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SWINE CONFINEMENT BUILDINGS: EFFECTS OF AIRBORNE PARTICLES AND SETTLED DUST ON AIRWAY SMOOTH MUSCLES

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Abstract: Swine confinement workers are exposed to various contaminants. These agents can cause airway inflammation and bronchoconstriction. This study was undertaken to evaluate if the bronchoconstrictive effects of swine barn air and settled dust are mediated by endotoxin, and if these effects are directly mediated on airway smooth muscles. Mouse tracheas where isolated and mounted isometrically in organ baths. Tracheas, with or without epithelium, were attached to a force transducer and tension was recorded. Concentrated swine building air at 68 EU/ml or settled dust extract at 0.01 g/ml were added for 20 minutes and tracheal smooth muscle contraction was measured. Direct role of LPS was assessed by removing it from air concentrates with an endotoxin affinity resin. Swine barn air and settled dust extract caused contraction of tracheal smooth muscle by 26 and 20%, respectively, of the maximal induced by methacholine. Removal of epithelium did not affect the contractile effects. LPS alone and LPS with peptidoglycans did not induce contraction. However, when endotoxin was removed from swine barn air concentrates, it lost 24% of its contractile effect. Concentrated swine barn air and settled dust have direct effects on airway smooth muscles. This effect is partially due to LPS but a synergy with other components of the environment of swine confinement buildings is required.

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Key words: airborne particles, airborne settled dust, swine buildings, airway smooth muscles, bronchoconstriction, endotoxin.

INTRODUCTION

Farming practice is the basic industry of mankind. Unfortunately, modern farming exposes farmers to hazards which may result in an array of adverse health outcomes. This work can be associated with high risks for environmental diseases, such as hypersensitivity pneumonitis, organic dust toxic syndrome or silo's lung, which decrease the quality of life and productivity of farmers. Among farmers, swine confinement building (SCB) workers are usually exposed to an environment highly contaminated with dust [13] and endotoxin [4]. Facilities are closed environments contaminated with toxic gas, such as ammonia and carbon dioxide, dust, and a large variety of microorganisms [7]. Repeated exposure to this environment results in different physiological effects, including pulmonary inflammation [5], broncochonstriction and bronchial responsiveness [20], hyperresponsiveness to methacholine [3], and a decrease in lung function [23]. The precise causative factors responsible for these effects are still unclear. Endotoxins, ubiquitous in swine buildings, have the potential to explain many of the effects resulting from exposure to SCB. They have the potential to induce the production of inflammatory mediators, which could explain the inflammatory effects and airway hyperresponsiveness, but not necessarily the bronchoconstriction [12].

Lipopolysaccharides (LPS) are components of the gram negative bacterial membrane. Interactions between LPS

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and cells are very complex. LPS is recognized by Toll-like receptor 4 (TLR-4) and, in the presence of other extracellular matrix proteins, activates several kinases leading to the translocation of NF-κB to the nucleus [15]. This process induces the production of inflammatory mediators which lead to pulmonary inflammation [15]. The activation of NF-kB also stimulates cyclooxygenase-2 (COX-2) and increases the synthesis of prostaglandins (PGs) [2]. This group of lipid mediators includes not only various pro-inflammatory molecules, but also the anti-inflammatory and muscle relaxant PGE_2 [11]. LPS exposure also leads to the synthesis of nitric oxide, another potent relaxant and anti-inflammatory substance [25]. Gram negative bacteria were found in all piggeries studied, but they usually represented only around 5% of the total microflora which is composed mostly of gram positive bacteria [27]. Gram positive bacteria produce peptidoglycans, which could also be implicated in the respiratory effects of exposure to the swine building environment. Leukotrienes, substances which have a direct effect on airway smooth muscles [17] and on migration of neutrophils [26], have also been found in SCB air.

This study was performed to evaluate the role of LPS and other potential compounds in the bronchoconstriction induced by exposure to swine buildings. In addition, since exposure to SCB increases airway responsiveness in naïve subjects [3], a secondary objective was to verify if swine barn air concentrates could modify non-specific airway responsiveness of trachea smooth muscles. To address these objectives, an isometric method which allows direct measurement of tracheal smooth muscle contraction was used. Finally, since airway epithelium produces many inflammatory cytokines and mediators in response to LPS, and potentially other substances found in SCB, we also verified if the integrity of the epithelial layer is required for the contractile effects induced by swine buildings contaminant extracts, or whether they act directly on airway smooth muscles.

