

Rhinovirus infection induces expression of type 2 nitric oxide synthase in human respiratory epithelial cells in vitro and in vivo

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Background: Human rhinovirus (HRV) infections are the predominant cause of the common cold and are associated with exacerbations of asthma. Nitric oxide (NO) may play an important role in host defense by means of its potent antiviral properties.

Objective: We sought to determine whether epithelial expression of type 2 nitric oxide synthase (NOS 2), which produces NO, is induced on rhinovirus infection in vitro and in vivo.

Methods: Primary cultures of human airway epithelial cells were infected with HRV-16, and NOS 2 mRNA expression was assessed by conventional and real-time RT-PCR and NOS 2 protein by using Western blot analysis. Human subjects were also infected with HRV-16 in vivo, and mRNA for NOS 2 was assessed in nasal epithelial scrapings obtained before and after infection.

Results: NOS 2 mRNA levels increased within 8 hours after HRV-16 infection of cultured cells and remained elevated up to 48 hours after infection. NOS 2 protein was elevated at 24 hours. Induction of NOS 2 did not occur with UV-inactivated HRV-16 but could be reproduced by using double-stranded RNA, indicating that induction was dependent on viral replication. Increased NOS 2 expression was also observed in nasal epithelial scrapings during symptomatic colds.

Conclusion: Increased epithelial expression of NOS 2 mRNA occurs as part of the host response to HRV infection in vitro and in vivo. Given the antiviral effects of NO, we speculate that increased host production of NO may play an important role in host defense during HRV infections. (*J Allergy Clin Immunol* 2001;107:235-43.)

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

It is becoming increasingly evident that nitric oxide (NO) plays a key role in innate immunity against a wide variety of pathogens, including viruses.^{1,2} The antiviral effects are broad-ranging and have been demonstrated for

Abbreviations used

C_T: Threshold cycle
HRV: Human rhinovirus
NOS: Nitric oxide synthase
TCID₅₀: Tissue culture infective dose

many different DNA and RNA viruses.^{3,4} We and others, for example, have shown that NO inhibits the replication of rhinoviruses⁵ and several other picornaviruses.⁶⁻⁸

Studies suggest that an important component of the antiviral response is the ability of the host to regulate NO production by using the enzyme nitric oxide synthase (NOS). There are 3 known isoforms of the enzyme, and most often, it is the inducible type 2 isoform (NOS 2) that has been associated with the host response to viral infections. In animals and cells viral infection has been shown to induce the expression of NOS 2.^{3,8-11} Inhibitors of NOS have been shown to increase viral load and decrease survival in virus-infected mice.^{7,12} Knockout mice deficient in NOS 2 have been reported to be more susceptible to viral infection.¹³⁻¹⁵ Although there is clearly evidence that NOS and the production of NO play an important role in host defense against viruses in animals, little is known about the antiviral effects in human subjects.

Rhinovirus infections are the predominant cause of the common cold, one of the most frequently experienced acute respiratory illnesses in human patients.¹⁶ Recent evidence also suggests that upper respiratory viruses, particularly rhinoviruses, are a major cause of acute exacerbations of asthma¹⁷⁻²⁰ and may be involved in chronic bronchitis,²¹ sinusitis,^{22,23} and otitis media.²⁴ We have hypothesized that NO plays an important beneficial role in the pathology of these diseases through its potent antiviral properties.²⁵ The present studies were undertaken to test the hypothesis that part of the host response to rhinovirus infection involves an increase in epithelial NOS 2. We demonstrate that human rhinovirus 16 (HRV-16), one of the major strains of rhinovirus, induces the expression of NOS 2 in primary human respiratory epithelial cells, the predominant target cell of rhinoviruses.^{26,27} Furthermore, we show, for the first time, that the expression of NOS 2 mRNA in nasal cells is markedly induced in human subjects during rhinovirus infections.

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METHODS

Viruses and cell lines

HRV-16 and WI-38 cells were purchased from the American Type Culture Collection (Rockville, Md). Additional HRV-16 viral stocks were generated by passage in WI-38 cells and purified by centrifugation through sucrose gradients, as previously described.⁵ For some experiments, HRV-16 was inactivated by UV exposure for 30 minutes, as previously described.²⁸

Rhinovirus inoculum for in vivo studies

A small seed stock of safety-tested HRV-16 inoculum was kindly provided by Dr Elliott Dick (University of Wisconsin) and was passaged through a human host to maintain virulence of the viral stock. Healthy volunteers with no detectable serum neutralizing antibody to HRV-16 were infected intranasally with 1000 tissue culture infective dose (TCID₅₀) units of the seed stock. Nasal lavage was performed on 5 sequential days after infection. The lavage obtained from one subject with a severe cold on the peak symptom day was selected and passaged once through WI-38 cells in roller tubes and then used to infect WI-38 cells in large roller bottles. The final cytopathic cell lysate was centrifuged, passaged through a 0.45- μ m filter, and stored in aliquots in sealed vials at -80°C . The inoculum was then subjected to an intensive battery of tests to confirm the absence of any contaminating bacteria, fungi, viruses, or mycoplasmas, according to published recommendations.^{29,30} The final preparation contained 1500 TCID₅₀ units/0.1 mL.

