S-nitrosothiols regulate cell-surface pH buffering by airway epithelial cells during the human immune response to rhinovirus

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Submitted 22 September 2005; accepted in final form 16 November 2005

Carraro, Silvia, Joseph Doherty, Khalequez Zaman, Iain Gainov, Ronald Turner, John Vaughan, John F. Hunt, Javier Márquez, and Benjamin Gaston. S-nitrosothiols regulate cell-surface pH buffering by airway epithelial cells during the human immune response to rhinovirus. Am J Physiol Lung Cell Mol Physiol 290: L827–L832, 2006; doi:10.1152/ajplung.00406.2005.—Human rhinovirus infection is a common trigger for asthma exacerbations. Asthma exacerbations and rhinovirus infections are both associated with markedly decreased pH and ammonium levels in exhaled breath condensates. This observation is thought to be related, in part, to decreased activity of airway epithelial glutaminase. We studied whether direct rhinovirus infection and/or the host immune response to the infection decreased airway epithelial cell surface pH in vitro. Interferon-γ and tumor necrosis factor-α, but not direct rhinovirus infection, decreased pH, an effect partly associated with decreased ammonium concentrations. This effect was 1) prevented by nitric oxide synthase inhibition; 2) independent of cyclic GMP; 3) associated with an increase in endogenous airway epithelial cell S-nitrosothiol concentration; 4) mimicked by the exogenous S-nitrosothiol, S-nitroso-N-acetyl cysteine; and 5) independent of glutaminase expression and activity. We then confirmed that decreased epithelial pH inhibits human rhinovirus replication in airway epithelial cells. These data suggest that a nitric oxide synthase-dependent host response to viral infection mediated by S-nitrosothiols, rather than direct infection itself, plays a role in decreased airway surface pH during human rhinovirus infection. This host immune response may serve to protect the lower airways from direct infection in the normal host. In patients with asthma, however, this fall in pH could be associated with the increased mucus production, augmented inflammatory cell degranulation, bronchoconstriction, and cough characteristic of an asthma exacerbation.

asthma; S-nitrosothiol; airway epithelium; glutaminase

ACUTE ASTHMA EXACERBATIONS are often triggered by viral upper respiratory tract infections (4, 36, 42). In adults and in children >2 yr old, the virus most commonly associated with asthma attacks is human rhinovirus (HRV) (12, 27, 40). Patients with acute asthma exacerbations have low pH (25) in their exhaled breath condensate (EBC). Notably, there is also a reduction in EBC pH during experimental upper airway infection with HRV (35). Indeed, the decrease in airway pH caused by HRV infection has been proposed to have a role in the pathophysiology of acute asthma attacks; exposure of airways to exogenous acid causes increased mucus production, impaired ciliary motility, cough, and bronchoconstriction (25, 26).

During an experimental HRV upper respiratory infection, HRV mRNA can be detected in the intrathoracic airways (16), but there are also modifications of lower airway reactivity and of bronchial inflammation that may be independent of direct lower airway infection (12, 13, 19). For example, cytokines produced by T helper (Th) 1 lymphocytes in response to viral infection can affect the lower airways (24), and it has been proposed that upper airway inflammation can also signal lower airway responses through neuronal signaling pathways (26). Thus it is possible that HRV infection could decrease airway pH, and so contribute to an acute asthma exacerbation, through a direct infection of bronchial epithelial cells and/or through the indirect signaling effects of inflammatory and neuronal mediators.

One common mechanism by which both inflammation and neuronal signaling could affect the airways following upper airway HRV infection is through upregulation or activation of nitric oxide synthases (NOS) (44). NOS activation forms both NO and S-nitrosothiols (SNOS) (17, 21, 48). S-nitrosothiol bioactivities can be mediated by homolytic cleavage to form NO, with downstream activation of guanylate cyclase to form cyclic guanosine monophosphate (cGMP). On the other hand, SNO effects can also be mediated by transnitrosation reactions involving S-nitrosylation of specific target cysteine residues (30, 41, 44). In eukaryotic organisms, NH3 is synthesized by glutaminase, S-nitrosogluthathione reductase (GSNOR) and other enzymes expressed by airway epithelial cells (24, 25, 39) that may have a role in maintaining pH homeostasis in the airways (26). Because glutaminase has an active site cysteine (5), and GSNOR is involved in airways nitrogen oxide metabolism (39), one mechanism by which NOS activation could lower pH could be through impaired NH3 production.

