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ORIGINAL RESEARCH

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 (O_3) dose and toxic effect between exercise levels in humans, and between humans and rats. Resting human subjects were exposed to labeled O_3 ($^{18}O_3$, 0.4 ppm, for 2 hours) and alveolar O_3 dose measured as the concentration of excess ^{18}O in cells and extracellular material of nasal, bronchial, and bronchoalveolar lavage fluid (BALF). We related O_3 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 (FEV_1) changes following O_3. Subjects exposed while resting had ^{18}O concentrations in BALF cells that were 1/5th of those of exercising subjects and directly proportional to the amount of O_3 breathed during exposure. Quantitative measures of alveolar O_3 dose and toxicity that were observed previously in exercising subjects were greatly reduced or non-observable in O_3 exposed resting subjects. Resting rats and resting humans were found to have a similar alveolar O_3 dose.

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

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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.[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 O_3 in the two studies reported here.

	Age, yr	Height, cm	Weight, kg
$^{18}O_3$ lavage study, 8 subjects			
Mean	26.4	183	87.9
SE	1.2	2	3.4
Physiology study, 68 subjects			
Mean	25.2	181	81.7
SE	0.4	1	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 $^{18}O_3$ 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 $^{18}O_3$ through a face mask (to conserve $^{18}O_2$) 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 O_3 analyzer (Monitor Labs model 8410 chemiluminescent

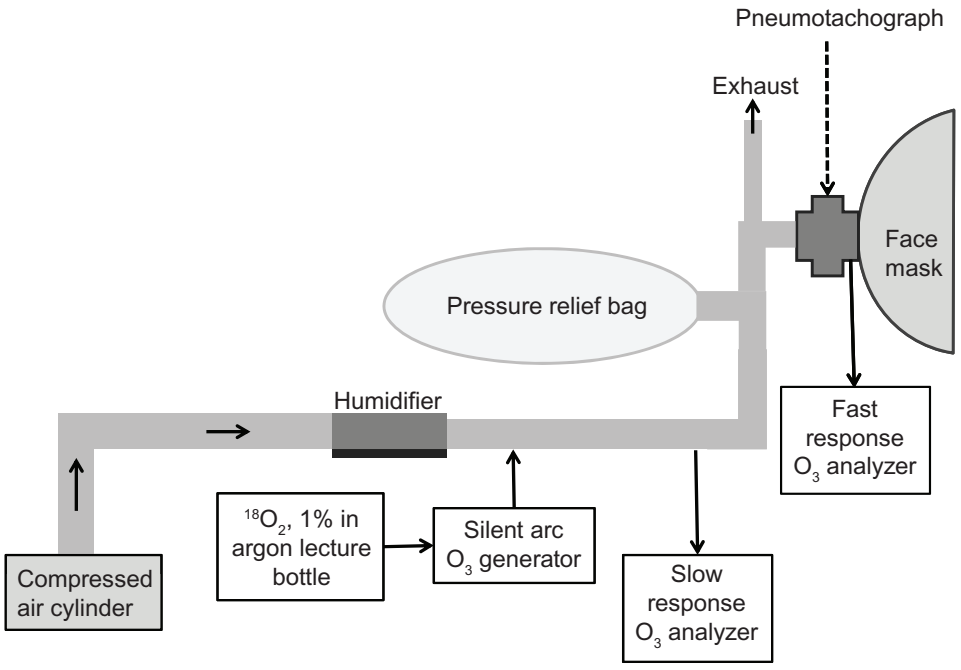

Figure 1. Schematic of face mask exposure of human subjects to $^{18}O_3$ with measurement of breathing airflow and O_3 uptake.
Notes: Subjects breathed into a modified silicone face mask. Inspired and expired flow rates and times as well as breath-by-breath O_3 removal from breathing air were measured for brief intervals during a 2 hour exposure to $^{18}O_3$ at a concentration of 0.4 ppm. $^{18}O_3$ concentration was maintained manually by adjusting the flow rate of an $^{18}O_2$-argon mixture through a silent arc O_3 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.

