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Progress in Assessing Air Pollutant Risks from *In Vitro* Exposures: Matching Ozone Dose and Effect in Human Airway Cells

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The authors certify that all research involving human subjects was done under full compliance with all government policies and the Helsinki Declaration.

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ABSTRACT

In vitro exposures to air pollutants could, in theory, facilitate a rapid and detailed assessment of molecular mechanisms of toxicity. However, it is difficult to ensure that the dose of a gaseous pollutant to cells in tissue culture is similar to that of the same cells during *in vivo* exposure of a living person. The goal of the present study was to compare the dose and effect of O_3 in airway cells of humans exposed *in vivo* to that of human cells exposed *in vitro*. Ten subjects breathed labeled O_3 ($^{18}O_3$, 0.3 ppm, 2 h) while exercising intermittently. Bronchial brush biopsies and lung lavage fluids were collected 1 h post exposure for *in vivo* data whereas *in vitro* data were obtained from primary cultures of human bronchial epithelial cells exposed to 0.25–1.0 ppm $^{18}O_3$ for 2 h. The O_3 dose to the cells was defined as the level of ^{18}O incorporation and the O_3 effect as the fold increase in expression of inflammatory marker genes (IL-8 and COX-2). Dose and effect in cells removed from *in vivo* exposed subjects were lower than in cells exposed to the same $^{18}O_3$ concentration *in vitro* suggesting upper airway O_3 scrubbing *in vivo*. Cells collected by lavage as well as previous studies in monkeys show that cells deeper in the lung receive a higher O_3 dose than cells in the bronchus. We conclude that the methods used herein show promise for replicating and comparing the *in vivo* dose and effect of O_3 in an *in vitro* system.

Key words: ozone; *in vivo* versus *in vitro* dose; extrapolation; epithelial cells; bronchoalveolar lavage

Recent proposals for identifying exposure hazards to toxic agents involve the identification of "toxicity pathways" in primary cultures of human cells. The possibility of extrapolating toxicity observed *in vitro* to human risk *in vivo* is attractive because it might allow a deeper understanding of the molecular basis of injury through use of "omics" techniques as well as the possibility of high throughput screening. Air pollutant gases and aerosols present a unique challenge to *in vitro* work because of difficulty replicating the dose and exposure conditions found *in vivo*. Few studies have attempted to re-create *in vitro* the dose and effect of an air pollutant observed *in vivo*. (National Research Council, 2007).

The goal of the present study was to contribute a proof-of-concept for direct extrapolation of air pollutant toxicity from *in*

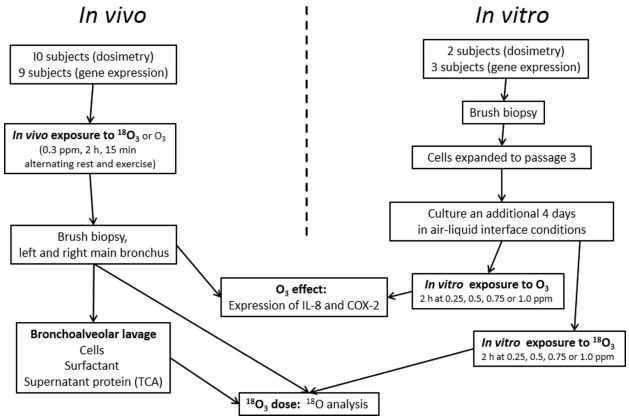

FIG. 1. Flow chart of the experimental design. The study involved both an *in vivo* and an *in vitro* exposure to $^{18}O_3$ of human epithelial cells. Bronchoalveolar lavage cells and constituents were also measured for comparison with the epithelial cells. Biological effects of O_3 consisted of measurements of IL-8 and COX-2 gene expression in cells harvested after exposure to unlabeled O_3. Cells were sampled 1 h after *in vivo* exposures and immediately following the *in vitro* exposures.

vitro cell cultures to *in vivo* human bronchial epithelial cells. Epithelial cells were chosen because they secrete mediators which initiate inflammatory events that are believed to be important in human risk assessments. They also can be expanded in air-liquid interface cultures and used in high throughput assays.

Ozone (O_3) was chosen as a prototype pollutant because it is one of the best-studied and ubiquitous of pure gaseous inhalants (U. S. E. P. A., 2013).

We showed previously that exposure to heavy oxygen-labeled ozone ($^{18}O_3$) results in incorporation of ^{18}O into cells and extracellular material obtained from bronchoalveolar lavage fluid (BALF) of humans and animals (Hatch *et al.*, 1994, 2013; Plopper *et al.*, 1998) creating a convenient micro-measurement of O_3 dose that is related to O_3 toxicity. We have also demonstrated that epithelial cells release mediators following O_3 exposure that are early markers of inflammation and that appear to have health consequences in the human population (Devlin *et al.*, 1996; Devlin *et al.*, 2012). The generation of these mediators can be quantified by gene expression assays. We here compare the $^{18}O_3$ dose and gene expression of two inflammatory mediators in cells obtained from human subjects exposed *in vivo* with the same measurements made in human primary cells expanded in culture and exposed in an air-liquid interface system *in vitro*.

MATERIALS AND METHODS

A flow chart depicting the study design is shown in Figure 1. *In vivo* exposures to $^{18}O_3$ (for dose measurement) and unlabeled O_3 for gene expression measurement were compared with *in vitro* exposures of the same length (2 h) that measured the same dose and effect endpoints. *In vitro* exposures to $^{18}O_3$ were performed on cells expanded from two subjects, and gene expression changes were evaluated in cells expanded from three subjects. Both the dose and effect *in vitro* studies employed the same range of $^{18}O_3$ or unlabeled O_3 concentrations. Cells were harvested at the termination of exposure in both the *in vivo* and *in vitro* studies.

