

Vanadium pentoxide (V₂O₅) induced mucin production by airway epithelium

Dongfang Yu,¹ Dianne M. Walters,² Lingxiang Zhu,³ Pak-Kei Lee,¹ and Yin Chen³

¹Division of Translational Biology, the Hamner Institutes for Health Research, ²Department of Physiology, Brody School of Medicine, East Carolina University, North Carolina; ³Department of Pharmacology and Toxicology, School of Pharmacy, University of Arizona, Arizona

Submitted 27 August 2010; accepted in final form 27 April 2011

Yu D, Walters DM, Zhu L, Lee P-K, Chen Y. Vanadium pentoxide (V₂O₅) induced mucin production by airway epithelium. *Am J Physiol Lung Cell Mol Physiol* 301: L31–L39, 2011. First published April 29, 2011; doi:10.1152/ajplung.00301.2010.—Exposure to environmental pollutants has been linked to various airway diseases and disease exacerbations. Almost all chronic airway diseases such as chronic obstructive pulmonary disease and asthma are caused by complicated interactions between gene and environment. One of the major hallmarks of those diseases is airway mucus overproduction (MO). Excessive mucus causes airway obstruction and significantly increases morbidity and mortality. Metals are major components of environmental particulate matters (PM). Among them, vanadium has been suggested to play an important role in PM-induced mucin production. Vanadium pentoxide (V₂O₅) is the most common commercial source of vanadium, and it has been associated with occupational chronic bronchitis and asthma, both of which are MO diseases. However, the underlying mechanism is not entirely clear. In this study, we used both in vitro and in vivo models to demonstrate the robust inductions of mucin production by V₂O₅. Furthermore, the follow-up mechanistic study revealed a novel v-raf-1 murine leukemia viral oncogene homolog 1-IKK-NF-κB pathway that mediated V₂O₅-induced mucin production. Most interestingly, the reactive oxygen species and the classical mucin-inducing epidermal growth factor receptor (EGFR)-MAPK pathway appeared not to be involved in this process. Thus the V₂O₅-induced mucin production may represent a novel EGFR-MAPK-independent and environmental toxicant-associated MO model. Complete elucidation of the signaling pathway in this model will not only facilitate the development of the treatment for V₂O₅-associated occupational diseases but also advance our understanding on the EGFR-independent mucin production in other chronic airway diseases.

v-raf-1 murine leukemia viral oncogene homolog 1; NF-κB; ROS; EGFR-independent

AIRWAY MUCUS OVERPRODUCTION (MO) has been considered as a major pathogenic factor in almost all chronic airway diseases such as chronic obstructive pulmonary disease and asthma (34, 36). The clinical manifestations of those diseases are determined by the interplay between underlying genetic factors and environmental exposures (17, 18). In the past decade, tremendous progresses have been made to understand the mechanistic basis of MO (26, 35). However, the studies on MO models caused by the exposure to environmental toxicants have been limited. Several oxidant gases such as SO₂ (5) and O₃ (11) have been shown to induce mucous cell metaplasia (MCM, the direct cause of MO) in rat. However, no underlying mechanisms have been demonstrated. In this field, the only well-studied MO models are the ones induced by tobacco smoke (33) or its components [e.g., acrolein (8)], in which

epidermal growth factor receptor (EGFR)-MAPK pathway appears to play a predominant role in mediating mucin production (8, 33).

Environmental exposure to respirable particles (particularly those particulate matters with the diameter smaller than 10 μm, PM₁₀) has been shown to significantly increase the risk and mortality of lung diseases (9, 25, 29, 30). Metals are major components of PM. Metal-rich combustion pollutant-residue oil fly ash (ROFA) was reported previously by two groups to induce epithelial mucin productions through either oxidant- (14) or phosphotyrosine-mediated pathways (22). Vanadium, which counts for almost 40% of the dry weight of ROFA, appeared to be responsible for the mucin induction (14, 22). Notably, other metals (e.g., iron, nickel), despite their abundant presence in ROFA, had no such effect (22). Thus vanadium may elicit a unique mucin-inducing effect on airway epithelia. However, the inconsistent chemical composition of ROFA from different sources has limited its use to establish a reproducible environmental toxicant-relevant MO model. Furthermore, the effect of other known/unknown substances in ROFA cannot be easily ruled out. Therefore, to further understand the effect of vanadium and to establish an environmental toxicant-related MO model, we decided to use a pure compound, V₂O₅. V₂O₅ is a widely used catalyst and is released into the environment during oil and coal combustion and from metallurgical works (12). Occupational exposures to V₂O₅ are significantly high in petrochemical, mining, and steel industries (12). Exposures to high levels of vanadium can also be the results of accidental or intentional burning of fuel oils, such as the incidence of Kuwait oil fires in 1991 (27). V₂O₅ exposure causes chronic bronchitis or asthma (13, 21), both of which are MO diseases. Thus V₂O₅, a widely prevalent occupational/environmental toxicant, may well preserve the mucin-inducing capability of vanadium compound.

MATERIALS AND METHODS

Chemicals, Inhibitors, Antibodies

V₂O₅, N-acetylcysteine (NAC), and dimethylthiourea (DMTU) were purchased from Sigma-Aldrich (St Louis, MO). Chemical inhibitors [AG1478, U0126, SP600125, SB203580, Raf inhibitor, caffeic acid phenethyl ester (CAPE), IKK inhibitor] were purchased from Calbiochem (EMD Biosciences, San Diego, CA). Anti-MUC5AC monoclonal antibody was purchased from Labvision (Now acquired by Thermo Scientific, Fremont, CA). Actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phospho-proteins [p-EGFR, p-ERK, p-P38, p-JNK, p-Jun, p-v-raf-1 murine leukemia viral oncogene homolog 1 (RAF1), IκB-α, p-P65 ser536] were purchased from Cell Signaling Technology (Danvers, MA).

Address for reprint requests and other correspondence: Yin Chen, Dept. of Pharmacology and Toxicology, School of Pharmacy, Univ. of Arizona, AZ 85721 (e-mail: ychen@pharmacy.arizona.edu).

Mouse Model of V₂O₅-Induced Lung Inflammation and Mucous Metaplasia

Female AKR mice (6–8 wk) were anesthetized and administered with 50 μ l PBS containing V₂O₅ through laryngeal aspiration as described elsewhere (38). The control mice were administered with PBS only. The appropriate protocols for V₂O₅ administrations were predetermined through various pilot studies (data not shown). In this study, mice were administered with V₂O₅ at 4 mg/kg body wt in two time points (*days 1* and *7*). Twenty-four hours later (*day 8*), mice were euthanized and lungs were perfused and fixed in paraformaldehyde and embedded in paraffin. Some slides were stained with hematoxylin and eosin (H&E) to evaluate tissue inflammation. Alcian-blue/periodic acid Schiff staining was used to examine mucous-producing (goblet) cells. For immunohistochemistry, sections were incubated with 1:100 diluted anti-MUC5AC antibody overnight at 4°C. An anti-mouse IgG antibody conjugated with Alexa488 green fluorescence dye (Invitrogen, Carlsbad, CA) was used for detection. Propidium iodide (red) was used to stain the nuclei. The images were acquired by a microscope (AxioObserver Z1; Carl Zeiss, Thornwood, NY).

