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Aryl Hydrocarbon Receptor Regulation of Induction of Inflammatory Mediators by Organic Dust Extract in Lung Epithelial Cells

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ARYL HYDROCARBON RECEPTOR REGULATION OF INDUCTION OF INFLAMMATORY MEDIATORS BY ORGANIC DUST EXTRACT IN LUNG EPITHELIAL CELLS

by

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LIST OF ABBREVIATIONS

AFO: Animal Feeding Operation

AhR: Aryl Hydrocarbon Receptor

ANOVA: Analysis of Variance

AP-1: Activator protein-1

ARNT: AhR Nuclear Translocator

AhRR: AhR repressor

BCP: bromochloropropane

BHLH: basic helix-loop-helix

BSA: Bovine Serum Albumin

CAFO: Concentrated Animal Feeding Operation

Cyp1A1: Cytochrome P450 Family 1 Subfamily A Member 1

DAPI: 4,6-diamidino-2-phenylindole

DE: Organic Dust Extract

ELISA: Enzyme Linked Immunosorbent Assay

EVs: Extracellular vesicles

HSP90: Heat shock protein

ICAM-1: Intracellular Adhesion Molecule 1

IDO1: Indoleamine 2,3-dioxygenase

IgG: Immunoglobulin G

IL-1β: Interleukin 1β

IL-6: Interleukin 6

IL-8: Interleukin 8

LPS: Lipopolysaccharide

MAPK: Mitogen activated protein kinase

NFκB: Nuclear factor κB

PAH: Polycyclic aromatic hydrocarbons

PAI-2: plasminogen activator inhibitor-2

PAS: PER-ARNT-SIM

PCR: Polymerase Chain Reaction

pro-IL-1β: Pro-Interleukin 1β

qRT-PCR: Quantitative Real Time Polymerase Chain Reaction

ROS: Reactive oxygen species

Sc: ScrambledSDS-PAGE: Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis

Src: Tyrosine kinase pp60

Stat-3: signal transducer/ activator of transcription 3

TBST: Tris-buffered saline containing 0.1% Tween 20

TCDD: 2,3,7,8-tetrachlorodibenzodioxin

TNF-α: Tumor Necrosis Factor α

XAP2/ AIP: AhR-Interacting Protein

ABSTRACT

Previous studies have indicated a link between respiratory symptoms and diseases and organic dust exposure in agricultural workers. The aryl hydrocarbon receptor (AhR), a transcription factor activated by a wide variety of exogenous and endogenous compounds, is known to be involved in the metabolism of toxic chemicals and recently in the modulation of immune and inflammatory responses. To better understand mechanisms of pathogenesis of respiratory symptoms and diseases, the role of AhR in the regulation of induction of inflammatory mediators by poultry organic dust extract in airway epithelial cells was studied. The role of AhR was studied by investigating the effects of organic dust extract on AhR nuclear translocation and the effects of AhR silencing on the organic dust extract induction of proinflammatory mediators IL-1β, ICAM-1, IL-6 and IL-8. Our studies showed that AhR translocation is increased by organic dust extract indicating AhR activation and AhR silencing inhibited induction of inflammatory mediators by organic dust extract. Collectively, our studies showed that AhR positively regulates induction of inflammatory mediators in airway epithelial cells by organic dust.

INTRODUCTION

CAFOs and Organic Dust

The United States Environmental Protection Agency (EPA) defines animal feeding operations (AFOs) as agricultural enterprises comprised of confined spaces in which animal production operations occur in confined spaces on small land areas [1]. Concentrated animal feeding operations (CAFOs) refer to large, concentrated animal feeding operations in which more than 1,000 animal units (an animal unit defined as the animal equivalent of 1,000 pounds live weight) are kept on site more than 45 days during the year [1]. Within CAFOs, animals are raised at high density to achieve efficient production and cost effectiveness; however, the indoor air tends to be contaminated with high concentrations of aerosolized dust due to lack of optimal ventilation [2]. Previous studies have indicated occupational exposure to organic dust is associated with acute and chronic respiratory diseases, each of which are characterized by enhanced lung inflammation [3].

The dust generated in CAFOs referred to as organic or agricultural dust is a complex mixture of plant, bacterial, and fungal products as well as gases such as methane, hydrogen sulfide, and ammonia [3, 4]. Early studies on respiratory health in CAFO workers considered the various constituents found in organic dust and have shown that the CAFO environment is rich in microbial life [5]. In particular, the concentrations of bacteria and fungi in the air in poultry buildings was higher than in buildings housing other animals [6]. A study conducted during the 1990's characterized the microbial presence within CAFOs across seasons and found slightly elevated culturable mold and total organism levels with fungal types *Cladosporium* and *Alternaria* being predominant in the summer and fall and yeasts *Penicillium* and *Fusarium* being predominant in the winter and spring [7]. Studies conducted to determine the components of poultry organic dust have identified the following constituents: waste, feed, litter, dander, ammonia, carbon dioxide,

pollen, grain mites, fungi, bacteria, viruses, and endotoxin [8, 9]. Research over the last few decades have shown that microbial exposures, especially to endotoxins, are related to adverse effects on respiratory health [5]. European multicenter studies were conducted to determine the prevalence and risk factors of respiratory diseases in farmers; the results of this study revealed workers in animal farms were at the highest risk of developing respiratory symptoms with the major risk factor being ventilation in the animal houses [10, 11]. To further assess the symptoms associated with poultry and swine farmers, another study was conducted in which the prevalence of respiratory symptoms and lung function were studied; the results of this study indicated that poultry farmers had lower lung function than pig farmers and the impairment of lung function was associated with inadequate ventilation of animal houses [12]. In consideration of adverse respiratory health effects associated with exposure to organic dust, it is important to understand the mechanisms underlying pathogenesis of respiratory symptoms and respiratory diseases. A better understanding of mechanisms of pathogenesis of respiratory symptoms and respiratory diseases may lead to the development of new and improved treatments.

Organic Dust Exposure and Respiratory Symptoms and Diseases

Occupational and environmental lung diseases continue to be major causes of respiratory impairment among workers and general population throughout the world [13]. Previous studies have suggested that occupational exposure to organic dust is associated with respiratory diseases such as asthma, hypersensitivity pneumonitis, bronchitis, chronic obstructive pulmonary disease, and organic dust toxic syndrome [3]. Considering each of these diseases are characterized by enhanced lung inflammation [14], efforts have been made to understand the mechanisms underlying inflammation and its role in causing cell and tissue injury.

In the body, acute inflammatory responses protect the host from systemic infection and restore tissue homeostasis in response to injury or pathogens [15]. In the lung, acute inflammation can significantly compromise vital gas exchange; to combat this, several mechanisms are employed to regulate the severity and duration of acute inflammation [15]. Within the respiratory system, the epithelium acts as a physical barrier against particulates and microbial pathogens while also regulating immune and inflammatory responses; in response to injury or pathogens, respiratory epithelia produce chemokines, cytokines, and other bioactive molecules [16]. To restore inflamed tissues to their basal state, inflammatory cells must be cleared, and leukocyte recruitment must be rescinded in which neutrophils undergo apoptosis and are then engulfed by phagocytic macrophages while other mediators suppress the progression of inflammation as well as promote tissue repair [17]. In some cases, dysregulation of inflammatory responses can occur due to persistent exposure to allergens, pathogens, or defects in genes that control inflammation, thus leading to the development of respiratory symptoms and diseases as described previously [17].