Here, we show that 1) HRV infection does not directly inhibit airway epithelial cell pH buffering in vitro; 2) interferon (IFN)-γ and tumor necrosis factor (TNF)-α inhibit airway epithelial cell pH buffering; 3) the pH effect of IFN-γ and TNF-α is mimicked by that of S-nitroso-N-acetyl cysteine (SNOAC), which is inhibited by the NOS inhibitor nitro-L-arginine methyl ester (L-NAME), and is cGMP independent; 4) IFN-γ and TNF-α exposure results in SNO production in airway epithelial cells; 5) glutaminase activity in the airway epithelium is primarily characterized of the renal isoform (5, 6); 6) however, the effects of IFN-γ, TNF-α, and SNOS are independent of glutaminase; and 7) decreasing airway epithelial pH inhibits HRV replication. These data suggest that airway host

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defense mechanisms that increase NOS activity could both retard HRV replication and exacerbate asthma, in part, through an SNO-associated decrease in airway pH.

METHODS

Materials and cell cultures. Reagents were purchased from Sigma (St. Louis, MO) unless otherwise stated. BEAS-2B and A549 cells (American Type Tissue Collection, Manassas, VA) were initially grown to confluence (ph 7.0) in LHC9 medium (Biofluids, Rockville, MD) and DMEM/F12 (Life Technologies, Gaithersburg, MD), respectively. For pH regulation experiments, the CO2-bicarbonate buffer system was minimized by medium deaeration-decarbonation with argon (30 min) as assayed using a blood gas analyzer (Rapid Lab 800, Bayer; PCO2 detection range 5–25 mmHg; bicarbonate detection with argon (30 min) as assayed using a blood gas analyzer (Rapid Lab). Medium deaeration-decarbonation was achieved by the method of Lowry.

In vitro viral infection. Both BEAS-2B and A549 cells express ICAM-1, to which HRV binds to cause infection (37, 45). Cells were infected with HRV-16 as previously described (28) using a multiplicity of infection (MOI) of 2 for the BEAS-2B cells and MOI of 5 for the A549 cells. An equal volume of medium alone was added to control wells. After 1 h of incubation at 34°C in a CO2-free incubator, the media were replaced with HCO3- buffer-depleted media as described (28). Samples were collected at specified time points after the infection. HRV infection was confirmed and quantitated in MRC-5 cells by quantitative viral culture as previously described (28).

Biochemistry and pharmacology. Selected wells were treated with TNF-α and IFN-γ (10 ng/ml each) with the N-s-nitrosylating agent SNOAC (1, 10, or 100 µM) and/or 1-NAME (1 mM) in HCO3- buffer-depleted media. During the experiments, the wells were incubated at 37°C in a CO2-free incubator. Ammonium concentration was determined spectrophotometrically (Shimadzu, Kyoto, Japan) by reaction with α-ketoglutarate, catalyzed by glutamate dehydrogenase, in the presence of NADPH (Diagnostic Chemicals, Charlottetown, Prince Edward Island, Canada) (34). pH was measured with an Orion model 98-02 microelectrode attached to a model 410A pH meter (Thermo Orion, Waltham, MA). Protein concentrations were determined by the method of Lowry.

Airway lining fluid levels of H+ and other electrolytes in vivo are difficult to recapitulate in vitro and 2) measure without altering the result (7, 24, 25). This issue is particularly challenging with regard to H+ because of the abundant buffering capacity of cells and cell culture medium. To measure changes in airway epithelial buffering, we have chosen to measure the change in pH of a fixed volume of buffer-free medium on the surface of cells; this system is, by necessity, somewhat arbitrary but has previously been shown to reflect relative changes in airway epithelial buffering by NH3 (24).

