Hog barn dust extract stimulates IL-8 and IL-6 release in human bronchial epithelial cells via PKC activation

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Romberger, D. J., V. Bodlak, S. G. Von Essen, T. Mathisen, and T. A. Wyatt. Hog barn dust extract stimulates IL-8 and IL-6 release in human bronchial epithelial cells via PKC activation. J Appl Physiol 93: 289–296, 2002; 10.1152/japplphysiol.00815.2001.—Hog barn workers have an increased incidence of respiratory tract symptoms and demonstrate an increase in lung inflammatory mediators, including interleukin (IL)-8 and IL-6. Utilizing direct kinase assays for protein kinase C (PKC) activation, we demonstrated that dust from hog confinement facilities, or hog dust extract (HDE), augments PKC activity of human airway epithelial cells in vitro. A 5% dilution of HDE typically stimulates an approximately twofold increase in human bronchial epithelial cell (HBEC) PKC activity compared with control medium-treated cells. This increase in PKC is observed with 15 min of HDE treatment, and kinase activity reaches peak activity by 1–2 h of HDE treatment before returning to baseline PKC between 6 and 24 h. The classic PKC inhibitor, calphostin C, blocks HDE-stimulated PKC activity and associated IL-8 and IL-6 release. Desensitization to HDE stimulation of PKC activation does not appear to occur because subsequent exposures to HDE after an initial exposure result in further augmentation of PKC. Detoxification of HDE with polymyxin B to remove endotoxin did not change PKC activation or IL-8 release, suggesting that endotoxin is not solely responsible for HDE augmentation of PKC. These data support the hypothesis that HDE exposure augments HBEC IL-8 and IL-6 release via a PKC-dependent pathway.

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IT IS INCREASINGLY RECOGNIZED that workers in animal confinement facilities, and hog confinement workers in particular, are at increased risk for the development of respiratory tract disease (6, 25, 35). Workers in hog confinement facilities frequently have airway symptoms and suffer from an increased incidence of chronic bronchitis (4, 11). Smokers who work in this environment are two to three times more likely to have significant symptoms (34). Importantly, persons acutely exposed to hog confinement facilities have evidence of airway inflammation characterized by neutrophilia with an associated increase of proinflammatory cytokines, including interleukin (IL)-8 and IL-6 (5, 38). This airway inflammatory response may be due in part to an upregulation of the responses of airway epithelial cells when exposed to the environmental conditions of hog barns. As the initial cells exposed to inhaled irritants, epithelial cells are thought to play a central role in airway inflammatory processes and have the ability to release a variety of mediators, including IL-8 and IL-6 (22, 32). Indeed, dust from hog confinement facilities has been shown to potentiate human bronchial epithelial cell IL-8 and IL-6 release in vitro (17, 20). IL-8 is an important chemokine involved in recruiting neutrophils to the airways and may be central in the neutrophilic response seen in airway secretions of persons exposed to hog barns. IL-6 also has a variety of proinflammatory properties and has been demonstrated to be augmented in both the blood and airway secretions of persons exposed to hog barns (38, 40).

The signaling pathways involved in hog barn dust mediation of epithelial cell inflammatory responses such as IL-8 and IL-6 release have not been clearly defined. Our laboratory previously observed that cigarette smoke promotes airway epithelial cell cytokine release via protein kinase C (PKC) activation (41). We hypothesized that hog barn dust extract (HDE) may also augment IL-8 and IL-6 release of airway epithelial cells via PKC activation. Our laboratory previously demonstrated that bronchial epithelial cells (BEC) possess several isoenzymes of PKC, namely PKC-α, -β2, -δ, -ε, and -ζ (42). Acetaldehyde activates PKC directly in bronchial epithelial cells (43) and is one component of cigarette smoke that results in cigarette smoke extract-stimulated PKC activity and IL-8 release (43). This cigarette smoke-stimulated release of IL-8 is inhibited by Gö-6976, an inhibitor specific for PKC-α (31).

