Nerve growth factor modulates human rhinovirus infection in airway epithelial cells by controlling ICAM-1 expression

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Submitted 21 November 2011; accepted in final form 8 March 2012

Othumpangat S, Regier M, Piedimonte G. Nerve growth factor modulates human rhinovirus infection in airway epithelial cells by controlling ICAM-1 expression. Am J Physiol Lung Cell Mol Physiol 302: L1057–L1066, 2012. First published March 16, 2012; doi:10.1152/ajplung.00365.2011.—Human rhinoviruses (HRV) are the most common agent of upper respiratory infections and an important cause of lower respiratory tract symptoms. Our previous research with other viral pathogens has shown that virus-induced airway inflammation and hyperreactivity involve neurotrophic pathways that also affect tropism and severity of the infection. The goals of this study were to analyze systematically the expression of key neurotrophic factors and receptors during HRV–16 infection of human airway epithelial cells and to test the hypothesis that neurotrophins modulate HRV infection by controlling the expression of a major cellular receptor for this virus, the intercellular adhesion molecule 1 (ICAM-1). Neurotrophins and ICAM-1 expression were analyzed at the mRNA level by real-time PCR and at the protein level by flow cytometry and immunocytochemistry. A small inhibitory RNA (siRNA) or a specific blocking antibody was utilized to suppress nerve growth factor (NGF) expression and measure its effects on viral replication and virus-induced cell death. Nasal and bronchial epithelial cells were most susceptible to HRV-16 infection at 33°C and 37°C, respectively, and a significant positive relationship was noted between expression of NGF and tropomyosin-related kinase A (TrkA) and virus copy number. ICAM-1 expression was dose dependently upregulated by exogenous NGF and significantly downregulated by NGF inhibition with corresponding decrease in HRV-16 replication. NGF inhibition also increased apoptotic death of infected cells. Our results suggest that HRV upregulates the NGF-TrkA pathway in airway epithelial cells, which in turn amplifies viral replication by increasing HRV entry via ICAM-1 receptors and by limiting apoptosis.

apoptosis; asthma; chronic obstructive pulmonary disease; neurotrophins; RNA interference; intercellular adhesion molecule 1

HUMAN RHINOVIRUSES (HRV) are the most common agent of upper respiratory infections in children and adults and are also believed to be an important cause of lower respiratory tract symptoms (22). However, several aspects of the pathophysiology of HRV infections remain unclear, including the higher incidence in the colder months of the year, the different susceptibility of upper and lower airways, and the mechanisms of virus-induced airway hyperreactivity. The majority of HRV-A and HRV-B genotypes (16) use the intercellular adhesion molecule (ICAM)-1 to gain entrance to human cells, whereas a minority uses the low-density lipoprotein receptor. The receptor for the recently identified HRV type C has not been determined yet but might be similar to ICAM-1 (12).

Our previous research with a different viral pathogen (respiratory syncytial virus, RSV) has shown that virus-induced airway inflammation and hyperreactivity involve neuroimmune mechanisms dependent on the expression of nerve growth factor (NGF) and its membrane-bound receptors tropomyosin-related kinase A (TrkA) and p75NTR, both in animal models (4, 13) and in humans (21). NGF controls multiple aspects of neuronal cells biology, including survival, growth, differentiation, and neurotransmission, and also exerts a number of direct and indirect (i.e., neurogenic-mediated) proinflammatory and immunomodulatory functions (19). Furthermore, activation of the NGF-TrkA pathway inhibits the programmed death of infected epithelial cells, favoring lytic cycles of viral replication and the spreading of infecting virions to neighboring cells (14).

