Respiratory syncytial virus induces airway insensitivity to β-agonists in BALB/c mice

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Traylor ZP, Yu EN, Davis IC. Respiratory syncytial virus induces airway insensitivity to β-agonists in BALB/c mice. Am J Physiol Lung Cell Mol Physiol 298: L437–L445, 2010. First published December 4, 2009; doi:10.1152/ajplung.00363.2009.—β-Adrenergic agonist insensitivity to β2-AR (β2-agonists) are frequently used to treat RSV bronchiolitis, primarily in an attempt to induce bronchodilation, thereby alleviating hypoxemia. However, it is not clear that these drugs, which are not without potential adverse effects, are clinically effective (14, 19). Recently, therefore, the American Academy of Pediatrics recommended that bronchodilators should not be used routinely in the management of bronchiolitis (2), although their widespread use persists.

The lack of efficacy of β-agonists as treatments for bronchiolitis has never been adequately explained. Some investigators have proposed that drug ineffectiveness may result from physical factors, such as impaired aerosol delivery to the small airways of young infants, particularly in the presence of bronchoconstriction (39). It has also been suggested that, in the presence of necrotic cell debris, inflammatory exudates, and edema fluid, β-agonists may be unable to diffuse from airways to airway smooth muscle (ASM) (31). Alternatively, or additionally, β-agonist insensitivity may result from a specific defect in drug-binding to ASM β2AR interaction or downstream signaling (8, 28). In support of this latter mechanism, we (8) recently demonstrated that RSV infection of BALB/c mice results in heterologous desensitization of bronchoalveolar epithelial (BAE) β2-AR to β-agonists. Likewise, in vitro infection of human ASM cells with RSV has been shown to induce β-agonist insensitivity (28). However, this study must be interpreted with some caution, since there is no evidence that RSV infects ASM in vivo. To date, therefore, in vivo effects of RSV on airway responsiveness to β-agonists have not been determined. We hypothesized that, in addition to its effects on BAE β2-AR, RSV infection induces airway desensitization to β-agonists and that this accounts for the poor utility of these drugs as bronchodilators in acute bronchiolitis. Results presented herein support this hypothesis, since they show that RSV infection induces functional β-agonist insensitivity in murine airways by the same paracrine, CXCRI2-mediated mechanism that we (8) previously described for BAE β2-AR.

MATERIALS AND METHODS

Reagents. Acetyl β-methacholine (MCh) (Sigma-Aldrich, St. Louis, MO), terbutaline (Sigma-Aldrich), rat anti-mouse CXCRI2 IgG2a MAb (MAB2164; R&D Systems, Minneapolis, MN), rat anti-mouse keratinocyte cytokine (KC) MAb (MAB4531; R&D Systems), normal rat IgG (R&D Systems), and recombinant murine KC/CXCL8 (453-KC; R&D Systems) were reconstituted in normal saline, aliquoted, and stored at −80°C. Aliquots of recombinant murine KC were heat-inactivated by boiling for 10 min in a water bath.

Animals. Eight- to ten-week-old pathogen-free female BALB/cAnNCr mice (National Cancer Institute, Frederick, MD), maintained in autoclaved microisolators, were used in these studies. Animals were given sterile autoclaved food and water ad libitum, monitored.
daily for signs of respiratory distress or other illness, and euthanized if these signs were detected. All mouse experiments were approved by The Ohio State University Institutional Animal Care and Use Committee.

Preparation of viral inocula. Viral stocks were grown in monolayers of HEp-2 human epithelial cells and purified by ultracentrifugation onto a 60% sucrose cushion (22). Viral titers were determined by serial dilution and plaque assay in Vero cells under agar (38). Virus preparations were checked for absence of mycoplasmal and endotoxin contamination. A mock-infected stock, identically prepared, served as a control to account for possible effects of cellular components in the inoculum.

UV inactivation of RSV. RSV stocks were inactivated by exposure to 1,800 mJ of radiation in a Stratalinker UV crosslinker (Stratagene, Cedar Crossing, TX), eliminating viral infectivity without altering the conformation of viral proteins and mediators (18). Loss of infectivity was confirmed by plaque assay as above.

Mouse infection protocol. Mice were infected intranasally with 10⁶ plaque-forming units (pfu) of RSV strain A2 (in 100 μl) under light anesthesia (0.87 mg ketamine/100 g body wt; 0.13 mg xylazine/100 g body wt ip). Control animals received an equal volume of media from mock-infected cells. Mice were placed in lateral recumbency, allowed to recover, and returned to their cage. For all studies, data for each experimental group were derived from a minimum of two independent infections.