In this study, we demonstrated that hog barn dust-stimulated release of airway epithelial cell IL-8 and IL-6 is indeed mediated via PKC-dependent mechanisms. This may offer new possibilities for designing more specific therapeutic targets aimed at controlling inflammation in hog production facility workers.
METHODS

Cell preparation. Human BEC (HBEC) were obtained from one of two sources. We utilized bronchoscopy brushings of patients undergoing bronchoscopy for clinical reasons, as previously published, after obtaining informed consent and with the approval of the Human Studies Subcommittee of the Research and Development Committee of the Omaha Veteran’s Affairs Medical Center (23). Cells were processed by using the technique of Kelsen et al. (14). Cells were passaged no more than seven times before use in experiments. With use of a cytokeratin stain, cells were found to be 95–98% epithelial in origin. Additionally, BEAS-2B cells (American Type Culture Collection, Manassas, VA), an SV40-transformed human bronchial epithelial cell line, were also examined. Airway epithelial cells from all 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% CO2-95% air. LHC-9-RPMI 1640 at 1:1 mix was used to support the growth of these cells, as previously described (2, 23). LHC-9 medium contains LHC basal medium (Biofluids, Rockville, MD), 0.5 μM phosphoethanolamine-ethanolamine (Sigma Chemical, St. Louis, MO), 0.11 mM calcium (Fisher, Springfield, NJ), 50 U/ml penicillin and streptomycin (Life Technologies, Grand Island, NY), 2 μg/ml fungizone (Life Technologies), trace elements, 5 μg/ml bovine insulin (Sigma Chemical), 5 ng/ml epidermal growth factor (Sigma Chemical), 10 μg/ml bovine transferrin (Sigma Chemical), 10 nM 3,3’,5-triiodothyronine (Biofluids), bovine pituitary extract (50 μg protein/ml; Pel Freeze, Rogers, AR), 0.2 μM hydrocortisone (Biofluids), 0.5 μg/ml epinephrine (Sigma Chemical), and 0.1 μg/ml retinoic acid (Sigma Chemical).

Preparation of HDE. Settled surface dust from hog confinement facilities was obtained for use in these experiments. The dust of each facility was processed separately, and experiments were repeated with different sources of dust. We obtained dust from two different facilities, each having ∼500–700 animals. Samples were collected in the winter. This extract was prepared in a fashion similar to that for grain dust extracts utilized in previously published work (36). HDE was prepared by placing 1 g of dust in 10 ml of Hanks’ balanced salt solution (Biofluids) without calcium. The mixture was vortexed and allowed to stand at room temperature for 1 h. The mixture was centrifuged for 10 min, and the supernatant was recovered and centrifuged again. The final supernatant was filter sterilized and used immediately. The extract made with hog feed from a matching swine confinement facility, or feed dust extract (FDE), was prepared in an identical fashion to the HDE.

The amount of endotoxin in the HDE was quantitated by using the limulus amebocyte lysate gel clot assay commercially available and was recorded on each extract prepared (3). PKC activity assay. PKC activity was determined in crude whole cell fractions of HBEC. The assay employed was a modification of procedures previously described (10) using 100 μM PKC substrate peptide (Peninsula, Belmont, CA), 12 mM Ca(C2H3O2)2, 8 μM phosphatidylt-r-serine, 24 μg/ml phorbol 12-myristate 13-acetate, 30 mM dithiothreitol, 150 μM ATP, 45 mM Mg(C2H3O2)2, and 10 μC/ml γ-32P]ATP (ICN Biomedicals, Costa Mesa, CA) in a Tris-HCl buffer (pH 7.5). Samples (50 μl) were added to 40 μl of the above reaction mixture and incubated for 15 min at 30°C. Spotting 50 μl of each sample onto P-81 phosphocellulose papers (Whatman, Clifton, NJ) halted incubations. Papers were then washed five times for 5 min each in phosphoric acid (75 mM), washed once in ethanol, dried, and counted in nonaqueous scintillant as previously described (24). Kinase activity was expressed in relation to total cellular protein assayed and calculated in picomoles of phosphate incorporated per minutes per milligram. Similar to previously published reports of variability in IL-8 release (18), baseline unstimulated values for PKC activity vary in HBEC between cell lines and between passages. The trends in PKC activation are consistent, whereas the empirical magnitude of the kinase activity may differ between cells from different donors and passages.

