

Respiratory syncytial virus induces insensitivity to β -adrenergic agonists in mouse lung epithelium in vivo

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Submitted 18 November 2006; accepted in final form 9 April 2007

Davis IC, Xu A, Gao Z, Hickman-Davis JM, Factor P, Sullender WM, Matalon S. Respiratory syncytial virus induces insensitivity to β -adrenergic agonists in mouse lung epithelium in vivo. *Am J Physiol Lung Cell Mol Physiol* 293: L281–L289, 2007. First published April 13, 2007; doi:10.1152/ajplung.00458.2006.—Respiratory syncytial virus (RSV) is the most common cause of bronchiolitis in infants and children worldwide. We wished to determine whether intratracheal administration of β -agonists improved alveolar fluid clearance (AFC) across the distal respiratory epithelium of RSV-infected mice. Following intranasal infection with RSV strain A2, AFC was measured in anesthetized, ventilated BALB/c mice by instillation of 5% BSA into the dependent lung. We found that direct activation of protein kinase A by forskolin or 8-bromo-cAMP increased AFC at *day 2* after infection with RSV. In contrast, short- and long-acting β -agonists had no effect at either *day 2* or *day 4*. Insensitivity to β -agonists was not a result of elevated plasma catecholamines or lung epithelial cell β -adrenergic receptor degradation. Instead, RSV-infected mice had significantly higher levels of phosphorylated PKC ζ in the membrane fractions of their lung epithelial cells. In addition, insensitivity to β -agonists was mediated in a paracrine fashion by KC (the murine homolog of CXCL8) and reversed by inhibition of either PKC ζ or G protein-coupled receptor kinase 2 (GRK2). These results indicate that insufficient response to β -agonists in RSV may be caused, at least in part, by impaired β -adrenergic receptor signaling, as a consequence of GRK2-mediated uncoupling of β -adrenergic receptors from adenylyl cyclase.

paramyxovirus; protein kinase C; G protein-coupled receptor kinase 2; CXCL8

RESPIRATORY SYNCYTIAL VIRUS (RSV) is the most common cause of lower respiratory tract disease in infants and children worldwide (44), is a frequent initiator of acute asthma exacerbations in young children, and has a disease impact comparable to that of nonpandemic influenza A in the elderly (8). Approximately 2–3% of all cases of RSV bronchiolitis result in severe hypoxia or a need for parenteral fluid supplementation that necessitates hospitalization (44). β -agonists are frequently used to treat RSV bronchiolitis, primarily because of their perceived ability to relax airway smooth muscle and cause bronchodilation, with the ultimate aim of alleviating hypoxemia. However, it is not clear that these drugs are clinically effective: meta-analyses have shown little or no overall benefit, regardless of viral bronchiolitis severity (15, 20). Their lack of efficacy has not been explained, although it has often been ascribed to difficulties associated with drug delivery to the small airways of young

infants, particularly in the presence of bronchoconstriction and inflammatory exudates or airway obstruction (28). β -agonists increase total body oxygen consumption, thereby increasing oxygen demands in infants hospitalized for respiratory compromise, and can potentially exacerbate ventilation-perfusion mismatch by inducing vasodilation without bronchodilation (12). Indeed, because of an aggregate preponderance of potential harm over therapeutic benefit, the American Academy of Pediatrics recently recommended that bronchodilators should not be used routinely in the management of bronchiolitis.

The process of alveolar fluid clearance (AFC) is crucial to efficient gas exchange in the lung (reviewed in Refs. 24 and 25), and patients with acute lung injury with intact AFC have lower morbidity and mortality than those with compromised AFC (45). More than 90% of pulmonary β -adrenergic receptors (β -AR) are actually expressed on alveolar epithelial cells rather than in bronchial epithelium or smooth muscle (3), and β -agonists have been shown to improve AFC in animal models of lung injury in which AFC is impaired (reviewed in Ref. 33). β -agonist prophylaxis has also been shown to be of value in reducing the incidence of high-altitude pulmonary edema (itself a consequence of impaired AFC secondary to hypoxia at high altitude) in susceptible mountaineers (42), and intravenous salbutamol treatment can reduce extravascular lung water in patients with acute lung injury (35).

Previously, we demonstrated that infection of BALB/c mice with RSV significantly impairs AFC at early time points after infection (by 43% from mock-infected values at *day 2*). This decrease in AFC, which is mediated by de novo synthesized UTP acting on P2Y purinergic receptors, was temporally associated with hypoxemia in RSV-infected mice (5). Our studies also showed that RSV-mediated nucleotide release, AFC inhibition, and the associated hypoxemia could be prevented by pretreatment of mice with the de novo pyrimidine synthesis inhibitor leflunomide (5). These findings suggest that bronchoalveolar edema, occurring as a consequence of reduced active Na⁺ transport by the respiratory epithelium, may be an unrecognized component of RSV disease that plays a role in development of hypoxemia, either by impairing alveolar gas exchange or by contributing to obstruction of small airways. As described above, results of previous studies indicate that the AFC deficit caused by RSV infection in this model should be corrected by β -agonists (33). We were therefore able to use our model as a functional in vivo assay to directly determine

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whether or not intra-alveolar instillation of short- and long-term-acting β -agonists can increase AFC after RSV infection. Having determined that β -agonists failed to increase AFC in RSV-infected mice, we designed a series of physiological and biochemical studies to identify the potential cellular mechanisms underlying this β -agonist insensitivity.

MATERIALS AND METHODS

Reagents. 8-Bromo-cAMP (Sigma-Aldrich, St. Louis, MO), β -agonists (Sigma-Aldrich), propranolol (Sigma-Aldrich), 14-22 amide (EMD Biosciences, La Jolla, CA), adenosine deaminase (Sigma-Aldrich), metRANTES (R&D Systems, Minneapolis, MN), anti-KC MAb (MAB453, R&D Systems), anti-KC pAb (AF-453-NA, R&D Systems), anti-CXCR2 MAb (MAB2164, R&D Systems), anti-CXCR4 pAb (TP503; Torrey Pines Biolabs, Houston, TX), and rat IgG_{2A} (MAB006, R&D Systems) were reconstituted in normal saline. Forskolin (Sigma-Aldrich), amiloride (Sigma-Aldrich), G protein-coupled receptor kinase 2 (GRK2) inhibitor (EMD Biosciences), and GF-109203X (EMD Biosciences) were reconstituted in DMSO. Indomethacin (Sigma-Aldrich) was reconstituted in ethanol. Fresh tert-butane stocks were prepared weekly.

