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Ozone-induced airway hyperresponsiveness is reduced in immature mice

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The young represent a population that may be particularly susceptible to O₃. Their lungs are still growing, and they are generally more active, have higher metabolic rates, and are therefore likely to inhale more O₃ relative to their lung size. It is also likely that their defense mechanisms, including those that might counter the detrimental effects of O₃, may not yet be fully developed (4, 12). Nevertheless, no studies of age-related differences in O₃-induced AHR have been reported, and relatively few studies have considered age as a variable in any other pulmonary response to O₃. In mice, the results of existing studies using outcomes such as death, alveolar damage, or ability to phagocytose bacteria all suggest that responses to O₃ are more severe in younger animals (3, 13, 33).

In adult rats and some strains of mice, O₃ causes a decrease in minute ventilation (V̇E), which occurs with a slight delay after the initiation of exposure and can be profound (1, 22, 30, 31). Because the dose of O₃ delivered to the lungs is the product of O₃ concentration, exposure time, and V̇E (23, 43), O₃-induced decreases in V̇E are likely to be protective. We have previously reported that the decrease in V̇E observed on exposure of adult (8- to 12-wk-old) rats to O₃ is not observed in immature (2- to 4-wk-old) rats (30). In addition, V̇E normalized for body weight is increased in immature vs. adult rats. The net effect of these differences is that the immature rats receive a much greater dose of O₃ normalized for body weight during exposure to the same inhaled concentration.

The purpose of this study was to determine whether O₃-induced changes in V̇E are different in immature and mature mice and whether such differences result in age-related differences in the effect of O₃ on airway responsiveness. To this end, we measured V̇E and the pattern of breathing in mice aged 2–12 wk during exposure to O₃ (0.3–3 parts/million (ppm) for 3 h) or filtered air and used these measurements to calculate the inhaled dose of O₃. Airway responsiveness to inhaled aerosolized methacholine was measured before and 3 h after cessation of the O₃ exposure. Because substantive age-related differences in O₃-induced AHR were observed and because inflammation is believed to

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OZONE (O₃) EXPOSURE CAUSES damage to lung and airway epithelial cells. The response to this injury is inflammation, including the generation of prostanooids, cytokines, and chemokines, as well as an influx of polymorphonuclear leukocytes (PMN) (2, 15, 21, 26–28, 44, 45). Inflammation leads to symptoms of respiratory irritation, loss of lung function, and, in many species, airway hyperresponsiveness (AHR) (6, 10, 17, 20, 26, 27, 31, 40, 44). In children, hospital admissions for asthma increase on days of high O₃ concentrations (11, 38). It is likely that O₃-induced AHR contributes to the ability of O₃ to trigger these asthmatic episodes.

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be important for the AHR that occurs in response to O₃, we also examined age-related differences in O₃-induced bronchoalveolar lavage (BAL) protein, tumor necrosis factor (TNF)-α, interleukin (IL)-6, macrophage inflammatory protein (MIP)-2, and PMN. BAL protein has been proposed as a sensitive indicator of O₃-induced lung damage (38a), whereas an influx of neutrophils is the primary cellular response to this injury (19). TNF-α, IL-6, and MIP-2 were used as indexes of inflammation because all three cytokines have been reported to increase after O₃ exposure and have been shown to be important for the response to O₃ (14, 31, 39, 45).

METHODS

Animals. This study was approved by the Harvard Medical Area Standing Committee on Animals. Male and female A/J mice aged 2, 4, 8, or 12 wk were purchased from Jackson Laboratory (Bar Harbor, ME). Two-week-old mice were housed with their mother until use. A/J mice were used because they are among the strains of mice that are most sensitive to O₃ (19, 29, 44). In addition, they are among the strains with the most robust airway responses to methacholine (8).

Monitoring ventilation. Mice were placed in Plexiglas restraining tubes, which served as nose-only-exposure flow plethysmographs, as previously described (30). The tubes were fitted with silicone rubber gaskets designed to fit snugly around the mouse’s neck and seal the head from the rest of the body. Different neck gasket sizes were used for the mice of different ages. Once the animal was in the tube, a large piston was moved into place behind the animal. For the younger mice, a Plexiglas spacer was introduced between the mouse and the piston. The piston served to prevent the animal from moving and to seal the body chamber from the outside air. Air displaced at the body surface as the animal breathed passed across a pneumotachograph (8-mm diameter fitted with a screen filter) attached to a differential pressure transducer (model 163PC01D75, Omega Engineering). The resulting flow signal was analyzed by a computer program (Buxco, Troy, NY), which computed Vt, tidal volume (VT), breathing frequency (f), and inspiratory and expiratory (TE) time on a breath-by-breath basis and reported the average of each of these values every minute. The cranial end of the tube was inserted through a port in the Plexiglas door of a stainless steel chamber (~145 liters in volume) into which O₃ was introduced. The mice were first exposed to filtered air for 45 min. The first 25 min of this period were used to adapt the animals to the plethysmographs. Baseline values were the average values obtained in the last 20 min of this 45-min period. The animals then either continued to be exposed to filtered air or the air in the exposure chamber was switched to O₃ (0.3–3 ppm). Exposure to air or O₃ then proceeded for an additional 3 h. In each mouse, averages of each ventilatory parameter were computed 10 min after initiation of O₃ and at every 20 min thereafter.

