IL-8 (18). A principal source of IL-6 and IL-8 production in the airways is the bronchial epithelial cell (14, 27).

Previously, we examined the effect of CAFO dust from hog confinement facilities on airway epithelial cell proinflammatory cytokine production. Hog barn dust stimulated a significant production and release of IL-6 and IL-8 in an endotoxin-independent manner (20). We also found that the release of these interleukins was regulated via the action of protein kinase C (PKC). In addition, we observed that hog barn dust extract enhanced the adhesion of lymphocytes to airway epithelial cells in a PKC-dependent manner (15). BEAS-2B cells also have been used in a model for soil-derived, dust-induced proinflammatory cytokine release (29). Thus exposure to organic dusts derived from hog CAFOs demonstrates an in vitro potential for stimulating airway inflammation. It is likely that exposure to other CAFOs could elicit proinflammatory airway responses as well, although the effect of dusts from cattle feedlots has not been extensively studied either in vivo or in vitro.

We hypothesized that dusts generated from cattle feedlots would also stimulate interleukin release in bronchial epithelial cells in a PKC-dependent manner. To test this hypothesis, we measured interleukin production in normal human bronchial

epithelial cells as well as in bronchial epithelial cell lines that express a dysfunctional specific PKC isoenzyme. Our findings suggest that cattle feedlot dusts have the potential to elevate proinflammatory cytokines in human airway cells.

METHODS

Preparation of feedlot dust extract. Airborne particulate matter was obtained on 8" × 10" glass-fiber filters that were exposed in high-volume, total suspended particulate (TSP) samplers (General Metal Works, Smyrna, GA) deployed downwind of large cattle feedlots across the Great Plains. Samplers were operated for 3–5 h at air flow rates of ~1.13 cubic meters per minute (m^3/min) during the well-documented "evening dust peak" (28), a diurnal phenomenon characteristic of cattle feedlots. Filters were preconditioned before exposure in a drying oven for ~18 h at 90°C. Following exposure in the high-volume sampler, filters were again conditioned in the drying oven and weighed on an analytical balance (model AG245; Mettler-Toledo, Columbus, OH) to determine the time-averaged mass concentration of TSP represented by the samples. Filters were folded exposed-side-in and archived in paper envelopes. No records of sampler locations were retained.

The dust was extracted from the filters by placing sections of the filters into 50-ml conicals containing 40 ml of distilled H_2O. Conicals were agitated for 72 h. The solution was frozen at −80°C until lyophilized. Feedlot dust extract (FLDE) was prepared by mixing 0.1 g of lyophilized material in 10 ml of Hanks' balanced salt solution (HBSS; Biosource International, Camarillo, CA) without calcium. The mixture was mixed on a stir plate, incubating at room temperature for 1 h and clarified twice by centrifugation (2,000 *g*). The second supernatant was filter-sterilized and used as 100% fresh FLDE for the subsequent experiments. Endotoxin levels in FLDE were assayed using the limulus amebocyte lysate assay commercially available (Sigma, St. Louis, MO) (19).

Cell culture. Normal human bronchial epithelial (NHBE) cells were purchased from Clonetics (Cambrex, East Rutherford, NJ). NHBE cells were passaged no more than six times before use in experiments. Additionally, BEAS-2B cells (American Type Culture Collection, Manassas, VA), a simian virus 40-transformed human bronchial epithelial cell line, were also used. Airway epithelial cells from both sources were plated on type I collagen (Vitrogen 100; Collagen, Palo Alto, CA)-coated dishes and maintained in culture in serum-free medium at 37°C in 5% CO_2-95% air. A mixture (2:1) of Laboratory for Human Carcinogenesis/Roswell Park Memorial Institute (LHC-9/RPMI 1640) media was used to support the growth of the BEAS-2B cells under serum-free conditions, as previously described (20). NHBE cells were grown under serum-free conditions using a 1:1 mixture of Bronchial Epithelial Cell Basal Medium (Cambrex) and DMEM. Confluent monolayers of cells were pretreated for 1 h with or without PKC inhibitors followed by 1–24 h of exposure to 1–10% FLDE at 37°C.

Interleukin assay. After cell treatment conditions, media supernatants were collected and assayed for the concentration of interleukins released using a sandwich ELISA (20). Flat-bottomed 96-well polystyrene ELISA plates (Thermo Electron, Milford, MA) were coated with 200 μl/well of purified (goat) anti-human IL-8 antibody diluted 1:500 (R&D Systems, Minneapolis, MN) or IL-6 antibody diluted 1:1,000 in Voller's buffer (pH 9.6) overnight at 4°C. After three washings in PBS-Tween 20 (PBS-Tween), undiluted culture supernatants and human recombinant IL-8 or IL-6 standards (R&D Systems) were applied to the plates and incubated at room temperature for 2 h. Plates were again washed three times with PBS-Tween and incubated with (rabbit) anti-human IL-8 antibody diluted 1:500 (Endogen, Woburn, MA) or IL-6 antibody (Calbiochem, La Jolla, CA) diluted 1:1,000 in PBS Tween/BLOTTO (0.2% instant nonfat milk in PBS-Tween) for 1 h. After three washes, human serum-absorbed peroxidase-conjugated (goat) anti-rabbit IgG (Rockland, Gilbertsville, PA)

was added at 1:2,000 (IL-6) or 1:1,000 (IL-8) in PBS-Tween/BLOTTO for 45 min. The plates were again washed three times, and 200 μl/well of peroxidase substrate [10 ng/ml orthophenylenediamine (Sigma), 0.003% H_2O_2 in dH_2O] was added. The reaction was terminated with 27.5 μl/well of 8 M sulfuric acid, and plates were read at 490 nm in an automated ELISA reader (Bio-Rad, Hercules, CA). Culture supernatants were also assayed for IL-4, -10, -13, -17, and -18, as well as IL-6 and IL-8, utilizing the SearchLight array (Pierce Biotechnology, Rockford, IL), a multiplexed sandwich ELISA.

