

Rhinovirus infection causes steroid resistance in airway epithelium through nuclear factor κ B and c-Jun N-terminal kinase activation

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Background: Although inhaled glucocorticoids are the mainstays of asthma treatment, they are poorly effective at treating and preventing virus-induced asthma exacerbations. The major viruses precipitating asthma exacerbations are rhinoviruses.

Objective: We sought to evaluate whether rhinovirus infection interferes with the mechanisms of action of glucocorticoids.

Methods: Cultured primary human bronchial or transformed (A549) respiratory epithelial cells were infected with rhinovirus 16 (RV-16) before dexamethasone exposure. Glucocorticoid receptor (GR) α nuclear translocation, glucocorticoid response element (GRE) binding, and transactivation/transrepression functional readouts were evaluated by using immunocytochemistry, Western blotting, DNA binding assays,

real-time quantitative PCR, coimmunoprecipitation, and ELISA techniques. Specific inhibitors of c-Jun N-terminal kinase (JNK) and of I κ B kinase (IKK) were used to investigate the involvement of intracellular signaling pathways.

Results: RV-16 infection impaired dexamethasone-dependent (1) inhibition of IL-1 β -induced CXCL8 release, (2) induction of mitogen-activated protein kinase phosphatase 1 gene expression, and (3) binding of GR to GREs in airway epithelial cells. This was associated with impaired GR α nuclear translocation, as assessed by means of both immunocytochemistry (54.0% $\pm$ 6.8% vs 24.7% $\pm$ 3.8% GR-positive nuclei after 10 nmol/L dexamethasone treatment in sham- or RV-16-infected cells, respectively; $P < .01$) and Western blotting. RV-16 infection induced nuclear factor κ B activation and GR α phosphorylation, which were prevented by inhibitors of IKK2 and JNK, respectively. In rhinovirus-infected cells the combination of JNK and IKK2 inhibitors totally restored dexamethasone suppression of CXCL8 release, induction of mitogen-activated protein kinase phosphatase 1 gene expression, and GR α nuclear translocation.

Conclusion: RV-16 infection of human airway epithelium induces glucocorticoid resistance. Inhibition of RV-16-induced JNK and nuclear factor κ B activation fully reversed rhinovirus impairment of both GR α nuclear translocation and the transactivation/transrepression activities of glucocorticoids. (*J Allergy Clin Immunol* 2013;■■■:■■■-■■■.)

Key words: Asthma, viral respiratory tract infections, glucocorticoids, transcription factors, kinases

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Asthma is a chronic inflammatory disease of the lower airways, and regular treatment with inhaled corticosteroids (ICSs) represents the mainstay of asthma management.¹ Viral infections have been identified as the most frequent triggers of asthma exacerbations and are associated with 80% to 85% of exacerbations in children and adults.^{2,3} Rhinoviruses are the most frequently identified viruses in patients with asthma exacerbations.^{4,5} A human model has been developed recently, confirming, in experimentally controlled conditions, that rhinovirus infection can exacerbate asthma.⁶

Although the precise mechanisms by which viral infections exacerbate asthma are not completely clear, there is evidence that rhinovirus can infect both the upper and lower respiratory tracts and that rhinovirus infection worsens pre-existing inflammation in asthmatic airways.^{2,7} Asthma exacerbations are characterized by increased airway inflammation that occurs despite anti-inflammatory treatment. Thus they can be considered episodes of transient failure of the mechanisms involved in containing and controlling airway inflammation. Notably, several studies show that high doses of ICSs or systemic corticosteroids are

Abbreviations used

EC ₅₀ :	Median effective concentration
E _{max} :	Maximal effective concentration
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
GR:	Glucocorticoid receptor
GRE:	Glucocorticoid response element
HBEC:	Human bronchial epithelial cell
ICAM-1:	Intercellular adhesion molecule 1
ICS:	Inhaled corticosteroid
IKK:	IκB kinase
JNK:	c-Jun N-terminal kinase
MKP-1:	Mitogen-activated protein kinase phosphatase
MOI:	Multiplicity of infection
NF-κB:	Nuclear factor κB
RV-16:	Rhinovirus 16
RV-1B:	Rhinovirus type 1B
siRNA:	Small interfering RNA

ineffective in the treatment/prevention of virus-induced acute asthma exacerbations, particularly in children.⁸⁻¹³ This is in line with experimental evidence that (1) ICSs do not prevent worsening of airway inflammation induced by experimental rhinovirus infection in asthmatic patients^{14,15}; (2) oral prednisone therapy does not provide any clinical benefit in experimental rhinovirus infection¹⁶; and (3) budesonide does not inhibit cytokine production in nasal lavage fluid during experimental rhinovirus infection in asthmatic and nonasthmatic subjects.¹⁷ The mechanisms by which viral infections make corticosteroid treatment ineffective are unknown. Glucocorticoids act by binding to cytosolic glucocorticoid receptor (GR) α, which, on binding, becomes activated and rapidly translocates to the nucleus. Within the nucleus, activated GRα switches on anti-inflammatory pathways through binding to glucocorticoid response elements (GREs) in the promoter regions of responsive genes, such as mitogen-activated protein kinase phosphatase (MKP) 1.¹⁸ Alternatively, activated GRs can repress the ability of proinflammatory signaling pathways, such as mitogen-activated protein kinases and nuclear factor κB (NF-κB), to enhance inflammatory gene expression by means of transrepression.¹⁹

The potential of viral infections to interfere with the anti-inflammatory activities of glucocorticoids has not been extensively investigated, and the underlying molecular mechanisms are unknown. Because respiratory epithelial cells are the major site for viral infection and replication, we have performed studies in these cells and demonstrate that rhinovirus infection induces corticosteroid resistance and that this process requires activation of the NF-κB and c-Jun N-terminal kinase (JNK) pathways.

METHODS**Viral stocks**

Rhinovirus 16 (RV-16; a major group rhinovirus, the receptor of which on the cell surface is intercellular adhesion molecule 1 [ICAM-1]) was obtained from the European Collection of Cell Cultures, which has incorporated the original viral strains from the MRC Common Cold Unit (Salisbury, United Kingdom). Viral stocks were prepared by means of infection of sensitive cell monolayers (Ohio HeLa cells), as described elsewhere.²⁰

For selected experiments rhinovirus type 1B (RV-1B; a minor group virus that binds to members of the low-density lipoprotein receptor family), also obtained from the European Collection of Cell Cultures, was used to evaluate whether the results were group/receptor restricted. Filtration of the virus from

inoculum to remove viral particles was performed, as previously described.²⁰ RV16 at a multiplicity of infection (MOI) of 5 and RV-1B at an MOI of 1 were used for all the experiments, unless otherwise stated. Filtered viral (f-RV) stocks were used as a negative control.

Cell culture

Ohio HeLa cells were obtained from the European Collection of Cell Cultures, and A549 cells, a type II respiratory cell line, were obtained from the American Type Culture Collection (Rockville, Md). Primary human bronchial epithelial cells (HBECs) were obtained from bronchial brushings of healthy nonsmoking volunteers and cultured, as previously described.^{21,22} These cells are more than 95% cytokeratin 18 immunoreactive, as assessed by means of immunocytochemistry. The study was approved by the local ethics committee of the University Hospital of Ferrara, and informed consent was obtained from each participant in accordance with the principles outlined in the Declaration of Helsinki.

Rhinovirus, medium alone, or f-RV inocula were added to the cells when approximately 80% confluent for 1 hour at room temperature with shaking. Viral preparations were removed, and 1 mL of medium containing dexamethasone (10^{-12} to 10^{-6} mol/L) or diluent alone was added. Further details on cell-culture methods are provided in the **Methods** section in this article's Online Repository at www.jacionline.org.

In selected experiments the involvement of the NF-κB and JNK pathways was evaluated by using pharmacologic tools: AS602868 (Serono, Geneva, Switzerland) or PS1145 (Sigma-Aldrich, Milan, Italy) as inhibitors of IκB kinase 2 (IKK2) (a kinase involved in NF-κB activation) and SP600125 (Sigma-Aldrich), a JNK inhibitor, were added 20 minutes before RV-16 infection. Further details are provided in the **Methods** section in this article's Online Repository.

Immunocytochemistry for GRα in airway cells

Immunocytochemistry with peroxidase detection for GRα was performed, as previously described, with minor modifications.²³ GRα nuclear translocation was evaluated 1 hour after dexamethasone or diluent alone, unless otherwise stated. Where specified, cells were infected with rhinovirus 1 hour before dexamethasone exposure. As previously described, a nucleus was counted as positively stained when more than 50% of the nuclear surface was stained with the tested antibody.²⁴ Details are provided in the **Methods** section in this article's Online Repository.

Western blotting for GRα in airway cells

Western blotting for GRα detection was performed, as previously described.²⁵ Western blot analyses were performed in nuclear and cytosolic fractions of cell cultures performed in 150-cm² flasks at approximately 80% confluence. GRα nuclear translocation was evaluated 1 hour after dexamethasone or diluent alone, unless otherwise stated. Where specified, cells were infected with rhinovirus 1 hour before dexamethasone exposure. Details on preparation of nuclear and cytosolic fractions and of the Western blotting technique are provided in the **Methods** section in this article's Online Repository.

CXCL8 ELISA

CXCL8 IL-8 levels were measured by means of sandwich ELISA, according to the manufacturer's recommendations (R&D Systems Europe, Abingdon, United Kingdom), in cell-culture supernatants at 24 hours after IL-1β stimulation (1 ng/mL). The minimum detectable concentration was 10 pg/mL. Details are provided in the **Methods** section in this article's Online Repository.

