1 **Title:** Acid exposure disrupts mucus secretion and impairs mucociliary transport in neonatal
2 piglet airways
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4 **One sentence summary**: Early life airway acidification produces mucus defects
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ABSTRACT

Tenacious mucus produced by tracheal and bronchial submucosal glands is a defining feature of several airway diseases, including cystic fibrosis (CF). Airway acidification as a driving force of CF airway pathology has been controversial. Here we tested the hypothesis that transient airway acidification produces pathologic mucus and impairs mucociliary transport. We studied pigs challenged with intra-airway acid. Acid had a minimal effect on mucus properties under basal conditions. However, cholinergic stimulation in acid-challenged pigs revealed retention of mucin 5B (MUC5B) in the submucosal glands, decreased concentrations of MUC5B in the lung lavage fluid, and airway obstruction. To more closely mimic a CF-like environment, we also examined mucus secretion and transport following cholinergic stimulation under diminished bicarbonate and chloride transport conditions *ex vivo*. Under these conditions, airways from acid-challenged pigs displayed extensive mucus films and decreased mucociliary transport. Pre-treatment with diminazene aceturate, a small molecule with ability to inhibit acid detection through blockade of the acid-sensing ion channel (ASIC) at the doses provided, did not prevent acid-induced pathologic mucus or transport defects but did mitigate airway obstruction. These findings suggest that transient airway acidification early in life has significant impacts on mucus secretion and transport properties. Further, they highlight diminazene aceturate as an agent that might be beneficial in alleviating airway obstruction.

2

INTRODUCTION

Cystic Fibrosis (CF) is a life-shortening autosomal recessive disorder caused by mutations in the gene encoding the *cystic fibrosis transmembrane conductance regulator (CFTR)*. CF airways are distinguished by frequent infection, inflammation and adherent mucus. Over time, these factors lead to progressive airway destruction and respiratory demise (6, 16, 41, 51). Several lines of evidence suggest that airway mucus is abnormal in early CF disease (22, 61). For example, pathologic findings in neonates with CF described bronchiole obstruction due to "thick" mucus produced by the tracheal and bronchial submucosal glands (61). Hyperconcentration of mucins due to excessive secretion of mucin5B (MUC5B) and mucin5AC (MUC5AC) cells has also been reported (2). In porcine models of CF, a failure of mucus detachment from airway submucosal glands was observed (22). Likewise, rats with CF display submucosal gland duct plugging prior to onset of airway infection (5), whereas ferrets with CF show excessive mucus accumulation even in a sterile airway environment (50).

The cause of abnormal mucus in CF has received significant attention (43). Numerous mechanisms have been proposed, including dehydration of the airway (7, 19, 21, 32) and an acidification of the airway caused by lack of CFTR-mediated bicarbonate transport (38, 40, 58). Recently, Boucher and colleagues demonstrated that the proinflammatory cytokine IL-1β predominated the CF airway and correlated with increased expression of MUC5B (15). We recently reported that acute airway acidification in neonatal piglets increased lung lavage concentrations of IL-1β and caused airway obstruction (45), however we did not examine mucus transport or mucus secretion properties. Thus, here we tested the hypothesis that transient airway acidification produces mucus with pathologic features and mucociliary transport defects.

3

81 Secondarily, we tested the hypothesis that pharmacological inhibition of acid detection would

82 mitigate acid-induced mucus deficits.

83 **MATERIALS AND METHODS.**

84

85 **Animals**

86 A total of 130 piglets (Yorkshire-Landrace, 2-3 days of age) were obtained from a commercial

87 vendor and fed commercial milk replacer (Liqui-Lean) and allowed a 24-hour acclimation period

88 prior to interventions. Data were collected from 17 separate cohorts of piglets across

89 approximately 1.5 - 2 years. At the conclusion of the experiments, animals were sedated with

90 ketamine (20 mg/kg), and xylazine (2.0 mg/kg), and intravenous propofol (2 mg/kg) (Henry

91 Schein Animal Health), followed by euthanasia with intravenous Euthasol (90mg/kg) (Henry

92 Schein Animal Health). The University of Florida Animal Care and Use Committee approved all

93 procedures. Care was in accordance with federal policies and guidelines.

94

95 **Airway Instillation**

96 After acclimation, piglets were anesthetized with 8% sevothesia (Henry Schein). The piglets'

97 airways were accessed with a laryngoscope; a laryngotracheal atomizer (MADgic) was passed

98 directly beyond the vocal folds as previously described (45, 48) to aerosolize either a 500 µl

99 0.9% saline control or 1% acetic acid in 0.9% saline solution to the airway. This procedure

100 results in widespread distribution of aerosolized solutions throughout the piglet airway, including

101 the lung (11, 12).

102

103 **Chemicals and Drugs**

4

104   USP grade acetic acid (Fisher Scientific) was dissolved 0.9% saline to final concentration of 1%

105   and sterilized with a 0.22 µm filter (Millex GP). The pH of the 1% acetic acid solution measured

106   2.6 using an Accumet AE150 pH probe (Fisher Scientific). The estimated pH once applied to the

107   airway surface is ~6.6 to 6.8 (45). Acetyl-beta-methacholine-chloride (Sigma) was dissolved in

108   0.9% saline for intravenous delivery and *ex vivo* application; details about concentrations are

109   provided in respective experimental sections below. Diminazene aceturate (Selleckchem) was

110   dissolved in 0.9% saline containing 5% DMSO (Fisher Scientific).

111

112   **Diminazene aceturate delivery**

113   Approximately 30 minutes prior to airway instillations, piglets received an intramuscular

114   injection of either vehicle (5% DMSO + 95% 0.9% saline) or vehicle containing 17.5 mg/ml

115   (~33.7 millimolar) diminazene aceturate. Diminazene aceturate was dosed at 3.5 mg/kg. This

116   dose was chosen based upon previous studies showing efficacy in trypanosome infections in

117   dogs (35) and is below the reported dose required to affect angiotensin converting enzyme 2

118   (ACE2) (8, 42). Using a conversion chart for dogs (1), we estimated the body surface area of a 2-

119   3 kg piglet to be 0.16-0.21 m^2. Based upon this conversion, the estimated final drug molarity in

120   the pig body was ~9 µM, which should provide near 100% block of ASIC1a, as it is 3 fold

121   greater than the IC50 of 3 µM (10, 29). The time point of 30 minutes prior to instillation was

122   selected based upon previous studies showing peak serum concentrations at 15 minutes and peak

123   interstitial fluid concentrations at 3 hours in adult rabbits (17).

124

125   **Bronchoalveolar lavage and ELISA**

5

126 Following euthanasia, the caudal left lung of each piglet was excised and the main bronchus

127 cannulated; three sequential 5 ml lavages of 0.9% sterile saline were administered as previously

128 described (45, 48). The recovered material was pooled, spun at 500 X g, and supernatant stored

129 at -80°C. A porcine MUC5AC (LSBio, LS-F45847-1) and porcine MUC5B (LSBio, LS-F45852-

130 1) ELISA was performed according to the manufacturer's instructions in duplicate. The ELISA

131 was read using a filter-based accuSkan FC micro photometer (Fisher Scientific). The limits of

132 sensitivity were <0.188 ng/ml and < 0.375 ng/ml for MUC5AC and MUC5B, respectively. The

133 intra-assay and inter-assay coefficient of variability were respectively <6.1% and <5.2% for

134 MUC5AC and <6.5% and <5.9% for MUC5B.

135

136 **Histology**

137 Lung tissues were fixed in 10% neutral buffered formalin (~7-10 days), processed, paraffin-

138 embedded, sectioned (~4 µm) and stained with Periodic-acid-Schiff stain (PAS) to detect

139 glycoproteins as previously described (45, 48). Digital images were collected with a Zeiss Axio

140 Zoom V16 microscope. Indices of obstruction were assigned as previously described (45, 48).

141

142 **Immunofluorescence in tracheal cross sections**

143 1-2 tracheal rings were removed post-mortem and embedded in Peel-A-Way embedding molds

144 containing Tissue-Tek OCT (Electron Microscopy Sciences). Molds were placed in a container

145 filled with dry ice until frozen and stored at -80 °C long-term. Tissues were sectioned at a

146 thickness of 10 µm and mounted onto SuperFrost Plus microscope slides (ThermoFisher

147 Scientific). Storage until immunofluorescence also occurred at -80 °C. We used

148 immunofluorescence procedures similar to those previously described (45, 47). Briefly,

6

149   representative cross-sections from a single cohort of pigs were selected and fixed in 2%

150   paraformaldehyde for 15 minutes. Tissues were then permeabilized in 0.15% Triton X-100,

151   followed by blocking in PBS Superblock (ThermoFisher Scientific) containing 2-4% normal

152   goat serum (Jackson Laboratories). Tissues were incubated with primary antibodies for 2 hours

153   at 37 °C. Tissues were washed thoroughly in PBS and incubated in secondary antibodies for 1

154   hour at room temperature. Tissues were washed and a Hoechst stain performed as previously

155   described (45). A 1:10 glycerol/PBS solution was used to cover the sections and cover glass

156   added. Sections were imaged on a Zeiss Axio Zoom V16 microscope. Identical microscope

157   settings within a single cohort of piglet tracheas were used and applied. Three to five images

158   encompassing the posterior, anterior, and lateral surfaces of the trachea were taken. Images were

159   exported and analyzed using ImageJ. The trachea surface epithelia and entire submucosal gland

160   regions were traced and the mean signal intensity MUC5B and MUC5AC recorded. Background

161   signal intensity was measured and subtracted manually. The final signal intensities were

162   averaged to identify a mean signal intensity for MUC5B and MUC5AC per sub-compartment for

163   each piglet. To analyze the amount of the trachea lumen that was ciliated, three images of the

164   trachea were assessed and the length of the epithelia that was ciliated was divided by the total

165   length of the epithelia and percent reported. The percent of each image was averaged and

166   reported per each piglet.

167

168   **Antibodies and lectins**

169   We used the following anti-mucin antibodies: rabbit anti-MUC5B (1:500; Santa Cruz, Cat.#

170   20119) and mouse anti-MUC5AC (clone 45M1) (1:1,000, ThermoFisher Scientific, Cat #

171   MA512178), mouse anti-acetyl alpha tubulin, clone 6-11B-1, 1:500 (EMD Millipore,

7

172 MABT868) (28), followed by goat anti-rabbit and goat anti-mouse secondary antibodies

173 conjugated to Alexa-Fluor 488 or 568 (ThermoFisher Scientific, 1:1,000 dilution). We used

174 WGA-rhodamine (Vector Laboratories) and Jacalin-FITC (Vector Laboratories) at 1:1,000

175 dilution. Tracheas were submerged in PBS and visualized using a Zeiss Axio Zoom V16

176 microscope.

177

178 *In vivo* **methacholine and ventilation**

179 We have previously described the ventilation and *in vivo* methacholine procedures in piglets (45,

180 48). Briefly, 48 h post instillation, animals were anesthetized with ketamine (20 mg/kg), and

181 xylazine (2.0 mg/kg), and intravenous propofol (2 mg/kg) (Henry Schein Animal Health). A

182 tracheostomy was performed, and a cuffless endotracheal tube (Coviden, 3.5–4.0 mm OD) was

183 placed. Piglets were connected to a flexiVent system (SCIREQ); paralytic (rocuronium bromide,

184 Novaplus) was administered. Piglets were ventilated at 60 breaths/min at a volume of 10 ml/kg

185 body mass. Methacholine was administered intravenously in approximately ~3 min intervals

186 with increasing concentrations (in mg/kg): 0.25, 0.5, 1.0, 1.5, 2.0. Piglets were ventilated to

187 ensure animal well-being and patency of airway tissues during cholinergic challenge.

