

Nitric oxide inhibits human rhinovirus-induced transcriptional activation of CXCL10 in airway epithelial cells

Rommy Koetzler, MD, MSc, Raza S. Zaheer, BSc, Shahina Wiehler, MSc, Neil S. Holden, PhD, Mark A. Gierbycz, PhD, and David Proud, PhD *Calgary, Alberta, Canada*

Background: Human rhinovirus (HRV) infections trigger exacerbations of asthma and chronic obstructive pulmonary disease. Nitric oxide (NO) inhibits HRV replication in human airway epithelial cells and suppresses HRV-induced epithelial production of several cytokines and chemokines.

Objective: We sought to delineate the mechanisms by which NO inhibits HRV-induced epithelial production of CXCL10, a chemoattractant for type 1 T cells and natural killer cells.

Methods: Primary human bronchial epithelial cells or cells of the BEAS-2B human bronchial epithelial cell line were exposed to HRV-16 in the presence or absence of the NO donor 3-(2-hydroxy-2-nitroso-1-propylhydrazino)-1-propanamine (PAPA NONOate). A cGMP analogue and an inhibitor of soluble guanylyl cyclase were used to examine the role of the cyclic guanosine monophosphate (cGMP) pathway in the actions of NO. BEAS-2B cells were transfected with CXCL10 promoter-luciferase constructs and the effects of PAPA NONOate were examined to study mechanisms of transcriptional regulation. Electrophoretic mobility shift assays were also used.

Results: PAPA NONOate inhibited HRV-16-induced increases in CXCL10 mRNA and protein. Inhibition of CXCL10 production occurred through a cGMP-independent pathway. PAPA NONOate inhibited HRV-16-induced CXCL10 transcription by blocking nuclear translocation, binding, or both of both nuclear factor κ B and IFN response factors (IRFs) to their respective recognition elements in the CXCL10 promoter.

Conclusions: NO inhibits HRV-16-induced production of CXCL10 by inhibiting viral activation of nuclear factor κ B and of IRFs, including IRF-1, through a cGMP-independent pathway. The broad-ranging inhibition of HRV-induced epithelial cytokine

and chemokine production by NO suggests a potential therapeutic utility of NO donors in viral exacerbations of asthma and chronic obstructive pulmonary disease. (*J Allergy Clin Immunol* ■■■■;■■■:■■■-■■■.)

Key words: Human rhinovirus, asthma, airway epithelial cell, CXCL10, IFN- γ -inducible protein of 10 kd, nitric oxide, IFN response factors, nuclear factor κ B

Human rhinovirus (HRV) infections are a major risk factor for exacerbations of asthma and chronic obstructive pulmonary disease (COPD).¹⁻³ The mechanisms by which HRV induces exacerbations of these diseases remain to be fully elucidated, but the airway epithelial cell is the primary site of HRV infection, and it has been demonstrated that infection can spread to the epithelium of the lower airways.⁴ HRV infection of epithelial cells does not lead to overt cytotoxicity but induces elaboration of a range of proinflammatory cytokines and chemokines that could contribute to the pattern of exaggerated inflammation that is characteristic of viral exacerbations.⁵⁻⁷ Moreover, a number of these epithelial products have been detected in increased amounts in airway secretions during *in vivo* HRV infections.^{1,6}

Current drugs are less than ideal for the treatment of virally induced exacerbations of airway diseases, and alternative therapies to modulate viral pathogenesis would be beneficial. One approach in this regard might be to supplement endogenous antiviral defenses. The free radical nitric oxide (NO) is an important component of the host defense not only against HRV but also against other viruses.⁸ HRV infection of epithelial cells induces expression of inducible nitric oxide synthase (iNOS) both *in vitro* and *in vivo*,⁹ and increased epithelial expression of iNOS during experimental HRV infections *in vivo* correlates with levels of exhaled NO. Interestingly, subjects with the highest levels of exhaled NO appear to clear virus more rapidly and to have lower symptom scores.¹⁰ The effects of NO might be mediated not only by its ability to inhibit replication of HRV in epithelial cells but also by inhibition of production of HRV-induced epithelial proinflammatory cytokines and chemokines.^{11,12} Understanding the molecular mechanisms by which NO inhibits HRV-induced cytokine and chemokine production might provide insights into selective therapies for virally induced exacerbations of airway diseases.

We examined HRV-induced production of CXCL10 (IFN- γ -inducible protein of 10 kd) as a model chemokine to delineate the mechanisms of action of NO. This chemokine, which is a chemoattractant for type 1 T lymphocytes and natural killer cells, is produced both *in vitro* and *in vivo* in response to HRV infection and replication⁶ and has been proposed as a novel biomarker for

From the Airway Inflammation Group, Institute of Infection, Immunity and Inflammation, University of Calgary.

Supported by grant no. 43923 from the Canadian Institutes of Health Research. Dr Proud is the recipient of a Canada Research Chair in Inflammatory Airway Diseases. Rommy Koetzler was the recipient of a ST*AR award from the American Academy of Allergy, Asthma & Immunology. Rommy Koetzler and Raza Zaheer are recipients of studentship awards from the Lung Association of Alberta and Northwest Territories.

Disclosure of potential conflict of interest: M. A. Gierbycz has received research support from Nycomed, AstraZeneca, and GlaxoSmithKline. D. Proud has received research support from AstraZeneca AB. The rest of the authors have declared that they have no conflict of interest.

Received for publication July 1, 2008; revised August 25, 2008; accepted for publication September 29, 2008.

Reprint requests: David Proud, PhD, Department of Physiology and Biophysics, University of Calgary Faculty of Medicine, 3330 Hospital Dr NW, Calgary, Alberta T2N 4N1, Canada. E-mail: dproud@ucalgary.ca.

0091-6749/\$34.00

© 2008 American Academy of Allergy, Asthma & Immunology

doi:10.1016/j.jaci.2008.09.041

Abbreviations used

AP-1:	Activator protein 1
BEGM:	Bronchial epithelial cell growth medium
cGMP:	Cyclic guanosine monophosphate
COPD:	Chronic obstructive pulmonary disease
dsRNA:	Double-stranded RNA
EMSA:	Electrophoretic mobility shift assay
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
HBE:	Primary human bronchial epithelial cell
HRV:	Human rhinovirus
IKK β :	Inhibitory κ B kinase β
iNOS:	Inducible nitric oxide synthase
IRF:	IFN regulatory factor
ISGF:	IFN-stimulated gene factor
ISRE:	IFN-stimulated response element
NF- κ B:	Nuclear factor κ B
NO:	Nitric oxide
ODQ:	1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one
PAPA NONOate:	3-(2-Hydroxy-2-nitroso-1-propylhydrazino)-1-propanamine
PMSF:	Phenylmethylsulfonyl fluoride
TCID:	Tissue culture infective dose

HRV-induced asthma exacerbations.¹³ We show that NO inhibits HRV-16-induced CXCL10 gene transcription by regulating nuclear translocation, binding, or both of both nuclear factor κ B (NF- κ B) and IFN regulatory factors (IRFs), including IRF-1, through a cyclic guanosine monophosphate (cGMP)–independent pathway.

METHODS

More details on all methods are provided in the [Methods](#) section in this article's Online Repository at www.jacionline.org.

Virus preparation and cell culture

Stocks of HRV-16 were propagated and purified as previously described.^{6,11} Primary human bronchial epithelial cells (HBES) and the BEAS-2B human bronchial epithelial cell line were cultured as described previously.⁶

Stimulation of epithelial cells

BEAS-2B cells and HBEs were exposed to $10^{4.5}$ and $10^{5.5}$ 50% tissue culture infective dose (TCID₅₀) units per milliliter of HRV-16, respectively, as previously described.¹⁴ Alternatively, cells were transfected with 0.1 μ g of synthetic double-stranded RNA (dsRNA) by using FuGene6. Cells were incubated in the absence or presence of 3-(2-hydroxy-2-nitroso-1-propylhydrazino)-1-propanamine (PAPA NONOate; Cayman Chemical Company, Ann Arbor, Mich; 100–1000 μ mol/L) for appropriate times at 34°C. Infected cells were also treated with 8-Bromo-cGMP (a cell-permeable cGMP analog) at concentrations ranging from 10 to 300 μ mol/L to determine whether cGMP would mimic the effects of PAPA NONOate. Cells were preincubated with vehicle or 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 3–30 μ mol/L) for 30 minutes and then infected with HRV-16 in the presence of PAPA NONOate to determine whether an inhibitor of soluble guanylyl cyclase could reverse the effects of PAPA NONOate in infected cells.

Assessment of CXCL10 protein and mRNA

Measurements of CXCL10 protein and mRNA were performed as previously described.⁶ A synthetic first-strand cDNA amplicon was used to generate standard curves to permit absolute quantification of mRNA.

CXCL10 promoter–luciferase constructs

The 972-bp CXCL10 promoter construct, corresponding to the sequence from –875 to +97 (relative to the transcriptional start site), and the 376-bp truncated construct (sequence from –279 to +97) of the 5'-flanking region of the human CXCL10 gene have been previously described.⁶ Point mutations in the putative activator protein-1 (AP-1), NF- κ B1 (κ B1), NF- κ B2 (κ B2), and IFN-stimulated response element (ISRE) recognition elements in the 376-bp truncated CXCL10 promoter were generated by means of site-directed mutagenesis. Reporter constructs containing 5 copies of the κ B1, κ B2, or ISRE sequences were produced by means of oligonucleotide synthesis. Reporter constructs containing 2 copies of ISRE and 2 copies of κ B1 (ISRE- κ B1-ISRE- κ B1), as well as constructs containing 2 copies of ISRE and 2 copies of κ B2 (ISRE- κ B2-ISRE- κ B2), were also generated. Transient transfection and luciferase assays were performed as previously described.⁶

Electrophoretic mobility shift assays

Nuclear extracts were prepared by using a modification of published methods.¹⁵ Oligonucleotide sequences containing the κ B1, κ B2, or ISRE recognition sequences were end-labeled with T4 polynucleotide kinase. Nuclear extracts were incubated with labeled probes before separation with 6% acrylamide gels, vacuum drying, and autoradiography. For supershift assays, antibodies to the Rel family members p50, p65, p52, and cRel, as well as to IRF-1, IRF-2, IRF-3, IRF-7, and IFN-stimulated gene factor (ISGF) 3 γ p48 (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif) were used.

