Biomarkers of Dose and Effect of Inhaled Ozone in Resting versus Exercising Human Subjects: Comparison with Resting Rats

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Abstract: To determine the influence of exercise on pulmonary dose of inhaled pollutants, we compared biomarkers of inhaled ozone (O₃) dose and toxic effect between exercise levels in humans, and between humans and rats. Resting human subjects were exposed to labeled O₃ (¹⁸O₃, 0.4 ppm, for 2 hours) and alveolar O₃ dose measured as the concentration of excess ¹⁸O in cells and extracellular material of nasal, bronchial, and bronchoalveolar lavage fluid (BALF). We related O₃ dose to effects (changes in BALF protein, LDH, IL-6, and antioxidant substances) measurable in the BALF. A parallel study of resting subjects examined lung function (FEV₁) changes following O₃. Subjects exposed while resting had ¹⁸O concentrations in BALF cells that were 1/5th of those of exercising subjects and directly proportional to the amount of O₃ breathed during exposure. Quantitative measures of alveolar O₃ dose and toxicity that were observed previously in exercising subjects were greatly reduced or non-observable in O₃ exposed resting subjects. Resting rats and resting humans were found to have a similar alveolar O₃ dose.

Keywords: ozone, inhalation toxicology, exercise, animal human extrapolation
Introduction

Ozone (O$_3$) pollution of ambient air is a significant public health problem worldwide, and adds to the pollutant burden of particulate matter and volatile organics. Mandatory emission controls on automobiles and other pollution sources have been reasonably effective in limiting the accumulation of O$_3$ in urban areas. However, the expense of O$_3$ regulations and the continued refinement of low-dose health effects of O$_3$ have kept it in the scientific and regulatory spotlight. Considerable attention is given to the U.S. National Ambient Air Quality standard for O$_3$, which is presently 0.075 ppm averaged over 8 hours. Justification for this standard derives from controlled O$_3$ exposures of exercising human subjects with support from human epidemiology and laboratory animal toxicology.$^1$

In this study we explore the influence of physical exercise in humans on the resultant alveolar dose and effect of inhaled O$_3$. To date, almost all clinical studies of O$_3$ effects in humans have been performed while subjects exercised during O$_3$ exposure. Here, we quantify the dose of O$_3$ to the lung alveoli during resting O$_3$ exposure, and compare this dose to that achieved during exercise. We also compare the human O$_3$ dose to that of similarly exposed resting rats.

Physical exercise during exposure increases the alveolar O$_3$ dose by switching the air flow to the mouth, where it is scrubbed less efficiently, and by increasing the amount of O$_3$ that enters the lung due to increased minute ventilation (Ve) and tidal volume. Early human clinical studies showed enhanced physiological effects of O$_3$ if subjects exercised during exposure.$^2$ Since exercise is a part of everyday life, the inclusion of exercise with O$_3$ exposure has been employed in almost all human clinical studies of O$_3$. For technical reasons, exercise has not been employed in most animal inhalation studies.

Ozone has become a prototype for the study of chemically reactive air pollutants. Although O$_3$ appears to react at the air-liquid interface of the entire respiratory tract, the target sites of greatest interest toxicologically appear to be the terminal airways and alveolar region. Terminal airways receive a proportionally higher dose of O$_3$ because of their small surface area and lack of mucus covering.$^4$ Alveolar epithelium is in close proximity to the blood, and it is believed that transport of O$_3$ reaction products to blood might contribute to enhancement of atherosclerotic plaque formation.$^5$

Studies with inhaled oxygen-18 labeled ozone ($^{18}$O$_3$) have shown that O$_3$ reacts chemically with constituents of airway lining fluid, leaving behind oxygen atoms bound to cellular and extracellular material.$^6-8$ Consistent with its known chemistry, O$_3$ has a broad spectrum of reactivity with most biomolecules it interacts with. We showed previously that the concentration of $^{18}$O labeled products in $^{18}$O$_3$ reactions in BALF cellular and extracellular constituents were related to O$_3$ induced toxic effects including increased BALF protein concentrations and neutrophil counts.$^6$ These results were observed for both humans and rats; however, resting rats had a much smaller accumulation of $^{18}$O and a corresponding lack of O$_3$ effects on BALF protein and neutrophil count, unless the $^{18}$O$_3$ exposure concentration was increased 5-fold to 2 ppm. The possibility that numerous studies of O$_3$ exposed laboratory rats might actually underestimate human dose and effect has been difficult to explain because rats have been assumed to breathe more air than humans and therefore should receive a higher alveolar O$_3$ dose. We show here that resting human subjects achieve a much lower alveolar O$_3$ dose than exercising subjects and that this dose is comparable to that of resting rats. The resting subjects also show fewer detectable O$_3$ induced cellular, biochemical, and physiological (FEV$_1$) effects than exercising subjects.

Methods

Experimental design and recruitment of subjects

Two experiments involving resting exposure to O$_3$ by human subjects and measurements made during or immediately after exposure are reported. The first was a 2 hour exposure to $^{18}$O$_3$ by face mask, followed by nasal, bronchial, and bronchoalveolar lavage. The second was a 2 hour chamber exposure to unlabeled O$_3$ in which 68 subjects were exposed to four different O$_3$ concentrations, and then examined physiologically for a change in FEV$_1$.

