6.6-Hour Inhalation of Ozone Concentrations from 60 to 87 Parts per Billion in Healthy Humans

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***Rationale*: Identification of the minimal ozone (O_3) concentration and/or dose that induces measurable lung function decrements in humans is considered in the risk assessment leading to establishing an appropriate National Ambient Air Quality Standard for O_3 that protects public health.**

***Objectives*: To identify and/or predict the minimal mean O_3 concentration that produces a decrement in FEV_1 and symptoms in healthy individuals completing 6.6-hour exposure protocols.**

***Methods*: Pulmonary function and subjective symptoms were measured in 31 healthy adults (18–25 yr, male and female, nonsmokers) who completed five 6.6-hour chamber exposures: filtered air and four variable hourly patterns with mean O_3 concentrations of 60, 70, 80, and 87 parts per billion (ppb).**

***Measurements and Main Results*: Compared with filtered air, statistically significant decrements in FEV_1 and increases in total subjective symptoms scores ($P < 0.05$) were measured after exposure to mean concentrations of 70, 80, and 87 ppb O_3. The mean percent change in FEV_1 ($\pm$standard error) at the end of each protocol was 0.80 $\pm$ 0.90, –2.72 $\pm$ 1.48, $-$5.34 $\pm$ 1.42, $-$7.02 $\pm$ 1.60, and $-$11.42 $\pm$ 2.20% for exposure to filtered air and 60, 70, 80, and 87 ppb O_3, respectively.**

***Conclusions*: Inhalation of 70 ppb O_3 for 6.6 hours, a concentration below the current 8-hour National Ambient Air Quality Standard of 75 ppb, is sufficient to induce statistically significant decrements in FEV_1 in healthy young adults.**

Keywords: ozone; clinical study; exposure assessment; human

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

The acute inhalation of ambient concentrations of ozone induces several health effects including airway irritation and inflammation, decrements in pulmonary function, and symptoms of respiratory discomfort.

What This Study Adds to the Field

This study identifies 70 ppb as the mean concentration of ozone averaged over 6.6 hours that results in a statistically significant decrement in FEV_1 and presents an empirically validated model that predicts the onset of pulmonary responses induced by any combination of ozone concentration, minute ventilation, and exposure duration.

Ozone is the primary oxidant found in photochemical air pollution and is one of the six criteria air pollutants identified in the 1971 United States Clean Air Act as adversely affecting public health. Human clinical exposure studies have played an important role in the risk assessment required to set the National Ambient Air Quality Standard (NAAQS) for ozone. These clinical exposure studies have been designed to expose defined subpopulations of individuals while varying ozone concentrations, exposure duration, and minute ventilations. A central question inherent in these studies has been as follows: What is the minimal mean ozone concentration that produces a statistically significant decrement in FEV_1 and other markers of response?

Early clinical studies focused on manipulating ozone concentration and/or minute ventilation ($\dot{V}E$) by adjusting exercise workloads, while limiting the duration of exposure to 2.5 hours or less (1–4). The minimal mean ozone concentration that produced a statistically significant decrement in FEV_1 in healthy male subjects in these early studies was shown to be 120 ppb using a 2.5-hour protocol and heavy intermittent exercise (15-min periods of rest and exercise with exercise $\dot{V}E$ of 65 L/min) (3) and 300 ppb using a 1-hour protocol and heavy continuous exercise (exercise $\dot{V}E$ of 66 L/min) (4). Subsequently, Schelegle and Adams (5) observed a statistically significant decrement in FEV_1 in endurance athletes who completed a 1-hour competitive simulation (mean $\dot{V}E$ of 86.6 L/min) while breathing 180 ppb ozone. More recently several investigators have extended exposure duration to 6.6 hours, using a protocol initially described by Folinsbee and colleagues (6). This protocol contains six 50-minute exercise periods with minute ventilation maintained at 8 L/min/L of FVC ($\dot{V}E$ of approximately 40 L/min). As noted by Folinsbee and colleagues (6) and McDonnell and colleagues (7), this level of exertion was "intended to simulate work performed during a day of heavy to severe manual labor in outdoor laborers." Folinsbee and colleagues (6), Horstman and colleagues (8), and Adams (9, 10) have used this 6.6-hour protocol to examine ozone-induced responses in healthy young adults exposed to ozone concentrations ranging from 40 to 120 ppb. Of these, the studies conducted by Adams (9–11) have played an important role in the 2007 exposure/risk assessment conducted by the United States Environmental Protection Agency and the establishment of a new NAAQS for ozone in 2008. Adams (9) observed statistically significant FEV_1 decrements and respiratory symptoms at 80 ppb. Although lung function decrements and respiratory symptoms in the 60 ppb ozone protocols were not statistically significant, the magnitude of the pulmonary function decrements at 60 ppb ozone at 40 L/minute were consistent with the trend observed at higher levels and some subjects had "notable" FEV_1 decrements (i.e., >10% FEV_1) at 60 ppb.