In Vivo $^{18}O_3$ Exposures of Human Subjects

For the dosimetry of ten subjects, eight (seven Caucasian and one Hispanic) were male and two (one Caucasian and one Hispanic) were female, and the following data apply: age 26 ± 1 years (19–33 years), body weight 80.8 ± 2.8 kg, height 176.9 ± 3.0 cm, and resting forced vital capacity (FVC) 5.55 ± 0.27 l. For the biological effects study of nine subjects, all were males (one Black, one Hispanic and seven Caucasian), with the following characteristics: age 23.7 ± 0.9 (20–29 years), body weight 86.1 ± 2.6 kg, height 182.0 ± 2.0 cm, and resting FVC 5.88 ± 0.2 l. The study protocol was approved by the University of North Carolina Committee on the Rights of Human Subjects (Institutional Review Board) as well as the U.S. Environmental Protection Agency. All individuals provided written informed consent and were recruited under a contract to the Westat Corp. (Rockville, MD). All subjects underwent a physical examination, with a complete blood count and differential, serum electrolytes, glucose, and liver enzymes. Female subjects had a negative urine pregnancy test. Volunteers were excluded from the study if they had a smoking history within 2 years of the study, or were not able to refrain from taking over-the-counter anti-inflammatory agents, vitamins, or antioxidants for at least a week prior to exposure. Participants who reported a recent respiratory tract infection (or who had symptoms) were not studied for a period of at least 6 weeks after symptoms had disappeared.

Each volunteer was exposed on a single occasion for 2 h to $^{18}O_3$. Subjects were seated on a bicycle ergometer with their forehead touching a pad on the top of a 6 inch diameter cone that blew $^{18}O_3$ downward across the face at a flow rate of 500 l/min. The ozone concentration delivered did not deviate from the target concentration by more than ±5%. A sampling tube (with a flow rate of 2–2.5 l/min) similar to a head-mounted microphone collected breathing air 1–2 inches in front of the face of the subject. $^{18}O_3$ concentrations were thus monitored in the breathing zone every 10 s and stored as 2 min averages. Mean concentrations in the breathing zone ranged from 0.21 to 0.33 ppm and were 30–50% lower than in the incoming airstream.

Throughout the exposure, volunteers underwent moderate intermittent exercise (15 min intervals) on the bicycle ergometer. The level of exercise was targeted to a minute ventilation (V_e) of 25 l/min/m^2 body surface area (~50 l/min/subject). Prior to the exposure day, all subjects participated in a training session to establish the workload that would be required to achieve this V_e and the same work load was targeted on the ergometer. Subjects breathed naturally by mouth or nose depending on the subjects' comfort level. On the study day, all subjects performed a pulmonary function test prior to the exposure using a Sensormedics Vmax 220 instrument and software (Sensormedics Corp., Yorba Linda, CA) according to American Thoracic Society guidelines. Spirometry was also conducted on all subjects immediately following the exposure. Decrements in FEV_1 were <20% for all subjects post- versus pre-exposure. All volunteers had forced expiratory volume in 1 s (FEV_1) and FVC baseline values of at least 80% of that predicted for their height, age, and gender. Subjects were outfitted with a telemetry unit for the duration of the $^{18}O_3$ exposure and bronchoscopy for continuous monitoring of heart rate and rhythm as well as finger oxidimetry for O_2 saturation. Each subject was exposed twice, once to air and once to O_3, separated by at least 2 weeks.

In vivo $^{18}O_3$ and O_3 generation. The $^{18}O_3$ and unlabeled O_3 exposures were conducted identically in a chamber (dimensions of 1.22 m wide × 2.44 m long × 1.83 m high) at the U.S. Environmental Protection Agency Human Studies Facility on the

campus of the University of North Carolina, Chapel Hill, NC. Air from an oil-less compressor was passed through an air purifier (ZEKS Nomonox model CDP480CD00, West Chester, PA). It was then humidified by passage over heated deionized water. $^{18}O_3$ was generated by passing a 1–2% stream of $^{18}O_2$ (Isotec, Inc., Miamisburg, OH; 99 atom %) in argon through a silent electric discharge (model CMGK-F 0.5 O_3 generator; Innovatec Gerätetechnik GmbH, Rheinbach, Germany). The $^{18}O_3$ concentration was controlled by altering the flow of $^{18}O_2$ into a constant flow of argon that passed through the generator. Chamber relative humidity and temperature were measured once a second and stored as 2 min averages that yielded an overall mean and SD of 39.1 ± 0.2% and 22.5 ± 0.1°C, respectively.

Exposures to unlabeled O_3 were performed by metering pure O_2, along with dilution air, into a silent arc O_3 generator. O_3 concentration was controlled by varying the power to the generator, but otherwise the exposures were done identically to the $^{18}O_3$ exposures.

Bronchoscopic sample collection. A bronchoscopy was conducted ~45 min following the completion of the 2 h exposure to $^{18}O_3$ as described previously (Ghio *et al.*, 1998) modified as follows. Immediately prior to the procedure, subjects were given intravenous fluids through a saline lock catheter placed in an antecubital vein to ensure they were hydrated. Primary bronchial epithelial cells were obtained by brush biopsy of both the right and left mainstem bronchus. Each bronchus was brushed six times in and out with a ~5 cm excursion, and then repeated with a second brush. The two cytology brushes from each side were kept separate and the epithelial cells dislodged by agitation and brief vortexing in 0.1 ml of cold Dulbecco's phosphate buffered saline. The dislodged cells were pelleted by centrifugation at 400 × g at 4°C. For ^{18}O measurements, the supernatant was removed and the remaining cell pellet was lysed by pipetting up and down in 0.1 ml of 0.05% SDS. This solution was then diluted by adding an additional 0.1 ml of water.

