Title: Neutrophilic oxidative stress mediates organic dust-induced pulmonary inflammation and airway hyperresponsiveness

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Abstract:

Background: Airway exposure to organic dust (OD) from swine confinement facilities induces airway inflammation dominated by neutrophils and airway hyperresponsiveness (AHR). One important neutrophilic innate defense mechanism is the induction of oxidative stress. Therefore, we hypothesized that neutrophils exacerbate airway dysfunction following OD exposure by increasing oxidant burden. Methods: BALB/C mice were given intranasal challenges with OD or PBS (one per day for three days). Mice were untreated or treated with a neutrophil depleting antibody, anti-Ly6G, or the antioxidant dimethylthiourea (DMTU) prior to OD exposure. Twenty-four hours after the final exposure, we measured airway responsiveness in response to methacholine (MCh), and collected BAL fluid to assess pulmonary inflammation and total antioxidant capacity. Lung tissue was harvested to examine the effect of OD-induced antioxidant gene expression and the effect of anti-Ly6G or DMTU. Results: OD exposure induced a dose-dependent increase of airway responsiveness, a neutrophilic pulmonary inflammation and secretion of keratinocyte cytokine. Depletion of neutrophils reduced OD-induced AHR. DMTU prevented pulmonary inflammation involving macrophages and neutrophils. Neutrophil depletion and DMTU were highly effective in preventing OD-induced AHR affecting large, conducting airways and tissue elastance. OD-induced an increase in total antioxidant capacity, and mRNA levels of NRF-2 dependent antioxidant genes, effects prevented by administration of DMTU and neutrophil depletion. Conclusion: Increase in oxidative stress and neutrophilia are critical in the induction of OD-induced AHR. Prevention of oxidative stress diminishes neutrophil influx and AHR, suggesting that mechanisms driving OD-induced AHR may be dependent on neutrophil-mediated oxidant pathways.
Keywords: Organic dust, anti-Ly6G, neutrophilia, oxidative stress, antioxidants

Introduction

The air in swine confinement facilities has been shown to contain high levels of organic dust and toxic inhalants including endotoxin, ammonia and hydrogen sulfide (2, 5, 21). Pulmonary dysfunction in swine farmers is not uncommon and is characterized by reduced expiratory flow rates, airway hyperresponsiveness (AHR) and pulmonary inflammation (1, 8, 20, 48, 49). Both acute and chronic exposure to these inhaled irritants is associated with increased prevalence of respiratory symptoms, pulmonary inflammation and airflow limitation as well as cough, wheezing, chest tightness, shortness of breath (19, 47, 48). Chronic exposure often results in increased prevalence of airway symptoms and chronic bronchitis (6, 11-13), whereas acute exposure induces flu-like symptoms, fever, intense airway inflammation and bronchial hyperresponsiveness to direct stimuli (19, 22).

The pulmonary inflammation associated with exposure to OD in humans is characterized by an increase in several cell types including macrophages, lymphocytes and neutrophils (6, 14, 18). Neutrophils, in particular, have been shown to be greatly increased in bronchoalveolar lavage (BAL) and nasal lavage fluid following OD exposure and may increase 70-fold or more following a single exposure to OD (17, 18). How this inflammation is linked to AHR is still unknown; however, it has been shown that neutrophil presence in the airways may directly impact on AHR in an oxidative stress-induced model of irritant-induced asthma using Cl2 gas (24). During the oxidative burst, neutrophils release myeloperoxidase (MPO) which catalyzes the production of hypochlorous acid, a potent oxidant, further suggesting a role for oxidative stress in neutrophil dominated pulmonary disease. MPO levels have also been associated with
increases in other markers of oxidative stress including 8-isoprostane and nitric oxide in chronic obstructive pulmonary disease (COPD) and allergic asthma (35, 36). In addition to elevated neutrophils in BAL and sputum, individuals exposed to OD also demonstrate increased levels of nitric oxide (NO) and 8-isoprostane in exhaled breath condensate following exposure to OD (9, 16, 21). However, studies dedicated to understanding the degree of oxidant burden and the specific pathological role of oxidant stress following OD have been limited. To address a role for oxidative stress we evaluated whether treatment using the antioxidant dimethylthiourea (DMTU) could ameliorate OD-induced AHR and inflammation. In addition to being a potent hydroxyl radical scavenger, DMTU acts by scavenging products of neutrophil-derived MPO, including hypochlorous acid(37), making it a relevant antioxidant to examine the contribution of neutrophil-derived oxidative stress.

