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Rhinovirus-Induced MMP-9 Expression Is Dependent on Fra-1, Which Is Modulated by Formoterol and Dexamethasone

Claire E. Tacon,*† Robert Newton,‡ David Proud,† and Richard Leigh*†

Matrix metalloproteinase-9 is implicated in airway inflammation and airway remodeling in asthma. We have previously confirmed that human rhinovirus-16 (HRV-16) infection increases MMP-9 expression both in vivo and in vitro. However, the role of the AP-1 sites within the MMP-9 promoter and the effect of commonly used asthma pharmacotherapies in modulating human rhinovirus (HRV)-induced MMP-9 production have not yet been elucidated. Experiments were performed in vitro in the human bronchial epithelial (HBE) cell line BEAS-2B and in primary HBE cells obtained from non-transplanted lungs. Using site-directed mutagenesis approaches, AP-1 sites were found to be necessary for HRV-induced MMP-9 promoter drive. EMSAs and supershift assays identified complexes consisting of Fos-related Ag-1 (Fra-1) in addition to other AP-1 subunits. Small interfering RNA approaches indicated that Fra-1 was induced upon HRV-16 infection in BEAS-2B cells and was necessary for MMP-9 expression in both BEAS-2B and primary HBE cells. Inhibition of MEK1/2 activity using PD98059 and U0126 reduced Fra-1 expression, DNA binding, MMP-9 promoter drive, and MMP-9 protein production. The long-acting β2-agonist formoterol and the glucocorticoid dexamethasone significantly reduced HRV-induced ERK phosphorylation, Fra-1, and MMP-9 expression in BEAS-2B cells. These data indicate that HRV-induced activation of the MEK/ERK MAPK pathway and Fra-1 expression are necessary for the upregulation of MMP-9 and can be modulated by two distinct but commonly used asthma pharmacotherapies. Together, these results offer insights into the mechanisms by which long-acting β2-agonists and glucocorticoids might reduce HRV-related asthma exacerbations. The Journal of Immunology, 2012, 188: 4621–4630.

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Abbreviations used in this article: BEBM, bronchial epithelial basa l medium; BEGM, bronchial epithelial growth medium; Fra-1, Fos-related Ag-1; HBE, human bronchial epithelial; HRV, human rhinovirus; HRV-16, human rhinovirus-16; ICS, inhaled corticosteroid; LABA, long-acting β2-adrenoceptor agonist; siRNA, small interfering RNA; TCID50, 50% tissue culture infective dose; TRE, 12-O-tetradecanoylphorbol-13-acetate response element.

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Materials and Methods

Reagents and Abs
HBSS, FBS, OptiMEM serum-free medium, DTT, and RNAi Max lipid transfection reagent were from Invitrogen Life Technologies (Burlington, ON, Canada), protease inhibitor mixture tablets were from Roche (Mississauga, ON, Canada), the pharmacological inhibitors PD98059 and U0126 were from Calbiochem EMD Biosciences (Gibbstown, NJ), Abs against
phospho-ERK1/2 (no. 9101), total ERK1/2 (no. 9120), and phospho-IκBα (no. 9246) were from Cell Signaling Technology (Beverly, MA). GAPDH Ab was from AbD Serotec (Raleigh, NC). HRP-conjugated anti-rabbit Ig Ab was from GE Healthcare Bio-Sciences (Piscataway, NJ). HRP-conjugated anti-mouse Ig Ab was from Jackson ImmunoResearch Laboratories (West Grove, PA), and Abs used in supershift experiments for Fos-related Ag-1 (Fra-1) (sc-22794X), c-Fos (sc-52X), c-Jun (sc-79X), ATF-2 (sc-187X), CREB-1 (sc-186X), and CREB-2 (sc-200X) were from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals and pharmacochemical compounds were purchased from Sigma-Aldrich (St. Louis, MO).

