ORIGINAL ARTICLE

Restoration of Corticosteroid Sensitivity in Chronic Obstructive Pulmonary Disease by Inhibition of Mammalian Target of Rapamycin

Akihisa Mitani, Kazuhiro Ito, Chaitanya Vuppusetty, Peter J. Barnes, and Nicolas Mercado

Airway Disease Section, National Heart and Lung Institute, Imperial College London, London, United Kingdom

Abstract

Rationale: Corticosteroid resistance is a major barrier to the effective treatment of chronic obstructive pulmonary disease (COPD). Several molecular mechanisms have been proposed, such as activations of the phosphoinositide-3-kinase/Akt pathway and p38 mitogen-activated protein kinase. However, the mechanism for corticosteroid resistance is still not fully elucidated.

Objectives: To investigate the role of mammalian target of rapamycin (mTOR) in corticosteroid sensitivity in COPD.

Methods: The corticosteroid sensitivity of peripheral blood mononuclear cells collected from patients with COPD, smokers, and nonsmoking control subjects, or of human monocytic U937 cells exposed to cigarette smoke extract (CSE), was quantified as the dexamethasone concentration required to achieve 30% inhibition of tumor necrosis factor-α–induced CXCL8 production in the presence or absence of the mTOR inhibitor rapamycin. mTOR activity was determined as the phosphorylation of p70 S6 kinase, using Western blotting.

Measurements and Main Results: mTOR activity was increased in peripheral blood mononuclear cells from patients with COPD, and treatment with rapamycin inhibited this as well as restoring corticosteroid sensitivity. In U937 cells, CSE stimulated mTOR activity and c-Jun expression, but pretreatment with rapamycin inhibited both and also reversed CSE-induced corticosteroid insensitivity.

Conclusions: mTOR inhibition by rapamycin restores corticosteroid sensitivity via inhibition of c-Jun expression, and thus mTOR is a potential novel therapeutic target for COPD.

Keywords: chronic obstructive pulmonary disease; corticosteroid resistance; mammalian target of rapamycin; rapamycin; c-Jun

At a Glance Commentary

Scientific Knowledge on the Subject: Peripheral blood mononuclear cells from patients with chronic obstructive pulmonary disease (COPD) are corticosteroid insensitive.

What This Study Adds to the Field: We provide evidence of a novel molecular mechanism of corticosteroid insensitivity in COPD, namely oxidative stress–induced c-Jun expression via mammalian target of rapamycin (mTOR) activation, and for the potential use of rapamycin, an mTOR inhibitor, for restoring corticosteroid sensitivity.

Chronic obstructive pulmonary disease (COPD) is associated with an inflammatory response to inhaled toxins, particularly cigarette smoke, and is a leading cause of mortality in the world (1). The majority of patients are elderly, and there is a growing evidence that COPD, particularly emphysema, presents accelerated aging of the lung (2–4). Nonprogrammed aging and cellular senescence may be accelerated by oxidative stress, which is markedly increased in COPD lungs (5, 6).

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Correspondence and requests for reprints should be addressed to Akihisa Mitani, M.D., Ph.D., Airway Disease Section, National Heart and Lung Institute, Dovehouse Street, London SW3 6LY, UK. E-mail: mitania-tky@umin.ac.jp

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Physiological and structural similarities between the aged lung and COPD lung have also been identified (7).

Long-acting bronchodilators are currently the mainstay of COPD therapy but do not significantly influence the underlying disease process. Glucocorticoids are the most effective therapy for many inflammatory diseases such as asthma. However, in patients with COPD, the clinical benefit of inhaled corticosteroids has been demonstrated only in the reduction of exacerbations, but not in reduction of symptoms or in the control of inflammation (8). We have previously reported that peripheral blood mononuclear cells (PBMCs) from patients with COPD are relatively corticosteroid-insensitive (9). This corticosteroid insensitivity in COPD lungs may be why even high doses of inhaled corticosteroids fail to reduce disease progression or mortality (8).

Glucocorticoids act by binding to and activating cytosolic glucocorticoid receptors (GRs). The activated GRs then translocate into the nucleus and induce the expression of a number of antiinflammatory genes by binding with glucocorticoid response elements (GREs) in the promoter regions of these genes. Activated GRs also bind to proinflammatory transcription factors and recruit corepressor proteins, resulting in the suppression of transcription of inflammatory genes, such as CXCL8 (10).

Several studies have investigated the molecular mechanisms of corticosteroid insensitivity in COPD. First, reduced histone deacetylase-2 (HDAC2) activity causes corticosteroid resistance because recruitment of HDAC2 is a major mechanism of inflammatory gene repression by glucocorticoids. Activation of phosphoinositide-3-kinase-δ (PI3Kδ) has been shown to reduce HDAC2 activity in smoking-induced inflammation (9, 11–13). PI3Kδ activity is increased and HDAC2 reduced in patients with COPD (9). Second, GR phosphorylation decreases its nuclear translocation, inducing corticosteroid insensitivity. Activation of p38 mitogen-activated protein kinase (p38MAPK)-α (14), p38MAPK-γ (15), or c-Jun N-terminal kinase (JNK) kinase induces GR phosphorylation, which impairs GR nuclear translocation (16). Third, increased expression of the dominant negative form of GR, GRβ, can reduce GR ligand-binding affinity, resulting in resistance to the antiinflammatory actions of glucocorticoid (17). Furthermore, increased proinflammatory transcription factors, such as activator protein (AP)-1, JNK, and STAT5 (signal transducer and activator of transcription-5), also account for glucocorticoid resistance. For example, excessive AP-1 binds GR and thus prevents its interaction with GREs (10, 18).

Mammalian target of rapamycin (mTOR), a serine/threonine kinase that plays a central role in cellular growth and metabolism as well as protein synthesis, is an important downstream effector of the PI3K/Akt pathway. mTOR is known to be activated in cancer, neurodegeneration, and diabetes (19). mTOR is present in two distinct complexes called mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Unique accessory proteins, regulatory-associated protein of mTOR (RAPTOR) and rapamycin-insensitive companion of mTOR (RICTOR), define mTORC1 and mTORC2, respectively. In yeast and mammals, the mTOR inhibitor rapamycin inhibits the ability of mTORC1, but not mTORC2, to phosphorylate its substrates (20). mTORC1 directly phosphorylates p70 S6 kinase (S6K), which is a serine/threonine protein kinase required for cell growth.

The mTOR inhibitor rapamycin has been considered a potential antiaging molecule (21). Treatment with rapamycin results in an extension of the life span of several species, including mice (22, 23). Similarly, transgenic mice with reduction of mTOR or S6K also demonstrate life span extension (24, 25). Although these results in mice may not necessarily translate to human aging pathways, recognition of COPD as a disease of accelerated aging has raised interest that mTORC1 might play a role in the pathogenesis of COPD.

Methods

For details, *see* METHODS in the online supplement. All data shown are expressed as means ± SEM.

Results

Corticosteroid Insensitivity in PBMCs from Patients with COPD

PBMCs were collected from nonsmoking healthy volunteers (HVs), smoking volunteers (SVs), and patients with mild to severe COPD; characteristics of the subjects are summarized in Table 1 (and *see* Table E1 in the online supplement). Reflecting the fact that the overall severity of COPD in the patients in this study was rather mild, only 1 patient of 13 was prescribed an inhaled corticosteroid. There was no patient taking oral corticosteroids, theophylline, nortriptyline, or statins (Table E1).