The current study further examines the minimal mean ozone concentration that produces a statistically significant decrement in FEV_1 and symptoms in healthy individuals completing five 6.6-hour exposure protocols with variable ozone concentration

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profiles. In addition, the variable ozone concentration profiles were specifically designed to examine the relative importance of mean ozone concentration over the entire exposure period versus peak dose rate in determining peak pulmonary function decrements and symptoms during and after exposure. This was accomplished by adjusting the mean and peak ozone concentrations for each exposure protocol. The mean ozone concentrations of the five protocols were 0, 60, 70, 80, and 87 ppb. Each of the four stepwise ozone concentration profiles (60, 70, 80, and 87 ppb ozone) had peak ozone concentrations in the fourth hour of exposure with peaks of 90, 90, 150, and 120 ppb, respectively. The dose of onset (Dos) of ozone-induced decrements in FEV_1 was derived from the combined individual subject FEV_1 and cumulative dose data, using an iterative process involving least-squares linear regression analysis (12). The derived value of Dos that represents the cumulative dose of ozone at which approximately half the subjects have developed pulmonary responses and half have not was then combined with other published (12) values of Dos to predict the onset of ozone-induced pulmonary responses in the healthy young subjects at multiple combinations of ozone concentration, exposure duration, and minute ventilations. The results of this study have been previously reported in the form of an abstract (13).

METHODS

Subject Recruitment

Thirty-one young adults (16 females and 15 males), ages 18–25 years, participated in the study. Subjects were solicited volunteers from the University of California, Davis, or surrounding community. Each subject was informed of study risks and subsequently signed a consent form approved by the Institutional Human Subjects Review Board (IRB) of the University of California, Davis. Individuals with a history of cardiovascular disease or respiratory ailments (i.e., asthma, seasonal allergies) were not allowed to participate in the study. Subjects were nonsmokers and did not live in a high air pollution area 6 months before participation in the study. All subjects were engaged in a regular program of aerobic training to ensure their ability to complete the exercise protocols. All subjects underwent preexperimental screening to determine normal pulmonary function (Table 1) and filled out a general health questionnaire, both of which were reviewed by the project physician before being included in the study. In addition, female subjects were asked to perform a urine pregnancy test to make sure they were not pregnant at the time of enrollment.

Experimental Design

All exposures were performed in a free-standing $9 \times 10 \times 8$ ft stainless steel environmental chamber (model 1328-M; Vista Scientific, Ivyland, PA). The chamber and its operation have been previously described by Adams (9). The five 6.6-hour chamber exposures completed by each subject were composed of six 50-minute exercise bouts at a mean equivalent ventilation rate of 20 L/minute/m^2 body surface area (BSA) (9). The 50-minute exercise bouts were done alternately on a cycle ergometer and treadmill. A 35-minute lunch break took place after completion of Hour 3 and was taken at rest in the chamber at the Hour 3 O_3 concentration. Temperature and relative humidity were maintained between 21 and 25°C and between 40 and 60%, respectively. The exposure regimens were composed of filtered air and four varying hourly O_3 concentrations averaging 60, 70, 80, and 87 ppb. The stepwise patterns of O_3 concentration are given in Table 2. Ozone concentration was monitored continuously with a Dasibi monitor (model 1003H; Dasibi Instruments Inc, Glendale, CA) calibrated according to the ultraviolet absorption photometric method, traceable to a National Institute of Standards and Technology standard photometer, at the Primate Research Center of the University of California, Davis. The protocols were conducted in single-blind fashion and completed by each subject in random order, with a minimum of 7 days intervening between protocols.

Subjects performed two to four forced expiratory maneuvers immediately before and after each experimental exposure, during the last 10 minutes of each hour and at 1 and 4 hours postexposure, using a portable computer-based spirometer (SpiroVision-3; FUTUREMED Inc., Granada Hills, CA). The FVC and FEV_1 values were selected on the basis of American Thoracic Society guidelines (14).

Minute ventilation ($\dot{V}_E$), tidal volume (V_T), breathing frequency (f), expired gas temperature, heart rate, and subjective symptoms were monitored as previously described (9). Subjects were asked to rate the severity of each of four symptoms: throat tickle, cough, shortness of breath, and pain on deep inspiration. Each symptom was rated according to a severity scale (ranging from 0, not present, to 40, severe) as previously described (15). Total subjective symptoms (TSS) score was calculated as the sum of the severity ratings for the four individual symptoms.

Statistical Procedures

The effect of exposure on FVC, FEV_1, and FEV_1/FVC was expressed as the percent change from the preexposure value. Similarly, f, V_T, and $\dot{V}_E$ are presented as percent change from the initial value obtained at 7–10 minutes of the first exercise period. TSS was analyzed as absolute changes from zero. All data are expressed as means (standard error). The effects of gas concentration and exposure time were determined by mixed model two-way analysis of variance (ANOVA) with repeated measures ($P < 0.05$) (SAS software; SAS Institute, Cary, NC), using

TABLE 1. FEMALE AND MALE SUBJECT PHYSICAL CHARACTERISTICS AND BASELINE PULMONARY FUNCTION MEASURES

Characteristic	Females (*n = 16*)	Males (*n = 15*)
Age, yr	21.4 (0.6)	21.4 (0.5)
Height, m	1.68 (0.02)	1.82 (0.02)
Weight, kg	65.1 (2.8)	81.0 (2.8)
BSA, m^2	1.73 (0.05)	2.04 (0.04)
FVC, L	4.16 (0.19)	5.72 (0.19)
FEV_1, L	3.43 (0.13)	4.67 (0.18)
FEV_1/FVC, %	82.8 (1.9)	81.7 (1.5)
FEF_{25-75}, L/s	3.35 (0.15)	4.48 (0.29)
PEF, L/s	7.04 (0.17)	9.96 (0.46)

Definition of abbreviations: BSA = body surface area; FEF_{25-75} = forced expiratory flow between 25 and 75% FVC; PEF = peak expiratory flow.

