THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 21, pp. 15366–15375, May 25, 2007

Corticosteroids and β_2 Agonists Differentially Regulate Rhinovirus-induced Interleukin-6 via Distinct Cis-acting Elements*

Received for publication, February 15, 2007, and in revised form, March 21, 2007 Published, JBC Papers in Press, March 29, 2007, DOI 10.1074/jbc.M701325200

Michael R. Edwards[‡1], **Jennifer Haas**[‡], **Rey A. Panettieri, Jr.**[§], **Malcolm Johnson**[¶], **and Sebastian L. Johnston**[‡]

From the [‡]*Department of Respiratory Medicine, National Heart and Lung Institute and Wright Fleming Institute of Infection and Immunity, Imperial College London, London W2 1PG, United Kingdom MRC Centre in Allergic Mechanisms of Asthma,* [§]*Department of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6160, and* [¶]*GlaxoSmithKline, Middlesex, TW8 9GS, United Kingdom*

Interleukin-6 (IL-6) is a proinflammatory cytokine up-regulated by rhinovirus infection during acute exacerbations of asthma and chronic obstructive pulmonary disease. The role of IL-6 during exacerbations is unclear; however, it is believed IL-6 could contribute to airway and systemic inflammation. In this study we investigate the effects of common asthma treatments fluticasone propionate and β_2 agonists salmeterol and salbutamol on IL-6 production in BEAS-2B and primary bronchial epithelial cells. Salmeterol and salbutamol enhanced rhinovirus- and IL-1β-induced IL-6 production; however, fluticasone treatment caused a reduction of IL-6 protein and mRNA. Combined activity of salmeterol and fluticasone at equimolar concentrations had no effect on rhinovirus or IL-1β induction of IL-6. The induction of IL-6 by salmeterol was dependent upon the β_2 receptor and could also be induced by cAMP or cAMP-elevating agents forskolin and rolipram. Using transfection of IL-6 promoter reporter constructs, dominant negative mutants, and electromobility shift assays, it was found that NF-κB was the only transcription factor required for rhinovirus induction of IL-6 gene expression. Salmeterol caused an augmentation of rhinovirus-induced promoter activation via a mechanism dependent upon the c/EBP and/or CRE (cyclic AMP response element) cis-acting sites. The suppressive effect of FP was dependent upon distinct glucocorticoid response element sequences proximal to the transcriptional start site within the IL-6 promoter. The data demonstrate that β_2 agonists can augment IL-6 expression by other stimuli in an additive manner via cyclic AMP and that the negative effect of steroids is mediated by glucocorticoid response elements within the IL-6 promoter.

Asthma and chronic obstructive pulmonary disease (COPD)[2] are inflammatory diseases of the airway. Recent evidence suggests a large proportion of exacerbations of both diseases are precipitated due to viral infections (1–7), and the most prominent respiratory virus associated with either disease is human rhinovirus (RV) (8–10). Rhinovirus infects the bronchial epithelium and induces a variety of proinflammatory cytokines, chemokines, and adhesion molecules, serving to attract inflammatory cells and prolong local inflammation within the airway (11, 12). Among the many cytokines induced, IL-6, a pleiotropic cytokine, is commonly associated with both diseases (13–15). Although IL-6 has inflammatory, anti-inflammatory, and immunomodulatory properties, its exact role in either disease remains unclear.

Adequate treatment of asthma and COPD exacerbations remains an important therapeutic goal. Inhaled corticosteroids (GCs) and long acting β_2 agonists (LABAs) are common treatments for asthma and COPD and exacerbations of these diseases, often used in combination. However, these treatments are only partially effective, reducing rates of asthma exacerbations by 40% and less so for COPD (16, 17). A thorough understanding of the actions and interactions of these treatments at the physiological, cellular, and molecular level is a major research objective, allowing a more careful application of these treatments to appropriate patients.

Several studies have demonstrated the anti-inflammatory potential of GCs; however, their mode of action has been vigorously debated (18). Recent evidence also demonstrates that LABAs can enhance the anti-inflammatory action of steroids (19–22). LABAs, however, can affect the expression of other genes via cAMP-dependent pathways, such as the induction of IL-6 in airway smooth muscle cells (ASM) (23). The human IL-6 promoter contains several different cis-acting sites proximal to the TATA box, including NF-κB, AP-1, CCAAT enhancer-binding protein (c/EBP), and a cyclic AMP response element (CRE), and all have been implicated in IL-6 transcription using a range of different stimuli (23–25).

Because bronchial epithelial cells express both glucocortcoid receptors (GR) and β_2 receptors and are target cells for combined GC and β_2 agonist therapy *in vivo*, we have investigated the modulation of RV- and IL-1β-induced IL-6 by pretreatment

* This work was funded by an unrestricted grant from GlaxoSmithKline. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom correspondence should be addressed: Dept. of Respiratory Medicine, National Heart Lung Institute, St. Marys Hospital, Imperial College, London Norfolk Place, London W2 1PG, United Kingdom. Tel.: 44-207-594-3764; Fax: 44-207-262-8913; E-mail: michael.edwards@ic.ac.uk.

[2] The abbreviations used are: COPD, chronic obstructive pulmonary disease; RV, rhinovirus; GC, corticosteroid; LABA, long acting β_2 agonist; ASM, airway smooth muscle; c/EBP, CCAAT enhancer-binding protein; CRE, cyclic-AMP response element; GR, glucocortcoid receptor; GRE, glucocorticoid response element; nGRE, negative GRE; SM, salmeterol; FP, fluticasone propionate; IKK, IκB kinase; SB, salbutamol; DN, dominant negative; CMV, cytomegalovirus; ELISA, enzyme-linked immunosorbent assay; NHBE, normal human bronchial epithelial cells.

with salmeterol (SM) and fluticasone propionate (FP) in primary bronchial epithelial cells and cell lines. RV induced IL-6 via IκB kinase (IKK)-β and NF-κB and was augmented via SM by CRE and c/EBP binding transcription factors in a cAMP-dependent manner. FP decreased RV-induced IL-6 via negative glucocorticoid response elements (GRE), proximal to the TATA box, confirming IL-6 as one of few steroid susceptible genes that is controlled via a negative GRE. Interestingly, in combination, RV induced IL-6 mRNA and protein levels were unaltered, showing that the effect of one agent negates the other. Finally, the data further demonstrate that β_2 agonist have biological effects other than suppressing gene transcription in collaboration with GCs.

EXPERIMENTAL PROCEDURES

Tissue Culture and Viruses—BEAS-2B cells were obtained from the European Collection of Cell Cultures (ECACC 95102433). Cells were grown in RPMI 1640 media supplemented with Glutamax (Invitrogen) with 10% fetal calf serum (FCS, Invitrogen) buffered with 1% sodium bicarbonate (Invitrogen) and 0.075% HEPES (Invitrogen). Cells were grown at 37 °C in a humidified incubator using 175-cm^2 flasks and split when confluent, approximately 3 times a week. RV serotypes 16 and 1B were grown in Ohio HeLa cells and titrated on confluent HeLa cells to ascertain $TCID_{50}$ (2). RV16 was $\sim 1 \times 10^7$, and RV1B was 10^7 $TCID_{50}$/ml. The identity of all RVs was confirmed by titration on HeLa cells and neutralization using serotype-specific antibody. UV inactivation was performed essentially as previously described (26, 27), and filtered virus was obtained by spinning HeLa cell supernatants containing RV through a 30-kDa membrane (Millipore, Stonehouse Gloustershire, UK) at 10,000 *g* in a microcentrifuge (Heraeus) for 5 min.

Plasmids, Site-directed Mutagenesis, and Reagents—Recombinant human IL-1β was purchased from R&D Systems (Abingdon, UK), dissolved in phosphate-buffered saline at 10 μg/ml, and stored at −20 °C. Salbutamol (SB), propranolol, forskolin, and rolipram were purchased from Sigma-Aldrich, dissolved in dimethyl sulfoxide (Me_2SO) at a concentration of 0.1 M, and stored at −20 °C. SM and FP (GlaxoSmithKline) were also dissolved in Me_2SO, at 0.1 M. Dibutryl-cAMP was also purchased from Sigma-Aldrich and made up at 0.1 M in water. Before use, stocks were dissolved in RPMI 1640 medium with 2% FCS (infection media) at the required concentrations. IL-6 promoter-reporter constructs consisted of various mutations or deletions of the full-length IL-6 promoter (−651 bp) fused to firefly luciferase (24). We constructed IL-6 ΔGRE 651 using a site-directed mutagenesis kit (Stratagene, La Jolla CA) and mutant oligos 5′-GACTGGAGATGTCTGAG**AA**AT**TC**TTC**GAATTC**CGAGGTCGACGGT-3′ and 3′-CTGACCTCTACAGACTC**TT**A**AG**AAGC**TTAAG**GCTCCAGCTGCCA-5, where mutations are presented underlined and in bold. IL-6 ΔCRE651 was made using the oligonucleotides 5′-GCGATGCTAAAGG**GATC**CACATTGCA-3′ and 3′-CGCTACGATTTCC**CTAG**GTGTAACGTG-5′, according to previously published methods (28, 29). All mutant constructs were verified by dideoxy terminator sequencing. A dominant negative (DN) mutant of IκB under control of the CMV promoter was purchased from Clontech (Oxford, UK). pcDNA3.1 and a construct encoding β-galactosidase constitutively expressed by the CMV promoter (pCMVSPORT-β-gal), were purchased from Invitrogen. All plasmids were grown in *Escherichia coli* XL-1 blue, and plasmid DNA was prepared using a Maxiprep method (Qiagen, Crawley, UK) and stored at −80 °C at 1 μg/μl.

