Effect of low-level CO₂ on innate inflammatory protein response to organic dust from swine confinement barns

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Abstract

Background: Organic hog barn dust (HDE) exposure induces lung inflammation and long-term decreases in lung function in agricultural workers. While concentrations of common gasses in confined animal facilities are well characterized, few studies have been done addressing if exposure to elevated barn gasses impacts the lung immune response to organic dusts. Given the well documented effects of hypercapnia at much higher levels we hypothesized that CO₂ at 8 h exposure limit levels (5000 ppm) could alter innate immune responses to HDE.

Methods: Using a mouse model, C57BL/6 mice were nasally instilled with defined barn dust extracts and then housed in an exposure box maintained at one of several CO₂ levels for six hours. Bronchiolar lavage (BAL) was tested for several cytokines while lung tissue was saved for mRNA purification and immunohistochemistry.

Results: Exposure to elevated CO₂ significantly increased the expression of pro-inflammatory markers, IL-6 and KC, in BAL fluid as compared to dust exposure alone. Expression of other pro-inflammatory markers, such as ICAM-1 and matrix metalloproteinase-9 (MMP-9), were also tested and showed similar increased expression upon HDE + CO₂ exposure. A chemokine array analysis of BAL fluid revealed that MIP-1γ (CCL9) shows a similar increased response to HDE + CO₂. Further testing showed CCL9 was significantly elevated by barn dust and further enhanced by CO₂ co-exposure in a dose-dependent manner that was noticeable at the protein and mRNA levels. In all cases, except for ICAM-1, increases in tested markers in the presence of elevated CO₂ were only significant in the presence of HDE as well.

Conclusions: We show that even at mandated safe exposure limits, CO₂ is capable of enhancing multiple markers of inflammation in response to HDE.

Keywords: Carbon dioxide, Hypercapnia, Barn dust

Background

Concentrated animal feeding operations (CAFOs) are a common feature of agriculture in developed countries. Exposure to the organic dusts within these facilities has been shown to cause a number of short and long-term problems for exposed workers. These problems include, but are not limited to, increased risk for asthma and COPD [1–3], chronic bronchitis [4], and a general decrease in lung function over time exposed [5].

The immune response to organic barn dusts is mediated primarily by the innate immune system. Previous work identifies endotoxin and peptidoglycans as key components of the dust that trigger innate immune responses in humans, cell cultures, and mouse models [6–9]. These bacterial components act through the TLR4 and TLR2 receptors, respectively [10, 11], and it has been shown that one or both of these receptors may be critical to the immune response generated to these dusts [10–12], though it is doubtful that these components are the only causes of this inflammation.

Response to hog barn dust extracts (HDE) typically involves expression of pro-inflammatory cytokines and chemokines such as TNF-α [8, 13], IL-6 [13–15], and IL-8 (KC in mice) [13–16]. This increased cytokine
expression, particularly through IL-8, results in increased infiltration of cells, particularly neutrophils, into the lung [9, 16]. Other changes to the lung will occur such as increased edema, and prolonged exposure can lead to lymphoid aggregate formation [9]. A host of other chemokines will also likely be induced in this response, but IL-6 and KC are the most often characterized markers of this inflammation.

HDE exposure does not occur in isolation of gasses in the barn air, and three gasses, in particular, are commonly recognized as being elevated in most CAFO operations: ammonia (NH₃), hydrogen sulfide (H₂S), and carbon dioxide (CO₂). Ammonia has potential negative effects on lung function and health of workers and animals in the CAFO environment [17, 18] though little is known. H₂S is recognized as a serious potential health hazard in CAFO operations, usually involving waste management and removal. H₂S in these workplace exposures is both an irritant and an asphyxiant, and if levels are high and sustained, fatal.

