

Human rhinovirus infection enhances airway epithelial cell production of growth factors involved in airway remodeling

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Background: Childhood human rhinovirus (HRV) infections are associated with an increased risk of asthma. We reasoned that HRV infections might be important in the pathogenesis of airway remodeling, thereby providing a mechanism by which these children are at risk of asthma.

Objective: We sought to determine whether HRV infection of airway epithelial cells regulates production of growth factors associated with airway remodeling and to determine whether vascular endothelial growth factor (VEGF) was upregulated in airways during HRV-induced natural colds.

Methods: Cultured human airway epithelial cells were infected with HRV. Amphiregulin, activin A, and VEGF protein levels were assayed by means of ELISA, and VEGF mRNA was quantified by using real-time RT-PCR. Pharmacologic inhibitors were used to assess the role of mitogen-activated protein kinase and nuclear factor κ B pathways. Nasal lavage samples from subjects with confirmed natural HRV infections were assayed for VEGF protein and compared with baseline levels and with control levels.

Results: HRV infection upregulated amphiregulin, activin A, and VEGF protein levels compared with control media ($P < .05$). VEGF gene expression was maximally induced 3 hours after infection. HRV-induced generation of VEGF was regulated by p38 mitogen-activated protein kinase and extracellular signal-regulated kinase 1/2 pathways but did not depend on nuclear factor κ B activation. In subjects with HRV infections, VEGF levels during peak cold symptoms were significantly higher than at baseline ($P = .005$) or in control subjects ($P < .01$).

Conclusion: HRV-16 infection upregulates amphiregulin, activin A, and VEGF in airway epithelial cells, and HRV infections *in vivo* upregulate airway VEGF production. (J Allergy Clin Immunol 2008;122:1000-1006.)

Key words: Human rhinovirus, asthma, airway remodeling, airway epithelial cell, vascular endothelial growth factor, amphiregulin, activin A, mitogen-activated protein kinase, nuclear factor κ B, I κ B kinase

The morbidity and occasional mortality associated with asthma are a consequence of the underlying airway hyperresponsiveness (AHR), leading to exaggerated bronchoconstriction in response to a variety of stimuli. Although the mechanisms underlying AHR are not yet fully characterized and are likely to be multifactorial, an evolving body of literature implicates airway remodeling as a contributing factor in the development and persistence of AHR.¹ Although the first symptoms of asthma can occur at any age, recurrent episodes of variable airflow limitation manifest before the age of 6 years in the majority of patients,² suggesting that asthma is likely to originate in childhood. A recent bronchial biopsy study in preschool children with new-onset respiratory symptoms demonstrated that features of airway remodeling were present even before the diagnosis of asthma was confirmed.³ However, indices of airway remodeling are not evident in infants (children <1 year old) with symptoms of airflow limitation, suggesting that airway remodeling likely occurs as a consequence of these episodes rather than being a congenital phenomenon in children at high risk of asthma.⁴

Respiratory viral infections are the most common cause of acute wheezing illnesses during infancy and childhood.⁵ Both longitudinal and retrospective studies have reported that children who have viral bronchiolitis or who wheeze during what appear to be viral infections during the first 2 to 3 years of life are at increased risk of subsequent asthma.^{6,7} Human rhinovirus (HRV) accounts for approximately 50% of all viral infections during infancy and early childhood, and HRV-associated wheezing during infancy is a significant risk factor for the subsequent development of asthma,⁸ raising the issue of whether HRV infections play a role in the pathogenesis of early airway remodeling. Despite the strong association of early childhood HRV infections with increased risk of asthma, however, there is little evidence as to how HRV infection and the resultant changes in epithelial biology might initiate or regulate cellular and molecular mechanisms that are involved in airway remodeling processes.

We hypothesized that HRV infection of airway epithelial cells is a major regulator of airway remodeling by generating key mediators that are involved in this remodeling process, and we therefore examined potential mechanisms by which HRV infection of airway epithelial cells both *in vitro* and *in vivo* might be

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Abbreviations used

AHR:	Airway hyperresponsiveness
EGF:	Epidermal growth factor
ERK:	Extracellular signal-regulated kinase
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
HBE:	Human bronchial epithelial
HRV:	Human rhinovirus
IKK:	I κ B kinase
IP-10:	IFN- γ -induced protein of 10 kd
JNK:	C-Jun N-terminal kinase
MAPK:	Mitogen-activated protein kinase
NF- κ B:	Nuclear factor κ B
p38:	p38 mitogen-activated protein kinase
VEGF:	Vascular endothelial growth factor

involved in the pathogenesis of airway remodeling. Although we acknowledge that there are a number of chemokines, cytokines, and growth factors involved in the pathogenesis of airway remodeling, we chose to focus our *in vitro* studies on HRV-induced epithelial cell production of 3 selected mediators that have been strongly implicated in the remodeling process. Amphiregulin, a member of the epidermal growth factor (EGF) family, is released in response to airway epithelial injury and engages EGF receptors to drive the altered repair process that is characteristic of airway remodeling.⁹ Activin A is a member of the TGF- β superfamily^{10,11} and has been implicated in the subepithelial fibrosis characteristic of airway remodeling. Expression of vascular endothelial growth factor (VEGF) and its receptors is increased in asthma,¹² and VEGF is the major proangiogenic activity in asthmatic airways.¹³ Increases in the size and number of airway wall blood vessels are characteristic of airway remodeling, even in mild asthma,¹⁴ and moreover, overexpression of VEGF in the airways of mice has been shown to result in both vascular and airway remodeling.¹⁵

METHODS**Materials**

The following were purchased: Ham's F-12 medium, Eagle's minimal essential medium, Hanks' buffered salt solution, penicillin-streptomycin-amphotericin B, L-glutamine, TRIzol, nonessential amino acids, gentamicin, FBS, deoxyribonucleoside triphosphates, oligo(dT), and Superscript III from Invitrogen Life Technologies (Burlington, Ontario, Canada); bronchial epithelial cell growth medium from BioWhittaker (Walkersville, Md); SB-203580, PD-98059, and SP-600125 from Calbiochem-Novabiochem (San Diego, Calif); TaqMan master mix, 20 $\times$ glyceraldehyde-3-phosphate dehydrogenase (GAPDH), RNase inhibitor, and reverse transcriptase from Applied Biosystems (Foster City, Calif); and all mitogen-activated protein kinase (MAPK) antibodies from Cell Signaling Technology (Danvers, Mass). All other chemicals were purchased from Sigma-Aldrich (St Louis, Mo). The I κ B kinase (IKK) β -selective inhibitors PS-1145 (N-[6-chloro-9H- β -carbolin-8-yl] nicotinamide) and ML-120B (N-[6-chloro-7-methoxy-9H- β -carbolin-8-yl]-2-methyl-nicotinamide) have been previously described by us¹⁶ and were provided as chemical-free bases by Millennium Pharmaceuticals (Cambridge Mass).