Cell culture and viral infection

The BEAS-2B cell line was a gift from Dr. Curtis Harris (National Cancer Institute, Bethesda, MD), and primary human bronchial epithelial (HBE) cells were obtained by protease digestion of non-transplanted normal human lung, as described previously (24). BEAS-2B and primary HBE cells were cultured as previously described (13). Cells were pretreated in bronchial epithelial basal medium (BEBM) prior to 2-h pretreatment with formoterol, forskolin, dexamethasone, or fluticasone propionate and 1-h pretreatment with the MEK inhibitors PD98059 or U0126, followed by human rhinovirus-16 (HRV-16) infection. In the case of the β2-receptor antagonist ICI 118551, cells were pretreated for 1.5 h before addition of formoterol. Propagation and purification of HRV-16 was performed as previously described (25, 26). BEAS-2B cells were infected with 10^3.5 50% tissue culture infective dose (TCID₅₀) U/ml and 10^3.5 TCID₅₀ U/ml used to infect primary cells, as approximately only 10% of primary cells are infected even with high doses of HRV (13, 27). The BEAS-2B cell line has previously been shown to be a robust model system in which to study the regulation of HRV-induced MMP-9 expression (13). The BEAS-2B cell line was therefore used for the majority of experiments, and the major findings were confirmed in primary HBE cells.

Determination of mRNA and protein expression using real-time PCR, ELISA, and Western blotting

MMP-9 mRNA and protein expression were analyzed using real-time PCR or ELISA, as previously described (13). Whole-cell lysates were collected in lysis buffer (1% Triton X-100 in 1× MES buffered saline, 5 mM EDTA pH 7.4, anti-protease tablets, 2 mM NaVO₄, 20 mM NaF, 50 mM NaF, and 1 mM phenylmethylsulfonyl fluoride), followed by sonication and centrifugation. Lysates were assayed for protein concentration using a DC Protein Assay (Bio-Rad Laboratories, Mississauga, ON, Canada). Equivalent amounts of protein were separated using SDS-PAGE, and proteins were transferred to a nitrocellulose membrane (Bio-Rad Laboratories). Membranes were blocked by incubation in 5% skim milk for 1 h followed by probing with specific Abs to phosphorylated IκBα (1:1000 dilution), Fra-1 (1:1000 dilution), phosphorylated ERK1/2 (1:1000 dilution), and ERK1/2 (1:1000 dilution) at 4˚C overnight. Membranes were washed and incubated for 1 h with the appropriate HRP-conjugated secondary Ab. Immune complexes were visualized with enhanced chemiluminescence substrate reagent (Bio-Rad Laboratories).

Promoter constructs and transfection and luciferase assays

Literature precedent and analysis with Genomatix MatInspector identified three AP-1 sites, −98/−91 (AP-1[1]), −552/−545 (AP-1[2]), −1671/−1664 (AP-1[3]), conforming to the 12-O-tetradecanoylphorbol-13-acetone response element (TRE) or AP-1 consensus sequence 5’-TGA(C/G)TCA-3’ within the −1273/−6 MMP-9 promoter region (28, 29) (Fig. 1A). Point mutations within the two proximal AP-1 sites at −98/−91 bp (mAP-1[1] and −552/−545 bp (mAP-1[2]), respectively, were introduced using site-directed mutagenesis within the −1017/−6 bp MMP-9 promoter luciferase construct. Forward primer sequences were as follows, with mutated residues depicted as lowercase letters: mAP-1[1] 5’-CTGACCCCTGTGATGCGACTCTGCTC-3’ and mAP-1[2] 5’-AAGAAGAATCGTATGAAAGGGTCT-3’ (30).

An NF-κB luciferase reporter construct was generated using custom-designed oligonucleotides (130 bp) and using forward primer 5’-ATGCGATTGCTTCTTGGTTTG-3’ and reverse primer 5’-CTGAGAAGTCCGTGTTTGGT-3’. The construct contained three copies of the promoter sequence between −635 and −607. This region contained the two adjacent NF-κB sites, previously shown to be important in HRV-induced MMP-9 transcription. These repeats were inserted upstream of the MMP-9-specific TATA (TTAAA) box in the final construct. These constructs were cloned into a pGL4.10[luc2]Vector (Promega, Madison, WI), and mutations and constructs were confirmed by DNA sequencing (University of Calgary DNA Sequencing). Promoter transcription and measurement of luciferase activity were performed in BEAS-2B cells as previously described (13).