Values represent means (SE).

TABLE 2. TARGET AND ACTUAL MEAN OZONE CONCENTRATIONS EXPRESSED AS PARTS PER BILLION FOR THE FIVE 6.6-HOUR EXPOSURE PROTOCOLS

Protocol	Target/ Actual	Exercise Period 1	2	3	4	5	6	Mean
FA	Target	0	0	0	0	0	0	0
	Actual	1 (0)	1 (0)	1 (0)	1 (0)	1 (0)	1 (0)	1 (0)
60 ppb†	Target	40	70	70	90	50	40	60
	Actual	43 (1)	72 (1)	73 (1)	91 (1)	54 (1)	42 (1)	63 (1)
70 ppb‡	Target	50	70	80	90	80	50	70
	Actual	52 (1)	73 (1)	82 (1)	92 (0)	81 (1)	54 (1)	72 (1)
80 ppb§	Target	30	70	100	150	80	50	80
	Actual	33 (1)	71 (1)	101 (1)	147 (1)	84 (1)	52 (1)	81 (1)
87 ppb‖	Target	40	80	90	120	100	90	87
	Actual	42 (1)	81 (1)	93 (1)	119 (1)	102 (1)	91 (1)	88 (1)

Definition of abbreviation: FA = filtered air.

Actual values represent means (SE); n = 31.

† Pattern is modified from Adams (9), with the ozone concentration in the third and fourth exercise period switched.

‡ Daily pattern observed in Little Rock, Arkansas metropolitan area (A. Lefohn, personal communication).

§ Pattern is the Adams (9) stepwise 80 ppb protocol and provides a means of direct comparison with previous published findings.

‖ Daily pattern observed in San Bernardino, California metropolitan area (A. Lefohn, personal communication).

the procedures described by Littell and colleagues (16). There are two steps in performing a mixed model two-way ANOVA with repeated measures (16). In the first step we determined the best fit of the data to one of several within-subject covariance structures using the Akaike information criterion (AIC) and the Schwarz Bayesian criterion (SBC). After fitting the data to unstructured, Toeplitz, compound symmetry, heterogeneous compound symmetry, heterogeneous first-order autoregressive, first-order autoregressive, and heterogeneous covariance structures both the AIC and SBC indicated that the Toeplitz covariance structure provided the best fit to the data. In the second step we analyzed the time and protocol effects by estimating and comparing means, initially using least-squares means and then using a Tukey adjustment (SAS Institute).

Having analyzed the data in a manner that allowed for the evaluation of the time and mean concentration effects we reanalyzed the data, focusing on the effect that each protocol had on the immediate postexposure FEV_1 compared with the filtered air protocol. To make this comparison we used both parametric and nonparametric tests. The parametric analysis followed the same procedures as those used for the whole data set described previously, except that a Dunnett's adjustment was used to compare means. The Dunnett's test limits the number of comparisons to those between the control group (in this case the filtered air protocol) and each experimental protocol. The nonparametric approach used the Friedman test, which is the nonparametric equivalent of a repeated measures ANOVA on ranks, with post hoc comparisons of mean ranks using Dunnett's test (17).

To further examine the time course of the FEV_1 response we identified the dose of onset (Dos), using a regression approach similar to the one we previously described and applied to breathing pattern data (12). In brief, we plotted the percent change in FEV_1, corrected for filtered air (FA) responses, for all the subjects combined against the cumulative dose of O_3 (micrograms) for each exposure protocol. Dos was determined by an iterative process involving least-squares linear regression (Microsoft Excel X; Microsoft Corporation, Redmond, WA). In each iteration step two lines were fit to the percent change in FEV_1 and cumulative dose. In the first iteration the first region (region 1) of the data that was fit included the first 31 data points. The second region (region 2) that was fit began at the 32nd point and included all the cumulative dose points greater than this to the end of the protocol. In the next iteration a point was added to region 1 and subtracted from region 2. This iterative process was continued until region 2 consisted of the data from the last 31 points. With each iteration step the slope, intercept, and correlation coefficient were calculated for each region. In addition, the difference in slope of regions 1 and 2, and the average correlation coefficient of regions 1 and 2, were calculated. The point at which the maximum in the correlation coefficient of region 1, the average correlation coefficient of regions 1 and 2, and the difference in slopes of region 1 and 2 occurred was determined and averaged to obtain the estimated Dos.

RESULTS

A summary of the male and female subjects' characterization data is given in Table 1. All the subjects were engaged in some form of regular aerobic activity. One male subject was a competitive cyclist at the time of the study. All the subjects were healthy and had normal pulmonary functions, with the baseline value of FEV_1/FVC% ranging from 69.8 to 96.2%.

The group mean exercise $\dot{V}_E$ and estimated overall mean $\dot{V}_E$ (includes estimated resting $\dot{V}_E$ [18]) values for the five 6.6-hour protocols are given in Table 3. Resting $\dot{V}_E$ was estimated using regression equations derived from the data of Aitken and colleagues (18) that relate resting $\dot{V}_E$ to body surface area for college-age males [resting $\dot{V}_E$ = 7.61(BSA)] and females [resting $\dot{V}_E$ = 8.05(BSA)]. There were no statistically significant differences in exercise $\dot{V}_E$ with regard to time of exposure or protocol. In addition, there was no statistically significant difference in the estimated overall mean $\dot{V}_E$ for the five protocols.