Transient Transfection of BEAS-2B Cells—Cells were seeded at 1.7×10^5 cells per well in 12-well plates (Nunc, Roskilde, Denmark), pre-coated with type IV calf collagen solution (Sigma-Aldrich), diluted 1/10 in phosphate-buffered saline, and left to grow for 48 h in RPMI 1640 with Glutamax (Invitrogen) supplemented with 10% FCS. Cells were transiently transfected with 1 μg of DNA per well consisting of either 0.8 μg of a IL-6 promoter-reporter construct and 0.2 μg of pCMVSPORT-β-gal, (Invitrogen). Cells were also transfected with 0.7 μg of the IL-6 reporter and 0.1 μg or either a DN of IκB or empty vector pcDNA3.1 and 0.2 μg of β-galactosidase. Transfection made use of 3 μl per well of Superfect (Invitrogen) according to the manufacturer's recommended protocol. DNA-Superfect complexes remaining on the cells for 3 h were washed off with 0.5 ml of phosphate-buffered saline/well, and 1 ml/well of RPMI 1640 media (Invitrogen) with 10% FCS, and the cells were incubated overnight at 37 °C. Cells were then placed in infection media for 4 h and treated with SM or FP or medium before infection with RV16. Protein lysates were harvested at 72 h post-infection.

RV Infection and IL-1β Treatment of Bronchial Epithelial Cells—For the induction and study of proteins, BEAS-2B cells were counted using trypan blue exclusion and seeded in 12-well plates (Nunc) at 1.7×10^5 cells/well in RPMI 1640 and 10% FCS medium and allowed to adhere for 24 h. BEAS-2B cells were then placed in infection media overnight. Monolayers were pretreated with SM, SB, and FP at various doses, diluted in infection media, or treated with medium for 1 h. All wells were normalized for the presence of Me_2SO. Cultures were then stimulated with 0.2 ml of RV16 or RV1B (m.o.i. of 1–4) or 1 ng/ml IL-1β (R&D Systems) for 1 h with shaking at room temperature. Viruses and IL-1β were then removed and replaced with 1 ml of infection media and incubated for 24 h. When using pharmalogical inhibitors, each inhibitor was diluted in infection medium and placed on the cells for 2 h before treatment with SM and SB or infection with RV16. All supernatants were stored at −80 °C for analysis. For induction of promoter activation, BEAS-2B cells were transfected as above and then placed in infection media for 4 h. Cells were then treated with SM and FP at the required concentrations or medium for 1 h and then with 0.2 ml of RV16 (m.o.i. 1–4) or medium for 1 h with shaking at room temperature. For RV-infected wells, infection media containing SM and or FP was then placed on the cells and incubated for 72 h to allow expression of the reporter gene.

Quantitative ELISA for IL-6—Supernatants were tested for the amounts of IL-6 by ELISA using commercially available paired antibodies and standards (R&D Systems) according to the manufacturer's recommendations. One hundred microliters of supernatant were tested in duplicate and compared with a standard curve, allowing quantification of each sample. The sensitivity of the assay was 7 pg/ml.

Reporter Gene Assays—Cellular extracts were prepared using commercially available reagents for the measurement of luciferase protein (Promega, Madison, WI). Luciferase

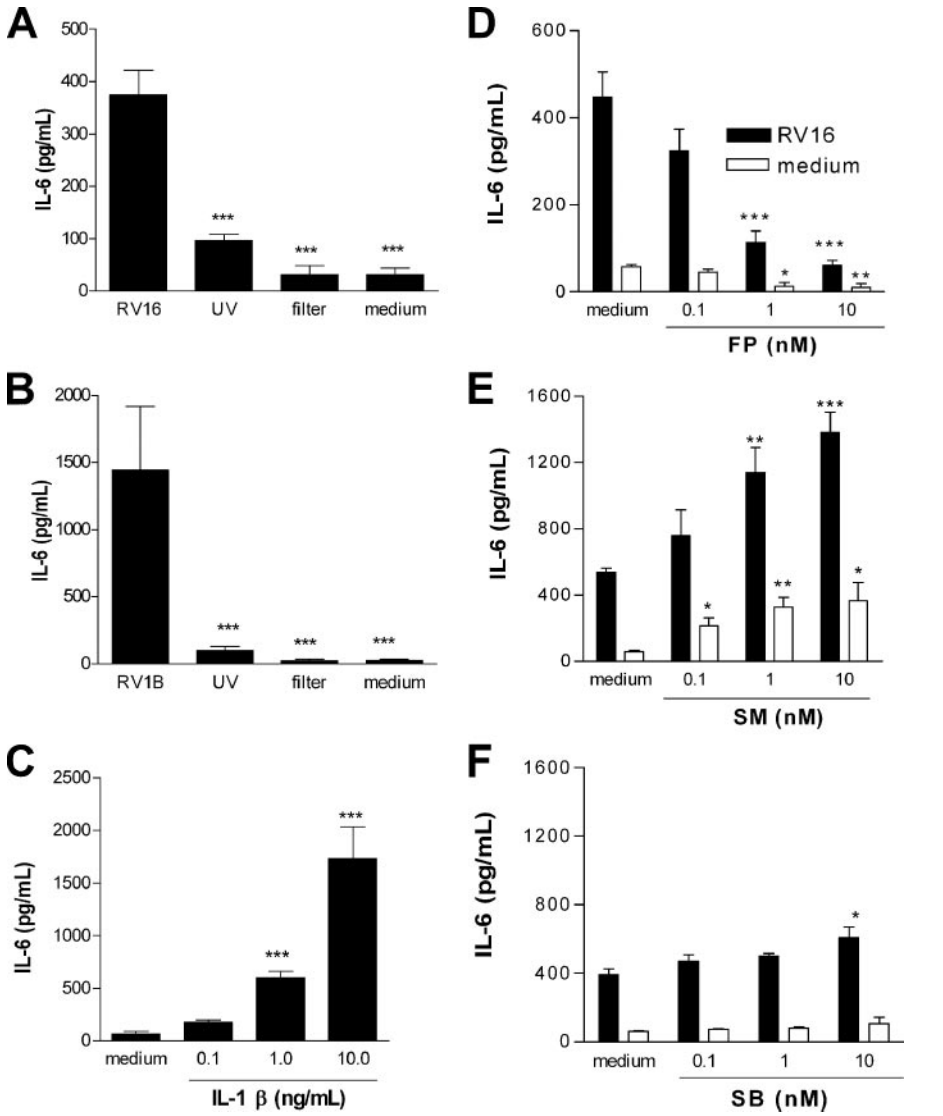

FIGURE 1. **Induction of IL-6 by rhinovirus and IL-1β and modulation by SM and FP in BEAS-2B cells.** RV16 (*A*) and RV1B (*B*) induced IL-6 protein, and this could be decreased either by filtration through a 30-kDa filter or by UV irradiation. ***, $p < 0.001$ *versus* RV infected. *C*, IL-1β caused a dose-dependent increase in IL-6 protein measured at 24 h. ***, $p < 0.001$ *versus* medium. RV16-induced IL-6 protein was suppressed by FP (*D*) or increased by SM (*E*) in a dose-dependent manner and also increased by SB (*F*) at 10 nM. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (*versus* RV16 infected or medium-treated-only cells). All IL-6 protein measurements were performed by ELISA; $n = 4$ experiments.

activity was measured using commercially available reagents (Promega) and a AutoLumat LB953 luminometer (Berthold Systems) for 10 s. All luciferase measurements were normalized to β-galactosidase expression using a commercially available enzymatic assay (Promega) at 420 nm in a Spectromax plate reader (Molecular Devices Ltd, Wokingham UK). Luciferase data were normalized by expressing relative luciferase units over β-galactosidase measurements (absorbance at 420 nm).

