

Role of nasal nitric oxide in the resolution of experimental rhinovirus infection

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Background: Human rhinovirus (HRV) infections are associated with exacerbations of asthma, chronic obstructive pulmonary disease, and sinusitis. Nitric oxide (NO) might play an important role in host defense through its potent antiviral properties. Previous studies have shown that HRV infection in human subjects increased nasal epithelial expression of type 2 nitric oxide synthase (NOS2), an isoform of the enzyme that produces NO.

Objective: We sought to investigate whether increases in exhaled NO (eNO) would accompany the increased NOS2 expression and would be associated with clearance of the virus.

Methods: Six human subjects were infected with HRV-16 intranasally. eNO from nasal and lower airways was measured by means of direct measurement at multiple controlled flow rates. eNO was monitored at baseline (day 1) and on days 2 to 5, 8, 14, and 42 after infection. Nasal lavages were performed on days 1 to 5 and 8, and nasal scrapings were performed on days 1 to 4. NOS2 mRNA expression in nasal cells was measured by using quantitative real-time RT-PCR. Viral shedding in nasal lavage fluid was monitored by using real-time RT-PCR and bioassay.

Results: Peak HRV titers and symptom scores were correlated on day 3, and HRV persisted until day 5 (n = 4) or day 8 (n = 2). Infection was associated with transient but significant increases in lymphocytes and monocytes in nasal lavage fluid. Significant increases in both nasal and lower airway eNO concentrations accompanied HRV infection and were positively correlated. Increased nasal eNO concentrations on day 3 were associated with increased expression of NOS2 mRNA in nasal scrapings. Symptom scores on day 4 were inversely correlated with the increases in nasal eNO concentration. **Conclusions:** We conclude that increased production of NO occurs as part of the host response to HRV infection and speculate that NO plays a beneficial role in viral clearance. (J Allergy Clin Immunol 2004;113:697-702.)

Key words: Rhinovirus, common cold, asthma, epithelial cells, inducible nitric oxide synthase, nitric oxide, host defense

Human rhinovirus (HRV) infections are the major cause of infection-related exacerbations of asthma and also lead

Abbreviations used

HRV: Human rhinovirus
eNO: Exhaled nitric oxide
NO: Nitric oxide
NOS: Nitric oxide synthase
TCID₅₀: Tissue culture infective dose

to secondary bacterial infections and exacerbations of other severe respiratory diseases, including chronic bronchitis and sinusitis. HRV infections have been shown to elicit antigen-specific humoral and cellular responses, but these tend to be detectable after the resolution of the clinical disease, suggesting that factors other than specific immunity are essential for host defense and the regulation of symptom severity during HRV infections. Recent studies suggest that among its many physiologic effects, the antiviral activity of nitric oxide (NO) might play an important role in host defense against respiratory viruses.

Upper respiratory tract viral infections have been associated with increases in lower airway exhaled NO (eNO) concentrations.¹⁻³ Studies have shown that symptomatic upper respiratory tract infections increase the concentration of NO in exhaled air 1 to 2 days after the onset of symptoms. Significant increases in eNO have also been observed on days 2 and 3 after experimental RV-16 infection and day 8 after inoculation of influenza but not during experimental infections with respiratory syncytial virus.⁴

Although increases in eNO have been observed in some studies, the precise role of NO in the pathogenesis of upper respiratory viral infections has not been elucidated. This might be due in part to the fact that NO can be produced by almost all mammalian cells and can participate in many cellular and organ functions.^{5,6} Much of the initial focus on the role of NO in the airways targeted its potential pro-inflammatory functions. In fact, the measurement of eNO has increasingly been used as a noninvasive marker of airway inflammation. The purpose of this study was to further examine the role of NO in the host response to HRV infections.

METHODS

Subjects

The institutional review board approved the study protocol, and informed consent was obtained from all subjects. Six nonasthmatic healthy adults not allergic to ragweed (5 male and 1 female patients)

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with no serum-neutralizing antibody to HRV-16 virus or cold symptoms in the previous 6 weeks were recruited.