Cell Culture and V₂O₅ Treatment With/Without Inhibitors

Differentiated primary cell culture. Human tracheobronchial tissues were obtained from National Disease Research Interchange, with an approved protocol. The University of Arizona approved all procedures involved in tissue procurement. We have, in the past, successfully established primary airway epithelial cultures from these tissues (2, 40). Normally, primary cells were plated on a Transwell (Corning Costar, Corning, NY) chamber (25 mm) at $1\text{--}2 \times 10^4$ cells/cm², in a Ham's F12:DMEM (1:1) supplemented with eight factors, including: insulin (5 μ g/ml), transferrin (5 μ g/ml), EGF (10 ng/ml), dexamethasone (0.1 μ M), cholera toxicant (10 ng/ml), bovine hypothalamus extract (15 μ g/ml), BSA (0.5 mg/ml), and all-*trans*-retinoic acid (30 nM). After a week in immersed culture condition, cultured cells were shifted to an air-liquid interface culture condition. Under the biphasic culture condition, high transepithelial resistance ($> 500 \Omega\text{-cm}^2$), multiple cell layers, cilia beating, and the formation of mucus-secreting granules were observed (2, 40). Normally, experiments were performed at *day 21* or 2 wk after the change of the culture condition from immersed to air-liquid interface. Medium was routinely changed once every other day. To count for the donor variation, all the primary cell data were repeated on at least three independent donors.

NCI-H292 cells. Cells were obtained from ATCC and cultivated on regular tissue culture dish in RPMI media plus 10% FBS.

V₂O₅ treatment. For differentiated primary cell culture, 200 μ l media containing desired concentration of V₂O₅ was only added on the apical side of cells, which mimicked luminal side of airway epithelia. For the monolayer NCI-H292 cells, V₂O₅ was diluted into culture media with desired concentration. When inhibitors or scavengers were applied, cells were pretreated for 1 h, and then V₂O₅ was added into the same well for various time points. Scavengers were freshly dissolved in the culture medium. All chemical inhibitors were dissolved in DMSO, and the appropriate inhibitory doses were pre-tested. The stock inhibitor solution was prepared at the concentration of 1,000 $\times$ of the highest working concentration, which represented a final 0.1% of DMSO in culture media at the highest inhibitor dose. In the preliminary test, DMSO had no effect on V₂O₅-induced effects at the concentration up to 1% of the culture media (data not shown). For convenience, once inhibitors were used, 0.1% DMSO was also included in both control and V₂O₅-treated only samples in the same set of experiments.

Real-Time PCR

Real-time PCR was performed as described previously (15). cDNA was prepared from 3 μ g of total RNA with Moloney murine leukemia

virus-reverse transcriptase (Promega, Madison, WI) by oligo-dT primers for 90 min at 42°C in a 20- μ l reaction solution and was then further diluted to 100 μ l with water for the following procedures. Two microliters of diluted cDNA were analyzed using 2 $\times$ SYBR Green PCR Master Mix by an ABI 5700 or ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA), following the manufacturer's protocol. Primers (Table 1) were used at 0.2 μ M. The PCR reaction was performed in 96-well optical reaction plates, and each well contained a 50 μ l of reaction mixture. The SYBR green dye was measured at 530 nm during the extension phase. The relative mRNA amount in each sample was calculated on the basis of $\Delta\Delta C_t$ method using housekeeping gene *GAPDH*. The purity of amplified product was determined from a single peak of a dissociation curve. Efficiency curves were performed for each gene of interest relative to the housekeeping gene, on the basis of the manufacturer's instructions. Results were calculated as fold induction over control, as described previously (15).

ELISA

Culture media were collected and determined by ELISA assay using 45M1 antibody on the basis of protocol described elsewhere (24). Purified human tracheal mucin was used as standard as described in our previous study (3).

Western Blot

Total cellular protein was collected on the basis of methods described previously (7). The sources of antibodies have been described in *Chemicals, Inhibitors, Antibodies*. Equal protein load was confirmed using the staining of anti-actin antibody.

siRNA and Transient Transfection

Control siRNA was purchased from Ambion (Austin, TX). On-target plus SMARTpool siRNA against RAF1 (siRAF) was purchased from Dharmacon (Lafayette, CO). siRNA was transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) on the basis of manufacturer's instruction. Successful knockdown of the target was confirmed by real-time RT-PCR and Western blot.

Nuclear Protein Extraction and EMSA

Nuclear protein extractions were prepared by using the NE-PER nuclear and cytoplasmic extraction kit (Pierce Biotechnology, Rockford, IL) according to the user's manual. Briefly, cells were washed twice with cold PBS, and cell pellets were collected by centrifugation at 500 g for 3 min. Ice-cold reagent cytoplasmic extract reagent I (CERI; 200 μ l) was added to the pellets and vigorously vortexed for 15 s. After incubation on ice for 10 min, 11 μ l of ice-cold CERI was

Table 1. Real-time primers

Gene	Primers
Human <i>MUC5AC</i>	
forward	GCCTTCACTGTACTGGCTGAG
reverse	TGGGTGTAGATCTGGTTCAGG
Human <i>IL-8</i>	
forward	GCTCTGTGTGAAGGTGCAGTT
reverse	TGTGGTCCACTCTCAATCACTC
Human <i>GAPDH</i>	
forward	CAATGACCCCTTCATTGACC
reverse	GACAAGCTTCCCGTTCTCAG
Mouse <i>Muc5ac</i>	
forward	AGAATATCTTTTCAAGACCCCTGCT
reverse	ACACCAGTGCTGAGCATACTTTT
Mouse <i>Gapdh</i>	
forward	AAGATTGTCAGCAATGCATCC
reverse	CTTCCACAATGCCAAAGTTGT

added into the traction and incubated on ice for 1 min. After centrifugation at $>16,000\text{ g}$ for 5 min, the supernatant was collected as the cytoplasmic fraction. The insoluble pellet, which contained nuclei, was resuspended in 100 μl ice-cold NER and vortexed for 15 s, incubated on ice with repeat vortexing every 5 min, for a total of 40 min. After 40 min, the reaction was centrifuged at $\sim 16,000\text{ g}$, and the supernatants were collected as the nuclear protein extraction. EMSA

was performed using biotin-labeled double-stranded consensus probes (NF- κ B, AGTTGAGGGGACTTTCCCAGGC). Detection of protein/oligonucleotide complex was performed with an EMSA gel-shift kit (Panomics, Fremont, CA). Briefly, nuclear protein (5 μg) was incubated with 10 ng/ μl of biotin-labeled oligonucleotide for 30 min at room temperature in 5 $\times$ binding buffer and 100 ng of poly(dI-dC). The specificity of the DNA/protein binding was determined in com-

