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Solitary cholinergic stimulation induces airway hyperreactivity and transcription of distinct pro-inflammatory pathways

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Abstract

Airway hyperreactivity is a hallmark feature of asthma and can be precipitated by airway insults, such as ozone exposure or viral infection. A proposed mechanism linking airway insults to airway hyperreactivity is augmented cholinergic transmission. In the current study, we tested the hypothesis that acute potentiation of cholinergic transmission is sufficient to induce airway hyperreactivity. We atomized the cholinergic agonist bethanechol to neonatal piglets and forty-eight hours later measured airway resistance. Bethanechol-treated piglets displayed increased airway resistance in response to intravenous methacholine compared to saline-treated controls. In the absence of an airway insult, we expected to find no evidence of airway inflammation; however, transcripts for several asthma-associated cytokines, including IL17A, IL1A, and IL8, were elevated in the tracheas of bethanechol-treated piglets. In the lungs, prior bethanechol treatment increased transcripts for IFN γ and its downstream target CXCL10. These findings suggest that augmented cholinergic transmission is sufficient to induce airway hyperreactivity, and raise the possibility that cholinergic-mediated regulation of pro-inflammatory pathways might contribute.

Keywords

airway hyperreactivity; piglet; cholinergic; inflammation

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Author contributions statement

LRR, DKM, and MAA conceived the study. LRR, DKM, and MAA wrote the manuscript. LRR, SPK, KRA, MVG, DKM and MAA performed experiments. LRR, KRA and SPK performed data analysis.

Introduction

Airway hyperreactivity is a common feature of asthma and is characterized by exaggerated airway narrowing in response to a variety of stimuli [1]. It is well established that airway insults (e.g. viral infection, ozone inhalation, antigen challenge) can precipitate or worsen asthma-like symptoms, including airway hyperreactivity, in “normal” (e.g. non-reactive) and asthmatic airways [2–4]. Enhanced cholinergic transmission is a proposed mechanism mediating insult-induced airway hyperreactivity [5]. Specifically, evidence suggests that inflammation inhibits presynaptic cholinergic receptors on nerve terminals innervating the airway, resulting in enhanced acetylcholine release, and augmented bronchoconstriction [3,4].

We hypothesized that if enhanced cholinergic transmission is a key factor mediating airway hyperreactivity [5], then cholinergic stimulation in the absence of an airway insult might also be sufficient to induce airway hyperreactivity. To examine this possibility, we challenged neonatal piglets with the non-selective muscarinic receptor agonist bethanechol. Because previous studies have shown that airway hyperreactivity can occur as early as 24–48 hours following an airway insult [3,4], we measured airway resistance 48 hours later. We selected the piglet model due to its similar anatomy, physiology, and development compared to humans [6], as well as our extensive expertise with the model [7,8].

Materials and methods

Animals and Ethic Statement

A total of 16 piglets (Landrace/Yorkshire breed, 2–3 days of age) were fed commercial milk replacer (Sowena Litter Life), and allowed a 36–48-hour to acclimate before interventions began. The University of Iowa Animal Care and Use Committee approved all procedures. Procedures were completed in accordance with federal policies and guidelines.

Airway instillation

After acclimation, piglets were anesthetized with 20mg/kg ketamine and 2.0mg/kg xylazine (Henry Schein Animal Health). Airways were accessed with a laryngoscope. A laryngotracheal atomizer (MADgic) was passed directly beyond the vocal folds to aerosolize either a 500 µl of 0.9% saline control or 8 mg/ml bethanechol chloride (Selleckchem) in 0.9% saline solution to the airway. The dose selected has previously been shown to acutely increase airway resistance in piglets [9]. Of the total 16 piglets that underwent instillation, 6 piglets were simply observed and euthanized. Histological specimens were examined from these piglets to evaluate overall tolerability of the bethanechol paradigm.

FlexiVent

Forty-eight hours post instillation, animals (n =10) were anesthetized with ketamine (20mg/kg) and xylazine (2.0mg/kg) and intravenous propofol (2mg/kg) (Henry Schein Animal Health). A tracheostomy was performed [10] and a cuffless endotracheal tube (Covidien, 4.0 mm OD) was placed. Piglets were connected to a flexiVent system (SCIREQ), and paralytic (rocuronium bromide, Novaplus) was administered. Piglets were ventilated at

60 breaths/min at a volume of 10ml/kg body mass. Increasing doses of methacholine were administered intravenously. Measurements for each dose were taken at 10 sec intervals over the course of approximately 5 mins. Airway resistance was measured using a single compartment model.