TABLE 3. GROUP MEAN EXERCISE MINUTE VENTILATION AND ESTIMATED OVERALL MINUTE VENTILATION* FOR THE FIVE EXPOSURE PROTOCOLS

	$\dot{V}_E$	
Protocol	Exercise	Estimated Overall
FA	39.3 (0.9)	33.4 (0.8)
60 ppb	38.5 (0.9)	32.8 (0.7)
70 ppb	38.6 (1.0)	32.8 (0.8)
80 ppb	38.9 (1.0)	33.1 (0.8)
87 ppb	38.4 (0.9)	32.7 (0.7)

Definition of abbreviation: $\dot{V}_E$ = minute ventilation.
Values represent means (SE).
* Inclusive of rest and lunch periods.

The group mean ozone concentrations during each exercise period, as well as the average ozone concentration for each protocol, are given in Table 2, whereas the mean cumulative inhaled dose (CD, μg) of ozone is plotted against time of exposure in Figure 1. The ozone concentrations (ppb) during each exercise period for all four ozone protocols were significantly greater than the background levels measured in the FA protocol. Comparing across protocols, whenever the target ozone concentration was set to be different compared with any other protocol the measured values for this comparison were found to be significantly different. The inhaled dose rate (DR, μg/min) during each exercise period for all four ozone protocols was significantly greater than the background levels measured in the FA protocol. In contrast to ozone concentration the DR during each exercise period was significantly different across ozone protocols only when the difference in the target ozone concentration was greater than or equal to 20 ppb. All possible comparisons of CD at the end of each protocol were significant different. However, the time during exposure at which the CD became significantly different between ozone protocols varied (Figure 1). The CD for the 60 ppb exposure protocol became significantly different from the 70, 80, and 87 ppb ozone exposure protocols during the fifth, fourth, and third exercise periods, respectively. The CD for the 70 ppb exposure protocol became significantly different from the 80 and 87 ppb ozone exposure protocols during the fourth exercise period. The CD for the 80 ppb exposure protocol became significantly different from the 87 ppb ozone exposure protocol during the fifth exercise period.

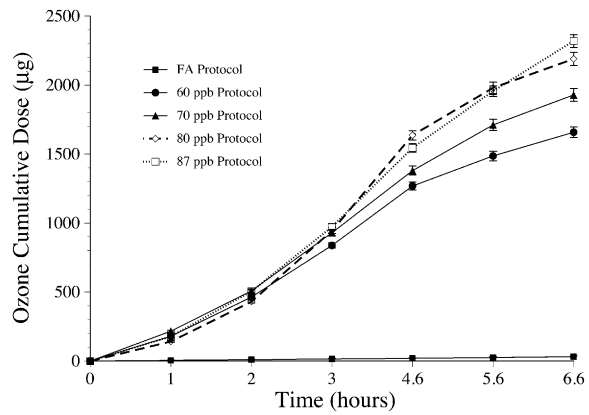

Figure 1. Diagram of mean group values for cumulative dose of ozone (micrograms) against time of exposure for each of the five protocols. Values represent means ± SEM.

Pulmonary Function and Symptom Responses

The mean responses for the percent change in FVC, percent change in FEV_1, percent change in FEV_1/FVC%, and TSS are shown in Figure 2. In comparison with the FA protocol statistically significant decrements in the percent change in FVC were present after the fourth and sixth exercise periods of the 80 ppb protocol and after the fifth and sixth exercise periods and 1 hour postexposure of the 87 ppb protocol (Figure 2A). In comparison with the FA protocol the 70, 80, and 87 ppb exposure protocols resulted in statistically significant decrements in the percent change in FEV_1 (Figure 2B). These statistically significant differences occurred after the sixth exercise period in the 70 ppb protocol; after the fourth, fifth, and sixth exercise periods and 1 hour postexposure of the 80 ppb protocol; and after the fifth and sixth exercise periods and 1 hour postexposure of the 87 ppb protocol. In comparison with the FA protocol, statistically significant decrements in the percent change in FEV_1/FVC% were present only after the fifth and sixth exercise periods of the 87 ppb protocol (Figure 2C). In comparison with the FA protocol the 70, 80, and 87 ppb exposure protocols resulted in statistically significant increases in TSS (Figure 2D). In each of these three exposure protocols the statistically significant increases in TSS occurred after the fifth and sixth exercise periods. In all the protocols pulmonary function and symptoms returned to preexposure levels within 4 hours of the end of exposure.

In comparison with the FA protocol the inhalation of ozone during the 60 ppb protocol did not result in a statistically significant decrement in percent change in FVC, percent change in FEV_1, percent change in FEV_1/FVC, or TSS at any time point. Examination of the percent change in FEV_1 shows that the maximal difference between the FA and 60 ppb protocol occurred after the sixth exercise period (Figure 2B). The magnitude of this difference was 3.52 ± 1.52% (mean ± SE) and was the result of 11 subjects who had FEV_1 decrements greater than 5% compared with the FA protocol (11.42 ± 2.62%). To increase the power of our analysis we limited the number of mean comparisons and narrowed the hypothesis being tested by restricting our analysis to immediate postexposure FEV_1 data and only comparing each ozone protocol with the filtered air protocol. In addition, we used both parametric and nonparametric tests in this restricted analysis. The distribution of the percent change in FEV_1 at 6.6 hours for each protocol is illustrated using histograms in Figure 3. While increasing the power of the analysis both parametric and nonparametric tests provided the same result as the more global two-way ANOVA with repeated measures (Table 4).