RNA Extraction and Quantitative Reverse Transcription-PCR—Total RNA was extracted (RNeasy kit, Qiagen), and 2 μg was used for cDNA synthesis (Omniscript RT kit, Qiagen). Quantitative PCR was carried out using specific primers and probes for IL-6 (sense, 5′-CCA GGA GCC CAG CTA TGA AC-3′; antisense, 5′-CCC AGG GAG AAG GCA ACT G-3′; probe, 5′-FAM CCT TCT CCA CAA GCG CCT TCG GT-6-carboxytetramethylrhodamine-3′) and 18 S rRNA (sense, 5′-CGC CGC TAG AGG TGA AAT TCT-3′; antisense, 5′-CAT TCT TGG CAA ATG CTT TCG-3′; probe, 5′-FAM ACC GGC GCA AGA CGG ACC AGA-6-carboxytetramethylrhodamine-3′). Reactions consisted of 12.5 μl of 2× Quanti-Tect Probe PCR Master Mix (Qiagen) and 300 nM sense and 900 nM antisense primer and 175 nM probe (IL-6) or 300 nM concentrations each of primer and 175 nM probe (18 S rRNA). Two μl of cDNA (18 S 2 μl diluted 1/100) was made up to 25 μl with nuclease-free water (Promega). Reactions were analyzed (ABI 7000 TaqMan, Foster City CA) at 50 °C for 2 min, 94 °C for 10 min, and 45 cycles of 94 °C for 15 s and 60 °C for 15 s. IL-6 expression was normalized to 18 S rRNA and presented as copies of IL-6 mRNA/μg of total RNA using a standard curve based on amplification with plasmid DNA.

Nuclear and Cytoplasmic Protein Harvest—BEAS-2B cells were grown in six-well plates and prepared as for reporter and protein experiments. Cells were pretreated SM, FP, or medium and then infected with RV16 for 20 min with shaking, and protein extracts were harvested at 30 min post-infection. Nuclear and cytoplasmic protein fractions were harvested using protein extraction reagents (Pierce) supplemented with protease inhibitors (Pierce).

Electromobility Shift Assay (EMSA)—Electromobility shift assay (EMSA) was performed using a nonradioactive EMSA kit (LightShift™, Pierce) according to the manufacturer's recommended protocol. Oligonucleotides were designed on the NF-κB site within the human IL-6 promoter including surrounding sequences; (forward, 5′-ATCAAATGTGGGATTTTCCCATGAG-3′; reverse, 5′-CTCATGGGAAAATCCCACATTTGAT-3′). Oligonucleotides with mutated NF-κB sites were also designed; forward, 5′-ATCAAATGTGGGATTTT**AGAC**TGAG-3′; reverse, 5′-CTCA**GTCT**AAAATCCCACATTTGAT-3′, with mutated nucleotides shown in underlined boldface type.

Statistics—All data were analyzed using one-way ANOVA at a 95% confidence interval and, if significant, pin-pointed with Bonferroni's multiple comparison test or pin-pointed using a two-tailed *t* test. Data were accepted as significantly different when $p < 0.05$.

RESULTS

Human Rhinovirus and IL-1β Stimulate IL-6 Protein in Bronchial Cells in Vitro—We have used RV16 and RV1B and the proinflammatory cytokine IL-1β as examples of proinflammatory agents that can lead to IL-6 production in bronchial epithelial cells. Both major RV16 and minor group RV1B viruses produce IL-6 in BEAS-2B cells after 24 h of culture (Fig. 1, *A* and *B*). The induction of IL-6 was due to virus replication and infection rather than other constituents of the HeLa supernatant as UV-inactivated, and filtered virus preparations gave significantly lower IL-6 production ($p < 0.01$). Fig. 1*C* shows that IL-1β induces IL-6 after 24 h of culture in a dose-dependent manner.

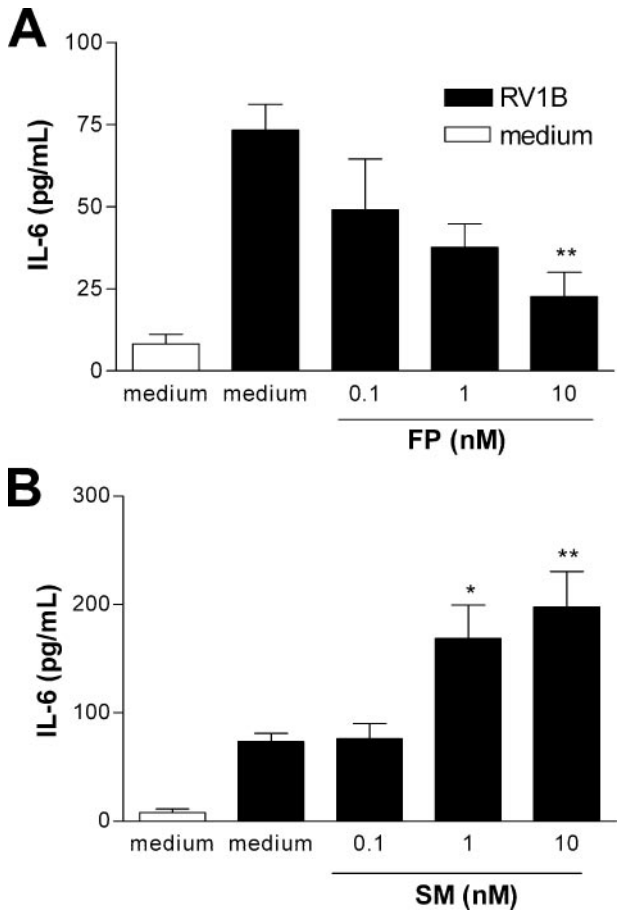

FIGURE 2. **Modulation of RV1B induced IL-6 by SM and FP in NHBE cells.** RV1B-induced IL-6 protein was suppressed by FP (*A*) and increased by SM (*B*) in a dose-dependent manner. *, $p < 0.05$; **, $p < 0.01$ (*versus* RV16-infected cells). All IL-6 protein measurements were performed by ELISA; $n = 5$ experiments.

Modulation of Rhinovirus and IL-1β Induced IL-6 by β_2 Agonists and GCs in Both BEAS-2B and NHBE Cells—The corticosteroid FP and β_2 agonists SM and SB were used to investigate whether or not asthma therapies were effective at alleviating RV- and IL-1β-induced IL-6 production. Fig. 1 demonstrates the ability of each treatment alone to affect spontaneous and RV16-induced IL-6 protein production after 24 h. FP suppressed both RV16-induced and spontaneous IL-6 in a dose-dependent manner ($p < 0.001$, *versus* control cells, Fig. 1*D*). In contrast, SM greatly augmented spontaneous and RV16-induced IL-6 production at 1 and 10 nM ($p < 0.01$ and $p < 0.001$, respectively, *versus* RV16 infected, untreated controls, Fig. 1*E*). At 10 nM SB gave modestly augmented RV16-induced IL-6 production ($p < 0.05$ *versus* RV16-infected, untreated control, Fig. 1*F*) but did not affect the basal level of IL-6 production in cultures treated only with medium. In primary NHBE cells, FP suppressed RV1B-induced IL-6 protein in a dose-dependent manner (Fig. 2*A*), whereas SM augmented RV1B-induced IL-6 protein in a dose-responsive manner (Fig. 2*B*).

BEAS-2B cells were then pretreated with different combinations of SM and FP at 1 or 0.1 nM (Table 1). At 1 nM SM augmented RV16-, RV1B-, and IL-1β-induced IL-6, whereas1 nM FP led to suppression of IL-6 with the same stimuli. When used in combination, the activities of SM and FP were diminished and had little affect when compared with RV16-, RVIB-, or IL-1β alone-treated cultures ($p > 0.05$). When the FP concentration was reduced to 0.1 nM, FP alone had little affect on virus and IL-1β-induced IL-6 protein. When used in combination with SM at 1 nM, the augmentative effects of SM were observed again despite the presence of the steroid and gave significant increases in IL-6 when using IL-1β ($p < 0.05$) and RV16 ($p < 0.001$) but not RV1B ($p > 0.05$). Similar results were observed with NHBE cells (Table 2) with 1 nM SM augmenting RV1B-induced IL-6 ($p < 0.001$) and 1 nM FP reducing RV1B-induced IL-6 ($p < 0.01$), and in combination, IL-6 levels were elevated significantly compared with untreated RV1B infected cells ($p < 0.01$).

SM and RV16 Induce IL-6 mRNA but with Different Kinetics—Time course experiments in BEAS-2B cells were designed to investigate the transcriptional induction of IL-6 in BEAS-2B cells. RV16 induced IL-6 mRNA at 24–72 h when compared with medium-treated cells ($p < 0.01$, Fig. 3*A*), still rising at 72 h. In contrast, SM induced IL-6 mRNA much earlier, significantly

TABLE 1

Modulation of IL-6 protein by SM or FP (0.1–1 nM) alone and in combination

BEAS-2B cells were pretreated with SM, FP, or SM and FP and treated with either IL-1β or RV. IL-6 protein was measured by ELISA.

	% IL-6 release in BEAS-2B cells						
		Pretreatment with 1 nMFP			Pretreatment with 0.1 nMFP		
	Untreated	SM	FP	SM+FP	SM	FP	SM+FP
				mean ± S.E.			
IL-1β	100	293.8 ± 71.3[a]	45.3 ± 2.7[c]	94.0 ± 17.3[d]	226.3 ± 43.9[a]	81.38 ± 11.3[d]	202.9 ± 34.1[a]
RV16	100	286.5 ± 64.9[a]	49.6 ± 11.9[b]	73.1 ± 18.3[d]	408.2 ± 32.6[c]	101.7 ± 11.9[d]	381.4 ± 18.3[c]
RV1B	100	402.3 ± 54.5[a]	37.5 ± 5.6[c]	106.8 ± 16.6[d]	284.9 ± 67.9v	98.7 ± 9.0[d]	218.7 ± 51.8[d]

[a] $p < 0.05$.
[b] $p < 0.01$.
[c] $p < 0.001$.
[d] NS, not significant.