Virus isolation. Viral replication in mouse lungs was quantified as plaque-forming units per gram lung tissue, as described previously (40).

Measurement of lung mechanics. Mechanical properties of the mouse lung were assessed using the forced oscillation technique, as previously described (5, 15, 16). Only female mice were used in these studies, since male mice exhibit exaggerated airway hyperresponsive (AHR) responses to MCh (4a). Each mouse was anesthetized with valium [1.75 mg/100 g body wt] followed by ketamine (45 mg/100 g body wt). Once at a surgical plane of anesthesia (assessed by toe pinch), the trachea was exposed surgically, a tracheotomy performed, and a trimmed sterile 18-gauge intravenous catheter was inserted caudally into the lumen. Pancuronium was then administered (0.08 μg/kg ip). The mouse was mechanically ventilated on a computer-controlled piston ventilator (flexiVent; SCIREQ, Montréal, Québec, Canada) with a tidal volume of 10 ml/kg at a frequency of 150 breaths/min against 2–3 cmH₂O positive end-expiratory pressure (PEEP). Following 2 total lung capacity (TLC) maneuvers to standardize volume history, pressure and flow data (reflective of airway and tissue dynamics) were collected during a series of standardized volume perturbation maneuvers. These data were used to calculate both total lung resistance (R) and elasance (E) using the single-compartment model and used to derive further parameters of respiratory function: Rₙ (Newtonian resistance, composed mostly of the flow resistance of the conducting pulmonary airways), G (tissue damping, reflective of resistance of peripheral airways and parenchyma), H (parenchymal tissue elasance), and η (G-to-H ratio) (16). The flexiVent calibration procedure removes cannula impedance from the reported data. Residual ineritance (I) is therefore negligible and is not reported herein.

Nebulization regimens. MCh dilutions in sterile normal saline were prepared fresh daily. For assessment of AHR and following initial baseline recordings of R and E with nebulization of saline only, mice on the flexiVent were exposed to increasing doses of MCh (0.1, 1, 10, 20, and 50 μg/ml). Each MCh dose was delivered over a 10-s period via an Aeroneb vibrating plate ultrasonic nebulizer in series with the inspiratory limb of the flexiVent Y-tube. Ten recordings of R and E were generated from 20 s up to 3 min following administration of each MCh dose. Average values of all 10 measurements for each parameter at each MCh dose were then calculated. A schematic of this nebulization regimen is shown in Fig. 1A.

For assessment of β-agonist responsiveness, a second, age-matched group of female mice were evaluated in parallel for each infection time point or treatment condition. In these animals, initial baseline measures of R and E with nebulization of saline were followed by exposure to the nebulized β-agonist terbutaline (100 μM) and then increasing doses of MCh (0.1, 1, 10, 20, and 50 μg/ml) with a further dose of β-agonist between each dose of MCh. A control group received saline in place of β-agonist. A schematic of this nebulization regimen is shown in Fig. 1B. To avoid loss of β-agonist bronchodilator effects, TLC maneuvers were not performed between nebulizer doses. However, the nebulizer was carefully cleaned and dried with a swab between each dose of MCh or terbutaline to eliminate carryover.

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Fig. 1. Schematic of lung function analysis study protocol. A: analysis of response to methacholine (MCh). Anesthetized, tracheotomized, uninfected, or respiratory syncytial virus (RSV)-infected mice are mechanically ventilated on a flexiVent computer-controlled piston ventilator. Following 2 total lung capacity (TLC) maneuvers to standardize volume history, mice are exposed to nebulized saline (S), and then 10 initial baseline values of total lung resistance (R) and elasance (E) are recorded (gray arrow). Mice are then exposed to increasing doses of MCh in saline (0.1, 1, 10, 20, and 50 μg/ml; M₀₁, M₁, M₁₀, M₂₀, and M₅₀, respectively), delivered by nebulizer. Ten recordings of R and E are generated following administration of each MCh dose (gray arrow). Finally, in some studies, mice are exposed to blocking antibodies to keratinocyte cytokine (KC) or CXC2R2 or to recombinant KC by nebulization immediately or after baseline data values are recorded following saline nebulization. B: analysis of response to MCh + terbutaline. flexiVent analysis is performed as in A except that mice are exposed to 100 μM terbutaline (T₁₀₀) by nebulizer before each MCh dose. Ten recordings of R and E are generated following administration of each MCh dose and each terbutaline dose (gray arrow).
Both the nebulizer and tubing were also thoroughly cleaned between animals to eliminate carryover from one animal to the next.