Study protocols for both experiments were approved by the Institutional Review Board at the University of North Carolina Medical School in Chapel Hill and the EPA; informed consent was obtained from all subjects before their participation in the study. Table 1 shows the physical characteristics...
Table 1. Characteristics of male subjects exposed while resting to air or O₃ in the two studies reported here.

|                        | 18O₂ lavage study, 8 subjects | Physiology study, 68 subjects |
|------------------------|-------------------------------|-------------------------------|
| Age, yr                | 26.4 ± 1.2                   | 25.2 ± 0.4                   |
| Height, cm             | 183                           | 181                           |
| Weight, kg             | 87.9 ± 3.4                   | 81.7 ± 1.5                   |

and age of the subjects in the two experiments. Paid volunteers were selected on the basis of being healthy, non-smoking 18–35 years of age, and with no history of asthma or allergic rhinitis. They were predominantly students recruited from colleges in the Chapel Hill-Durham area of North Carolina. No attempt was made to catalogue ambient pollution levels at the time of our controlled exposures because the subjects lived in a low-industry area with relatively low ambient pollution. Subjects were excluded if they had cold or flu-like symptoms during the previous 6 weeks.

Resting 18O₂ exposure study #1

Eight male subjects were enrolled in the first study; they ranged in age from 21–32 years and in weight from 70–103 kg (Table 1). They were exposed on two separate occasions separated by at least 2 weeks. Ozone exposures reported here were performed during September to early December; they were compared to exercising subjects in a published study which were exposed in the same laboratory during July and August three years earlier. Subjects were asked to avoid exposure to environmental tobacco smoke or to other irritating substances such as paint fumes, and to avoid taking vitamin C or E supplements or NSAIDS for at least 48 hours prior to each exposure. Exposures took place during the morning and subjects ate no food after midnight the day prior to their exposure. Subjects breathed 18O₃ through a face mask (to conserve 18O₂) while resting in a seated position. No attempt was made to control or target the resting level of breathing in the subjects. As shown in Figure 1, subjects breathed into a silicone face mask that had been modified by blocking the air intake filtration ports and installing PTFE tubing to the front of the mask. Flow rates of breathing air were measured by a pneumotachograph (Hans Rudolph, Kansas City, MO, model 4700) that transmitted the signal via a preamplifier to a computer. A rapid response O₃ analyzer (Monitor Labs model 8410 chemiluminescent

Figure 1. Schematic of face mask exposure of human subjects to 18O₂ with measurement of breathing airflow and O₃ uptake. Notes: Subjects breathed into a modified silicone face mask. Inspired and expired flow rates and times as well as breath-by-breath O₃ removal from breathing air were measured for brief intervals during a 2 hour exposure to 18O₂ at a concentration of 0.4 ppm. 18O₂ concentration was maintained manually by adjusting the flow rate of an 18O₂-argon mixture through a silent arc O₃ generator (rate was 3–10 mL/min). Cylinder air maintained at 50% relative humidity flowed past the face mask at a rate of 60 L/min.
O₃ analyzer, flow rate 300 mL/min) measured inspired and expired O₃ concentrations during each breath at randomly selected sampling times during the exposure. The air supply for the face mask came from a compressed cylinder that was humidified to 50% before flowing past the face mask at a rate of 60 L/min. A 60 L Teflon pressure relief bag equalized the air pressures during the breathing cycle. ¹⁸O₃ was generated as previously described⁶ by passing a mixture of 1% ¹⁸O₂ (99% purity, Isotec, Miamisburg, OH) in argon through a small electric arc O₃ generator taken from a NO/NO₂ air monitor (Bendix, Lewisburg, WV, modified to 3–10 mL/min flow rate). The efficiency of conversion of O₂ to O₃ in this system was 2%–4%. Oxygen-18 labeled ozone concentration in the breathing air was monitored by a slow response (Dasibi) monitor (flow rate 500 mL/min) and maintained to 0.4 ppm ± 2.0% by manually controlling the flow of the ¹⁸O₂/argon mixture through the arc generator. We showed previously that the small enrichment in ¹⁸O₂ in breathing air, which occurs due to inefficient ¹⁸O₃ generation from ¹⁸O, results in an insignificant enrichment of ¹⁸O in the tissues.

**Nasal, bronchial, and bronchoalveolar lavage fluid collection**

Nasal lavage (NL) was performed within one hour post exposure; five consecutive 0.2 mL sprays of sterile saline were injected into each nostril, then expelled into a small cup. This procedure was repeated 7 times, making the total saline instilled equal to 14 mL.

The bronchial lavage (BL) procedure consisted of one 20 mL instillation which was withdrawn prior to the BALF collection from the same lobe and consisted of 4 subsequent washes of 50 mL volume. The bronchial lavage followed by BALF collection was done on the middle lobe and was then repeated in the lingula. Thus, the total instilled saline for BL was 40 mL and the total for BALF was 400 mL. Clinical details of the BAL procedure have been previously described.⁹

**Preparation of lavage fluids and blood for analysis**

The first two aliquots of BALF were combined, and they along with the BL and NL fluid were centrifuged at 400 g for 10 minutes to pellet the cells. BALF surfactant fraction was obtained by centrifuging the cell-free supernatant of the combined lavage fluids at 27,000×g for 30 min (4°C). BALF, BL and NL supernatants were brought to 3% perchloric acid (PCA) by adding 60% acid. All PCA samples were centrifuged at 20,000 g for 20 minutes at 5°C to pellet the protein. The PCA pellets were re-suspended in 0.25 N NaOH and analyzed for protein using the Coomassie blue binding method⁸ and using bovine serum albumin as a standard. Cells from all BALF washes were combined and resuspended in RPMI and then counted. One million cells were then pelleted and suspended into 0.3 mL of 3% PCA.