Corticosteroid effect was evaluated as the dexamethasone concentration inducing 30% inhibition of tumor necrosis factor (TNF)-α–induced CXCL8 production in PBMCs (Dex-IC_{30}). We used IC_{30} instead of IC_{50} because the value of percentage inhibition at maximal concentration (E_{max}) was sometimes below 50% and IC_{50} could not be calculated. The value for each patient is presented as log(Dex-IC_{30}) and groups of patients are compared. The log(Dex-IC_{30}) value in HVs was −8.00 ± 0.09, and log (Dex-IC_{30}) in COPD was significantly higher (−7.63 ± 0.08; $P < 0.01$), indicating that PBMCs in COPD are 2.3-fold less sensitive to steroid treatment compared with those of HVs (Figure 1A). Furthermore, the E_{max} value in COPD was also significantly

Table 1. Profile of Healthy Volunteers, Healthy Smoking Volunteers, and Patients with Chronic Obstructive Pulmonary Disease

	HVs (*n = 11*)	SVs (*n = 11*)	Patients with COPD (*n = 13*)
Age, yr	62.2 ± 2.2	52.3 ± 3.1*	65.4 ± 1.8
Sex, M/F	5/6	8/3	5/8
FVC, % predicted	112.8 ± 6.1	103.4 ± 4.2	99.1 ± 6.0
FEV_1/FVC, %	73.9 ± 2.4	77.1 ± 1.9	54.2 ± 3.4†
FEV_1, % predicted	101.9 ± 4.9	96.6 ± 3.2	65.8 ± 5.3†
Stage, 1/2/3/4	—	—	3/7/3/0
Smoking, pack-years	0	31.3 ± 6.8†	25.2 ± 4.8†

Definition of abbreviations: COPD = chronic obstructive pulmonary disease; HVs = healthy volunteers; SVs = smoking volunteers.
*$P < 0.05$ (compared with HVs).
†$P < 0.001$ (compared with HVs).

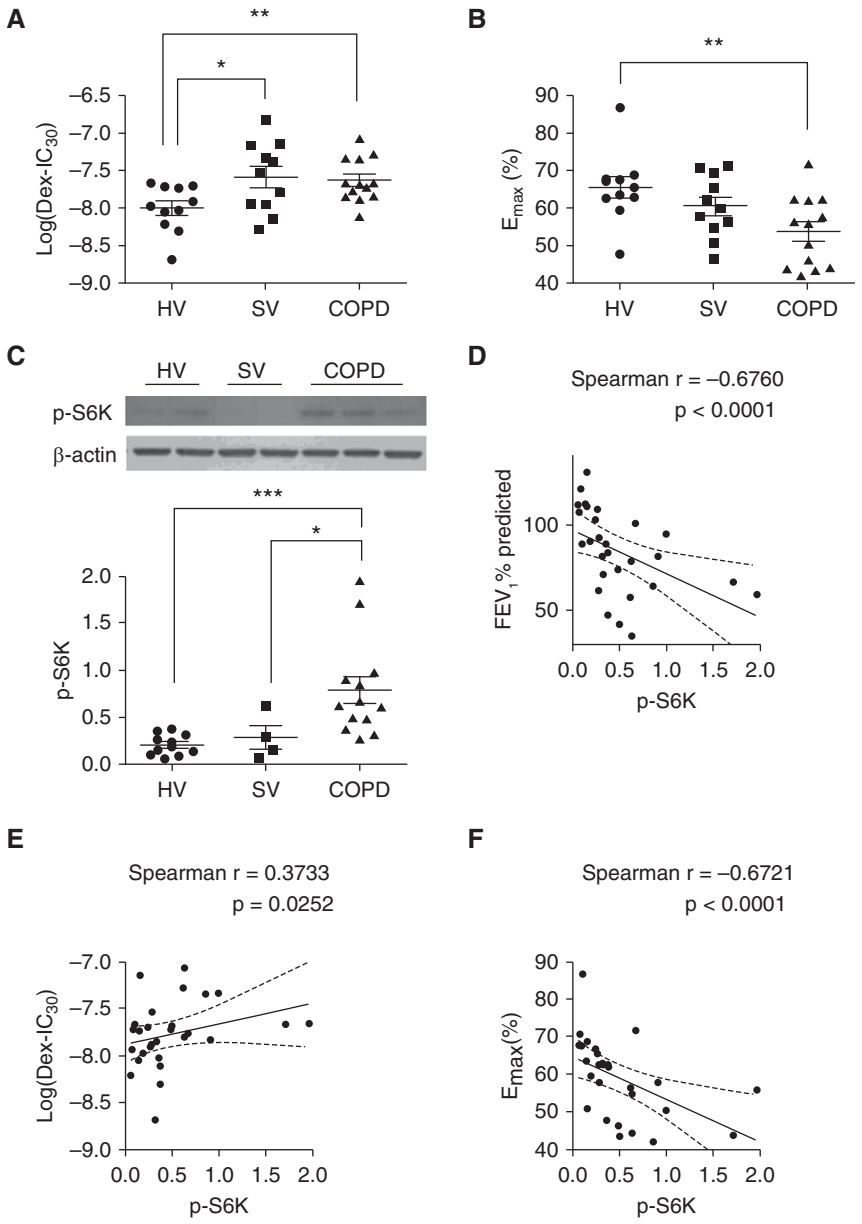

Figure 1. Analysis of peripheral blood mononuclear cell (PBMC) samples from healthy volunteers (HVs), smoking volunteers (SVs), and patients with chronic obstructive pulmonary disease (COPD). (*A* and *B*) PBMCs were seeded in the presence of various concentrations of dexamethasone (Dex) for 1 hour before overnight stimulation with tumor necrosis factor-α at 1 ng/ml. CXCL8 expression in supernatant was measured by ELISA. The rate of inhibition of CXCL8 by dexamethasone was calculated, and corticosteroid sensitivity was measured as (*A*) the concentration inducing 30% inhibition (IC_{30}) and (*B*) percent inhibition at maximal concentration (E_{max}), and plotted individually. (*C*) Mammalian target of rapamycin complex 1 (mTORC1)/p70 S6 kinase (S6K) activity was evaluated as phosphorylated S6K (p-S6K). (*D–F*) Correlation of mTORC1/S6K activity with (*D*) FEV_1% predicted, (*E*) log(IC_{30}-Dex), and (*F*) E_{max} was analyzed by Spearman correlation test. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (compared with NT if not indicated otherwise). NT = not treated.

lower than that in HVs (53.7 ± 2.6 vs. 65.4 ± 2.8%; $P < 0.01$) (Figure 1B). SVs also displayed less corticosteroid sensitivity, as represented by IC_{30}, compared with the HV group ($P < 0.05$), but the difference in E_{max} value was not statistically significant.

mTORC1 activity, which was assessed by S6K phosphorylation, was significantly increased in COPD PBMCs compared with HV and SV PBMCs (Figure 1C), negatively correlated with disease severity measure as FEV_1% predicted (Figure 1D), and correlated with steroid sensitivity measured as log(Dex-IC_{30}) (Figure 1E) and E_{max} (Figure 1F), suggesting a positive association between mTORC1/S6K activity and corticosteroid insensitivity.

Improvement of Corticosteroid Sensitivity in COPD PBMCs by Rapamycin

Pretreatment with rapamycin (20 nM) inhibited mTORC1/S6K activity almost completely (Figure 2A). Rapamycin also displayed a small but significant antiinflammatory effect against CXCL8 release by TNF-α in all three groups (Figure 2B). The corticosteroid effect was increased by pretreatment with rapamycin both in the HV and COPD groups, with a significant decrease in the value of log(Dex-IC_{30}) (Figure 2C). The EC_{50} (dexamethasone dose inducing inhibition halfway between minimal and maximal inhibition) was also decreased by pretreatment with rapamycin in COPD PBMCs (Table 2). On the other hand, E_{max} values were not improved by rapamycin (Figure 2D). This clearly showed that rapamycin improved only corticosteroid sensitivity, not maximal inhibition.