Epithelial cell culture

Primary human bronchial epithelial cells and primary human adenoid cells were obtained by means of protease digestion of human tissue, as previously described.³¹ Primary bronchial epithelial cells were grown in culture medium consisting of Dulbecco's modified Eagle's medium/F12 with 5% heat-inactivated FBS, penicillin (100 U/mL), streptomycin (100 U/mL), amphotericin B (250 ng/mL), and L-glutamine (2 mmol/L). This medium is hereafter referred to as *complete medium*. Primary adenoid epithelial cells were grown in serum-free bronchial epithelial cell growth medium (BEGM) (Clnetics, San Diego, Calif). Cells were plated at a density of 1.5×10^5 cells per well or 1×10^6 cells per flask on 6-well plates (Costar, Cambridge, Mass) or 75-cm² tissue culture flasks (Costar). For primary human bronchial epithelial cells, the plasticware was precoated with rat tail collagen (type VII; Sigma, St Louis, Mo). As previously reported, the epithelial nature of primary cultures was established by using cytokeratin staining.³²

Viral infection of epithelial cells

Subconfluent (60%-80%) monolayers of epithelial cells were washed 3 times with HBSS. Purified HRV-16 was added to the cells at a concentration of 10^4 TCID₅₀/mL HBSS. The cells were incubated with the virus at 34°C for 1 hour and washed 3 times with HBSS, and then fresh complete medium was added to the cells. The cells were incubated at 34°C , and at various times after infection, supernatants were transferred to sterile Eppendorf tubes and stored at -80°C for later analysis of nitrite production and viral content. RNA was extracted from the cells at various times after infection and stored at -80°C for later analysis of NOS 2 mRNA expression.

Polyinosinic-polycytidilic acid and cytokine treatment of epithelial cells

Subconfluent (60%-80%) monolayers of epithelial cells were washed 3 times with HBSS. Poly (I-C) was added to the cells at a concentration of 1 to 30 $\mu\text{g/mL}$ complete medium. The cells were incubated at 34°C , and at various times after treatment, supernatants were removed and stored at -80°C for later analysis of cytokine

protein production. RNA was extracted from the cells at various times after treatment and stored at -80°C for later analysis of NOS 2 mRNA expression. In some experiments cells were treated with a mixture of cytokines consisting of TNF- α (30 U/mL), IFN- γ (100 U/mL), and IL-1 (1 ng/mL), hereafter referred to as *cytomix*.

Chemiluminescence NO analyzer

NO production by epithelial cells was measured by using a Sievers 270B NO analyzer interfaced through a DT2821 A to D board to a personal computer. Data acquisition and analysis were performed by using custom-developed software.³³ The stable oxidation products of NO, nitrite and nitrate, were determined in sample supernatants from cells infected with HRV-16 for various times. Nitrite was measured by adding the cell culture supernatant (100 μL) to glacial acetic acid containing 1% potassium iodide. Nitrate was measured by using 2 N HCl containing 1% vanadium chloride. The sample concentrations were determined by comparing the integral of the signal over time compared with that produced by sodium nitrite or sodium nitrate standards.

Clinical study protocol

The study protocol was approved by the institutional review board, and informed consent was obtained from all subjects. Four healthy adults (3 women and 1 man) with reciprocal serum neutralizing antibody titers of 4 or less to the challenge HRV-16 virus were recruited from the Baltimore area. Subjects were required to come to the laboratory on 4 consecutive days. After baseline nasal lavage and nasal scraping, viral challenges were done by means of intranasal administration of a safety-tested inoculum of rhinovirus type 16. 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 a pipette while the subjects had their heads tilted back and also 3 sprays per nostril (70 μL per spray) from a metered-dose spray while sitting in a normal position. This gave a total dose of about 1000 TCID₅₀ HRV-16.

Nasal scrapings were performed on day 0 and on day 2 or 3. Each subject had 2 scrapes in one nostril on the first session and 2 scrapes in the opposite nostril on the second session. Nasal epithelial cells were collected from the inferior nasal turbinate by scraping the area 2 times with a curette (Rhino Probe) and rinsing the probe 2 times in a sterile Eppendorf tube containing 200 μL of lactated Ringers solution. The cells were centrifuged at 300g for 5 minutes at room temperature. The RNA was isolated by using a QIAGEN RNeasy kit (QIAGEN, Valencia, Calif).

Nasal lavages were done with 10 mL of lactated Ringers solution in the morning on days 0 to 3. Recovered lavage fluids were transferred to 15-mL tubes, placed in aliquots, and stored at -80°C for subsequent assay of IL-8 and viral titers.