Dose at Onset

We were able to obtain reliable estimates of Dos, using the pooled FEV_1 from the 80 and 87 ppb ozone exposure protocols and when all of the FEV_1 data was combined, but not from the

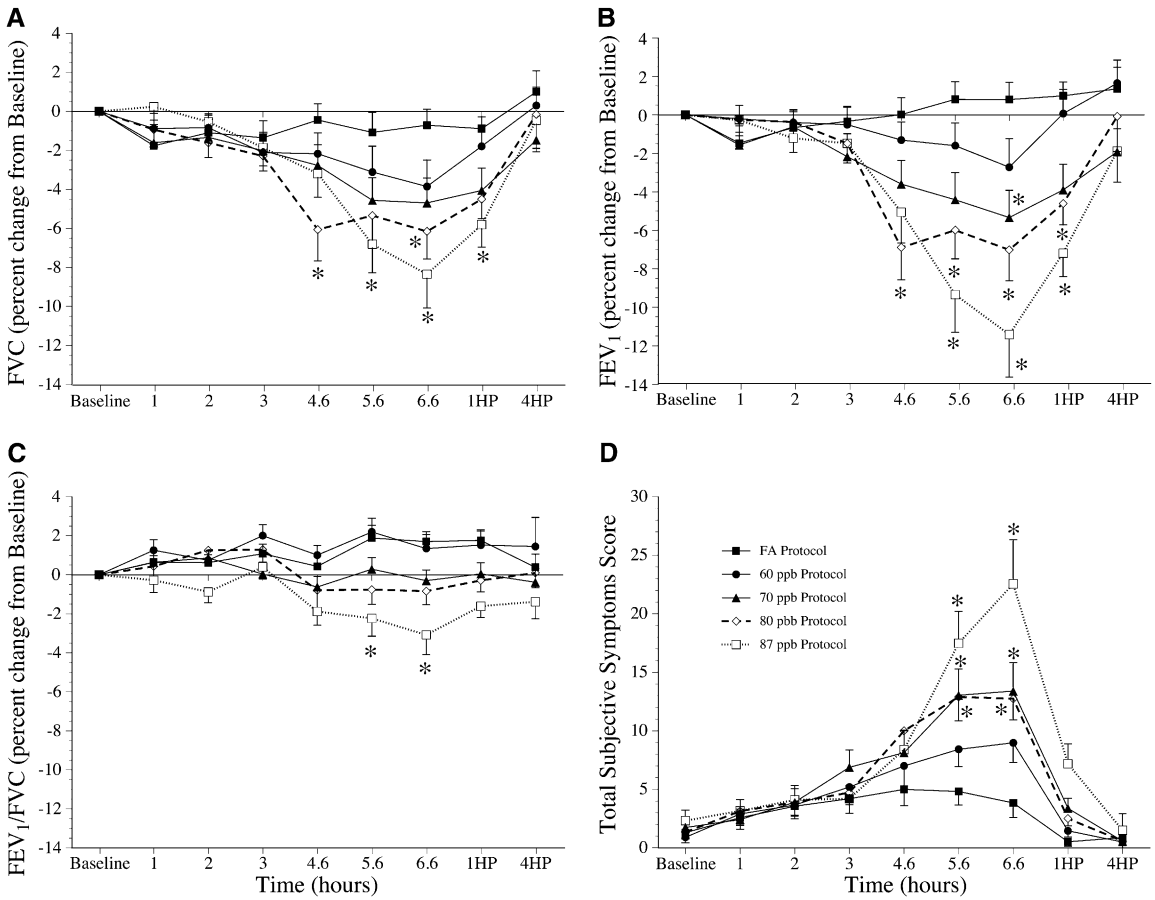

Figure 2. Mean responses of 31 healthy young adult subjects to various time–concentration profiles during, and 1 and 4 hours after, 6.6-hour ozone exposure. (*A*) FVC; (*B*) FEV_1; (*C*) FEV_1/FVC, all percent change from baseline; and (*D*) total subjective symptoms scores. *Asterisks* indicate a statistically significant difference from filtered air (FA) at the same time point. *Note:* Only a portion of the subjects completed the 4-hour postexposure measurements: FA (n = 17); 60 ppb (n = 15); 70 ppb (n = 15); 80 ppb (n = 15); and 87 ppb (n = 13). Values represent means ± standard error of the mean.

