Nucleotide-mediated inhibition of alveolar fluid clearance in BALB/c mice after respiratory syncytial virus infection

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Davis, Ian C., Wayne M. Sullender, Judy M. Hickman-Davis, J. Russell Lindsey, and Sadis Matalon. Nucleotide-mediated inhibition of alveolar fluid clearance in BALB/c mice after respiratory syncytial virus infection. Am J Physiol Lung Cell Mol Physiol 286: L112–L120, 2004. First published August 29, 2003; 10.1152/ajplung.00218.2003. —Respiratory syncytial virus (RSV) is the most common cause of lower respiratory tract disease in infants and children worldwide. Intranasal infection of BALB/c mice with RSV strain A2, but not ultraviolet-inactivated RSV, for 2 or 4 days reduced basal alveolar fluid clearance (AFC), a seminal function of bronchoalveolar epithelium, and caused loss of AFC sensitivity to amiloride inhibition. Reduced AFC was temporally associated with increased lung water content but was not a consequence of increased epithelial permeability or cell death. Reduced AFC was also not due to decreased transcription of epithelial Na⁺ channel subunit genes in lung tissue. RSV-mediated inhibition of AFC 2 days after infection was rapidly prevented by addition to the instillate of P2Y receptor antagonists (suramin and XAMR-0721) or enzymes that degrade UTP, but not those that degrade ATP. After UTP degradation, AFC returned to control levels but was no longer sensitive to amiloride. UTP at nanomolar concentrations recapitulated the AFC inhibitory effect of RSV in normal mice and mice infected with RSV for 6 days, indicating that normalization of AFC at this time point is a consequence of cessation of UTP release, rather than P2Y receptor desensitization. We conclude that RSV infection of the bronchoalveolar epithelium results in reduced AFC as a consequence of autocrine feedback inhibition mediated by UTP. These studies are the first to demonstrate AFC inhibition by an important pulmonary viral pathogen. Reduced AFC may result in formation of an increased volume of fluid mucus, airway congestion, and rhinorrhea, all features of severe RSV disease.

epithelial sodium channel; UTP; ATP; purinergic receptor; lung

THE PNEUMOVIRUS respiratory syncytial virus (RSV) is the most common cause of lower respiratory tract disease in infants and children worldwide (4), but the pathogenesis of bronchiolitis and severe pneumonia in primary RSV infection remains poorly understood (35). Interactions between RSV and the bronchoalveolar epithelium have not been well characterized. In particular, the effects of RSV infection on ion transport, which is a seminal function of bronchoalveolar epithelial cells, have not been investigated.

The dominant ion transport process of normal alveolar epithelial cells is active, amiloride-sensitive transport of Na⁺ from the alveolar lining fluid (ALF) to the interstitium (29). Transepithelial movement of Na⁺ creates an osmotic gradient, which causes water to move passively from the air space to the interstitium. This process of alveolar fluid clearance (AFC) is crucial to clearance of air space fluid and maintenance of a thin ALF, which permits efficient gas exchange and normal mucociliary clearance function.

The importance of active Na⁺ transport to this process of AFC has been clearly demonstrated experimentally (29). Moreover, morbidity and mortality are lower in patients with acute lung injury or acute respiratory distress syndrome with normal AFC than in those with compromised AFC (30). Finally, patients with systemic pseudohypoaldosteronism, caused by loss of function mutations in the genes encoding epithelial Na⁺ channel (ENaC) subunits, completely lack electrogenic Na⁺ transport in the upper and lower airways, resulting in a doubling of ALF volume, persistent rhinorrhea, and recurrent respiratory illness (18).

We hypothesized that inhibition of epithelial Na⁺ transport by RSV may contribute significantly to the pathogenesis of RSV bronchiolitis. We found that replicating RSV reduces the AFC capacity of the bronchoalveolar epithelium in vivo without inducing detectable epithelial cell death or an increase in alveolar permeability to albumin. RSV-mediated inhibition of AFC was not due to reduced ENaC subunit gene transcription but was mediated by UTP acting on P2Y receptors (P2YR) on bronchoalveolar epithelial cells. These studies are the first to show that RSV inhibits AFC by inducing 5′-nucleotide release into the alveolar space and that UTP can modulate Na⁺ transport across bronchoalveolar epithelial cells in vivo.

MATERIALS AND METHODS

Preparation of viral inocula. Viral stocks were grown in monolayers of Hep-2 cells. Cells and media were harvested when 80–90% cytopathic effect was observed and centrifuged to remove cell debris. The medium was further clarified by ultracentrifugation through 35% sucrose (31). Virions were resuspended in fresh medium, aliquoted, and rapidly frozen at −80°C. Viral titers were determined by serial dilution and plaque assay in Vero cells under agar (43). Plaque-forming units (pfu) per milliliter of original sample were calculated. Virus preparations were checked for mycoplasma contamination by PCR using the Mycoplasma Plus PCR primer set (Stratagene, La Jolla, CA) and HotStarTaq DNA polymerase (Qiagen, Valencia, CA) in accordance with the manufacturers’ instructions. Endotoxin content of viral stocks was determined by a standard Limulus amoebocyte assay. Stocks in which mycoplasma or endotoxin contamination was detected were discarded. A mock-infected stock, prepared in an identical fashion, served as a control to account for possible effects of cellular components in the viral inoculum.