TABLE 2

Modulation of IL-6 protein by SM or FP (0.1–10 nM) alone and in combination

NHBE cells were pretreated with SM, FP, or SM and FP and infected with RV1B. IL-6 protein was measured by ELISA. NS, not significant.

[FP]	% IL-6 release in NHBE cells (pretreatment with FP and SM at 1 nM)			
	RV1B	SM	FP	SM+FP
nM	*mean ± S.E.*			
0.1	100	294.9 ± 57.5[a]	89.3 ± 11.9	442.4 ± 123.3[a]
1	100	353.0 ± 23.9[c]	51.5 ± 6.7	180.2 ± 25.0[a]
10	100	313.1 ± 25.1[b]	46.7 ± 2.2	110.9 ± 42.7[d]

[a] $p < 0.05$.
[b] $p < 0.01$.
[c] $p < 0.001$.
[d] NS, not significant.

different from medium-treated cells, and peaked at 8 h post-treatment ($p < 0.01$, Fig. 3*B*).

SM Induction of IL-6 Is cAMP- and β-Receptor-dependent—The SM induction of IL-6 was blocked using the β-receptor antagonist propranolol in a dose-dependent manner (Fig. 4*A*). Dibutryl cAMP augmented RV16-induced IL-6 and also induced IL-6, statistically significant at 1 mM ($p < 0.01$ compared with RV16-infected and $p < 0.05$ compared with medium-treated Fig. 4*B*). Furthermore, the adenylate cyclase activator forskolin also induced IL-6 in a dose-dependent manner (Fig. 4*C*). Finally the phosphodiesterase inhibitor rolipram also induced IL-6 in BEAS-2B cells (Fig. 4*D*). Rolipram and cAMP also induced IL-6 mRNA at 8 and 24 h post-treatment in BEAS-2B cells when compared with cells treated with medium (Table 3).

Importance of NF-κB in Rhinovirus-induced IL-6—Because various data implicate the transcription factor NF-κB in RV-induced proinflammatory cytokine gene expression (25, 30), we investigated the role of NF-κB in RV-induced IL-6 production. The role of NF-κB was first assessed using mutated or deleted constructs of the human IL-6 promoter (depicted in Fig. 5*A*) in transient transfection experiments in BEAS-2B cells. RV16 up-regulated the IL-6 promoter 24–72 h post-infection (data not shown), with maximal promoter activation occurring at 72 h. RV16 up-regulated a −651-bp fragment of the human IL-6 promoter compared with medium ($p < 0.001$), and this induction was abrogated when using promoter constructs with an NF-κB mutation (ΔNF-κB), both NF-κB and c/EBP mutation (ΔNF-κB/ΔcEBP, $p < 0.001$ compared with induction with the −651-bp fragment, Fig. 5*B*). There was no difference between induction with the −651-bp fragment and the −160-bp fragment or with the IL-6 promoter with a mutated AP-1 site (ΔAP-1) or a promoter with mutated c/EBP site (Δc/EBP). IL-6 promoter activation was also inhibited using a DN mutant of IκB, which prevents NF-κB nuclear translocation (Fig. 5*C*). IL-6 promoter activation was inhibited in the presence of the IκB DN compared with cells transfected with IL-6 promoter and empty vector control ($p < 0.001$). Using EMSA- on RV16-infected nuclear extracts and oligonucleotides designed on the NF-κB site within the human IL-6 promoter, a protein-oligonucleotide band shift was observed that was out-competed with excess unlabeled oligonucleotide (50× and 100× excess). However, the band was not competed by unlabeled oligonucleotide having mutations within the NF-κB binding site (Fig. 5*D*).

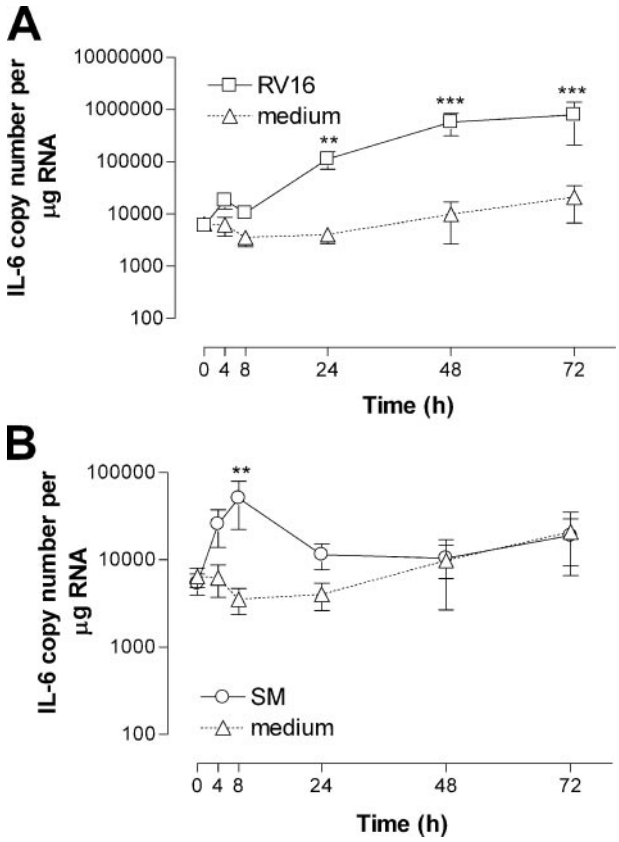

FIGURE 3. **Time course of RV16 and SM-induced IL-6 mRNA synthesis in BEAS-2B cells.** *A*, RV1B induced IL-6 mRNA in a time-dependent manner, peaking at 48–72 h. *B*, SM induced IL-6 mRNA in a time-dependent manner, peaking at 8 h. **, $p < 0.01$; ***, $p < 0.001$ (*versus* medium-treated cells). All IL-6 mRNA measurements were performed by quantitative reverse transcription-PCR normalized to constitutive 18 S rRNA expression; $n = 5$ experiments.

Finally, the crucial role of NF-κB was confirmed, and a role for the IκB kinase IKK-β using a pharmalogic inhibitor of IKK-β, AS602868, which reduced RV16-induced IL-6 protein release in a dose-dependent manner ($p < 0.001$ *versus* infected, untreated control; Fig. 5*E*).

FP and SM Regulate Rhinovirus-induced IL-6 via Distinct Cis-acting Elements—Because FP down-regulated IL-6 protein levels, whereas SM induced IL-6 protein and mRNA, we used IL-6 promoter-reporter constructs to determine whether these effects were mediated by distinct cis-acting elements. FP reduced IL-6 promoter activation in a dose-dependent manner (Fig. 6*A*). Similar results were observed when using a −651-bp fragment of the IL-6 promoter or a −160-bp fragment ($p < 0.05$ compared with RV16 infected, Fig. 6*B*). To determine whether a TATA proximal negative GRE (nGRE) was involved in FP-mediated suppression of the IL-6 promoter activation, we produced a −651-bp fragment of the promoter with a mutated GRE (*−651 ΔGRE*, Fig. 6*C*). When compared with the parental −651-bp fragment, this mutated promoter was not steroid-responsive ($p > 0.05$ compared with the −651-bp promoter) but was still RV16-inducible (Fig. 6*D*).

These same IL-6 promoter constructs were used to determine any cis-acting sites capable of mediating SM augmentation of RV16-induced IL-6. SM enhanced RV16-induced