Subjects were given a symptom questionnaire to fill out twice daily, morning and evening, on days 0 to 4, regarding the following 8 symptoms: sneezing, stuffy nose, runny nose, sore throat, cough, headache, chills or fever, and malaise. Symptoms were rated for severity as follows: 0, none; 1, mild; 2, moderate; and 3, severe. As previously reported, criteria for a cold required a total symptom score of 5 or greater over 4 days after challenge, the belief of the subject that a cold had occurred, or both.³⁴

Titration of HRV-16

WI-38 cells were cultured in 96-well, flat-bottomed plates, as previously described.⁵ Confluent monolayers were inoculated with serial 10-fold dilutions of supernatants from infected epithelial cells or 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 by using a spectrophotometer.

Serum-neutralizing antibodies were measured by incubating 2-fold serial dilutions of serum from human subjects with 25 TCID₅₀ units of purified HRV-16 and testing for blockade of WI-38 cell cytotoxicity.

Quantification of cytokines

Levels of cytokines in nasal lavage fluids were determined by using specific ELISA. Measurements of IL-8 were performed by a previously described ELISA sensitive to 30 pg of cytokine per milliliter.²⁸

RNA extraction

Total cellular RNA was extracted from epithelial cells by using RNeasy B, as previously described.⁵ Total RNA was extracted from nasal scrapings by using a QIAGEN RNeasy kit. The integrity of each RNA sample was assessed by using electrophoresis of an aliquot (0.5 µg) on a 1% agarose gel with 0.5 µg of ethidium bromide per milliliter of buffer. RNA was stored at -80°C.

Semiquantitative conventional RT-PCR

RNA (1 µg) was reverse transcribed to cDNA at 42°C for 30 minutes. After inactivation of the reverse transcriptase, an aliquot was subjected to PCR in the presence of specific primers for NOS 2 (35 cycles) and the housekeeping gene encoding hypoxanthine phosphoribosyl transferase, *HPRT* (30 cycles), by using the following conditions: denaturation at 95°C for 45 seconds, annealing at 56°C for 2 minutes, and extension at 72°C for 2 minutes. The primer sequences were as follows: NOS 2 forward, 5'GCCTCGCTCTGGAAAGA3'; NOS 2 reverse, 5'TCCATGCAGACAACCTT3'; hypoxanthine phosphoribosyl transferase forward, 5'CGAGATGTGATGAAGGAGATGG3'; and hypoxanthine phosphoribosyl transferase reverse, 5'GGATTATACTGCCTGACCAAGG3'. These primers produced amplicons of 500 and 325 bp, respectively. To establish the specificity of responses, negative controls were used in which input RNA was omitted or in which RNA was used but reverse transcriptase was omitted.

Quantitative real-time RT-PCR

Using the Applied Biosystems Model 7700 Sequence Detector, RNA (400 ng) was reverse transcribed to cDNA, followed by PCR amplification in the presence of specific primers for NOS 2 and a fluorescently labeled probe that were selected by using Primer Express software (NOS 2 primers: forward, 5'ACATTGCTGTGATC-CATAGTTTTC3'; reverse, 5'CCCACGGGACCGGTATTC3'; probe: 6-carboxy-fluorescein-CATGGACCACCACTCGGCTGC-AG-6-carboxytetramethylrhodamine (PE Biosystems, Foster City, Calif). The primers and probe for the housekeeping gene encoding glyceraldehyde 3-phosphate dehydrogenase, *GAPDH*, were obtained from PE Biosystems.

Using the Sequence Detector software, the reporter dye emission is compared with the quenching dye emission generating a peak normalized reporter fluorescence value.³⁵ Peak normalized reporter fluorescence values are plotted versus the cycle number. The computer algorithm then calculates the threshold cycle (C_T), the point at which the amplification plot crosses a defined fluorescence threshold, set by the algorithm at 10 times the SD of the baseline value obtained during the first 10 to 15 cycles.

The comparative C_T method was used to determine relative quantities of NOS 2 in cell samples. As recommended by the manufacturer's technical bulletin, a validation experiment was performed to determine whether the efficiencies of the amplifications for NOS 2 and glyceraldehyde 3-phosphate dehydrogenase were approximately equal. According to the manufacturer, the slope of the plot of absolute value of the log input RNA amount versus C_T should be less than 0.1. We analyzed input RNA ranging from 5 to 400 ng, and the slope of absolute value of the log input RNA

amount versus C_T was 0.0422. It was therefore valid to determine the relative quantitation for NOS 2 in an unknown sample by using the following equation:

$$2^{-\Delta\Delta C_T}$$

where $\Delta\Delta C_T = \Delta C_{T,q} - C_{T,cb}$. C_T is defined as the average C_T value for NOS 2 minus the average C_T value for glyceraldehyde 3-phosphate dehydrogenase for a given sample, q is the unknown sample, and cb is the calibrator. Each sample was analyzed in duplicate.

