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Using gene expression profiling to evaluate cellular responses in mouse lungs exposed to V_2O_5 and a group of other mouse lung tumorigens and non-tumorigens

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ABSTRACT

Many compounds test positive for lung tumors in two-year NTP carcinogenicity bioassays in B6C3F1 mice. V_2O_5 was identified as a lung carcinogen in this assay, leading to its IARC (International Agency for Research on Cancer) classification as group 2b or a "possible" human carcinogen. To assess potential tumorigenic mode of action of V_2O_5, we compared gene expression and gene ontology enrichment in lung tissue of female B6C3F1 mice exposed for 13 weeks to a V_2O_5 particulate aerosol at a tumorigenic level (2.0 mg/m^3). Relative to 12 other compounds also tested for carcinogenicity in 2-year bioassays in mice, there were 1026 differentially expressed genes with V_2O_5, of which 483 were unique to V_2O_5. Ontology analysis of the 1026 V_2O_5 differentially expressed genes showed enrichment for hyaluronan and sphingolipid metabolism, adenylate cyclase functions, c-AMP signaling and PKA activation/signaling. Enrichment of lipids/lipoprotein metabolism and inflammatory pathways were consistent with previously reported clinical findings. Enrichment of c-AMP and PKA signaling pathways may arise due to inhibition of phosphatases, a known biological action of vanadate. We saw no enrichment for DNA-damage, oxidative stress, cell cycle, or apoptosis pathway signaling in mouse lungs exposed to V_2O_5 which is in contrast with past studies evaluating *in vivo* gene expression in target tissues of other carcinogens (arsenic, formaldehyde, naphthalene and chloroprene).

1. Introduction

Vanadium pentoxide (V_2O_5) is used in the manufacture of metal alloys (e.g., steel and titanium) and as an oxidation catalyst in the production of industrial chemicals (e.g., sulfuric acid, phthalic anhydride, and acetaldehyde) (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2006). While vanadium compounds are poorly absorbed after oral administration, respiratory exposure to particulates in industrial settings has irritant activity (ACGIH, 2009). With respect to toxicity, inhalation exposure to V_2O_5 increased the incidence of lung tumors as well as alveolar histolytic cellular infiltration, chronic inflammation, alveolar and bronchiolar hyperplasia in male and female B6C3F1 mice exposed to 1–4 mg/m^3 of a respirable particulate for two years (NTP, 2002; Paramanik and Rajalakshmi, 2013; Ress et al., 2003; Thomas et al., 2009). While both inflammatory and hyperplastic lesions were also found in lungs of F344N rats, there was no significant increase in lung tumors in the rats studied concurrently.

Cellular oxidative damage appears as a feature in the development of some cancers (Georgakilas 2012; Klaunig et al., 2010; Klaunig et al., 2011; Lee et al., 2012; Valavanidis et al., 2013). A high cellular oxidative load in lung tissues may result from an environmental perturbation such as particulate exposure and can disrupt the normal cellular redox state (Georgakilas 2012; Klaunig et al., 2010; Klaunig et al., 2011). Vanadium compounds formed in tissues from V_2O_5, including vanadate, may induce oxidative stress *in vivo* (Lee et al., 2012; Kulkarni et al., 2014). For instance, V_2O_5 nanoparticles in a nose-only inhalation exposure in male Wistar

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rats showed transient oxidative stress and VO_2 nanoparticles produce persistent oxidative stress (Kulkarni et al., 2014). Inhalation of V_2O_5 in B6C3F1 mice showed evidence of weak oxidative stress, but showed little evidence of oxidative stress at low concentrations (0.25 and 1 mg/m (NTP, 2002)) (Schuler et al., 2011). The role of oxidative stress in mouse lung tumors formed on V_2O_5 exposures remains unresolved.

In the NTP V_2O_5 mouse cancer assay, B6C3F1 animals were exposed to 1, 2 and 4 mg/m (NTP, 2002) V_2O_5 for 2 years and showed an increased incidence of lung tumors. The overall incidence of alveolar/bronchiolar adenomas and carcinoma in females was: controls = 1/50, 1 mg/m^3 = 32/50, 2 mg/m^3 = 35/50, 4 mg/m^3 = 32/50 (NTP, 2002). While all exposures (i.e., 1, 2, or 4 mg/m^3) caused significant increases in tumor incidence in the NTP study, we chose to use 2 mg/m (NTP, 2002) V_2O_5 because it gave a maximal response for lung tumors in both sexes of mice and was the intermediate concentration tested (NTP, 2002; Thomas et al., 2009). All 10 of the V_2O_5 treated animals we examined histologically had "*alveolar epithelial hyperplasia, inflammation and degeneration of the bronchial epithelium*" (Thomas et al., 2009). These tissue changes were consistent with those observed by NTP in mice following a 3-month exposure over a range of 2–16 mg/m^3 (NTP, 2002) Our study duration was 13 weeks (Thomas et al., 2009).