Real-time quantitative PCR detection of MKP-1 mRNA

Commercially available kits were used to extract total cellular RNA (Rneasy; Qiagen, Crawley, United Kingdom) and to perform reverse transcription (Omniscript RT, Qiagen). The gene transcript level of MKP-1

(CL-100), the transcription of which is induced by glucocorticoids,²⁶ and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were quantified by means of real-time PCR by using a QuantiTect SYBR Green PCR kit (Qiagen) with a Rotor-Gene 3000 PCR machine (Corbett Research, Cambridge, United Kingdom). MKP-1 mRNA levels were evaluated at 4 hours after 10^{-8} mol/L dexamethasone stimulation in A549 cell and HBEC lysates. In some experiments cells were infected with RV-16 1 hour before dexamethasone exposure. Details are provided in the Methods section in this article's Online Repository.

GR-GRE binding

Nuclear extracts were prepared in A549 cells with a nuclear protein extraction kit (Active Motif, Rixensart, Belgium). Activated GR in nuclei was detected by evaluating its binding to GRE by using an ELISA-based assay (TransAM Transcription Factor Assay kit; Active Motif), according to the manufacturer's recommendations. GR-GRE binding was evaluated at 1 hour after 10^{-8} mol/L dexamethasone exposure in cells preinfected for 1 hour with RV or sham-infected cells.

GR-NF- κ B association by coimmunoprecipitation

Nuclear and cytoplasmic lysates were prepared, as described in detail in the Methods section in this article's Online Repository. The NF- κ B subunit p65 was then immunoprecipitated with 10 μ L of antibody against p65 using A/G agarose slurry, as shown previously.²⁷ GR-p65 coimmunoprecipitation was evaluated in cells exposed to 1 hour of RV-16 (or shame) infection followed by 1 hour of dexamethasone (10^{-8} mol/L) stimulation.

Serine 226 phosphorylated GR

Activated GR phosphorylated at serine 226 was detected by using SDS-PAGE/Western blotting analysis with anti-GR (Ser226) antibody (Abcam, Cambridge, United Kingdom) in whole-cell lysates after 1 hour of RV-16 (or shame) infection in the presence or absence of IKK2 (PS1145, Sigma-Aldrich) or JNK (SP600125, Sigma-Aldrich) inhibitors. Band density was normalized to that of total GR by using Gelworks ID intermediate software (Ultraviolet Products, Cambridgeshire, United Kingdom).

JNK-small interfering RNA assay

RNA interference was used to specifically suppress expression of JNK-1 or JNK-2 isoforms in A549 cells. Further details are provided in the Methods section in this article's Online Repository.

Statistical analysis

Data from 3 or more independent experiments are presented as means $\pm$ SEMs, except where stated, and were compared by using GraphPad Prism 4 software (GraphPad Software, La Jolla, Calif; <http://www.graphpad.com>). Statistical analysis was made by using 1-way ANOVA with Bonferroni post-test correction for repeated measurements or by using the 1-sample Student *t* test or *t* test with the Welch correction, where appropriate. *P* values of less than .05 were considered significant. Further details are provided in the Methods section in this article's Online Repository.

RESULTS

Rhinovirus infection induces glucocorticoid insensitivity

IL-1 β significantly induced CXCL8 production in A549 cells (1413 ± 93 vs 109 ± 11 pg/mL, $P < .001$) and HBECs (166.3 ± 25 vs 32.6 ± 6.6 pg/mL, $P < .05$) at 24 hours. This was inhibited by dexamethasone in both A549 cells ($P < .001$, ANOVA; Fig 1, A) and HBECs ($P < .001$, ANOVA; Fig 1, C) in a concentration-dependent manner. RV-16 infection resulted in a rightward shift of

the concentration-response curve for dexamethasone inhibition of IL-1 β -stimulated CXCL8 production, with the inhibitory concentration of 50% (IC₅₀) increased approximately 66-fold in A549 cells (IC₅₀: 19 ± 5.5 vs 0.29 ± 0.086 nmol/L for RV-16-infected and uninfected cells, respectively; $P < .01$; Fig 1, B) and approximately 96-fold in HBECs (IC₅₀: 535 ± 429 vs 5.6 ± 2.9 nmol/L for RV-16-infected and uninfected cells, respectively; $P < .01$; Fig 1, D). RV-16 infection also resulted in a significant increase in the median effective concentration (EC₅₀) by approximately 38-fold in A549 cells (0.23 ± 0.081 vs 8.7 ± 1.4 nmol/L for uninfected and RV-16-infected cells, respectively; $P < .05$; Table I) and by approximately 9.8-fold in HBECs (0.98 ± 0.33 vs 9.6 ± 1.4 nmol/L for uninfected and RV-16-infected cells, respectively; $P < .05$), as well as a significant reduction of maximal effective concentration (Emax) in A549 cells (86 ± 2.2 vs 73 ± 3.2 nmol/L for uninfected and RV-16-infected cells, respectively; $P < .05$; Fig 1, B, and Table I) and trend toward a reduction of Emax in HBECs (60 ± 5.2 vs 51 ± 4.7 nmol/L for uninfected and RV-16-infected cells, respectively). These data indicate that RV-16 infection reduces the ability of dexamethasone to inhibit IL-1 β -induced CXCL8 production (transrepression) in airway epithelial cells.

Dexamethasone (10^{-8} mol/L) also significantly induced MKP-1 mRNA expression over baseline values after 4 hours of incubation in A549 cells (Fig 1, E) and HBECs (Fig 1, F). Pretreatment of cells with RV-16 resulted in a significant attenuation of the ability of dexamethasone to induce MKP-1 mRNA expression both in A549 cells (6.5 ± 1.0 -fold vs 4.4 ± 0.5 -fold induction of mRNA levels, $P < .05$; Fig 1, E) and HBECs (4.80 ± 0.41 -fold vs 3.82 ± 0.26 -fold induction of mRNA levels, $P < .05$; Fig 1, F). Furthermore, RV-16 inhibited 10^{-8} mol/L dexamethasone-induced GR-GRE binding ($P < .001$) in a titer-dependent manner (Fig 1, G). Similar results were observed with the minor group virus RV-1B when tested under the same experimental conditions (see the Results section in this article's Online Repository at www.jacionline.org). This indicates that the observed effects are ICAM-1-receptor independent (see Fig E1, A and B, in this article's Online Repository at www.jacionline.org).

Rhinovirus infection impairs GR α nuclear translocation

Given that glucocorticoid function was impaired in the presence of RV-16 infection, we next assessed whether rhinovirus infection affects GR α nuclear translocation, which is the crucial upstream step of glucocorticoid action.

Immunocytochemistry with an anti-GR α antibody demonstrated that dexamethasone induced GR α nuclear translocation in a concentration-dependent manner in A549 epithelial cells ($P < .001$, ANOVA; Fig 2, A). Suboptimal concentrations of dexamethasone were used in subsequent experiments. In addition, 10^{-8} mol/L dexamethasone-induced GR α nuclear translocation was maximal at 30 to 60 minutes in A549 cells and at 60 minutes in primary normal HBECs (data not shown). Thus an incubation time of 60 minutes was selected for subsequent experiments. RV-16 infection resulted in a titer-dependent inhibition of dexamethasone-induced GR α nuclear translocation in A549 cells, ranging from 7% inhibition at an MOI of 0.1 to 86% inhibition at an MOI of 200 ($P < .001$, ANOVA; Fig 2, B and C). Cells stimulated with filtered viral inocula from which virus was removed did not affect GR nuclear translocation (Fig 2, C).