O₃ exposure. O₃ was generated by passing dry 100% O₂ through ultraviolet light and mixing it with filtered room air in the chamber. Air within the chamber was drawn continuously through a sampling port, and O₃ concentration was measured by an O₃ chemiluminescent analyzer (model 49, ThermoElectron Instruments, Hopkinton, MA), which was calibrated by an ultraviolet photometric O₃ calibrator (model 49PS, ThermoElectron Instruments).

Whole body plethysmography. To measure airway responsiveness, mice were placed awake and unrestrained in a whole body plethysmograph (Buxco). To prevent buildup of CO₂, a constant bias flow was provided through the system. Whole body plethysmography works as follows. As the mice breathe, pressure fluctuations in the plethysmograph are measured with reference to a similar chamber. These fluctuations represent differences between nasal flow and thoracic flow. With bronchoconstriction, there are changes in the shape of the pressure excursions, particularly during expiration, which can be quantified by the algorithm for enhanced pause (Penth), as described by others (16, 37). The algorithm for Penth is

\[ \text{Penth} = \frac{\text{TE} - \text{Tr} \times \text{PEP}}{\text{Tr}} \]

(Note that TE includes any end-expiratory pause or apnea that may occur.) PIP and PEEP are peak inspiratory and peak expiratory pressures, respectively. The total area under the box pressure vs. time curve during expiration is calculated, and the time required from the start of expiration to reach 64% of this area is determined [relaxation time (Tr)]. During methacholine challenge, Penth has been demonstrated empirically to correlate with pulmonary resistance (7, 16, 35, 37), to be markedly reduced by bronchodilators, and is consequently believed to represent airway narrowing. Methacholine-induced changes in Penth are not substantively altered by tracheostomy but are attenuated by bronchodilators. Hence, changes in Penth are believed to represent changes in the lower airways rather than in the nose and pharynx (9, 16, 35).

Dose-response curves to inhaled aerosolized methacholine were obtained as follows. Aerosols of saline and then of methacholine chloride dissolved in saline were delivered to the chamber for 1 min. The concentrations of methacholine used increased in half-log intervals from 0.1 to 30 mg/ml. Because the peak response to methacholine occurred between 3 and 7 min after the exposure, the average Penth value obtained over this time interval was used to measure the response to methacholine. Ten minutes were allowed to elapse between aerosol administrations. Aerosols were generated from an acorn nebulizer at an airflow of 10 l/min and introduced through a port at the top of the chamber.

BAL. Mice were killed with an overdose of halothane. The trachea was cannulated with a tubing adaptor, and the lungs were lavaged twice with phosphate-buffered saline (1 ml) instilled and then slowly withdrawn over 30 s. The recovered BAL fluid was placed on ice until centrifuged at 400 g at 4°C for 10 min. The supernatant was frozen and subsequently analyzed for protein concentration by using the Bradford technique. An aliquot of the supernatant was recentrifuged at 60,000 g at 4°C for 30 min and subsequently analyzed for TNF-α, IL-6, and MIP-2 by using enzyme immunoassay kits (Endogen, Woburn, MA for IL-6 and TNF-α, and R&D Systems, Minneapolis, MN for MIP-2). Cell pellets were suspended in saline, and the total number of cells were counted with a hemocytometer. Aliquots of cells were also centrifuged onto glass slides at 800 rpm for 5 min (Cytospin 2, Shandon, Sewickley, PA), air dried, and stained with Wright-Giemsa (LeukoStat, Fisher Scientific, Pittsburgh, PA). Cell differentiation was determined by counting 300 cells under ×400 magnification.