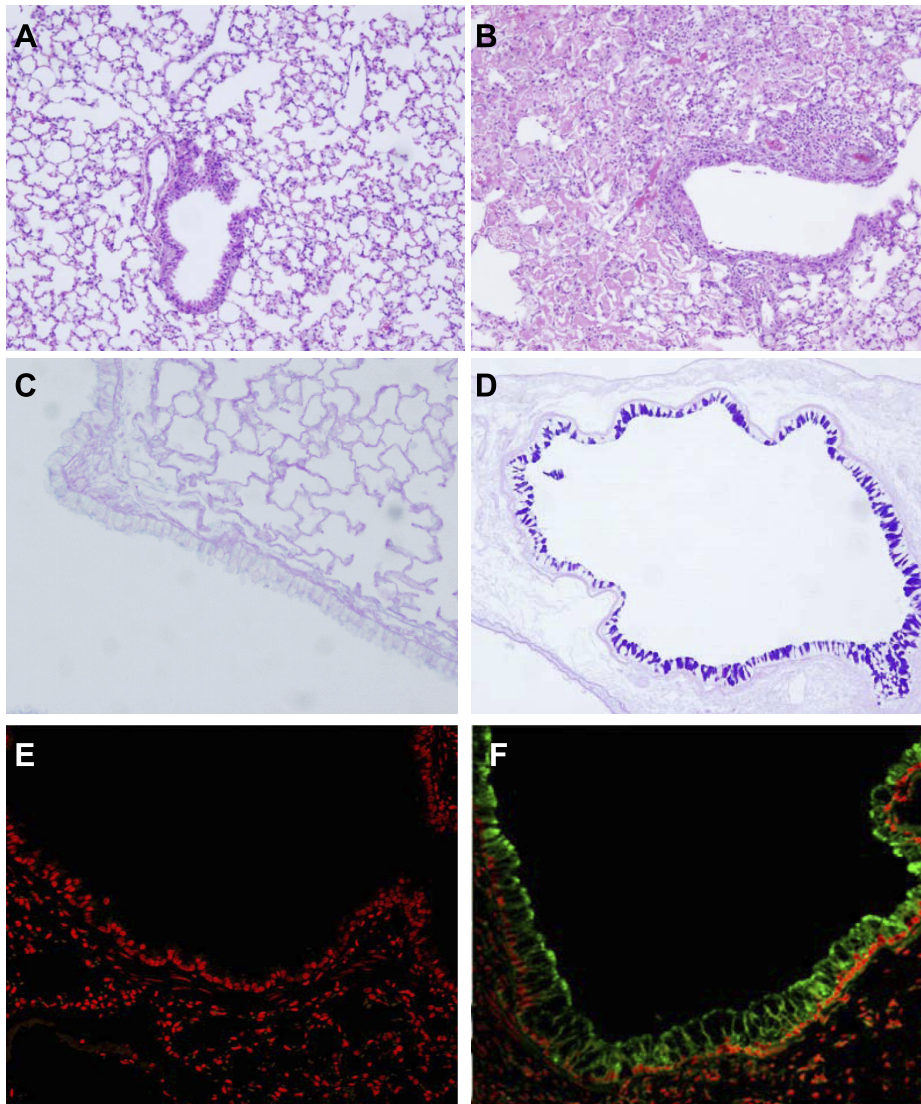


Fig. 1. V₂O₅-induced airway inflammation and mucous cell metaplasia in mouse airways. All images are the representative image from 5 independent experiments. Detailed protocol is described in MATERIALS AND METHODS. A: hematoxylin and eosin (H&E) staining of PBS-treated mice. B: H&E staining of V₂O₅-treated mice. C: Alcian-blue/periodic acid Schiff (PAS) staining of PBS-treated mice. D: Alcian-blue/PAS staining of V₂O₅-treated mice. E: Muc5ac staining of the airways of PBS-treated mice. F: Muc5ac staining of the airways of V₂O₅-treated mice. The airway sections were incubated with anti-Muc5ac monoclonal antibody and detected by immunofluorescence. Green, anti-Muc5ac antibody; red, nucleus staining by propidium iodide. G: real-time PCR result of Muc5ac expression from whole lung. C, PBS control; V₂O₅, V₂O₅-treated mice. * $P < 0.05$, $n = 5$.

petition reactions in which a 50-fold molar excess of unlabeled oligonucleotide was added to the binding reaction (data not shown). Products of binding reactions were resolved by electrophoresis on a 6% polyacrylamide gel by using 0.5× TBE buffer followed by electroblotting to a nylon membrane (Schleicher and Schuell, Keene, NH). After incubation in blocking buffer for 15 min at room temperature, the membrane was incubated with streptavidin-horseradish peroxidase conjugate for 30 min at room temperature. The membrane was washed and visualized with SuperSignal chemiluminescence reagent (Pierce Biotechnology). The specific NF- κ B binding was quantified by Image J (<http://rsbweb.nih.gov/ij/>). The fold induction was calculated by dividing the intensity of NF- κ B binding bands in the treated cells by those in the controls.

Statistical Analysis

Experimental groups were compared using a two-sided Student's *t*-test, with significance level set as $P < 0.05$. When data were not distributed normally, significance was assessed with the Wilcoxon matched-pairs signed-ranks test, and $P < 0.05$ was considered to be significant. Matlab 6.0 with statistics toolbox (MathWorks, Natick, MA) was used for analyses of the data.

RESULTS

V₂O₅ Induced Lung Inflammation and Airway MCM

As shown in Fig. 1, the administration of V₂O₅ by laryngeal aspiration induced severe airway inflammation (Fig. 1B) and MCM (Fig. 1D), whereas the control animals receiving PBS were completely lacking inflammation and MCM (Fig. 1, A and C). Furthermore, data from both immunofluorescence (Fig. 1F) and real-time PCR (Fig. 1G) demonstrated that major airway mucin-Muc5ac was highly elevated in the mice challenged by V₂O₅ but not in the control mice (Fig. 1E). Therefore, V₂O₅ significantly induced MCM and mucin production in mouse model.

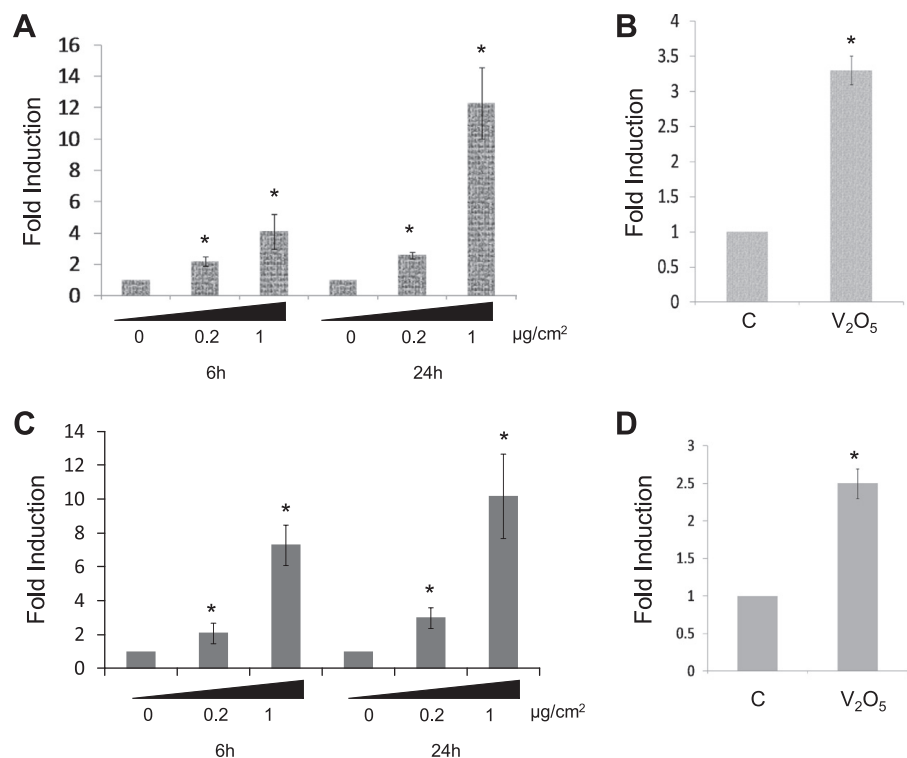
V₂O₅ induced MUC5AC Production in Primary Cells and NCI-H292 Cells

Then, we tested whether V₂O₅ could directly induce mucin gene expression *in vitro*. Indeed, V₂O₅ directly induced major airway mucin-MUC5AC expression both in human primary airway cells (Fig. 2A) and in a widely used epithelial cell model, NCI-H292 cell (Fig. 2C). Those inductions were clearly dose dependent (Fig. 2, A and C). At high dose (1 μ g/cm²), the induction was time dependent, particularly in primary cells. However, longer time treatment appeared not to significantly increase MUC5AC expression at a lower dose (0.2 μ g/cm²). Consistently, the increase of mRNA further led to the increase of MUC5AC secretion in both cells (Fig. 2, B and D). Because the condition and the response in either cell model were similar, we decided to use NCI-H292 as our cell model to further explore the underlying molecular mechanism.