Preparation of viral inocula and infection of mice. Preparation of viral stocks and intranasal infection of 8- to 12-wk-old pathogen-free BALB/c mice of either sex with endotoxin- and mycoplasma-free RSV strain A2 (10^6 PFU in 100 μ l) were performed as previously described (5). All mouse procedures were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

AFC measurements. AFC was measured as previously described. All reagents were added to the AFC instillate from stock solutions directly before instillation, in a minimal volume of solvent (1-10 μ l/ml). Previous studies have demonstrated that measured declines in AFC are not a consequence of instillate dilution by intrapulmonary edema fluid (14, 18).

Measurement of plasma catecholamines. EDTA plasma was collected from mice, euthanized following administration of an identical anesthetic regimen to that used in AFC procedures. Epinephrine and norepinephrine levels were measured using the CatCombi ELISA (RDI, Concord, MA).

Alveolar cell isolation and cytoplasmic and membrane fraction preparation. Alveolar cells were isolated from BALB/c mice using an adaptation of the method of Warshamana et al. (46). Cell cytoplasm and membrane fractions were prepared as follows. Briefly, control and RSV-infected cells were lysed in 500 μ l of lysis buffer (50 mM Tris·HCl, pH 7.5, 2 mM EDTA, 2 mM EGTA, and 0.2 mM Na₃VO₄) supplemented with 1 \times protease inhibitor cocktail (BD Pharmingen, San Diego, CA) and then centrifuged at 16,000 g for 20 min at 4°C to separate cytosolic and membrane fractions. The membrane pellet was then lysed in the above buffer plus 1% Triton X-100, 0.5% Nonidet P-40, and 150 mM NaCl, and cleared by centrifugation at 16,000 g for 10 min. The supernatant containing membrane proteins was then carefully removed. Protein concentrations in all preparations were measured by the BCA method using BSA as a standard. All protein samples were stored at -80°C before use.

Western blotting protocol. Alveolar cell membranes and cytoplasmic proteins were separated by SDS-PAGE, and Western blots were performed using a standard protocol. Blots were probed with rabbit antibodies to PKC ζ (sc-216; Santa Cruz Biotechnology, Santa Cruz, CA) and then stripped and reprobed for phospho-PKC ζ (sc-12894-R; Santa Cruz Biotechnology) and β -actin (Cell Signaling Technology, Danvers, MA). Bound primary antibodies were detected with HRP-conjugated goat anti-rabbit secondary antibodies and enhanced chemiluminescence (GE Healthcare Life Sciences, Piscataway, NJ), followed by exposure to X-ray film. Band intensity was measured using AlphaEaseFC on a FluorChem imager (Alpha Innotech, San Leandro, CA) and normalized to β -actin levels.

Peripheral lung total cell membrane protein preparation. Mice were euthanized using an identical anesthetic regimen to that for AFC studies, and a thoracotomy was performed. The right ventricle was cannulated, and the pulmonary circulation was flushed with ice-cold PBS until no visible evidence of blood remained. The peripheral lung tissue from 15–20 mice was pooled for each group (uninfected, day 2, or day 6) and then homogenized in lysis buffer. Total cell membrane protein was isolated, and cell membrane fractions were stored at -80°C until used. The assay was repeated three times for each time point.

β_2 -AR saturation binding assay. Saturation binding was performed as previously described (21). Briefly, 100 μ g of whole peripheral lung cell membrane proteins were incubated with incremental concentrations (1–50 nM) of the β_2 -AR-specific inverse agonist [³H]ICI-118,551 at 37°C for 1 h with gentle shaking. The reaction was terminated by rapid vacuum filtration using a cell harvester (Molecular Devices Micro 96, Sunnyvale, CA) through presoaked Printed Filtermat B filters (Wallac, Tuku, Finland), which were washed five times and then counted with a micro-beta counter. Nonspecific binding was determined by incubating 100 μ g of protein with 200 μ M unlabeled ICI-118,551 before addition of 50 nM [³H]ICI-118,551 and harvesting. Specific binding was determined by subtracting nonspecific binding from total binding. Triplicate measurements were made for each concentration of [³H]ICI-118,551 tested. Scatchard analysis was performed using Prism statistical software (GraphPad, San Diego, CA).

β_2 -AR competition binding assay. Competition binding assays were performed as previously described (21). Total peripheral lung cell membranes were prepared as described above for β_2 -AR saturation binding assays, except two additional centrifugations were performed to ensure the removal of endogenous GTP. Membranes (100 μ g) were incubated with 10 nM [³H]ICI-118,551 and 24 incremental concentrations of the β_2 -AR-specific agonist procaterol (10^{-10} to 2×10^{-3} M) for 1 h at 37°C. All reactions were performed in duplicate. Competition data were evaluated with one- and two-binding site models by an iterative least-squares technique.

Statistical analyses. Descriptive statistics were calculated using Instat software (GraphPad). Gaussian data distribution was verified by the method of Kolmogorov and Smirnov. Differences between group means were analyzed by ANOVA, with Tukey-Kramer multiple comparison post tests. Student's *t*-test was used to compare group means for data in Fig. 2B only. *P* < 0.05 was considered statistically significant. All data are presented as means \pm SE.

