Nitric oxide inhibits IFN regulatory factor 1 and nuclear factor-κB pathways in rhinovirus-infected epithelial cells

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Background: Nitric oxide (NO) has previously been shown to inhibit human rhinovirus (HRV) replication in airway epithelial cells and to inhibit rhinovirus-induced epithelial cytokine and chemokine production independently of its effects on viral replication by modulating nuclear translocation and binding of transcription factors.

Objective: To define the molecular mechanisms by which NO inhibits HRV-16–induced epithelial production of CXCL10 by affecting nuclear translocation and binding of nuclear factor-κB (NF-κB) and IFN regulatory factor 1 (IRF-1).

Methods: Cultured human airway epithelial cells were infected with HRV-16 in the absence or presence of a NO donor, or were preincubated with 2 highly selective inhibitors of inhibitor of κB kinase (IKK)β and then infected with HRV-16. Effects on the NF-κB and IRF-1 pathways were examined by using electrophoretic mobility shift assays, Western blotting, and real-time RT-PCR.

Results: Nitric oxide directly inhibited the binding of both recombinant NF-κB p50 protein and recombinant IRF-1 to their recognition sequences from the CXCL10 promoter. NO also inhibited phosphorylation of the NF-κB inhibitor, IκBα, in HRV-16–infected cells. In addition, both NO and inhibitors of IKKβ inhibited viral induction of IRF-1 mRNA and protein.

Conclusions: Nitric oxide blocks rhinovirus-mediated activation and nuclear translocation of both NF-κB and IRF-1. NO also directly inhibits the binding of each of these transcription factors to their respective recognition sites in the CXCL10 promoter. In addition, the ability of HRV-16 to induce epithelial expression of IRF-1 is dependent, at least in part, on viral activation of NF-κB. (J Allergy Clin Immunol 2009;124:551-7.)

Key words: *Human rhinovirus, asthma, airway epithelial cells, CXCL10, nitric oxide, IFN regulatory factors, nuclear factor-κB*

Abbreviations used

COPD:	Chronic obstructive pulmonary disease
EMSA:	Electrophoretic mobility shift assay
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
HBEs:	Primary human bronchial epithelial cells
HRV:	Human rhinovirus
IκB:	Inhibitor of κB
IKK:	Inhibitor of κB kinase
iNOS:	Inducible nitric oxide synthase
IRF:	IFN regulatory factor
ISRE:	IFN-stimulated response element
NF-κB:	Nuclear factor-κB
NO:	Nitric oxide
PAPA NONOate:	3-(2-Hydroxy-2-nitroso-1-propylhydrazino)-1-propanamine
rh:	Recombinant human
TBK-1:	TRAF family member-associated NFκB activator binding kinase 1

Upper respiratory viral infections are associated with about 50% of exacerbations of chronic obstructive pulmonary disease (COPD) and trigger 50% to 85% of acute exacerbations of asthma.[1-3] In both diseases, human rhinovirus (HRV) is the dominant viral pathogen detected during exacerbations.[1-3] HRV primarily infects airway epithelial cells of both the upper and lower airways,[4] and triggers the production of a number of cytokines and chemokines that could exacerbate airway inflammation.[3,5,6] The molecular basis by which HRV infection induces many of these chemokines is not yet well defined.

Although HRV infection of the airway epithelium can trigger production of proinflammatory cytokines and chemokines, the epithelium also responds to HRV infection by producing a variety of antimicrobial molecules that could contribute to antiviral defenses. In addition to inducing production of proteins such as human β-defensin-2 and viperin,[7,8] HRV infection of epithelial cells induces expression of inducible nitric oxide synthase (iNOS) both *in vitro* and *in vivo*.[9] During an *in vivo* experimental HRV infection study, epithelial iNOS expression correlated with levels of exhaled nitric oxide (NO). Interestingly, subjects producing the greatest levels of exhaled NO in response to infection had lower symptom scores and appeared to clear virus more rapidly.[10] Further evidence supporting a role for NO in the host antiviral response to HRV infection is provided by observations that NO inhibits HRV replication in epithelial cells.[11,12] NO also inhibits HRV-induced epithelial production of a number of cytokines and chemokines.[11-13]

We have recently shown that inhibition of HRV-16–induced epithelial production of the chemokine CXCL10 by NO occurs independently of effects on viral replication, and is mediated, at least in part, at the level of transcription.[13] NO blocks CXCL10 production by inhibiting nuclear translocation and/or binding of nuclear factor-κB (NF-κB) and IFN regulatory factors (IRFs),

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including IRF-1, through a cyclic guanosine monophosphate–independent pathway.[13]

We now extend these studies by delineating the molecular mechanisms by which NO inhibits HRV-16–induced NF-κB and IRF-1–dependent CXCL10 transcription. We demonstrate that NO not only directly inhibits the ability of the NF-κB p50 (NF-κB1) protein to bind to its recognition sequence in the CXCL10 promoter but also reduces HRV-16–induced phosphorylation of inhibitor of κB (IκB) α. This latter effect would limit nuclear translocation of NF-κB.

