Adaptation to Ozone in Rats and Its Association with Ascorbic Acid in the Lung


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Ozone (O$_3$) adaptation is a well-known, but poorly understood phenomenon that has been demonstrated in humans and laboratory animals. This study examined pulmonary function and bronchoalveolar lavage fluid (BALF) parameters in O$_3$-adapted F-344 rats to explore possible mechanisms of adaptation. Of particular interest was ascorbic acid (AA), an antioxidant reported to be protective against O$_3$ injury and found to be increased in O$_3$-adapted rats. Adaptation was induced by exposure to 0.25 ppm O$_3$, 12 hr/day for 6 or 14 weeks and evaluated with a challenge test, one that reexposed rats to 1.0 ppm O$_3$ and measured attenuation in the O$_3$ effect on frequency of breathing. Pulmonary function was assessed 1 day postexposure and adaptation and BALF were evaluated 1, 2, and 7 days postexposure. Results showed that forced vital capacity increased over time but decreased due to exposure and that the 14-week, O$_3$-exposed rats had an increase in forced expiratory flow rate. All of the O$_3$-exposed rats that were tested demonstrated adaptation on Postexposure Days 1, 3, and 7, but it was diminished on Day 7. Adaptation was also more pronounced in rats exposed for 14 weeks. Except for AA, BALF levels of total protein, potassium, lysozyme, uric acid, and a-tocopherol were unaffected by O$_3$ exposure. Lactic acid dehydrogenase, alkaline phosphatase, glucose-6-phosphate dehydrogenase, and total glutathione were also assayed but were always below detectable limits. Ascorbic acid concentrations were elevated on Days 1, 3, and 7, showing postexposure patterns similar to those found for adaptation. Significant correlation was found between AA concentration and the magnitude of adaptation ($r = 0.91$, $p < 0.002$). We conclude that AA may play an important role in mechanisms associated with O$_3$ adaptation in rats.

Repeated daily exposure to ozone (O$_3$) can induce an adaptive condition in which functional, biochemical, and cellular responses to O$_3$ are attenuated with additional exposure. In general, the nature of O$_3$ adaptation, its development and endurance, appears to be similar for humans and rats in that it will occur with daily exposure concentrations between 0.2 and 0.4 ppm O$_3$, over 3 to 5 days in humans (Farrell et al., 1979; Dimeo et al., 1981) and in rats (Tepper et al., 1989). Long-term urban-type oxidant exposures also induce and maintain an adaptive state both in humans (Hackney et al., 1977; Linn et al., 1988) and in rats (Wiester et al., 1995). However, the postexposure persistence of adaptation is less certain. It may endure for less than 2 weeks (Horvath et al., 1981; Kulle et al., 1982; Evans et al., 1985) or for as long as a few months (Stockinger and Wagner, 1956; Linn et al., 1988).

Adaptation (sometimes referred to as “tolerance”) has been defined using tests that evaluate different aspects of O$_3$-induced lung dysfunction or injury. Adaptable lung responses include O$_3$ effects on spontaneous breathing in rats (Tepper et al., 1989; Wiester et al., 1995), on forced expiratory measures of pulmonary function in humans (Kulle et al., 1982; Farrell et al., 1979; Horvath et al., 1981) and in rats (Tepper et al., 1989), on bronchoalveolar lavage fluid (BALF) components in humans (Devlin et al., 1993) and in rats (Tepper et al., 1989; Van Bree et al., 1989), and on lung tissue in rats (Evans et al., 1985; Flopper et al., 1994).

At the present time, it is not clear how adaptation develops or which cellular or biochemical mechanisms are involved. An earlier study from this laboratory showed adaptation in rats after 12 or 18 months of daily exposure to an environmental-type O$_3$ profile (Wiester et al., 1995). Results from an extensive examination of the exposure effects in these rats (Chang et al., 1992; Norwood et al., 1989; Tepper et al., 1991; Selegrade et al., 1990) did not provide any obvious link to adaptation except for the increase in ascorbic acid in lung tissue and in BALF (Norwood et al., 1989). A number of studies have suggested that antioxidants may participate in adaptation and, if true, could provide a common mechanism for all of the various adaptable responses.

Inhaled O$_3$ can injure lung tissue by initiating the autooxi-
Adaptation of polyunsaturated fatty acids, inactivating enzymes, and oxidizing hemoglobin (Menzel, 1976). Ascorbic acid in the extracellular lining fluid may potentially protect the underlying cells from oxidative damage by directly reacting with O₃ as well as interacting with and preventing depletion of other antioxidant substrates (Giampaiva et al., 1985).