Remaining cells from the above treatment conditions were assayed for mRNA expression changes for IL-6 and IL-8.

IL-6 and IL-8 mRNA expression. Cells were grown to near confluence on collagen-coated six-well tissue culture dishes and maintained in serum-free LHC-9/RPMI 1640 (1:1) growth medium. Cells (1.5 × 10^6/condition) were challenged with 1% or 5% FLDE dissolved in growth medium for 1, 2, 6, or 24 h. At the end of the challenge period, cell layers were rinsed twice with HBSS and trypsinized at 37°C, and cell pellets were suspended in RNA*later* (Applied Biosystems, Foster City, CA) and stored at −20°C until analyzed. Total RNA was extracted from ~7.5 × 10^5 cells/condition using the MagMAX-96 Total RNA Isolation kit (Applied Biosystems) with contaminating genomic DNA removed by DNase treatment. Quality of the isolated RNA was measured spectrophotometrically (ND-1000; NanoDrop Technologies, Wilmington, DE), and the nucleic acid-to-protein (260/280) ratios fell between 1.8 and 2.3 for all samples. Reverse transcription was performed using 40 ng of purified mRNA template, 1× RT buffer, 5.5 mM $MgCl_2$, 500 mM deoxyNTP mixture, 2.5 mM random hexamers, 0.4 U/ml RNase inhibitor, and 1.25 U/ml reverse transcriptase (Applied Biosystems Gene Expression Assay kit). Thermal cycling conditions recommended by the manufacturer were as follows: incubation at 25°C for 10 min, transcription at 48°C for 30 min, and enzyme inactivation at 95°C for 5 min. Real-time PCR was then employed to quantify human IL-6 and IL-8 message. IL-6 (hs00174131_m1)- and IL-8 (hs00174103_m1)-specific primer/probe sets were combined with TaqMan Universal Master Mix and buffer and were subjected to 40 cycles in an Applied Biosystems 7500 Real Time PCR System. Thermal cycling parameters included two set-up steps at 50°C for 2 min and 95°C for 10 min followed by 40 denaturing and annealing cycles (95°C for 15 s and 60°C for 1 min each). Fluorescence values were recorded for each sample. To control for unequal loading of mRNA, all values were normalized to endogenous ribosomal RNA, and final results were expressed as fold change compared with unmanipulated control samples.

Cell viability assay. Media supernatant (50 μl) from cell monolayers treated with FLDE, PKC inhibitors, or media alone were sampled for cell viability assays. In addition, confluent 60-mm dishes were lysed as a positive control for lactate dehydrogenase (LDH) release. Cell viability was determined by a commercially available kit (Sigma) to measure LDH release according to the manufacturer's instructions.

PKC isoform assay. After media supernatants were removed from treated cells, the cell monolayers were flash frozen in cell lysis buffer as described (36). The cells were scraped with a cell lifter, sonicated, and centrifuged at 10,000 *g* for 30 min at 4°C. The supernatant was removed (cytosolic fraction), and the pellet was resuspended in cell lysis buffer containing 0.01% Triton X-100 and sonicated again (particulate fraction). PKC isoform activity was determined in crude whole cell cytosolic and particulate fractions of NHBE and BEAS-2B similar to methods described previously (32, 36). Airway epithelial cells contain the α, β, δ, ε, and ζ PKC isoforms (1). To measure PKC isoform activity specifically, 24 μg/ml PMA, 30 mM dithiothreitol, 150 μM ATP, 45 mM Mg-acetate, a PKCε isoform-specific substrate peptide, and 10 μCi/ml [γ-^{32}P]ATP were mixed in a Tris·HCl buffer (pH 7.5). Chilled (4°C) cell lysate (cytosolic or particulate) samples (20 μl) were added to 40 μl of the reaction mix and incubated for 15 min at 30°C. This mixture (60 μl) was then spotted onto P-81 phosphocellulose papers (Whatman, Clinton, NJ) to halt incubation,

and papers were subsequently washed five times in 75 mM phosphoric acid for 5 min, washed once in 100% ethanol for 1 min, dried, and counted in nonaqueous scintillant (National Diagnostics, Atlanta, GA). PKC activity was expressed in relation to the total amount of cellular protein assayed as picomoles of phosphate incorporated per minutes per milligram.