RESULTS

Effect of protein kinase A activation on AFC. Previously, we demonstrated that AFC is significantly inhibited at day 2 and day 4 after infection of BALB/c mice with RSV (5). For our current studies of β -agonist effects on impaired AFC after RSV infection, we concentrated predominantly on the day 2 time point, when AFC is most suppressed. The mean AFC rate over 30 min in RSV-infected mice is 21% at day 2 and 27% at day 4. Thus AFC is reduced by 43% and 26%, respectively, at these time points, from the mean rate in mock-infected animals [36%, which is identical to the rate in uninfected animals (5)]. There was no difference between day 2 AFC values derived from three separate infections using the viral inoculum preparation used in all the experiments described in the current study ($21.8 \pm 2.3\%$, *n* = 10) and those we reported previously using two historically distinct viral preparations [$22 \pm 1\%$, *n* = 25 (5)]. Likewise, day 4 AFC values derived using the current inoculum ($26.3 \pm 1.6\%$, *n* = 16) were comparable to those we previously reported with a distinct virus preparation [$27.4 \pm 2.1\%$, *n* = 9 (5)].

Stimulatory effects of β-agonists on AFC have been shown to be mediated via activation of adenylyl cyclase (AC), which generates cAMP and thereby stimulates cAMP-dependent protein kinase (protein kinase A or PKA, reviewed in Ref. 19). AC can be activated directly using the bacterial toxin forskolin. Data shown here indicate that, when added to the AFC instillate, forskolin (50 μM) increased mean AFC by 53% in RSV-infected mice at day 2, thereby restoring AFC to its level in mock-infected mice (Fig. 1A). The PKA activator 8-bromo-cAMP (50 μM) had a comparable effect on AFC at day 2. The stimulatory effect of forskolin on AFC was significantly inhibited (by 27%) by concomitant addition of the epithelial Na⁺ channel (ENaC) inhibitor amiloride (1.5 mM) or the PKA inhibitor 14-22 amide (100 μM) to the instillate (Fig. 1B). Forskolin only increased amiloride-insensitive AFC by 8% at day 2, suggesting that the majority of its stimulatory effect on AFC was due to activation of ENaC.

Effect of β-adrenergic agonists on AFC. Unlike forskolin, the β₂-AR agonist terbutaline (1, 10, or 100 μM) had no significant effect on AFC at day 2 (Fig. 2A). Terbutaline (1 mM) did stimulate AFC at this time point (increasing AFC by 42% to 30.6 ± 0.67% over 30 min, n = 13), but AFC still remained significantly lower (P < 0.05) than in mock-infected animals [34.8 ± 1.5, n = 11 (5)]. Salmeterol (100 μM) (a highly potent, long-acting β₂-AR agonist), isoproterenol (a nonspecific β-AR agonist), and procaterol (a short-acting β₂-AR agonist) likewise had no stimulatory effect on AFC at day 2 after RSV infection (Fig. 2A). Responsiveness to 100 μM terbutaline was also absent at day 4 after infection (Fig. 2B).

These data demonstrate that PKA is functional in RSV-infected mice and that direct activation of PKA by forskolin or 8-bromo-cAMP restores AFC to normal levels, primarily by stimulating Na⁺ reabsorption via amiloride-sensitive ENaC-like channels in the bronchoalveolar epithelium. On the basis of this finding, and on previous reports using comparable β-agonist doses (10, 36), upstream activation of β-AR by β-agonists should therefore result in PKA activation and hence the same increase in AFC. However, β-agonists had no effect on AFC in RSV-infected mice, except at very high doses. Our subsequent studies therefore focused on determining the underlying cause of this insensitivity to β-agonists following

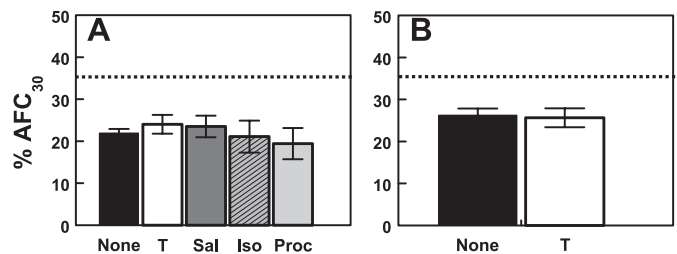


Fig. 2. Effect of β-adrenergic agonists on AFC in RSV-infected mice. A: effect of 100 μM terbutaline (T; n = 17), 100 μM salmeterol (Sal; n = 14), 100 μM isoproterenol (Iso; n = 8), and 100 μM procaterol (Proc; n = 7) on AFC at day 2 (no treatment, None; n = 10). B: effect of 100 μM terbutaline (T; n = 9) on AFC at day 4 (no treatment, None; n = 16). Dotted line indicates mean AFC rate in untreated, mock-infected animals (n = 11).

RSV infection and ultimately demonstrate that this phenomenon is not a simple dose effect, since lower concentrations of β-agonists can stimulate AFC provided that the intracellular milieu is appropriately modulated.

Role of endogenous catecholamines in β-agonist insensitivity in RSV-infected mice. β₂-AR can become desensitized as a result of prolonged exposure to exogenous β-agonists or high levels of endogenous catecholamines (9, 32). We therefore examined the possibility that RSV infection might induce sufficient physiological stress to trigger activation of the adrenal medulla and thereby chronically elevate systemic endogenous catecholamine (norepinephrine and epinephrine) levels. This might be sufficient to induce β₂-AR internalization or sequestration, with loss of receptors from the cell surface. However, as shown in Fig. 3A, RSV infection had no effect on plasma catecholamine levels, which remained at control levels at day 2 (Fig. 3A). To support these findings, we examined the effect of β-agonists on AFC in RSV-infected adrenalectomized BALB/c mice, in which the majority of the catecholamine response is absent (although central pathways remain intact). Normal, adrenalectomized mice showed a reduced rate of AFC compared with control animals (Fig. 3B), an effect that has been reported previously (7). Infection of adrenalectomized mice with RSV for 2 days resulted in a reduction in AFC to a final level identical to that seen in intact mice after infection (21%). However, like intact RSV-infected animals, adrenalectomized, RSV-infected mice showed no increase in AFC in response to 100 μM terbutaline at day 2. Together, these findings indicate that β-AR desensitization at day 2 after RSV