RNA isolation and qRT-PCR

RNA from the trachea was isolated using RNeasy Lipid Tissue kit (Qiagen), with DNase digestion using RNase-free DNase (Qiagen). RNA concentrations were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific). RNA (1000 ng) was reverse transcribed using Superscript VILO Master Mix (ThermoFisher). Briefly, RNA and master mix were incubated for 10 mins at 25°C, followed by 60 mins at 42°C, followed by 5 mins at 85°C. Inflammatory-directed quantitative real-time (qRT)-PCR arrays (Qiagen, PASS-011ZF) were completed according the manufacturer's instructions, using fast SYBR green master mix (Applied Biosystems). The qRT-PCR array has 5 housekeeping genes that can be used to for quantification with standard CT methods [8]. For inflammatory-directed qRT-PCR arrays, RNA was reversed transcribed for each animal. For analysis, equal amounts of individual tracheal cDNA was pooled for each treatment group (n = 5 control piglets, n = 5 bethanechol piglets). Three separate sets of pooled cDNA per group were generated and used for qRT-PCR arrays. Statistical analysis for tracheal tissues was performed on the technical replicates (n = 3). To confirm the array findings, we also performed qRT-PCR for *IL17A* (Ss03391803_m1), *IL8* (Ss03392437_m1), and *IL1a* (Ss03391335_m1) with β -actin (Ss0337653_uH) as a housekeeping gene on the individual tracheal cDNA (n = 5 bethanechol, n = 5 control) using TaqMan Universal PCR master mix II, no UNG (Applied Biosystems) and primer and probe sets from ThermoFisher. Standard CT methods were used for analysis [8]. For inflammatory-directed qRT-PCR arrays of the lung, RNA was reversed transcribed for each animal and assayed separately (n = 5 saline-treated piglets, n = 5 bethanechol-treated piglets). Statistical analysis was performed on the individual qRT-PCR arrays. To examine *muc5AC*, *muc5B* transcript abundance, we utilized previously published primer sequences [11]. *FoxJ1* primers were developed with the following sequences: *FoxJ1 forward* 5'-ATA TGG CCG AGA GCT GGC TA-3'; *FoxJ1 reverse* 5'-CCT TGG CGT TGA GAA TGG AG-3'. Actin was used as a housekeeping gene utilizing previously described primers sequences [8]. Data were acquired using fast SYBR green master mix (Applied Biosystems). Standard CT methods were used for analysis [8]. Melting curves were implemented for all SYBR green primer sets. All PCR was performed on a Lightcycler 480 (Roche).

Chemicals

Bethanechol chloride (Selleckchem) was dissolved in 0.9% saline. Acetyl-beta-methacholine-chloride (Sigma) was dissolved in 0.9% saline for flexiVent studies.

Bronchoalveolar lavage, ELISAs, and cell count analysis

The caudal left lung of each piglet was excised and the main bronchus cannulated; three sequential 5-ml lavages of 0.9% sterile saline were administered. The recovered material was pooled, spun at 500 × g, and supernatant removed. Cells were counted on a hemocytometer. ELISAs for porcine IL17A (ThermoFisher, ESIL17A), IL8 (R&D Systems,

P8000), CXL10 (Ray Biotech, ELP-IP10-1) and IFN γ (ThermoFisher, KSC4021) were performed according to the manufacturer's instructions, and quantified with an accuSkan FC (Fisher Scientific). The lower limit of sensitivity was 14pg/ml, 6.7 pg/ml, 0.4ng/ml, 2pg/ml for IL17A, IL8, CXCL10, and IFN γ , respectively.

Histology

Trachea and lung tissues were fixed in 10% neutral buffered formalin (~7–10 days) then routinely processed, paraffin-embedded, sectioned (~4 μ m) and stained with H&E and dPAS stains. Tissues were examined by a pathologist (DKM) masked to conditions. Digital images were collected with specialized equipment (BX51 microscope and DP73 digital camera, Olympus) and software (CellSens Pathology Edition, Olympus). Indices of inflammation were assigned as previously described [12]. Histological examination included 3 animals per treatment group that did not undergo flexiVent procedures, in addition to those that did.

Statistical Analysis

A two-way ANOVA was performed to assess airway resistance in response to intravenous methacholine and to assess inflammation via quantitative real-time PCR arrays. For airway resistance, a Sidak's multiple comparison test was applied. For the PCR arrays, a false discovery rate using the two-stage step-up method of Benjamin, Krieger, and Yekutieli was applied. An unpaired Student's t-test was used to assess basal airway resistance, cytokine concentrations, and TaqMan primer and probe qPCR. For histopathological scoring, a non-parametric Mann Whitney test was performed between groups. All tests were carried out using GraphPad Prism 7.0a. Statistical significance was determined as $p < 0.05$.

Results and discussion

We first examined airway mechanics. Basal airway resistance measurements were not different between treatment groups (Figure 1A). Piglets that were exposed to bethanechol 48 hours prior to testing exhibited greater airway resistance in response to intravenous methacholine compared to saline controls (Figure 1B).

Previous studies suggest that airway insults augment cholinergic transmission secondary to inflammation [3,4]. Because there was no inciting airway insult, we anticipated airway inflammation to be absent in the bethanechol-treated piglets. However, inflammatory-directed qRT-PCR arrays revealed elevated transcripts for several pro-inflammatory markers in the tracheas of bethanechol-treated piglets compared to saline controls (Table 1). When correcting for false discovery rate (FDR), elevations in three cytokines (IL17A, q value < 0.0001 ; IL1A, q value = 0.031; IL8, q value < 0.0001) were statistically significant (Table 1). Primer and probe sets with a β -actin as a reference gene confirmed elevations in IL17A, IL8, and IL1A (Table 1, gray shaded rows). Because the lungs of asthmatics are often affected by inflammation [13], we also examined lung tissues with inflammatory-directed quantitative real-time PCR arrays. Doing so revealed that prior bethanechol treatment increased mRNA expression of IFN γ (q value < 0.0001) and its downstream target CXCL10 (q value = 0.0047) (Table 2).

We further examined inflammation by analyzing bronchoalveolar lavage fluid of bethanechol-treated piglets and saline-treated piglets. We found no differences in the total number of cells (Figure 1C). Granulocytes were similarly lacking in both groups (data not shown). We also examined protein concentrations of IL17A and IL8, the two cytokines that showed the greatest induction at the transcript level in the trachea (Table 1), and IFN γ and CXCL10. Concentrations of IL17A were below the limit of reliable detection (Figure 1D). IL8 levels were within the limit of sensitivity, but no differences were detected between treatment groups (Figure 1E). Concentrations of CXCL10 were below detection (data not shown); IFN γ was detected, but no differences were observed (Figure 1F). Histological examination also revealed no differences in inflammation (Figure 1G,H) or sloughed epithelium [14] (Figure 1I,J) between treatment groups in either the trachea or the lung. Edema and macrophages were also examined in the lung; however, no differences were detected between treatment groups (Figure 1K,L). Thus, bethanechol induced changes in inflammatory pathways that were only detectable at the transcriptional level.