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UV inactivation of RSV. Aliquots of RSV stocks were inactivated by exposure to 1,800 mJ of radiation in a Stratalinker UV cross-linker (Stratagene), eliminating viral infectivity without altering the conformation of viral proteins and mediators (17). UV inactivation reduced RSV infectivity to 1 pfu/ml (a 7-log-fold reduction).

Animals. Eight- to 12-wk-old BALB/c mice of either gender, maintained in autoclaved microisolators, were used in these studies. The pathogen-free status of the animals was monitored by culture for mycoplasmal, viral, fungal, and bacterial pathogens (Charles River Biotechnical Services, Wilmington, MA) (36). Animals were given sterile autoclaved food and water ad libitum and monitored daily for signs of respiratory distress or other illness. To ensure that respiratory function was not compromised by buildup of ammonia in the litter (39), bedding was changed every 2–3 days. All mouse experiments were performed in animal biosafety level 2 facilities at the University of Alabama at Birmingham with use of procedures approved by its Institutional Animal Care and Use Committee and in accordance with federal guidelines.

Mouse infection protocol. Mice were infected intranasally dropwise with 10⁵ pfu of RSV strain A2 (in 100 µl, with 50 µl administered to each nostril) under light anesthesia with ketamine (0.87 mg/100 g body wt ip; Abbott Laboratories, Chicago, IL) and xylazine (0.13 mg/100 g body wt ip; Vedco, St. Joseph, MO). Mock-infected mice received an equal volume of autologous Hb-2 cells. Uninfected animals received no instillate. In certain experiments, mice were infected with 100 µl of UV-inactivated RSV strain A2. Mice were placed in lateral recumbency, allowed to recover, and returned to their cage. In some experiments, mice were individually marked and subsequently weighed on a daily basis. For all studies, data for each experimental group were derived from a minimum of two independent infections.

Virus isolation and quantitative culture. Viral replication in lungs of infected mice was quantified as previously described (43, 44). The number of plaque-forming units of virus per gram of lung tissue was calculated.

Lung histopathology. Uninfected, mock-infected, and RSV-infected mice were euthanized, and the larynx, trachea, and lungs were removed en bloc. The lungs were inflated with 10% formalin in 70% ethanol and fixed. After fixation, each of the five lobes was trimmed along its airway and vascular arborization, embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin. Histological changes (exudate, epithelial change, lymphoid hyperplasia, and parenchymal lesions) were evaluated separately for each lobe in a blinded fashion by an independent observer and allocated a score on a scale of 0 (normal tissue) to 4 (severe pathology) according to the procedure of Cartner et al. (3).

Detection of apoptotic cells in lung tissue. Apoptotic cells in thin sections of lung tissue were detected using the ApopTag peroxidase in situ oligo ligation kit (Intergen, Norcross, GA) in accordance with the manufacturer’s instructions. Positive control sections (provided with the kit) were assayed simultaneously with test sections to verify the efficacy of the assay.

Bronchoalveolar lavage. Bronchoalveolar lavage (BAL) was performed on uninfected, mock-infected, and RSV-infected mice. Mice were euthanized (as above), and a trimmed sterile 18-gauge Surflo intravascular catheter (Terumo Medical, Elkton, MD) was inserted caudally into the lumen of the exposed trachea. The lungs were then lavaged in situ with two separate 1-ml washes of sterile normal saline. Lactate dehydrogenase (LDH) content was evaluated using LDH colorimetric end-point assay kit (Sigma-Aldrich, St. Louis, MO).

AFC measurements. AFC was measured as previously described (14). Amlodipine-sensitive AFC (ΔAFCa) was calculated as the percentage of mean basal AFC that was blocked by amlodipine (Sigma-Aldrich). Protein concentrations in AFC samples were measured using the bicinchoninic acid protein assay (Pierce Endogen). A standard curve was prepared by assaying known concentrations of BSA in 0.9% NaCl.

Measurement of AFC assumes that the concentration of protein is not reduced significantly by the presence of alveolar fluid. To test this assumption, in some studies, the alveolar instillate was withdrawn 30 s after its instillation, and its protein concentration was measured. The quantity of alveolar fluid leaving the alveolar space within this time period should be minimal, so changes in albumin concentration reflect the amount of alveolar edema.