Virus preparations and cell lines

Stocks of HRV-16 were generated and purified to remove ribosomes and soluble factors, as described previously.¹⁰ Stocks of HRV-1A were propagated in H1 HeLa cells (American Type Culture Collection, Rockville, Md) and purified in the same manner. Viral titers were determined by using WI-38 or H1 HeLa cells grown in 96-well plates, as described previously.¹⁷

Epithelial cell cultures

Primary human bronchial epithelial (HBE) cells and the BEAS-2B human bronchial epithelial cell line were cultured as described previously.¹⁸⁻²⁰ The BEAS-2B cell line has been used extensively for studies of the production of epithelial cytokines and growth factors in response to a range of stimuli, including HRV. Here we used this cell line for experiments requiring large numbers of cells and to determine whether it will be a useful model for subsequent mechanistic studies requiring transfection approaches that cannot be performed in primary cells.

Viral infection of epithelial cells

Cells were exposed to HRV-16 or HRV-1A and incubated at 34°C for specified periods of time. BEAS-2B cells were infected with 10^{4.5} 50% tissue culture-infective dose U/mL of each virus (multiplicity of infection, approximately 0.1), whereas HBE cells were infected with 10^{5.5} 50% tissue culture-infective dose U/mL (multiplicity of infection, approximately 1). The higher dose used in HBE cells is needed to induce measurable responses because it has been shown that, even at very high virus doses, no more than 10% of primary cells are infected.²¹ Studies with selective MAPK inhibitors were performed as previously described.²² Small-molecule, highly selective IKK β inhibitors were dissolved in dimethyl sulfoxide and diluted in cell-culture medium and preincubated with cells for 1.5 hours before the addition of HRV-16. Cells were then incubated with HRV-16 in the presence of the inhibitors for a further 24 hours.

Real-time RT-PCR

Gene expression for *VEGF* was quantified by using real-time RT-PCR, as described in the [Methods](#) section of the Online Repository at www.jacionline.org.

ELISAs

Measurements of activin A, amphiregulin, VEGF, and IFN- γ -induced protein of 10 kd (IP-10) were performed by using matched antibody pairs (R&D Systems, Minneapolis, Minn), according to manufacturer's protocol. IL-8 was measured by using a previously described ELISA.¹⁸

Western blotting for MAPK pathways

Western blots to assess activation of MAPK pathways were performed as described in the [Methods](#) section of the Online Repository.

Clinical study design and methods

Previously healthy nonsmoking subjects (mean age, 35 years) reported to the research clinic within 36 hours after the onset of cold symptoms. Subjects completed a validated symptom questionnaire and underwent nasal lavage, as described previously.²³ Symptom scores and nasal lavage were repeated on the 2 subsequent days. Subjects returned to the clinic for a retrospective baseline visit 4 weeks after symptom resolution, when all outcome measures were repeated. Control subjects (mean age, 31 years) without cold symptoms were assessed, and outcomes were measured at a single visit. All subjects provided informed consent, and the protocol was approved by the Conjoint Health Ethics Board of the Faculties of Medicine, Nursing, and Kinesiology, University of Calgary.

Real-time RT-PCR for HRV

Viral RNA from nasal lavage specimens was isolated as described in the [Methods](#) section in the Online Repository.

Statistical analysis

For normally distributed data, between-group comparisons were made by using 1-way ANOVA, with appropriate post-hoc multiple comparison testing

