Am J Transl Res 2020;12(6):2805-2813
www.ajtr.org /ISSN:1943-8141/AJTR0110267

Original Article

Methacholine induced airway contraction in porcine precision cut lung slices from indoor and outdoor reared pigs

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Received March 3, 2020; Accepted April 20, 2020; Epub June 15, 2020; Published June 30, 2020

Abstract: Repetitive exposure to bioaerosols in swine production facilities (SPF) promotes respiratory dysfunction in workers and animals. An adequate understanding of the impact of the SPF environment on pulmonary physiology is needed. However, there is currently no sufficient *ex vivo* model to investigate the cause for agriculture-related lung disease. The precision cut lung slices (PCLS) technique represents a practical and useful procedure for *ex vivo* studies. Our goal was to use the PCLS technique to develop a model of agriculture-related lung diseases using a physiologically relevant animal model, the domesticated pig. Freshly prepared pig lung tissue cores were sectioned into 300 μm slices and viability was measured by lactate dehydrogenase activity and live/dead staining. Airway contractility in response to a methacholine (MCh) dose gradient (10^{-7}-10^{-4} M) was measured. After the last MCh dose, PCLS were incubated with 1 mM chloroquine to allow airways to relax. Time-lapse images were taken every minute for 35 minutes and used to determine airway lumen area changes. Porcine PCLS remained viable and demonstrated metabolic activity for three days. PCLS from indoor and outdoor pigs contracted in response to MCh exposure and relaxed when incubated with chloroquine. Notably, PCLS of indoor pigs showed greater airway constriction in response to 10^{-5} M MCh exposure compared to outdoor pig PCLS ($P<0.05$). These data suggest that exposure to the indoor pig production environment may be associated with hyperresponsiveness in swine airways, and support future studies to investigate lung response to inflammatory substances using the porcine PCLS model.

Keywords: Precision cut lung slice, methacholine, airway contractility, pig, swine production facility

Introduction

Exposure to concentrated animal feeding operations (CAFOs) including swine production facilities (SPF), has been linked to respiratory symptoms and disease in animals and facility workers [1-3]. Inhalation of noxious substances including organic dusts, chemicals, gases, and bioaerosols within CAFOs triggers airway inflammation and lung disease [1, 4-6]. Swine producers, farmers and residents in rural areas have emerged as a population with increased susceptibility to chronic obstructive pulmonary disease (COPD), particularly chronic bronchitis, as a result of these environmental and occupational exposures [7-11]. A complete understanding of cellular and molecular mechanisms governing the natural history of these conditions is lacking. Robust research models are needed to address this lack of knowledge. However, currently available *ex vivo* models do not adequately represent natural exposures within animal production facilities and thus do not permit thorough investigation of lung response by chronically exposed lungs. Therefore, improved *ex vivo* models of environmental and occupational lung exposures are needed.

To develop improved *ex vivo* models of agriculture-related exposures, the basic biology of the lung and exposures within CAFO-style facilities must be considered. The domestic pig has been used as both anatomical and physiological model of humans for over a century. In recent decades, pigs have been used to model pulmonary diseases including asthma and cystic fibrosis [12, 13]. Farm raised pigs represent a unique model because they experience similar exposures to their producers [3]. The tracheal of pigs raised indoors have larger lumen size,

display greater goblet cell density within the airway epithelial mucosa and have diminished antioxidant capacity compared to pasture raised pigs [14, 15]. These changes in the tracheal epithelium are likely due to environmental exposures to organic dust and other substances within the indoor SPFs. These distinctions within the airways of indoor and outdoor reared pigs make ideal models for studying lung responses regulated by environmental exposures.