Rhinovirus inoculum for in vivo studies

A safety-tested HRV-16 inoculum was prepared and subjected to an intensive battery of tests to confirm the absence of any contaminating bacteria, fungi, viruses, or mycoplasmas, according to published recommendations.^{7,8} The final preparation contained 1500 tissue culture infective dose (TCID₅₀) units/0.1 mL.⁹

Clinical study protocol

Subjects came to the laboratory on 5 consecutive days and on days 8, 14, and 42 after HRV-16 inoculation. eNO measurements were made at every visit, followed by nasal lavage on days 1 to 5 and 8 and nasal scraping on days 1 to 4. Viral challenges were done on day 1. The virus stock at a concentration of 1500 TCID₅₀ units/0.1 mL was diluted 1:20 in HBSS containing 0.5% gelatin. Volunteers were inoculated twice intranasally during a 1-hour period with 0.250 mL per nostril delivered by means of pipette while the subjects had their heads tilted back and also 3 sprays per nostril (70 μ L per spray) administered as a metered-dose spray while the subjects sat in a normal position. This provided a total dose of approximately 1000 TCID₅₀ units of HRV-16. Nasal epithelial cells were collected from the nasal turbinate by scraping twice with a curette (Rhino Probe) and rinsing in 200 μ L of lactated Ringers solution. Each day (days 1-4), a different area of the nostril was scraped to avoid introduction of artifact through the scraping procedure. The cells were centrifuged at 300g for 5 minutes at room temperature.

Nasal lavages were done with 10 mL of lactated Ringers solution. Recovered volumes were recorded; aliquots were taken for cell counts by hemacytometer, differential cell counts were performed on 500 cells with Diff-Quik (Baxter)—stained cytocentrifuge preparations (Cytospin, Shandon Southern Instruments), and the remaining fluids were stored at -80°C for subsequent assay of viral titers. Symptom questionnaires were completed twice daily on days 0 to 8 regarding 8 symptoms (sneezing, stuffy nose, runny nose, sore throat, cough, headache, chills-fever, and malaise) rated for severity on a scale of 0 to 3 (none, mild, moderate, and severe). The higher of the 2 scores is reported in the results. Criteria for a cold required a total symptom score of greater than 5 over 4 days after challenge, the belief of the subject reporting that a cold had occurred, or both.¹⁰

Measurement of eNO

Measurements were made according to American Thoracic Society guidelines¹¹ for direct online measurement of eNO by using a chemiluminescence analyzer (NOA280, Sievers) with a lower limit of detection of 1 ppb. For lower airway and nasal eNO measurement, subjects exhaled from a mouthpiece or nasal mask through needle restrictors at pressures of 10 or 20 cm H₂O to maintain constant flows of 9, 27, 41, and 78 mL/s. Each eNO value was measured in triplicate, and the SD was less than 5% of the mean.

Because nasal eNO measurements performed in this manner include lower airway NO, the wall concentration of NO from the nose was extrapolated by performing a linear regression of nasal eNO versus flow by using 3 or 4 flows where reproducible measures were available. Nasal NO wall concentration was defined by extrapolating this regression to 0 flow. Similarly, lower airway NO wall concentration was determined by means of linear regression to 0 flow, as previously reported.¹² For data analysis, values for nasal and lower airway eNO extrapolated to 0 flow were used.

Titration of HRV-16

WI-38 cells (American Type Culture Collection) were cultured in 96-well, flat-bottomed plates, as previously described.¹³ Confluent monolayers were inoculated with serial 10-fold dilutions of nasal

lavage fluids from infected subjects. After incubation at 34°C for 5 days, cytotoxicity was assessed by staining with crystal violet and reading absorbance at 570 nm with a spectrophotometer.

Serum neutralizing antibodies were measured by incubating 2-fold serial dilutions of serum from human subjects with 150 TCID₅₀ units of purified HRV-16 and testing for blockade of WI-38 cell cytotoxicity.