O_3 analyzer, flow rate 300 mL/min) measured inspired and expired O_3 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. $^{18}O_3$ was generated as previously described[8] by passing a mixture of 1% $^{18}O_2$ (99% purity, Isotec, Miamisburg, OH) in argon through a small electric arc O_3 generator taken from a NO/NO_2 air monitor (Bendix, Lewisburg, WV, modified to 3–10 mL/min flow rate). The efficiency of conversion of O_2 to O_3 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 $^{18}O_2$/argon mixture through the arc generator. We showed previously that the small enrichment in $^{18}O_2$ in breathing air, which occurs due to inefficient $^{18}O_3$ generation from $^{18}O_2$, results in an insignificant enrichment of ^{18}O 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 method[9] and using bovine serum albumin as a standard. Cells from all BALF washes were combined and re-suspended in RPMI and then counted. One million cells were then pelleted and suspended into 0.3 mL of 3% PCA.

Samples for ^{18}O 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 previously[11] 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).

^{18}O analysis of blood and lavaged constituents

Isotope ratio mass spectrometry was used to measure amounts of excess ^{18}O 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 ^{18}O values from the air-exposed subjects were subtracted from the values measured in the $^{18}O_3$ exposed subjects to obtain the excess ^{18}O due to the $^{18}O_3$ exposure. Hereafter, we will dispense with the distinction of 'excess ^{18}O' and simply refer to it as '^{18}O.' We have shown in previous studies that the lyophilization procedure traps the portion of $^{18}O_3$ 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.[12] Total glutathione (GSx, consisting of the sum of GSH and GSSG) was analyzed by enzymatic recycling.[13] BALF cells and supernatants were also analyzed for alpha tocopherol concentrations according to a published method.[14]

Resting ozone exposure: study #2, physiology

Subjects were exposed in whole-body inhalation chambers according to methods outlined previously,[15] 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 O_3. FEV_1 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 FEV_1 measurement was done in triplicate with the largest value of the three measurements reported for that subject. The baseline FEV_1 values measured pre-exposure to air or O_3 averaged 4.48 ± 0.082 L (N = 68). FEV_1 percent change was determined using the following formula: $[(\text{pre-}O_3 - \text{post-}O_3)/\text{pre-}O_3] \times 100$.

Statistics

Two-tailed pairwise comparisons were made of data from air versus O_3 exposures with $P \leq 0.05$ assigned significance. Comparisons of the resting data with previously published exercising O_3 exposure data are exploratory, with no corrections made for multiple comparisons. The FEV_1 study employed three methods: (1) linear regression of the FEV_1 changes against the O_3 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.[16]

Results

Breathing and ozone uptake measurements

Breathing frequency, tidal volume, and percentage of $^{18}O_3$ uptake from breathing air during exposure of the resting subjects to air or 0.4 ppm $^{18}O_3$, 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 $^{18}O_3$ 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.[6] Comparing the volume of air breathed during the 2 hour exposure to 0.4 ppm $^{18}O_3$ 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 ^{18}O in BALF supernatants and cells

The ^{18}O accumulated by BALF cells, BALF supernatant, and NL of resting subjects exposed to $^{18}O_3$

Table 2. Breathing measurements and percentage O_3 uptake in 8 resting subjects exposed by face mask to $^{18}O_3$.

	Breaths measured	Inspired breaths		Expired breath	Total breath time, sec	Ve, L/min	O_3 uptake, %
		Volume, L	Time, sec	Time, sec			
Air							
Mean	235	0.59	1.6	2.8	4.4	8.11	
SE	3	0.13	0.1	0.3	0.3	0.48	
N	8	8	8	8	8	8	
O_3							
Mean	135	0.66	1.7	3.0	4.6	8.66	79.8
SE	3	0.15	0.2	0.5	0.5	0.47	1.8
N	5	5	5	5	5	5	5

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).[6]

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.

	Bronchoalveolar lavage		Nasal lavage
	Cell pellet	Surfactant	
Excess oxygen-18, ug/g dry weight			
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 O_3 exposure.

Table 6 shows that serum-opsonized Candida albicans was engulfed by ~20% fewer phagocytes in BALF from 0.4 ppm 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 O_3.