Assessment of airway responsiveness to stimulants is normally observed in intubated and ventilated small animal models. While such models have their advantages, they are costly [7]. The precision cut lung slices (PCLS) technique permits analysis of airways, including bronchioles, and allows investigation of the native microenvironment. Porcine PCLS generated from farm raised pigs represent a powerful model for understanding airway responsiveness to stimuli, including organic dust and other inflammatory substances, which uniquely represent exposures in rural settings. Methacholine (MCh) is a synthetic compound used to measure how 'reactive' airways are due to its ability to cause airway narrowing and bronchospasm. Clinically, the methacholine challenge test is used to help diagnosis asthma and is commonly used in animal studies, including pigs, to induce bronchospasm [13]. MCh and the bronchodilator chloroquine (ChQ), have both been used, with success, to evaluate airway constriction in PCLS [16, 17]. The purpose of this study was to create PCLS from farm raised pigs and to investigate whether a difference in airway reactivity exists between pigs raised indoors and outdoors. Endpoint assessments included PCLS viability, metabolic activity and response to methacholine challenge. The results reported here demonstrate that airways within PCLS from indoor reared pigs are more reactive than PCLS from pigs raised outdoors.

Materials and methods

Collection of porcine lungs and preparation of precision cut lung slices (PCLS)

Pig lower respiratory tracts (larynx, trachea, and lungs) were collected from local meat processors using a protocol approved by the North Carolina Agricultural and Technical State University Institutional Animal Care and Use Committee. Lung specimens were from freshly harvested, market weight pigs (at least 150 pounds) from indoor (n=4) or outdoor (n=4) swine production facilities and kept preserved on wet ice in plastic bags during transport to lab. Upon arrival, specimens were processed immediately by removing the trachea and cutting away any excess connective tissue and lymph nodes using sterile surgical instruments.

Figure 1 is a schematic (top) and pictorial overview (bottom) of the four steps encompassing our pig PCLS preparation process (i.e., day 0-1). In step 1 the left lung lobe was isolated by cutting at the primary bronchus (**Figure 1A**, **1B**). The left lung lobe was then gently suctioned using a serological pipette (according to size of 1° bronchi) to remove any excess blood, clots or emboli that may prevent injection of low melting point agarose (LMP) (ISC BioExpress, Kaysville, UT). In step 2, i.e., immediately after suctioning, the lung was quickly re-inflated with air manually to using a fresh serological pipette tip attached to a pipet aid and then injected with 4% LMP agarose solution (4% agarose was dissolved in a 1:1 ratio of deionized water to 2X HBSS (Gibco, Gaithersburg, MD)) as shown in **Figure 1C**. Lungs were gently re-inflated prior to injecting agarose to ensure even and maximal filling of the airways and alveolar tissue. Injected agarose naturally collected within distal airways and alveolar regions, producing golf ball-sized swelling that were used to create the lung cores described below. The lung was then clamped shut with sterile hemostats at the primary bronchus opening and wrapped in three layers of sterile gauzed soaked in ice cold 1X HBSS and placed on wet ice at 4°C for 1 hour. Gauze layers were used to keep lung tissue moist and viable while the agarose solidified. When the agarose had solidified, excess lung tissue was cut away using a scalpel and cores from distal portion of lung were cut into 5 × 1 × 1 cm cuboidal lung cores using a sterile blade (**Figure 1D**). The lung cores were embedded in 2.5% LMP agarose in 15 mL conical tubes and allowed to firm for 30 minutes at 4°C (**Figure 1E**). In step 3 the embedded lung cores were mounted onto the microtome stage and sliced at 300 μm thickness on an oscillating tissue slicer (Electron Microscopy Sciences, Hatfield,