Inhibition of Cigarette Smoke Extract–activated mTORC1/S6K Pathway by Rapamycin

To investigate the underlying mechanisms whereby rapamycin improved corticosteroid sensitivity in COPD cells, a human monocytic cell line (U937) was stimulated with cigarette smoke extract (CSE) and the activities of the PI3K/Akt and mTORC1/S6K pathways were measured. PI3K/Akt activity, measured as the relative ratio of phosphorylated Akt at Ser-473 to total Akt, was quickly (2.5 min) and transiently activated after CSE stimulation (Figure 3A). This was immediately followed by activation (5 min) of mTORC1, which is downstream of PI3K/Akt (Figure 3B). Pretreatment with 20 nM rapamycin for 2 hours, which does not have any effect on cell viability, totally inhibited mTORC1/S6K activity, but not PI3K/Akt activity (Figures 3C and 3D). This justified the use of 20 nM rapamycin in our experiments.

Restoration of CSE-induced Corticosteroid Insensitivity in U937 Cells by Rapamycin

High concentrations of rapamycin decreased TNF-α–induced CXCL8 production (maximum, 30% at 20 nM)

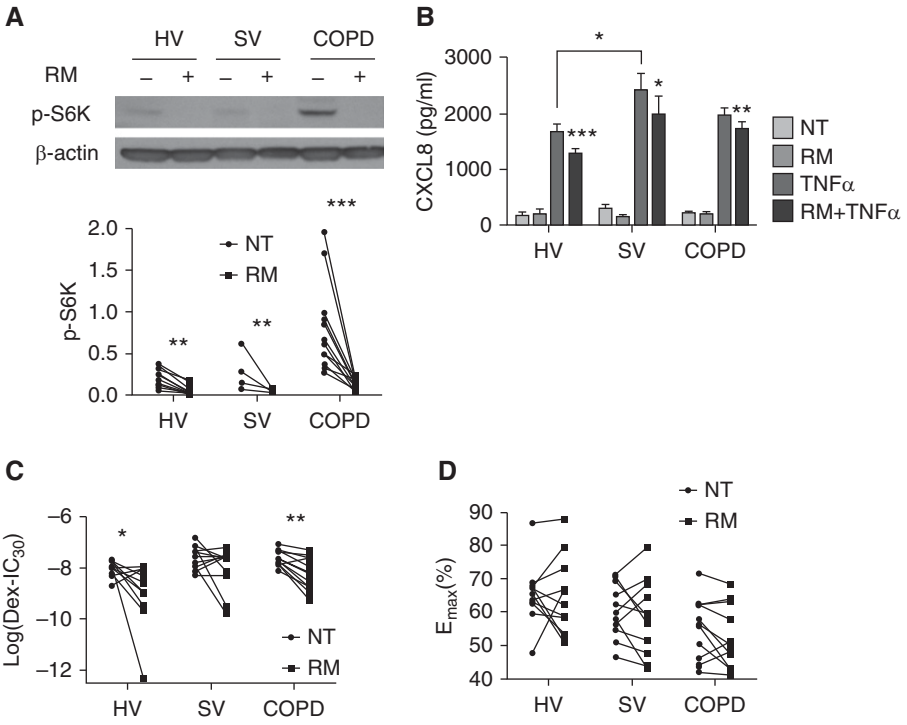

Figure 2. Effect of rapamycin (RM) treatment of peripheral blood mononuclear cell (PBMC) samples from healthy volunteers (HVs), smoking volunteers (SVs), and patients with chronic obstructive pulmonary disease (COPD). (*A*) PBMCs were incubated with 20 nM RM for 2 hours, and mammalian target of rapamycin complex 1/p70 S6 kinase (S6K) activity was calculated. (*B*) CXCL8 release caused by overnight tumor necrosis factor (TNF)-α stimulation with or without RM pretreatment. (*C* and *D*) PBMCs were treated with RM for 4 hours before measuring corticosteroid sensitivity. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (compared with NT if not indicated otherwise). Dex-IC_{30} = dexamethasone concentration inducing 30% inhibition; E_{max} = percent inhibition at maximal concentration; NT = not treated.

(Figure 4A). However, rapamycin did not affect basal corticosteroid sensitivity in U937 cells (Figure 4B). When U937 cells were pretreated with CSE, the IC_{30} and EC_{50} values for dexamethasone on TNF-α–induced CXCL8 production were increased from 4.3 and 6.1 nM to 56.1 and 33.3 nM, respectively. CSE pretreatment also decreased the E_{max} value from 61.8 ± 5.6 to 45.5 ± 5.6%. Rapamycin treatment before CSE (20 nM, 2 h) decreased significantly the IC_{30} and EC_{50} to 13.2 and 11.7 nM (from 56.1 and 33.3 nM), respectively, and increased E_{max} to 56.6 ± 4.8% (from 45.5 ± 5.6%). This confirms that rapamycin partially reverses the corticosteroid insensitivity caused by CSE in U937 cells ($P < 0.01$) (Figure 4C and Table 3).

Restoration of CSE-induced Corticosteroid Sensitivity in BEAS2B Cells by Rapamycin

Corticosteroid sensitivity in BEAS2B cells was measured, using the inhibition rate of IL-1β–induced granulocyte–macrophage colony–stimulating factor by dexamethasone (Figure 4D). When the cells were incubated with CSE for 3 hours, the IC_{30} and EC_{50} values for dexamethasone were increased from 22.8 to more than 1,000 nM and from 11.6 to 13.7 nM, respectively, and the E_{max} value decreased from 42.0 ± 0.5 to 10.5 ± 1.8%. Incubation with rapamycin before CSE stimulation decreased the IC_{30} and EC_{50} values to 69.9 and 11.7 nM, respectively, and increased E_{max} to 33.2 ± 0.2% ($P < 0.05$) (Figure 4D and Table 4). These results show that rapamycin restored CSE-induced corticosteroid resistance in BEAS2B cells as well as in U937 cells.

Inhibition of CSE-induced c-Jun by Rapamycin

As it has been reported that HDAC2 activity is reduced in COPD via activation of PI3Kδ (12, 26), we investigated the effect of rapamycin on the activity of immunoprecipitated HDAC2 in CSE-treated U937 cells. We confirmed that CSE decreased HDAC2 activity significantly (approximately 20%) as previously shown (9, 13), but rapamycin pretreatment did not affect HDAC2 activity and protein levels in the presence or absence of CSE (*see* Figures E1A and E1B).

We also investigated the effect of rapamycin on the expression of proinflammatory transcription factors by measuring p65 (nuclear factor [NF]-κB), c-Jun, and c-Fos, the last two forming AP-1, as NF-κB and AP-1 have previously been shown to be involved in corticosteroid sensitivity (10, 18). We found that neither CSE nor rapamycin altered p65 protein expression (Figure E1C). Rapamycin also failed to decrease

Table 2. Results of Steroid Sensitivity Test with or without Rapamycin Pretreatment

Treatment	Log(Dex-IC_{30})	Log(Dex-EC_{50})	E_{max}
HVs			
NT	−8.00 ± 0.09	−7.99 ± 0.11	65.4 ± 2.8
RM	−9.01 ± 0.37*	−8.75 ± 0.39	64.5 ± 3.5
SVs			
NT	−7.59 ± 0.14†	−7.70 ± 0.15	60.4 ± 2.5
RM	−8.03 ± 0.26	−9.56 ± 0.71	58.4 ± 3.5
Patients with COPD			
NT	−7.63 ± 0.08‡	−7.77 ± 0.07	53.7 ± 2.6‡
RM	−8.22 ± 0.17§	−9.25 ± 0.33§	50.1 ± 2.9

Definition of abbreviations: COPD = chronic obstructive pulmonary disease; Dex-EC_{50} = dexamethasone dose inducing inhibition halfway between minimal and maximal inhibition; Dex-IC_{30} = dexamethasone concentration inducing 30% inhibition of tumor necrosis factor-α–induced CXCL8 production in peripheral blood mononuclear cells; E_{max} = percentage inhibition at maximal concentration; HVs = healthy volunteers; NT = not treated; RM = rapamycin; SVs = smoking volunteers.
*$P < 0.05$ (compared with NT).
†$P < 0.05$ (compared with HVs).
‡$P < 0.01$ (compared with HVs).
§$P < 0.01$ (compared with NT).

