Inflammation and remodelling in experimental models of COPD - Mechanisms and therapeutic perspectives

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Chapter 4

ROLE FOR TAK1 IN CIGARETTE SMOKE-INDUCED PRO-INFLAMMATORY SIGNALLING AND IL-8 RELEASE BY HUMAN AIRWAY SMOOTH MUSCLE CELLS

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Abstract

Chronic obstructive pulmonary disease (COPD) is an inflammatory disease, characterized by a progressive decline in lung function. Airway smooth muscle (ASM) mass may be increased in COPD, contributing to airflow limitation and pro-inflammatory cytokine production. Cigarette smoke (CS), the major risk factor of COPD, causes ASM cell proliferation as well as interleukin-8 (IL-8)-induced neutrophilia. In various cell types, TGF-β-activated kinase 1 (TAK1) plays a crucial role in MAP kinase and NF-κB activation as well as IL-8 release induced by IL-1β, TNF-α and lipopolysaccharide. The role of TAK1 in CS-induced IL-8 release is not known. The aim of this study was to investigate the role of TAK1 in CS-induced NF-κB and MAP-kinase signaling and IL-8 release by human ASM cells. Stimulation of these cells with CS extract (CSE) increased IL-8 release and ERK 1/2 phosphorylation, as well as Iκ-Bα degradation and p65 NF-κB subunit phosphorylation. CSE-induced ERK 1/2 phosphorylation and Iκ-Bα degradation were both inhibited by pretreatment with the specific TAK1 inhibitor (LL-Z-1640-2; 100 nM). Similarly, expression of dominant-negative TAK1 inhibited CSE-induced ERK 1/2 phosphorylation. In addition, inhibitors of TAK1 and the NF-κB (SC-514; 50 μM) and ERK 1/2 (U 0126; 3 μM) signaling inhibited the CSE-induced IL-8 release by ASM cells. These data indicate that TAK1 plays a key role in CSE-induced ERK 1/2 and NF-κB signaling as well as in IL-8 release by human ASM cells, and identify TAK1 as a potential novel target for the inhibition of CS-induced inflammatory responses involved in the development and progression of COPD.

Introduction

Chronic obstructive pulmonary disease (COPD) is an inflammatory disease characterized by progressive airflow obstruction. Pulmonary infiltration of inflammatory cells, including neutrophils, may contribute to structural changes of the lung that are involved in the decline of lung function, including small airway remodeling (1). Small airway remodeling in COPD is characterized by peribronchiolar fibrosis and mucus cell hyperplasia, as well as increased airway smooth muscle (ASM) mass, particularly in severe disease (1-5).

By its contractile function, ASM is a key regulator of airway diameter. However, ASM cells can also act as synthetic cells, releasing inflammatory cytokines and contributing to inflammatory responses in the lung. Cigarette smoke (CS), the major risk factor of COPD, has been shown to induce the release of interleukin-8

(IL-8) from ASM cells, which may lead to increased numbers of neutrophils in the airways (6-8). The proportion of airways containing neutrophils - as well as other inflammatory cells - has been found to correlate with COPD severity (1). The mechanisms involved in IL-8 release by ASM cells have, however, not yet been elucidated.

The serine/threonine kinase TGF-β-activated kinase 1 (TAK1) is a member of the mitogen activated protein kinase kinase kinase (MAP3K) family (MAP3K7). Initially, TAK1 was identified as a mediator of transforming growth factor-β (TGF-β) and bone morphogenetic protein (BMP) signaling (9), but has since emerged as a key player in interleukin-1 (IL-1)- (10), tumor necrosis factor-α (TNF-α)- (11) and Toll-like receptor (TLR) (12) signaling. TAK1 activates both nuclear factor-kappa B (NF-κB) (13, 14) and MAP kinase (9) signaling pathways, including the ERK 1/2 pathway (15-18). TAK1 has been found to play a major role in various immune responses (12, 19-21); however, its role in pro-inflammatory cytokine release by ASM cells or in CS-induced signaling is currently unknown. In this study, we present evidence that TAK1 is importantly involved in CS-induced IL-8 release from ASM cells.