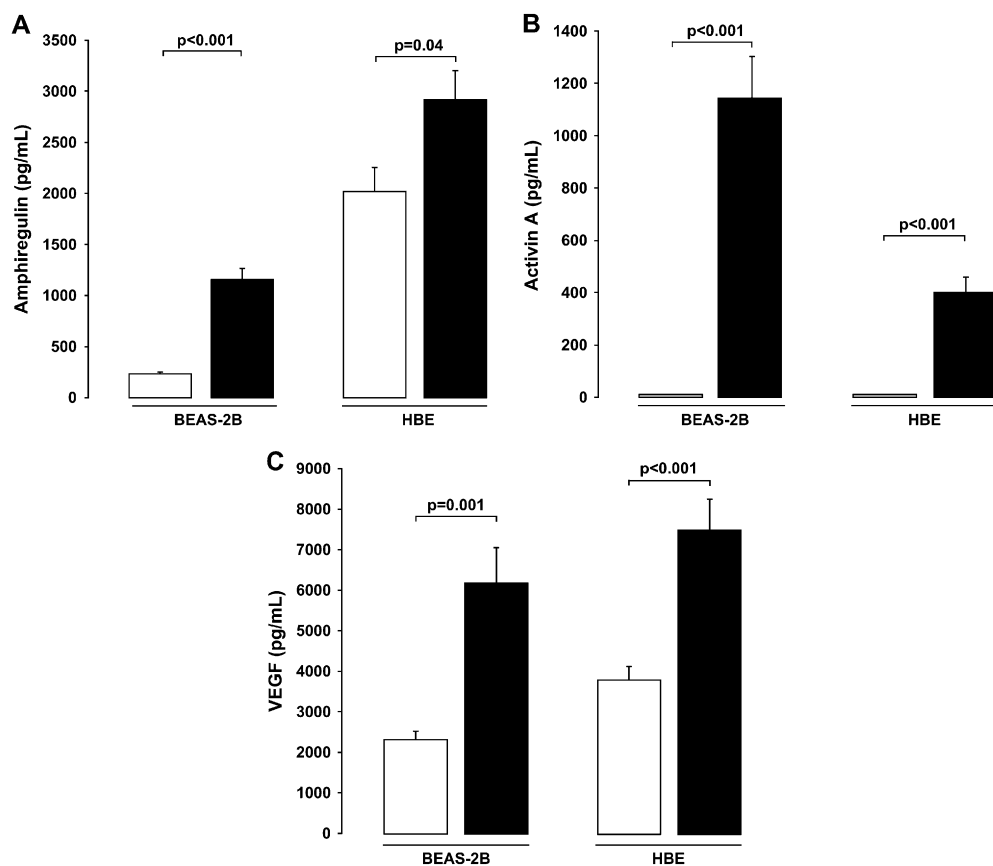


FIG 1. HRV-16 infection (solid bars) significantly upregulated amphiregulin (A), activin A (B), and VEGF (C) protein production measured by means of ELISA compared with that seen in noninfected control subjects (open bars) in BEAS-2B and HBE cells. Data are presented as means $\pm$ SEM ($n = 6-9$).

done by using the Dunnett test to assess for significant effects. For data that were not normally distributed, Kruskal-Wallis or Friedman ANOVA was used, followed by post-hoc analysis by using the Wilcoxon matched-pairs signed-rank test. Within-group comparisons were done by using paired t tests. Correlations between continuous clinical variables were assessed by using the Spearman rank-order correlation coefficient (ρ [p]). All comparisons were 2-tailed, and P values of less than .05 were considered significant.

RESULTS

HRV-16 infection of human airway epithelial cells upregulates production of amphiregulin, activin A, and VEGF

HRV-16 infection of both BEAS-2B and HBE cells resulted in significant upregulation of amphiregulin and activin A production (Fig 1, A and B). In addition, HRV-16 infection resulted in significant upregulation of the proangiogenic factor VEGF in both BEAS-2B and HBE cells (Fig 1, C). Despite significant basal expression of amphiregulin and VEGF in BEAS-2B and HBE cells, HRV-16 infection led to significant increases above these levels in both cell types. There was no basal production of activin A, but robust induction was seen in response to HRV-16 infection. We also measured TGF- β 1 protein levels after HRV infection of both BEAS-2B and HBE cells and confirmed that HRV infection resulted in upregulation of TGF- β 1 protein. However, the magnitude of this upregulation, as detected by using the commercial R&D ELISA kit, was relatively modest (data not shown), and we did not pursue this further.

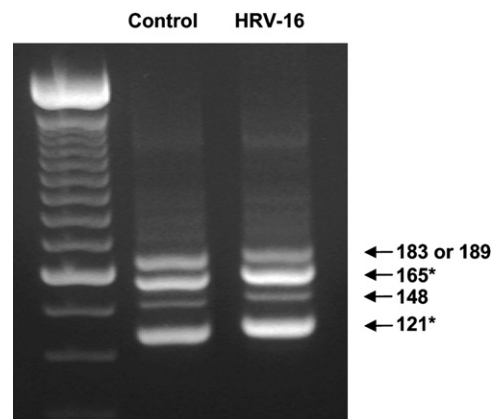


FIG 2. VEGF isoforms visualized by means of agarose gel electrophoresis. Total cellular RNA was extracted from control and HRV-16-infected BEAS-2B cells and used to perform RT-PCR by using primers capable of detecting all known VEGF isoforms. A molecular weight marker is included on the left. Asterisks indicate the isoforms increased by HRV-16 infection. Data are representative of 2 experiments.

VEGF expression increases rapidly after HRV-16 infection, is also induced by minor group HRV-1A, and selectively reflects the 121- and 165-amino-acid isoforms

Time-course studies performed in BEAS-2B cells showed a trend toward HRV-16-induced upregulation of VEGF protein