MATERIALS AND METHODS

Air sampling. Air samples and settled dust were collected from a swine confinement building near Quebec City, Canada. Air sampling was performed with All glass impingers (AGI-30) (Ace Glass Inc., Vineland, NJ, USA) [10]. Two impingers were placed in the middle of the SCB which housed 3 months old pigs. The sampling lasted 70 minutes with the pump set at 12.5 l/min. For control, air was bubbled under a biological hood with the same instrumental settings. After sampling, the volume of liquid remaining in the impinger was noted and adjusted to 25 ml with sterile HBSS-Ca^{2+}. The liquid from both impingers was immediately pooled, aliquoted, and frozen at -20°C waiting endotoxin measurements by Limulus Amebocyte Lysate assay (LAL) (BioWhittaker; Walkersville, MD) described in detail by Zucker *et al.* [27]. Tests were also performed with swine building settled dust from the same building. Settled dust was collected from the space between the pens into a 50 ml sterile centrifuge tube by scooping up enough settled dust to completely fill the tube. The settled dust was returned to the laboratory and frozen at -20°C until used for the experiment. Settled dust was then solubilised at 0.01 g/ml, as described previously with minor modifications [16]. Solubilisation was carried out in Krebs bicarbonate buffer (mM: NaCL, 112; KCL, 4.96; KH_2PO_4, 1; $MgSO_4 \cdot 7H_2O$, 1.2; $CaCl_2 \cdot 2H_2O$, 2.52; $NaHCO_3$, 29.76; $C_6H_{12}O_6$, 10.42 in distillated water), and centrifuged 3 times.

Animals and isometric experiments. All the animal experiments were conducted according to the Helsinki recommendations. The protocol was approved by the Animal Ethic Committee of Laval University. Tracheas were isolated from female BALB/c mice (18–20 g) from Charles River (St-Constant, PQ, Canada), and the tracheas removed and mounted in 5 ml organ baths for the isometric studies, as described previously [9]. Mouse serum at a final concentration of 2% was added to the baths to provide the LPS binding protein necessary for LPS to be coupled to its receptor. Viable tracheal rings were incubated with 2.5 ml of bubbled swine barn air, 2.5 ml of settled dust extract or 2.5 ml of control buffer (HBSS-Ca^{2+}1.5 mM) for 20 minutes. Tension was recorded at a rate of 6 measurements per second, with AcqKnowledge 3.7.3 software. The preparations were then washed and the tracheas allowed to re-equilibrate for 30 minutes. In the experiments with swine barn air, cumulative dose-responses to methacholine were assessed as follows: once tension had returned to baseline values, cumulative doses (10^{-8}–10^{-5}M) of methacholine were added to the baths, and tensions recorded to verify if hyperresponsiveness was observed. The results are expressed as a percentage of the initial maximal methacholine contraction (C_{max}). To assess if contractions were induced by LPS, isometric experiments were performed with LPS from *Escherichia coli* 026:B6 (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 1 µg/ml. LPS of air samples was also removed by passage through an endotoxin affinity resin (END-X B15, Associates of Cape Cod, Falmouth, MA, US), and endotoxin free supernatant used.

Isometrics studies were also performed with other substances present in swine barn air which could be acting in synergy with LPS from *Escherichia coli* 026:B6 to induce contraction. Peptidoglycan (PG) from staphylococcal species (Toxin Technology, Sarasota, FL, USA) at 10 µg/ml was used alone and in combination with 1 µg/ml LPS. Leukotriene B4 (50 ng/ml) alone and with LPS (1 µg/ml) were also tested.

To ensure that the contractile effect induced by swine settled dust was a direct effect on smooth muscle, epithelium was removed in some experiments, as previously described [9].

Nitric oxyd (NO) synthesis was also blocked by adding the NO inhibitor N^G-nitro-L-arginine methyl ester (L-NAME; Sigma-Aldrich; 10^{-6} M) 10 min before extract addition of settled dust.

Tracheas were used only once, since repeated treatment to methacholine can affect their contractile properties.