V₂O₅-Induced Mucin Production Was not Affected by ROS Scavengers

Vanadium compound has been shown to induce reactive oxygen species (ROS) (39), and ROS-mediated signaling has been involved in mucin production (14, 26, 35). Thus we tested two commonly-used ROS scavengers (NAC and DMTU) in our cell culture model. Both compounds had no inhibitory effect on V₂O₅-induced mucin MUC5AC expression in the cells (Fig. 3A), which is consistent with the previous report that ROFA-induced MUC5AC promoter activity was not affected by DMTU (22). The lack of inhibition appeared not to be caused by the dysfunction of ROS-mediated signaling in these cells, because both scavengers could effectively block V₂O₅-induced IL-8 expression (Fig. 3B).

Fig. 2. V₂O₅-induced MUC5AC expression in airway epithelial cells. * $P < 0.05$ when the V₂O₅-treated cells were compared with the control, $n = 5$. A: primary airway epithelial cells were treated with either 0.2 μ g/cm² or 1 μ g/cm² for 6 and 24 h. MUC5AC expression was determined by real-time PCR. B: ELISA assay of MUC5AC secretion in the apical media of primary cells. C: NCI-H292 cells were treated with either 0.2 μ g/cm² or 1 μ g/cm² for 6 and 24 h. MUC5AC expression was determined by real-time PCR. D: ELISA assay of MUC5AC secretion in the culture media of NCI-H292 cells.



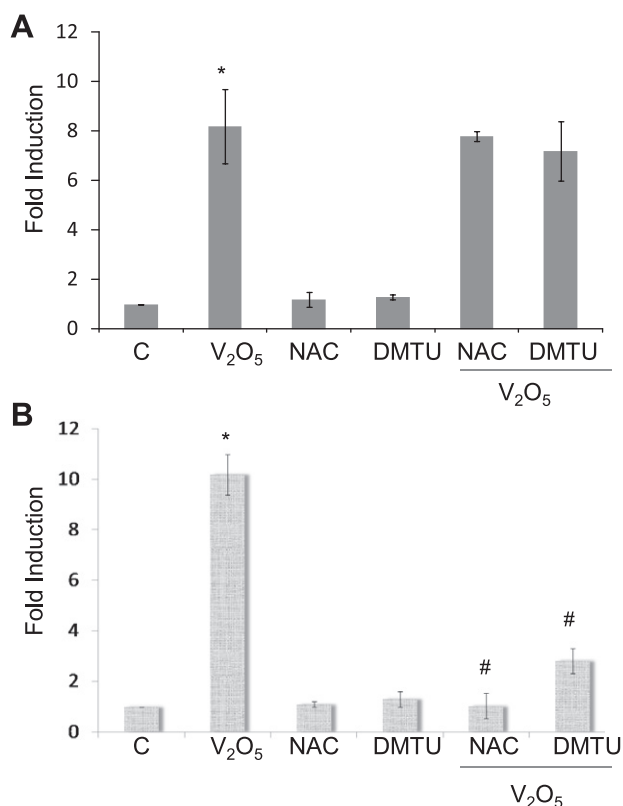


Fig. 3. V₂O₅-induced MUC5AC expression was independent of reactive oxygen species (ROS). NCI-H292 cells were either pretreated with ROS scavenger (freshly dissolved in culture medium before use) or medium for 1 h. Then, 1 $\mu\text{g}/\text{cm}^2$ V₂O₅ was added. 6 h later, RNA samples were collected for real-time PCR analysis. Dimethylthiourea (DMTU) was used at 40 mM, and N-acetylcysteine (NAC) was used at 10 mM. * $P < 0.05$, when the V₂O₅-treated cells were compared with the control. # $P < 0.05$, when the inhibitor + V₂O₅-treated cells were compared with V₂O₅-treated cells. $n = 5$. A: V₂O₅-induced MUC5AC expression was not inhibited by DMTU or NAC. B: V₂O₅-induced IL-8 expression was significantly inhibited by DMTU or NAC.

V₂O₅-Induced Mucin Production was not Dependent on EGFR-MAPK Pathways

V₂O₅ activated EGFR and MAP kinases (ERK, P38 and JNK) as shown in Fig. 4A. Because most of the mucin-inducing substances, particular the environmental toxicants, utilize EGFR-MAPK pathway, we tested whether the inhibitors targeting this pathway could block V₂O₅-induced mucin expression. As shown in Fig. 4A, the activations of EGFR and ERK were completely inhibited by AG1478, suggesting that V₂O₅-induced ERK activation was mediated by EGFR. However, the activations of P38 and JNK appeared to be independent of EGFR activation (Fig. 4A). When MEK1/2 inhibitor-U0126 was used, activations of both ERK and P38 were inhibited (Fig. 4B), suggesting that both kinases were activated by MEK1/2. Specific JNK inhibitor-SP600125 was used to inhibit JNK, and the effect was demonstrated by a complete inhibition of its substrate c-JUN phosphorylation (Fig. 4C).

Surprisingly, none of these inhibitors had any effect on V₂O₅-induced MUC5AC expression (Fig. 4D). Because all those inhibitors worked as expected (Fig. 4, A–C), the lack of inhibition on MUC5AC expression was not due to the inactivity of those inhibitors. Furthermore, V₂O₅-induced IL-8 was completely blocked by AG1478 and U0126 (Fig. 4E), suggest-

ing that EGFR-MAPK pathway was intact in the cells. SP600125 by itself appeared to drastically induce IL-8 independent of V₂O₅ (data not shown), which needs further investigation. Nonetheless, the classical mucin-inducing EGFR-MAPK, although activated by V₂O₅, was not responsible for MUC5AC expression.

V₂O₅-Induced Mucin Production was Dependent on RAF1-IKK-NF- κ B Pathway

To find out what pathway was really responsible for the V₂O₅-induced mucin expression, we screened a set of inhibitors against various protein kinases (e.g., PKA, PKG, PKC, etc.) and found that only NF- κ B and RAF1 inhibitors had significant and reproducible inhibitions on MUC5AC expression (Fig. 5A). For RAF1, both the specific RAF1 inhibitor (RAF_inh) (Fig. 5A) and the siRNA (siRAF) knockdown (Fig. 5B) were used. For NF- κ B, both the direct NF- κ B inhibitor CAPE and the upstream I κ B kinase inhibitor IKK_inh were used (Fig. 5A). RAF1 activation was demonstrated by the time-dependent increase of RAF1 phosphorylation (Fig. 6A). The activation of NF- κ B was demonstrated by the time-dependent decrease of I κ B- α and increase of phosphorylated P65 (Fig. 6B), both of which were mediated by IKK activation (23). This was further confirmed by the increase of binding to NF- κ B consensus sequence in cell nuclear extract (Fig. 6D) upon V₂O₅ treatment. Data also indicated that either RAF1 inhibition (RAF_inh/siRAF) or NF- κ B inhibition (CAPE/IKK_inh) could significantly block the nuclear NF- κ B binding (Fig. 6, D and E). As shown in Fig. 6D, V₂O₅ induced 2.5-, 4.1-, and 4.6-fold activation of NF- κ B at 1, 3, and 6 h, and the treatment of RAF_inh could reduce the activation to 1.3-, 1.4-, and 2.1-fold at the different time points. Direct inhibition of NF- κ B activation by CAPE or its upstream kinase by IKK_inh could reduce the activation to 0.6- and 0.8-fold, respectively. When RAF1 knockdown was used, siRAF could reduce NF- κ B activation from 2.9-fold to 0.7-fold (Fig. 6E). The effectiveness of siRAF was demonstrated in Fig. 6C, where both the total RAF1 protein and the phosphorylated RAF1 (p-RAF) were significantly decreased by siRAF transfection. Thus RAF1-IKK-NF- κ B pathway appeared to mediate V₂O₅-induced MUC5AC expression.