5′-Nucleotide binding to purinoceptors was inhibited by addition of suramin (Sigma-Aldrich) or XAMR-0721 (Calbiochem, La Jolla, CA) to the AFC instillate. Hexokinase (grade IV, 2 U/ml; Sigma-Aldrich) was added to convert ATP to ADP, which then was used to metabolize ATP to ADP in the presence of 10 µM glucose. Arylhydrolase (grade VII, 2 U/ml; Sigma-Aldrich) was used to degrade UTP to UDP in the presence of 10 µM UDP-glucose pyrophosphorylase (UDP-G-PP, type X, 2 U/ml; Sigma-Aldrich). Each of these substances was added to the AFC instillate. Hexokinase (grade IV, 2 U/ml; Sigma-Aldrich) was used to degrade ATP to ADP and to instillates to metabolize ATP to ADP in the presence of 10 µM glucose. Arylhydrolase (grade VII, 2 U/ml; Sigma-Aldrich) was used to degrade UTP to UDP in the presence of 10 µM UDP-glucose 1-phosphate and 2 µM inorganic pyrophosphatase. During inhibitor studies, basal AFC was concomitantly measured in pairs of untreated AFC-infected mice to confirm infection and inhibition of AFC by the virus.

Measurement of lung wet-to-dry weight ratio. Lung wet-to-dry weight ratio was measured as previously described (28). Briefly, mice were euthanized and exsanguinated, and their lungs were removed, weighed, and dried in an oven at 55°C for 7 days. After they were dried, the lungs were weighed, and dried to a constant weight. The wet-to-dry weight ratio was then calculated as an index of intrapulmonary fluid accumulation. No correction for blood content was made. Lung water content was calculated as the difference between wet and dry weight. Percent changes in mean lung water content at each time point were calculated relative to the mean water content of lungs from mock-infected mice.

Measurement of alveolar barrier function by radiolabeled albumin efflux. AFC measurement assumes that there is no significant flux of albumin across the alveolar epithelium during the experimental period. To test this assumption, isosomotic NaCl containing 5% BSA and 1 µCi of 125I-labeled albumin (ICN Pharmaceuticals, Costa Mesa, CA) was instilled into the lungs of mice from the various experimental groups. AFC was measured, and 125I translocation was determined as previously described (14).

Isolation of RNA from mouse lung tissue. Total RNA was isolated from 30 mg of fresh lung tissue for each mouse using the RNeasy Mini kit (Qiagen) according to the manufacturer’s instructions.

Reverse transcription. cDNAs were generated by reverse transcription using the Omniscript RT kit (Qiagen) according to manufacturer’s instructions. Negative control reactions (for genomic DNA contamination) were performed in the absence of reverse transcriptase.

Preparation of plasmid standards for real-time PCR. Total RNA was isolated from rat alveolar type II cells and reverse transcribed as described above. The α-, β-, and γ-enac and gapdh gene segments were amplified by PCR using specific primers derived from published rodent gene sequences: ATCGGCTTTCAACTGTGCA (forward primer) and CAGGGCTTCTCTCGTGAGC (reverse primer) for γ-enac (156-bp product), TCAGGGCCGGTGGACGAC (forward primer) and GCACAGACCGAGCCCC (reverse primer) for β-enac (180-bp product), GATACCTGACTGGCCGAC (forward primer) and GCCGGCTACTGCTGGTC (reverse primer) for γ-enac (288-bp product), and TGGCCTTCACAGCTTGCT (forward primer) and TGAGAGCTATGAGTACCACCC (reverse primer) for gapdh (300-bp product).

The PCR products were visualized by electrophoresis through a 1% agarose gel in Tris-boric acid-EDTA to confirm that a single product was obtained for each primer set. A melting curve program was also run on each primer pair, and only one melting temperature (Tm) was observed, suggesting one product for each reaction. PCR products were then cloned into pGEM-T Easy (Promega). The sequence of each insert was verified by sequencing the plasmid. Each plasmid was then used to generate a standard curve for real-time PCR using serial 10-fold dilutions of plasmid DNA (from 100 to 0.01 pg/reaction).
Real-time PCR. Real-time PCR was performed using the SYBR green PCR core kit (Applied Biosystems) in accordance with the manufacturer’s instructions. After a hot start (3 min at 95°C), 2.5 μl of each cDNA were subjected to 40 cycles of PCR (30 s each at 95°C Tm, 55°C annealing temperature (Ta), and 72°C elongation temperature (Te) per cycle, except for β-enac: 60°C Ta and 68°C Te) in 25-μl reaction volume on a Bio-Rad iCycler using AmpliTaq Gold DNA polymerase (Applied Biosystems) and 100 pmol of intron-spanning primers (see above). Reactions containing RNase-free water in place of cDNA were also performed as negative controls for contamination with junk DNA. PCR products were also visualized by electrophoresis (see above) to verify size and absence of nonspecific product. Three separate PCR were performed per animal for each gene product. In each reaction, five cDNA replicates were subjected to PCR. Data were pooled and analyzed using iCycler software (Bio-Rad). Levels of enac subunit mRNA are expressed as a relative ratio to levels of gapdh mRNA.

Statistical analyses. Michaelis-Menten kinetics were calculated using Enzfitter software (Biosoft, Ferguson, MO). Descriptive statistics (means and SE) were calculated using Instat software (GraphPad, San Diego, CA). Differences between group means were analyzed by ANOVA or Student’s t-test, with appropriate post tests. Correlations were calculated by Pearson’s linear correlation analysis, P < 0.05 was considered statistically significant. Values are means ± SE.