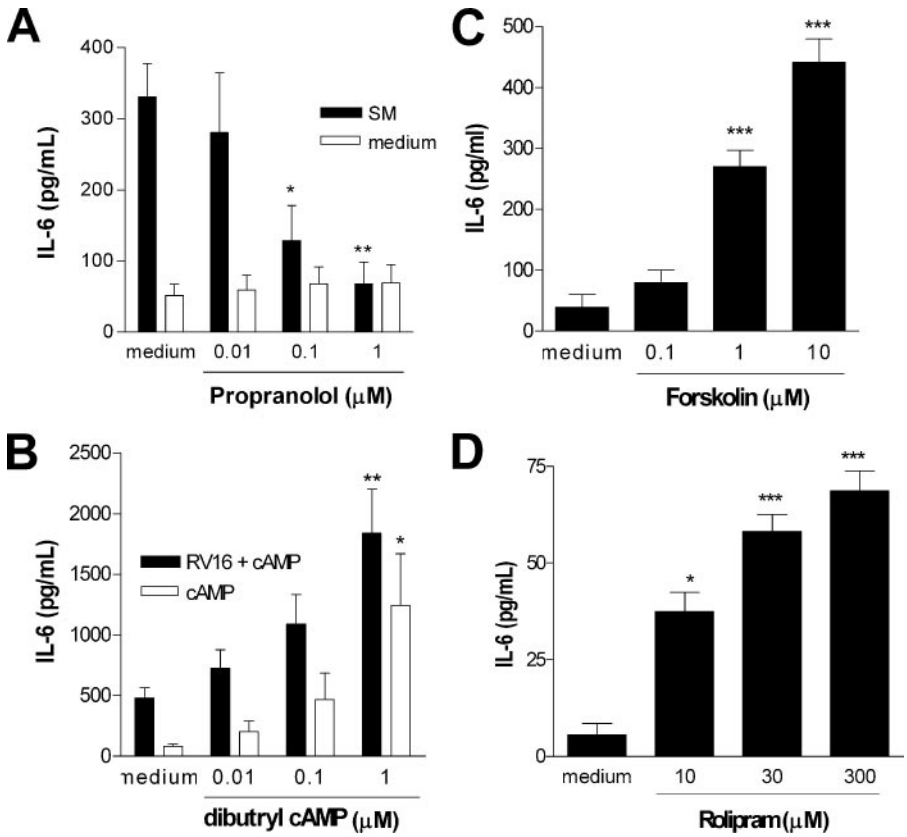

FIGURE 4. **Induction of IL-6 by SM is β-receptor-dependent and requires cAMP in BEAS-2B cells.** *A*, SM-induced IL-6 protein at 24 h was blocked in a dose-dependent manner by propranolol a β-receptor antagonist; *, $p < 0.05$; **, $p < 0.01$ (*versus* medium-retreated SM-treated). *B*, cAMP induced IL-6 protein and augmented RV16-induced IL-6 in a dose-dependent manner; *, $p < 0.05$; **, $p < 0.01$, (*versus* medium-treated only or RV-infected only). The adenylate cyclase activator forskolin (*C*) and the phosphodiesterase inhibitor rolipram (*D*) induced IL-6 protein at 24 h in a dose-dependent manner. *, $p < 0.05$; ***, $p <$ (0.001 *versus* medium-treated cells). All IL-6 protein measurements were performed by ELISA; $n = 3–4$ experiments.

TABLE 3

Induction of IL-6 mRNA by cAMP or rolipram

BEAS-2B cells were treated with medium, rolipram, or cAMP for 8 or 24 h, and IL-6 mRNA was measured by TaqMan reverse transcription-PCR.

Treatment	Copy number of IL-6 mRNA per μg of total RNA	
	8 h	24 h
	mean ± S.E.	
Medium	6,581 ± 1,294	9,541 ± 2,328
Rolipram	60,615 ± 20,587[a]	74,275 ± 17,090[b]
cAMP	119,099 ± 45,724[b]	153,032 ± 38,364[c]

[a] $p < 0.05$.
[b] $p < 0.01$.
[c] $p < 0.001$.

IL-6 promoter activation using the −651-bp construct ($p < 0.05$, Fig. 7*A*) but not the −160-bp construct (lacking AP-1 and CRE) or −651-bp constructs with a mutated NF-κB, NF-κB, and c/EBP or c/EBP sites ($p > 0.05$). To confirm the importance of the CRE or c/EBP sites, −651-bp constructs with mutated CRE sites were designed (*−651ΔCRE*, Fig. 7*B*). Further experiments suggested that it is the c/EBP site that is required for the SM augmentation of RV16-induced IL-6, as there was no significant augmentation of RV16-induced reporter activation with 651 Δc/EBP ($p > 0.05$); however, the 651 ΔCRE construct was still responsive to SM treatment ($p < 0.01$, Fig. 7*C*).

DISCUSSION

IL-6 is a pleiotropic cytokine associated with inflammatory lung diseases including asthma and COPD. The biological effects of IL-6 include B-lymphocyte maturation, monocyte-macrophage differentiation, induction of the acute phase response in hepatocytes, and other immunomodulatory properties. In this study we have analyzed the transcriptional regulation of the IL-6 gene by rhinovirus and the modulation of this gene by both GCs and β_2 agonists.

The IL-6 promoter is well characterized and consists of several cis-acting sites that have been shown to be responsive to various stimuli. Several studies have shown the NF-κB, AP-1, c/EBP (NF-IL-6), and CRE sites to bind their respective trans-activating proteins in a range of model systems (23, 24); therefore, the human IL-6 promoter is an excellent model system of a complex human gene relevant to human disease. Our data and others (25, 31) show that RV requires NF-κB for IL-6 promoter inducibility, and we have further shown that NF-IL6 or AP-1 is not required in BEAS-2B cells. The NF-κB or Rel family of transcription factors are cytosolic proteins implicated in the regulation of more than 100 different genes (32). Activation of NF-κB occurs via phosphorylation, ubiquitination, and then degradation of its cytosolic inhibitor, IκB, via several different kinases, notably IKK-α/β. Once free from its inhibitor, NF-κB translocates to the nucleus and binds to recognition sites within promoters of NF-κB-responsive genes. The activation of NF-κB by RV is well documented (31, 33, 34), and NF-κB has also been implicated in the transcriptional regulation of many genes induced by RV (26, 30, 34–36). The present study also supports evidence that IL-6 transcription via RV occurs in a IKK-β-dependent manner.

RV-induced IL-6 was sensitive to pretreatment with the GC, FP. GCs act through a range of different mechanisms and can either induce or down-regulate the expression of many different genes. Induction of GC responsive genes, such as mitogen-activated kinase phosphatase-1 and Toll-like receptor-2, occurs via GRE elements within promoters of affected genes (37–39). Suppression of gene transcription is the most well studied action of GCs, and this may occur through several postulated mechanisms. GCs have been shown to make protein-protein interactions with various proinflammatory transcription factors, notably NF-κB and AP-1 in the process of transrepression (40–43), thus preventing the transcription factors from bind-