Nuclear extraction procedure and EMSA

BEAS-2B cells were infected with HRV, as outlined earlier, and nuclear extracts were obtained as previously described (31). A double-stranded oligonucleotide containing an AP-1 transcription factor binding site with the identical consensus sequence to the two proximal AP-1 sites, but identical to the identical flanking sequence to the AP-1[1] site (underlined, 5’-CTGACCCCTGTGATGCGACTCTGCTC-3’), was used for EMSA and supershift assays as described previously (31). The modulation of Fra-1 AP-1 DNA binding was investigated using a TransAM kit containing a generic AP-1 consensus sequence that was identical to the consensus sequence present in both the AP-1[1] and AP-1[2] sites. Briefly, 10–15 μg nuclear extract was loaded onto 96-well plates precoated with oligonucleotides containing the AP-1 consensus sequence. A Fra-1–specific primary Ab was used to detect Fra-1 bound to the AP-1 sequence, and a secondary anti-IgG HRP-conjugated Ab provided a colorimetric readout, which was measured using a spectrophotometer. Data were expressed as a percentage of Fra-1 DNA binding relative to virally stimulated cells.

Small interfering RNA knockdown of Fra-1

BEAS-2B cells at 40–50% confluence or HBE cells at 70–80% confluence were transfected with 3 nM Fra-1–specific (A, S100420420; B, S100420427; C, S100420434; Qiagen) or Medium GC control small interfering RNA (siRNA) (Invitrogen, Burlington, ON, Canada) for 24 h at 37˚C with RNAi Max lipid transfection reagent (Invitrogen) in bronchial epithelial growth medium (BEGM) without antibiotics. Cells were allowed to recover for 24 h in BEGM prior to a 1-h pretreatment in BEBM and HRV-16 infection for 24 h.

Statistical analysis

Data were plotted as mean (±SEM) and analyzed using one-way ANOVA, with appropriate post hoc multiple comparison testing for between-group comparisons. All comparisons were two-tailed, and p values <0.05 were considered significant.

Results

AP-1 is necessary for HRV-induced MMP-9 transcription

The MMP-9 promoter contains three AP-1 (or TRE) consensus sites, with the consensus sequence “TGAGTCA” (Fig. 1A) (28, 29). Data from our previous publication established that the AP-1[3] site was not necessary for HRV-induced MMP-9 expression (13). We therefore synthesized luciferase promoter constructs in which mutations were introduced in one or both of the AP-1[1] or AP-1[2] sites: mAP-1[1], mAP-1[2], and mAP-1[1,2], respectively. Mutation of the AP-1[1] site reduced both control basal and virally induced promoter drive relative to the −1017/−6 bp wild-type construct (Fig. 1B) but did not alter the promoter drive when expressed as a fold increase above the respective basal levels (Fig. 1C). Mutation of the AP-1[2] site enhanced basal and viral drive (Fig. 1B) but reduced the fold increase in promoter drive relative to the −1017/−6 bp construct (Fig. 1C). Mutation of both AP-1[1] and AP-1[2] (mAP-1[1,2]) reduced both basal and viral drive (Fig. 1B), as well as the fold increase in promoter drive (Fig. 1C). Together, these data indicate that these two AP-1 sites are involved in MMP-9 promoter drive.