Samples for ¹⁸O determination were lyophilized and individual analyses containing 0.3–0.8 mg of protein were weighed into silver cups for oxygen-18 analysis.

Venous blood was drawn from subjects prior to exposure and within an hour after exposure. A one mL sample of the heparinized blood was centrifuged to separate the red cells from the plasma and lyophilized. Oxygen-18 labeled ozone determination was made on both the plasma and the red cell fractions of the dried blood.

**Analysis for BALF cytokines, LDH, and BALF cell phagocytosis**

Methods have been published previously¹¹ for most of the cytokines, LDH, and BALF cell phagocytosis assays. ELISA techniques were used for assay of elastase,⁹ interleukin-8 (kit from R & D, Minneapolis, MN) and tissue plasminogen activator (tPA, Enzyme Research Labs, South Bend, IN).

**¹⁸O analysis of blood and lavaged constituents**

Isotope ratio mass spectrometry was used to measure amounts of excess ¹⁸O in lyophilized samples of lavage fluids and in the red blood cell and plasma fraction of the dried venous blood, per published methods.⁶ Natural abundance ¹⁸O values from the air-exposed subjects were subtracted from the values measured in the ¹⁸O₃ exposed subjects to obtain the excess ¹⁸O due to the ¹⁸O₃ exposure. Hereafter, we will dispense with the distinction of “excess ¹⁸O” and simply refer to it as ¹⁸O.” We have shown in previous studies that the lyophilization procedure traps the portion of ¹⁸O₃ reaction products that form adducts with tissue molecules.
Antioxidant analyses
Supernatants originating from PCA homogenizations were assayed by HPLC-EC for uric acid and ascorbic acid, per previously published methods. Total glutathione (GSx, consisting of the sum of GSH and GSSG) was analyzed by enzymatic recycling. BALF cells and supernatants were also analyzed for alpha tocopherol concentrations according to a published method.

Resting ozone exposure: study #2, physiology
Subjects were exposed in whole-body inhalation chambers according to methods outlined previously, but instead of exercising, they were exposed while resting in a seated position. They breathed nasally as they normally would under resting conditions. The sequence of exposures was randomized and neither volunteers nor investigators were informed of the exposure; each individual experienced only one exposure, whether to air or to a given concentration of O₃. FEV₁ was measured three times in all subjects as follows: (1) prior to exposure, (2) at the intermediate time of 1 hour, and (3) at the end of the 2-hour of exposure of the same subject. Each FEV₁ measurement was done in triplicate with the largest value of the three measurements reported for that subject. The baseline FEV₁ values measured pre-exposure to air or O₃ averaged 4.48 ± 0.082 L (N = 68). FEV₁ percent change was determined using the following formula: [(pre-O₃ – post-O₃)/pre-O₃] × 100.

Statistics
Two-tailed pairwise comparisons were made of data from air versus O₃ exposures with P ≤ 0.05 assigned significance. Comparisons of the resting data with previously published exercising O₃ exposure data are exploratory, with no corrections made for multiple comparisons. The FEV₁ study employed three methods: (1) linear regression of the FEV₁ changes against the O₃ exposure concentration to determine whether the slope of the regression line differed from zero, (2) ANOVA followed by Dunnett’s test, and (3) Williams test for non-parametric data.

Results
Breathing and ozone uptake measurements
Breathing frequency, tidal volume, and percentage of ¹⁸O uptake from breathing air during exposure of the resting subjects to air or 0.4 ppm ¹⁸O₃, is shown in Table 2. Oxygen-18 labeled ozone exposure (compared to air) produced no significant change in the Ve or inspired or expired airflow measurements. The percentage uptake of ¹⁸O from breathing air was 79.9%; it was in close agreement among the 5 subjects examined. We compared these measurements to our previous study of exercising subjects (see Table 3). The Ve of our resting subjects was lower (8.3 L/min) than the resting Ve of subjects we reported from our earlier intermittent exercise regimen (13.5 L/min) in which subjects alternated 15 minute periods of rest and exercise. Comparing the volume of air breathed during the 2 hour exposure to 0.4 ppm ¹⁸O₃ in the earlier intermittent exercise study with the volume of air breathed in the present study showed a 4.7-fold higher volume with exercise than with resting exposure (Table 3).

Excess ¹⁸O in BALF supernatants and cells
The ¹⁸O accumulated by BALF cells, BALF supernatant, and NL of resting subjects exposed to ¹⁸O₃

| Breath | Inspired breaths | Expired breaths | Total breath | O₂ uptake, % |
|--------|-----------------|-----------------|--------------|--------------|
|        | Volume, L       | Time, sec       | time, sec    | L/min        |
| Air    | 0.59            | 1.6             | 2.8          | 4.4          |
| SE     | 0.13            | 0.1             | 0.3          | 0.3          |
| N      | 8               | 8               | 8            | 8            |
| O₃     | 0.66            | 1.7             | 3.0          | 4.6          |
| Mean   | 0.15            | 0.2             | 0.5          | 0.5          |
| SE     | 5               | 5               | 5            | 5            |
| N      | 5               | 5               | 5            | 5            |

Table 2. Breathing measurements and percentage O₂ uptake in 8 resting subjects exposed by face mask to ¹⁸O₃.
Table 3. Comparison of ventilation and air volumes breathed per exposure in resting and exercising subjects exposed to 0.4 ppm $^{18}$O$_3$ for 2 hr.