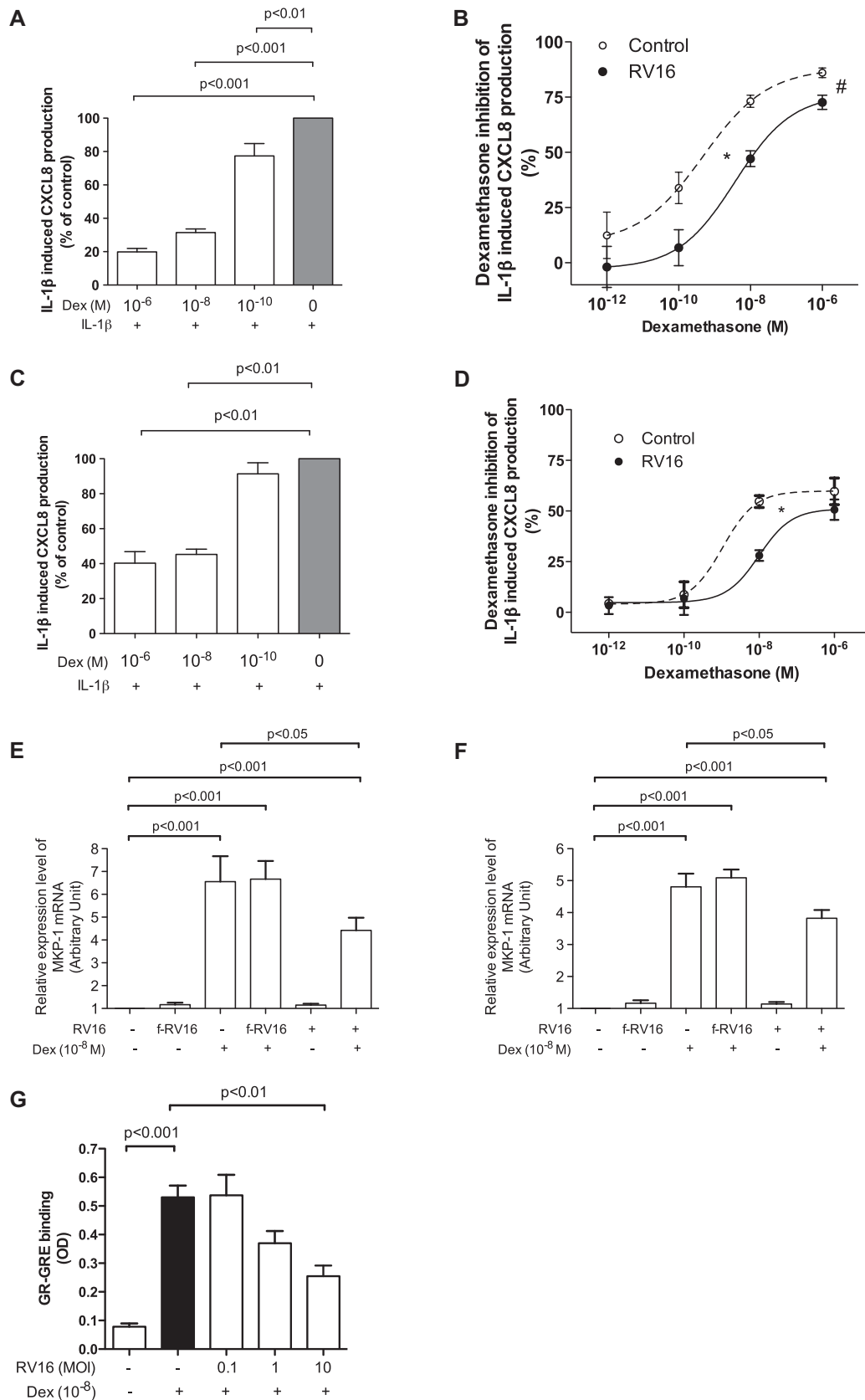


FIG 1. Rhinovirus infection induces dexamethasone insensitivity. **A** and **C**, IL-1 β -induced CXCL8 production in A549 cells (Fig 1, A) and HBECs (Fig 1, C). **B** and **D**, Dexamethasone inhibition of IL-1 β -induced CXCL8 production in A549 cells (Fig 1, B) and HBECs (Fig 1, D) in the presence (continuous line) or absence (dashed

TABLE I. Rhinovirus prevents dexamethasone inhibition of IL-1 β -induced CXCL8 production: effects of NF- κ B and JNK inhibitors

Condition (n = 3)	Dexamethasone	
	EC ₅₀ (nmol/L)	E _{max} (%)
No infection	0.23 $\pm$ 0.081	86 $\pm$ 2.2
RV-16	8.7 $\pm$ 1.4*	73 $\pm$ 3.2*
RV-16 + SP600125	1.0 $\pm$ 0.67†	74 $\pm$ 5.9
RV-16 + PS1145	9.7 $\pm$ 0.96	82 $\pm$ 1.3†
RV-16 + PS1145 + SP600125	0.17 $\pm$ 0.030§	83 $\pm$ 4.5

E_{max} represents the maximal percentage of dexamethasone inhibition of IL-1 β -induced CXCL8 production.

PS1145, IKK2 inhibitor; SP600125, JNK inhibitor.

* $P < .05$ versus no infection by using the paired t test with the Welch correction.

† $P < .05$.

‡ $P < .01$.

§ $P < .001$ versus RV-16 infection control with Bonferroni correction.

RV-16–dependent impairment of dexamethasone-induced GR α nuclear translocation was still seen at 24 hours after infection (Fig 2, D).

This RV-16–dependent reduction in nuclear GR expression in response to dexamethasone stimulation was confirmed by means of Western blotting ($P < .05$ vs dexamethasone-treated cells; Fig 2, E and F). Importantly, the same effects were observed in primary HBECs in which RV-16 pretreatment for 1 hour reduced GR α nuclear translocation induced by 10^{-8} mol/L dexamethasone (Fig 2, G).

Furthermore, similar results were observed with the minor group virus RV-1B, indicating that this effect is also not ICAM-1 receptor specific (see Fig E1, C).

Rhinovirus-induced impairment of dexamethasone-induced GR nuclear translocation is NF- κ B dependent

NF- κ B activity, as determined based on nuclear p65 by using immunocytochemistry, was found on an average of $7.2\% \pm 1.0\%$ of A549 cells in resting conditions (Fig 3, A and B). After 1 hour of RV-16 infection, there was a 7.7-fold increase in the percentage of airway epithelial cells expressing nuclear p65 (Fig 3, A and B). Western blot analysis documented that 1 hour after RV-16 infection, a significant proportion of p65 was still present in the cytoplasmic compartment (see Fig E2 and the Results section in this article's Online Repository at www.jacionline.org). These results were confirmed by using a DNA-binding assay (data not shown). RV-16–mediated activation of NF- κ B was abolished by means of pretreatment with the IKK2 inhibitors AS602868 (Fig 3, A) and PS1145 (Fig 3, B). Although neither AS602868 (Fig 3, C) nor PS1145 (see Fig E3 in this article's Online Repository at www.jacionline.org) alone had any effect on dexamethasone-induced GR α nuclear translocation, they were both able to partially restore GR nuclear translocation that had been inhibited by RV-16 pretreatment (Fig 3, C, and Fig E3). This suggests that

rhinovirus-induced impairment of GR nuclear translocation is, at least in part, NF- κ B dependent.

Because the direct interaction between activated NF- κ B and GR α has been reported as one of the mechanisms impairing GR α nuclear translocation,^{28,29} we investigated whether viral infection affects the binding between NF- κ B and GR α . Using coimmunoprecipitation, we found minimal GR α -p65 association in the absence of concomitant exposure to dexamethasone (activated GR) and RV-16 (activated NF- κ B; Fig 3, D). RV-16 infection enhanced GR α -p65 association after dexamethasone exposure in both cytoplasmic and nuclear extracts but occurred more effectively in the cytosol (Fig 3, D). This suggests that rhinovirus induction of glucocorticoid insensitivity is mediated, at least in part, through inhibition of GR α nuclear translocation by NF- κ B p65.

Rhinovirus-induced impairment of dexamethasone-induced GR nuclear translocation is JNK dependent

RV-16–induced impaired GR nuclear translocation was only partially restored by IKK inhibitors. Therefore we explored other mechanisms able to modulate GR nuclear translocation. Activation of JNK has been reported to inhibit GR function.^{30,31} Phosphorylated JNK (the active form) was found in less than 5% of the A549 cells under resting conditions by using immunocytochemistry (Fig 4, A). After 1 hour of RV-16 infection, there was a 21-fold increase in the percentage of cells expressing phosphorylated JNK ($P < .001$). This induction was inhibited by the presence of the pan-JNK inhibitor SP600125 (Fig 4, A). Western blot analysis demonstrated that SP600125 partially reversed RV-16–dependent inhibition of dexamethasone-induced GR α nuclear translocation (Fig 4, B and C).

JNK1 phosphorylates GR on Ser226,³¹ and Ser226 hyperphosphorylation inhibited GR nuclear translocation.^{32,33} Western blot analysis demonstrated that RV-16 infection resulted in increased GR Ser226 phosphorylation and that this change was significantly reduced by SP600125 but not by PS1145 (Fig 4, D and E). This suggests that rhinovirus inhibition of GR α nuclear translocation is mediated, at least in part, through JNK activation and phosphorylation of GR on Ser226. To evaluate which JNK isoform is involved in RV-16–induced corticosteroid insensitivity, we used small interfering RNA (siRNA) specifically designed to knock down JNK-1 or JNK-2 expression. We found that JNK-1 knock down abolished RV-16–induced inhibition of dexamethasone-induced GR-GRE binding. Conversely, no effect was documented when cells were treated with JNK-2 siRNA (Fig 4, F). These data showed that the JNK-1, but not the JNK-2, isoform was involved in RV-16–induced corticosteroid insensitivity.

Rhinovirus-induced glucocorticoid resistance is reversed by the combination of JNK and IKK inhibitors

Given that both JNK and NF- κ B inhibitors independently significantly reversed the RV-16–induced attenuation of GR

line) of rhinovirus infection. * $P < .01$ and # $P < .05$ for IC₅₀ and E_{max} in infected versus uninfected cells, respectively. E and F, Dexamethasone-induced MKP-1 mRNA expression in A549 cells (Fig 1, E) and HBECs (Fig 1, F) in the presence or absence of RV-16 infection. G, Dexamethasone-induced GR-GRE binding in A549 cells. f-RV, Virus removed by means of filtration.