RESULTS

Effects of RSV on body weight in BALB/c mice. Although mock infection resulted in a slight (2.5%) decline in body weight 1 day after challenge (presumably a consequence of anesthesia and reduced appetite), RSV caused a significant (~10%) decline in body weight 1–3 days after infection, which recovered to preinfection levels (Fig. 1A). Loss of weight was associated with ruffling of fur, hunched posture, and reduced activity and may have been related to the high levels of TNF-α detectable in BAL fluid 2 days after infection (116 ± 21 pg/ml, n = 8). No TNF-α was detected in BAL fluid 0, 4, 6, or 8 days after infection (n = 8 per group). Weight loss was comparable to that in previous studies (12, 46).

RSV replication in lungs of BALB/c mice. Intranasal infection was confirmed by plaque assay of homogenized whole lung from infected mice. Virus replication was undetectable 1 day after infection but was detected 2 days after infection. Replication was significantly higher 4 days after infection and began to decline by 6 days after infection (Fig. 1B). Viral replication was undetectable 8 days after infection. Kinetics of viral replication in our animal model system were similar to those previously described for BALB/c mice (12).

Effects of RSV on alveolar epithelial integrity. An independent observer, scoring histopathology in a blinded fashion, found no evidence of any epithelial cell death or sloughing of epithelium at any time after RSV infection. Some very mild lymphoid hyperplasia and parenchymal changes were noted 2 days after infection (mean scores for both <0.06, n = 6), together with occasional slight hypertrophy of alveolar lining cells. By 6 days after infection, there was significant lymphoid infiltration around major vessels (mean score 0.47 ± 0.03, n = 6), together with some interstitial edema and parenchymal hypercellularity (predominantly infiltrating lymphocytes, mean score 0.37 ± 0.1).

LDH was not detectable in BAL from mock-infected mice or 2 or 4 days after infection but was significantly elevated 6 days after infection (90 ± 35 U/ml, n = 7, P < 0.05) and 8 days after infection (158 ± 25 U/ml, n = 5, P < 0.0005), which may reflect death of infiltrating leukocytes.

Likewise, measurements of alveolar permeability to solute [flux of 125I-labeled BSA from the instillate to the bloodstream over a 30-min period (25)] confirmed that RSV does not induce significant increases in alveolar permeability, which would be indicative of alveolar epithelial cell death. The flux of intracheally instilled 125I-labeled BSA into the blood (<0.03%, n = 7–9 per group) and recovery of 125I-labeled BSA in the AEC aspirate (70–80% when corrected for AEC, n = 5–7 per group) were unaffected by RSV infection at any time point.

Because RSV might induce bronchoalveolar epithelial cell apoptosis, rather than necrosis, levels of apoptotic epithelial cells in lung sections were evaluated after specific immunostaining using the ApopTag in situ oligo ligation kit. There was no evidence of bronchoalveolar epithelial cell apoptosis 2–6 days after infection in lung tissue sections, despite successful staining of positive control tissue sections (n = 9 per time point).

Effects of RSV on AFC in BALB/c mice. AFC was evaluated in live mice with normal oxygenation and acid-base balance (14). Mean percent basal AFC over 30 min (AFC30 basal) in mock-infected mice was not different from that in uninfected mice (Table 1). AFC30 basal was also normal 1 day after infection but was significantly depressed (by 43% from mock-infected values) 2 days after infection (data derived from 6 separate infection studies). By 4 days after infection, AFC30 basal was still significantly depressed (by 26% from mock-infected values), although it was recovering toward nor-
Table 1. Effects of RSV infection on AFC in BALB/c mice

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<tr>
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<th>nbasal</th>
<th>AFC30 basal</th>
<th>nAmil</th>
<th>AFC30 Amil</th>
<th>ΔAmil</th>
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<tr>
<td>Uninfected</td>
<td>6</td>
<td>37.55 ± 3.10</td>
<td>7</td>
<td>14.65 ± 1.59</td>
<td>61</td>
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<td>Mock-infected</td>
<td>8</td>
<td>37.21 ± 1.20</td>
<td>10</td>
<td>12.65 ± 1.43</td>
<td>66</td>
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<td>RSV</td>
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<td>1 day</td>
<td>17</td>
<td>35.71 ± 1.48</td>
<td>8</td>
<td>21.92 ± 3.64</td>
<td>39</td>
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<tr>
<td>2 days</td>
<td>23*</td>
<td>21.19 ± 0.94</td>
<td>7</td>
<td>22.82 ± 1.92</td>
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<tr>
<td>4 days</td>
<td>9</td>
<td>27.40 ± 2.12</td>
<td>8</td>
<td>28.34 ± 3.93</td>
<td>-3</td>
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<tr>
<td>6 days</td>
<td>7</td>
<td>41.36 ± 1.63</td>
<td>7</td>
<td>17.37 ± 3.53</td>
<td>58</td>
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<tr>
<td>8 days</td>
<td>8</td>
<td>38.38 ± 1.48</td>
<td>5</td>
<td>16.59 ± 1.28</td>
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<td>UV-RSV</td>
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<td>2 days</td>
<td>6</td>
<td>32.32 ± 2.06</td>
<td>6</td>
<td>13.90 ± 2.22</td>
<td>57</td>
</tr>
<tr>
<td>4 days</td>
<td>6</td>
<td>32.57 ± 2.38</td>
<td>7</td>
<td>13.28 ± 2.14</td>
<td>59</td>
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Values are means ± SE. RSV, respiratory syncytial virus; nbasal, number of mice in which basal alveolar fluid clearance (AFC) was evaluated; AFC30 basal, mean %basal AFC after 30 min; nAmil, number of mice in which AFC was evaluated in the presence of 1.5 mM amiloride; AFC30 Amil, mean %AFC after 30 min in the presence of 1.5 mM amiloride; ΔAmil, mean %AFC inhibited by 1.5 mM amiloride; UV-RSV, UV-inactivated RSV. *Data derived from 6 separate infection studies. **P < 0.005 vs. mock-infected mice. †P < 0.0005 vs. mock-infected mice.