188
189 **Mucus secretion assay** *ex vivo*

190 We adopted methods described by Ostedgaard et al (36). Briefly, 3-4 rings of trachea were

191 removed post-mortem and the outside of the tracheas were wrapped in gauze soaked with 5 ml of

192 the following: 135 mM NaCl, 2.4 mM K_2HPO_4, 0.6 mM KH_2PO_4, 1.2 mM $CaCl_2$, 1.2 mM

193 $MgCl_2$, 10 mM dextrose, 5 mM HEPES, pH 7.4 (NaOH), 1.5 mg/ml of methacholine, and 100

194 μM bumetanide. We determined that the amount of methacholine the tissue was exposed to was

195 0.15 mgs total (e.g., external surface of the trachea is covered by approximately 100 μl). Thus,

8

196   the methacholine dose delivered to the trachea was 0.15 mg/4.71 cm^2 (tissue dimensions of

197   trachea: radius = 0.5 cm, height = 1.5 cm). Using a conversion chart for dogs (1), we estimated

198   the body surface area of a 2-3 kg piglet to be 0.16-0.21 m^2. Therefore, the estimated dose of

199   methacholine delivered to the tissue was ~6-7 fold higher than the cumulative *in vivo* dose of

200   methacholine to account for diffusion of methacholine across tissues layers and in the absence of

201   blood circulation. Tracheas were then placed in a temperature-controlled humidified incubator

202   for 3 hours. Following stimulation, tracheas were fixed overnight in 4% paraformaldehyde and

203   permeabilized for 30 minutes using a triton solution (0.15%), followed by blocking in

204   SuperBlock PBS. Jacalin-FITC and wheat-germ agglutin-rhodamine were used to visual mucus

205   (3, 36) and incubated overnight with tracheas at concentration of 1:1,000. Tracheas were then

206   washed, cut upon the posterior surface, and pinned to wax covered petri-dishes followed by

207   submersion in PBS. Tracheas were imaged with Zeiss Axio Zoom V16 posterior to anterior

208   using identical microscope settings. Images were assigned scoring indices for sheet formation

209   and strand formation by two observers blinded to conditions. The number of dilated submucosal

210   gland ducts was also measured by two observers blinded to conditions. Scoring for sheet

211   formation follows: 1 = no sheet formation; 2 = 1-25% of image field shows sheet formation; 3 =

212   26-50% of image field shows sheet formation; 4 = 51-75% of image field shows sheet formation;

213   5 = 76-100% of image field shows sheet formation. Sheet formation was defined as the loss of

214   discrete cellular packets of mucus. Secondary analysis and validation of scoring was performed

215   with IMARIS software (detailed below). Scoring for strand formation was similar to sheet

216   formation. Scoring for strand formation follows: 1 = no strand formation; 2 = 1-25% of image

217   field shows 3 = 26-50% of image field shows a strand; 4 = 51-75% of image field shows a

218   strand; 5 = 76-100% of image field shows a strand. Secondary analysis consisting of tracing the

9

219   strand area and expressing it a percentage of the total image field of view was also performed on

220   a subset of images in ImageJ.

221

222   *Ex vivo* **mucus transport assays**
223
224   Mucociliary transport was measured using methods similar to those described by Hoegger et al

225   (22). Briefly, 3 rings of tracheas were submerged in 5 ml of prewarmed solution containing the

226   following: 138 mM NaCl, 1.5 mM KH_2PO_4, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 2.67 mM KCl, 8.06

227   mM $Na_2HPO_4.7H_20$, 10 mM HEPES, pH 7.4 (NaOH), and 100 μM bumetanide. Tracheas were

228   placed onto a heated stage and kept at 37 °C. Images were acquired every 1 minute for 35

229   minutes. After 5 minutes of baseline measurements, methacholine was administered directly into

230   the solution covering the basolateral and apical sides of tracheas at a dose of 0.004 mg/ml. This

231   dose matched the estimated accumulative dose of methacholine administered to piglets *in vivo*

232   *(e.g., 3.75 mg/kg = 3.75 mg/L = 0.00375 mg/ml).* Mucus transport was assessed for an additional

233   30 minutes, with the exception of one set of tracheas, in which only an additional 25 minutes was

234   measured. In one additional sample, the microscope malfunctioned after 5 minutes of recording

235   post methacholine stimulation. IMARIS software was used to track mucus transport across time

236   measured. Details about IMARIS software and processing are highlighted below.

237

238   **IMARIS computer-assigned particles for measurement of mucus transport and mucus**

239   **sheet formation**

240   Computer particles based upon signal intensity above background were automatically generated

241   with IMARIS software (Bitplane). The particles were then tracked through time using a custom

242   IMARIS algorithm that utilized principles of the well-validated algorithms published by

10

243   Jaqaman and colleagues (24). For each trachea, the average mean speeds of the computer-

244   assigned particles were reported. The length of the particle track was also computed

245   automatically and the mean track length of all the particles per trachea were calculated and

246   reported. For determination of sheet formation, an image was chosen at random from a subset of

247   tracheas stained with lectins. Particles were assigned to the images automatically as described

248   above. The number of particles (representing the jacalin-labeled mucus with intensity above

249   background) were reported. In tracheas that exhibit robust mucus sheet formation, the number of

250   computer-assigned particles is decreased due to a loss of discreet cellular packets of jacalin-

251   labeled mucus.

252

253   **Statistical analysis**

254   Our previous work indicated that airway acidification elicited sex-independent airway

255   obstruction (45). Thus, in the current study, we hypothesized that airway acidification would

256   have sex-independent effects on airway mucus and mucociliary transport. To test this prediction,

257   we initially performed a two-way ANOVA (sex as one factor, treatment as the other factor), but

258   observed no significant interactions between sex and treatment, indicating that sex did not affect

259   the response to treatments. Thus, stratification based upon sex was not strongly justified (59),

260   and therefore we grouped the data to better represent the population. Two-way ANOVA analyses

261   stratified by sex are available in Table 1. For parametric data that compared four groups, we used

262   a two-way ANOVA test, with treatment as one factor and drug as the other factor, followed by a

263   Sidak multiple comparison test. Post-hoc multiple comparison tests were run in a classic 2x2

264   manner, comparing cell means for across rows and columns. Similar to our previous studies, for

265   non-parametric data that compared four groups, we used a one-way ANOVA (Kruskal-Wallis)

266   test followed by Dunn's multiple comparison test (45, 48). For all experiments, we reported the

11

267 following post-hoc comparisons, due to relevance of the questions being asked in our study: acid

268 vs saline; acid vs acid + diminazene aceturate, and saline vs saline + diminazene aceturate. For

269 analyses that compared two groups, we used a two-tailed unpaired Student's t-test. All tests were

270 carried out using GraphPad Prism 7.0a. Statistical significance was determined as $P < 0.05$.

271

272 **RESULTS**

273 **Airway mucin secretion in acid-challenged piglet is largely unaffected under basal**

274 **conditions**

275 We performed multi-level analyses of the major secretory gel-forming mucins, MUC5AC and

276 MUC5B (20, 27).We examined basal secretion by measuring the amount of MUC5AC and

277 MUC5B in the bronchoalveolar lavage fluid. Detectable levels of proteins were found in all

278 treatment groups. No statistically significant differences were noted in MUC5AC concentrations

279 (treatment, $F_{1, 27} = 2.63$, $p = 0.12$; drug, $F_{1, 27} = 1.86$, $p = 0.18$; interaction, $F_{1, 27} = 1.12$, $p = 0.29$,

280 Figure 1A). Post hoc comparisons indicated a trend for decreased concentrations of MUC5B in

281 acid-challenged piglets ($p = 0.068$) compared to saline-treated controls, whereas decreased

282 concentrations of MUC5B were observed in saline-treated animals that received diminazene

283 aceturate compared to saline-treated animals that did not receive diminazene aceturate

284 (treatment, $F_{1, 27} = 21.66$, $p < 0.0001$; drug, $F_{1, 27} = 37.9$, $p < 0.0001$; interaction, $F_{1, 27} = 67.11$, p

285 < 0.0001, Figure 1B).

286

287 Bronchoalveolar lavage fluid is limited in that it primarily captures non-adherent proteins and is

288 a mixed fluid retrieved from alveolar and bronchial spaces. Therefore, we measured MUC5AC

289 and MUC5B protein expression in tracheal cross sections using antibody-specific labeling and

12

290 signal intensity analyses (15). We observed no significant differences in MUC5AC signal

291 intensity within and on the tracheal surface across treatment groups (treatment, $F_{1, 54} = 1.98$, p =

292 0.17; drug, $F_{1, 54} = 0.84$, p = 0.36; interaction, $F_{1, 54} = 3.64$, p = 0.06, Figure 1C). Main effects of

293 treatment ($F_{1, 54} = 15.00$, p = 0.0003) and drug ($F_{1, 54} = 12.11$, p = 0.001) were observed for

294 MUC5B signal intensity on the tracheal surface, but no interactions were noted ($F_{1, 54} = 0.99$, p =

295 0.32, Figure 1D). Analysis of MUC5AC expression in the submucosal glands revealed a

296 significant main effect of treatment ($F_{1, 54} = 8.69$, p = 0.0047), but no effect of drug ($F_{1, 54} = 0.29$,

297 p = 0.59) or interaction ($F_{1, 54} = 1.23$, p = 0.27) were noted (Figure 1E). Similarly, analysis of

298 MUC5B expression in the submucosal glands revealed a significant main effect of treatment ($F_{1,}$

299 $_{54} = 5.84$; p = 0.019), but no effect of drug ($F_{1, 54} = 0.22$, p = 0.64) or interaction ($F_{1, 54} = 1.66$, p =

300 0.20) was observed (Figure 1F). These data suggested that acid challenge minimally affected

301 basal mucin production and secretion in the airway.

302

303 **MUC5B secretion in response to cholinergic stimulation is modified in acid-challenged**

304 **piglet airways**

305 Previous studies suggested that CF airways are characterized by abnormal submucosal gland

306 secretion and ductal plugging. Thus, we stimulated submucosal gland secretion by administering

307 the cholinergic agonist methacholine and assessed mucin secretion. No significant differences in

308 MUC5AC concentrations in the bronchoalveolar lavage fluid from methacholine-stimulated

309 piglets were found (treatment, $F_{1, 23} = 0.68$, p = 0.42; drug, $F_{1, 23} = 2.05$, p = 0.17; interaction, $F_{1,}$

310 $_{23} = 1.68$, p = 0.21, Figure 2A). In contrast, MUC5B concentrations were significantly decreased

311 in acid-challenged piglets compared to saline-treated controls (treatment, $F_{1, 23} = 11.13$, p =

312 0.0029; drug, $F_{1, 23} = 50.97$, p < 0.0001; interaction, $F_{1, 23} = 59.55$, p < 0.0001, Figure 2B).

13

313   Treatment with diminazene aceturate did not prevent acid-induced defects (Figure 2B) but did

314   decrease MUC5B concentrations in saline-treated pigs compared to saline-treated pigs without

315   diminazene aceturate (Figure 2B).