A complex of Fra-1, c-Jun, and JunD binds to the AP-1 sites

To confirm AP-1 nuclear localization and DNA binding to the AP-1 consensus sites, we used EMSA and supershift assays utilizing an oligonucleotide identical to the AP-1[1] site. Nuclear extracts were collected from BEAS-2B and primary HBE cells at various time points after viral infection for time-course studies. One band was noted at all time points in both BEAS-2B (Fig. 1D) and primary cells (Fig. 1F). Supershift assays were performed at the time point at which the greatest HRV induction of binding was seen in BEAS-2B cells (24 h postinfection), which is the time point at which maximal HRV-16 induction of MMP-9 mRNA was observed in BEAS-2B cells (13). Abs to Fra-1, c-Jun, and JunD in both BEAS-2B (Fig. 1E) and primary HBE cells (Fig. 1G) resulted
in a shift in the complex, whereas Abs to c-Fos, ATF-2, CREB-1, and CREB-2 did not result in a shift (Fig. 1E, 1G), indicating that the AP-1 complex(es) binding to the AP-1 sites 24 h after HRV infection is composed of Fra-1, c-Jun, and JunD. We also investigated, using TransAM kits, the modulation of Fra-1, c-Jun, and JunD binding in control and virally infected cells at an early (3 h) and late (24 h) time point (Supplemental Fig. 1). In both BEAS-2B and primary HBE cells, there was consistent DNA binding of c-Jun and JunD in control and virally infected cells at both time points. Fra-1 DNA binding was present in uninfected control cells. HRV-16 infection of BEAS-2B cells resulted in a substantial induction of Fra-1 DNA binding but did not alter Fra-1 DNA binding in primary HBE cells.

**Fra-1 is necessary for HRV-induced MMP-9 expression**

To determine whether Fra-1 is necessary for HRV-induced MMP-9 expression, we used two distinct Fra-1 (FOSL1) specific siRNAs. Fra-1 was induced upon HRV infection in BEAS-2B cells (Fig. 2A) but was not further induced upon viral infection of primary HBE cells compared with control uninfected primary HBE cells (Fig. 2B). Nonetheless, Fra-1 knockdown was seen 24 h after viral infection in BEAS-2B (Fig. 2A) and primary HBE cells (Fig. 2B). Using a Fra-1–specific TransAM kit, Fra-1 binding to an oligonucleotide containing the AP-1 consensus sequence was also significantly reduced in the presence of the two different Fra-1–specific siRNA duplexes in both BEAS-2B (Fig. 2E) and primary HBE (Fig. 2F) cells. These data indicate that Fra-1 is necessary for MMP-9 expression.

**Activation of MEK is necessary for Fra-1 expression and DNA binding**

Fra-1 expression has been reported to be dependent on the MEK/ERK MAPK pathway (23, 32). In addition, we have shown chronic activation of this MAPK pathway upon HRV infection (12, 33). As reported earlier, Fra-1 expression is induced upon HRV infection of BEAS-2B cells but is constitutively present in primary HBE cells. Thus, we next sought to determine whether the MEK/ERK MAPK pathway was playing a role in Fra-1 expression and consequently in HRV-induced MMP-9 expression. The pharmacological inhibitors of MEK1/2, PD98059 (1–30 μM) and U0126 (0.3–10 μM), which have been shown to inhibit HRV-induced ERK phosphorylation (33), both decreased HRV-induced Fra-1 expression in BEAS-2B cells in a concentration-dependent manner (Fig. 3A, 3B). Although HRV infection of primary cells did not modulate the high basal levels of ERK phosphorylation, both PD98059 and U0126 inhibited ERK phosphorylation and Fra-1 expression in both uninfected and infected primary HBE cells (Fig. 3C). Together, these data indicate that MEK activity is necessary for Fra-1 expression. Using a Fra-1–specific TransAM kit, we then sought to determine whether the reduction in Fra-1 expression upon treatment with MEK inhibitors resulted in a decrease in Fra-1 DNA binding. PD98059 and U0126 significantly
reduced DNA binding of Fra-1 in BEAS-2B (Fig. 3D) and in primary HBE (Fig. 3E) cells. These data indicate that MEK activity is necessary for HRV-induced (in BEAS-2B cells) or constitutive (in primary HBE cells) Fra-1 expression.