Antioxidants have been shown to protect the lung from oxidant injury (Bendich et al., 1986; Heffner and Repine, 1989; Pagnoto and Epstein, 1969) and are inducible with exposure (Tepper et al., 1989; Van Bree et al., 1989; Norwood et al., 1989; Slade et al., 1989; Kodavanti et al., 1995) and, therefore, could impart protection from further challenge.

This study investigated the relationship between the magnitude of O₃ adaptation in rats and the associative changes in lavegable antioxidants over 1 week following 6 and 14 weeks of daily exposure to low levels of O₃ to determine if antioxidants, in particular AA, could play a key role in this phenomenon.

METHODS

Animals. One hundred and thirty-six Fischer 344 60-day-old male rats [CDF (F-344) CrlBR, VAF + animals, Charles River Breeding Laboratories, Inc., Kingston, NY] were weighed and ear-tagged upon arrival. Control and exposure groups were weight-matched and housed in stainless steel wire cages. The cages were placed in barrier-maintained 14.2-m² walk-in environmentally controlled exposure rooms 12 days prior to the start of the exposure. The exposure has been previously described (Davies et al., 1987). The lights were turned on at 6:00 AM and off at 6:00 PM. Food (Purina Rodent Lab Chow, St. Louis, MO) and water were available ad libitum and cages were cleaned each day between 9 and 11 AM.

Health evaluations were performed on sentinel animals prior to the study and at the 6- and 14-week time points. The evaluations included murine virus antibody determination, nasopharyngeal/tracheal/lung washing for respiratory tract pathogens, lung tissue histopathology, endoparasite monitoring, and ectoparasite fecal Pseudomonas detection. No evidence of respiratory tract infectious agents was found during the study.

Daily exposure. For the development of O₃ adaptation, a low ambient level of exposure was selected that would be environmentally relevant and cause minimal injury to the lung. An earlier study indicated that exposure to an urban-like concentration profile (0.06 ppm for 13 hr/day, 7 days/week; Monday–Friday, peak to 0.25 ppm O₃ over 9 hr, for 12 or 18 months) resulted in O₃ adaptation in rats, which was observed on the day that they were removed from the exposure (Wieser et al., 1995). Although it was not certain how long it took for adaptation to develop, other studies have shown that exposure to higher concentrations for a week or less can induce adaptation (Tepper et al., 1989; Van Bree et al., 1989). For these reasons, we exposed rats to 0.25 ppm O₃, 12 hr/day for 6 or 14 weeks (between 11:00 AM and 11:00 PM), anticipating that rats may adapt after 6 weeks and probably would after 14 weeks. Ozone was generated by passing cylinder-supplied oxygen (National Specialty Gas Co., Raleigh, NC) through a silent arc O₃ generator (Model OSV-0, Ozone Research and Equipment Corp., Phoenix, AZ). Chamber air was monitored using Bendix Model 8002 (RFOA-0176) analyzers that were calibrated at 2-week intervals using a UV standard. Chamber air was changed eight times per hour which was adequate to maintain a uniform O₃ distribution. Samples for analysis were withdrawn from inside the rat cages at 21 different sites. Ranges for daily averages of O₃ concentrations, chamber temperatures, and relative humidities were 0.218–0.262 ppm O₃, 21.5–24.2°C, and 49.08–61.71%, respectively. Control rats were maintained under similar living conditions and received filtered air.

Pulmonary function. Thirty-nine rats were evaluated for effects of O₃ on pulmonary function on Day 1 following exposures of 6 and 14 weeks, which was 10–16 hr postexposure (Table 1). Prior to testing, each rat was weighed, anesthetized with halothane (2–5% inhaled), intubated with a tracral cannula, and placed in a whole-body pressure plethysmograph. Plethysmographic measurements of static lung volume in rodents in this study system have been described (Raub et al., 1982). Briefly, vital capacity (VC) was measured between airway pressures of −15 and +30 cm H₂O. Total lung capacity (TLC) was obtained by gas dilution methods and residual volume (RV) was computed as the difference between TLC and VC. End expiratory volume (EEV) was measured using an application of Boyle’s law. Multibreath diffusing capacity of carbon monoxide (DLCO) was also obtained by a modified gas dilution technique (Takezawa et al., 1980).

Respiratory system compliance (CR) was measured during slow deflation (3 ml/sec) from TLC to RV. The data are reported as the slope (and intercept) from a nonlinear least-squares regression of the deflation wing of the quasi-static pressure–volume curve.

Small airway integrity was evaluated by examining forced expiration (Tepper et al., 1987). The lung was slowly inflated to +30 cm H₂O and then rapidly evacuated at −40 cm H₂O pressure. From the resultant maximum expiratory flow–volume curve, forced vital capacity (FVC), peak flow, and flow at 50% (FEE50), 25% (FEE25), and 10% (FEE10) of the remaining FVC were analyzed for treatment differences. Additionally, the delta flow at 25% (ΔFEE25) of the remaining FVC was calculated by subtracting the predicted value of FEE25 (FEE25-pred) from the actual FEE25. This value indicates the deviation from the normal convexity of the effort-independent portion of the curve and has been shown to decrease with O₃ exposure (Tepper et al., 1989).