In the present study, we first measured systematically the effects of infection with the rhinovirus strain HRV-16 on the mRNA and protein expression of the key neurotrophic factors NGF and brain-derived neurotrophic factor (BDNF), their respective cognate receptors TrkA and TrkB, and the pan-neurotrophic p75NTR receptor. This analysis was conducted using real-time RT-PCR and fluorescence-activated cell sorting (FACS) of primary human epithelial cell lines derived from nasal, tracheal, or bronchial airways and incubated at the optimal temperature for HRV growth (33°C) or at the human body core temperature (37°C). As we noted a significant positive relationship between expression of the NGF-TrkA axis and HRV copy number, we extended these studies to test the hypothesis that NGF binding to its high-affinity receptor controls ICAM-1 expression on target cells and consequently affects the degree of HRV internalization. For this, we manipulated NGF expression in nasal epithelial cells cultured at 33°C using recombinant human protein, a targeted small inhibitory RNA, or a specific blocking antibody. Finally, we studied the effect of NGF inhibition on the death of HRV-infected cells by apoptosis or necrosis, as an additional mechanism by which this neurotrophin can modulate viral growth.

METHODS AND METHODS

Virus. The HRV-16 stock used for this study was a kind gift of Dr. Ronald B. Turner (University of Virginia, Charlottesville, VA). Cultivation and harvesting of the viral stock was performed at West Virginia University in Morgantown, WV as described previously (7). Aliquots of the final viral suspension were snap frozen in liquid nitrogen and stored at −80°C until use. The titer of the virus stock used in this study was 2.6 × 106 50% median tissue culture infective dose per ml (TCID50/ml) and was calculated by adding serial dilutions of the stock to nasal epithelial cell monolayers in 96-well plates and using the following formula: (number of infectious units) × (inverse...
of dilution)/(volume of inoculum). To confirm that the observed effects were caused by actively replicating virus and not by passive exposure to constituents of the virion or culture medium, selected experiments were repeated using aliquots of HRV-16 irradiated with a UV light source for 20 min to inactivate the viral nucleic acid.

Cell lines. Human airway epithelial cells derived from Caucasian or black donors of both sexes with ages ranging from 16 to 65 yr were purchased from PromoCell, Heidelberg, Germany (nasal cells) and from Cell Applications, San Diego, CA (tracheal and bronchial) and were maintained in six-well BD Falcon Multiwell tissue culture plates (BD Biosciences, Franklin Lakes, NJ) using the media and supplements provided by the respective vendor (14). Each experiment was repeated using cells from different donors throughout the study to control for host genetics and environment. The number of passage times for each airway section was matched in each experiment, and it never exceeded five passages. As 100% confluency alters the cell morphology, we performed pilot studies in cells that were 70–80% confluent at the time of the infection with HRV-16 at different multiplicity of infection (MOI), and a MOI of 1 was selected to obtain adequate infection rate in a 48-h period. The initial experiments were carried out in parallel using cell lines derived from nasal, tracheal, and bronchial airways at the optimal temperature for HRV growth (33°C) and at the human body core temperature (37°C). For the subsequent, more mechanistic experiments we focused on the nasal cells cultured at 33°C.

RT-PCR analysis. Total RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA) following the manufacturer’s recommendations. Total RNA (1 μg) was used as the template for cDNA synthesis (Applied Biosystems, Foster City, CA), and the cDNA was used for quantitative real-time PCR analysis using the SYBR green master mix (Applied Biosystems) with an ABI 7500 cycler (Applied Biosystems). Primers for the neurotrophic factors NGF and BDNF and for their TrkA, TrkB, and p75NTR receptors were purchased from SuperArray (Rockville, MD). The hypoxanthine phosphoribosyltransferase 1 (HPRT1) gene was used as the housekeeping control for transcript normalization. Relative changes in gene expression were calculated with the following formula: fold change $= 2^{(\Delta \Delta Ct)} = 2^{-\Delta \Delta Ct}$ (treated samples) $- \Delta \Delta Ct$ (control samples); where $\Delta \Delta Ct = Ct$ (detected gene) $- Ct$ (HPRT1), and Ct is the threshold number. HRV-16 titration was carried out using a PCR kit from PrimerDesign (Southampton, United Kingdom), and the virus copy number was extrapolated from a standard curve derived from serial dilutions of the positive control provided by the vendor.