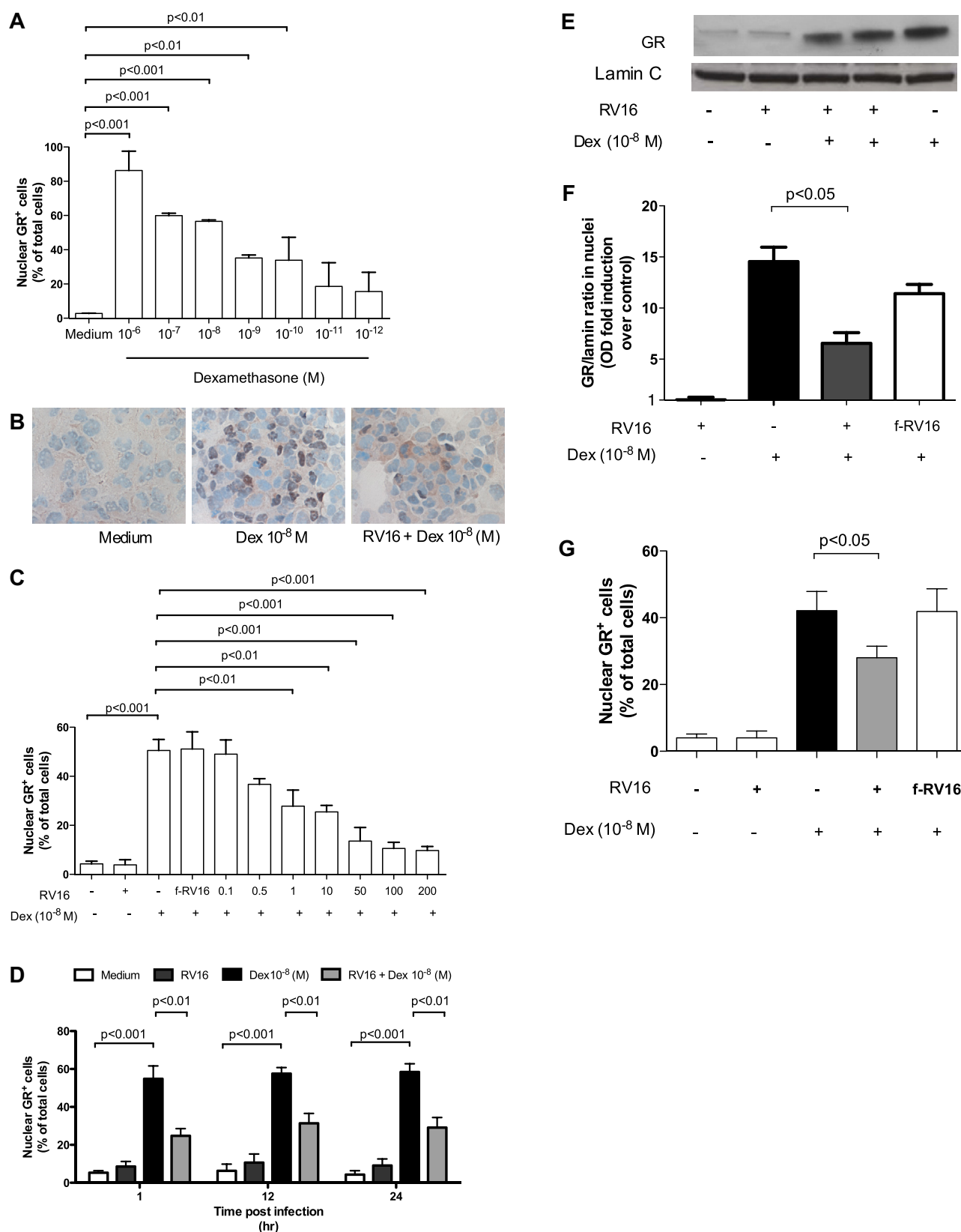


FIG 2. Rhinovirus infection impairs dexamethasone-induced GR α nuclear translocation. **A-D** and **G**, Immunocytochemical evaluation of GR α nuclear translocation induced by dexamethasone (representative staining; Fig 2, **A** and **B**) in the presence or absence of RV-16 infection (Fig 2, **C**) at different time points after RV-16 (Fig 2, **D**) in A549 cells and primary epithelial cells (Fig 2, **G**). **E** and **F**, Results (means $\pm$ SEMs; Fig 2, **F**) and representative blots (Fig 2, **E**) of GR α Western blot analysis in nuclear extracts of A549 cells. Control refers to unstimulated and uninfected cells. *f-RV*, Virus removed by means of filtration.

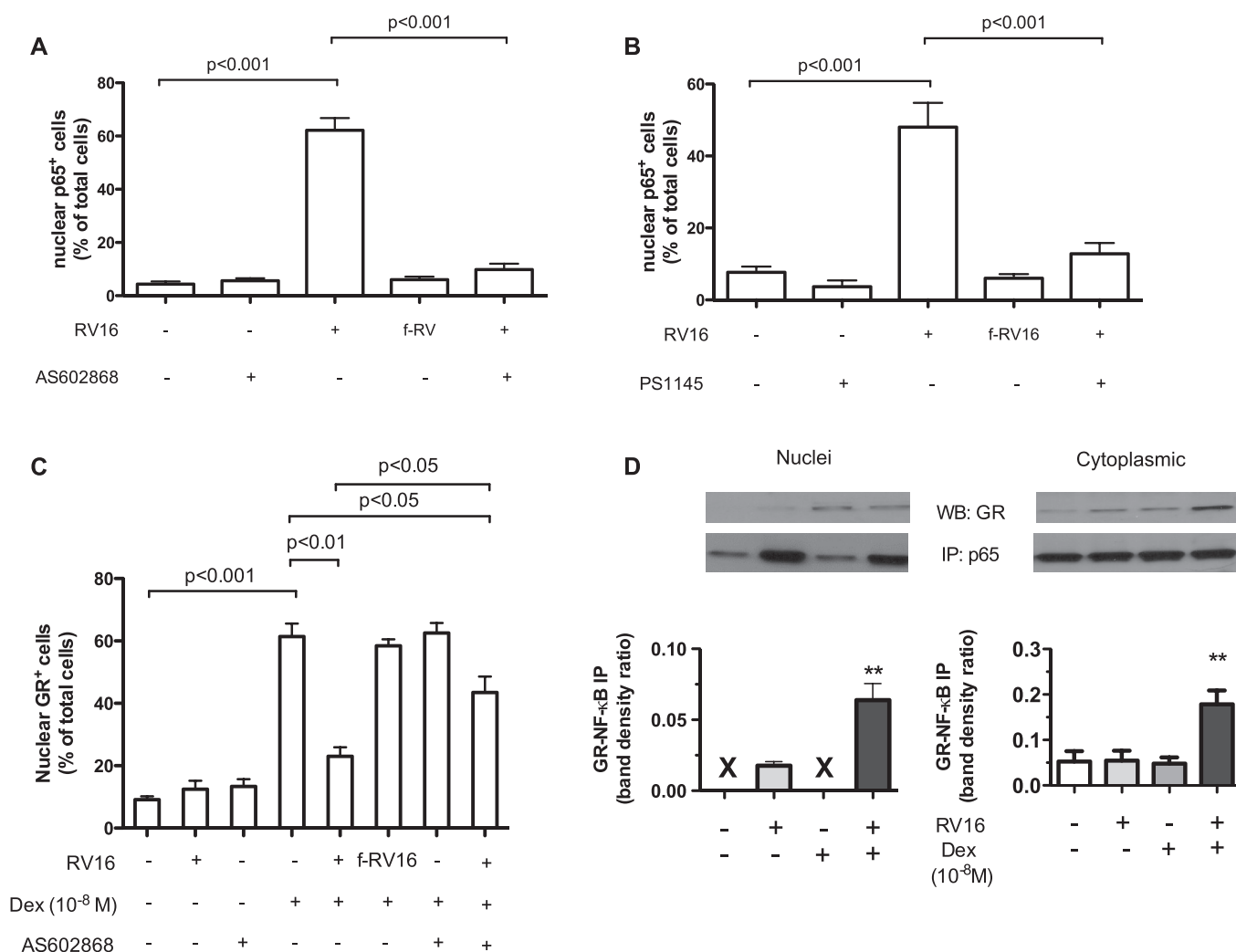


FIG 3. Rhinovirus-induced impairment of dexamethasone-induced GR nuclear translocation is partially NF-κB dependent. **A** and **B**, Immunocytochemical evaluation of nuclear p65 after RV-16 infection with or without IKK2 inhibitors (AS602868 [Fig 3, A] and PS1145 [Fig 3, B]) in A549 cells. **C**, Immunocytochemical evaluation of GRα nuclear translocation induced by dexamethasone in the presence of RV-16 with or without AS602868. **D**, Coimmunoprecipitation of GR associated to precipitated p65 in nuclear and cytoplasmic extracts of A549 cells. *Lower films*, Western blotting of immunoprecipitated p65 (IP: p65); *upper films*, Western blotting for GR bound to the immunoprecipitated p65 (WB: GR). The results of the graphs are expressed as the ratio between band density readings of GR and p65 (means ± SEMs). X, The ratio between GR and p65 was not calculated in the absence of RV-16-induced p65 nuclear translocation. ***P* < .01 versus all other conditions. *f-RV*, Virus removed by means of filtration.

nuclear translocation by dexamethasone, we investigated the functional consequences of the inhibition of both JNK and NF-κB pathways in our experimental setting. Either the JNK inhibitor SP600125 or the NF-κB inhibitor PS1145 alone partially reversed the RV-16-induced impairment of GR-GRE binding induced by dexamethasone (10⁻⁸ mol/L; Fig 5, A). In contrast, the combination of both JNK and NF-κB inhibitors fully reversed the RV-16-induced reduction in GR-GRE binding (Fig 5, A). In addition, SP600125 and PS1145 both alone and in combination restored the RV-16-mediated inhibition of MKP-1 mRNA induction by dexamethasone (Fig 5, B).

SP600125 partially reversed the RV-16-mediated attenuation of dexamethasone inhibition (EC₅₀) of IL-1β-induced CXCL8 production, whereas PS1145 improved the maximal inhibition (E_{max}) only without affecting the EC₅₀ (Table I). The combination

of both the JNK and NF-κB inhibitors totally restored dexamethasone sensitivity and maximal inhibition (Table I and Fig 5, C).