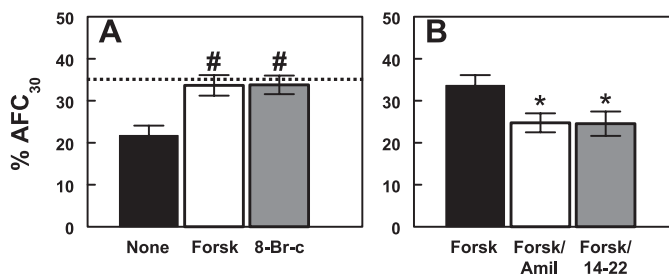


Fig. 1. Effect of protein kinase A activation on alveolar fluid clearance (AFC) in respiratory syncytial virus (RSV)-infected mice. A: effect of 50 μM forskolin (Forsk; n = 10) and 50 μM 8-Br-cAMP (8-Br-c; n = 9) on AFC at day 2 (no treatment, None; n = 10). B: effect of 1.5 mM amiloride (Forsk/Amil; n = 8) and 100 μM 14-22 amide (Forsk/14-22; n = 10) on forskolin-stimulated AFC at day 2 (forskolin-stimulated, Forsk; n = 10). Dotted line indicates mean AFC rate in untreated, mock-infected animals (n = 11). #P < 0.005 compared with untreated, infected mice at day 2. *P < 0.05 compared with forskolin-treated, RSV-infected mice at day 2.

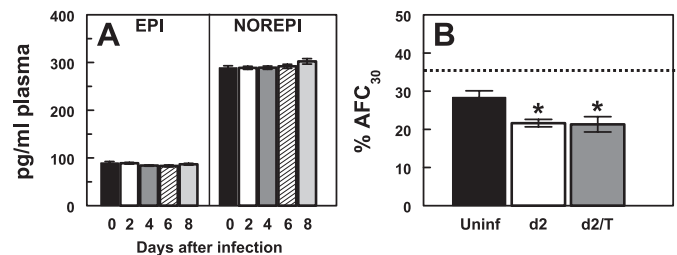


Fig. 3. Role of endogenous catecholamines in β-agonist insensitivity in RSV-infected mice. A: effect of RSV infection on plasma epinephrine (EPI) and norepinephrine (NOREPI) levels at day 0 to day 8 after infection (n = 12–16/group). B: effect of RSV infection for 2 days (d2; n = 8) and RSV infection for 2 days + 100 μM terbutaline (d2/T; n = 10) on AFC in adrenalectomized mice (uninfected, Uninf; n = 9). Dotted line indicates mean AFC rate in untreated, mock-infected intact mice (n = 11). *P < 0.05 compared with uninfected, adrenalectomized mice.

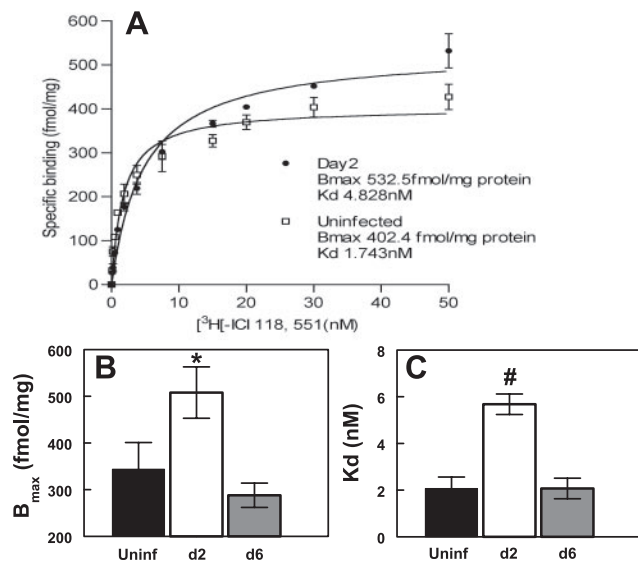


Fig. 4. Effect of RSV infection on β_2 -adrenergic receptor (β_2 -AR) expression in the peripheral lung. **A**: representative Scatchard plots for β_2 -AR density and affinity in peripheral lung homogenates from mice infected with RSV for 2 days compared with uninfected mice (mean data from 2 experiments). **B**: effect of RSV infection for 2 and 6 days on β_2 -AR density in mouse peripheral lung homogenates (Uninf, uninfected). **C**: effect of RSV infection for 2 and 6 days on β_2 -AR ligand affinity in mouse peripheral lung homogenates (Uninf, uninfected). $N = 3$ replicate assays per group (15–20 mice per group per replicate). * $P < 0.05$, # $P < 0.005$ compared with untreated mice (by paired ANOVA).

infection is not due to receptor sequestration or degradation mediated by endogenous catecholamines.

Effect of RSV infection on membrane-bound β_2 -AR characteristics. To further investigate effects of RSV on β -AR function, we quantified membrane-bound β_2 -AR levels in homogenates of peripheral lung tissue using a saturation binding method with the highly β_2 -AR-specific inverse agonist ICI-118,551 (Fig. 4A, data plot from 2 pooled experiments). Interestingly, infection with RSV for 2 days actually resulted in a 32% increase in β_2 -AR density (B_{max} , Fig. 4B). However, the K_d (the concentration at which 50% of receptors are bound) was fourfold greater at day 2 compared with uninfected mice (Fig. 4C). The precise binding site(s) of ICI-118,551 to the β_2 -AR are not known, thus the K_d may not be an accurate index of receptor affinity for ligand. Mice infected with RSV for 6 days had normal β_2 -AR density and ligand affinity. AFC rate is normal at this time point (5).

Effect of protein kinase inhibition on β -agonist sensitivity in RSV-infected mice. The results of our Scatchard analyses were incompatible with β_2 -AR desensitization due to receptor internalization or degradation. However, β_2 -AR can also become desensitized as a result of serine phosphorylation by GRK2 (reviewed in Ref. 19). This process, which is rapid and readily reversible, uncouples β_2 -AR from its signal transduction pathway so that β -agonist binding no longer results in AC activation. Using AFC as a functional readout, we therefore examined the effects of GRK2 inhibition on β -agonist sensitivity after RSV infection. Coinstitution of 100 μ M terbutaline with a specific cell-permeable GRK2 inhibitor (100 μ M, EMD Biosciences) resulted in restoration of terbutaline sensitivity and increased AFC to its level in uninfected mice (Table 1). In the absence of terbutaline, the GRK2 inhibitor had no effect on AFC at day 2 after infection. Thus GRK2 blockade prevented desensitization of β_2 -AR to β -agonists. The increase in AFC induced by terbutaline following GRK2 inhibition could be blocked by 14-22 amide or amiloride, confirming that it results from activation of PKA and upregulation of Na^+ transport via amiloride-sensitive ENaC pathways. Inhibition of GRK2 also restored terbutaline sensitivity at day 4 after infection (increasing AFC over 30 min from $25.6 \pm 1.7\%$, $n = 9$, to $33.2 \pm 1.2\%$, $n = 8$).