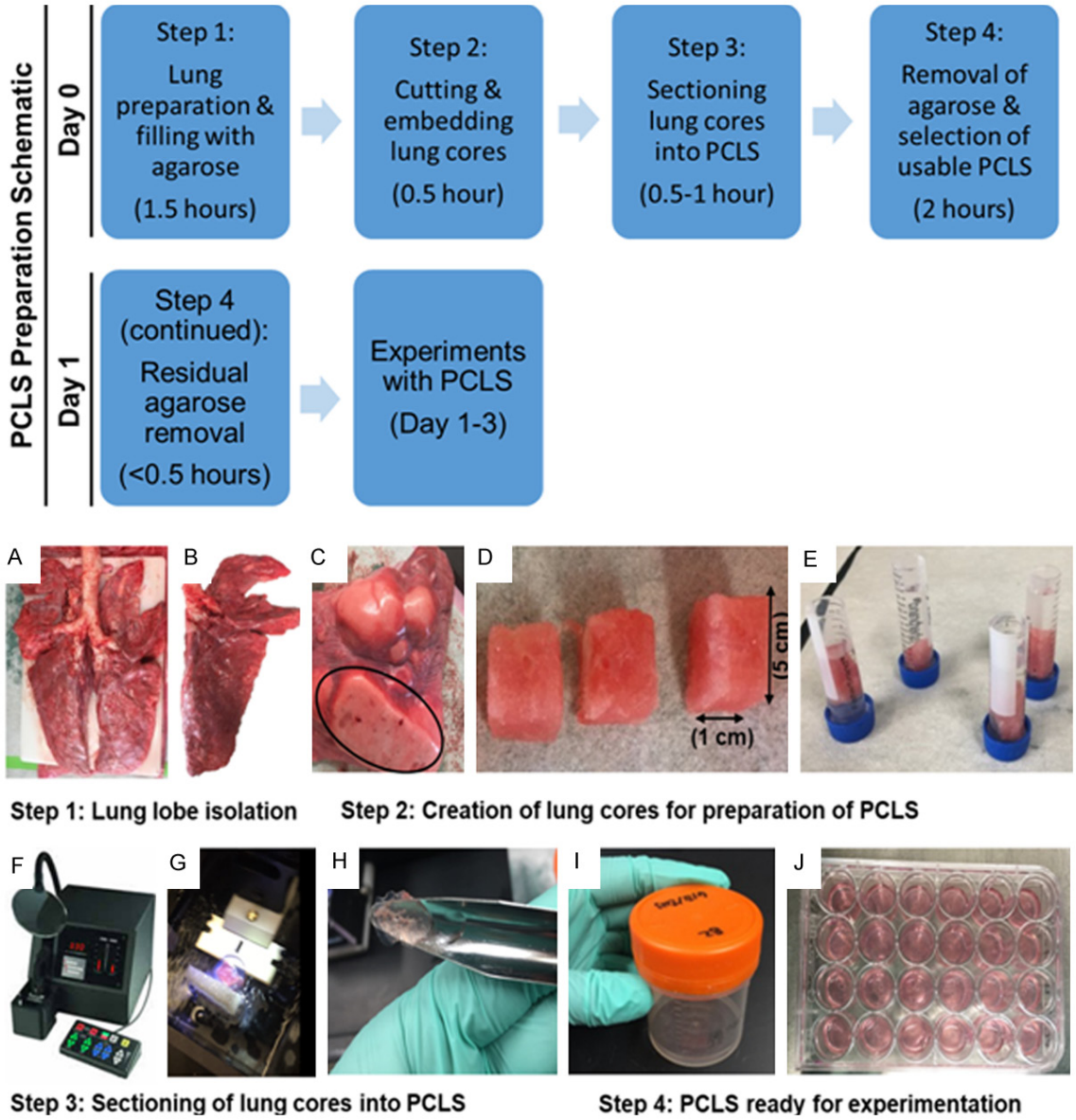

Figure 1. PCLS preparation workflow. Schematic and pictorial overview of the 4-step process for preparation of porcine PCLS including lungs acquisition and lobe isolation; creation of cores; slicing into PCLS; and removal of agarose from lung slices prior to experimentation.

PA) filled with 1X HBSS (**Figure 1F-H**). This thickness is sufficient to allow visualization of airways and maintain tissue reactivity. In step 4 pig PCLS were placed in a sterile specimen cup with fresh 1X HBSS for washing steps (**Figure 1I**, **1J**). A laminar flow hood was used to handle PCLS to maintain sterile tissue culture techniques.