To determine the mechanism of airway desensitization to $\beta$-agonist, mice were exposed to nebulized rat anti-CXCR2 or anti-KC MAb (both 50 $\mu$g/ml, equivalent to 0.2 mg/kg) following initial baseline saline nebulization. Controls received nebulized normal rat IgG (50 $\mu$g/ml). Antibody nebulization was followed by nebulization with increasing doses of MCh or MCh + terbutaline, as described above.

In other studies, normal, uninfected BALB/c mice were exposed to nebulized recombinant murine KC or its heat-inactivated equivalent (both 50 $\mu$g/ml) following initial baseline saline nebulization.

**Calculation of mean %ΔR values.** Group mean values for the percentage change in $R$ elicited by exposure to a given dose of MCh (%ΔR) were determined by measuring $R$ values at each MCh dose for each individual mouse in the group, calculating %ΔR at each MCh dose as the percentage change in $R$ vs. its baseline value for that mouse and then calculating the mean %ΔR value for the group at each MCh dose by combining individual %ΔR values from all mice in the group.

**Calculation of mean %ΔR values.** Group mean values for the maximal increase in $R$ elicited by exposure to the 50 mg/ml MCh dose ($R_{MAX}$) were determined by measuring $R_{MAX}$ values for each individual mouse in the group and then calculating the mean $R_{MAX}$ for that group.

**Calculation of ΔTERBUT values.** To determine responses to terbutaline at a given MCh dose (20 or 50 mg/ml) at a particular time point, the percentage change in $R$ from its baseline value was calculated for that MCh dose for each mouse that received nebulized MCh only (%ΔR$_{MCH}$). A mean %ΔR$_{MCH}$ value for that group of mice (minimum 6 mice per group) was then calculated. The percentage change in $R$ from its baseline value was then calculated for that MCh dose for each mouse at that time point in a second group that received nebulized MCh + terbutaline (%ΔR$_{MCH} + \tau$) and a mean %ΔR$_{MCH} + \tau$ value calculated. %ΔTERBUT for that time point was then calculated as: %ΔTERBUT = (Mean %ΔR$_{MCH}$ − Mean %ΔR$_{MCH} + \tau$) × 100 / Mean %ΔR$_{MCH}$.

**Systemic antibody administration protocol.** Rat anti-mouse CXCR2 IgG$_{2A}$ MAb or normal rat IgG (both 0.2 mg/kg, in 100 $\mu$l vol) were administered to mice by intraperitoneal injection 12 h before infection and 36 h postinfection with RSV, as in previous studies (4).

**Bronchoalveolar lavage.** Mice were euthanized, the trachea exposed surgically, and a trimmed sterile 18-gauge intravenous catheter inserted caudally into the lumen. The lungs were then lavaged in situ with 1 ml of sterile saline. Cell viability was determined via trypan blue exclusion, and cell types were differentiated on cytospin preps with 1 ml of sterile saline. Cell viability was determined via trypan blue exclusion, and cell types were differentiated on cytospin preps with 1 ml of sterile saline. Cell viability was determined via trypan blue exclusion, and cell types were differentiated on cytospin preps with 1 ml of sterile saline.

**Measurement of BAL KC content.** Bronchoalveolar lavage (BAL) KC content was measured by ELISA (R&D Systems), performed in accordance with manufacturer’s instructions.

**Lang wet-to-dry weight ratio.** Lung wet-to-dry weight ratio was measured as previously described (7). 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 again. The wet-to-dry weight ratio was then calculated as an index of intrapulmonary fluid accumulation. No correction for blood content was made.

**Statistical analyses.** Descriptive statistics (mean and standard error) were calculated using InStat software (GraphPad, San Diego, CA), whereas MCh dose-response curves were analyzed using Prism software (GraphPad). Gaussian data distribution was verified by the method of Kolmogorov and Smirnov. For two-group comparisons, a two-sample t-test was used, and for more than two groups, ANOVA was used to assess significance, with a post hoc Tukey-Kramer multiple-comparison posttest. All data are presented as means ± SE. $P < 0.05$ was considered statistically significant.