MEK1/2 inhibitors attenuate HRV-induced MMP-9 transcription and protein expression

To confirm that activation of MEK1/2 is necessary for HRV-induced MMP-9 transcription, we used a 21017/26 bp MMP-9 promoter luciferase construct. Both PD98059 and U0126 significantly attenuated viral-drive of the promoter in BEAS-2B cells (Fig. 4A). HRV-induced MMP-9 protein expression was also concentration-dependently decreased by PD98059 and U0126 in BEAS-2B cells (Fig. 4B). Similar data were obtained in primary HBE cells (Fig. 4C). The MEK/ERK pathway is therefore necessary for Fra-1 expression and downstream HRV induction of MMP-9 transcription and expression. These results, however, do not exclude the possibility that the MEK/ERK pathway may have additional effect(s) on HRV-induced MMP-9 expression, independent of effects on Fra-1 expression.

Formoterol and dexamethasone inhibit HRV-induced MMP-9 protein and mRNA expression

We next sought to determine whether commonly used asthma therapies, namely LABAs or glucocorticoids, could inhibit HRV-induced MMP-9 expression. Both formoterol (1 pM to 10 nM) (Fig. 5A) and dexamethasone (0.3 nM to 1 μM) (Fig. 5D) reduced HRV-induced MMP-9 protein expression in a concentration-dependent manner in BEAS-2B cells (EC50 = 1.7 × 10⁻¹¹ M and 2.2 × 10⁻⁸ M, respectively). Formoterol (10 nM) (Fig. 5C) and dexamethasone (1 μM) (Fig. 5D) also reduced HRV-induced MMP-9 mRNA in BEAS-2B cells but did not alter HRV titer (data not shown). HRV-induced MMP-9 protein (data not shown) and mRNA expression in BEAS-2B (Fig. 5C, 5D) cells were also inhibited by the adenylyl cyclase activator forskolin and the glucocorticoid fluticasone propionate. In addition, the β₂-adrenergic receptor antagonist ICI 118551 (100 nM) reversed the formoterol-induced decrease in MMP-9 expression in BEAS-2B cells (Fig. 5E). Together, these data indicate that the repression of MMP-9 expression by glucocorticoids is class, but not compound, specific and that the effects of formoterol are mediated via β₂-adrenergic receptor signaling, most likely via a cAMP pathway.

Formoterol and dexamethasone in combination inhibit HRV-induced MMP-9 protein

Current asthma guidelines advocate that treatment be initiated with inhaled corticosteroid (ICS) monotherapy, but that if patients continue to experience symptoms despite being adherent to ICS, they should be treated with ICS–LABA combination therapies (34, 35). We were therefore interested to investigate whether the combination of formoterol and dexamethasone repressed HRV-induced MMP-9 expression to a greater extent than dexamethasone alone. BEAS-2B cells were treated with dexamethasone (0.3 nM to 1 μM) alone or in combination with 30 pM formoterol (Fig. 6A). The EC50 of both concentration-response curves are similar (1.8 × 10⁻⁸ M and 1.1 × 10⁻⁸ M, respectively), and the addition of formoterol to the highest concentration of dexamethasone (1 μM) did not result in any further significant attenuation of HRV-induced MMP-9 expression compared with that of either treatment alone (Fig. 6B, using same data as Fig. 6A).
Formoterol and dexamethasone decrease HRV-induced MMP-9 transcription

To determine whether formoterol and dexamethasone act at a transcriptional level to repress virally induced MMP-9 expression, we used the −1017 bp and −681 bp MMP-9 promoter luciferase-reporter constructs as previously described (13). For formoterol (10 nM) and forskolin (10 μM) (Fig. 7A), as well as dexamethasone (1 μM) and fluticasone propionate (300 nM) (Fig. 7B), significantly decreased viral drive of the 1017 bp construct in BEAS-2B cells. No significant differences in inhibition of promoter drive were seen between the 1017 bp and 681 bp constructs, indicating that the region of DNA between the truncations is not necessary for repression of virally induced MMP-9 promoter drive by cAMP elevators or glucocorticoids.