PCLS were then transferred to a new sterile specimen cup and filled with sterile 1X HBSS and incubated at 37°C for 1 hour and rocked for 10 minutes at room temperature (RT) 2X's to allow agarose to melt. PCLS were then soaked in a soaking media (125 ml DMEM (ATCC, Manassas, VA), 63 mg DTT (Sigma, St. Louis, MO) and 1.3 mg DNase (Sigma)) by gently inverting specimen cup and allowing tissue to rest for 1-2 minutes. The inversion and rest steps were repeated for a total of 5 minutes. The DNase/DTT solution was used to lyse and neutralize microbes to reduce bacterial contamination during incubation. Slices were then washed in washing media (500 mL DMEM

(ATCC), 5 mL Pen-Strep (Gibco), 500 µL Amphotericin B (Thermo Fisher Scientific, Waltham, MA)) by gently inverting the specimen cup for 1-2 minutes 3X's (for a total of 6 minutes) to remove any contaminants and residual melted agarose. PCLS with at least 1-2 airways, were then selected and placed into 12 well plates with 1.5 mL of porcine complete media (250 mL DMEM (ATCC), 250 mL Ham's F-12 (Corning, Corning, NY), 100 U/mL Nystatin (Sigma), 100 U/0.1 mg/mL Pen-Strep (Gibco), 0.3 mg/mL Gentamicin (Lonza, Morrison, NJ), 1 µg/mL Amphotericin B (ATCC), 5 ng/mL EGF (Sigma), 0.5 ng/mL Hydrocortisone (Sigma), 5 µg/mL Transferrin (Sigma), 1.5 µg/mL BSA (Sigma), 5 µg/mL Insulin (Sigma), 20 ng/mL Triiodothyronine (Sigma), 0.13 mg/mL bovine pituitary extract, BPE (made in house, bovine pituitaries from Pel-Freez, Rogers, AR), 4% FBS (Atlanta Biologicals, Flowery Branch, GA), 15 ng/mL Retinoic Acid (Sigma)) and placed in incubator at 37°C to melt agarose. The media was changed every 30 minutes for two hours to remove melted agarose and PCLS were incubated overnight to melt any remaining agarose. The next day (i.e., day 1), media was immediately changed three more times to remove any residual melted agarose. PCLS were visually inspected for small airways with intact epithelium and ciliary beating. Only PCLS with intact airways were used for experimentation. Immediately following last media change experiments began (i.e., day 1), unless stated otherwise. PCLS were maintained in porcine complete media at 37°C in humidified air conditioned with 5% CO_2 up to three days.

PCLS cell and tissue viability assessment

Cell viability of PCLS was measured by lactate dehydrogenase (LDH) release into culture medium 1-3 days post-PCLS preparation. Cell-free supernatant was collected by centrifugation and LDH was measured using a commercially available cytotoxic kit according to manufacturer instructions (Roche, Pleasanton, CA). LDH activity was calculated according to manufacturer instructions and reported as a percentage of total LDH activity in Triton X-100-lysed PCLS, i.e., % cytotoxicity. Metabolic activity of PCLS was assessed after 1 and 3 days in culture using the LIVE/DEAD viability/cytotoxicity stain reagents according to manufacturer instructions (Molecular Probes, Eugene, OR). Briefly, following the last wash, PCLS were stained with 4 mM calcein-AM and 2 mM ethidium homodimer-1 (EthD-1) in 1X DPBS in a humidified incubator at 37°C for 30 minutes. Images were taken using an EVOS FI using green fluorescent protein and Cy3 light cubes to illuminate calcein-AM and EthD-1, respectively.

Porcine PCLS airway contraction using MCh

Immediately after removal of agarose from PCLS (i.e., day 1), single lung slices containing small airway lumen area (1,000-5,000 µm^2) with an intact epithelium, evidenced by cilia beating, were placed into a 24-well plate. Porcine complete media (500 µL) was placed onto slices for 5 minutes and aspirated off. Then PCLS were incubated sequentially for 5 minutes each with 500 µL of increasing doses of MCh (10^{-7}-10^{-4} M) (Sigma) and aspirated off before placing the next dose on. The tissue was then rinsed with 500 µL of fresh porcine media, aspirated, and 500 µL of 1 mM of Chloroquine (Sigma) was placed onto slice for 10 minutes. Time-lapse images were taken every minute for 35 minutes at 40X magnification using an EVOS FL Inverted Microscope (Life Technologies, Carlsbad, CA). Per slice, one airway was measured and three PCLS were analyzed per animal (n=3 per housing type).