CO₂, on the other hand, may be elevated as high as 5000 ppm in some facilities [19], but perceived effects are often mild and do not include irritation [20]. As such, it is often not considered a potential problem in the barn environment and has thus received little to no study. Studies with cell cultures and animal models have yielded mixed results as to the pro- or anti-inflammatory nature of hypercapnia [21–23]. In almost every case, the levels used in these studies far exceed CO₂ levels that may be encountered in a workplace environment. We hypothesized that the combination of CO₂ gas exposure enhances the inflammatory response to HDE exposure.

Given that elevation of other gasses in this environment showed potential to enhance lung inflammatory symptoms [18], that CO₂ could alter response to dust extract exposures in bronchial epithelial cells [24] and that levels as low as 1000 ppm were capable of inducing changes in cognitive function [25], we hypothesized that the elevation of CO₂ gas exposure may enhance the inflammatory response to HDE exposure. We therefore show that 8 h CO₂ permissible exposure limits used in these studies far exceed CO₂ levels that may be encountered in a workplace environment. We hypothesized that the combination of CO₂ gas exposure enhances the inflammatory response to HDE exposure.

Methods

**Hog barn dust extracts**

HDE extracts were prepared from settled dust samples combined from two separate swine confinement facilities and have been characterized as to content of protein, endotoxin, and muramic acid [28]. The bacterial composition has also been characterized [29]. Dust extracts were prepared as previously described [15]. Dust (1 g) was mixed with 10 ml HBSS without calcium and incubated for 1 h at room temperature before centrifugation for 10 min, with media being decanted and sterile-filtered for use, for a final concentration of approximately 0.105 g/ml dust. No stability problems are noted in such extracts for at least a year or more after storage. Extracts were used at a concentration of 12.5% v/v or about 0.005 g/ml dust and prepared no earlier than 2 weeks before use.

**Animals**

All procedures were approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center (protocol number 04-059-08). Female, 6–8-week-old C57BL/6 mice (Charles River, Wilmington, MA) were acclimated for one week after arrival. The animals were group-housed, and their diet consisted of commercial rodent chow and water *ad libitum*. Animals were assigned randomly to each treatment group: saline, saline + hypercapnia, HDE instillation (12.5%), or HDE instillation + hypercapnia. All mice (8 per group) were instilled nasally one time [11] with 40 μl of treatment. Mice were treated for 6 h in an exposure chamber, with controlled fresh air ventilation and a fan to circulate air within the box. Mice exposed to elevated CO₂ were given CO₂ in the same box. Levels of CO₂ were assayed using a CO₂ monitor (Extech CO210, Extech, Nashua, NH) and the chamber was manually ventilated to maintain levels for normal CO₂ (400 ppm), and hypercapnia (5000 or 7500 ppm). All treatment groups were split over two separate occasions, separated by no more than two weeks (4 animals/group/occasion; *n* = 8).

Sacrifice of animals was done within 1 min of withdrawal from exposure chamber, and staged so that BAL and lung excision did not exceed 30 min after completion of treatment.
Serum collection
Blood was taken from animals at time of sacrifice by car-
diac puncture in serum collection tubes (Microtainer,
Becton Dickson, Franklin Lakes, NJ) using an 18-gauge
needle (Becton Dickson). Samples were centrifuged 10
min at 7000 × g and serum stored at -80 °C until used
for ELISA. Blood was not taken from the 7500 ppm CO₂
exposed group by mistake.

Bronchoalveolar lavage (BAL) collection
Lungs were lavaged as detailed previously [9]. Briefly,
lungs were washed three times with 1 ml sterile saline
each time. BAL fluid was centrifuged 1750 × g for 10
min and supernatant samples stored at -80 °C until used.
Cells were resuspended in 1 ml PBS, counted, and 1.5 ×
10³ cells adhered to glass slides via cytospin. Cells were
stained using a Diff-Quik kit (Siemens Healthcare Diag-
nostics, Newark, DE) and cover slips mounted. A differen-
tial count of at least 200 cells was made based on
morphometric criteria and expressed as absolute cell
numbers (mean +/- SEM).