Quantitative real-time RT-PCR

Total RNA was extracted from nasal scrapings and nasal lavage fluid with a QIAGEN RNeasy Kit (QIAGEN). By using the Applied Biosystems Model 7700 Sequence Detector, RNA (100 ng) was reverse transcribed to cDNA, followed by PCR amplification in the presence of specific primers and a fluorescently labeled probe that were selected by using Primer Express software. Nitric oxide synthase 2 (NOS2) primers as published⁹ and the HRV-16 primers were as follows: forward, 5'GGG TTT TCA TCT ATG CTA TTG AGA TCA T3'; reverse, 5'AGT TGG CGT ATG TAT TGA GGG TAA G3'; probe, FAM-CTG AAC AAC AAG GCC AAA TTC AAA TCT CTA AAC A-TAMRA (PE Biosystems). The primers and probe for the housekeeping gene *GAPDH* were obtained from PE Biosystems.

Absolute quantitation of NOS2 mRNA was achieved by using relative standard curves (ABI Prism 7700 Sequence Detection Bulletin no. 2). A standard curve for NOS2 was prepared by using a first-strand NOS2 cDNA (referred to hereafter as the NOS2 amplicon), which corresponded to a 122-bp sequence in the NOS2 gene from 1457 to 1578 that included the binding sites for the forward and reverse NOS2 primers. The NOS2 amplicon was used in a range of 0.01 fg to 10 pg. Quantities of NOS2 mRNA in nasal scraping cells were expressed relative to *GAPDH*. A standard curve for HRV-16 was prepared by using RNA isolated from dilutions of a standard HRV-16 preparation (log TCID₅₀ ranged from 5-2).

Statistical analysis

Comparisons between groups were made by means of ANOVA. When a significant difference between groups was observed, pairwise comparisons of the means were performed with the least-significant-difference test. For data that were not normally distributed, analysis was performed by using Friedman ANOVA, followed by post hoc analysis with the Wilcoxon matched-pairs signed-rank test. Differences were considered significant for all statistical tests at *P* values of less than .05. Linear regressions were performed with StatView SE+ Graphics (Abacus Concepts, Inc). The equation for the line and the 95% confidence bands for the true mean of *Y* are given.

Materials

The following reagents were purchased from the indicated suppliers: Dulbecco modified Eagle medium, Eagle minimal essential medium, Ham F-12 medium, HBSS, L-glutamine, and penicillin-streptomycin-amphotericin B (Fungizone; BioFluids); FBS (GIBCO BRL); RNAzol B (Tel-Test, Inc); agarose (FMC Bioproducts); TaqMan Gold RT-PCR kit (PE Biosystems); and RNeasy kit (QIAGEN). All other chemicals were purchased from Sigma Chemical Company.

RESULTS

HRV-16 infection and cold symptoms

All 6 subjects completed the study and became infected as determined on the basis of self-report and greater than 4-fold increases in serum neutralizing antibody titers. Specifically, serum neutralizing antibody titers were increased by 4-fold (*n* = 2), 8-fold (*n* = 1), 16-fold (*n* = 1), or 32-fold (*n* = 2) 6 weeks after infection.

Quantitation of HRV-16 in the nasal lavage fluid by means of bioassay and real-time RT-PCR (Table I) were

TABLE I. HRV-16 titer in nasal lavage fluid

Day	Subject 1		Subject 2		Subject 3		Subject 4		Subject 5		Subject 6	
	RT-PCR	Bioassay	RT-PCR	Bioassay	RT-PCR	Bioassay	RT-PCR	Bioassay	RT-PCR	Bioassay	RT-PCR	Bioassay
1	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0.6	1.3	0.9	1.95	2.9	2.4	2.3	2.7	3.6	3.4
3	0.9	<1.3*	2.8	2.6	2.9	2.5	5.3	4.35	5.4	4.9	4.3	4.1
4	1.6	1.4	3.6	3.4	2.3	1.5	3.4	2.4	3.1	1.9	2.3	1.4
5	2.3	1.4	2.3	2.6	1.2	<1.3*	2.5	1.45	2.0	1.3	0	2.25
8	0	0	0	0	1.2	<1.3*	2.3	<1.3*	0	0	0	0

*Less than 1.3 but greater than 0. Numbers represent log TCID₅₀ units (10^X/mL) for nasal lavage samples collected at baseline (day 1) and days 2 to 8 after HRV-16 infection. The correlation coefficient for these independent measurements of viral titers was 0.884 ($P < .0001$).

highly correlated ($r = 0.88$, $P = .0001$), demonstrating that viral RNA detected by means of RT-PCR was isolated from intact virus capable of infecting WI38 cells. Viral shedding was detected within 1 to 2 days of inoculation in all subjects (day 2, $n = 5$; day 3, $n = 1$). Viral titers peaked on day 3 and were significantly correlated with peak symptom scores ($P = .01$, Fig 1). Peak symptom scores were also correlated with the fold induction in neutralizing antibody titer; that is, the subjects with the highest symptom scores had the largest increase in antibody responses ($P = .02$). Subjects had negative results for virus by day 8 ($n = 4$) or day 14 ($n = 2$).