DISCUSSION

Despite the abundant studies carried out on the mechanism underlying MO, most of them have been focused on the pathogens or downstream inflammatory mediators (35). The studies on MO induced by environmental toxicant exposures have been very limited. Gases [such as SO₂ (5) and O₃ (11)] and tobacco smoke (33) [including its components such as acrolein (8)] have been shown to induce MO in various in vitro and in vivo models, but the underlying molecular mechanisms are not entirely clear. EGFR-MAPK-mediated pathway has been proposed to mediate the tobacco smoke- or acrolein-induced mucin production (8, 33). In fact, EGFR-MAPK-dependent pathways are so popular that most of the mucin-inducing events have eventually been traced back to these pathways (34, 35). IL-13-induced MUC5AC expression in vitro is probably the only example showing that the contribution of EGFR has been explicitly ruled out (41), but this

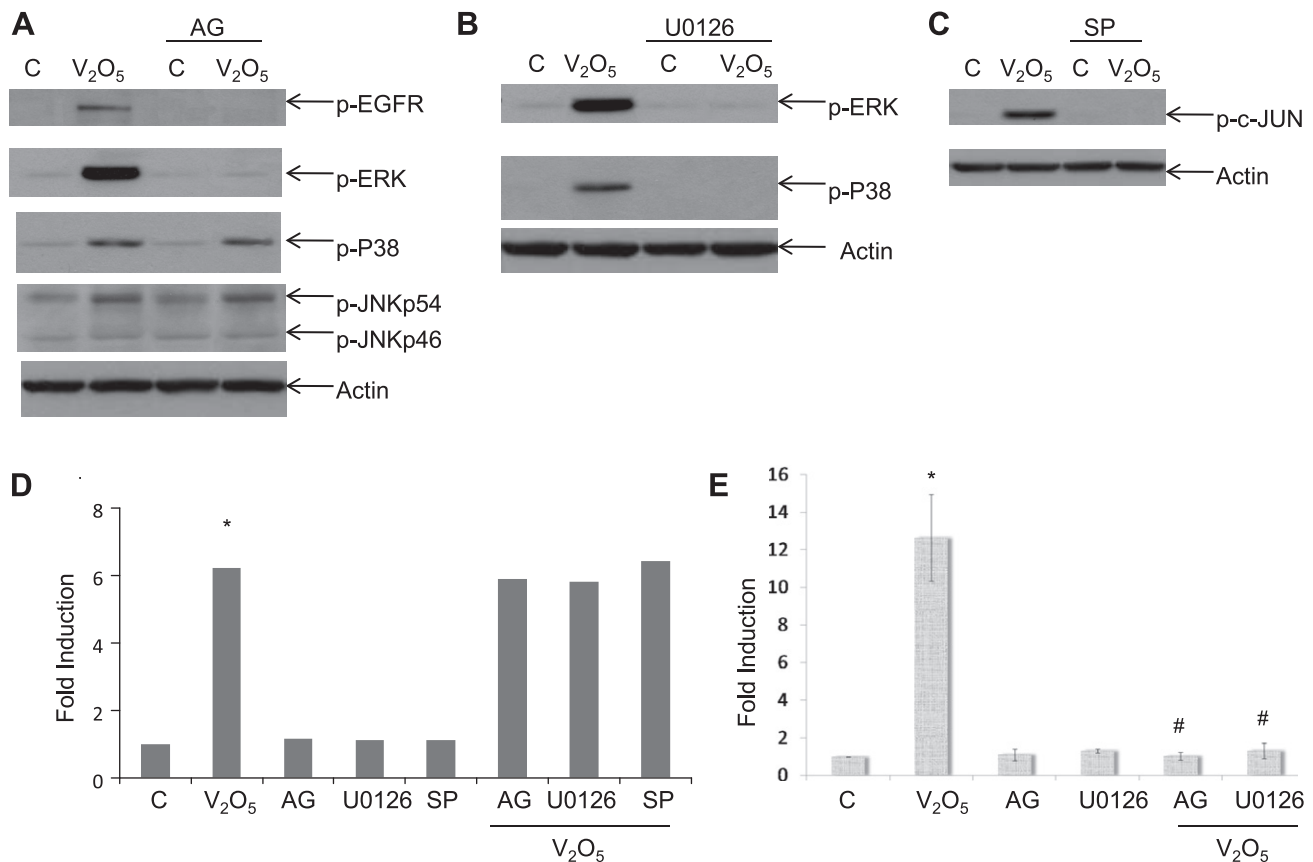


Fig. 4. V₂O₅-induced MUC5AC expression was independent of epidermal growth factor receptor (EGFR) and MAPK activations. NCI-H292 cells were either pretreated with inhibitors or DMSO (the solvent) for 1 h. Then, 1 $\mu\text{g}/\text{cm}^2$ V₂O₅ was added. 6 h later, protein or RNA samples were collected for analysis. EGFR inhibitor-AG1478 (AG), MEK inhibitor-U0126, and JNK inhibitor-SP600125 (SP) were used at 10 μM . **A:** Western blot analysis of the activations (phosphorylation) of EGFR (p-EGFR), ERK (p-ERK), P38 (p-P38), and JNK (p-JNK54/46). Actin was used as a loading control. **B:** Western blot analysis of the activations (phosphorylation) of ERK and P38. Actin was used as a loading control. **C:** Phosphorylation of c-JUN (p-c-JUN) was used to demonstrate the effect of SP. Actin was used as a loading control. **D:** V₂O₅-induced MUC5AC expression was not inhibited by AG1478 (AG), U0126, and SP600125 (SP). MUC5AC expression was determined by real-time PCR. * $P < 0.05$. $n = 5$. **E:** V₂O₅-induced IL-8 expression was completely inhibited by AG1478 (AG) and U0126. * $P < 0.05$ when the treated cells were compared with the control. # $P < 0.05$ when the inhibitor + V₂O₅-treated cells were compared with the V₂O₅-treated cells. $n = 5$.

pathway may still play significant role in mediating the IL-13-induced mucin production in vivo (31, 32).

EGFR- and MAPK-independent pathway in mucin induction has rarely been discussed before. In the case of nontypeable *Haemophilus Influenzae* lipoprotein P6-induced MUC5AC expression, a TGF- β -activating kinase-1 (TAK1)-IKK-NF- κ B pathway has been found to play a critical role in regulating mucin expression (1). Because TAK1 sometime functions as MAPKKK, this example demonstrated for the first time that a MAPKKK-like kinase can regulate mucin gene expression independent of MAPK. This is very similar to what we have found in the present study, because RAF1 is a MAPKKK and is able to directly activate NF- κ B (20).