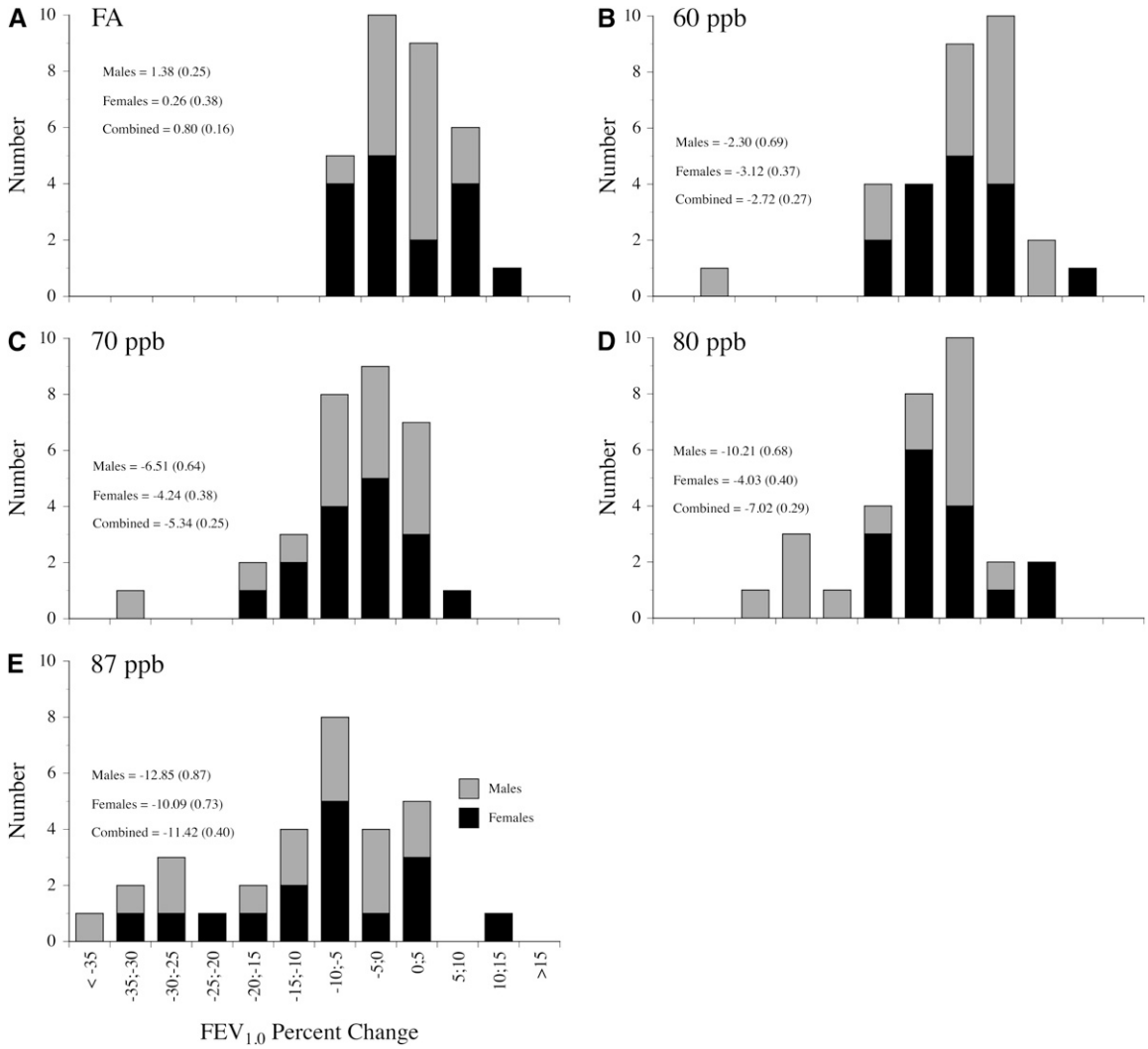

Figure 3. Histograms of percent change in FEV_1 after 6.6-hour exposure to (*A*) filtered air, (*B*) 60 ppb O_3, (*C*) 70 ppb O_3, (*D*) 80 ppb O_3, and (*E*) 87 ppb O_3 for both female subjects (*solid column portions*) and male subjects (*shaded column portions*). Values represent means (SEM).

pooled FEV_1 data from the 60 and 70 ppb ozone exposure protocols. The inability to estimate Dos using the FEV_1 data from the 60 and 70 ppb ozone exposure protocols is most likely because less than one third of the subjects had changes in FEV_1 greater than 5% in either of these protocols. The estimated values of Dos from the 80 and 87 ppb ozone exposure protocols are 1,374 and 1,326 μg of ozone, respectively. The estimated value of Dos from all of the FEV_1 data combined was 1,362 μg of ozone.

TABLE 4. PROBABILITY VALUES OBTAINED BY THREE STATISTICAL APPROACHES TO ANALYZE FEV_1 RESPONSE AFTER FOUR 6.6-HOUR EXPOSURE PROTOCOLS COMPARED WITH FILTERED AIR

	Statistical Test		
Comparison	Two-way ANOVA with Tukey-Kramer	One-way ANOVA with Dunnett's Test	Friedman's Test with Dunnett's Test
FA vs. 60 ppb	0.8	0.2	>0.05
FA vs. 70 ppb	0.0016	0.0023	<0.01
FA vs. 80 ppb	<0.0001	0.0002	<0.001
FA vs. 87 ppb	<0.0001	<0.0001	<0.0001

Definition of abbreviation: ANOVA = analysis of variance.

DISCUSSION

The U.S. Clean Air Act defines a primary air quality standard to protect the public health, while allowing for an adequate margin of safety. An Ozone NAAQS was first established on April 30, 1971 and subsequently reviewed and revised in 1979, 1997, and 2008. The NAAQS for ozone established in 1971 and 1979 were based on a peak 1-hour average. Subsequently, in 1997, in an effort to address the broad multiple-hour elevations seen in ambient ozone concentration in some urban and suburban environments, the NAAQS for ozone was revised to an 8-hour average concentration of 0.08 ppm for the fourth highest average over 3 years. More recently the NAAQS for ozone was revised to an 8-hour average concentration of 0.075 ppm for the fourth highest average over 3 years. In the current study the mean 6.6-hour ozone concentration of the four ozone protocols brackets the current NAAQS. We observed statistically significant decrements in FEV_1 and TSS associated with the 70, 80, and 87 ppb protocols, but not the 60 ppb protocol. In addition, there were statistically significant decrements in FVC in the 80 and 87 ppb protocols and in FEV_1/FVC in the 87 ppb protocol. These findings lower the mean ozone concentration at which statistically significant decrements in FEV_1 have been observed during a 6.6-hour exposure protocol to 70 ppb.