| Body weight, kg | N | Tidal Vol., L | Freq, breaths/min | Minute ventilation, L/min | Mean total air breathed, L/exposure | Reference |
|----------------|---|--------------|--------------------|--------------------------|------------------------------------|-----------|
| Resting exposure (120 min) | 87.9 ± 3.4 | 8 | 0.59 ± 0.13 | 13.7 ± 0.9 | 8.3 ± 0.4 | 998 | Present study |
| Resting periods (60 min total) | 76.2 ± 2.5 | 8 | | | 13.5 ± 0.1 | 810 | Hatch et al, 1994 |
| Exercising periods (60 min total) | | | | | 64.6 ± 3.2 | 3876 |
| Total | | | | | | 4676 |
| Ratio: exercise/resting | | | | | | 4.7 |

Note: Values are mean ± S.E.

is shown in Table 4. Results reported previously for subjects exposed identically but with concurrent intermittent exercise are included for comparison. The concentration of $^{18}$O in BALF cells was 5.1-fold greater with exercising exposure than with resting exposure. The BALF extracellular fraction showed concentrations 2-fold higher than following resting exposure. The dried material of NL fluid was about twice as concentrated after resting exposure as it was after exercising exposure, suggesting that mouth breathing during exercise drew exposure away from the nose. The variability of the $^{18}$O data appeared to be about the same for resting as for exercising exposures. For comparison to rats, Table 4 also shows the previously reported $^{18}$O accumulated in BALF cells and fluids of F344 rats exposed while at rest to the same 0.4 ppm 2 hour exposure regimen. BALF cells from resting rats and resting humans accumulated about the same concentration of $^{18}$O, while the BALF surfactant of the rats incorporated less than half the concentration found in the resting humans. Blood plasma and pelleted red blood cells did not show a detectable $^{18}$O increase due to $^{18}$O$_3$ exposure, similar to results observed previously in exercising subjects (data not shown).

### Table 4. $^{18}$O concentration in bronchoalveolar lavage and nasal lavage of human subjects exposed for 2 hours to 0.4 ppm $^{18}$O$_3$: intermittent exercise versus rest during exposure.

| Excess oxygen-18, ug/g dry weight | Bronchoalveolar lavage | Nasal lavage |
|----------------------------------|------------------------|--------------|
|                                  | Cell pellet | Surfactant | Surfactant | Surfactant |
| Resting                          | 5.6 ± 1.7 (6) | 26.4 ± 2.4 (3) | 377 ± 62 (5) |
| Exercise (1)                     | 28.4 ± 5.5 (8) | 51.6 ± 7.9 (8) | 192 ± 58 (8) |
| Exercise/ resting                | 5.07 | 1.95 | 0.51 |
| Resting F344 rat (1)             | 7.5 ± 1.6 (6) | 10.9 ± 1.4 (8) | NM |

Notes: Shaded values are newly reported here. (1) From Hatch et al, 1994. All enrichments in $^{18}$O are significantly elevated above baseline. Means ± standard error are given for (N) subjects or rats. Abbreviation: NM, not measured.

### BALF fluid changes in cellular and biochemical markers

We measured a slight but significant 19% decrease in total cells recovered in BALF fluid, as well as a slight increase (0.9% to 1.3%) in PMNs recovered in resting subjects exposed to $^{18}$O$_3$ (Table 5). None of the other cellular changes were significant. Data from seven different cytokines and other biochemical indicators in BALF supernatant indicated no significant change due to $^{18}$O$_3$ exposure (Table 1 supplementary). Table 2 (supplementary) shows that BALF supernatant protein as well as ascorbate, urate, and total glutathione (GSx) were not significantly altered by the resting $^{18}$O$_3$ exposure.

Table 6 shows that serum-opsonized Candida albicans was engulfed by ~20% fewer phagocytes in BALF from 0.4 ppm $^{18}$O$_3$ exposed resting subjects. This effect was not observed for other types of opsonization due to greater variability of responses. Phagocytosis expressed as Candida particles per cell was not affected by resting exposure to $^{18}$O$_3$. 

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Table 5. BAL cell numbers and differential following resting exposure of 8 human subjects to O₃.

|          | BAL vol recovered, ml | Total cells x 10⁶ | % cells |
|----------|-----------------------|--------------------|---------|
|          |                       |                    | Macs    | PMNs   | Lymphos | Monos | Epith | Eos  |
| Air      | Mean                  | 229.9              | 43.6    | 87.3%  | 0.9%    | 8.9%   | 1.5%  | 0.3%  | 0.3%  |
|          | SE                    | 10.9               | 6.0     | 2.9%   | 0.3%    | 2.8%   | 0.4%  | 0.1%  | 0.1%  |
| O₃       | Mean                  | 240.3              | 35.4    | 90.8%  | 1.3%    | 6.6%   | 1.2%  | 0.4%  | 0.1%  |
|          | SE                    | 9.3                | 5.8     | 1.8%   | 0.2%    | 1.7%   | 0.5%  | 0.2%  | 0.1%  |
| O₃/air   | P value,              | 1.05               | 0.81    | 1.04   | 1.52    | 0.75   | 0.76  | 1.32  | 0.46  |
|          | Note: *P = 0.04 by 2-tail t-test of means. |