316

317   Assessment of MUC5B and MUC5AC expression in tracheal cross sections revealed no

318   statistically significant effect of acid on surface MUC5AC (treatment, $F_{1,31} = 1.083$, $p = 0.31$;

319   drug, $F_{1,31} = 3.48$, $p = 0.072$; interaction, $F_{1,31} = 0.71$, $p = 0.41$, Figure 2C). Main effects of

320   treatment ($F_{1,31} = 12.55$, $p = 0.013$) and drug ($F_{1,31} = 11.04$, $p = 0.0023$), as well as an

321   interaction ($F_{1,31} = 15.46$, $p = 0.0004$) were noted for MUC5B surface expression (Figure 2D).

322   However, post hoc comparisons revealed no statistically significant differences between acid-

323   challenged pigs and saline-challenged pigs (Figure 2D). In contrast, saline-challenged piglets

324   treated with diminazene aceturate showed a significant elevation in surface MUC5B compared to

325   saline-challenged pigs without diminazene aceturate treatment (Figure 2D). No differences were

326   observed in MUC5AC labeling in the submucosal gland (treatment, $F_{1,31} = 3.99$, $p = 0.055$; drug,

327    $F_{1,31} = 0.002$, $p = 0.961$; interaction, $F_{1,31} = 0.342$, $p = 0.563$, Figure 2E). In contrast, MUC5B

328   labeling tended to be greater in the submucosal gland of acid-challenged piglets and was

329   significantly elevated in the submucosal glands of saline-treated piglets provided diminazene

330   aceturate (treatment, $F_{1,31} = 1.46$, $p = 0.236$; drug, $F_{1,31} = 5.69$, $p = 0.0234$; interaction, $F_{1,31} =$

331   21.25, $p < 0.0001$, Figure 2F). Combined, these data suggested three key findings: 1) acid

332   challenge impacted stimulated submucosal gland secretion; 2) diminazene aceturate did not

333   prevent the effects of acid; and 3) in the absence of acid challenge, diminazene aceturate

334   modified mucus secretion properties.

335

Diminazene aceturate mitigates acid-induced mucus obstruction

Obstruction of the small airways is common in CF, and alterations in mucus biophysical

properties and composition can lead to airway obstruction. Thus, we examined the small airways

for evidence of obstruction using standard histological techniques (45). Similar to our previous

studies (45), we found that acid challenge induced airway obstruction under both basal ($F_{3, 60}$ =

13.81; p = 0.0032, Figure 3A-3E) and methacholine-stimulated conditions ($F_{3, 31}$ = 10.21; p =

0.0169, Figure 4A-4E). Diminazene aceturate significantly attenuated airway obstruction in both

conditions (Figure 3, Figure 4).

Intra-airway acid induces formation of mucus sheets and mucus strands

All of our *in vivo* studies were performed under normal physiologic conditions. However, in CF,

there is a diminished bicarbonate and chloride transport. Thus, to more closely mimic a CF

environment, we investigated mucus morphology and secretion properties *ex vivo* in tracheal

segments stimulated with methacholine under diminished bicarbonate and chloride transport

conditions. As a surrogate marker for MUC5AC and MUC5B, we stained mucus with jacalin

(36) and wheat germ agglutin (WGA)(36), respectively.

In saline-treated piglet airways, mucus was found in discreet packets that decorated the surface

like ornaments (Figure 5A). In contrast, mucus in acid-challenged piglets formed film-like sheets

(Figure 5B). Acid-challenged pigs showed greater mucus sheet formation for jacalin-labeled

mucus ($F_{3, 53}$ = 34.69; p < 0.0001, Figure 5E). A main treatment effect was observed for WGA-

labeled mucus ($F_{3, 53}$ = 53.31; p = 0.0011, Figure 5F) but post-hoc comparisons revealed no

significant differences between acid-challenged and saline-challenged pigs. In a smaller cohort

15

359 of samples, we also assessed mucus sheet formation using IMARIS software and found a

360 decrease in the number of discreet mucus particles in acid-challenged pigs compared to saline-

361 treated controls (t_{16}= 4.416, p = 0.0004, Figure 5G). Diminazene aceturate did not significantly

362 alter or prevent the formation of sheet-like structures (Figure 5C-E).

363

364 Mucus strands emanating from submucosal glands are characteristic of CF airways (14, 36).

365 Thus, we examined tracheal segments stimulated *ex vivo* with methacholine for strand formation

366 (Figure 6). A significant main effect of treatment was observed for the abundance of strands

367 formed by mucus labeled with jacalin ($F_{3, 53}$ = 13.42; p = 0.0038, Figure 6E), but post-hoc

368 comparisons revealed no significant differences between acid-challenged and saline-challenged

369 pigs. However, a significant increase in strand formation for WGA-labeled mucus was observed

370 in acid-challenged piglets compared to saline-treated controls ($F_{3, 53}$ = 18.62; p = 0.0003, Figure

371 6F). In a smaller cohort of samples, we performed a secondary analysis that consisted of tracing

372 the strand area manually in image J and expressing it as a percentage of the total image field of

373 view. Consistent with our scoring method, manual tracing also indicated significantly more

374 strands in acid-challenged piglets compared to saline-treated controls (t_{16}= 2.22, p = 0.0412,

375 Figure 6G). Diminazene aceturate did not significantly alter mucus strand formation (Figure 6C-

376 F).

377

378 Dilated submucosal gland ducts, due to plugging and obstruction of the submucosal gland, are

379 commonly observed in CF airways (61). Thus, we also examined the tracheal surfaces for the

380 presence of dilated submucosal gland duct openings. Submucosal gland duct openings were only

381 marginally visible in saline-treated piglets (Figure 7A). In stark contrast, acid-challenged piglets

16

382   displayed a significant elevation in dilated submucosal gland duct openings (Figure 7B).

383   Statistical analysis revealed a main effect of treatment ($F_{1, 53}$ = 36.45; p < 0.0001), but no effect

384   of drug ($F_{1, 53}$ = 2.72; p = 0.11) or interaction ($F_{1, 53}$ = 2.72; p = 0.11). The lack of interaction

385   precluded any post hoc testing between groups.

386

387   **Mucociliary transport is impaired in acid-challenged airways**

388   CF airways are distinguished by impaired mucociliary transport (5, 22, 25, 34). Thus, we studied

389   freshly excised tracheal segments stimulated with methacholine under diminished bicarbonate

390   and chloride transport conditions. We assessed mucociliary transport using methods developed

391   by Hoegger and colleagues (22), in which tracheas are submerged in a physiologic solution

392   containing fluorescent nanospheres that bind and attach to mucus, allowing for real-time

393   visualization of mucus production and movement. To measure the movement of fluorescently-

394   labeled mucus, we utilized IMARIS computer assigned particle-tracking that uses validated

395   algorithms (24) (Figure 8A-E). We found that the average speed of mucus was decreased in acid-

396   challenged pig airways (treatment, $F_{1, 60}$ = 3.69, p = 0.059; drug, $F_{1, 60}$ = 3.03, p = 0.087;

397   interaction, $F_{1, 60}$ = 4.002, p = 0.05, Figure 8F). Because speed is equal to distance over time, we

398   also examined computer assigned particle track length and found it was decreased in acid-

399   challenged airways (treatment, $F_{1, 60}$ = 2.68, p = 0.107; drug, $F_{1, 60}$ = 0.802, p = 0.3740;

400   interaction, $F_{1, 60}$ = 6.198, p = 0.0156, Figure 8G). Finally, we noted that under submerged

401   conditions, there was very little mucus that accumulated on the airway surface in acid-challenged

402   pigs. Therefore, at the conclusion of the experiment, we measured the signal intensity of

403   fluorescently labeled mucus on the airway surface (Figure 8H). We found a main effect of

17

404   treatment ($F_{1, 60}$ = 18.4, p < 0.0001), but no effect of drug ($F_{1, 60}$ = 0.4256, p = 0.5166) or an

405   interaction ($F_{1, 60}$ = 0.9036, p = 0.3459), thus precluding any post hoc comparisons.

406

407   Because mucociliary transport can also be decreased due to defects in cilia number and/or

408   function, we also measured the percent of the trachea lumen that was ciliated using antibody

409   labeling (Figure 9A-D). We found a main effect of treatment ($F_{1, 52}$ = 12.92, p = 0.0007), but no

410   effect of drug ($F_{1, 52}$ = 0.0499, p = 0.824), or an interaction ($F_{1, 52}$ = 0.0625, p = 0.8036), were

411   observed (Figure 9E).

412

413   **DISCUSSION**

414   Early in CF pathogenesis, the airway is acidic (4, 5, 38). Although mucus abnormalities precede

415   airway infection and inflammation (5, 15, 22), whether transient airway acidification is sufficient

416   to produce pathologic mucus and decrements in mucociliary transport is controversial. Thus, we

417   interrogated the effect of early life airway acidification on mucus properties by studying neonatal

418   piglets challenged with intra-airway acid or saline control. Secondarily, to investigate potential

419   mechanisms, we blocked detection of acid with diminazene aceturate (53).

420

421   Our data showed a marginal effect of transient acidification on basal mucin expression and

422   secretion under physiologic conditions. Upon *in vivo* methacholine stimulation, acid-challenged

423   pigs had less MUC5B in the bronchoalveolar lavage fluid and tended to have more MUC5B

424   retained in the submucosal gland compared to saline-treated controls, suggesting a defect at the

425   level of the submucosal gland. To more closely mimic a CF-like environment, we investigated

426   mucus secretion properties under diminished bicarbonate and chloride transport conditions.

18

427   Under these conditions, we observed abnormal mucus secretion in acid-challenged pig airways,

428   characterized by extensive mucus sheets and mucus strands. These features are similar to those

429   described in newborn CF pig airways (36, 58). Acid-challenged pigs also displayed decreased

430   mucociliary transport under diminished bicarbonate and chloride transport conditions. Thus,

431   combined, these data suggest that transient airway acidification produces abnormal mucus

432   secretion and transport, mimicking several features of CF.

433

434   Direct measurements of airway pH in children with CF have suggested that pH is not different

435   compared to children that do not have CF (33, 54), yet other studies suggest that the airways are

436   acidified in human neonates with CF (4). These findings have raised controversy whether

437   acidification is an initiating factor in CF pathogenesis. In our model, the acidification procedure

438   we utilize leads to a mild decrement in airway pH (45), resulting in airway pH values similar to

439   what has been reported in animal models of CF (58). While we did not observe a significant

440   increase in mucin production that others have reported in CF (15), we did find other similarities,

441   including evidence for defective submucosal gland secretion, airway obstruction and impaired

442   mucociliary transport (5, 22, 52, 61). It is likely that the cause of decreased mucociliary transport

443   was multifactorial, with both a loss of cilia and change in mucus secretion contributing. It is also

444   possible that acidic pH caused a sustained change in mucus viscosity (58), which could also

445   decrease mucociliary transport.

446

447   To interrogate potential mechanisms, we studied diminazene aceturate. Diminazene aceturate is a

448   widely used drug for the treatment of protozoan diseases with reportedly minimal adverse side

449   effects (13). Its low cost and availability in numerous regions of the world make it an attractive

19

450   drug. Diminazene aceturate blocks the acid-sensing ion channel 1a (ASIC1a) (53), which is

451   present in nerves innervating the airway (18, 30), and throughout the central nervous system

452   (39). Since airway nerves are critical in detecting noxious stimuli and regulating mucus

453   secretion, we hypothesized that detection of acid through ASIC1a might be critical in our model.