It is well established that exposure to organic dust is a risk factor for the development of respiratory symptoms and diseases [3]. As animal production industry has grown worldwide in recent years employing increasing numbers of workers, the prevalence of respiratory diseases among these workers is expected to increase. As a general principle, the concentration of animals in proximity increases the likelihood of microorganism transmissions among the group and the infection of surrounding life, even if they are of a different species [18]. Due to the nature of CAFOs and insufficient ventilation, the indoor air tends to be contaminated with high concentrations of organic dust. A study conducted to determine the effects of working conditions for animal confinement workers found that workers had both acute and chronic respiratory

symptoms likely due to the inhalation of endotoxins, ammonia, and microorganisms found in the dust [19]. Studies conducted to evaluate inflammatory responses in bronchial epithelial, monocytic, and macrophage cells in response to various organic dusts reported induction of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-8 (IL-8), intracellular adhesion molecule-1 (ICAM-1), and interleukin-1β (IL-1β) [20-25]. Mice exposed to swine facility dust extract showed increased neutrophil and macrophage counts in bronchoalveolar lavage fluid with responses dampening after repetitive exposure; however, it was also noted that a persistently elevated presence of inflammatory mediators within the lavage fluid was noted in response to repetitive dust exposure [26]. Similar findings were reported in which healthy, non-smoking subjects exposed to swine dust during work hours had significant increases in neutrophil counts in nasal and bronchoalveolar lavage fluids [27].

Multiple studies have described the inflammatory responses to organic dust exposure with a special emphasis being placed on the induction of inflammatory mediators and cytokines; however, mechanisms mediating their induction are not well understood. A study conducted to assess the inflammatory responses of pig farmers, smokers, and healthy non-smokers to swine organic dust hypothesized that those regularly exposed to organic material (e.g., pig farmers and smokers) developed a tolerance [28]. The results of this study confirmed their hypothesis in which pig farmers demonstrated a reduction in respiratory symptoms, bronchial responsiveness, and markers of airway inflammation in addition to an attenuation of lung function and markers of low-grade, on-going inflammation when compared to controls [28]. When comparing smokers to controls, mixed results were obtained in which some subjects responded similarly to pig farmers and others like non-smokers, thus suggesting swine organic dust exposure and tobacco smoke exposure do not activate the same adaptive mechanisms [28]. Another study yielded similar results

in which naïve subjects had the most pronounced inflammation when compared to swine confinement building workers thus suggesting a level of tolerance to the endotoxins and other substances present in the dust for swine confinement workers [29]. Based on these studies and others, it is evident that organic dust is linked to the development of respiratory symptoms and diseases; however, the mechanisms by which they occur are still unclear.

Aryl Hydrocarbon Receptor

The aryl hydrocarbon receptor (AhR) was first identified in 1976 as a dioxin-binding protein [30]. Since its discovery, AhR has been identified as a member of the basic helix-loop-helix (bHLH)/ PER-ARNT-SIM (PAS) superfamily of transcription factors and is regarded as well-conserved protein with a ubiquitous presence and variable expression levels throughout mammalian life [31]. AhR has been widely studied in its role as a transcription factor responding to environmental toxic chemicals. In its non-activated form, AhR is found in the cytosol bound by its chaperone, heat shock protein 90 (HSP90), the AhR-interacting protein (XAP2/ AIP), tyrosine kinase pp60 (Src) and its cochaperone, p23 [31-33]. Once bound to a ligand, AhR dissociates from XAP2/ AIP and undergoes conformational changes that expose its nuclear localization signal, thus leading to nuclear translocation [31-33]. Once in the nucleus, AhR forms a complex with aryl hydrocarbon receptor nuclear translocator (ARNT) which then binds to AhR response elements (AHRE/ DRE/ XRE) DNA sequences to regulate the transcription of target genes [31-33]. Following activation, AhR transcriptional activity is controlled via negative feedback regulation by the AhR repressor (AhRR), ligand-induced proteolytic degradation by the ubiquitin-proteasome pathway, and enhanced metabolism of the activating ligand [34]. A schematic diagram depicting AhR activation is shown in Figure 1. Activation of AhR is also known to control cell signaling

pathways important for homeostasis and has also been implicated in the modulation of inflammatory response and development of diseases [35, 36].

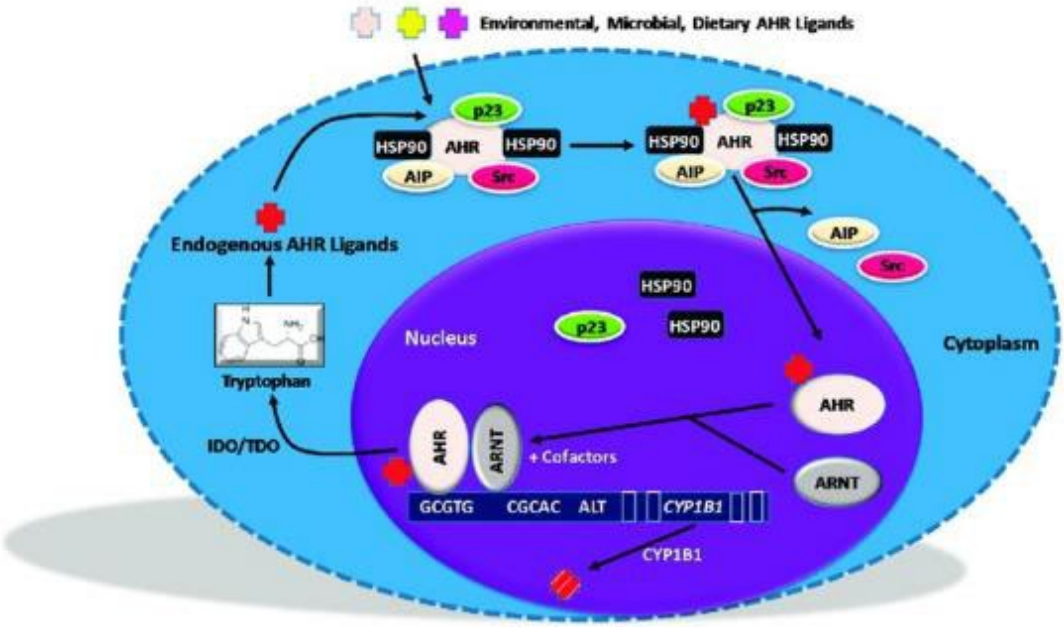

Figure 1. AhR activation mechanism.
A schematic of the mechanism of AhR activation by endogenous or exogenous ligands. Binding of the activating ligand to cytosolic AhR causes dissociation from XAP2/ AIP and a conformational change that exposes the nuclear localization signal. AhR is translocated to the nucleus where it forms a complex with ARNT which then binds to AhR response element sequences to regulate the transcription of target genes. Wang Z, Snyder M, Kenison JE, Yang K, Lara B, Lydell E, Bennani K, Novikov O, Federico A, Monti S, Sherr DH. How the AHR Became Important in Cancer: The Role of Chronically Active AHR in Cancer Aggression. *International Journal of Molecular Sciences*. 2021; 22(1):387. https://doi.org/10.3390/ijms22010387