To measure PCLS response to MCh and chloroquine treatments, airway area was measured using ImageJ (National Institutes of Health, Bethesda, MD) in the same manner as Rosner et al. 2014. We selected values corresponding to the maximum constriction in response to each MCh treatment and maximum dilation in response to ChQ. Averages of the magnitude of each MCh and ChQ value divided by the baseline (media) was used to compute the normalized airway contraction value. Normalized airway lumen area values were then converted into percent control by multiplying the normalized value by 100 (each MCh dose or 1 mM ChQ area/Baseline area x 100) and analyzed via GraphPad Prism 5 for three lung slices/animal.

Statistical analysis

Each pig was considered a biological replicate. All results were expressed as the mean of n= 3-4 biological replicates. Differences in mean LDH activity levels in PCLS culture media from indoor and outdoor pigs were determined by

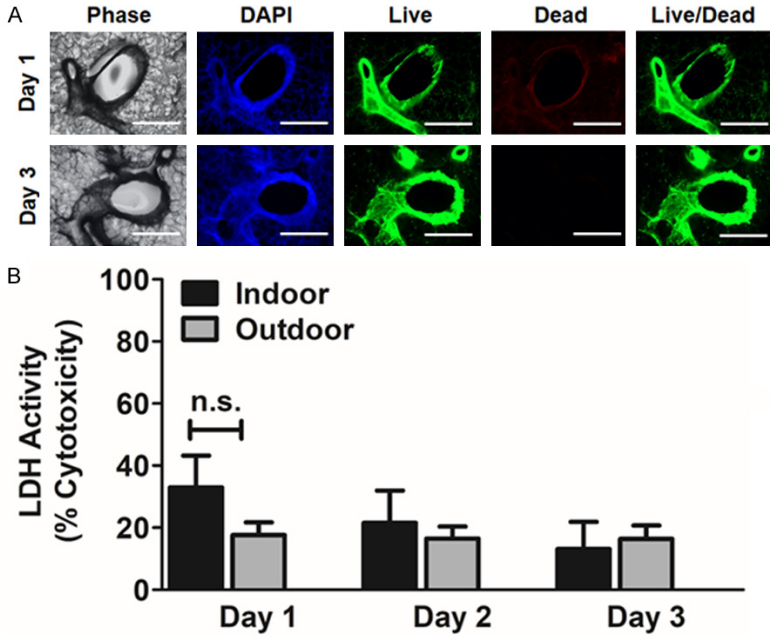

Figure 2. Porcine PCLS are viable for three days. A. Representative images of phase contrast, DAPI and live-dead stained indoor pig PCLS depicting viable airways, cells and surrounding tissue. All images were taken at 4X magnification, scale bar =1,000 μm. Representative of n=3 animals per housing type. B. LDH activity (% cytotoxicity) in PCLS culture media relative to total LDH activity from triton-x lysed PCLS. Data reported as mean ± SEM. n.s., not significant. n=4.

T-tests with Welch's Correction for unequal variances. For airway contraction analysis, repeated measures one-way and two-way analysis of variance (ANOVA) with Bonferroni post-test were applied to detect significant differences between airway lumen area means of PCLS from farm raised pigs using Graphpad Prism software version 5. Within group (indoor or outdoor compared to baseline) differences were analyzed by one-way ANOVA. Whereas between group differences (i.e., pigs raised in the indoor and outdoor production environments) were analyzed via two-way ANOVA. For all analyses, a two-sided significance level of $P<0.05$ was used and data are reported as mean ± SEM.

Results

Porcine PCLS remain viable after three days ex vivo

To assess PCLS cell and tissue viability, a live/dead test, which assesses cell membrane integrity and intracellular esterase activity, was used. Viable cells/tissue is capable of converting the colorless calcein-AM dye into a detectable green fluorescent product. Where-as the nuclei acid of dead/dying cells is stained by EthD-1 which emits red fluorescence. Overall tissue integrity and viability was evaluated daily by visual inspection for cilia bea-ting. During the course of the three-day study, PCLS tissue remained viable, with intact airways containing beating cilia metabolic activity as depicted in **Figure 2A**. LDH levels in culture media was measured to determine cell membrane integrity. LDH leakage is an intracellular enzyme that is released when the cell membrane is damaged. Not surprisingly, LDH activity was detected in culture media on day 1 and remained stabilize at day 2 and 3 of PCLS culture (**Figure 2B**). There was no significant difference in LDH activity levels between PCLS from indoor and outdoor reared pigs. The vast majority of the PCLS cells, airway epithelia and lung tissue were viable.

