Vanadium pentoxide (V$_2$O$_5$) induced mucin production by airway epithelium

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Yu D, Walters DM, Zhu L, Lee P-K, Chen Y. Vanadium pentoxide (V$_2$O$_5$) 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 (V$_2$O$_5$) 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 V$_2$O$_5$. Furthermore, the follow-up mechanistic study revealed a novel v-raf-1 murine leukemia viral oncogene homolog 1 (RAF1)-IKK-NF-$\kappa$B pathway that mediated V$_2$O$_5$-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 V$_2$O$_5$-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 V$_2$O$_5$-associated occupational diseases but also advance our understanding of the EGFR-independent mucin production in other chronic airway diseases.

v-raf-1 murine leukemia viral oncogene homolog 1; NF-$\kappa$B; 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 SO$_2$ (5) and O$_3$ (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 $\mu$m, 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, V$_2$O$_5$. V$_2$O$_5$ is a widely used catalyst and is released into the environment during oil and coal combustion and from metallurgical works (12). Occupational exposures to V$_2$O$_5$ 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). V$_2$O$_5$ exposure causes chronic bronchitis or asthma (13, 21), both of which are MO diseases. Thus V$_2$O$_5$, a widely prevalent occupational/environmental toxicant, may well preserve the mucin-inducing capability of vanadium compound.

MATERIALS AND METHODS

Chemicals, Inhibitors, Antibodies

V$_2$O$_5$, 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) and to establish an environmental toxicant-related MO model, we decided to use a pure compound, V$_2$O$_5$. V$_2$O$_5$ is a widely used catalyst and is released into the environment during oil and coal combustion and from metallurgical works (12). Occupational exposures to V$_2$O$_5$ 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). V$_2$O$_5$ exposure causes chronic bronchitis or asthma (13, 21), both of which are MO diseases. Thus V$_2$O$_5$, a widely prevalent occupational/environmental toxicant, may well preserve the mucin-inducing capability of vanadium compound.

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Mouse Model of V2O5-Induced Lung Inflammation and Mucous Metaplasia

Female AKR mice (6–8 wk) were anesthetized and administered with 50 μl 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 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 1–2 × 10⁴ cells/cm², in a Ham’s F12:D-MEM (1:1) supplemented with eight factors, including: insulin (5 μg/ml), transferrin (5 μg/ml), EGF (10 ng/ml), demethylasone (0.1 μM), cholera toxicant (10 ng/ml), bovine hypothyrotalumus extract (15 μg/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 Ω·cm²), 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 every other day. To count for the donor variation, all the primary cells 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. The culture condition, high transepithelial resistance (> 500 Ω·cm²), cells were treated with 1% of the culture media (data not shown). 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).

**Real-Time PCR**

Real-time PCR was performed as described previously (15). cDNA was prepared from 3 μg 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-μl reaction solution and was then further diluted to 100 μl with water for the following procedures. Two microliters of diluted cDNA were analyzed using 2× 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 μM. The PCR reaction was performed in 96-well optical reaction plates, and each well contained a 50 μl 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 ΔΔCt, the ratio of a single peak of a dissociation curve. Efficiency curves were performed for each gene of interest relative to the 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 μl) was added to the pellets and vigorously vortexed for 15 s. After incubation on ice for 10 min, 11 μl of ice-cold CERI was added to the pellets and vigorously vortexed for 15 s. After incubation on ice for 10 min, 11 μl of ice-cold CERI was added to the pellets and vigorously vortexed for 15 s.
added into the traction and incubated on ice for 1 min. After centrifugation at >16,000 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 ~16,000 g, and the supernatants were collected as the nuclear protein extraction. EMSA was performed using biotin-labeled double-stranded consensus probes (NF-κB, 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× binding buffer and 100 ng of poly(dI-dC). The specificity of the DNA/protein binding was determined in com-