Cytokine release assay. IL-8 and IL-6 levels in culture supernatants were quantified by using a sandwich ELISA. Ninety-six-well flat bottomed polystyrene microtiter plates (Dynatech, Chantilly, VA) were coated with 200 μl/well of purified (goat) anti-human IL-8 antibody (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 the plates were washed three times in PBS-Tween 20, 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 120 min. Plates were washed three times with PBS-Tween 20 followed by the addition of (rabbit) anti-human IL-8 antibody (Endogen, Woburn, MA) or IL-6 antibody (Calbiochem, La Jolla, CA) diluted 1:1,000 in PBS-Tween 20-Blotto (0.2% instant nonfat milk in PBS-Tween 20) for 60 min. 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 o-phenylenediamine (Sigma Chemical), 0.003% H2O2 in distilled H2O] was added. The reaction was terminated with 27.5 μl/well of 8 M sulfuric acid, and plates were read at 492 nm in an automated ELISA reader (Bio-Rad, Hercules, CA).

Materials. RPMI 1640, DMEM, and MEM were purchased from GIBCO BRL (Rockville, MD). The calphostin C was purchased from Calbiochem. Lipo polysaccharide (LPS) utilized was from Escherichia coli purchased from Sigma Chemical. All other reagents not specified were purchased from Sigma Chemical.

Statistical analysis. All experiments were performed three separate times, and the data presented represent means ± SE of triplicate experiments with n = 9 samples, unless otherwise noted. The constitutive amount of PKC does vary depending on the passage of epithelial cell utilized in the experiment. We expressed PKC as fold changes in activation comparing control, untreated cells with the various agents. Data were analyzed for statistical significance in each experiment by ANOVA utilizing Statview statistical program.

RESULTS

Our laboratory’s previous finding that cigarette smoke induced IL-8 release of airway epithelial cells in a PKC-dependent manner (41) prompted us to hypothesize that HDE stimulates the release of IL-8 in HBEC via a similar signaling pathway. Similar to cigarette smoke extract, a 5% dilution of HDE stimulates an approximately twofold increase in HBEC PKC activity compared with control medium-treated cells (Fig. 1B). The increase in PKC is observed with 15 min HDE of treatment. PKC is expressed as fold activation of untreated cells (Fig. 1B).
of 3 separate experiments with triplicate samples in each experiment; \( n = 9 \) (\( P = 0.0006 \) by ANOVA). This increase in PKC activation persists at 1 h (1.8 ± 0.15-fold activation; \( P = 0.002 \)) and 2 h (1.9 ± 0.27-fold activation; \( P = 0.005 \)) after HDE exposure and is inhibited by pretreatment of airway epithelial cells with the classic PKC inhibitor calphostin C (increase of PKC of cells pretreated with 1 \( \mu \)M calphostin C followed by HDE: at 15 min = 0.6 ± 0.1-fold, at 1 h = 0.7 ± 0.2-fold, at 2 h = 0.6 ± 0.1-fold; pretreated with calphostin alone and with no HDE = 0.9 ± 0.2-fold). The kinase activity reaches peak activity within 1–2 h of HDE treatment and persists for 4–6 h before returning to baseline PKC levels between 6 and 24 h (see Fig. 2B) (increase in activation: at 6 h = 2.7 ± 0.34-fold, 24 h = 1.3 ± 0.36-fold, 48 h = 1.36 ± 0.14-fold, 72 h = 1.23 ± 0.12-fold). The effects of HDE on PKC activity were similar in time, dose, and magnitude in HBEC, the transformed human cell line BEAS-2B, and bovine BEC, suggesting that other BEC may serve as a good model for human BEC mechanisms (data not shown). Furthermore, no changes were observed in either cAMP-dependent protein kinase (PKA) or cGMP-dependent protein kinase activity in HBEC exposed to 5% HDE for up to 24 h (data not shown).