Fig. 2. Effect of RSV infection on lung water content. Lung water content was measured by wet-to-dry lung weight ratio (n = 8 per group). Values are means ± SE. *P < 0.05; **P < 0.005 compared with mock-infected mice.

Fig. 3. Effects of RSV on epithelial Na⁺ channel (ENaC) subunit gene expression. Infection with RSV for 2 (n = 4) or 4 (n = 3) days had no effect on α-enac-to-gapdh, β-enac-to-gapdh, or γ-enac-to-gapdh mRNA ratios compared with mock-infected animals (Fig. 3; n = 2–6).

Effects of purinergic receptor antagonists on RSV-mediated inhibition of AFC 2 days after infection. We investigated whether 5'-nucleotides, acting via P2YR, might mediate the inhibitory effects of RSV on AFC30 basal. These studies concentrated on 2 days after infection, as much as inhibition of AFC30 basal by RSV was maximal at this time. Addition of suramin to the AFC instillate completely reversed the inhibitory effect of RSV on AFC30 basal 2 days after infection and restored AFC30 basal to normal levels (Fig. 4A). ΔAmil was decreased to only 22%, and amiloride-insensitive AFC was significantly increased. Similarly, XAMR-0721 completely normalized AFC30 basal 2 days after infection (Fig. 4B). As after suramin treatment, ΔAmil was reduced to only 6%, and amiloride-insensitive AFC was significantly increased. Neither suramin nor XAMR-0721 had an effect on AFC30 basal in mock-infected mice (data not shown). These data show that the inhibitory effect of RSV on AFC 2 days after infection is mediated by a P2YR agonist.

mean AFC30 basal and mean wet-to-dry weight ratios at all time points.

Effects of RSV on ENaC subunit gene expression. Infection with RSV for 2 (n = 4) or 4 (n = 3) days had no effect on α-enac-to-gapdh, β-enac-to-gapdh, or γ-enac-to-gapdh mRNA ratios compared with mock-infected animals (Fig. 3; n = 2–6).

Fig. 2. Effect of RSV infection on lung water content. Lung water content was measured by wet-to-dry lung weight ratio (n = 8 per group). Values are means ± SE. *P < 0.05; **P < 0.005 compared with mock-infected mice.

Fig. 3. Effects of RSV on epithelial Na⁺ channel (ENaC) subunit gene expression. Ratios of α-enac-to-gapdh, β-enac-to-gapdh, and γ-enac-to-gapdh mRNA in lung homogenates from mock-infected mice (n = 2–6) and mice infected with RSV for 2 (n = 4) or 4 (n = 3) days were measured by real-time PCR after reverse transcription to cDNA. For each gene product, 3 separate PCR were performed per animal. In each reaction, 5 cDNA replicates were subjected to PCR. Values are means ± SE.
Effects of purinolytic enzymes on RSV-mediated inhibition of AFC 2 days after infection. Addition of apyrase to the AFC instillate completely restored AFC₃₀basal to normal levels, although ΔAmil was reduced to 22% and amiloride-insensitive AFC was significantly increased (Fig. 5A). However, RSV-mediated inhibition of AFC₃₀basal was unaffected by addition of hexokinase (with glucose) to the instillate, suggesting that ATP does not mediate the inhibitory effect of RSV on AFC 2 days after infection (n = 10; data not shown). In contrast, addition of UDP-G-PP to the AFC instillate (with inorganic pyrophosphatase and glucose 1-phosphate) reversed the inhibitory effect of RSV on AFC₃₀basal (Fig. 5B). Moreover, ΔAmil was reduced to only 14%, and amiloride-insensitive AFC was significantly increased. Importantly, in the absence of inorganic pyrophosphatase, which provides the energy to drive the otherwise fully reversible UTP degradation reaction, UDP-G-PP had no effect on RSV-mediated inhibition of AFC₃₀basal, indicating that the effects of UDP-G-PP are specific and energy dependent (data not shown). Neither apyrase nor UDP-G-PP had an effect on AFC₃₀basal in mock-infected mice (data not shown). Taken together, these data show that the inhibitory effect of RSV on AFC 2 days after infection is mediated by UTP acting via the P2YR.