To determine which glutaminase isoform was active in airway epithelial cells, BEAS-2B cells were trypsinized and eluted with PBS plus fetal calf serum (5%) for assay as previously described (38). Cells were pelleted and washed twice with TES buffer (25 mM Tris-HCl, 0.2 mM EDTA, 0.33 M sucrose, pH 8). Cells were lysed with two freeze-thaw cycles, and samples of 25 µl were added to 35 µl of J) a mixture of 99 mM potassium phosphate, 171 mM glutamine, 171 mM glutamate; 2) a mixture of 99 mM potassium phosphate, 171 mM glutamate; 3) deionized water; or 4) in these conditions in the presence of 10 µM SNOAC. The samples were incubated for either 30 or 60 min at 37°C. The reaction was terminated by adding 10 µl of 10% trichloracetic acid, kept on ice for 15 min, and then centrifuged. Ammonium concentration in the supernatant was determined as described above.

Immunoblotting. Immunoblotting was performed as described previously (30). In brief, whole cell extracts were prepared in 1% Nonidet P-40 (Sigma) lysis buffer containing 50 mM Tris-HCl buffer at pH 8.0, 1% Nonidet P-40, 150 mM NaCl, 2 µM leupeptin, 1 µM aprotinin, 1 µM pepstatin, 1 µM phenylmethylsulfonyl fluoride (PMSF), and 2 µM Na2VO4 (Boehringer, Mannheim, Indianapolis, IN and Roche Diagnostics, Mannheim, Germany). Insoluble material was recovered and sheared through a 25-gauge needle. Protein was quantified by the BCA protein assay kit. Protein (100 µg) separated by 10% SDS/polyacrylamide gel in 1× electrode buffer, was transferred on pure nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) in Tobin Transfer buffer. Blots were blocked in Tris-buffered saline/Tween 20 containing 5% (wt/vol) nonfat dried milk and probed overnight (4°C) with a 1:200 dilution of rabbit polyclonal anti-human glutaminase antibody (6), followed by horseradish peroxidase-conjugated goat anti-rabbit antibody (1:3,000; Pierce). Glutaminase was visualized by enhanced chemiluminescence (ECL; Amersham Biosciences, Piscataway, NJ) using Hyperfilm (Amersham Bioscience). Membranes were stripped and reprobed with antibody to actin (1:100; Sigma Immuno Chemicals, St. Louis, MO). The gel films were scanned on a Molecular Dynamics Densitometer using ImageQuan.

Cellular S-nitrosothiol assay. We collected cells treated with TNF-α and IFN-γ and/or L-NAME by scraping in 1 ml of protease inhibitor buffer [2 µM leupeptin, 1 µM aprotinin, 1 µM pepstatin, 1 µM PMSF, and 2 µM Na2VO4 plus 1 mM EDTA and N-ethyl maleimide in PBS, pH 7.4]. The cells underwent centrifugation at 13,000 rpm for 10 min at 4°C. The cell pellet was frozen (−80°C) and then was resuspended immediately before assay in 50 µl of protease inhibitor buffer plus 1% Triton X-100 and assayed for SNO content. The SNO assay involved reductive chemiluminescence in 100 µM cysteine and saturated CuCl2 as previously described (10).

Statistical analysis. Data are expressed as means ± SE, if normally distributed, and as median and interquartile range if not. Differences between two groups of cells were evaluated by the t-test for independent samples if normally distributed or with Mann-Whitney test if not. Differences among treatment groups were evaluated by ANOVA or ANOVA on ranks with appropriate pairwise comparisons (Holm-Sidak or Dunn’s test). Differences were considered significant at P values < 0.05.