PCLS from indoor reared pigs have greater contractile response to MCh than PCLS from pasture raised pigs

To measure contractile response of PCLS from indoor pigs and outdoor pigs a MCh dose-response curve was evaluated. PCLS from indoor and outdoor reared pigs were responsive to increasing doses of MCh. **Figure 3A** depicts representative images of indoor and outdoor pig PCLS airways following exposure to media (baseline), the highest MCh dose (10^{-4} M) and 1 mM ChQ treatment. Upon subsequent exposure to ChQ we observed dilation of the lumen of the airways of both indoor and outdoor animals. To quantify the impact of housing and MCh treatment on pig PCLS airway constriction, normalized lumen areas were converted into percent control values. As shown in **Figure 3B**, airways of PCLS from indoor pigs constricted in a dose-dependent manner in response to MCh treatment when compared to

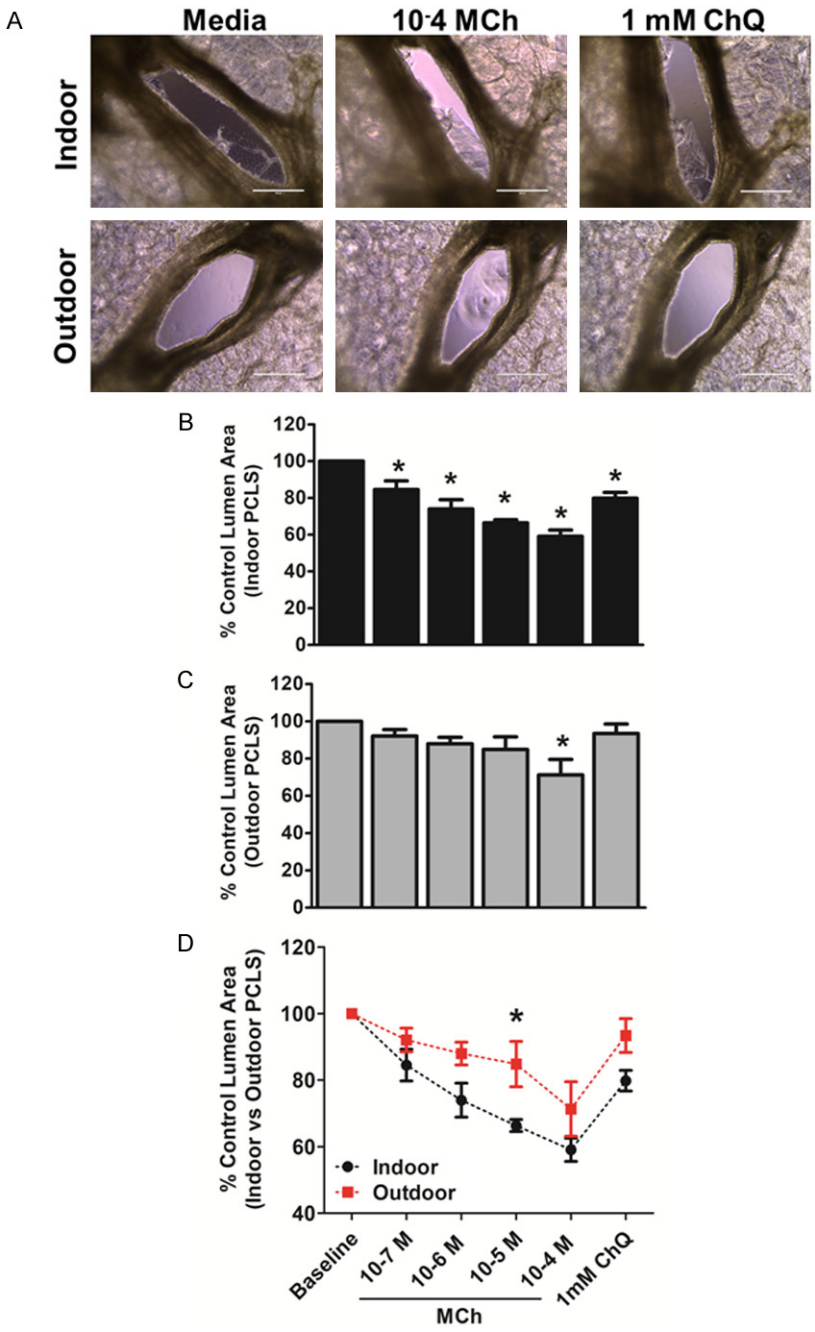

Figure 3. Indoor PCLS showed greater airway constriction compared to outdoor PCLS. A. Representative images of the constriction and relaxation of indoor and outdoor PCLS. Indoor and outdoor PCLS were both stimulated with increasing doses of methacholine for 5 minutes per dose and subsequently stimulated with a relaxing agonist, chloroquine, for 10 minutes. Representative images of indoor and outdoor pig PCLS exposed to media (baseline), 10^{-4} MCh and 1 mM ChQ from three lung slices per indoor and outdoor animal. n=3 per housing type. Scale bar, 100 µm. B-D. Percent control measurements of respective airway lumen areas of indoor and outdoor pig PCLS in response to MCh and ChQ treatment. PCLS airway lumen area for each dose of MCh and ChQ treatment were divided by baseline (media) and multiplied by 100 to get the normalized percent control lumen area. B. Percent control normalized lumen of indoor pig PCLS. *P<0.05, baseline vs. all MCh doses and ChQ. C. Percent control normalized lumen of indoor pig PCLS. *P<0.05, baseline vs. 10^{-4} M MCh. D. Comparison of airway lumen area of PCLS from indoor and outdoor pigs indicated a housing effect at 10^{-5} M MCh. *P<0.05, indoor vs outdoor at 10^{-5} M MCh. MCh, methacholine; ChQ, chloroquine. Data reported as mean ± SEM of 3 outdoor pigs and 3 indoor pigs.