Bronchoalveolar lavage. On Postexposure Days 1, 3, and 7, and after both 6 and 14 weeks of O₃ exposure, a second group of rats was anesthetized with an ip injection of urethane (1.25 g/kg) and tranorally intubated (Table 1). Air was partially evacuated from the lung by connecting the tracheal cannula to a negative-pressure reservoir (−23 cm H₂O) for approximately 10 breaths (Fig. 1). The lung was then connected to a positive-pressure reservoir (+23 cm H₂O) and allowed to fill with 37°C normal saline for 60 sec. The lung was again connected to the negative-pressure reservoir and BALF was evacuated at −23 cm H₂O for 90 sec. Afterward, the rat was disconnected from its tracheal cannula so that the BALF, remaining in the cannula and tubing, would flow into the sample tube. Gravimetric measurements of infused saline volumes were compared to gas dilution measure-
FIG. 1. The bronchoalveolar lavage system. The saline infusion portion of the system consists of a saline reservoir with two glass tubes that penetrate the stopper. The bottom of the tubes are placed 23 cm above the chest of the rat. For lavage, saline flows from the reservoir into the rat lung or to waste via one two-way and one three-way stopcock. The stainless steel three-way stopcock has an added 20-gauge tube, 1 cm long. The tube was placed distal to the male hub that holds the tracheal cannula. The tube serves to connect the lung to the negative pressure reservoir and the BALF collection container. Negative pressure, provided by house vacuum, is controlled at $-23$ cm H$_2$O using a pressure switch. The tracheal cannula, suitable for ~300-g rats, is made from a 13-gauge tubing adaptor (Becton-Dickinson and Co., Rutherford, NJ) extended to 4 cm, with polyethylene tubing.

ments of TLC and RV. The saline measurements were based on the assumption that, at transpulmonary pressures between $\pm 23$ cm H$_2$O, 1 g fluid occupies 1 ml of lung space. Formulas for the fluid lung volume measurements are as follows: $RV = [\text{rat weight after lavage}] - [\text{rat weight before lavage}]$, $TLC = \text{weight of collected BALF} + RV$. Fluid values of TLC were determined to be equivalent to gas dilution volumes of TLC (see Appendix).

**BALF analysis.** After removing an aliquot for a differential cell count, BALF was centrifuged at 400g for 15 min at 4°C. A 1-ml sample of cell-free supernatant was mixed with 35 $\mu$A of 60% perchloric acid for assay of ascorbic acid (AA), uric acid (UA), and total glutathione (GSH). This mixture was centrifuged at 20,000g for 20 min at 4°C and the supernatant was stored at $-80$°C. AA and UA were analyzed by liquid chromatography with electrochemical detection (LCEC) according to Kutnink et al. (1985). $\alpha$-Tocopherol was also analyzed by LCEC by electrochemical detection (Vanderwoude et al., 1984). Data were collected and analyzed using a Nelson Analytical 3000 Series chromatography data system (Cupertino, CA).

The following assays were modified for use on the Centrifichem System 500 centrifugal spectrophotometer (Baker Instruments, Allentown, PA) and chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Total GSH content was determined by DTNB-GSSG reductase recycling assay, a modification of the method of Anderson (1985). The reagent contained 10 mg of reduced nicotinamide adenine dinucleotide phosphate (NADPH), 6 mM 5,5’-dithiobis-2-nitrobenzoic acid (DTNB), glutathione reductase, in a 0.143 M sodium phosphate, 6.3 mM ethylenediamine tetracetic acid (EDTA), pH 7.4 buffer. Sample concentration of GSH was determined from a standard curve. Protein concentration was determined using the Bio-Rad method (Bio-Rad Laboratories, Richmond, CA). Sample protein concentration was determined from a standard curve using bovine serum albumin (BSA) standards. Alkaline phosphatase and lactate dehydrogenase (LDH) were determined using commercially prepared kits. Lysozyme activity was determined by measuring the initial rate of lysis of a 1.38 mg/ml Micrococcus lysodeikticus cell wall suspension in a 0.05 m sodium phosphate buffer at pH 6.0. A standard curve using hen egg-white lysozyme (EWL) was employed. The method was modified from Konstan et al. (1982). Glucose-6-phosphate dehydrogenase (G-6-PDH) assays were performed using 1.2 mM glucose 6-phosphate, 0.15 mM NADP, and 50 mM triethanolamine–HCl, pH 7.5, as modified from Mustafa et al. (1977).