Statistical analysis

For normally distributed data, between-group comparisons were made by means of 1-way ANOVA, with appropriate *post-hoc* analysis using the Fisher least significant difference test. For nonparametric data, Kruskal-Wallis ANOVA was used, followed by the Wilcoxon matched-pairs signed-rank test. *P* values of less than .05 were considered significant.

RESULTS**NO inhibits HRV-induced CXCL10 production from human airway epithelial cells**

PAPA NONOate significantly inhibited HRV-16–induced CXCL10 mRNA expression and protein production from BEAS-2B cells in a concentration-dependent manner (Fig 1, A and B). Similarly, 500 μ mol/L PAPA NONOate inhibited HRV-16–induced CXCL10 mRNA expression and protein production from HBEs (Fig 1, C and D). PAPA NONOate did not affect epithelial cell viability, as assessed by means of lactate dehydrogenase release, either in the presence or absence of HRV-16 at any concentration or time (data not shown).

Experiments were conducted with PAPA NONOate that had been left at room temperature overnight to deplete all of the NO content to confirm that the observed inhibition was specifically caused by NO. The inactive compound did not inhibit CXCL10 production (data not shown). In addition, we confirmed that a second, structurally unrelated NO donor, sodium nitroprusside, also significantly inhibited CXCL10 production from both HBEs and BEAS-2B cells (see Fig E1 in this article's Online Repository at www.jacionline.org).

NO can inhibit production of CXCL10 independently of effects on viral replication

We have shown that NO inhibits HRV replication in epithelial cells,¹¹ and therefore NO-mediated inhibition of CXCL10 production could be occurring solely as a result of the inhibition of viral replication. To assess this, we used synthetic dsRNA as a

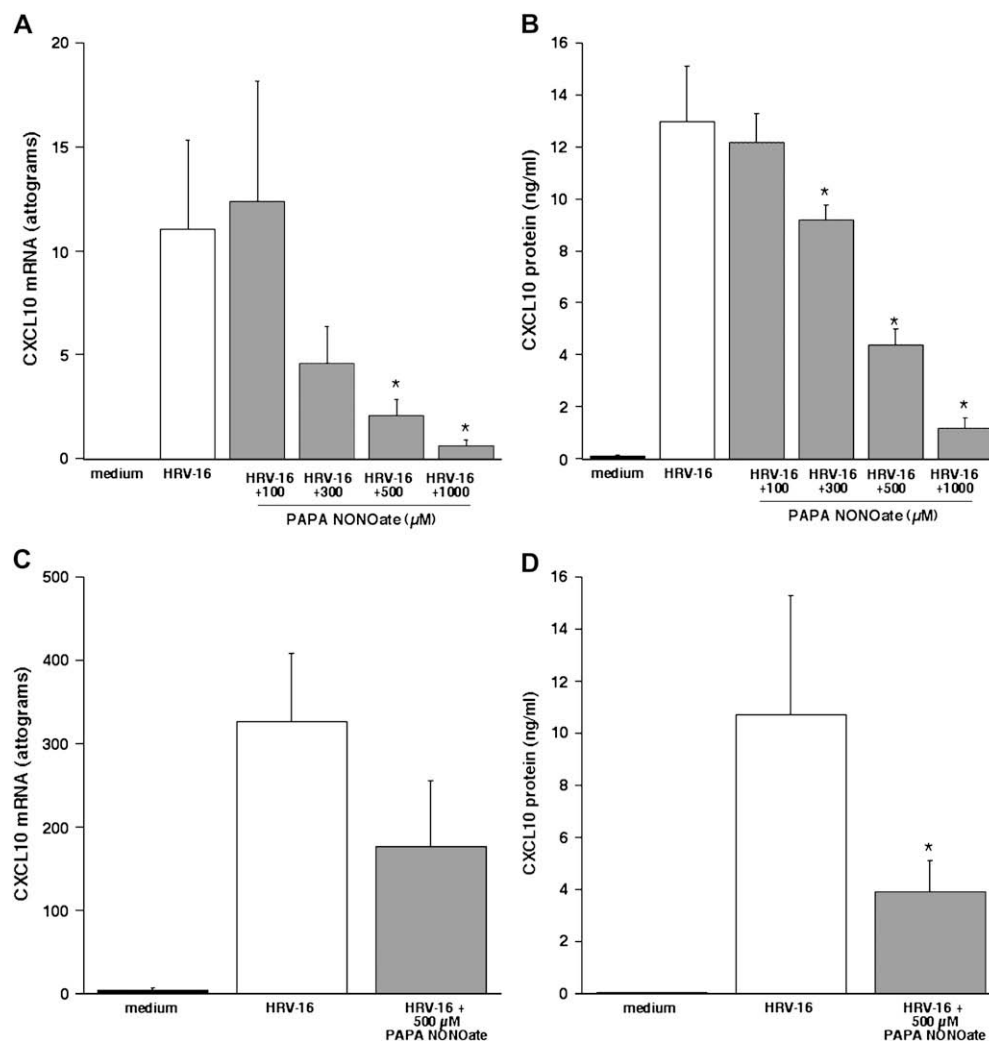


FIG 1. PAPA NONOate inhibits induction of CXCL10 mRNA and protein in HRV-16-infected epithelial cells. **A and B,** In BEAS-2B cells ($n = 7$) induction of CXCL10 mRNA and protein was inhibited in a concentration-dependent manner ($P < .05$, ANOVA). Asterisks indicate significant inhibition compared with HRV-16 alone by means of appropriate *post-hoc* tests. **C and D,** PAPA NONOate (500 μ mol/L) also inhibits CXCL10 production in HBEs ($n = 9$).

substitute for the dsRNA formed during viral replication. We have shown that dsRNA mimics the effects of HRV-16 in stimulating CXCL10 production.⁶ PAPA NONOate caused a significant concentration-dependent inhibition of dsRNA-induced CXCL10 mRNA and protein production from BEAS-2B cells. Similarly, PAPA NONOate significantly inhibited dsRNA-induced CXCL10 mRNA and protein production from HBEs (see Fig E2 in this article's Online Repository at www.jacionline.org). Thus NO can inhibit epithelial production of CXCL10 independently of effects on viral replication.

Inhibition of HRV-induced CXCL10 production by NO is independent of cGMP

HRV-16-infected cells were treated with the cell-permeable cGMP analog 8-bromo-cGMP (10-300 μ mol/L) to determine whether cGMP mediated the effects of PAPA NONOate. As shown (Fig 2, A), 8-bromo-cGMP had no effect on HRV-16-induced production of CXCL10 from BEAS-2B cells. Similarly, an inhibitor of soluble guanylyl cyclase, ODQ (3-30 μ mol/L), did

not reverse the PAPA NONOate-mediated inhibition of CXCL10 protein production in BEAS-2B cells (Fig 2, B), despite inhibiting PAPA NONOate-mediated increases in intracellular cGMP levels in a concentration-dependent fashion (see Fig E3 in this article's Online Repository at www.jacionline.org). Similar data were obtained when 8-bromo-cGMP and ODQ were used in HBEs (see Fig E4 in this article's Online Repository at www.jacionline.org).

Transcriptional regulation of HRV-16-induced CXCL10 production by NO

We have shown that HRV-16 infection induces transcriptional activation of CXCL10.⁶ To determine whether NO inhibits HRV-induced CXCL10 production at the level of transcription, we examined the effects of PAPA NONOate on HRV-16-induced activation of a 972-bp CXCL10 promoter-luciferase construct (IFN- γ -inducible protein of 10 kd pGL3) and of a truncated 376-bp construct (Fig 3, A). As we reported previously, although HRV-16 induced responses with both promoter constructs, slightly greater activation was seen with the 972-bp promoter.⁶

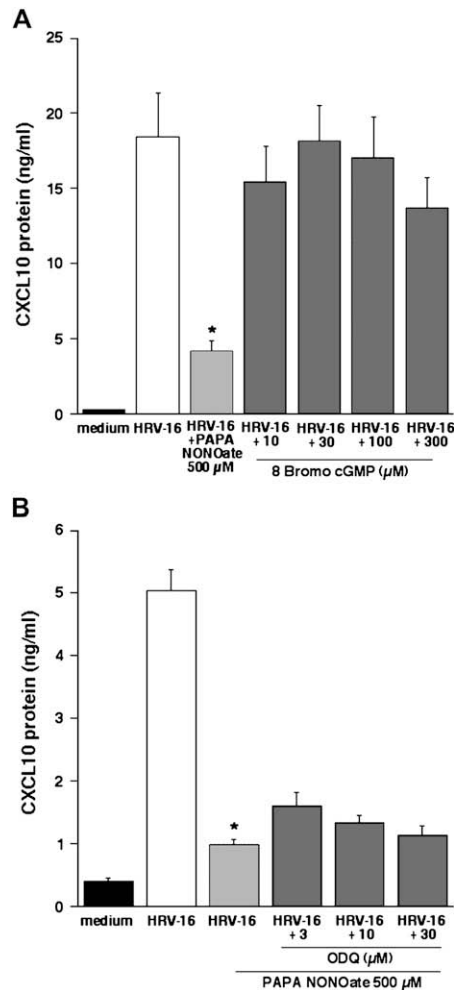


FIG 2. Inhibition of HRV-16-induced CXCL10 in epithelial cells is not mediated through cGMP. **A**, The cell-permeable analog 8-bromo-cGMP did not mimic the effects of PAPA NONOate (500 μ mol/L) in BEAS-2B cells ($n = 4$). **B**, Similarly, an inhibitor of soluble guanylyl cyclase (ODQ) did not reverse the effects of PAPA NONOate ($n = 4$). Asterisks indicate significant inhibition compared to HRV-16 alone.