Protocol. Two cohorts of mice were used in these experiments. In the first cohort, mice aged 2, 4, 8, and 12 wk were used. Airway responsiveness to inhaled, aerosolized methacholine was assessed in each mouse on the day before O₃
exposure. O₃ exposure was at 0.3, 0.5, 1.0, 2.0, or 3.0 ppm for 3 h. Each mouse was exposed to only 1 concentration of O₃. All mice were exposed to O₃ in head-out plethysmographs as described above. Ventilatory parameters were measured throughout the exposure. Immediately after cessation of O₃, the mice were removed from these chambers and placed in whole body plethysmographs. Penh was monitored every 15 min for the next 3 h, at which time airway responsiveness was again measured.

In the second cohort, we sought to determine whether age-related differences in O₃-induced AHR were the result of differences in O₃-induced airway injury and inflammation. Two-week-old and 8-wk-old mice were exposed to O₃ (2 ppm) or to filtered air for 3 h. Four or 24 h after cessation of O₃ exposure, the mice were killed and BAL was performed. Two time points were chosen for the following reason. In this strain of mouse, BAL PMN were not significantly elevated 4 h after cessation of O₃ but were increased at 24 h. In contrast, BAL protein and cytokines were elevated at 4 h but returned to baseline by 24 h (see RESULTS). In this cohort, we used 8-wk-old mice to represent the adult response because the ventilatory response to O₃ was fully developed by this age and AHR was observed. We compared these mice with 2-wk-old mice because the O₂ dose was greatest in these youngest animals and AHR was not observed.

Statistics. To assess O₂-induced changes in airway responsiveness, the dose of methacholine required to cause an increase in Penh of 2 units above the baseline value (EC₂Penh) was calculated by log-linear interpolation between the two doses bounding the point at which a 2-unit increase occurred. ANOVA performed with Bonferroni post hoc analysis was used to compare baseline ventilatory parameters, O₂-induced changes in Penh, and log EC₂Penh, as well as age and O₃ effects on the total volume of air inhaled during O₃ exposure. In assessing ventilatory pattern changes within groups over time during O₃ exposure, repeated-measures ANOVA was conducted to correct for the lack of independence of correlated observations. Differences between air- and O₃-exposed mice in BAL protein, cytokines, and inflammatory cells were assessed by t-tests. All statistical analyses were carried out with the use of SAS software (SAS Institute, Cary, NC).

RESULTS

Baseline ventilatory parameters. There was a marked, almost sixfold increase in body weight in mice between 2 and 12 wk of age (P < 0.001; Table 1). Baseline Vₑ (i.e., Vₑ measured before the onset of O₃ exposure) also increased significantly with increasing age (P < 0.001), but there was a consistent reduction in Vₑ normalized for body weight (Vₑ/g) with increasing age (P < 0.001). The latter observation is consistent with the greater metabolic rate of younger animals (4–25). Age-related differences in Vₑ were primarily the result of age-related differences in Vₜ, which also increased significantly with age (P < 0.001) until 8 wk of age. In contrast, f did not vary consistently with age, although it was significantly greater in 4- and 8-wk-old mice than in 2- and 12-wk-old mice, which were not significantly different.

Effect of O₃ exposure on ventilation. The effect of O₃ on Vₑ in 8-wk-old mice is shown in Fig. 1A. There was a significant effect of O₃ concentration on O₃-induced changes in Vₑ (P < 0.001 by repeated-measures ANOVA). Relative to air exposure, exposure to O₃ for 3 h caused a decrease in Vₑ at all O₃ concentrations >0.3 ppm. At 3.0 ppm O₃, the decrease in Vₑ was significant (P < 0.001) within 30 min of the onset of O₃ exposure and declined to as little as 28% of the baseline Vₑ by the end of the 3-h exposure period. At lower O₃ concentrations, the effects were progressively smaller in magnitude, but even 1 ppm O₃ caused an ~50% decrease in Vₑ. The decreases in Vₑ occurred as the result of decreases in both Vₜ (Fig. 1B) and f (Fig. 1C). The change in f was primarily the result of an increase in end-expiratory pause (Fig. 1D), whereas inspiratory time was not significantly altered.

Figure 2 shows the changes in Vₑ induced by 2 ppm O₃ in all four age groups. Animal age had a significant effect on O₃-induced changes in Vₑ. Follow-up analysis indicated that the responses were not different among the 4-, 8-, and 12-wk-old mice, which all had prominent decreases in Vₑ with exposure to O₃. In contrast, decreases in Vₑ induced by O₃ were significantly less in 2-wk-old mice than in the more mature animals (P < 0.001). Results of data obtained at other O₃ concentrations are summarized in Fig. 3.