Cloning and transfection of human PKC mutants in airway epithelial cells. Mutant PKCα-expressing cells were generated on BEAS-2B cells to produce dominant-negative (DN) versions of PKCα using the tetracycline-responsive promoter expression system (Clontech, Palo Alto, CA), as previously described (7). BEAS-2B cells were grown to 90% confluency in six-well tissue culture dishes. Cells were transfected with the pTet-On expression vector encoding reverse tetracycline transactivator protein (rtTA) as well as a neomycin resistance gene using a cationic lipid technique, Lipofectamine-2000 (Invitrogen, Carlsbad, CA). Cells were propagated and selected for neomycin resistance using G418 (400 μg/ml; Calbiochem, San Diego, CA). Antibiotic-resistant clones were then transfected by electroporation with a DN variant made by in vitro mutagenesis, substituting an alanine for lysine at position 368. Expression of PKCα from this vector was under the control of the tetracycline response element (21). In addition, BEAS-2B cells were simultaneously cotransfected with a plasmid encoding the "humanized" green fluorescent protein (GFP) reporter to facilitate cell selection. All PKCα plasmid vectors were generously provided by Dr. Dan Rosson (Lankenau Medical Research Center, Wynnewood, PA).

To generate DN PKCε, mutation within the ATP-binding site of PKCε was created at amino acid position 437 using site-directed mutagenesis, rendering the kinase inactive (9, 12, 17, 31). The PKC DN cDNA was similarly cloned into the Clontech pEGFP-N1 vector, a gift of Dr. Christer Larsson. This construct was transfected using Lipofectamine 2000 into live BEAS-2B cells. Stable transfectants were selected for 3 wk with media containing 200 μg/ml G418. Isolated stable clones were extracted using Corning cloning cylinders (Corning, Acton, MA), and GFP-positive clones were sorted by fluorescence-activated cell sorting to enrich the population of transfected cells.

Reagents. To assess the effect of chemical inhibition of various isoforms of PKC, confluent, submerged cell monolayers were treated with the PKCα inhibitor Gö-6976 (1 μM), the PKCζ inhibitor rottlerin (20 μM), the PKCζ inhibitor myristylated PKCζ (1 μM), or the novel PKC inhibitor Ro 31-8220 (BioMol, Plymouth Meeting, PA). Optimal PKCε activity inhibition occurred at 10 μM Ro 31-8220 as previously determined (25). Specific PKC isoform substrate peptides were for PKCε (ERMRPRKRQGSVRRRV; Calbiochem), PKCα (VRKRTLRRL; Bachem, Torrance, CA), PKCδ (Calbiochem), and PKCζ (SIYRRGSR-RWRKL; Biosource, Camarillo, CA). All other reagents not specified were purchased from Sigma.

Statistical analysis. All quantitative experiments were performed in triplicate, and each experiment was repeated a total of three times. All data were analyzed using GraphPad Prism (version 4.00 for Windows; GraphPad Software, San Diego, CA) and represented as means ± SE. Therefore, each data point graphically presented represents $n = 9$ to generate the standard error of the mean. Data were analyzed for statistical significance using ANOVA. Significance was accepted at the 95% confidence interval.

RESULTS

The effect of FLDE exposure on proinflammatory cytokine release in NHBE cells was examined. Confluent monolayers of NHBE cells were exposed to increasing concentrations of FLDE from 1–10%, and supernatant media was collected 1–24 h. The media was subsequently assayed for IL-6 and IL-8 using ELISAs specific for these interleukins. A significant increase in IL-6 release was detected in response to 1% and 5% FLDE ($P < 0.05$) at 6 h, but an additional significant ($P < 0.05$)

elevation in IL-6 was observed in response to 24-h treatment with 1% and 5% FLDE (Fig. 1A). Likewise, 1% and 5% FLDE stimulated significant increases in IL-8 release at 6- and 24-h treatment (Fig. 1B). These increases in interleukin protein release were temporally preceded by a rapid elevation of mRNA for both IL-6 and IL-8 at 1-h exposure to either 1% or 5% FLDE (Fig. 1, *A* and *B*, *insets*). A purified concentration of endotoxin equivalent to that in our preparation of 10% FLDE failed to stimulate any release of IL-6 or IL-8 in our assays (data not shown). No significant media release of IL-4, -10, -13, -17, or -18 was detected in response to any concentration or time point of FLDE assayed (data not shown). No cell death was observed in response to 10% FLDE (data not shown). These data demonstrate that an extract of cattle feedlot dust can elicit the specific release of IL-6 and IL-8 from the bronchial epithelium.