**RESULTS**

**Effect of RSV infection on basal lung mechanics and responsiveness to MCh.** To determine effects of RSV infection on airway responsiveness to $\beta$-agonists, we first evaluated changes in lung function parameters elicited by exposure to increasing nebulized doses of the muscarinic agonist MCh. Comparison was made between uninfected BALB/c mice, mock-infected mice, and mice at 2, 4, or 8 days postinfection (d.p.i.) with RSV. Infection time points were based on our previous studies showing that RSV maximally impairs BAE responses to $\beta$-agonists at 2 d.p.i. (8), replicates maximally in the lung at 4 d.p.i. (7), and undergoes viral clearance by 8 d.p.i. (5).

Baseline $R$ values in normal BALB/c mice were similar to those reported previously (3). Baseline changes in lung function comparable with those we (5) reported previously were found in the current study (data not shown): a significant increase in baseline airway resistance (R) at 2 d.p.i., but not at other time points after infection, as well as nonsignificant increases in Newtonian (central airway) resistance ($R_N$), tissue damping (G), and tissue elastance (H) at this time point. Mock infection for 2 days had no impact on airway responsiveness to MCh (data not shown). Likewise, despite increasing baseline $R$ at 2 d.p.i., RSV did not induce AHR to nebulized MCh at this or any other time point after infection. AHR was absent irrespective of whether we compared the percentage change in $R$ from its baseline for each animal (%ΔR; Fig. 2A) or maximal mean $R$ values ($R_{MAX}$; elicited by exposure to 50 mg/ml MCh) for animals in each group (Fig. 2B). This finding is in agreement with previous studies using the A2 viral strain in BALB/c mice (27, 30).

**Effect of RSV infection on airway responsiveness to nebulized $\beta$-agonist.** In the absence of any bronchoconstrictive stimulus, we found that nebulization of terbutaline had no...
effect on baseline $R$ values in either uninfected or RSV-infected mice (Fig. 3A). To determine whether airways remained capable of bronchodilation in response to inhaled $\beta$-agonists following RSV infection, we therefore compared $\%\Delta R$ MCh dose-response curves between groups of mice receiving only nebulized MCh and mice receiving both MCh and terbutaline by nebulizer at each time point following RSV infection. We then calculated what percentage of the maximal bronchoconstrictive response to MCh was inhibited by terbutaline ($\Delta_{\text{TERTBUT}}$).

We found that in normal, uninfected mice, terbutaline was a relatively effective bronchodilator, as representative tracings of changes in $R$ in response to MCh or MCh + terbutaline from 2 separate mice, analyzed consecutively on the same day, demonstrate (Fig. 3B). Quantitatively, terbutaline nebulization inhibited 43% of the mean bronchoconstrictive response to 20 mg/ml MCh and 26% of the bronchoconstrictive response to 50 mg/ml MCh in uninfected mice, i.e., in the presence of terbutaline, group mean $\%\Delta R$ was 43 and 26% lower, respectively, at the 20 and 50 mg/ml MCh doses than in its absence (Fig. 3, C–E). Nebulization of terbutaline induced similar reductions in bronchoconstrictive response to MCh in mice infected with RSV at 4 or 8 d.p.i. However, nebulization of an equal volume of saline only had no effect on response to MCh in normal mice (data not shown).

In contrast to its efficacy in uninfected mice or mice infected with RSV for longer time periods, the bronchodilatory effect of terbutaline was significantly attenuated in mice infected with RSV at 2 d.p.i. At this time point, terbutaline inhibited only 14% of the bronchoconstrictive response to 20 mg/ml MCh and 11% of the bronchoconstrictive response to 50 mg/ml MCh (Fig. 3, C–E, and representative tracings from 2 separate mice, analyzed consecutively on the same day, in Fig. 3F). However, when mice were infected with UV-inactivated virus for 2 days, the bronchodilatory response to terbutaline was not impaired: terbutaline inhibited 42 and 29% of the bronchoconstrictive response to 20 and 50 mg/ml MCh, respectively, in these animals ($n = 4$ per group). This finding supports our hypothesis that, as with its effect on BAE cells, RSV induces airway insensitivity to $\beta$-agonists at early time points after infection and that this effect requires viral replication.