FEV₁ changes in resting subjects exposed to O₃

A scatter plot of FEV₁ changes observed in each individual subject exposed to air or to 4 different concentrations of O₃ is shown in Figure 2. FEV₁ was assessed at an interim 1 hour point, at the completion of the 2 hour resting inhalation of air, and at four different concentrations of O₃. A linear regression of all data for each time period was performed and the slopes of the regression tested for significance against a zero slope. A slope of approximately –6.5% per ppm O₃ was observed for both measurement at 1 and 2 hours. Linear regression indicated that the slope was not significantly different from zero at one hour and marginally significant after 2 hours of O₃ exposure (P = 0.053). This significant result was dependent on inclusion of an outlier (judged by Grubb’s test) at 0.25 ppm. Further experiments could possibly unmask effects observed at 0.4 ppm exposure where the subjects appeared to separate into two groups: responders and non-responders. Figure 3 shows a comparison of the resting data obtained against our earlier published study involving exercising subjects (intermittent 15 minutes on and 15 minutes off to Ve ~ 65 L/minute maximum).¹⁷ Non-parametric Williams test reported previously on the exercising study indicated that an O₃ concentration of 0.12 ppm represented the lowest dose that was significantly different from control. Application of the same test to the resting O₃ exposure yielded no significant effect for any O₃ concentration. Tests at individual exposure concentrations indicated borderline significance (0.3 ppm, p = 0.049) but only if not corrected for multiple comparisons. Similarly, ANOVA followed by Dunnett’s test correcting for multiple comparisons yielded no significance for all O₃ concentrations in resting subjects.

Comparison of O₃ effect markers: resting versus exercising

A side-by-side comparison summary of O₃ effects observed during resting versus exercising exposures is presented in Table 7. The change in the mean values observed for O₃ exposure compared to air exposure is represented as either an O₃ minus Air value or an O₃ to Air ratio if that was more appropriate. The FEV₁ percent change and the fold increase in neutrophils was about 5-fold greater during exercise than during resting O₃ exposure. The mean decrement in BALF cell recovery appeared to be similar following exercising and resting exposures. BALF protein was increased 2 fold with exercising exposure to O₃ and was unchanged with resting exposure to O₃.
Figure 2. The percentage change in FEV$_1$ in individual resting subjects exposed to four concentrations of O$_3$ and to air plotted against the O$_3$ inhaled concentration. **Notes:** FEV$_1$ was measured pre-exposure, and after 1 and 2 hours of exposure in the same subjects. The regression trend lines had a similar slope. The 2 hour O$_3$ exposure line appeared to have a slope be significantly different from zero slope ($P = 0.053$).

**Discussion**
The goal of this study was to measure O$_3$ dose and effect in such a way that would improve the basis for extrapolating O$_3$ dose and effect between exercise levels in humans, as well as between rats and humans. New data presented here include: (1) fractional removal of O$_3$ from breathing air, (2) $^{18}$O$_3$ dose measurements made in nasal lavage fluid and BALF, (3) O$_3$ induced cellular and biochemical effects measurements in the same BALF, and (4) pulmonary function (FEV$_1$) measurements made in a parallel group of resting subjects. These data provide a basis for extrapolating alveolar O$_3$ dose between resting and exercising humans, and between resting rats and resting humans.

Figure 3. Comparison of O$_3$-induced FEV$_1$ changes (mean ± S.E.) observed after 2 hours of exposure to various concentrations of O$_3$ while at rest or while exercising intermittently (15 minute intervals) at a level of 65 L/min Ve. **Note:** Asterisks indicate the lowest concentration of O$_3$ exposure at which a significant change from air exposed occurred (by Williams test for non-parametric data).
Table 7. Comparison between resting and exercising effects and dose 1–2 hours following 0.4 ppm, 2 hr \( \text{O}_3 \) exposure.

| Measurement | Resting exposure | Exercising exposure | Exercise/rest | Reference for exercising exposure |
|-------------|------------------|---------------------|--------------|-----------------------------------|
| \( \text{O}_3 \) minus air | | | | |
| FEV, \% decrement | \( \sim 2 \) | 10.3* | 5.0 | 1 |
| BALF cell \( ^{18}\text{O} \) | 5.6* | 28.4* | 5.1 | 1 |
| BAL surfactant \( ^{18}\text{O} \) | 26.4* | 51.6* | 2.0 | 1 |
| Nasal lavage \( ^{18}\text{O} \) | 377* | 192* | 0.5 | 1 |
| \( \text{O}_3 \)/air ratio | | | | |
| BALF cells recovered, \% decrement | 19* | 28* | 1.5 | 1 |
| BALF neutrophils, fold increase | 1.52* | 7.6* | 5.0 | 1, 2 |
| BALF protein, fold increase | 0.99 | 1.9* | 2.0 | 1, 2 |
| BALF cell phagocytosis (serum opsonized), \% decrement | 20* | 23 | 1.2 | 2 |
| BALF cell phagocytosis (IgG opsonized), \% decrement | 1 | 26* | 26 | 2 |
| BALF cell phagocytosis (unopsonized), \% decrement | 24 | 45* | 1.9 | 2 |
| BALF IL6, fold increase | 1.1 | 7.3* | 6.9 | 2 |
| BALF LDH, fold increase | 1.2 | 1.5* | 1.3 | 2 |
| BALF a1-AT, fold increase | 1.1 | 1.7* | 1.5 | 2 |
| BALF C3a fold increase | 2.3 | 1.4 | 0.6 | 2 |

Notes: *Significant effect of \( \text{O}_3 \) compared to respective air exposed. 1Hatch et al, 1994; 2Devlin et al, 1996.