Because our previous studies revealed that CAFO dust obtained from hog confinement facilities stimulated the release
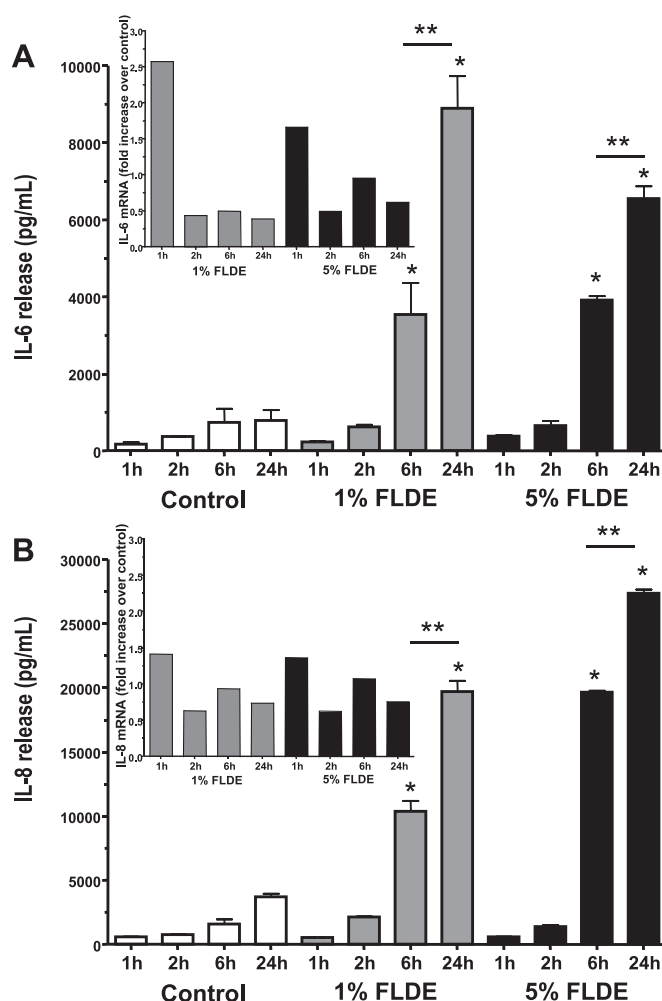

Fig. 1. Feedlot dust extract (FLDE) stimulates IL-6 and IL-8 mRNA production and protein release in a concentration-dependent manner in normal human bronchial epithelial cells. FLDE (1% in gray bars; 5% in black bars) stimulated a significant (*$P < 0.05$ vs. media control) release of both IL-6 (*A*) and IL-8 (*B*) at 6 h of treatment. At 24-h treatment, both 1% and 5% FLDE were significantly (**$P < 0.05$) increased over the 6-h treatment. By 1-h treatment with 1% and 5% FLDE, increases in mRNA for both IL-6 (*A, inset*) and IL-8 (*B, inset*) were detected.

of IL-6 and IL-8 in a PKC-dependent manner (20), we assayed the effect of FLDE on PKC activity in NHBE cells. FLDE-stimulated isoenzyme activity was assayed for PKCα, PKCδ, PKCε, and PKCζ, isoforms previously identified in airway epithelium (34). A rapid activation of PKCα was observed after a 1-h exposure with 1% and 5% FLDE (Fig. 2A). A subsequent activation of PKCε was observed after a 6-h exposure with 1% and 5% FLDE (Fig. 2B). No significant activation of PKCδ or PKCζ was observed under these same treatment conditions (data not shown). These data reveal that FLDE activates PKCα and PKCε concurrent with the release of IL-6 and IL-8.

The impact of pharmacological inhibition of PKC on FLDE-stimulated release of interleukins was assayed next. NHBE cells were pretreated for 1 h in the presence or absence of 1 μM of the PKCα-specific inhibitor, Gö-6976. We previously found this concentration of Gö-6976 to be effective at inhibiting PKCα activity in airway epithelium (35). Gö-6976 did not inhibit IL-6 (Fig. 3A) or IL-8 (Fig. 3C) release after a 6-h exposure to 5% FLDE. IL-6 and IL-8 release was also not significantly decreased in the presence of Gö-6976 after a 24-h exposure to FLDE (data not shown). Direct assay of NHBE PKCα activity levels demonstrated that FLDE failed to activate PKCα in the presence of Gö-6976 (Fig. 3E). Next, NHBE cells were pretreated for 1 h in the presence or absence of 10 μM of the novel PKC class inhibitor Ro 31-8220. We previously used this concentration of Ro 31-8220 to effectively inhibit PKCε

activity in bronchial epithelium (25). Ro 31-8220 significantly inhibited IL-6 (Fig. 3B) and IL-8 (Fig. 3D) release after a 6- and 24-h exposure to 5% FLDE. Likewise, the direct assay of NHBE PKCε activity levels demonstrated that FLDE failed to activate PKCε in the presence of Ro 31-8220 (Fig. 3F). Interestingly, the PKCδ inhibitor rottlerin and the PKCζ inhibitor myristolated PKCζ inhibitor peptide failed to inhibit FLDE-stimulated release of IL-6 or IL-8, even though these inhibitors both decreased their respective isoform activities (data not shown). These data show that whereas FLDE activates both PKCα and PKCε, only the inhibition of PKCε results in the inhibition of IL-6 and IL-8 release.

Because pharmacological kinase inhibitors can lack specificity or induce unexpected side effects, we created cell lines from the immortalized BEAS-2B cell line that express a DN PKCα or PKCε to confirm our results using inhibitor drugs. To characterize these BEAS-2B mutant cell lines, we performed direct kinase activity and translocation assays. The DN PKCα cell line made in BEAS-2B cells demonstrated a significant reduction in calcium-activated PKCα activity, whereas the DN PKCε cell line retained a calcium-activated PKCα response similar to that of wild-type BEAS-2B cells (Fig. 4A). Conversely, a significant decrease in total PKCε activity was detected in the DN PKCε cell line, but not in the DN PKCα cells (Fig. 4B). Functionally, no stimulated translocation of PKCε from cytosol to membrane in response to the PKC-activating phorbol ester, PMA, was observed in DN PKCε cells (Fig. 4C). These control experiments characterize these DN cell lines to be functionally lacking their specific PKC isoform activities and demonstrate their usefulness in assaying specific PKCα- and PKCε-stimulated responses.