The induction of this unconventional pathway may be due to the unique characteristic of vanadium compound, which is in essence a phosphatase inhibitor (28). In addition, high-valence vanadium such as V₂O₅ has also been shown to induce ROS generation (39). In our study using ROS scavengers, ROS-mediated signaling appeared not to be responsible for the mucin induction, but it did play an important role in cytokine (i.e., IL-8) induction. This finding is consistent with the previous report that ROFA-induced MUC5AC promoter activity

was not affected by DMTU (22). However, because IL-8 is a neutrophil chemoattractant and neutrophil-derived elastase has been shown to induce mucin expression (10), the potential role of ROS in V₂O₅-induced mucin production in vivo cannot be completely ruled out, which will require further study.

In the past, there were only two reported molecular studies regarding the effect of vanadium-containing ROFA on mucin production (14, 22). One study mainly dealt with Muc2 induction in a guinea pig epithelial model and demonstrated the involvement of ROS-dependent pathway (14). However, different from the guinea pig airway (4), there is only limited MUC2 expression in the human airway (6, 16). The regulation of Muc2 by ROS in the guinea pig epithelial model may reflect the interspecies difference between rodent and human. The other study focused on MUC5AC and cellular phosphorylation event (22). Similarly, the significant contribution of NF- κ B was also found. However, different from our study, various kinases such as PKA, PKC, EGFR, and MAPK (not P38) were demonstrated to be involved in the increase of mucin promoter activity. Two main differences exist between the present and previous studies. One is the use of different model compounds (V₂O₅ vs. Na₃VO₄), and the other is the use of different

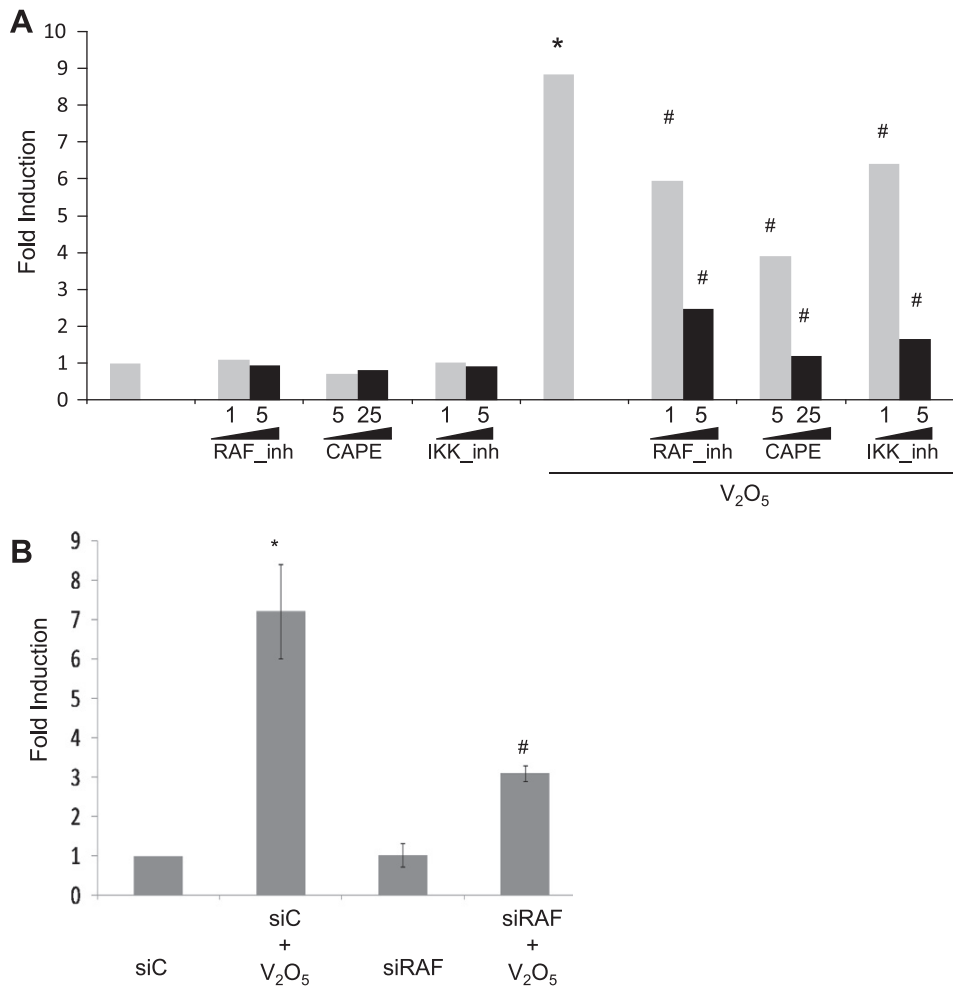


Fig. 5. V₂O₅-induced MUC5AC expression was dependent on v-raf-1 murine leukemia viral oncogene homolog 1 (RAF1) and NF- κ B. **A:** V₂O₅-induced MUC5AC expression was inhibited by RAF1 inhibitor (RAF_inh), NF- κ B inhibitor (caffeic acid phenethyl ester, CAPE), and IKK inhibitor (IKK_inh). NCI-H292 cells were either pretreated with inhibitors or DMSO (the solvent) for 1 h. Then, 1 μ g/cm² V₂O₅ was added. 6 h later, RNA samples were collected for real-time PCR analysis. RAF_inh was used at either 1 or 5 μ M; CAPE was used at either 5 or 25 μ g/ml; IKK_inh was used at either 1 or 5 μ M. * P < 0.05 when the V₂O₅-treated cells were compared with the control. # P < 0.05 when the inhibitor + V₂O₅-treated cells were compared with the V₂O₅-treated cells. n = 5. **B:** siRNA against RAF1 inhibited V₂O₅-induced MUC5AC expression. NCI-H292 cells were transfected either with control siRNA (siC) or with siRNA against RAF1 (siRAF) for overnight. Then, 1 μ g/cm² V₂O₅ was added. 6 h later, RNA samples were collected for real-time PCR analysis. * P < 0.05 when the treated cells were compared with the control. # P < 0.05 when the siRAF + V₂O₅-treated cells were compared with the V₂O₅-treated cells. n = 5.

measurements (mRNA vs. promoter-reporter). As discussed in the introduction, V₂O₅ is the most common commercial form of vanadium and is used in a variety of manufacturing processes. Na₃VO₄ is an experimental reagent. Although Na₃VO₄ is part of the products when V₂O₅ or ROFA is suspended in physiological fluids, it may not represent the whole characteristic of those particulate compounds. Furthermore, promoter activity is merely an indirect indicator of the true mRNA level. Their relationships could be very obscure if different promoter regions were responsive to different treatments or there were posttranscriptional controls (37). Nonetheless, further studies will be needed to address these discrepancies.

Because the present model uses particulates, one of the major controversial areas is how to correlate the *in vitro* dose with the real-life exposure. Although it is difficult to calculate the exact dose deposited in the lung, one previous study has shown that the occupational exposure of ROFA ranges between 3.6 to 25.1 mg/m³ (19). In the rat study, it has been shown that ~3.74 μ g ROFA/cm² was deposited onto the trachea in a 6-h period when exposed to ROFA at 12 mg/m³ (14). Thus, considering that the ROFA contains almost 40% of vanadium, our *in vitro* dose (i.e., 1 μ g/cm²) is at the low boundary of occupational exposures. For the mouse model, we used laryngeal respiration method. Because of the rapid delivery of V₂O₅ into the deep lung and the proportions of tracheal vs. bronchial vs. bronchiolar deposition are unclear, it is

difficult to estimate the *in vivo* dose in mice by simple extrapolation. Direct measurement using radioisotope labeling in a future study will be required to determine the actual airway deposition of V₂O₅ in this model.