454

455   We found that diminazene aceturate had a negligible effect in preventing acid-induced mucus

456   defects, despite being used at doses known to inhibit ASIC1a (10, 29). In contrast, diminazene

457   aceturate treatment in saline-challenged controls lead to a significant increase in MUC5B on the

458   airway surface, but less MUC5B in the bronchoalveolar lavage fluid. Although unbuffered saline

459   is acidic (44, 46), our previous studies showed that application of unbuffered saline to the apical

460   surface of porcine airway epithelial cells *in vitro* had a negligible effect on airway surface liquid

461   pH (45). However, we cannot exclude the possibility that unbuffered saline induces a transient

462   activation of ASIC1a in the airway, as current techniques may underestimate the change in pH. If

463   true, then the effect of diminazene aceturate on MUC5B in saline controls is potentially via

464   inhibition of ASICs, although other mechanisms could also be involved (8, 42).

465

466   The lack of effect in acid-challenge conditions suggests that either the dosing or delivery of

467   diminazene aceturate was suboptimal. For example, our previous studies demonstrated that acid

468   application to porcine airway epithelial cells *in vitro* produced a transient acidification of the

469   airway surface liquid from 7.5 to ~6.6 (45). Previous studies showed that the ASIC1a decay in

470   response, or desensitization, is pH-dependent, with slower desensitization correlating with proton

471   concentration (31). Specifically, when ASIC1a was subjected to a drop in pH from 7.4 to 6.6,

472   subsequent application of solutions of pH 7.2 and 7.4 prolonged the desensitization period

20

473   compared to solutions of pH 7.6 and 8.0. This property translates into prolonged channel

474   activation at low proton concentrations (31). This might be particularly important in our

475   paradigm, in which pH likely returns to baseline over the course of 10 minutes (45). Therefore, it

476   is possible that we underestimated the dose required to cause effective and sustained blockade of

477   ASIC1a in the trachea, which is expected to receive a greater and more homogeneous exposure

478   to acid (45). Alternatively, this finding might suggest that ASIC1a is not involved in acid-

479   mediated mucus defects at the trachea level.

480

481   We observed a strong protective effect of diminazene aceturate in the intrapulmonary airways. It

482   is possible that ASIC1a is more concentrated in the small airways compared to the large airways.

483   The lack of commercially available antibodies that detect ASIC1a in the pig preclude

484   investigations focused on addressing this question. Although we are not certain of the

485   mechanisms by which potential activation of ASICs in the intrapulmonary airways could lead to

486   airway obstruction, our previous work illustrated that this acid-challenge model induces neural

487   remodeling at the level of nodose ganglia and in the brainstem. These changes were associated

488   with airway obstruction and predicted to involve Aδ and c-fibers (26, 45, 60). Consistent with

489   that, we previously found that elimination of ASIC1a in mice disrupted sensory nerve function,

490   leading to a decreased amount of the neuropeptide substance P in the lung lavage fluid of

491   ovalbumin-sensitized mice compared to wild-type controls (47). Recent work demonstrates that

492   substance P in vagal sensory neurons drives mucus cell metaplasia (57). Therefore, if ASICs

493   were indeed involved in the acid-mediated airway obstruction, then it is conceivable that

494   substance P in c-fibers innervating the intrapulmonary airways played a role. Additionally,

495   although our previous work suggests negligible expression of ASIC1a in murine airway cells

21

496   (45), it is possible that ASIC1a is expressed in non-neuronal cells in the small airways of the pig.

497   An additional consideration is that the effects of diminazene aceturate were not ASIC1a-related

498   at all and perhaps due to off-target effects, such as activity on the (ACE2) (8, 42). Although the

499   diminazene aceturate dose used in this study was below the reported dose required to cause

500   activity on ACE2, it is a possibility that we must consider.

501

502   We previously examined inflammation extensively in acid-challenged piglets using

503   inflammatory-directed gene arrays and ELISAs, and found evidence for transcriptional

504   inflammation, as well as an elevation in IL-1β protein levels (45). Recently Boucher and

505   colleagues reported that IL-1β predominates the CF airway and correlates with mucin production

506   (9). Thus, it is possible that IL-1β contributes to some of the acid-mediated defects we report

507   here.

508

509   We found a main effect of acidification on cilia abundance in the trachea. Although we did not

510   investigate mechanisms responsible for this observation, deciliation in response to other airway

511   irritants, such as viral infection and smoke, have been reported (55). Similarly, epithelial damage

512   following severe bronchospasm has also been reported (37). Thus, it is possible that both

513   chemical (pH) and mechanical (bronchoconstriction) forces contributed to the observed effect of

514   acidification on airway ciliation.

515

516   Our studies demonstrated that acidification impaired mucus secretion and decreased mucociliary

517   transport. While we do not know whether a change in the biophysical properties of

518   mucins/mucus contributed, strong evidence suggests that mucin folding, secretion, and viscosity

22

519   are pH-dependent (49, 58) . For example, recently Hughes and colleagues reported that when

520   MUC5B is subjected to an acidic pH and high calcium environment, it forms dense structures

521   (23). Such structures are likely to impact the mucus barrier and modify the transition from a

522   condensed form of mucin to a more expanded form of mucin. However, biophysical changes are

523   typically reversible and flexible (23, 49, 58). Thus, in our experimental model, it is anticipated

524   that an effect of acidification on mucin/mucus biophysical properties would have occurred

525   acutely during the initial exposure to acid and likely reversed by the time the studies were

526   conducted (48 hours later). This suggests that perhaps the effect of acidification on mucus

527   secretion and mucus transport in our studies was not due to an acute change in biophysical

528   properties. Alternatively, if biophysical properties were altered in our model, then they might

529   have been more long-term or sustained.

530

531   Our study has limitations. We did not assess mucus secretion under basal and stimulated

532   conditions within the same subject. This was largely due to the inability to take airway samples

533   from a subject before and after methacholine stimulation. Further, our study was transient and

534   therefore lacked information regarding long-term consequences of airway acidification.

535   However, the transient nature of our study might also be an advantage, because it allowed for the

536   effects of acidification to be isolated from potential secondary complications, such as infection

537   and/or prolonged inflammation. We also did not identify a mechanism responsible for acid-

538   mediated defects in mucus secretion and mucus transport, but our studies highlight the

539   submucosal gland as a target of airway acidification. Although not measured here, it is possible

540   that a reduction in ciliary beat frequency, either due directly to acid (56), or secondarily due to

541   changes in mucus composition, contributes. We also did not study mucus transport under normal

23

542 chloride and bicarbonate conditions; thus, we cannot make any conclusions regarding the

543 specificity of acidification in driving airway pathology in a CF-like environment. Finally, as

544 mentioned above, we did not study the biophysical or biochemical properties of mucus (e.g.,

545 glycosylation, viscosity); future studies focused on these qualities will be of value.

546

547 In summary, early life airway acidification produced mucus with pathologic features, airway

548 obstruction, and decreased mucociliary transport. Diminazene aceturate mitigated acid-induced

549 airway obstruction. Thus, these findings suggest that even transient airway acidification early in

550 life might have profound impacts on mucus secretion and transport properties. Further, they

551 highlight diminazene aceturate as a potential agent beneficial for alleviating some features of CF

552 airway disease.

553

554 **Figures and Figure Legends**

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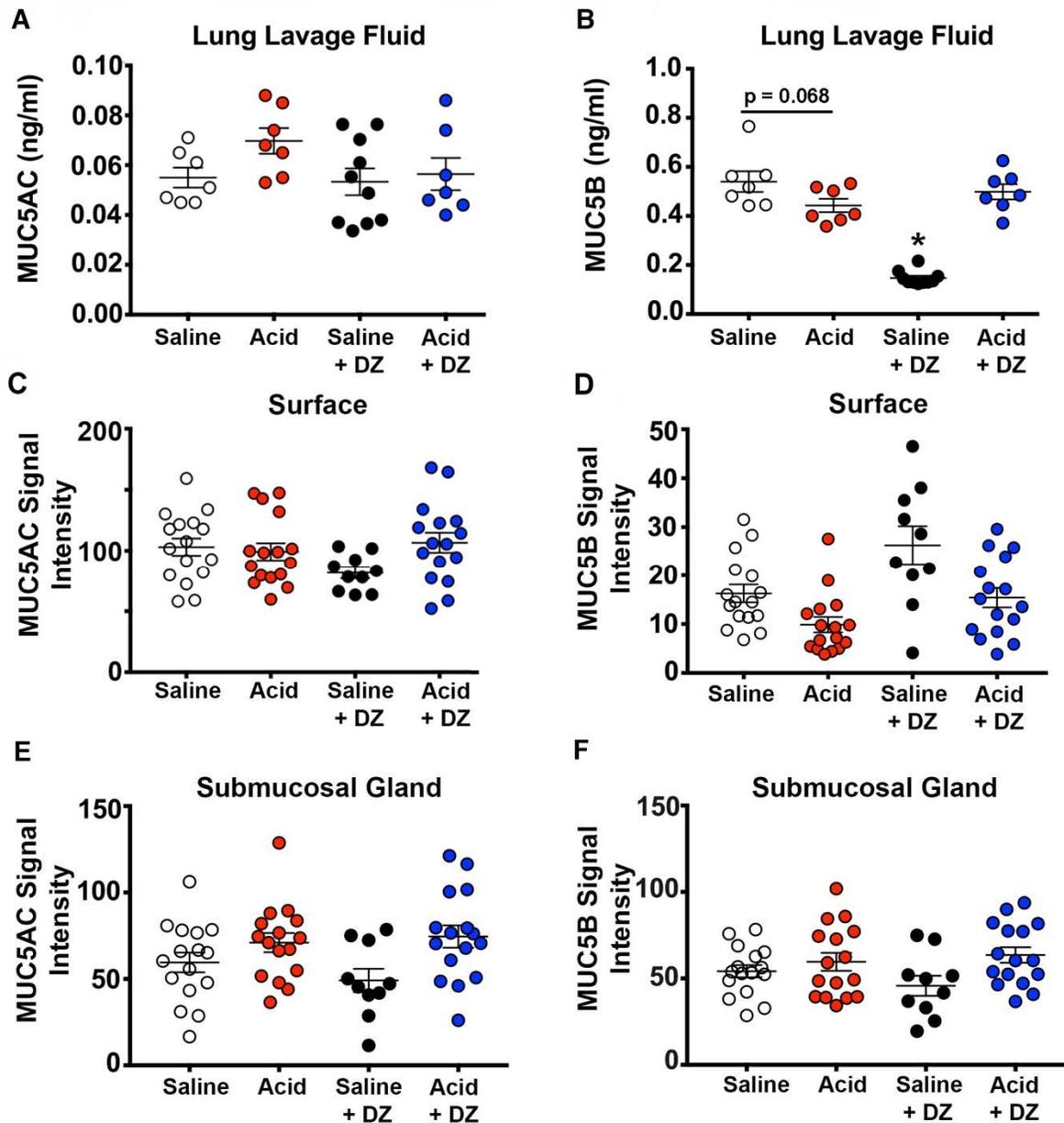
Fig. 1. Mucin in piglet airways under basal conditions. Bronchoalveolar lavage fluid

concentrations of MUC5AC (A) and MUC5B (B). MUC5AC and MUC5B staining signal

intensity of the surface epithelia (**C, D**) and submucosal gland (**E, F**). For panels A and B, n = 7

saline-challenged piglets (4 females, 3 males), n = 7 acid-challenged pigs (4 females, 3 males), n

= 10 saline-challenged pigs + diminazene aceturate (5 females, 5 males), n = 7 acid-challenged +

diminazene aceturate pigs (4 females, 3 males). For panels C-F, n =16 saline-challenged piglets

25

562   (8 females, 8 males), n = 16 acid-challenged pigs (8 females, 8 males), n = 10 saline-challenged

563   + diminazene aceturate (5 females, 5 males), n = 16 acid-challenged + diminazene aceturate pigs

564   (8 females, 8 males). Data points represent the mean fluorescent intensity for each piglet

565   calculated from 3-5 images analyzed (encompassing the anterior, middle and posterior regions of

566   the trachea). Abbreviations: DZ, diminazene aceturate; MUC5B, mucin 5B; MUC5AC, mucin

567   5AC. * $p < 0.05$ compared to saline-challenged pigs. Data were assessed with a parametric two-

568   way ANOVA followed by Sidak post hoc test. Mean ± S.E.M shown.