Similar to the reports of others (20, 39), we detected a subsequent increase in the release of both IL-6 (Fig. 1A) and IL-8 (Fig. 1C) after treatment of HBEC with HDE. We also found that a 1-h preincubation of HBEC with 1 \( \mu \)M of the PKC-specific inhibitor, calphostin C, resulted in the inhibition of HDE-stimulated PKC activation as well as release of both IL-6 and IL-8 (Fig. 1, A–C). PKC inhibition by calphostin C results in attenuation of HDE-induced IL-6 as early as 15 min that

Fig. 1. Time course of interleukin (IL)-6 and IL-8 release and protein kinase C (PKC) activation of hog dust extract (HDE)-exposed bronchial epithelial cells (BEC). Monolayers of BEC were treated with control medium (○), 5% HDE (●), 1 \( \mu \)M calphostin C (CC; △), or a combination of 5% HDE and 1 \( \mu \)M CC (○) for 0.25–2.0 h, and IL-6 (A) and IL-8 (C) in supernatants of BEC cultures were measured by ELISA. PKC activity was assayed in cell layers (B). Kinase assays were performed as described (see METHODS). PKC is expressed in fold activation of PKC compared with unstimulated cells. Values are means ± SE of triplicate experiments; \( n = 9 \) samples. *Significant difference between HDE and HDE + CC, \( P < 0.05 \) (by ANOVA).

Fig. 2. Lack of desensitization of PKC to HDE. Monolayers of BEC were either treated once with medium containing 5% HDE (●) or reexposed to medium containing 5% HDE for 15 min at each subsequent time point (○) ranging from 6 to 72 h. Culture supernatants were either harvested for IL-8 (A) or IL-6 (C). BEC fractions were assayed for PKC activation (B). Values are means ± SE of triplicate experiments; \( n = 9 \) samples. *Statistically significant difference between treated once and HDE exposed, \( P < 0.05 \) (by ANOVA).
persists at 2.0 h [means ± SE; n = 9; 15-min time point: control = none detected, HDE = 58.6 ± 3.2 pg/ml; calphostin C = none detected; HDE + calphostin C = 20 ± 11 pg/ml; 1.0-h time point: control = 69 ± 40 pg/ml; HDE, 442 ± 109 pg/ml; calphostin C = none detected; HDE + calphostin C = 228 ± 90 pg/ml; 2.0-h time point: control = 553 ± 45 pg/ml; HDE, 9,775 ± 2,378 pg/ml; calphostin C = none detected, HDE + calphostin C = 199 ± 95 pg/ml; P < 0.0001 for control vs. HDE, HDE vs. HDE + calphostin C at 2.0 h (ANOVA)]. The attenuation of HDE-induced IL-8 is not seen until 1 h and persists at 2.0 h [1-h time point: control = 425 ± 183 pg/ml, HDE, 1,413 ± 109 pg/ml, calphostin C = 189 ± 95 pg/ml, HDE + calphostin C = 1,023 ± 87 pg/ml; 2.0-h time point: control = 1,471 ± 715 pg/ml, HDE = 7,603 ± 1,137 pg/ml, calphostin C = 386 ± 127, HDE + calphostin C = 924 ± 119 pg/ml; P < 0.0001 for control vs. HDE, HDE vs. HDE + calphostin C at 2.0 h (ANOVA)]. Similar attenuation of IL-8 and IL-6 were observed when Go-6976, an inhibitor specific for the PKC-α isozyme, was utilized in place of calphostin C (data not shown). This suggests that HDE stimulation of airway epithelial cell PKC augments release of both IL-6 and IL-8.

We observed a concentration-dependent effect of HDE in that exposure of BEC to 1% HDE for 24 h results in a 2.0 ± 0.2-fold stimulation of IL-8 release (means ± SE, 9 different experiments) and that 5% HDE results in a 4.1 ± 0.9-fold increase (P = 0.02 by Student’s t-test). Data from the representative experiment show control BEC with 1,428 ± 81 pm IL-8 (means ± SE; n = 3) in culture supernatant with 1% HDE exposure resulting in a 1.9-fold increase (2,694 ± 204 pm), 2.5% HDE in a 3.9-fold increase (5,565 ± 219 pm), 5% HDE in a 5.7-fold increase (8,232 ± 330 pm), and 10% HDE in a 8.9-fold increase of IL-8 (12,660 ± 1,614 pm).