Materials and Methods

Cells

Primary human bronchial smooth muscle cells, immortalized by stable expression of human telomerase reverse transcriptase (hTERT) were cultured in Dulbecco's Modified Eagle Medium DMEM supplemented with 50 U/ml streptomycin, 50 μg/ml penicillin, 1.5 μg/ml amphotericin B and 10% vol/vol fetal bovine serum (FBS; Gibco BRL Life Technologies, Paisley, UK).

Cigarette Smoke Extract

Cigarette smoke extract (CSE) was prepared by combusting two University of Kentucky 3R4F research cigarettes (filters removed) using a peristaltic pump (Watson Marlow 323 E/D) and passing the smoke through 25 ml of FBS-free DMEM supplemented with penicillin, streptomycin and amphotericin B at a rate of 5 min / cigarette. The obtained solution is referred to as 100 % strength.

Interleukin-8 determination

hTERT immortalized human ASM cells were plated in 24-well cluster plates and grown to confluence. Upon confluence, cells were washed two times with sterile phosphate-buffered saline (PBS) and made quiescent by incubation in serum-

free medium, supplemented with ITS (5 µg/ml insulin, 5 µg/ml transferrin, and 5 ng/ml selenium) for 24 h. Cells were then washed with PBS and stimulated with CSE (5 or 15%) in serum-free medium. Inhibitors of TAK1 (LL-Z1640-2, 100nM), IκB kinase 2, IKK2 (SC-514; 50 µM) and mitogen-activated protein kinase kinase 1 MEK 1/2 (U 0126; 3 µM) were added 30 min before stimulation. Supernatants were collected 24 h after CSE stimulation and stored at -20°C until use. IL-8 levels were determined by using a specific sandwich enzyme-linked immunosorbent assay (ELISA) (Sanquin, Amsterdam, The Netherlands), according to the manufacturers' instructions.

Transfection of plasmid DNA

Cells were grown to 95% confluence on 6-well plates, washed twice with PBS and then transfected using a mixture of 10 µl Lipofectamine 2000 and 2 µg DNA encoding a hemmagluttinine (HA)-tagged, dominant-negative TAK1 mutant (TAK1 K63W) or GFP, as control, for 6 h in 600 µl DMEM without serum and antibiotics. After 6 h, cells were washed twice with PBS and the medium was changed to DMEM supplemented with antibiotics and 10% FBS. Subsequently, the cells were then cultured for another 18 h. Dominant-negative TAK1 was a kind gift from Dr. B.J. Eggen, (Department of Developmental Genetics, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, The Netherlands), with permission of Prof. K. Matsumoto, (Department of Molecular Biology, Graduate School of Science, Nagoya University, Japan)

Western blot analysis

Cells were plated in 6-well cluster plates and grown to confluence. Upon confluence, cells were washed two times with sterile PBS and made quiescent by incubation in serum-free medium, supplemented with ITS for 24 h. Cells were then washed with PBS and stimulated with 15% CSE in serum-free DMEM. To obtain total cell lysates, cells were washed once with ice-cold PBS and subsequently lysed in ice-cold RIPA buffer (composition: 50 mM Tris, 150 mM NaCl, 1% Igepal CA-630, 1% deoxycholic acid, 1 mM NaF, 1 mM Na_3VO_4, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 7 µg/ml pepstatin A, 5 mM 2-glycerophosphoric acid, pH 8.0). Lysates were stored at -20 °C until further use. Protein content was determined according to Bradford(22). Homogenates containing 20 µg of protein per lane were then subjected to immunoblot analysis, using specific antibodies against IκBα, p-p65, p-ERK 1/2 (Thr^{202}/Tyr^{204}), total ERK1/2, and β-actin or GAPDH as loading control. The antibodies were visualized using enhanced chemiluminescence. Photographs of the blots were analyzed by densitometry.