**Effect of systemic blockade of CXCR2 on RSV-induced airway insensitivity to nebulized $\beta$-agonist.** Previously, we (8) demonstrated that BAE $\beta_2$-AR desensitization occurs at 2–4 d.p.i. following RSV infection by a paracrine and heterologous mechanism involving activation of G protein-coupled CXCR2 chemokine receptors by their major ligand, the proinflammatory chemokine KC (the murine homolog of CXCL8), which is released into the lung in increased amounts following RSV infection. We therefore investigated the role of CXCR2 acti-

![Image](https://example.com/figure3.png)

**Fig. 3.** Effect of RSV infection on airway responsiveness to nebulized $\beta$-agonist. A: effect of nebulized terbutaline (100 $\mu$M) on baseline $R$ values in uninfected mice and RSV-infected mice at 2, 4, or 8 d.p.i. $n = 6$–8 per group. B: representative tracings from 2 separate mice, analyzed consecutively on the same day, show increases in $R$ with time in response to nebulization of 10, 20, or 50 mg/ml MCh (bold line) and attenuation of MCh responses by nebulized terbutaline (T; thin line). C: box and whisker plots showing the percentage change in $R$ from baseline ($\%\Delta R$) in individual mice following nebulization of 20 mg/ml MCh in the absence (●) or presence (○) of 100 $\mu$M terbutaline. $\%\Delta R$ values are shown for uninfected mice and RSV-infected mice at 2, 4, or 8 d.p.i. D: box and whisker plots showing the percentage change in $R$ from baseline ($\%\Delta R$) in individual mice following nebulization of 50 mg/ml MCh in the absence (●) or presence (○) of 100 $\mu$M terbutaline. $\%\Delta R$ values are shown for uninfected mice and RSV-infected mice at 2, 4, or 8 d.p.i. E: percentage of the maximal mean bronchoconstrictive response to 20 or 50 mg/ml MCh inhibited by nebulized terbutaline (100 $\mu$M) in uninfected mice and RSV-infected mice at 2, 4, or 8 d.p.i. $n = 6$–8 per group. F: representative tracings from 2 separate RSV-infected mice at 2 d.p.i., analyzed consecutively on the same day, showing increases in $R$ with time in response to nebulization of 10, 20, or 50 mg/ml MCh (bold line) and lack of attenuation of MCh responses by nebulized terbutaline (thin line). $\%\Delta_{\text{TERTBUT}}$, percentage change in $R$ after terbutaline.
Effect of systemic blockade of CXCR2 on RSV-induced airway desensitization in RSV-infected mice at 2 d.p.i.

Table 1. Effect of systemic blockade of CXCR2 on viral replication and indices of lung inflammation in RSV-infected mice at 2 d.p.i.

<table>
<thead>
<tr>
<th></th>
<th>Uninfected</th>
<th>Untreated</th>
<th>IgG-Treated</th>
<th>Anti-CXCR2-Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung virus titer, pfu/g</td>
<td>N.D.</td>
<td>4,086±667</td>
<td>4,128±386</td>
<td>4,162±755</td>
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<tr>
<td>Neutrophils, ×10⁶/ml</td>
<td>0.02±0.004</td>
<td>0.56±0.05δ</td>
<td>0.49±0.08ξ</td>
<td>0.2±0.05ξ</td>
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<tr>
<td>BAL KC, pg/ml</td>
<td>68±4</td>
<td>1,153±56ξ</td>
<td>1,176±46ξ</td>
<td>1,199±125ξ</td>
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<td>Wet-to-dry weight ratio</td>
<td>3.9±0.14</td>
<td>4.78±0.19ξ</td>
<td>4.75±0.13*</td>
<td>4.91±0.16δ</td>
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<tr>
<td>Basal R, cmH₂O s⁻¹ ml⁻¹⁻¹</td>
<td>0.78±0.03</td>
<td>0.9±0.05*</td>
<td>0.87±0.05*</td>
<td>0.82±0.04</td>
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</tbody>
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Values are means ± SE. *P < 0.05, †P < 0.005, ‡P < 0.0005, compared with untreated, respiratory syncytial virus (RSV)-infected group. §P < 0.005, compared with untreated, RSV-infected group. d.p.i., Days postinfection; pfu, plaque-forming units; N.D., not detected; BAL, bronchoalveolar lavage; KC, keratinocyte cytokine; R, total lung resistance.

Fig. 4. Effect of systemic blockade of CXCR2 on RSV-induced airway insensitivity to nebulized β-agonist. A: effect of treatment with rat IgG or anti-CXCR2 antibody (both 0.2 mg/kg, in 100 μl vol ip, 12 h before infection and 36 h postinfection) on bronchoconstrictive responses to MCh in RSV-infected mice at 2 d.p.i. B: percentage of the maximal mean bronchoconstrictive response to 50 mg/ml MCh inhibited by nebulized terbutaline (100 μM) in uninfected mice, RSV-infected mice at 2 d.p.i. (D2), and RSV-infected mice at 2 d.p.i. treated with rat IgG or anti-CXCR2 antibody. n = 6–8 per group.