Because mucus obstruction and goblet cell hyperplasia are key features of asthma [15], we also quantified the transcript abundance of two major secreted mucin glycoproteins, *muc5AC* and *muc5B* [16]. Prior bethanechol treatment had no effect on *muc5AC* or *muc5B* mRNA expression in either the trachea (Figure 2A, B) or lung (Figure 2C,D). Transcript abundance of *foxJ1*, a marker of goblet cell progenitors [17], also revealed no differences in the trachea (Figure 2E) or lung (Figure 2F) of bethanechol-treated piglets compared to saline-treated piglets. Consistent with this, we observed no differences in histological indices of airway obstruction, mucus, or goblet cell number (Figure 2G–M).

Previous data suggest a causal role for cholinergic signaling in the manifestation of insult-induced airway hyperreactivity [3,5]. In the current study, we have expanded upon those findings by demonstrating that cholinergic stimulation in the absence of an inciting insult is sufficient to evoke airway hyperreactivity. Moreover, while previous studies in mice suggest that repeated cholinergic stimulation exaggerates airway narrowing in lung slices *ex vivo* [18], our studies in the neonatal piglet suggest that repeated stimulation is not necessary to evoke airway pathophysiology.

We discovered that bethanechol-mediated airway hyperreactivity was associated with an increase in inflammatory transcripts (IL17A, IL1A, IL8, CXCL10, IFN γ) that are of known or proposed significance in asthma pathogenesis [19–22]. Although we did not anticipate this finding, previous studies have suggested a pro-inflammatory role for cholinergic signaling in the airway. Indeed, muscarinic receptor activation has been shown to increase the release of IL6 and IL8 from airway smooth muscle cells [23]. Activation of muscarinic receptors on CD4⁺ CD62L⁺ T cells has also been shown to facilitate differentiation into Th17 cells [24], which express IL17A [25]. Thus, while previous studies suggest that augmented cholinergic transmission secondary to inflammation underlies airway hyperreactivity [3–5], our data suggest that cholinergic transmission *per se* is capable of inducing transcription of asthma-associated pro-inflammatory mediators. Therefore, it is possible that cholinergic-mediated modulation of the inflammatory network is an underappreciated factor in insult-induced airway hyperreactivity.

We found elevations in transcripts for IFN γ in the lung following acute cholinergic potentiation. Of interest, Jacoby and colleagues demonstrated that IFN γ increases the release of acetylcholine in cultured parasympathetic neurons [26]. It was proposed that a mechanism by which viral infections precipitate airway hyperreactivity is through IFN γ -mediated augmentation of acetylcholine release. If true, then our data might suggest a possible feedforward circuit, in which cholinergic potentiation can increase IFN γ to further augment release of acetylcholine.

Although bethanechol treatment increased transcripts for pro-inflammatory mediators, overt inflammation in the form of infiltrating immune cells or increased concentrations of cytokines in the bronchoalveolar lavage fluid was not detected. Histological sections of both the trachea and the lung also revealed no evidence of inflammation. Perhaps repeated or prolonged cholinergic stimulation [23] might be required to elicit such changes. Alternatively, a single cholinergic stimulation in a primed or compromised airway might reveal detectable or enhanced inflammation.

It has been reported that repeated challenge with the cholinergic agonist methacholine is sufficient to induce airway remodeling in mild asthmatics [27]. For example, Grainge and colleagues found that epithelial TGF- β and Ki67, submucosal thickness, and goblet cell percentage were increased following a three-day challenge with methacholine. When methacholine was co-delivered with a bronchodilator, the airway remodeling was prevented, suggesting that bronchoconstriction, and not the methacholine itself, was responsible for the airway remodeling. In our studies, we delivered a single dose of bethanechol that elicited bronchoconstriction [9]. Although we did not observe airway remodeling in our piglets, our findings are consistent with previous work suggesting that bronchoconstriction contributes to asthma pathology [27].

In summary, our data suggest that a single cholinergic event is sufficient to evoke airway hyperreactivity. They also suggest that cholinergic signaling can induce transcription of several pro-inflammatory cytokines that are associated with asthma and airway hyperreactivity. Given these findings, it is possible that mitigating cholinergic transmission might be of even greater clinical significance than previously appreciated.