We also provide the first demonstration that NO directly inhibits the ability of IRF-1 to bind to the IFN-stimulated response element (ISRE) in the CXCL10 promoter. Moreover, we demonstrate that NO inhibits HRV-16–induced expression of IRF-1 mRNA and protein. Finally, we show that HRV-16–induced expression of IRF-1 is also reduced by inhibitors of IκB kinase (IKK) β, demonstrating functional cross-talk between the NF-κB and IRF-1 pathways.

METHODS

Materials

The following reagents were purchased: Ham's F12 medium, Earle's minimal essential medium, HBSS, penicillin-streptomycin-amphotericin B, L-glutamine, TRIzol reagent, sodium pyruvate, nonessential amino acids, gentamicin, FBS, oligo(dT), Superscript III, and deoxyribonucleotide triphosphates (Invitrogen Life Technologies, Burlington, Ontario, Canada); Taqman master mix and 20X glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Applied Biosystems, 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 Chemical Co, Ann Arbor, Mich); Complete Mini (a protease inhibitor cocktail; Roche Applied Science, Indianapolis, Ind); NF-κB recombinant human (rh) p50 (Promega, Madison, Wis); and rhIRF-1 (ProSpec—Tany TechnoGene Ltd, Rehovot, Israel). The IKKβ-selective inhibitors PS-1145 (N-[6-chloro-9H-β-carbolin-8-ly] nicotinamide) and ML120B (N-[6-chloro-7-methoxy-9H-β-carbolin-8-ly]-2-methyl-nicotinamide) have been previously described,[14] and were provided as chemical-free bases by Millennium Pharmaceuticals (Cambridge, Mass). All other chemicals were purchased from Sigma-Aldrich (St Louis, Mo) unless stated otherwise.

Virus preparations and cell culture

Stocks of HRV-16 were propagated and purified as previously described.[11,13] Primary human epithelial cells (HBEs) were obtained from normal nontransplanted human lungs as previously described.[13,15] HBEs and the BEAS-2B human epithelial cell line were cultured as described previously.[13]

Stimulation of epithelial cells

Before stimulation, cells were cultured overnight in bronchial epithelium cell growth medium from which hydrocortisone had been withdrawn. BEAS-2B cells were infected with $10^{4.5}$ 50% tissue culture infective dose units/mL (multiplicity of infection ~ 0.1) of HRV-16, whereas HBEs were infected with $10^{5.5}$ 50% tissue culture infective dose units/mL (multiplicity of infection ~ 1.0) of HRV-16. IKKβ inhibitors were dissolved in dimethyl sulfoxide, diluted in cell culture medium, and preincubated with BEAS-2B cells or HBEs for 90 minutes before the addition of HRV-16. Cells were then incubated with HRV-16 in the presence of the inhibitors for a further 24 hours. Supernatants were collected for assay of CXCL10 protein. For IRF-1 experiments, cells were preincubated with PS-1145 or ML120B for 90 minutes and then exposed to HRV-16, or cells were infected with HRV-16 in the presence or absence of the NO donor PAPA NONOate at 500 μmol/L. IRF-1 mRNA and protein expression was examined after a 6-hour incubation in BEAS-2B cells. Neither PAPA NONOate nor the selective IKKβ inhibitors affected epithelial cell viability at the concentrations used, as assessed by means of lactate dehydrogenase release (data not shown).

ELISA

Protein levels for CXCL10 were assayed, according to the manufacturer's protocol(R&D Systems), by ELISA using matched antibody pairs. The 2 IKKβ inhibitors did not interfere in the CXCL10 ELISA.

Real-time RT-PCR

Gene expression for IRF-1 and the housekeeping gene GAPDH was quantified using the Applied Biosystems model 7900 Sequence Detector with the primer and probe gene expression assay kits available from Applied Biosystems. Samples were treated with DNase I (Ambion, Austin, Tex) before use to minimize the contribution of genomic DNA to amplification. Briefly, 1 μg RNA was reverse-transcribed with oligo(dT) and Superscript III, and 1 μL cDNA was used as the template in the real time PCR reaction. We confirmed that the efficiency of IRF-1 amplification was comparable to that of GAPDH (~100%), using previously described methods,[16] allowing us to assess fold induction of genes data by using the ΔΔCt method as previously described.[9]