FACS analysis. Cells were isolated by trypsinization and were stained for intracellular and surface detection of target proteins using specific antibodies. NGF, BDNF, and TrkA polyclonal antibodies

Fig. 1. Neurotrophin gene expression after human rhinovirus (HRV)-16 infection at 33°C. Human nasal (A), tracheal (B), and bronchial (C) epithelial cells were infected with HRV-16 at a multiplicity of infection (MOI) of 1 for 48 h at 33°C. Gene expression of neurotrophic factors and receptors and HRV-16 copy number (D) were measured by RT-PCR. The hypoxanthine phosphoribosyltransferase 1 (HPRT1) gene was used as the housekeeping control for transcript normalization. Data are presented as the means ± SE (n = 6). *P < 0.05; **P < 0.01; ***P < 0.001 compared with noninfected cells. ###P < 0.001 compared with different cell type. NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; Trk, tropomyosin-related kinase.
were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). ICAM-1 (CD54) mouse monoclonal antibody and the matching isotype control were purchased from BD Biosciences. The isotype control rabbit IgG was purchased from Southern Biotechnology (Birmingham, AL). Secondary fluorescent antibodies labeled with Alexa 488 and Alexa 546 were purchased from Invitrogen (Carlsbad, CA). Data were acquired and analyzed using FACSCalibur (BD Biosciences). In addition, cytospin preparations of stained cells were mounted on glass slides with Prolong Gold anti-fade reagent (Invitrogen) and imaged with a Zeiss LSM510 confocal microscope using the AxioImager Z1 system (Carl Zeiss, Jena, Germany).

NGF inhibition. Interactions between NGF and ICAM-1 expression during HRV-16 infection were investigated by silencing NGF gene expression with a specific siRNA (NGF.siRNA; On-Target plus; Dharmacon, Chicago, IL), which was labeled with DyLight red fluorescent dye DY547 (cy3) to identify successfully transfected cells (14). Exponentially growing cells were transiently transfected with either 50 \( \mu \text{g}/\text{ml} \) of NGF.siRNA or with the scrambled siRNA control (SCR.siRNA) using Lipofectamine 2000 (Invitrogen). Forty-eight hours after transfection, HRV-16 was added to the cultures at MOI of 1 for 48 h. These cells were analyzed for the expression of ICAM-1 protein by FACS, and their RNA was amplified by RT-PCR to measure HRV-16 copy number.

Cell death analysis. To evaluate whether NGF plays a role in the programmed death of host cells during HRV infection, the cells were transfected with either NGF.siRNA or SCR.siRNA for 48–60 h, and the efficiency of NGF gene silencing was monitored by RT-PCR (14). All cells were then infected with HRV-16 at MOI of 1 for 48 h, resuspended in 500 \( \mu \text{L} \) of 1× annexin buffer, and stained with 5 \( \mu \text{L} \) of annexin V-FITC (BD Biosciences) and 5 \( \mu \text{L} \) of propidium iodide (PI; BD Biosciences) for 10 min. After fixation with 1% paraformaldehyde, 500 \( \mu \text{L} \) of 1× buffer was added to each sample, and FACS analysis was performed with a FACSCalibur instrument.

Statistical analysis. Data are expressed as means ± SE. RT-PCR data are the average of three independent experiments performed in duplicate. Geometric mean fluorescent intensity was calculated from the FACS measurements obtained in four independent experiments. The paired Student’s t-test (24) was used to analyze differences between infected and noninfected cells, and between cells treated with NGF-specific or scrambled siRNA. One-way ANOVA (24) was

![Graphs showing neurotrophin gene expression](https://example.com/fig2.png)

Fig. 2. Neurotrophin gene expression after HRV-16 infection at 37°C. Human nasal (A), tracheal (B), and bronchial (C) epithelial cells were infected with HRV-16 at an MOI of 1 for 48 h at 37°C. Gene expression of neurotrophic factors and receptors and HRV-16 copy number (D) were measured by RT-PCR. The HPRT1 gene was used as the housekeeping control for transcript normalization. Data are presented as the means ± SE (n = 6). *P < 0.05; **P < 0.01; ***P < 0.001 compared with noninfected cells. #P < 0.05; ##P < 0.001 compared with different cell type.
performed with the software SigmaStat version 3.5 for Windows (Systat Software, Point Richmond, CA) to compare HRV copy numbers among different cell lines, and post hoc pairwise multiple comparisons between means were performed using the Holm-Sidak method (23). Linear regression was performed using the R statistical software (20) to analyze the strength of the relationships between neurotrophins, the logarithm of HRV-16 copy number, and ICAM-1. The F-test was used to assess the regression relation for all analyses. Differences having a \( P \) value <0.05 were considered significant.