In summary, we have established both *in vivo* and *in vitro* vanadium-induced MO models. Our mechanistic study has demonstrated a surprising EGFR-MAPK-independent, but RAF1-IKK-NF- κ B-dependent, pathway in mediating mucin production by V₂O₅. This finding is, not only environmentally and occupationally relevant due to the wide prevalence of vanadium compounds, but also can serve as a useful model to understand the EGFR-MAPK-independent mechanism underlying mucin production in other diseases.

ACKNOWLEDGMENTS

Present address of D. Yu: Cystic Fibrosis/Pulmonary Research and Treatment Center, University of North Carolina at Chapel Hill, NC 27599.

GRANTS

The study was supported in part by Long Range Initiative (LRI) of American Chemistry Council and NIH grant RO1AI061695.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

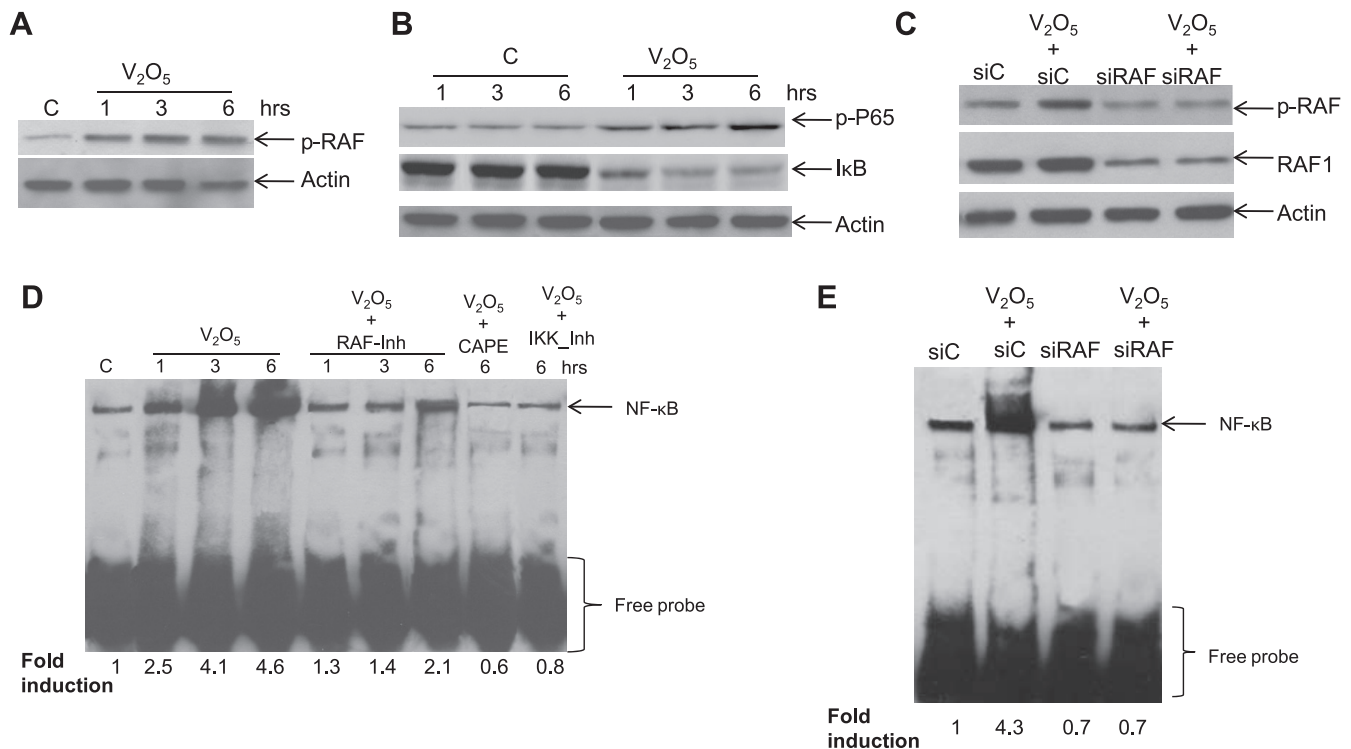


Fig. 6. RAF1 regulated NF-κB activation by V₂O₅. All these images are the representative image from 3 independent experiments. Actin was used as a loading control. **A**: Western analysis of the activation (phosphorylation) of RAF1 (p-RAF) in NCI-H292 cells with V₂O₅ treatment at different time as indicated. **B**: Western analysis of the degradation of IκB-α (IκB) and the phosphorylation of p65 (p-P65) at Ser536 in NCI-H292 cells with V₂O₅ treatment at different time as indicated. **C**: Western analysis of siRNA (siRAF) effect on both the total RAF1 protein (RAF1) and the activation (phosphorylation) of RAF1 (p-RAF). **D**: EMSA assay on the activation of NF-κB (increase of NF-κB binding in nuclear extract). RAF_inh, RAF1 inhibitor (5 μM); CAPE, NF-κB inhibitor (25 μg/ml); IKK_inh, IKK inhibitor (5 μM). The intensity of specific NF-κB binding and the fold induction were calculated as described in MATERIALS AND METHODS. **E**: EMSA assay on the activation of NF-κB upon siRNA (siRAF) knockdown.