In this study, the aim was to elucidate the relationship among oxidative stress, pulmonary neutrophilia and AHR following OD exposure. The specific focus was to explore how neutrophils might affect endogenous antioxidant pathways and to understand the impact of neutrophil-mediated oxidative stress on airway physiology in different lung compartments. For this, we exposed mice to OD on three consecutive days followed by evaluation of respiratory system mechanics, pulmonary inflammation, antioxidant gene expression, and histological analysis of inflammatory response. To evaluate the role of neutrophils we used a granulocyte depleting antibody, anti-Ly6G (4, 26, 42). The role of oxidative stress was evaluated using an antioxidant, dimethylthiourea (DMTU).
Methods

Mice

BALB/C male mice (8 - 10 weeks) from Charles River (Wilmington, MA, USA) were housed in a conventional animal facility at McGill University. All mice were treated in accordance with the guidelines of the Canadian Council for Animal Care and protocols were approved by the Animal Care Committee of McGill University.

Organic dust collection, preparation and LPS quantification

OD used was collected and processed from a single barn containing 800-900 pigs and used for all experiments. Sedimented dust on horizontal surfaces, 1 – 1.5 meters above the floor, was collected by brushing into plastic bags. The dust was size classified using a method slightly modified from Cooper et al as previously described (7). Briefly, dust was gradually aerosolized using a continuous focal air jet at 10 L/min and then diluted to 25 L/min before being passed through a separating cyclone with a 50% cut off at 5 µm. The cyclone effluent was filtered through a 140 mm membrane filter, whereas the dust fraction precipitated in the cyclone was discarded. The filtered dust fraction was collected and immediately stored at -20°C. Using the PreciseInhale (Inhalation Sciences, Huddinge, Sweden) exposure platform the mass median aerodynamic diameter of the aerosolized dust was found to be 2.7 µm. The dust was dissolved in sterile phosphate buffered saline and was sonicated for 30 minutes before intranasal installation.

While the exact composition of OD is not known, there is a potential contribution of LPS which may influence pulmonary response. Therefore, LPS levels in OD were measured using Pierce™ LAL Chromogenic Endotoxin Quantitation Kit (Life Technologies, Burlington, ON, Canada) according to manufacturer’s instructions. The amount of LPS present in OD was
determined to be 0.428 μg/100 μg OD, which was the dose administered for each intranasal installation.

Experimental protocol

All experiments for this study were performed using the same number and interval of organic dust exposures. Mice were exposed to organic dust three times, at 24 hour intervals. On days 1-3, mice were lightly anesthetized using inhaled isoflurane and then treated intranasally (i.n) with 40 μl of either PBS or organic dust. Twenty-four hours following the third exposure, respiratory system mechanics were performed followed by collection of BAL and lung tissue. An initial experiment was performed to assess how varying doses of organic dust affected changes in pulmonary inflammation and airway responsiveness to methacholine MCh. For this purpose, we exposed mice to 25, 50, 100 or 250 μg of organic dust followed by assessment of respiratory mechanics and pulmonary inflammation. Based on the results of this experiment, we established 100 μg as a suitable dose of organic dust, eliciting substantial changes in AHR and neutrophilia and all subsequent experiments were performed using this dose.

For neutrophil depletion, mice were injected intraperitoneally (i.p.) with 100 μg/mouse of anti-Ly6G (Bio X Cell, West Lebanon, NH, USA) in 100 μl sterile phosphate buffered saline (PBS) 24 hours prior to the first organic dust exposure and again 72 hours later. Control group mice were injected i.p. with an isotype control antibody rat IgG2a (Bio X Cell, West Lebanon, NH, USA) at a concentration of 100 μg/mouse in 100 μl sterile PBS at the same time points as anti-Ly6G.

To assess the role of oxidative stress following organic dust exposure, experiments were performed using the antioxidant DMTU. DMTU was prepared fresh prior to each organic dust
exposure and a concentration of 100 mg/kg in 500 μL of sterile phosphate buffered saline (PBS) was administered i.p. one hour before each organic dust exposure. Control group mice were injected i.p. with 500 μL PBS only one hour prior to OD exposure. Following exposure animals were returned to the animal facility and allowed food and water \textit{ad libitum}.

\textit{Bronchoalveolar lavage}

Following assessment of respiratory mechanics, mice were euthanized with an excess of sodium pentobarbital (150 mg/kg, i.p.). 1 mL of sterile saline was instilled into the lungs via a tracheal cannula and the fluid recovered was placed in a 1.5 mL tube (BD Biosciences, Mississauga, ON, Canada) kept on ice. The volume recovered did not differ significantly among the groups. BAL fluid was centrifuged at 1500 rpm for 5 min at 4°C and the supernatant was retained for chemokine analysis. The cell pellet was re-suspended in 500 μL sterile saline. Total live and dead cells were counted by hemacytometer and trypan blue exclusion. Cytospin slides were prepared using a cytocentrifuge (Shandon, Pittsburgh, PA, USA) and stained with DiffQuik. Differential cell counts were determined based on a count of 300 cells/slide.