PKC, which is known to be activated during RSV infection (30), can serine phosphorylate GRK2, promoting its translocation from the cytosol to the plasma membrane and increasing its activity (34). Addition of the general PKC inhibitor bisindolylmaleimide 1 (GF-109203X, 100 μ M) or a cell-permeable inhibitor of the PKC ζ isoform (myristoylated PKC ζ inhibitory peptide, 2 μ g/ml) to the AFC instillate again resulted in a significant increase in AFC in response to concomitant terbutaline stimulation at day 2 (Table 2). In contrast, an inhibitor of the PKC α/β isoforms (Gö6976, 10 μ M) or a cell-impermeable inhibitor of the PKC ζ isoform (unmyristoylated PKC ζ inhibitory peptide, 2 μ g/ml) had no effect on terbutaline sensitivity. In the absence of terbutaline, none of these PKC inhibitors had any effect on AFC in RSV-infected mice at day 2.

Effect of RSV infection on PKC ζ phosphorylation in mouse lung alveolar cells. To confirm that infection with RSV results in activation of PKC ζ , mouse lung alveolar cells from three uninfected mice and three infected animals were analyzed by Western blotting using specific antibodies. When normalized to β -actin levels, RSV infection reduced total PKC ζ levels in both the cytosolic (not shown) and membrane fraction from alveolar cells. In contrast, infection with RSV caused a signif-

Table 1. Effect of GRK2 inhibition on terbutaline-sensitive alveolar fluid clearance at day 2 after RSV infection

Treatment	Conc., μ M	n	%AFC ₃₀ *	Δ Terbutaline (%)†
None		10	21.8 \pm 2.3	
Terbutaline	100	17	24 \pm 2.3	10
GRK2i	100	10	23.4 \pm 2.1	
GRK2i + terbutaline	100+100	8	32.9 \pm 2.2‡§	50
GRK2i + terbutaline + amiloride	100+100+1,500	7	19.6 \pm 1.9	-11
GRK2i + terbutaline +14-22 amide	100+100+100	6	19.9 \pm 2.1	-9

GRK2i, G protein-coupled receptor kinase 2 inhibitor; Conc., concentration; AFC, alveolar fluid clearance. n = Number of mice in which AFC was evaluated. *Mean %AFC \pm SE. †%Change in mean AFC with terbutaline. ‡%AFC₃₀ in mock-infected mice is 34.8 ± 1.5 ($n = 11$) (from Ref. 4). § $P < 0.05$ vs. untreated AFC at day 2.

Table 2. Effect of PKC inhibition on terbutaline-sensitive AFC at day 2 after RSV infection

Treatment	Conc.	n	%AFC ₃₀ *	ΔTerbutaline (%)†
None		10	21.8±2.3	
Terbutaline	100 μM	17	24±2.3	10
PKC inhibitor (GF-109203X)	100 μM	6	19.7±1.9	
GF-109203X + terbutaline	100 μM+100 μM	7	33.7±2.1‡§	54
PKCα/β1 inhibitor (Gö6976)	10 μM	9	19.8±2.5	
Gö6976 + terbutaline	10 μM+100 μM	11	23.8±2.5	8
Myristoylated PKCζ inhibitor (mPKCζi)	2 μg/ml	8	23.2±2	
mPKCζi + terbutaline	2 μg/ml+100 μM	8	33.3±1.1 ^a	52
Unmyristoylated PKCζ inhibitor + Terb.	2 μg/ml+100 μM	6	23.8±1.3	9

RSV, respiratory syncytial virus; Terb., terbutaline. n = Number of mice in which AFC was evaluated. *Mean %AFC ± SE. †%Change in mean AFC with terbutaline. ‡%AFC₃₀ in mock-infected mice is 34.8 ± 1.5 (n = 11) (from Ref. 4). §P < 0.05, ^aP < 0.005 vs. untreated AFC at day 2.

icant increase in activated Thr410 phospho-PKCζ levels in the membrane fraction at day 2 (Fig. 5, A and B). Thus the ratio of phosphoPKCζ:PKCζ in the membrane fraction of alveolar cells from RSV-infected mice increased by ~300% at day 2 compared with uninfected mice. Although activation of several PKC isoforms, including PKCζ, has been demonstrated previously in vitro following RSV infection (1, 30, 41), this is the first demonstration of PKCζ activation in RSV-infected mouse lung ex vivo.

Effect of RSV infection on β₂-adrenergic receptor phosphorylation. The above findings suggest that activated GRK2 desensitizes β₂-AR either directly by receptor phosphorylation or indirectly by phosphorylation or sequestration of a stimulatory G protein α-subunit (G_sα), either of which will prevent activation of AC in response to β-agonists. In our next series of experiments, we therefore performed competition binding assays (with ICI-118,551 and procaterol) to quantify the fraction of high- and low-affinity receptors in pooled homogenates of peripheral lung tissue. Comparison of curve fit analysis from two separate sets of experiments for both RSV and uninfected controls was most consistent with a two-site binding site model (Fig. 6). However, no significant differences were detected in the fraction of low-affinity (phosphorylated) β₂-AR between uninfected mice (logEC₅₀ -7.59 ± 0.22, n = 2) and RSV-infected mice at day 2 (logEC₅₀ -7.97 ± 0.16, n = 2). Likewise, infection with RSV for 2 days had no effect on the fraction of high-affinity (unphosphorylated) β₂-AR detected by this technique (logEC₅₀ -5.93 ± 0.06 at day 2 vs. -5.79 ± 0.13 in uninfected mice). These data suggest that β₂-AR insensitivity is not a result of increased phosphorylation of membrane-bound receptors.