To examine the implications of the age-related differences in both baseline specific Vₑ and O₃-induced changes in Vₑ on O₃, we integrated Vₑ/g over the 3-h exposure period to calculate the total volume normalized for body weight inhaled during this period (Fig. 3) and multiplied this total volume by O₃ concentration to calculate O₃ dose normalized for body weight (Fig. 4). There was a significant effect of both age (P < 0.001) and O₃ concentration (P < 0.001) on the total volume of air normalized for body weight that was inhaled during the 3-h exposure period. Overall, this

| Table 1. Age, body weight, and baseline ventilatory parameters |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
|                             | 2 (n = 50)                  | 4 (n = 48)                  | 8 (n = 48)                  | 12 (n = 42)                |
| Weight, g                   | 5.4 ± 0.17                  | 14.5 ± 0.17                 | 23.4 ± 0.17                 | 27.2 ± 0.18                |
| Vₑ, ml/min                  | 26.1 ± 1.5                  | 53.9 ± 1.5                  | 56.7 ± 1.5                  | 50.7 ± 1.6                 |
| Vₑ/g, ml/min · 1-g⁻¹        | 4.94 ± 0.14                 | 3.72 ± 0.15                 | 2.43 ± 0.15                 | 1.87 ± 0.16                |
| Vₜ, ml                      | 0.097 ± 0.006               | 0.167 ± 0.006               | 0.189 ± 0.006               | 0.198 ± 0.006              |
| Vₑ/g, ml/g                  | 0.018 ± 0.0004              | 0.012 ± 0.0004              | 0.0081 ± 0.0004             | 0.0073 ± 0.0005            |
| f, breaths/min              | 275 ± 5.9                   | 325 ± 6.0                   | 303 ± 6.0                   | 261 ± 6.4                  |

Results are means ± SE of data from the number (n) of animals in each group and include both the air- and O₃-exposed mice. Vₑ, minute ventilation; Vₜ, tidal volume; f, breathing frequency; Vₑ/g and Vₑ/g, specific ventilation and specific tidal volume, respectively.
volume decreased progressively from air-exposed values at all concentrations of O₃ greater than 0.3 ppm except in 2-wk-old mice, in which it was not significantly less than during air exposure except at 3 ppm O₃. In addition, because of the higher specific VE observed in the younger animals, at all concentrations of O₃, the total volume of air normalized for body weight, which was inhaled during the exposure period, was greatest in 2-wk-old, smaller in 4-wk-old (P < 0.001), and smaller still in 8- and 12-wk-old mice (P < 0.001), which were not significantly different except during air exposure. When the total volume inhaled during the 3-h exposure period normalized for body weight was multiplied by O₃ concentration to obtain the total inhaled dose of O₃ normalized for body weight (Fig. 4), a statistically significant age-related effect was observed (P < 0.001 by ANOVA). Normalized O₃ dose was significantly greater in the 2-wk-old mice than in adult (8- and 12-wk-old) mice at all O₃ concentrations except 0.3 ppm. Normalized O₃ dose was also significantly greater in 4-wk-old mice than in adult mice at O₃ concentra-

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**Fig. 1.** Effect of air or increasing concentrations of ozone [O₃; 0.3–3.0 parts/million (ppm)] on minute ventilation (VE; A), tidal volume (VT; B), breathing frequency (f; C), and end-expiratory pause (EEP; F) in 8-wk-old mice. A–C: results are expressed as percent changes from baseline values (mean values in the 20-min period immediately before O₃ exposure). Results are means ± SE (n = 6–12 animals in each group). O₃ begins at time (t) = 0 min and ends at t = 180 min. Repeated-measures ANOVA indicated a significant effect of O₃ concentration on each of the 4 ventilatory parameters (P < 0.001) in each case. 

**Fig. 2.** Effect of animal age on changes in VE induced by exposure to 2 ppm O₃ for 3 h. Results are expressed as percent changes from baseline values (mean values in the 20-min period immediately before O₃ exposure). Results are means ± SE (n = 11–12 animals in each group). O₃ begins at t = 0 min and ends at t = 180 min. Repeated-measures ANOVA indicated a significant age effect (P < 0.001). 

**Fig. 3.** Total volume inhaled during exposure to air (0 ppm) or increasing concentrations of O₃. Results are means ± SE (n = 6–12 animals in each group). ANOVA indicated significant effects of both age (P < 0.001) and O₃ concentration (P < 0.001). 

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tions >1 ppm. The importance of the decrease in \( V_E \) that occurs with exposure to \( O_3 \) on total \( O_3 \) dose is particularly apparent when comparing the \( O_3 \) dose at \( O_3 \) concentrations of 2 vs. 3 ppm. Despite a 50% greater inhaled concentration, there was no difference in \( O_3 \) dose at 3 ppm vs. 2 ppm \( O_3 \) in any of the age groups.