AhR has been shown to play an essential role in the modulation of responses to microbial pathogens with lipopolysaccharide (LPS) stimulation being a frequently used model [36, 37]. In one study using an LPS-sepsis mice model, AhR was found to control infection and modulate septic shock [37]. In this study, it was determined that LPS induces proinflammatory cytokines to initiate tryptophan degradation by indoleamine 2,3-dioxygenase (IDO1) to generate catabolites that activate AhR [36]. AhR activation resulted in the generation of regulatory T-cells and the

regulation of inflammatory responses considering AhR knockout mice expressed higher proinflammatory cytokine levels when compared to wildtype mice [36]. Another study revealed that AhR knockout mice were hypersensitive to LPS-induced septic shock, mainly due to macrophage dysfunction [37]. In response to LPS, AhR knockout mice secreted enhanced levels of IL-1β in bone marrow-derived macrophages through regulation of plasminogen activator inhibitor-2 (PAI-2) [37]. Recent evidence suggests that AhR responds to multiple exogenous and endogenous signals derived from diet, host metabolism, the intestinal microbiome, and the surrounding environment [38-40]. AhR has also been implicated in lung inflammation and lung disease [41, 42]. A study conducted by Chiba et al. reported human bronchial epithelial cells express AhR and its activation induced mucin production through reactive oxygen species (ROS) [41].

Considering its role as a modulator in disease and immune settings, and its implications in lung inflammation and disease, we sought to determine the role of AhR in the induction of lung inflammatory mediators by organic dust extract.

RESEARCH HYPOTHESIS

Unpublished studies from our laboratory indicated that siRNA-mediated knockdown of AhR protein levels decreased the induction of pro IL-1β, ICAM-1, IL-6, and IL-8 protein levels in Beas2B bronchial epithelial cells. This data suggested that AhR could be involved in the regulation of lung inflammatory responses, therefore we sought to understand the role of AhR in the induction of inflammatory mediators by organic dust extract.

We hypothesized that organic dust activates AhR to modulate inflammatory responses in lung epithelial cells.

Experimental Objectives

Objective I: Determine the effects of AhR knockdown on the induction of inflammatory mediators by organic dust extract.

Objective II: Determine the effects of AhR antagonist, CH223191, on the induction of inflammatory mediators by organic dust extract.

Objective III: Determine the effects of organic dust extract on AhR activation and AhR expression levels.

MATERIALS AND METHODS

Organic Dust Extract Preparation

Dust settled on vertical surfaces of buildings was collected from a poultry farm in East Texas after 8 weeks of animal growth. The farm is an indoor facility equipped with tunnel ventilation in which chickens are floor-raised. The dust collected was placed in sterile plastic tubes and stored at -70°C until extraction. Organic dust extract was prepared, according to methods described previously [25], by incubating dust (1g/10 mL) in serum-free F12K medium containing penicillin (100 U/mL), streptomycin (100 µg/mL), and amphotericin B (0.25 µg/mL) in an ultrasound water bath at room temperature for 10 minutes with periodic agitation. The suspension was cleared by centrifugation at 800 g for 5 minutes at 4°C followed by centrifugation at 10,000 x g for 10 minutes at 4°C. The supernatant obtained was filtered using a 0.2 µ syringe filter and the filtrate was stored at -20°C. The concentration of this extract was arbitrarily considered as 100%. Protein concentration of dust extract was determined by Bradford method using bovine serum albumin (BSA) as the protein standard

Cell Culture and Treatment

Beas2B bronchial epithelial cells (ATC CRL-9609) were grown on plastic culture dishes coated with fibronectin, bovine type I collagen, and BSA in LHC-9 medium (Invitrogen) or BEGM medium (Lonza) in a humidified environment of 95% room air and 5% carbon dioxide. When cells reached 70-80% confluence, growth medium was replaced with serum-free RPMI 1640 medium (Gibco) overnight and cells were treated with dust extract and AhR chemical agonist and antagonist in the same medium.

siRNA Transfection of Cells

Beas2B cells (~40,000 per well) were plated on coated 6-well plastic culture dishes in 2 mL LHC-9 or BEGM medium 24 hours prior to transfection. Scrambled (Sc) and AhR siRNAs (Sigma-Aldrich) were diluted in serum-free OptiMEM or serum-free RPMI 1640 medium (0.125 mL) for a final concentration of 33 nM and Lipofectamine 3000 (Invitrogen) (3.5 μL in 0.125 mL OptiMEM or serum-free RPMI 1640 medium) in separate tubes. The samples were incubated at room temperature for 5 minutes before mixing and incubation continued at room temperature for 10-15 minutes. Beas2B cell medium was replaced with fresh medium and the siRNA-Lipofectamine complexes were added dropwise to the cells with gentle swirling. Cells were incubated for 6 hours before changing to fresh complete medium and cells were grown for 48 -72 hours to achieve knockdown of target proteins.

ELISA

IL-6 and IL-8 levels in cell culture medium were determined by colorimetric sandwich enzyme linked immunosorbent assay (ELISA) (R and D Systems) according to the manufacturer's instructions.

MTS Cell Viability Assay

Beas2B cells (~ 10, 000 cells/well) were plated in 96-well culture plates and grown for 24 hours prior to transfection with control and AhR siRNAs according to the procedure described in the previous paragraph. Cells were grown for ~60 hours and then treated with medium only or 0.25% dust extract for 3 h. Cell viability was analyzed by MTS assay (Promega) according to the manufacturer's protocol.

Immunofluorescence Confocal Imaging

Beas2B cells (~10,000 per well) were grown on coverslips (12 mm borosilicate glass) placed in 24-well culture plates. Cell culture medium was replaced with serum-free RPMI 1640 medium overnight prior to treatments. After treatment, cells were washed two times with 400 μL wash buffer (PBS containing 0.1% BSA). Washing consisted of adding wash buffer, incubation for 5 minutes at room temperature, then cells were subjected to fixation by incubation in 2% paraformaldehyde prepared in PBS for 20 minutes at room temperature. The cells were again washed twice with wash buffer. For immunostaining, cells were washed twice with wash buffer and incubated with 400 μL blocking buffer (PBS containing 3% goat serum and 0.3% Triton X-100) for 45 minutes at room temperature. After blocking, cells were washed once with wash buffer and incubated with 150 μL primary antibody diluted in PBS containing 3% goat serum overnight at 4◦C. Cells were washed two times with wash buffer and incubated with 400 μL 1 μg/ml fluorescent dye-conjugated secondary antibody in PBS containing 3% goat serum for 1 hour at room temperature. Cells were rinsed two times with 400 μL wash buffer and coverslips were removed from the wells and excess buffer carefully removed with blotting paper. 20 μL of 4,6-diamidino-2-phenylindole (DAPI) in mounting medium (Permount) (Southern Biotech) was pipetted onto the microscopic slide and coverslip was placed on the slide with the cells facing the slide. Microscope slides were wrapped in aluminum foil to protect from light and stored at 4°C until visualization using a confocal microscope. AhR fluorescence intensity within the nucleus was quantified using ImageJ software (NIH). Typically, 5-10 cells from 2-3 fields from each experiment were quantified for AhR staining.