\textit{Measurements of respiratory system mechanics and airway responsiveness to methacholine}

Twenty-four hours following the third organic dust exposure, respiratory system mechanics were measured using a small animal ventilator (FlexiVent; Scireq, Montreal, QC, Canada). Prior to measurements, mice were sedated (xylazine hydrochloride, 10 mg/kg, i.p.) and anesthetized (pentobarbital, 50 mg/kg, i.p.). Once anesthetized, mice were tracheotomised using an 18-gauge cannula and connected to the ventilator. Muscle paralysis was induced with pancuronium bromide (0.2 mg/kg i.p.). The mice were ventilated in a quasi-sinusoidal fashion with the following settings: a tidal volume of 10 ml/kg, maximum inflation pressure of 30 cm H$_2$O, a
positive end-expiratory pressure of 3 cmH\textsubscript{2}O, and a frequency of 150/min. After an equilibration period of 3 minutes of tidal ventilation, two lung inflations to a trans-respiratory pressure of 25 cm H\textsubscript{2}O were performed and baseline measurements were taken. Airway responsiveness was estimated using a 3 second, broadband low frequency forced oscillation maneuver containing 13 mutually prime frequencies between 1 and 20.5 Hz to estimate Newtonian resistance ($R_n$), tissue damping ($G$) and tissue elastance ($H$).

Baseline was established as the average of three perturbations. MCh was administered using an in-line nebulizer (Aeroneb Lab, standard mist model; Aerogen Ltd., Galway, Ireland), and progressively doubling doses ranging from 6.25 to 50 mg/ml were administered over ten seconds and synchronous with inspiration. Six measurements were made at each dose of MCh to establish the peak response. The highest value for each parameter after MCh was kept for analysis subject to a coefficient of determination above 0.85.

\textit{Semi-quantitative analysis of airway inflammation}

Twenty-four hours following the third organic dust exposure, the pulmonary circulation was flushed with sterile saline \textit{via} the right ventricle until the effluent was clear. After removal, the left lung was fixed by intra-tracheal perfusion with 10\% buffered formalin at a constant pressure of 25 cm H\textsubscript{2}O for a period of 24 hours. Sections were cut at a thickness of 5 μm from paraffin-embedded lung tissue using a microtome and stained with hematoxylin and eosin. Sections were evaluated for inflammatory cell infiltration around airways and scored in a blinded fashion. Scoring was performed on each airway on a scale of 1-4 using the following criteria: 1-no inflammation detectable beneath the basement membrane; 2-inflammation 1-5 cells thick beneath the basement membrane; 3-inflammation more than five cells thick but without
extension into the surrounding parenchyma; 4-inflammation extended beyond the airways into
the parenchyma. A minimum of 6 airways per mouse was assessed with 6-7 mice per group.

*Evaluation of keratinocyte-derived cytokine (KC) levels in BAL*

To assess whether OD affected levels of the neutrophil chemoattractant KC and if DMTU could
affect these changes, we evaluated KC levels by ELISA in BAL using a Mouse KC Quantikine
ELISA Kit (R&D Systems, Minneapolis, MN, USA) according to manufacturer's instructions.

*Total antioxidant capacity*

Bronchoalveolar lavage fluid supernatant was stored at -80°C. Total antioxidant capacity (TAC)
was assessed using a colorimetric assay according to manufacturer's instructions (BioVision,
Milpitas, CA, USA).

*RNA Isolation*

Twenty-four hours following organic dust exposure, the right lung lobe was placed in a 1.5 mL
tube, snap frozen in liquid nitrogen and stored at -80°C. Subsequently, RNA was extracted using
RNeasy Mini kit (Qiagen, Mississauga, ON, Canada) according to manufacturer's instructions.

*Reverse Transcription and Real-Time quantitative PCR*

Following RNA isolation, cDNA was synthesized with Oligo (dT) primers and Super-Script™ II
reverse transcriptase (Invitrogen, Burlington, ON, Canada) according to the manufacturer's
instructions. A total of 250 ng total RNA was reverse-transcribed into cDNA.

For quantitative real-time PCR, SYBR® Green PCR Master Mix (Applied Biosystems,
Foster City, CA) using the Applied Biosystems StepOne Plus Real-Time PCR System.
Transcripts for the following genes were evaluated; KC, NRF-2, SOD-1, GPX-2, HO-1, NQO-1, SOD-3, Catalase, NOX-1, TXN-1. Primer sequences are listed in Table 1.