Bronchoalveolar lavage was conducted after the collection of the brush biopsies. Three 50 ml aliquots of sterile saline were sequentially injected into the right middle lung lobe. After each aliquot of saline was instilled it was gently aspirated back into the same syringe. The unfiltered lavage fluid was placed on ice in 50 ml conical tubes. Mean percent recovery volumes (range) of BALF from each lavage were as follows: wash no. 1; 29.4 ± 7.0% (14–38%), wash no. 2; 67.4 ± 9.2% (52–84%), wash no. 3; 77.8 ± 11.4% (58–92%). Samples were immediately centrifuged at 1000 × g for 10 min at 4°C to pellet the cell fraction. BALF supernatants from all three aliquots were pooled and stored at −80°C for subsequent analysis of $^{18}O/^{16}O$ content. Cell pellets from each aliquot were individually lysed by the addition of 0.1 ml of 0.05% SDS and the resulting cell lysates were dispersed by pipetting up and down and then diluted with an equal volume of water.

Primary Human Epithelial Cell Cultures and $^{18}O_3$ Exposures

Cell collection and culture. Two dose-response *in vitro* $^{18}O_3$ exposure experiments were performed to measure ^{18}O and three to measure mRNAs' coding for pro-inflammatory mediators. Each concentration had three technical replicates that were averaged. Each experiment used cells originating from bronchial brush biopsies from a separate volunteer that participated in the *in vivo* exposure experiments. Three wells (technical replicas) were exposed. Cells were expanded to passage three in bronchial epithelial growth medium (Lonza, San Diego, CA). This media contained a background level of 0.83 mg/ml protein, 0.16nM retinoic acid, and no ascorbic acid. Cells were plated on Costar Trans-Clear filter supports with a 0.2μm pore size (Costar Corp., Cambridge, MA) and inserted into 12-well tissue culture plates (22.1 mm diameter wells; Corning Inc., Wilkes-Barre, PA). Each filter support was pre-coated by addition of 0.2 ml of 40 ug/ml collagen solution. Culture media under the interface (1 ml volume) was enriched to 0.1uM retinoic acid after the cells reached 100% confluence. Air-liquid interface culture conditions were initiated 24–48 h after the addition of retinoic acid by removing the apical medium. The cells were maintained at air-liquid interface conditions by replacing the basolateral medium (containing 0.1uM retinoic acid) every 48 h for 4 days. The last medium exchange was always done immediately prior to the $^{18}O_3$ exposure. At this stage of passage, cells were not yet producing a measurable mucus layer on their surface.

In vitro $^{18}O_3$ exposure of epithelial cells. Cells were exposed to four $^{18}O_3$ concentrations and an air control (0.0, 0.25, 0.50, 0.75, or 1.0 ppm $^{18}O_3$) for 2 h on the same day. No lid was present and atmospheres consisted of filtered air or $^{18}O_3$ in 5% CO_2 and maintained at 37°C and 88% relative humidity. Previous studies by us and others have shown that these concentrations do not cause more than minimal (5% or less) decreases in cell viability as measured with lactate dehydrogenase release assay. The exposure chambers were 30 × 30 × 40 cm stainless steel boxes with airflow passing from top to bottom through porous shelves. They were housed inside Nuaire Incubators (Plymouth, MN) that kept the temperature constant and provided the necessary CO_2 analyzer. Air flowing through the chambers originated from an oil-less compressor that was passed through a purifier (Aacdo Medical Inc., Cleves, OH) and a humidifier (Pure Steam, Chaska, MN) and pumped from the chamber using a Fox Venture Eductor (Dover, NJ). $^{18}O_3$ was generated similar to the *in vivo* exposures except that the $^{18}O_3$ generator was taken from a Bendix NO/NOx analyzer (Lewisburg, WV). Chamber concentrations of $^{18}O_3$ were monitored using TECO 49i O_3 analyzers (Thermo Science, Franklin, MA) and controlled by altering the flow rate of an argon/$^{18}O_2$ mixture passing through the O_3 generator.

In both *in vivo* and *in vitro* exposures, there was a small excess (100–200 ppm) of $^{18}O_2$ resulting from incomplete conversion of $^{18}O_2$ to $^{18}O_3$ (conversion efficiency is 1–2%). We have shown that $^{18}O_2$ exposure at 21% results in ^{18}O incorporation, but that $^{18}O_3$ incorporation is 142,000- to 210,000-fold higher on a per ppm basis (Supplementary table 1). This difference in incorporation appears to be the result of higher chemical reactivity of the $^{18}O_3$ and is evident even though the incorporation of $^{18}O_2$ into cells also includes uptake resulting from normal metabolic processes.

Tissue and BALF Fraction Preparation for ^{18}O Analysis

BALF cell pellets were resuspended in 0.2 ml of 0.025% SDS. BALF supernatants were combined and centrifuged for 1 h at 22,000 × g to obtain a white "surfactant pellet." The resulting dried supernatant contained proteins and lipids mixed in a high NaCl matrix that could interfere with measurement of ^{18}O. For this reason, we precipitated the protein by adding trichloroacetic acid (TCA) to a final concentration of 10% and centrifuged again (40,000 × g for 1 h at 4°C). The surfactant and TCA pellets were resuspended in 0.4 ml of the 0.025% SDS solution.