SDS-PAGE Electrophoresis and Western Blot Analysis

After treatment, cell medium was collected and cleared by centrifugation at ~ 800 x g for 5 minutes at 4◦C and stored at -70◦C. Beas2B cells were rinsed once with cold PBS and lysed using 80-100 µL cell lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, (and freshly added protease and phosphatase inhibitor cocktail) per well of 6-well cell culture dishes and collected by scraping into 1.5 mL tubes. Cell lysate was incubated on ice for 15 minutes and cleared by centrifugation at ~ 15,000 x g for 10 minutes at 4°C. Protein concentrations were determined by Bradford Assay; equal amounts of protein (10-30 µg) were separated by sodium dodecyl-sulfate polyacrylamide chain reaction (SDS-PAGE) on 10% NuPAGE Bis-Tris gels (Life Technologies) alongside molecular weight protein markers using MOPS running buffer. Separated proteins were transferred to Hybond-PVDF membranes (0.2 µM; GE Healthcare) by electroblotting. Membranes were blocked by incubation in 5% non-fat dry milk in 1x tris-buffered saline containing 0.1% Tween 20 (TBST) with shaking at room temperature for 1 hour. Membranes were washed in TBST and incubated with primary antibody in TBST containing 5% BSA or 5% non-fat dry milk overnight at 4°C with shaking. Membranes were washed with TBST and incubated with secondary antibody diluted in TBST for 1 hour at room temperature with shaking. List of antibodies used is shown in Table 1. Membranes were washed with TBST, reacted with fluorescent/chemiluminescent substrate, and scanned using an imager (Bio-Rad Chemidoc MP Imager). Protein bands were quantified using ImageLab software. Membranes were stripped of bound antibodies and probed for other proteins of interest and finally actin for normalization.

Table 1. List of antibodies used in western blot analysis

Antibody	Company	Catalog No.
AhR (D5S6H)	Cell Signaling Technology	83200
β-Actin	Santa Cruz Biotechnology Inc.	sc-47778
ICAM-1	Santa Cruz Biotechnology Inc.	sc-8439
IL-1β (D3U3E)	Cell Signaling Technology	1273
Anti-mouse IgG, HRP-linked	Cell Signaling Technology	7076
Anti-rabbit IgG, HRP-linked	Cell Signaling Technology	7074

RNA Isolation and Real-Time Quantitative RT-PCR (qRT-PCR)

After treatment, total RNA was isolated from Beas2B cells using TRI-Reagent (Molecular Research Center) according to the manufacturer's instructions. Cells were lysed with TRI reagent (1 mL/ well, 6-well dish) and samples were either stored at -80°C or immediately used for RNA isolation. For 1 mL cell lysate, 100 µL bromochloropropane (BCP) was added, mixed vigorously by hand, and incubated at room temperature for 10 minutes. The samples were then centrifuged at 15,000 x g for 15 minutes at 4°C; the aqueous layer was carefully transferred to a new 1.5 mL tube and equal amount of isopropanol and 2 µL of 5 µg/µL glycogen were added, then the samples were incubated at room temperature for 10 minutes. Samples were centrifuged at 15,000 x g for 8 minutes at 4°C, supernatant was removed, then pellets were washed two times with 1 mL 75% cold ethanol and centrifuged at 7,500 rpm for 5 minutes at 4°C. After washing, the pellets were air-dried and dissolved in 45 µL DNase/ RNase free water. Isolated RNA was digested with TURBO DNase (Ambion) to remove genomic DNA and precipitated by adding 0.3 M sodium acetate pH 5.2 and 2.5 volumes of ethanol; RNA pellets were washed with 70% ethanol, dried,

and dissolved in nuclease-free water. RNA was quantified by measuring absorbance at 260 nm and purity was determined by measuring A_{260}/A_{280} and A_{260}/A_{230} ratios using a nanodrop spectrophotometer (ThermoScientific). RNA was reverse transcribed using iScript reverse transcription supermix (Bio-Rad) by incubation in a thermal cycler with the following conditions – 5 minutes at 25°C, 30 minutes at 42°C, and 5 minutes at 85°C. The levels of mRNAs were determined by real-time quantitative polymerase chain reaction (PCR) with TaqMan probe assays (Bio-Rad/Life Technology) using CFX 96 Real-Time PCR Detection System (Bio-Rad) with the following reaction conditions – 95°C for 30 seconds, 95°C for 5 seconds, and 60°C for 30 seconds with 40 cycles total. TaqMan probes for target genes are shown in Table 2. mRNA levels were normalized to actin mRNA levels and normalized gene expression data (ΔΔCt) relative to control (untreated) sample set to 1 was obtained using CFX Manager Software (Bio-Rad).

Table 2. Gene expression IDs of TaqMan assays for quantification of RNA levels

Gene Symbol	**Gene Name**	**Assay ID**
AhR	Aryl hydrocarbon receptor	Hs001629233_m1A
Cyp1A1	Cytochrome P450 1A1	Hs01054797_g1
ACTB	β-actin	Hs1060665z_g1

Statistics

Each experiment was performed at least three times independently and the data was analyzed using GraphPad Prism 9 software. Two-tailed paired t-tests or one-way analysis of variance (ANOVA) as appropriate were used to analyze statistical significance between treatment groups; p value of <0.05 was considered as statistically significant.

RESULTS

Effects of AhR Knockdown on the Induction of Inflammatory Mediators by Organic Dust Extract

As AhR is activated by a wide variety of chemical compounds and the availability of AhR antagonists targeting these compounds is limited, we thought that siRNA mediated AhR silencing would be a better approach in discerning its role in the organic dust induction of inflammatory mediators. AhR protein levels were reduced in Beas2B cells by siRNA transfection prior to treatment with 0.25% organic dust extract. Transfection with Sc siRNAs were included as controls. Results indicated that AhR protein levels were significantly reduced by AhR siRNA transfection (Figure 2) and AhR knockdown significantly reduced the induction of pro-IL-1β, ICAM-1, IL-6, and IL-8 protein levels by dust extract (Figure 3). MTS assays were performed on cells transfected with scrambled or AhR siRNA to determine effects on cell viability. Results indicated that transfection with scrambled or AhR siRNA did not adversely affect cell viability (Figure 5).

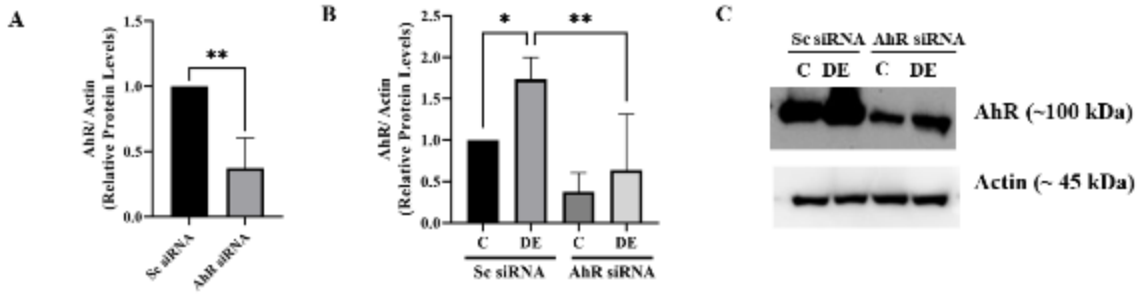

Figure 2. AhR protein levels are reduced via transfection.
Scrambled (Sc) or AhR siRNAs were transfected into Beas2B cells using Lipofectamine 3000 and cells were maintained for 48-60 hours to ensure significant decrease in AhR protein levels. Cells were treated with either medium (C) or 0.25% organic dust extract (DE) for 3 hours. AhR and actin protein levels were determined via western immunoblotting. AhR protein levels were normalized to actin protein levels. Data shown are mean ± SE (n = 3-5). *p < 0.05 and **p < 0.01.