Effect of KC inhibition on β-agonist sensitivity in RSV-infected mice. Since RSV only infects a relatively small proportion of respiratory epithelial cells (23), generalized desensitization of β₂-AR is unlikely to occur as a result of a direct effect of viral gene products on infected cells and is more likely

to be mediated by a soluble factor. Because infection with RSV does not elevate circulating catecholamine levels and thereby induce homologous β₂-AR desensitization, we investigated the possibility that, by binding to its receptors on epithelial cells, another soluble mediator induces heterologous β₂-AR desensitization following RSV infection. We have shown previously that, quantitatively, the predominant soluble mediator in the lung at days 2 and 4 after infection is the CXC chemokine KC (keratinocyte cytokine, the murine homolog of CXCL8/IL-8), which is present at log-fold higher concentrations in the bronchoalveolar lavage (BAL) than other cytokines such as TNF-α and IL-1β [BAL KC concentration 913 ± 36 pg/ml at day 2 vs. 80 ± 21 pg/ml in mock-infected mice (4)]. BAL KC levels also return to normal at days 6 and 8, when AFC has normalized. Mice express only functional CXCR2 receptors for KC, whereas humans also possess a CXCR1 receptor subtype that can bind CXCL8 (29). CXCR2 is coupled to inhibitory Gα protein subunits and can activate PKC (38). We therefore investigated the possibility that cross talk signals from activation of CXCR2 by KC might account for β-agonist insensitivity at day 2 following RSV infection and found that addition to the AFC instillate of a neutralizing monoclonal antibody to KC did indeed result in restoration of terbutaline sensitivity at day 2 (Table 3). This antibody had no effect on AFC in the absence of terbutaline, and an isotype-matched irrelevant antibody had no effect on AFC in either the presence or absence of terbutaline. A polyclonal neutralizing anti-KC antibody had a similar effect on terbutaline sensitivity at day 2. Likewise, addition to the AFC instillate of a neutralizing MAb to CXCR2, which alone had no effect on AFC, also resulted in partial restoration of terbutaline sensitivity at day 2, whereas a neutralizing polyclonal antibody to the chemokine receptor CXCR4 (species matched to that used against KC) had no such effect. These findings suggest that β₂-AR desensitization may occur as a consequence of cross talk from CXCR2 chemokine receptors activated by KC.

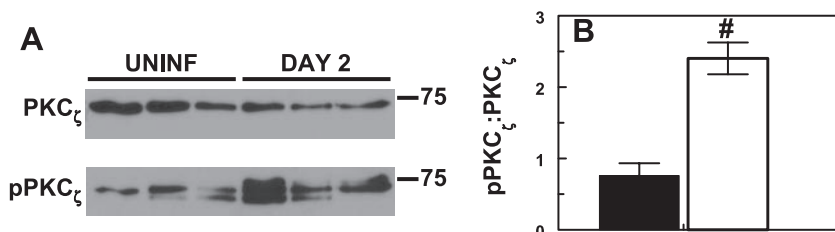


Fig. 5. Effect of RSV infection on PKCζ phosphorylation in mouse lung alveolar cells. A: representative Western blot showing total and phospho-PKCζ (both ~73 kDa) in the membrane fraction in uninfected and RSV-infected mice at day 2. B: effect of RSV infection for 2 days on the mean ratio of phospho-PKCζ:total PKCζ (measured by densitometry) in the membrane fraction from alveolar cells (n = 3/group). #P < 0.005 compared with uninfected mice.

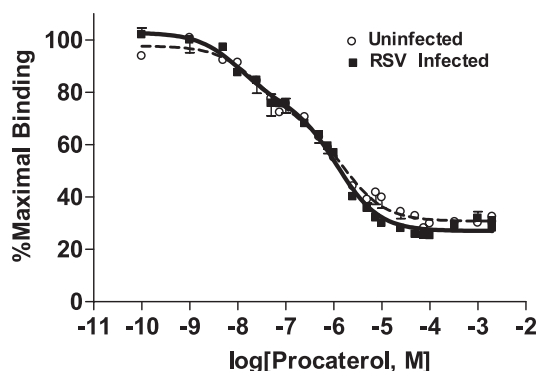


Fig. 6. Effect of RSV infection on β_2 -AR phosphorylation. Results are of competition binding assays on total peripheral lung cell membranes from uninfected mice and mice infected with RSV for 2 days using [3 H]ICI-118,551 and the β_2 -AR-specific agonist procateterol. $N = 2$ replicate assays/group (20 mice per group per replicate).

DISCUSSION

Previously, we demonstrated that infection of BALB/c mice with RSV results in reduced AFC at *days 2–4* after infection (5). We have restricted our current studies predominantly to a comparison of the effects of forskolin and β -agonists on AFC at *day 2* after RSV infection, the time point at which AFC is most impaired in our model, and therefore, at least in terms of its ion transport function, the epithelium is functionally most “injured.” Although it has not yet been formally demonstrated that impaired AFC is a component of human RSV disease, a significant subset of hospitalized infants with RSV that requires mechanical ventilation actually exhibits restrictive lung disease that fulfills clinical criteria for classification as acute respiratory distress syndrome (12, 13). Impaired AFC is therefore likely to be present at least in this group of infants (45). Moreover, the AFC response to β -agonists provides a useful *in vivo* readout of epithelial β -AR (and associated signaling apparatus) function, regardless of the overall contribution of impaired AFC to the pathogenesis of RSV bronchiolitis.

Initially, we found that forskolin stimulates AFC at *day 2* after RSV infection, when AFC is most impaired, and returns AFC to its level in normal mice. This increase in AFC, which is comparable to that reported by other investigators using β -agonists in injured lung models (reviewed in Ref. 33), can be completely inhibited by 14-22 amide or amiloride, indicating

that forskolin, by activating PKA, is stimulating transepithelial Na^+ transport predominantly through ENaC-like channels in RSV-infected mice (although we cannot exclude effects on the $\text{Na}^+-\text{K}^+-\text{ATPase}$). These channels are the rate-limiting step in epithelial Na^+ reabsorption, which is the main driving force for AFC. In contrast, low doses of either short- or long-acting, β_2 -specific, or nonspecific, β -agonists that had been shown in other studies to stimulate AFC (39) did not increase AFC at *day 2* and *day 4* post-RSV infection. Although we did find some improvement in AFC at *day 2* with 1 mM terbutaline, such a high dose is unlikely to be of either physiological or clinical relevance and would be highly likely to provoke significant side effects such as tachycardia and arrhythmias (26, 40).