Using these isoform-specific PKC DN BEAS-2B cell lines, we examined the effect of FLDE on the stimulated release of IL-6 and IL-8. Treatment with 5% FLDE for 6 and 24 h stimulated a significant IL-6 release in wild-type BEAS-2B cells (Fig. 5A). The DN PKCα-expressing cell line demonstrated no differences from these wild-type cells in response to FLDE. Similarly, the FLDE-stimulated IL-8 release, although decreased, remained significantly stimulated in PKCα DN-expressing cells (Fig. 5C). Direct PKC activity assay revealed that PKCα was not activated by FLDE in the DN PKCα cells (Fig. 5E). However, in the DN PKCε-expressing cell line, a significant reduction in IL-6 (Fig. 5B) and IL-8 (Fig. 5D) in response to 6- and 24-h FLDE was observed in contrast to the levels of stimulated IL-6 and IL-8 in wild-type BEAS-2B at the same time points. Again, direct PKC isoform activity assays in these same cells showed no PKCε activation in response to FLDE in PKCε DN cells (Fig. 5F). These data confirm that the FLDE-stimulated release of IL-6 and IL-8 requires the activation of PKCε in bronchial epithelial cells.

DISCUSSION

Our findings suggest, for the first time, that PKCε regulates the stimulation of IL-6 and IL-8 release in response to FLDE in NHBE and BEAS-2B cells. Using direct measurements of interleukin release and kinase activation, we found that both a PKCε inhibitor as well as expression of DN PKCε blocks the stimulation of IL-6 and IL-8 release by extracted feedlot dust. We used two approaches to confirm that the DN-transfected constructs expressed in the BEAS-2B cell line were indeed
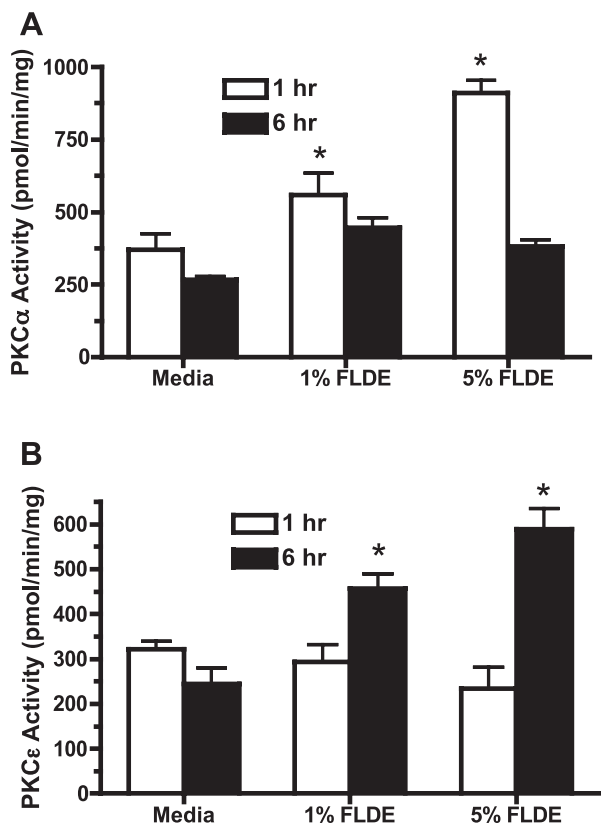