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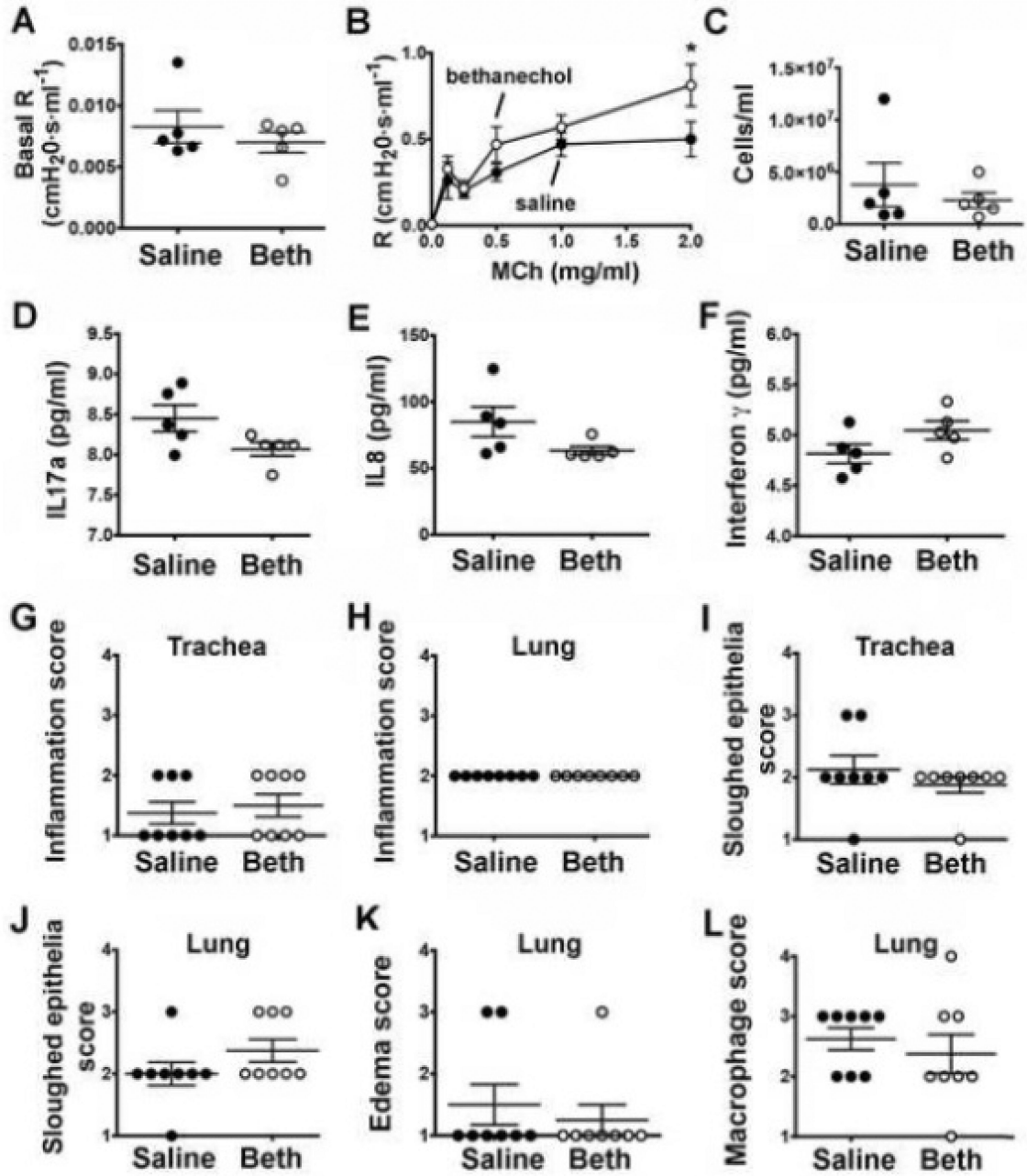


Figure 1. Airway resistance and inflammation markers in neonatal piglets forty-eight hours post bethanechol challenge. (A) Baseline airway resistance prior to administering methacholine. (B) Airway resistance in response to increasing doses of methacholine; n = 5 piglets each group. (C) Number of cells/ml observed in the bronchoalveolar lavage fluid of saline-treated and bethanechol-treated piglets. Bronchoalveolar lavage fluid concentrations of IL17A (D) IL8 (E) and IFN γ mediated. Histological scoring by a pathologist masked to treatment for inflammation (G, H), sloughed epithelium (I, J), edema (K) and macrophages (L). Scoring parameters for inflammation, edema and sloughed epithelium are as follows: 1= within

normal limits; 2 = mild, uncommon, focal; 3 = moderate, multifocal; 4 = extensive, coalescing changes. Scoring parameters for macrophages are as follows: 1= within normal limits; 2 = accumulation within airway (<33%); 3 = accumulation within airway (34–66%); 4 = accumulation within airway (>67%). For panels A-F, n = 5 piglets per group; for panels G-L, n = 8 piglets per group. Data are mean \pm SEM. For *, $p < 0.05$ for treatment effect using a two-way ANOVA. An unpaired Student's t-test was used to assess basal airway resistance and bronchoalveolar lavage fluid cells cytokine concentrations. A non-parametric Mann Whitney test was performed to examine histological scoring. All tests were carried out using Prism 7.0a. Abbreviations: R, resistance; MCh, methacholine; Beth, bethanechol; IL17a, interleukin 17a; IL8, interleukin 8; CXCL10, C-X-C motif chemokine 10; IFN γ , interferon gamma.