DISCUSSION

Our study shows that rhinovirus infection inhibits glucocorticoid mechanisms of action, inducing partial glucocorticoid resistance. Rhinovirus infection impaired both the transactivation and transrepression activities of dexamethasone, implying that rhinovirus infection targets an upstream aspect of GR activation. The proinflammatory pathways JNK and NF-κB were both activated by rhinovirus and are key elements in rhinovirus-induced steroid insensitivity. Inhibition of both JNK and NF-κB activation is required to fully restore all aspects of glucocorticoid responsiveness.

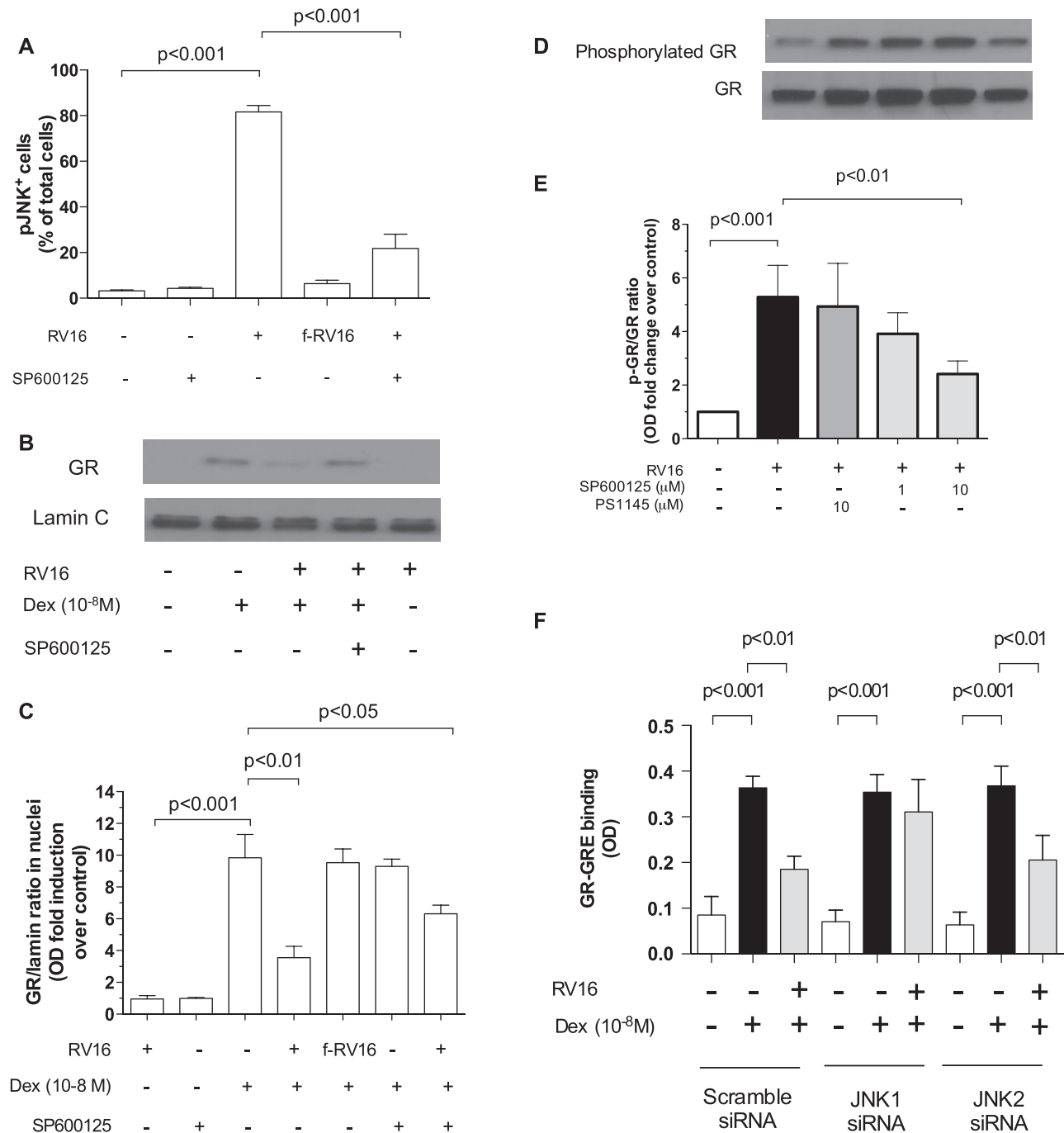


FIG 4. Rhinovirus-induced impairment of GR nuclear translocation is partially JNK dependent. **A**, Immunocytochemistry evaluation of phosphorylated JNK in A549 cells after RV-16 infection with or without pan-JNK inhibitor SP60012. **B** and **C**, Results (mean $\pm$ SEM; Fig 4, C) and representative blots (Fig 4, B) of GR α Western blot analysis in nuclear extracts of A549 induced by dexamethasone after RV-16 infection with or without JNK inhibitor SP60012. Control refers to unstimulated and uninfected cells. **D** and **E**, Results (means $\pm$ SEMs; Fig 4, E) and representative blots (Fig 4, D) of Western blot analysis of phosphorylated GR Ser226/GR α ratio in whole-cell lysates of A549 after RV-16 infection with or without the JNK inhibitor SP60012 or the IKK2 inhibitor PS1145. **F**, GR-GRE binding in nuclear extracts of A549 cells stimulated with dexamethasone after RV-16 infection without (Scramble) or with JNK-1 or JNK-2 siRNA. f-RV, Virus removed by means of filtration.

These findings unveil a novel molecular mechanism for rhinoviruses, the most important triggers of asthma exacerbations, to impair the ability of glucocorticoids to control airway

inflammation. A transient steroid insensitivity would lead to an uncontrolled inflammatory response and to exacerbations of asthma that are poorly responsive to steroid treatment.

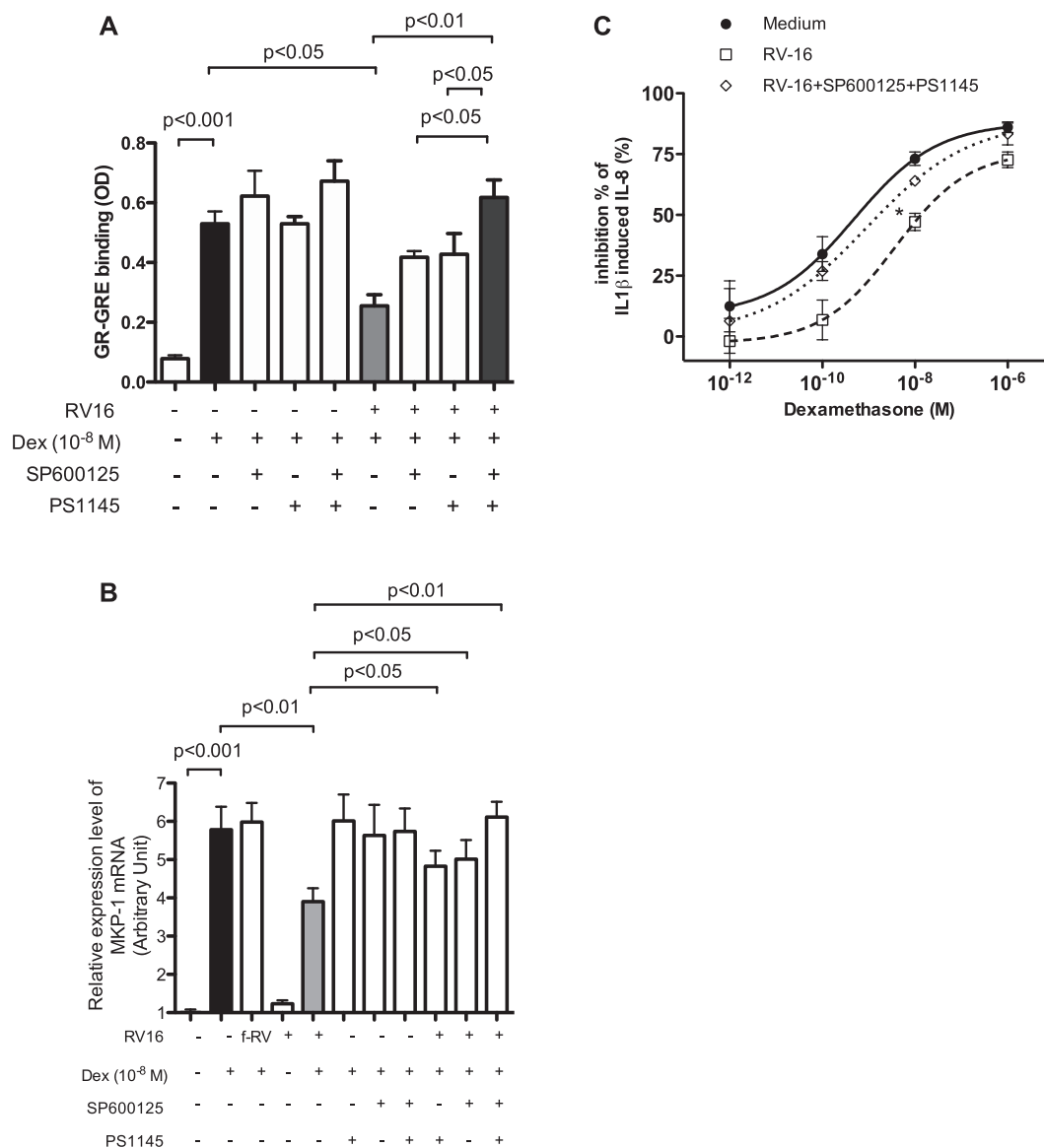


FIG 5. Rhinovirus-induced attenuation of dexamethasone activity is reversed by a combination of JNK and IKK2 inhibitors. **A**, GR-GRE binding in nuclear extracts of A549 cells stimulated with dexamethasone after prior infection of cells with RV-16 in the presence (+) or absence of the JNK inhibitor SP600125 and the IKK2 inhibitor PS1145. **B**, Dexamethasone-induced MKP-1 mRNA expression after 4 hours in A549 cells after prior infection of cells with RV-16 in the presence (+) or absence of SP600125 and PS1145. **C**, Ability of dexamethasone to inhibit IL-1 β -induced CXCL8 production, which is inhibited by RV-16 pretreatment, is restored by the combination of SP600125 and PS1145.