Western blotting

For experiments using the selective IKKβ inhibitors, cells were preincubated in basal medium and then incubated with PS-1145 or ML120B for 90 minutes before infection with HRV-16. Alternatively, cells were infected with HRV-16 in the presence or absence of the PAPA NONOate at 500 μmol/L. After 6 hours, supernatants were removed, and cells were washed with HBSS and lysed in ice-cold lysis buffer (1% Triton X-100 in 1x MBS [pH 7.4], 1x Complete Mini [a protease inhibitor cocktail], 1 mmol/L phenylmethylsulfonyl fluoride, 2 mmol/L sodium vanadate, 20 mmol/L sodium pyrophosphate, and 50 mmol/L sodium fluoride) by scraping. The lysate was then incubated on ice for 10 minutes, sonicated, and centrifuged. Protein concentrations for the Triton X-100–soluble lysates were quantified with a DC Protein Assay (BioRad, Montreal, Quebec, Canada). Equivalent amounts of protein (20 μg per lane) were separated by means of SDS-PAGE, and proteins were transferred to a nitrocellulose membrane. Membranes were blocked with 5% skimmed milk for 1 hour and probed with a 1:2000 dilution of anti–IRF-1 antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif) overnight at 4°C. Membranes were then washed and incubated for 1 hour with a 1:2000 dilution of horseradish peroxidase–conjugated antirabbit immunoglobin. Proteins were visualized with enhanced chemiluminescent substrate reagent (Amersham—GE Healthcare Bio-Sciences Inc, Baie d'Urfe, Quebec, Canada). Each membrane was stripped and reprobed with an antibody against GAPDH (AbD Serotec, Raleigh, NC) to control for variations in loading. Lysates from BEAS-2B cells as well as HBEs infected with HRV-16, treated with PAPA NONOate, and incubated for 3 hours, were also used, and membranes were probed with a 1:1000 dilution of antibody to serine 32/36 phosphorylated IκBα (Cell Signaling Technology, Danvers, Mass).

Electrophoretic mobility shift assay

Nuclear extracts were prepared as previously described.[13] We have previously shown that 2 recognition sequences for NF-κB, referred to as κB1 and κB2, as well as an ISRE are important both for rhinovirus-induced drive of the CXCL10 promoter and for the inhibitory effects of NO.[13] The following oligonucleotides containing recognition sequences of κB1: 5′TGCAACA TGGGACTTCCCCAGGAAC3′; of κB2: 5′GGAGC AGAGGGAAATTC CGTAACTT3′; and of the ISRE: 5′TGTTTTGGAAAGTGAAA CCTAA TTC3′ sites in the CXCL10 promoter were made. Oligonucleotides end-labeled with T4 polynucleotide kinase (New England BioLabs Inc, Ontario, Canada) and [γ-^{32}P]ATP (PerkinElmer, Boston, Mass). For binding reactions,