After infection, there was an increase in the total number of inflammatory cells recovered by means of nasal lavage (Fig 2). The majority of these cells were neutrophils, which increased on day 3 and remained increased through day 5. These changes did not reach statistical significance; however, there were significant increases in lymphocytes on days 3, 4, and 5 and in monocytes on days 2 and 3 ($P < .05$).

HRV infection increases eNO concentrations in the nose and lower airway

Individual nasal and lower airway eNO concentrations obtained at baseline are shown in Table II. Values for direct measurements at a flow of 27 mL/s and from extrapolation to a flow of 0, representing an estimate of wall concentration, are shown. As expected, extrapolation at a flow of 0 resulted in higher eNO concentrations. Lower respiratory NO wall concentrations ranged from 13 to 162 ppb, which are within the range published by Silkoff et al¹² for healthy subjects. Nasal NO wall concentrations ranged from 64 to 434 ppb and were 3- to 5-fold higher in the nose than in the lower airway, which is in agreement with measurements made by other investigators.¹⁴⁻¹⁶

After infection, both nasal and lower airway respiratory tract eNO concentrations increased (Fig 3). When all measurements of baseline eNO were compared with those after infection, nasal and lower airway eNO concentrations were significantly increased over baseline values ($P = .0006$ and $P = .038$, respectively). For all measurements ($n = 48$), nasal and lower airway eNO concentrations were positively correlated ($P = .0001$, data

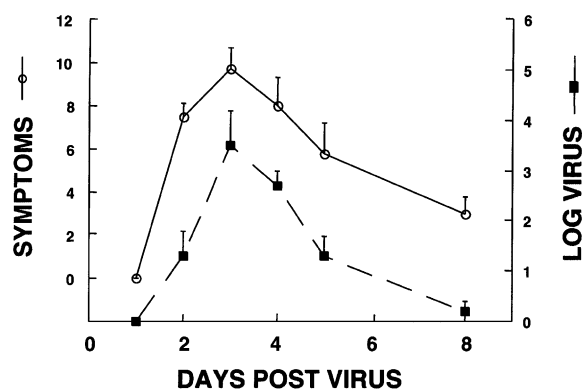


FIG 1. Symptom scores and viral titers for nasal lavage samples: see the "Methods" section (mean $\pm$ SEM, $n = 6$).

