Reactive Species Mediate Inhibition of Alveolar Type II Sodium Transport during Mycoplasma Infection

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Rationale: *Mycoplasma pneumoniae* is a significant cause of pneumonia in humans.

Objectives: To determine the impact of mycoplasma infection and the host inflammatory response on alveolar type II (ATII) cell ion transport *in vivo* and *in vitro*.

Methods: Mice were infected with *M. pulmonis* for measurements of alveolar fluid clearance (AFC) *in vivo* and isolation of ATII cells. ATII cells were infected *in vivo* for determination of epithelial Na⁺ channel (ENaC) total and cell surface protein levels by biotinylation and Western blot and *in vitro* for whole cell patch clamp recording and measurement of nitric oxide (NO) production by chemiluminescence.

Results: Mycoplasma infection significantly inhibited AFC at 24 h and total and amiloride-sensitive AFC by 48 h postinfection (pi). In contrast, infected myeloperoxidase-deficient mice had similar basal and amiloride-sensitive AFC values to uninfected control mice at 48 h pi. Addition of forskolin restored total and amiloride-sensitive AFC to control values at 48 h pi. ATII cells isolated from infected mice demonstrated normal α , β , and γ ENaC total protein levels; however, infected whole-lung cell-surface levels of γ ENaC were significantly decreased. Patch-clamp recordings demonstrated a significant decrease in total and amiloride-sensitive Na⁺ currents at 24 h pi. ATII cells demonstrated a significant increase in the production of NO at 24 h pi and inhibition of NO by ATII cells before infection reversed the decrease in total Na⁺ currents.

Conclusions: These data indicate that mycoplasma infection results in decreased AFC and functional ENaC via the production of reactive oxygen nitrogen intermediates.

Keywords: alveolar fluid clearance; amiloride; chemiluminescence; epithelial sodium channels; nitric oxide synthase; patch clamp

In the United States, mycoplasmas account for up to 20 to 30% of the 4 million cases of pneumonia reported annually and cause significant morbidity and mortality. Murine respiratory infection with *Mycoplasma pulmonis* reproduces the essential features of human respiratory mycoplasmosis. This model has revealed that reactive oxygen and nitrogen species (RONS) produced by the alveolar macrophage (AM) and surfactant protein A, secreted by the alveolar type II (ATII) cells, are essential for early clearance of respiratory mycoplasmas *in vivo* and *in vitro* (1, 2). However, even with significant production of RONS in response to mycoplasma infection, susceptible individuals may develop serious illness. In this context, adherence of mycoplasma species to the respiratory epithelium is necessary for effective coloniza-

tion and subsequent development of disease (3). Because of the broad spectrum of clinical manifestations and the special testing required to distinguish infection, the role of *M. pneumoniae* as a cause of severe disease in the lungs and other organs is underdiagnosed and often inappropriately treated (4). Understanding the basic mechanisms of mycoplasma pathogenesis *in vivo* could have a significant impact on patient care.

Active transport of Na⁺ from the airway lumen to the interstitium by epithelial cells creates an osmotic gradient for movement of water from the airspace to the interstitium (5) and is therefore vital for clearance of fluid from the airspaces. Active Na⁺ transport is dependent on the activity of the apical, rate-limiting, amiloride-sensitive epithelial Na⁺ channels (ENaCs), the basolateral ouabain-sensitive Na⁺/K⁺ ATPase, and basolateral K⁺ channels (6). Previously it was reported that *M. pulmonis* inhibited amiloride-sensitive Na⁺ transport in mouse tracheal epithelium *in vitro* (7). It is unknown if mycoplasma infection compromises Na⁺ transport across the alveolar epithelium and, if so, by which mechanisms and which transporters are affected. We hypothesized that mycoplasma infection impairs alveolar epithelial ion transport by up-regulating the production of RONS, which in turn damage ENaCs.

RONS have been shown to be involved in the development of pulmonary epithelial injury in a variety of pathologic situations, including pneumonia (8, 9). RONS have been shown to inhibit the activity of ENaCs (10–13) and the ATII cell Na⁺/K⁺ ATPase (14–16).

Activated neutrophils also secrete myeloperoxidase (MPO) into the alveolar space, which catalyzes the reaction of nitrite, hydrogen peroxide, and chloride to produce new intermediates capable of nitrating, oxidizing, and chlorinating proteins (17); these events have been shown to decrease ENaC function (18). To determine the impact of RONS and MPO on ion transport during *M. pulmonis* infection *in vivo*, we measured alveolar fluid clearance (AFC) in infected and uninfected mice lacking MPO (MPO^{−/−}). To identify the mechanism by which mycoplasmas damage Na⁺ transport *in vivo*, we measured total and cell-surface α , β , and γ ENaC protein levels in ATII cells and whole lungs from uninfected and mycoplasma-infected mice. In addition, we attempted to reverse the effects of mycoplasma infection by increasing intracellular cAMP levels by intratracheal administration of forskolin. Finally, to determine if mycoplasmas modify ENaCs in the absence of an inflammatory response, we isolated ATII cells from B6 mice, infected them *in vitro*, patched them in whole-cell mode, and measured whole-cell currents in the presence and absence of amiloride and steady-state levels of NO in the medium by chemiluminescence. The patch-clamp measurements were also performed across ATII cells pretreated with the inducible NO synthase (iNOS) inhibitor 1400W. Exposure of ATII cells to mycoplasmas *in vitro* confirms that these cells are capable of producing significant amounts of nitric oxide (NO) in response to pathogens and that NO or its congeners are responsible for the down-regulation of Na⁺ currents. These studies are the first to identify inhibition of ion transport in the lower airway by a bacterial pathogen *in vivo* and offer a putative

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mechanism for mycoplasma-mediated inhibition of amiloride-sensitive Na^+ transport. Likewise, we report for the first time measurements of ENaC subunit levels on the surface of the whole lung after *in vivo* biotinylation and identify electrophysiologic alterations of ion transport by a bacterial pathogen.