PAPA NONOate inhibited HRV-16-induced activation of both constructs. Importantly, the degree of inhibition observed was essentially the same with each construct (Fig 3, B). This suggests that all of the key elements needed for NO-induced inhibition are contained within the 376-bp promoter, and therefore this was used as the template for additional studies of point mutations to identify potentially important transcription factor binding sites mediating the actions of NO. We have previously reported that the 2 proximal NF- κ B recognition sequences, κ B1 and κ B2, are involved in HRV-16-induced drive of the CXCL10 promoter, with κ B1 playing a more prominent role.⁶ This was confirmed in our present studies (Fig 3, C). Mutation of the κ B1 site led to a significant abrogation of activation. This was significantly further reduced in the presence of PAPA NONOate. Mutation of the κ B2 site also significantly reduced viral drive, although to a lesser extent, and this was significantly further inhibited in the presence of PAPA NONOate. We extended these studies by also examining the potential role of the ISRE and AP-1 sites in HRV-16-induced activation of the CXCL10 promoter. Mutation

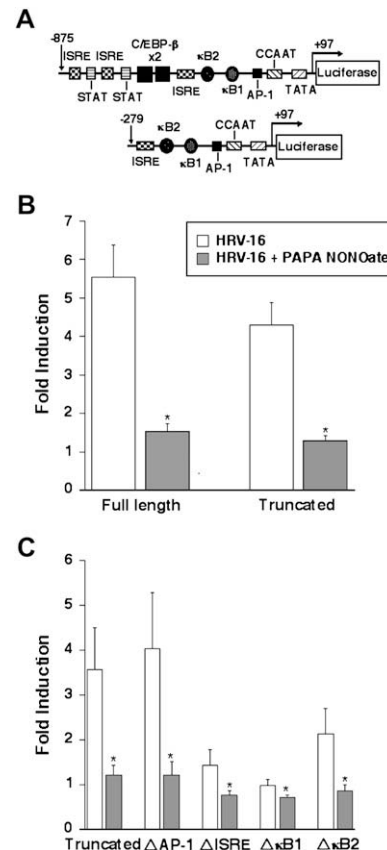


FIG 3. PAPA NONOate inhibits HRV-16-induced activation of CXCL10 promoter-luciferase constructs. **A**, Diagrammatic representations of full-length (972-bp) and truncated (376-bp) promoters. **B**, PAPA NONOate (solid bars) inhibits activation of full-length and truncated constructs to a similar degree. Asterisks indicate significant inhibition compared with HRV-16 alone (open bars, $n = 7$). **C**, Mutation of individual NF- κ B and ISRE, but not AP-1, recognition sequences in the truncated promoter reduces HRV-16-induced promoter activation. Asterisks indicate significant inhibition by PAPA NONOate (solid bars) compared with virus alone (open bars, $n = 6$). C/EBP, CCAAT/enhancer binding protein.

of the AP-1 site had no significant effect on the ability of HRV-16 to induce promoter activation, and inhibition by the NO donor was comparable with that seen with the wild-type promoter. By contrast, mutation of the ISRE site led to significantly reduced viral drive, and this residual activity was further inhibited by the NO donor. Control studies were also performed with a pGL3 basic promoter containing only a TATA box. HRV-16 did not induce activation of this promoter, and PAPA NONOate had no effect on the basal activity of this construct (data not shown). Taken together, these data suggest that the κ B1, κ B2, and ISRE sites play a role both in viral induction of the promoter and in the inhibitory actions of NO. To establish whether NO-mediated inhibition of CXCL10 promoter activation was secondary to effects on viral replication, we examined the activation of these constructs with dsRNA. PAPA NONOate inhibited dsRNA-induced promoter activation. Furthermore, mutation of the AP-1 site had no significant effect on drive, whereas the κ B1, κ B2, and ISRE sites again appeared to play a role both in dsRNA induction of the promoter and in the inhibitory actions of NO (see Fig E5 in this article's Online Repository at www.jacionline.org). To further investigate the role of the κ B1, κ B2, and ISRE sites in viral responses and the

effects of NO, we generated reporter constructs containing 5 copies of either the κ B1 recognition sequence ($5\times \kappa$ B1), the κ B2 sequence ($5\times \kappa$ B2), or the ISRE sequence ($5\times$ ISRE). Constructs with 5 copies of a mutated form of each sequence were also produced. HRV-16 induced activation of both the $5\times \kappa$ B1 and $5\times \kappa$ B2 constructs (Fig 4, A) but not their mutated counterparts (data not shown). Importantly, activation of $5\times \kappa$ B1 and $5\times \kappa$ B2 constructs was significantly reduced in the presence of PAPA NONOate, further supporting that the inhibitory actions of NO include effects on NF- κ B activation, binding, or both. Interestingly, HRV-16 infection did not induce activation of the $5\times$ ISRE-luciferase construct. Previous studies have shown strong cooperativity between NF- κ B and ISRE sites in the RANTES promoter.¹⁶ Thus it was possible that the ISRE site contributes to activation of the CXCL10 promoter only when present in conjunction with the NF- κ B site or sites. To test this possibility, we examined constructs containing 2 copies of ISRE and 2 copies of κ B1 (ISRE- κ B1-ISRE- κ B1), as well as constructs containing 2 copies of ISRE and 2 copies of κ B2 (ISRE- κ B2-ISRE- κ B2). These promoters were modestly induced after HRV-16 infection, and the NO donor significantly inhibited this response (Fig 4, B). Interestingly, the ISRE- κ B2-ISRE- κ B2 construct, in which sequences were identical and in the same alignment as in the endogenous promoter, was induced more than the ISRE- κ B1-ISRE- κ B1 construct, which was not in the same context as in the endogenous promoter. Both the AP-1 and κ B2 sequences were mutated in the 376-bp promoter to determine whether activation of the endogenous promoter occurred when only ISRE and κ B1 were left intact. HRV-16 infection triggered more robust induction of this construct, and this was completely abrogated in the presence of PAPA NONOate (Fig 4, C).

Electrophoretic mobility shift assay analysis of nuclear translocation, binding, or both, of NF- κ B and IRFs

Labeled oligonucleotides containing the specific NF- κ B1, NF- κ B2, or ISRE recognition sequences from the promoter were used in electrophoretic mobility shift assay (EMSA) studies to further evaluate the actions of NO on nuclear translocation, binding, or both of transcription factors to the CXCL10 promoter. Initial time-course experiments demonstrated that HRV-16 infection induced specific binding to all 3 recognition sequences, with NF- κ B activation slightly preceding ISRE responses (see Fig E6 in this article's Online Repository at www.jacionline.org). HRV-16 infection induced 2 specific bands with oligonucleotides containing NF- κ B recognition sequences and 3 specific bands with the ISRE oligonucleotide (see Fig E6). HRV-16-induced specific binding to the NF- κ B1 oligonucleotide was inhibited in the presence of PAPA NONOate (Fig 5, A). Supershift analysis showed that the upper band was shifted by antibodies to both p65 and p50, suggesting it represents the canonical p65-p50 heterodimer, which we have previously reported to be induced in HRV-16-infected cells,¹⁷ whereas the lower band was shifted only by antibodies to p50, suggesting it might represent p50 homodimers. Antibodies to p52 and cRel were without effect (Fig 5, B). Identical inhibition data and supershift results were seen with the NF- κ B2 oligonucleotide (see Fig E7 in this article's Online Repository at www.jacionline.org). Incubation of nuclear proteins from HRV-16-infected epithelial cells with the ISRE oligonucleotide led to the formation of 3 specific bands. In the presence of

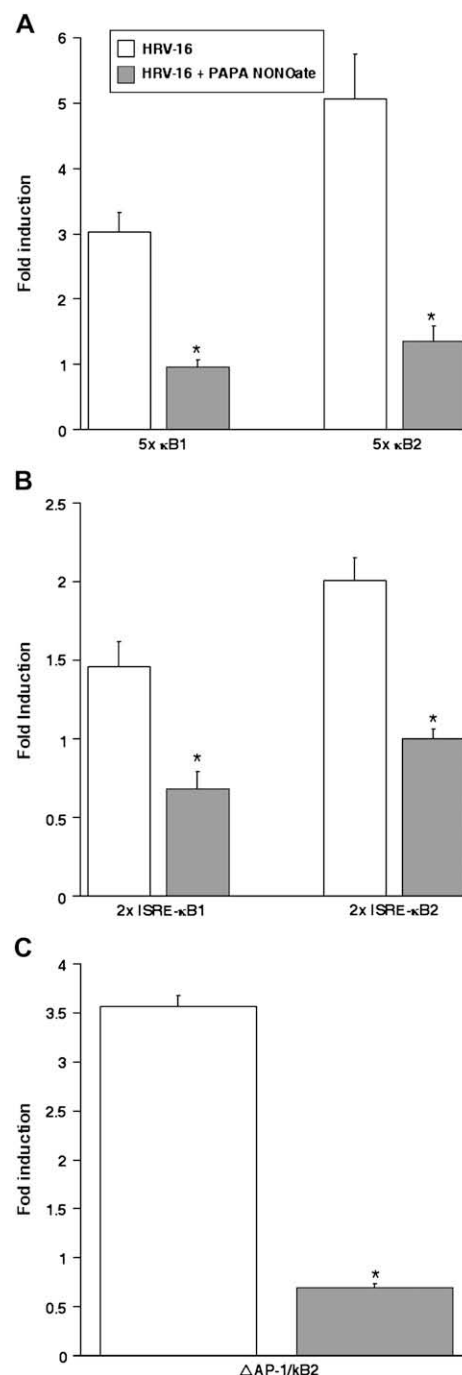


FIG 4. PAPA NONOate inhibits HRV-16-induced activation of reporter constructs. **A**, PAPA NONOate significantly inhibits HRV-16-induced activation of constructs containing 5 copies of either the NF- κ B1 or NF- κ B2 recognition sequences ($n = 6$). **B**, PAPA NONOate significantly inhibits HRV-16-induced activation of constructs containing 2 sequential copies of ISRE-NF- κ B1 or ISRE-NF- κ B2 sequences ($n = 6$). **C**, PAPA NONOate significantly inhibits HRV-16-induced activation of the truncated promoter containing mutations in both the AP-1 and NF- κ B2 recognition sequences ($n = 6$). In each case asterisks indicate significant inhibition by PAPA NONOate (solid bars) compared with virus alone (open bars).