Immediately following the *in vitro* exposures, cells were analyzed for ^{18}O content. This time point was chosen to minimize metabolism of the ^{18}O signal by cells and to coincide with the same time point in which ozone-induced changes in mRNAs coding for pro-inflammatory proteins have been observed. Cells were lysed by the addition of 0.1 ml of 0.05% SDS to the api-

cal surface. The cells were dislodged from the filter support using a pipette tip and the lysate transferred to a cryovial. The filter support was rinsed with an additional 0.1 ml of water and this was pooled with the original 0.1 ml of cell lysate for a final SDS concentration of 0.025% in the final sample. Epithelial cell SDS lysates from both the *in vivo* and *in vitro* $^{18}O_3$ exposures and BALF cells from the *in vivo* exposures were stored at −80°C, then lyophilized and stored with desiccant at 4°C.

Analysis of ^{18}O in lyophilized samples has been described previously (Hatch *et al.*, 1994; Santrock *et al.*, 1989). Briefly, lyophilized samples weighing 0.5–1.5 mg were placed into silver cups (3 × 5 mm), crimped, and then placed into an elemental analyzer (Carlo Erba Instruments, Italy) where all oxygen in the samples was converted to CO under a stream of pure helium. The elemental analyzer quantified the CO which yielded a percentage (per dry weight) of total elemental oxygen in each sample. The helium/CO effluent of the elemental analyzer was captured and passed by continuous flow through a heated (110°C) column of granular I_2O_5 (to convert CO to CO_2), then a cryogenic trap (−60°C to remove produced I_2), and then bled by capillary into the vacuum of an isotope ratio mass spectrometer (SIRA 10, VG instruments) where the ratios between CO_2 masses were determined. $^{18}O_3$ exposed samples were compared with their natural abundance controls inserted into each run.

Quantitative RT-PCR for Detection of Gene Expression Changes

Gene expression changes of the inflammatory markers IL-8 and COX-2 in human airway epithelial cells were quantified using reverse transcription polymerase chain reaction (RT-PCR). Total RNA was isolated using an RNeasy Mini Kit according to manufacturer's instructions (Qiagen, Valencia, CA). RNA quantity was determined on a Nanodrop ND-1000 and RNA integrity was assessed using RNA6000 Nano chips on a Bioanalyzer 2100 (Agilent). Total RNA (100–200 ng) was reverse transcribed to generate cDNA using the High Capacity cDNA Reverse Transcription kit (Life Technologies Applied Biosystems, Foster City, CA). Quantitative fluorogenic amplification of cDNA was performed using the ABI StepOnePlusTM Real-Time PCR System (Applied Biosystems) with iTaq Universal Probes Supermix (Bio-Rad), primer/probe sets for the target gene of interest multiplexed with b-actin (ACTB) primer/probe sets as the normalizing housekeeping gene (20× VIC/MGB, Primer Limited, Applied Biosystems). Values from triplicate technical replicates for each sample were averaged and data analyzed using the DDCt method to obtain fold change values following ozone exposure with respect to air exposure values.

Data Analysis

The "incorporation of ^{18}O" is defined here as the measure of O_3 dose. $A^{18}O/^{16}O$ ratio was derived from the relative CO_2 masses obtained from the mass spectrometer as a unitless "delta value." The enrichment in ^{18}O in the $^{18}O_3$ exposed samples was quantified and units changed to umoles of ^{18}O/mole of total oxygen by subtracting the mean of the background natural abundance of ^{18}O from all samples. This background, consisting of ∼0.2 atom % of all oxygen, was obtained from analysis of air-exposed cells in the case of *in vitro* data or from blood plasma samples of the subjects in the case of *in vivo* exposures. The denominator for the enrichment in ^{18}O was then converted to per gram dry weight by use of the mean elemental percentage of oxygen (obtained as output from the elemental analyzer). This value was obtained from 10 subjects, each exposed to both clean air and ozone, and paired t-tests were used to determine statistical significance ($p < 0.05$). For *in vitro* exposure experiments, one-way ANOVA was used to calculate significance.

Analysis of gene expression performed on *in vivo* exposed samples involved paired t-test with each subject serving as his/her own control. Analysis of *in vitro* gene expression data was performed by ANOVA followed by Dunnett's test of individual means.

RESULTS

Methodology Findings

The cells obtained by brush biopsy were estimated to be greater than 95% epithelial cells. We originally explored trying to determine the percentage of different types of epithelial cells in the biopsies but they come off the brush in clumps making this impossible. The only cells that grow from these biopsies are the basal cells. Ciliated cells, mucus cells, clara cells, etc. are all terminally differentiated and do not divide in culture. Cell counts and differentials obtained from BALF are shown in Supplementary table 2. Percentage of polymorphonuclear leukocytes (PMNs) was increased by O_3 exposure at both the 1 h and 24 h BALF collection times.

We were able to obtain sufficient sample from a single culture well of confluent epithelial cells, or from the brush biopsy from one main bronchus. BALF cells and surfactant pellets yielded larger samples than were obtained from TCA pellets of BALF. Our ^{18}O analysis required 0.5–1.5 mg of dry weight with a total oxygen percentage >10%. The bronchial biopsies barely met these requirements and for this reason the gene expression changes could not have been measured in the same samples as the ^{18}O dose. The detection limit for the ^{18}O assay depends on (1) the quantity of elemental oxygen in the sample (accuracy improves with higher oxygen/sample) and (2) number of replicate samples (accuracy improves with higher number of samples analyzed due to both "memory" from the previous samples and increased statistical power with larger sample numbers). Dry weights of brush biopsy samples are shown in Supplementary table 3. The ^{18}O values for the left and right bronchial brush biopsies were averaged because there was no correlation between the two measurements. The use of SDS for lysis and dispersion of samples prevented sticking of dry sample to the walls of the tubes that facilitated weighing the samples into silver sampling cups after lyophilization. The contribution of SDS to the dry weight of the samples was ∼10% for the BALF cell and surfactant fractions, and 5% for the other samples.