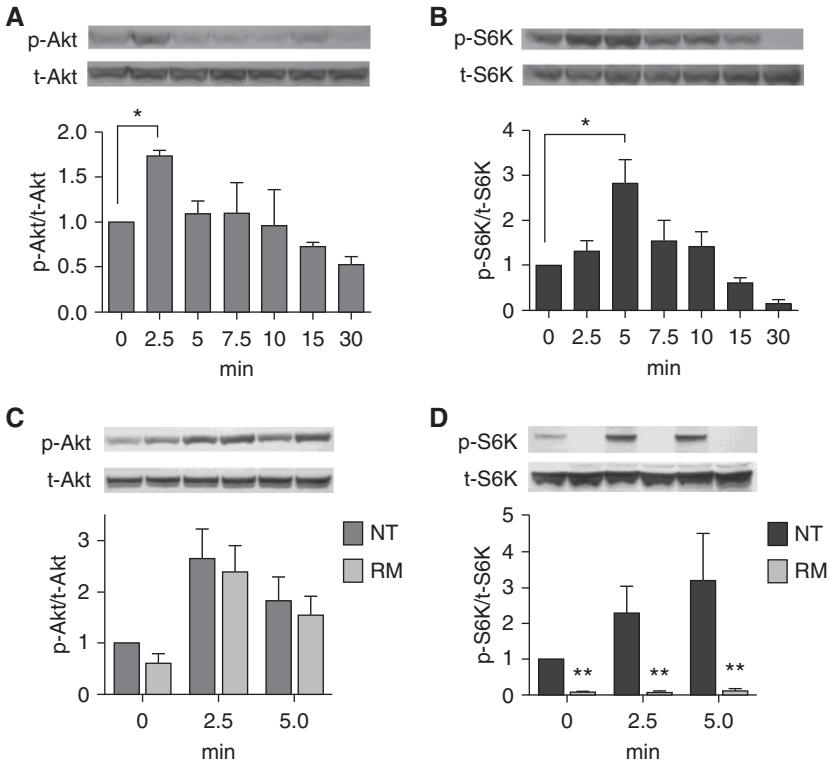

Figure 3. Effect of cigarette smoke extract (CSE) on phosphoinositide-3-kinase/Akt and mammalian target of rapamycin complex 1/p70 S6 kinase (S6K) activities in U937 cells. (*A* and *B*) U937 cells were treated with CSE at various time points (2.5 to 30 min), and (*A*) phosphorylated Akt (p-Akt) levels and (*B*) phosphorylated S6K (p-S6K) levels were determined by Western blotting. (*C* and *D*) After pretreatment with 20 nM rapamycin (RM) for 2 hours, cells were stimulated with CSE for up to 5 minutes. (*C*) p-Akt and (*D*) p-S6K levels were calculated. $^{*}P < 0.05$; $^{**}P < 0.01$ (compared with NT if not indicated otherwise). NT = not treated; t-Akt = total Akt; t-S6K = total S6K.

p65 nuclear localization induced by CSE and TNF-α (Figure E1D). Similarly, c-Fos protein expression was not altered by CSE and rapamycin (Figure E1E).

However, the expression of c-Jun, another AP-1 component, was significantly increased 4 hours after CSE stimulation (Figure 5A). Interestingly, rapamycin pretreatment significantly decreased both basal and CSE-induced c-Jun protein expression (Figure 5A). Furthermore, rapamycin was shown to decrease c-Jun expression in a concentration- and time-dependent manner without CSE stimulation (Figure 5B).

In addition, pretreatment with PI3Kδ inhibitor IC87114, which decreased mTORC1/SK6 activity as well as Akt activity (Figures 5C and 5D), also decreased the expression of c-Jun (Figure 5E) and CSE-induced c-Jun protein expression as observed with rapamycin (Figure 5F). These data suggest that CSE up-regulates c-Jun expression via activated PI3Kδ/Akt and mTORC1/SK6 pathways, and that inhibition of these pathways mediates c-Jun inhibition.

Corticosteroid Sensitivity Improved by c-Jun Knockdown

The expression of c-Jun protein was reduced to almost half at 8 hours after c-Jun small interfering RNA transfection and was inhibited by 30% at 24 hours (Figure 6A). Using the 8-hour time point, the effect of dexamethasone (10^{-7} M) on TNF-α–induced CXCL8 was evaluated by ELISA. In agreement with previous results, CSE decreased the percent inhibition by dexamethasone of CXCL8 (from 60.1 ± 2.9 to 26.0 ± 4.9%), whereas in c-Jun knockdown cells, the percent inhibition of CXCL8 was only slightly reduced (from 58.1 ± 3.3 to 47.3 ± 6.9%) (Figure 6B), suggesting that c-Jun has an essential role in this model of corticosteroid insensitivity.

c-Jun Protein Stability Reduced by Rapamycin

In U937 cells, CSE stimulation increased phosphorylation of JNK (p-JNK) and of c-Jun at Ser-63 and Ser-73, which are reported to cause increased c-Jun mRNA (27), but rapamycin did not affect this (Figures 7A–7C). Transcription of c-Jun was also significantly increased by CSE treatment, but rapamycin had only a little effect on the amount of c-Jun mRNA (Figure 7D).

Next we measured c-Jun protein stability in the presence of a 100-μg/ml concentration of cycloheximide, a protein translational inhibitor. U937 cells were treated with cycloheximide and rapamycin simultaneously, and 2 hours after these treatments they were exposed to CSE at the 0-hour time point. In the nontreated cells, c-Jun protein expression was decreased to almost 50% at the 2-hour time point, and reached 39.9 ± 28.3% of baseline at 4 hours. However, in CSE-treated cells, c-Jun protein was not altered up to 4 hours after exposure (Figure 7E). Conversely, in rapamycin-pretreated cells, c-Jun protein was rapidly reduced to about 70% during the first 2 hours in cycloheximide-treated cells. Rapamycin also reduced c-Jun protein expression stabilized by CSE stimulation (68.6 ± 24.2% reduction at the 4-h time point) compared with CSE-stimulated cells without rapamycin pretreatment ($P < 0.01$) (Figure 7E).

When the proteasome inhibitor MG132 was added, rapamycin-induced down-regulation of c-Jun expression was impaired (Figure 7F), suggesting rapamycin controlled c-Jun expression in a proteasome-dependent manner.

Increased c-Jun Expression in COPD PBMCs

We examined c-Jun protein expression in PBMCs from patients with COPD, HVs, and SVs. c-Jun protein was significantly increased in PBMCs of patients with COPD compared with HV PBMCs, but not SV PBMCs (Figure 8A). In addition, we found that mTORC1 activity, as measured by p-S6K, and c-Jun expression were positively correlated (Figure 8B) in all samples, confirming our previous *in vitro* data using U937 cells. Furthermore, c-Jun expression was positively correlated with log(Dex-IC_{30}) (Figure 8C) and E_{max} (Figure 8D), indicating c-Jun has an important role in corticosteroid sensitivity in PBMCs.