These findings are consistent with the results of Adams (9), who reported statistically significant decrements in FEV_1 using a 6.6-hour protocol with a mean ozone concentration of 80 ppb, but not 40 or 60 ppb. Adams (9) obtained the same result while using both constant ozone concentrations of either 60 or 80 ppb over the exposure period, and variable stepwise ozone concentration profiles similar but not identical to those used in the current study. There has been some concern expressed in the literature (19) that the univariate two-way ANOVA with repeated measures followed by Scheffé's post hoc test used by Adams in 2006 (9) indicating no statistically significant effect on FEV_1 in the 60 ppb protocols lacked sufficient statistical power to guarantee that this finding did not represent a false negative (type II error). It has been suggested that limiting the analysis to the immediate postexposure FEV_1 data and restricting the mean comparisons to the filtered air control would increase the power of the analysis and allow for the detection of significant differences when differences between means are small (18). We recognize the validity of this suggestion and agree that limiting the scope of the hypothesis being tested can increase the power of the statistical approach; however, we also recognize that when doing so caution should be exercised to ensure that the approach used is consistent with the original study design. In this case the individual subject FEV_1 responses induced by each exposure protocol are not independent and therefore any analysis should consider the effect of multiple comparisons. To address these concerns in the current paper we analyzed our data using a mixed-model two-way ANOVA with repeated measures followed by Tukey's post hoc test and then analyzed the immediate postexposure FEV_1 data using both parametric and nonparametric approaches. The mixed-model two-way ANOVA with repeated measures followed by Tukey's post hoc test provides the ability to directly address the covariance structure of the data and greatly enhances the ability to analyze repeated measures data by providing valid standard errors and efficient and powerful comparisons of means within a global analysis (16). This greatly improves the ability to examine time effects within protocols and protocol effects at multiple time points. Our analysis of the immediate postexposure FEV_1 data using both parametric and nonparametric statistics, while correcting for the inherent multiple comparisons in the original study design, optimized the power of the analysis by limiting the mean comparisons to those between the filtered air protocol and the 60, 70, 80, and 87 ppb exposure protocols. Furthermore, we also used a nonparametric statistic that is appropriate if the within-subject variance is not normally distributed (17). We obtain a similar result regardless of our statistical method, with the 60 ppb exposure protocol not being significantly different from filtered air, whereas the 70, 80, and 87 ppb exposure protocols were significantly different from filtered air. Although recognizing the consistency of our statistical analyses we point out, as did Adams (11), that there is a subset of responsive subjects that did respond to the 60 ppb protocol in excess of a 10% decrement in FEV_1. In addition, it is important to note that the previous study conducted by Adams (11) and the current study use a 6.6-hour protocol, which is 1.4 hours less than the 8-hour NAAQS and that if the 60 ppb ozone protocols were extended greater decrements might be achieved. A counterpoint to this possibility is the fact the mean overall ventilations in Adams (9) and this study are equal to or greater than mean ventilations that might be encountered during a day of heavy to severe manual labor among the construction workers observed by Linn and colleagues (20) and that this represents the higher end of ventilations that might be encountered in the normal population for this prolonged period.

In the current study, the variable stepwise profile of ozone concentration differed from protocol to protocol in such a way that the peak ozone concentration did not correlate with the mean ozone concentration over the entire protocol (Table 2). The clearest example of this and the one with demonstrated consequences are the 80 and 87 ppb protocols in which the peak 1 hour (4.6 to 5.6 h) values were 150 and 120 ppb ozone, respectively. The 80 ppb protocol also started and ended at a lower ozone concentration (30 and 50 ppb ozone) than the 87 ppb protocol (40 and 90 ppb ozone). The net result was that the cumulative dose for these two protocols did not become significantly different from each other until the final hour of exposure (*see* Figure 1), with the dose rate becoming significantly greater in the 80 ppb protocol during the fourth exercise period and then becoming significantly less during the fifth and sixth exercise periods. This pattern of exposure resulted in decrements in FEV_1 becoming statistically significant 1 hour earlier (4.6 vs. 5.6 h) in the 80 ppb protocol compared with the 87 ppb protocol (Figure 2B). This pattern then resulted in a plateau in FEV_1 decrements in the 80 ppb protocol, whereas FEV_1 decrements continued to increase in the 87 ppb protocol (Figure 2B). These observations, in combination with a delay in onset of response of approximately 3 hours (Figure 2) in the face of an increasing cumulative dose, suggest that there exists a complex interaction between time and dose rate at the level of the individual subject and cohort studied.