Fractional removal of ozone from breathing air

Past studies have examined the fractional removal of \( \text{O}_3 \) from breathing air to arrive at estimates of \( \text{O}_3 \) dose to the respiratory tract. Exercising subjects have been reported to remove a smaller fraction of \( \text{O}_3 \) from the breathing air than resting subjects;\(^18\) however, when that fraction is multiplied by the increased volume of air breathed during exercise, the \( \text{O}_3 \) retained in the lung is definitely increased by exercise.\(^{19}\) Our result of \( \sim 79\% \) of removal of \( \text{O}_3 \) from breathing air agrees with the 73%–76% removal measured at the face of subjects breathing at rest in a previous study.\(^{20}\) The fractional uptake of \( \text{O}_3 \) from breathing air by the whole body, or by the nasal or thoracic regions, has been measured previously either by a facial exposure similar to ours or by placement of catheters into the posterior pharynx. Our result is lower than the \( \sim 88\% \) uptake measured by integration of breath \( \text{O}_3 \) concentrations at the posterior pharynx, which was reported in previous studies\(^{21,22}\) that cited as possible reasons for their higher percentage uptake a larger tidal volume in their subjects compared to the Wiester study. Our resting subjects had a tidal volume (0.59 L) similar to that reported by Wiester et al (0.63–0.64 L) and lower than that reported by Gerrity et al (0.75–0.83 L).

\( ^{18}\text{O}_3 \) dose measurements in lavaged fluids

We have demonstrated here that human subjects exposed while at rest to \( ^{18}\text{O}_3 \) accumulate \( ^{18}\text{O} \) in BALF cells and surfactant material in lower concentrations than exercising subjects. The \( ^{18}\text{O} \) label that remains in the tissue after lyophilization appears to be the result of oxygen addition reactions of \( ^{18}\text{O}_3 \) with biomolecules. Accumulation of \( ^{18}\text{O} \) in BALF cells and surfactant material suggests that \( ^{18}\text{O}_3 \) penetrates into the alveolar region of the lung during resting exposure. BALF cells and surfactant reside at the air-liquid interface and appear (from histological evidence) to have an alveolar origin.\(^{23}\) The fact that lung parenchyma following lavage contains very little \( ^{18}\text{O} \) following \( ^{18}\text{O}_3 \) exposure, as seen in a study involving rhesus monkeys,\(^4\) suggests that the reaction of \( ^{18}\text{O}_3 \) is concentrated at the air-liquid interface. This finding is in agreement with physicochemical modeling predictions that suggest that \( \text{O}_3 \), because of its high chemical reactivity, does not penetrate far into the surface fluid or epithelial cells.\(^{24}\) We have not yet been able to detect excess \( ^{18}\text{O} \) in human blood following
either resting or exercising exposure. This inability is probably due to the difficulty in detecting the label after such a large dilution into the large systemic volume of blood.

**Ozone-induced cellular and biochemical effects measurements**
Our ability to measure both O3 dose and effect in the same BALF cells and fluids makes it possible to determine the relationship between dose and effect in the same subject. Results suggest that the sensitivity for detecting excess 18O is greater than the sensitivity for detecting many of the biological effects in BALF at early post exposure times. Our present finding that resting exposure to O3 produced few statistically significant biological effects in BALF highlights the low-dose nature of alveolar O3 exposure, even at the relatively high inhaled O3 concentration of 0.4 ppm. We detected small but significant decreases in BALF cell recovery and neutrophil counts following resting exposure (Table 5). Other indicators previously measured in BALF during exercise were not detectable here after resting exposure. Many of the O3 effect markers examined here immediately post exposure would have been greater if measured 16–24 hours post exposure.11 In agreement with our lack of cellular effects following resting O3 exposure, a previous report showed a lack of effect of resting O3 exposure on BALF cell DNA single strand breaks, as opposed to a positive effect if O3 exposure occurred during exercise.25

Airway antioxidants participate in the reactions of inhaled O3, and measurement of changes in antioxidants can provide insight on where O3 reacts. Our results showed only a non-significant lowering of NL ascorbate by O3 at zero hour post O3 exposure (Table 2, supplementary). Two published studies measured antioxidants under a less vigorous exercise regimen targeting 20 L/min per m2 body surface area rather than the present 35 L/min per m2 with exposure to 0.2 ppm of O3 for 2 hours. The first found significant increases in dehydroascorbic acid in bronchial lavage and BALF six hours post exposure.26 The second found insignificant changes in NL fluid antioxidants at zero and six hours post exposure and 26%–100% elevations in BALF and BL concentrations of GSx, ascorbate, and uric acid at six hours post exposure.27 Thus, although previous studies do not exactly match our exposure scenario, they do confirm the difference in response during rest and exercise.

**Ozone induced pulmonary function (FEV1) changes**
Our regression of FEV1 changes versus four resting concentrations of O3 up to 0.4 ppm showed a slope that appears to be different from zero (Fig. 2). Previously published reports that looked at FEV1 changes immediately following resting 2 hour O3 exposures and which found no significant decrements at O3 concentrations lower than 0.5 ppm were probably due to the smaller number of subjects examined.28,29 In addition to a lower delivered O3 dose in resting exposures, the inability to detect significant alterations in FEV1 may be due to higher variability of response incident to a less targeted control of breathing during rest than is possible during exercising exposures.