the baseline/media control (*p*-value <0.05). Conversely, compared to baseline area only the 10^{-4} M MCh dose caused significant constriction of PCLS airways from outdoor reared pigs (**Figure 3C**). Interestingly, MCh dose-response curves demonstrated that indoor pig PCLS airways constricted significantly more than outdoor pig PCLS airways in response to 10^{-5} M MCh dose (*p*-value <0.05) indicating a housing effect (**Figure 3D**). As summarized in **Table 1**, indoor pig PCLS airways overall showed greater sensitivity to MCh evidenced by smaller airway lumen means compared to means of outdoor pig PCLS airways.

Discussion

Swine production facility workers are exposed to a variety of airway stimulants, including organic dust, in the indoor production environment. Inhaling these particulates leads to airway inflammation and long-term exposure puts individuals at high chances of developing lung diseases, including chronic bronchitis, nonatopic asthma, and pulmonary fibrosis. There are currently no cures for

Table 1. PCLS airway lumen means and standard deviations following MCh and ChQ treatments

	Indoor PCLS Airway Area (% Control)		Outdoor PCLS Airway Area (% Control)	
	Mean	SD	Mean	SD
Baseline (control)	100.0	0.0	100.0	0.0
10^{-7} M MCh	84.5	8.3	92.1	6.2
10^{-6} M MCh	73.9	8.9	88.0	5.9
10^{-5} M MCh	66.3	3.1	84.8	11.8
10^{-4} M MCh	59.1	6.0	71.3	14.3
1 mM ChQ	79.8	5.4	93.4	8.8

SD, standard deviation; MCh, methacholine; ChQ, chloroquine.

these diseases and current therapeutic options do not completely relieve symptoms [18, 19]. Thus, there is a need for models and applications that are better suited for investigating the effects of inhalation exposures. The present study showed that the porcine PCLS model is a viable option for studying airway response. PCLS viability was demonstrated by maintenance of tissue integrity, beating cilia and airway contractile and relaxant responses to stimulants.

