

Ozone Adaptation in Rats after Chronic Exposure to a Simulated Urban Profile of Ozone¹

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Studies in both humans and rats have indicated that certain pulmonary responses induced by exposure to an acute provocative concentration of ozone (O₃) will eventually attenuate if the exposure is repeated on a daily basis. This phenomenon is commonly referred to as O₃ adaptation. Whether or not a "state" of adaptation develops due to long-term low level O₃ exposure is unknown. Two human studies have reported adaptation in subjects living in Los Angeles during periods when ambient O₃ concentrations have been relatively high. At present, however, we are not aware of comparable information from rats. This study assessed O₃ adaptation in rats following chronic (12 or 18 months) exposure and after a 4-month recovery period. A chronic exposure pattern, similar to that found in an urban area during the summer (0.06 ppm O₃ for 13 hr/day, 7 days/week; Monday-Friday, peak to 0.25 ppm O₃, over 9 hr), was used. To assess whether adaptation had occurred and/or persisted, awake rats were challenged with high provocative concentrations of O₃ for up to 2 hr. During a challenge, rats were monitored for typical O₃-induced alterations in spontaneous breathing parameters (e.g., increase in breathing frequency and decrease in tidal volume). Adaptation was defined as attenuation of breathing response during the challenge in rats chronically exposed to O₃ as compared to that in "control" rats (chronically exposed to air). Adaptation was found in the rats within 8 hr following the chronic O₃ exposure but not after the 4-month recovery period. Spontaneous breathing parameters that were significantly attenuated in the chronically exposed rats were breathing frequency, tidal volume, inspiratory and expiratory times, and maximum expiratory flow. We conclude that rats demonstrated adaptation to O₃ after long-term exposure to an urban-type O₃ profile and that the adaptation was not seen 4 months postexposure. These results suggest that exposure to environmental O₃ in

Los Angeles air may have been responsible for the adaptation found in residential subjects. © 1995 Society of Toxicology.

Several studies report that, in humans, the magnitude of the spirometric decrements from an acute provocative exposure of ozone (O₃) will diminish or attenuate if the exposure is repeated on a daily basis (U.S. Environmental Protection Agency, 1986). The phenomenon, an acquired resistance to the nonlethal effects of O₃, has been referred to as adaptation (Mustafa and Tierney, 1978). Besides spirometry, other parameters have shown adaptation, including cellular and biochemical markers in bronchoalveolar lavage (BAL) fluid (Devlin *et al.*, 1993) and airway responsiveness (Dimeo *et al.*, 1981). These studies indicate that with repeated daily exposure, subjects tend to have an augmented pulmonary response on the second exposure day, but a decreasing responsiveness on successive days. However, a more recent study, using different exposure conditions (0.12 ppm O₃, 6.6 hr/day for 5 days), found that the O₃-induced increase in airway responsiveness did not attenuate with repeated exposure (Folinsbee *et al.*, 1990). Subjects in the latter study exercised for 5 hr at a ventilation of 40 liter/min during each exposure.

Adaptation to O₃ has also been reported in rats receiving acute repeated daily exposures of O₃. Tepper *et al.* (1989) exposed rats to 0.35, 0.5, or 1.0 ppm O₃ for 2.25 hr for 5 consecutive days with CO₂ administered periodically during the O₃ exposures to stimulate breathing, thereby increasing the O₃ dose rate. The study found that, by the fifth day, O₃ effects on the spontaneous breathing parameters, frequency of breathing (*f*) and tidal volume (*V_T*), were attenuated. In addition, a flow limitation in the smaller airways, noticed after the second day in the 0.5-ppm O₃ exposure group, had also disappeared by the fifth day. Despite apparent attenuation of these pulmonary physiologic parameters, the 0.5-ppm O₃-exposed rats continued to show a progressive pattern of lung epithelial damage, inflammation in terminal airways, and a sustained increase in lavageable protein over the 5 days. Furthermore, rats exposed to

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1.0 ppm did not show attenuation of any of their respiratory responses. This study suggests that the attenuation of respiratory responses and cellular and biochemical responses are not coincident and are likely to be dependent on exposure parameters. On the other hand, Van Bree *et al.* (1989) examined cellular and biochemical responses in lungs of rats exposed to 0.4 ppm O₃ for 12 hr for 3 consecutive nights. They reported that the rats developed acute pulmonary injury and inflammation following the first nocturnal exposure; however, the injury appeared to be reversible, despite continuous exposure.

Even though the human and rat studies are largely in agreement with respect to O₃ adaptation when using relatively acute repeated exposure protocols, one cannot reliably extrapolate the data to predict adaptation after chronic exposure to urban levels of O₃ in either humans or rats.

At present, we are aware of only two studies of humans associating O₃ adaptation with long-term exposure to O₃ as found in polluted urban areas. One study (Hackney *et al.*, 1977a) reported that subjects living in Los Angeles, surmised to have been exposed to high urban concentrations of O₃, were less responsive to an experimental O₃ challenge than were Canadian subjects who lived in an area of very low O₃ concentrations. These investigators suggested that environmental exposure to O₃, and possibly other oxidants present in the Los Angeles air, may have provided these subjects with some degree of adaptation. The other study (Linn *et al.*, 1988) reported that the response to O₃ is a persistent individual characteristic that shows seasonal variability. Subjects who responded to experimental O₃ exposures in the spring appeared to have lost their O₃ sensitivity in the autumn. The authors suggested that these subjects adapted to the high ambient O₃ exposures during the summer/fall and thus had decreased responsiveness in the fall.

The objective of our investigation was to determine whether a long-term exposure to environmentally relevant O₃ concentrations would induce O₃ adaptation in laboratory rats and, if so, whether adaptation persisted. Rats were exposed to O₃ using an urban-like concentration profile for 12 and 18 months and were subsequently tested for adaptation. A portion of the rats, from the 18-month exposure group, were held in filtered air for recovery and were tested 4 months postexposure.

METHODS

Animals

Male, 60-day-old Fischer 344 rats [CDF (F-344) CrI/Br, VAF+, Charles River Breeding Laboratories, Inc., Kingston, NY] were used for both the 12- and 18-month exposures. In order to save time, rats scheduled to be exposed for 18 months and those scheduled for 12 months were purchased separately and delivered to two different facilities. All rats were otherwise treated identically, except where noted.

On arrival, rats were ear-tagged, weighed, and housed in individual wire cages with food (Purina Rodent Lab Chow, St. Louis, MO) and water *ad*

libitum. They were quarantined in barrier-maintained exposure facilities for 10 days, and then randomly assigned to either filtered air or O₃ exposure groups (Table 1).