RESULTS

Direct HRV-16 infection does not affect airway epithelial cell pH in vitro. BEAS-2B cells in LHC9 medium and A549 in DMEM/F-12 medium at initial pH of 7.0 were infected with HRV16, while an equal number of control cells were treated with buffer. Seventy-two hours after the infection, the viral titer in the infected cells was 101.75 tissue culture infectious dose 50 (TCID50) per 20 µl for the BEAS-2B cells and 101.0 TCID50 per 20 µl for the A549 cells, while there was no virus in uninfected cells. At each time point there was no significant difference in medium pH values between cells infected with HRV-16 and control cells (n = 6 each, P = not significant [NS]). Similarly, there was no significant difference in medium NH4+ concentration between infected and control cells (n = 6 each, P = NS). HRV16 infection did not affect glutaminase expression in either cell line.}

Th1 cytokine-stimulated decrease in airway epithelial pH is NOS dependent. BEAS-2B cells in modified LHC9 medium at initial pH of 7 were treated with IFN-γ and TNF-α (10 ng/ml each). Consistent with our previous report (24), cells (five wells each) treated with the cytokines had, relative to control cells, a greater fall in pH (−0.30 ± 0.006 in treated vs. −0.28 ± 0.006 in control, P = 0.008 at 48 h) and a lower medium [NH4+] (627 ± 18 in treated vs. 707 ± 19 in control, P = 0.015). In previous studies, we have also observed induction of NOS2 in BEAS-2B cells by IFN-γ and TNF-α, particularly in early cell passages. We hypothesized that IFN-γ
and TNF-α might affect the pH response through an inducible NOS (iNOS)-dependent mechanism. Indeed, in freshly purchased cells, there was a more robust decrease in pH with IFN-γ and TNF-α, and this effect was inhibited by the NOS inhibitor L-NAME (Fig. 1, \( P < 0.05 \)). Of note, the effect of L-NAME alone on pH (\( P = 0.05 \) at 100 \( \mu \)M 8-BrcGMP) did not differ from control. Moreover, the effect was not mimicked by a cell-permeable cGMP analog, 8-bromoguanosine 3',5'-cyclic monophosphate (8-BrcGMP), that is known to mimic guanylate cyclase activation in airway epithelial cells in vitro (47) (Fig. 1, \( P = NS \)). IFN-γ and TNF-α treatment also resulted in an increased epithelial cell concentration of endogenous SNO (Fig. 2A, \( P > 0.05 \)), and the cell-permeable SNO, SNOAC, caused a decrease in pH (Fig. 2B). There was a similar effect on [\( \text{NH}_4^+ \)], but, importantly, it was not always in parallel with the effect on pH (Fig. 2C), suggesting that SNOAC and Th1 cytokines may affect pH both by \( \text{NH}_3 \)-dependent and \( \text{NH}_3 \)-independent mechanisms. Indeed, multiple buffering modalities (26) may explain the relative heterogeneity of results within and between cell types, a heterogeneity that could have introduced \( \beta \)-error with regard to identifying differences in pH and \( \text{NH}_3 \). Cells were >90% viable by Trypan blue after 5 h under all conditions.

Expression of glutaminase isoforms. Both kidney (KGA) and liver (LGA) isoforms of glutaminase are expressed in the airway (24), but the physiological activity in the airway, as well as its regulation by Th1 cytokines, is most characteristic of KGA. In BEAS-2B cells, \( \text{NH}_4^+ \) production was stimulated by glutamine and phosphate, and this effect was inhibited by glutamate (Fig. 3), suggesting that the principal airway epithelial glutaminase activity is identical to that previously described for KGA (38). SNOAC treatment of the isolated KGA enzyme did not inhibit its activity (Fig. 3). Furthermore, neither SNOAC (data not shown) nor the combination of IFN-γ and TNF-α (10 ng/ml each) (Fig. 3B) substantially affected expression of glutaminase (\( n = 3 \) each, \( P = NS \) by densitometry), suggesting that neither the effect of SNOAC nor

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**Fig. 1.** Th1 cytokines decrease airway epithelial cell surface pH in a nitric oxide synthase (NOS)-dependent and cGMP-independent fashion. Cells were exposed to TNF-α and IFN-γ (10 ng/ml each) with or without 1 mM nitro-l-arginine methyl ester (l-NAME) for 5 h or 100 \( \mu \)M 8-BrcGMP for 5 h (\( *P < 0.05 \), \( n = 15 \) each).