**Effect of \( O_3 \) exposure on airway responsiveness.** To determine the functional consequences of this increase in \( O_3 \) dose normalized for body weight in the younger animals, we measured airway responsiveness to inhaled methacholine on the day before and then again 3 h after cessation of \( O_3 \) exposure in mice in each age group. Baseline Penh measured on the day before \( O_3 \) exposure was not significantly different among the four age groups. Figure 5 shows baseline airway responsiveness measured before \( O_3 \) exposure in each of the age groups. Twelve-week-old mice were more sensitive to inhaled methacholine than any of the other age groups, which were not different from each other. Log \( EC_{2}Penh \) averaged 0.74 ± 0.07 in 12-wk-old mice, which was significantly less (\( P < 0.001 \)) than the average log \( EC_{2}Penh \) obtained in 2-, 4-, and 8-wk-old mice (1.21 ± 0.06, 1.15 ± 0.05, and 1.13 ± 0.06, respectively; \( n = 30–36 \) in each group).

Table 2 shows the change in log \( EC_{2}Penh \) induced by exposure to \( O_3 \) in 2-, 4-, 8-, and 12-wk-old mice. Note that a negative value (decrease in \( EC_{2}Penh \)) indicates an increase in responsiveness. Data from the 2- and 3-ppm exposures were combined because the \( O_3 \) dose was not different for these 2 concentrations (Fig. 4). ANOVA indicated a significant effect of age (\( P < 0.001 \)) on the change in log \( EC_{2}Penh \) induced by \( O_3 \). Two- and 4-wk-old mice had no statistically significant change in log \( EC_{2}Penh \) after exposure to any concentration of \( O_3 \). In contrast, in 8- and 12-wk-old mice, there was a statistically significant decrease in log \( EC_{2}Penh \) at 2.0/3.0 ppm \( O_3 \) (\( P < 0.001 \) in each case) but not at lower \( O_3 \) concentrations. In addition, the change in log \( EC_{2}Penh \) observed at 2.0/3.0 ppm \( O_3 \) was greater in 8- and 12-wk-old mice than in 2- and 4-wk-old mice (\( P < 0.001 \) in each case).

Figure 6 shows Penh values measured at 15-min intervals in the 3 h after cessation of \( O_3 \) (3 ppm) exposure but before the measurement of airway responsiveness. Penh was elevated over baseline (pre-\( O_3 \)) values within 30 min after cessation of \( O_3 \) (the first time point we measured) in all four age groups, and, for the most part, Penh did not change substantively over the next 3 h. Therefore the effects of \( O_3 \) dose and age on Penh were assessed by using values measured 3 h after cessation of \( O_3 \) (Fig. 7). As we had done for the measurements of airway responsiveness, data from the 2 and 3 ppm exposures were combined because the \( O_3 \) dose was not different for these two concentrations (Fig. 4). ANOVA indicated a significant dose effect (\( P < 0.001 \)). Follow-up tests indicated that, overall, Penh was signficantly elevated above baseline (0 ppm) values at 1 ppm \( O_3 \) and above but not at lower doses. ANOVA also indicated a significant age effect (\( P < 0.05 \)). Follow-up tests indicated that, overall, \( O_3 \)-induced changes in Penh were greater in 8- and 12-wk-old mice than in 2-wk-old mice (\( P < 0.05 \) in each case), whereas 2- and 4-wk-old mice were not significantly different.

**Effect of \( O_3 \) exposure on pulmonary injury and inflammation.** To determine whether there were age-related differences in the magnitude of \( O_3 \)-induced injury or inflammation that might account for the differences in \( O_3 \)-induced AHR, we performed BAL after exposure of 2- and 8-wk-old mice to air or \( O_3 \) (2 ppm for 3 h) and measured BAL protein, TNF-\( \alpha \), IL-6, and MIP-2. BAL was performed 4 or 24 h after cessation of \( O_3 \) exposure. Results for the 4-h time point are shown in Fig. 7. By 24 h, protein and cytokine values had returned to control values measured in air exposed mice. In control air-exposed mice, BAL protein, IL-6, and TNF-\( \alpha \) were higher in 8-wk-old than in 2-wk-old mice (Fig. 8). This is likely the result of age-related
differences in the dilution of the BAL fluid, since the same volume was used for the BAL in both age groups, but the lungs of the 2-wk-old mice are much smaller. Indeed the three- to fourfold lower concentrations of protein, IL-6, and TNF-α in the air-exposed 2-wk-old compared with 8-wk-old mice are approximately accounted for by the fourfold difference in body weight. O₃ exposure resulted in greater lung injury in 2-wk-old than in 8-wk-old mice. In 2-wk-old mice, O₃ exposure resulted in an ~100% increase in BAL protein (P < 0.05), whereas in 8-wk-old mice, O₃ exposure resulted in only a 40% increase in BAL protein, which was not statistically significant. In contrast, O₃ resulted in a greater release of inflammatory cytokines in 8-wk-old than in 2-wk-old mice. BAL MIP-2 were not significantly increased by O₃ in 2-wk-old mice, but O₃ did cause a statistically significant increase in MIP-2 in 8-wk-old mice. Similarly, BAL IL-6 levels were increased after O₃ exposure in both 8- and 2-wk-old mice, but the effect was greater in the 8-wk-old (325 ± 50% increase) than in the 2-wk-old mice (135 ± 50% increase; P < 0.01). BAL TNF-α levels increased in both 2- and 8-wk-old mice, but the effect did not reach statistical significance in either age group. After O₃ exposure, BAL PMN increased to approximately the same extent in 2-wk-old and 8-wk-old mice (Table 3).