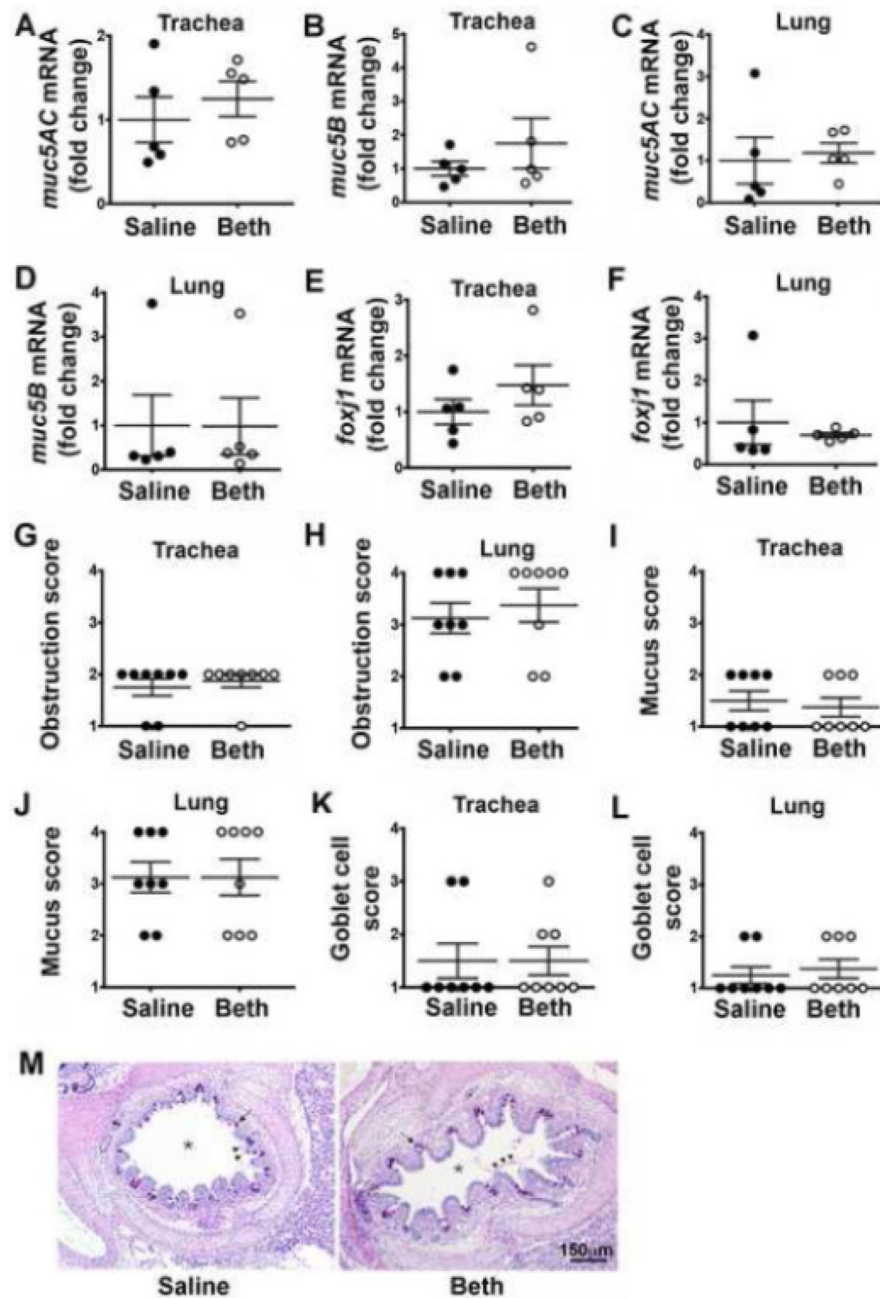


Figure 2. Mucin expression and mucus obstruction in neonatal piglet airways forty-eight hours post bethanechol challenge. Fold change mRNA expression of *muc5AC* (A, C), *muc5B* (B, D), and *foxj1* (E, F). Histological scoring for airway obstruction (G, H), mucus (I, J), and goblet cells (K, L). Scoring parameters for goblet cells are as follows: 1= within normal limits; 2 = mild, uncommon, focal; 3 = moderate, multifocal; 4 = extensive, coalescing changes. Scoring parameters for obstruction and mucus are as follows: 1= within normal limits; 2 = accumulation within airway (<33%); 3 = accumulation within airway (34–66%); 4 = accumulation within airway (>67%). (M) Lung cross-section stained with dPAS; asterisk (*)

indicates airway; arrow indicates goblet cells; arrowheads point to mucus. For panels A–F, n = 5 piglets per group; for panels G–L, n = 8 piglets per group. Data are mean \pm SEM. For *, p < 0.05 for treatment effect using a two-way ANOVA. An unpaired Student's t-test was used to assess mRNA fold changes. A non-parametric Mann Whitney test was performed to examine histological scoring. All tests were carried out using Prism 7.0a. Abbreviations: Beth, bethanechol; muc5AC, mucin5AC; muc5B, mucin5B; foxj1, forkhead box J1.

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Table 1
List of transcripts queried through inflammatory-directed PCR arrays in piglet tracheal tissues