Elemental Oxygen Percentages

Oxygen percentages that were used in converting the units of ^{18}O dose as described above were as follows (mean ± SE): *in vitro* epithelial cells expt. 1, 17.0 ± 0.7; *in vitro* epithelial cells expt. 2, 17.2 ± 1.2; *in vivo* bronchial brush biopsies, 12.7 ± 0.9; BALF cells, 16.2 ± 1.1; BALF supernatant TCA pellet, 11.9 ± 0.8; BALF surfactant pellet, 18.0 ± 0.2; plasma 1, 23.8 ± 1.7; Plasma 2, 21.4 ± 1.1.

Comparison of In Vitro and In Vivo $^{18}O_3$ Incorporation

$^{18}O_3$ exposure resulted in significant incorporation of ^{18}O following the *in vivo* study and each $^{18}O_3$ concentration in the *in vitro* study (Fig. 2). The mean ± SE ^{18}O values of bronchial brush biopsies taken after *in vivo* $^{18}O_3$ exposure are plotted along with the concentration response of ^{18}O data from the *in vitro* exposure. *In vivo* exposed cells showed lower ^{18}O incorporation than *in vitro* exposed cells at the same $^{18}O_3$ concentration. For example, the ^{18}O incorporation *in vivo* was 64% lower than that seen at the

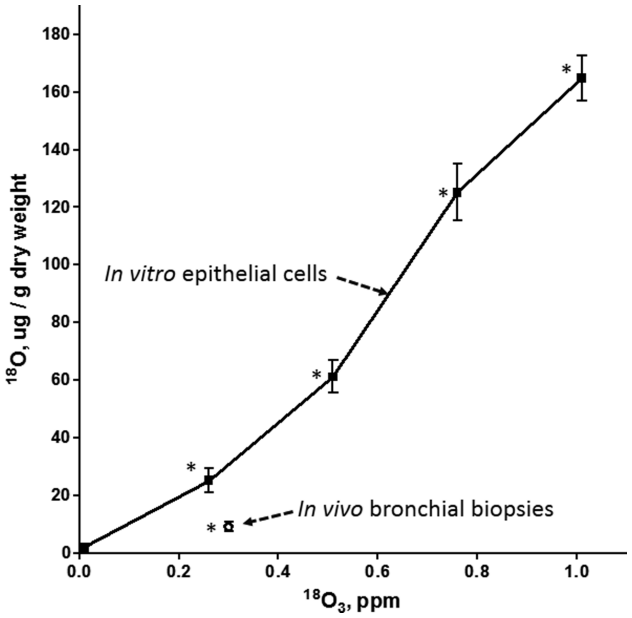

FIG. 2. *In vitro* and *in vivo* dose comparison: ^{18}O incorporation into bronchial brush biopsies collected from human subjects exposed for 2 h to 0.3 ppm $^{18}O_3$ while exercising intermittently or human primary epithelial cells exposed *in vitro* to a range of $^{18}O_3$ concentrations (mean ± SE). Standard error bars are within the data point for the *in vivo* data ($N = 10$ human subjects for *in vivo* data and 5 culture wells derived from two replicate *in vitro* exposures to each $^{18}O_3$ concentration for *in vitro* data). Each mean shown is significantly elevated (* denotes $p < 0.05$) relative to natural background ^{18}O (zero $^{18}O_3$).

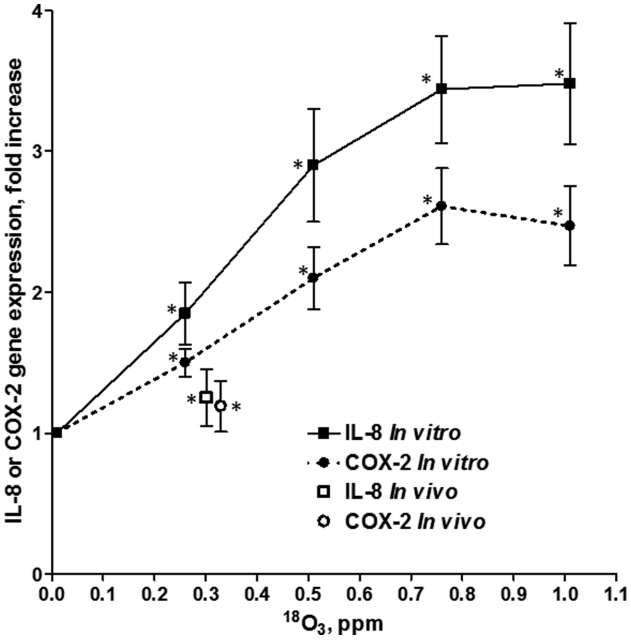

FIG. 3. *In vitro* and *in vivo* biological effects comparison of O_3 exposures under conditions similar to the dose comparison in Figure 2. Shown are the fold increases in expression of IL-8 or COX-2 genes (means ± SE) in bronchial brush biopsies obtained from nine human subjects exposed *in vivo* to O_3 compared with their response following exposure to clean air. Also shown are fold increases of primary epithelial cells exposed *in vitro* to each O_3 concentration (means ± SE for three subjects and three replicates per subject) compared with their response following exposure to clean air. Asterisks denote significance ($p < 0.05$).

same $^{18}O_3$ level (0.3 ppm) on the dose response curve of the *in vitro* exposed cells. We were unable with the present sample size to observe any relationships between ^{18}O incorporation and subject age, sex, or race. Variability observed in the *in vitro* data could have been the result of (1) differences between the subjects from which cells were obtained, (2) variation in the two ^{18}O analysis runs, or (3) between the replicate culture wells exposed at each $^{18}O_3$ concentration. The variability in natural abundance of ^{18}O (zero ppm $^{18}O_3$) was small compared with variability among $^{18}O_3$ exposed cells.