Fig. 2. FLDE activates protein kinase C (PKC) in a concentration-dependent manner in normal human bronchial epithelial cells. PKCα is significantly (*P < 0.05 vs. media control) activated by 1–5% FLDE at 1-h exposure (A), whereas PKCε is significantly activated at 6-h FLDE exposure to 1–5% FLDE (B). PKCα activity is significantly elevated at 1 h by 5% vs. 1% (*P < 0.05).
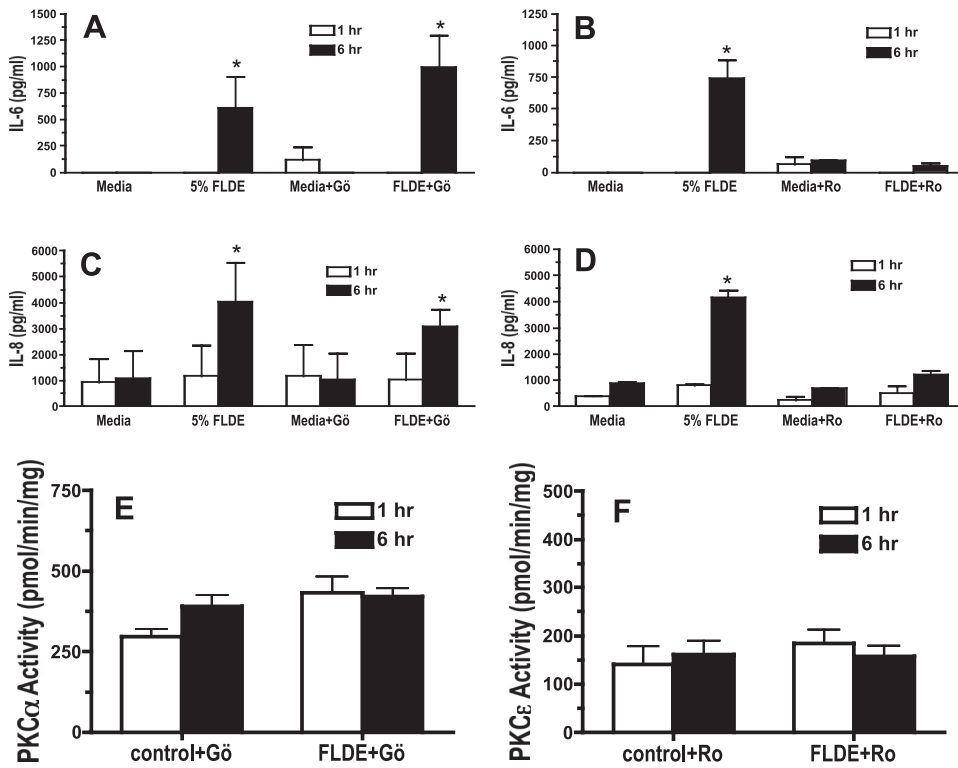
Fig. 3. Inhibition of PKCε, but not PKCα, blocks FLDE-stimulated increases in IL-6 and IL-8 in normal human bronchial epithelial cells. Preincubation for 1 h with 1 μM Gö-6976 (Gö), a PKCα-specific inhibitor, before stimulation with 5% FLDE did not inhibit the stimulated release of IL-6 (*A*) or IL-8 (*C*) even though Gö-6976 effectively blocked FLDE-stimulated increases in PKCα (*E*). Preincubation for 1 h with 1 μM Ro 31-8220 (Ro), a PKCε inhibitor, before stimulation with 5% FLDE inhibited the stimulated release of IL-6 (*B*) and IL-8 (*D*). Ro 31-8220 also inhibited FLDE-stimulated increases in PKCε (*F*). *$P < 0.05$ vs. media control.

capable of altering the function of our target enzyme. First, we directly assayed the kinase activity of the DN cells. Because the classic PKC isoforms require calcium binding to the regulatory region of the enzyme before catalytic activation can occur, we assayed the total in vitro PKCα activity in the presence and absence of calcium. Unlike PKC from the wild-type BEAS-2B cells, PKC from the DN PKCα cell line failed to respond to exogenous calcium. Second, we assayed the translocation of activated kinase to the membrane in both wild-type and PKCε DN cells. It is established that phorbol
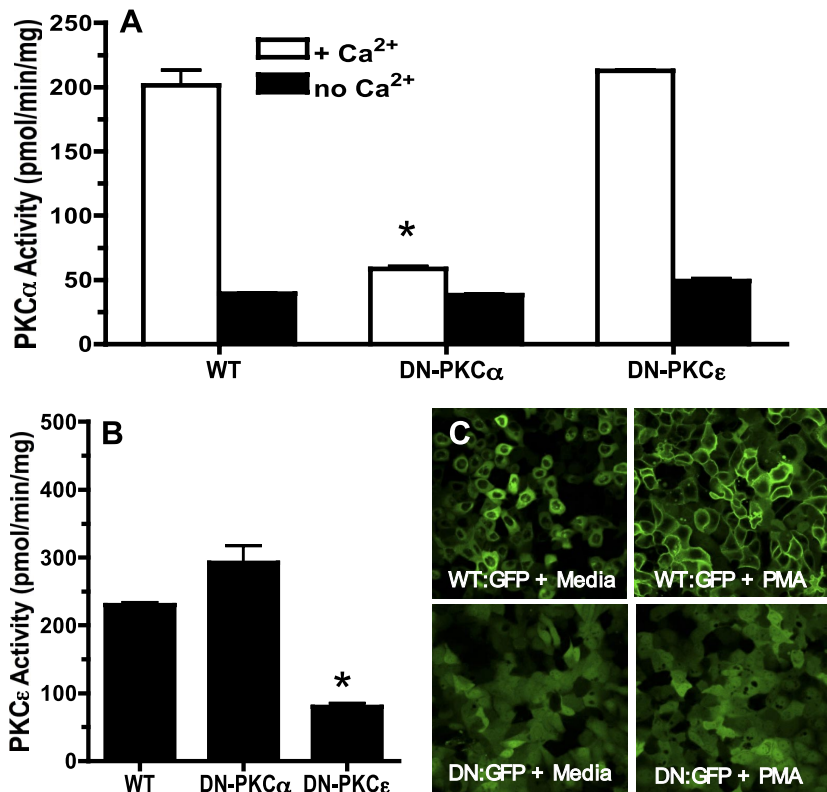