Data analysis

All data represent means ± S.E.M. from separate experiments. The statistical significance of differences between data was determined by one-way ANOVA, followed by a Bonferroni multiple comparison test. Differences were considered to be statistically significant when $P<0.05$.

Results

CSE-induced IL-8 production by ASM cells

First, we evaluated CS-induced IL-8 production by ASM cells, by determining the effects of CSE (5 and 15%) on IL-8 release by hTERT-immortalized human ASM cells. CSE induced a profound, concentration-dependent increase in IL-8 release by these cells (Figure 1). These findings were confirmed in primary human tracheal smooth muscle cells (data not shown).

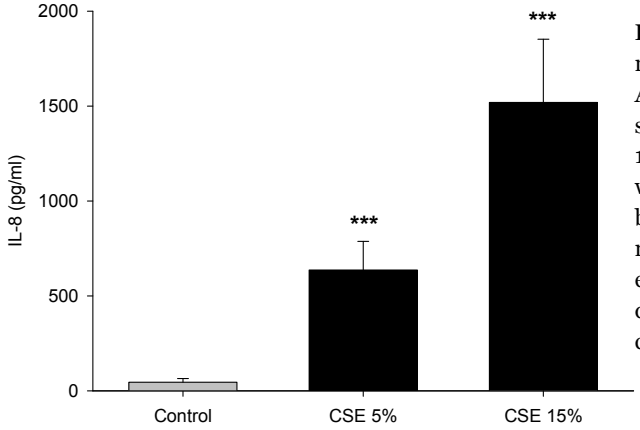

Figure 1: CSE induces IL-8 release by hTERT human ASM cells. Cells were stimulated with CSE (5 or 15%) for 24 h. Supernatants were collected and analyzed by ELISA. Data represent means ± S.E.M. of 8 experiments performed in duplicate. ***P<0.005 *vs* control.

CSE-induced NF-κB and ERK 1/2 pathway activation

To investigate the effects of CS on NF-κB and ERK 1/2 pathway activation, hTERT human ASM cells were stimulated with 15% CSE for 0.5 - 2 h (NF-κB) or for 5 min - 2 h (ERK 1/2). Cell lysates were analysed by western blotting for IκBα, and the phospho-p65 NF-κB subunit (NF-κB pathway) or phospho-ERK 1/2. CSE induced a significant decrease in IκBα abundance after 1 and 2 h of stimulation (Figure 2A) as well as an increase in phosphorylation of the p65 NF-κB subunit after 2h (Figure 2B). CSE induced phosphorylation of ERK 1/2, reaching significance after 60 min, which was sustained for up to at least 2 h (Figure 3). These data indicate that CSE induces activation of both the NF-κB and ERK 1/2 pathways.