PCR samples contained 9 μl of Power SYBR® Green PCR Master Mix and 1 μl of cDNA with a final primer concentration of 0.5 μM. The run method included a holding stage (95 °C for 10 min), a cycling stage (45 cycles at 95 °C for 15 s, 60 °C for 30 s, 72 °C for 25s) and a melting curve stage (95 °C for 15 s, 60 °C for 45 s, 95 °C for 30 s). Analysis was performed using StepOne control with s(9) used as a reference gene. For all PCRs, normalization done by using a calibrator sample to allow for comparison between multiple PCRs. The calibrator sample used came from a mouse treated with PBS-PBS.

Statistical analysis

Data for BAL, gene expression analysis, TAC assay, chemokine analysis and inflammatory scoring were analyzed using one-way ANOVA and the post hoc analysis with Newman-Keuls test comparing all pairs of data. Experiments evaluating respiratory system mechanics were analyzed using repeated measures ANOVA and post hoc analysis was performed with a Bonferroni multiple-comparison test. A p value of <0.05 was considered significant.
Results

*OD exposure increases airway responsiveness in a dose-dependent manner*

To establish a mouse model that in which exposure to OD would induce AHR, OD was given once a day for three consecutive days using four different doses (25, 50, 100 and 250 µg).

Generally, an evident dose-dependent increase was obtained in all the measured parameters, with a non-significant increase for the lowest doses and an effect for the two highest doses that was significantly increased from baseline but not different from each other. (Figure 1A-C). Exposure to 100 µg and 250 µg of OD increased $R_n$ at the three highest doses of MCh (Figure 1A). $G$ was increased following 250 µg of OD at the two highest doses of MCh and at the highest dose of MCh with 100 µg and (Figure 1B). $H$ was increased at the two highest doses following 100 µg and 250 µg OD (Figure 1C).

*Changes in BAL cellularity of mice exposed to organic dust*

Following measurement of respiratory system mechanics, BAL was collected. We observed a dose-dependent increase in total inflammatory cell counts (Figure 2A). Macrophages were increased at 100 µg dose of OD compared to baseline (Figure 2B). Neutrophils were significantly increased at all doses of OD, compared to PBS exposed mice (Figure 2C). Neither eosinophils nor lymphocytes increased following OD exposure (Figure 2D & E). Epithelial cell shedding was increased at the highest dose of OD (Figure 2F).

*Depletion of neutrophils in BAL with Anti-Ly6G*

As we sought to study the influence of neutrophils following OD, we used a granulocyte depletion antibody, anti-Ly6G, to deplete neutrophils. Following exposure to OD neutrophils in
BAL fluid were decreased by 64.2% following anti-Ly6G treatment compared to mice anti-
Ly6G + PBS (Figure 3C). Treatment with anti-Ly6G prior to OD exposure reduced total cell
numbers in BAL compared to IgG2a+PBS and IgG2a+OD treated groups (Figure 3A).
Macrophages were increased in IgG2a+OD mice compared to IgG2a+PBS treated mice, but not
compared to anti-Ly6G+PBS or anti-Ly6G+OD treated mice (Figure 3B). There were no
statistically significant differences among any groups in regard to eosinophils (Figure 3D).
Lymphocytes were increased in IgG2a+OD compared to mice exposed to PBS but were
unaffected by anti-Ly6G treatment (Figure 3E). There were no differences among any groups
with regard to the number of epithelial cells observed in BAL (Figure 3F).

**Anti-Ly6G treatment reduces OD-induced AHR**

Anti-Ly6G prevented increases in $R_n$ (Figure 4A) but did not prevent increases in $G$ (Figure 4B).
Neutrophil depletion completely prevented OD-induced increases in $H$ (Figure 4C). Anti-Ly6G
alone had no effect on control group mice exposed to PBS (Figure 4A-C).

**DMTU prevents OD-induced airway inflammation**

The anti-oxidant DMTU was used to address whether oxidative processes were involved in OD-
induced inflammation and AHR and to determine if outcomes related to neutrophil depletion
were dependent on oxidative processes. Semi-quantitative evaluation of lung sections showed
that OD exposure substantially increased inflammatory cell influx around airways compared to
unexposed mice (Figure 5A). Treatment with DMTU prior to OD exposure prevented
inflammatory cell infiltration to levels comparable to those of control mice (Figure 5A). Figure
5, panels B-E show representative examples of inflammatory cell infiltration around airways
from various treatment groups. PBS + PBS is shown in Figure 5B, DMTU + PBS is shown in Figure 5C, PBS + OD airway is shown in Figure 5D, and DMTU + OD is shown in Figure 5E.