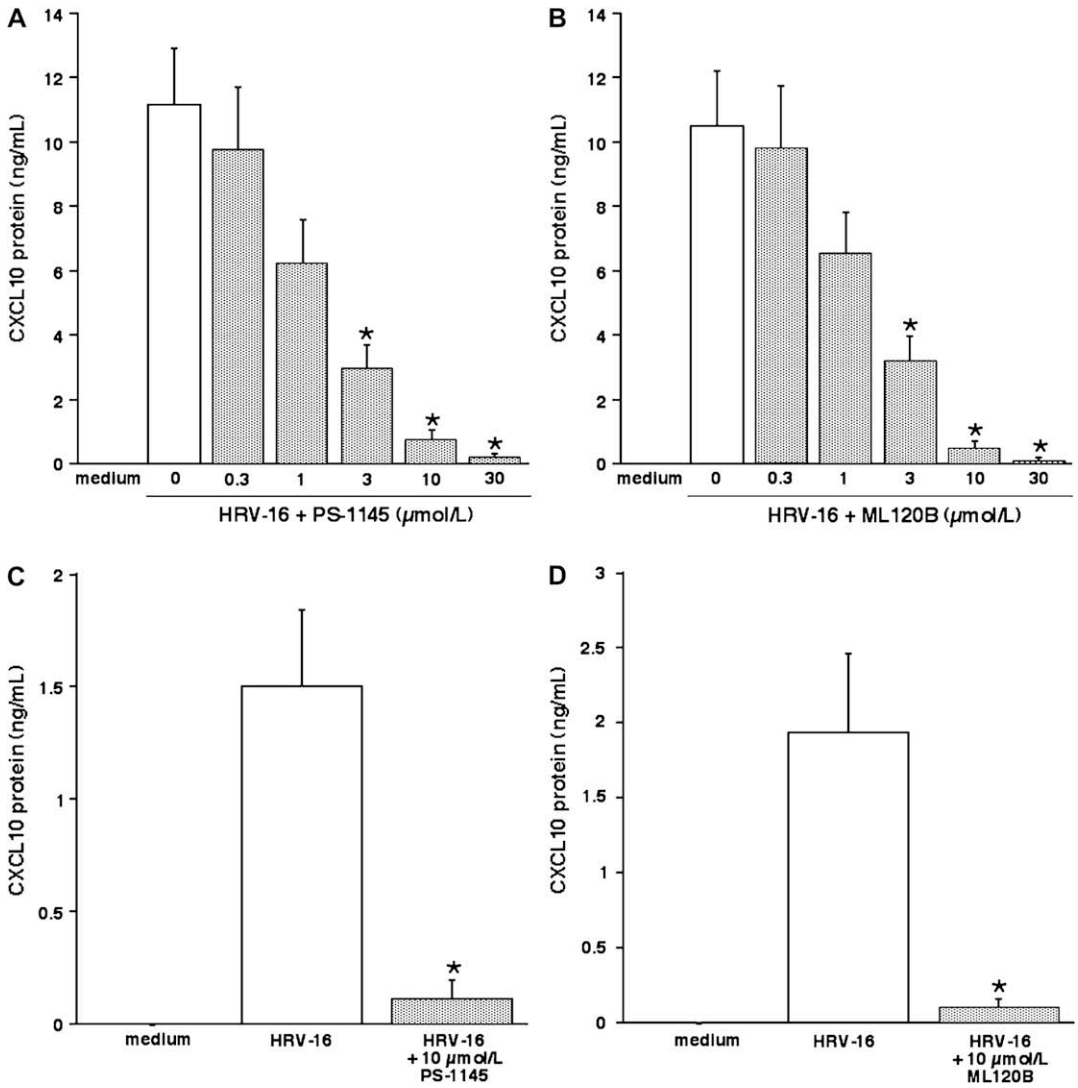

FIG 1. Selective inhibitors of IKKβ inhibit HRV-16–induced production of CXCL10 from airway epithelial cells. Both PS-1145 **(A)** and ML120B **(B)** inhibit, in a concentration-dependent manner, HRV-induced production of CXCL10 from BEAS-2B epithelial cells (n = 6). PS-1145 **(C)** and ML120B **(D)**, at a concentration of 10 μmol/L, also inhibit HRV-16–induced CXCL10 production from primary cultures of HBEs (n = 3). *Statistical significance compared with HRV-16 alone.

5 μg nuclear extracts were incubated with 4 μL binding buffer (20% glycerol, 5 mmol/L $MgCl_2$, 2.5 mmol/L EDTA, 1 μg poly dI:dC, 250 mmol/L NaCl, 50 mmol/L TRIS-HCl and 0.01 mol/L dithiothreitol), and 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 phenymethylsulfonyl fluoride, and 0.01 mol/L DTT) was used to bring the mixture to 14 μL. The samples were then incubated for 20 minutes, labeled probe was added, and samples were incubated at 4°C for 1 hour. Samples were fractionated on 6% acrylamide gels before vacuum drying and autoradiography.

Statistical analysis

For normally distributed data, between-group comparisons were made by means of 1-way ANOVA, with appropriate *post hoc* analysis using the Student-Newman-Keuls method. The Student *t* test was also performed when appropriate. For nonparametric data, Kruskal-Wallis ANOVA was used, followed by the Wilcoxon matched-pairs signed-rank test. The Mann-Whitney *U* test was also performed when applicable. *P* values of less than .05 were considered significant.

RESULTS

Effect of PS-1145 and ML120B on HRV-induced CXCL10 production in airway epithelial cells

Because viruses have been reported to activate not only the canonical NF-κB pathway but also the IKKε/TANK binding kinase 1 (TBK-1) pathway, we used 2 highly selective inhibitors to determine the role of the canonical pathway. Both PS-1145 and ML120B have been shown to be selective for IKKβ with little or no effect on IKKε.[17,18] Both drugs significantly inhibited HRV-16–induced CXCL10 protein production from BEAS-2B cells in a concentration-dependent manner (Fig 1, *A and B*). At the higher concentrations, HRV-16–induced CXCL10 production was almost completely inhibited. Similarly, when the 2 inhibitors were used at 10 μmol/L in HRV-16–infected primary HBEs, almost complete inhibition of CXCL10 protein release was observed with both compounds (Fig 1, *C and D*). Thus, further studies focused on the canonical NF-κB pathway.

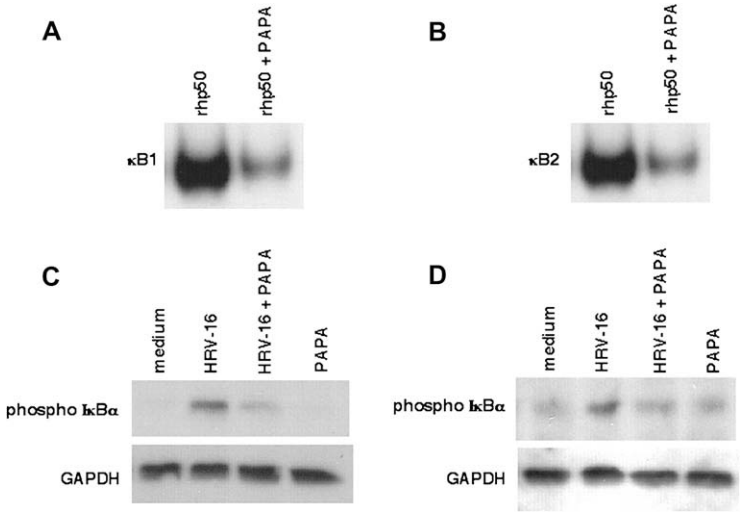

FIG 2. PAPA NONOate inhibits NF-κB DNA binding and IκBα phosphorylation. Preincubation of rh p50 for 90 minutes with 500 μmol/L of PAPA NONOate inhibited its ability to bind to either the κB1 **(A)** or κB2 **(B)** recognition sequences from the CXCL10 promoter. PAPA NONOate also prevented the HRV-16–induced phosphorylation of IκBα observed 3 hours after infection of BEAS-2B **(C)** or HBEs **(D)**. All experiments are representative of $n \geq 3$.