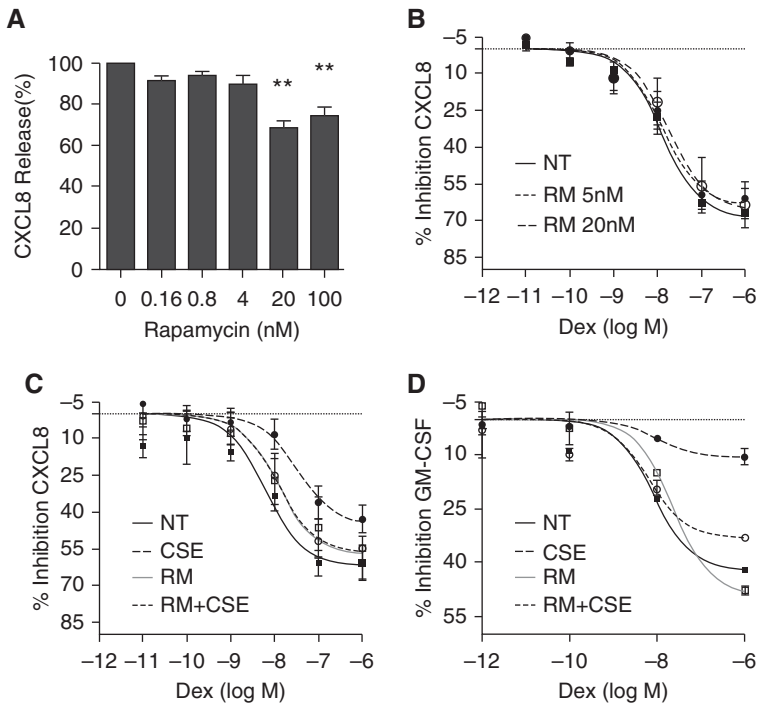

Figure 4. Rapamycin (RM) improved steroid sensitivity in U937 cells, as well as in BEAS2B cells. (*A*) Rate of inhibition of tumor necrosis factor-α–induced CXCL8 release by various concentrations of RM (0–100 nM) in U937 cells. (*B*) Corticosteroid sensitivities after pretreatment with various concentrations of RM (5 and 20 nM). (*C*) U937 cells were treated with RM for 2 hours and incubated with cigarette smoke extract (CSE) for another 2 hours. Cells were washed with phosphate-buffered saline and seeded in starvation medium, and corticosteroid sensitivity was measured on the basis of the rate of CXCL8 inhibition by dexamethasone (Dex). (*D*) Corticosteroid sensitivity in BEAS2B cells was also measured, based on the rate of inhibition of IL-1β–induced granulocyte–macrophage colony–stimulating factor by dexamethasone. $^{**}P < 0.01$. NT = not treated.

Increased mTORC1/S6K Activity and c-Jun Expression in Peripheral Lung Tissues from Patients with COPD

Peripheral lung tissue samples from HVs, SVs with normal lung function, and patients with COPD GOLD (Global Initiative for Chronic Obstructive Lung Disease) stage 1 (C1) and stage 2 (C2) were obtained from a tissue bank. The clinical features are summarized in Table 5. mTORC1/S6K activities in peripheral lung tissue from C1 and C2 were significantly increased compared with those from HVs and SVs (Figure 9A). c-Jun expression was also increased in the C1 and C2 groups, although there was no significant difference compared with the HV group (Figure 9B). Interestingly, mTORC1 activity was negatively correlated with disease severity measured as FEV_1% predicted (Figure 9C). There results reinforced the evidence demonstrated in PBMCs and U937 cells, suggesting the importance of mTORC1 activity and c-Jun expression in the pathogenesis of COPD.

Discussion

Corticosteroid resistance is an important characteristic of COPD. PBMCs from patients with COPD were found to be less corticosteroid responsive compared with cells from healthy volunteers, thus confirming previous results from our group (9).

In this study we focused on mTORC1/S6K activity because it lies downstream of the PI3K/Akt pathway, which has been previously shown to be involved in corticosteroid resistance in COPD (9, 13). We found for the first time that mTORC1/S6K activity is increased in patients with COPD and that it also negatively correlated with COPD disease severity as measured by FEV_1% predicted. In addition, mTORC1/S6K activity positively correlated with corticosteroid insensitivity as measured by the inhibitory effect of dexamethasone on release of inflammatory mediators (Dex-IC_{30}), suggesting that the mTORC1/S6K pathway plays a role in the development of corticosteroid resistance. Importantly, the mTOR inhibitor rapamycin also improved corticosteroid sensitivity (Dex-IC_{30}) in PBMCs from healthy volunteers and patients with COPD. The fact that rapamycin reduced Dex-EC_{50}, and consequently increased corticosteroid sensitivity (Dex-IC_{30}) but did not affect E_{max}, suggests that rapamycin would not increase the maximal effect of corticosteroids, which means that increasing the dose of corticosteroids may be as effective as rapamycin, although high doses of corticosteroids may be required.

Oxidative stress, such as that generated by H_2O_2 and CSE, may play an important role in the development of corticosteroid resistance in COPD and severe asthma (10). In this study, we used human monocytic U937 cells and showed that CSE stimulated the mTORC1/S6K pathway shortly after the activation of PI3K/Akt, and decreased corticosteroid

Table 3. Effects of Rapamycin on Corticosteroid Sensitivity in U937 Cells

	Dex-IC_{30} (*nM*)	Dex-EC_{50} (*nM*)	E_{max} (%)
NT	4.3	6.1	61.8 ± 5.6
CSE	56.1	33.3	45.5 ± 5.6
RM	15.6	11.6	57.4 ± 6.7
RM+CSE	13.2	11.7	56.6 ± 4.8

Definition of abbreviations: CSE = cigarette smoke extract; Dex-EC_{50} = dexamethasone dose inducing inhibition halfway between minimal and maximal inhibition; Dex-IC_{30} = dexamethasone concentration inducing 30% inhibition of tumor necrosis factor-α–induced CXCL8 production in peripheral blood mononuclear cells; E_{max} = percentage inhibition at maximal concentration; NT = not treated; RM = rapamycin.

Table 4. Effects of Rapamycin on Corticosteroid Sensitivity in BEAS2B Cells

	Dex-IC_{30} (*nM*)	Dex-EC_{50} (*nM*)	E_{max} (%)
NT	22.8	11.6	42.0 ± 0.5
CSE	>1,000	13.7	10.5 ± 1.8
RM	35.3	21.9	48.0 ± 1.0
RM+CSE	69.9	10.2	33.2 ± 0.2

Definition of abbreviations: CSE = cigarette smoke extract; Dex-EC_{50} = dexamethasone dose inducing inhibition halfway between minimal and maximal inhibition; Dex-IC_{30} = dexamethasone concentration inducing 30% inhibition of tumor necrosis factor-α–induced CXCL8 production in peripheral blood mononuclear cells; E_{max} = percentage inhibition at maximal concentration; NT = not treated; RM = rapamycin.