Slides for cell counts were prepared using a Shandon Cytospin (Southern Products Ltd., Astmoor, Runcorn, Cheshire) and stained with LeukoStat (Fisher Scientific Co., Pittsburgh, PA). Cell-free BALF was assayed for potassium (K$^+$) using Astra-4 (Beckman Instrument Inc., Brea, CA).

**Assessment of adaptation.** Rats naive to O$_3$ exposure typically increase frequency of breathing ($f$) when acutely challenged. Rats previously exposed to O$_3$ show an attenuation in the magnitude of this response (Tepper et al., 1989) and/or a delay in the onset of the effect on frequency (Wiester et al., 1995). The time from the beginning of the challenge exposure until $f$ doubled was used to determine whether rats were adapted to O$_3$. It was assumed that the time to double $f$ was proportional to the magnitude of adaptation.

Acute O$_3$ challenges were performed on Days 1, 3, and 7 after both the 6- and 14-week exposures (Table 1). For the acute challenge, O$_3$ was generated by passing O$_2$ through a series of ultraviolet lamps. Compressed zero-grade air was added to obtain the desired concentration (1.0 ppm) at an airflow of 2.7 lpm. Ozone concentration was monitored using an ultraviolet O$_3$ monitor (Dasibi Environmental, Glendale, CA) that was periodically...
O$_3$ ADAPTATION AND ASCORBIC ACID

TABLE 2

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Air</th>
<th>Ozone</th>
<th>Air</th>
<th>Ozone</th>
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<td>10</td>
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<td>10</td>
<td>9</td>
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<tr>
<td>Body weight (g)</td>
<td>284 ± 5</td>
<td>272 ± 5</td>
<td>338 ± 5*</td>
<td>331 ± 5*</td>
</tr>
<tr>
<td>TLC (ml)</td>
<td>10.8 ± 0.2</td>
<td>10.4 ± 0.2</td>
<td>12.2 ± 0.2a</td>
<td>11.8 ± 0.2a</td>
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<tr>
<td>RV (ml)</td>
<td>1.17 ± 0.09</td>
<td>1.28 ± 0.09</td>
<td>1.20 ± 0.09</td>
<td>1.35 ± 0.09</td>
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<tr>
<td>$C_n$ (ml/cm H$_2$O)</td>
<td>0.54 ± 0.03</td>
<td>0.60 ± 0.03</td>
<td>0.66 ± 0.04a</td>
<td>0.71 ± 0.03a</td>
</tr>
<tr>
<td>$V$ (ml)</td>
<td>3.12 ± 0.15</td>
<td>3.87 ± 0.13</td>
<td>3.05 ± 0.17</td>
<td>3.07 ± 0.06</td>
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<tr>
<td>DL$_{co}$ (ml/min/mm Hg)</td>
<td>0.15 ± 0.01</td>
<td>0.14 ± 0.004</td>
<td>0.16 ± 0.01*</td>
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<tr>
<td>FVC (ml)</td>
<td>8.94 ± 0.23</td>
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<td>10.44 ± 0.23</td>
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<tr>
<td>PEF (ml/sec)</td>
<td>115 ± 3</td>
<td>113 ± 4</td>
<td>125 ± 2a</td>
<td>124 ± 6a</td>
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<tr>
<td>FEF$_{50}$ (ml/sec)</td>
<td>81.4 ± 4.04</td>
<td>88.7 ± 2.64</td>
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<tr>
<td>FEF$_{25}$</td>
<td>40.1 ± 2.74</td>
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<td>FEF$_{10}$ (ml/sec)</td>
<td>21.6 ± 1.19</td>
<td>21.3 ± 1.68</td>
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<td>29.4 ± 2.0a</td>
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</table>

*Effects due to exposure.
aEffects due to time.
*Effects due to exposure × time.

TABLE 2

Effects of Ozone Exposure on Pulmonary Function

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*Effects due to exposure × time.

calibrated against a primary reference standard. Steady-state exposure concentrations never exceeded 10% of the target value.

Rats were exposed nose-only while being restrained in tubes that had been converted into flow plethysmographs. Differential pressure (pressure inside the plethysmograph referenced to inside the exposure chamber) was sensed by a pressure transducer (Validyne, Northridge, CA) and amplified (Hewlett-Packard, Chelmsford, MA), and the resultant signal was digitized and analyzed by commercially available software (Po-Ne-Mah, Simsbury, CN). Baseline $f$ measurements in filtered air were obtained during the first 15 min after which 1.0 ppm O$_3$ was introduced. Exposure continued until each rat doubled baseline $f$. When this occurred, the rat was quickly removed from the O$_3$ exposure and immediately subjected to the lavage procedure described above.