Our data suggest that β -agonist insensitivity in RSV-infected mice does not result from receptor internalization or degradation due to chronic elevation of endogenous catecholamines and is not characterized by any dramatic shift in β_2 -AR binding kinetics. Rather, it appears to result from β_2 -AR desensitization by GRK2, which on activation by PKC ζ uncouples β_2 -AR from their normal AC stimulation pathway. In turn, PKC ζ activation may result from ligation of CXCR2 by KC, released in response to RSV infection (Fig. 7). Moreover, β -agonist insensitivity is relatively prolonged, since it persists until *day 4* after infection, and can be accounted for by the same mechanism throughout this period.

Several lines of evidence support our contention that β_2 -AR desensitization in RSV-infected mice occurs as a result of receptor uncoupling rather than internalization or degradation because of phosphorylation. First, blockade of PKC ζ , GRK2, or CXCL8 restores responsiveness to terbutaline comparable to that induced by forskolin, indicating that such blockade effectively recouples β_2 -AR to PKA. Second, RSV is known to trigger phosphorylation, sustained activation, and cytoplasm-to-membrane translocation of PKC ζ (30, 41), which is consistent with this mechanism. Third, membrane β_2 -AR expression increases at *day 2*, which is incompatible with β -agonist desensitization as a result of either receptor internalization or degradation. Fourth, terbutaline insensitivity in RSV-infected mice is rapidly reversible (inhibitors of GRK2, PKC, and CXCL8 are only present during the 30-min AFC procedure), indicating that β -agonist insensitivity cannot be a consequence of β_2 -AR degradation. Moreover, the ability to resensitize β_2 -AR to 100 μM terbutaline after GRK2, PKC, or CXCL8

Table 3. Effect of CXCL8 (KC) neutralization on terbutaline-sensitive AFC at *day 2* after RSV infection

Treatment	Conc.	n	%AFC $_{30}$ *	Δ Terbutaline (%)†
None		10	21.8 \pm 2.3	
Terbutaline	100 μM	17	24 \pm 2.3	10
Rat IgG $_{2A}$	1 $\mu\text{g}/\text{ml}$	7	23.8 \pm 1.1	
Rat IgG $_{2A}$ + terbutaline	1 $\mu\text{g}/\text{ml}$ + 100 μM	8	22.4 \pm 1.8	-6
Anti-KC MAb‡	1 $\mu\text{g}/\text{ml}$	13	21 \pm 1.3	
Anti-KC MAb + terbutaline	1 $\mu\text{g}/\text{ml}$ + 100 μM	20	32 \pm 0.94§ ^c	53
Anti-KC pAb ^a	1 $\mu\text{g}/\text{ml}$	7	21.2 \pm 2	
Anti-KC pAb + terbutaline	1 $\mu\text{g}/\text{ml}$ + 100 μM	13	30.8 \pm 1.9 ^b	45
Anti-CXCR2 MAb‡	1 $\mu\text{g}/\text{ml}$	13	22.3 \pm 2.1	
Anti-CXCR2 MAb + terbutaline	1 $\mu\text{g}/\text{ml}$ + 100 μM	9	28.7 \pm 0.8 ^b	29
Anti-CXCR4 pAb ^a	1 $\mu\text{g}/\text{ml}$	8	23.7 \pm 3.1	
Anti-CXCR4 pAb + terbutaline	1 $\mu\text{g}/\text{ml}$ + 100 μM	10	24.5 \pm 2.9	4

n = Number of mice in which AFC was evaluated. *Mean %AFC \pm SE. †Change in mean AFC with terbutaline. ‡Rat IgG $_{2A}$ isotype. §%AFC $_{30}$ in mock-infected mice is 34.8 \pm 1.5 ($n = 11$) (from Ref. 4). ^aRabbit anti-rat polyclonal antibody (pAb). ^b $P < 0.05$, ^c $P < 0.005$ vs. untreated AFC at *day 2*.

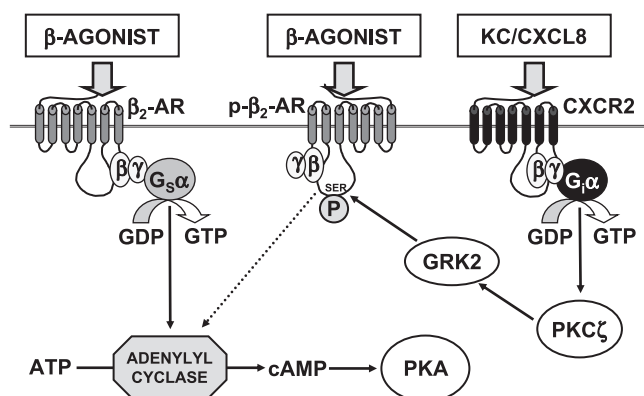


Fig. 7. Proposed mechanism for heterologous desensitization of β_2 -AR by KC following RSV infection. Normally, binding of β -agonist to β_2 -AR results in hydrolysis of GTP by the stimulatory α -subunit of the receptor-associated heterotrimeric G protein ($G_s\alpha$), which then dissociates and activates adenylyl cyclase. Adenylyl cyclase in turn catalyzes formation of cAMP from ATP. cAMP then activates cAMP-dependent protein kinase (protein kinase A, PKA), which stimulates AFC via effects on epithelial Na^+ channel and the Na^+ - K^+ -ATPase (and possibly CFTR). However, following RSV infection, KC/CXCL8 is produced. Upon binding to its receptors (CXCR2), and in humans also CXCR1), KC promotes hydrolysis of GTP by the inhibitory α -subunit of the receptor-associated heterotrimeric G protein ($G_i\alpha$), which then activates PKC ζ . In turn, PKC ζ phosphorylates and activates G protein-coupled receptor kinase 2 (GRK2), which either serine phosphorylates β_2 -AR (p- β_2 -AR), uncoupling them from $G_s\alpha$, or which can directly sequester $G_s\alpha$ (not shown). Upon binding of β -agonist, these phosphorylated receptors are unable to activate adenylyl cyclase, and no AFC response is detected.