Although the modulatory effects of purinergic receptor antagonists and pyrimidinolytic enzymes on the amiloride sensitivity of AFC could be explained by direct interaction of these agents with amiloride, we could find no spectrophotometric evidence [at 361-nm absorbance (14)] that these agents interfere with amiloride activity or solubility over a 60-min period. Similarly, because addition of P2YR antagonists and enzymes that degrade UTP restored AFC₃₀basal but not amiloride sensitivity, it is possible that these treatments were inducing Na⁺ transport mechanisms unrelated to ENaC. However, addition of 10 mM NiCl [which inhibits ENaC by a mechanism different from amiloride (40)] to the AFC instillate, in the presence of apyrase and amiloride, reduced AFC₃₀basal by 83% (to 6.5 ± 2.2%, n = 7), indicating that the AFC₃₀basal normalization induced by addition of apyrase to the AFC instillate 2 days after infection is predominantly mediated by ENaC-like channels, albeit with decreased sensitivity to amiloride.

Effects of exogenous UTP on AFC in normal mice. To confirm that UTP alone can recapitulate the inhibitory effects of RSV on AFC, we instilled 5% BSA containing 10-fold dilutions of UTP into the lungs of normal mice and determined AFC₃₀basal. Final doses of UTP from 1 nM to 10 nM had a significant inhibitory effect on AFC₃₀basal, but 1 nM UTP had no effect (Fig. 6A; n = 3–4 per group). UTP at 1 nM and 100 μM induced significantly greater inhibition of AFC₃₀basal than that caused by RSV (62 and 70%, respectively), whereas 1 μM–10 nM UTP caused inhibition of AFC₃₀basal similar to that induced by infection with RSV for 2 or 4 days (42–36%). Effects of <1 nM UTP were not determined. Using a Line-Weaver-Burk plot, we found Kₘ for the binding of UTP (1 μM–1 nM) to its receptor to be 1.4 ± 0.6 nM. Vₘₐₓ was 39.3 ± 3.2% inhibition of AFC₃₀basal. Because maximal inhibition of AFC₃₀basal was only 43% (2 days after infection), Vₘₐₓ indicates that RSV-mediated inhibition of AFC₃₀basal can be completely accounted for solely by the action of UTP on P2YR.

To further examine the effects of UTP, we added 500 nM UTP to AFC instillates administered to uninfected BALB/c mice and determined AFC₃₀basal and AFC₃₀Amil. UTP at 500 nM significantly reduced AFC₃₀basal (by 39%) and reduced ΔAmil to 27% (Fig. 6B). Amiloride-insensitive AFC was not significantly different from that in untreated mice, indicating that UTP specifically induced loss of amiloride-sensitive AFC. The effects of 500 nM UTP on AFC₃₀basal and AFC₃₀Amil were not significantly different from those induced by infection with RSV for 2 or 4 days. Addition of 500 nM UTP to the AFC instillate administered to normal, uninfected BALB/c mice, therefore, fully recapitulated the inhibitory effects of RSV on AFC₃₀basal and AFC₃₀Amil 2 and 4 days after infection.

Effects of exogenous UTP on AFC 6 days after infection. Despite the presence of significant viral replication 6 days after infection, AFC₃₀basal returned to normal levels. To investigate whether normalization of AFC₃₀basal was a consequence of P2YR desensitization, we added 500 nM UTP to AFC instillates administered to uninfected BALB/c mice and determined AFC₃₀basal and AFC₃₀Amil. Addition of 500 nM UTP to AFC instillates administered to normal, uninfected BALB/c mice, therefore, fully recapitulated the inhibitory effects of RSV on AFC₃₀basal and AFC₃₀Amil 2 and 4 days after infection.
increased AFC 24 h after infection, and this increase, which was not mediated by an entirely novel mechanism involving activation of P2YR. Moreover, to our knowledge, this is the first in vivo demonstration of modulation of respiratory epithelial Na⁺ transport by UTP or of altered 5’-nucleotide responses to infection with any pulmonary pathogen. Reduced capacity to clear alveolar and airway fluid may therefore be an unrecognized component of the pathogenesis of RSV bronchiolitis, because it will lead to an increase in the volume of the ALF. In turn, this volume increase may result in compromised gas exchange, altered mucociliary clearance, and rhinorrhea, all features of RSV disease (4). Indeed, the similarities in clinical presentation between RSV infection and systemic pseudohypaldosteronism, which results in a complete absence of electrogenic Na⁺ transport in upper and lower airways, are striking, given that both diseases are characterized by rhinorrhea, chest congestion, cough, tachypnea, wheezing, and crackles (18).

Our data indicate that the inhibitory effect of RSV on AFC requires the onset of active viral replication, because it is not manifest during the eclipse phase of the replication cycle (day 1). Moreover, unlike the inhibitory effect of influenza virus on Na⁺ transport in vitro, which requires only cell surface binding of hemagglutinin (20), the inhibitory effect of RSV cannot be mimicked by challenge with UV-inactivated virus, which retains antigenicity (and presumably cell binding capacity) (17) but lacks the ability to replicate. It is not clear why AFC returns to normal (almost supernormal) levels 6 days after infection, which is, however, considerably delayed compared with the time at which AFC returns to normal in murine RSV infection, it is possible that the inhibitory effects of RSV on AFC may persist for a longer period in infected humans.