Effects of NO on the NF-κB pathway

To determine whether NO directly modified binding of NF-κB to the recognition sequences in the CXCL10 promoter, 10 ng rh p50 was incubated in the presence or absence of PAPA NONOate (500 μmol/L) for 90 minutes, and then the ability of the protein to bind to oligonucleotides containing either the κB1 or κB2 sites in the CXCL10 promoter was examined by electrophoretic mobility shift assay (EMSA). In the absence of PAPA NONOate, rh p50 bound strongly when incubated with either the κB1 and κB2 oligonucleotides (Fig 2, *A and B*). Marked inhibition of binding was observed, however, after incubation with PAPA NONOate (Fig 2, *A and B*).

Phosphorylation of IκBα targets this protein for subsequent ubiquitination and degradation, permitting translocation of NF-κB to the nucleus. To determine whether NO also regulates nuclear translocation of NF-κB, therefore, Western blotting studies using phospho-specific anti-IκBα antibody were performed. On the basis of earlier time course studies showing that HRV-induced IκBα phosphorylation peaks at 3 hours,[19] this time point was selected for study. PAPA NONOate markedly inhibited HRV-induced IκBα phosphorylation at 3 hours (Fig 2, *C*). Similar results were seen in primary HBEs (Fig 2, *D*).

Effect of NO on the IRF-1 pathway

We recently provided the first demonstration that NO also inhibits HRV-16–induced production of CXCL10 by inhibiting nuclear translocation and/or binding of IRFs, including IRF-1.[13] To determine whether binding of IRF-1 to the ISRE recognition sequence in the CXCL10 promoter is directly affected by NO, 10 ng rhIRF-1 was incubated in the presence or absence of PAPA NONOate for 90 minutes, and the ability to bind to an oligonucleotide containing the CXCL10 ISRE sequence was evaluated by EMSA. In the absence of the NO donor, a strong band was observed, indicating IRF-1 binding. A substantial inhibition of this band was seen, however, when IRF-1 protein was incubated with PAPA NONOate (Fig 3, *A*).

Initial time course experiments demonstrated that HRV-16 infection induced expression of IRF-1 in BEAS-2B cells, with both IRF-1 mRNA and protein expression peaking at about 6 hours (data not shown). This time course of induction corresponds with the peak of IRF-1 DNA binding observed in our earlier EMSA studies.[13] To determine whether NO can also modulate IRF-1 induction, cells were exposed to HRV-16 in the presence or absence of PAPA NONOate, and cellular levels of IRF-1 mRNA and protein were examined 6 hours later. In cells exposed to PAPA NONOate, significant inhibition of HRV-16–induced IRF-1 mRNA expression (Fig 3, *B*) and protein production (Fig 3, *C*) was observed.

Inhibition of HRV-16–induced IRF-1 expression by the selective IKKβ inhibitors PS-1145 and ML120B

It is established that IRFs and NF-κB can cooperate to regulate the transcription of genes, including CCL5 and CXCL10.[13,20,21] There is also a precedent for NF-κB to play a role in the regulation of IRF-1 expression,[22] but this has never been examined in the setting of HRV infection. To determine whether NF-κB plays a role in HRV-16–induced epithelial expression of IRF-1, epithelial cells were preincubated either with the IKKβ inhibitors PS-1145 or ML120B (10 μmol/L), or with vehicle control, and HRV-induced expression of IRF-1 mRNA and protein was examined. PS-1145 significantly inhibited HRV-16–induced epithelial IRF-1 mRNA expression (Fig 4, *A*). In a more limited number of experiments, ML120B also consistently inhibited HRV-16–induced IRF-1 mRNA expression, but this did not reach statistical significance (Fig 4, *B*). In Western blotting experiments, both PS-1145 and ML120B consistently inhibited HRV-16–induced epithelial expression of IRF-1 protein (Fig 4, *C*).