### DISCUSSION

Our results indicate that specific ventilation (i.e., Vₑ/g) is greater in immature than in adult mice (Table 1), consistent with a greater metabolic rate in younger animals. Our results also indicate that O₃ exposure causes a concentration-related decrease in Vₑ in mice and that the reduction in Vₑ is less marked in 2-wk-old than in 4-, 8-, or 12-wk-old mice (Fig. 2). Because the inhaled dose of O₃ is the product of O₃ concentration, exposure time, and Vₑ (23, 43), the net effect of the greater metabolic rate and the failure to decrease Vₑ with O₃ in 2-wk-old mice is that the inhaled dose of O₃, relative to body weight, is about three to four times greater in these mice than in adult (8- to 12-wk-old) mice (Fig. 4). Four-week-old mice have an O₃ dose normalized for body weight that is intermediate between the immature and adult animals (Fig. 4). The ability of O₃ to reduce ventilation is also attenuated in immature compared with adult rats (30).

There are some technical issues that may have influenced our results. Because the plastic plethysmographs that we used to expose the animals restrain them, their activity is reduced. The insulating potential of the plethysmographs may also make thermoreg-

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**Table 2. Effect of age on changes in log EC₂Penh induced by O₃ in mice**

<table>
<thead>
<tr>
<th>O₃ Concentration, parts/million</th>
<th>2 wk</th>
<th>4 wk</th>
<th>8 wk</th>
<th>12 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3</td>
<td>0.01 ± 0.22</td>
<td>−0.24 ± 0.20</td>
<td>−0.25 ± 0.20</td>
<td>−0.28 ± 0.20</td>
</tr>
<tr>
<td>0.5</td>
<td>0.07 ± 0.22</td>
<td>−0.14 ± 0.20</td>
<td>−0.36 ± 0.20</td>
<td>−0.11 ± 0.20</td>
</tr>
<tr>
<td>1.0</td>
<td>0.17 ± 0.20</td>
<td>−0.34 ± 0.20</td>
<td>−0.34 ± 0.20</td>
<td>−0.23 ± 0.20</td>
</tr>
<tr>
<td>2.0/3.0</td>
<td>0.08 ± 0.13</td>
<td>−0.01 ± 0.12</td>
<td>−0.78 ± 0.14†</td>
<td>−0.55 ± 0.11†</td>
</tr>
</tbody>
</table>

Results are means ± SE of data from 6–18 mice in each group. A decrease in the dose of methacholine required to increase enhanced pause 2 units above baseline (EC₂Penh) indicates an increase in airway responsiveness. *P < 0.001 compared with 0 (i.e., no change in responsiveness). †P < 0.001 compared with 2- and 4-wk-old mice.

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**Fig. 6. Changes in Penh measured during the first 3 h after cessation of O₃ exposure.** Preexposure values were measured on the day before O₃ exposure. Results are means ± SE (n = 6–12 animals in each group).

**Fig. 7. Effect of age and O₃ concentration on Penh measured 3 h after the cessation of O₃ exposure.** Data at 0 ppm reflect Penh values measured in the baseline period before methacholine inhalation on the day before O₃ exposure. Results are means ± SE of data from 6–12 animals in each group, except in the case of the 0-ppm data, which are means ± SE of data from 30–42 animals in each group. ANOVA indicated a significant effect of both age (P < 0.05) and O₃ concentration (P < 0.001) on Penh.

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ulation difficult. Both thermoregulation and activity may influence metabolism and, consequently, $V_E$. However, we do not know of any reason why use of these tubes should have differentially affected the younger vs. older animals, since the size of the tube was adjusted for the size of the animal.