Exacerbations are the major cause of morbidity, mortality, and health care costs for asthmatic patients, and current preventive and therapeutic options are limited. Regular treatment with inhaled glucocorticoids is of unquestioned benefit in asthma management, except when respiratory viruses are the trigger.³ Indeed, several randomized controlled trials questioned the efficacy of both inhaled and systemic glucocorticoids in the prevention/treatment of virus-induced acute asthma episodes, particularly in children.⁸⁻¹³ Human experimental rhinovirus challenge models confirm that ICSs do not prevent worsening of airway inflammation in asthmatic patients.^{14,15} These data therefore reflect the clinical expression of a glucocorticoid-resistant system associated with acute viral episodes.⁸⁻¹³

In the absence of glucocorticoid stimulation, the GR resides in the cytosol in the inactivated form complexed with a variety of proteins.³⁴ Glucocorticoid binding to the cytoplasmic GR results in its release from its chaperone proteins, GR activation, and rapid nuclear translocation. Nuclear import of GR α is mediated through its interaction with importins, and this process is under tight control.²⁹ The ability of GR to associate with the importin machinery is regulated by its phosphorylation status.²⁹ Within the nucleus, GR α induces transcription of genes (transactivation) by binding to specific DNA elements (GREs) at the regulatory sequences of steroid-responsive genes (GR-GRE binding, genomic effect).³⁵ Glucocorticoids can also reduce inflammatory gene transcription (transrepression) induced by proinflammatory

transcription factors, such as NF- κ B or activator protein 1, through association between these factors and GR α ³⁵ and/or through recruitment of nuclear receptor corepressors, repression of coactivator complexes and other mechanisms (nongenomic effects).²⁹

Here we show that rhinovirus infection attenuates GR α nuclear translocation in response to dexamethasone and reduced corticosteroid sensitivity in airway and lung epithelial cells. Nuclear cytoplasmic shuttling of GR α is associated with phosphorylation on Ser226, and we demonstrated that rhinovirus infection resulted in a JNK-dependent increase in GR Ser226 phosphorylation. In particular, we found that JNK-1 isoform activation is involved in rhinovirus-induced corticosteroid insensitivity. Because JNK activation, but not induction, occurs quickly after virus/virus surrogate stimulation,^{36,37} JNK activation, rather than increased expression of JNK-1, is likely involved in the rhinovirus-induced corticosteroid insensitivity observed under our experimental conditions. JNK inhibition partially reversed the reduction in dexamethasone efficacy (EC₅₀) induced by rhinovirus infection without affecting the maximal dexamethasone response. This suggests that specific changes in GR posttranslational modifications can have marked effects on distinct aspects of GR function.¹⁹ In contrast, we were unable to detect any modulation of the impaired dexamethasone efficacy induced by rhinovirus infection with IKK2 inhibition, but this was able to restore the maximal dexamethasone response (E_{max}). In addition, NF- κ B activation by rhinovirus infection was associated with corticosteroid resistance and linked with inhibition of corticosteroid-induced GR nuclear import through the formation of an inhibitory GR-p65 complex. A previous study has reported that activated NF- κ B-p65 can physically interact with activated GR and that this interaction is sufficient to transrepress GR activity.³⁸ Overall, the differences in GR function modulated by these inflammatory pathways indicate that the mechanism by which rhinovirus infection regulates steroid sensitivity is a complex, multifactorial process using distinct signaling pathways that impinge on some, but not all, steroid functions.

The findings of our study indicate a strategy through which rhinovirus infection can overcome the anti-inflammatory defensive shield of the airways but also indicate approaches that might reverse this process. On the one hand, rhinovirus infection switches on several proinflammatory signaling cascades, including the JNK and NF- κ B pathways, leading to an enhanced inflammatory response. In addition, the products of this activation interfere with the anti-inflammatory actions of glucocorticoids. This self-potentiating 2-pronged attack undermines the ability of glucocorticoids to contain airway inflammation, resulting in uncontrolled airway inflammation and an asthma exacerbation that is poorly responsive to steroid therapy.

Rhinovirus has been reported to induce JNK and NF- κ B activation through different mechanisms, including (1) oxidative pathways,^{39,40} (2) direct activation through the induction of Toll-like receptor pathways,⁴¹ and (3) indirect activation through the virus-induced release of proinflammatory cytokines, such as TNF- α ,^{42,43} and/or antiviral molecules, such as type I interferons.^{21,22,44} Rhinovirus infection induces an intracellular prooxidative state, which can be involved also in the activation of the stress kinase JNK1.^{45,46} In addition, JNK activation is reported to occur quickly after polyinosinic-polycytidylic acid stimulation,³⁶ suggesting a role for Toll-like receptors also in this pathway.

Our data showing that rhinovirus induction of glucocorticoid resistance can be completely reversed by inhibition of both the JNK and NF- κ B pathways identifies new therapeutic approaches for asthma exacerbations and more in general for rhinovirus-associated diseases for which no effective treatment is currently available. These new approaches to treatment would in themselves be anti-inflammatory, but they would have the additional important advantage of restoring glucocorticoid sensitivity in a context in which virus-induced glucocorticoid resistance clearly plays an important role in exacerbation pathogenesis.

Key messages

- Rhinovirus infection of human airway epithelium induces glucocorticoid resistance.
- Rhinovirus infection of human airway epithelium switches on several proinflammatory signaling cascades, including activation of the JNK and NF- κ B pathways.
- Rhinovirus induction of glucocorticoid resistance in the human airway epithelium can be reversed by inhibition of both the JNK and NF- κ B pathways.

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METHODS

Viral stocks

RV-16 (a major group rhinovirus, the receptor of which on the cell surface is ICAM-1) was obtained from the European Collection of Cell Cultures, which has incorporated the original viral strains from the MRC Common Cold Unit (Salisbury, United Kingdom). Viral stocks were prepared by means of infection of sensitive cell monolayers (Ohio HeLa), as described elsewhere.^{E1} Median tissue culture infective dose per milliliter values were determined, and rhinovirus serotype was confirmed by mean of neutralization with serotype-specific antibodies (American Type Culture Collection).^{E2} For selected experiments, RV-1B (a minor group virus that binds to members of the low-density lipoprotein receptor family), also obtained from the European Collection of Cell Cultures, was used to evaluate whether the results were group/receptor restricted.

RV-16 at an MOI of 5 was used for all the experiments, unless otherwise stated. At variance, RV-1B at an MOI of 1 was used for all the experiments (unless otherwise stated) because of the toxicity of higher RV-1B concentrations.^{E3}

Filtration of the virus from inoculum (f-RV), to remove viral particles was performed, as previously described.^{E1} Filtered viral (f-RV) stocks were used as a negative control.

Cell culture

Ohio HeLa cells were obtained from the European Collection of Cell Cultures, and A549 cells, a type II respiratory cell line, were obtained from American Type Culture Collection. A549 cells (an alveolar basal epithelial cell line) were used because they represent a respiratory cell line widely used/well standardized to investigate both *in vitro* experimental rhinovirus infection^{E4,E5} and corticosteroid response pathways.^{E6}

A549 cells were grown in minimal essential medium supplemented with 10% FCS. Cells were seeded in 6-well plates for functional assays (ELISA and RT-PCR) in 8-well slides for immunohistochemistry and in 150-cm² flasks for Western blot assays. A549 cells were placed overnight in serum-free medium before stimulation with rhinovirus, drugs, or both. Rhinovirus infection was then performed when cells were approximately 80% confluent.

Primary HBECs were obtained from bronchial brushings of healthy nonsmoking volunteers and cultured, as previously described.^{E7,E8} These cells were more than 95% cytokeratin 18 immunoreactive, as assessed by using immunocytochemistry. HBECs were grown into hormonally supplemented bronchial epithelial growth medium (Clonetics, San Diego, Calif) containing 50 U/mL penicillin and 50 mg/mL streptomycin. Experiments in HBECs were conducted in triplicate. HBECs were obtained from 12 healthy nonsmoking patients. Nine samples provided effective cell cultures. The HBECs harvested by means of bronchial brushing during bronchoscopy were freshly seeded in 25-cm² flasks. When subconfluent, HBECs were split and seeded in 75-cm² flasks. When subconfluent, HBECs were split and resuspended at 2×10^5 cell/mL. Suspended cells were seeded in 6-well plates (2.5 mL per well) for functional assays (ELISA and real-time quantitative PCR) and in 8-well slides for immunohistochemistry. Rhinovirus infection was then performed when cells (at passage 3) were approximately 80% confluent. In accordance with standard protocols,^{E9} bronchial epithelial growth medium was replaced by bronchial epithelial basal medium without hormonal (including hydrocortisone) supplementation 24 hours before any experimental procedure was started.