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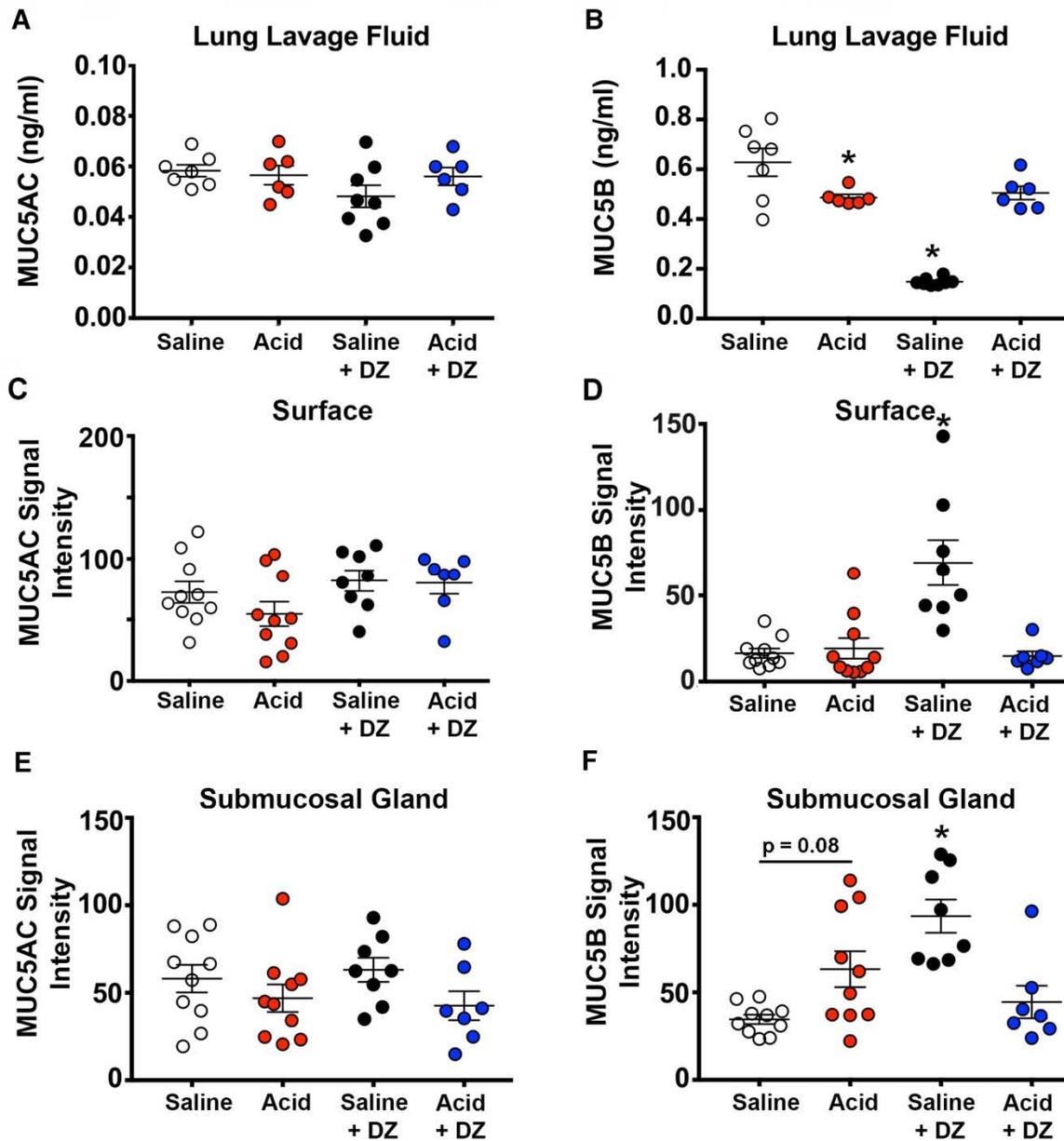
Fig. 2. Mucin in piglet airways under methacholine-stimulated conditions. Bronchoalveolar lavage fluid concentrations of MU5AC (**A**) and MUC5B (**B**). MUC5AC and MUC5B staining signal intensity of the surface epithelia (**C, D**) and submucosal gland (**E, F**). For panels A and B, n =7 saline-challenged piglets (4 females, 3 males), n = 6 acid-challenged pigs (3 females, 3 males), n = 8 saline-challenged + diminazene aceturate (4 females, 4 males), n = 6 acid-challenged + diminazene aceturate pigs (3 females, 3 males). For panels C-F, n = 10 saline-

27

581   challenged piglets (5 females, 5 males), n = 10 acid-challenged pigs (5 females, 5 males), n = 8

582   saline-challenged + diminazene aceturate (4 females, 4 males), n = 7 acid-challenged +

583   diminazene aceturate pigs (4 females, 3 males). Data points represent the mean fluorescent

584   intensity for each piglet calculated from 3-5 images analyzed (encompassing the anterior, middle

585   and posterior regions of the trachea). Abbreviations: DZ, diminazene aceturate; MUC5B, mucin

586   5B; MUC5AC, mucin 5AC. * $p < 0.05$ compared to saline-challenged pigs. Data were assessed

587   with a parametric two-way ANOVA followed by Sidak post hoc test. Mean ± S.E.M shown.

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Fig. 3. **Airway obstruction under basal conditions.** Representative histological lung cross sections stained with Periodic acid-Schiff stain (PAS) in piglets challenged with **(A)** saline, **(B)** acid, **(C)** saline-challenged + diminazene aceturate, or **(D)** acid-challenge + diminazene aceturate under non-stimulated conditions. Arrows highlight airway and asterisk highlights airway lumen. Scale bar in panel A applies to panels B-D. **(E)** Obstruction score assigned to lung cross sections. n =18 saline-challenged piglets (9 females, 9 males), n = 18 acid-challenged pigs (9 females, 9

600   males), n = 10 saline-challenged + diminazene aceturate (5 females, 5 males), n = 18 acid-

601   challenged + diminazene aceturate pigs (9 females, 9 males). Abbreviations: DZ, diminazene

602   aceturate. * p < 0.05 compared to saline-challenged pigs, # p < 0.05 compared to acid-challenged

603   piglets. Data were assessed with a non-parametric one-way ANOVA (Kruskal-Wallis) followed

604   by Dunn's multiple comparison test. Mean ± S.E.M shown.

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Fig. 4. Airway obstruction under methacholine-stimulated conditions.

Representative histological lung cross sections stained with PAS in piglets delivered

methacholine in vivo following challenge with **(A)** saline, **(B)** acid, **(C)** saline-challenged +

diminazene aceturate or **(D)** acid-challenged + diminazene aceturate. Arrows highlight airway

and asterisk highlights airway lumen. Scale bar in panel A applies to panels B-D. **(E)**

Obstruction score assigned to lung cross sections. n =10 saline-challenged piglets (5 females, 5

31

614   males), n = 10 acid-challenged pigs (4 females, 6 males), n = 8 saline-challenged + diminazene

615   aceturate (4 females, 4 males), n = 7 acid-challenged + diminazene aceturate pigs (4 females, 3

616   males). Abbreviations: DZ, diminazene aceturate. * $p < 0.05$ compared to saline-challenged pigs,

617   # $p < 0.05$ compared to acid-challenged piglets. Data were assessed with a non-parametric one-

618   way ANOVA (Kruskal-Wallis) followed by Dunn's multiple comparison test. Mean ± S.E.M

619   shown.