The health of the rats was monitored over the duration of the chronic exposure. Serum samples, taken from 5% of the rats upon arrival and from sentinel rats during the chronic exposure, were free of antibodies to reovirus Type 3, pneumonia virus of mice, encephalomyelitis virus, Sendai virus, mouse adenovirus, sialodacryoadenitis, Toolan H-1, Kilham rat virus, lymphocytic choriomeningitis, and rat coronavirus. Lung washes from these animals were free of *Mycoplasma pulmonis*, and bacterial cultures from the nasopharynx, trachea, and gut revealed no abnormal findings. Rats were also free of ecto- and endoparasites.

Chronic Exposure Facilities and Regimen

Exposures were carried out in automatic inhalation exposure facilities designed for chronic exposure of rodents to gaseous pollutants, a complete description of which has been reported by Davies *et al.* (1987).

The 12-month exposure groups were housed in Hazleton-2000 exposure chambers and the 18-month groups were placed in 14.2-m³ walk-in environmental rooms. Ozone concentrations, temperature (74 ± 3°F, SD), humidity (60 ± 10%, SD), and lighting (0600–1800 hr) were automatically monitored and controlled. These parameters were maintained under similar conditions in both chamber types. Air changes in the Hazleton-2000 chambers (37.4/hr) and in the environmental rooms (8/hr) were adequate to maintain equivalent O₃ exposures.

Ozone was generated by passing cylinder-supplied oxygen (National Specialty Gas Co.) through a silent arc O₃ generator (Ozone Research and Equipment Corp., Model 05V-0) and was monitored using continuous chemiluminescent analyzers (Bendix Model 8002) (RFOA-0176-007). Analyzers were calibrated at 2-week intervals using a uv standard (Federal Register, 1976, 1979).

The O₃ exposure consisted of a background concentration of 0.06 ppm with a concentration spike reaching 0.25 ppm on each of the 5 week days (Fig. 1). Only the background concentration was maintained over Saturday and Sunday. The concentration spike increased from 0.06 to 0.25 ppm and back to 0.06 ppm over a 9-hr period. A down time of 2 hr was included in each day for servicing. Integration of the concentration spike showed that the exposure corresponded to a square wave that averaged 0.19 ppm.

The O₃ concentration profile (Fig. 1) continued through Friday of the final week for both the 12- and 18-month studies. On the last day (Sunday) of the 18-month study, some of the rats were placed in filtered air for recovery. All of the 12-month rats and the remaining 18-month rats were tested Monday through Friday during the week following their respective exposures. During these testing periods, the background exposure pattern (0.06 ppm O₃, 23 hr/day) was maintained. Rats designated for recovery were held for 4 months and were tested during the week following this period. On each test day, rats were selected at random and removed from the exposure chambers at 8:30 AM and tested between 11:00 AM and 4:00 PM, approximately 2.5–7.5 hr after leaving the chambers. All rats were tested so that the investigators were blind to previous exposure (O₃ or air), and each exposed group was randomly distributed over time on each testing day.

Tests for Adaptation

Two different challenge testing procedures were used. In Procedure I, individual rats were exposed to 1.0 ppm O₃ for 1 or 2 hr. In Procedure II, four rats were simultaneously exposed to 0.5 ppm O₃ for 2.25 hr with CO₂ added to stimulate ventilation. Reasons for using two procedures were (1) Procedure I was used to test adaptation and to measure percentage O₃ uptake, and (2) Procedure II was used because it had been previously used in our laboratory to differentiate between O₃-adapted and nonadapted rats following different exposure scenarios (Tepper *et al.*, 1989). In Procedure I, 12-month rats were exposed to the challenge for 2 hr to accommodate

TABLE 1
Experimental Groups of Rats and Challenge Protocols

Chronic exposure groups ^a	Challenge protocol			Number of rats/test group
	Type of exposure	Time	Test groups ^b	
Procedure I				
12-A ^c	Air	120 min	12-A/air	4
12-A	1.0 ppm O ₃	120 min	12-A/O ₃	4
12-OZ ^c	Air	120 min	12-OZ/air	4
12-OZ	1.0 ppm O ₃	120 min	12-OZ/O ₃	4
18-A	Air	60 min	18-A/air	4
18-A	1.0 ppm O ₃	60 min	18-A/O ₃	4
18-OZ	1.0 ppm O ₃	60 min	18-OZ/O ₃	5
Procedure II				
18-A	0.5 ppm O ₃ /CO ₂	135 min	18-A/O ₃ + CO ₂	4
18-OZ	0.5 ppm O ₃ /CO ₂	135 min	18-OZ/O ₃ + CO ₂	5
18-A-R	0.5 ppm O ₃ /CO ₂	135 min	18-A-R/O ₃ + CO ₂	4
18-OZ-R	0.5 ppm O ₃ /CO ₂	135 min	18-OZ-R/O ₃ + CO ₂	4

^a Months of chronic exposure: 12 or 18. A, OZ, and R, type of chronic exposure; air, ozone, and recovery in air for 4 months, respectively.

^b Group designation for challenge exposures; Air, air; O₃, ozone; CO₂, 8.0% carbon dioxide added at 15-min intervals.

^c Rats were challenged for 2 hr to accommodate another investigator because the number of chronically exposed rats was very limited.

another investigator and 18-month rats were challenged for 1 hr. Challenge protocols are presented in Table 1.

Some of the equipment and measurement techniques were similar for both procedures. In both procedures, awake rats were individually restrained in acrylic holders (Wiester *et al.*, 1987a), placed in stainless-steel head-out plethysmographs, and exposed to the challenge gas. Spontaneous breathing parameters, collected during the challenge exposures, were derived from thoracic wall excursions inside the plethysmograph, as sensed by an attached pressure transducer (Validyne Engineering Corp.). The resultant analog signal, calibrated in milliliters, was continuously recorded on a polygraph chart and also digitized by an analog-to-digital converter every 4 msec during a 4-sec sample period. Each period was automatically triggered at preselected times. During an epoch, four breaths were analyzed by a computer (PDP 11/03, Digital Equipment Corp.) and averaged by a customized FORTRAN IV program. Measurements of V_T , f , expiratory

minute volume (\dot{V}_E), maximum inspiratory and expiratory flows ($\dot{V}_{I_{max}}$ and $\dot{V}_{E_{max}}$), and inspiratory and expiratory times (T_I and T_E) were computed (Tepper *et al.*, 1988). Following an experiment, polygraph tracings were examined and sample periods showing animal movement artifacts were deleted from the data set.