Fig. 5. Effect of aerosol blockade of CXCR2 and KC on RSV-induced airway insensitivity to nebulized β-agonist. A: effect of administration of anti-KC or anti-CXCR2 antibodies (both 50 μg/ml) by nebulization immediately before airway function studies on bronchoconstrictive responses to MCh in RSV-infected mice at 2 d.p.i. B: percentage of the maximal mean bronchoconstrictive response to 50 mg/ml MCh inhibited by nebulized terbutaline (100 μM) in uninfected mice, RSV-infected mice at 2 d.p.i., and RSV-infected mice at 2 d.p.i. treated with rat IgG, anti-KC antibody, or anti-CXCR2 antibody (all 50 μg/ml) by nebulization. n = 6–8 per group.
zation significantly improved the ability of the bronchodilator terbutaline to attenuate the maximal bronchoconstrictive response to 50 mg/ml MCh in mice infected with RSV at 2 d.p.i. (Fig. 5B). Taken together, these findings demonstrate that, as we found previously for BAE β2-AR (8), airway desensitization to β-agonists occurs at 2 d.p.i. in RSV-infected mice by a paracrine and heterologous mechanism involving activation of G protein-coupled CXCR2 chemokine receptors by the proinflammatory chemokine KC.

Effect of KC on β-agonist responsiveness in normal, uninfected BALB/c mice. To confirm the role of KC as mediator of heterologous β2-AR desensitization in RSV-infected mice, we treated uninfected mice with recombinant murine KC by nebulization immediately before airway function analysis. Acute airway exposure to KC (50 µg/ml) had no effect on bronchoconstrictive responses to MCh alone (Fig. 6A). However, KC nebulization significantly attenuated the bronchodiatoral effect of terbutaline in normal mice (Fig. 6B). In KC-treated mice, terbutaline inhibited only 20% of the bronchoconstrictive response to 20 mg/ml MCh and 13% of the bronchoconstrictive response to 50 mg/ml MCh (compared with 43 and 26% of the bronchoconstrictive response to 20 and 50 mg/ml MCh, respectively, in untreated mice). However, when KC was inactivated by heat treatment, it no longer induced β-agonist insensitivity. Taken together, these data indicate that presence of this chemokine in the airways alone is sufficient to induce β-agonist insensitivity.

DISCUSSION

β-Agonists are frequently used to treat RSV bronchiolitis, primarily in an attempt to induce bronchodilation, thereby alleviating hypoxemia (36). However, it is not clear that these drugs are clinically effective (14, 19), and their routine use in management of bronchiolitis was recently discouraged by the American Academy of Pediatrics (2). Moreover, β-agonists are not without detrimental effects: they can increase total body oxygen consumption, thereby raising oxygen demand in infants hospitalized for respiratory compromise. β-Agonists can also potentially exacerbate ventilation-perfusion mismatch by inducing vasodilation without bronchodilation, if they desensitize airway but not vascular β2-AR, and can induce arrhythmias (13). In addition, they are expensive: the cost of essentially ineffective therapy for bronchiolitis was estimated to be $37.5 million for the year 1999 in the United States alone (19) and is undoubtedly now much higher. A clearer understanding of the underlying causes of β-agonist treatment failure in RSV bronchiolitis would therefore be of considerable benefit.

Some previous authors have proposed that β-agonist insensitivity in children with bronchiolitis may be a consequence of physical factors: impaired drug delivery to the small airways of young infants, particularly in the presence of bronchoconstriction and inflammatory airway narrowing (39), or the inability of β-agonists to diffuse from airways to smooth muscle, due to the presence of necrotic cell debris, inflammatory exudates, and edema fluid (31). Alternatively, or additionally, other investigators have provided evidence that β-agonist insensitivity may result from some specific defect in drug-receptor interaction or receptor signaling (28). In support of this latter hypothesis, we (8) previously demonstrated that BAE β2-AR are desensitized to β-agonists in RSV-infected BALB/c mice. We found that BAE β-agonist insensitivity in RSV-infected mice does not result from receptor internalization or degradation due to chronic elevation of endogenous catecholamines (which does not occur in response to RSV infection) and is not characterized by any dramatic shift in β2-AR binding kinetics. Rather, BAE β-agonist insensitivity appears to result from heterologous β2-AR desensitization, induced by ligation of CXCR2 by the chemokine KC, released in response to RSV infection. In the current study, we extended these findings to show that airway responses to β-agonists are similarly attenuated and by the same mechanism. Based on our data, we propose that heterologous, KC-mediated desensitization of β2-AR on BAE and ASM may account for the poor utility of β-agonists as therapies for both edema/hypoxemia and bronchoconstriction/wheezing in RSV bronchiolitis. Furthermore, based on our previous findings (8), we hypothesize that KC induces its effects via CXCR2-mediated activation of PKCζ and G protein-coupled receptor kinase 2 (GRK2), which phosphorylates β2-AR and thereby uncouples them from adenyl cyclase activation.