Table 5. BAL cell numbers and differential following resting exposure of 8 human subjects to O_3.

	BAL vol recovered, ml	Total cells × 10e6	% 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%
O_3								
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%
O_3/air	1.05	0.81	1.04	1.52	0.75	0.76	1.32	0.46
P value, O_3 vs. air		0.04		0.02				

FEV_1 changes in resting subjects exposed to O_3

A scatter plot of FEV_1 changes observed in each individual subject exposed to air or to 4 different concentrations of O_3 is shown in Figure 2. FEV_1 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 O_3. 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 O_3 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 O_3 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 O_3 concentration of 0.12 ppm represented the lowest dose that was significantly different from control. Application of the same test to the resting O_3 exposure yielded no significant effect for any O_3 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 O_3 concentrations in resting subjects.

Table 6. Bronchoalveolar lavage cell phagocytosis of Candida albicans particles following resting exposure to O_3.

	Air	O_3	Air	O_3	Air	O_3
Percentage of macrophages that phagocytized particles						
Mean	23.7	17.9	55.7	55.2	69.6	55.4
SE	4.4	6.6	14.8	14.8	29.8	15.2
N	7.0	7.0	7.0	7.0	6.0	7.0
O_3/air		0.76		0.99		0.80*
Number of Candida particles per cell						
Mean	1.4	1.0	6.5	6.6	3.5	3.7
SE	0.2	0.2	0.4	0.6	0.4	0.6
N	7	7	7	7	6	7
O_3/air		0.69		1.02		1.05

Note: *$P = 0.04$ by 2-tail *t*-test of means.

Comparison of O_3 effect markers: resting versus exercising

A side-by-side comparison summary of O_3 effects observed during resting versus exercising exposures is presented in Table 7. The change in the mean values observed for O_3 exposure compared to air exposure is represented as either an O_3 minus Air value or an O_3 to Air ratio if that was more appropriate. The FEV_1 percent change and the fold increase in neutrophils was about 5-fold greater during exercise than during resting O_3 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 O_3 and was unchanged with resting exposure to O_3.

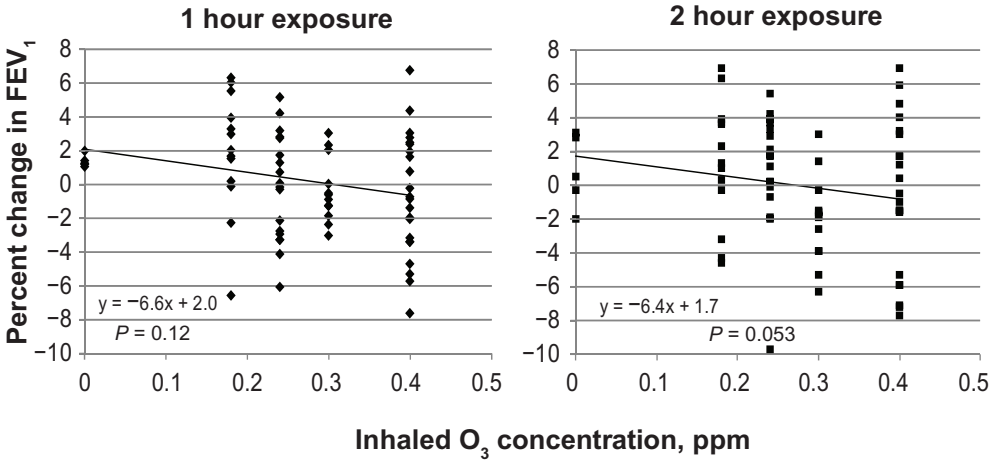

Figure 2. The percentage change in FEV_1 in individual resting subjects exposed to four concentrations of O_3 and to air plotted against the O_3 inhaled concentration.
Notes: FEV_1 was measured pre-exposure, and after 1 and 2 hours of exposure in the same subjects. The regression trend lines had a similar 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.