Procedure I. Details of this procedure have previously been reported (Wiester *et al.*, 1987b; 1988). Briefly, after the rat was placed in the plethysmograph, a glass cone-shaped chamber, designed for nose-only exposure (the apex of the cone, where the nose was located, opened into the side of a vertical glass tube), was placed over the rat's head and sealed around the neck to the plethysmograph.

Ozone was produced by passing oxygen through a uv generator. The 1.0-ppm O₃ concentration was achieved by dilution, using cylinder air (Zero-grade air, Union Carbide Corp., Linde Division, Danbury, CT). The air mixture flowed upward past the nose through the out-flow tube at a rate sufficient to prevent rebreathing of downstream air. Flows were measured using a Gilibrator primary flow calibrator (Gilian Instrument Corp., West Caldwell, NJ). While the rat breathed spontaneously into the system, the downstream concentrations of O₃, O₂, and CO₂ (upstream concentrations were established prior to putting the rat in the system) were monitored once each minute using a photometric O₃ analyzer (Model 8810, Monitor Labs Inc., San Diego, CA), a TCM2 Tc oxygen monitor, and a TCM20 carbon dioxide monitor (Radiometer America Inc., Westlake, OH), respectively.

During each challenge, the rat first breathed zero-grade air for 30 min in order to acclimate it to the testing apparatus. Then, the challenge exposure gas (1.0 ppm O₃ or zero air) was administered for 1 or 2 hr. Along with the spontaneous breathing parameters, oxygen consumption ($\dot{V}O_2$), carbon dioxide production ($\dot{V}CO_2$), and O₃ uptake data were obtained once each minute. Each 1-min measurement of a spontaneous breathing parameter represented mean data from four breaths. Immediately following a challenge, the rat received a lethal intraperitoneal injection of pentobarbital sodium. Data collection continued for 5 min after the cessation of breathing. These nonbreathing gas measurements provided an estimate of O₃ lost to the surface of the face and confirmation that upstream concentrations of O₃, O₂, and CO₂ remained relatively stable over the entire challenge pe-

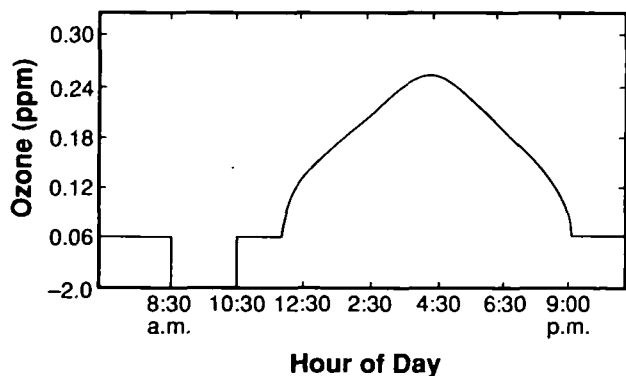


FIG. 1. The simulated urban profile of O₃ concentrations, used during the 12- and 18-month O₃ exposures, are plotted. Rats were exposed to the profile for 5 days each week and to a background level (0.06 ppm) on the remaining 2 days. Integration of the spike portion of the curve determined that the exposure was equivalent to a square wave averaging 0.19 ppm O₃.

riod. Although these changes were usually very small, they were entered into a reanalysis program to correct the data.

Procedure II. In this procedure, rats were challenged four at a time. Four plethysmographs were attached to the door of a 0.33-m³ Rochester exposure chamber so that when rats were placed in plethysmographs and the door closed, the rats' heads were inside the chamber. The challenge regimen consisted of filtered air for 15 min, followed by 0.5 ppm O₃ for 2.25 hr, and then back to filtered air for another 15 min. At four times (15 min each time) during the O₃ challenge, 8% CO₂ was administered concurrently with the O₃. Detailed descriptions of exposure and breathing measurement techniques are reported in Tepper *et al.* (1988, 1989). Spontaneous breathing parameters were collected and analyzed during the last 3 min of each 15-min interval. Each of the 11 samples for one rat was the average of 4–16 breaths.

Data Analysis

Baseline data (Table 2) were analyzed using a multivariate analysis of variance (MANOVA) model. Effects from the chronic O₃ exposure, the exposure plus 4 months of recovery, and those due to time alone were examined.

Challenge data. To normalize pulmonary measurements among rats, baseline values were determined for each rat and the successive measure-

ments, collected during the challenge, were adjusted by these baseline values (the averaged baseline value was subtracted from each successive measurement). Baseline-adjusted measurements are identified with delta (Δ). For Procedure I, the 1-min breathing parameter data were averaged within successive 5-min intervals over the challenge time. These mean values were paired with the midpoint times of their respective intervals. Data from Procedure II were averaged at each of the 11 (3 min) data collection periods. These mean data were paired with respective 15-min time points. A repeated measures analysis of variance was used to determine differences in response between rat groups along a time axis (Figs. 2, 6, and 7). Testing for changes and absolute differences in response between groups was performed through subtesting within the overall MANOVA. The experiment-wise error rate was maintained ($p \leq 0.05$) by adjusting the significance level of each comparison using a Bonferroni adjustment.

Chronic exposure groups were also compared by analysis of the cumulative dose-response curves. The following methods of calculations provided cumulative dose estimates for each rat as the challenge exposure progressed (Figs. 4, 5, and 8).

Procedure I:

$$\text{O}_3 \text{ dose } (\mu\text{g}/\text{min}) = \dot{V}_E \times \text{O}_3 \text{ concentration } (\mu\text{g}/\text{ml}) \times \% \text{O}_3 \text{ uptake.}$$

Dose was accumulated over each successive minute of the challenge period. The cumulative dose was then divided into incremental increases at