Western blot analysis

Cells were suspended in PIPES (piperazine-N, N'-bis [2-ethane sulfonic acid]) buffer with 20 mmol/L EDTA, and an equal volume of 2× TRIS-glycine sample buffer (Novex/Invitrogen, Carlsbad, Calif) was added, followed by protease inhibitor cocktail (PharMingen, San Diego, Calif) at a final concentration of 1×. Protein extracts were separated on 8% polyacrylamide gels and transferred onto nitrocellulose filters (Novex/Invitrogen). Membranes were blocked for 30 minutes with 3% nonfat dry milk in 0.05% Tween-20 in PBS (PBS-T). The blots were rinsed in PBS-T and incubated with rabbit anti-human type 2 NOS antibody (NO53; Merck Research Laboratories, Rahway, NJ) at 1:1000 in PBS-T with 1% nonfat dry milk for 2 hours. The blots were washed 3 times with PBS-T (10 minutes) and then incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:5000; Amersham Pharmacia, Piscataway, NJ) in PBS-T-containing 1% nonfat dry milk for 1 hour. The blots were washed 6 times in PBS-T (5 minutes), followed by detection of immunoreactive proteins by chemiluminescence (Super Signal West Dura; Pierce, Rockford, Ill).

Statistical analysis

Data are expressed as the mean ± SEM. Comparisons of basal NOS 2 expression in nasal cells were made by using the Student *t* test for paired samples. Comparisons of the levels of nitrite and nitrate in supernatants from infected and control cells and of IL-8 levels in nasal lavage fluid after HRV-16 infection were made by using 1-way ANOVA. When significant variance ratios were obtained, pairwise comparisons of the means were performed with the least significant difference multiple-range test. Differences were considered significant for *P* values of less than .05.

Materials

Merck Research Laboratories kindly provided the rabbit anti-human type 2 NOS antibody. The following reagents were purchased from the indicated suppliers: Dulbecco's modified Eagle's medium; Eagle's minimal essential medium; Ham's F-12 medium; HBSS; L-glutamine; penicillin-streptomycin-amphotericin B (Fungizone; BioFluids, Rockville, Md); FBS (GIBCO BRL, Grand Island, NY); RNeasy B (Tel-Test, Inc, Friendswood, Tex); agarose (FMC Bioproducts, Rockland, Me); TaqMan Gold RT-PCR kit (PE Biosystems); RNeasy kit (QIAGEN); TRIS-glycine sample buffer, nitrocellulose filters (Novex/Invitrogen); protease inhibitor cocktail (PharMingen); and horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham Pharmacia). TNF-α, IFN-γ, and IL-1 were obtained from Upstate Biotechnology Inc (Lake Placid, NY). Synthetic double-stranded RNA polyinosinic-polycytidylic acid (poly [I-C]) and all other chemicals were purchased from Sigma Chemical Company.

RESULTS

HRV-16 infection of epithelial cells induces expression of NOS 2 mRNA and protein

HRV-16 infection of cultured primary human bronchial epithelial cells induced the expression of mRNA for NOS

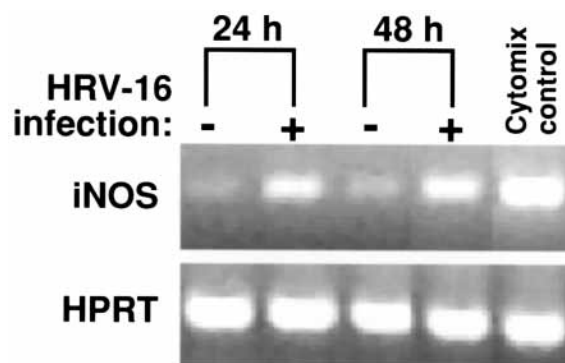


FIG 1. Induction of mRNA for NOS 2 in primary human bronchial epithelial cells at 24 and 48 hours after infection with HRV-16. Data are from a representative experiment ($n = 5$). Cells treated for 24 hours with cytomix (30 U of TNF- α /mL, 100 U of IFN- γ /mL, and 1 ng of IL-1 β /mL) were used as positive controls.

TABLE I. Relative gel band areas (arbitrary densitometric units), as assessed by RT-PCR for NOS 2 mRNA in primary human epithelial cells isolated from 5 different lungs

Experiment	Densitometric value					
	24 h			48 h		
	Control	HRV-16	Fold $\uparrow$	Control	HRV-16	Fold $\uparrow$
1	21	120	6	41	120	3
2	ND	19	$\uparrow$	ND	108	$\uparrow$
3	ND	30	$\uparrow$	ND	ND	$\uparrow$
4	241	289	1.2	407	455	1.1
5	ND	5	$\uparrow$	6	99	16

ND, Not detected.

2 at 24 and 48 hours after infection (Fig 1). Relative gel band areas in arbitrary densitometric units for NOS 2 mRNA were assessed by means of conventional RT-PCR in primary human epithelial cells isolated from 5 different lungs. Table I shows that HRV-16 induced expression of NOS 2 in 5 of 5 experiments at 24 hours and 4 of 5 experiments at 48 hours. Cytomix treatment, the positive control, elevated NOS 2 mRNA by over 100-fold.