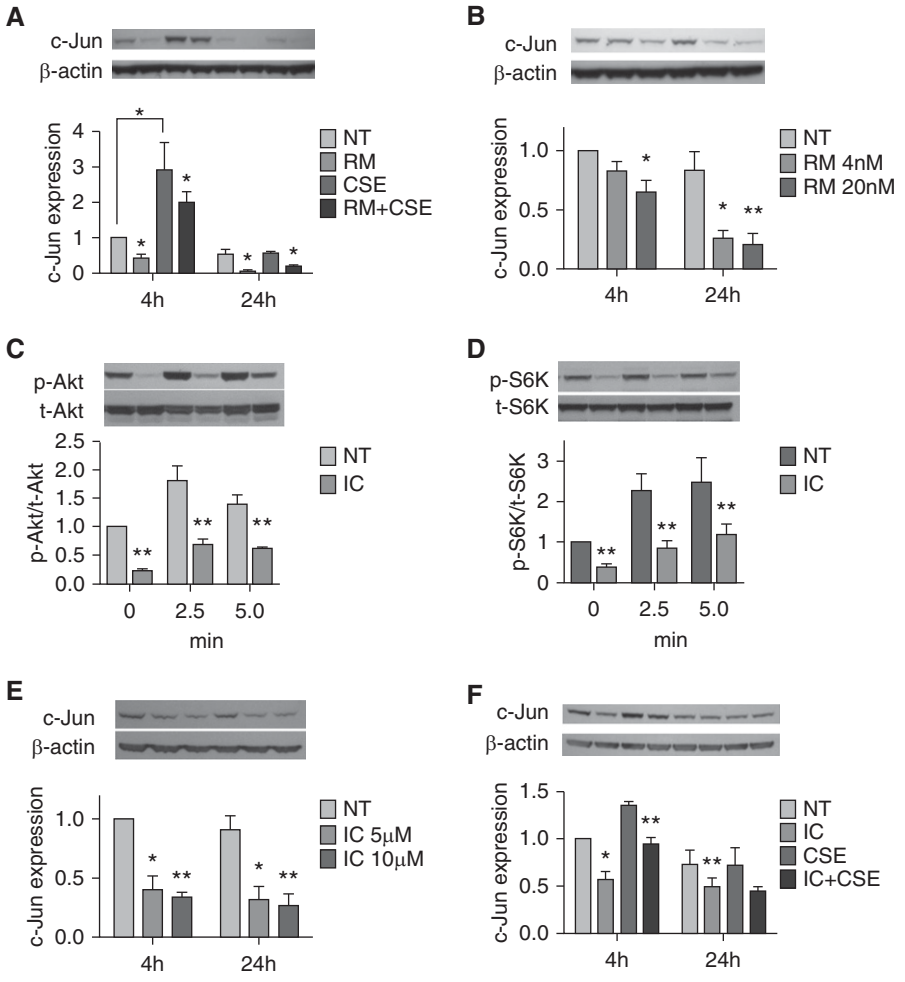

Figure 5. Rapamycin (RM) and phosphoinositide-3-kinase-δ (PI3Kδ) inhibitor IC87114 (IC) decrease c-Jun expression. (*A*) U937 cells were incubated in the medium at various time points after cigarette smoke extract (CSE) stimulation with or without RM pretreatment for 2 hours. c-Jun expression was measured by Western blotting. (*B*) c-Jun expression in U937 cells, which were incubated with 4 or 20 nM RM, at various time points. (*C* and *D*) Cells were treated with 10 μM IC before CSE stimulation, and (*C*) PI3K/Akt activity and (*D*) mammalian target of rapamycin complex 1/p70 S6 kinase (S6K) activity were calculated. (*E* and *F*) c-Jun expression after (*E*) treatment with 5 or 10 μM IC, and (*F*) CSE stimulation with 10 μM IC pretreatment. *$P < 0.05$; **$P < 0.01$ (comparison of activities with and without RM [or IC] pretreatment). NT = not treated; p-Akt = phosphorylated Akt; p-S6K = phosphorylated S6K; t-Akt = total Akt; t-S6K = total S6K.

sensitivity. Rapamycin totally inhibited mTORC1 activity, and reversed corticosteroid resistance caused by CSE, suggesting CSE decreased steroid sensitivity via the mTORC1/S6K pathway as well as the upstream PI3K/Akt pathway.

We analyzed the effects of CSE on possible targets involved in corticosteroid insensitivity and found that CSE increased c-Jun protein in our model. AP-1, a heterodimer of c-Fos and c-Jun, is activated by various proinflammatory stimuli, such as TNF-α, through the JNK pathway, and in turn controls a number of cellular processes including proliferation, differentiation, and apoptosis (27). Excessive accumulation of AP-1 has been identified as one mechanism of glucocorticoid resistance, because overexpressed AP-1 binds to GR, which prevents GR from interacting with GREs and other transcription factors, resulting in steroid resistance (28, 29). Knockdown of c-Jun confirmed that ablation of c-Jun prevents CSE-induced corticosteroid insensitivity in U937 cells. Hence CSE-induced corticosteroid insensitivity is mediated via increased c-Jun expression. Interestingly, rapamycin treatment decreased the expression of c-Jun in U937 cells. The inhibition of PI3Kδ/Akt also had the same effect on c-Jun, reinforcing the fact that this pathway is important in the regulation of c-Jun.

Previously we have shown that CSE-induced corticosteroid insensitivity in U937 cells was in part caused by reduced HDAC2 activity and that nortriptyline reversed corticosteroid sensitivity by inhibiting PI3Kδ and restoring HDAC2 activity levels (13). Although we confirmed that CSE reduced HDAC2 activity levels, rapamycin did not have any effect on HDAC2 activity. This suggests that the PI3K/Akt pathway causes corticosteroid resistance by at least two different pathways in CSE-treated U937 cells (Figure 10).

It has been reported that rapamycin inhibits phytohemagglutinin-induced c-Jun expression in human T cells (30), and that starvation, which inhibits mTOR activity, also reduces c-Jun expression (31). However, the underlying mechanism has not been elucidated. JNK activity itself is known to affect corticosteroid sensitivity. Wan and colleagues reported that the selective JNK inhibitor SP-600125

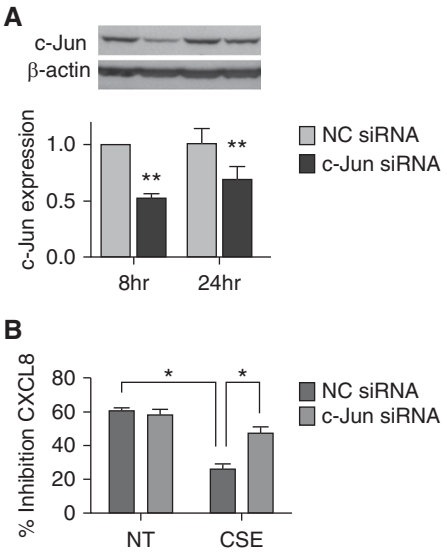

Figure 6. c-Jun knockdown improved corticosteroid sensitivity. (*A*) c-Jun small interfering RNA (siRNA) was transfected into U937 cells, using GenomONE-Neo EX (Cosmo Bio USA, Carlsbad, CA). The effect of c-Jun siRNA on expression of c-Jun was measured by Western blotting. (*B*) Eight hours after transfection with c-Jun siRNA corticosteroid sensitivity was measured, using the rate of inhibition of tumor necrosis factor-α–induced CXCL8 at 10^{-7} M dexamethasone. $^{*}P < 0.05$; $^{**}P < 0.01$ (compared with negative control [NC] if not indicated otherwise). CSE = cigarette smoke extract; NT = not treated.