Gene Name	saline			Bethanechol			Mean Diff.	q value	P Value
	Mean	SEM	n	Mean	SEM	n			
AIMP1	1	0.090	3	1.178	0.031	3	-0.1789	0.9966	0.8458
BMP2	1	0.079	3	1.139	0.165	3	-0.1393	0.9966	0.8797
C5	1	0.123	3	1.382	0.061	3	-0.3823	0.9966	0.6778
CCL1	1	0.088	3	0.882	0.277	3	0.1179	0.9966	0.898
CCL17	1	0.238	3	0.847	0.278	3	0.1529	0.9966	0.868
CCL2	1	0.060	3	1.429	0.114	3	-0.4296	0.9966	0.6406
CCL20	1	0.261	3	1.540	0.272	3	-0.5407	0.9966	0.5568
CCL21	1	0.136	3	0.886	0.116	3	0.1135	0.9966	0.9018
CCL22	1	0.263	3	0.506	0.091	3	0.4938	0.9966	0.5915
CCL3L1	1	0.135	3	3.141	0.351	3	-2.142	0.3242	0.0204
CCL4	1	0.081	3	2.330	0.101	3	-1.331	0.9966	0.1487
CCL5	1	0.086	3	1.336	0.075	3	-0.337	0.9966	0.7142
CCL8	1	0.201	3	2.043	0.498	3	-1.043	0.9966	0.2573
CCR1	1	0.119	3	1.180	0.211	3	-0.1807	0.9966	0.8443
CCR10	1	0.177	3	1.053	0.116	3	-0.05312	0.9966	0.954
CCR2	1	0.325	3	1.306	0.294	3	-0.3062	0.9966	0.7393
CCR3	1	0.398	3	0.936	0.397	3	0.06304	0.9966	0.9454
CCR4	1	0.222	3	2.091	0.192	3	-1.092	0.9966	0.2359
CCR5	1	0.226	3	1.291	0.263	3	-0.2918	0.9966	0.7512
CCR7	1	0.049	3	1.083	0.163	3	-0.0831	0.9966	0.928
CD40LG	1	0.182	3	1.491	0.169	3	-0.4917	0.9966	0.5931
CD70	1	0.173	3	1.585	0.167	3	-0.5859	0.9966	0.5244
CSF1	1	0.076	3	1.159	0.147	3	-0.1596	0.9966	0.8623
CSF2	1	0.118	3	2.626	0.278	3	-1.626	0.7294	0.0778
CSF3	1	0.067	3	0.953	0.370	3	0.04611	0.9966	0.96
CXCL10	1	0.003	3	1.096	0.253	3	-0.09634	0.9966	0.9166

Gene Name	saline			Bethanechol			Mean Diff.	q value	P Value
	Mean	SEM	n	Mean	SEM	n			
CXCL11	1	0.110	3	0.964	0.272	3	0.03556	0.9966	0.9692
CXCL12	1	0.023	3	0.902	0.054	3	0.09731	0.9966	0.9158
CXCL2	1	0.074	3	1.641	0.184	3	-0.6414	0.9966	0.4858
CXCL9	1	0.202	3	1.274	0.219	3	-0.2749	0.9966	0.7651
CXCR2	1	0.153	3	2.591	0.645	3	-1.591	0.7294	0.0844
CXCR4	1	0.088	3	1.302	0.100	3	-0.3025	0.9966	0.7423
FASLG	1	0.073	3	1.789	0.081	3	-0.7899	0.9966	0.3908
FLT3LG	1	0.086	3	0.930	0.071	3	0.06991	0.9966	0.9394
IFNG	1	0.405	3	1.340	0.482	3	-0.341	0.9966	0.7109
IL10	1	0.112	3	1.821	0.125	3	-0.8215	0.9966	0.3722
IL10RA	1	0.057	3	1.206	0.150	3	-0.2066	0.9966	0.8224
IL10RB	1	0.134	3	1.213	0.326	3	-0.2132	0.9966	0.8167
IL12B	1	0.158	3	1.132	0.336	3	-0.11329	0.9966	0.8852
IL13	1	0.327	3	0.557	0.153	3	0.4428	0.9966	0.6304
IL15	1	0.149	3	0.977	0.313	3	0.02237	0.9966	0.9806
IL16	1	0.106	3	0.947	0.089	3	0.05269	0.9966	0.9543
IL17A	1	0.204	3	8.988	0.726	3	-7.988	<0.0001	<0.0001
IL17F	1	0.078	3	3.264	0.189	3	-2.264	0.3038	0.0143
IL18	1	0.144	3	1.358	0.225	3	-0.3588	0.9966	0.6966
IL18RA	1	0.313	3	1.834	0.868	3	-0.8341	0.9966	0.3649
IL1A	1	0.238	3	4.029	1.723	3	-3.03	0.0308	0.0011
IL1B	1	0.117	3	3.101	0.222	3	-2.102	0.3242	0.0229
IL1RN	1	0.051	3	1.825	0.402	3	-0.8259	0.9966	0.3696
IL21	1	0.434	3	1.299	0.533	3	-0.3	0.9966	0.7444
IL23A	1	0.166	3	0.968	0.161	3	0.03178	0.9966	0.9724
IL27	1	0.203	3	0.926	0.211	3	0.07378	0.9966	0.9361
IL2RG	1	0.099	3	1.412	0.322	3	-0.4126	0.9966	0.6538
IL4	1	0.090	3	0.881	0.091	3	0.1182	0.9966	0.8977