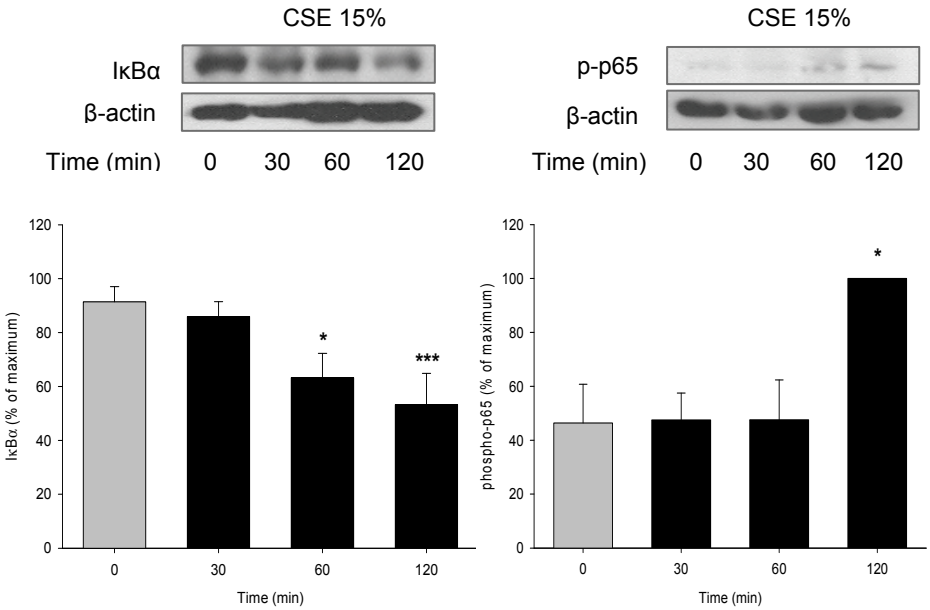

Figure 2: CSE induces IκBα degradation and p65 NF-κB phosphorylation in hTERT human ASM cells. Cells were treated with 15% CSE for up to 2 h. Cell lysates were analyzed by immunoblotting for IκBα and phospho-p65; β-actin was used to correct for protein loading. Densitometry data are normalized to the maximal response in each individual experiment and are means ± S.E.M. of 4-6 experiments. Representative blots are shown. *P<0.05; ***P<0.001 *vs* t=0.

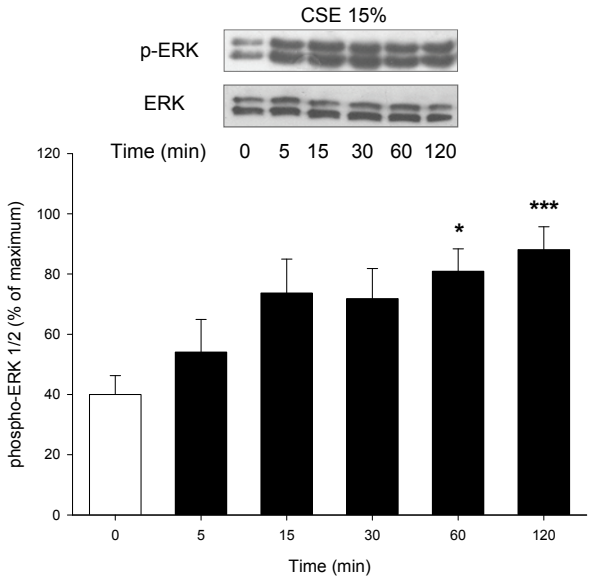

Figure 3: CSE induces ERK 1/2 phosphorylation in hTERT human ASM cells. Cells were treated with CSE 15% up to 2 h. Cell lysates were analyzed by immunoblotting for phospho-ERK 1/2; ERK 1/2 was used to correct for protein loading. Densitometry data are normalized to the maximal response in each individual experiment and are means ± S.E.M. of 3-5 experiments. Representative blots are shown. *P< 0.05; ***P< 0.001 *vs* t=0.

Involvement of TAK1 in CSE-induced NF-κB and ERK 1/2 pathway activation

In order to investigate the involvement of TAK1 in NF-κB and ERK 1/2 signaling activation, cells were stimulated with 15% CSE, in the absence or presence of the TAK1 inhibitor LL-Z-1640-2. In addition, cells were transfected with dominant negative TAK1 and stimulated with CSE as well. LL-Z-1640-2 profoundly inhibited the CSE-induced decrease in IκBα abundance (Figure 4A) as well as the increase in ERK 1/2 phosphorylation (Figure 4B). Similarly, transfection with dominant negative TAK1 abolished the CSE-induced increase in ERK 1/2 phosphorylation at t = 2h (Figure 5). These data indicate that TAK1 plays a major role in the activation of NF-κB and ERK 1/2 pathways by CSE.