RESULTS

We used RT-PCR to investigate whether HRV-16 infection modulates gene expression of key neurotrophic factors and their receptors in human nasal, tracheal, and bronchial epithelial cells. At the optimal temperature for HRV-16 replication (33°C), the virus increased significantly expression of NGF \((P < 0.001)\) and BDNF \((P < 0.01)\), as well as the TrkA receptor \((P < 0.05)\), only in nasal epithelial cells (Figure 1A). TrkA also increased in tracheal cells (Fig. 1B; \(P < 0.05\)) but without changes in its cognate ligand, whereas the only change measured in bronchial cells was a decrease in TrkB (Fig. 1C; \(P < 0.05\)). HRV-16 replication at 33°C (Fig. 1D) was significantly more efficient in nasal cells compared with both tracheal \((P < 0.001)\) and bronchial cells \((P < 0.001)\), and it was more efficient in bronchial cells compared with tracheal cells \((P < 0.001)\).

The pattern of neurotrophin gene expression in HRV-infected cells was exquisitely temperature dependent. In fact, at core body temperature (37°C), only BDNF and its cognate receptor TrkB were upregulated in infected nasal cells, whereas NGF was actually downregulated (Fig. 2A; \(P < 0.001\)). However, at this temperature, infected tracheal cells had mildly increased NGF and p75

\( ^\text{NTR} \) (Fig. 2B; \(P < 0.01\)), and bronchial cells had markedly increased NGF and TrkA (Fig. 2C; \(P < 0.001\)). HRV replication at 37°C was generally less efficient than at 33°C by approximately one order of magnitude (Fig. 2D), but it followed the same pattern of the NGF-TrkA axis expression being significantly more efficient in bronchial cells compared with both tracheal \((P < 0.001)\) and nasal cells \((P < 0.001)\); it was also slightly more efficient in tracheal cells compared with nasal cells \((P < 0.05)\). As a result, the linear regression of the logarithm of HRV-16 copy number on NGF \((r^2_{\text{adj}} = 0.71, P < 0.001)\) and TrkA \((r^2_{\text{adj}} = 0.90, P < 0.001)\) mRNA/HPRT1 showed significant positive relationships.

Next, we studied whether the protein expression of neurotrophic factors and receptors was concordant with transcript levels in the same cell lines exposed to HRV-16 or sterile medium. FACS analysis of nasal epithelial cells cultured at 33°C confirmed significant increases in NGF (Fig. 3A; \(P < 0.01\)) and TrkA (Fig. 3B; \(P < 0.05\)) proteins after infection with HRV-16. In the same cells, we also observed a significant increase of ICAM-1 protein after infection with HRV-16 (Fig. 3C; \(P < 0.01\)). The linear regression of ICAM-1 on NGF showed a significant positive relationship between the two \((r^2_{\text{adj}} = 0.95, P < 0.05)\), and a similar relationship was found for ICAM-1 on TrkA \((r^2_{\text{adj}} = 0.99, P < 0.01)\).

To determine whether the effects of HRV-16 on neurotrophin expression are induced by viral components, or rather require active viral replication, cells were exposed to UV-inactivated HRV-16 (UV-HRV) at an MOI of 1. UV-HRV had no significant effect on the expression of any neurotrophic factor or receptor gene compared with control cells incubated with sterile medium (Fig. 4A). This observation was confirmed by FACS (Fig. 4B), as NGF, TrkA, and ICAM-1 protein expression in cells exposed to UV-HRV was not different from control cells.