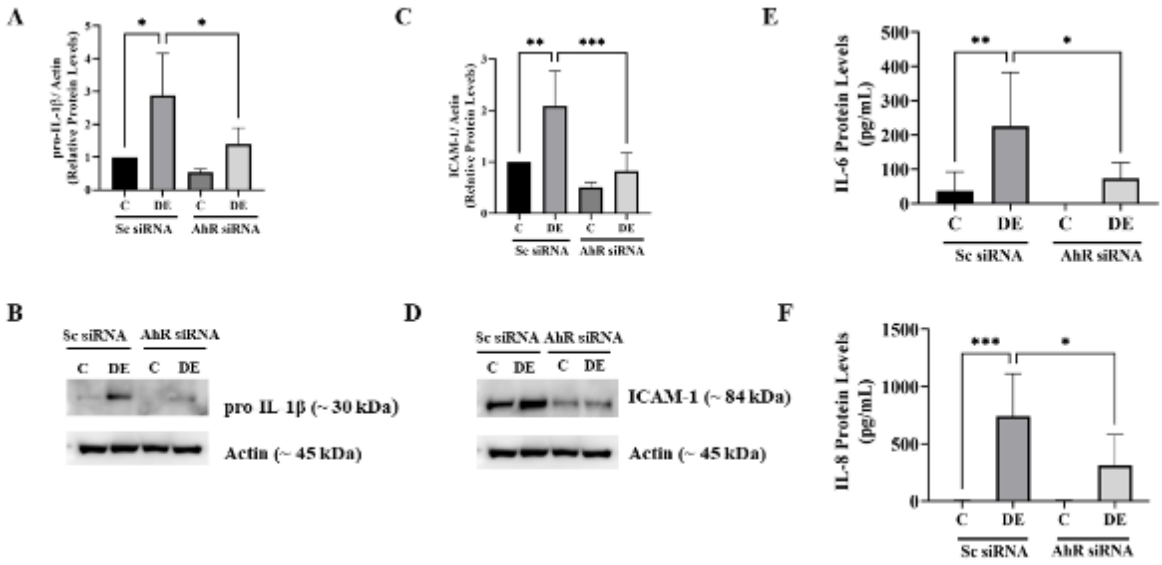

Figure 3. AhR knockdown reduces the induction of inflammatory mediators pro-IL-1β, ICAM-1, IL-6, and IL-8.
Scrambled (Sc) or AhR siRNAs were transfected into Beas2B cells using Lipofectamine 3000 and cells were maintained for 48-60 hours to ensure significant decrease in AhR protein levels. Cells were treated with either medium (C) or 0.25% organic dust extract (DE) for 3 hours. Protein levels of pro-IL-1β (A) and ICAM-1 (C) were determined via western immunoblotting and were normalized to actin protein levels. IL-6 and IL-8 levels in the medium were determined via ELISA (E and F). Data shown are mean ± SE (n = 4-6). *p < 0.05, **p < 0.01, and ***p < 0.001.

As a positive control, the effects of 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), a well-known AhR agonist, on induction of cytochrome P450 family 1 subfamily A member 1 (Cyp1A1) mRNA in Beas2B cells transfected with Sc and AhR siRNAs were determined. Results showed that whereas TCDD robustly induced Cyp1A1 mRNA in scrambled siRNA transfected cells, it failed to induce Cyp1A1 mRNA in AhR siRNA transfected cells (Figure 4 B). Likewise, AhR antagonist CH223191 significantly blocked Cyp1A1 mRNA induction by TCDD (Figure 4 C) validating that AhR is functional in Beas2B cells. MTS assay results revealed that dust extract treatment did not adversely affect the viability of Sc and AhR siRNA transfected cells, indicating that the inhibition of Cyp1A1 mRNA levels in AhR siRNA transfected cells is due to reduced AhR levels and not due to reduced cell viability (Figure 5).

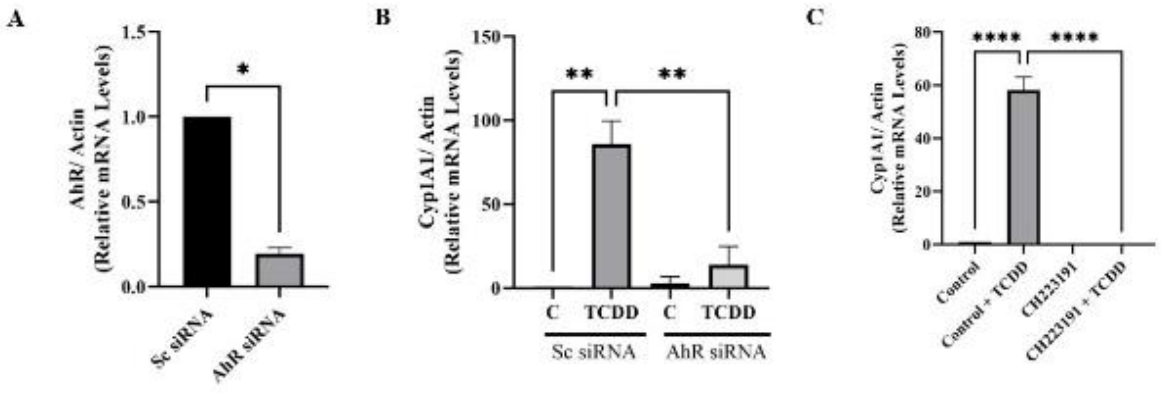

Figure 4. AhR knockdown and treatment with AhR antagonist CH223191 reduces induction of Cyp1A1 by TCDD. Sc or AhR siRNAs were transfected into Beas2B cells using Lipofectamine 3000 and cells were maintained for 48-60 hours to ensure significant decrease in AhR protein levels (A and B) or cells were treated with 10 µM CH223191 for 1 hour prior to treatment (C). Cells were treated with either medium (C) or 10 nM TCDD for 48 hours. AhR, Cyp1A1, and actin mRNA levels were determined via qRT-PCR and AhR and Cyp1A1 mRNA levels were normalized to actin mRNA levels and arbitrary values were assigned with normalization to control samples (A-C). Data shown are mean ± SD (n = 2-3). *p < 0.05, **p <0.01, and ****p < 0.0001.