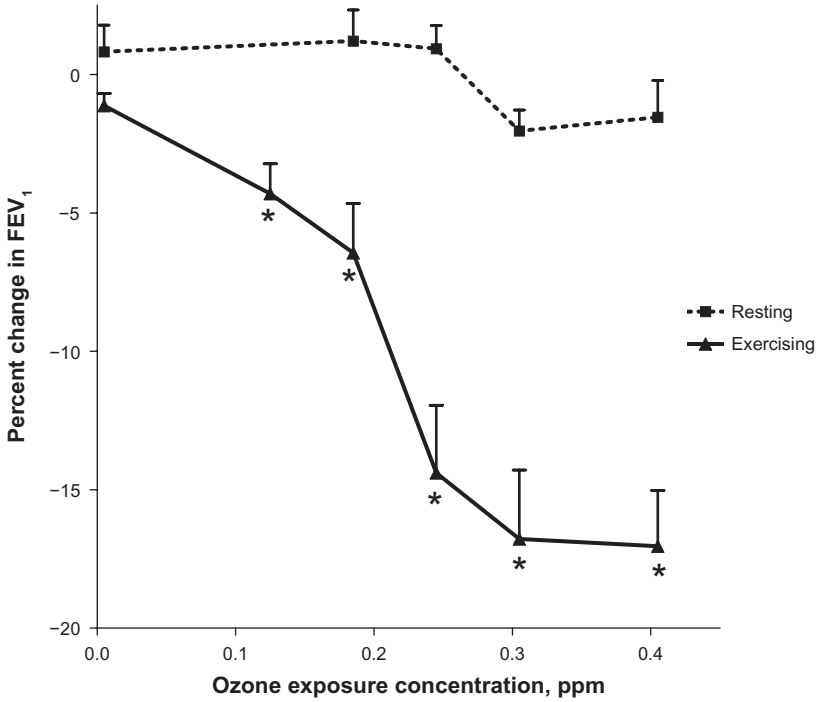

Figure 3. Comparison of O_3-induced FEV_1 changes (mean ± S.E.) observed after 2 hours of exposure to various concentrations of O_3 while at rest or while exercising intermittently (15 minute intervals) at a level of 65 L/min Ve.
Note: Asterisks indicate the lowest concentration of O_3 exposure at which a significant change from air exposed occurred (by Williams test for non-parametric data).[16]

Table 7. Comparison between resting and exercising effects and dose 1–2 hours following 0.4 ppm, 2 hr O_3 exposure.

Measurement	Resting exposure	Exercising exposure	Exercise/ rest	Reference for exercising exposure
O_3 minus air				
FEV_1 % decrement	~2	10.3*	5.0	1
BALF cell ^{18}O	5.6*	28.4*	5.1	1
BAL surfactant ^{18}O	26.4*	51.6*	2.0	1
Nasal lavage ^{18}O	377*	192*	0.5	1
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 O_3 compared to respective air exposed. [1]Hatch et al, 1994; [2]Devlin et al, 1996.

Fractional removal of ozone from breathing air

Past studies have examined the fractional removal of O_3 from breathing air to arrive at estimates of O_3 dose to the respiratory tract. Exercising subjects have been reported to remove a smaller fraction of 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 O_3 retained in the lung is definitely increased by exercise.[19] Our result of ~79% of removal of 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 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 ~88% uptake measured by integration of breath by breath 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).

$^{18}O_3$ dose measurements in lavaged fluids

We have demonstrated here that human subjects exposed while at rest to $^{18}O_3$ accumulate ^{18}O in BALF cells and surfactant material in lower concentrations than exercising subjects. The ^{18}O label that remains in the tissue after lyophilization appears to be the result of oxygen addition reactions of $^{18}O_3$ with biomolecules. Accumulation of ^{18}O in BALF cells and surfactant material suggests that $^{18}O_3$ 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}O following $^{18}O_3$ exposure, as seen in a study involving rhesus monkeys,[4] suggests that the reaction of $^{18}O_3$ is concentrated at the air-liquid interface. This finding is in agreement with physicochemical modeling predictions that suggest that 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}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 O_3 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 ^{18}O 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 O_3 produced few statistically significant biological effects in BALF highlights the low-dose nature of alveolar O_3 exposure, even at the relatively high inhaled O_3 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 O_3 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 O_3 exposure, a previous report showed a lack of effect of resting O_3 exposure on BALF cell DNA single strand breaks, as opposed to a positive effect if O_3 exposure occurred during exercise.[25]