PAPA NONOate, formation of all 3 bands was inhibited (Fig 6, A). In supershift experiments the upper band was completely shifted by using an antibody to IRF-1 (Fig 6, B). The 2 lower bands were unaffected by antibodies to IRF-1, IRF-2, IRF-3,

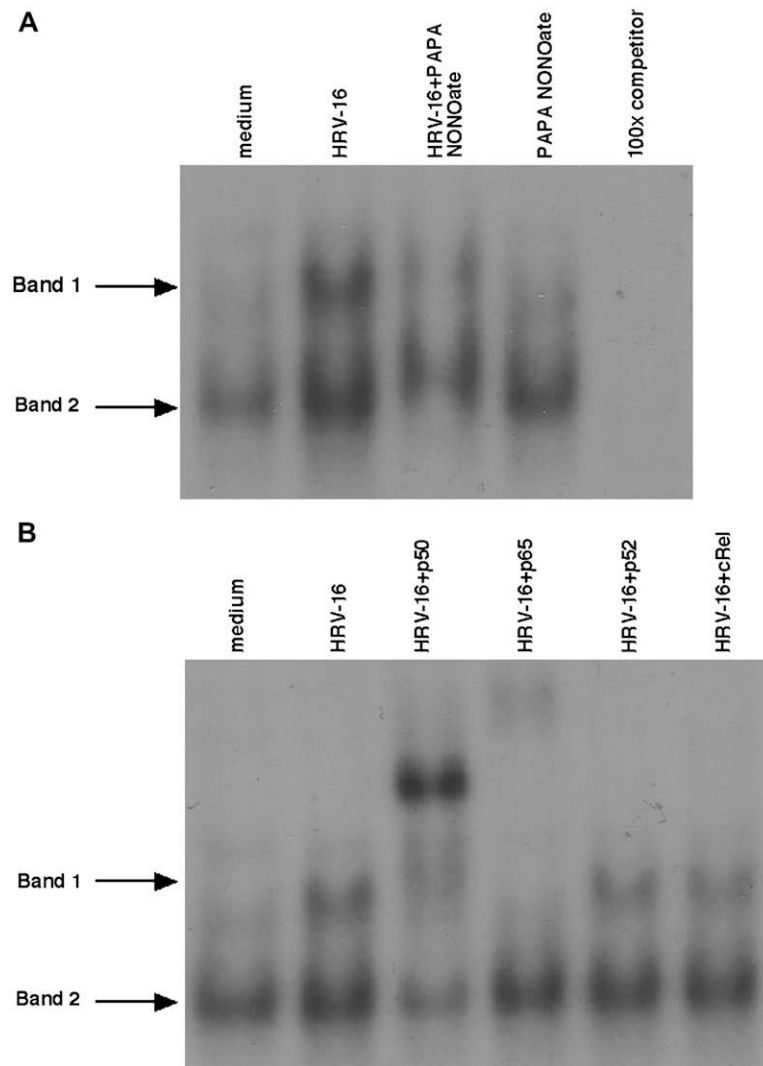


FIG 5. Effects of PAPA NONOate on nuclear translocation, binding, or both of NF- κ B in HRV-16-infected BEAS-2B cells. **A**, HRV-16 infection induced 2 bands on incubation of nuclear extracts with the NF- κ B1 oligonucleotide. Induction of both bands was inhibited by PAPA NONOate. **B**, Supershift experiments demonstrate that antibody to p65 shifts only the upper band, whereas antibody to p50 shifts both bands. Antibodies to p52 and cRel were without effect. Data are representative of 3 experiments.

IRF-7, or ISGF-3 γ p48 (Fig 6, B), and additional studies will be needed to identify the constituents of these bands.

DISCUSSION

NO is an important component of the host defense to HRV infection.⁸ Patients with the greatest increases in levels of exhaled NO in the upper airways during HRV infections clear virus more rapidly and have the lowest symptoms,¹⁰ whereas asthmatic subjects with the largest increase in lower airway exhaled NO levels during viral infections had the smallest increases in airway hyperresponsiveness.¹⁸ The protective effects of NO can be mediated not only through its ability to reduce HRV replication in epithelial cells but also through inhibition of HRV-induced epithelial production of proinflammatory cytokines and chemokines.^{11,12} We now show that NO inhibits, in a concentration-dependent manner, HRV-induced epithelial production of CXCL10, a chemoattractant for type 1 lymphocytes and natural killer cells that has

been implicated as a novel biomarker for viral exacerbations of asthma.¹³ The ability of NO to also inhibit dsRNA-induced CXCL10 production indicates that the inhibitory effects of NO can occur independently of effects on viral replication. Therefore we used CXCL10 as a model to delineate the mechanisms by which NO inhibits HRV-induced chemokine production.

Although inhibition by NO of some cytokines, such as IFN- α from human dendritic cells, is mediated through cGMP,¹⁹ inhibition of HRV-induced production of CXCL10 clearly occurs independently of this second messenger. Not only was a cell-permeable cGMP analog unable to mimic the effects of NO, but an inhibitor of soluble guanylyl cyclase was unable to block NO-mediated inhibition of CXCL10 when used at concentrations that substantially inhibited PAPA NONOate-induced cGMP accumulation.

HRV-16-induced production of CXCL10 is regulated at the level of transcriptional activation, in part through the actions of NF- κ B.⁶ Mutation studies now show that the ISRE site is also

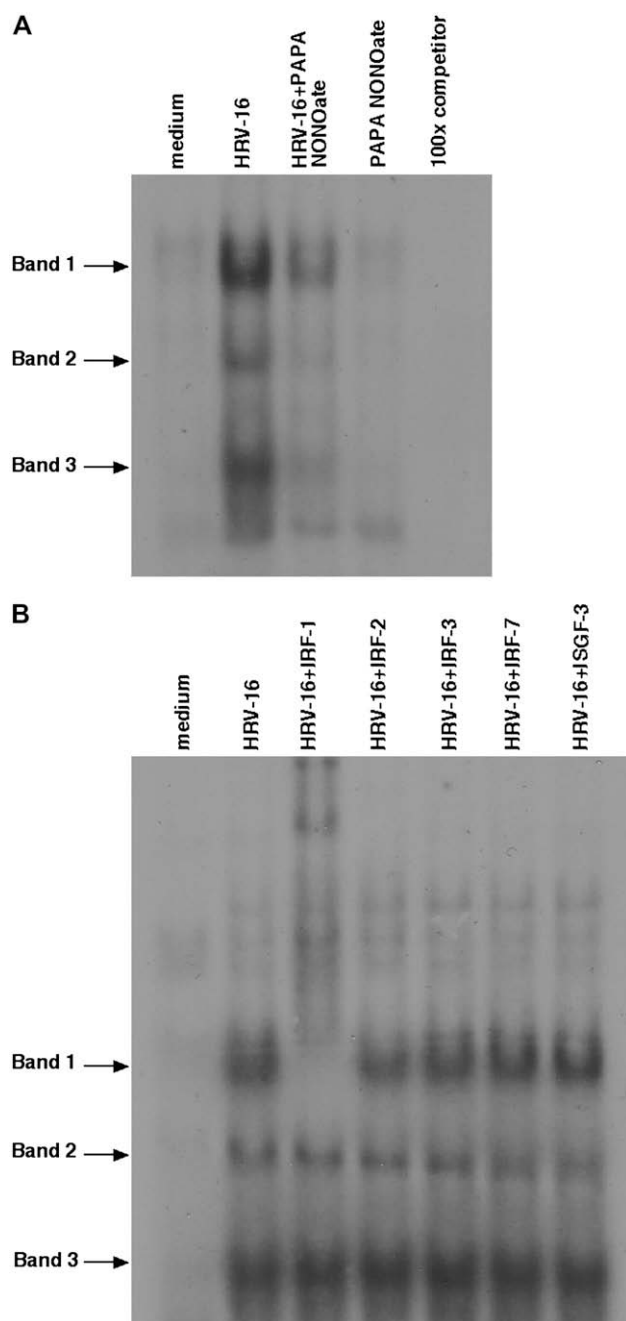


FIG 6. Effects of PAPA NONOate on nuclear translocation, binding, or both of IRFs in HRV-16-infected BEAS-2B cells. **A**, HRV-16 infection induced 3 bands on incubation of nuclear extracts with the ISRE oligonucleotide. Induction of all 3 bands was inhibited by PAPA NONOate. **B**, Supershift experiments demonstrate that antibody to IRF-1 completely shifts the upper band. Antibodies to IRF-2, IRF-3, IRF-7, or ISGF-3 were without effect on any band. Data are representative of 3 experiments.

important for HRV-16-induced CXCL10 promoter activation. We also showed that PAPA NONOate inhibits virally induced CXCL10 transcription and that the truncated form of the CXCL10 promoter contains the essential elements involved in NO-mediated inhibition. Again, the ability of NO to also inhibit dsRNA-induced promoter activation suggests that inhibition can occur independently of effects on viral replication. There is ample precedent for NO to modulate gene transcription by means of

direct modulation of transcription factor function,²⁰ and therefore we examined the role of each transcription factor binding site in the inhibition of HRV-induced CXCL10 transcription. Although NO can inhibit the DNA binding ability of AP-1,²¹ mutation of the AP-1 site in the CXCL10 promoter had no effect either on viral or dsRNA-induced promoter activation or on the degree of inhibition by NO. By contrast, the NF- κ B and ISRE sites clearly play the dominant role in HRV-16-induced and dsRNA-induced activation of the promoter, and therefore modulation of these sites is likely to be key in the actions of NO. Constructs containing multiple copies of either the NF- κ B1 or NF- κ B2 sites responded to HRV-16, and their activation was inhibited in the presence of PAPA NONOate. Moreover, EMSA studies showed a clear reduction in nuclear translocation, binding, or both of both p65-p50 heterodimers and p50 homodimers to oligonucleotides containing NF- κ B1 and NF- κ B2 recognition sequences from the CXCL10 promoter. NO exerts complex modulatory roles on the NF- κ B pathway through S-nitrosylation events.²² S-nitrosylation of p21ras by NO enhances activation of NF- κ B.²³ Inhibition of the effects of NF- κ B by NO can result from direct S-nitrosylation of the Cys-62 residue of p50, which prevents DNA binding and gene transcription.²⁴ In addition, S-nitrosylation of Cys-179 of inhibitory κ B kinase β (IKK β) inhibits the activity of this enzyme, preventing downstream activation of NF- κ B by means of this canonical pathway.²⁵ Because viruses can also activate the IKK-related kinases tank-binding kinase-1 and IKK ϵ ,²⁶ these enzymes also might contribute to NF- κ B activation in HRV-infected cells. Further studies will be required to identify the mechanisms underlying inhibition by NO of NF- κ B nuclear translocation, binding, or both in HRV-infected epithelial cells. Regardless of the mechanisms involved, this activity likely contributes to the ability of NO to inhibit HRV-induced production of a number of cytokines, such as IL-8, IL-6, and GM-CSF,^{11,12} all of which have NF- κ B recognition sequences in the promoter regions of their genes.