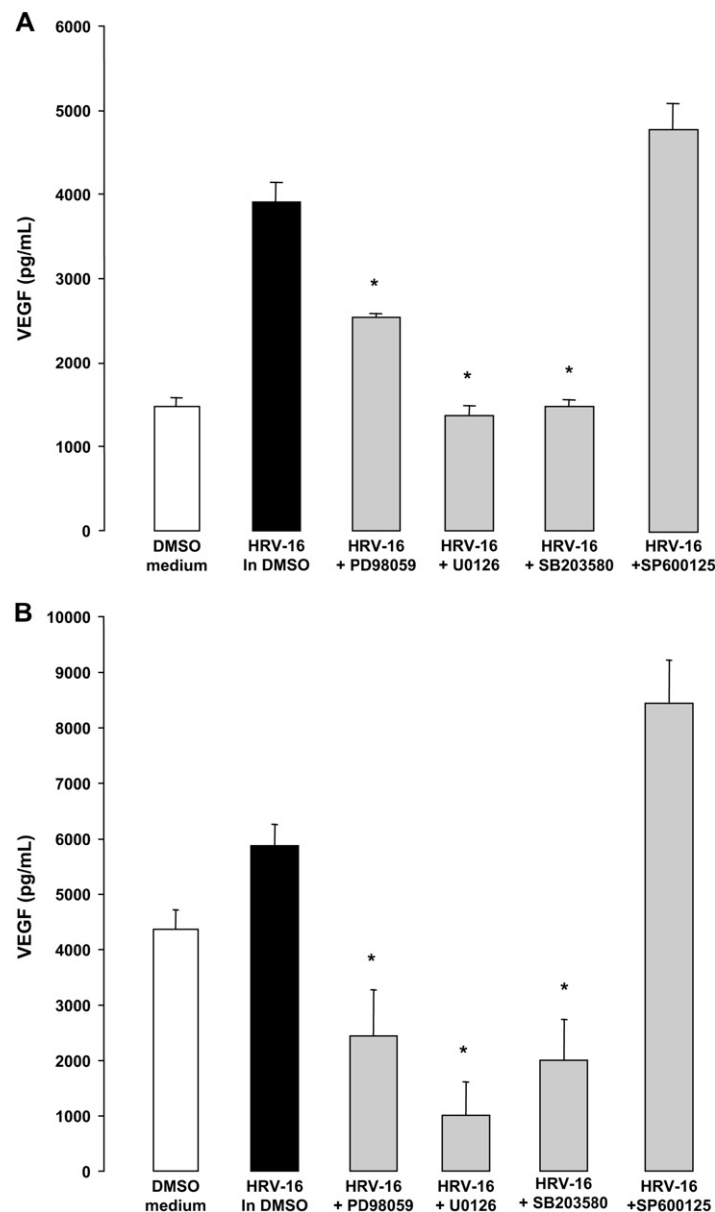


FIG 3. Effects of selective inhibitors of the ERK1/2 (PD-98059 or U0126), p38 (SB-203580), or JNK (SP-600125) MAPK pathways on VEGF protein production in BEAS-2B (A) and primary HBE (B) cells after HRV-16 infection. Data are presented as means $\pm$ SEM ($n = 4-6$ per condition). Asterisks indicate significant inhibition ($P < .05$) compared with HRV-16 plus dimethyl sulfoxide (DMSO).

level at 4 to 6 hours after infection, with levels significantly increased at 8 hours and maximally increased at 24 hours (see Fig E1 in the Online Repository at www.jacionline.org).

VEGF gene expression in BEAS-2B cells was induced early after HRV-16 infection and was significantly upregulated 3 hours after infection in BEAS-2B cells ($P = .01$, see Fig E2 in the Online Repository at www.jacionline.org). At 24 hours, mRNA levels in BEAS-2B cells had returned to near-baseline levels. By contrast, in primary HBE cells mRNA expression for VEGF remained increased by 2.6 ± 0.7 -fold above baseline at 24 hours ($n = 3$, data not shown). Levels of VEGF in cells that were lysed immediately after HRV-16 infection were at or less than the limit of detection, indicating that rapid viral induction of VEGF was not due to the existence of preformed intracellular pools (data

not shown). We confirmed that infection of epithelial cells with a minor group rhinovirus (HRV-1A) also upregulated VEGF protein production. In 3 experiments HRV-1A significantly increased VEGF production from a basal level of 1634 ± 191 pg/mL (mean $\pm$ SEM) to 4375 ± 210 pg/mL ($P = .001$).

Several isoforms of VEGF that differ in matrix association and receptor binding can be generated as a result of alternative splicing of the single VEGF gene.²⁴ RT-PCR experiments showed that epithelial cells constitutively expressed mRNA for the 121-, 148-, 165-, and 189-amino-acid isoforms. Densitometric analysis showed that HRV-16 led to selective increases in expression of mRNA only for the 121-amino-acid (141% of control value) and 165-amino-acid (137% of control value) isoforms (Fig 2).