A reduced ventilatory response to CO$_2$ has been reported in some strains of mice exposed to O$_3$ (34) and may have contributed to the marked decreases in $V_E$ that occurred with O$_3$ exposure in these A/J mice. However, it is more likely that the decrease in $V_E$ induced by O$_3$ is the result of a decrease in metabolic rate since, in rats and mice, oxygen consumption, core body temperature, heart rate, and activity also decrease during O$_3$ exposure (1, 18, 22, 32, 42), but arterial PCO$_2$ is unchanged (36). Thus the observed age-related differences in the ventilatory response to O$_3$ (Fig. 2) suggest that the ability of O$_3$ to reduce metabolism may be reduced in immature mice. We do not know what accounts for this difference. However, it is unlikely to represent a general inability of immature mice to lower their metabolic rates because immature rats are capable of reducing metabolic rate in response to other stimuli, such as hypoxia, and indeed do so to a greater extent than adults (25).

Relatively few studies have considered age as a factor in any aspect of the response to O$_3$ and the results vary with the species used. In mice, the results of existing studies suggest that some responses to O$_3$ are more severe in younger animals (4, 30). Stockinger (33) reported that the lethal dose of O$_3$ was lower in younger than in older mice, and Bils (3) reported that O$_3$-induced alveolar damage was greater in 4-day-old than in 1- or 2-mo-old animals. Gilmour et al. (13) reported that young (5-wk-old) mice had a more severely compromised ability to phagocytose bacteria after O$_3$ exposure (0.4–0.8 ppm for 3 h) than did older (9-wk-old) mice. Although the inhaled dose of O$_3$ and the dose delivered to the tissues might not necessarily be the same, it is possible that part of the apparently increased susceptibility to O$_3$ reported in the studies cited above could be the result of the greater inhaled dose of O$_3$ normalized for body weight in the younger animals (Fig. 4). The increased dose of O$_3$ delivered to the lungs of the younger mice is also likely to account for the increased BAL protein (Fig. 3B), tumor necrosis factor (TNF)-1$\alpha$ (Fig. 3A), interleukin (IL)-6 (Fig. 3C), and macrophage inflammatory protein (MIP)-2 (Fig. 3D) in air- and O$_3$-exposed mice aged 2 or 8 wk. Mice were exposed to 2 ppm O$_3$ for 3 h, and bronchoalveolar lavage was performed 4 h after the cessation of exposure. Results are means ± SE ($n = 9–19$ mice in each group). *P < 0.05 compared with air-exposed mice of the same age.

Table 3. BAL differentials in 2- and 8-wk-old mice exposed to air or O$_3$

<table>
<thead>
<tr>
<th></th>
<th>Air</th>
<th>O$_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 wk old</td>
<td>8 wk old</td>
</tr>
<tr>
<td>Total cells ($\times 10^4$)</td>
<td>$2.6 \pm 0.3$</td>
<td>$5.8 \pm 0.6$</td>
</tr>
<tr>
<td>Macrophages, %</td>
<td>$98.6 \pm 1.0$</td>
<td>$85 \pm 5.0$</td>
</tr>
<tr>
<td>Neutrophils, %</td>
<td>$0.6 \pm 0.5$</td>
<td>$6.2 \pm 3.7^*$</td>
</tr>
<tr>
<td>Epithelial cells, %</td>
<td>$1.6 \pm 1.9$</td>
<td>$2.9 \pm 1.6$</td>
</tr>
</tbody>
</table>

Mice were exposed to 2 ppm O$_3$ for 3 h and killed 24 h later. For cell percentages, results are geometric means ± SE of data from 3–10 mice in each group. BAL, bronchoalveolar lavage. *P < 0.05 compared with age-matched air-exposed mice.
protein in these mice, we did not observe a greater susceptibility to the induction of AHR by O\textsubscript{3} in immature mice. In fact, O\textsubscript{3} exposure, even at concentrations as high as 2–3 ppm, did not induce AHR in either 2- or 4-wk-old mice, whereas 8- and 12-wk-old mice did develop AHR in response to O\textsubscript{3} (Table 2). These results suggest that the immature mice are in fact less sensitive to O\textsubscript{3}-induced AHR than adult mice because, for a greater inhaled dose, they developed a lesser response. Similarly, it is likely that immature mice are less sensitive to O\textsubscript{3}-induced pulmonary cytokine formation because, despite a greater inhaled dose normalized for body weight, they did not have as substantial an increase in BAL IL-6 and MIP-2 (Fig. 8, C and D). To our knowledge, this is the first report of age-related differences in O\textsubscript{3}-induced AHR in any species and the first report of age-related differences in O\textsubscript{3}-induced pulmonary inflammation in immature compared with juvenile mice.