Airway antioxidants participate in the reactions of inhaled O_3, and measurement of changes in antioxidants can provide insight on where O_3 reacts. Our results showed only a non-significant lowering of NL ascorbate by O_3 at zero hour post O_3 exposure (Table 2, supplementary). Two published studies measured antioxidants under a less vigorous exercise regimen targeting 20 L/min per m^2 body surface area rather than the present 35 L/min per m^2 with exposure to 0.2 ppm of O_3 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 (FEV_1) changes

Our regression of FEV_1 changes versus four resting concentrations of O_3 up to 0.4 ppm showed a slope that appears to be different from zero (Fig. 2). Previously published reports that looked at FEV_1 changes immediately following resting 2 hour O_3 exposures and which found no significant decrements at O_3 concentrations lower than 0.5 ppm were probably due to the smaller number of subjects examined.[28,29] In addition to a lower delivered O_3 dose in resting exposures, the inability to detect significant alterations in FEV_1 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 $^{18}O_3$ 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 O_3 between different levels of physical activity. 'Effective dose' was first defined as the product of concentration, Ve, and exposure time by Silverman et al[30] 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 O_3 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 $^{18}O_3$ exposure study[6] 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 $^{18}O_3$ dose is a measurement closer to the pulmonary target site for O_3 than previous estimates of 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}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 Ve levels or the duration that might be experienced by the sizable population that now participates in marathons and other high Ve activities. It would not be uncommon for people participating in such activities to achieve a 4-fold higher average 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 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 O_3 dose can be achieved by comparing the ^{18}O content in BALF cells obtained from humans and rats similarly exposed to $^{18}O_3$. In our previous study, rats had to be exposed to 2.0 ppm $^{18}O_3$ 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 O_3 dose. The finding that human resting BALF ^{18}O dose approximates that of the resting rat BALF ^{18}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 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 O_3 reactions in the lung and therefore serves as a shield to BALF cells, causing them to retain less $^{18}O_3$;[7,8] (2) the ability of rats to lower body temperature and Ve during O_3 exposure;[33] (3) a higher nasopharyngeal removal of O_3 in rats;[34] and (4) a lower whole-body percent retention of 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 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 O_3, and suggest a similar effect of exercise on the alveolar dose of other chemically reactive gases with properties similar to O_3.

Conclusion

Results confirm that exercise contributes greatly to both the dose and effect of O_3 measured by indicators in BALF. Quantification of $^{18}O_3$ reaction products in BALF cells has provided a basis for extrapolation of acute O_3 dose between resting and exercising exposures. The comparison between resting and exercising O_3 effects, along with the dose measurements in each type of exposure provide an improved understanding of low-dose O_3 effects. Results confirm the use of Ve as a factor in the extrapolation of inhaled dose of 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 O_3. The similarity of alveolar 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

Conceived and designed the experiments: GH and RD. Analysed the data: GH, JB, WM, ES, JS, RS, KC and RD. Wrote the first draft of the manuscript: GH; Contributed to the writing of the manuscript: GH, JM, JB, WM, ES, JS, RS, KC, RD. Agree with manuscript results and conclusions: GH, JM, JB, WM, ES, JS, RS, KC, RD. Jointly developed the structure and arguments for the paper: GH, WM, RD. Made critical revisions and approved final version: GH, JM, JB, WM, ES, JS, RS, KC, RD. All authors reviewed and approved of the final manuscript.

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

The research described in this article has been reviewed by the National Health and Environmental Effects Research Laboratory, United States Environmental Protection Agency and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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[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[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_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.[37]

Appendix 2

The relationship between body weight and Ve across species has been reported as 379 M^0.8, where M = body weight in kg and Ve is in milliliters.[38] The same author reports that the wet lung weight in grams varies by the relationship 11.3 M^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 $^{18}O_3$.

	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							
Mean	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_3							
Mean	2.7	26.5	104	81.7	434	2.0	4.4
SE	0.3	12.5	13	45.3	171	0.35	0.56
O_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.