**Extrapolation between exercise levels in humans**
We found that the fold change in BALF cell 18O3 reaction product concentration roughly correlates with the average Ve between different exercise levels; this lends support to the use of Ve as a factor in extrapolating pulmonary dose of O3 between different levels of physical activity. ‘Effective dose’ was first defined as the product of concentration, Ve, and exposure time by Silverman et al30 and has often been used as a default assumption since. A recent meta-analysis of 23 published human exposure studies showed strong associations between total BALF protein and neutrophilia responses, and O3 dose defined as the product of exposure concentration, ventilation, and time.31

The increase in Ve which accompanies exercise is due to increases in both breathing frequency and tidal volume, and it would therefore be valuable to define the relative contribution of each. Our earlier 18O3 exposure study6 did not measure tidal volume or frequency; however, a study which did measure these parameters under similar conditions suggests that the exercise periods saw a 3.8-fold increase in tidal volume and a 2.3-fold increase in breathing frequency.17 This study also employed male subjects of similar age (22.5 ± 3.1 year), weight (76.2 ± 7.5 kg), and Ve (66.2 ± 7.6 L/min) as our previous study (see Table 3). They reported tidal
volumes of 2.2 L and breathing frequencies averaging 31 breaths/minute during the 15 minute intermittent exercising periods.

The \( ^{18}\text{O} \) dose is a measurement closer to the pulmonary target site for \( \text{O}_3 \) than previous estimates of \( \text{O}_3 \) dose, which were obtained by measurement of removal of the gas from breathing air as it passed through the nasal or thoracic regions.\(^{21,22} \) Our resting and exercising \( ^{18}\text{O} \) dose measurements of BALF cells can be used to create a two-point regression line from which to make a crude extrapolation to higher exercise levels. Although human controlled exposure studies to date have had a reasonable level of activity for normal people, they do not reach the \( \text{Ve} \) levels or the duration that might be experienced by the sizable population that now participates in marathons and other high \( \text{Ve} \) activities. It would not be uncommon for people participating in such activities to achieve a 4-fold higher average \( \text{Ve} \) (to 120 L/min) for a 2-fold longer time (4 hour) than has yet been investigated in human clinical studies. There is a need for further research at low \( \text{O}_3 \) concentrations during continuous high exercise levels. There is also a need for a further expansion of the sample size and time points measured post exposure.

**Extrapolation between rats and humans**

We report here and in our previous study\(^6 \) that a direct comparison between rat and human alveolar \( \text{O}_3 \) dose can be achieved by comparing the \( ^{18}\text{O} \) content in BALF cells obtained from humans and rats similarly exposed to \( ^{18}\text{O}_3 \). In our previous study, rats had to be exposed to 2.0 ppm \( ^{18}\text{O}_3 \) in order to achieve a BALF cell dose similar to exercising humans exposed to 0.4 ppm. It is apparent from the present study that the exercise level of the human subjects accounted for their higher BALF \( ^{18}\text{O} \) dose. The finding that human resting BALF \( ^{18}\text{O} \) dose approximates that of the resting rat BALF \( ^{18}\text{O} \) dose is unexpected because rats are known to have a higher ratio of body surface area/body volume and breathe more air; they should therefore experience a higher \( \text{O}_3 \) dose than humans. Allometric relationships predict that a resting rat lung would be exposed to 2.8 times the volume of inhaled air per wet lung weight than a resting human lung (see Appendix 2). We offered previously as an explanation for lower than expected dose to the rat lung the fact that rats are nocturnal and are therefore exposed during their dormant period (our daytime). Other reasons might include the following: (1) an approximately 8-fold higher BALF ascorbate concentration in rats compared to humans,\(^{32} \) as ascorbate appears to quench \( \text{O}_3 \) reactions in the lung and therefore serves as a shield to BALF cells, causing them to retain less \( ^{18}\text{O}_3 \);\(^7,8 \) (2) the ability of rats to lower body temperature and \( \text{Ve} \) during \( \text{O}_3 \) exposure;\(^13 \) (3) a higher nasopharyngeal removal of \( \text{O}_3 \) in rats;\(^34 \) and (4) a lower whole-body percent retention of \( \text{O}_3 \) from breathing air in rats.\(^21,35,36 \) A complete discussion of these differences is beyond the scope of the present paper; however, it is apparent that moderate exercise in humans is able to increase alveolar \( \text{O}_3 \) dose to levels much higher than that seen in similarly exposed resting rats. We therefore confirm with quantitative evidence the important contribution of physical exercise to the alveolar dose of \( \text{O}_3 \), and suggest a similar effect of exercise on the alveolar dose of other chemically reactive gases with properties similar to \( \text{O}_3 \).

**Conclusion**

Results confirm that exercise contributes greatly to both the dose and effect of \( \text{O}_3 \) measured by indicators in BALF. Quantification of \( ^{18}\text{O} \) reaction products in BALF cells has provided a basis for extrapolation of acute \( \text{O}_3 \) dose between resting and exercising exposures. The comparison between resting and exercising \( \text{O}_3 \) effects, along with the dose measurements in each type of exposure provide an improved understanding of low-dose \( \text{O}_3 \) effects. Results confirm the use of \( \text{Ve} \) as a factor in the extrapolation of inhaled dose of \( \text{O}_3 \) at different levels of physical activity, and suggest that higher and more continuous activity levels will yield significant effects at even lower ambient levels of \( \text{O}_3 \). The similarity of alveolar \( \text{O}_3 \) dose and effect between resting human and resting rats strengthens the extrapolation of rat inhalation data to humans.

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Author Contributions
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Competing Interests
All of the authors were employed by the United States Environmental Protection Agency at the time of the completion of the study (some are now retired). There are no conflicts of interest.