METHODS

Mycoplasmas

The University of Alabama CT strain of *M. pulmonis* was used in all experiments (19). For *in vivo* experiments, mice were inoculated intranasally with 10^7 cfu/50 μl . Control mice received an equal volume of sterile broth. Colony-forming units in all inocula were confirmed by enumeration after serial dilution, inoculation of agar plates, and incubation for 7 d.

Animals

B6 mice were obtained from the Frederick Cancer Research and Development Center, National Cancer Institute (Frederick, MD). MPO knockout ($\text{MPO}^{-/-}$) and littermate matched control animals ($\text{MPO}^{+/+}$) were a generous gift from Dr. Bruce Freeman (University of Alabama at Birmingham, Birmingham, AL). Mice were used at 8 to 12 wk of age (20–25 g body weight). All experiments were performed according to the Institutional Animal Care and Use Committee guidelines at the University of Alabama.

Quantitative Lung Cultures

Mice were killed at 72 h postinfection (pi). The lungs were removed aseptically, individually minced, and sonicated for 1 min in sterile broth (Becton Dickinson, Cockeysville, MD). Tenfold serial dilutions were plated onto mycoplasma agar, and the total number of colony-forming units in the lungs of each animal was determined after incubation for 7 d (20).

AFC

AFC was measured as an indication of Na^+ transport across the distal and alveolar lung spaces as previously described (21). Mice were anesthetized and placed on a heating pad to allow maintenance of normal body temperature. The trachea was cannulated and connected to a mouse respirator (model 687; Harvard Apparatus, Holliston, MA), and mice were ventilated with 100% O_2 with a tidal volume of 0.2 ml and frequency of 160 ± 1 breaths/min. Heart rate and rhythm were monitored continuously via a three-lead electrocardiogram (Physio-Control Corp., Redmond, WA). NaCl (0.3 ml; $\sim 30\%$ of total lung capacity) containing 5% fatty-acid-free bovine serum albumin (BSA) was instilled into the tracheal cannula, and room air was infused into the catheter to clear the dead space and position the fluid in the alveolar space. Each mouse was ventilated for 30 min, and the alveolar fluid was aspirated. AFC, expressed as a percentage of total instilled volume, was calculated from the following relationship as described previously (22): $\text{AFC} = (1 - C_i/C_{30})/0.95$, where C_i and C_{30} are the protein concentration of the instillate before instillation and of the alveolar sample at 30 min, respectively. In some mice, amiloride (1.5 mM), forskolin (100 μM), or amiloride plus forskolin were added to the instillate (Sigma Aldrich, St. Louis, MO).

Fluid-filled Lung

AFC was performed using fluid-filled lung (FFL) as described previously (23). Uninfected and mycoplasma-infected mice were killed, and the trachea were exposed and cannulated. Iso-osmotic NaCl (0.7 ml) containing 5% fatty-acid-free BSA (Sigma, St. Louis, MO) with or without amiloride (0–1,000 μM) or benzamil (100 μM ; Sigma Aldrich) was instilled into the lungs, aspirated after 15 min, and AFC calculated.

Cell Isolation

ATII cells were isolated using an adaptation of the method of Warshamana and colleagues (24). Mice were killed, the pulmonary artery was cannulated, and the lungs were perfused with saline. The trachea was cannulated, and dispase (Becton Dickinson, San Jose, CA)

was injected into the lungs followed by 1% low-melting-point agarose to prevent the isolation of Clara cells and upper airway epithelial cells. The lungs were cooled on ice, removed, rinsed with saline, and placed in dispase to digest at room temperature. Lung tissue was teased apart, and the resulting cell suspension was filtered through sterile nylon mesh. AMs and lymphocytes were removed by treatment with biotin-labeled rat polyclonal anti-murine CD45 antibody (Becton Dickinson) and biotin-labeled rat polyclonal anti-murine CD16/CD32 antibodies (Becton Dickinson). Final cell suspensions were pelleted by centrifugation, resuspended in 10% fetal bovine serum in Dulbecco's modified Eagle medium, counted using a hemocytometer, and plated on glass coverslips (1×10^6 cells/coverslip) in the presence or absence of 1400W, a specific iNOS inhibitor. Cytospins were performed for Papanicolaou staining. On average, a mouse yields $4\text{--}8 \times 10^6$ ATII cells ($> 90\%$ purity) by this method. AMs were isolated by bronchoalveolar lavage as previously described (1, 2).

Papanicolaou Staining

Cytospins were dried overnight, stained in Harris' hematoxylin, rinsed, and incubated in a supersaturated lithium carbonate solution. After rinsing, slides were dehydrated through serial ethanol to xylene and mounted under Permount (Fisher Scientific, Suwannee, GA). ATII cells are identified by the presence of dark blue, often refractile, inclusions (25). ATII cell yields (as % of total cells) were 95 to 98% in uninfected and 75 to 95% in infected mice (depending on duration and severity of infection) as detected by this method.

Patch Clamp

Whole-cell currents were obtained from mouse ATII cells plated on coverslips and mounted in a flow-through chamber on the stage of an inverted microscope (DMIRB; Leica, Heidelberg, Germany). Recording pipettes were constructed from borosilicate glass capillaries (Warner Instruments, Inc., Hamden, CT) using a Narishige PC-10 microelectrode puller (Narishige Scientific Instrument Laboratory, Tokyo, Japan) and were fire polished with an MF-830 microforge (Narishige). The pipettes were partially filled with internal standard pipette solution (*see below*) and had tip resistances of 3 to 5 M Ω for whole-cell recordings. Membrane capacitance was measured using pClamp 8.0 software (Axon Instruments, Union City, CA). Membrane capacitance was compensated before the onset of recordings. Currents were recorded using an Axopatch 200B patch-clamp amplifier (Axon Instruments, Union City, CA) and were low-pass filtered at 5.0 kHz (LPF-8; Warner Instruments). All experiments were performed at room temperature (20–22°C).