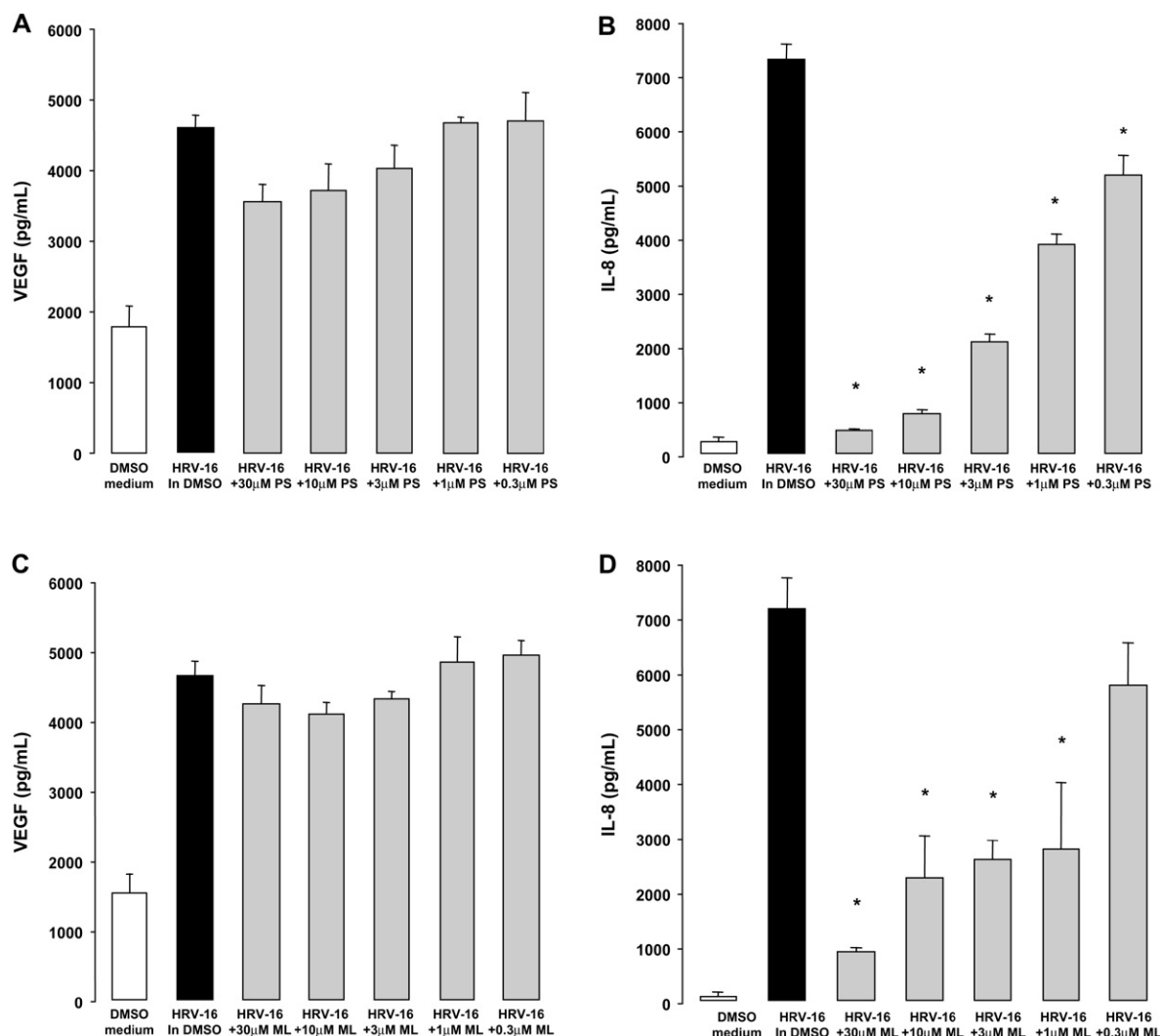


FIG 4. The small-molecule, highly selective IKK β inhibitors PS-1145 (A and B) and ML-120B (C and D) each dose-dependently inhibit HRV-16-induced production of IL-8 from BEAS-2B epithelial cells but did not inhibit VEGF production (n = 3). Asterisks indicate significant inhibition ($P < .05$) compared with HRV-16 plus dimethyl sulfoxide (DMSO).

HRV-induced upregulation of VEGF is mediated through the p38 mitogen-activated protein kinase and extracellular signal-regulated kinase 1/2 MAPK pathways but is independent of nuclear factor κ B activation

MAPK pathways have been implicated in early HRV signaling.^{22,25} Western blotting confirmed that HRV-16 infection results in rapid (within 15 minutes) activation of all 3 major MAPK pathways (p38 mitogen-activated protein kinase [p38], extracellular signal-regulated kinase [ERK] 1/2, and C-Jun N-terminal kinase [JNK]), with maximal activation at approximately 30 to 60 minutes (see Fig E3 in the Online Repository at www.jacionline.org). We then used selective inhibitors of each pathway in both BEAS-2B and primary HBE cells. An inhibitor of the p38 pathway, SB-203580, significantly attenuated VEGF protein production in both BEAS-2B and primary cells ($P < .01$), as did either of the selective ERK1/2 pathway inhibitors PD-98059 or U0126 ($P < .01$, Fig 3). By contrast, the JNK pathway inhibitor SP-600125 had no effect on VEGF production (Fig 3).

HRV infection induces production of several epithelial cytokines through nuclear factor κ B (NF- κ B)-dependent transcription. Interestingly, neither of the 2 highly selective low-molecular-weight inhibitors of IKK β , PS-1145 and ML-120B, significantly inhibited HRV-induced production of VEGF (Fig 4, A and C), but both drugs significantly reduced HRV-induced production of IL-8 from the same cells in a dose-dependent fashion (Fig 4, B and D).

Naturally acquired HRV infections are associated with increased airway production of VEGF

Real-time RT-PCR confirmed that 26 (65%) of 40 previously healthy subjects presenting with colds had HRV infections. By contrast, HRV was not detectable in any of the 15 asymptomatic control subjects or in any of the previously ill subjects when they returned after 4 weeks. In patients with HRV infections, peak viral titers significantly correlated with peak patient cold symptoms ($\rho = 0.80$, $P < .001$) and with maximal

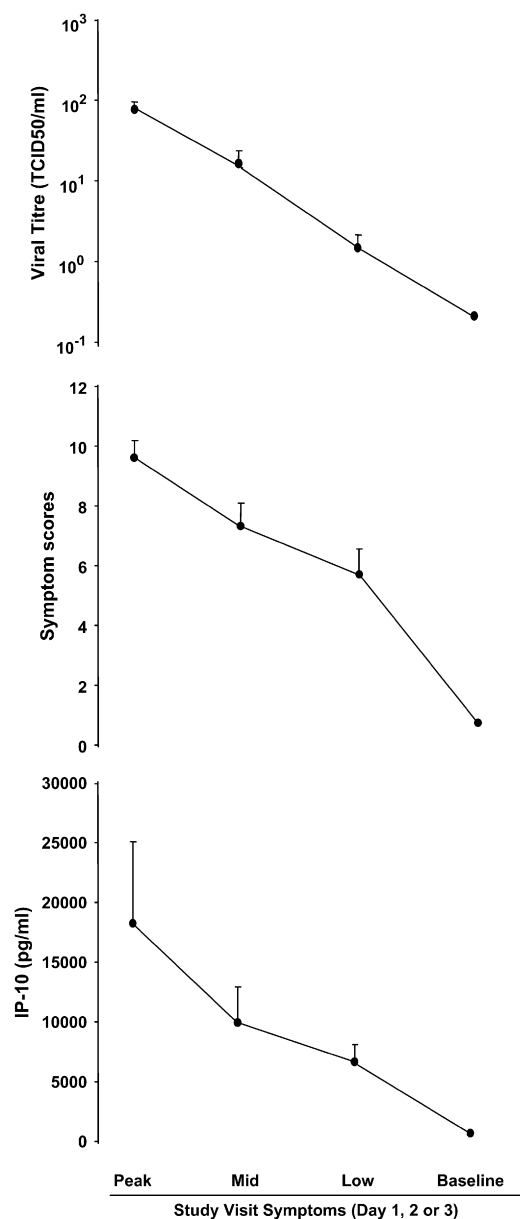


FIG 5. Patient symptom scores recorded during the 3 consecutive days of the study were ranked as peak (day of maximal symptoms), mid, and low. Measurements were done on each patient at each time point. Peak symptoms scores were significantly correlated with the highest HRV viral titers ($P < .001$) and with maximal IP-10 protein levels ($P < .001$). TCID50, 50% Tissue culture-infective dose.

levels of the chemokine IP-10 (CXCL10; $\rho = 0.78$, $P < .001$; Fig 5). We assessed VEGF protein levels in nasal lavage fluids from all 26 patients with confirmed rhinovirus infections during peak illness and at the 4-week baseline visit. In these subjects VEGF levels measured at the time of maximum cold symptoms (median, 165 pg/mL) were significantly higher ($P = .005$) than at baseline (median, 127 pg/mL; Fig 6) or in comparison with VEGF levels in asymptomatic control subjects (median, 136 pg/mL; $P < .01$). Although peak VEGF levels in nasal secretions did not correlate with cold symptoms, they did correlate significantly with peak viral titers ($\rho = 0.35$, $P = .03$) and with IP-10 levels ($\rho = 0.70$, $P < .001$).