Selective IKKβ inhibitors inhibit nuclear translocation and/or binding of IRF-1 in HRV-infected cells

To determine whether reduced IRF-1 expression observed in the presence of IKKβ inhibitors is associated with reduced nuclear translocation and binding of IRF-1, epithelial cells were

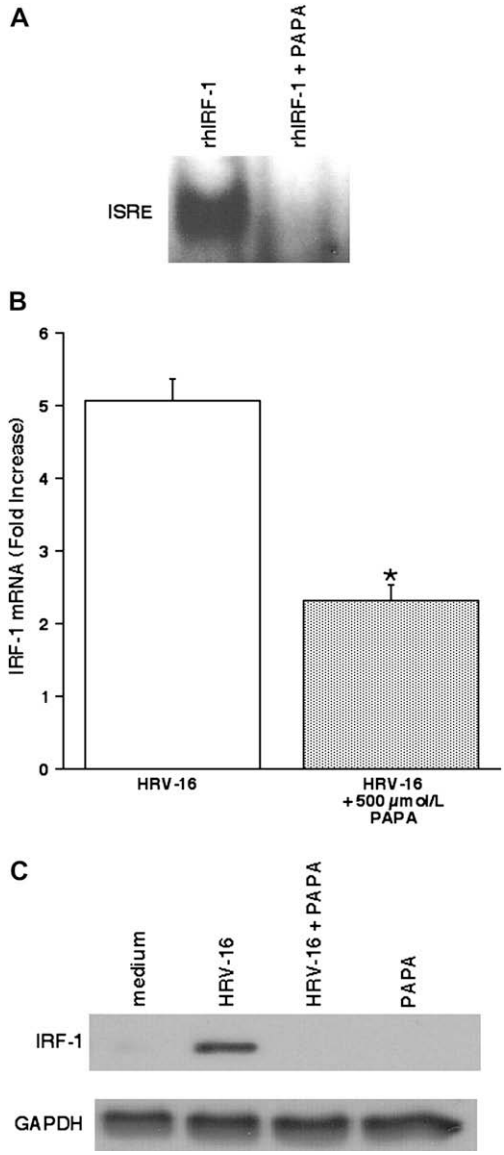

FIG 3. PAPA NONOate inhibits IRF-1 DNA binding and HRV-16–induced IRF-1 expression. **A,** Preincubation of rhIRF-1 for 90 minutes with 500 μmol/L of PAPA NONOate inhibits its ability to bind to the ISRE recognition sequence in the CXCL10 promoter (representative of n = 3). **B,** PAPA NONOate inhibits HRV-16–induced epithelial increases in IRF-1 mRNA in BEAS-2B cells (n = 9). *Statistical significance compared to HRV-16 alone. **C,** PAPA NONOate also inhibits HRV-16–induced expression of IRF-1 protein (representative of n = 3).

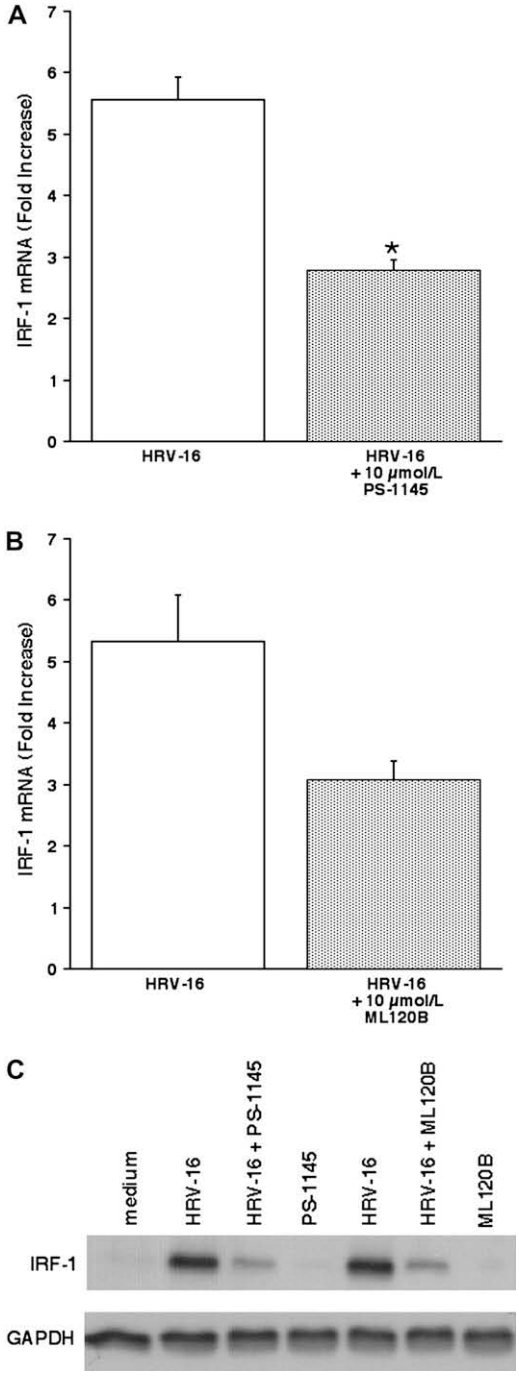

FIG 4. Reduction of HRV-16–induced epithelial expression of IRF-1 by selective inhibitors of IKKβ. Both PS-1145 (**A;** n = 6) and ML120B (**B;** n = 3) inhibit HRV-16–induced increases in IRF-1 mRNA in BEAS-2B cells. Asterisk indicates statistical significance compared to HRV-16 alone. **(C)** PS-1145 and ML120B partially inhibit HRV-16–induced expression of IRF-1 protein (representative of n = 3).