**Fig. 2.** NOS activation produces S-nitrosothiols (SNOs) that can decrease airway epithelial pH and \( \text{NH}_4^+ \). A: Th1 cytokine exposure results in SNO production by airway epithelial cells that is inhibited by l-NAME (\( *P < 0.05 \), \( n = 6 \) each). B: S-nitroso-N-acetyl cysteine (SNOAC) decreases airway surface epithelial pH. Cells at an initial pH of 7 were treated with SNOAC (\( n = 5 \) each). After 2 and 3 h, pH was decreased in all treatment wells with an increased effect at 100 \( \mu \)M SNOAC. At 4 and 5 h, pH was decreased only in wells that had been treated with 100 \( \mu \)M SNOAC (\( \dagger P < 0.05 \) comparing individual treatments to controls; \( \ddagger P < 0.01 \), comparing individual treatments to controls), though there was some discordance between change in \( \text{NH}_4^+ \) levels and pH, particularly at 1 and 10 \( \mu \)M.
the Th1 cytokine response is mediated through an effect on glutaminase expression.

**Low pH inhibits HRV16 replication in airway epithelial cells.** It is proposed that the distal airways may be acidic (26). Indeed, the pH of lamellar bodies is ~5 before they enter the alveolar space (46). We have proposed that this low pH is buffered in the more proximal airway by the activity of glutaminase and other enzymes (26). Therefore, we measured whether attenuated buffering on the airway epithelial surface could affect HRV replication in airway epithelial cells. One-hour exposure of BEAS-2Bs to HRV16 at pH 5 attenuated viral replication as assayed at 48 and 72 h (*P < 0.05 and ¶P < 0.01, respectively; Fig. 4). Cells were >90% viable at all time points.

**DISCUSSION**

Acute asthma exacerbations are often triggered by viral upper respiratory infections. HRV is the virus most frequently associated with acute asthma attacks in asthmatic patients >2 yr old (40, 42). During asthma exacerbations, both the EBC pH and the EBC [NH₄⁺] decrease (24, 25). There is also a fall in EBC pH, mimicking the effect seen in asthma exacerbations, during an HRV infection of the upper airways (35). Because airway acidification can lead to cough, bronchoconstriction, increased airway mucus production, impaired ciliary motility, and release of inflammatory mediators from eosinophils (15, 22, 25, 26), it is proposed that this HRV-associated decreased airway epithelial pH may contribute to the pathophysiology of HRV-associated asthma exacerbations.

During experimental nasal HRV infection, HRV mRNA is detectable in the lower airways (16). However, the role of direct HRV infection in causing asthma exacerbations is debatable. With regard to the potential role of pH in HRV-associated asthma exacerbations, we did not find a direct effect of the virus on airway epithelial cell medium pH or on NH₄⁺ levels, despite a significant infection in vitro. Thus HRV...
infection of bronchial epithelial cells does not appear directly to affect epithelial pH regulatory activity in these cell types.

On the other hand, consistent with our previous results (24), we found that IFN-γ and TNF-α reduced airway epithelial cell culture medium pH and [NH₄⁺]. These cytokines can be produced following Th1 lymphocyte activation in response to HRV and other infections (13, 19). We and others have previously shown that IFN-γ and TNF-α increase iNOS expression and activity in BEAS-2B cells, A549 cells, and other epithelial cell lines (3, 29). Indeed, this NOS induction appears to have important immune effector functions (33).

Here, we have shown for the first time that J/NOS inhibition prevents the IFN-γ and TNF-α-induced fall in airway epithelial pH, suggesting that NOS activity can mediate, in part, the fall in epithelial pH caused by Th1-stimulated airway epithelial NOS activation is cGMP independent, is associated with increased cellular SNO production, and is mimicked by the exogenous SNO, SNOAC. NOS-derived SNOs could inhibit pH regulatory enzymes through a mechanism involving cysteine S-nitrosylation (14, 17, 30, 44).