Just before the start of the experiment, each coverslip was rinsed with external solution with the following ionic composition (in mM): 140 glutamic acid (sodium salt), 4.5 KCl, 3.6 CaCl_2 , 2.4 glutamic acid (magnesium salt), 10 glucose, and 10 *N*-2-hydroxyethylpiperazine-*N'*-ethane sulfonic acid (pH 7.4). Pipettes were backfilled with internal solution with the following ionic composition (in mM): 135 glutamic acid (potassium salt), 2 MgCl_2 , 10 NaCl, 4 Na_2ATP , and 10 *N*-2-hydroxyethylpiperazine-*N'*-ethane sulfonic acid. Recordings were obtained under constant bath perfusion.

Whole-cell currents were elicited by using a step-pulse protocol from -100 to $+100$ mV in 20-mV increments for a 500- μs duration from a holding potential of -40 mV. Current-voltage (*I*-*V*) relationships were constructed by averaging the current values between 400 and 500 ms from the start of recording with the Clampfit Program (Axon Instruments) and plotted using Origin Software (Microcal Software, Northampton, MA). Only those cells with stable baseline currents were included in the results presented here. More specifically, at least three superimposable *I*-*V* relationships were required to proceed with the experiment. In some instances, spontaneous increases in whole-cell currents were observed. In the event of this occurrence, the experiment was discarded. After confirming stable whole-cell currents, cells were perfused with whole-cell external solution containing amiloride (2 μM). In some cases, dimethyl sulfoxide was used as vehicle. For those cells, control currents were obtained in whole-cell external solution containing the same concentration (vol%) of dimethyl sulfoxide as that present in the amiloride solution.

Biotinylation

Biotinylation of lung tissues was performed *in vivo* using a cell-surface protein biotinylation and purification kit (Pierce, Rockford, IL). Briefly, mice were killed, and the caudal vena cava was transected immediately to remove excess blood. The lungs were perfused via the pulmonary artery with a 21-gauge catheter and 0.9% NaCl until white. An 18-gauge cannula was inserted into the trachea, and the lungs were filled with EZ-link sulfo-NHS-SS-biotin (Pierce) for 30 min at room temperature. The fluid was removed, and fresh biotin was instilled every 10 min. The reaction was quenched, and the lung lobes were removed, rinsed in cold 0.9% NaCl, and homogenized in lysis buffer containing protease inhibitors. Clarified protein lysate was mixed with NeutrAvidin gel (Pierce) to isolate biotinylated proteins, which were eluted with 50 mM dithiothreitol sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Protein samples were concentrated using an ultrafree-0.5 concentrator with a molecular weight cutoff of 10 kD (Millipore, Bradford, IL). Protein from four mice was combined for a single sample, and equal amounts were loaded onto gels for Western blots performed as described below. Bands were normalized to total biotinylated protein as identified by probing blots for streptavidin.

Western Blotting

Freshly isolated ATII cells were resuspended in lysis buffer containing 50 mM Tris, 1% Nonidet P-40, 76 mM NaCl, 10% glycerol, 2 mM ethyleneglycol-*bis*-(β -aminoethyl ether)-*N,N'*-tetraacetic acid, and 10 μ g/ml each of phenylmethylsulfonyl fluoride, *N*-*p*-Tosyl-phenylalanine chloromethyl ketone (TPCK) (Sigma-Aldrich), *N* _{α} -Tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK) (Sigma-Aldrich), leupeptin, and antipain. The cells were sonicated for 30 s on ice in 1-ml Eppendorf tubes using an ultrasonic liquid processor (XL2015; Misonix, Inc., Farmingdale, NY). The material was spun at $15,000 \times g$ for 10 min, and the supernatant was stored at -80°C until it was used for Western blotting for α , β , and γ ENaCs as previously described (23). Briefly, the membrane lysate was separated on 7.5% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and immunostained with α (Affinity Bioreagents, Deerfield, IL) β , and γ (Chemicon International, Temecula, CA) ENaC polyclonal antibodies followed by a secondary antibody (anti-rabbit horseradish peroxidase conjugate) and visualized using electrochemiluminescence reagents.

NO Production

NO production by ATII cells and AMs was quantified by reductive denitrosation of samples using a mixture of iodine/iodide in glacial acetic acid and subsequent detection of the liberated NO as nitrite using a tri-iodide-dependent, ozone-based chemiluminescence detector (Eco Medic CLD 88sp; ECO PHYSICS, Ann Arbor, MI). Data were analyzed using ACD/SpecManager software (Advanced Chemistry Development Inc., Toronto, ON, Canada) (26). All samples were run in parallel in the presence of 0.5% sulfanilamide to definitively identify nitrite. Nitrate was reduced to nitrite in all samples before measurements using *Escherichia coli* reductase.

Statistics

All experiments had a minimum of three samples per group. Unless otherwise stated, experiments were performed twice. Parametric data were analyzed by analysis of variance followed by Tukey's multigroup comparison of the means after log conversion or by paired *t* test. Unparametric data were analyzed by Kruskal-Wallis analysis of variance and Pearson's correlation of the means (Analytical Software, St. Paul, MN); *p* values of 0.05 or less were considered significant.

RESULTS

Effects of *M. pulmonis* Infection on AFC In Vivo

B6 mice were instilled with sterile broth or infected with 10^7 cfu of *M. pulmonis*, and AFC was measured 24, 48, and 72 h later. Total AFC was decreased by 46% ($p < 0.0001$) as compared with mock-infected mice at 24 h pi. Approximately the same degree

Figure 1. Effect of *M. pulmonis* on alveolar fluid clearance (AFC) and amiloride sensitivity in anesthetized, ventilated B6 mice. Mice were inoculated with sterile broth or mycoplasmas (10^7 cfu), and AFC was performed 24, 48, or 72 h later. AFC measurements were performed on ventilated mice, and percent clearance was calculated from changes in instilled bovine serum albumin (BSA) concentration during a 30-min period. In all cases, the instillate contained 5% fat-free BSA in NaCl with the osmolality adjusted to that of plasma (321 mOsm/l H_2O). Amiloride (1.5 mM) was added to the instillate as indicated. Data are means $\pm$ SE; $n = 5$ –8 mice for each group. * Denotes statistical significance at $p < 0.05$.