Repetitive exposure to farm animal production buildings places farm workers at increased risk of developing chronic bronchitis, COPD and asthma like syndromes [2, 20-22]. In human COPD and asthma, the smaller distal airways are significant sites of inflammation and abnormal lung function [23]. Presently, these distal airway sites are difficult to clinically test by non-invasive means. Functionally, PCLS allows for a study of smaller distal airways that are involved in disease process of COPD and asthma. In fact, PCLS works best with the smaller distal airways in that they are more sensitive to either allergen stimulation [24] or stimulation by methacholine [25] *ex vivo*. Methacholine, a non-selective muscarinic receptor agonist, is frequently used in clinical tests to determine bronchial hyperreactivity, such as that caused by asthma or COPD. In children and adults with a history of childhood asthma, the degree of airway responsiveness to stimuli, including methacholine, is linked to disease severity [26-28]. Here we demonstrate for the first time that PCLS generated from indoor raised pigs are more sensitive to MCh than PCLS from outdoor reared pigs. Percent control analysis of normalized lumen area showed that indoor pig PCLS were more responsive to MCh and constricted to a greater degree at a lower concentration than outdoor pig PCLS. Indoor pig lung slices were overall more sensitive to the stimulants. We speculate this to be a result of the repeated exposure to respirable particles, such as organic dust and gases within the swine production facilities, since it is known that exposures to organic dust from swine production facilities cause airway hyperresponsiveness [29].

There are a few important limitations to the present study. Although all pigs in this study were market weight (≥150 lbs.), exact weights, ages and sexes are unknown, potentially leading to some variability in the size of the lungs harvested. To account for this disparity, we made lung cores of similar size and from the same area of the lungs in all pigs. Likewise, PCLS airways with similar lumen area were used. While documented differences in response to stimuli exist between PCLS from adult animals compared to newborn animals, it is speculated that differences among adult animals of the same species, apart from dysfunction related to exposure, are not expected [30]. There is also historic disparity in the PCLS tissue studies themselves. Researchers from other labs reported marked variation in the response of lung slices within the same animal as well as in different species [16, 31]. Minshall et al. demonstrated a heterogeneity of responses in explanted airways from human donors [32]. Given the enhanced airway sensitivity to methacholine demonstrated by PCLS from indoor raised pigs compared to PCLS derived from pasture raised pigs, careful consideration is essential for selecting the appropriate animal model. Finally, it was beyond the scope of this study to examine PCLS response to environmental stimulants. Despite these limitations, the porcine PCLS model represents an ideal tool for studying lung and airway responses for several days, especially for evaluating the response of chronically exposed to environmental pollutants.

In conclusion, airway hyperresponsiveness is a hallmark of chronic lung diseases such as those resulting from repetitive environmental and occupational exposures. PCLS models have emerged as an ideal tool for *ex vivo* study of lung disease and dysfunction. The porcine PCLS model we have developed is a physiologi-

cally relevant tool studying lung and airway response to stimulants, including those experienced within agricultural settings. Moreover, pigs and humans have similar lung tissues. Porcine PCLS models described in this report represent powerful tools because indoor pigs have been 'naturally exposed' to indoor air pollution for a lifetime and their lung tissue bears consequential adaptations; while outdoor pigs have not had this exposure. PCLS from these two pig populations are an ideal resource for studying lung dysfunction, including occupational lung disease and comparative studies requiring 'affected and normal' groups. These pig lung slice models can be easily adapted for a myriad of *ex vivo* comparative lung response studies.

Acknowledgements

The project described was supported in part by grants from the North Carolina Agromedicine Institute, the Unites States Department of Agriculture, National Institute of Food and Agriculture under Grant number NC.X322-5-19-120-1; the National Center for Advancing Translational Sciences, National Institutes of Health (NIH) via the North Carolina Translational and Clinical Sciences (NC TraCS) Institute under Grant number 1UL1TR001111; and the NIH/National Heart, Lung, Blood Institute under Grant number: SC1HL150742. The content is solely the responsibility of the authors and does not necessarily represent the official views of the USDA or NIH. The authors thank Sara D. Tatum, Caressa L. Gerald and Kimberly L. Raiford for their contributions and insight to this work.

Disclosure of conflict of interest

None.

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