Comparison of In Vitro and In Vivo O_3 Biological Effects

Figure 3 shows the fold increases (±SE) in gene expression of IL-8 and COX-2 in bronchial brush biopsies from *in vivo* O_3 exposures and primary epithelial cell cultures exposed *in vitro* to the same range of O_3 concentrations as was used for the $^{18}O_3$ study. Similar to the ^{18}O data, the *in vivo* gene expression was lower than that observed following *in vitro* exposure. The IL-8 increase in expression in the *in vivo* exposed cells fell about 30% below that observed *in vitro* and the *in vivo* COX-2 response fell about 17% below the response observed *in vitro*. The *in vitro* expression of IL-8 was higher than the expression of COX-2 but both genes were induced in a concentration-related manner that leveled off at 0.75 and 1.0 ppm O_3. Although epithelial cells obtained from subjects exposed *in vivo* showed elevations in gene expression smaller than were observed at the lowest dose (0.25 ppm) used in the *in vitro* study, the increases in both genes were significant by paired t-test of the nine subjects studied.

Comparison of ^{18}O in Bronchial Brush Biopsies to Three BALF Fractions

^{18}O incorporation measured after *in vivo* exposure to $^{18}O_3$ resulted in higher ^{18}O incorporation into cell and surfactant fractions of BALF than bronchial brush biopsies from the same subjects (Fig. 4). The BALF surfactant incorporated the highest levels of ^{18}O, which had a mean value slightly higher than the BALF cells, fourfold higher than BALF TCA pellet, and about eightfold higher than the mean of the left and right bronchial brush biopsies. A subject-by-subject plot of the relationship between the four tissue pools is shown in Supplementary figure 1.

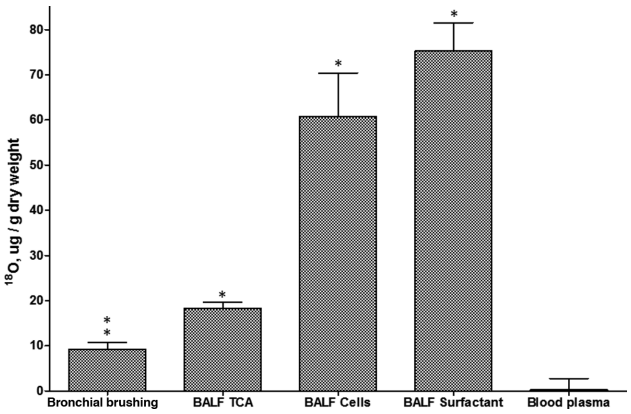

FIG. 4. Comparison of ^{18}O incorporation into epithelial cells (shown in Fig. 2) and BALF constituents of the same subjects following exposure to $^{18}O_3$ (0.3 ppm, 2 h with intermittent exercise). Means ± SE are shown for 10 subjects. All $^{18}O_3$ exposed tissues had significantly elevated ^{18}O when compared with the natural abundance ^{18}O concentration of blood plasma.

Relationships Between Different In Vivo Sample ^{18}O Measurements
No relationship was observed between the ^{18}O incorporation in the left and right bronchial biopsies. A search for relationships across the 10 human subjects in the measured values for ^{18}O incorporation in different tissue compartments yielded the result that BALF surfactant ^{18}O increased in proportion to BALF cell ^{18}O ($R^2 = 0.73$; see Supplementary fig. 2) but not between other BALF constituents ($R^2 < 0.5$). The concentration of $^{18}O_3$ measured at the mouthpiece of the subjects (which ranged from 0.21 to 0.33 ppm) did not appear to be correlated with the ^{18}O accumulation in any of the individual tissue or BALF constituents or any combination of the sum of the ^{18}O concentrations in BALF.

DISCUSSION

We achieved a proof-of-concept for obtaining comparable measurements of O_3 dose and effect between *in vivo* exposed epithelial cells and *in vitro* exposed primary epithelial cells exposed in an air-liquid interface culture. Prior to this, few, if any, equivalent measurements for an air pollutant gas had been identified and compared under both *in vivo* and *in vitro* conditions. Problems have included finding equivalent denominators applicable to both types of exposure. For example, measurements of released cytokines or LDH depend on equivalent volumes of extracellular fluid that cannot be controlled equally under *in vivo* and *in vitro* conditions. Although the main goal of the study was to compare the dose and response in epithelial cells, some valuable insights were obtained about the relative O_3 dose in different cell types. Epithelial cell production of inflammatory mediators appears to be easily measured by gene expression assays, and primary human epithelial cells appear to be a good model that could be expanded in air-liquid interface cultures used in high throughput assays.

This is the first report of ^{18}O incorporation into human bronchial epithelial cells *in vivo* or into any type of cell exposed to $^{18}O_3$ *in vitro*. Our previous studies examined BALF constituents of humans and rats exposed *in vivo* to $^{18}O_3$ but did not include epithelial cells (Hatch *et al.*, 1994). It was interesting that both the O_3 dose and effect observed *in vivo* always fell below that observed in the *in vitro* exposures at the same air concentration of O_3. Previous studies that measured O_3 air concentrations in the posterior pharynx of exercising human subjects found that fractional removal of O_3 in the nose is ~40% (Gerrity *et al.*, 1994). Thus, scrubbing of O_3 in the upper airways might explain the lower dose and effect of O_3 *in vivo* compared with *in vitro*. Other factors that could have influenced the relative dose and effect between *in vivo* and *in vitro* exposures include the following. (1) Brush biopsy of cells *in vivo* probably removed some underlying cells that would have a lower exposure to O_3 than the surface cells, (2) there may have been differences in the competition of the $^{18}O_3$ reaction with antioxidants that do not form addition products with $^{18}O_3$ (see below), or (3) a longer (1 h vs. minutes) delay following *in vivo* exposure compared with *in vitro* exposure could have resulted in some clearance of ^{18}O or a return to normal gene expression.