TABLE 2
Average Baseline (Prechallenge) Values for All Chronic Exposure Groups

Group Number/group	Rats tested using Procedure I			
	12-A 8	12-OZ 8	18-A 8	18-OZ 5
Body weight (g)	473 ± 7	468 ± 8	396 ± 8 ^b	408 ± 10 ^b
V _T (ml)	3.3 ± 0.2	3.1 ± 0.2	3.9 ± 0.1 ^b	3.7 ± 0.2 ^b
f (breaths/min)	89 ± 3	87 ± 4	85 ± 2	83 ± 2
\dot{V}_E (ml/min)	292 ± 23	274 ± 19	329 ± 12	311 ± 22
T _i (sec)	0.31 ± 0.01	0.36 ± 0.01	0.30 ± 0.02	0.31 ± 0.02
T _e (sec)	0.37 ± 0.02	0.37 ± 0.02	0.40 ± 0.02	0.39 ± 0.01
$\dot{V}_{i_{\max}}$ (ml/sec)	14.8 ± 1.4	12.9 ± 0.7	18.1 ± 1.4 ^b	17.3 ± 1.7 ^b
$\dot{V}_{e_{\max}}$ (ml/sec)	13.5 ± 1.1	13.8 ± 1.1	16.5 ± 0.8 ^b	15.7 ± 1.5 ^b
\dot{V}_{O_2} (ml/min STPD)	9 ± 0.6	9 ± 0.8	10 ± 0.4	10 ± 0.2
\dot{V}_{CO_2} (ml/min SPTD)	10 ± 0.4	9 ± 0.8	8 ± 0.2	10 ± 0.8
Group Number/group	Rats tested using Procedure II			
	18-A 4	18-OZ 5	18-A-R 4	18-OZ-R 4
Body weight (g)	422 ± 15	415 ± 7	400 ± 4 ^b	374 ± 12 ^b
V _T (ml)	2.9 ± 0.3	3.1 ± 0.1	2.6 ± 0.1 ^b	2.4 ± 0.1 ^b
f (breaths/min)	89 ± 3	94 ± 6	97 ± 9	87 ± 5
\dot{V}_E (ml/min)	252 ± 16	286 ± 11	249 ± 22 ^c	206 ± 4 ^c
T _i (sec)	0.29 ± 0.03	0.27 ± 0.02	0.25 ± 0.02	0.29 ± 0.02
T _e (sec)	0.40 ± 0.03	0.38 ± 0.03	0.39 ± 0.03	0.42 ± 0.04
$\dot{V}_{i_{\max}}$ (ml/sec)	15.2 ± 1.2	17.5 ± 1.1	15.6 ± 1.1	13.8 ± 0.6
$\dot{V}_{e_{\max}}$ (ml/sec)	11.5 ± 1.2	11.6 ± 0.7	10.2 ± 1.6	9.7 ± 1.1

Note. V_T, tidal volume; f, breathing frequency; \dot{V}_E , expiratory minute volume; T_i and T_e, inspiratory and expiratory times; $\dot{V}_{i_{\max}}$ and $\dot{V}_{e_{\max}}$, inspiratory and expiratory maximum flows; \dot{V}_{O_2} , oxygen consumed; \dot{V}_{CO_2} , carbon dioxide produced. Data represent means ± SE. Data were obtained at the end of the challenge procedure acclimation period. Significant if $p \leq 0.05$.

^a Effect due to exposure. (Deleted in proof.)

^b Effect due to time.

^c Effect due to exposure × time.

5- μg intervals. Breathing parameter values associated with each dose interval were averaged and these mean values were paired with the midpoints of their respective cumulative dose intervals.

Procedure II:

$$\text{O}_3 \text{ dose } (\mu\text{g}/15 \text{ min}) = \dot{V}_E \times 15 \times \text{O}_3 \text{ concentration } (\mu\text{g}/\text{ml}).$$

Dose was accumulated over each successive 15 min during the O_3 exposure using \dot{V}_E values obtained at the beginning of each measurement sequence (see Fig. 6). The resultant cumulative dose was divided into incremental increases at 10- μg intervals. Mean breathing parameter values (as stated above) were paired with their respective dose intervals.

A repeated measures analysis of variance was used to determine differences between chronic O_3 -exposed and chronic air-exposed rats along the cumulative dose axis (Figs. 5 and 8). Data treatment was similar to that performed on the time-response data.

RESULTS

Analysis of baseline measurements (prechallenge) indicates that, overall, there were no detectable effects from the chronic O_3 exposures on body weight, spontaneous breathing patterns, or exchange of respiratory gases, although \dot{V}_E in Procedure II rats showed a significant effect due to exposure \times time (Table 2). Data for f , T_i , T_e , $\dot{V}\text{O}_2$, and $\dot{V}\text{CO}_2$ were similar among the treatment groups. Time-related effects were detected in measurements of V_T , \dot{V}_{max} , and \dot{V}_{cmax} in rats examined in Procedure I and in V_T for those examined in Procedure II. Body weights among the various temporal groups of rats differed because of differences in housing between facilities and the fact that they derived from different breeding groups, given the number of animals needed to conduct the study. When checked against growth charts provided by the breeder, all groups were within 95% of their normal weight range.

Procedure I

Twelve- and 18-month air-exposed and air-challenged rats (12-A/air and 18-A/air, respectively) served as procedural controls. During the first hour of challenge, Δf and ΔV_T data were not statistically different between 12-A/air and 18-A/air rats. These air challenge data sets were plotted and are enclosed within the gray bands in Fig. 2. The boundaries of these bands represent ± 1 SE around the mean value. Since only the 12-month rats were challenged for 2 hr, the gray bands represent 12-A/air data during the second hour. Although not included in the plots, the Δf and ΔV_T data (mean ± 1 SE) obtained from 12-OZ/air rats also fitted within the respective gray bands over the 2-hr challenge period. The procedural control data indicate that as rats were held in the challenge apparatus and breathing air, they experienced a slight increase in f and a more pronounced but gradual decrease in V_T over the 2-hr period. The fact that the 12-OZ/air rat data also fitted within the band suggests that the 12-month O_3 exposure did not influence the procedural effects on Δf or ΔV_T .

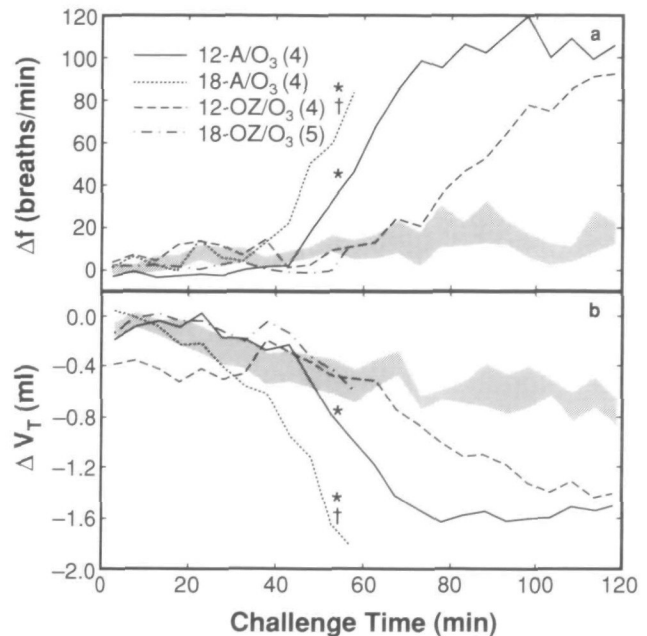


FIG. 2. Procedure I. Line plots in (a) represent Δf and those in (b) represent ΔV_T (V_T , tidal volume; f , breathing frequency) response patterns over time for groups of rats challenged with 1.0 ppm O_3 . The gray bands show the range of data (± 1 SE around the mean) obtained from procedural control rats (12- and 18-A/air). All groups are represented during the first hour, and data collected during this period were analyzed. The Δf and ΔV_T responses were significantly attenuated in 12- and 18-OZ/ O_3 rats after 50 min (*). Significant differences (+) were also found between age groups in that 12-A/ O_3 rats were less responsive to O_3 than 18-A/ O_3 rats. * $p < 0.05$; + $p < 0.05$. Numbers in parentheses are the number of rats per group.