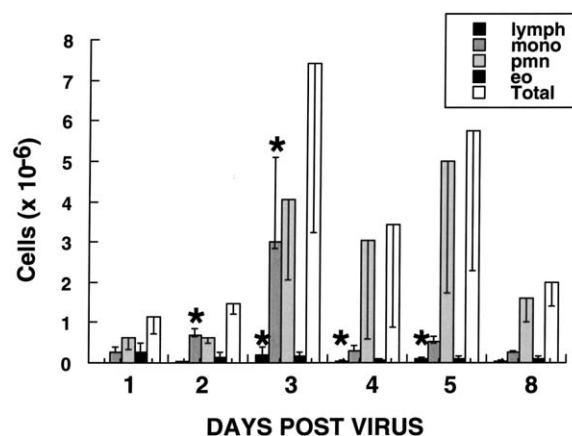


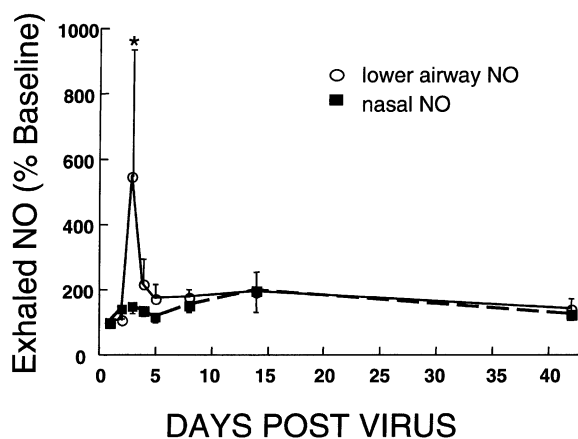
FIG 2. Differential counts performed on cells recovered from nasal lavage fluid: *lymph*, lymphocytes; *mono*, monocytes; *pmn*, neutrophils; *eo*, eosinophils (mean $\pm$ SEM, $n = 6$). * $P < .05$.

not shown). For each subject, increased eNO concentrations occurred on multiple days after infection; however, peak increases in eNO occurred on different days within the group (day 3 for 3 subjects, day 5 for 1 subject, and day 8 for 2 subjects). For nasal eNO, concentrations on no single day were significantly increased over baseline values, whereas day 3 levels were significantly increased for lower airway eNO (Fig 3). Nasal viral titers (days 1-8) were correlated with lower

TABLE II. Baseline eNO measurements (in parts per billion)

Subject no.	Lower airways eNO (ppb)		Nasal eNO (ppb)	
	Flow 27 mL/s	Flow 0*	Flow 27 mL/s	Flow 0*
1	11	13	133	170
2	85	108	171	218
3	25	31	107	143
4	140	162	349	434
5	21	21	59	132
6	4	13	41	64
Median	23	53	120	156
Range	4-140	13-162	41-349	64-434

*The concentration of eNO at 0 flow was calculated (x-axis intercept) from a 3-point linear regression of the concentration of eNO (in parts per billion) plotted against flow (9, 27, or 41 or 27, 41, or 78 mL/s).

**FIG 3.** Comparison of the wall concentration of eNO measured from the lower airways and nose (see the "Methods" section) of subjects during experimental HRV infection. Data expressed as a percentage of the baseline value (mean $\pm$ SEM, $n = 6$).

airway eNO concentrations ($r^2 = 0.71$, $P = .04$) but not with nasal eNO, suggesting that higher viral loads in the nose are more likely to have effects on the lower airway.

Increased nasal NO concentrations were associated with increases in *NOS2* mRNA in cells recovered by means of nasal scraping

Quantitative measurements of *NOS2* mRNA were made by using real-time RT-PCR in which a standard curve generated by using an amplicon was used to determine the amount of *NOS2* mRNA in nasal scraping cells relative to a set amount of the housekeeping gene *GAPDH*. A first-strand *NOS2* cDNA amplicon corresponding to the sequence in the *NOS2* gene that is recognized by the primers used for real-time RT-PCR to generate a standard curve was synthesized and shown by means of real-time PCR to be linear, in a range from 10 pg to 0.1 fg (Fig 4, A).

By using this quantitative approach, we assessed correlations between absolute levels of *NOS2* mRNA expression in epithelial cells from the nose relative to the concentrations of eNO before and after HRV infection. After normalizing for total input RNA, levels of *NOS2*

mRNA expressed at baseline ranged from 1.2 to 88 fg (18 ± 10 fg, mean $\pm$ SE) and trended toward significant increases to 100 ± 37 fg on day 3 and 70 ± 40 fg on day 4 ($P = .07-.08$). At baseline, the amount of *NOS2* mRNA expressed in nasal cells and nasal eNO concentrations were correlated ($r^2 = 0.8$, $P = .017$, $n = 6$). Absolute levels of *NOS2* mRNA in nasal scraping cells for all measurements on days 1 through 4 ($n = 23$) were correlated with nasal eNO concentrations ($r^2 = 0.25$, $P = .014$). Moreover, Fig 4, B, shows the significant correlation between increases in nasal *NOS2* mRNA and nasal eNO concentrations on day 3 after infection ($P = .015$, $n = 5$, insufficient RNA obtained from one subject). The findings support the notion that nasal eNO increases are related to HRV-induced *NOS2* induction in nasal epithelium.