The ISRE sequence in the CXCL10 promoter has been shown to play a role in promoter activation induced by IFN- γ or synthetic dsRNA.^{27,28} Our data are the first to demonstrate that the ISRE site is also important in HRV-16-induced promoter activation. Although mutation of the ISRE site markedly abrogated HRV-16-induced promoter activation, a synthetic construct containing 5 copies of just this site was not responsive to HRV-16 infection, implying that the ISRE contribution to promoter activation might be dependent on its context. There is precedent for cooperativity between the ISRE and NF- κ B sites for binding of their respective ligands in the RANTES promoter,¹⁶ and a construct with 2 sequential copies of the ISRE-NF- κ B2 sequence exactly as they occur in the endogenous CXCL10 promoter was induced by HRV-16 and was inhibited in the presence of the NO donor. A construct with 2 copies of an ISRE-NF- κ B1 sequence was less responsive, perhaps because these 2 sites are not found directly adjacent in the endogenous promoter. Thus retaining the secondary structure as found in the endogenous promoter might be critical. In support of this, mutating both the AP-1 and NF- κ B2 sites in the endogenous promoter, leaving only functional ISRE and NF- κ B1 sites in their original context, led to robust HRV-induced activation, which was markedly inhibited in the presence of PAPA NONOate. Nuclear extracts from HRV-16-infected epithelial cells formed 3 specific bands on interaction with a labeled oligonucleotide containing the ISRE site. Formation of all of these bands was inhibited by PAPA NONOate. The uppermost band was entirely shifted with an antibody to IRF-1. Although

IFN-stimulated gene factor 3 (ISGF3), a complex of IRF-9 (also known as ISGF-3 γ p48) with signal transducers and activator of transcription 1 and 2, has been implicated in mouse and human CXCL10 promoter induction in response to some stimuli.^{27,29} Antibodies to ISGF-3 γ p48 did not affect any of the bands. Indeed, the identity of the 2 lower bands remains unclear because they were not affected by antibodies to IRF-1, IRF-2, IRF-3, IRF-7, or ISGF-3 γ p48. To our knowledge, this is the first demonstration that NO can modulate gene activation by regulating nuclear translocation, binding, or both of IRFs, such as IRF-1. Further studies are needed to identify the mechanisms by which NO modulates activation of IRF-1 and to identify the other proteins that associate with the ISRE site.

In summary, NO inhibits HRV-16-induced CXCL10 gene transcription by regulating nuclear translocation, binding, or both of both NF- κ B and IRFs, including IRF-1, through a cGMP-independent pathway. This is the first description of NO modulating IRF-1 function. The ability of NO to inhibit both HRV-induced proinflammatory chemokine production and viral replication suggests it is an important component of host defense to HRV infection. Interestingly, the ability of NO to inhibit both NF- κ B and IRF-1 nuclear translocation/DNA binding might limit the role of endogenous NO in this regard because NF- κ B and IRF-1 have also been linked to transcriptional activation of iNOS,^{30,31} and therefore feedback inhibition can occur. Given this limitation, we speculate that topical administration of NO donors might be beneficial to supplement the endogenous host response during exacerbations of asthma and COPD triggered by HRV infections.

Clinical implications: The ability of NO to inhibit HRV-induced cytokine and chemokine production suggests that NO donors might provide a potential therapeutic approach to viral exacerbations of asthma and COPD.

REFERENCES

- Proud D, Chow C-W. Role of viral infections in asthma and COPD. *Am J Respir Cell Mol Biol* 2006;35:513-8.
- Khetsuriani N, Kazerouni NN, Erdman DD, Lu X, Redd SC, Anderson LJ, et al. Prevalence of viral respiratory tract infections in children with asthma. *J Allergy Clin Immunol* 2007;119:314-21.
- Traves SL, Proud D. Viral-associated exacerbations of asthma and COPD. *Curr Opin Pharmacol* 2007;7:252-8.
- Mosser AG, Vrtis R, Burchell L, Lee W-M, Dick CR, Weisshaar E, et al. Quantitative and qualitative analysis of rhinovirus infection in bronchial tissues. *Am J Respir Crit Care Med* 2005;171:645-51.
- Subauste MC, Jacoby DB, Richards SM, Proud D. Infection of a human respiratory epithelial cell line with rhinovirus. Induction of cytokine release and modulation of susceptibility to infection by cytokine exposure. *J Clin Invest* 1995;96:549-57.
- Spurrell JCL, Wiehler S, Zaheer RS, Sanders SP, Proud D. Human airway epithelial cells produce IP-10 (CXCL10) in vitro and in vivo upon rhinovirus infection. *Am J Physiol Lung Cell Mol Physiol* 2005;289:L85-95.
- Donninger H, Glashoff R, Haitchi H-M, Syce JA, Ghildyal R, Bardin PG. Rhinovirus induction of the CXC chemokine epithelial-neutrophil activating peptide-78 in bronchial epithelium. *J Infect Dis* 2003;187:1809-17.
- Proud D. Nitric oxide and the common cold. *Curr Opin Allergy Clin Immunol* 2005;5:37-42.
- Sanders SP, Siekierski ES, Richards SM, Porter JD, Imani F, Proud D. Rhinovirus infection induces expression of type 2 nitric oxide synthase in human respiratory epithelial cells in vitro and in vivo. *J Allergy Clin Immunol* 2001;107:235-43.
- Sanders SP, Proud D, Siekierski ES, Yachechko R, Liu MC. Role of nasal nitric oxide in the resolution of experimental rhinovirus infection. *J Allergy Clin Immunol* 2004;113:697-702.
- Sanders SP, Siekierski ES, Porter JD, Richards SM, Proud D. Nitric oxide inhibits rhinovirus-induced cytokine production and viral replication in a human respiratory epithelial cell line. *J Virol* 1998;72:934-42.
- Sanders SP, Kim J, Connolly KR, Porter JD, Siekierski ES, Proud D. Nitric oxide inhibits rhinovirus-induced GM-CSF production in bronchial epithelial cells. *Am J Respir Cell Mol Biol* 2001;24:317-25.
- Wark PAB, Buchieri F, Johnston SL, Gibson PG, Hamilton L, Mimica J, et al. IFN- γ -induced protein 10 is a novel biomarker of rhinovirus-induced asthma exacerbations. *J Allergy Clin Immunol* 2007;120:586-93.
- Leigh R, Oyelusi W, Wiehler S, Koetzler R, Zaheer RS, Newton R, et al. Human rhinovirus infection enhances airway epithelial cell production of growth factors involved in airway remodeling. *J Allergy Clin Immunol* 2008;121:1238-45.
- Nasuhara Y, Adcock IM, Catley M, Barnes PJ, Newton R. Differential I κ B kinase activation and I κ B α degradation by interleukin-1 β and tumor necrosis factor- α in human U937 monocytic cells. Evidence for additional regulatory steps in κ B-dependent transcription. *J Biol Chem* 1999;274:19965-72.
- Génin P, Alagarté M, Roof P, Lin R, Hiscott J. Regulation of RANTES chemokine gene expression requires cooperativity between NF- κ B and IFN-regulatory factor transcription factors. *J Immunol* 2000;164:5352-61.
- Kim J, Sanders SP, Siekierski ES, Casolaro V, Proud D. Role of NF- κ B in cytokine production induced from human airway epithelial cells by rhinovirus infection. *J Immunol* 2000;165:3384-92.
- de Gouw HW, Grunberg K, Schot R, Kroes AC, Dick EC, Sterk PJ. Relationship between exhaled nitric oxide and airway hyperresponsiveness following experimental rhinovirus infection in asthmatic subjects. *Eur Respir J* 1998;11:126-32.
- Morita R, Uchiyama T, Hori T. Nitric oxide inhibits IFN- α production of human plasmacytoid dendritic cells partly via a guanosine 3'5'-cyclic monophosphate-dependent pathway. *J Immunol* 2005;175:806-12.
- Kröncke K-D, Fehsel K, Suschek C, Kolb-Bachofen V. Inducible nitric oxide synthase-derived nitric oxide in gene regulation, cell death and cell survival. *Int Immunopharmacol* 2001;1:1407-20.
- Nikitovic D, Holmgren A, Spyrou G. Inhibition of DNA binding by nitric oxide involving conserved cysteine residues in Jun and Fos. *Biochem Biophys Res Commun* 1998;242:109-12.
- Marshall HE, Hess DT, Stamler JS. S-nitrosylation: physiological regulation of NF- κ B. *Proc Natl Acad Sci U S A* 2004;101:8841-2.
- Lander HM, Hajjar DP, Hempstead BL, Mirza UA, Chait BT, Campbell S, et al. A molecular redox switch on p21(ras). Structural basis for the nitric oxide-p21(ras) interaction. *J Biol Chem* 1997;272:4323-6.
- Matthews JR, Botting CH, Panico M, Morris HR, Hay RT. Inhibition of NF- κ B DNA binding by nitric oxide. *Nucleic Acids Res* 1996;24:2236-42.
- Reynaert NL, Ckless K, Korn SH, Vos N, Guala AS, Wouters EFM, et al. Nitric oxide represses inhibitory κ B kinase through S-nitrosylation. *Proc Natl Acad Sci U S A* 2004;101:8945-50.
- Hiscott J, Nguyen TL, Arguello M, Nakhaei P, Paz S. Manipulation of the nuclear factor- κ B pathway and the innate immune response by viruses. *Oncogene* 2006;25:6844-67.
- Majumder S, Zhou LZ-H, Chaturvedi P, Babcock G, Aras S, Ransohoff RM. p48/STAT-1 α -containing complexes play a predominant role in induction of IFN- γ -inducible protein, 10kDa (IP-10) by IFN- γ alone or in synergy with TNF- α . *J Immunol* 1998;161:4736-44.
- Wu C, Ohmori Y, Bandyopadhyay S, Sen G, Hamilton T. Interferon-stimulated response element and NF κ B sites cooperate to regulate double-stranded RNA-induced transcription of the IP-10 gene. *J Interferon Res* 1994;14:357-63.
- Nakaya T, Sato M, Hata N, Asagiri M, Suemori H, Noguchi S, et al. Gene induction pathways mediated by distinct IRFs during viral infection. *Biochem Biophys Res Commun* 2001;283:1150-6.
- Uetani K, Der SD, Zamanian-Daryoush M, de la Motte C, Lieberman BY, Williams BRG, et al. Central role of double-stranded RNA-activated protein kinase in microbial induction of nitric oxide synthase. *J Immunol* 2000;165:988-96.
- Paludan SR, Malmgaard L, Ellermann-Eriksen S, Bosca L, Mogensen SC. Interferon (IFN)-gamma and Herpes simplex virus/tumor necrosis factor-alpha synergistically induce nitric oxide synthase 2 in macrophages through cooperative action of nuclear factor-kappa B and IFN regulatory factor-1. *Eur Cytokine Netw* 2001;12:297-308.