To measure β-agonist effects on airway function in mice, we administered a bronchoconstrictive stimulus (the muscarinic agonist MCh) and then, in a separate, age-matched group of mice, determined the ability of the β-agonist terbutaline to attenuate airway constriction in response to this agent. This approach was necessary because large airways in the mouse are almost completely relaxed at baseline, and so β-agonists will cause virtually no additional decrease in lung resistance (16). Indeed, our data confirmed this (Fig. 3A). Therefore, to demonstrate a β-agonist bronchodilator effect of sufficient magnitude that any RSV-induced changes in bronchodilatory response could be detected, we had to first induce bronchoconstriction. Importantly, to measure such RSV-induced changes in β-agonist responses, we also had to ensure that RSV did not significantly alter bronchoconstrictive responses to MCh.
which was in fact the case (Fig. 2A). Finally, we utilized separate groups of mice to determine responses to MCh and MCh + terbutaline at each time point to ensure that MCh + terbutaline dose-response curves were not altered by prior MCh exposure (and vice versa) and to limit the duration of lung function analysis per animal. However, to reduce variability between groups, we analyzed MCh and MCh + terbutaline responses for each time point on the same day in mice infected in parallel and randomized to either dose-response group. Although we could induce strong bronchodilatory responses to a single nebulized dose of terbutaline in normal mice, we administered terbutaline before each MCh dose to maximize bronchodilatory responses under all conditions. Importantly, bronchodilatory responses to a single nebulized dose of terbutaline in normal mice were of similar magnitude to, but not greater than, those induced by the multiple terbutaline doses we administered between MCh nebulizations in our experimental regimen (data not shown). This indicates that administration of multiple terbutaline doses did not itself induce β2-AR desensitization.

As noted above, because of the necessity to induce bronchoconstriction before measuring the bronchodilatory effect of β-agonist, we were unable to measure airway responses to both MCh and MCh + terbutaline in the same mouse. We were therefore unable to “match” animals between the MCh and MCh + terbutaline nebulization groups in any meaningful way and so could only calculate a single value for the overall effect of terbutaline (ΔTERBUT) at each postinfection time point (see MATERIALS AND METHODS). However, it is important to note that, at each time point and for each of these two nebulization groups, all of which had a minimum group size of 6, a mean %ΔR value for each MCh dose was derived. This value was calculated by determining %ΔR values at each MCh dose vs. baseline on an individual animal basis (Fig. 3, C and D) and then deriving the group mean %ΔR values used in the calculation of ΔTERBUT from all these individual values. We feel that this approach has good statistical validity and sufficient power to detect treatment effects despite ultimately resulting in a single value for the effect of terbutaline on R (ΔTERBUT).

Finally, it is important to note that this calculation relies on the fact that, unlike the Line 19 strain, the A2 strain of RSV does not induce AHR in mice (20). If the RSV strain used in our studies had induced an exaggerated response to MCh, it would artificially skew the data so that RSV would appear to reduce β-agonist responsiveness compared with controls simply because the denominator of the %ΔTERBUT calculation would be higher in RSV-infected mice. Moreover, AHR might also make it more difficult to achieve bronchodilation with terbutaline: certainly, we found that, on a percentage basis, terbutaline was a less effective bronchodilator in normal mice at 50 mg/ml MCh (where it attenuated bronchoconstriction by only 26%) than when airways were less constricted by exposure to 20 mg/ml MCh (where it attenuated bronchoconstriction by 43%).