preincubated for 90 minutes with PS-1145 or ML120B (10 μmol/L) and then infected with HRV-16. After 6 hours, nuclear extracts were prepared and used for EMSA studies. As we previously reported, incubation of nuclear proteins from HRV-16–infected BEAS-2B cells with the ISRE oligonucleotide led to formation of 3 specific bands. The uppermost band can be completely shifted by using an antibody to IRF-1, whereas the identity of the protein components of the lower 2 bands have not yet been identified.[13] In cells incubated in the presence of PS-1145 or ML120B, formation of all 3 bands was inhibited (Fig 5, *A*). This was not a direct effect of the inhibitors on the ability of proteins to bind to the oligonucleotide, because preincubation of rhIRF-1 with the inhibitors did not affect complex formation with the recombinant protein (Fig 5, *B*).

DISCUSSION

We have recently shown that NO inhibits HRV-16–induced CXCL10 gene transcription by regulating nuclear translocation,

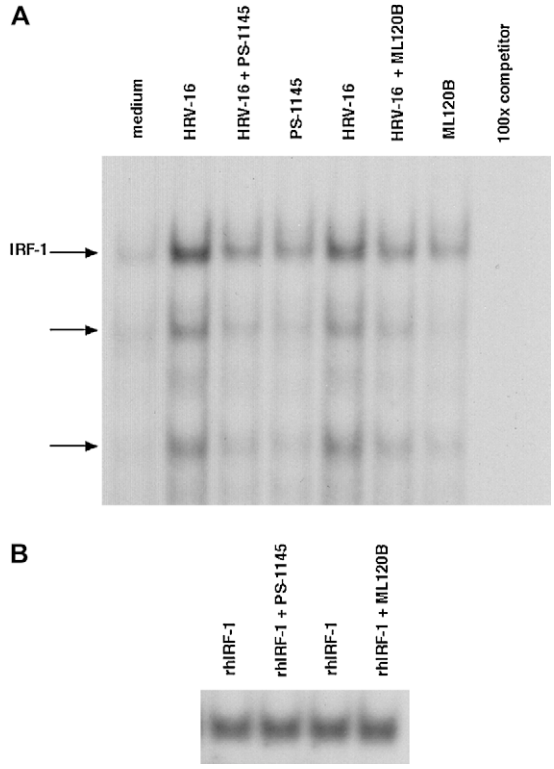

FIG 5. Reduced nuclear translocation and binding of IRF-1 in HRV-16–infected epithelial cells treated with selective inhibitors of IKKβ. **A,** Both PS-1145 and ML120B inhibited HRV-16–induced nuclear translocation and binding of IRF-1 in BEAS-2B cells (representative of n = 3). **B,** Neither inhibitor directly affected binding of rhIRF-1 to the ISRE recognition sequence from the CXCL10 promoter (representative of n = 3).

binding, or both, of both NF-κB and IRFs, including IRF-1, through a cyclic guanosine monophosphate–independent pathway.[13] We have now extended our studies to delineate further the mechanisms involved in the regulation of these transcription factor pathways.

Activation of the NF-κB pathway can occur via the canonical pathway involving activation of the IKK complex, or via activation of the IKK-related kinases, IKKε and TBK-1. Both pathways are known to be activated by viruses.[23] To determine the relative role of these activation pathways in rhinovirus-induced production of CXCL10, we used 2 structurally distinct inhibitors of IKKβ. The dose-dependent, virtually complete inhibition of CXCL10 production by these inhibitors clearly demonstrated that the canonical, IKK-dependent activation pathway plays an essential role in HRV-induced production of CXCL10 in both primary HBEs and BEAS-2B cells. This is consistent with a recent report that another inhibitor of IKKβ blocked HRV-induced production of CXCL8 from BEAS-2B cells.[24]

To evaluate whether NO could alter binding of NF-κB to recognition sequences derived from the CXCL10 promoter, rh p50 protein was incubated with PAPA NONOate, and the ability to form complexes with oligonucleotides containing either the κB1 or κB2 recognition sequences from the CXCL10 promoter was evaluated. The marked inhibition of binding to both the κB1 and κB2 recognition sequences observed is consistent with an earlier report that NO can reduce DNA binding by direct S-nitrosylation of the Cys-62 residue of p50.[25]