Real-time RT-PCR analysis confirmed that HRV-16 infection induced the expression of mRNA for NOS 2 in primary human bronchial cells. Two samples of RNA that were analyzed by conventional RT-PCR (experiments 4 and 5, 48 hours, Table I) were also analyzed by using real-time RT-PCR. HRV-16 infection resulted in 2-fold and 10-fold increases in NOS 2 mRNA by using real-time RT-PCR compared with an estimated 1.1-fold and 16-fold increase by using conventional RT-PCR. Real-time RT-PCR analysis of RNA harvested from HRV-16-infected adenoid epithelial cells at 24 hours showed a 3.0 ± 0.9 -fold elevation in NOS 2 mRNA expression ($n = 3$). Real-time RT-PCR analysis of RNA harvested from HRV-16-infected epithelial cells at 2, 4, 8, and 24 hours showed elevations in NOS 2 expression by 8 hours after infection (data not shown).

Western blot analysis of airway epithelial cell lysates using an antibody to NOS 2 demonstrated that HRV-16 infection and cytomix treatment induced the expression of NOS 2 protein (Fig 2).

Supernatants were collected at 24 and 48 hours after infection and assessed for viral content by using WI-38 cell cytotoxicity to quantify HRV-16. The supernatants from HRV-16-infected cells contained $10^{3.6 \pm 0.3}$ and $10^{3.75 \pm 0.3}$ TCID₅₀ per milliliter of virus at 24 and 48 hours, respectively ($n = 4$).

Supernatants from epithelial cell cultures were analyzed by using a chemiluminescence NO analyzer to measure nitrite and nitrate, the stable oxidation products of NO. Levels of nitrite (0.8 ± 0.3 μ mol/L) and nitrate (8.9 ± 0.7 μ mol/L) in supernatants from infected cells at 24 hours ($n = 6$) were not different from the levels in control cell supernatants ($n = 5$, 0.7 ± 0.2 μ mol/L nitrite; 7.8 ± 1.1 μ mol/L nitrate). Cells treated with cytomix for 24 hours had elevated levels of nitrite (3.5 ± 1.5 μ mol/L) and nitrate (40.6 ± 18.8 μ mol/L, $n = 4$; $P < .05$).

Double-stranded RNA induces expression of NOS 2

The earliest time point of elevated NOS 2 mRNA expression in HRV-16-infected epithelial cells was consistent with the time in the replication cycle of rhinovirus when new virus is detectable. Furthermore, real-time RT-PCR analysis of RNA harvested from epithelial cells infected with UV-inactivated virus did not show elevations in NOS 2 expression, suggesting that the induction of NOS 2 gene expression in primary human epithelial cells was dependent on viral replication; mean $C_T \pm SD$

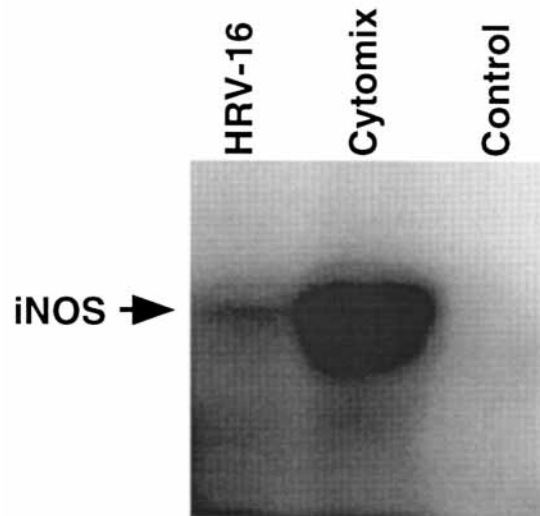


FIG 2. Western blot analysis for NOS 2 protein in primary human epithelial cells at 24 hours after infection with HRV-16. Data are from a representative experiment ($n = 2$). Cells treated for 24 hours with cytomix (30 U of $\text{TNF-}\alpha$ /mL, 100 U of $\text{IFN-}\gamma$ /mL, and 1 ng of $\text{IL-1}\beta$ /mL) were used as positive controls. Signal for NOS 2 was evident at 130 kd, as determined by molecular size markers.

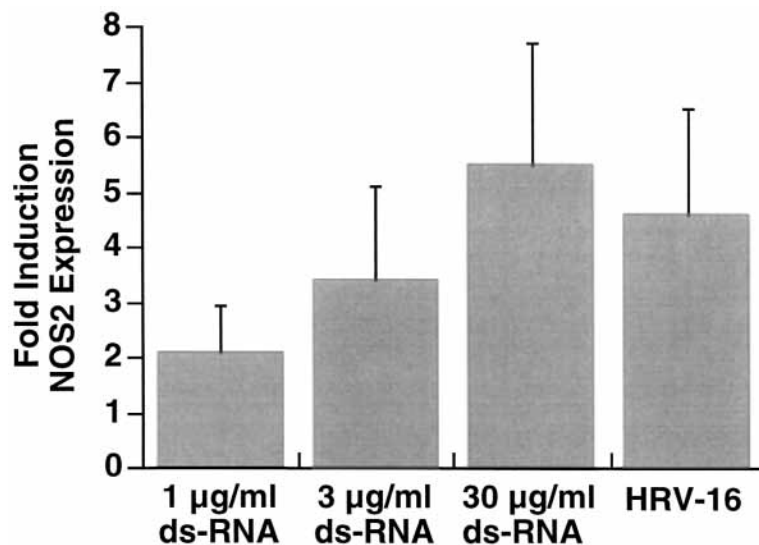


FIG 3. Real-time RT-PCR analysis of NOS 2 expression in primary epithelial cells infected with HRV-16 or treated with double-stranded RNA for 24 hours. Numbers represent the fold induction relative to the baseline control subjects ($n = 3$ for all bars except 1 µg/mL double-stranded RNA, where $n = 2$).