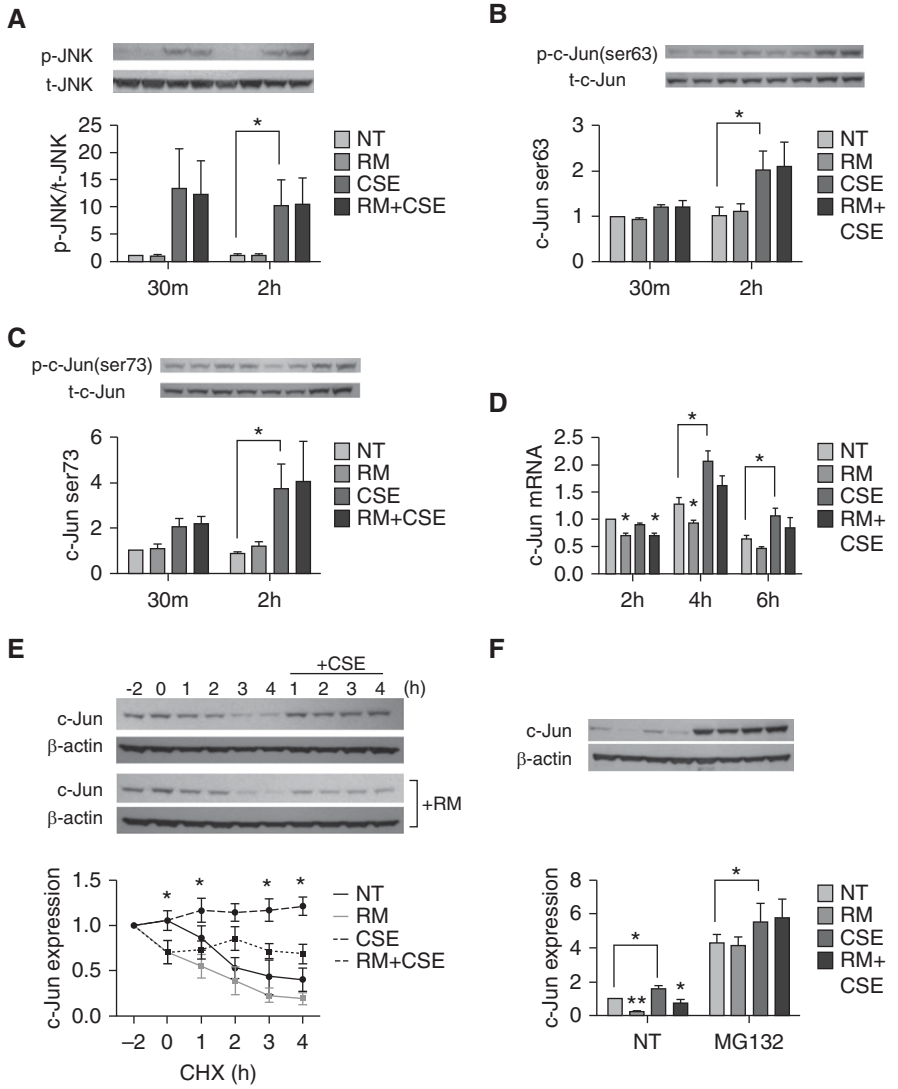

Figure 7. Rapamycin (RM) decreased c-Jun stability. (*A–C*) U937 cells were stimulated with cigarette smoke extract (CSE) at various time points (30 min and 2 h) with or without RM pretreatment. c-Jun N-terminal kinase (JNK) activity was evaluated as (*A*) phosphorylated JNK (p-JNK) level, and the phosphorylation status of (*B*) c-Jun Ser-63 and (*C*) c-Jun Ser-73 was measured. (*D*) Quantitative reverse transcriptase–polymerase chain reaction of c-Jun after CSE stimulation with or without RM. (*E*) c-Jun protein stability was measured with cycloheximide (CHX) at 100 μg/ml. In the presence of CHX, the cells were treated with RM and CSE, using the same procedure as described previously (RM was added at –2 h, and CSE was added at 0 h). Statistical analysis was performed between the CSE and RM+CSE groups. (*F*) Proteasome inhibitor MG132 was added at a concentration of 4 μM. Again, the cells were treated with CSE for 2 hours with RM pretreatment. $^{*}P < 0.05$; $^{**}P < 0.01$ (compared with NT if not indicated). NT = not treated; t-c-Jun = total c-Jun; t-JNK = total JNK.

increased GR binding to the GRE in mouse hippocampal HT22 cells (32). In addition, JNK1 hyperactivation in another corticosteroid-insensitive disease, severe asthma, is known to cause GR phosphorylation and defective GR nuclear translocation (33). We found that CSE increased c-Jun protein stability by inducing JNK-mediated phosphorylation of c-Jun at Ser-63 and Ser-73. Furthermore, CSE also induced c-Jun transcription levels, which might be the result of the phosphorylation of c-Jun (34). But our study showed that rapamycin did not affect the phosphorylated status of JNK or of c-Jun in U937 cells. Rapamycin slightly reduced c-Jun transcription, but not enough to inhibit CSE-induced up-regulation of transcription levels. The mTORC1/S6K pathway also plays critical roles in translational regulation by eIF4E (eukaryotic translation initiation factor 4E) (35–37). But knockdown of eIF4E or eIF4G did not affect c-Jun protein translation, revealing that c-Jun protein is not controlled by this cascade (*see* Figure E2). Instead, rapamycin was shown to reduce the protein half-life of c-Jun, either in the presence or absence of CSE. This was further confirmed by the fact that an inhibitor of the proteasome prevented rapamycin reduction of protein stability. Thus rapamycin seems to induce proteasomal degradation of c-Jun. Hence CSE-induced corticosteroid insensitivity is mediated via c-Jun transcription up-regulation and an increase in protein stability. Further work will be needed to establish how the activation of mTOR mediates an increase in c-Jun.

The expression of c-Jun was significantly higher in PBMCs from patients with COPD than in those from

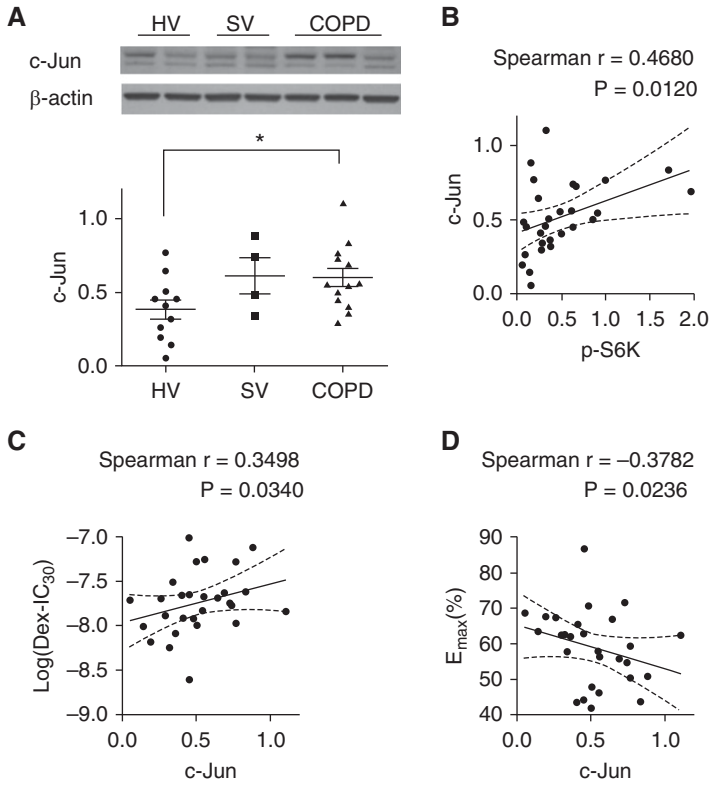

Figure 8. Expression of c-Jun in peripheral blood mononuclear cell samples from healthy volunteers (HVs), smoking volunteers (SVs), and patients with chronic obstructive pulmonary disease (COPD). (*A*) c-Jun expression was measured by Western blotting and plotted individually. (*B–D*) Correlations between c-Jun expression and (*B*) mammalian target of rapamycin complex 1/p70 S6 kinase (S6K) activity and between c-Jun and (*C* and *D*) corticosteroid sensitivity were analyzed by Spearman correlation test. $^*P < 0.05$. Dex-IC_{30} = dexamethasone concentration inducing 30% inhibition; E_{max} = percent inhibition at maximal concentration; p-S6K = phosphorylated S6K.

healthy volunteers, consistent with our results so far. The positive correlations between mTORC1/S6K activity, c-Jun expression, and log(Dex-IC_{30}) also suggest that activated S6K increases c-Jun expression, which in turn causes steroid resistance. The fact that mTORC1 activity and c-Jun expression were also increased in peripheral lung tissue from patients with COPD reinforces the clinical relevance of these findings.