of inhibition was seen at 48 and 72 h pi ($p < 0.01$; Figure 1). In control mice, 73% of AFC was inhibited by amiloride. In contrast, amiloride inhibited only 29% of AFC at 24 h pi ($p = 0.13$) and had no effect on AFC at 48 and 72 h pi (Figure 1). To better assess the effects of mycoplasma infection on amiloride-sensitive AFC, we performed measurements with fluid-filled lungs as described (23). Mice were inoculated with sterile broth or mycoplasmas and killed at 48 h pi (after complete loss of amiloride sensitivity) for AFC. As seen in Table 1 and Figure 2, in this model amiloride (0–25 μM) inhibited AFC in a dose-dependent fashion in mock-infected B6 mice with a K_i of 1.95 μM . This value is similar to the K_i of amiloride inhibition of Na^+ short-circuit current across confluent monolayers of ATII cells (0.85 μM) (11). On the other hand, amiloride significantly increased AFC by two to threefold in mice infected for 48 h. This ability of amiloride to increase AFC in the FFL method was consistent with the small but not significant increase in AFC observed at 48 and 72 h pi after the addition of amiloride in anesthetized ventilated mice (Figure 1). Benzamil (100 μM), a benzyl derivative of amiloride, and a selective and potent inhibitor of Na^+ channels, inhibited AFC of both uninfected and mycoplasma-infected mice to the same extent as 100 μM amiloride (see Table 1).

Effects of MPO on Mycoplasma-mediated Alterations in AFC

MPO is known to catalyze the production of oxidizing, nitrating, and chlorinating intermediates that may modify ion transporters, kill pathogens, and modulate the function of ion channels. Therefore, MPO $^{-/-}$ and littermate-matched MPO $^{+/+}$ mice were infected with 10^7 cfu *M. pulmonis* and AFC, and bacterial lung loads were measured after 24 h. There was no difference in bacterial clearance between B6 ($1.6 \times 10^6 \pm 2.3 \times 10^6$ cfu/ml) and

TABLE 1. INHIBITION OF ALVEOLAR FLUID CLEARANCE BY AMILORIDE OR BENZAMIL IN UNINFECTED OR MYCOPLASMA-INFECTED LUNGS OF B6 MICE

Amiloride or Benzamil (μM)	AFC (% instillate cleared per 30 min)			
	Uninfected (n)		48-h pi (n)	
	Amiloride	Benzamil	Amiloride	Benzamil
0	40 $\pm$ 3.7 (11)	ND	7.5 $\pm$ 1.5 (11)	ND
1	26 $\pm$ 4.1 (7)*	ND	16 $\pm$ 3.2 (8)*	ND
10	19.4 $\pm$ 6.1 (5)*	ND	23 $\pm$ 3.7 (6)*	ND
15	16 $\pm$ 4.2 (7)*	ND	15 $\pm$ 3.2 (8)*	ND
25	9.3 $\pm$ 2.5 (6)*	ND	22 $\pm$ 6.1 (6)*	ND
100	10 $\pm$ 4.6 (6)*	12.7 $\pm$ 4.1 (4)*	15 $\pm$ 3.9 (6)	13 $\pm$ 3.8 (7)
1,000	8.6 $\pm$ 1.94 (9)*	ND	16 $\pm$ 2.6 (9)*	ND

Definition of abbreviations: AFC = alveolar fluid clearance; ND = not determined; pi = postinfection.

Values are means $\pm$ SE; n = number of mice in each group.

*p < 0.05 compared with the 0- μM concentration within the same group.

B6.MPO^{-/-} ($1.58 \times 10^6 \pm 1.3 \times 10^6$ cfu/ml) mice after 24 h pi, confirming that MPO contributes little to early mycoplasma clearance. In contrast, at 24 h pi (data not shown) and 48 h pi, MPO^{-/-} mice had AFC levels equivalent to uninfected basal levels ($44 \pm 5\%$ MPO^{+/+} uninfected, n = 7, vs. $40 \pm 3.6\%$ MPO^{-/-} 48 h pi, n = 5) and retained significant amiloride-sensitive AFC (39%; p = 0.04; Figure 3).

Effects of cAMP on Mycoplasma-mediated Alterations in AFC

Addition of forskolin (100 μM) to the instillate restored AFC at 48 h pi to uninfected levels (from $25 \pm 2.6\%$ in the absence of forskolin [n = 9] to $36 \pm 3.44\%$ in the presence of forskolin [n = 5]). This increase was the result of an up-regulation of amiloride-sensitive pathways because forskolin also restored amiloride-sensitive AFC to uninfected levels (from $30 \pm 2.3\%$

48 h pi + amiloride [n = 8] to $12 \pm 4\%$ 48 h pi + amiloride + forskolin [n = 5]; Figure 4).

Effects of *M. pulmonis* on ENaC Protein Expression

α , β , and γ ENaC protein levels were measured in ATII cells isolated from B6 mice instilled with sterile broth or infected with 10^7 cfu of *M. pulmonis* for 24, 48, and 72 h pi. Protein levels of α , β , and γ ENaCs remained unchanged (n = 3; Figure 5). Specificity of ENaC bands was shown by inhibition with competing peptide (data not shown). To determine if *M. pulmonis* infection decreased levels of ENaCs in the cell membrane, biotinylation of cell-surface proteins was used to measure expression of ENaC subunits *in vivo*. Levels of α ENaCs were unchanged after 48 h of infection; however, there was a small but significant decrease ($\sim 30\%$, p = 0.02) in cell-surface γ ENaC

Figure 2. Amiloride inhibits AFC in uninfected but not in mycoplasma-infected mice *in vivo*. B6 mice were inoculated with sterile broth or mycoplasmas (10^7 cfu) and used in experiments at 48 h postinoculation. The K_i for amiloride was calculated from the dose-response curve in uninfected mice using Microcal Origin 6.0 (Microcal Software, Inc., Northampton, MA) after normalization of data to include the total amiloride-sensitive AFC and the following equation: $I_{AFC} = (1/(1 + IC_{50}/Amil))^H$, where 1 is the percentage of clearance in the absence of amiloride, I_{AFC} is the clearance after exposure to a defined concentration of amiloride (Amil), and H is the Hill coefficient. Data are means $\pm$ SEM.