Disclaimer
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Disclosures and Ethics
As a requirement of publication the authors have provided signed confirmation of their compliance with ethical and legal obligations including but not limited to compliance with ICMJE authorship and competing interests guidelines, that the article is neither under consideration for publication nor published elsewhere, of their compliance with legal and ethical guidelines concerning human and animal research participants (if applicable), and that permission has been obtained for reproduction of any copyrighted material. This article was subject to blind, independent, expert peer review. The reviewers reported no competing interests.

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Supplementary Data
Appendix 1
The approximate Ve of marathon runners was reported by Mahler\textsuperscript{37} to be 86.2% of maximal ventilatory volume (MVV). MVV was reported to be 180 and 176 L/min in trained runners and control subjects, respectively. Thus, the Ve of trained marathon runners can be estimated as 0.86*180 = 155 L/min. Since our earlier human clinical study\textsuperscript{6} employed alternating 15 minute periods of rest (Ve = 13.5 L/min) during half of the 2 hour exposure time, the average Ve for the two hours of O\textsubscript{3} exposure would have been 39 L/min (see Table 3). It appears that the exercise induced Ve of marathon runners could attain the level of 3.97 (155/39) times higher than our earlier ‘exercising’ human subjects and sustain that level for over twice the time. Less trained runners would experience a lower exposure level because they do not sustain the high Ve possible in the trained athletes; however, in a race event they would run for a longer time. The main difference between trained and untrained runners appears to be that the trained runners are able to sustain a Ve/MVV ratio that is 24% higher than untrained runners. They also consume oxygen at a 55% higher rate and for a longer time.\textsuperscript{37}

Appendix 2
The relationship between body weight and Ve across species has been reported as 379 M\textsuperscript{0.8}, where M = body weight in kg and Ve is in milliliters.\textsuperscript{38} The same author reports that the wet lung weight in grams varies by the relationship 11.3 M\textsuperscript{0.99}. Substituting values for a 0.3 kg rat and a 70 kg human yields the following: (11.342 mL/min)/758 g = 15.0 mL/min/g for human and 145/3.43 = 42.3 for the rat. Thus, a resting rat would be predicted to have an exposure 2.82-times higher than a resting human (42.3/15 = 2.82).

Table S1. Bronchoalveolar lavage fluid cytokines and enzymes following resting exposure to \textsuperscript{18}O\textsubscript{3}.

|          | IL-6, pg/mL | IL-8, pg/mL | tPA, IU/mL | Elastase, uM/hr | C3a, ng/mL | a1-AT, ug/mL | LDH, U/mL |
|----------|-------------|-------------|------------|-----------------|------------|--------------|-----------|
| Air      | 2.6         | 14.0        | 112        | 46.4            | 189        | 1.80         | 3.79      |
| SE       | 0.2         | 0.7         | 20         | 19.7            | 111        | 0.30         | 0.24      |
| O\textsubscript{3} | Mean      | 2.7         | 26.5       | 104             | 81.7       | 434          | 2.0       |
| SE       | 0.3         | 12.5        | 13         | 45.3            | 171        | 0.35         | 0.56      |
| O\textsubscript{3}/air | 1.06       | 1.90        | 0.93       | 1.76            | 2.30       | 1.10         | 1.17      |

Notes: No significant changes due to ozone exposure were detected in any of the measurements (2 tailed paired t test). N = 7 subjects in all groups.
## Table S2. Protein and antioxidant changes in lavage fluids following resting exposure to $^{18}$O$_3$.

|                      | Protein, ug/mL | Ascorbate, uM | Urate, uM | GSH, uM | Alpha tocopherol, nM |
|----------------------|----------------|---------------|-----------|---------|----------------------|
| **Nasal lavage fluid** |                |               |           |         |                      |
| Air                  |                |               |           |         |                      |
| Mean                 | 686            | 6.00          | 59.2      | 3.50    | NM                   |
| SE                   | 163            | 2.54          | 7.6       | 1.56    |                      |
| $O_3$                |                |               |           |         |                      |
| Mean                 | 727            | 3.82          | 46.9      | 3.75    | NM                   |
| SE                   | 244            | 1.38          | 8.3       | 0.84    |                      |
| $O_3$/air            | 1.06           | 0.64          | 0.79      | 1.07    |                      |
| **Bronchial lavage fluid** |                |               |           |         |                      |
| Air                  |                |               |           |         |                      |
| Mean                 | 36.6           | 0.35          | 0.35      | 0.48    | NM                   |
| SE                   | 3.4            | 0.05          | 0.04      | 0.04    |                      |
| $O_3$                |                |               |           |         |                      |
| Mean                 | 41.7           | 0.46          | 0.77      | 0.66    | NM                   |
| SE                   | 4.6            | 0.12          | 0.33      | 0.12    |                      |
| $O_3$/air            | 1.14           | 1.31          | 2.17      | 1.37    |                      |
| **Bronchoalveolar lavage fluid** |            |               |           |         |                      |
| Air                  |                |               |           |         |                      |
| Mean                 | 117            | 0.46          | 1.16      | 0.70    | 5.1                  |
| SE                   | 13             | 0.06          | 0.17      | 0.10    | 2.5                  |
| $O_3$                |                |               |           |         |                      |
| Mean                 | 116            | 0.54          | 1.23      | 0.74    | 2.8                  |
| SE                   | 16             | 0.06          | 0.14      | 0.09    | 1.2                  |
| $O_3$/air ratio      | 0.99           | 1.16          | 1.07      | 1.06    | 0.55                 |

**Abbreviation:** NM, not measured.