METHODS

Materials

The following reagents were purchased from the indicated suppliers: Ham's F12 medium, Earles minimal essential medium (EMEM), HBSS, penicillin-streptomycin-amphotericin B, and L-glutamine; TRIzol reagent, sodium pyruvate, nonessential amino acids, gentamicin, FBS, and deoxyribonucleoside triphosphates (Invitrogen Life Technologies, Burlington, Ontario, Canada); Taqman master mix, 20× glyceraldehyde-3-phosphate dehydrogenase (GAPDH), RNase inhibitor, and reverse transcriptase (Applied Biosystems, Foster City, Calif); Pfu polymerase (Stratagene, Foster City, Calif); antibody pairs for CXCL10 and recombinant human CXCL10 (R&D Systems, Minneapolis, Minn); 3-(2-hydroxy-2-nitroso-1-propylhydrazino)-1-propanamine (PAPA NONOate, Cayman); the firefly luciferase reporter plasmid pGL3-basic, the Renilla luciferase plasmid pRL-null, genomic DNA, passive lysis buffer, and the dual-luciferase reporter assay system (Promega, Madison, Wis); and FuGene6 (Roche Diagnostics, Laval, Quebec, Canada). All other chemicals were purchased from Sigma-Aldrich (St Louis, Mo).

Virus preparations and cell culture

HRV-16 was purchased from the American Type Culture Collection (Rockville, Md). HRV-16 viral stocks used for experiments were generated by propagation in WI-38 cells and were purified by means of centrifugation through a sucrose gradient, as previously described.^{E1}

Viral titers were determined by incubating confluent monolayers of WI-38 cells in 96-well plates with serial dilutions of the virus-containing supernatants in EMEM with 10% FBS. Plates were incubated at 34°C for 5 days. The medium was removed, and the cells were washed with HBSS and then fixed with methanol for 1 minute. The methanol was removed, and the cells were incubated with 0.1% crystal violet in distilled water for 20 minutes. Plates were washed, and the absorbance at 570 nm was determined with a plate reader. The dilution of supernatant required to infect 50% of the monolayers was determined and expressed as log TCID₅₀ units.^{E2} The BEAS-2B cell line was a gift from Curtis Harris (National Cancer Institute, Bethesda, Md). Normal human lungs not used for transplantation were obtained from a tissue retrieval service (IIAM, Edison, NJ). HBEs were obtained by means of protease digestion of these normal human lungs, as previously described.^{E3} In brief, airways were dissected away from parenchyma and vessels and cut into segments. The airways were then incubated at 4°C in medium containing 1 mg/mL pronase for 36 hours. Airway segments were then cut longitudinally to expose the luminal surface of the airways, and epithelial cells were dislodged from the luminal surface by spraying the surface with medium delivered from a 5-mL syringe. Recovered cells were counted and frozen in aliquots for use in primary cultures. Cells were grown in 6-well plates in serum-free epithelial growth medium (BEGM; Lonza, Allendale, NJ). Before stimulation, cells were cultured overnight in BEGM from which hydrocortisone had been withdrawn, and this hydrocortisone-free medium was used for all experimental exposures.

Stimulation of epithelial cells

BEAS-2B cells were infected with 10^{4.5} TCID₅₀ units/mL (multiplicity of infection, ~0.1) of HRV-16, whereas HBEs were infected with 10^{5.5} TCID₅₀ units/mL (multiplicity of infection, approximately 1.0) HRV-16. The higher dose used in HBEs is needed to ensure robust responses because it has been reported that, even at high virus doses, no more than 10% of primary cells are infected.^{E4} Cells were exposed to HRV-16 in the absence or presence of PAPA NONOate (100–1000 μmol/L) and incubated for appropriate times at 34°C. Based on preliminary time-course experiments, CXCL10 mRNA expression from HRV-infected BEAS-2B cells was examined at 6 hours after infection, whereas supernatant was collected at 24 hours for assay of CXCL10 protein. For primary cells, supernatant and mRNA were examined 24 hours after infection. For studies using synthetic dsRNA, cells were transfected for 1 hour with 0.1 μg of poly I:C in the absence or presence of PAPA NONOate using FuGene6 in basal medium, as per the manufacturer's protocol. Cells were then washed, and fresh medium, with or without PAPA NONOate, was added. CXCL10 mRNA and protein expression were examined after a 7-hour incubation in both BEAS-2B cells and HBEs. Infected cells were also treated with 8-Bromo-cGMP (a cGMP analog) at concentrations ranging from 10 to 300

μmol/L to determine whether cGMP would mimic the effects of PAPA NONOate. To determine whether an inhibitor of soluble guanylyl cyclase could reverse the effects of PAPA NONOate in infected cells, cells were preincubated with vehicle or 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 3–30 μmol/L) for 30 minutes and then infected with HRV-16 in the presence of PAPA NONOate. Measurements of cGMP accumulation were performed in cells pretreated for 30 minutes with phosphodiesterase V inhibitor II (Calbiochem, San Diego, Calif). Samples were acetylated and assayed by using an enzyme immunoassay (Cayman), according to the manufacturer's instructions.

Assessment of CXCL10 protein and mRNA

Protein levels for CXCL10 were assayed by means of ELISA by using matched antibody pairs according to the manufacturer's protocol (R&D Systems). Treatment of cells with PAPA NONOate has no effect on the measurements of CXCL10 by means of ELISA. CXCL10 mRNA expression analysis was performed by means of real-time RT-PCR by using the Applied Biosystems model 7900 Sequence Detector. Input RNA (400 ng) was reverse-transcribed into cDNA, followed by PCR amplification in the presence of specific primers and probe (Applied Biosystems). Primer and probe sequences have been described previously.^{E5} Expression of the housekeeping gene GAPDH was also assayed with reagents obtained from Applied Biosystems. We previously confirmed that the efficiency of amplification for CXCL10 was comparable with that of GAPDH.^{E5} A first-strand cDNA standard of the amplicon incorporating the primer sequences was synthesized to permit absolute quantification (University of Calgary DNA Services, Calgary, Alberta, Canada). Data were expressed as attograms calculated from the standard curve after correction for minor variations in housekeeping gene levels. The sample with the lowest cycle threshold value (ie, highest input DNA) for GAPDH was selected as a reference to accomplish this. For all other samples, the difference in the number of cycles for GAPDH compared with this reference was calculated. Assuming 100% efficiency, a value of 2 to the power of the cycle difference for GAPDH was used to establish variations of input DNA. For example, a cycle difference of 0.5 cycles between a sample and the reference would indicate a 2^{0.5}-fold (1.41-fold) difference in input DNA, and this would be used to correct the CXCL10 level in attograms. In fact, in all experiments the maximum range of variability in GAPDH levels was on the order of 0.5 cycles.

CXCL10 promoter constructs

The 972-bp CXCL10 promoter–luciferase construct, corresponding to the sequence from –875 to +97 (relative to the transcriptional start site) of the 5'-flanking region of the human CXCL10 gene and the 376-bp truncated construct (sequence from –279 to +97) have been described previously.^{E5} Point mutations in the putative AP-1, κB1, κB2, and ISRE promoter sites were generated in the truncated construct by using standard site-directed mutagenesis techniques. Bold-face, lowercase characters in the following sequences denote mutation sites in forward primers: AP-1, 5'-CCAGCAGGTTTGTCTAAG **ata**ACTGTAATGC-3'; κB1 5'-GCAACATGtGACTTCaCCAGG-3'; κB2 5'-GCAGAGtGAAATTaCGTAACCTGG-3'; and ISRE 5'-GTTTGGGA **c**AGTGAcACCTAATTC-3'. Constructs with 5 copies of the κB1 recognition sequence (TGGGACTTCCCCA), κB2 sequence (GGGAAATTCCTG), or ISRE sequence (GGAAAGTGAACCTA) were synthesized. Constructs containing ISRE-κB1-ISRE-κB1 and ISRE-κB2-ISRE-κB2 were also synthesized. Successful generation of all constructs was confirmed by means of sequencing (University of Calgary DNA sequencing facility).

Transient transfection and luciferase assay

Subconfluent (40% to 50%) monolayers of BEAS-2B cells were transiently transfected with 1 μg of each CXCL10 promoter construct and 0.1 μg of pRL-null vector for 5 hours at 37°C by using FuGene6 in basal medium as per the manufacturer's protocol. After 5 hours, medium was aspirated, and cells were allowed to recover overnight in BEGM (without hydrocortisone) containing 5% FBS. The next day, cells were washed and exposed to 10⁵ TCID₅₀ units/mL of HRV-16 in the presence or absence of PAPA NONOate (500 μmol/L) at 34°C for 24 hours. For experiments using synthetic dsRNA, promoter transfection and overnight recovery were performed as above. Cells were

then transfected for 1 hour with 0.1 μ g of Poly I:C using FuGene6, washed, and incubated in fresh medium with or without 500 μ mol/L PAPA NONOate at 37°C for 7 hours. In each type of experiment, cells were then lysed in passive lysis buffer, followed by scraping, sonication, and centrifugation. Firefly and Renilla luciferase activity were measured with the dual-luciferase reporter assay system. Firefly luciferase activity was normalized to Renilla luciferase activity, and data were expressed as fold increase of stimulated cells over control. Within each experiment, results were averaged from triplicate wells.