620

621

A *In vivo* Saline

B *In vivo* Acid

C *In vivo* Saline + Diminiazene Aceturate

D *In vivo* Acid + Diminiazene Aceturate

E Sheet Index

F Sheet Index

G

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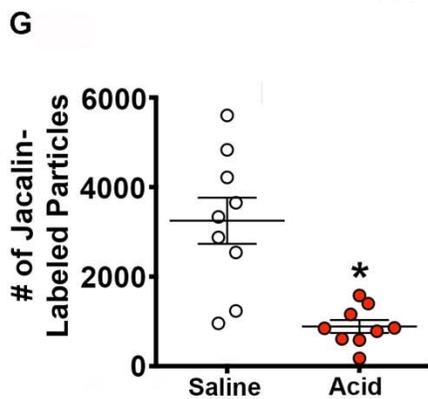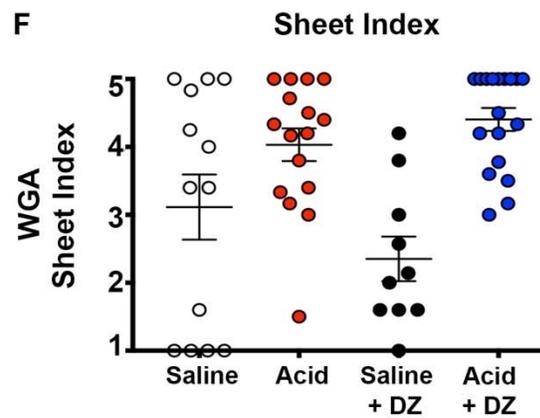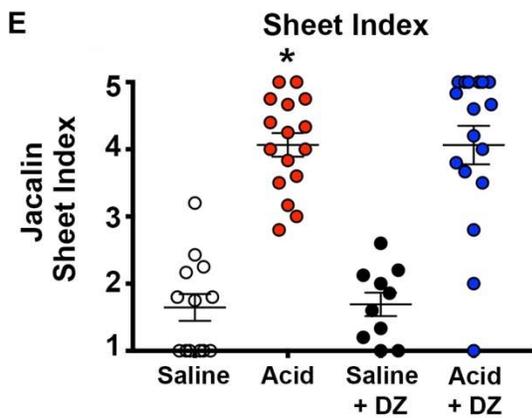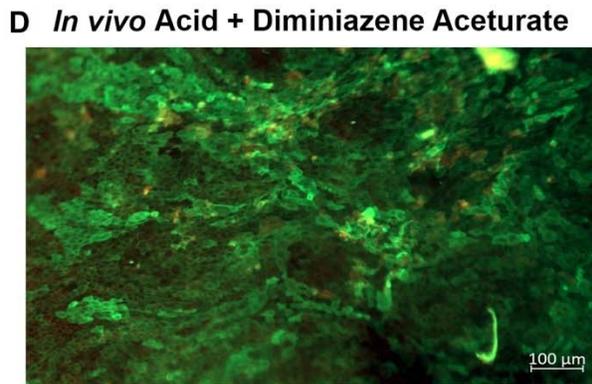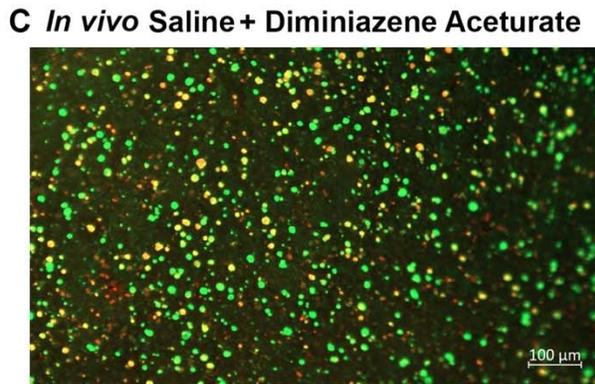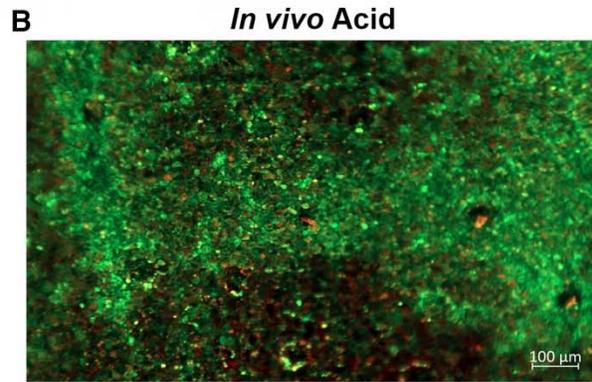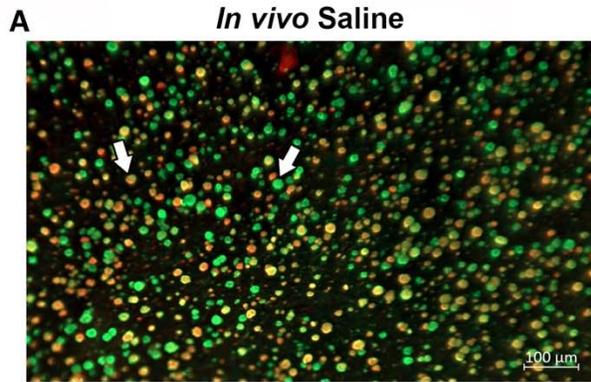
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Fig. 5. Mucus sheet formation under diminished bicarbonate and chloride transport. (**A**) Representative image of an *ex vivo* trachea from a saline-challenged piglet stimulated with methacholine. Discrete entities of mucus were observed and visualized by jacalin lectin (green) and wheat germ agglutinin lectin (red) staining. Arrows highlight examples of lectin labeling. Representative images of methacholine-stimulated *ex vivo* tracheas from an acid-challenged piglet (**B**), a saline-challenged + diminazene aceturate (**C**), and acid-challenged + diminazene aceturate (**D**). Sheet index for jacalin-labeled mucus **(E)** and wheat germ agglutinin-labeled mucus **(F). (G)** The numbers of jacalin-labeled mucus particles analyzed using IMARIS software as an additional measurement of mucus sheet formation in acid-challenged piglets. n = 9 saline-challenged piglets (4 females, 5 males), n = 9 acid-challenged pigs (4 females, 5 males). For panels A-F, n =13 saline-challenged piglets (7 females, 6 males), n = 16 acid-challenged pigs (8 females, 8 males), n = 10 saline-challenged + diminazene aceturate (5 females, 5 males), n = 18 acid-challenged + diminazene aceturate pigs (9 females, 9 males). Data points represent the mean score for each piglet calculated from 5-7 analyzed images (encompassing the anterior, middle and posterior regions of the trachea). Abbreviations: WGA, wheat germ agglutinin; DZ, diminazene aceturate. * $p < 0.05$ compared to saline-challenged pigs. For panels E-F, data were assessed with a non-parametric one-way ANOVA (Kruskal-Wallis) followed by Dunn's multiple comparison test. For panel G, data were assessed by an unpaired students t-test. Mean ± S.E.M shown.

34

A *In vivo* Saline

B *In vivo* Acid

C *In vivo* Saline + Diminiazene Aceturate

D *In vivo* Acid + Diminiazene Aceturate

E Strand Index

F Strand Index

G

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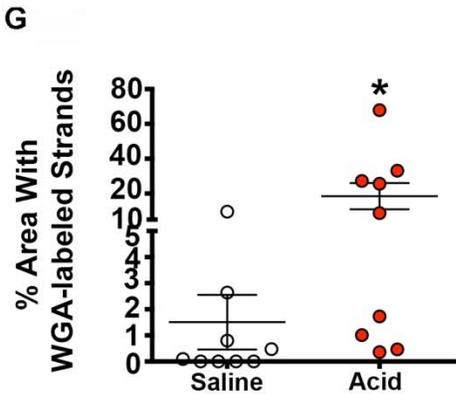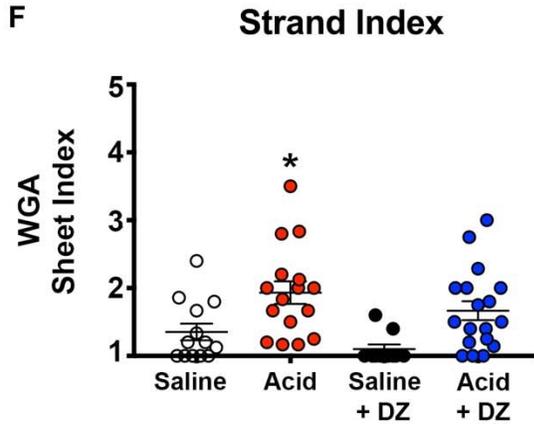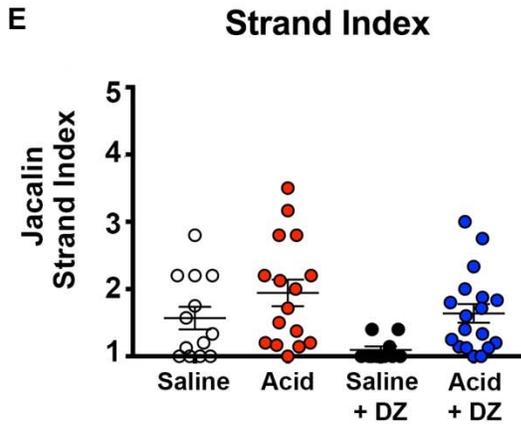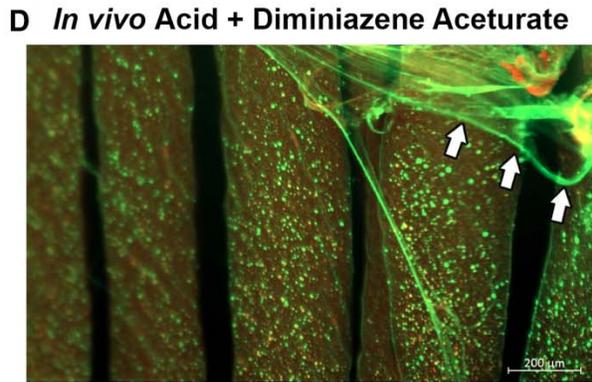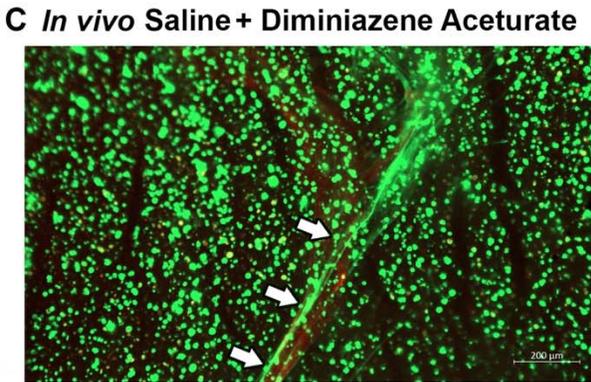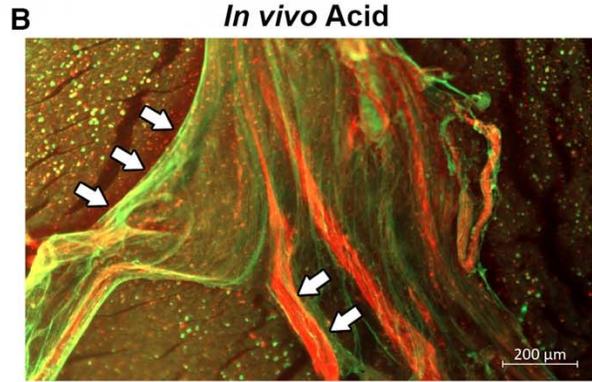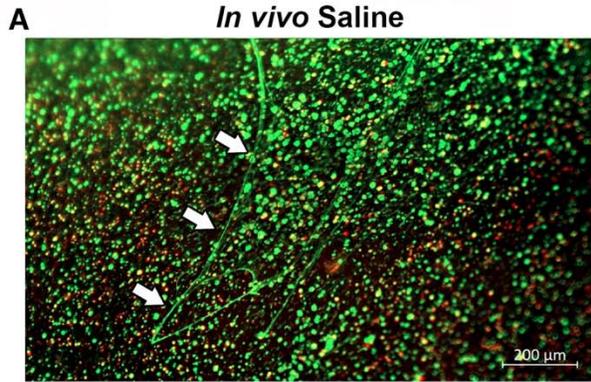
647 **Fig. 6. Mucus strand formation in acid-challenged piglets under diminished chloride and**

648 **bicarbonate transport conditions** (**A**) Representative image of an *ex vivo* trachea from a saline-

649 challenged piglet stimulated with methacholine. Mucus is labeled with jacalin lectin (green) and

650 wheat germ agglutinin lectin (red) staining. Arrows highlight an example of a mucus strand.

651 Representative images of *ex vivo* trachea from a saline-challenged (**A**)**,** acid-challenged (**B**)

652 saline-challenged provided diminazene aceturate (**C**), and acid-challenged pig provided

653 diminazene aceturate (**D**) piglet airways stimulated with methacholine. Strand index for jacalin-

654 labeled mucus **(E)** and wheat germ agglutinin-labeled mucus **(F). (G)** The percent (%) of field of

655 view occupied by WGA-labeled strands. n = 9 saline-challenged piglets (4 females, 5 males), n =

656 9 acid-challenged pigs (4 females, 5 males). For panels A-F, n =13 saline-challenged piglets (7

657 females, 6 males), n = 16 acid-challenged pigs (8 females, 8 males), n = 10 saline-challenged +

658 diminazene aceturate (5 females, 5 males), n = 18 acid-challenged + diminazene aceturate pigs (9

659 females, 9 males). Data points represent the mean score for each piglet calculated from 5-7

660 analyzed images (encompassing the anterior, middle and posterior regions of the trachea).

661 Abbreviations: WGA, wheat germ agglutinin; DZ, diminazene aceturate. $p < 0.05$ compared to

662 saline-challenged pigs. For Panels E-F, data were assessed with a non-parametric one-way

663 ANOVA (Kruskal-Wallis) followed by Dunn's multiple comparison test. For panel G, data were

664 assessed by an unpaired students t-test. Mean ± S.E.M shown.