Statistics. A multivariate analysis of variance (MANOVA) (SAS 516, SAS Institute, Cary, NC) was used to evaluate the overall effect of O$_3$ exposure on the dependent variables (i.e., responses in pulmonary function and BALF). The independent variables used in the analysis were the challenge (air and O$_3$), exposure (air, 1, 3, and 7 days post-O$_3$ exposure), and week (6 and 14). Upon finding an overall O$_3$ effect, an ANOVA was performed on each response variable to determine which were affected and the nature of the effect. Where significant effects on an individual response were found, pairwise comparisons were made among combinations of levels of the independent variables. Pearson correlation coefficients were calculated to evaluate the relationship between adaptation and the remaining lung parameters. Type 1 error rate for each ANOVA was set at 0.05. The significance levels of multiple comparisons were adjusted, using a modified Bonferroni correction, to maintain the Type 1 error rate of 0.05. Data are displayed as the means ± SE.

RESULTS

Six- and 14-Week O$_3$ Exposure Effects

Pulmonary function, Day 1. Forced vital capacity increased over time, but was reduced in the O$_3$-exposed rats at 6 and 14 weeks, resulting in a significant time × exposure effect (Table 2). A significant O$_3$ effect was observed at 14 weeks as an increase in FEF$_{25}$ and the calculated ΔFEF$_{25}$. No other changes in pulmonary function, related to O$_3$ exposure, were noted. Comparison between the 6- and 14-week rats showed significant time-related effects on body weight, TLC, $C_n$, DL$_{co}$, PEF, and FEF$_{10}$.

Adaptation, Days 1, 3, and 7 postexposure. Prior to the acute O$_3$ challenge, baseline resting values for $f$ were not different among the various groups of rats, averaging 88 ± 2 SE breaths/min. During a challenge exposure the O$_3$ effect on $f$ eventually caused a twofold increase (i.e., $f$ response) and the time required for this to occur was recorded in minutes. These time periods were averaged for each treatment group and are plotted in Fig. 2. Data show that rats previously exposed to O$_3$ had significant delays in $f$ response times averaging ∼15–50 min, indicating that they had developed some degree of resistance to the effects of O$_3$ or were adapted. These times were significantly greater for all of the O$_3$-exposed rats, compared to air-exposed rats. Delays at 14 weeks were more pronounced on Days 1 and 3 and appeared to be longer than that seen for 6-week rats; however, the differences did not reach significance. There appeared to be a diminished $f$ response by Day 7 postexposure.

BALF, Days 1, 3, and 7 postexposure (from nonchallenged rats). Among the various BALF components assayed on Day 1, only AA was affected by O$_3$ (Table 3). However, it is not clear how LDH, alkaline phosphatase, G-6-PDH, or GSH responded because they were below detectable limits. With the methodology used in this study, minimal detectable concentrations were LDH ≤20 U/liter, alkaline phosphatase ≤0.0001 U/liter, G-6-PDH ≤50 nm NADP + reduced/min/ml, and GSH <6.2 μg/ml. Significant time effects (i.e., con-
FIG. 2. Adaptation, expressed as a delay in $f$ response time, occurred in all groups of O$_3$-exposed rats when compared to air-exposed controls. The time required for each group to respond to the challenge O$_3$ exposure is shown on the $X$ axis. Data points show the means ± SE for each 6- and 14-week treatment group with the open circle and triangle representing air-exposed rats and the solid circles and triangles representing O$_3$-exposed rats. *Different from the respective air-exposed group ($p < 0.05$).

concentrations were greater in 14-week rats) were seen for K$^+$, lysozyme, and uric acid, whereas exposure × time effects were found for AA. α-Tocopherol, not measured in 6-week rats, was not affected by 14 weeks of O$_3$ exposure. Total protein was not affected by exposure or time. Other than AA, BALF concentrations for the various components were not different for Days 1, 3, or 7 (data for Days 3 and 7 are not shown).

Ascorbic acid levels in BALF were significantly increased following exposure and remained elevated for 1 week (Fig. 3). They were highest on Days 1 and 3 postexposure and, overall, concentrations were greater for 14-week rats than for 6-week rats.

FIG. 3. Ascorbic acid concentrations in BALF following the 6-week (solid circles) or 14-week (solid triangles) low-level O$_3$ exposure. Values are presented as means ± SE. a, significantly different ($p < 0.05$) from the corresponding air controls; b, difference between the 6- and 14-week O$_3$-exposed rats.

BALF, immediately following O$_3$ challenge, Days 1, 3, and 7 postexposure. The 1.0 ppm O$_3$ challenge exposure caused a decrease in the AA concentration for all rat groups (Fig. 4). These decreases reached significance on Postexposure Days 1 and 3 for both 6- and 14-week rat groups. The challenge caused UA to increase, however not significantly, in 14-week rats but had no effect on 6-week rats. There was no challenge exposure effect on total protein, K$^+$, lysozyme, or α-tocopherol and the measures of LDH, alkaline phosphatase, G-6-PDH, and GSH remained below detectable limits (data not shown).