Data analyses. Results are expressed as means of C_{max} ± SE. We determined the statistical differences between mean values using an ANOVA unpaired t-test. A p value of <0.05 was considered significant.

RESULTS

Isometric studies with swine bubbled air and settled dust. Final endotoxin concentration from swine barn air in the bath was 34 EU/ml, compared with only 0.06 in control air. Both swine barn air and settled dust induced tracheal contraction (Fig. 1). Control air bubbled under a laboratory hood had no effect (data not shown) and neither did the control buffer. Endotoxin content of control buffer was similar to that of air bubbled under the biological hood, and was used as control for further experiments. Bubbled swine barn air induced a maximal contraction of 26% ± 6 of C_{max}. This contraction persisted for up to one hour. The contraction induced by air concentrate was significantly different from controls for points between 5–20 min ($p \leq 0.005$) (Fig. 2). Settled dust induced a maximal contraction of 20% ± 5 of C_{max}, which decreased spontaneously within 10 min. This relaxation was blocked when tracheas were pre-treated with L-name, an NO synthesis inhibitor, before adding the settled dust extract in different isometric studies (Fig. 3). Cumulative dose-responses to methacholine were similar for tracheas pre-treated or not with settled dust. Both untreated and settled dust treated tracheas respectively reached maximal contractions of 152% and 168% of C_{max} (Fig. 4).

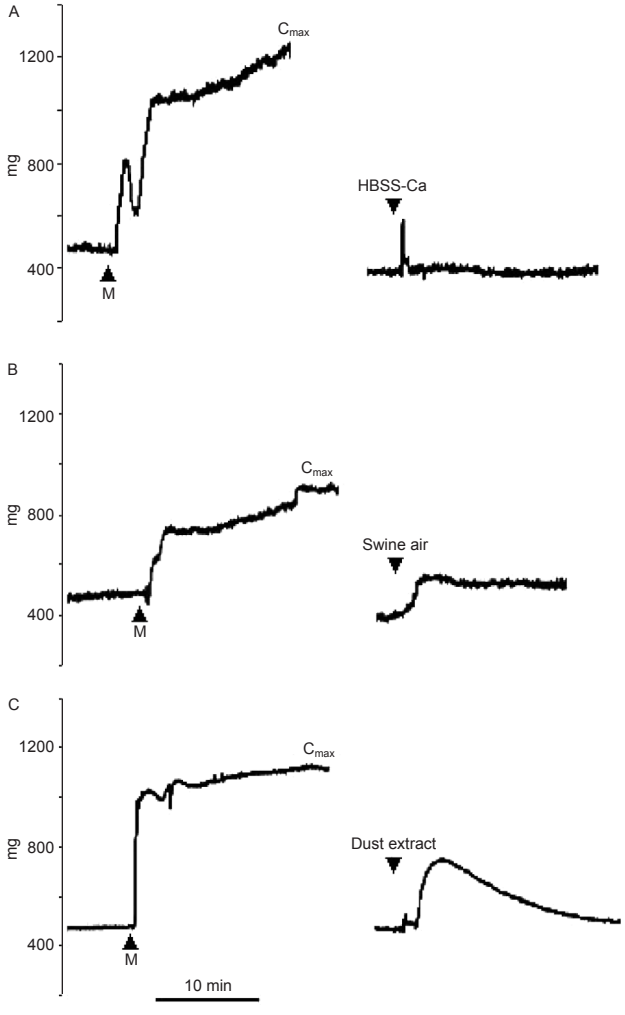

Figure 1. Representative tracing of isometric experiments performed with HBSS-Ca^{2+} (a), swine barn air (b) or settled dust extract (c) on tracheas. Data expressed as a mean of 6 measurement per second registered by AcqKnowledge 3.7.3 software in time. Data expressed in milligrams of tension in function of time. M: methacholine 10^{-5}M.