REFERENCES

- Chen R, Lim JH, Jono H, Gu XX, Kim YS, Basbaum CB, Murphy TF, Li JD. Nontypeable Haemophilus influenzae lipoprotein P6 induces MUC5AC mucin transcription via TLR2-TAK1-dependent p38 MAPK-AP1 and IKKβ-IκBα-NF-κB signaling pathways. *Biochem Biophys Res Commun* 324: 1087–1094, 2004.
- Chen Y, Zhao YH, Di YP, Wu R. Characterization of human mucin 5B gene expression in airway epithelium and the genomic clone of the amino-terminal and 5'-flanking region. *Am J Respir Cell Mol Biol* 25: 542–553, 2001.
- Chen Y, Zhao YH, Wu R. Differential regulation of airway mucin gene expression and mucin secretion by extracellular nucleotide triphosphates. *Am J Respir Cell Mol Biol* 25: 409–417, 2001.
- Chorley BN, Crews AL, Li Y, Adler KB, Minniccozi M, Martin LD. Differential Muc2 and Muc5ac secretion by stimulated guinea pig tracheal epithelial cells in vitro (Abstract). *Respir Res* 7: 35, 2006.
- Clark JN, Dalbey WE, Stephenson KB. Effect of sulfur dioxide on the morphology and mucin biosynthesis by the rat trachea. *J Environ Pathol Toxicol Oncol* 4: 197–207, 1980.
- Davies JR, Svitacheva N, Lannefors L, Kornfalt R, Carlstedt I. Identification of MUC5B, MUC5AC and small amounts of MUC2 mucins in cystic fibrosis airway secretions. *Biochem J* 344: 321–330, 1999.
- Deng J, Chen Y, Wu R. Induction of cell cornification and enhanced squamous-cell marker SPRR1 gene expression by phorbol ester are regulated by different signaling pathways in human conducting airway epithelial cells. *Am J Respir Cell Mol Biol* 22: 597–603, 2000.
- Deshmukh HS, Shaver C, Case LM, Dietsch M, Wesselkamper SC, Hardie WD, Korfhagen TR, Corradi M, Nadel JA, Borchers MT, Leikauf GD. Acrolein-activated matrix metalloproteinase 9 contributes to persistent mucin production. *Am J Respir Cell Mol Biol* 38: 446–454, 2008.
- Dockery DW, Speizer FE, Stram DO, Ware JH, Spengler JD, Ferris BG Jr. Effects of inhalable particles on respiratory health of children. *Am Rev Respir Dis* 139: 587–594, 1989.
- Fischer BM, Voynow JA. Neutrophil elastase induces MUC5AC gene expression in airway epithelium via a pathway involving reactive oxygen species. *Am J Respir Cell Mol Biol* 26: 447–452, 2002.
- Harkema JR, Hotchkiss JA. Ozone- and endotoxin-induced mucous cell metaplasias in rat airway epithelium: novel animal models to study toxicant-induced epithelial transformation in airways. *Toxicol Lett* 68: 251–263, 1993.
- IARC Working Group on the Evaluation of Carcinogenic Risks to Humans (2003: Lyon France), and International Agency for Research on Cancer. *Cobalt in Hard Metals and Cobalt Sulfate, Gallium Arsenide, Indium Phosphide and Vanadium Pentoxide*. Lyon: IARC, 2006, p. xiv, p. 330.
- Irsigler GB, Visser PJ, Spangenberg PA. Asthma and chemical bronchitis in vanadium plant workers. *Am J Ind Med* 35: 366–374, 1999.
- Jiang N, Dreher KL, Dye JA, Li Y, Richards JH, Martin LD, Adler KB. Residual oil fly ash induces cytotoxicity and mucin secretion by guinea pig tracheal epithelial cells via an oxidant-mediated mechanism. *Toxicol Appl Pharmacol* 163: 221–230, 2000.
- Kao CY, Chen Y, Thai P, Wachi S, Huang F, Kim C, Harper RW, Wu R. IL-17 markedly up-regulates beta-defensin-2 expression in human airway epithelium via JAK and NF-κB signaling pathways. *J Immunol* 173: 3482–3491, 2004.
- Kirkham S, Sheehan JK, Knight D, Richardson PS, Thornton DJ. Heterogeneity of airways mucus: variations in the amounts and glycoforms of the major oligomeric mucins MUC5AC and MUC5B. *Biochem J* 361: 537–546, 2002.
- Kleeberger SR, Cho HY. Gene-environment interactions in environmental lung diseases. *Novartis Found Symp* 293: 168–178; discussion 178–183, 2008.
- Kleeberger SR, Peden D. Gene-environment interactions in asthma and other respiratory diseases. *Annu Rev Med* 56: 383–400, 2005.
- Lees RE. Changes in lung function after exposure to vanadium compounds in fuel oil ash. *Br J Ind Med* 37: 253–256, 1980.

20. Li S, Sedivy JM. Raf-1 protein kinase activates the NF-kappa B transcription factor by dissociating the cytoplasmic NF-kappa B-I kappa B complex. *Proc Natl Acad Sci USA* 90: 9247–9251, 1993.
21. Liu Y, Woodin MA, Smith TJ, Herrick RF, Williams PL, Hauser R, Christiani DC. Exposure to fuel-oil ash and welding emissions during the overhaul of an oil-fired boiler. *J Occup Environ Hyg* 2: 435–443, 2005.
22. Longphre M, Li D, Li J, Matovinovic E, Gallup M, Samet JM, Basbaum CB. Lung mucin production is stimulated by the air pollutant residual oil fly ash. *Toxicol Appl Pharmacol* 162: 86–92, 2000.
23. Perkins ND. Integrating cell-signalling pathways with NF-kappaB and IKK function. *Nat Rev Mol Cell Biol* 8: 49–62, 2007.
24. Phillips JE, Case NR, Celly C, Chapman RW, Hey JA, Minnicozzi M. An enzyme-linked immunosorbent assay (ELISA) for the determination of mucin levels in bronchoalveolar lavage fluid. *J Pharmacol Toxicol Methods* 53: 160–167, 2006.
25. Pope CA 3rd, Schwartz J, Ransom MR. Daily mortality and PM10 pollution in Utah Valley. *Arch Environ Health* 47: 211–217, 1992.
26. Rose MC, Voynow JA. Respiratory tract mucin genes and mucin glycoproteins in health and disease. *Physiol Rev* 86: 245–278, 2006.
27. Sadiq M, Mian AA. Nickel and vanadium in air particulates at Dhahran (Saudi Arabia) during and after the Kuwait oil fires. *Atmospheric Environ* 28: 2249–2253, 1994.
28. Samet JM, Stonehuerner J, Reed W, Devlin RB, Dailey LA, Kennedy TP, Bromberg PA, Ghio AJ. Disruption of protein tyrosine phosphate homeostasis in bronchial epithelial cells exposed to oil fly ash. *Am J Physiol Lung Cell Mol Physiol* 272: L426–L432, 1997.
29. Schwartz J. Air pollution and daily mortality: a review and meta analysis. *Environ Res* 64: 36–52, 1994.
30. Schwartz J, Morris R. Air pollution and hospital admissions for cardiovascular disease in Detroit, Michigan. *Am J Epidemiol* 142: 23–35, 1995.
31. Takeyama K, Dabbagh K, Jeong Shim J, Dao-Pick T, Ueki IF, Nadel JA. Oxidative stress causes mucin synthesis via transactivation of epidermal growth factor receptor: role of neutrophils. *J Immunol* 164: 1546–1552, 2000.
32. Takeyama K, Dabbagh K, Lee HM, Agusti C, Lausier JA, Ueki IF, Grattan KM, Nadel JA. Epidermal growth factor system regulates mucin production in airways. *Proc Natl Acad Sci USA* 96: 3081–3086, 1999.
33. Takeyama K, Jung B, Shim JJ, Burgel PR, Dao-Pick T, Ueki IF, Protin U, Kroschel P, Nadel JA. Activation of epidermal growth factor receptors is responsible for mucin synthesis induced by cigarette smoke. *Am J Physiol Lung Cell Mol Physiol* 280: L165–L172, 2001.
34. Turner J, Jones CE. Regulation of mucin expression in respiratory diseases. *Biochem Soc Trans* 37: 877–881, 2009.
35. Voynow JA, Gendler SJ, Rose MC. Regulation of mucin genes in chronic inflammatory airway diseases. *Am J Respir Cell Mol Biol* 34: 661–665, 2006.
36. Voynow JA, Rubin BK. Mucins, mucus, and sputum. *Chest* 135: 505–512, 2009.
37. Voynow JA, Young LR, Wang Y, Horger T, Rose MC, Fischer BM. Neutrophil elastase increases MUC5AC mRNA and protein expression in respiratory epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 276: L835–L843, 1999.
38. Walters DM, Breyse PN, Wills-Karp M. Ambient urban Baltimore particulate-induced airway hyperresponsiveness and inflammation in mice. *Am J Respir Crit Care Med* 164: 1438–1443, 2001.
39. Wang L, Medan D, Mercer R, Overmiller D, Leonard S, Castranova V, Shi X, Ding M, Huang C, Rojanasakul Y. Vanadium-induced apoptosis and pulmonary inflammation in mice: Role of reactive oxygen species. *J Cell Physiol* 195: 99–107, 2003.
40. Wu R, Zhao YH, Chang MM. Growth and differentiation of conducting airway epithelial cells in culture. *Eur Respir J* 10: 2398–2403, 1997.
41. Zhen G, Park SW, Nguyenvu LT, Rodriguez MW, Barbeau R, Paquet AC, Erle DJ. IL-13 and epidermal growth factor receptor have critical but distinct roles in epithelial cell mucin production. *Am J Respir Cell Mol Biol* 36: 244–253, 2007.