Figure 3. Contribution of myeloperoxidase (MPO) to *M. pulmonis*-mediated alterations of AFC *in vivo*. MPO^{+/+} and littermate MPO^{-/-} mice were infected with 10^7 cfu *M. pulmonis*, and AFC was measured 48 h later. AFC measurements were performed on ventilated mice, and percentage of clearance was calculated from changes in instilled BSA concentration during a 30-min period. Data are means $\pm$ SE; n = 5–9 animals for each group. In all cases, the instillate contained 5% fat-free BSA in NaCl with the osmolality adjusted to that of murine plasma (322 mOsm/kg H₂O). Amiloride (1.5 mM) was added to the instillate as indicated. Black bars, uninfected; hatched bars, amiloride; gray bars, 48-h postinfection; cross-hatched bars, 48-h postinfection amiloride. * Significant difference from uninfected same strain control; † significant difference from 48-h infected group (p = 0.04).

Figure 4. Effect of forskolin on *M. pulmonis*-mediated alterations of AFC *in vivo*. B6 mice were inoculated with sterile broth or mycoplasmas (10^7 cfu), and AFC was measured 48 h later. AFC measurements were performed on ventilated mice, and percentage of clearance was calculated from changes in instilled BSA concentration in the presence or absence of forskolin (100 μ M) during a 30-min period. Data are means $\pm$ SE; $n = 5$ –9 animals for each group. In all cases, the instillate contained 5% fat-free BSA in NaCl with the osmolality adjusted to that of murine plasma (322 mOsm/l H_2O). Amiloride (1.5 mM) was added to the instillate as indicated. * Significant difference from uninfected; † significant difference from 48-h infected ($p < 0.05$).

expression in infected lungs as compared with uninfected control specimens ($n = 3$ –4; Figure 6).

Effects of *M. pulmonis* on AII Cell Whole-Cell Currents

Average current–voltage relationships from whole-cell recordings of mouse AII cells inoculated with sterile broth or infected with mycoplasmas are presented in Figure 7. Data are presented as pA/pF to ensure that cell size, as reflected by changes in capacitance, did not contribute to alterations in current with mycoplasma infection. Capacitance measurements were not different for uninfected and infected cells (16.54 ± 2.0 pF, $n = 26$, vs. 14.78 ± 0.71 pF, $n = 20$, respectively). Whole-cell currents from uninfected mouse AII cells were outwardly rectified; the addition of amiloride (2 μ M into the bath solution) decreased the inward ($n = 7$; Figures 7A and 8) and outward currents (Figure 8). In contrast, AII cells infected with mycoplasmas for 24 h had considerably lower inward currents (Figure 7B), which were insensitive to amiloride ($n = 10$; Figures 7C and 8). Comparison of infected to uninfected current–voltage relationships showed that mycoplasmas inhibited whole-cell currents to levels comparable to amiloride inhibition in uninfected cells (Figure 7D).

Production of NO by Mouse AII Cells *In Vitro*

Mouse AII cells (2×10^6) were plated on a 24-well plate and cultured for 24 h before inoculation with sterile broth or mycoplasmas. Mouse AMs were collected, plated, and inoculated at the same time as a positive control. Media controls were plated and inoculated at the same time in the absence of cells to allow for the subtraction of any nitrite contamination from reagents. Cell supernatants were collected after 24 h pi for detection of NO by chemiluminescence. Infection with mycoplasmas increased AII and AM NO production by 40% ($p = 0.0009$) and 800%, respectively ($n = 6$; Figure 9).

Effects of *M. pulmonis* and NO on AII Cell Whole-Cell Currents

To determine the contribution of increased NO production to decreased whole-cell basal currents during mycoplasma infec-

Figure 5. α , β , and γ epithelial sodium channel (ENaC) subunit levels in alveolar type II (AII) cells from B6 mice infected with *M. pulmonis*. Western blotting studies in proteins extracted from AII cells isolated from B6 mice inoculated with sterile broth (U = uninfected) or infected with mycoplasmas for 24, 48, or 72 h. Equal amounts of protein, extracted from AII cells, were separated using 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, immunostained with anti-epithelial Na^+ channel antibodies followed by anti-rabbit horseradish peroxidase conjugate as the secondary antibody, and developed by electrochemiluminescence. (A) Representative blot. Number adjacent to the arrow indicates the molecular weight of the noted band (kD). Identification of bands was confirmed by competitive inhibition with immunizing peptide (data not shown). (B) Combined densitometry of all blots normalized to actin levels. $n = 3$ for α and γ ; $n = 2$ for β .

tion, AII cells were incubated in the presence or absence of the specific iNOS inhibitor 1400W (10 μ M) before and after infection with *M. pulmonis*. Infection of AII cells with mycoplasmas resulted in a 300% decrease in inward basal currents, whereas inhibition of NO in infected AII cells caused a significant increase in whole-cell inward currents by 100% in mycoplasma-infected AII cells (Figure 10).

DISCUSSION

M. pneumoniae has been identified as the primary causative agent of serious pneumonia requiring hospitalization among adults in as high 29% of cases in 10 prospective studies (27, 28), with the incidence of *M. pneumoniae* as a cause of pneumonia requiring intensive care unit placement reportedly as high as

Figure 6. *In vivo* cell surface levels of ENaC subunits in B6 mice infected with *M. pulmonis*. Biotinylation of lung cell-surface proteins was performed *in vivo* using EZ-link sulfo-NHS-SS-biotin (Pierce Chemical, Rockford, IL) on B6 mice inoculated with sterile broth (U = uninfected) or infected with mycoplasmas for 48 h. NeutrAvidin gel (Pierce Chemical) was used to isolate biotinylated proteins from clarified protein lysates, and proteins were eluted with dithiothreitol sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer. Equal amounts were loaded onto gels for Western blot analysis of α , β , and γ ENaCs, and bands were normalized to total biotinylated protein as identified by probing blots for streptavidin. (A) Representative blot. Number adjacent to the arrow indicates the molecular weight of the noted band (kD). Identification of bands was confirmed by competitive inhibition with immunizing peptide (data not shown). (B) Combined densitometry of all blots normalized to total biotinylated protein. $n = 3$ for α , and $n = 4$ for γ . Black bars, control; hatched bars, 48 h postinfection. * Significant difference from uninfected mouse levels; $p = 0.02$.