Gene Name	saline			Bethanechol			Mean Diff.	q value	P Value
	Mean	SEM	n	Mean	SEM	n			
IL4R	1	0.053	3	1.034	0.078	3	-0.03445	0.9966	0.9701
IL5	1	0.098	3	1.025	0.067	3	-0.02593	0.9966	0.9775
IL5RA	1	0.331	3	0.852	0.327	3	0.1474	0.9966	0.8727
IL6	1	0.121	3	2.672	0.188	3	-1.672	0.7294	0.0698
IL6R	1	0.147	3	1.218	0.120	3	-0.2183	0.9966	0.8125
IL6ST	1	0.159	3	1.141	0.213	3	-0.1418	0.9966	0.8775
IL7	1	0.063	3	1.191	0.082	3	-0.1912	0.9966	0.8354
IL7R	1	0.073	3	1.448	0.107	3	-0.4489	0.9966	0.6256
IL8	1	0.464	3	18.028	13.101	2	-17.03	<0.0001	<0.0001
IL9	1	0.386	3	1.336	0.656	3	-0.3367	0.9966	0.7144
LIF	1	0.054	3	1.115	0.126	3	-0.1154	0.9966	0.9002
OSM	1	0.145	3	1.277	0.134	3	-0.2772	0.9966	0.7632
IL17B	1	0.057	3	1.192	0.526	3	-0.1923	0.9966	0.8345
IL33	1	0.093	3	0.743	0.029	3	0.256	0.9966	0.7808
LOC100519468	1	0.225	3	0.777	0.212	3	0.2225	0.9966	0.8089
IL9R	1	0.046	3	0.917	0.155	3	0.08256	0.9966	0.9285
LOC100621682	1	0.121	3	0.687	0.086	3	0.3126	0.9966	0.734
IL2RB	1	0.673	3	2.514	0.931	3	-1.515	0.7761	0.1004
LTA	1	0.178	3	0.981	0.234	3	0.01811	0.9966	0.9843
LTB	1	0.138	3	0.845	0.229	3	0.1544	0.9966	0.8667
MIF	1	0.165	3	1.126	0.122	3	-0.1268	0.9966	0.8904
NAMPT	1	0.277	3	1.526	0.188	3	-0.5269	0.9966	0.5669
SPP1	1	0.112	3	1.597	0.240	3	-0.5979	0.9966	0.5159
TGFB2	1	0.164	3	1.371	0.044	3	-0.3711	0.9966	0.6867
TNF	1	0.095	3	2.584	0.460	3	-1.584	0.7294	0.0858
TNFRSF11B	1	0.251	3	1.257	0.271	3	-0.2576	0.9966	0.7795
TNFRSF13B	1	0.241	3	1.192	0.327	3	-0.1922	0.9966	0.8345
TNFSF4	1	0.140	3	1.355	0.136	3	-0.3557	0.9966	0.6991

Gene Name	saline			Bethanechol			Mean Diff.	q value	P Value
	Mean	SEM	n	Mean	SEM	n			
VEGFA	1	0.082	3	1.076	0.083	3	-0.07657	0.9966	0.9337
TNFRSF10	1	0.107	3	1.094	0.022	3	-0.09462	0.9966	0.9181
IL17A	1	0.155	5	41.91	19.85	5	-40.91	N/A	0.0733
IL1A	1	0.158	5	2.094	0.401	5	-1.094	N/A	0.0349
IL8	1	0.152	5	2.701	0.529	5	-1.701	N/A	0.0150

* data in shaded rows were obtained using TaqMan primer and probes with β -actin as reference gene. In instances where the n is less than 3, the transcript was either not detected due to a technical error (i.e. the well was not loaded with cDNA), or because the transcript was not expressed at levels that were distinguishable from background.

List of transcripts queried through inflammatory-directed PCR arrays in piglet lung tissues

Table 2

Gene Name	saline			Bethanechol			Mean Diff.	q value	P Value
	Mean	SEM	n	Mean	SEM	n			
AIMP1	1	0.040	4	1.234	0.176	5	-0.234	0.7991	0.5042
BMP2	1	0.072	5	0.771	0.053	5	0.2288	0.7991	0.4885
C5	1	0.170	5	1.115	0.075	5	-0.1153	0.8612	0.7270
CCL1	1	0.154	5	1.444	0.447	5	-0.4441	0.7991	0.1789
CCL17	1	0.213	5	0.618	0.074	5	0.382	0.7991	0.2476
CCL2	1	0.191	5	0.690	0.086	5	0.3098	0.7991	0.3483
CCL20	1	0.161	5	0.841	0.108	5	0.1588	0.7991	0.6306
CCL21	1	0.208	5	0.652	0.089	5	0.3472	0.7991	0.2933
CCL22	1	0.180	5	0.750	0.104	5	0.25	0.7991	0.4492
CCL3L1	1	0.267	5	1.242	0.151	5	-0.2423	0.7991	0.4632
CCL4	1	0.137	5	1.670	0.110	5	-0.67	0.6139	0.0428
CCL5	1	0.088	5	1.003	0.125	5	-0.003831	>0.9999	0.9907
CCL8	1	0.297	4	1.489	0.245	5	-0.4897	0.7991	0.1624
CCR1	1	0.194	5	1.032	0.040	5	-0.03234	0.9923	0.9220
CCR10	1	0.173	5	0.638	0.110	5	0.3612	0.7991	0.2743
CCR2	1	0.258	5	0.717	0.085	5	0.2823	0.7991	0.3927
CCR3	1	0.124	5	0.835	0.126	5	0.1648	0.7991	0.6178
CCR4	1	0.207	5	0.676	0.111	5	0.3231	0.7991	0.3280
CCR5	1	0.303	5	0.953	0.083	5	0.04667	0.9674	0.8876
CCR7	1	0.164	5	0.692	0.107	5	0.3079	0.7991	0.3513
CD40LG	1	0.095	5	0.561	0.061	5	0.4384	0.7991	0.1846
CD70	1	0.278	5	1.212	0.122	5	-0.2121	0.7991	0.5207
CSF1	1	0.099	5	0.793	0.058	5	0.2063	0.7991	0.5322
CSF2	1	0.353	5	1.118	0.242	5	-0.1184	0.8612	0.7200
CSF3	1	0.169	5	0.597	0.075	5	0.403	0.7991	0.2226
CXCL10	1	0.338	5	2.400	0.464	5	-1.401	0.0011	<0.0001