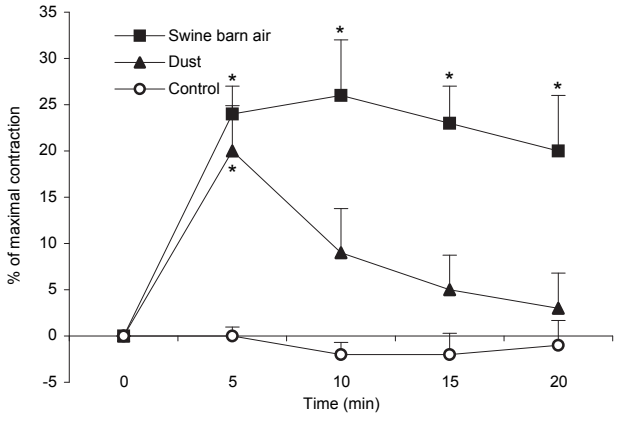

Figure 2. Effects of addition of 2.5 ml of swine barn air (■; n = 4) or 2.5 ml of settled dust extract (▲; n = 6) or HBSS-Ca^{2+} (○; n = 6) on BALB/c mice tracheas. Each point represents mean ± SE of percentage of maximal contraction induced by methacholine precontraction. * significantly different at $p \leq 0.005$ compared to control.

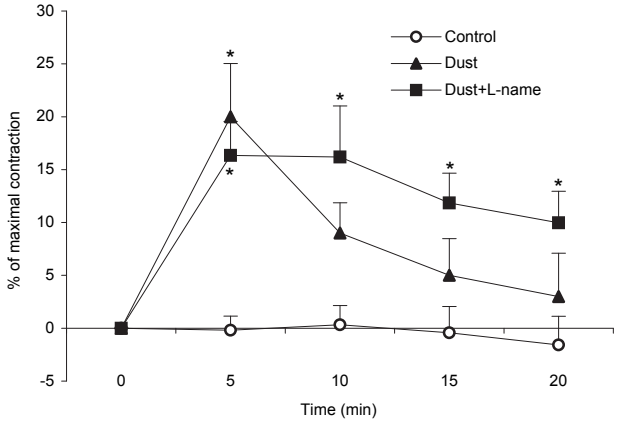

Figure 3. Effects of pre-incubation of tracheas with L-name 10^{-5}M (■; n = 5) or with HBSS-Ca^{2+} (▲; n = 6) 20 min before addition of 2.5 ml of settled dust extract compared to control tracheas incubated only with HBSS-Ca^{2+} (○; n = 6). Each point represents mean ± SE of percentage of maximal contraction induced by methacholine precontraction. * significantly different at $p \leq 0.05$ compared to control.

Implication of LPS in contraction. Purified LPS alone did not induce tracheal contraction, even when a very high

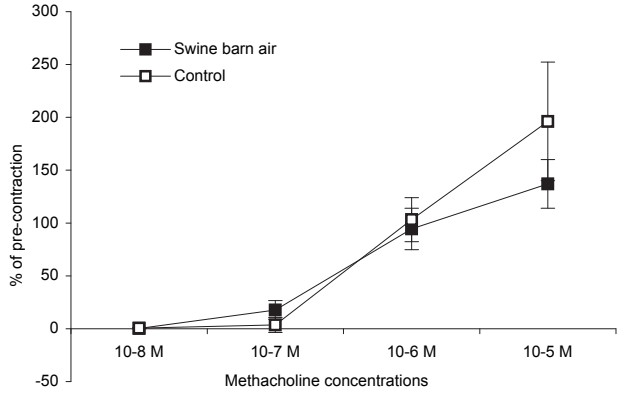

Figure 4. Dose-response curves for contraction of pretreated BALB/c tracheas with swine barn air (■; n = 4) or HBSS-Ca^{2+} (□; n = 6) by methacholine 10^{-8}–10^{-5}M. Each point represents mean ± SE of percentage of maximal contraction induced by methacholine precontraction. The black line shows the 100% of pre-contraction. No significant differences observed.

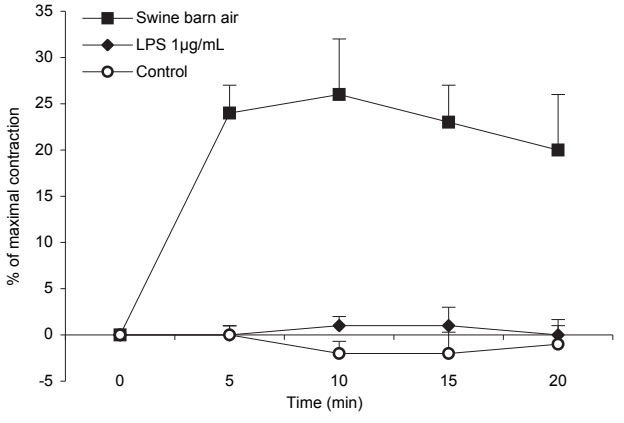

Figure 5. Differences between addition of 2.5 ml of swine barn air (■; n = 4) and of LPS 1 µg/ml (□; n = 5). Each point represents mean ± SE of percentage of maximal contraction induced by methacholine precontraction. * significantly different for $p \leq 0.005$.