inhibition demonstrates that terbutaline insensitivity after RSV infection cannot be a consequence of inhibition of β_2 -AR by the ketamine used in our AFC anesthetic regimen (43) or a simple dose effect artifact of the AFC procedure itself. In addition, the ability to increase AFC in response to both short-term treatment with either forskolin or β -agonists (when β_2 -AR phosphorylation is blocked) demonstrates that the AFC deficit is solely functional and not a consequence of cell death, which is not a feature of RSV infection in polarized epithelia (48). Finally, the lack of effect of inhibitors of GRK2, PKC, and CXCL8 on AFC in the absence of terbutaline further confirms our findings from adrenalectomized mice that endogenous catecholamines play no role in β_2 -AR desensitization and indicate that desensitization is a specific effect of RSV rather than a result of a physiological catecholamine response to the stress of infection.

Our findings suggest that desensitization of epithelial β_2 -AR can occur by a paracrine mechanism, induced by activation of epithelial CXCR1/2 G protein-coupled receptors by the proinflammatory chemokine CXCL8 following RSV infection. Although GRK2 generally preferentially phosphorylates agonist-occupied β_2 -AR (homologous desensitization), agonist-independent phosphorylation of β_2 -AR (heterologous desensitization) has been demonstrated in other systems. For example, the chemokine CCL17 can induce agonist-independent phosphorylation of β_2 -AR in human peripheral blood T cells (16). Thus, even though infection with RSV does not elevate circulating catecholamine levels, β_2 -AR desensitization can still occur in the absence of ligand as a consequence of cross activation of PKC ζ and GRK2 by binding of CXCL8 to its receptors. CXCL8 mainly promotes neutrophil chemotaxis and survival (22), and an effect of this chemokine on

β -agonist sensitivity has not been reported previously. However, we have found previously that KC is quantitatively the predominant proinflammatory mediator present in the mouse lung at days 2 and 4 (5), and an elevated plasma, nasal lavage, or lung level of CXCL8 may be an indicator of increased disease severity in children with RSV (reviewed in Ref. 44). Unfortunately, no studies of CXCL8 to date have stratified disease severity on the basis of persistence or absence of β -agonist sensitivity, so a relationship between the two in infants with RSV has not been demonstrated. Interestingly, however, 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 (27), suggesting that, if mediated by the same mechanism, β_2 -AR desensitization might be much more prolonged in human subjects than in our mouse model. Moreover, it is possible that other CXCR1/2 ligands that are known to be induced by RSV infection, such as CXCL2 [MIP-2 (37)] and CXCL5 [ENA-78 (49)], may also contribute to β_2 -AR desensitization by the same mechanism.

The increased K_d noted in the saturation binding assays from day 2-infected mice (Fig. 4C) are suggestive of decreased receptor affinity for ligand. However, these data must be interpreted in the context of the absence of data regarding where, and to how many, sites on the β_2 -AR ICI-118,551 binds. Thus this K_d may not be a useful index of β_2 -AR phosphorylation. To address this methodologic limitation, we performed competition binding studies using a highly specific partial agonist (procaterol) and ICI-118,551 (Fig. 6). These data are highly consistent with a receptor with two conformational states (phosphorylated and non-phosphorylated). As can be seen in Fig. 6, this method shows no significant difference in the proportion of high-affinity receptors between day 2-infected and sham-infected mice (40.6% vs. 35.2%). It is possible that dilution factors related to the analysis of whole lung homogenates may have impeded our ability to detect differences in epithelial β_2 -AR phosphorylation levels following RSV infection. However, a more likely possibility is that β_2 -AR desensitization is not a direct consequence of β_2 -AR phosphorylation by GRK2, but results instead from sequestration of $G_s\alpha$ by activated GRK2, as has been described for the glutamate receptor (reviewed in Ref. 6). To our knowledge, phosphorylation-independent regulation of β_2 -AR by GRK2 has not been reported previously.

A recent report demonstrated that in vitro infection of human airway smooth muscle cells with RSV resulted in insensitivity to β -agonists, which was associated with a reduction in β_2 -AR density (31). However, this study must be interpreted with some caution, since there is no evidence that RSV infects smooth muscle in vivo. Finally, it should be noted that influenza (17), rhinovirus (11), and parainfluenza virus (2) have been shown to desensitize β -AR in airway smooth muscle. To our knowledge, however, this remains the first report demonstrating loss of sensitivity to β -agonists by respiratory epithelium following viral infection.

In conclusion, our data indicate that bronchoalveolar epithelial β_2 -AR are desensitized to β -agonists for a prolonged period following RSV infection, so that even with optimal drug delivery (which our AFC model permits), appropriate physiological responses to β -agonists are blunted. Furthermore, desensitization appears to result from KC-mediated PKC ζ activation and stimulation of GRK2, which uncouples β_2 -AR,

thereby preventing appropriate activation of AC. Although there are undoubtedly differences between the murine model of RSV infection and the human disease [including the presence of β_2 -AR polymorphisms in humans that have recently been shown to affect responses to salmeterol (47)], and although other factors may also contribute to the lack of β -agonist effect, such direct in vivo evidence that β_2 -AR desensitization following RSV infection may account for the modest utility of β -agonists in therapy for bronchiolitis has not previously been reported.

ACKNOWLEDGMENTS

We acknowledge the excellent technical assistance of Glenda C. Davis. Present address of I. C. Davis: Department of Veterinary Biosciences, The Ohio State University, Columbus, OH 43210. Present address of J. M. Hickman-Davis: Department of Veterinary Preventive Medicine, The Ohio State University, Columbus, OH 43210.

GRANTS

This work was funded by National Institutes of Health Grants HL-31197 and HL-51173 (S. Matalon), RR-17626 (I. C. Davis), and HL-066211 and HL-071042 (P. Factor).

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