Following analysis of airway inflammation, we assessed the effects of DMTU on BAL fluid cellularity. Treatment with DMTU prior to OD exposure reduced total inflammatory cell influx compared to mice treated with PBS+OD (Figure 6A). PBS+OD mice had elevated numbers of macrophages in BAL. DMTU+OD treated mice had unaltered macrophage numbers in BAL compared to PBS+OD treated mice (Figure 6B). Neutrophil influx was markedly reduced in DMTU+OD treated mice compared to PBS+OD group, but remained higher than baseline levels (Figure 6C). Eosinophils were increased following OD exposure, in both DMTU+OD and PBS+OD groups compared to control groups (Figure 6D). Lymphocytes were increased in the PBS+OD group only (Figure 6E). Epithelial cell shedding was increased following OD exposure but was not reduced by DMTU (Figure 6F).

DMTU treatment prevents synthesis and release of neutrophil chemoattractant KC

Following the observation that DMTU treatment prevented OD-induced increases in neutrophil influx into BAL fluid, we chose to evaluate if DMTU treatment influenced levels of the neutrophil chemoattractant KC. We found that DMTU treatment prevents OD-induced increase in KC levels in mRNA isolated from lung homogenate (Figure 7A). DMTU treatment also prevented the increase in KC levels in BAL fluid compared to PBS+OD treated mice which demonstrated significantly increased KC levels compared to control groups (Figure 7B).

Antioxidant treatment partially prevents OD-induced AHR

We found that the antioxidant DMTU, given prior to OD exposure, resulted in complete abrogation of AHR in the large conducting airways at the highest level of MCh, as measured by
Rn (Figure 8A). However, OD-induced increases in $G$ were not affected by DMTU (Figure 8B). Treatment with DMTU prevented OD-induced changes in $H$, maintaining levels similar to PBS treated mice (Figure 8C).

Neutrophils increase oxidant burden following OD

To address whether neutrophil presence affected oxidant burden following OD, we evaluated total antioxidant capacity (TAC) in BAL and antioxidant gene expression in lung tissue following treatment with either DMTU or anti-Ly6G, both of which significantly attenuated pulmonary neutrophilia. OD evoked an increase TAC compared to control, and this increase was prevented by both DMTU and anti-Ly6G treatment (Figure 9A). NRF-2 expression increased in response to OD exposure, and this increase was prevented by DMTU treatment, but not anti-Ly6G (Figure 9B). OD exposure increased expression of heme-oxygenase 1 ($HO-1$) mRNA compared to control, an effect prevented by both DMTU and anti-Ly6G treatment (Figure 9C). Superoxide dismutase 3 ($SOD-3$) levels were not increased by OD exposure. However, both DMTU and anti-Ly6G reduced expression (Figure 9D). Glutathione peroxidase 2 ($GPX-2$) and NAD(P)H dehydrogenase [quinone] 1 ($NQO-1$) mRNA levels both increased following OD exposure and this increase was prevented by DMTU and anti-Ly6G (Figure 9E & F). mRNA levels of superoxide dismutase 1 ($SOD-1$), catalase, thioredoxin 1 ($TXN-1$), and NADPH oxidase 1 ($NOX-1$) did not change following OD and were not affected by either DMTU or anti-Ly6G (Figure 9G-J).
Discussion

In the present study, we have shown that OD exposure induces AHR, pulmonary neutrophilia and increased oxidative stress in mice in a dose-dependent fashion. Depletion of neutrophils prior to OD exposure prevented AHR in large, conducting airways and in the peripheral lung, but not in small airways. Treatment using the antioxidant DMTU prevented OD-induced increases in the neutrophil chemoattractant KC, reduced pulmonary neutrophilia and attenuated AHR in large conducting airways and peripheral lung in a similar fashion as was seen following neutrophil depletion. OD exposure induced an increase in oxidative stress and increased gene expression of NRF-2, and several NRF-2 dependent antioxidant enzymes. Both anti-Ly6G and DMTU were effective at preventing OD-induced increase in antioxidant enzyme gene expression for NRF-2 dependent genes. Interestingly, OD exposure had no effect on NRF-2 independent antioxidant enzymes, and these genes remained unaffected by both neutrophil depletion and DMTU treatment. These data demonstrate that OD-induced AHR is exacerbated by the presence of pulmonary neutrophils, particularly in large airways, and that AHR may be mediated, in part, via oxidative stress mechanisms.