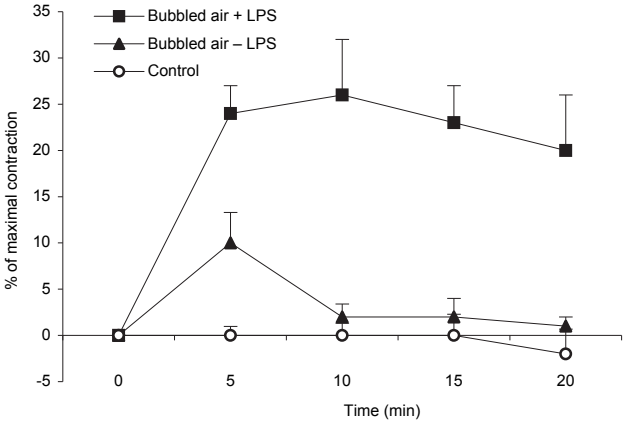

Figure 6. Addition of swine barn air (■; n = 4) and of swine barn air rendered endotoxin free (▲; n = 6) after passage on high affinity endotoxin resin compared to control tracheas (○; n = 6). Each point represents mean ± SE of percentage of maximal contraction induced by methacholine precontraction. No significant difference between contraction noted after addition of swine barn air without LPS and control tracheas.

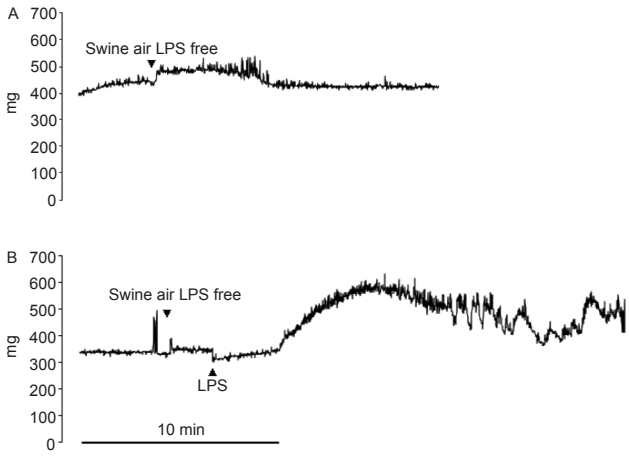

Figure 7. Representative tracing obtained by AcqKnowledge 3.7.3 from isometric experiments with swine barn air without endotoxin (A), and with swine barn air without endotoxin in which LPS 34 EU/ml is added (B). Contractile capacity of swine barn air is restored by LPS addition. Data expressed in milligrams of tension in function of time.

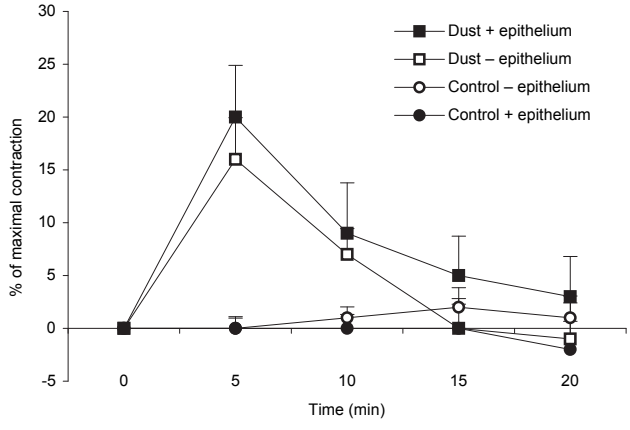

Figure 8. Differences of contraction when 2.5 ml of settled dust extract is added in the bath of tracheas with (■; n = 6) or without epithelium (□; n = 6), compared to control tracheas with epithelium (●; n = 6) and without epithelium (○; n = 5). Control tracheas are treated with HBSS-Ca^{2+}. Each point represents mean ± SE of percentage of maximal contraction induced by methacholine precontraction. No significant difference between contraction noted on tracheas with or without epithelium.