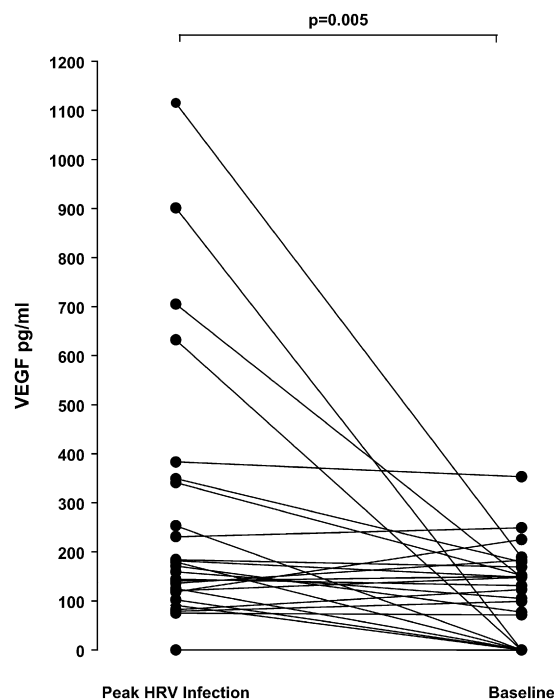


FIG 6. Levels of VEGF protein in nasal lavage fluid from 26 subjects with natural HRV infections both at the time of peak symptoms and at baseline 4 weeks after symptom resolution. Horizontal bars represent mean values.

DISCUSSION

Repeated HRV-induced respiratory illnesses during infancy and early childhood are strongly associated with an increased risk of subsequent asthma.⁸ Our current data support the hypothesis that early childhood HRV infections also might play a role in the development of the airway remodeling that manifests even before the confirmed diagnosis of asthma.^{3,4} HRV-16 infection of either the BEAS-2B epithelial cells or primary HBE cells leads to marked upregulation of the profibrotic mediators amphiregulin and activin A. In addition, we show a robust induction of VEGF on HRV infection of both the BEAS-2B and primary HBE cell lines. Other investigators have confirmed that HRV infection of airway epithelium both *in vitro* and *in vivo* results in robust upregulation of IL-11 production,²⁶ suggesting that HRV infection of airway epithelial cells is capable of upregulating a number of mediators implicated in the airway remodeling process.

These results address conflicting observations in the recent literature. Thus although Psarras et al²⁷ observed that infection with several serotypes of HRV increased VEGF protein generation from BEAS-2B cells and from bronchial epithelial cells derived from a single healthy subject, De Silva et al²⁸ found no upregulation of VEGF protein from commercially available bronchial epithelial cells (Clonetics, San Diego, Calif). Moreover, while Lee et al²⁹ observed epithelial induction of VEGF in response to respiratory syncytial virus infection, induction of VEGF was not detected from A549 cells or bronchial epithelial cells (Clonetics) on infection with HRV-14. Several factors could potentially explain these discrepant observations, including the use of different epithelial cell preparations and culture conditions and the fact that these studies propagated rhinovirus in a variety of cell lines and all used crude cell lysates for epithelial infection. Thus molecules derived from the different cell lines used for viral

propagation could have influenced the results obtained. Our current studies are the first to use purified rhinovirus and demonstrate induction of VEGF not only in BEAS-2B cells but also in primary HBE cells derived from multiple donors in response to both major and minor group serotypes. We also examined the time course and mechanisms underlying HRV-16–induced production of VEGF. In contrast to Psarras et al,²⁷ who did not observe a significant induction of VEGF from BEAS-2B cells until 36 hours after exposure to crude HRV-1B, we found that induction of *VEGF* mRNA expression occurred within 3 hours after infection with purified HRV-16, with significant protein induction within 8 hours, implying that early viral receptor–mediated signaling plays a role in VEGF induction. Moreover, the minor group virus HRV-1A induced marked VEGF protein production from primary HBE cells within 24 hours after infection, although full time-course studies were not conducted. RT-PCR demonstrated that HRV-16 infection selectively upregulated mRNA for the 121- and 165-amino-acid isoforms of VEGF. We were unable to detect specific isoforms of VEGF in our samples by means of Western blotting. This technique might not have been sufficiently sensitive. However, the ELISA used to detect VEGF in nasal lavage fluid (R&D Systems) is known to detect predominantly the 121 and 165 isoforms of VEGF protein and only weakly detects the 148 and 189 isoforms. Thus our ELISA data would support the production of the 121 and 166 isoforms from HRV-infected epithelial cells. These isoforms bind weakly (165 amino acids) or not at all (121 amino acids) to heparin or heparan sulfate proteoglycans and are the dominant soluble isoforms found in biologic fluids.¹⁷ The equal induction of mRNA expression of the 121 and 165 isoforms in bronchial epithelial cells by HRV-16 contrasts with data that respiratory syncytial virus did not increase mRNA for any isoform of VEGF in A549 cells yet induced release predominantly of the 165-amino-acid protein.²⁹

There have been no prior studies of the signaling mechanisms involved in HRV-induced epithelial production of VEGF. Because MAPK pathways have been implicated in early rhinovirus signaling,^{15,18} we examined the role of these pathways in HRV-16–induced production of VEGF. Western blotting confirmed early activation of all 3 major MAPK pathways. In contrast to a report that induction of VEGF in BEAS-2B cells in response to IL-31 was equally blunted by inhibition of any of the 3 MAPK pathways,³⁰ HRV-16–induced VEGF production in both BEAS-2B and primary HBE cells was attenuated on inhibition of either the p38 or ERK1/2 pathways but was not affected by inhibition of the JNK pathway.