Rhinovirus, medium alone, or f-RV inocula were added to the cells for 1 hour at room temperature with shaking. Virus preparations were removed, and 1 mL of medium containing dexamethasone (10^{-12} to 10^{-6} mol/L) or diluent alone was added.

In selected experiments the involvement of the NF- κ B and JNK pathways was evaluated by using pharmacologic tools: AS602868 (1 μ mol/L; Serono, Geneva, Switzerland) or PS1145 (1 μ mol/L, Sigma-Aldrich) as inhibitors of IKK2 (a kinase involved in NF- κ B activation) and SP600125 (1 μ mol/L, Sigma-Aldrich), a JNK inhibitor, were added 20 minutes before RV-16 infection. Off-target effects of the IKK2 inhibitors cannot be excluded,^{E10} although the 2 IKK inhibitors we used are known to be relatively specific to

IKK2.^{E10,E11} According to Bain et al,^{E12} the major off-target effects of PS1145 at 10 μ mol/L are extracellular signal-regulated kinase, glycogen synthase kinase-3 β , and Pim kinases, which are not affected by AS602868 at 1 μ mol/L (M. Dreano, personal communication to Ian M. Adcock; ie, the concentration we used in our experimental condition). Therefore we believe that this makes it unlikely that the results of the study were off-target effects of these IKK2 inhibitors.

All experiments were performed in at least triplicate.

The study was approved by the local ethics committee of the University Hospital of Ferrara, and informed consent was obtained from each participant in accordance with the principles outlined in the Declaration of Helsinki.

Immunocytochemistry for GR α in airway cells

Immunocytochemistry with peroxidase detection was performed, as previously described, with minor modifications,^{E13} in A549 cells and in HBECs. Eight-well chambers were fixed with cold acetone/methanol (10 minutes) and washed (PBS, 20 minutes, 22°C), and endogenous peroxidase activity was blocked by 3% hydrogen peroxide/methanol before cell membranes were permeabilized with 0.1% saponin. Nonspecific binding was blocked (5% normal goat serum, 20 minutes, 22°C) before incubation with the specific rabbit polyclonal anti-human GR α (sc-1003, www.scbt.com; 1:50 dilution, 1 hour, 22°C), as described previously.^{E14} Negative control slides were incubated with normal rabbit nonspecific immunoglobulins (Santa Cruz Biotechnology, Santa Cruz, Calif) at the same concentration as the primary antibody. Sections were then washed and incubated with goat anti-rabbit biotinylated antibody (Vector Laboratories, Burlingame, Calif) for 30 minutes at room temperature and developed with ABC reagent (Vector ABC Kit, Vector Laboratories). Slides were then incubated with chromogen—fast diaminobenzidine as a chromogenic substance and counterstained in hematoxylin. Negative control slides were included in each staining run. GR α^+ cells were quantified for cytoplasmic staining, nuclear staining, or both by 2 independent blinded observers; 400 cells staining brown, indicating GR α immunoreactive cells, were counted on each well. The mean intraobserver and interobserver coefficients of variance with counting were less than 10%. According to previous literature,^{E15} a nucleus was counted as positively stained when more than 50% of the nuclear surface was stained with the tested antibody. Hematoxylin counterstaining identified nuclear contour. The total nuclear surface and its 50% value have been calculated for each cell counted by using automated software (NIS-Elements, <http://www.nis-elements.com>).

Preparation of nuclear and cytosolic fractions

Nuclear and cytosolic proteins from airway epithelial cells were separated by using a commercial Nuclear Extraction kit (Active Motif), according to the manufacturer's instructions, as previously described.^{E16} Protein content was determined photometrically with the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, Calif).

Western blotting for GR α and p65 in airway cells

Western blotting was performed, as previously described.^{E17} One hundred micrograms per lane of nuclear and cytosolic cell lysate proteins were run on 4–12% Novex Bis-Tris gels (Invitrogen, Carlsbad, Calif) and transferred to nitrocellulose filters (Hybond-ECL; GE Healthcare, Fairfield, Conn) and blocked with 5% nonfat dry milk in TBS-Tween (0.05%). Rabbit polyclonal anti-human GR α (sc-1003, www.scbt.com; 1:500 dilution) or anti-human NF- κ B subunit p65 (sc-372, www.scbt.com; 1:500 dilution) was incubated for 1 hour at room temperature. Washing was performed with PBS-Tween (0.1%). Secondary antibody incubation was performed for 1 hour at room temperature. Blots were developed with ECL (GE Healthcare). Filters were reprobbed for lamin C as a loading control for nuclear samples and for actin for cytoplasmic samples. Western blot assays were performed in nuclear extracts for GR and in cytoplasmic extracts for p65. The GR or p65 bands were quantified by using densitometry with Grab-It and VisionWorks LS software (UVP, Cambridge, United Kingdom) and expressed as a ratio with the corresponding lamin C or actin optical density value of the same lane.

CXCL8 ELISA

To evaluate corticosteroid repression of inflammatory gene expression, we measured CXCL8 (IL-8) levels in the supernatants of epithelial cells exposed to IL-1 β (1 ng/mL) for 24 hours in the presence/absence of dexamethasone (10^{-8} mol/L). Rhinovirus (or shame) pretreatment was carried out 1 hour before IL-1 β and dexamethasone/vehicle stimulation to assess whether rhinovirus infection interferes with this corticosteroid anti-inflammatory pathway. CXCL8 levels had been assessed in each control condition of this experimental setting. These values had been used to normalize the results of the tested conditions in each experiment to obtain the net inhibitory effect of rhinovirus over dexamethasone anti-inflammatory activity. CXCL8 levels were measured by using sandwich ELISA, according to the manufacturer's recommendations (R&D Systems Europe).

Real-time quantitative PCR detection of MKP-1 mRNA

The gene transcript levels of MKP-1 (CL-100), the transcription of which is induced by glucocorticoids,^{E18} and the housekeeping gene GAPDH were quantified by using real-time PCR with a QuantiTect SYBR Green PCR kit (Qiagen) and a Rotor-Gene 3000 PCR machine (Corbett Research). The primer pairs of MKP-1 and GAPDH were bought from Qiagen. The specificity of the desired PCR products was determined by using melting-curve analysis. Variations in cDNA concentrations between different samples were corrected by using the housekeeping gene, in which the GAPDH concentration in each cDNA sample was calculated and the cDNA was diluted to contain equal amounts of GAPDH. Standard curves for GAPDH were generated by performing a dilution series of the untreated control cDNA. The relative amount of gene transcript present after different treatments was calculated and normalized by dividing the calculated value for the gene of interest by the housekeeping gene value.

GR-NF- κ B association by coimmunoprecipitation

Coimmunoprecipitation was conducted, as previously described.^{E17} Briefly, nuclear and cytoplasmic lysates were precleared with 20 μ L of A/G agarose (a 50:50 mix, Santa Cruz Biotechnology) and 2 μ g of normal IgG for 1 hour. After microcentrifugation, 20 μ L of A/G agarose conjugated with 5 μ g of mouse IgG₁ antibody anti-human NF- κ B subunit p65 (F-6P [SC-8008], Santa Cruz Biotechnology) was used to precipitate the NF- κ B subunit p65 in the nuclear and cytoplasmic lysates for 4 hours at 4°C with rotation. The immune complexes were pelleted by means of gentle centrifugation (2500 rpm) and washed 3 times with 1 mL of lysis buffer. After a final wash with IP buffer, the buffer was aspirated completely and resuspended in Laemmli sample buffer for Western blot analysis. The precipitated p65 and the GR associated with precipitated p65 was evaluated by means of Western blotting by using anti-NF- κ B-p65 rabbit IgG antibody (C-20 [SC-372], Santa Cruz Biotechnology) for NF- κ B and rabbit polyclonal anti-human GR α (sc-1003, Santa Cruz Biotechnology) for GR. Under the experimental conditions of the study, p65 immunoprecipitation is not optimized for quantitative comparisons.

RNA interference of JNK1 and JNK2

siRNAs of JNK1 and JNK2 were purchased from Qiagen, and nonspecific control duplex (scrambled oligonucleotide: sc, 47% GC content) was also purchased from Dharmacon (Colorado Springs, Colo). The siRNA sequences (100 nmol/L each) were transfected to A549 cells after 24 hours of FCS starvation by using HiPerfect transfection reagent (Qiagen), according to the manufacturer's instructions. A549 cells were incubated for 24 hours and then infected with RV-16 for 1 hour. Dexamethasone was treated for 4 hours after RV-16 infection, and then cells were collected for the GR-GRE binding assay.

Statistical analysis

Data from 3 or more independent experiments are presented as means $\pm$ SEMs, except where stated, and were compared by using GraphPad Prism 4 software (<http://www.graphpad.com>). For multiple comparisons, statistical analysis was performed by using 1-way ANOVA with post-test analysis (Bonferroni correction) for parametric variables or by using Kruskal-Wallis

analysis with Dunn post-test analysis for nonparametric variables by using GraphPad Prism 4 software. The differences between 2 groups in *in vitro* data were analyzed by using the Student *t* test or the *t* test with Welch correction, where appropriate. *P* values of less than .05 were considered significant. The pharmacologic parameters EC₅₀ (molar concentration of a stimulatory agonist, inhibitory agonist, or both that produces 50% of the maximal possible effect of that agonist), IC₅₀ (molar concentration of an antagonist that reduces the response to an agonist by 50%), and Emax (maximal effective concentration)^{E19} were calculated by using GraphPad Prism 4 software (<http://www.graphpad.com>).