Acute, short term exposure to OD has been shown to induce AHR in humans. Similarly exposure of OD dust once daily for three consecutive days induced a marked increase in AHR in mice. Previous studies using comparable protocols of OD exposure have demonstrated AHR in rats, by head-out plethysmography (5), and mice, measuring total lung resistance by invasive ventilator based-techniques (34). Using constant phase model to assess respiratory system mechanics, the present study showed that OD caused dose-dependent AHR in $R_n$, $G$ and $H$. These data demonstrate that OD exposure affects the responses to MCh in proximal parts of the lung represented by $R_n$, providing information about large airway responses. Distal lung
compartments, represented by $G$, a reflection of large and small airway narrowing and $H$, which represents altered dynamic lung elastance and increases due to de-recruitment of lung tissue from airway closure (40, 41), were also increased by OD-treatment.

Analysis of inflammatory cell profiles revealed dose-dependent increase in neutrophils, with a similar trend for macrophages although they were increased only at 100 μg OD compared to control mice. Similarly, OD exposure induces pulmonary inflammation in humans typified by an increase in neutrophils and macrophages in BAL fluid (19, 29) (32). Animals exposed to OD display severe neutrophilia (34) and when studying the kinetics, previous studies have shown that repeated OD exposure in mice results in a marked increase in pulmonary neutrophil populations which peak 24 hours following OD exposure (33). Due to the efficacy of 100 μg OD in inducing AHR as well as increased macrophage and neutrophil numbers in BAL in the present study, this dose was well-suited for all subsequent experiments as both macrophages and neutrophils have been shown to be important in mediating the OD response (32).

As severe pulmonary neutrophilia is a hallmark of OD exposure in both human and animal models mostly as assessed by BAL fluid analyses (3, 10, 20) we sought to address the importance of neutrophils using a granulocyte depletion antibody, anti-Ly6G. Despite not reaching 100% reduction in neutrophils, the anti-Ly6G effect on cell depletion was selective for neutrophils. Neutrophil depletion prevented OD-induced increases in $R_n$, as well as $H$, suggesting a role for neutrophils in both the large airway response and elastance. Interestingly, neutrophil depletion was ineffective at reducing $G$, suggesting a differential role for neutrophils among lung compartments following OD exposure. The effect of neutrophil depletion primarily in large, conducting airways is supported by Manni et al. who explored the role of neutrophils in a Th17-rich model of allergic asthma in the mouse and demonstrated a reduction of $R_n$ in mice.
treated with anti-Ly6G (23). In an oxidative stress-dependent model of irritant induced asthma using Cl$_2$ gas, anti-Ly6G was shown to be effective at preventing AHR in large, conducting airways, but not in peripheral lung (24). Neutrophil depletion in mice challenged with LPS, a pro-inflammatory component of OD (45), has also shown peripheral airways to be unaffected by presence of neutrophils (38). Furthermore, neutralization of IL-1β, a cytokine induced by OD exposure and involved in neutrophil recruitment, prevented increased respiratory system resistance and reduced neutrophilic inflammation (15, 41). Taken together, our data and previous studies suggest that the role of neutrophils is likely differentially regulated depending on stimulus in central and peripheral lung compartments, and may mediate mechanisms primarily related to central airway function and peripheral tissue properties following OD exposure.

It is well-established that activated neutrophils increase oxidant burden and exacerbate disease via production of reactive oxygen and nitrogen species (30, 31, 46). Therefore, to further evaluate the role neutrophils in the context of oxidant burden, we utilized an antioxidant, DMTU, which has been shown to prevent pulmonary neutrophilia following inhalation of chlorine gas (25). We found that DMTU prevented OD-induced airway inflammation in lung tissue and prevented neutrophilia. Furthermore, DMTU prevented OD-induced increases in gene expression of KC, a potent neutrophil chemoattractant, in lung homogenate and prevented KC release in BAL fluid, suggesting that neutrophil recruitment may be related to OD-induced oxidative stress. In addition to preventing neutrophil influx and changes in KC, DMTU completely prevented AHR in $Rn$ and $H$. However, DMTU was unable to prevent AHR in $G$. Similarly, Shalaby et al., demonstrated that DMTU prevented neutrophil accumulation in BAL fluid as well as AHR in both the central and peripheral lung compartments in an allergic model of asthma in mice exposed to birch pollen extract (39). In a model of irritant-induced asthma
using Cl₂ gas, DMTU was effective also in reducing AHR in both peripheral and central lung (25). Our findings suggest a role for oxidative stress, likely associated with neutrophil populations, following OD exposure. However, it appears that reducing oxidative burden may be effective only in some lung compartments, and that the mechanism of OD-induced AHR is complex and multi-factorial.