To explore the relationship between NGF and ICAM-1 gene expression, we first infected the cells with HRV-16 and monitored the relative mRNA concentrations over time by RT-PCR. Indeed, HRV-16 induced a time-dependent parallel increase in NGF and ICAM-1 expression. NGF transcripts increased significantly after 4 h of incubation \((P < 0.05)\; \text{Fig. 5A}\), reached a maximal fivefold increase after 7 h \((P < 0.001)\), and remained elevated throughout the experiment \((P < 0.05)\). Similarly, ICAM-1 transcripts increased progressively during incubation and reached a maximal threefold increase at 8 h \((P < 0.001)\; \text{Fig. 5B}\). A parallel increase in the rate of viral replication was also observed during the same incubation period (data not shown). NGF-dependent stimulation of ICAM-1 expression was confirmed by exposing noninfected cells to increasing concentrations of exogenous recombinant human NGF.
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rhNGF, which resulted in a significant and dose-dependent increase of ICAM-1 protein measured by FACS (Fig. 5C).

Immunocytochemical analysis with confocal microscopy confirmed sharply increased expression of FITC-labeled (green) ICAM-1 both on the surface and within the cytoplasm of cells infected with HRV-16 after SCR.siRNA transfection, with noninfected control cells having only minimal baseline expression of ICAM-1 primarily localized to their surface (Fig. 6). In contrast, cells made NGF deficient by transfecting DY547-labeled (red) NGF.siRNA had minimal ICAM-1 expression both at baseline and after exposure to HRV-16.

To study whether NGF control of ICAM-1 expression has repercussions on HRV-16 infection, cells were transfected with DY547-labeled NGF.siRNA or with SCR.siRNA before exposure to HRV-16 or sterile medium. In noninfected cells, NGF.siRNA silenced more than 70% of NGF gene expression (P < 0.001; Fig. 7A). HRV-16 infection induced strong NGF expression in cells transfected with SCR.siRNA, but this effect was prevented by transfection with NGF.siRNA (P < 0.001). Silencing of NGF gene expression resulted in almost complete downregulation of ICAM-1 protein in cells exposed to HRV-16 compared with cells nontransfected or transfected with SCR.siRNA (P < 0.001; Fig. 7B). In turn, the dearth of ICAM-1 receptors on target cells was associated with significantly lower HRV-16 copy number (P < 0.001; Fig. 7C).

In separate experiments, a specific blocking antibody was used to prevent NGF binding to its receptors expressed on the cell surface. This again resulted in significant downregulation of ICAM-1 expression (P < 0.001; Fig. 8A) and reduced HRV-16 copy number (P < 0.001; Fig. 8B) compared with control cells treated with the IgG isotype control antibody although the inhibitory effect of the antibody on both ICAM-1 and virus replication was less potent than the effect of gene silencing by RNA interference.

Finally, we used simultaneous analysis of annexin V-FITC staining and PI uptake to compare the effect of NGF depletion on the death by apoptosis or necrosis of HRV-16-infected cells. Compared with NGF-expressing cells infected with HRV-16 after transfection with SCR.siRNA (Fig. 9A), NGF-depleted infected cells showed a significant increase in apoptosis (Fig. 9D).
whereas the percentage of necrotic cells did not change (\( P < 0.75 \)). Consequently, after the silencing of the NGF gene, a smaller proportion of cells remained alive and available to support viral replication (Fig. 9C; \( P < 0.01 \)).

**DISCUSSION**

This study provides the first evidence that presence of replicating HRV-16 in human airway epithelial cells modifies the expression of key neurotrophic genes, particularly those encoding NGF and its high-affinity receptor TrkA. In turn, upregulation of the NGF-TrkA pathway induces expression of ICAM-1 receptors on the host cell surface and thus promotes further viral entry and replication. Among cell lines derived from the human respiratory tract, nasal epithelial cells are especially prone to HRV-16 infection at its optimum temperature of multiplication (33°C), a temperature that can be found in the upper respiratory tract where common colds develop. This tropism may derive from the concomitant upregulation of NGF and TrkA expression in the infected nasal cells, and it is virtually abolished when the NGF gene is silenced by RNA interference.

BDNF and its cognate receptor TrkB, as well as the p75NTR pan-neurotrophic receptor, also showed significant changes during HRV infection and may affect viral kinetics. However, their expression pattern did not predict the rate of viral replication in our model as well as NGF and TrkA did. Also, HRV replication was maximal when both NGF and TrkA were simultaneously upregulated, whereas mismatched increases in either NGF or TrkA had negligible effects. As TrkA is a membrane-bound receptor expressed on the target cell surface, this observation suggests the involvement of autocrine and/or paracrine activity. Finally, UV inactivation of the viral nucleic acid hindered the effect of HRV-16 on NGF-TrkA axis (and ICAM-1) expression, indicating that these biological effects require active viral replication and expression of the viral genome.