EMSA

Nuclear extracts were performed by using a modification of a previously described method.^{E6} In brief, cells were scraped and centrifuged at 5000g for 5 minutes. The supernatant was removed, the pellet was resuspended in 50 μ L of Gough buffer (0.01 mol/L of Tris-HCl, 0.15 mol/L NaCl, 1.5 mmol/L $MgCl_2$, 0.65% Nonidet P-40, 0.5 mmol/L phenylmethylsulfonyl fluoride [PMSF], and 0.01 mol/L DDT), vortexed for 15 seconds, left on ice for 10 minutes, and centrifuged at 12,000g for 2 minutes. The supernatant was then saved (cytoplasmic fraction), and the pellet was resuspended in 15 μ L of Buffer C (0.02 mol/L HEPES [pH 7.9], 25% glycerol, 0.4 mol/L NaCl, 1.5 mmol/L $MgCl_2$, 0.5 mmol/L PMSF, and 0.01 mol/L DDT), subjected to agitation every 15 minutes for 2 hours, and centrifuged at 12,000g for 10 minutes. Samples were kept on ice during the whole procedure. The supernatant (nuclear fraction) was transferred to a fresh tube containing 35 μ L of Buffer D (0.02 mol/L HEPES [pH 7.9], 20% glycerol, 0.05 mol/L KCl, 0.2 mmol/L EDTA [pH 8.0], 0.5 mmol/L PMSF, and 0.01 mol/L DDT), and kept at $-80^\circ C$.

The probes were end-labeled with T4 polynucleotide kinase (New England BioLabs, Inc, Ontario, Canada) and [γ - ^{32}P]ATP (PerkinElmer, Boston, Mass). For binding reactions, 5 μ g of nuclear extracts was incubated with 4 μ L of binding buffer (20% glycerol, 5 mmol/L $MgCl_2$, 2.5 mmol/L EDTA, 1 μ g poly dI:dC, 250

mmol/L NaCl, 50 mmol/L Tris-HCl, and 0.01 mol/L DTT), and Buffer D was used to bring the mixture to 14 μ L. The samples were then incubated for 20 minutes, labeled probe was added, and samples were incubated for 1 hour. For supershift experiments, samples were incubated for 2 hours with appropriate antibodies before addition of the probe. Samples were fractionated on 6% acrylamide gels before vacuum drying and autoradiography. The forward oligonucleotide sequences used were as follows: NF- κ B1, 5'TGCAACATGGGACTTCCCCAGGAAC3'; NF- κ B2, 5'GGAGCAGAGGGAAATTCCGTAACCTT3'; and ISRE, 5' TGTTT TGGAAAGTGAAACCTAATTC.

REFERENCES

- E1. Gern JE, Vrtis R, Kelly EAB, Dick EC, Busse WW. Rhinovirus produces nonspecific activation of lymphocytes through a monocyte-dependent mechanism. *J Immunol* 1996;157:1605-12.
- E2. Sanders SP, Siekierski ES, Porter JD, Richards SM, Proud D. Nitric oxide inhibits rhinovirus-induced cytokine production and viral replication in a human respiratory epithelial cell line. *J Virol* 1998;72:934-42.
- E3. Churchill L, Chilton FH, Resau JH, Bascom R, Hubbard WC, Proud D. Cyclooxygenase metabolism of endogenous arachidonic acid by cultured human tracheal epithelial cells. *Am Rev Respir Dis* 1989;140:449-59.
- E4. Mosser AG, Brockman-Schneider R, Amineva S, Burchell L, Sedgewick JB, Busse WW, et al. Similar frequency of rhinovirus-infectable cells in upper and lower airway epithelium. *J Infect Dis* 2002;185:734-43.
- E5. Spurrell JCL, Wiehler S, Zaheer RS, Sanders SP, Proud D. Human airway epithelial cells produce IP-10 (CXCL10) in vitro and in vivo upon rhinovirus infection. *Am J Physiol Lung Cell Mol Physiol* 2005;289:L85-95.
- E6. Nasuhara Y, Adcock IM, Catley M, Barnes PJ, Newton R. Differential I κ B kinase activation and I κ B α degradation by interleukin-1 β and tumor necrosis factor- α in human U937 monocytic cells. Evidence for additional regulatory steps in κ B-dependent transcription. *J Biol Chem* 1999;274:19965-72.

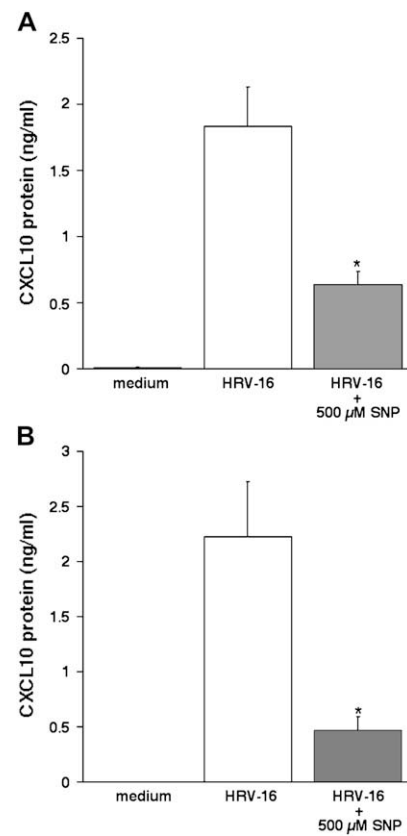


FIG E1. Sodium nitroprusside (SNP) inhibits HRV-16-induced epithelial production of CXCL10. **A**, BEAS-2B cells were exposed to HRV-16 in the presence or absence of 500 μ mol/L SNP, and CXCL10 protein was measured after 24 hours ($n = 5$). **B**, SNP also inhibited HRV-16-induced CXCL10 production from primary HBEs ($n = 5$).

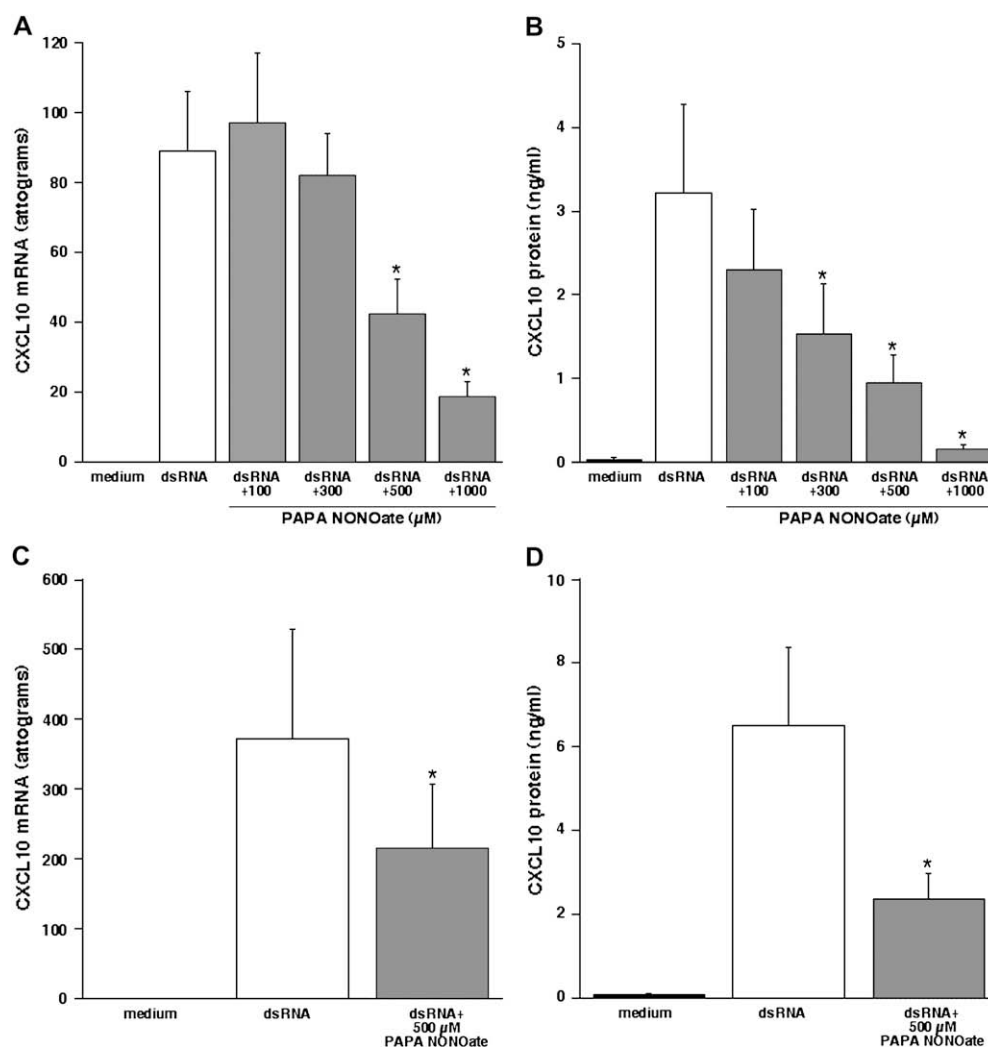


FIG E2. PAPA NONOate inhibits dsRNA-mediated induction of CXCL10 mRNA and protein in epithelial cells. **A and B,** In BEAS-2B cells ($n = 6$) induction of CXCL10 mRNA and protein was inhibited in a concentration-dependent manner ($P < .05$, ANOVA). Asterisks indicate significant inhibition compared with dsRNA alone by using appropriate *post-hoc* tests. **C and D,** PAPA NONOate (500 μ mol/L) also inhibits CXCL10 production in HBEs ($n = 8$).

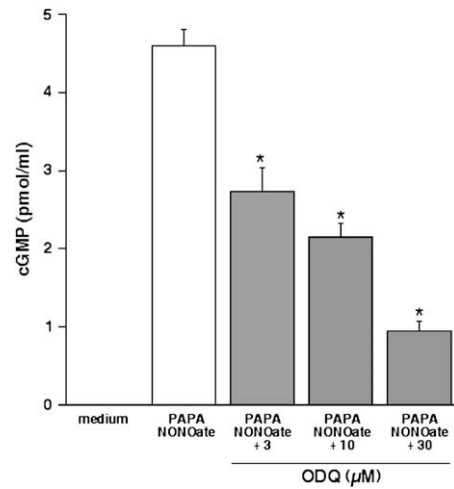


FIG E3. The inhibitor of soluble guanylyl cyclase, ODQ, dose-dependently inhibits PAPA NONOate-induced accumulation of cGMP in BEAS-2B cells. Cells pretreated with an inhibitor of phosphodiesterase V were exposed to PAPA NONOate (500 $\mu\text{mol/L}$) in the presence or absence of ODQ (3-30 $\mu\text{mol/L}$). Accumulation of cGMP was measured after 2 hours ($n = 4$).