Following the establishment that DMTU was effective in ameliorating lung compartment-specific AHR and neutrophilia, we next sought to explore specific antioxidant pathways affected by OD. We chose to examine NRF-2, a transcription factor which mediates the production of several antioxidant enzymes. First, we established that OD exposure elicits oxidative stress as evidenced by an increase in total antioxidant capacity (TAC) in BAL. Next, we found that OD induced an increase in gene expression of *NRF-2* and *NRF-2*-dependent antioxidant genes; *GPX-2*, *NQO-1* and *HO-1*. Interestingly, OD exposure did not elicit changes in expression levels of *NRF-2*-independent antioxidant genes. Treatment with both anti-Ly6G and DMTU prevented OD-induced increase in TAC and reduced gene expression of *HO-1*, *GPX-2* and *NQO-1*. Presumably, treatment with DMTU bolstered the antioxidant capacity of the lung such that the oxidant burden was lessened to a degree sufficient to prevent an increased endogenous response. In humans, OD exposure is associated with increases in oxidative stress markers (28, 44). Poole *et al.*, demonstrated that overexpression of dimethylarginine dimethylaminohydrolase, which inactivates the nitric oxide inhibitor asymmetric dimethylarginine by hydrolyzing it into citrulline and dimethylamine inhibitor was effective in attenuating neutrophilia induced by OD exposure in mice (3). Taken together, our data and human study data suggest that exposure to OD exposure induces up-regulation of antioxidant pathways, specifically NRF-2, and these changes are associated with neutrophil presence.
The mechanism for organic dust-induced neutrophil-mediated AHR observed in human and animal models is still unknown. As the exact composition of organic dust is undefined, it is not unreasonable to postulate that LPS plays a role. Human subjects exposed for three hours to dust in a pig barn (63 ng LPS/m$^3$) implied a total LPS exposure of approximately 0.19 $\mu$g and induced a greater increase in bronchial responsiveness than did inhalation of 53 $\mu$g of pure LPS in healthy subjects (43). Also, in vitro experiments have shown that organic dust is a more potent stimulus for IL-8 release in airway epithelial cells than LPS, glucan and grain dust; 100 $\mu$g/ml of organic dust induced four times more IL-8 release than LPS (100 $\mu$g/ml) in A549 cells (27). Moreover, Poole et al. demonstrated that treating mice with LPS (100 $\mu$g/ml, i.n.) had minimal effects on airway inflammation compared to OD exposure, noting that this concentration of LPS exceeded 250x the concentration found in OD (33). Interestingly, the concentration of LPS in the OD used in the current study is 0.428 $\mu$g LPS/100 $\mu$g OD, nearly identical to concentrations of LPS found in other in vivo studies using OD, suggesting that the LPS concentration in OD is relatively consistent from farm to farm. Thus, these data does not favor the assumption that LPS is mainly responsible for the pulmonary pro-inflammatory effects and the increase in bronchial responsiveness observed following exposure to dust from pig barns.

In this study, we found marked similarities in patterns of pulmonary response following neutrophil depletion and DMTU treatment. Amelioration of AHR in large conducting airways and peripheral lung compartments, but not in small airways was seen following both anti-Ly6G and DMTU. Additionally, the decrease in TAC as well as the pattern of antioxidant gene expression following DMTU was nearly identical to the pattern observed by anti-Ly6G treatment. mRNA analysis for antioxidant genes revealed OD induced a clear pattern of gene expression changes related to NRF-2, whereas NRF-2 independent genes were unaffected.
Taken together, these data suggest that OD exposure increases oxidant burden resulting in recruitment of neutrophils, and that neutrophils have distinct roles within the pulmonary microenvironments which affect AHR. While further examination of a variety of antioxidant therapies and precise nature of the mechanisms leading to augmented oxidant burden remains to be explored, OD-induced airway dysfunction appears to be linked to the NRF-2 pathway, suggesting a specific mechanism by which OD affects the antioxidant response.


Figure Legends

Figure 1. OD exposure increases airway responsiveness in a dose-dependent manner
Twenty-four hours following the last exposure to OD, respiratory system mechanics in response to inhaled MCh were evaluated. (A) Newtonian resistance. (B) Tissue damping (C) Tissue elastance (A-C) Neither 25 μg nor 50 μg induced any significant changes in respiratory system mechanics for any parameter in comparison to control conditions. * - 250 μg OD compared to control; # - 100 μg compared to control. */#p<0.05, ***/### p<0.001; n = 6-9 per group.