Our gene expression analysis of V_2O_5, utilized whole mouse genome Affymetrix microarray data from a previous study intended to determine if there were aggregate gene expression features that could statistically predict the likelihood of a chemical exposure progressing to tumors in a two-year bioassay (Thomas et al., 2009). In this earlier study, we included a total of 26 chemicals tested in NTP bioassay studies and had both positives and negatives for mouse lung tumor groups. Here, we conduct a more detailed examination of the ontologic pathways affected by V_2O_5 and by the group of compounds from our earlier study. Our primary goal was to understand the biological perturbations following 90-day V_2O_5 exposure at tumorigenic levels, in order to assess whether any of these perturbations were consistent with genotoxicity or oxidative stress and to compare the V_2O_5 responses with those of the other compounds tested for lung tumors in bioassay studies. In addition, we now have experience with gene expression analysis in target tissues for compounds whose cellular effects are better characterized, i.e., arsenic, formaldehyde, naphthalene, and chloroprene and it was of interest to evaluate whether cell response signatures similar to those seen with these other compounds were present with V_2O_5.

2. Materials and methods

2.1. Inhalation exposure of V_2O_5 aerosol

A general description of study design has been published (Thomas et al., 2009). Details specific for the V_2O_5 study follow. Female B6C3F1 mice (Charles River Laboratories, Raleigh, NC) were exposed to a V_2O_5 respirable particulate aerosol at target concentrations of either 0 (air-only control) or 2.0 mg/m^3, 6 h/day, 5 days/week, for 13 weeks. The whole body exposures were performed in 1 m^3 stainless steel and glass inhalation chambers (H1000, Lab Products, Seaford, DE). Animals remained housed in the inhalation chambers during the non-exposure periods; food and water were provided *ad libitum*. Inhalation chamber airflow, static pressure, temperature, and relative humidity were monitored throughout the 13-week study during both exposure and non-exposure periods.

One kilogram of V_2O_5 (CAS no. 1314-62-1) was obtained from Sigma Aldrich (catalog no. 221899-1 KG; lot/batch no. 06712BA). V_2O_5 purity was certified by Sigma Aldrich to be >99%. The inhalation exposure atmosphere was generated by aerosolizing V_2O_5 using a dry powder generator (Wright Dust Feeder Model WDF-II, Waltham, MA). The V_2O_5 was packed in a Wright Dust Feeder generation cup at a pressure of 3000 psi using a hydraulic press (Model No. 3912, Carver, Inc., Wabash, IN). The air delivery pressure through the Wright Dust Feeder was maintained at 20–25 psi. The V_2O_5 particulates were carried from the dry powder generator into a mixing/settling chamber with an approximate volume of 38 L. Particles leaving the mixing/settling chamber were further diluted with clean filtered air that entered directly into the inhalation exposure chamber. The WDF-II rotation speed was adjusted to produce the target particle concentration of 2.0 mg/m^3. Daily inhalation exposure concentrations of V_2O_5 were monitored using an optical particle sensor (Real-Time Aerosol Sensor, Model RAM-S, MIE Inc., Bedford, MA). The detection limit of the optical particle sensor was 0.03 mg/m^3. Exposure concentrations were determined approximately every 30 min during each six-hour exposure. Gravimetric filter samples were also collected from the V_2O_5 exposure chamber to confirm instrument calibrations and V_2O_5 exposure concentrations. An optical particle sizing spectrometer (Aerodynamic Particle Sizer, Model APS 3321, TSI, Inc. St. Paul, MN) was used to measure particle size and particle size distributions. Particle size data were collected from the V_2O_5 exposure chamber prior to the start of the 13-week study and during the last two weeks of exposure.

Daily exposure means of V_2O_5 concentration, airflow, static pressure, temperature, and relative humidity were calculated. Additionally, a grand mean of the daily means was calculated for each exposure parameter. Results indicated a mean and standard deviation V_2O_5 exposure concentration of 2.0 (±0.3) mg/m^3. The mass median aerodynamic diameter and geometric standard deviation of the V_2O_5 particle were 1.41 μm and 1.41 μm, respectively. Mean exposure chamber airflow, static pressure, temperature, and relative humidity were 225 L/min, -0.20 inches H_2O, 73.7°F, and 52%, respectively.

2.2. Genomic analysis of differential gene expression

The original Affymetrix array data files (i.e., CEL files, containing the array scanner fluorescence intensity data used for differential gene expression analysis) for the 26 chemicals analyzed in this study were previously deposited in the National Center for Biotechnology Information Gene Expression Omnibus, accession no. GSE17933 (Thomas et al., 2009). For this analysis, the Affymetrix CEL files were normalized in Partek Genome Suite ver. 6.6 using the Gene Chip Robust Multi-array Average background adjustment (GCRMA) algorithm and background correction, which uses sequence composition of the probes on the Affymetrix array to correct for probe binding affinity and thus potential non-specific binding artifacts in the intensity signal of the scanned array features (Wu et al., 2004). As previously noted for this data set there was a batch effect evident in this data related to the year of the different series of animal exposure experiments (Thomas et al., 2009). An ANOVA variance subtraction was used to remove this batch affect prior to differential gene expression analysis (Supplemental Fig. 1). The batch affect corrected data was then analyzed by ANOVA, with orthogonal linear contrasts for each individual chemical relative to its specific vehicle controls (Table 1 in Thomas et al, 2009). Differential gene expression was determined by a statistical threshold of a False Discovery Rate (FDR) corrected p-value less than 0.05 (i.e., FDR<0.05), a magnitude of change threshold of linear fold change (FC) being greater than +1.5 fold or less than −1.5 fold (i.e., |FC|>1.5), and by the simultaneous application of both these thresholds (Benjamini and Hochberg, 1995). Only genes which were simultaneously significant for both a