Of concern in studies of this kind is the possibility that the observed effects are simply a consequence of viral cytopathic effect. Our evidence indicates that the decline in AFC 2 days after infection is not a consequence of destruction of the epithelial barrier by a lytic RSV infection. Most importantly, the ability of purinergic receptor antagonists and enzymes that catabolize UTP to reverse the inhibitory effect of RSV on AFC 2 days after infection indicates that the respiratory epithelium retains its potential for normal AFC and is not necrotic, merely functionally impaired. Our experimental findings are in agreement with those from the majority of studies on the cytopathicity of RSV (12, 13, 38, 49). Although significant epithelial cell death was reported in tissue specimens from fatal cases of RSV (1, 32), much of this cell death may result from the inflammatory/immune response to the virus (45). Epithelial necrosis may well be a late-stage component of RSV bronchiolitis in mice and humans, but it does not appear to account for this early inhibitory effect of RSV on AFC. Even if RSV were

![Image](http://ajplung.physiology.org/)

**DISCUSSION**

Despite the fact that fluid and mucus accumulation in airways and lung tissue is a major component of most respiratory infections (27), the effect of pathogens on respiratory epithelial Na⁺ transport and AFC has not been studied in detail. Several lung pathogens, including Mycoplasma pulmonis (21), Pseudomonas aeruginosa (11, 42), Mycobacterium tuberculosis (50), and pneumotropic influenza A virus (20), have been shown to inhibit Na⁺ transport by tracheal, bronchial, or alveolar epithelial cells in vitro. However, in vivo correlates of these in vitro defects are limited. Instillation of Escherichia coli endotoxin into the lungs of rats resulted in a significant increase in AFC at 24 and 40 h, although no mechanism was defined (10). Similarly, induction of *P. aeruginosa* pneumonia in rats increased AFC 24 h after infection, and this increase, which was inhibited by amiloride, was at least partially mediated by TNF-α (37).

Our study is the first to demonstrate that any human respiratory viral pathogen has physiologically significant inhibitory effects on AFC in vivo, effects that are qualitatively and quantitatively very different from the stimulatory effects of gram-negative bacteria on AFC and are mediated by an entirely novel mechanism involving activation of P2YR. Moreover, to our knowledge, this is the first in vivo demonstration of modulation of respiratory epithelial Na⁺ transport by UTP or of altered 5’-nucleotide responses to infection with any pulmonary pathogen. Reduced capacity to clear alveolar and airway fluid may therefore be an unrecognized component of the pathogenesis of RSV bronchiolitis, because it will lead to an increase in the volume of the ALF. In turn, this volume increase may result in compromised gas exchange, altered mucociliary clearance, and rhinorrhea, all features of RSV disease (4). Indeed, the similarities in clinical presentation between RSV infection and systemic pseudohypaldosteronism, which results in a complete absence of electrogenic Na⁺ transport in upper and lower airways, are striking, given that both diseases are characterized by rhinorrhea, chest congestion, cough, tachypnea, wheezing, and crackles (18).

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**Fig. 6.** Effects of exogenous UTP on AFC in normal BALB/c mice. A: effect of 10-fold dilutions of UTP (1 mM–1 nM) on AFC30 basal compared with untreated mice (U; n = 3–4 per group). B: effect of 500 nM UTP on AFC30Amil (n = 6 for AFC30basal; n = 5 for AFC30Amil). AFC30 basal and AFC30 Amil after amiloride for uninfected mice are listed in Table 1. Values are means ± SE. *P < 0.05; **P < 0.005; ***P < 0.0005 compared with basal AFC in untreated animals.
to cause significant damage to the alveolar barrier which the presence of an inflammatory response does not per se imply (10), such damage does not automatically result in compromised AFC. Indeed, AFC was increased in bleomycin-injured rats, despite the presence of a significant increase in alveolar epithelial permeability to protein (7). Similarly, AFC was increased in rats with P. aeruginosa pneumonia, despite histological evidence of injury to the alveolar epithelium (37).

AF30 basal values in BALB/c mice are very similar to those reported for C57BL/6 mice (33 ± 6%) by use of the same technique (14) and for C57BL/6 [27 ± 5% (9)] and CD-1 [3.7 ± 0.32 ml·h⁻¹·g dry lung wt⁻¹ (16)] mice by in situ and isolated perfused lung methods, respectively. Likewise, ∆Amil in uninfected BALB/c mice is identical to that previously reported for C57BL/6 mice (61%) (14), although it is lower than that reported for CD-1 mice (90%) (8, 9). Maximal AFC rates appear to be significantly lower in human lungs (14%/h) than in the mouse (48). Nevertheless, in patients with acute lung injury, a reduction in maximal AFC by 30–50%, which is similar to the 43% and 26% inhibition induced by RSV in the mouse 2 and 4 days after infection, respectively, was associated with a significant reduction in survival (48). This suggests that an RSV-mediated decline in AFC might have an impact on clinical outcome as significant as that of acute lung injury. The specific loss of amiloride-sensitive AFC after RSV infection is comparable to that seen after exposure of rats to hypoxia for >12 h (47).