DISCUSSION

Rats exposed daily to low levels of O$_3$ for 6 or 14 weeks were significantly less sensitive to O$_3$ challenge (i.e., O$_3$-

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**TABLE 3**

Effects of Ozone on Bronchoalveolar Lavage Fluid Obtained on Day 1 Postexposure

<table>
<thead>
<tr>
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</tr>
<tr>
<td>No./group</td>
<td>5</td>
<td>6</td>
<td>6</td>
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</tr>
<tr>
<td>Total protein (mg/ml)</td>
<td>0.14 ± 0.02</td>
<td>0.14 ± 0.01</td>
<td>0.11 ± 0.02</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>K$^+$ (mg/dl)</td>
<td>0.92 ± 0.06</td>
<td>0.92 ± 0.10</td>
<td>1.18 ± 0.11$^*$</td>
<td>1.08 ± 0.07$^*$</td>
</tr>
<tr>
<td>Lysozyme (μg/ml)</td>
<td>49.2 ± 2.9</td>
<td>43.4 ± 1.5</td>
<td>52.7 ± 2.6$^*$</td>
<td>51.5 ± 1.2$^*$</td>
</tr>
<tr>
<td>Ascorbic acid (ng/ml)</td>
<td>3389 ± 270</td>
<td>5618 ± 437$^*$</td>
<td>4432 ± 299</td>
<td>9433 ± 662$^{**}$</td>
</tr>
<tr>
<td>Uric acid (ng/ml)</td>
<td>212 ± 54</td>
<td>311 ± 65</td>
<td>645 ± 102$^*$</td>
<td>691 ± 147$^*$</td>
</tr>
<tr>
<td>α-Tocopherol (ng/ml)</td>
<td>—</td>
<td>—</td>
<td>1292 ± 65</td>
<td>1361 ± 83</td>
</tr>
</tbody>
</table>

*Note. Air, filtered air; ozone, 0.25 ppm O$_3$ 12 hr/day. Values represent means ± SE. Significance, $p < 0.05$.

$^*$ Effects of exposure.

$^*$ Effects of time.

$^*$ Effects of exposure × time.
adapted) than rats previously exposed to filtered air. Adaptation persisted for at least 3 days but was diminished by the seventh day. Rats exposed to O₃ for 14 weeks appeared to be less sensitive to O₃ than rats exposed for 6 weeks, suggesting that there may be a quantitative aspect to adaptation.

The low-level O₃ exposure for 6 or 14 weeks did not affect body weight or the pulmonary function measures of TLC, RV, f, C₅₀, EEV, DL₅₀, PEF, FEF₅₀, or FEF₁₀₀. Two significant exposure effects on pulmonary function were found: an increase in ΔFEF₂₅ at 14 weeks and an exposure × time effect on FVC, resulting in a decrease in FVC. These effects, though suggestive of a restrictive lung lesion that is likely to be predominantly interstitial, would seem unlikely to modify O₃ sensitivity and, therefore, would not relate to adaptation.

Also, the exposure did not affect concentrations of total protein, K⁺, lysozyme, UA, or α-tocopherol in BALF. Concentrations of LDH, alkaline phosphatase, G-6-PDH, and GSH were consistently below detectable limits in BALF obtained from the O₃- or air-exposed rats. Although some of these constituents (e.g., α-tocopherol, G-6-PDH, and GSH) would possibly be elevated in lung tissues, our concern was directed at the air–fluid interface of the lung lumen where O₃ most likely reacts and triggers the irritant f response. In contrast, AA concentrations were significantly increased in BALF obtained from O₃-exposed rats on Postexposure Days 1, 3, and 7. In addition, AA was significantly increased in BALF after 14 weeks of O₃ exposure, when compared to 6 weeks. The patterns for the week-long postexposure changes in AA (Fig. 3) were similar to those seen for adaptation (Fig. 2).

To test for a possible relationship between the f response time and the concentration of AA in BALF, a correlation analysis was performed. Because the acute challenge exposure affected AA levels in BALF, both the response time and the prechallenge concentration of AA could not be measured in the same animal. Therefore, for the analysis, the f response times were averaged for each group of challenged rats and the AA concentrations were averaged for each group of nonchallenged rats and the groups were paired for treatment and time, respectively (e.g., average f response time for 14-week O₃-exposed rats on Day 1 was 84.7 min and the AA concentration was 9433 ng/ml), resulting in eight pairs of data. Analysis indicated that the time required for a rat to exhibit an f response was significantly correlated with the concentration of AA in BALF (r = 0.91, p < 0.002) as shown in Fig. 5 and suggests a strong association between AA concentration and adaptation in rats.