The p38 and ERK1/2 MAPK pathways have been linked to activation of the transcription factor NF- κ B,³¹ and NF- κ B activation plays a role in the generation of most of the HRV-induced epithelial genes studied to date.^{12,13,32} Using 2 selective inhibitors of IKK β , we confirmed dose-dependent inhibition of HRV-induced production of IL-8, another chemokine induced early after viral infection that is known to be dependent on NF- κ B activation.³² By contrast, neither compound significantly inhibited HRV-induced production of VEGF from the same cells. This lack of dependence on NF- κ B activation for VEGF production by HRV is consistent with the apparent lack of NF- κ B recognition sequences in the human VEGF promoter³³ and implies that novel mechanisms underlie induction of HRV-induced VEGF expression. Further studies are needed to delineate these pathways.

To examine viral induction of VEGF *in vivo*, we studied previously healthy subjects with natural rhinovirus infections. Levels

of VEGF in nasal secretions were significantly higher at the time of peak symptoms compared with either samples obtained 4 weeks after symptom resolution from the same subjects or levels in nasal secretions of healthy asymptomatic subjects. These data contrast with those of De Silva et al,²⁸ who found no significant increase in VEGF protein levels in nasal aspirates from patients with confirmed HRV infections compared with noninfected control subjects but agree with the findings of a significant increase in VEGF protein levels in nasopharyngeal secretions of 9 children during asthma exacerbations associated with the presence of HRV compared with levels in the same subjects at baseline.²⁷ We extended these observations by demonstrating that the levels of VEGF in nasal secretions correlate with viral peak viral titers ($\rho = 0.35$, $P = .03$) and with IP-10 protein levels ($\rho = 0.70$, $P < .001$).

In summary, we have demonstrated that HRV infection of airway epithelial cells upregulates production of amphiregulin, activin A, and VEGF. We provide the first evidence that generation of VEGF is associated with early and selective induction of mRNA for the 121- and 165-amino-acid isoforms of the protein and is dependent on activation of the p38 and ERK1/2, but not the JNK, MAPK pathways. HRV-induced production of VEGF is not dependent on activation NF- κ B. In addition, we show that VEGF production in airway secretions is increased *in vivo* during natural HRV infections. Together, these data provide insights into mechanisms by which HRV infections might cause airway remodeling.

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Clinical implications: These data provide insights into mechanisms by which HRV infections might cause airway remodeling.

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METHODS

Real-time RT-PCR

Gene expression for *VEGF* was quantified by using the Applied Biosystems model 7900 Sequence Detector (Foster City, Calif) with the primer and probe gene expression kit available from Applied Biosystems (Foster City, Calif). Samples were treated with DNase I (Ambion, Austin, Tex) before use to minimize the contribution of genomic DNA to amplification. Briefly, 1 μ g of RNA was reverse transcribed with oligo(dT) and Superscript III, and 1 μ L of cDNA was used as the template in the real-time PCR reaction. Analysis of the housekeeping gene *GAPDH* was performed on each sample by using a primer and probe set obtained from Applied Biosystems. We also confirmed that the efficiency of *VEGF* amplification was comparable with that of *GAPDH*, allowing us to assess fold induction of genes, as described previously.^{E1} Efficiency values were measured by using the cycle threshold slope method. This involves using a sample with high levels of the mRNA of interest (*VEGF*) and constructing dilution curves over a 5- to 6-log range. Both *VEGF* and *GAPDH* are assessed in the same sample. A plot of cycle threshold versus log cDNA concentration is constructed. With this method, the expected slope for a 10-fold dilution series of template DNA is -3.32 . Slopes for both *VEGF* and *GAPDH* very closely matched this theoretic value. Statistical comparisons of slopes for *VEGF* and *GAPDH* from 3 such experiments were performed, and no significant differences were found.

Western blotting for MAPK pathways

Subconfluent BEAS-2B cells were preincubated in bronchial epithelial basal medium for 1 hour and then stimulated with HRV-16 in bronchial epithelial cell growth medium without hydrocortisone, epinephrine, EGF, and bovine pituitary extract to reduce background MAPK activation. After 30 minutes, 1 hour, and 3 hours of culture, supernatants were removed, and cells were washed with Hanks' buffered salt solution and lysed in ice-cold lysis buffer (1% Triton X-100, 5 mmol/L EDTA [pH 7.4], 50 mU/mL aprotinin, 10 μ g/mL leupeptin, 10 μ mol/L pepstatin, 1 mmol/L phenylmethylsulfonyl fluoride, 2 mmol/L sodium orthovanadate, 20 mmol/L sodium pyrophosphate, and 50 mmol/L sodium fluoride) for 30 minutes on ice with gentle agitation. Cells were scraped, sonicated, and centrifuged. Protein concentrations for the Triton X-100-soluble lysates were quantified with a DC Protein Assay (Bio-

Rad, Montreal, Quebec, Canada). Equivalent amounts of protein (30 μ g per lane) were separated by means of SDS-PAGE, and proteins were then transferred to a polyvinylidene difluoride membrane. Membranes were blocked with 5% skimmed milk for 1 hour and probed with a 1:750 dilution of phospho-specific anti-p44/42, anti-p38, or anti-JNK antibody overnight at 4°C. Membranes were washed and then incubated for 1 hour with a 1:4000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG antibody (General Electric Healthcare BioSciences, Piscataway, NJ). Proteins were visualized with ECL substrate reagent (General Electric Healthcare BioSciences). Equal loading was further determined by stripping each membrane and reprobing with an antibody against total p44/42, p38, or JNK protein.

Real-time RT-PCR for HRV

Viral RNA from nasal lavages was isolated with the QIAamp Viral RNA Mini Kit (Qiagen, Mississauga, Ontario, Canada) and assayed by using a quantitative real-time RT-PCR technique targeting the highly conserved 5' untranslated region of the HRV genome. After analysis of all available 5' untranslated region sequences for HRV serotypes, 2 forward and 2 reverse primers were designed to cover minor variations between different HRV serotypes and used in each reaction with a single fluorescent probe as follows: forward primer 1, AGC CTG CGT GGC TGC C; forward primer 2, AGC CTG CGT GGC GGC; reverse primer 1, ACA CCC AAA GTA GTC GGT CCC; reverse primer 2, ACA CCC AAA GTA GTT GGT CCC A; and probe (FAM/MGB), TCC GGC CCC TGA AT.