As this virus may infect in vivo the lower respiratory tract of children and adults (6) and has been shown in vitro to replicate in human bronchial epithelial cells even better than in nasal cells (11), we repeated our experiments at the core temperature of the human body (37°C), which is also the homeostatic temperature of the lower respiratory tract. Indeed, at this temperature, bronchial cells yielded much more virus than tracheal and nasal cells, indicating that the tropism of HRV-16 for the different sections of the respiratory tract is temperature dependent in a fashion consistent with its clinical manifestations. More importantly, the correlation observed between the patterns of viral replication and expression of the NGF-TrkA axis in host cells, together with the evidence that the viral receptor ICAM-1 is regulated by the same neurotrophic pathway, provides a plausible molecular mechanism to explain both tropism and clinical manifestations of HRV infection.

It remains to be determined the exact biological cascade by which temperature differentially modulates gene expression of the NGF-TrkA pathway in different sections of the respiratory tract. Our recent finding that epithelial NGF expression is

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**Fig. 5.** Time and dose dependence of NGF and ICAM-1 expression. NGF (A) and ICAM-1 (B) transcript levels were measured by RT-PCR in human nasal epithelial cells infected with 1 MOI of HRV-16 for 48 h at 33°C. Data are presented as the means ± SE (n = 6). *\( P < 0.05 \); **\( P < 0.01 \); ***\( P < 0.001 \) compared with the 0-h time point. C: human nasal epithelial cells exposed to increasing concentrations of recombinant human NGF (rhNGF) for 24 h at 33°C were stained for ICAM-1 and analyzed by FACS. Geometric MFI data are presented as the means ± SE (n = 4 experiments). *\( P < 0.05 \); **\( P < 0.01 \); ***\( P < 0.001 \) compared with control cells not exposed to rhNGF.
controlled via epigenetic mechanisms (15) provides a suitable model to explain these interactions between environmental conditions and molecular events at the airway interface, which we are currently pursuing. Furthermore, the relatively high HRV copy numbers measured in bronchial cells incubated at 33°C despite low NGF-TrkA levels suggest the existence of alternative mechanisms for viral entry although it is unlikely that such temperature could be achieved in the lower airways in vivo.

Although the antiviral effect of NGF inhibition by siRNA is quite potent in vitro and may have significant clinical activity if replicated in vivo, it is obvious that many other factors play important roles in modulating HRV infectivity and could interact or interfere with the mechanism described in this study. Also, we know little about the level of differentiation of the cells used in this study and whether that they had tight junctions, and this could influence the results because nonpolarized cells may show different expression of membrane proteins than polarized cells.

NGF and ICAM-1. NGF is the prototypical member of a family of neurotrophic factors that includes BDNF and the neurotrophins (NT) 3 and 4 (10). Two types of membrane receptors mediate neurotrophic signaling: the three members of the tropomyosin-related kinase family of receptor tyrosine kinases (TrkA, TrkB, and TrkC), which exhibit selectivity for NGF, BDNF/NT4, and NT3 respectively; and the p75NTR receptor, which is a member of the tumor necrosis factor (TNF) receptor superfamily and binds at low affinity all four neurotro-

Fig. 6. Inhibition of ICAM-1 expression by NGF gene silencing. Human nasal epithelial cells were transfected with a NGF-specific small inhibitory RNA (NGF.siRNA) labeled with DY547 (red) or scrambled siRNA control (SCR.siRNA) for 48 h. After the confirmation of the knockdown by RT-PCR analysis, the cells were exposed to 1 MOI of HRV-16 or sterile medium for additional 48 h. Next, the cells were stained using an ICAM-1-specific antibody and counterstained with a fluorescently labeled (Alexa 488) secondary antibody (green). Photographs were taken using a confocal microscope having a fluorescence emission of 488 and 546 nm for the detection of ICAM-1 and NGF.siRNA, respectively.
phins but can also bind at high affinity the NGF precursor (pro-NGF) and regulate the affinity of TrkA for its cognate ligand. In addition to its neurotrophic functions, NGF modulates the activity of a variety of nonneural cell types involved in immune and inflammatory responses, thus functioning as a powerful and eclectic neuroimmunomodulator (19).