Fig. 4. Dominant negative (DN) PKC-expressing cell lines are isoform specific. Cell lines DN for PKCα and PKCε (DN-PKCα and DN-PKCε) were constructed in an immortalized human bronchial epithelial cell line (BEAS-2B). Wild-type (WT) cells demonstrated a 4.5-fold increase in total PKCα activity in response to 10 μM CaCl$_2$ (*A*). Calcium-stimulated PKCα activity was present in DN-PKCε cells, but not in DN-PKCα cells (*$P < 0.05$ vs. WT control). DN-PKCε cells demonstrated a significant (*$P < 0.05$) decrease in PKCε activity compared with WT or DN-PKCα cells (*B*). As visualized by confocal microscopy, no stimulated translocation of PKCε from cytosol to membrane in response to 15 min of 20 ng/ml of the PKC-activating phorbol ester, PMA, was observed in DN-PKCε cells (*C*).
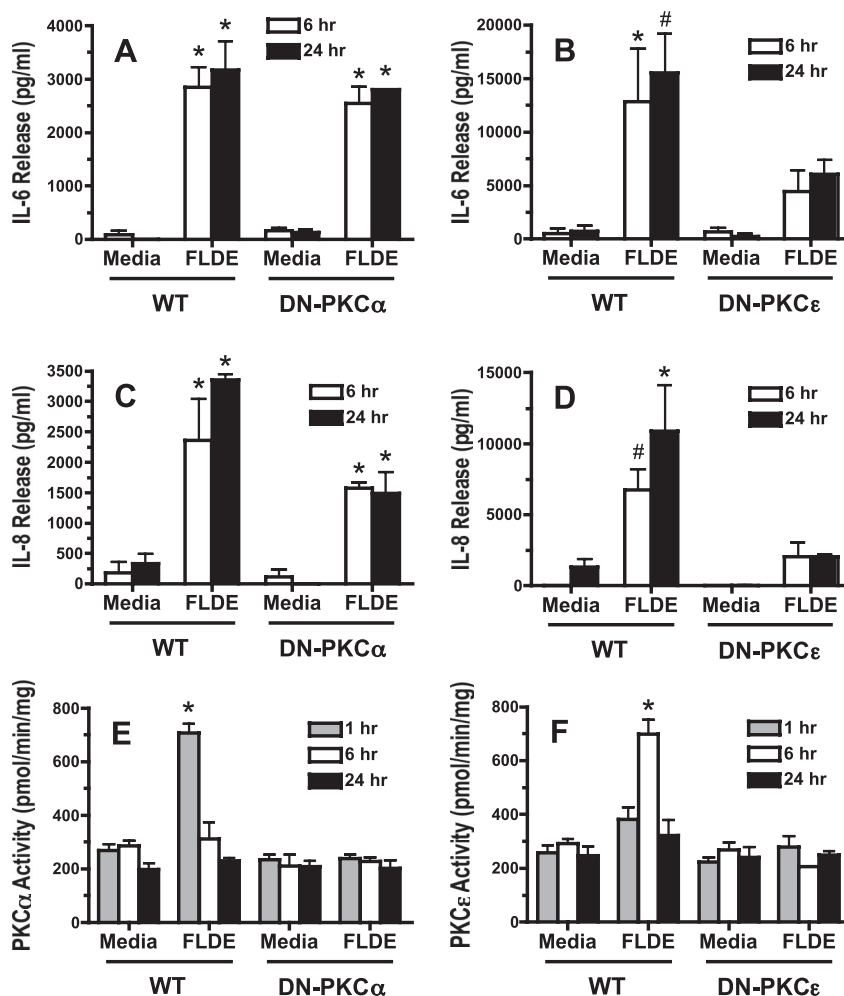
Fig. 5. FLDE fails to stimulate IL-6 and IL-8 release in a bronchial epithelial cell line (BEAS-2B) expressing a DN PKCε. No differences in 5% FLDE-stimulated release of IL-6 are observed between WT BEAS-2B cells and a DN-PKCα-expressing cell line (*A*). A significant, although reduced, amount of IL-8 is released in response to 5% FLDE-stimulated DN-PKCα cells (*C*). Significant inhibition of 5% FLDE-stimulated IL-6 (*B*) and IL-8 release (*D*) is observed in a DN-PKCε-expressing cell line. FLDE fails to activate PKCα or PKCε in the DN cell lines (*E* and *F*). #$P < 0.01$ vs. media control; *$P < 0.05$ vs. media control.

esters bind to the diacylglycerol binding site of the regulatory region and induce PKCε to translocate to the cell membrane. Therefore, we tagged both our wild-type and DN PKCε constructs with a GFP so that the localization of PKCε could be visualized by confocal microscopy. In this manner, we demonstrated that wild-type GFP-PKCε-expressing cells rapidly translocated to the cell membrane in response to phorbol ester, but DN GFP-PKCε-expressing cells failed to perform this enzyme activation function. Furthermore, GFP had no affect on kinase activity. Therefore, we have provided evidence that the DN cells used in our study indeed have functionally inhibited PKC isoform activity before using them in a novel assay involving FLDE.