Several studies support the hypothesis that ozone-induced rapid shallow breathing and decrements in inspiratory capacity and FEV_1 are mediated by lung C-fibers (21–23) and could be expected to have similar time courses. Using breathing pattern data collected from 97 healthy male and female subjects during ozone exposure protocols of shorter duration, higher ozone concentrations, and continuous exercise of greater intensity than those used in the current study, we identified a distinct delay and response phase in the development of ozone-induced tachypnea (12). We found that the delay phase was dependent on reaching a dose of onset (Dos) and that the value of Dos was not influenced by ozone concentration or duration of exposure and only mildly influenced by changing $\dot{V}_E$. The consequence of this relationship is that if $\dot{V}_E$ is held constant the higher the mean ozone concentration the shorter the time to reach the threshold for the onset of response. In addition, we observed that the magnitude of tachypnea that developed after Dos was reached correlated with dose rate and not the cumulative or effective inhaled dose. Also of considerable importance was the observation that the magnitude of Dos was not correlated with the magnitude of tachypnea. We proposed that the development of decrements in FVC and FEV_1 may follow a similar time course and cited previous 6.6-hour exposure protocols to support this possibility. The plot of the group mean decrement in FEV_1 versus cumulative inhaled dose (Figure 4) supports the notion that a Dos is clearly present in the FEV_1 data in the current study. In addition, the plateau of FVC and FEV_1 decrements in the 80 ppb protocol, despite the fact that cumulative inhaled dose continues to increase, supports the notion that after Dos is reached the magnitude of response is a function of dose rate.

Using a similar regression analysis approach for deriving Dos from breathing frequency data, we determined Dos on the basis of the combined individual FEV_1 data. The derived Dos, using all the pooled FEV_1 data, was 1,362 μg of ozone. This value of Dos is greater than the values of Dos derived in our previous analysis (12). This difference may be related to numerous factors, for example, the plot of Dos from this and our previous analysis against $\dot{V}_E$ further suggests that Dos is a function of $\dot{V}_E$ (Figure 5A). Dos is not only useful in providing a better understanding of the kinetics of ozone-induced pulmonary

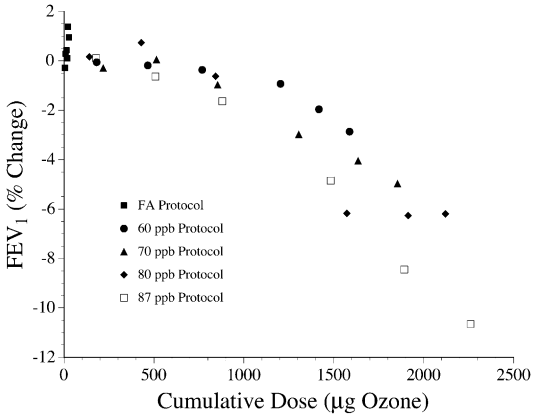

Figure 4. Scatter plot of group mean decrements in FEV_1 against the total cumulative dose of ozone (micrograms). Note that there is an inflection in the data between 1,300 and 1,400 μg of ozone. Values represent means ± SEM.

responses but provides insights into a component that contributes to the individual or group responsiveness to ozone. Given the relationship between Dos and $\dot{V}_E$ (Figure 5A) it is possible to predict the average maximal dose of ozone at which there is no pulmonary function decrement for exposures varying greatly in ozone concentration, duration of exposure, and minute ventilation (Figure 5B). The three-dimensional surface defined by this relationship (Figure 5B) provides a tool for predicting the maximal ozone exposure that approximately half of healthy individuals could experience without demonstrating functional responses. However, this relationship needs to be further validated, especially with studies using lower minute ventilations in combination with ambient ozone concentrations. It is also of critical importance to gain a better understanding of how the airway environment changes at the onset of decrements in lung function and subjective symptoms.

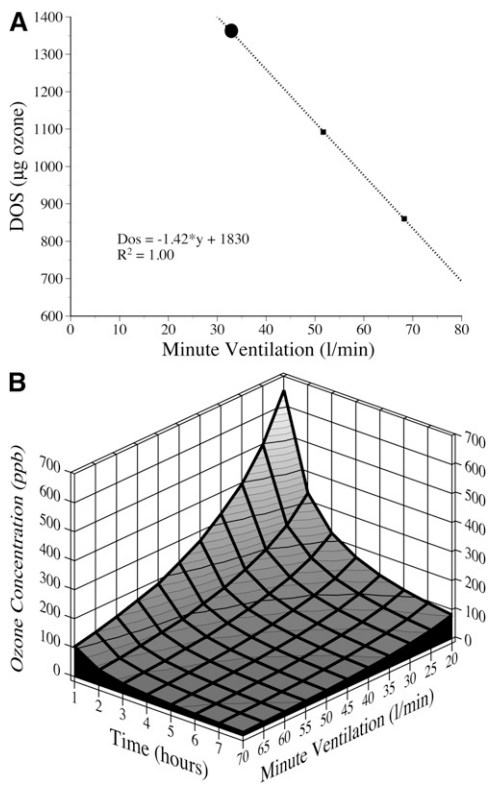

Figure 5. (*A*) Plot of dose of onset (Dos) derived previously by Schelegle and colleagues (12) (*solid squares*) and in the current study (*solid circle*) plotted against mean minute ventilation. (*B*) The three-dimensional surface defined by the relationship between Dos and minute ventilation that predicts the average cumulative dose of ozone in an average young healthy adult, below which no appreciable FEV_1 decrement occurs for exposures varying greatly in ozone concentration, duration of exposure, and minute ventilation. Dos is the cumulative dose of ozone at which approximately half the subjects have developed pulmonary responses and half have not.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript

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