ICAM-1 is the receptor used by a large number of HRV types to bind the surface of target airway epithelial cells (3). The present study provides several lines of evidence showing that NGF controls ICAM-1 expression on human airway epithelial cells. First, ICAM-1 expression in human airway epithelial cells infected with HRV-16 showed excellent correlation with the expression of both NGF and TrkA and followed the same time course. Second, exogenous addition of recombinant NGF to human airway epithelial cells induced a significant, dose-dependent increase in ICAM-1 protein, which has also been reported in human endothelial cells (18). Third, ICAM-1 expression was virtually abolished in human airway epithelial cells where the NGF gene was silenced by RNA inhibition, as shown by both FACS and immunocytochemical analysis. ICAM-1 was also inhibited by inactivation of the NGF protein on the surface of HRV-infected cells with a specific blocking antibody, albeit less efficiently than with the gene-silencing strategy, again suggesting that at least part of the effect of NGF involves autocrine and/or paracrine binding to its TrkA receptor expressed on the target cell surface.

The interactions between NGF and ICAM-1 shown in this study suggest a novel mechanism to explain the different clinical expression of HRV infections in atopic and asthmatic patients (2). In fact, a number of studies have demonstrated chronically increased expression of NGF and other neurotrophins in the upper and lower airways of subjects with atopy and/or asthma (19), and in light of the present data it is possible to link this observation with the increase in ICAM-1 expression found in the airway epithelium of atopic subjects (1), which favors both HRV infections and the migration and activation of cellular effectors of allergic inflammation. In addition, NGF represents an essential link between the epithelial lining and the dense subepithelial neural network innervating the mucosa of upper and lower airways, and it is essential for the development and amplification of neurogenic-mediated inflammation and hyperreactivity during and after the infection (17). This can explain many of the symptoms evoked by HRV both in the upper and lower respiratory tract (e.g., sneezing, nasal obstruction and discharge, sore throat, cough) and may contribute to the reported epidemiological link between early-life HRV infections and childhood asthma (5).

**NGF and apoptosis.** NGF is also known to prevent programmed death of multiple cell types through binding of its TrkA receptor (8) and upregulation of the antiapoptotic Bcl-2 family members (9). Confirming our previous findings with RSV (14), the present study shows that NGF protects HRV-infected cells from apoptotic death without affecting necrosis. The relevance of this finding is related to the role of apoptosis as an innate host cell defense mechanism, which in contrast to necrosis limits viral replication, propagation, and inflammation. Thus our previous and present data suggest that, as part of their evolutionary strategy for survival, viruses induce NGF expression to keep airway epithelial cells alive and permit completion of their replicative cycle. It should be noted, however, that the magnitude of the proapoptotic effect measured in HRV-infected cells after silencing the NGF gene was much smaller than the same effect measured in RSV-infected cells (14).

Therefore, we hypothesize that NGF modulates HRV infection by two distinct mechanisms. The predominant mechanism involves increased expression of ICAM-1 receptors on the surface of target epithelial cells, which translates into direct potentiation of viral entry and replication. A secondary mechanism is the reduced apoptotic death of infected cells, which allows the virus to complete its replicative cycle. We believe that the combination of data presented in this study makes the alternative hypotheses quite unlikely; i.e., if the virus would

![Graph A](image1.png)

**Graph A:** No siRNA, SCR.siRNA, and NGF.siRNA for NGF mRNA/HPRT1 (fold change) in control and HRV-16 infected human nasal epithelial cells. Data are presented as the means ± SE (n = 4 experiments), ***P < 0.001 compared with the SCR.siRNA control.

![Graph B](image2.png)

**Graph B:** ICAM-1 protein (MPF) in control, HRV-16 infected and noninfected human nasal epithelial cells transfected with NGF-specific small inhibitory RNA (NGF.siRNA) or scrambled siRNA control (SCR.siRNA) for 48 h and subsequently exposed to HRV-16 for additional 48 h. Data are presented as the means ± SE (n = 4 experiments), ***P < 0.001 compared with the SCR.siRNA control.