Formoterol and dexamethasone do not reduce NF-κB activation, DNA binding, or transactivation

We have reported that NF-κB is necessary for HRV-induced MMP-9 transcription and that phosphorylated IκBα and NF-κB DNA binding are significantly induced upon viral infection of BEAS-2B cells (13). We therefore measured phosphorylated IκBα as a marker of NF-κB activation. Neither formoterol nor dexamethasone had any effect on virally induced phosphorylation of IκBα at the time points examined in BEAS-2B cells (Fig. 8A). EMSA was performed using oligonucleotides with an identical sequence to the functionally active MMP-9 NF-κB sites (13) to determine whether NF-κB DNA binding was reduced. Neither formoterol nor dexamethasone affected HRV-induced NF-κB DNA binding in BEAS-2B cells (Fig. 8B). Finally, we used an NF-κB luciferase reporter construct, consisting of three repeats of the two NF-κB sites, to determine whether formoterol or dexamethasone was affecting the transactivation potential of NF-κB. Neither formoterol nor dexamethasone reduced virally induced drive of this reporter construct (Fig. 8C). These data indicate that formoterol and dexamethasone do not affect HRV-induced NF-κB activation, DNA binding, or transactivation.

Formoterol and dexamethasone modulate Fra-1 expression and DNA binding by abrogating HRV-induced activation of ERK1/2

Having established the importance of Fra-1 in HRV-induced MMP-9 upregulation, we next examined whether formoterol or dexamethasone affected Fra-1 expression. Both formoterol and dexamethasone inhibited viral induction of Fra-1 in BEAS-2B cells (Fig. 9A). Formoterol and dexamethasone also inhibited Fra-1 DNA binding, as assessed in BEAS-2B cells using EMSA and a Fra-1–specific TransAM kit (Fig. 9B). Western blotting also
showed that formoterol and dexamethasone reduced virus-induced ERK1/2 phosphorylation at 10 h after HRV infection in BEAS-2B cells (Fig. 9C). It is therefore likely, given the importance of the MEK/ERK pathway for Fra-1 expression, that formoterol and dexamethasone prevent Fra-1 expression, in part, by repressing activation of this MAPK pathway.

Discussion

HRV infections are a major trigger for acute exacerbations of asthma and have also now been implicated in the pathogenesis of airway remodeling (9, 21). It is thought that HRV infection causes disease exacerbations by altering airway epithelial cell biology in a manner that promotes airway inflammation and remodeling by upregulating epithelial production of mediators involved in the inflammatory and remodeling processes (9, 21). In this regard, we have reported that HRV infection selectively upregulates airway epithelial cell production of MMP-9 without affecting its associated tissue inhibitor (TIMP-1) (13). Increased expression of MMP-9 is present in the airways of asthmatic individuals (18, 36), and the subsequent disruption of the MMP-9/TIMP-1 ratio is considered to be a key factor involved in the pathogenesis of airway inflammation and remodeling, both in experimental models (37) and in asthma (21). The understanding of the pathways and mechanisms resulting in an increase in MMP-9 expression is therefore important and may aid the development of new targeted therapies or more rational approaches to the use of existing ones for the management of asthma.