The Δf and ΔV_T responses to 1.0 ppm O_3 in Procedure I for 12- and 18-month rats (i.e., 12-A/ O_3 , 12-OZ/ O_3 , 18-A/ O_3 , 18-OZ/ O_3) are also plotted in Fig. 2. After 50 min of challenge, both the Δf (Fig. 2a) and ΔV_T (Fig. 2b) responses were significantly attenuated in 12-OZ/ O_3 and 18-OZ/ O_3 rats when compared to their respective 12-A/ O_3 and 18-A/ O_3 groups. These results indicate that chronic O_3 exposure induced adaptation. Significant differences in response to the O_3 challenge were found in both the Δf and ΔV_T responses between 12-A/ O_3 and 18-A/ O_3 rats, indicating that the 12-month rats were less sensitive to the O_3 challenge than 18-month rats.

In Fig. 2 the plots show that by the end of the second hour the Δf and ΔV_T responses were comparable in magnitude in both treatment groups (i.e., 12-A/ O_3 and 12-OZ/ O_3) that were represented during that period. These data indicate that the diminished O_3 sensitivity, seen during the first hour in the 12-OZ/ O_3 group, could be overcome with continued high-level O_3 exposure.

To examine this apparent adaptation more closely, plots using the Δf response were constructed for individual rats (Figs. 3a and 3b). These plots show that, by the end of the first hour, all of the 12-A/ O_3 and 18-A/ O_3 rats (Fig. 3a) but

none of the 12-OZ/O₃ and 18-OZ/O₃ rats (Fig. 3b) experienced an increase in Δf . By the end of the second hour, while the 12-A/O₃ rats continued to exhibit a strong response, only two of the four 12-OZ/O₃ rats (animals D and C) exhibited a comparable response. Interestingly, rats A and B remained resistant to the challenge for up to 2 hr.

To see if any or all of these apparent differences in O₃ sensitivity could be attributed to the accumulation of the O₃ dose during the challenge, the Δf data were plotted against the cumulative dose of O₃ for individual rats (Figs. 4a and 4b). These plots indicate that, when the cumulative O₃ dose is taken into account, the 12-A/O₃ and 18-A/O₃ rats are similar in O₃ sensitivity (Fig. 4a). In addition, the two rats that failed to show a response with 2 hr of challenge (rats A and B in Fig. 3b) did not accumulate as much O₃ during the 2-hr time period as the responders (rats C and D).

Cumulative dose-response curve data, over the first hour of the challenge for rats in the 12- and 18-month exposure groups, were tested (12-A/O₃ vs 18-A/O₃, and 12-OZ/O₃ vs 18-OZ/O₃) and were not different. For statistical analysis of the curves, rat data for the 12- and 18-month exposures were combined. These data, illustrated using Δf and ΔV_T responses, are shown in Fig. 5. Both Δf and ΔV_T were significantly attenuated in O₃-preexposed rats after accumulating 12–15 μg O₃. This insensitivity to O₃ continued until the

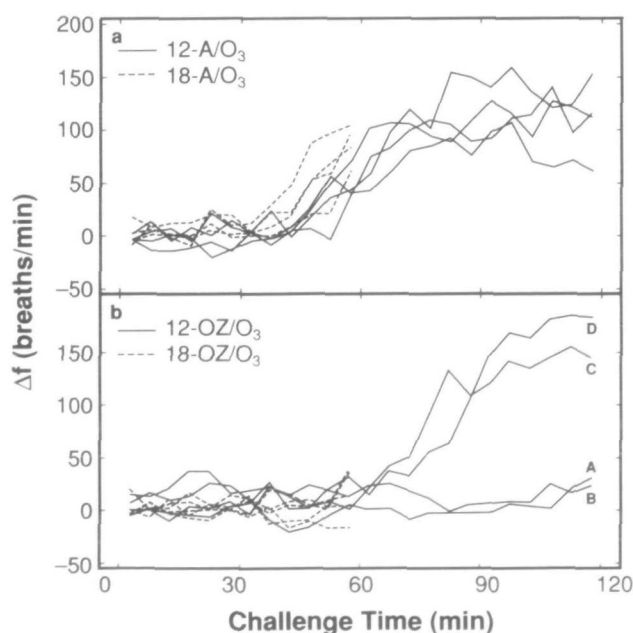


FIG. 3. Procedure I. Plots show Δf (f , breathing frequency) data for individual rats over time using a 1.0-ppm O₃ challenge. (a) By the end of the first hour, all eight 12- and 18-A/O₃ rats had responded and none of the 12- or 18-OZ/O₃ rats had responded to the challenge. (b) Rats A and B did not respond even after 2 hr. The two rats that did respond during the second hour, C and D, responded with the same vigor (rate and magnitude) as rats in (b). Three out of the four 18-A/O₃ rats in (a) responded before the 12-A/O₃ rats.