It is established that airway epithelial cells contain the PKC isoforms α, β, δ, ε, and ζ. Both the classic calcium-dependent isoform PKCα as well as the novel isoform PKCδ have been implicated in the regulation of interleukin release in airway epithelial cells. PKCα has been reported to regulate stretch-induced IL-8 secretion in A549 cells (37) and cigarette smoke (33) or aldehyde-stimulated IL-8 release (35) in bronchial epithelial cells. PKCδ has been reported to regulate TNFα-stimulated IL-8 release (16) in the 16HBE14o⁻ human bronchial cell line. It was therefore surprising to us to find that neither PKCα nor PKCδ activation was responsible for FLDE-stimulated IL-6 and IL-8 release in bronchial epithelium. The

difference in isoform activation times made it attractive to speculate that PKCα was activated early (1 h) by FLDE, and this activation led to a sequential activation of PKCε at 6 h. However, we found that although FLDE was associated with the activation of PKCα, similar to the effect that hog barn dust has on bronchial epithelial cells (20), PKCα activation was not associated with FLDE-stimulated IL-6 or IL-8 release.

Our findings also differ from previous reports that IL-8 is stimulated via the activation of PKCα in that we observe no change in FLDE-stimulated IL-6 and IL-8 whether we use normal cells treated with the PKCα inhibitor or mutant cells that express a DN PKCα. This observation may be unique to FLDE stimulation. However, we observed no inhibition of FLDE-stimulated IL-6 or IL-8 in response to the PKCδ inhibitor rottlerin even though rottlerin effectively inhibited PKCδ activity in NHBE and BEAS-2B. In fact, we have been unable to block the stimulated IL-8 release in response to cigarette smoke, aldehyde-adducted proteins, or TNFα by inhibiting PKCδ (data not shown). This finding is in contrast with those of Page et al. (16) where DN PKCδ-expressing cells were shown to block TNF-stimulated IL-8 release, although direct measurements of PKCδ activity were not performed in that study.

PKCε regulates diverse functions in the airway epithelium. PKCε has been shown to increase PMA-stimulated migration

and cell shape changes in human alveolar A549 cells (3). PKCε has been reported to facilitate cystic fibrosis transmembrane regulator activity via RACK1 binding in the human tracheal Calu-3 cell line (13) as well as the regulation of the sodium, potassium-ATPase in rat alveolar cells (8). We have recently implicated PKCε in the attachment of ciliated epithelial cells to the basal epithelium (25). Clearly, this isoenzyme plays many roles in airway epithelial signaling depending on the specific cell type and stimulatory agent involved. Adding to this complexity is the observation that interleukin release can be mediated by other PKC isoforms, such as the stimulation of cigarette smoke-induced IL-8 release via PKCα (11). Due to the nature of auto-downregulation in response to activation of PKC, it is not surprising that multiple PKC isoforms would demonstrate overlapping functions. Under such a scenario, the stimulation of proinflammatory cytokines may take place in cells exposed to cigarette smoke via PKCα after such a cytokine response by FLDE leading to downregulated PKCε has occurred. Such a scenario of dual cigarette smoke and organic dust exposure would be an interesting hypothesis to test using in vivo models.

Studies of the signal transduction mechanisms regulating airway inflammation in response to inhaled agricultural occupational dusts have important clinical implications. We have observed that PKC isoenzymes, especially PKCε, are critically involved in FLDE stimulation of epithelial cell IL-8 and IL-6 release, suggesting that the isoenzymes may represent novel targets for therapies aimed at modulating organic dust-induced airway inflammation. Dusts from different types of animal confinement feeding operations cause similar respiratory symptoms, but different dusts may elicit inflammatory mediator release using multiple signaling pathways. Defining how dusts are different in terms of cell signaling and additionally what components of dusts are responsible for these differences may assist in improved monitoring of occupational settings.

It is important to relate the dust material used in this study with realistic exposure conditions. Sweeten et al. (28) reported net downwind concentrations (5 h averaging time) of TSP ranging from 97 to 1,687 μg/m^3 and averaging 700 $\pm$ 484 μg/m^3 (mean $\pm$ 1 SD) on three commercial cattle feed yards in the Texas panhandle (net downwind concentrations are computed as the downwind concentration minus the upwind, or background, concentration, and they reflect the net increase in aerosol concentration attributable to the source area). Their measurements were taken between 15 and 61 m downwind of the feed pens. The corresponding 5-h concentrations of PM$_{10}$ (particles having an aerodynamic equivalent diameter of 10 μm or less) ranged from 11 to 866 μg/m^3 with an average of 285 $\pm$ 214 μg/m^3. Actual TSP concentrations measured in the monitoring events from which the present samples were taken ranged from 100 to 4,000 μg/m^3 at a distance of ~15 m downwind from the source boundary. In Sweeten et al. (28), as well as the present study, samples were collected along or near the center line of the plume at ground level under atmospheric conditions of very high to extreme dust potential. For the samples analyzed in the present study, those conditions included low afternoon relative humidity (<25%), high afternoon temperatures (>32°C), and low wind speed (<6.7 m/s).

In summary, an extract of dust obtained from ambient air downwind of cattle feeding operations stimulates airway epithelial cells' release of inflammatory cytokines, IL-8 and IL-6.

We have also determined that this dust augments epithelial cell PKCα and PKCε activation. Using both isoenzyme inhibitors and DN epithelial cells for PKCα and PKCε, we observed that PKCε is critical in the regulation of feedlot dust-induced IL-8 and IL-6. Further studies are needed to define the component(s) of cattle feedlot dust responsible for stimulation of IL-8 and IL-6 via a PKCε-dependent pathway.

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