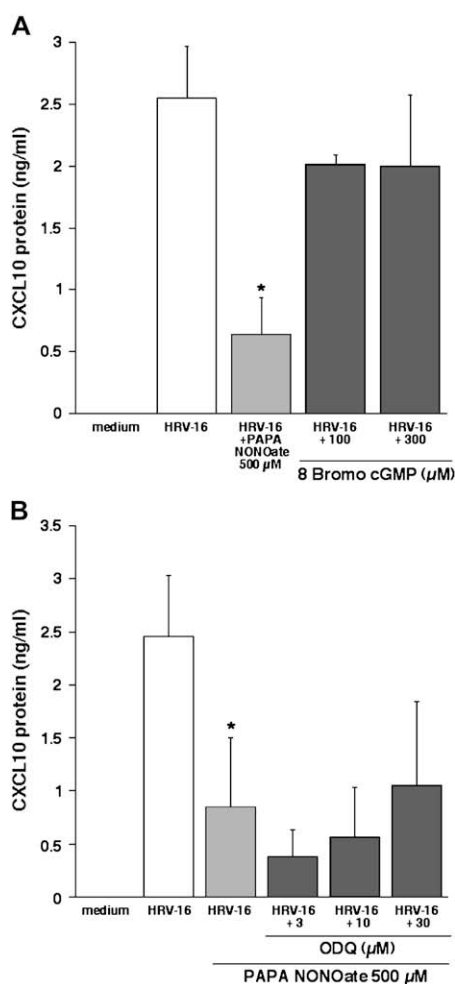


FIG E4. Inhibition of HRV-16-induced CXCL10 in HBEs is not mediated through cGMP. **A**, Exposure of primary HBEs to the cell-permeable analog 8-bromo-cGMP (100-300 $\mu\text{mol/L}$) did not mimic the ability of PAPA NONOate (500 $\mu\text{mol/L}$) to inhibit HRV-16-induced CXCL10 production ($n = 3$). **B**, Similarly, preincubation of primary HBEs with ODQ (3-30 $\mu\text{mol/L}$), an inhibitor of soluble guanylyl cyclase, did not reverse the PAPA NONOate-mediated inhibition of HRV-16-induced CXCL10 production ($n = 3$). Asterisks indicate significant inhibition compared to HRV-16 alone.

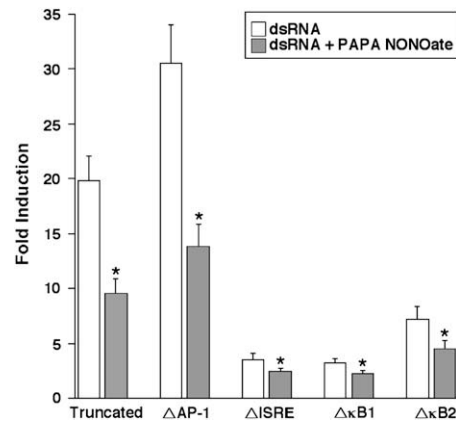


FIG E5. PAPA NONOate inhibits dsRNA-induced activation of CXCL10 promoter-luciferase constructs. PAPA NONOate inhibits dsRNA-induced activation of the 376-bp truncated construct. Mutation of individual NF- κ B and ISRE, but not AP-1, recognition sequences in the truncated promoter reduces dsRNA-induced promoter activation. Asterisks indicate significant inhibition by PAPA NONOate (solid bars) compared with dsRNA alone (open bars, $n = 6$).

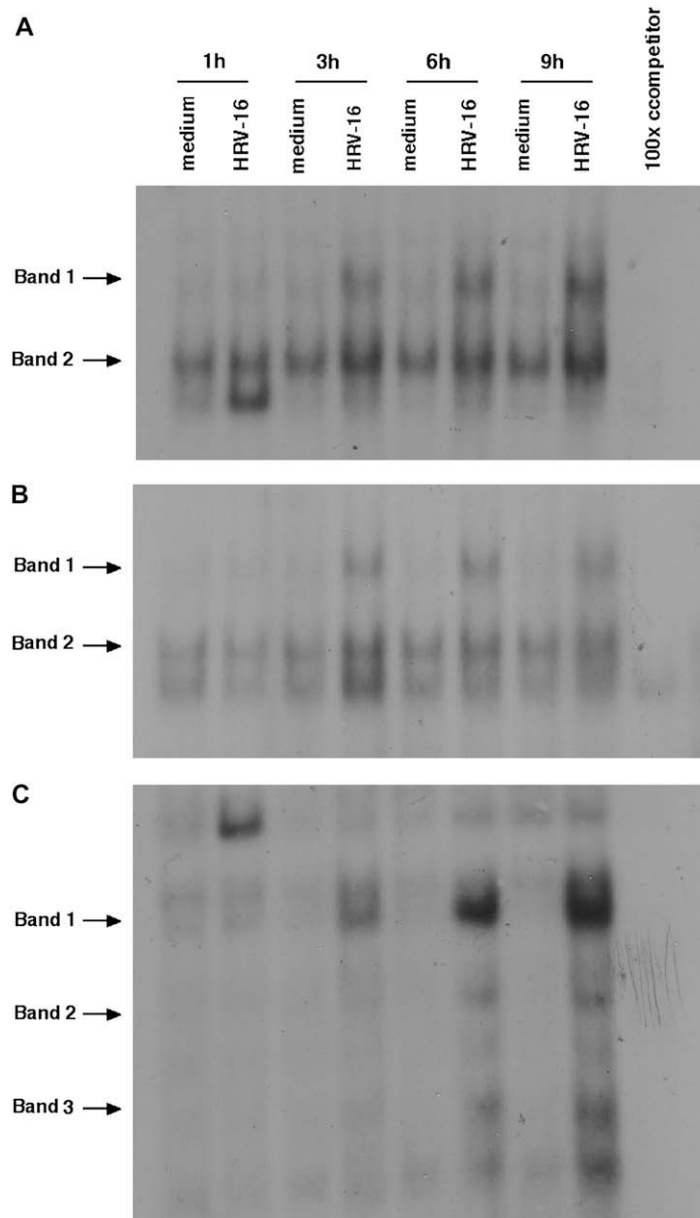


FIG E6. EMSA time-course experiments. Time courses of HRV-16-induced binding of nuclear proteins to oligonucleotides containing NF- κ B1 oligonucleotide (**A**), NF- κ B2 oligonucleotide (**B**), or ISRE oligonucleotide (**C**). Specific bands are shown by arrows. Data are representative of 3 experiments.

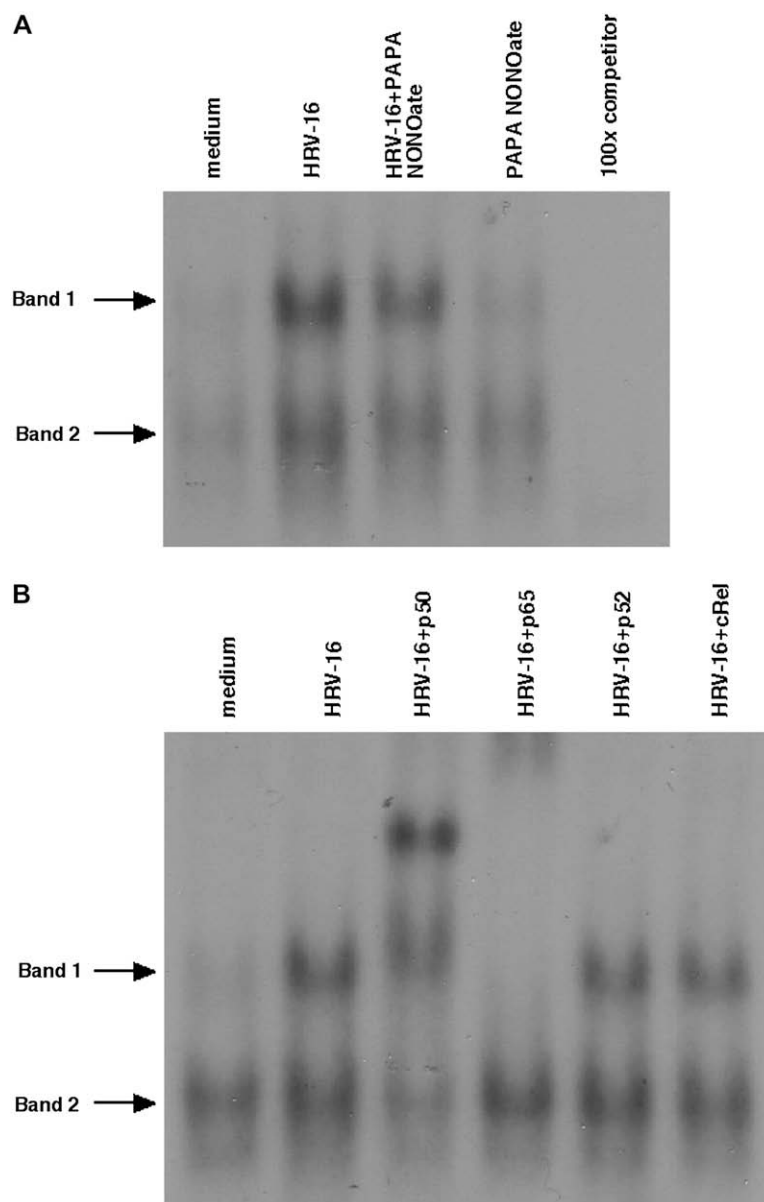


FIG E7. Effects of PAPA NONOate on nuclear translocation, binding, or both of NF- κ B in HRV-16-infected BEAS-2B cells. **A**, HRV-16 infection induced 2 bands on incubation of nuclear extracts with the NF- κ B2 recognition sequence. Induction of both bands was inhibited with PAPA NONOate. **B**, Supershift experiments demonstrate that antibody to p65 shifts only the upper band, whereas antibody to p50 shifts both bands. Antibodies to p52 and cRel were without effect. Data are representative of 3 experiments.