A range of HRV serotypes (HRV-37, HRV-39, HRV-51, HRV-89, HRV-1A, HRV-2, HRV-14, and HRV-16) showing sequence diversity was assayed to confirm that this assay detects all known HRV sequences. All serotypes were detected with comparable sensitivity. Purified HRV-16 of known titer was used to generate a standard curve that was run in each assay, allowing quantification of HRV over the range from 1 to 10^5 50% tissue culture-infective dose U/mL of original nasal lavage fluid.

REFERENCE

- E1 Ip WK, Wong CK, Li ML, Li PW, Cheung PF, Lam CW. Interleukin-31 induces cytokine and chemokine production from human bronchial epithelial cells through activation of mitogen-activated protein kinase signalling pathways: implications for the allergic response. *Immunology* 2007;122:532-41.

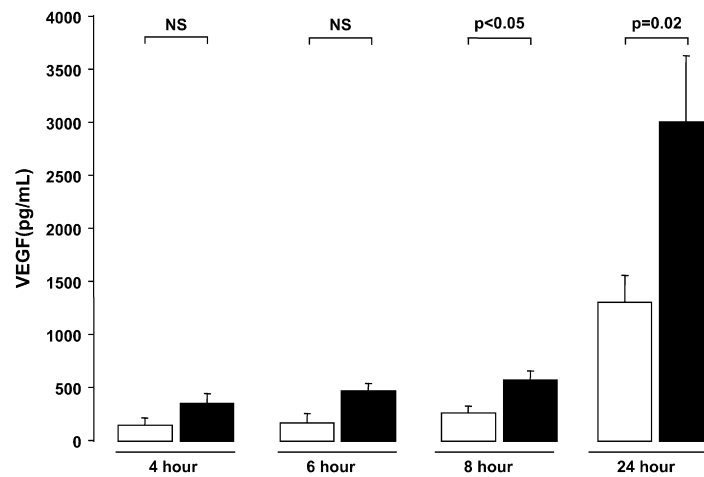


FIG E1. HRV-16 infection (*solid bars*) upregulated VEGF protein production in BEAS-2B cells in a time-dependent manner, with significant increases compared with levels in noninfected control subjects (*open bars*) first evident 8 hours and maximally evident 24 hours after infection. Data are presented as means $\pm$ SEMs ($n = 4$). NS, Not significant.

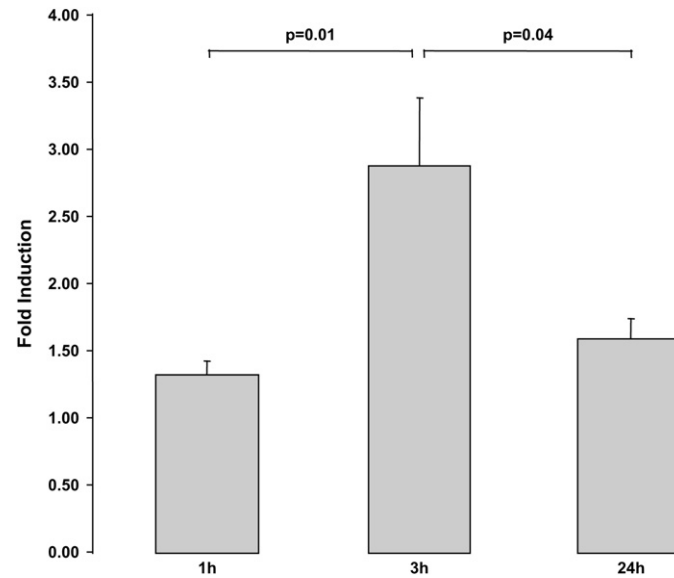


FIG E2. Real-time RT-PCR analysis of *VEGF* mRNA in BEAS-2B cells at 1, 3, and 24 hours after HRV-16 infection indicate that maximal fold induction occurred 3 hours after infection. Data are expressed relative to appropriate controls. Data are presented as means $\pm$ SEMs ($n = 4$).

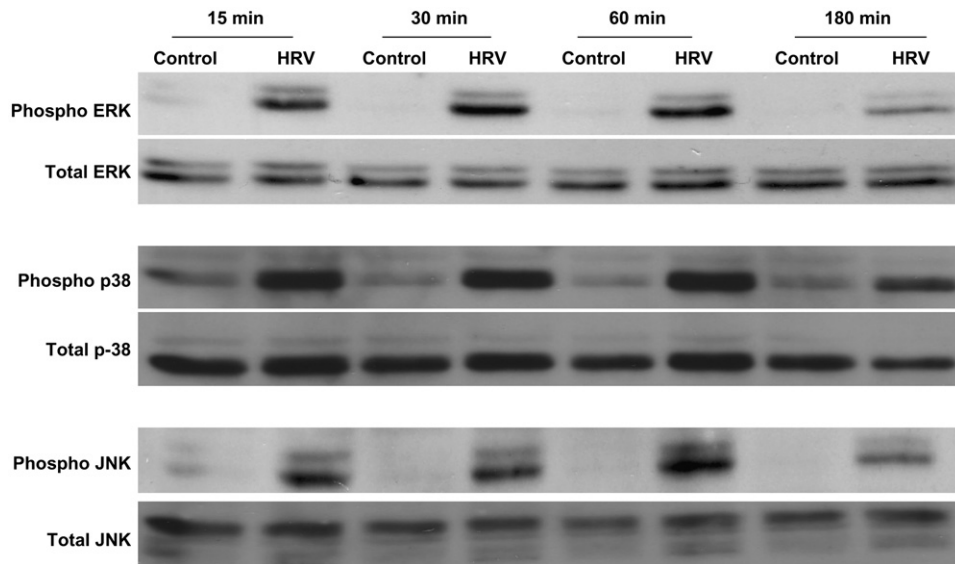


FIG E3. HRV -16 infection of BEAS-2B cells upregulated the ERK, p38, and JNK MAPK pathways in a time-dependent manner, with maximal induction occurring at approximately 30 to 60 minutes. Data are representative of 3 experiments.