However, the ability of NO to reduce DNA binding of NF-κB does not exclude the possibility that HRV-induced activation of the NF-κB pathway could also, and independently, be blunted by NO. NF-κB is retained in the cytoplasm as a complex with the inhibitory protein IκBα. Phosphorylation of IκBα is a key step in the activation pathway, because this targets IκBα for ubiquitination and proteasomal degradation, permitting NF-κB to translocate to the nucleus and participate in gene transcription. Our data showing that increased IκBα phosphorylation induced on HRV-16 infection is inhibited by PAPA NONOate clearly demonstrate that NO inhibits HRV-induced activation and nuclear translocation of NF-κB. Inhibition of HRV-induced IκBα phosphorylation could be mediated via the previously reported ability of NO to inhibit the activity of IKKβ,[26] and/or via effects on upstream kinases that induce phosphorylation of the IKK complex. Regardless, our data show that NO inhibits both nuclear translocation and DNA binding of NF-κB to the CXCL10 promoter. These effects on the NF-κB pathway likely contribute to the demonstrated ability of NO to inhibit HRV-induced epithelial production of a number of NF-κB–dependent cytokines, including GM-CSF, CXCL8, and IL-6.[11,12]

Although there is literature precedent for NO to affect the NF-κB pathway, to our knowledge, our data represent the first delineation of the effects of NO on modulating HRV-induced effects on the IRF-1 pathway. We show for the first time that NO can directly inhibit binding of IRF-1 to the ISRE recognition sequence from the CXCL10 promoter, presumably via S-nitrosylation of IRF-1. In addition, we provide the first demonstration that NO inhibits HRV-induced IRF-1 mRNA and protein expression in epithelial cells. Given that NO can inhibit replication of HRV in epithelial cells,[11] the possibility exists that the effects of NO on inhibiting HRV-induced expression of IRF-1 may be mediated to some degree by reductions in viral replication. Regardless, it is clear that NO can inhibit induction of IRF-1 protein as well as the DNA-binding capacity of this transcription factor.

Although induction of IRF-1 expression by type I and type II IFNs is known to be mediated largely via Janus kinase–signal transducer and activator of transcription signaling,[27] there also is a precedent for NF-κB to play a role in regulation of IRF-1 expression induced by other stimuli.[22,27] Our data showing that PS-1145 and ML120B both reduced, but did not totally eliminate, HRV-16–induced IRF-1 mRNA and protein expression demonstrate for the first time that HRV-induced IRF-1 expression is partially dependent on NF-κB activation.

It must be noted that both NO donors and inhibitors of IKKβ appeared to be more effective at inhibiting IRF-1 protein expression compared with mRNA levels. It is known that NO can modify protein-protein interactions via S-nitrosylation, so it is conceivable that NO donors modify antigenic epitopes of IRF-1 limiting detection on Western blots. This seems unlikely also to be the case with inhibitors of IKKβ. Other possibilities include the fact that IRF-1 protein expression may also be regulated by posttranscriptional mechanisms that are susceptible to regulation via NO and by the NF-κB pathway. Alternatively, interventions may be more readily able to reduce protein levels below the limit of detection of Western blotting while still permitting detection of mRNA by more sensitive real-time RT-PCR techniques. Ultimately, however, it is the loss of IRF-1 protein that is important in terms of transcriptional regulation.

We also must acknowledge potential limitations of our studies. Our current protocols have examined the effects of NO

on transcription factor pathways at only a single optimal time point selected on the basis of initial time-course studies. It is possible, therefore, that responses may not be permanently inhibited but rather delayed. In our *in vitro* model, however, it is not feasible to assess this because PAPA NONOate loses its NO content with time, so that any delayed response may be a result of the loss of NO from the system. In addition, our experiments rely on the use of pharmacologic inhibitors of IKKβ. The issue of the specificity of such compounds is always a concern. The particular compounds used, however, have been studied quite extensively in this regard.[17,18] Moreover, we have tried to ensure specificity of responses by using 2 structurally discrete compounds.

In summary, NO appears to be an important component of the host defense against HRV infection. Not only can NO inhibit replication of HRV in epithelial cells, but independent of these effects on HRV replication, NO can also inhibit HRV-induced generation of a variety of cytokines and chemokines that could contribute to an overexuberant inflammatory response that may trigger exacerbations of lower airway disease. We establish that NO can block HRV-induced generation of chemokines, such as CXCL10, not only by inhibiting activation and nuclear translocation of NF-κB but also by directly inhibiting the ability of NF-κB to bind to recognition sequences in the gene promoter. In addition, for the first time, we show that NO not only directly inhibits binding of IRF-1 to the CXCL10 promoter but also blocks induction of IRF-1 in HRV-infected epithelial cells. This latter effect is mediated in part by blocking NF-κB-mediated induction of IRF-1. Interestingly, NF-κB and IRF-1 are involved in transcriptional activation of iNOS,[28,29] so the ability of NO to block activation and binding of the transcription factors may serve as a negative feedback loop that may limit the antiviral effects of endogenous NO. It is feasible, therefore, that topical administration of NO, or of NO donors, may be of benefit in supplementing endogenous antiviral defenses during HRV-induced exacerbations of COPD and asthma.

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

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