The results of the current study provide novel evidence that the two AP-1 sites within the first 552 bp of the MMP-9 promoter are
necessary, along with an NF-κB site (13), for HRV-induced MMP-9 transcription. For the sake of full clarity, the promoter data were expressed as relative light units (as in Fig. 1B) and as fold increase relative to uninfected control cells (as in Fig. 1C) because of the variation in the basal drive upon mutation of the AP-1[1] and AP-1[2] sites. We believe that doing so minimizes any potential ambiguity in the conclusions that may be drawn when only one figure is displayed. We hypothesize that the modulation of basal promoter drive upon mutation of the AP-1 sites is due to abolishment of both activating as well as repressive factors. In support of this hypothesis, EMSA analysis using nuclear extracts obtained from BEAS-2B cells 24 h after mock or viral infection indicated that a radiolabeled oligonucleotide with an identical sequence to the mutation present in the mAP-1[1] and mAP-1[2] promoter sites abolished both basal and virally induced DNA binding (data not shown). A single complex was identified on EMSA using an oligonucleotide with an identical sequence to the AP-1[1] site and nuclear extracts obtained from virally stimulated cells. Supershift assays identified that the AP-1 family members Fra-1, c-Jun, and JunD formed part of this complex, and the ability of Fra-1 to bind the AP-1 sequence was further confirmed using a Fra-1 TransAM kit. Fra-1 siRNA was then used to confirm a role for Fra-1 in HRV-induced MMP-9 expression. An increase in AP-1 transactivation has previously been reported in response to transfection of a construct expressing the rhinovirus 3C protease (38), although to our knowledge this is the first report to show that Fra-1 expression is inducible upon HRV infection of BEAS-2B cells. Whereas we saw a significant increase in Fra-1 expression in BEAS-2B cells, little to no induction of Fra-1 above baseline levels was noted upon HRV infection of primary HBE cells. Knockdown of Fra-1, using siRNA in both BEAS-2B and primary HBE cells, resulted in a significant reduction in Fra-1 AP-1 DNA binding and MMP-9 protein expression in both BEAS-2B and primary HBE cells; this indicates that Fra-1 is necessary for MMP-9 transcription. These data are consistent with previous reports examining MMP-9 expression in response to different stimuli in different cell types (23, 30, 39, 40). Using MEK inhibitors, we have established that activation of MEK1/2 is necessary for Fra-1 expression and HRV-induced MMP-9 expression. The observation that Fra-1 expression is dependent on the activation of the MEK/ERK MAPK pathway is consistent with observations from previous studies (23, 41); however, we cannot rule out that MEK inhibitors may be having additional Fra-independent effects on MMP-9 transcription or expression. Although the focus of this study was to delineate the role of MEK in controlling Fra-1 expression, the MEK/ERK MAPK pathway may also be responsible for posttranscriptional modifications of Fra-1, which may affect Fra-1 DNA binding or transactivation potential (32, 42). Inhibitors of the p38 and JNK MAPK pathways, namely SB203580 and SP600125, did not inhibit HRV-induced MMP-9 expression (data not shown), indicating that MMP-9 expression is selectively dependent on activation of the MEK/ERK MAPK pathway upon HRV infection. Although we noted differences in the inducibility of Fra-1 upon HRV infection in the two model systems used in this study, Fra-1 expression was dependent on activation of the MEK/ERK MAPK pathway in both BEAS-2 and primary HBE cell types. The high level of basal Fra-1 in primary HBE cells is most likely due to the constitutive MEK/ERK activation observed in this cell type, as basal levels of Fra-1 can be repressed by the MEK inhibitors (PD98059 and U0126). Our previous study reported that NF-κB is also necessary for HRV-induced MMP-9 expression and that NF-κB activation and DNA binding is induced upon HRV infection of both BEAS-2B and primary HBE cells (13). Thus, these results lead us to conclude that HRV-induced MMP-9 expression in both BEAS-2B and primary HBE cells is dependent on the activation of NF-κB, as well as on MEK/ERK MAPK-mediated Fra-1 expression.

Although LABAs and ICS are commonly used asthma pharmacotherapies, their effects on virally induced airway inflammation and on airway remodeling remain to be fully elucidated. Despite this clinical uncertainty, both LABAs and ICS continue to be recommended as appropriate treatments for patients experiencing worsening asthma symptoms (34, 43). We found that the LABA formoterol and the glucocorticoid dexamethasone inhibit HRV-induced MMP-9 expression. We have previously shown that HRV replication is necessary for the increase in HRV-induced MMP-9 expression (13), but viral titer assays confirmed that the repression of MMP-9 expression observed in this study was not due to inhibition of viral replication by formoterol or dexamethasone (data not shown). This lack of effect on viral replication is in agreement with data reported in other in vitro (26) and in vivo (44, 45) studies. Formoterol and dexamethasone also had no effect on TIMP-1 protein in the presence or absence of virus (data not shown). These data indicate that LABAs and glucocorticoids, by
decreasing virally induced MMP-9 expression, aid in restoring the MMP-9/TIMP-1 ratio.