for control and UV-inactivated HRV-16 was 24.28 ± 0.12 and 24.29 ± 0.23 , respectively. Because processes that are dependent on viral replication are often mediated by double-stranded RNA, experiments were performed to determine whether synthetic double-stranded RNA induced NOS 2 gene expression in primary human bronchial epithelial cells. In each of 3 experiments, increases in NOS 2 gene expression were seen in cells exposed to increasing concentrations (1-30 µg/mL) of poly (I-C), a synthetic form of double-stranded RNA for 24 hours (Fig 3). Induction of NOS 2 mRNA by poly (I-C) was also observed at 4 and 48 hours (data not shown).

HRV-16 infection induces NOS 2 expression in nasal scrapings from human subjects

Because the protocol to examine the expression of NOS 2 in human subjects would require scraping cells from the right and left nostrils, we first examined whether there was a significant difference in NOS 2 mRNA expression in the left versus the right nostril at baseline. Cells were obtained from 4 healthy volunteers by nasal scraping. Total RNA was extracted from the samples and subjected to real-time RT-PCR analysis for NOS 2 expression. Paired t test analyses demonstrated

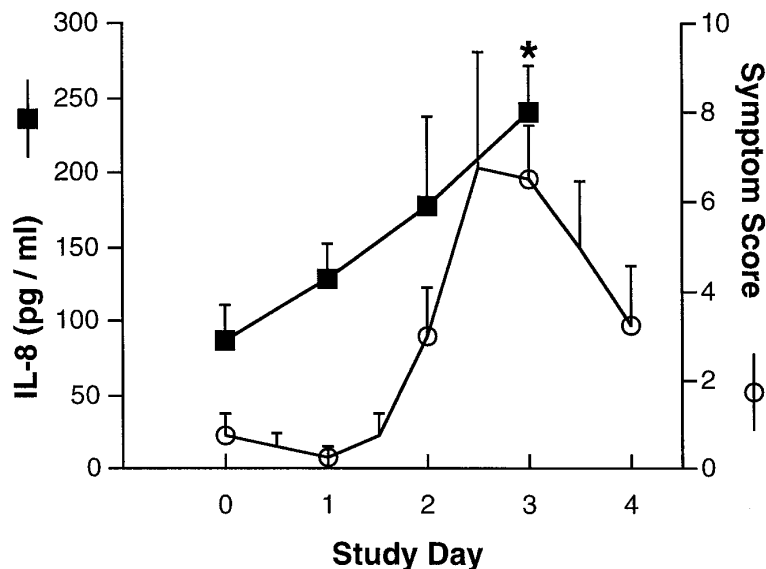


FIG 4. Symptom scores and levels of IL-8 in nasal lavage fluid recovered from subjects with HRV-16 infections. The baseline lavage fluid (day 0) was obtained before inoculation, and days 1 to 3 were after HRV-16 infection. The numbers represent the means $\pm$ SEM ($n = 4$).

TABLE II. Peak cold scores and nasal cell NOS 2 expression, as assessed by real-time RT-PCR from study subjects infected with HRV-16

Subject No.	Sex	Peak cold score	NOS 2 (fold induction)
1	F	5 (day 3)	1.3
2	F	13 (day 2)	2.4
3	M	5 (day 3)	10.3
4	F	9 (day 2)	18.3

that there was not a significant difference in baseline NOS 2 expression in cells from one nostril versus those from the other ($P = .5627$). Cell differential analysis of nasal scrapings with modified Wright's stain indicated that 95% or more of the cells were epithelial cells.

Healthy control subjects were infected with HRV-16 and were followed for 5 days. HRV-16 was not detected in the nasal lavage fluid before inoculation (Day 0) but was detected in the nasal lavage fluid on day 2 (3 subjects) or day 3 (1 subject). All subjects had increases in cold symptoms that peaked in the evening of day 2 or the morning of day 3 and then declined by day 4 (Table II and Fig 4). As shown in Fig 4, IL-8 in the nasal lavage fluid increased on all days after inoculation, reaching significance on day 3 ($P < .05$). Real-time RT-PCR analysis of NOS 2 mRNA expression in nasal scraping cells from one representative subject are shown in Fig 5. There was an 18-fold induction in NOS 2 mRNA expression during the cold in this subject. All 4 subjects had an increase in NOS 2 mRNA expression after HRV-16 infection, suggesting that the induction of NOS may play a role in the host response to rhinovirus infections (Table II).