The AA concentration in BALF was significantly reduced immediately after an acute O₃ challenge exposure without evidence of O₃ effects on the other end points that were assayed in this experiment. An estimate for the rate of loss of AA during an effective exposure (f doubled) was computed for normal (i.e., O₃-naïve) rats during exposure to 1.0 ppm O₃. Ascorbic acid potentially decreased at a rate of ~35 ng/min in lung lining fluid (as reflected in BALF) in these rats. The rate of loss did not appear to be related to

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FIG. 4. Concentrations of ascorbic acid in BALF immediately following O₃ (solid circles) or air challenge (open circles). Values represent the means ± SE with *indicating a significant (p = 0.05) difference between the O₃-challenged group and its respective control.

FIG. 5. Relationship between the concentration of ascorbic acid in BALF and the time required for a rat to manifest an f response (double breathing frequency) during challenge with 1.0 ppm O₃.
the initial AA concentration, which ranged between ~3390 and ~9430 ng/ml for all groups of unchallenged rats. However, the threshold of the f response may be related to the initial AA concentration (Fig. 4) because greater concentrations of AA were found after the challenge in the O3-adapted rats, compared to O3-naive rats.

Although it is not clear how AA in the lung lining fluid could modify the expression of an O3-induced effect (e.g., delay the f response) or what the biological links between AA and adaptation may be, our results indicate that AA is very sensitive to O3 exposure and that there is a substantial association between the AA in BALF and adaptation. It is not known whether a delay in the f response to the challenge was related to a decrease in cell injury because epithelial lining cells were not directly examined. We measured protein levels in BALF, an index of injury (Hu et al., 1982), immediately following the challenge and found no effect, although this particular assay is far more sensitive to an acute exposure if delayed 10–15 hr (Hu et al., 1982).

The potential for an inhaled irritant gas, like O3, to generate resistance against its own toxic action is partially governed by its solubility in tissue fluids. The less soluble of irritant gases, referred to as deep lung irritants, readily reach the bronchiolar and alveolar regions and are typically capable of producing adaptation (Fairchild, 1967). This suggests that O3 adaptation may be initiated within the peripheral lung. Morphological evidence in rats may be in agreement with this statement. Evans et al. (1985) associated O3 adaptation with a decrease in the surface area of type I epithelial cells along with an increase in cellular volume with respect to that surface. They suggested that adaptation exists when the exposed cell surface area is small enough so that the antioxidant mechanism, within the cell, can protect it. Interestingly, a study by Barry et al. (1985) exposed Fischer 344 male rats to 0.25 ppm O3, 12 hr/day for 6 weeks, similar to the 6-week experiment in our study, and reported significant increases in type I cells in the proximal alveolar region that were smaller in volume, covered less surface area, and thicker than type I cells from air-exposed controls.

Plopper et al. (1994) exposed rats to 0.0, 0.12, 0.5, or 1.0 ppm O3 6 hr/day, 5 days/week, for 20 months and killed them 7 days postexposure. Although adaptation was assumed, based on our results, it is probable that the rats had adapted and were still O3-adapted when killed. They reported that the terminal bronchiolar epithelium of the centriacinar regions was reorganized in favor of the nonciliated cell population and suggested that the reorganization plays a role in adaptation produced by long-term exposure. In addition, they found that regional differences in epithelial response were site-specific and correlated with the predicted O3 dose (Overton and Miller, 1988) at those sites.

In a large chronic study in rats, adaptation was found after 12 or 18 months of exposure to an ambient pattern of O3 (Wiester et al., 1995). Lung tissue from the proximal alveolar region of the 18-month rats, examined by Chang et al. (1992), had a 17% increase in the volume of type I epithelium and a 46% increase in type I cell numbers, each with less surface area per cell. A hyperplastic response in type II cells was found with a 91% increase in volume of type II epithelium. These epithelial responses were diminished after the 4-month recovery period, a time when adaptation also was not seen (Wiester et al., 1995). Norwood et al. (1989) reported that the AA/protein ratio was increased by 85% in BALF cells after 12 months and by 151% after 18 months. In addition, the AA/protein ratio in BALF was increased by 133% after 18 months.

The above studies suggest that adaptive mechanisms for O3 in the rat may be mediated via the nonciliated epithelial cells located in the peripheral lung. Although a specific site for an AA response to O3 is not known, its synthesis, storage, and/or transport activities may be related to the observed epithelial changes.