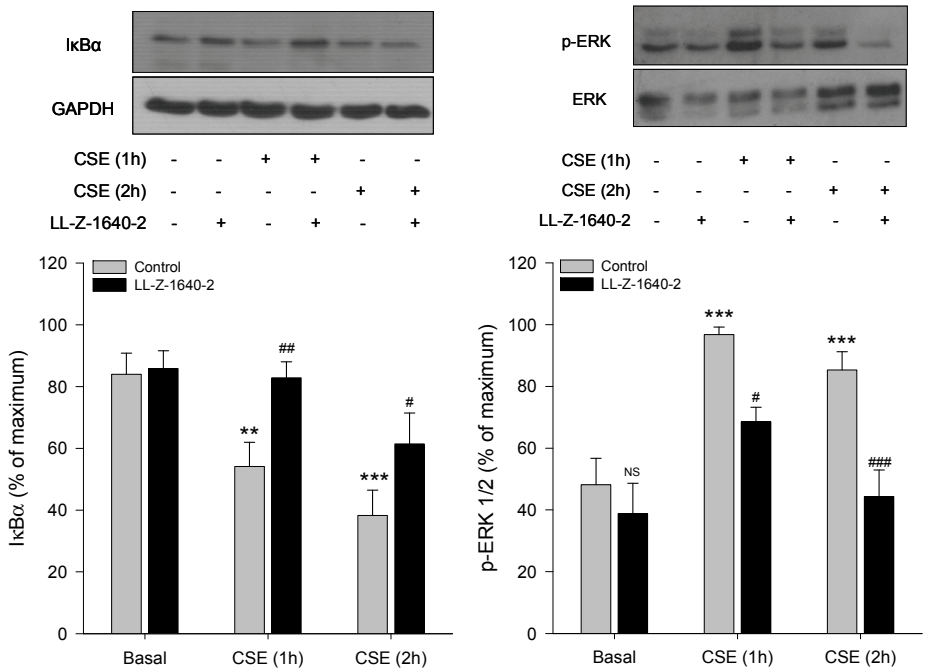

Figure 4: CSE-induced IκBα degradation (A) and ERK 1/2 phosphorylation (B) are inhibited by LL-Z-1640-2 (100nM) in hTERT human ASM. Cells were treated with CSE for 1 or 2 h in absence or presence of LL-Z-1640-2. Cell lysates were analyzed by immunoblotting for IκBα or phospho-ERK 1/2; GAPDH or ERK 1/2 was used to correct for protein loading, respectively. Densitometry data are normalized to the maximal response in each individual experiment and are means ± S.E.M. of 6-7 experiments. Representative blots are shown. **P<0.01, ***P<0.001 *vs* untreated basal, #P<0.05, ##P<0.01, ###P<0.001 vs respective CSE treatment in the absence of inhibitor.

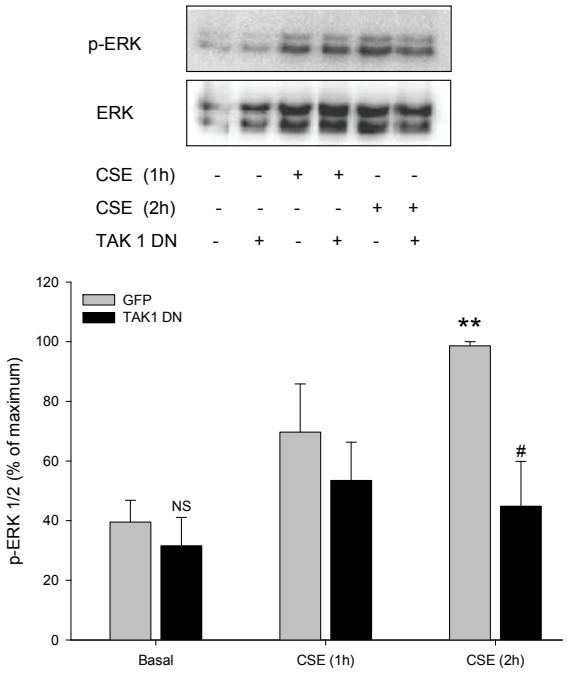

Figure 5: CSE-induced ERK 1/2 phosphorylation is inhibited by TAK1 DN protein expression in hTERT human ASM. Cells transfected with TAK1 DN or GFP were treated with CSE for 1 or 2 h. Cell lysates were analyzed by immunoblotting for phospho-ERK 1/2; ERK 1/2 was used to correct for protein loading. Densitometry data are normalized to the maximal response in each individual experiment and are means ± S.E.M. of 4-5 experiments. Representative blots are shown. **P<0.01, NS: not significant *vs* untreated GFP-transfected cells, #P<0.05 *vs* CSE treatment of GFP-transfected cells.