Although HDE-stimulated PKC activation subsides by 24 h of treatment with a single dose, PKC can be reactivated by additional doses of HDE (Fig. 2B). PKC activity is activated by 15 min of exposure to fresh media containing 5% HDE, regardless of the cells’ previous exposure to HDE at 24, 48, and 72 h [means ± SE; increase in activation with additional 15 min of HDE exposure: at 6 h = 2.3 ± 0.19-fold, 24 h = 2.5 ± 0.27-fold, 48 h = 2.6 ± 0.22-fold, and 72 h = 2.4 ± 0.03-fold compared with increase in activation at 6 h = 2.7 ± 0.34-fold, 24 h = 1.3 ± 0.36-fold, 48 h = 1.36 ± 0.14-fold, and 72 h = 1.23 ± 0.12-fold with 1 exposure only to HDE; P = 0.0006, P = 0.0007, and P = 0.0012 at 24, 48, and 72 h, respectively (ANOVA)]. Similarly, amounts of IL-8 released are greater after additional HDE doses compared with a single dose at the 24- (P = 0.0013) and 48-h time points (P = 0.0024) but not at 72 h (P = 0.54) (Fig. 2A). Furthermore, IL-6 levels are unchanged with subsequent additional exposure to HDE (Fig. 2C). This suggests that desensitization or tolerance to the effects of HDE does not occur in BEC regarding PKC activation and IL-8 release within the first 48 h of in vitro exposure to HDE. However, by 72 h after a single exposure to HDE, PKC can still be reactivated by additional exposure to HDE, whereas there is no further increase in IL-8 release. This suggests that a change in PKC isoforms may occur and that isoforms with less effect on IL-8 release may be activated after multiple exposures to HDE. In contrast to IL-8, tolerance to the PKC induction of IL-6 occurs within the first 24 h and persists at 72 h.

The effects of environmental dusts are often attributed to bacterial endotoxins present in the samples. In an attempt to identify whether endotoxin is the primary component of HDE responsible for PKC signaling leading to IL-8 and IL-6 release, we assayed the effects of endotoxin by using LPS on our system. Because the amount of endotoxin varies within a sample of dust from any given hog confinement facility, we routinely measure the amount of endotoxin in the HDE utilized in our experiments. The same concentration of endotoxin [40 endotoxin units (EU)/ml] contained in a given 5% HDE sample does not stimulate PKC activation (Fig. 3B). PKC activity in HDE-treated cells after 1, 3, and 6 h was increased by 2.9 ± 1.0, 3.3 ± 0.27, and 2.8 ± 0.83-fold, respectively (means ± SE; n = 9). In contrast, there was no significant change in PKC activation in cells treated with 40 EU/ml of LPS (1.1 ± 0.35-fold at 1 h, 0.87 ± 0.13-fold at 3 h, and 1.7 ± 0.7-fold at 6 h; P value significant for comparison between HDE-stimulated change in PKC activation compared with LPS exposed cells at P = 0.039 for 1 h and P = 0.005 for 3 h but not significantly different at 6-h comparison). Cells exposed to both HDE and LPS simultaneously demonstrated PKC activation that was very similar to cells exposed to HDE alone. Although LPS alone did augment IL-8 release slightly, 5% HDE stimulated a significantly greater increase in IL-8 at 3 and 6 h (Fig. 3A) (means ± SE; control cells = 185 ± 113 pg/ml; HDE at 1 h = 1,171 ± 298 pg/ml, at 3 h = 7,993 ± 610 pg/ml, at 6 h = 22,245 ± 4,338 pg/ml; LPS at 1 h = 183 ± 113 pg/ml, at 3 h = 336 ± 214 pg/ml, at 6 h = 1,144 ± 495; P = 0.001, HDE vs. LPS at 3 h; P < 0.0001, HDE vs. LPS at 6 h). Similarly, LPS alone also augmented IL-6 release, but HDE (with the same amount of endotoxin present as in the LPS sample) stimulated a significantly greater amount of IL-6 (Fig. 3C) (control cells = 392 ± 156 pg/ml; HDE at 1 h = 905 ± 277 pg/ml, at 3 h = 10,234 ± 2,763 pg/ml, at 6 h = 13,437 ± 2,531 pg/ml; LPS at 1 h = 385 ± 102 pg/ml, at 3 h = 1,384 ± 343 pg/ml, at 6 h = 1,844 ± 247 pg/ml; P = 0.0002 for HDE vs. LPS at 3 h and P < 0.001 at 6 h). These data demonstrate that 5% HDE exposure results in a significantly greater increase in airway epithelial cell PKC activation and subsequent IL-8 and IL-6 release than in cells exposed to LPS alone with the amount of endotoxin matched to that found in the HDE. When LPS alone was used in experiments at higher concentrations than the 40 EU/ml utilized in this sequence of experiments, a dose-response increase in IL-8 was observed (data not shown).