7% (28). Subclinical mycoplasma infections may be associated with serious secondary sequelae, such as asthma and chronic obstructive pulmonary disease. This study is directed at understanding one aspect of the mechanisms by which mycoplasmas and the resulting inflammatory response damages the alveolar epithelium, which may contribute to the pathogenesis of mycoplasma pneumonia. Pathologic studies on patients with diagnosed *M. pneumoniae* pneumonia demonstrate significant neutrophilic exudate within the bronchioles, lymphoplasmacytic infiltrate around airways, and hyperplasia of ATII cells (29, 30). Significant pulmonary complications may occur, including pleural effusion, pneumatocele, lung abscess, pneumothorax, bronchiectasis, chronic interstitial fibrosis, and respiratory distress syndrome (reviewed in Reference 27). Likewise, bronchiolitis obliterans with fibrin deposits within intraalveolar lesions and respiratory insufficiency have been reported as a commonly unrecognized complication of *M. pneumoniae* infection (27, 30–32).

For more than 20 yr, the mouse has provided a widely accepted animal model for the study of antimycoplasma defenses (19, 33–36). The pathologic features of *M. pulmonis* respiratory infection in the mouse directly mirror those reported for *M. pneumoniae* in humans (29, 30, 37). The results of our *in vivo* studies indicate (1) that infection of B6 mice with mycoplasmas decreases Na^+ -dependent AFC across the distal lung and alveolar epithelium and (2) that this effect is mediated by RONS, primarily via products of MPO catalyzed reactions (most likely nitrogen dioxide [38]). In addition, our *in vitro* studies indicate that infection of ATII cells with mycoplasmas down-regulates epithelial Na^+ channel function via an iNOS-related mechanism.

Active Na^+ transport across the alveolar epithelium requires the coordinated entry of Na^+ ions through amiloride-sensitive channels located in the apical membranes of ATI and ATII cells, the extrusion of Na^+ ions across the basolateral membrane by the $\text{Na}^+\text{-K}^+\text{-ATPase}$, and the exit of K^+ ions through basolateral K^+ channels. Although the entry of Na^+ ions through amiloride sensitive channels is thought to be a rate-limiting process, injury to any of these transporters may compromise vectorial ion transport and the removal of fluid from the lung (reviewed in Reference 39). Our *in vitro* measurements showing that infection of mouse ATII cells with mycoplasmas decreases amiloride-sensitive whole-cell currents indicate that amiloride-sensitive Na^+ channels have been injured. In this case, injury occurred in the absence of MPO-catalyzed reactions. However, mycoplasma infection did increase NO production by these cells (most likely through the up-regulation of iNOS), and NO has been shown to inhibit amiloride-sensitive Na^+ transport *in vitro* and *in vivo* (11, 12, 40).

Previously, we reported similar levels of nitrotyrosine content in the lungs of B6 and B6.iNOS $^{-/-}$ mice infected with mycoplasmas and that pretreatment of B6.iNOS $^{-/-}$ mice with Cytoxin (to decrease circulating and lung inflammatory cells) before infection significantly decreased MPO, neutrophil infiltration, nitrotyrosine formation, and tissue damage (41). These studies also demonstrated that peroxidases released by inflammatory cells—not NO by iNOS—was the limiting factor for nitrotyrosine formation. iNOS has been demonstrated to colocalize with MPO (42), allowing for the simultaneous presence of nitrogen oxides to act as a substrate for MPO. Peroxidases have been demonstrated to catalyze the nitration of tyrosine residues in the presence of H_2O_2 and nitrite (43), and nitrite has been shown to act as a substrate for MPO resulting in the nitration, oxidation, and chlorination of proteins (44). In the present study, the lack of MPO as a substrate for nitration reactions in mycoplasma-infected MPO $^{-/-}$ mice allowed for the return of AFC and amiloride sensitivity to near normal levels (*in vivo* relevance of protein nitration reviewed in Reference 45), indicating the importance of MPO and the products of the reactions it catalyzes to apical Na^+ transporters.

The ability of RONS to modulate ion-channel activity has become an area of intense interest. Recently, Lazrak and colleagues (46) and Jain and colleagues (47) reported that NO suppressed the activity of a cation channel in the apical membrane of A549 and freshly isolated rat ATII cells through a cGMP-dependent protein kinase pathway. Furthermore, NO donors added to the apical compartments of Ussing chambers containing rat ATII cell monolayers inhibited Na^+ transport (11) through cGMP-independent mechanisms. Excessive production of reactive oxygen species may result in post-translational modification of ENaC proteins through nitration or oxidation of limited domains or specific key amino acid residues (13, 18, 48, 49). Likewise, reactive oxygen species may directly alter channel protein activity (11) or cytoskeletal structures, which are required for proper Na^+ channel function (50). Mycoplasma infection

Figure 7. Effect of *M. pulmonis* infection on basal and amiloride-sensitive whole-cell current-voltage (I-V) relationships. Whole-cell currents were recorded from mouse ATII cells plated on glass coverslips for 24 h and inoculated with sterile broth or mycoplasmas for 24 h. (A) I-V relationship of basal and amiloride sensitive currents from uninfected ATII cells. (B) I-V relationship of basal currents from uninfected and infected ATII cells. (C) I-V relationship of basal and amiloride-sensitive currents from infected ATII cells. (D) I-V relationship of basal current from infected and basal and amiloride-sensitive current from uninfected ATII cells. Data are means $\pm$ SE. $n = 7$ separate cell patches for uninfected cells and $n = 10$ for infected cells.

causes an acute inflammatory response characterized by significant accumulation of neutrophils and nitration of tissues (41).