AP-1 has been shown to be involved in the corticosteroid resistance of severe asthma. It has been reported that c-Fos expression was increased in PBMCs and bronchial biopsies, and that high-dose prednisolone did not reduce the number of cells expressing phosphorylated c-Jun (29). On the other hand, in mice a lack of c-Jun specifically in lung alveolar epithelial cells results in progressive emphysema and worsens cigarette smoke–induced lung inflammation (38). These studies suggest that the role of c-Jun in COPD is complex. Excessive expression of c-Jun causes corticosteroid resistance, although it is critical for maintaining lung alveolar cell homeostasis, and loss of its expression could contribute to lung inflammation.

Overall, our data reveal that inhibition of mTORC1/S6K is a potential novel strategy for restoration of corticosteroid sensitivity in COPD. Rapamycin is already in clinical use as an immunosuppressive for other diseases. For example, it is administered to patients with lymphangioleiomyomatosis (LAM) because it prevents the growth of LAM-derived smooth muscle cells (39). However, tolerance and safety concerns limit the chronic treatment of patients with rapamycin. In the case of COPD, inhalation therapy might be a solution to reducing adverse effects. Studies in combination therapy with theophylline are also warranted, because we previously reported that a low concentration of theophylline

Table 5. Characteristics of Patients from Whom Peripheral Lung Tissue Was Obtained

	HVs (*n* = 3)	SVs (*n* = 10)	C1 (*n* = 7)	C2 (*n* = 9)
Age, yr	68.7 ± 3.5	65.8 ± 4.0	68.4 ± 2.7	62.7 ± 2.5
Sex, M/F	1/2	4/6	5/2	4/5
FEV_1/FVC, %	82.1 ± 3.0	73.9 ± 0.7*	62.7 ± 1.9*	61.5 ± 2.6*
FEV_1, % predicted	101.5 ± 8.4	95.9 ± 5.0	88.5 ± 1.7	65.4 ± 5.8*†
Smoking, pack-years	0	57.9 ± 10.3*	52.6 ± 11.6*	54.3 ± 12.8*

Definition of abbreviations: C1, C2 = chronic obstructive pulmonary disease GOLD (Global Initiative for Chronic Obstructive Lung Disease) stages 1 and 2, respectively; HVs = healthy volunteers; SVs = smoking volunteers.
*$P < 0.05$ (compared with HVs).
†$P < 0.01$ (compared with C1).

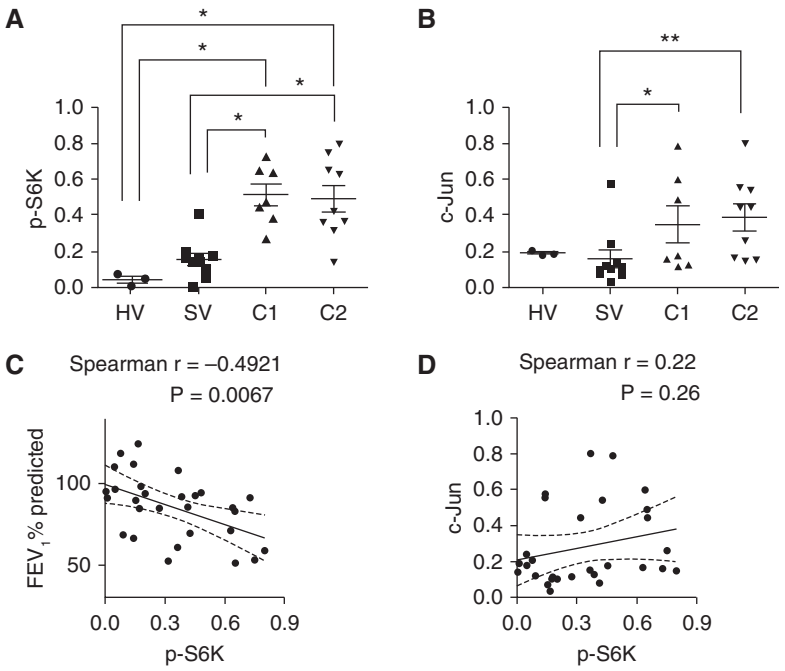

Figure 9. Mammalian target of rapamycin complex 1 (mTORC1)/p70 S6 kinase (S6K) activity and expression of c-Jun in peripheral lung tissue from healthy volunteers (HVs), smoking volunteers (SVs), and patients with chronic obstructive pulmonary disease GOLD category 1 (C1) and category 2 (C2). (*A*) mTORC1/S6K activity was calculated on the basis of phosphorylated S6K (p-S6K) expression and plotted individually. (*B*) c-Jun expression was also measured by Western blotting. (*C* and *D*) Correlations between mTORC1/S6K activity and (*C*) FEV_1% predicted and between mTORC1/S6K activity and (*D*) c-Jun expression were analyzed by Spearman correlation test. $^*P < 0.05$; $^{**}P < 0.01$.

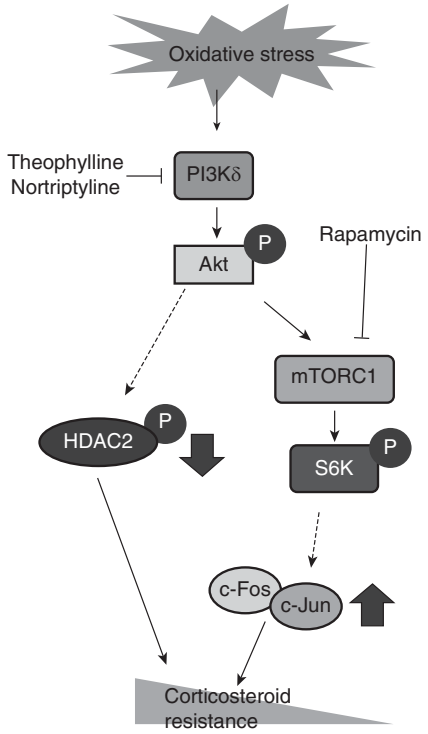

Figure 10. A scheme of how mammalian target of rapamycin may be involved in the development of steroid resistance. HDAC2 = histone deacetylase-2; mTORC1 = mammalian target of rapamycin complex 1; P = phosphorylated; PI3Kδ = phosphoinositide-3-kinase-δ; S6K = p70 S6 kinase.

improves corticosteroid sensitivity via inhibition of PI3Kδ activity and restoration of HDAC2 activity (9). In addition, numerous rapamycin analogs (rapalogs) are being developed, such as temsirolimus, everolimus, and ridaforolimus; they are expected to work similarly to rapamycin as mTOR inhibitors but may be less toxic (40). The second generation of mTOR inhibitors includes ATP-competitive mTOR kinase inhibitors, which block both mTORC1 and mTORC2 (41). Their effect on corticosteroid sensitivity is unknown. Further investigations including animal models are needed.

In conclusion, the mTOR inhibitor rapamycin restores corticosteroid resistance via decreased c-Jun expression, suggesting that the targeting of mTORC1 might be a potential new treatment for COPD. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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