When HDE is applied to a polymyxin B column to remove endotoxin from the sample, no differences in PKC are detected between HDE and detoxified HDE (Fig. 4B). There was a 1.9 ± 0.15-fold activation with-
out detoxification and 2.0 ± 0.05-fold with detoxification (15 min); 2.0 ± 0.4-fold without detoxification and 2.3 ± 0.5-fold with detoxification (2 h); 2.3 ± 0.3-fold without detoxification and 2.2 ± 0.3-fold with detoxification (19 h) (means ± SE; triplicate experiments). There were also no significant differences in IL-8 released (Fig. 4A) [445 ± 124 pg/ml without detoxification and 850 ± 230 pg/ml with detoxification (15 min); 5,120 ± 1,040 pg/ml without detoxification and 3,107 ± 783 pg/ml with detoxification (2 h); 27,074 ± 3,766 pg/ml without detoxification and 31,474 ± 4,333 pg/ml with detoxification (19 h); no statistical differences by ANOVA] or IL-6 [247 ± 102 pg/ml at 15 min with detoxification and 141 ± 90 pg/ml without detoxification; 3,172 ± 908 pg/ml without detoxification and 4,841 ± 1,739 pg/ml with detoxification (2 h); 10,787 ± 2,742 pg/ml without detoxification and 9,791 ± 2,161 pg/ml with detoxification (19 h); no statistical differences by ANOVA]. There was no detectable endotoxin present in the HDE samples after detoxification within the limits of our assay (0.06 EU/ml). This suggests that the effects observed with HDE are not likely caused solely by endotoxin present in the dust extract.

A component of the dust from the hog barns is derived from the feed dust (8). For this reason, we also examined the effects of FDE alone on PKC, IL-6, and IL-8. The feed was obtained from one of the same barns used for the HDE samples. No significant increase in release of IL-6 was detected in response to FDE at any time point assayed (Fig. 5C) [268 ± 93 pg/ml, control cells at 15 min and (respectively) 476 ± 264, 12 ± 12, and 2,202 ± 683 pg/ml, 1% FDE at 15 min, 1 h, and 24 h; 11 ± 11, 12 ± 12, and 740 ± 188 pg/ml, 5% FDE at 15 min, 1 h, and 24 h; 61 ± 46, 15 ± 15, and 862 ± 235 pg/ml, 10% FDE at 15 min, 1 h, and 24 h; 274 ± 140, 462 ± 141, and 867 ± 244 pg/ml 20% FDE at 15 min, 1 h, and 24 h; means ± SE of duplicate experiments]. IL-8 release was also not significantly altered by FDE exposure (Fig. 5A) [250 ± 250 pg/ml, untreated cells at 15 min and 1,720 ± 573 at 24 h and (respectively) 402 ± 402 pg/ml, 554 ± 385, and 2,674 ± 1,181 pg/ml 1% FDE at 15 min, 1 h, and 24 h; 502 ± 402, 519 ± 419, and 3,274 ± 2,219 pg/ml 5% FDE at 15 min, 1 h, and 24 h; 957 ± 254, 632 ± 404, and 2,859 ± 1,339 pg/ml 10% FDE at 15 min, 1 h, and 24 h; 1,344 ± 198, 1,328 ± 208, and 3,743 ± 2,357 pg/ml 20% FDE at 15 min, 1 h, and 24 h]. In addition, there was no significant activation of PKC in HBEC by any concentration of FDE at any time point assayed (Fig. 5B). The PKC values represent the mean of triplicate experiments. This suggests that feed dust is not a major source of endotoxin or PKC activator.
component of the HDE in terms of PKC activation and subsequent IL-6 and IL-8 stimulation.