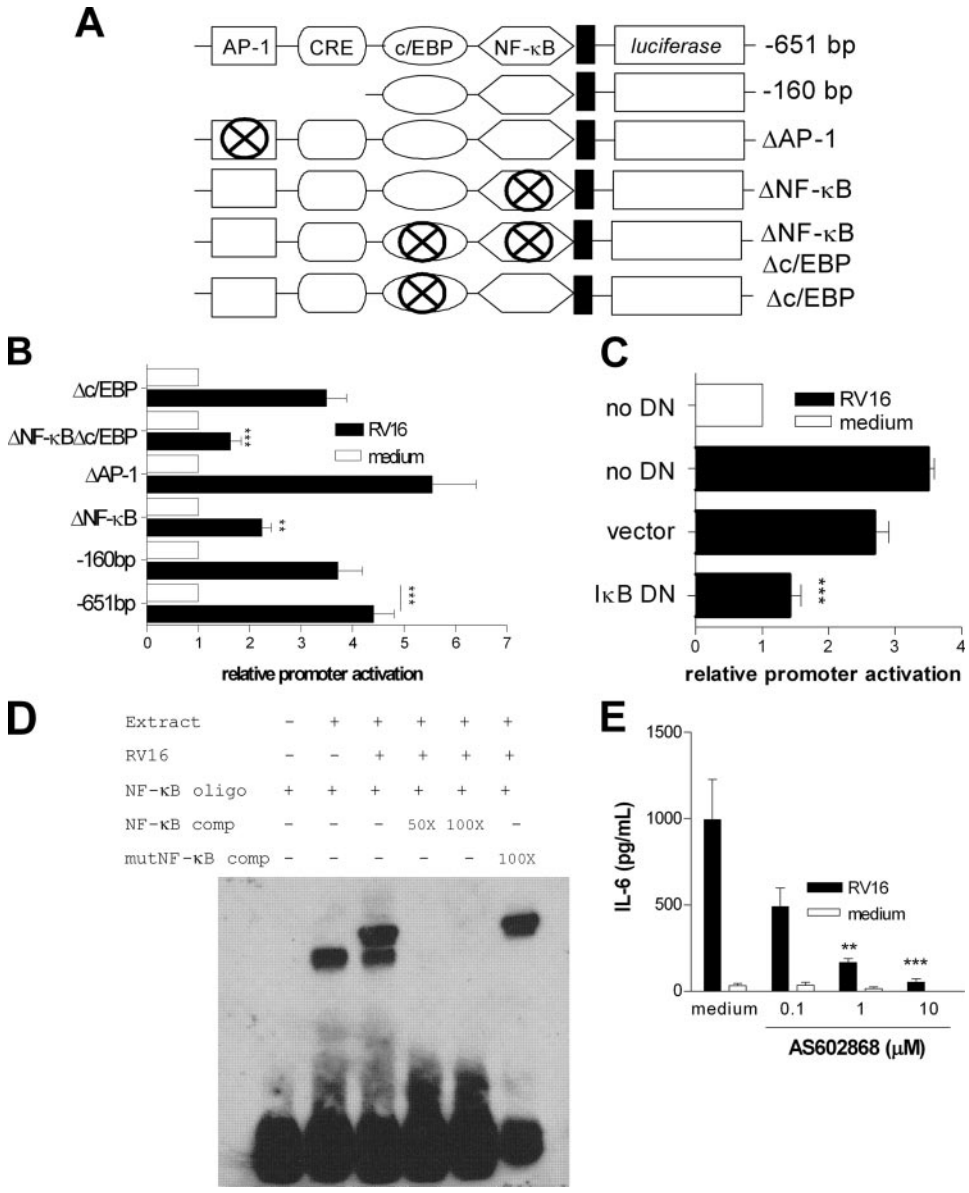

FIGURE 5. **Importance of NF-κB in RV16-induced IL-6 in BEAS-2B cells.** *A*, schematic diagram of TATA proximal cis-acting sites within the human IL-6 promoter. The wild type 651-bp parental promoter was mutated or deleted to give the corresponding reporter promoter constructs with altered transcription factor binding sites. *B*, the 651-bp promoter was induced by RV16 at 72 h post-infection, and deletion of NF-κB sequences but not AP-1 CRE or c/EBP caused a loss of promoter activity. **, $p < 0.01$; ***, $p < 0.001$ (*versus* 651-bp promoter or as indicated). *C*, transfection of the 651-bp promoter with an IκB DN but not empty vector caused suppression of IL-6 promoter activation. ***, $p < 0.001$ *versus* empty vector control. *D*, protein extracts from RV16-infected cells but not medium-treated cells caused a shift in electrophoretic pattern of a labeled oligonucleotide designed on the NF-κB binding sequence from the IL-6 promoter. *E*, the IKK-β inhibitor AS602868 suppressed RV16 and basal IL-6 protein at 24 h in a dose-dependent manner. **, $p < 0.01$; ***, $p < 0.001$ *versus* (RV16-infected-only cells or medium-treated-only cells). All luciferase measurements were normalized to constitutive β-galactosidase measurements and IL-6 protein was performed by ELISA $n = 4–6$ experiments.

ing their cis-acting sites and recruiting co-activators to the transcription initiation complex. GCs may also modulate chromatin by enhancing histone deacetylation (44) and decreasing histone acetylation (45), causing DNA to remain protein bound and preventing transcription factor access to unwound DNA and, hence, accessible cis-acting sites.

Careful scrutiny of the IL-6 promoter has identified at least two nGREs, at −170 bp relative to the transcriptional start site and one close to the TATA box and transcription start site (46). Upon steroid treatment, these sites confer steroid sensitivity in HeLa cells, indicating IL-6 is different from most genes in that promoter-specific nGRE sites act to down-regulate transcription rather than enhance it (46). We have confirmed this initial study by showing that suppression of RV-induced IL-6 occurs via a single nGRE site near the TATA box and transcription start site (−5 to +10). Mutation of this site using site-directed mutagenesis resulted in a loss of FP sensitivity to the −651-bp fragment of the IL-6 promoter. A construct (−160-bp fragment) lacking another GRE site located further upstream of the TATA box at position −170 bp was still FP-sensitive, indicating that the downstream TATA proximal site was crucial in mediating GC suppression of RV-induced IL-6. An nGRE element at this position could function by binding the GR dimer and by blocking RNA polymerase activity at the transcription start site via steric hindrance or by preventing association of transcription factors with their co-activators CBP or p300 and, hence, association of the IL-6 enhancersome. Further experiments are required to establish the finer details of how this unique sequence interacts with the GR and affects IL-6 transcription.

Because GCs are used in conjunction with LABAs, we were interested in the effects of SM on IL-6 alone and in conjunction with FP. We found that SM and to a lesser extent SB- and cAMP-elevating agents to be efficient inducers of IL-6 protein and mRNA. This was not due to a general toxic response of bronchial epithelial cells to SM or other agents, because in the same experiments SM had no effect on IL-1β- and RV-induced CXCL8/IL-8 or RANTES (regulated on activation normal T cell expressed and secreted)/CCL5, and in combination with FP, the combination significantly down-regulated these genes superior to FP treatment alone both in BEAS-2B cells and primary bronchial epithelial cells (22). In the present study, SM induced IL-6 in both bronchial epithelial cell lines and primary bronchial epithelial cells, indicating this was not simply a feature of the BEAS-2B cell line.

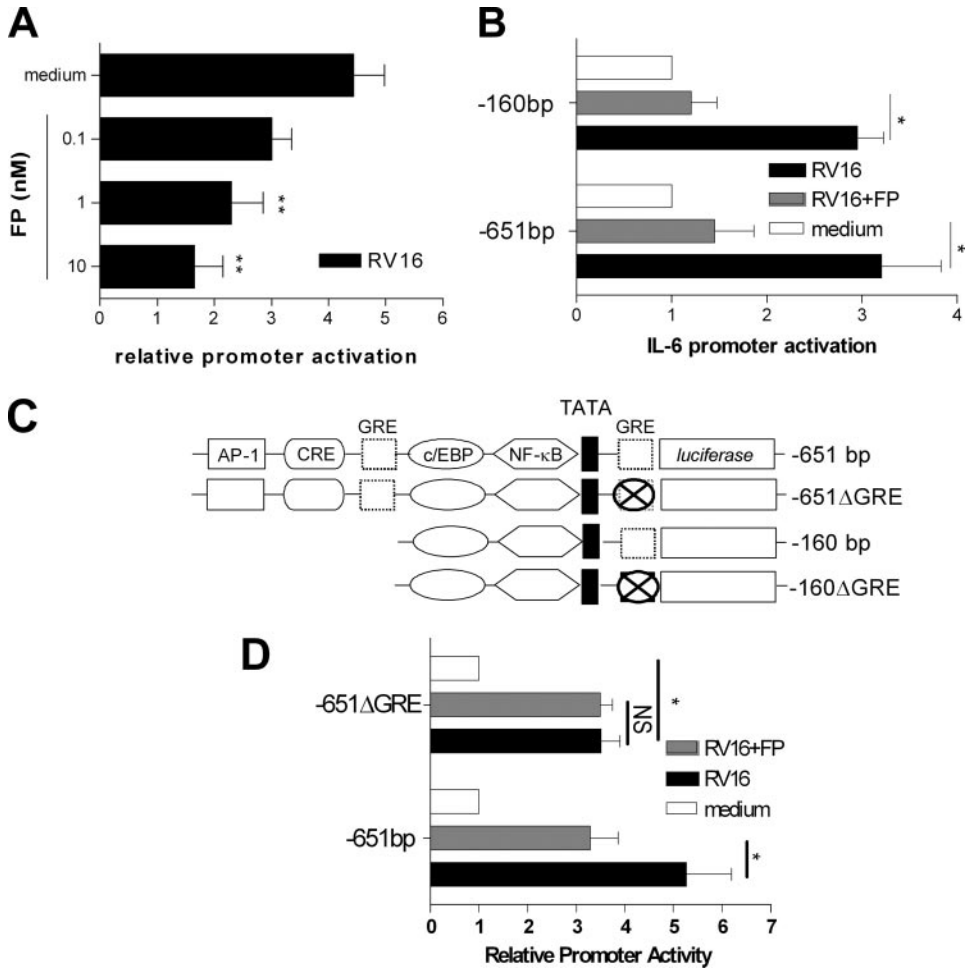

FIGURE 6. **Modulation of RV16-induced IL-6 promoter activation by FP in BEAS-2B cells.** *A*, FP suppressed RV16-induced IL-6 promoter activation in a dose-dependent manner. *B*, FP suppressed promoter activation using either a 651-bp parental promoter or a truncated 160-bp fragment. *C*, schematic diagram of the human IL-6 promoter showing the location of two GRE-like sequences. The TATA proximal sequence was mutated by site-directed mutagenesis producing the 651 ΔGRE construct. *D*, the 651 ΔGRE construct failed to respond to FP treatment and did not suppress RV16-induced IL-6 promoter activation. All luciferase measurements were normalized to constitutive β-galactosidase measurements; $n = 4–5$ experiments.