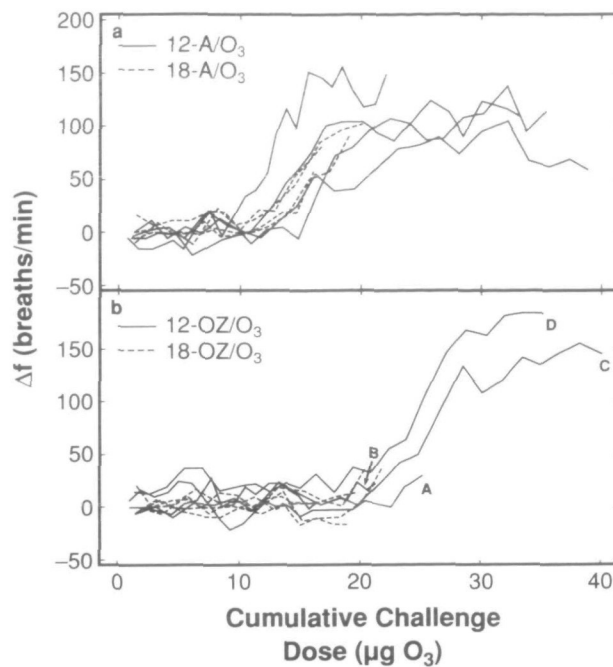


FIG. 4. Procedure I. Cumulative dose-response curves for Δf (f , breathing frequency) in individual rats, challenged with 1.0 ppm O₃, show that 12- and 18-A/O₃ rats are equally sensitive to O₃ (a). In addition, rats A and B (the two nonresponders in Fig. 3b) apparently did not acquire a sufficient O₃ dose to reach a response in 2 hr.

cumulative dose reached 20–23 μg . Response parameters $\Delta \dot{V}_E$, ΔT_i , ΔT_e , $\Delta \dot{V}_{i\max}$, and $\Delta \dot{V}_{e\max}$, $\Delta \dot{V}_{O_2}$, and $\Delta \dot{V}_{CO_2}$ were also tested within the dose-response analysis (data not shown). Among these parameters, significant attenuation in response was found in ΔT_i , ΔT_e , and $\Delta \dot{V}_{e\max}$ in rats preexposed to O₃. In addition, attenuation in response was always seen at the same cumulative dose levels.

In Procedure II, CO₂ administered during the O₃ challenge to stimulate breathing was effective in all four groups (i.e., 18-A/O₃ + CO₂, 18-OZ/O₃ + CO₂, 18-OZ-R/O₃ + CO₂, and 18-A-R/O₃ + CO₂) (Fig. 6). Analysis determined that cumulative ventilation ($\dot{V}_E \times \text{time}$) was equivalent among the groups.

The response to O₃ challenge was attenuated in the 18-OZ/O₃ + CO₂ rats as compared to 18-A/O₃ + CO₂ rats for both Δf and ΔV_T (Fig. 7). Analogous plots of ΔT_i , ΔT_e , and $\Delta \dot{V}_{e\max}$ (not shown) showed the same attenuation in the 18-OZ/O₃ + CO₂ rats. However, analysis determined that none of these differences reached statistical significance. After 4 months of recovery, there was no evidence of O₃ adaptation in 18-OZ-R/O₃ + CO₂ rats (Fig. 7).

Acute O₃ responses in Procedure II rats were reexamined using a dose-response approach similar to that used for the Procedure I rats (as discussed above). These data, represented by Δf and ΔV_T , are shown in Fig. 8. Both responses were significantly attenuated in the 18-OZ/O₃ + CO₂ group

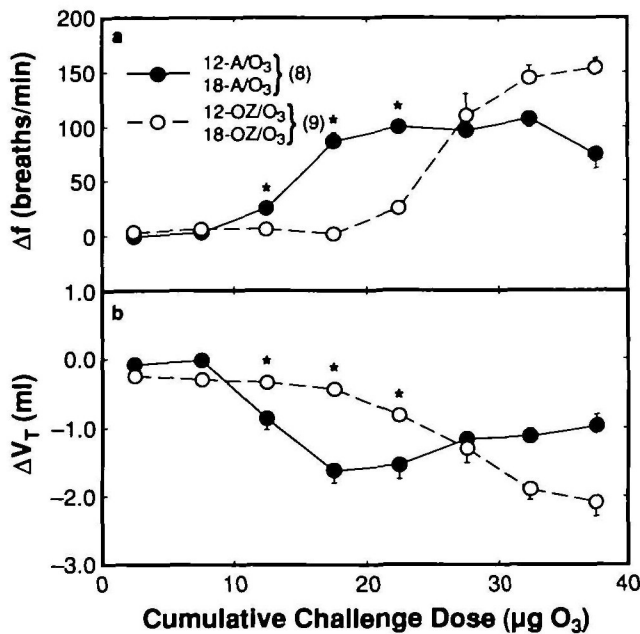


FIG. 5. Procedure I. Group data for Δf and for ΔV_T (f , breathing frequency; V_T , tidal volume) were combined and plotted against cumulative doses for rats challenged with 1.0 ppm O_3 . Analysis of these data shows statistically significant attenuation in O_3 responses for rats previously subjected to the long-term low-level O_3 exposures. Data represent means \pm SE; * $p < 0.05$.

after rats had inhaled 20–25 $\mu g O_3$. Significant attenuation was also seen in ΔT_i and ΔT_e at ~ 15 and $\sim 25 \mu g O_3$ and in $\Delta \dot{V}_{e_{max}}$ at $\sim 25 \mu g O_3$ (data not shown). No effects were observed in $\Delta \dot{V}_E$ or $\Delta \dot{V}_{i_{max}}$. Attenuation was not apparent in any of the responses for 18-OZ-R/ O_3 + CO_2 rats.

DISCUSSION

This work provides evidence indicating that long-term exposure of laboratory rats to O_3 at concentrations found in the urban environment can induce O_3 adaptation. Our findings complement results from two human studies that re-

ported decreased responsiveness to O_3 , using spirometric measurements, in subjects living in Los Angeles (Hackney *et al.*, 1977a; Linn *et al.*, 1988). These authors speculated that their subjects had probably acquired some degree of adaptation after exposure to the high ambient summer levels of O_3 that often occur in this city. How closely the rat and human responses parallel each other is not known. However, among humans, rats, and other laboratory animals, there are a number of common lung responses to provocative concentrations of O_3 . In our study, adaptation was detected using some of these common responses, and the testing for adaptation was done, as it is in human subjects, in unanesthetized spontaneously breathing rats. Thus, the adaptation found in our rats may reflect the adaptation seen in humans after long-term urban exposure. We assessed responsiveness to O_3 in rats by acutely challenging them to a provocative concentration of O_3 and measuring O_3 -induced changes in spontaneous breathing parameters, primarily the increase in f and the decrease in V_T . These O_3 responses are common to both humans (Folinsbee *et al.*, 1975, 1977) and animals (Lee *et al.*, 1979; Murphy *et al.*, 1964; Tepper *et al.*, 1988).

After the rats were chronically exposed for 12 or 18 months to an urban profile of O_3 concentrations, their O_3 responsiveness was significantly attenuated, indicating that they had adapted to O_3 . Attenuation was seen in measures of f , V_T , T_i , T_e , and $\dot{V}_{i_{max}}$ but not in \dot{V}_E , $\dot{V}_{e_{max}}$, $\dot{V}O_2$, or $\dot{V}CO_2$. Similar results were obtained from challenge procedures I and II using parallel groups of chronically exposed animals.