Lung collection
After BAL collection, lungs were excised from animals.
The left lung was tied off at the primary bronchus and
removed, flash frozen in liquid nitrogen and stored at
-80 °C for mRNA collection. A cannula was inserted in
the trachea of the remaining lung and cinched with a su-
ture. Lungs were hung in a bath of 10% formalin fixative
as 0.8 ml of this fixative was allowed to enter the lungs
via the cannula for 24 h under a pressure of 15 cm H₂O.
The fixed lung was then embedded in paraffin for later
immunohistochemical staining.

Tracheal cell collection
A separate exposure was done with 10 mice/group at
normal CO₂ (400 ppm), and hypercapnic (7500 ppm)
CO₂ levels. The trachea was saved from each animal,
opened, and the internal surface scraped gently with a
cell scraper into cell lysis buffer (Qiagen, Chatsworth
CA) and processed for RNA as per whole lung tissue
(described below). Cell lysate samples were pooled from
2 mice to yield enough mRNA for testing.

ELISAs
Cytokine and chemokine quantitation of BAL fluid was
done by enzyme linked immunosorbant assay kits to
IL-6, KC, and CCL9 (R&D Systems, Minneapolis, MN)
according to manufacturer's instructions. Broad spectrum
testing of BAL fluid for chemokine expression was accom-
plished using a dot blot array kit (ARY020, R&D Systems,
Minneapolis, MN).

Wet/Dry ratio
Four additional mice per group were exposed as de-
scribed previously and lungs removed after treatment.
Lungs were weighed at time of removal and then
dried overnight in a drying oven at 60 °C and visu-
ally checked for dryness at the end of this time be-
fore being re-weighed. Ratio was calculated of wet to
dry weight.

RNA purification and RT-PCR analysis
RNA was purified from lung tissue samples using a
Qiagen spin miniprep kit according to manufacturer's
instructions, including additional DNAse digestion
(Qiagen, Chatsworth CA). Initial homogenization of
tissue was done in 350 μl of lysis buffer from the
miniprep kit with 2.0 mm stainless steel beads (Next
Advance, Averill Park, NY) and placed in a Bullet
Blender Storm 24 magnetic bead beater (Next Ad-
vance, Averill Park, NY) for 3 m at 4 °C. RNA was
quantified by NanoDrop ND-1000 (Thermo Scientific,
Wilmington, DE).

cDNA synthesis was performed using the Taqman re-
verse transcription kit (Applied Biosystems, Branchburg,
NJ) with 200 ng of template mRNA. cDNA synthesis
(RT-PCR) reactions contained the following reagents: 1X
TaqMan RT buffer, 5.5 mM MgCl₂, 500 μM of each
dNTP, 2.5 μM random hexamers, 0.4 U/μl RNase inhibi-
tor, and 1.25 U/μl MultiScribe reverse transcriptase as
per kit instructions (Applied Biosystems, Branchburg,
NJ). Samples were incubated at 25 °C for 10 min, then
48 °C for 30 min, and 95 °C from 5 min in a thermocy-
clcer (MJ Mini; Bio-Rad, Hercules, CA).

RT-PCR was performed using probes to CCL9 (Life
Technologies, Mm00441260), MMP-9 (Mm00600163),
and ICAM-1 (Mm00516023). Ribosomal RNA was used
as an endogenous control. PCR was conducted using an
ABI PRISM 7500 Sequence Detection System (Applied
Biosystems). Reactions were carried out for 2 min at 50 °
C, 10 min at 95 °C, followed by 40 cycles at 95 °C for 15
s and 60 °C for 1 min each. All reactions were carried
out in duplicate. For relative comparison of targets to
the ribosomal RNA endogenous control, we analyzed
cycle threshold (CT) value of real-time PCR data with
the ΔΔCt method.

ICAM-1 tissue staining
ICAM-1 staining was localized in lung tissue as previ-
ously reported [9]. Briefly, tissue was blocked overnight
at 4 °C, with primary antibody added at 1:75 (rat anti-
mouse ICAM-1; Rockland Immunotechnologies, Gilbertsville,
PA) and incubated for 1 h at room temperature, followed
by secondary antibody (rat anti-CD54, 1:300; Biolegend,
San Diego, CA) for 2 h.
Statistical analysis
All data was analyzed using GraphPad Prism (GraphPad Software, San Diego, CA). Error bars represent the mean +/- SEM. Statistical significance was determined using ANOVA with follow-up Bonferroni test, with \( p \leq 0.05 \) confidence interval being considered significant.