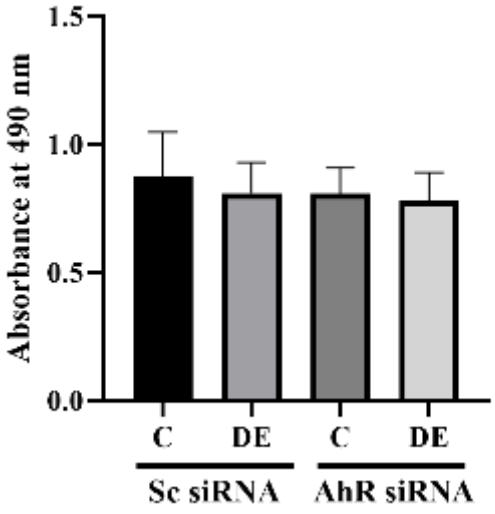

Figure 5. Transfection with Sc or AhR siRNAs does not affect cell viability.
Scrambled (Sc) or AhR siRNAs were transfected into Beas2B cells using Lipofectamine 3000 and cells were maintained for 48-60 hours to ensure significant decrease in AhR protein levels. Cells were treated with medium (C) or 0.25% organic dust extract (DE) for 3 hours. Cell viability was assessed by MTS assay. Data shown are mean ± SE (n = 3).

Effects of AhR Antagonist CH223191 on the Induction of Inflammatory Mediators by Organic Dust Extract

CH223191 functions as a select AhR antagonist against halogenated aromatic hydrocarbons such as TCDD [46]. As TCDD and dioxin-like compounds are widely detected in the environment in air, water, soil, and food [47], we investigated the effects of CH223191 on AhR activation by dust extract to determine if dioxin-like compounds that may be present in organic dust could contribute to AhR activation. Beas2B cells were first incubated with CH223191 (10 or 20 μM) prior to treatment with 0.25% organic dust extract. As CH223191 is prepared in DMSO, treatments with corresponding concentrations of DMSO were employed to confirm the effects seen were not due to DMSO itself. Results showed that CH223191 did not suppress induction of inflammatory mediators by dust extract (Figure 6). To confirm that CH223191 was functional, its effects on TCDD induction of Cyp1A1 mRNA levels in Beas2B cells were determined. It is known that TCDD is a strong inducer of Cyp1A1 [46]. Results showed that TCDD markedly induced Cyp1A1 mRNA levels that were suppressed effectively by CH223191 (Figure 4C). These results indicated that AhR activating ligands present in organic dust extract are different from dioxin and dioxin-like compounds.

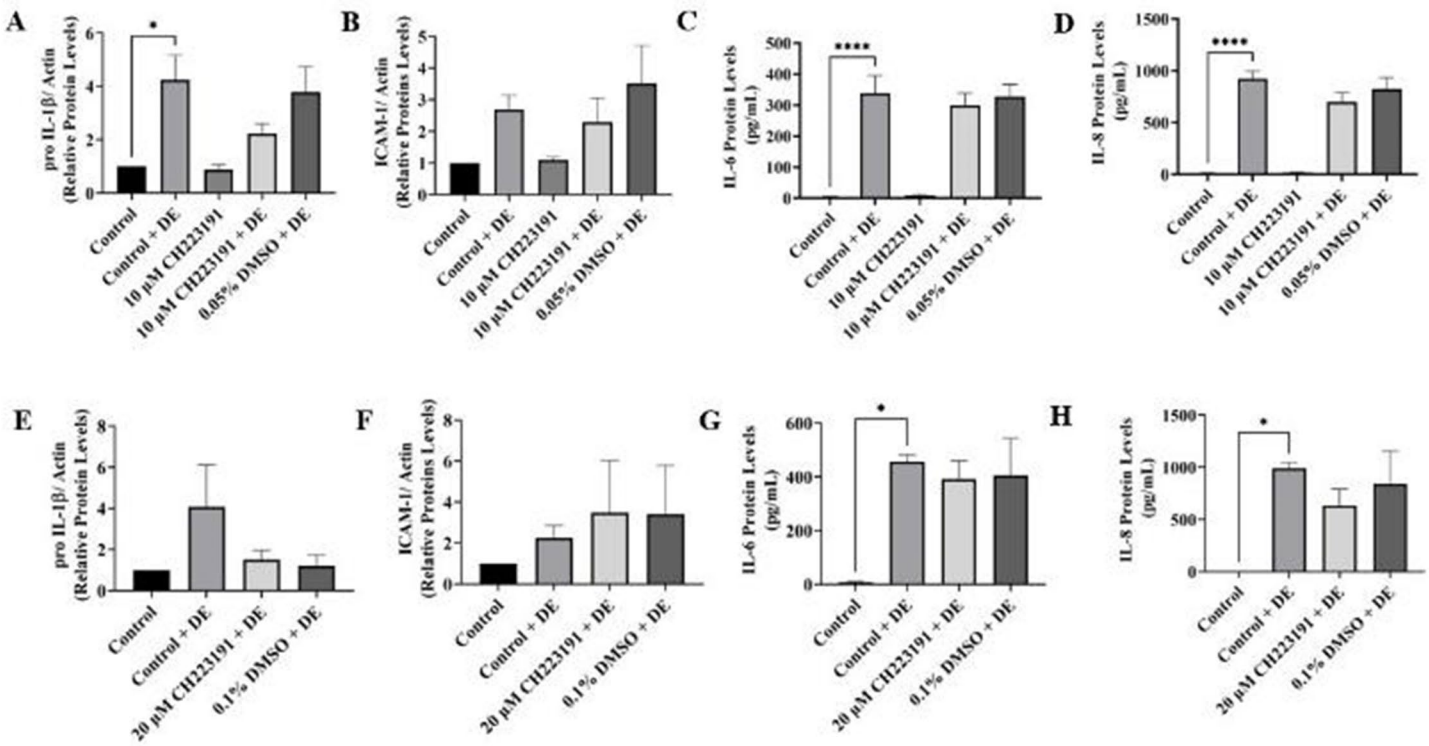

Figure 6. AhR antagonist CH223191 has no effect on the induction of inflammatory mediators.
Beas2B cells were treated with either 10 or 20 μM CH223191 or corresponding concentration of DMSO for 1 hour before treatment with medium (C) or 0.25% organic dust extract (DE) for 3 hours. Protein levels of pro-IL-1β and ICAM-1 were determined by western immunoblotting and normalized to actin protein levels and (A, B, E, and F). IL-6 and IL-8 levels in the medium were determined via ELISA (C, D, G, and H). Data shown are mean ± SE (n = 3 - 6). *p < 0.05 and ****p <0.0001.

Effects of Organic Dust Extract on AhR Activation and Expression

Our studies showed that AhR acts as a positive regulator of inflammatory mediators induced by organic dust extract. This suggested that AhR activation by organic dust extract likely mediates its effects as a positive regulator. We determined the effects of organic dust extract on AhR activation by analyzing nuclear AhR translocation by immunofluorescence confocal microscopy. Beas2B cells were treated with medium only or medium containing 0.25% dust extract for 1,3, 6 and 24 h and AhR levels were visualized by immunofluorescence confocal microscopy. Results showed that the nuclear content of AhR increased as a function of treatment time; modest increase in nuclear AhR staining was apparent at 1 h after treatment and the intensity of AhR staining increased steadily with treatment time (Figure 7 A, Figure 8). Staining with non-immune immunoglobulin G (IgG) of the same isotype as AhR antibody produced very low background indicating the specificity of AhR antibody (Figure 7 B). Cytosolic AhR staining appeared to increase with time suggesting that dust extract may increase AhR protein levels (Figure 7 A).