In this study, neither body weight nor spontaneous breathing parameters were affected by the chronic exposure to the urban-like profile of O_3 . A previous report concerning rats from the same chronic exposure groups indicated that pulmonary function was compromised by the exposure (Tepper *et al.*, 1991). In that report, a cohort of unanesthetized rats, each with an intrapleural catheter surgically implanted 1 day prior to testing, was evaluated after 1, 3, 13, 52, and 78 weeks of the same urban profile O_3 exposure.

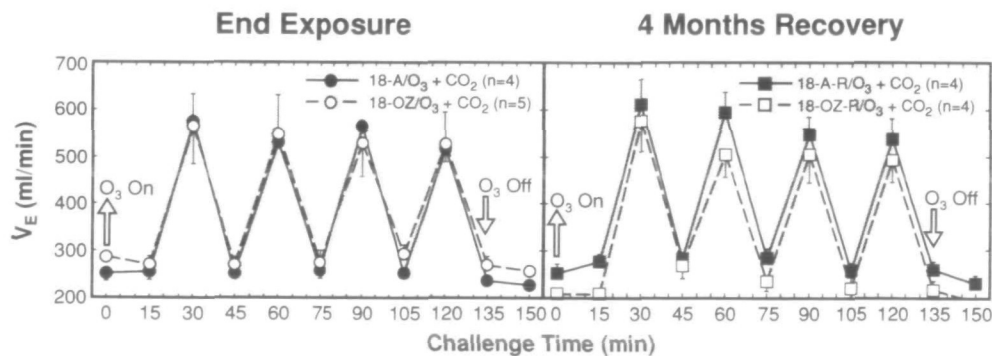


FIG. 6. Procedure II. Plots showing \dot{V}_E (expiratory minute volume) responses for groups of rats challenged with 0.5 ppm O_3 with 8% CO_2 administered at 15-min intervals (gray columns) to stimulate breathing. Results are expressed as means \pm SE.

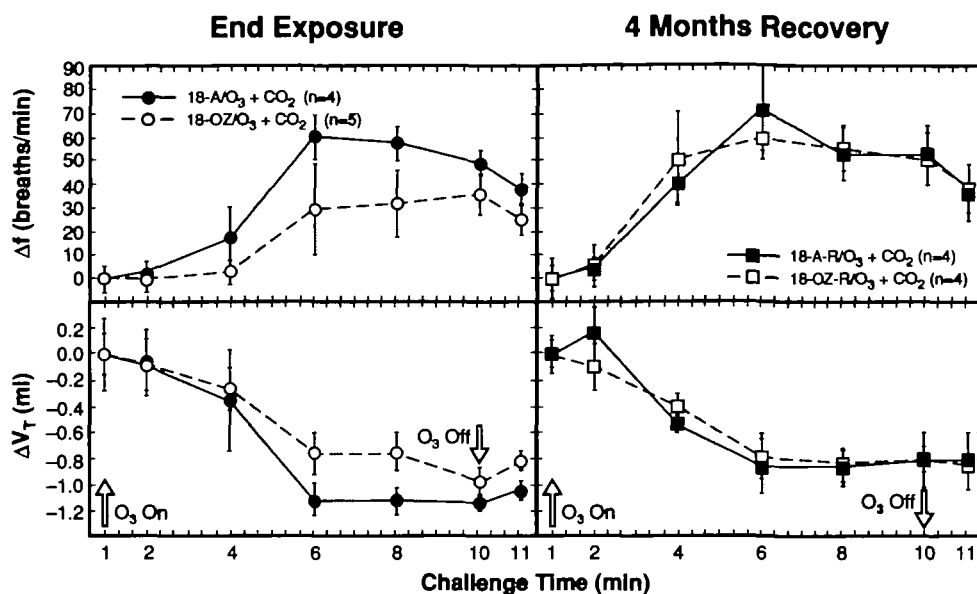


FIG. 7. Procedure II. The Δf and ΔV_T (f , breathing frequency; V_T , tidal volume) responses in rats challenged with 0.5 ppm O₃ plus CO₂ are plotted over time. In the 18-OZ/O₃ + CO₂ group, both responses were attenuated following the chronic exposure, although differences between 18-OZ/O₃ + CO₂ and 18-A/O₃ + CO₂ rats did not reach significance. Recovered rats (18-OZ-R/O₃ + CO₂) showed no effect of the prior O₃ exposure. Results are expressed as means \pm SE.

These rats were challenged with 4 and 8% CO₂ in the same apparatus used in the present study (Procedure II). Tepper *et al.* (1991) found an overall increase in expiratory resistance and a T_i -dependent reduction in f . In the present study, resistance was not measured (because pleural catheters were not implanted), but a T_i -dependent reduction in f

was observed, although it was not statistically significant. This difference may have been due to the difference in data analysis which included the CO₂-produced effects and/or the introduction of an intrapleural catheter for measurement of breathing mechanics in the study by Tepper *et al.* (1991).

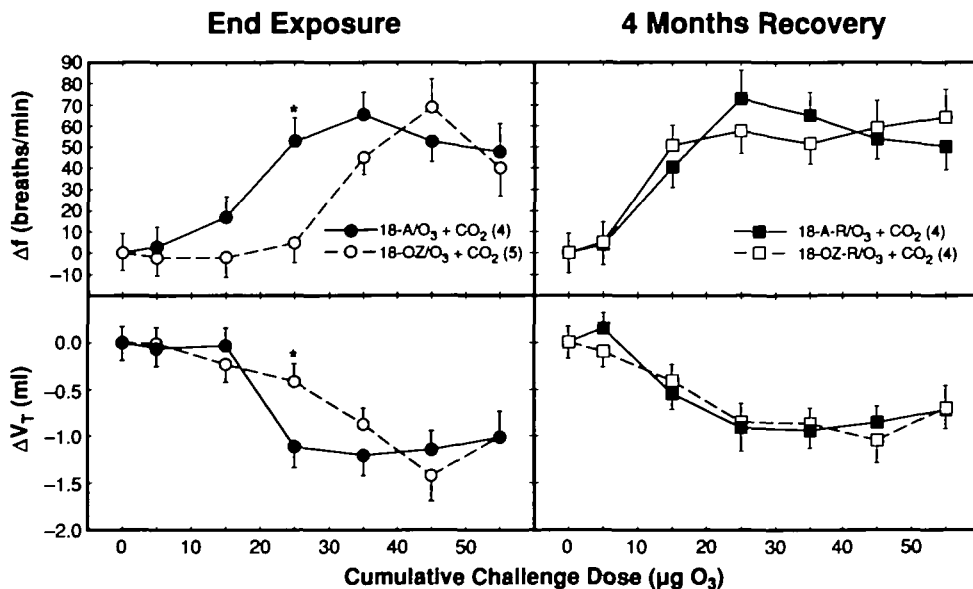


FIG. 8. Procedure II. The Δf and ΔV_T (f , breathing frequency; V_T , tidal volume) responses in rats to challenge with 0.5 ppm O₃ plus CO₂, plotted against the cumulative dose, indicate that both responses were significantly attenuated in the 18-O₃/O₃ + CO₂ rat group by the time they had accumulated 25 μ g O₃. Neither response was attenuated following recovery. Data represent mean values \pm SE; * p \leq 0.05. Numbers in parentheses are the number of rats per group.