**DISCUSSION**

Airway inflammation occurs as a result of exposure to hog confinement facilities (15, 25, 37). This inflammatory response is likely critical to the subsequent development of workers’ respiratory symptoms and airflow obstruction (4, 28). Although not completely characterized, the airway inflammatory response in persons in hog barns involves release of cytokines, including IL-8, that appear to originate in part from epithelial cells of the respiratory tract (1, 5, 38). In vitro studies have shown that hog barn dust is a particularly potent stimulus of airway epithelial cell IL-8 and IL-6 release (20, 39). In this study, we report that an increase in airway epithelial cell PKC activation mediates, at least in part, hog barn dust-induced IL-8 and IL-6 release in vitro. We have shown that exposure to HDE results in airway epithelial cell PKC activation and that the PKC inhibitor calphostin C blocks HDE augmentation of HBEC IL-8 and IL-6 release as well as PKC activation.

The mechanisms regulating hog barn dust-induced airway epithelial cell release of IL-8 and IL-6 are poorly understood. Our results demonstrate that PKC activation is clearly involved. This is similar to our laboratory’s previous report that cigarette smoke extract in vitro augments PKC activation and is required for subsequent complement (C5a)-induced bronchial epithelial cell IL-8 release (41). Monick et al. (19) reported that respiratory syncytial virus infection of lung epithelial cells results in activation of various PKC isoforms that leads to activation of extracellular signal-related kinase and associated IL-8 release. In contrast, ozone has been shown to modulate airway epithelial cell IL-8 expression in a PKC-independent but protein tyrosine kinase- and PKA-dependent manner (13). Taken together, these results support the complexity involved in how various environmental agents induce airway epithelial cell cytokine release.

There is much interest in determining which component(s) of the hog barn environment is most responsible for airway inflammation and respiratory tract symptoms. Dust, endotoxin, and ammonia have all been associated with chronic respiratory illness in swine confinement workers (7, 21, 33). Endotoxin is clearly an important component of hog barn dust and is well recognized as a primary inflammatory stimulus in grain dusts (27). Healthy subjects exposed to hog barns demonstrate airflow obstruction and an increase in serum neutrophils and IL-6 that is most strongly associated with endotoxin in the environment (44). In an animal model of corn dust exposure, endotoxin was shown to be primarily responsible for the inflammatory response (12). Endotoxin-hyporesponsive mice have reduced biological response to corn dust (26). Recently, George et al. (9) demonstrated that endotoxin-sensitive mice, and not genetically endotoxin-hyporesponsive mice with a mutation in the Toll-like receptor-4, develop airway remodeling with subchronic exposure to corn dust. However, it is not clear whether endotoxin alone is responsible for all the changes induced by all grain dusts or hog barn dust. Von Essen et al. (36) observed that airway epithelial cell neutrophilic chemotactic activity induced by various grain dusts was independent of endotoxin in the samples. Others have observed that endotoxin does not appear to be solely responsible for pulmonary inflammation or symptoms in persons exposed to hog barns (5, 15). This correlates with our in vitro data suggesting that endotoxin alone is not solely responsible for hog barn dust-induced epithelial cell IL-8 or IL-6 release (Fig. 4). In addition, we observed that HDE was a more potent stimulus of airway epithelial cell IL-8 and IL-6 release than LPS (at concentrations similar to those found in the particular sample of HDE) alone (Fig. 3). This is similar to observations by Palmberg et al. (20) that hog barn dust...
was more potent than LPS in augmenting IL-8 release of airway epithelial cells. The FDE we utilized did not stimulate either PKC activity, IL-6, or IL-8 (Fig. 5). These data are also similar to observations by Palmberg et al. (20) that both glucans and grain dust were weaker stimuli than swine dust and LPS in their experiments. In our experiments, epithelial cell exposure to peptidoglycan alone, a gram-positive bacterial component present in hog barn dust, did not show significant stimulation of HBEC IL-8 or IL-6 release (data not shown). We have not identified the primary component of HDE that is responsible for the augmentation of PKC, IL-8, or IL-6. It is likely that multiple components act together to modulate changes in epithelial cell PKC activation and cytokine release. In the barns, gases, including ammonia, can bind to dust particles and potentiate the inflammatory response. Dissecting the component of HDE that binds to airway epithelial cells and subsequently signals PKC activation will require intensive analysis of this complex organic material. We have preliminary data to suggest that scavenger receptors on the airway epithelial cell membrane may be instrumental in PKC-mediated IL-8 production (data not shown).