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**Fig. 1.** V$_2$O$_5$-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 V$_2$O$_5$-treated mice. **C:** Alcian-blue/periodic acid Schiff (PAS) staining of PBS-treated mice. **D:** Alcian-blue/PAS staining of V$_2$O$_5$-treated mice. **E:** Muc5ac staining of the airways of PBS-treated mice. **F:** Muc5ac staining of the airways of V$_2$O$_5$-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; **V$_2$O$_5$:** V$_2$O$_5$-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-κB binding was quantified by Image J (http://rsbweb.nih.gov/ij/). The fold induction was calculated by dividing the intensity of NF-κB 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 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).

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**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 \( \mu \)g/cm\(^2\) or 1 \( \mu \)g/cm\(^2\) 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 \( \mu \)g/cm\(^2\) or 1 \( \mu \)g/cm\(^2\) 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.
V$_2$O$_5$ Induced Mucin Production was Dependent on EGFR-MAPK Pathways

V$_2$O$_5$ 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 V$_2$O$_5$-induced mucin expression. As shown in Fig. 4A, the activations of EGFR and ERK were completely inhibited by AG1478, suggesting that V$_2$O$_5$-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 V$_2$O$_5$-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, V$_2$O$_5$-induced IL-8 was completely blocked by AG1478 and U0126 (Fig. 4E), suggesting that EGFR-MAPK pathway was intact in the cells. SP600125 by itself appeared to drastically induce IL-8 independent of V$_2$O$_5$ (data not shown), which needs further investigation. Nonetheless, the classical mucin-inducing EGFR-MAPK, although activated by V$_2$O$_5$, was not responsible for MUC5AC expression.

V$_2$O$_5$-Induced Mucin Production was Dependent on RAF1-IKK-NF-κB Pathway

To find out what pathway was really responsible for the V$_2$O$_5$-induced mucin expression, we screened a set of inhibitors against various protein kinases (e.g., PKA, PKG, PKC, etc.) and found that only NF-κB 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-κB, both the direct NF-κB inhibitor CAPE and the upstream IkB 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-κB was demonstrated by the time-dependent decrease of IkB-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-κB consensus sequence in cell nuclear extract (Fig. 6D) upon V$_2$O$_5$ treatment. Data also indicated that either RAF1 inhibition (RAF_inh/siRAF) or NF-κB inhibition (CAPE/IKK_inh) could significantly block the nuclear NF-κB binding (Fig. 6, D and E). As shown in Fig. 6D, V$_2$O$_5$ induced 2.5-, 4.1-, and 4.6-fold activation of NF-κB 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-κB 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-κB 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-κB pathway appeared to mediate V$_2$O$_5$-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 SO$_2$ (5) and O$_3$ (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 or conventional MAPK (i.e., ERK, P38 and JNK)-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...
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-κB 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-κB (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 V₄O₆ 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 chemotactant 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-κB 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 conditions.
measurements (mRNA vs. promoter-reporter). As discussed in the introduction, V$_2$O$_5$ is the most common commercial form of vanadium and is used in a variety of manufacturing processes. Na$_3$VO$_4$ is an experimental reagent. Although Na$_3$VO$_4$ is part of the products when V$_2$O$_5$ 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/m$^3$ (19). In the rat study, it has been shown that ~3.74 µg ROFA/cm$^2$ was deposited onto the trachea in a 6-h period when exposed to ROFA at 12 mg/m$^3$ (14). Thus, considering that the ROFA contains almost 40% of vanadium, our in vitro dose (i.e., 1 µg/cm$^2$ V$_2$O$_5$) is at the low boundary of occupational exposures. For the mouse model, we used laryngeal respiration method. Because of the rapid delivery of V$_2$O$_5$ 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 V$_2$O$_5$ 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-κB-dependent, pathway in mediating mucin production by V$_2$O$_5$. 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.

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GRANTS

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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); IκK_inh, IκK 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.

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