To our knowledge, only one previous study to date has examined the effects of β-agonists on airway function during RSV infection; in this report, chronic systemic treatment with salmeterol was shown to reduce AHR in RSV-infected ovalbumin-sensitized mice (35). However, one shortcoming of this study was that lung mechanics were evaluated using the enhanced pause parameter (Penh), which is derived from unrestrained whole body plethysmography. In mice, Penh data must be interpreted with some caution, since this parameter is primarily related to ventilatory timing and contains little information regarding airway function (1). In contrast, the flexiVent computer-controlled piston ventilator system, which we used in the current study, permits accurate determination of both central and peripheral airway resistance and lung compliance using the forced oscillation technique (16) together with the ability to deliver by nebulization multiple doses of bronchoconstrictors and bronchodilators directly to the airways. Unlike whole body plethysmography, however, it is invasive and so does not permit serial measurements from the same animal.

Only a limited number of studies to date have employed the flexiVent to determine the effects of RSV on airway function in mice (3, 27, 42), and no previous studies have evaluated β-agonist responses in RSV-infected animals using this system.

We (8) showed previously that RSV infection does not alter either plasma catecholamine levels or whole lung homogenate β2-AR levels in mice. Herein, we show that terbutaline insensitivity in RSV-infected mice could be rapidly reversed by administration of blocking antibodies to KC or CXCR2 immediately before lung function analysis, which suggests that, like BAE β-agonist insensitivity, airway β-agonist insensitivity is unlikely to be a consequence of β2-AR degradation. Moreover, the ability to resensitize airways to terbutaline after KC or CXCR2 neutralization demonstrates that terbutaline insensitivity after RSV infection cannot be a consequence of inhibition of β2-AR by the chemokine used in our anesthetic regimen (34) or a simple dose-effect artifact of the MCh and terbutaline challenge regimen. Furthermore, the ability to reverse β-agonist insensitivity by systemic or local blockade of CXCR2, without altering viral replication, BAL KC levels, or lung edema (Table 1), indicates that β-agonist insensitivity is not a direct function of viral burden, although the lack of effect of UV-irradiated virus clearly demonstrates that viral replication is necessary for its induction. Finally, the ability of nebulized recombinant KC, but not heat-inactivated KC, to induce terbutaline insensitivity in uninfected mice demonstrates that presence of this chemokine in the airways is sufficient to induce β2-AR desensitization and indicates that desensitization is a specific consequence of RSV-induced airway inflammation rather than a result of a physiological catecholamine response to the stress of infection.

We (6) and others (17, 24) have previously shown (and show herein, Table 1) that KC is the predominant inflammatory mediator present in the BAL and lung tissue of mice at early time points following RSV infection. In children with RSV, an elevated plasma, nasal lavage, or lung level of CXCL8 may be an indicator of increased disease severity (36). KC/CXCL8 mainly promotes neutrophil chemotaxis and survival (21), but this chemokine can also directly alter ASM function: CXCL8 provokes bronchoconstriction in guinea pigs in vivo (11) and contraction of human ASM, which expresses CXCR2, in vitro (12). However, to our knowledge, CXCR2 expression has not been demonstrated on ASM in mice. Moreover, RSV Line 19-induced AHR was prevented by neutralization of CXCR2 and found to be absent in CXCR2−/− mice (20, 25). However, we did not find that recombinant KC induced AHR in normal mice (Fig. 5A). It is therefore tempting to speculate that the bronchoconstrictive effects of CXCL8 reported in these studies might actually reflect impaired bronchodilatory responses to
endogenous catecholamines. Importantly, BAL CXCL8 levels are elevated for a far longer period in infants hospitalized for severe bronchiolitis than are KC levels in lungs from RSV-infected mice (23). This suggests that β-agonist insensitivity following RSV infection might be much more prolonged in human subjects than in our mouse model and that the relatively early and brief effect that we report herein is more a reflection of the rather limited nature of RSV infection in the mouse than it is indicative of the clinical situation in children with bronchiolitis.

In conclusion, our data indicate that, in a similar fashion to those of BAE β2-AR, airway responses to β-agonists are acutely attenuated following RSV infection, so that even with optimal drug delivery via nebulizer, appropriate physiological bronchodilatory responses to β-agonists are blunted. Furthermore, desensitization of BAE and airway β-agonist responses occurs by the same mechanism, involving heterologous β2-AR uncoupling, induced by binding of the proinflammatory chemokine KC to its receptor, CXCR2. Whereas other physical factors, such as airway obstruction with inflammatory sequestrations and cellular debris, undoubtedly also contribute to the overall poor efficacy of β-agonists in children with bronchiolitis, these results support our previous finding (8) that RSV infection induces a specific β2-AR signaling defect, which may account for the poor therapeutic utility of β-agonists as bronchodilators in this condition (26).

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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