A variety of testing methodologies and endpoints have been used to assess adaptation in both animals and humans. There is no clear evidence that adaptation in terms of the spontaneous breathing parameters correlates with, or is mechanistically similar to, spirometrically determined adaptation. There is, however, some evidence that during a provocative O₃ challenge in humans, an increase in f and a decrease in V_T occur concurrently with the decrements in the spirometric measurements. In humans, Folinsbee *et al.* (1977) showed that O₃ exposure caused changes in spontaneous breathing parameters as well as changes in pulmonary function. Subjects exposed to 0.75 ppm O₃ for 2 hr (15-min light exercise alternated with 15-min rest) showed a marked increase in f and a decrease in V_T along with significant reductions in forced vital capacity, forced expiratory volume in 1 sec, maximal expiratory flow at 50% of vital capacity, expiratory reserve volume, and inspiratory capacity. Adaptation in rats (Tepper *et al.*, 1989) has been previously expressed as an attenuation in O₃-induced changes in f and V_T using daily repeated exposures to 0.5 ppm O₃. Changes of forced expiratory data, similar to spirometric changes observed in humans, were also observed. The fact that acute exposure to high concentrations of O₃ causes similar changes in f and V_T in both humans and animals suggests that measurements of spontaneous respiratory responses may be as useful as the spirometric or the airway responsiveness measurements in assessing O₃ adaptation. In humans, Folinsbee *et al.* (1980) exposed subjects to 0.5 ppm O₃ on a daily basis and observed reduced effects in spirometric parameters by the third day and no effects on the fourth day. However, they found no significant changes in the spontaneous respiratory pattern that could be attributed to O₃.

Rats exposed to urban-type O₃ for 18 months and then recovered for 4 months breathing filtered air showed no evidence of O₃ adaptation. In humans, environmentally induced attenuation in O₃ effects may be more persistent. Linn *et al.* (1988) found that the attenuated O₃ responses seen in sensitive subjects in the autumn (late in the high O₃ season) were still attenuated in the winter. Yet Horvath *et al.* (1981), testing subjects exposed to 0.5 ppm O₃ on 5 consecutive days and adapted by the fifth day, found that adaptation lasted, on average, less than 2 weeks and was shortest for the more sensitive subjects. A study in our laboratory showed that adaptation in rats lasted for at least 1 week (Wiester *et al.*, 1991).

At the present time, mechanisms providing O₃ adaptation in humans or in animals have not been wholly explained by either cellular or biochemical studies, although adaptative changes have been reported in these lung parameters. In humans, Devlin *et al.* (1993) showed that acute exposure (0.4 ppm O₃, 2 hr/day)-induced inflammation, measured by the presence of neutrophils in BAL fluid, was completely attenuated by the fifth consecutive exposure

day. Other endpoints (e.g., increases in lactic acid dehydrogenase and elastase content in BAL fluid and a decrease in macrophage phagocytosis) were attenuated but to a lesser degree. In mice, Canning *et al.* (1991) found that O₃-induced reduction in the phagocytic activity of macrophages and increased levels of protein and prostaglandins, recoverable in BAL fluid, tend to resolve with continued O₃ exposure (0.5 ppm O₃ for 14 days). In rats exposed to 0.5 ppm O₃ for 5 days, Tepper *et al.* (1989) reported that even though the respiratory responses to O₃ adapted with repeated exposure over time, there was a progressive pattern of lung epithelial damage, inflammation in terminal airways, and a sustained increase in bronchoalveolar lavageable protein. Van Bree *et al.* (1989), however, examined biochemical and cellular changes in the lungs of rats exposed to 0.4 ppm O₃ for 12 hr for 3 consecutive nights and found that the acute pulmonary injury and inflammation, seen after the first exposure night, were reversible despite continuous exposures.

Adaptation appears to be a series of physiological and biochemical adjustments, shared by a number of species. Although there has been much speculation about various mechanisms that may contribute to O₃ adaptation, data from some studies suggest that antioxidants may play a role. Tepper *et al.* (1989) found that lung ascorbate and glutathione were increased in the BAL fluid from O₃-adapted rats. Jackson and Frank (1984) observed that preexposure of rats to 0.8 ppm O₃ for 7 days made the animals cross-tolerant to hyperoxia. This tolerance corresponded with an increase in lung tissue activity of antioxidants [i.e., superoxide dismutase (SOD), glutathione peroxidase, glucose 6-phosphate dehydrogenase, and catalase]. In the present study, rats chronically exposed for 12 months had significantly increased concentrations of glutathione in whole lung homogenate; however, SOD and nonprotein sulfhydryls were not affected (Grose *et al.*, 1989). In addition, Norwood *et al.* (1989) found that antioxidants in BAL fluid and in lung tissue were changed in rats from these same exposures. Specifically, α -tocopherol in surfactant was decreased after 12 and 18 months, but was increased in lavaged cells and in lung tissue homogenate after 18 months. The ascorbic acid/protein ratio was increased by 85% in lavaged cells after 12 months and by 151% after 18 months. This ratio was also increased in the BAL fluid itself by 133% after 18 months. Wiester *et al.* (1991) recently reported data suggesting a relationship between O₃ adaptation in rats and an increase in ascorbic acid levels in the BAL fluid.

In summary, the rats in this study developed statistically significant O₃ adaptation after long-term exposure to an urban-type O₃ profile. These changes were apparent without concurrent O₃-associated changes in body weight or resting breathing parameters. This adaptation appeared to be reversible because it was not seen after rats had been allowed to recover for 4 months in filtered air. Adaptation

was most clearly observed when the data were normalized for the cumulative O₃ dose, suggesting that some type of competitive antioxidant mechanism may be induced with chronic exposure that is overcome with a sufficient challenge exposure.

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