Gene Name	saline			Bethanechol			Mean Diff.	q value	P Value
	Mean	SEM	n	Mean	SEM	n			
CXCL11	1	0.290	5	1.415	0.216	5	-0.4154	0.7991	0.2087
CXCL12	1	0.120	5	0.942	0.109	5	0.05758	0.9511	0.8616
CXCL2	1	0.181	5	0.903	0.071	5	0.09672	0.8645	0.7696
CXCL9	1	0.398	5	1.851	0.452	5	-0.8515	0.2902	0.0101
CXCR2	1	0.087	5	1.191	0.212	5	-0.192	0.7991	0.5611
CXCR4	1	0.090	5	1.178	0.200	5	-0.1787	0.7991	0.5884
FASLG	1	0.089	5	1.242	0.240	5	-0.2425	0.7991	0.4628
FLT3LG	1	0.044	5	0.754	0.054	5	0.2458	0.7991	0.4568
IFNG	1	0.239	5	3.509	1.534	5	-2.509	<0.0001	<0.0001
IL10	1	0.218	5	1.484	0.160	5	-0.4848	0.7991	0.1424
IL10RA	1	0.144	5	0.895	0.072	5	0.1046	0.8645	0.7514
IL10RB	1	0.157	5	0.696	0.071	5	0.3039	0.7991	0.3575
IL12B	1	0.243	5	0.879	0.187	4	0.1208	0.8612	0.7301
IL13	1	0.122	5	0.698	0.195	4	0.3011	0.7991	0.3901
IL15	1	0.292	5	0.439	0.131	5	0.5602	0.7991	0.0902
IL16	1	0.128	5	0.807	0.105	5	0.1925	0.7991	0.5599
IL17A	1	0.342	5	0.558	0.201	5	0.4418	0.7991	0.1812
IL17F	1	0.184	5	0.899	0.240	5	0.1001	0.8645	0.7618
IL18	1	0.203	5	1.199	0.348	5	-0.1994	0.7991	0.5460
IL18RA	1	0.432	5	0.547	0.130	5	0.4526	0.7991	0.1708
IL1A	1	0.252	5	0.530	0.095	5	0.4697	0.7991	0.1553
IL1B	1	0.100	5	0.753	0.102	5	0.2468	0.7991	0.4549
IL1RN	1	0.054	5	0.829	0.072	5	0.1704	0.7991	0.6060
IL21	1	0.353	5	0.774	0.110	5	0.2254	0.7991	0.4949
IL23A	1	0.261	5	0.810	0.197	5	0.19	0.7991	0.5652
IL27	1	0.198	5	0.803	0.079	5	0.1967	0.7991	0.5514
IL2RG	1	0.190	5	0.904	0.140	5	0.09518	0.8645	0.7732
IL4	1	0.220	5	0.579	0.096	5	0.4208	0.7991	0.2029

Gene Name	saline			Bethanechol			Mean Diff.	q value	P Value
	Mean	SEM	n	Mean	SEM	n			
IL4R	1	0.123	5	0.814	0.090	5	0.1852	0.7991	0.5751
IL5	1	0.238	5	0.714	0.130	5	0.2852	0.7991	0.3880
IL5RA	1	0.422	5	0.481	0.048	5	0.5188	0.7991	0.1165
IL6	1	0.269	5	0.713	0.198	5	0.2869	0.7991	0.3851
IL6R	1	0.121	5	0.822	0.132	5	0.1779	0.7991	0.5900
IL6ST	1	0.311	5	0.720	0.052	5	0.2797	0.7991	0.3972
IL7	1	0.226	5	0.977	0.153	5	0.02259	0.9977	0.9455
IL7R	1	0.222	5	0.716	0.145	5	0.283	0.7991	0.3915
IL8	1	0.399	3	0.305	0.188	3	0.6947	0.7991	0.1035
IL9	1	0.488	5	0.247	0.041	5	0.7522	0.4951	0.0230
LIF	1	0.218	5	0.656	0.058	5	0.3433	0.7991	0.2988
OSM	1	0.128	5	0.801	0.117	5	0.1985	0.7991	0.5478
IL17B	1	0.496	5	0.380	0.046	5	0.6193	0.7514	0.0611
IL33	1	0.128	5	1.327	0.360	5	-0.3271	0.7991	0.3221
LOC100519468	1	0.187	5	0.626	0.072	5	0.3738	0.7991	0.2579
IL9R	1	0.117	5	0.773	0.131	5	0.2267	0.7991	0.4924
LOC100621682	1	0.160	5	0.577	0.092	5	0.423	0.7991	0.2005
IL2RB	1	0.533	5	0.317	0.061	5	0.6826	0.6139	0.0390
LTA	1	0.139	5	0.654	0.064	5	0.346	0.7991	0.2950
LTB	1	0.238	5	0.841	0.115	5	0.1586	0.7991	0.6311
MIF	1	0.145	5	0.828	0.076	5	0.1719	0.7991	0.6028
NAM1PT	1	0.284	5	0.630	0.086	5	0.3695	0.7991	0.2634
SPP1	1	0.082	5	1.276	0.304	5	-0.2763	0.7991	0.4028
TGFB2	1	0.109	5	0.822	0.084	5	0.1775	0.7991	0.5910
TNF	1	0.223	5	1.116	0.142	5	-0.1167	0.8612	0.7239
TNFRSF11B	1	0.116	5	0.882	0.129	5	0.1172	0.8612	0.7226
TNFRSF13B	1	0.427	5	0.827	0.149	5	0.173	0.7991	0.6005
TNFSF4	1	0.078	5	0.984	0.105	5	0.01504	0.9997	0.9637

Gene Name	saline		Bethanechol			Mean Diff.	q value	P Value
	Mean	SEM	n	Mean	SEM			
VEGFA	1	0.122	5	0.979	0.090	5	0.02062	0.9502
TNFRSF10	1	0.214	5	0.724	0.067	5	0.2758	0.7991

* In instances where the n is less than 5, the transcript was either not detected due to a technical error (i.e. the well was not loaded with cDNA), or because the transcript was not expressed at levels that were distinguishable from background.