RESULTS

RV-1B infection induces glucocorticoid insensitivity and impairs GR α nuclear translocation

RV-1B infection resulted in a rightward shift of the concentration-response curve for dexamethasone inhibition of IL-1 β -stimulated CXCL8 production, with the EC₅₀ increased approximately 40-fold in A549 cells (11.4 ± 1.7 vs 0.30 ± 0.098 nmol/L for RV-1B-infected and uninfected cells, respectively; Fig E1, A). Pretreatment of cells with RV-1B resulted in a significant attenuation of the ability of dexamethasone to induce MKP-1 mRNA expression in both A549 cells (6.5 ± 1.0 vs 3.3 ± 0.5 mRNA expression over control after dexamethasone stimulation in the absence or presence of RV-1B, respectively; *P* < .05; Fig E1, B). In addition, RV-1B infection led to titer-dependent inhibition of dexamethasone-induced GR nuclear translocation in A549 cells (*P* < .001, ANOVA; Fig E1, C).

Low-density lipoprotein receptor is the surface cellular receptor for the rhinovirus minor group to which RV-1B belongs, whereas ICAM-1 is the surface cellular receptor of the rhinovirus major group to which RV-16 belongs. Because rhinovirus infection induced corticosteroid insensitivity, irrespective of the rhinovirus group (RV-16/major vs RV-1B/minor) and specific receptor, we concluded that the rhinovirus-induced corticosteroid insensitivity occurs irrespective of the surface cellular receptor involved.

Evaluation of p65 in the cytoplasmic compartment after rhinovirus infection

A significant proportion of NF- κ B subunit p65 was detectable by means of Western blot analysis at 1 hour after RV-16 infection in the cytoplasmic compartments of A549 cells. The amount of p65 in the cytoplasmic compartment of A549 cells after 1 hour of RV-16 infection was significantly lower compared with that seen in uninfected cells (*P* < .01, Fig E2).

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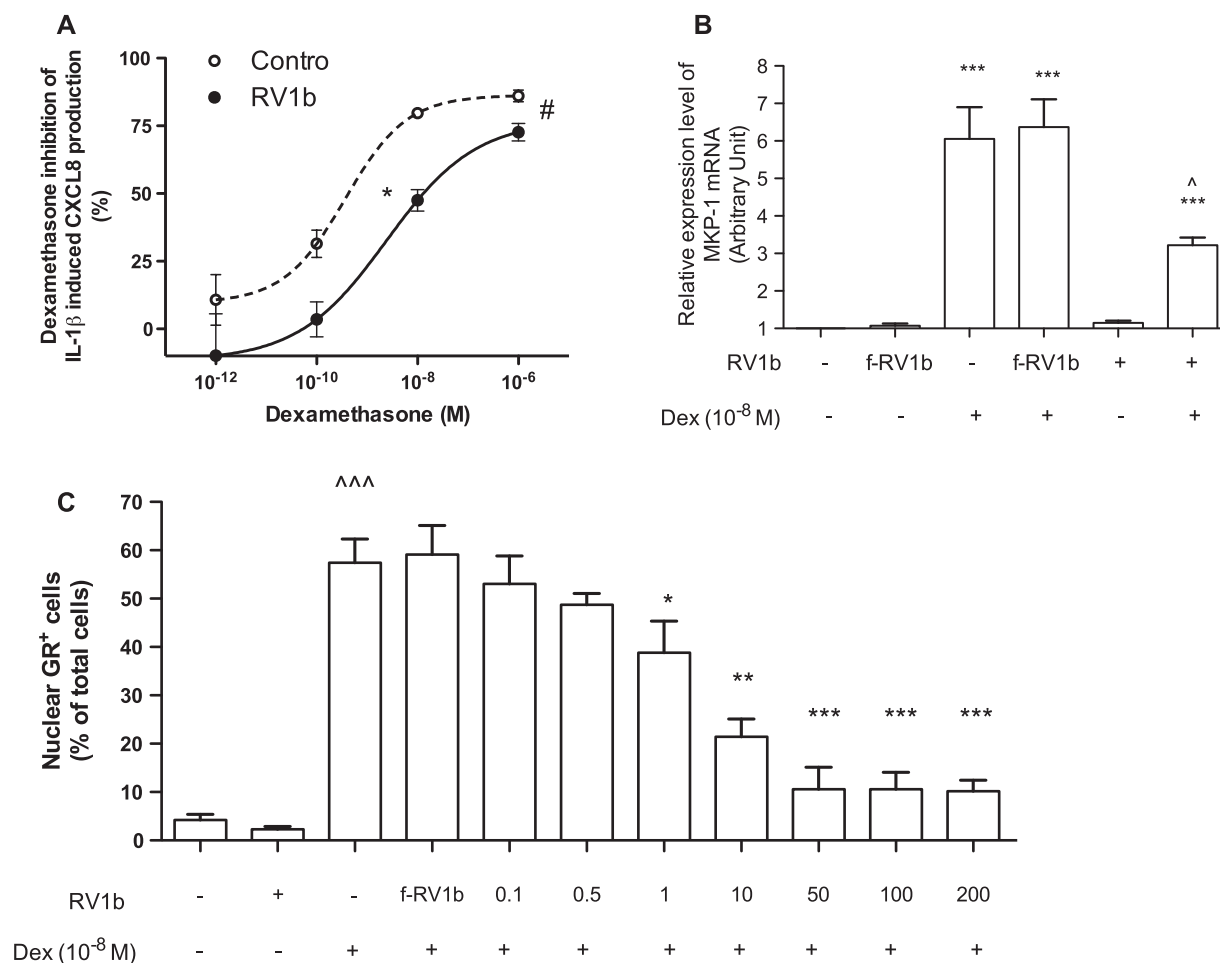


FIG E1. RV-1B infection induces glucocorticoid insensitivity and impairs GR α nuclear translocation. **A**, Dexamethasone inhibition of IL-1 β -induced CXCL8 production in A549 cells in the presence (*continuous line*) or absence (*dashed line*) of RV-1B. * $P < .01$. **B**, Dexamethasone-induced MKP-1 mRNA expression in A549 cells in the presence (+) or absence (–) of RV-1B. In selected experiments cells were exposed to medium in which virus was removed by means of filtration (f-RV). *** $P < .001$ versus control (untreated and unexposed cells). ^ $P < .05$ versus dexamethasone-treated cells. **C**, RV-1B infection led to titer-dependent inhibition of dexamethasone-induced GR nuclear translocation in primary bronchial epithelial cells ($P < .001$, ANOVA). ^^^ $P < .001$ versus untreated and uninfected cells. *** $P < .001$ versus dexamethasone-treated uninfected cells. ** $P < .01$ versus dexamethasone-treated uninfected cells.

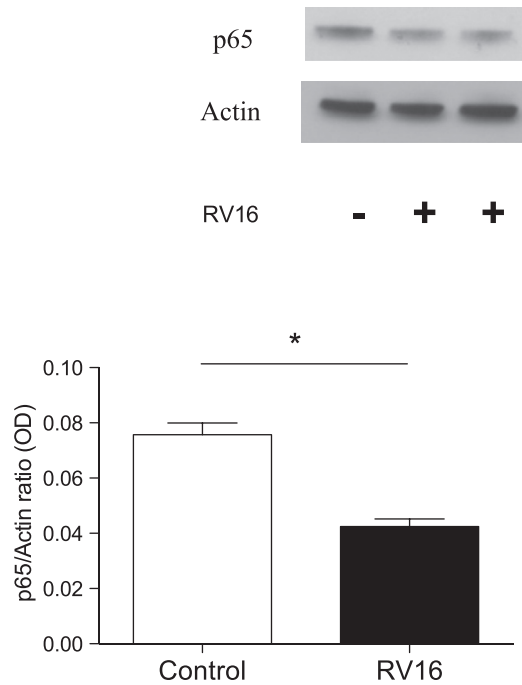


FIG E2. Western blot analysis of p65 in the cytoplasmic compartment of A549 cells after RV-16 infection. Western blot analysis of NF- κ B p65 in cytoplasmic extracts of A549 cells exposed to RV-16 infection for 1 hour. A representative blot of 3 independent experiments is shown in the *upper panel*. A graphic representation of the densitometric data defined as means $\pm$ SEMs is shown in the *lower panel*. * $P < .01$.

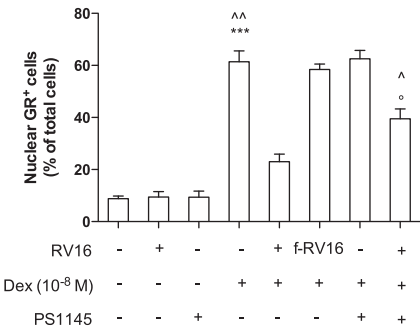


FIG E3. RV-16-induced impairment of GR nuclear translocation is NF- κ B dependent. A549 cells positive for nuclear GR α by means of immunocytochemical staining after exposure to dexamethasone plus RV-16 alone or RV-16 in the presence of the IKK2 inhibitor PS1145. *** P < .001 versus untreated cells. ^^ P < .01 versus dexamethasone-treated cells. ^ P < .05 versus dexamethasone-treated cells. ° P < .05 versus dexamethasone-treated and infected cells.