Though upregulation of iNOS is considered characteristic both of uncontrolled asthma (9) and of response to viral infection, it is also possible in vivo that there is a neural reflex response to upper airway HRV infection leading to activation of neuronal NOS (nNOS) in nonadrenergic, noncholinergic neurons and that nNOS signals glutaminase inhibition through a mechanism involving cysteine S-nitrosylation (14, 17, 30, 44).

We hypothesized that one pH-regulatory target of SNOs might be glutaminase (5). Airway epithelial cells express glutaminase (24), and our current data are the first to suggest that this enzyme activity is mainly that of the kidney (KGA) isoform (Fig. 3). In the kidney, this enzyme catalyzes glutamate and NH₄⁺. This activity contributes to the pH buffering in the kidney by two mechanisms. One involves dissociation of NH₄⁺ into NH₃ and H⁺; NH₃ diffuses into the lumen and binds protons, buffering the pH, whereas H⁺ is eventually eliminated through the basolateral Na⁺/H⁺ exchange (39). The second involves downstream breakdown of product glutamate to form HCO₃⁻ (and other buffers) (18). However, neither the expression nor the activity of glutaminase was inhibited by SNOAC, suggesting that the NH₄⁺ production in the intact cells that was inhibited by SNOAC was a different enzyme, perhaps GSNO (39). Work is ongoing to characterize this biochemistry. Importantly, our data also suggest that there are mechanisms, independent of NH₃, by which Th1 cytokines can regulate epithelial pH.

The contents of alveolar type II cell lamellar bodies are secreted into the airways at low pH (46). Furthermore, the distal airways and gas exchange units are heavily populated with acidifying macrophages (20), and the alveolar Pco₂ is ~40 mmHg. The airway lining fluid in distal human airways may therefore be acidic; indeed, this acidity may have an antimicrobial effect in the distal airway as it does in the stomach. During mucociliary transport of airway lining fluid to the proximal airway, airway pH may then be buffered by glutaminase, in addition to other mechanisms such as HCO₃⁻ transport, H⁺/Na⁺ exchange, and epithelial carbonic anhydrase activity (4, 7, 8). Thus, since both asthma attacks and HRV infections are associated with a reduction in EBC pH (to ~5), inhibition of pH buffering could be both an antimicrobial defense in the more proximal airway and a mechanism by which HRV infections trigger acute asthma exacerbations. Indeed, nebulization of mild base has been reported to improve lung function during asthma exacerbations (1, 2).

Consistent with previous results (23), we show here that decreased pH inhibits HRV infection in airway epithelial cells in vitro. This suggests the possibility that cytokine/NOS-associated airway epithelial pH buffering could help prevent spread of HRV in the lower airway; HRV rarely causes true pneumonia in immunocompetent hosts. Of note, NOS activation itself, presumably independently of pH, can inhibit viral replication, both through S-nitrosylation-based inhibition of viral cysteine proteases (31, 43) and through impaired host-defense gene regulation (49). Furthermore, a fall in pH results in J/NOS inhibition (24, 43) and 2) conversion of nitrite to NO (25), both of which could augment the antiviral effect of NOS products.

In conclusion, we have found that airway epithelial cell pH can be affected, in part, by Th1 cytokines; downstream, this effect appears to be a cGMP-independent NOS effect that may involve SNO signaling. Therefore, we suggest that the HRV infection of the upper airways may affect the activity of pH-regulatory mechanisms indirectly through the immune response, causing a decrease in airway epithelial pH. In non-asthmatic subjects, this decrease in pH can provide an innate host defense, inhibiting viral replication in the lower airways. In asthmatic patients, however, this mechanism may also have a role in triggering asthma symptoms and could be involved in the pathophysiology of HRV infection-induced asthma exacerbations.

GRANTS

This work was supported by National Institutes of Health Grants PO1-AI-50989-04 and 2 R01 HL-59373 to University of Virginia Asthma Center; MedImmune Grant SYN087-MD; and Grant SAF 2004-02339 from the Spanish government.

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