Higher nasal NO responses to HRV are associated with reduced cold symptoms

Higher levels of nasal eNO expressed as a percentage of baseline values (Fig 5) or as an absolute change in parts per billion from baseline (data not shown, $r^2 = 0.78$, $P = .019$) were associated with lower cold symptoms on day 4, although not across all days. Furthermore, the 4 subjects who had cleared the virus by day 8 had the largest increases in nasal eNO concentrations on day 4. These findings support the hypothesis that NO plays an important antiviral role in the resolution of the cold.

DISCUSSION

We have previously demonstrated that HRV infection increases epithelial expression of *NOS2* mRNA in human subjects.⁹ We now show that HRV-induced expression of *NOS2* mRNA in nasal cells recovered from human subjects after experimental HRV infection is associated with increases in eNO from both the upper and lower airways. More importantly, increases in exhaled nasal NO levels are inversely related to symptom scores, suggesting that NO produced in response to HRV infection might play a role in the resolution of the cold.

Although there have been previous studies demonstrating increases in eNO concentrations after respiratory viral infection,¹⁻³ this is the first to link changes in eNO concentrations with symptoms of the viral infection. Moreover, there are conflicting results regarding changes in eNO concentrations after viral infection, with some studies failing to detect changes in NO values either by means of direct measurement of the expired gases⁴ or by means of indirect measurement of the oxidation products of NO: nitrite, nitrate, or both.^{4,17} The present study followed the American Thoracic Society guidelines for measurement of eNO and monitored concentrations of NO from the lower airways and nose at 3 different low-flow rates. These measures allow the wall concentration of NO to be calculated by using a 3-point linear regression extrapolated to 0 flow.^{11,12} It is well established that the concentration of eNO is highly dependent on flow rate, and our data clearly support this. The data from the present

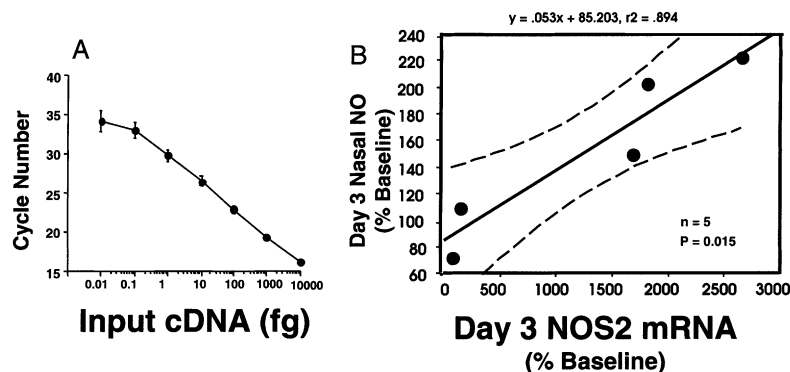


FIG 4. A, Standard curve for real-time RT-PCR of *NOS2* by using first-strand cDNA standard (mean $\pm$ SEM, $n = 3$). B, Correlation of nasal *NOS2* mRNA and increases in concentration of nasal eNO on day 3 after HRV infection ($n = 5$, insufficient sample in one subject).

study suggest that measurements at the lowest possible flow rates will give the highest concentrations of eNO, thereby maximizing sensitivity of the measurements. By using this approach, we suggest that this study has detected changes that might have been considered nondetectable with previous methodologies.⁴

Despite the fact that this study was limited to the enrollment of only 6 subjects, there are several significant correlations of the data that support an important role for NO in the innate immune response to HRV infections. First, by using a relative standard curve to quantify *NOS2* mRNA, our study demonstrates that there is a significant correlation between increases in nasal *NOS2* mRNA and increases in the concentration of nasal eNO that occur after HRV infection. These data provide direct evidence that the increases in nasal eNO concentration are related to virally induced increases in *NOS2* expression in cells that are recovered by means of nasal scraping. Although previous studies suggest that greater than 95% of these cells are epithelial cells,⁹ we cannot rule out a contribution from inflammatory cells. However, in vitro studies demonstrate that HRV infection leads directly to *NOS2* mRNA induction in human airway epithelial cells,⁹ supporting the concept that the increases in *NOS2* mRNA in nasal scrapings are occurring in the airway epithelium.