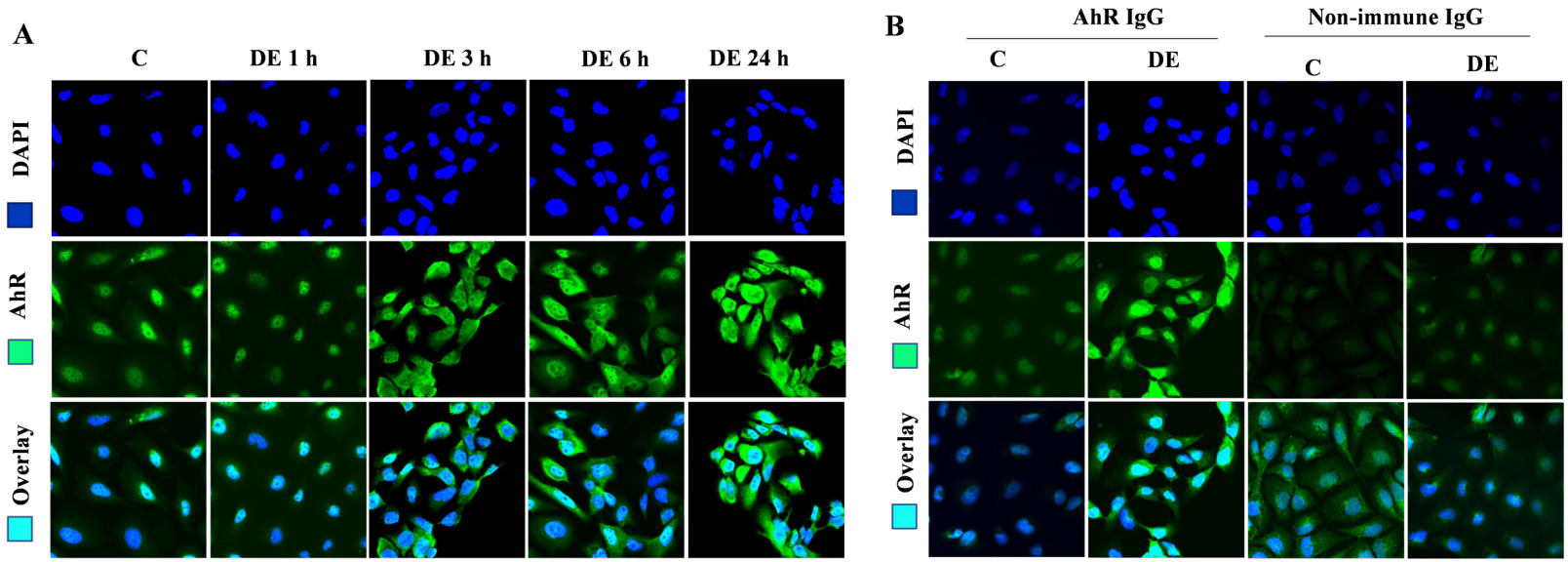

Figure 7. Organic dust extract activates AhR.

Beas2B cells were grown on coverslips were treated with either media (C) or 0.25% organic dust extract (DE) for 1, 3, 6, and 24 hours (A) or were treated with media (C) or 0.25% organic dust extract (DE) for 6 hours (B). After treatment, cells were fixed onto coverslips, stained following the immunocytochemsitry staining protocol, and mounted onto microscope slides for analysis using a confocal fluorescence microscope. AhR staining is indicated by green fluorescenc, nuclear staining by DAPI is indicated by blue fluorescence and overlay is observed as cyan (n = 3).

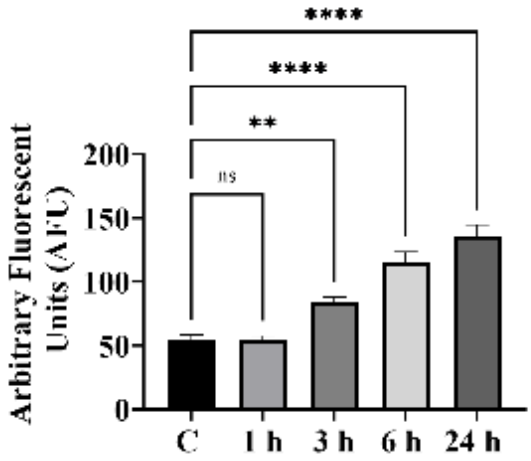

Figure 8. Organic dust extract increases AhR nuclear translocation.
Beas2B cells grown on coverslips were treated with medium (C) or 0.25% organic dust extract for 1, 3, 6, and 24 hours. AhR immunostaining was visualized by confocal fluorescence microscopy. Nuclear AhR intensity was quantified using ImageJ. Data shown are mean ± SE (n = 40). ns, no significant, **$p < 0.01$, and ****$p < 0.0001$.

Increase in AhR protein levels by dust extract could positively affect nuclear translocation of AhR to modulate the expression of inflammatory mediators. To determine if dust extract increases AhR expression, we investigated the effects of dust extract on AhR protein and AhR mRNA levels by western blotting and qRT-PCR, respectively. Results showed that treatment with dust extract appeared to increase AhR protein and AhR mRNA levels, but the changes were not statistically significant (Figures 9 and 10).

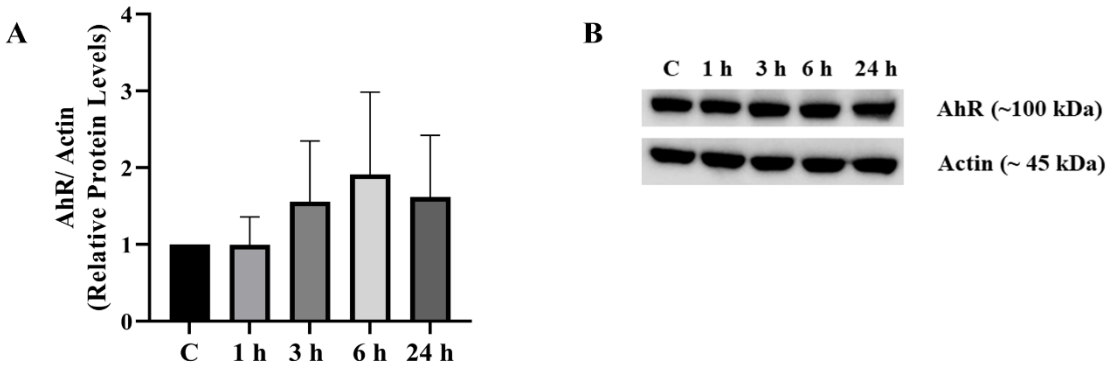

Figure 9. Organic dust extract has no effect on AhR protein levels.
Beas2B cells were treated with medium (C) 0.25% organic dust extract for 1, 3, 6, and 24 hours. AhR and actin protein levels were determined by western immunoblotting and AhR levels were normalized to actin levels. Data shown are mean ± SE (n = 4).