Cytoadherence of mycoplasmas to the epithelium is regarded as the primary virulence factor, with adherence-deficient mutants

being avirulent (51). Mycoplasmas are unique among bacteria in that they lack a rigid cell wall, so that upon attachment the bacterial membrane is closely associated with the cytoplasmic membrane of the host. Fusion of mycoplasmas with the host cell has been reported with delivery of mycoplasma cellular content into the host cell and insertion of mycoplasma membrane components into the host cell membrane (52). Despite the fact that adherence to the respiratory epithelium is necessary for effective

Figure 8. Effect of *M. pulmonis* infection on amiloride-sensitive whole-cell currents. Mouse ATII cells were plated on glass coverslips for 24 h and inoculated with sterile broth or mycoplasmas for 24 h. Whole-cell currents were recorded at -100 mV to $+100$ mV. Data are means $\pm$ SE. $n = 7$ separate cell patches for uninfected cells, and $n = 10$ for infected cells. Solid squares, infected Δ amiloride (difference current before and after perfusion with $2 \mu\text{M}$ amiloride); solid circles, control Δ amiloride.

Figure 9. Production of NO by ATII cells *in vitro*. Mouse ATII cells and alveolar macrophages (AMs) were inoculated with sterile broth or mycoplasmas at a multiplicity of infection of 1:3 (mycoplasma/cell) at 24 h postinoculation. Medium was collected for NO determination at 24 h postinoculation. Data are presented as a percentage of control set at 100% (means $\pm$ SE, $n = 6$).

Figure 10. Effect of *M. pulmonis* infection and NO on basal whole-cell current–voltage relationships. Whole-cell currents were recorded from mouse ATII cells plated on glass coverslips for 24 h and inoculated with sterile broth or mycoplasmas for 24 h in the presence or absence of the iNOS inhibitor 1400W (10 μ M). Graph represents percentage of change in current (pA/pF) showing the percentage difference between mycoplasma infected (100%; black bars) and uninfected basal currents (data from Figure 7: infected ATII cells set at 100%, $n = 10$; uninfected ATII cells, $n = 7$; narrow-hatched bars) and the increase in basal current with treatment of infected cells with 1400W (infected ATII cells set at 100%, $n = 6$; infected cells + 1400W, $n = 6$; wide-hatched bars).

colonization and development of disease (3), the impact of mycoplasma infection on respiratory epithelial ion transport (which governs mucus and fluid homeostasis) is poorly understood. Previous *in vitro* studies have demonstrated that the murine pathogen *M. pulmonis*, but not the nonpathogenic *M. fermentans*, rapidly inhibits amiloride-sensitive Na^+ absorption and cholinergic-stimulated Cl^- secretion by mouse tracheal epithelial cells, which is consistent with mucus accumulation and impaired pulmonary clearance in this disease (7). Similarly, *M. hypopneumoniae* infection of cultured bronchial epithelial cells has been shown to disrupt K^+ channels resulting in ciliostasis (53). Likewise, *Pseudomonas aeruginosa* rhamnolipids have been shown to inhibit amiloride-sensitive Na^+ transport by cultured ovine tracheal epithelium (54), whereas *P. aeruginosa* hemolysin blocks active Na^+ uptake and Cl^- secretion by canine bronchial epithelium *in vitro* (55). However, the interpretation of *in vitro* results with respect to *in vivo* disease is difficult (i.e., what percent inhibition of amiloride-sensitive Na^+ absorption *in vitro* is necessary for mucus accumulation, edema formation, and lung dysfunction during infection *in vivo*). There is little *in vivo* data on the impact of live pathogens on ion transport properties of the lung. In contrast to previous *in vitro* results, infection of rats with *P. aeruginosa* resulted in an amiloride sensitive increase in AFC *in vivo*, which was attributed to an increase in tumor necrosis factor α (56). Similar to mycoplasma infection, intranasal infection with respiratory syncytial virus A2 resulted in reduced basal AFC *in vivo* by 2 d and a loss of sensitivity to amiloride by 2 and 4 d after infection; however, this was shown to be caused by increased release of 5' nucleotides from the respiratory epithelium (22). Regardless, *in vivo* correlation for mycoplasma disruption of ion transport *in vitro* and the mechanism(s) involved has not been identified.

Studies have shown that under normal conditions, AFC is totally dependent on the movement of Na^+ ions across the alveolar epithelium and that amiloride may inhibit 60–90% of this transport in the mouse (57, 58). Mycoplasma infection inhibited up to 46% of AFC over a 30-min period after 24 h of infection. Amiloride-sensitive AFC was decreased at 24 h pi, with a com-

plete loss of amiloride-sensitive AFC apparent at 48 and 72 h pi. These data support previous *in vitro* data demonstrating a loss of amiloride-sensitive ion transport in mouse tracheal epithelial cells infected with *M. pulmonis* for 48 h. At 48 and 72 h pi, AFC was increased in the presence of amiloride. Ion channels have been shown to be important for pathogenesis of some simple parasites and viruses (e.g., Influenza virus). *M. fermentans* has been shown to have a K^+ channel with similar properties to eukaryotic K^+ channels, so it is possible (although unlikely) that K^+ may substitute for Na^+ upon the addition of amiloride and therefore normalize AFC. Likewise, it was recently reported that edema fluid is capable of inducing amiloride-insensitive α ENaC-independent channel activity in rat distal lung epithelium (59).