Extracellular ATP and UTP may be extremely labile and likely to be present in the ALF at very low concentrations. Extracellular ATP may be rapidly degraded by ecto-ATPases (23), but fewer data regarding the presence of enzymes capable of degrading UTP in the ALF are available. It is therefore possible that UTP, although likely to be present in the ALF in 10-fold-lower concentrations than ATP (5), may actually be more stable than ATP and, therefore, of greater physiological significance.

We can infer from our inhibitor studies and from the effects of exogenous UTP on AFC30 basal in normal mice that UTP levels in the ALF are elevated after RSV infection, although our finding that doses of UTP as low as 10 nM can recapitulate the effect of RSV in normal mice suggests that only small elevations in UTP levels in the ALF may be sufficient to inhibit AFC. In previous studies, steady-state UTP concentrations in nasal airway surface liquid (ASL) were ~40 nM (5), and it is unlikely that such low concentrations would be detectable after the dilution of ASL that occurs with BAL. Intracellular UTP levels in bronchoalveolar epithelial cells are likely to be considerably lower. In normal, resting human lymphocytes, intracellular UTP concentrations have been measured at 100 pmol/10⁶ cells (6); no similar data are available for respiratory epithelial cells. The calculated Kₐ for UTP-P2YR interaction, which is likely to be an overestimate because of ongoing degradation of instilled UTP, indicates that the affinity of UTP for its receptors on bronchoalveolar epithelial cells appears to be high. Again, this finding is supported by previous studies in which concentrations of UTP as low as 100 nM could trigger intracellular Ca²⁺ waves in P2Y₂ receptor-expressing murine nasal epithelial cells (15).

Release of 5’-nucleotides from respiratory epithelial cells has been demonstrated in vitro (although never in response to infection with a pulmonary pathogen), but the underlying pathway for nucleotide release remains undefined (15, 22). It is not clear whether RSV induces increased UTP release through normal cellular pathways or whether it induces release via virus-specific mechanisms. Although LDH was undetectable in BAL 2 and 4 days after infection, it is possible that UTP release is solely a consequence of a low level of epithelial cell death (although no inhibition of AFC is evident 6 days after infection, when LDH levels in BAL are significantly elevated). Alternatively, elevated ALF UTP levels may result from UTP release by RSV-infected alveolar macrophages or infiltrating inflammatory cells.

Once released, ATP, its metabolites, and UTP have been shown to modulate ENaC activity in respiratory epithelial cells in vitro. ATP and UTP have been shown to inhibit respiratory epithelial Na⁺ absorption in vitro (for review see Ref. 24) via interaction with the P2Y₂ purinoceptor (33), which can be expressed on apical and basolateral epithelial cell surfaces (15). In vivo, UTP administered at pharmacological doses (100 µM) to human subjects has also been shown to induce chloride secretion by nasal epithelium (19) and, when given by aerosol, to promote mucociliary clearance, although, interestingly, in the presence of amiloride, it also induced mild hypoxemia (34).

Downstream signaling events mediating ENaC downregulation have not been fully defined. P2YR are G protein-coupled and act via the inositol phosphate pathway to stimulate Ca²⁺ release from intracellular stores but can also act via multiple secondary signal transduction pathways, including protein kinase C (PKC) (2). Activation of PKC has been shown to reduce ENaC activity and modify its subunit composition, although the isoforms of PKC involved have not been defined (26, 41). It is possible that a change in ENaC subunit stoichiometry, induced by RSV infection, underlies the difference in amiloride sensitivity of AFC between normal mice (∆Amil = 60%) and RSV-infected mice in which AFC30 basal has been “normalized” by degradation or blockade of UTP (∆Amil < 20%). An alternative possibility is that agents that degrade or block UTP also induce non-ENaC expression. However, given the varying nature of these agents and the fact that they do not increase AFC30 basal in mock-infected mice, which they should if they are inducing new channels, this seems unlikely. Moreover, although it is possible that NiCl is causing blockade of basolateral K⁺ channels or the Na⁺–K⁺-ATPase, and thereby inhibiting AFC, we believe that it is unlikely that NiCl, when added to the apical aspect of the alveolus in the AFC instillate, could reach the basolateral cell surface in sufficient concentration to have an inhibitory effect on these transporters. The inhibitory effects of NiCl on AFC are therefore more likely to be a consequence of inhibition of amiloride-sensitive or -insensitive Na⁺ channels in the apical membrane.

In conclusion, we have shown for the first time that RSV has a significant and detrimental inhibitory effect on AFC in the mouse. Moreover, we have found that this effect appears to be mediated by UTP, presumably released by the bronchoalveolar epithelium in response to infection. The underlying mechanism and the full pathophysiological consequences of this nucleotide release remain to be determined. Finally, our data suggest that P2YR antagonists may be useful in the treatment of severe RSV bronchiolitis to improve AFC and, hence, gas exchange in the lung.
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