Many of the initial studies regarding airway inflammation in response to hog barns have been done in naïve subjects exposed acutely to the environment of the facilities. Pronounced airway neutrophilia and IL-8 are associated with such acute exposures (5, 15). Airway neutrophilia is more variable in hog barn workers who have chronic exposure in that some studies report no change in the cellularity of bronchoalveolar lavage fluid, whereas other studies observe an increase in neutrophils, although not as pronounced as in naive persons (16, 25). This may suggest that chronic exposure modifies the inflammatory response differently than acute exposure. In our in vitro system, reexposure to HDE for an additional 15 min after a prior exposure caused a further increase in PKC and IL-8 at 24 and 48 h, suggesting little early desensitization to the effects of the dust. By 72 h, repeat exposure to HDE no longer resulted in an increase in IL-8, implying that desensitization to IL-8 may occur with chronic exposure. In contrast to IL-8, desensitization to IL-6 release appears to occur within the first 24 h (Fig. 2C). Obviously, this in vitro system does not necessarily model a repeated work environment in vivo. However, chronic exposure studies with HDE currently underway may have implications for mechanisms regulating the differences in inflammatory responses seen in workers compared with naïve subjects.

As noted previously, repeated exposures to HDE did not appear to desensitize PKC activation (Fig. 2B) despite evidence of possible desensitization of IL-8 and IL-6. It is possible that HDE with its multiple components could activate various PKC isoforms at different exposure times and that these isoforms might variably regulate subsequent cytokine release. For example, Monick et al. (19) demonstrated that respiratory syncytial virus infection of lung epithelial cells (cell line A549) results in activation of the PKC-ζ isoform within 10 min of infection that correlates with early virus binding and a sustained activation of PKC-β1, -δ, -ε, and -ζ isoforms occurring 24–28 h postinfection. Bronchial epithelial cells have several isoforms of PKC, including PKC-α, -β2, -δ, -ε, and -ζ (42), and the effect of HDE on activation of these individual isoforms is not yet clearly defined. We have observed that an inhibitor that is more specific for PKC-α, Gö-6976, appeared to have similar results as calphostin C in attenuating HDE-augmented changes in PKC and IL-8 (data not shown).

There is evidence of a systemic inflammatory response in association with inhalation of hog barn dust as well as an airway response (30, 40). Wang et al. (40) described an increase in TNF-α and IL-6 in serum of naïve subjects exposed to hog barns for only 3 h. Although maximum IL-6 generally occurred after maximum TNF-α levels, in many subjects there was an early rise in IL-6, suggesting that multiple origins of IL-6 may be involved in response to hog barn dust. Senthilselvan et. al. (29) observed that the serum IL-6 response in subjects exposed to a swine confinement environment utilizing dust suppression with canola oil was reduced compared with those in a more typical barn setting. Thus inhalation of dusts in swine confinement facilities appears to stimulate systemic responses that may mediate inflammatory responses outside the lung.

In summary, we have shown that exposure to HDE initially results in activation of PKC in airway epithelial cells in vitro. This kinase activation mediates the HDE-stimulated release of both IL-8 and IL-6 release. Endotoxin alone does not appear to be completely responsible for HDE effects on PKC activation and subsequent IL-8 and IL-6 release. Modulation of PKC activation of the epithelium may represent an additional pathway to regulate airway inflammation of workers in environments such as hog barns.

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