Levels of *NOS2* mRNA and eNO increased in all 6 subjects after HRV infection. When all measurements of eNO after infection were compared with baseline values, there were significant increases in both lower airway and nasal eNO concentrations, but the absolute magnitude and day of peak increase varied from subject to subject. By using all eNO measurements, nasal and lower airway eNO concentrations were correlated. When analyzed by day, only lower airway eNO concentrations on day 3 showed a significant increase over baseline. These findings are consistent with those of previous studies demonstrating that HRV infection in the nose might result in infection in the lower airway as well.¹⁸⁻²⁵

In the present study symptoms occurred within a day of viral infection, and the time to peak symptoms was 2 to 4 days. There was a highly significant relationship between symptoms and viral load, with both peaking after 2 days.

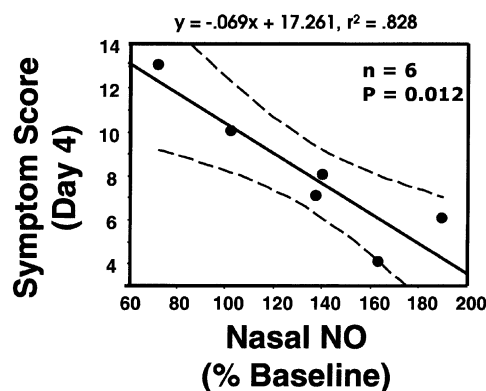


FIG 5. Symptom scores on day 4 were inversely correlated with the concentration of nasal eNO ($n = 6$).

This is similar to the onset of common cold symptoms in naturally acquired colds, which have been reported to occur within 1 to 2 days after viral infection and peak in 2 to 4 days.^{26,27} Gwaltney et al²⁸ have shown that the duration of a typical cold is about 7 days, but some might last 2 weeks. Virus shedding can be detected at the time of cold symptoms and can continue after the resolution of the symptoms.

Interestingly, we found that nasal eNO concentrations correlated inversely with symptoms on day 4, suggesting that higher levels of nasal NO might be associated with resolution of symptoms. Four of 6 subjects had cleared the virus by day 8, and these were the subjects who also had the largest changes in nasal eNO concentrations on day 4. Although these data suggest that NO plays a role in viral clearance, this is complicated by the fact that the induction of *NOS2* is linked to viral replication.⁹ No correlation between nasal eNO concentration and viral titer was found. We know from the present study that levels of lower airway NO correlated directly with viral load, suggesting that the higher the viral load in the nose, the larger the NO response in the lower airways. This would be consistent with our hypothesis that induction of *NOS2* mRNA is linked to viral replication and with previous reports suggesting that lower airway infection with HRV is more likely in those with high nasal titers of virus.²³

Collectively, the data support the hypothesis that replicating HRV signals an induction of *NOS2* mRNA, leading to an increase in NO. We speculate that NO working through its antiviral properties^{13,29} feeds back to inhibit viral replication, possibly by means of S-nitrosylation of viral proteases,³⁰⁻³² leading to viral clearance and resolution of symptoms. We should emphasize that the latter is a speculation because the current study cannot rule out the possibility that NO might be a marker of inflammation. Nevertheless, it should be noted that the range of concentrations of NO that we have previously shown to inhibit both HRV replication and HRV-induced cytokine production in vitro²⁹ were equivalent to a range of 100 to 200 ppb. Thus levels of NO seen during experimental HRV infections, particularly in the nose, clearly fall within the antiviral range. Moreover, it is well established that NOS activation might in fact lead to the production of additional biologically active molecules, such as peroxynitrite or S-nitrosoglutathione that might also be playing a role in HRV infections.

In summary, experimental HRV infection in human subjects leads to an upregulation of *NOS2* mRNA in nasal cells from infected individuals and, importantly, an increase in levels of eNO from the nose and lower airways. These data suggest that increased levels of NOS2 activity accompany increased *NOS2* mRNA expression. Our data on the induction of NO during HRV infection, together with its previously demonstrated ability to inhibit viral replication and cytokine production from infected epithelial cells, provides strong support for the hypothesis that NO participates in the host response to HRV infection in human subjects. An improved understanding of NO and its role in rhinovirus infections might provide new targets for therapeutic interventions to treat colds and their related complications.

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