The amiloride-sensitive ENaCs are the main pathway for Na^+ entry for lung airway and alveolar epithelium. In the present *in vivo* studies, a high dose of amiloride (1.5 mM) was added to the instillate based on previous studies using ventilated mice that demonstrated decreased alveolar concentrations of amiloride over a 30-min period. A static FFL method was therefore used to determine if mycoplasma infection was shifting the K_i for amiloride such that amiloride-sensitive AFC would be difficult to detect given the limitations of dosage in the ventilated mouse model. This method allows for enhanced uniform distribution of amiloride within the alveolar space and permits the use of lower concentrations of amiloride for the inhibition of fluid clearance. AFC was measured at 48 h pi because this was when there was a complete loss of amiloride-sensitive AFC. Using the FFL method, no amiloride-sensitive AFC was detected in infected mice even at 1,000 μM amiloride. However, consistent with the data generated with the ventilated mouse model, there was a significant increase in fluid clearance at several doses of amiloride (10, 15, 25, and 1,000 μM as compared with 0 μM) in the infected mice, which may reflect the presence of increased edema fluid with blocked Na^+ resorption and concomitant up-regulation of amiloride-insensitive channels (59). Alternatively, it has been previously reported that amiloride can stimulate channel activity when the chemical composition of the extracellular environment is altered (60). Although the ability of amiloride to significantly increase (if only slightly) AFC in infected mice seems to be counterintuitive, the fact that benzamil stimulates the same response suggests that this is a real phenomenon and indicates the presence of a set of unidentified channels capable of contributing to fluid clearance during times of stress with appropriate stimulation. Forskolin, an agent that increases intracellular cAMP levels, also corrected the mycoplasma-mediated inhibition of AFC by increasing amiloride-sensitive fluid clearance. Previous studies in a variety of species have shown that intratracheal instillation of agents that increase cAMP up-regulate the amiloride sensitive (57) or the amiloride-insensitive fraction of AFC (61). Patch-clamp studies of uninfected primary rat ATII cells *in vitro* indicate that increased cAMP increases the open time and probability of amiloride-sensitive single channels (62, 63), although recently forskolin was reported to up-regulate amiloride-insensitive AFC in ventilated uninfected B6 mice *in vivo* (21). Similarly, hypoxia was shown to decrease Na^+ channel activity in rat ATII cells by reducing levels of ENaC subunits, an effect that was corrected by the stimulation of cAMP with terbutaline (64, 65). The ability of forskolin to correct AFC inhibition during mycoplasma infection is consistent with the insertion of new channels to the epithelial cell surface or an increase in the opening time of functional ENaCs. Taken together, these studies suggest that increased concentrations of β_2 -adrenergic agonists in situations involving hypoxia (64, 65), acid aspiration (66), and certain bacterial pneumonias may prove to

be clinically relevant for improving outcome in patients with alveolar edema.

Western blot analysis of ATII cells isolated from infected or uninfected mice demonstrated no significant alterations in ENaC subunit levels. These data are consistent with previous reports showing that inhibition of AFC during RSV infection was not related to alterations in gene expression (22). The Western blot data, combined with our earlier finding that mycoplasma infection decreases amiloride sensitive AFC at 48 and 72 h pi, suggest two possibilities: (1) disruption of the interaction among α , β , and γ ENaCs or (2) post-translational modification of ENaC (nitration or oxidation). Although total protein levels remained constant, cell-surface levels of γ ENaCs were significantly decreased, suggesting at least a partial explanation for decreased functionality of ENaCs. Biotinylation studies with rat (64, 67) and mouse (68) ATII cells have used confluent or semiconfluent monolayers of cells *in vitro*. In the present study, we biotinylated cell-surface proteins *in situ*, thus ensuring accurate orientation of cells and circumventing any impact that isolation and *in vitro* culture techniques may have on the composition and orientation of cell surface proteins. This is the first demonstration of *in situ* biotinylation of lung cell-surface proteins and should allow for more accurate determination of apical cell membrane composition. Unfortunately, we were unable to detect β ENaC subunit proteins using this technique, presumably due to the small amount of this protein present in a sample.

There are limited studies detailing the effect of pathogens on the electrophysiology of lung cells. Recently it was reported that influenza virus (strain A/PR/8/34) caused rapid inhibition of apical Na^+ channels in rat ATII cells *in vitro* and decreased amiloride-sensitive fluid clearance *in vivo* based on wet/dry ratios (18). However, these studies must be interpreted carefully because this strain of influenza cannot replicate or produce an active infection. Infection of primary mouse ATII cells *in vitro* with the pathogenic strain *M. pulmonis* caused significant reduction of whole-cell basal currents and a complete loss of amiloride-sensitive currents after 24 h of infection. These data correlate with earlier reports of dose- and time-dependent mycoplasma-mediated inhibition of amiloride-sensitive transport in mouse tracheal epithelium (7). Because vectorial Na^+ transport across the alveolar epithelium involves a number of transporters and pumps, it is possible that mycoplasma-induced injury to K^+ channels and the Na^+/K^+ ATPase could affect Na^+ channel function. However, for the purposes of this article, patch-clamp studies were designed specifically to characterize Na^+ channel function only. The reversal potentials generated in the whole-cell patch-clamp studies indicate that the detected channels were cation channels with equal permeability to Na^+ and K^+ ions. In spite of this, mouse ATII cells infected *in vitro* lack significant interaction with inflammatory cells thought to be responsible for decreased AFC with infection *in vivo*. Correlation of *in vitro* data to the *in vivo* disease state is often difficult. Primary ATII cells have been shown to up-regulate iNOS and NO production in response to hyperoxia (21), diesel particles (69), and endotoxin (56). The ability of mycoplasmas to significantly increase NO production after 24 h indicates that damage from reactive nitrogen species to ENaC under these conditions remains a possibility. The ability of the iNOS inhibitor 1400W to partially reverse the mycoplasma-mediated inhibition of whole-cell basal currents indicates that NO is important for this effect. The fact that 1400W does not completely reverse the inhibition of basal current suggests that mycoplasmas alone may contribute to epithelial cell ion transport dysfunction during infection. Likewise, ATII cells in culture miss a number of the primary antioxidant properties of the whole lung and are exposed to a significantly greater multiplicity of infection than would be expected *in vivo*. These

latter differences may explain the difference in timing after mycoplasma infection between *in vivo* (48 h, AFC) and *in vitro* (24 h, patch clamp) for loss of amiloride-sensitive channels.

Inhibition of fluid transport with the concomitant accumulation of mucus and fluid in the lungs correlates well with the pattern of disease in severe mycoplasma pneumonia. The fact that mycoplasmas alter ENaC function is supported by AFC measurements in ventilated mice *in vivo* and by whole-cell patch-clamp data *in vitro*. This study provides new evidence on the ability of pathogens to affect ion transport and supports the concept that RONS produced during the inflammatory response can modify the Na^+ transport properties of the respiratory epithelium.

Conflict of Interest Statement: None of the authors have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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