665

666

Fig. 7. Dilated submucosal gland duct openings on airway surface in acid-challenged piglets visualized by lectin staining. (A) Arrows highlight example of a duct opening with wheat-germ agglutinin-stained mucus emanating in a saline-challenged piglet. Green is jacalin-labeled mucus. **(B)** Arrows highlight examples of a dilated duct openings with mucus emanating in an acid-challenged piglet. **(C)** Arrows highlight example of a duct opening with mucus emanating in a saline-challenged piglet treated with diminazene aceturate. **(D)** Arrows highlight examples

37

674   of a dilated duct openings with mucus emanating in acid-challenged piglets treated with

675   diminazene aceturate **(E)** Number of dilated submucosal gland ducts normalized to area. n =13

676   saline-challenged piglets (7 females, 6 males), n = 16 acid-challenged pigs (8 females, 8 males),

677   n = 10 saline-challenged + diminazene aceturate (5 females, 5 males), n = 18 acid-challenged +

678   diminazene aceturate pigs (9 females, 9 males). Data points represent the mean score for each

679   piglet calculated from 5-7 analyzed images (encompassing the anterior, middle and posterior

680   regions of the trachea). Abbreviations: DZ, diminazene aceturate. Data were assessed with a

681   parametric two-way ANOVA followed by Sidak post hoc test. Mean ± S.E.M shown.

A

IMARIS-Assisted Particle Assignment
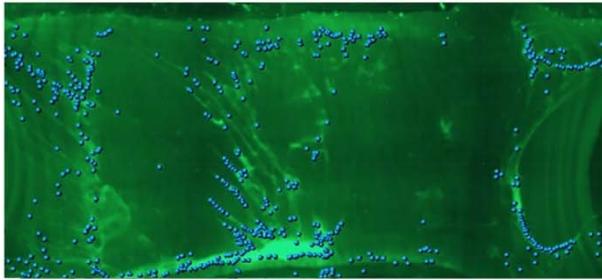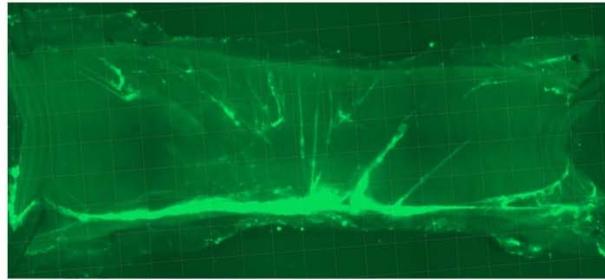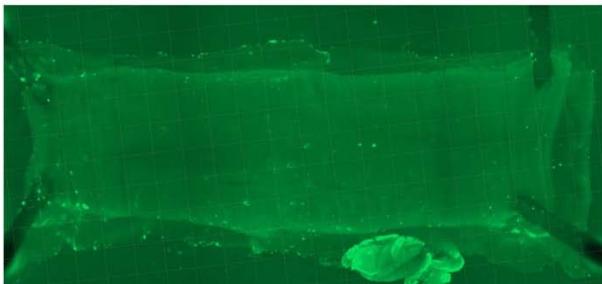
B

***In vivo* Saline**

C

***In vivo* Acid**

D

***In Vivo* Saline + Diminiazene Aceturate**

E

***In Vivo* Acid + Diminiazene Aceturate**

F

G

H

682
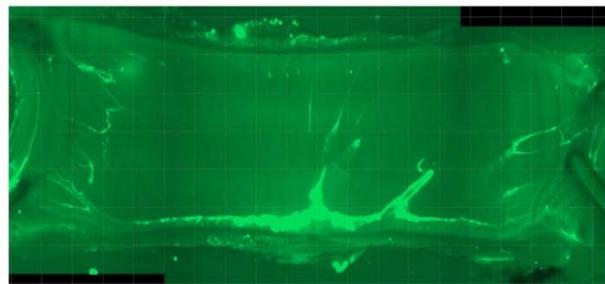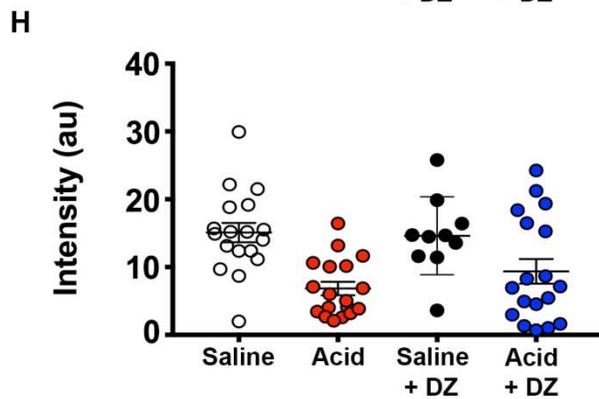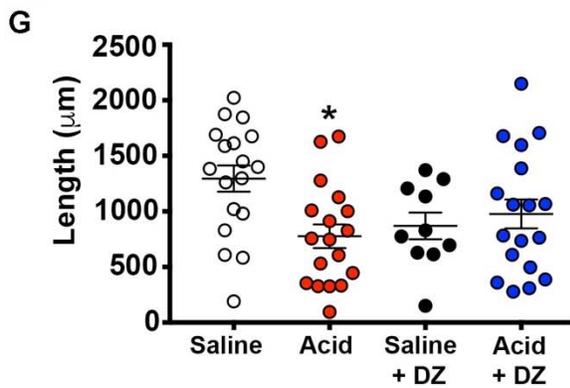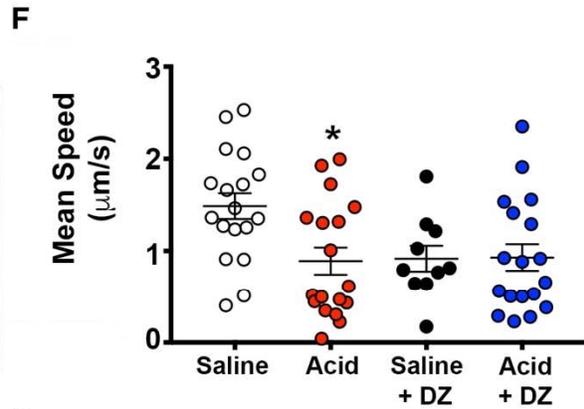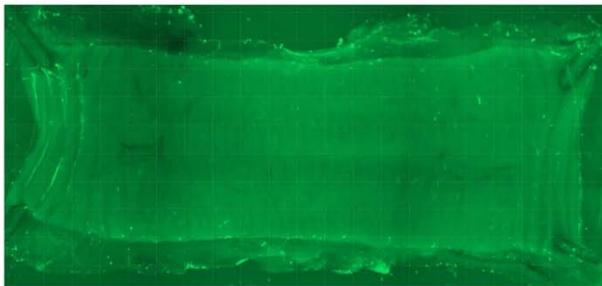
Fig. 8. Mucus transport in piglet airways under diminished chloride and bicarbonate transport. (A) Representative image of an *ex vivo* piglet trachea stimulated with methacholine. Mucus is visualized in real-time with fluorescent nanospheres (bright green). Mucus often forms strands. Computer particles are assigned based upon fluorescence intensity and appear as blue/aqua in color. Representative images of tracheas at the conclusion of the experiment post methacholine stimulation in saline-challenged (**B**), acid-challenge (**C**), saline-challenged + diminazene aceturate (**D**), and acid-challenged + diminazene aceturate (**E**) piglet airways. (**F**) Mean mucus transport speed. (**G**) Computer assigned particle-track length. (**H**) Quantification of the signal intensity of fluorescently labeled mucus on the airway surface at the conclusion of the experiment. n =18 saline-challenged piglets (9 females, 9 males), n = 18 acid-challenged pigs (9 females, 9 males), n = 10 saline-challenged + diminazene aceturate (5 females, 5 males), n = 18 acid-challenged + diminazene aceturate pigs (9 females, 9 males). Abbreviations: DZ, diminazene aceturate; au, arbitrary units. * $p < 0.05$ compared to saline-challenged pigs. Data were assessed with a parametric two-way ANOVA followed by Sidak post hoc test. Mean ± S.E.M shown.

A *In vivo* Saline
B *In vivo* Acid
50µm
C *In vivo* Saline + Diminiazene Aceturate
D *In vivo* Acid + Diminiazene Aceturate
E
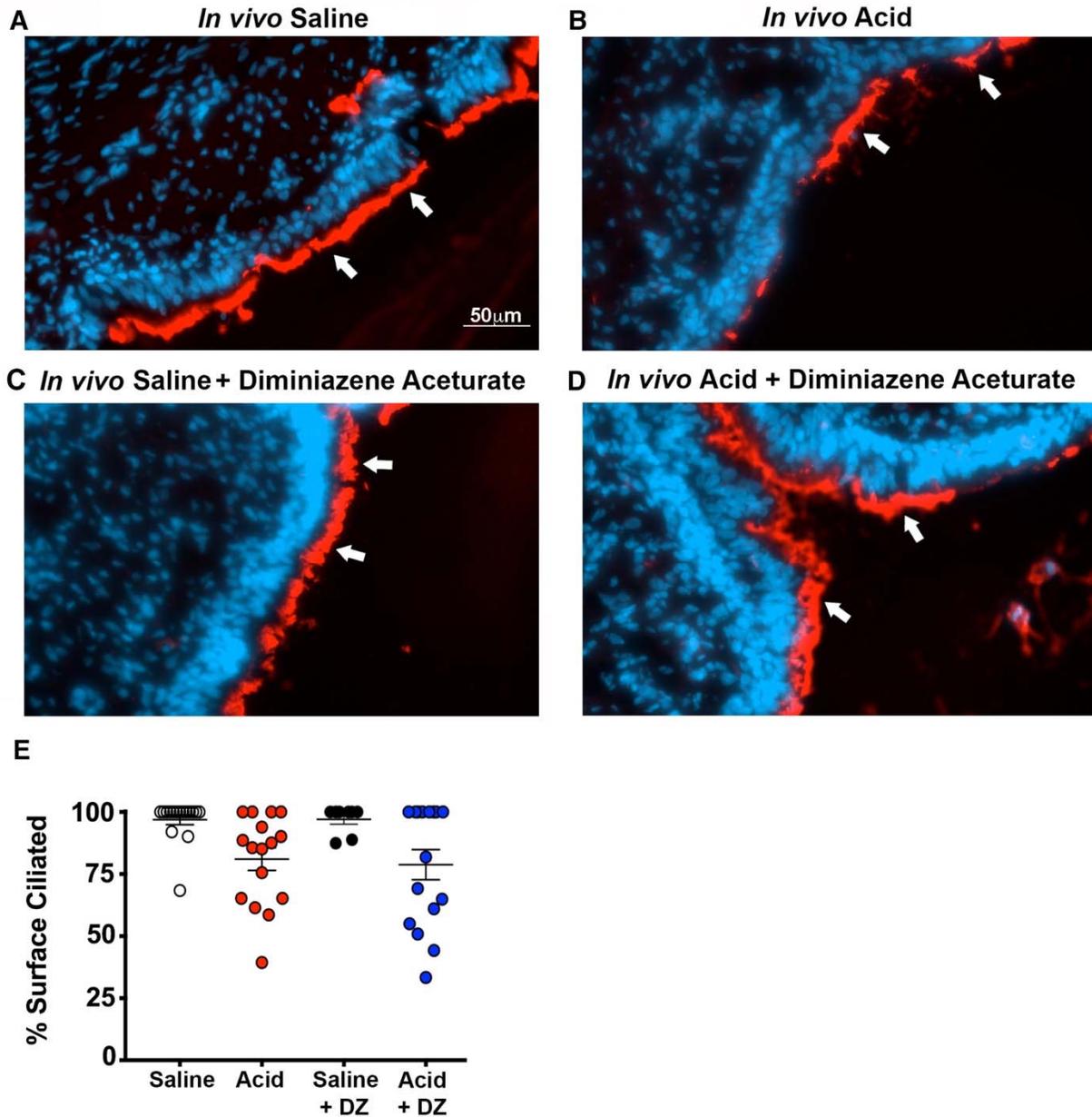
699
Fig. 9. Percent of trachea ciliated. Representative images of cilia detected by antibody staining

of acetylated tubulin (shown in red, highlighted by arrows) in tracheas from saline-challenged

(**A**), acid-challenged (**B**), saline-challenged treated + diminazene aceturate (**C**), and acid-

challenged + diminazene aceturate (**D**) piglets. (**E**) Quantification of ciliated trachea lumen

expressed as a percentage (%) of the length of the epithelia that was ciliated divided by the total

length of the epithelia. n =16 saline-challenged piglets (8 females, 8 males), n = 16 acid-

41

706   challenged pigs (8 females, 8 males), n = 10 saline-challenged + diminazene aceturate (5

707   females, 5 males), n = 16 acid-challenged + diminazene aceturate pigs (8 females, 8 males).