DISCUSSION

The initial host responses to viral infections are rapid and nonspecific innate immune responses. Increasing evidence suggests that the free radical NO is an important effector molecule in these early responses to viruses. We previously demonstrated that NO inhibits not only rhinovirus replication but also rhinovirus-induced production of proinflammatory cytokines, suggesting that NO may play an important antiviral role in rhinovirus infections.⁵ The present studies now demonstrate that one factor in the host response to rhinovirus infection is the induction of the expression of the NOS 2 isoform of the enzyme that produces NO in human epithelial cells, the primary cellular target of rhinovirus infections.

Significant induction of NOS 2 expression was observed in primary human epithelial cells infected with rhinovirus. Increased levels of NOS 2 mRNA and protein were detected in primary human bronchial epithelial cells and primary human adenoid cells, indicating that virus-induced upregulation of NOS 2 occurs in epithelial cells from both the upper and lower airways. Elevations in NOS 2 mRNA levels were detected as early as 8 hours after infection and were still elevated at 24 and 48 hours after infection. Because the replication cycle of picornaviruses, such as rhinovirus, takes 8 to 10 hours, the timing of the induction suggested that the signals transducing the expression of NOS 2 may be linked to the replication of the virus. UV-inactivated virus did not induce NOS 2 expression, further suggesting that the NOS 2 mRNA expression in primary human epithelial cells was dependent on viral replication. During the replication of rhinovirus, which is a single-stranded, positive-sense RNA virus, host cells contain double-stranded RNA. The present studies demonstrated that synthetic double-stranded

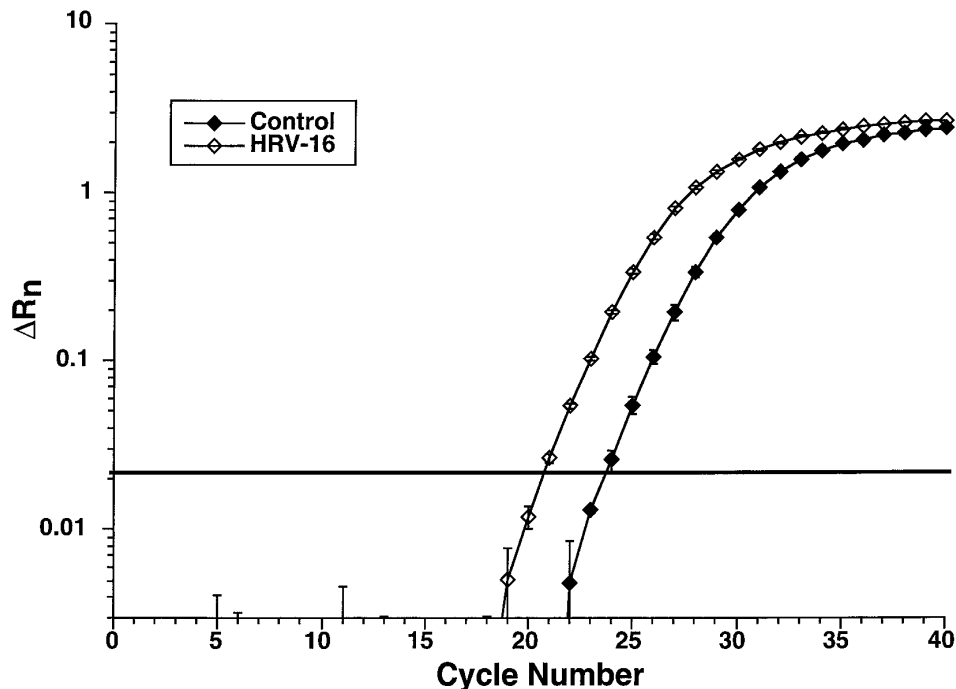


FIG 5. Real-time RT-PCR analysis of NOS 2 expression in cells from nasal scrapings performed before and during infection with HRV-16. Data are from a representative experiment ($n = 4$). Nasal scrapings were performed on day 0 at baseline (before cold) and on day 2 or 3 after infection (during cold).

RNA (poly [I-C]) increased the expression of NOS 2 mRNA in a dosedependent fashion in primary human epithelial cells. These results are consistent with the observation that viral replication is necessary to trigger the induction of NOS 2 mRNA expression. Other members of the Picornavirus family, including poliovirus⁶ and coxsackievirus,^{11,14,36-38} have been shown to induce NOS, although it is not yet known whether double-stranded RNA is involved in the induction by these viruses.

Although various isoforms of NOS have been implicated in the innate immune response to many viruses, not only picornaviruses, the pathways involved in the signal transduction of the response have not been fully defined. Because RNA and DNA viruses induce NOS gene expression, particularly NOS 2 expression, in numerous cell types, it is likely that there will be multiple mechanisms of induction, possibly involving both transcriptional and translational control.³⁹ The promoter region of the NOS 2 gene contains potential binding sites for several cytokine-induced transcription factors,⁴⁰ and the role of these sites in virally induced upregulation of NOS 2 varies, depending on the host cell and the type of virus. For example, respiratory syncytial virus, a single-stranded, negative-sense RNA virus, has recently been shown to elevate the expression of NOS 2 in the human A549 type 2 alveolar epithelial cell line.⁹ The induction was rapid, within 4 hours of infection, and was shown to involve the transcriptional activator interferon regulatory factor 1. Hepatitis B virus, a DNA virus, upregulated the NOS 2 expression in human hepatocytes, and the effect