Figure 2. OD induces a dose-dependent increase in inflammation in BAL
Twenty-four hours following the last exposure to OD, BAL fluid was collected and evaluated. (A) Total inflammatory cell counts (B) Macrophages (C) Neutrophils (D-E) Neither eosinophils nor lymphocytes increased following OD exposure. (E) Epithelial cells *p<0.05, **p<0.01, *** p<0.001; n = 6-9 per group.

Figure 3. Anti-Ly6G depletes neutrophils in BAL
Anti-Ly6G was used to deplete neutrophils prior to OD exposure. (A) Total cells (B) Macrophages (C) Neutrophils (D) Eosinophils (E) Lymphocytes (F) Epithelial cells *p<0.05, **p<0.01, *** p<0.001; n = 6 per group.

Figure 4. Anti-Ly6G partially prevents OD-induced AHR
Anti-Ly6G was used to deplete neutrophils prior to OD exposure. (A) Newtonian resistance. (B) Tissue damping. (C) Tissue elastance (A-C) Isotype control antibody IgG2a + PBS and anti-Ly6G + PBS were not different for any parameter. * - difference between IgG2a+OD and anti-Ly6G + OD. * p<0.05, *** p<0.001; n = 6-8 per group.

Figure 5. DMTU prevents OD-induced airway inflammation

Airway inflammation was evaluated in a blinded, semi-quantitative fashion on lung sections stained with H & E. (A) Inflammation score (B) PBS + PBS (C) DMTU + PBS (D) PBS + OD (E) DMTU+OD exposed airway. **p<0.01, *** p<0.001; a minimum of 6 airways per mouse were assessed with 6-7 mice per group.

Figure 6. DMTU prevents OD-induced pulmonary inflammation

The anti-oxidant DMTU was used to establish whether oxidative processes were involved in OD-induced inflammation. (A) Total cells (B) Macrophages (C) Neutrophils (D) Eosinophils (E) Lymphocytes (F) Epithelial cells (A-F) DMTU+PBS had no effect on inflammatory cells or epithelial cell shedding. *p<0.05, **p<0.01, *** p<0.001; n = 6-9 per group.

Figure 7. DMTU prevents OD-induced changes in KC.

KC was evaluated by PCR or ELISA following OD exposure with and without DMTU. (A) mRNA levels of KC (B) KC levels in BAL. * p<0.05, **p<0.01, *** p<0.001; n = 5-6 per group.

Figure 8. Antioxidant treatment partially attenuates OD-induced AHR.

Twenty-four hours following last OD exposure, mice were evaluated for respiratory system mechanics in response to inhaled MCh. (A) Newtonian (B) Tissue damping (C) Tissue elastance
(A-C) DMTU+PBS was not different than PBS+PBS for any parameter. **p<0.01, *** p<0.001 - difference between PBS+OD and DMTU+OD; n = 6 per group.

Figure 9. Antioxidant treatment and neutrophil depletion reduce oxidative burden after OD

Antioxidant pathways was measured (A) in BAL and (B-J) as mRNA expression in the lung 24 hours following the last administration of OD relative expression compared to calibrator. (A) Total antioxidant capacity (TAC), (B) NRF-2 (C) HO-1 (D) SOD-3 (E) GPX-2 (F) NQO-1 (G-J) mRNA levels of SOD-1, catalase, TXN-1, and NOX-1 did not change following OD and were not affected by either DMTU or anti-Ly6G. * p<0.05, ** p<0.01, n=6 per group.
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<tr>
<th>Gene</th>
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<td>TXN-1</td>
<td>TGAAGCTGATCGAGACAGAAG</td>
<td>AGAAAGCTCCACCACGACAGC</td>
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</tbody>
</table>
Figure 1.
Figure 2.

A. **Total Cell Count**

B. **Macrophages**

C. **Neutrophils**

D. **Eosinophils**

E. **Lymphocytes**

F. **Epithelial Cells**
Figure 3.

A. **Total Cell Count**

B. **Macrophages**

C. **Neutrophils**

D. **Eosinophils**

E. **Lymphocytes**

F. **Epithelial Cells**
Figure 4.
Figure 5.

A. Inflammation Score

B. PBS + PBS
C. DMTU + PBS
D. PBS + OD
E. DMTU + OD

Inflammation score
Figure 6.

A. Total Cell Count

B. Macrophages

C. Neutrophils

D. Eosinophils

E. Lymphocytes

F. Epithelial Cells
Figure 7.

A. mRNA expression of KC relative to calibrator

B. pg/mL KC
Figure 8.

A. Newtonian Resistance

B. Tissue Damping

C. Tissue Elastance
Figure 9.