The effect of SM on IL-6 induction became clearer after careful examination of the IL-6 promoter and also consideration of the literature on IL-6 induction derived from airway smooth muscle. Two studies have investigated the effects of β_2 agonists on IL-6 in ASM and also showed these agents to be strong inducers of IL-6 (23). This can be explained by the IL-6 promoter, which is cAMP-responsive. At position −166 to −154, a CRE element exists of sequence AGGACGTCACAT that confers LABA inducibility in ASM cells (23). The IL-6 promoter also contains a c/EBP or NF-IL6 site (ATTGCACAAT, −154 to −146) that may also respond to cAMP-mediated pathways (47). These two sites both bind bZIP transcription factors; CRE binds ATF/CREB family members, whereas c/EBP binds c/EBP family members. Initial experiments clearly showed that IL-6 was induced by cAMP- or cAMP-elevating agents and that promoters truncated to −160 bp resulted in diminished ability of SM to enhance RV16-induced IL-6 promoter activation in BEAS-2B cells. Further experiments identified the c/EBP site rather than the CRE site of this region to be absolutely required for SM augmentation of IL-6 promoter activation. It was surprising that the −160-bp fragment (lacking CRE), but not 651 ΔCRE, gave diminished IL-6 promoter inducibility by SM. These discrepancies could be explained by the −160-bp fragment and the 651 ΔCRE not having exactly the same sequence with respect to nucleotides surrounding the CRE element, such that surrounding sequences missing in the −160-bp fragment, but intact in the 651 ΔCRE mutant, could influence transcription factor binding at the c/EBP site. Interestingly, this region (−173 to −145) may contain more than one CRE element, as originally proposed by Ray *et al.* (46), and this region is perhaps more complex than depicted by the figures presented in this study and by other studies (23, 24). The importance of the CRE and c/EBP sites is further complicated by the fact that CREB has weak affinity for c/EBP and that c/EBP may also bind CRE-like sequences (48, 49). In fact, CRE and c/EBP sites have been found in close proximity in a number of genes (49–51) and can act in concert to promote gene transcription; hence, the biology of these two sequences is often linked. Further studies are required to identify the transcription factors that mediated cAMP induction of IL-6 and how these transcription factors interact with CRE or c/EBP sequences.

Because cAMP and cAMP-elevating agents forskolin, an adenylate cyclase activator, and rolipram, a phosphodiesterase inhibitor, induced IL-6 protein and mRNA in BEAS-2B cells, the data point to a cAMP-responsive kinase that may induce transcriptional activation of the IL-6 gene. β_2 agonists act via adenylate cyclase, inducing intracellular cAMP, and this leads to activation of several downstream pathways, including protein kinase A. Because we did not study the entire pathway leading to IL-6 gene expression, it is unclear whether protein kinase A or another kinase is responsible for inducing c/EBP or CRE binding transcription factors. Because protein kinase A can activate both CREB and c/EBP (51) transcription factors, the role of protein kinase A in SM-induced IL-6 seems likely.

LABAs are powerful bronchodilators, and their use in asthma and COPD is largely because of this effect. In conjunction with steroids, β_2 agonists may also exhibit anti-inflammatory and anti-proliferative effects, and recent *in vitro* evidence suggests they are capable of exhibiting this property when used alone (19, 21, 22, 47). There is much more to be explored regarding the biology and immunoregulatory properties of β_2

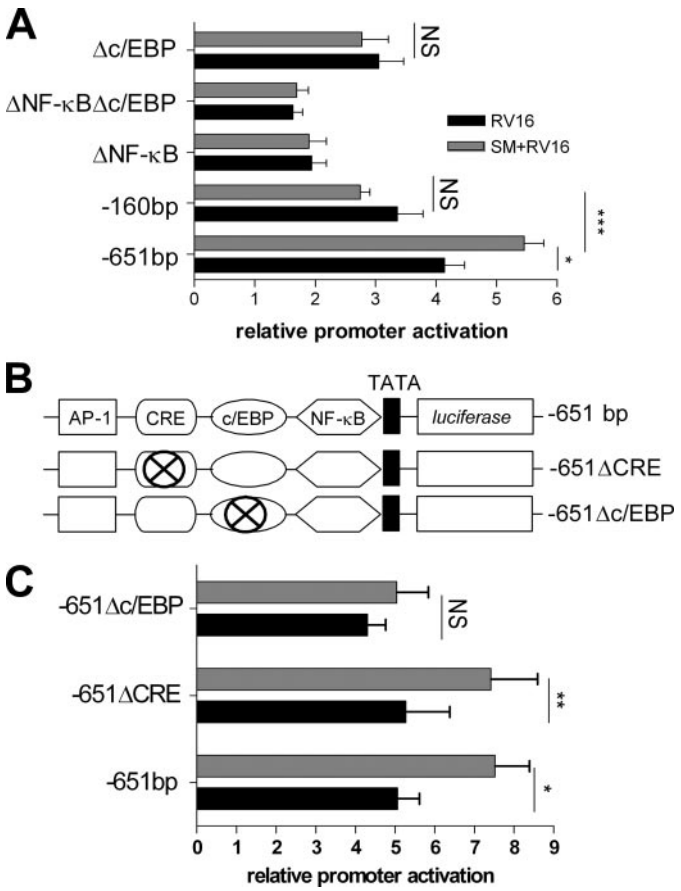

FIGURE 7. **Modulation of RV16-induced IL-6 promoter activation by SM in BEAS-2B cells.** *A*, SM augmented RV16-induced IL-6 promoter activation at 72 h using the 651-bp construct. SM did not augment RV16-induced IL-6 promoter activation using the 160-bp construct or fragments containing a mutation in the c/EBP site (651 Δc/EBP). *B*, schematic diagram showing the 651 ΔCRE construct. *C*, SM augmented RV16-induced IL-6 promoter activation using the 651 construct and the 651 ΔCRE construct but not the 651 Δc/EBP construct. All luciferase measurements were normalized to constitutive β-galactosidase measurements; $n = 6–8$ experiments. *NS*, not significant.

agonists; this study and others have demonstrated that certain cAMP-responsive genes may be up-regulated by β_2 agonists. Considering the beneficial properties of β_2 agonists in asthma, it is possible that some of the beneficial effect may be due to the up-regulation of as yet unidentified CRE responsive genes as well as the anti-inflammatory and bronchodilator effect. Further studies are required to investigate which genes involved in asthma and COPD are regulated by β_2 agonists.

Whether IL-6 plays a helpful or harmful role in the context of disease remains largely unanswered. Classically, IL-6 is cited as a proinflammatory cytokine, the overproduction of which is implicated in the pathology of many inflammatory disorders. There is good evidence to suggest that IL-6 contributes to the pathogenesis of rheumatoid arthritis and inflammatory bowel diseases, such as Crohn disease. For example, IL-6 knock-out mice are resistant to antigen-induced experimental arthritis (52). Elevated IL-6 serum levels also correlate with the severity of Crohn disease (53). The role of IL-6 in respiratory diseases, however, is not so clear. IL-6 plays a role in O_3-induced lung injury and pulmonary inflammation yet is not involved in O_3-induced airway hyperresponsiveness (54). Furthermore, transgenic mice overexpressing IL-6 had no effect on lung function (55). Meanwhile, in the endotoxic lung, IL-6 may have an anti-inflammatory role as mice deficient in IL-6 have higher levels of proinflammatory cytokines tumor necrosis factor-α and macrophage inflammatory protein-2 (56) and inflammatory cells after exposure to an aerosolized allergen (57). The above data suggest that whereas IL-6 may have some inflammatory properties *in vivo*, this does not affect airway function in the above models. Also, our data provide evidence that steroids and β_2 agonists should be given together rather than as separate treatments, as steroids potentially down-regulate potential proinflammatory genes induce by β_2 agonists.

In summary, GCs and β_2 agonists have differing effects on IL-6 transcription in bronchial epithelial cells. GCs suppress IL-1β and RV-induced IL-6 via a unique mechanism involving an nGRE element proximal to the TATA box. In contrast, β_2 agonists and cAMP elevating agents induce IL-6 and augment RV- and IL-1β-induced IL-6 via c/EBP and/or CRE sites within the IL-6 promoter. The data suggest that not all proinflammatory genes are affected in the same manner by GCs and β_2 agonists and that an understanding of the transcriptional regulation of proinflammatory genes can assist in understanding how these two common asthma treatments affect their expression.