Inhibition of TAK1, IKK2 and ERK1/2 inhibits CSE-induced IL-8 release

To evaluate the functional roles of TAK1, NF-κB and ERK 1/2 in CSE-induced IL-8 release, cells were stimulated with 15% CSE in the absence or presence of LL-Z-1640-2 (100 nM), SC-514 (50 μM) and U0126 (3 μM), inhibitors of TAK1, IKK2 and MEK1/2, respectively. The CSE-induced IL-8 release was abolished by LL-Z-1640-2 (Figure 6) and strongly inhibited by SC-514 and U0126 (Figure 7), indicating that TAK1 and the downstream NF-κB and ERK 1/2 signaling pathways play a key role in CSE-induced IL-8 release.

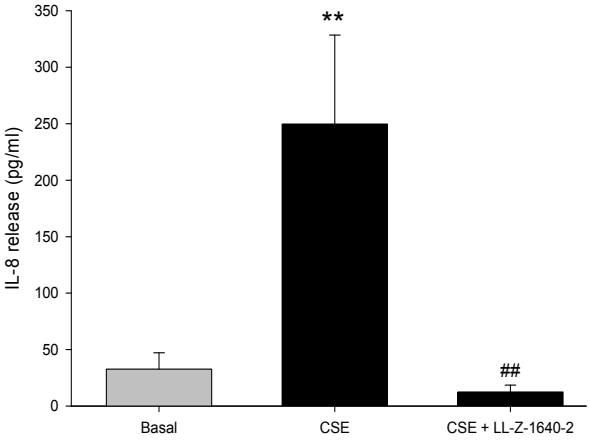

Figure 6: CSE-induced IL-8 release by hTERT human ASM cells is inhibited by LL-Z-1640-2 (100nM). Cells were treated with CSE for 24 h in the absence or presence of LL-Z-1640-2. Cell supernatants were analyzed by ELISA. Data are means ± S.E.M. of 6 experiments each performed in duplicate. **P<0.01 *vs* basal, ##P<0.01 *vs* CSE treatment in the absence of inhibitor.

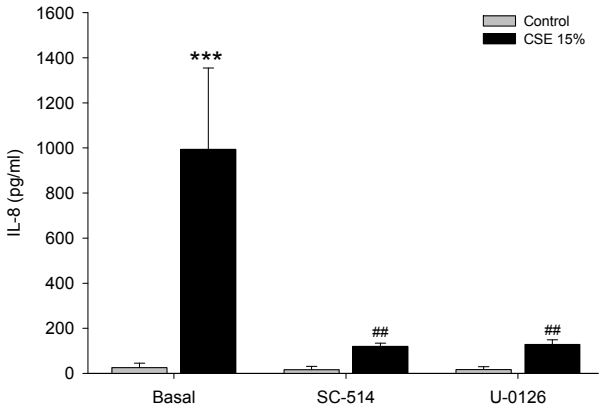

Figure 7: IKK2 and ERK 1/2 inhibitors suppress CSE-induced IL-8 release by hTERT human ASM cells. Cells were stimulated with CSE (15%) for 24 h, in presence or absence of IKK2 (SC-514, 50 μM) or ERK 1/2 (U-0126, 3 μM) inhibitors. Supernatants were collected and analyzed by ELISA. Data represent means ± S.E.M. of 4 experiments performed in duplicate. ***P<0.001 *vs* untreated control; ##P<0.01 *vs* untreated CSE.