Results
BAL cell numbers are unaffected by hypercapnia
Counts of cells harvested from BAL of mouse lungs showed that nasal instillation of barn dust induced an increase in total cell numbers in the lung (Fig. 1a), and a shift in the population from predominantly macrophages to predominantly neutrophils (Fig. 1b), as has been seen in previous studies [9, 24]. Additional exposure to elevated CO\(_2\) conditions induced no discernable changes in either number or type of cells present in the alveolar space, both in saline as well as barn dust exposed animals, even when CO\(_2\) was increased to 7500 ppm.

Cytokine expression in response to barn dust is increased with hypercapnia
BAL was tested first for common markers of inflammation, IL-6 and KC. Both IL-6 (Fig. 2a) and KC (Fig. 2b) were increased in response to barn dust as has been reported previously [5, 13–15] though only IL-6 was significantly elevated by six hours. Exposure to 5000 and 7500 ppm CO\(_2\) caused significant increases in both IL-6 and KC in HDE-exposed animals compared to saline. This effect was only seen with exposure to HDE, as saline-treated mice showed no similar cytokine increases in response to elevated CO\(_2\). No changes were noted in the serum for either (results not shown).

Fig. 1 Effect of HDE on BAL cell counts. Exposure to HDE caused an increase in total BAL cell numbers (a) and a shift from predominantly macrophages to neutrophils (b). Increasing CO\(_2\) levels (5000 and 7500 ppm) from ambient air (air) had no significant effect on BAL cell number or type in BAL samples. Bar graphs represent standard deviation with error bars shown (\( N = 8 \) mice/group, \( N = 4 \) mice/group for 7500 ppm groups). Statistical significance denoted by asterisks (\(^* p < 0.05\), \(^{**} p < 0.01\)) as compared to respective saline treatment group.

Fig. 2 CO\(_2\) enhances HDE stimulated cytokine expression in BAL. BAL fluid was tested by ELISA for (a) IL-6 and (b) KC. HDE instillation induces both cytokines. Increasing CO\(_2\) levels (5000 and 7500 ppm) in HDE-treated mice significantly increased production of both cytokines vs ambient air (air). Bar graphs represent standard deviation with error bars shown (\( N = 5-8 \) mice/group). Statistical significance denoted by asterisks (\(^* p < 0.05\), \(^{**} p < 0.01\), \(^{***} p < 0.001\), \(^{****} p < 0.0001\))
To look for other possible chemokine targets that may be altered in response to the combination of HDE with elevated CO$_2$, we tested BAL samples using a chemokine dot blot array. Numerous chemokines were altered by HDE (data not shown), however one chemokine, CCL9 showed what appeared to be a noticeable change in response to CO$_2$. To confirm this result, we tested BAL for CCL9 using ELISA to quantitate the results (Fig. 3a). CCL9 expression was significantly increased by exposure to HDE, and significantly increased over those levels when mice were exposed to HDE plus CO$_2$. The levels of CCL9 were increased further still at 7500 ppm CO$_2$ plus HDE, though none of these increases was reflected in serum samples (Fig. 3b), which were uniformly high, as has been shown in other studies [30]. Examination of mRNA showed that increase in CCL9 occurred at the mRNA level as well in lung tissue (Fig. 3c).

CO$_2$ does not induce significant lung leak
As increased CCL9 levels in the lung may reflect increased leak from the blood, additional mice were treated as in previous experiments, and whole lungs were collected for measure of wet-to-dry ratio. There was no significant increase in wet-to-dry ratio of the lungs of HDE treated or HDE + CO$_2$ (7500 ppm) treated animals, suggesting extra fluid had not accumulated in the lungs due to leak (Fig. 4).