Our results indicate that the effects of O3 exposure on AA levels in BALF are confounding (i.e., the acute challenge exposures caused AA levels to decrease and the 6- and 14-week daily exposures caused AA to increase). These effects may be related to each other with regard to the genesis and persistence of adaptation in rats. The following discussion offers a hypothetical explanation for this relationship. Although AA does appear to participate in O3 adaptation, it is probably not the only antioxidant that is active in this respect (Tepper et al., 1989; Jackson and Frank, 1984) nor does it necessarily protect the lung from all O3 effects (Tepper et al., 1989; Folinbee et al., 1994). Slade et al. (1989), using guinea pigs, suggested that the degree of protection afforded lung tissue by AA is dependent on the concentration of O3 and that AA may only provide protection at lower O3 concentrations like those encountered in the environment. Similarities between humans and rats with respect to protection by AA from O3 toxicity and AA participation in adaptation are not known. Although most animal species synthesize AA, humans, other primates, and guinea pigs do not and require dietary supplementation. However, AA is stored in the body; the half-life in humans is ~16 days and in guinea pigs ~4 days. Kodavanti et al. (1995) have shown in guinea pigs that with daily O3 exposure even AA-deficient animals will have increased levels of AA in BALF relative to deficient controls. Bui et al. (1992) studied cigarette smokers (exposed daily to a variety of oxidizing agents) and found that AA levels in BALF were significantly elevated over AA levels found in nonsmokers. These studies suggest that even though the source of AA is different among species, AA may serve a similar function. A number of additional functional, cellular, and biochemical O3 responses have also been proposed as mechanisms responsible for adaptation (Gertner et al., 1983; Rao et al., 1985; Dungworth et al., 1975).
Results showed that the lavage technique measurements of TLC were not different from gas dilution measurements for rats exposed to air or O₃ for 6 or 14 weeks. Lavage technique measurements of RV were in agreement with the gas dilution measurements at 6 but not at 14 weeks. Unlike body weight and TLC, the gas dilution measurements of RV were not increased in 14-week rats. However, measurements obtained using the lavage system were significantly larger after 14 weeks. The reasons for these findings in RV are not clear but may be related to differences between how air (gas dilution) or liquid (lavage) influences the elastic recoil properties of the lung at −23 cm H₂O.

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APPENDIX

The lavage technique generated measurements of TLC and RV. These calculations assumed that the total volume of saline that entered the degassed lung at +23 cm H₂O was equivalent to TLC and that the volume of liquid remaining in the lung, after evacuation at −23 cm H₂O, was equivalent to RV. These assumptions were tested by comparing the lavage measurements to those obtained using the gas dilution technique, for similarly treated rats (Table 4).

In summary, the magnitude and persistence of adaptation, induced by daily repeated low-level exposure to O₃ in rats, were significantly and positively correlated with the concentration of AA (an antioxidant reported to protect the lung against O₃ injury) in BALF. These data suggest an associative relationship between increased levels of lavageable AA in the lung and adaptation in rats.

REFERENCES


TABLE 4
Comparison between the Gas Dilution Technique and the Lavage System Gravimetric Technique for Measurement of TLC and RV in Rats

<table>
<thead>
<tr>
<th>Exposure Duration (weeks)</th>
<th>Air 6</th>
<th>Ozone 6</th>
<th>Air 14</th>
<th>Ozone 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt (g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gas dilution</td>
<td>283 ± 5 (10)</td>
<td>272 ± 5 (10)</td>
<td>338 ± 5* (10)</td>
<td>331 ± 5* (9)</td>
</tr>
<tr>
<td>Lavage</td>
<td>284 ± 5 (12)</td>
<td>274 ± 5 (12)</td>
<td>325 ± 5* (12)</td>
<td>333 ± 5* (12)</td>
</tr>
<tr>
<td>TLC (ml)</td>
<td>10.8 ± 0.2 (10)</td>
<td>10.4 ± 0.2 (10)</td>
<td>12.2 ± 0.2* (10)</td>
<td>11.8 ± 0.2* (9)</td>
</tr>
<tr>
<td>RV (ml)</td>
<td>10.1 ± 0.3 (5)</td>
<td>10.0 ± 0.3 (6)</td>
<td>11.9 ± 0.2* (6)</td>
<td>11.9 ± 0.3* (6)</td>
</tr>
<tr>
<td>Gas dilution</td>
<td>1.17 ± 0.12 (10)</td>
<td>1.28 ± 0.09 (10)</td>
<td>1.20 ± 0.09 (10)</td>
<td>1.35 ± 0.09 (9)</td>
</tr>
<tr>
<td>Lavage</td>
<td>1.07 ± 0.09 (5)</td>
<td>1.08 ± 0.11 (6)</td>
<td>1.73 ± 0.11* (6)</td>
<td>1.55 ± 0.11* (6)</td>
</tr>
</tbody>
</table>

* Effects of procedure (gas dilution vs lavage).
+ Effects of time (6 weeks vs 14 weeks).