Discussion

In this study, we demonstrated that TAK1 plays a key role in CSE-induced activation of NF-κB and ERK 1/2 and subsequent IL-8 release by human ASM cells. This is the first study implicating the involvement of TAK1 in CS-induced cellular signaling and pro-inflammatory cytokine release.

CS has previously been shown to induce IL-8 release from ASM cells (6-8). This may involve oxidative stress, as the CS-induced IL-8 release was inhibited by glutathione pre-treatment and associated with increased expression of heme oxygenase-1 (7). CS exposure has previously also been shown to induce NF-κB and MAP kinase pathways in various cells, including ASM cells (23-27). Although the IL-8 gene promoter region contains binding sites for NF-κB and AP1 (28), and these pathways were shown to be involved in CS-induced IL-8 release from human airway epithelial cells, lung fibroblasts and macrophages, as well as in neutrophils (23, 25, 29, 30), their contribution to CS-induced IL-8 release by ASM cells has only recently been demonstrated (26). Our data show that the CSE-induced activation of NF-κB and ERK 1/2 pathways is mediated by TAK1 and is crucial for IL-8 release in ASM cells.

TAK1 can be activated by a wide variety of stimuli. Pathogen-associated molecular patterns, such as lipopolysaccharide (LPS), and the cytokine IL-1β activate TAK1 via the adaptor protein MyD88. This leads to activation of IRAK4 and IRAK1 and subsequent formation of the TRAF6-TAK1 complex, which results in the activation of TAK1 (10, 31, 32). TNF-α receptor-induced TAK1 activation is mediated by the TRAF2/TRAF5 adaptor (11, 32). In addition to these receptor-specific stimuli, cellular stress, such as osmotic stress or hypoxia, have also been shown to activate TAK1 (33, 34).

TAK1 has been demonstrated to activate of NF-κB and MAP kinase pathways, which may lead to increased production of pro-inflammatory cytokines, including IL-6, IL-8 and TNF-α (19, 35-37). TAK1 has previously been shown to mediate IL-8 release form various cell types in response to pro-inflammatory stimuli, including IL-1β (38), TNF-α (15, 39) and LPS (15, 36). A role for TAK1 in CS-induced cellular responses has not previously been reported. However, it has been suggested that TLRs may mediate CS-induced signaling. Thus, CSE-induced IL-8 release was shown to be dependent on TLR4 and TLR9 activation in macrophages and neutrophils, respectively (24, 40). In addition, CS-induced neutrophilia and inflammatory cytokine production are attenuated in mice lacking TLR4 or the downstream MyD88 adapter (41, 42). These data suggest that TLRs may play a role in CS-induced inflammatory responses and provide a possible mechanism for the CS-mediated activation of TAK1. Although LPS was found to be present in CS (43), it may not be the cause of CSE-induced TLR4 activation since the LPS concentration in CSE was very low and CSE-induced cytokine release from macrophages was not affected by neutralization of LPS with polymyxin B (24, 27, 41).

Interestingly, diesel exhaust particles (DEP) have been shown to induce NF-κB activation in a TAK1-dependent manner in rat lung epithelial cells (44). DEP exposure, much like exposure to CS, induces an increase of lung neutrophils, macrophages and T-lymphocytes in experimental animals (45) and was recently shown to increase COPD mortality in railroad workers (46). Similarly, silica exposure, which has also been associated with the development of COPD (47), was shown to activate the NF-κB pathway in a TAK1-dependent manner in lung fibroblasts (48). Collectively, these and our data indicate that TAK1 may be importantly involved in inflammatory responses induced by environmental stimuli on various cell types that have been implicated in the development of COPD.

In conclusion, our observations indicate that TAK1 plays a major role in CS-induced IL-8 release by ASM cells, and identify this enzyme as a potential novel target for the inhibition of inflammatory responses that play a role in the development and progression of COPD.

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