ICAM-1 expression
ICAM-1 expression is reported to be either increased [26] or decreased [27] in response to hypercapnia. To address this, we performed immunohistochemical staining of mouse lungs. We observed a visible increase in ICAM-1 expression in the bronchial epithelium exposed to saline + 5000 ppm CO$_2$ alone, which was even more pronounced at 7500 ppm (Fig. 5a-c). ICAM-1 expression was also increased by HDE alone. The addition of CO$_2$ exposure caused further increases (Fig. 5d-f). To quantify these visual observations, we examined ICAM-1 mRNA expression. CCL9 mRNA expression in whole lung homogenates showed a similar pattern to what was seen with immunohistochemical staining (Fig. 5g). There was no change in ICAM-1 mRNA in saline-treated animals with increased CO$_2$, but in HDE + CO$_2$ treated animals, there were increases in mRNA which became significantly elevated at the 7500 ppm level over that of HDE treatment alone. This was true in both whole lung (Fig 5g) as well as tracheal epithelium (Fig. 5h) for mRNA samples.

MMP-9 mRNA expression
A feature of obstructive lung disease is an increase in MMP-9. We suspected that hypercapnia plus an inflammatory stimulus may have a similar effect. Testing for
mRNA expression in whole lung mRNA showed that MMP-9 was not increased by hypercapnia alone, and HDE treatment alone did not induce a significant increase in mRNA (Fig. 6). Combined HDE + hypercapnia treatment, however, showed a significant elevation of MMP-9 mRNA that appeared to be CO₂ dose-dependent (Fig. 6).

Discussion
Workers in CAFOs are exposed to a mixture of organic dusts and gasses in barns. These gasses are produced from the animals living in the building as well as gasses released from wastes and bacterial action. While changes to ventilation may impact dust and gasses in the air, other dust remediation steps such as cleaning or sprinkling [12] may reduce dust, but not gasses. These problems may be greater in colder climates where ventilation must be balanced against heat loss and energy costs.

Of the gasses often studied in CAFOs, three are commonly increased as a result of biological processes in these facilities; NH₃, H₂S, and CO₂ [31–33].

Fig. 4 Mean wet: dry ratio of mouse lung after HDE instillation and/or CO₂ (7500 ppm) treatment. Lungs were weighed post-necropsy, desiccated and weighed to determine wet: dry ratios to examine lung leak. No significant changes from ambient air (air) were noted for any treatments. Bar graphs represent standard deviation with error bars shown (N = 3 mice/group)

Fig. 5 ICAM-1 Expression in bronchial epithelium is altered by HDE and CO₂ exposure. Saline treated mice (a-c) showed increased ICAM-1 staining at 5000 ppm (b) and 7500 ppm CO₂ (c). The same pattern was observed in HDE-treated mice (d-f) with ambient air (d), 5000 ppm CO₂ (e), or 7500 ppm CO₂ (f). ICAM-1 mRNA harvested from whole lung (g) and tracheal epithelium (h) showed no significant increases in response to CO₂ exposure, but HDE with CO₂ treatment induced significant increases of ICAM-1 mRNA. Bar graphs represent standard deviation with error bars shown (5G N = 7-8 mice/group, 5H N = 4 mice/group). Statistical significance denoted by asterisks (*p < 0.05, **p < 0.01, ***p < 0.001)
CO$_2$ while known to cause headaches at higher levels has no current association with known occupational disease. CO$_2$ is a commonly elevated gas in some work environments such as wastewater treatment [34], and potentially small airtight spaces with several people in them [35]. Many well-ventilated facilities will not reach the 8 h time weighted average exposure (TWA) OSHA limit (5000 ppm), but some facilities tested have been shown to reach these levels [33]. Work by other groups into the effects of permissive hypercapnia show CO$_2$ induced significant increases. Bar graphs represent standard deviation with error bars shown (N = 8 mice/group). Statistical significance denoted by asterisks (*$p < 0.05$, **$p < 0.01$) as compared to all saline treatment groups.