It is likely that the bronchial brush biopsy samples measured here gave a low approximation of epithelial cell dose compared with the distal airways that appear to be the main targets for O_3 in the human lung. A prior study of resting Rhesus monkeys showed that *in vivo* exposure (0.4 or 1.0 ppm $^{18}O_3$ for 2 h) resulted in bronchial cell levels of ^{18}O similar to those observed here for bronchial cells of exercising humans (Plopper *et al.*, 1998): the bronchial cells of monkeys incorporated 4–7 ug ^{18}O/g dry while the human bronchial cells incorporated ~10 ug ^{18}O/g dry. The smallest airways of the monkey (respiratory bronchioles) had a 2–4-fold higher ^{18}O incorporation than cells of the main bronchus suggesting that human terminal airways would be higher as well. This higher dose deeper in the lung is supported by the 4–8-fold higher ^{18}O incorporation observed here in the BALF constituents. Histopathology data on O_3 injury to epithelium and O_3 dosimetry uptake models also predict the highest O_3 dose and effect in the small terminal airways of all species (Barry *et al.*, 1985; Miller et al, 1978). It would not be possible to biopsy tissues of these small airways in human subjects. The above-mentioned references also demonstrate that although we examined only one dose of $^{18}O_3$ in the present *in vivo* study, a dose response behavior should be expected. Taken together, our results suggest that the dose response we observed here in the *in vitro* exposed epithelial cells covered a reasonable range of doses for human airway epithelial cells exposed *in vivo*.

In addition to the epithelial cell data, we learned that BALF constituents experience a high dose of $^{18}O_3$ compared with bronchial epithelial cells. This difference might be due to the larger surface area exposed or biochemical differences in reactivity of the $^{18}O_3$. We have observed in other studies that low ascorbate is related to high $^{18}O_3$ levels in BALF (Gunnison and Hatch, 1999; Kari *et al.*, 1997). The lack of ascorbate in the culture media used here would have resulted in a loss of ascorbate from cells during culture (Lane *et al.*, 2013) which would have increased the incorporation of $^{18}O_3$ in *in vitro* cells compared with the cells exposed *in vivo* that are known to be bathed in extracellular ascorbate (Slade *et al.*, 1993; van der Vliet *et al.*, 1999). In an earlier study, we reported a 30–50% lower ^{18}O incorporation into human BALF cells and surfactant pellets than we observed here. This was in spite of the fact that the earlier study involved a higher $^{18}O_3$ concentration (0.4 ppm, 2 h rather than 0.3 ppm, 2 h) and higher targeted minute ventilation (60 l/min/subject vs. 50 l/min/subject in the present study). One reason for this difference might be that the earlier study measured the $^{18}O_3$ concentration at a site distant from the breathing zone of the subjects so that surfaces for reaction with $^{18}O_3$ within the exposure chamber could have reduced the actual concentrations inhaled. In the present study, $^{18}O_3$ was measured in the breathing zone to better ensure that losses of $^{18}O_3$ on surfaces would not occur. The *in vitro* exposures might also be affected by this phenomenon, however, there appeared to be fewer surfaces for reaction with $^{18}O_3$ in the *in vitro* chambers than in the *in vivo* chambers. Another finding is that our earlier study employed dialysis of the BALF supernatant to separate the protein/lipid fraction from the NaCl present in the saline lavage—a necessity because high levels of NaCl can interfere with the ^{18}O assay. The earlier reported ^{18}O levels in the dialyzed BALF high speed supernatant were higher than the present results found in the TCA-precipitated BALF high speed supernatant. It appears that TCA treatment may have dissociated the ^{18}O adduct, or failed to concentrate the same extracellular materials in BALF as did dialysis.

Limitations of the present study include the small number of *in vitro* replicate exposures as well as of human subjects contributing to the cultures. In addition, we observed significant intra- and inter-subject variations in ^{18}O in cells removed by brush biopsy. Part of this variation may be attributable to the fact that not all the inhaled ozone will react with the surface of airway epithelial cells that are then removed by brush biopsy. Some will react with compounds present in lung lining fluid such as mucin or antioxidants and these compounds may differ in concentration and among people or even in the same person

on a day-to-day basis. This is also true to some extent within a subject. It is not known if mucin or antioxidants are distributed evenly throughout the airways. Another factor that may be contributing to variability is that airway cells are not a monolayer and there is a possibility that some of the cells not directly opening into the airway and therefore not receiving a dose of ozone (e.g., basal cells) would be removed during brush scraping to varying degrees from one individual to another or even from different locations within an individual. It is not known if the makeup of cell composition (e.g., % of ciliated cells, mucin secreting cells, clara cells, basal cells, etc.) is homogenous throughout the airway. Finally, some variability was probably due to the limited mass of sample that made it impossible to obtain more than one ^{18}O analysis for each bronchus sampled. Although the BALF cells sampled after *in vivo* exposure were obtained in a higher sample mass than the tissue mass of bronchial brush biopsies, we believe that the overall contribution of epithelial cells to the inflammatory response would be greater because of a larger surface area exposed to O_3 and a higher secretion of inflammatory cytokines in epithelial cells than other cells (Devlin *et al.*, 1994). Future studies should compare the sensitivity to injury of alveolar macrophages and epithelial cells and also determine the contribution of mucus or epithelial lining fluid covering the cells to the resulting dose and effect of O_3. Another potential limitation is the possibility that the subjects studied had been exposed previously to ozone and as such might have experienced epigenetic effects that could alter the cell responses following *in vitro* ozone exposure. No attempt was made to control for this possibility because there are so many other outside influences that could also induce epigenetic effects, and because we presently have no evidence for this type of change in the scenario we have studied here.