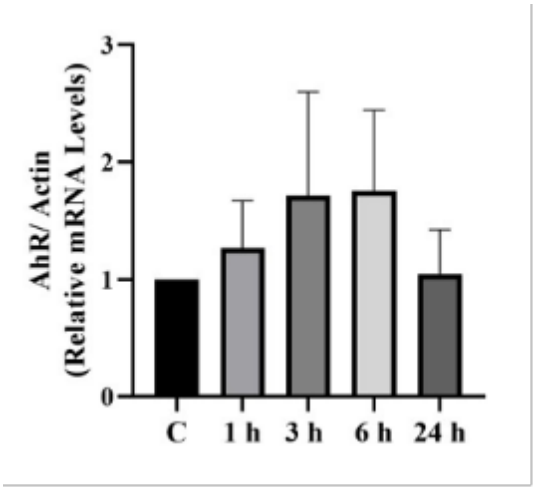

Figure 10. Organic dust extract has no effect on AhR mRNA levels.
Beas2B cells were treated with medium (C) 0.25% organic dust extract for 1, 3, 6, and 24 hours. AhR and actin mRNA levels were determined by qRT-PCR and AhR levels were normalized to actin levels. Data shown are mean ± SE (n = 4).

DISCUSSION

Agricultural workers employed by CAFOs are at an increased risk of developing acute and chronic respiratory symptoms and diseases caused by exposure to airborne organic dust generated by these operations [3]. Respiratory symptoms and respiratory diseases are characterized by enhanced lung inflammation that can be caused by dysregulation of inflammatory responses in the lung and/or persistent exposure to the offending agent [14]. Previous studies have indicated that organic dust exposure is associated with increased production of inflammatory mediators such as TNF-α, IL-6, and IL-8 by inflammatory cells and epithelial cells in the lung [26, 27]. In vitro cell culture studies of lung epithelial and monocytic cells have shown that extracts of organic dusts induce chemokines, cytokines and proteins implicated in inflammatory responses [20-25]. As there is a strong association between exposure to organic dust and development of respiratory symptoms and respiratory diseases [3, 5], it is necessary to identify constituents of organic dust responsible for eliciting inflammation and to understand mechanisms mediating induction of inflammation.

Organic dust contains a wide variety of compounds originating from the animals, animal feed and waste, microbes and microbial products [3, 4,]. Although LPS is found abundantly in organic dust, its role in inducing inflammation is not well understood. Neutralizing LPS or depleting LPS from organic dust extracts did not reduce the ability of the dust extracts to induce inflammatory mediators in lung cells [22, 25]. Additionally, lung inflammatory markers in workers could not be correlated with the concentrations of LPS in the animal confinement facility [43]. Recently, serine-like proteases [44, 45] and bacterial extracellular vesicles (EVs) [48] from organic dust were found to induce lung inflammatory responses indicating that they may be involved in the development of lung injury. Previous studies have shown that activation of protein kinase C [25, 48] and mitogen activated protein kinase (MAPK) [25] signaling pathways were found to mediate

induction of expression of inflammatory mediators by organic dust. Induction of inflammatory mediator protein levels were associated with similar increases in levels of mRNAs suggesting that transcriptional mechanisms play key roles in the induction [25]. Induction of expression of inflammatory mediators was found to be dependent on nuclear factor κB (NFκB), activator protein-1 (AP-1) and signal transducer/ activator of transcription 3 (Stat-3) transcription factors [25, 48] which suggests that combinatorial/cooperative effects of these factors control induction.

The role of AhR in the control of lung inflammation induced by organic dust is not known. As AhR is activated by a wide array of compounds including toxic chemicals, metabolites, and microbial products [38-40], we hypothesized that organic dust being a complex mixture of a wide variety of compounds may play a role in the induction of inflammatory mediator expression. AhR silencing via siRNA knockdown showed that reduced AhR levels inhibited induction of inflammatory mediator protein levels indicating that AhR serves a positive role in their induction (Figure 3). The viability of AhR siRNA transfected cells was not reduced (Figure 5) indicating that the negative effects on inflammatory mediators are indeed due to reduced AhR levels and not the result of reduced cell viability. Consistent with AhR's positive role, dust extract increased AhR nuclear translocation suggesting that AhR could control induction of inflammatory gene expression at the transcriptional level. It remains to be studied if AhR interacts directly with the promoters of inflammatory genes induced by organic dust.

Treatment with dust extract appeared to increase AhR protein and AhR mRNA levels in a time dependent manner but the changes were not statistically significant. However, immunofluorescence confocal studies indicated an increase in cytosolic AhR staining in dust extract treated cells particularly at later time points (3, 6 and 24 h) indicating an increase in AhR protein expression (Figure 7A). Increase in cytosolic AhR staining was not uniform across Beas2B

cells indicating heterogeneity in AhR expression which may explain lack of significant effects on AhR protein levels assessed by western blotting analysis of cell cultures (Figure 9). Phenotypic heterogeneity in in vitro cell cultures is a common phenomenon even though the cells are genetically identical to begin with [50]. It has even been suggested that the occurrence of phenotypic heterogeneity in cell cultures is a rule rather than an exception [50]. Stochastic interactions between cells and variations in the immediate milieu of each cell pertaining to nutrients, oxygen, and molecules secreted by the cells were suggested to be responsible for the development of phenotypic heterogeneity. AhR activators present in organic dust extracts are yet to be identified. Lack of effect of CH223191 to inhibit dust extract induction of inflammatory mediators (Figure 6) suggested that AhR activators present in dust extract may be distinct from dioxin and dioxin-like compounds. As bacteria are present abundantly in bioaerosols generated in poultry CAFOs [6], bacterial metabolites and bacterial bioactive compounds may serve as AhR activators. Pigmented virulence factors such as phenazines from Pseudomonas aeruginosa and naphthoquinone phthiocol from Mycobacterium tuberculosis were found to activate AhR [51]. Activation of AhR was found to regulate inflammatory cytokine and chemokine levels as well as Cyp1A1 mRNA indicating that AhR is important for defense against bacterial infections [51]. AhR regulates the expression of several LPS-responsive genes in mice, and AhR deficiency was found to sensitize mice to endotoxin shock [36]. A metabolite of tryptophan, L-kynurenine was identified as an endogenous AhR ligand mediating LPS effects [36].

Several environmental stimuli like diesel and gasoline exhaust particles [52], urban particulate matter [53], and atmospheric particulate matter activate [54] AhR. polycyclic aromatic hydrocarbons (PAH) and their derivatives occurring in urban particulate matter were found to be major contributors of AhR activation [54]. In U937 macrophages and human aortic endothelial

cells, $PM_{2.5}$ urban particulate matter and dietary compounds such as cholesterol, fructose, glucose, palmitic acid and triglycerides were found to induce Cyp1A1 as well as proinflammatory cytokine mRNAs via AhR activation suggesting that toxic chemicals found in the environment and dietary compounds may contribute to the development of cardiovascular diseases [55].

In summary our studies have shown that AhR serves as a positive regulator of induction of proinflammatory mediators by organic dust extract. Enhanced AhR nuclear translocation by organic dust extract suggested that AhR likely functions as a positive regulator of transcription of IL-1β, ICAM-1, IL-6 and IL-8 genes induced by organic dust extract. Further studies are required to elucidate AhR's role in the control of induction of proinflammatory mediators by organic dust extract. Future studies using primary lung epithelial cells and wild type mice and mice lacking AhR will be particularly useful in the understanding of AhR's role in the modulation of lung inflammatory responses induced by organic dust extract.

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This thesis was typed by Maxine Stenhouse.