Although immature mice are less susceptible than adults to the induction of AHR by O\textsubscript{3} and to the release of certain cytokines, they are more susceptible to O\textsubscript{3}-induced pulmonary injury and O\textsubscript{3}-induced death, as described above. Taken together, the data suggest that there are differences in age-related susceptibility to O\textsubscript{3} depending on the outcome indicator examined. This would not be surprising, since it is clear that the mechanistic basis for AHR and pulmonary injury induced by O\textsubscript{3} are not the same. For example, we and others have reported that AHR induced by acute exposure to O\textsubscript{3} is attenuated in TNF receptor-deficient mice, whereas BAL protein is not affected (6, 31).

Because TNF has been shown to be required for O\textsubscript{3}-induced AHR in mice (6, 31), we hypothesized that a greater ability of O\textsubscript{3} to induce TNF-\(\alpha\) might account for the greater O\textsubscript{3}-induced AHR observed in the older mice. Our results do not support this hypothesis. Although TNF-\(\alpha\) did, on average, increase the BAL of both 2- and 8-wk-old mice, the increase was not statistically significant and there were no apparent differences between age groups (Fig. 8B). It is, however, possible that there are age-related differences in the expression of TNF receptors or the soluble TNF receptor, the endogenous inhibitor of TNF, which might account for the differences observed. It is also clear that production of other cytokines, such as IL-6 and MIP-2, is increased in the adult animals (Fig. 8, C and D). We do not know to what extent these cytokines may be also important in O\textsubscript{3}-induced AHR.

It is also possible that the magnitude of O\textsubscript{3}-induced AHR might depend on the baseline responsiveness of the animals. However, we think this an unlikely explanation for the inability of O\textsubscript{3} to induce AHR in the younger animals. Baseline airway responsiveness was greater in the 12-wk-old animals than in the other age groups, and these animals did develop AHR. However, 2-, 4-, and 8-wk-old mice all had equivalent baseline airway responsiveness (Fig. 5), and 8-wk-old mice also developed AHR after O\textsubscript{3} exposure, whereas 2- and 4-wk-old mice did not (Table 2). We do not know at what time between 4 and 8 wk of age O\textsubscript{3}-induced AHR develops.

O\textsubscript{3} exposure at concentrations of 1 ppm or more caused an increase in baseline Penh in mice of all age groups (Figs. 6 and 7). The change in Penh was greater in the 8- and 12-wk-old mice than in the 2-wk-old mice. Data from a number of investigators strongly support the hypothesis that during methacholine-induced bronchospasm, Penh is an index of airway narrowing (7, 16, 35, 37). It is likely that at least part of the change in Penh induced by O\textsubscript{3} is also the result of airway narrowing, since data from a variety of species, including mice, indicate that pulmonary resistance increases, though not substantively, with O\textsubscript{3} exposure (17, 20, 26, 40). Furthermore, McGraw et al. (24) reported that O\textsubscript{3}-induced changes in Penh were attenuated by the bronchodilator albuterol. However, it is also possible that some of the increase in Penh induced by O\textsubscript{3} is a reflection of changes in the pattern of breathing rather than airway narrowing. The algorithm that computes Penh (see METHODS) is such that certain changes in the pattern of breathing would increase Penh, and breathing pattern did change during exposure to the higher concentrations of O\textsubscript{3} (Fig. 1).

We do not know to what extent the results reported here can be extrapolated to humans. Of importance, humans do not have a robust hypothermic response after exposure to toxic agents such as O\textsubscript{3} (41). Because, in rodents, this response reduces V\textsubscript{E} and consequently reduces the inhaled dose of O\textsubscript{3}, the toxic effects of O\textsubscript{3} might be expected to be higher in humans than in rodents for the same inhaled concentration. There may also be differences between mice and humans in the degree of postparturition development of systems, which are involved in sensing or responding to inhaled irritants such as O\textsubscript{3}. Such species differences in development further complicate extrapolation of our results to humans.

In summary, our results indicate that 2-wk-old mice have a higher V\textsubscript{E}/g but a reduced ventilatory response to O\textsubscript{3} compared with adult mice. These changes in ventilation result in a marked increase in the inhaled dose of O\textsubscript{3} normalized for body weight in the immature mice. Consistent with the increased dose, immature mice have increased BAL protein, consistent with increased pulmonary injury after O\textsubscript{3} exposure. In contrast, O\textsubscript{3}-induced AHR and O\textsubscript{3}-induced BAL cytokine production are reduced in immature compared with older mice. The results indicate that factors other than dose and injury are responsible for the reduced susceptibility to O\textsubscript{3}-induced AHR observed in immature mice.

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REFERENCES