Fig. 6 HDE plus CO$_2$ induces MMP-9 mRNA expression. Whole lung mRNA was tested for MMP-9 expression. While HDE alone did not increase MMP-9 mRNA expression, the addition of 5000 and 7500 ppm CO$_2$ induced significant increases. Bar graphs represent standard deviation with error bars shown (N = 8 mice/group). Statistical significance denoted by asterisks (*$p < 0.05$, **$p < 0.01$) as compared to all saline treatment groups.

Another important marker of increased inflammation is ICAM-1 expression. This cell receptor is vital for migration of neutrophils to the lung [43], and given the lack of significant neutrophil increase, we wondered if CO$_2$ had any effect on this receptor. Somewhat surprisingly, we did see clear increases in tissue staining for the receptor in the bronchial epithelium with saline + CO$_2$.
alone. Because HDE predictably induced ICAM-1 so significantly in these bronchial cells [44], it was not clear if addition of CO2 enhanced this effect or not. An examination of lung mRNA showed that mRNA was indeed increased with HDE + CO2, but not so with saline + CO2. As whole lung RNA samples may not reflect tracheal mRNA we isolated tracheal epithelial cell mRNA from animals as well, showing a similar pattern of expression to whole lung samples. This may suggest that a process in ICAM-1 protein production may be responsible for the increases we see with CO2 treatment alone. This may also help to explain the modest increase in ICAM-1 mRNA with HDE treatment alone, despite its clear increase in response to HDE [44].

Finally, we decided to look at MMP-9 due to mention of an unpublished observation that CCL9 increased MMP-9 in the lung [39] and its roles in neutrophil migration and tissue remodeling in the lung [45, 46]. Similar to the other chemokines we examined, there was a clear increase in MMP-9 mRNA in lung tissue as CO2 was increased. Similar to other factors examined such as CCL9, this increase was only apparent when HDE was present.

Conclusions
These results raise a number of questions with relation to workplace ventilation. Does ventilation need to be considered with relation to common illnesses and contaminants of workplaces and how they may interact? While elevated CO2 exposure alone in most cases appeared insufficient to induce changes, the lung appears to alter several responses when elevated CO2 is present in addition to an innate immune stimulus. In this respect, CO2 might function as a tuning mechanism of innate immunity, or perhaps an indicator of dysfunction, requiring a different or elevated response. Another question remaining regards possible acidosis in the exposed animals, and if this is a factor in altered immune responses. While blood gas sampling was not done, the anesthesia protocol at the end of the experiment had animals out of the chamber long enough to likely skew such readings. This remains a technical issue to address in future studies. What our work clearly does show is that the effects of gas exposures at levels seen in work environments, particularly in CAFOs, can depend on responses to other elements present, in particular organic dust.

While this work is in mice and uses an established laboratory-optimized dust injury model, we do note that dust extracts may not mimic a true dust exposure as encountered in the barn. Work will also have to be done with regards to which signaling pathways are affected by these exposures. We do still feel this work shows the importance of further work in mixed environmental exposures and for testing of workers to dusts and gas exposures in the work environment.

Abbreviations
BAL: Bronchoalveolar lavage; CAFO: Concentrated animal feed operation; CCL9: CC chemokine ligand 9; CO2: Carbon dioxide; COPD: Chronic obstructive pulmonary disease; H2S: Hydrogen sulfide; KC: Keratinocyte chemoattractant; MMP-9: Matrix metalloproteinase-9; NH3: Ammonia; TLR: Toll-like receptor

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Availability of data and materials
The datasets developed and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors’ contributions
DS conceived of the study, analyzed data, and carried out most of the experiments and writing of the manuscript. JMD provided technical help and aided with experiments. KLB provided further technical help with regards to mRNA techniques and analysis. DJR and TAW assisted with experimental design, manuscript writing, and guidance of the project. All authors have read and approved of the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Consent for publication
Not applicable.

Ethics approval
All procedures were approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center (protocol number 05-059-08).

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