In summary, our results suggest that *in vitro* exposure conditions can be made to reasonably match the *in vivo* dose of O_3 to epithelial cells exposed *in vivo* and that comparable measures of dose and effect can be obtained. These studies add confidence to the possibility of extrapolating effects observed *in vitro* to effects that might occur in human airways *in vivo*.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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REFERENCES

Barry, B. E., Miller, F. J. and Crapo, J. D. (1985). Effects of inhalation of 0.12 and 0.25 ppm ozone on proximal alveolar region of juvenile and adult rats. *Lab. Invest.* **53**, 692–704.

Devlin, R. B., Duncan, K. E., Jardim, M., Schmitt, M. T., Rappold, A. G. and Diaz-Sanchez, D. (2012). Controlled exposure of healthy young volunteers to ozone causes cardiovascular effects. *Circulation* **126**, 104–111.

Devlin, R. B., McDonnell, W. F., Becker, S., Madden, M. C., McGee, M. P., Perez, R., Hatch, G., House, D. E. and Koren, H. S. (1996). Time-dependent changes of inflammatory mediators in the lungs of humans exposed to 0.4 ppm ozone for 2 hr: A comparison of mediators found in bronchoalveolar lavage fluid 1 and 18 hr after exposure. *Toxicol. Appl. Pharm.* **138**, 176–185.

Devlin, R. B., McKinnon, K. P., Noah, T., Becker, S. and Koren, H. S. (1994). Ozone-induced release of cytokines and fibronectin by alveolar macrophages and airway epithelial cells. *Am. J. Physiol.* **266**(6 Pt 1), L612–L619.

Gerrity, T. R., McDonnell, W. F. and House, D. E. (1994). The relationship between delivered ozone dose and functional responses in humans. *Toxicol. Appl. Pharm.* **124**, 275–283.

Ghio, A. J. B. M., Chall, A. N., Levin, D. G. and Bromberg, P. A. (1998). Bronchoscopy in healthy volunteers. *J. Bronchol.* **5**, 185–194.

Gunnison, A. F. and Hatch, G. E. (1999). O_3-induced inflammation in prepregnant, pregnant, and lactating rats correlates with O_3 dose estimated by ^{18}O. *Am. J. Physiol.* **276**, L332–L340.

Hatch, G. E., McKee, J., Brown, J., McDonnell, W., Seal, E., Soukup, J., Slade, R., Crissman, K. and Devlin, R. (2013). Biomarkers of dose and effect of inhaled ozone in resting versus exercising human subjects: comparison with resting rats. *Biomarker Insights* **8**, 53–67.

Hatch, G. E., Slade, R., Harris, L. P., McDonnell, W. F., Devlin, R. B., Koren, H. S., Costa, D. L. and McKee, J. (1994). Ozone dose and effect in humans and rats—A comparison using ^{18}O labeling and bronchoalveolar lavage. *Am. J. Respir. Crit. Care Med.* **150**, 676–683.

Kari, F., Hatch, G., Slade, R., Crissman, K., Simeonova, P. P. and Luster, M. (1997). Dietary restriction mitigates ozone-induced lung inflammation in rats: A role for endogenous antioxidants. *Am. J. Respir. Cell Mol. Biol.* **17**, 740–747.

Lane, D. J. R., Chikhani, S., Richardson, V. and Richardson, D. R. (2013). Transferrin iron uptake is stimulated by ascorbate via an intracellular reductive mechanism. *Biochim. Biophys. Acta* **1833**, 1527–1541.

Miller, F. J., Menzel, D. B and Coffin, D. L. (1978) Similarity between man and laboratory-animals in regional pulmonary-deposition of ozone. *Environ. Res.*, **17**, 84–101.

National Research Council (2007). *Toxicity Testing in the 21st Century: A Vision and a Strategy*. The National Academies Press, Washington, D.C.

Plopper, C. G., Hatch, G. E., Wong, V., Duan, X., Weir, A. J., Tarkington, B. K., Devlin, R. B., Becker, S. and Buckpitt, A. R. (1998). Relationship of inhaled ozone concentration to acute tracheobronchial epithelial injury, site-specific ozone dose and glutathione depletion in rhesus monkeys. *Am. J. Respir. Cell Mol. Biol.* **19**, 387–399.

Santrock, J., Hatch, G. E., Slade, R. and Hayes, J. M. (1989). Incorporation and disappearance of ^{18}O in lungs from mice exposed to 1 ppm $^{18}O_3$. *Toxicol. Appl. Pharm.* **98**, 75–80.

Slade, R., Crissman, K., Norwood, J. and Hatch, G. (1993). Comparison of antioxidant substances in bronchoalveolar lavage cells and fluid from humans, guinea-pigs, and rats. *Exp. Lung*

Res. **19**, 469–484.

U. S. E. P. A. (2013) *Air quality criteria for ozone and related photochemical oxidants: Integrated Science Assessment*. Research Triangle Park, NC. Available at: http://www.epa.gov/ttn/naaqs/standards/ozone/s_o3_2008_isa.html Accessed June 30, 2014.

van der Vliet, A., O'Neill, C. A., Cross, C. E., Koostra, J. M., Volz, W. G., Halliwell, B. and Louie, S. (1999). Determination of low-molecular-mass antioxidant concentrations in human respiratory tract lining fluids. *Am. J. Physiol.* **276**, L289–L296.