Using promoter luciferase constructs, we found that formoterol and dexamethasone modulate virally induced MMP-9 transcription. The repression of inflammatory gene transcription by glucocorticoids is generally attributed to the action of glucocorticoids on proinflammatory transcription factors, such as NF-κB and AP-1 (46). Using Western blotting, EMSAs, and luciferase promoter constructs, we report that formoterol and dexamethasone do not modulate NF-κB activation, DNA binding, or transactivation. These results are consistent with previous studies that have shown no effect of LABAs and glucocorticoids on either NF-κB activation, DNA binding, or transactivation in HBE cells (47–49). Formoterol and dexamethasone did, however, reduce Fra-1 expression and DNA binding as well as ERK1/2 phosphorylation. Given these data, the inhibition of the MEK/ERK pathway may represent a potential mechanism by which these compounds inhibit Fra-1 and MMP-9 expression. Other studies have reported that LABAs have no effect on AP-1 transactivation (50), whereas glucocorticoids repress AP-1–dependent transactivation (51). These divergent results are likely attributable to the specific stimuli used or the specific outcomes examined. Our data highlight potential roles for formoterol and dexamethasone in the repression of secondary inflammatory gene expression via effects on primary response genes and identify an area that may be exploited to optimize a more rational use of LABA–ICS combination therapy.

The repression of MMP-9 expression by formoterol is likely due to signaling via the β2-adrenergic receptor, as formoterol-induced inhibition of MMP-9 protein expression is reversed by a β2-adrenergic receptor antagonist. Activation of adenylyl cyclase using forskolin also mimicked the effects induced by formoterol, indicating that an increase in cAMP may be necessary for inhibition of MMP-9 expression. Glucocorticoid-dependent repression of inflammatory genes has been proposed to occur via two distinct mechanisms; the direct inhibition of inflammatory gene expression, termed transrepression, and the induction of anti-inflammatory genes, which then negatively regulate inflammatory gene expression, referred to as transactivation (52). This study indicates
that both formoterol and dexamethasone act to repress ERK phosphorylation and suggests that these two distinct pharmacotherapies may be acting via a common mechanism of action.

We also investigated whether a combination of formoterol and dexamethasone was able to repress MMP-9 expression to a greater degree than that seen with dexamethasone alone. No alterations of the EC50 of the dose-response curves were noted. We also observed that the addition of formoterol to the maximal concentration of dexamethasone did not significantly decrease HRV-induced MMP-9 expression below that seen with the maximum concentration of dexamethasone alone. This may be due to the potent antioxidant effects of formoterol and the induction of dexamethasone alone. These studies constitute the initial investigations into the effects of dexamethasone and formoterol on HRV-induced MMP-9 expression, and as such, we chose to use the concentration of dexamethasone (1 μM) that has previously been shown to produce optimal transactivation of anti-inflammatory genes in airway epithelial cells (53–55). Future studies will investigate the concentration dependence of the inhibition and whether glucocorticoid action is dependent on transrepression and/or transactivation.

In summary, this study extends our current knowledge of how HRV infections might cause worsening airway inflammation and promote airway remodeling in asthma by identifying that the AP-1 action is dependent on transrepression and/or transactivation. These studies suggest that both formoterol and dexamethasone act to repress ERK phosphorylation and that ERK phosphorylation is necessary for HRV-induced MMP-9 expression in airway epithelial cells. As such, we chose to use the concentration of dexamethasone (1 μM) that has previously been shown to produce optimal transactivation of anti-inflammatory genes in airway epithelial cells (53–55). Future studies will investigate the concentration dependence of the inhibition and whether glucocorticoid action is dependent on transrepression and/or transactivation.

Disclosures
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References
Erk1/2 pathway-mediated phosphorylation of a unique C-terminal destabilizer.