708   Scale bar in panel A applies to panels B-D Abbreviations: DZ, diminazene aceturate. Data were

709   assessed with a parametric two-way ANOVA followed by Sidak post hoc test. Mean ± S.E.M

710   shown.

711

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A Lung Lavage Fluid

B Lung Lavage Fluid

C Surface

D Surface

E Submucosal Gland

F Submucosal Gland

A *In vivo* Saline

B *In vivo* Acid

C *In vivo* Saline + Diminiazene Aceturate

D *In vivo* Acid + Diminazene Aceturate

E

Obstruction Score plot with y-axis labeled "Obstruction Score" ranging from 1 to 4, and x-axis categories: Saline, Acid, Saline + DZ, Acid + DZ

A *In vivo* Saline

B *In vivo* Acid

C *In vivo* Saline + Diminiazene Aceturate

D *In vivo* Acid + Diminazene Aceturate

E

A *In vivo* Saline

B *In vivo* Acid

C *In vivo* Saline + Diminiazene Aceturate

D *In vivo* Acid + Diminiazene Aceturate

E Sheet Index

Jacalin Sheet Index

Saline   Acid   Saline + DZ   Acid + DZ

F Sheet Index

WGA Sheet Index

Saline   Acid   Saline + DZ   Acid + DZ

G

# of Jacalin-Labeled Particles

Saline   Acid

A *In vivo* Saline

B *In vivo* Acid

C *In vivo* Saline + Diminiazene Aceturate

D *In vivo* Acid + Diminiazene Aceturate

E Strand Index

F Strand Index

G

A *In vivo* Saline

B *In vivo* Acid

C *In vivo* Saline + Diminiazene Aceturate

D *In vivo* Acid + Diminiazene Aceturate

E

A IMARIS-Assisted Particle Assignment

B *In vivo* Saline

C *In vivo* Acid

D *In Vivo* Saline + Diminiazene Aceturate

E *In Vivo* Acid + Diminiazene Aceturate

F

G

H

A   *In vivo* Saline

B   *In vivo* Acid

50μm

C *In vivo* Saline + Diminiazene Aceturate

D *In vivo* Acid + Diminiazene Aceturate

E

Table 1. Results separated by sex under non-stimulated or methacholine-stimulated conditions. Data are shown as Mean ± SEM.

| Variables | Groups | | | | | | | | Two-way ANOVA P-values | | |
| | Female | | | | Male | | | | | | |
	Female Saline	Female Acid	Female Saline + DZ	Female Acid + DZ	Male Saline	Male Acid	Male Saline + DZ	Male Acid + DZ	Sex	Treatment	Sex* Treatment
Under non-stimulated conditions											
Bronchoalveolar lavage concentrations of MUC5AC	0.052 ± 0.0045	0.071 ± 0.0072	0.058 ± 0.0071	0.064 ± 0.0097	0.059 ± 0.0076	0.064 ± 0.0056	0.049 ± 0.0084	0.047 ± 0.0049	0.2749	0.2687	0.5291
Bronchoalveolar lavage concentrations of MUC5B	0.55 ± 0.073	0.42 ± 0.027	0.15 ± 0.017	0.48 ± 0.043	0.52 ± 0.039	0.47 ± 0.056	0.15 ± 0.008	0.53 ± 0.049	0.5560	<0.0001	0.7604
Surface MUC5AC mean signal intensity	111.7 ± 11.52	110.4 ± 10.75	84.44 ± 8.48	125.8 ± 9.91	94.38 ± 7.97	87.77 ± 8.04	79.05 ± 4.71	87.29 ± 9.32	0.0039	0.1159	0.4325
Surface MUC5B mean signal intensity	15.64 ± 2.29	11.92 ± 2.74	21.97 ± 2.84	16.91 ± 3.20	16.91 ± 2.98	7.823 ± 1.37	30.55 ± 7.21	13.89 ± 2.49	0.8849	0.0003	0.2582
Gland MUC5AC mean signal intensity	68.58 ± 8.14	71.97 ± 3.97	58.35 ± 7.71	79.62 ± 8.98	50.44 ± 7.12	70.03 ± 10.79	39.97 ± 10.06	69.47 ± 9.58	0.0559	0.034	0.7429
Gland MUC5B mean signal intensity	55.43 ± 5.28	63.23 ± 5.85	44.2 ± 9.34	63.43 ± 7.09	52.89 ± 5.07	55.95 ± 8.84	47.25 ± 7.93	63.65 ± 5.99	0.7426	0.1079	0.9023
Mean lung obstruction scores	1.44 ± 0.18	2.22 ± 0.28	1.8 ± 0.37	1.56 ± 0.24	1.33 ± 0.17	2.33 ± 0.24	1.40 ± 0.25	1.67 ± 0.17	0.6708	0.0011	0.7333
Mean percentage of trachea ciliated	100 ± 0	89.81 ± 4.17	96.86 ± 3.14	75.02 ± 9.84	93.81 ± 3.91	72.22 ± 7.03	97.20 ± 2.79	82.51 ± 7.54	0.4673	0.0033	0.2130
Under methacholine-stimulated conditions											
Bronchoalveolar lavage concentrations of MUC5AC	0.058 ± 0.0035	0.059 ± 0.0073	0.048 ± 0.0059	0.058 ± 0.0016	0.058 ± 0.0035	0.054 ± 0.0033	0.048 ± 0.0074	0.054 ± 0.0073	0.5453	0.2716	0.9659

Variables	Groups								Two-way ANOVA P-values		
	Female				Male						
	Female Saline	Female Acid	Female Saline + DZ	Female Acid + DZ	Male Saline	Male Acid	Male Saline + DZ	Male Acid + DZ	Sex	Treatment	Sex* Treatment
Bronchoalveolar lavage concentrations of MUC5B	0.63 ± 0.059	0.50 ± 0.023	0.14 ± 0.006	0.45 ± 0.011	0.63 ± 0.121	0.47 ± 0.008	0.15 ± 0.008	0.56 ± 0.031	0.5186	<0.0001	0.6148
Surface MUC5AC mean signal intensity	66.12 ± 15.34	39.98 ± 7.345	94.73 ± 7.075	77.52 ± 15.36	78.4 ± 9.44	69.19 ± 16.92	69.05 ± 13.6	83.36 ± 9.613	0.5620	0.1421	0.2151
Surface MUC5B mean signal intensity	13.79 ± 3.35	14.77 ± 6.38	57.12 ± 8.11	16.34 ± 4.85	19.06 ± 4.37	23.73 ± 10.53	81.42 ± 25.62	12.8 ± 1.02	0.2480	<0.0001	0.6511
Gland MUC5AC mean signal intensity	39.41 ± 8.16	38.92 ± 7.33	63.23 ± 10.25	45.17 ± 6.60	76.74 ± 6.20	54.81 ± 13.85	62.99 ± 10.84	39.41 ± 19.59	0.1231	0.2080	0.1771
Gland MUC5B mean signal intensity	32.24 ± 3.52	58.52 ± 15.89	77.1 ± 7.04	50.58 ± 15.41	36.86 ± 4.15	67.89 ± 14.55	109.9 ± 13.83	36.3 ± 8.52	0.3398	0.0002	0.3351
Mean lung obstruction scores	2.40 ± 0.24	3.25 ± 0.48	2.25 ± 0.48	2.00 ± 0.71	1.80 ± 0.31	3.18 ± 0.32	1.50 ± 0.29	2.00 ± 0.58	0.1645	0.0151	0.8601
Mean jacalin-labeled sheet index scores	1.81 ± 0.33	3.70 ± 0.26	1.40 ± 0.23	4.27 ± 0.43	1.45 ± 0.21	4.43 ± 0.16	1.98 ± 0.20	3.84 ± 0.34	0.5727	<0.0001	0.1370
Mean WGA-labeled sheet index scores	2.98 ± 0.66	4.08 ± 0.26	1.92 ± 0.48	4.18 ± 0.28	3.28 ± 0.76	3.98 ± 0.42	2.78 ± 0.39	4.63 ± 0.17	0.2365	0.0001	0.7673
Mean Jacalin-labeled strand index scores	1.60 ± 0.25	1.94 ± 0.37	1.16 ± 0.09	1.54 ± 0.13	1.53 ± 0.24	1.95 ± 0.18	1.03 ± 0.03	1.74 ± 0.25	0.9954	0.0175	0.8943
Mean WGA-labeled strand index scores	1.19 ± 0.11	1.83 ± 0.25	1.20 ± 0.13	1.83 ± 0.17	1.54 ± 0.22	2.04 ± 0.24	1.00 ± 0	1.53 ± 0.22	0.8631	0.0061	0.3713
Mean number of dilated submucosal gland ducts per mm^2	0 ± 0	1.86 ± 0.25	0 ± 0	4.42 ± 1.23	0 ± 0	3.79 ± 1.19	0 ± 0	5.45 ± 1.09	0.2557	<0.0001	0.6654

| Variables | Groups | | | | | | | | Two-way ANOVA P-values | | |
| | Female | | | | Male | | | | | | |
	Female Saline	Female Acid	Female Saline + DZ	Female Acid + DZ	Male Saline	Male Acid	Male Saline + DZ	Male Acid + DZ	Sex	Treatment	Sex* Treatment
Mean speed (μm/s) of mucus transport	1.32 ± 0.22	0.90 ± 0.19	0.93 ± 0.22	0.71 ± 0.14	1.66 ± 0.16	0.87 ± 0.23	0.89 ± 0.20	1.15 ± 0.24	0.2464	0.0103	0.5626
Mean length (μm) of mucus particle track	1162.0 ± 201.0	789.7 ± 168.7	816.7 ± 141.5	680.6 ± 115.0	1428.0 ± 121.3	762.8 ± 140.4	922.1 ± 208.0	1273.0 ± 191.7	0.0580	0.0117	0.2510
Mean intensity of fluorescently labeled mucus particles on the tracheal surface	14.46 ± 1.08	7.21 ± 1.37	17.41 ± 2.53	9.44 ± 2.55	15.71 ± 2.71	6.46 ± 1.51	11.82 ± 2.13	9.28 ± 2.74	0.4149	0.0103	0.5669