![Graph C](image3.png)

**Graph C:** HRV copy number in control, HRV-16 infected and noninfected human nasal epithelial cells transfected with NGF-specific small inhibitory RNA (NGF.siRNA) or scrambled siRNA control (SCR.siRNA) for 48 h and subsequently exposed to HRV-16 for additional 48 h. Data are presented as the means ± SE (n = 4 experiments), ***P < 0.001 compared with the SCR.siRNA control.

Fig. 7. Inhibition of HRV-16 infection by NGF gene silencing. NGF transcripts (A), ICAM-1 protein (B), and HRV-16 copy number (C) in HRV-infected and noninfected human nasal epithelial cells transfected with NGF-specific small inhibitory RNA (NGF.siRNA) or scrambled siRNA control (SCR.siRNA) for 48 h and subsequently exposed to HRV-16 for additional 48 h. Data are presented as the means ± SE (n = 4 experiments). ***P < 0.001 compared with the SCR.siRNA control.
upregulate ICAM-1 independently from NGF activity, or if ICAM-1 would initiate NGF overexpression upon viral entry into target cells, then NGF inhibition would not be able to affect ICAM-1 expression and virus replication. If correct, our hypothesis may have important translational implications, as the pharmacological inhibition of neurotrophic factors or receptors using strategies based on RNA interference could offer a promising new approach for the management of this common respiratory infection by reducing viral entry and improving the efficiency of viral clearance.

In conclusion, our data show for the first time that HRV-16 infection upregulates the prototypical neurotrophin NGF and its cognate receptor TrkA in airway epithelial cells, and this in turn potentiates HRV-16 internalization by increasing expression of the virus ICAM-1 receptor on target cells. NGF also exerts an antiapoptotic effect that delays the clearance of infected cells and favors completion of the viral replicative cycle. Pharmacological inhibition of the NGF-TrkA pathway may have important antiviral effects and could be beneficial in the management of common colds, as well as in the prevention of asthma and chronic obstructive pulmonary disease exacerbations.

ACKNOWLEDGMENTS

We thank Dr. Ronald B. Turner for providing the HRV-16 stock and for critical review of the final manuscript. We also thank Cheryl Walton and Lennie Samsell for invaluable technical assistance. Image acquisition and data analysis were performed in part by the WVU Microscope Imaging Core Facility. FACS experiments were performed in the WVU Flow Cytometry Core Facility.

GRANTS

This work was supported in part by grant RO1 HL-61007 from the National Institutes of Health and by the WVU Pediatric Research Institute endowment to Dr. Giovanni Piedimonte. Some of the findings reported in this paper were presented at the International Conference of the American Thoracic Society in 2010 (New Orleans, LA) and 2011 (Denver, CO).

Fig. 8. Inhibition of HRV-16 infection by anti-NGF antibody. ICAM-1 protein (A) and HRV-16 copy number (B) in human nasal epithelial cells treated with anti-NGF antibody (5 μg/ml) or with a matched isotype antibody control for 2 h and infected with HRV-16 for additional 48 h. Cells were stained for ICAM-1 using a specific antibody and subsequently counterstained with a FITC-labeled secondary antibody. HRV-16 copy number was measured by RT-PCR. Data are presented as the means ± SE (n = 4 experiments). ***P < 0.001 compared with the IgG isotype control.

Fig. 9. Effect of NGF gene silencing on virus-induced cell death. Human nasal epithelial cells were transfected with SCR.siRNA (A) or NGF.siRNA (B) for 48 h and then infected with 1 MOI of HRV-16 for additional 48 h. Next, the cells were analyzed by FACS after simultaneous staining with annexin V FITC-conjugated antibody for apoptosis and propidium iodide for necrosis. Numbers in each quadrangle represent the percentage of total cell population (n = 4 experiments). Geometric MFI for the same experiments is summarized in the bar graph (C). **P < 0.01 compared with the SCR.siRNA control.
REFERENCES