was enhanced by proinflammatory cytokines, such as TNF- α and IFN- γ .¹⁰ Hepatitis B virus has been reported to activate members of the nuclear factor κ B family of transcription proteins, which are known to be important in the upregulation of NOS 2⁴¹⁻⁴³ and the innate immune antimicrobial response in epithelial cells.⁴⁴ Because other studies have shown that double-stranded RNA stimulates NOS 2 expression by mechanisms that involve the activation of nuclear factor κ B,^{45,46} it is possible that there are some common pathways of NOS 2 induction shared by both RNA and DNA viruses.

The combination of cytokines IL-1, TNF- α , and IFN- γ increased the levels of nitrite and nitrate, an indirect measurement of NO, produced by primary human epithelial cells in vitro. There were, however, no significant differences in the levels of nitrite and nitrate between control and HRV-16-infected epithelial cells, despite the fact that there was a significant induction in NOS 2 protein. These results may reflect an inability to detect changes relative to the magnitude of NOS 2 induction. For example, HRV-16 infection resulted in 2- to 16-fold inductions in NOS 2 mRNA expression, whereas cytomix treatment elevated NOS 2 by over 100-fold. The relative fold inductions of NOS 2 protein were also greater for cytomix-treated cells. In contrast, cytomix treatment resulted in only a 5-fold increase in nitrite and nitrate levels in the supernatants. If the larger fold inductions in NOS 2 mRNA and protein expression in cytomix-treated cells were associated with a 5-fold increase in nitrite and nitrate levels, then the smaller fold inductions associated

with virus infection may not have reached detectable levels. It is possible that although there was a significant increase in NOS 2 protein expression in HRV-16-infected cells, there may not have been an elevation in the function of NOS 2 protein, NO production, or both. The latter may occur if the infected cells lack essential cofactors or substrates necessary for NO production. This is improbable, however, because the cytomix-treated cells had significantly elevated nitrite and nitrate levels, indicating the cells were grown under conditions that provided the essential cofactors. Alternatively, it is possible that viral infection was associated with a functional upregulation of NO production, but the site of production was in close proximity to the site of reactivity with intracellular targets, such as superoxide anions, and thus did not lead to the extracellular accumulation of the NO oxidation products nitrite and nitrate.

One of the more significant findings of the present study was that NOS 2 expression was elevated in nasal cells obtained from human subjects infected with HRV-16. All 4 subjects had elevated levels of NOS 2 during their colds compared with precold levels. There was a range of induction from about 1.5- to 18-fold, suggesting that there was variability in the response. Because multiple scraping of the epithelium in the nose may conceivably lead to abnormal replication of the cells, the present studies were limited to sampling one nostril before the cold and one during the cold, and it was not possible to obtain samples from the same individual at different times after infection. For these reasons, it was not possible to determine the time at which the peak elevation in NOS 2 expression occurred. From the *in vitro* experiments, increases in NOS 2 were observed as early as 8 hours after infection and in most cases remained elevated at 48 hours. On the basis of these results, the nasal samples were obtained about 48 hours after infection, a time at which NOS 2 mRNA expression was expected to be elevated and when cold symptoms were clearly evident. The increased NOS 2 mRNA expression in nasal samples is consistent with the *in vitro* findings and suggests that the induction is directly related to the virus. The present studies, however, cannot rule out the possibility that mediators generated secondary to viral infection, such as IL-1, may contribute to the induction of NOS 2 mRNA expression *in vivo*.

The upregulation of NOS 2 mRNA expression suggests that NO may play a role in host defense against rhinovirus infections. However, the present *in vivo* studies have not demonstrated that there is a functional upregulation of the production of NO from nasal cells expressing the NOS 2 mRNA. Other studies, however, have shown that elevations of NOS 2 gene expression *in vivo* are accompanied by increased NOS protein expression and NO production.^{10,36} Furthermore, there have been some *in vivo* studies demonstrating that exhaled NO increases after natural and experimental upper respiratory tract infections.^{47,48} Although there was not a correlation between cold scores and fold induction of NOS 2, the number of subjects in the present study was too small

to provide enough data for this analysis. It is clear from animal studies with inhibitors of NOS and murine knock-out mice that NOS 2 is a critical component of the antiviral response for many viruses. Future studies will examine the relationship between elevations in NOS 2 gene expression and host defense against rhinoviruses.

In conclusion, our data demonstrate, for the first time, that NOS 2 mRNA is induced in human nasal cells during rhinovirus infections. The data further demonstrate that the signal transduction pathways leading to the upregulation of NOS 2 are linked to viral replication. We suggest that the induction of NOS 2 may play an important role in the innate immune response to rhinovirus infections. Knowing that upper respiratory tract infections, particularly rhinovirus infections, are a major trigger for acute asthma attacks, it is tempting to speculate that NO may play a protective role in virally induced exacerbations of asthma.

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