REFERENCES

1. Nicholson, K. G., Kent, J., and Ireland, D. C. (1993) *BMJ* **307,** 982–986
2. Johnston, S. L., Pattemore, P. K., Sanderson, G., Smith, S., Lampe, F., Josephs, L., Symington, P., O'Toole, S., Myint, S. H., Tyrrell, D. A., and Holgate, S. T. (1995) *BMJ* **310,** 1225–1229
3. Corne, J. M., Marshall, C., Smith, S., Schreiber, J., Sanderson, G., Holgate, S. T., and Johnston, S. L. (2002) *Lancet* **359,** 831–834
4. Wark, P. A., Johnston, S. L., Moric, I., Simpson, J. L., Hensley, M. J., and Gibson, P. G. (2002) *Eur. Respir. J.* **19,** 68–75
5. Johnston, N. W., Johnston, S. L., Duncan, J. M., Greene, J. M., Kebadze, T., Keith, P. K., Roy, M., Waserman, S., and Sears, M. R. (2005) *J. Allergy Clin. Immunol.* **115,** 132–138
6. Papi, A., Bellettato, C. M., Braccioni, F., Romagnoli, M., Casolari, P., Caramori, G., Fabbri, L. M., and Johnston, S. L. (2006) *Am. J. Respir. Crit. Care Med.* **173,** 1114–1121
7. Wilkinson, T. M., Donaldson, G. C., Johnston, S. L., Openshaw, P. J., and Wedzicha, J. A. (2006) *Am. J. Respir. Crit. Care Med.* **173,** 871–876
8. Rakes, G. P., Arruda, E., Ingram, J. M., Hoover, G. E., Zambrano, J. C., Hayden, F. G., Platts-Mills, T. A., and Heymann, P. W. (1999) *Am. J. Respir. Crit. Care Med.* **159,** 785–790
9. Rawlinson, W. D., Waliuzzaman, Z., Carter, I. W., Belessis, Y. C., Gilbert, K. M., and Morton, J. R. (2003) *J. Infect. Dis.* **187,** 1314–1318
10. Seemungal, T. A., Harper-Owen, R., Bhowmik, A., Jeffries, D. J., and Wedzicha, J. A. (2000) *Eur. Respir. J.* **16,** 677–683
11. Fleming, H. E., Little, F. F., Schnurr, D., Avila, P. C., Wong, H., Liu, J., Yagi, S., and Boushey, H. A. (1999) *Am. J. Respir. Crit. Care Med.* **160,** 100–108
12. Pizzichini, M. M., Pizzichini, E., Efthimiadis, A., Chauhan, A. J., Johnston, S. L., Hussack, P., Mahony, J., Dolovich, J., and Hargreave, F. E. (1998) *Am. J. Respir. Crit. Care Med.* **158,** 1178–1184
13. Grunberg, K., Smits, H. H., Timmers, M. C., de Klerk, E. P., Dolhain, R. J., Dick, E. C., Hiemstra, P. S., and Sterk, P. J. (1997) *Am. J. Respir. Crit. Care Med.* **156,** 609–616
14. Wilkinson, T. M., Hurst, J. R., Perera, W. R., Wilks, M., Donaldson, G. C., and Wedzicha, J. A. (2006) *Chest* **129,** 317–324
15. Hurst, J. R., Perera, W. R., Wilkinson, T. M., Donaldson, G. C., and Wedzicha, J. A. (2006) *Am. J. Respir. Crit. Care Med.* **173,** 71–78
16. Pauwels, R. A., Lofdahl, C. G., Postma, D. S., Tattersfield, A. E., O'Byrne, P., Barnes, P. J., and Ullman, A. (1997) *N. Engl. J. Med.* **337,** 1405–1411
17. Calverley, P., Pauwels, R., Vestbo, J., Jones, P., Pride, N., Gulsvik, A.,

Anderson, J., and Maden, C. (2003) *Lancet* **361,** 449–456
18. Pelaia, G., Vatrella, A., Cuda, G., Maselli, R., and Marsico, S. A. (2003) *Life Sci.* **72,** 1549–1561
19. Korn, S. H., Jerre, A., and Brattsand, R. (2001) *Eur. Respir. J.* **17,** 1070–1077
20. Pang, L., and Knox, A. J. (2000) *Am. J. Respir. Cell Mol. Biol.* **23,** 79–85
21. Pang, L., and Knox, A. J. (2001) *FASEB J.* **15,** 261–269
22. Edwards, M. R., Johnson, M. W., and Johnston, S. L. (2006) *Am. J. Respir. Cell Mol. Biol.* **34,** 616–624
23. Ammit, A. J., Lazaar, A. L., Irani, C., O'Neill, G. M., Gordon, N. D., Amrani, Y., Penn, R. B., and Panettieri, R. A., Jr. (2002) *Am. J. Respir. Cell Mol. Biol.* **26,** 465–474
24. Eickelberg, O., Pansky, A., Mussmann, R., Bihl, M., Tamm, M., Hildebrand, P., Perruchoud, A. P., and Roth, M. (1999) *J. Biol. Chem.* **274,** 12933–12938
25. Zhu, Z., Tang, W., Ray, A., Wu, Y., Einarsson, O., Landry, M. L., Gwaltney, J., Jr., and Elias, J. A. (1996) *J. Clin. Investig.* **97,** 421–430
26. Papi, A., and Johnston, S. L. (1999) *J. Biol. Chem.* **274,** 9707–9720
27. Johnston, S. L., Papi, A., Bates, P. J., Mastronarde, J. G., Monick, M. M., and Hunninghake, G. W. (1998) *J. Immunol.* **160,** 6172–6181
28. Malkoski, S. P., and Dorin, R. I. (1999) *Mol. Endocrinol.* **13,** 1629–1644
29. Vanden Berghe, W., De Bosscher, K., Boone, E., Plaisance, S., and Haegeman, G. (1999) *J. Biol. Chem.* **274,** 32091–32098
30. Zhu, Z., Tang, W., Gwaltney, J. M., Jr., Wu, Y., and Elias, J. A. (1997) *Am. J. Physiol.* **273,** L814–L824
31. Kim, J., Sanders, S. P., Siekierski, E. S., Casolaro, V., and Proud, D. (2000) *J. Immunol.* **165,** 3384–3392
32. Karin, M., Yamamoto, Y., and Wang, Q. M. (2004) *Nat. Rev. Drug Discov.* **3,** 17–26
33. Funkhouser, A. W., Kang, J. A., Tan, A., Li, J., Zhou, L., Abe, M. K., Solway, J., and Hershenson, M. B. (2004) *Pediatr. Res.* **55,** 13–18
34. Laza-Stanca, V., Stanciu, L. A., Message, S. D., Edwards, M. R., Gern, J. E., and Johnston, S. L. (2006) *J. Virol.* **80,** 8248–8258
35. Papi, A., and Johnston, S. L. (1999) *J. Biol. Chem.* **274,** 30041–30051
36. Spurrell, J. C., Wiehler, S., Zaheer, R. S., Sanders, S. P., and Proud, D. (2005) *Am. J. Physiol.* **289,** L85–L95
37. Hermoso, M. A., Matsuguchi, T., Smoak, K., and Cidlowski, J. A. (2004) *Mol. Cell. Biol.* **24,** 4743–4756
38. Abbinante-Nissen, J. M., Simpson, L. G., and Leikauf, G. D. (1995) *Am. J. Physiol.* **268,** L601–L606
39. Usmani, O. S., Ito, K., Maneechotesuwan, K., Ito, M., Johnson, M., Barnes, P. J., and Adcock, I. M. (2005) *Am. J. Respir. Crit. Care Med.* 172, 704–712
40. De Bosscher, K., Vanden Berghe, W., Vermeulen, L., Plaisance, S., Boone, E., and Haegeman, G. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 3919–3924
41. Tao, Y., Williams-Skipp, C., and Scheinman, R. I. (2001) *J. Biol. Chem.* **276,** 2329–2332
42. Adcock, I. M., Nasuhara, Y., Stevens, D. A., and Barnes, P. J. (1999) *Br. J. Pharmacol.* **127,** 1003–1011
43. Tuckermann, J. P., Reichardt, H. M., Arribas, R., Richter, K. H., Schutz, G., and Angel, P. (1999) *J. Cell Biol.* **147,** 1365–1370
44. Ito, K., Barnes, P. J., and Adcock, I. M. (2000) *Mol. Cell. Biol.* **20,** 6891–6903
45. Ito, K., Jazrawi, E., Cosio, B., Barnes, P. J., and Adcock, I. M. (2001) *J. Biol. Chem.* **276,** 30208–30215
46. Ray, A., LaForge, K. S., and Sehgal, P. B. (1990) *Mol. Cell. Biol.* **10,** 5736–5746
47. Roth, M., Johnson, P. R., Rudiger, J. J., King, G. G., Ge, Q., Burgess, J. K., Anderson, G., Tamm, M., and Black, J. L. (2002) *Lancet* **360,** 1293–1299
48. Flammer, J. R., Popova, K. N., and Pflum, M. K. (2006) *Biochemistry* **45,** 9615–9623
49. Tsukada, J., Saito, K., Waterman, W. R., Webb, A. C., and Auron, P. E. (1994) *Mol. Cell. Biol.* **14,** 7285–7297
50. Wardlaw, S. A., Zhang, N., and Belinsky, S. A. (2002) *Mol. Pharmacol.* **62,** 326–333
51. McCauslin, C. S., Heath, V., Colangelo, A. M., Malik, R., Lee, S., Mallei, A., Mocchetti, I., and Johnson, P. F. (2006) *J. Biol. Chem.* **281,** 17681–17688
52. Boege, U., Kobasa, D., Onodera, S., Parks, G. D., Palmenberg, A. C., and Scraba, D. G. (1991) *Virology* **181,** 1–13
53. Holtkamp, W., Stollberg, T., and Reis, H. E. (1995) *J. Clin. Gastroenterol.* **20,** 123–126
54. Johnston, R. A., Schwartzman, I. N., Flynt, L., and Shore, S. A. (2005) *Am. J. Physiol.* **288,** L390–L397
55. Kuhn, C., III, Homer, R. J., Zhu, Z., Ward, N., Flavell, R. A., Geba, G. P., and Elias, J. A. (2000) *Am. J. Respir. Cell Mol. Biol.* **22,** 289–295
56. Xing, Z., Gauldie, J., Cox, G., Baumann, H., Jordana, M., Lei, X. F., and Achong, M. K. (1998) *J. Clin. Investig.* **101,** 311–320
57. Qiu, Z., Fujimura, M., Kurashima, K., Nakao, S., and Mukaida, N. (2004) *Clin. Exp. Allergy* **34,** 1321–1328