concentration was added to the bath (Fig. 5). When endotoxin was removed from swine barn air samples no tracheal contraction was seen (Fig. 6). Results of LAL assay for swine barn air after passage on resin were negative. To assure that endotoxin affinity resin did not remove any other substances in the air samples we reintroduced LPS in the endotoxin free supernatant. Contractile properties of samples were restored when 34 EU/ml of LPS were added to the bath (Fig. 7). Neither PG nor leukotriene B4 alone, or in combination with LPS, induced any contraction (data not shown).

Implication of epithelium. Contractile effect of settled dust extracts was epithelium independent since removal of epithelium did not prevent contraction (Fig. 8).

DISCUSSION

Results of this study show that swine confinement building airborne and sedimented contaminants can induce smooth muscle contraction in mouse trachea.

Swine barn air and settled dust induced different patterns of tracheal contraction. The contraction induced by addition of swine barn air lasted up to one hour, while the contraction induced by settled dust spontaneously waned over 10 minutes, suggesting that there are differences in their composition.

Incubation of tracheas with swine barn air or settled dust extract did induce contraction of the tracheas, but did not modify the response to methacholine. This is different from that observed in humans in whom swine building exposure induced bronchial hyperresponsiveness [24] as well as bronchoconstriction [8]. A possible explanation is that we used isolated murine tracheal rings, thus the overall pulmonary milieu was absent. Interactions between inflammatory and epithelial cells within the lung are probably necessary for the increase in airway hyperresponsiveness.

In these experimental settings, LPS alone did not directly induce tracheal contraction [1, 14]. Even when we used doses two orders of magnitude more concentrated than the levels of endotoxin present in swine barn air, we did not induce tracheal contraction. However, when LPS was removed from the air sample with very specific columns the contractile effect of air was abolished. But when a similar concentration of endotoxin to that present in the air sample was added to the bath, with the endotoxin free air samples the contractile property of the sample were restored. Our interpretation for these findings is that even if LPS alone does not induce smooth muscle contraction it is an essential component of swine building air to induce this effect. LPS may not be the mediator inducing tracheal contraction but seems to be an enhancer of other substances present in swine barn air. This possibility is demonstrated with some molecules such as bradykinin [1, 14], and can increase their spasmogenic potential. It is also demonstrated that exposure to LPS can induce airway hyperresponsiveness to adenosine [21] and methacholine [6]. LPS thus acting in synergy with co-factors present in swine barn air still needs to be identified.

There are many substances present in swine confinement building air that could interact with LPS and induce bronchoconstriction, but neither substance that we tested – LTB4 and peptidoglycans – could explain the tracheal contraction noted. This indicates that a mixture of numerous substances are possibly needed to induce the contraction of tracheal smooth muscles.

Maximal contractions reached with or without epithelium had similar kinetics. This could be explained by the presence of LPS receptor on epithelium as well as on airway smooth muscle cells [19]. Contractile effects of swine settled dust therefore does not require the presence of epithelium nor mediators secreted by it.

The reversal of the spontaneous relaxation by L-name, a specific inhibitor of NO synthesis, indicates that NO is responsible for the relaxation, but does not confirm that NO was induced by endotoxins in our experiments. Endotoxin levels were higher in settled dust extract than in swine barn air samples, and exposure to high concentrations of endotoxin are known to induce NO synthesis and this NO can causes smooth muscle relaxation [22].

Since all the experiments were performed with the same strain of Balb/c naive mice, we believe that comparison between groups of experiments is valid. Other strengths of the study were the application of a simple technique that was easily standardized and the quick evaluation of effects. This rapid evaluation avoided the spontaneous increase in contractility of tracheas that appears after hours in the baths. We therefore believe that this study using mice tracheas revealed effects that are relevant for the understanding of human reactions to inhaled air in SCBs.

In summary, LPS does not induce tracheal contraction, but plays an essential role in contraction induced by swine barn air and settled dust. It must act in synergy with other substances present in swine barn air, substances that still need to be identified. Contractions induced by swine building contaminants are mouse epithelium, independently and interacting directly with mouse smooth muscle cells.

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