Tumor Necrosis Factor Receptor 2 Contributes to Ozone-induced Airway Hyperresponsiveness in Mice

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The purpose of this study was to determine whether tumor necrosis factor (TNF) contributes to airway hyperresponsiveness (AHR) and migration of polymorphonuclear leukocytes (PMN) into the airways following exposure to ozone (O_3). Wild-type mice, TNF p55 or p75 receptor knockout mice (p55 TNFR $-/-$ and p75 TNFR $-/-$), as well as double receptor knockout mice (p55/p75 TNFR $-/-$), were exposed to O_3 . Three hours after cessation of O_3 , airway responses to inhaled methacholine were determined by whole body plethysmography using changes in enhanced pause (Penh) as an index of airway narrowing. In wild-type mice, O_3 exposure (0.5 ppm, 3 h) caused a significant increase in airway responsiveness as indicated by a 1.2 log leftward shift in the methacholine dose-response curve. In contrast, in p55/p75 TNFR $-/-$ mice, O_3 caused only a 0.5 log shift in the dose-response curve ($p < 0.05$ compared with wild-type). Similar results were obtained in p75 TNFR $-/-$ mice. In contrast, O_3 -induced airway hyperresponsiveness was not different in WT and p55 TNFR $-/-$ mice. During O_3 exposure (1 pm, 3 h), minute ventilation ($\dot{V}_E$) decreased by $64 \pm 4\%$ in wild-type, but only $24 \pm 5\%$ in p55/p75 TNFR $-/-$ mice, indicating that despite their reduced O_3 -induced AHR, the TNFR-deficient mice actually inhaled a greater dose of O_3 . Similar results were obtained in p75 $-/-$ mice, whereas changes in $\dot{V}_E$ induced by O_3 were the same in wild-type and p55 $-/-$ mice. PMN numbers in bronchoalveolar lavage fluid recovered 21 h after cessation of exposure to O_3 (2 ppm, 3 h) were significantly increased compared with after air exposure but were not different in wild-type and p55/p75 TNFR $-/-$ mice. Our results indicate that TNF contributes to the AHR but not the PMN emigration induced by acute O_3 exposure.

Keywords: whole body plethysmography; polymorphonuclear leukocytes; minute ventilation; knockout mice; methacholine

Ozone (O_3) exposure causes damage to epithelial cells both in the airways and the alveoli. The response to this injury includes generation of numerous inflammatory mediators including cytokines and chemokines (1–4), an influx of polymorphonuclear leukocytes (PMN) (1, 5–11), symptoms of respiratory irritation, and loss of lung function (11–14). In many species, including mice, acute exposure to O_3 also causes airway hyperresponsiveness (1, 5–10). Airway hyperresponsiveness (AHR) is a defining feature of asthma, and hospital admissions for asthma increase on days of high O_3 concentrations (15–17). O_3 -induced AHR and/or inflammation is likely to contribute to the ability of O_3 to trigger episodes of asthma.

Data from many investigators suggest that O_3 -induced airway inflammation contributes to O_3 -induced AHR, but the precise component of inflammation required for AHR is still not firmly established. Early reports suggested that O_3 -induced

migration of leukocytes into the airways was critical for the development of AHR (8, 10). However, more recent studies, including studies in mice, have not borne this out (1, 6, 7, 9). There is, however, reason to believe that tumor necrosis factor- α (TNF- α) may contribute to O_3 -induced AHR. First, synthesis of TNF- α is increased in alveolar macrophages and lung epithelial cells following O_3 exposure of whole animals, and cultured macrophages or epithelial cells also express TNF- α in response to O_3 (3, 4, 18). Second, genetic studies have identified a locus on mouse chromosome 17 that confers susceptibility to O_3 -induced inflammation. The TNF- α gene is close to this locus (19). Third, administration of TNF- α antibodies to rats prior to O_3 exposure significantly attenuates other aspects of the response to O_3 such as increases in macrophage adherence, in tracheal permeability, and in neutrophil migration (19–21). Finally, exogenous administration of TNF- α has been shown to cause AHR in humans and rats (22, 23).

The purpose of this study was to examine the role of TNF- α in O_3 -induced AHR. To that end, we measured the effect of O_3 on airway responsiveness in wild-type mice and mice genetically deficient in either the p55, the p75, or both TNF receptors. Because it has been proposed that TNF- α contributes to O_3 -induced neutrophil influx into the airways (19), perhaps through effects on the expression of adhesion molecules (24), we also examined O_3 -induced influx of PMN into the airways in wild-type and TNF receptor-deficient mice.

METHODS

Animals

This study was approved by the Harvard Medical Area Standing Committee on Animals. Breeding pairs of mice genetically deficient in either the p55 or the p75 TNF receptor or both receptors were kindly provided by Dr. Jacques Peschon at Immunex (Seattle, WA) (25–27). The mice do not exert any overt phenotype when unchallenged, live a normal life-span, and breed normally. The mice have been backcrossed to a C57BL/6 background through six generations. Consequently, we used C57BL/6 mice as wild-types. Mice were studied at 7–9 wk of age. For measurements of O_3 -induced AHR, we used p55 $-/-$ mice from Jackson Labs (28). These mice have also been backcrossed to a C57BL/6 background. The p55-deficient mice from Immunex and those from Jackson Labs were created using different targeting vectors. However, both mutations completely prevented the expression of p55.

Protocol

Three cohorts of mice were used in these studies. In the first cohort, airway responsiveness to inhaled aerosolized methacholine was assessed in each mouse on two occasions: once on the day before O_3 exposure and then again 3 h after cessation of O_3 exposure. O_3 exposure was at 0.5 ppm for 3 h. In the second cohort, minute ventilation ($\dot{V}_E$) was measured during O_3 exposure (1 ppm, 3 h) in mice exposed in nose-only exposure plethysmographs. The purpose of these experiments was to determine whether differences in O_3 -induced AHR between wild-type and knockout animals were the result of differences in the inhaled dose of O_3 resulting from differences in $\dot{V}_E$ during exposure. In the third cohort, mice were exposed to air or to O_3 (2 ppm, 3 h); 21 h after cessation of exposure, the mice were killed by an over-

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dose of halothane and a bronchoalveolar lavage was performed. Blood was also obtained in these groups of mice, and blood PMN was assessed.

Ozone Exposure

Exposure to O₃ or filtered room air was conducted in a stainless-steel chamber with a Plexiglas door on the front (approximately 145 L in volume). O₃ was generated by passing dry 100% oxygen through ultraviolet light, and mixing it with filtered room air in the chamber. Chamber atmosphere was drawn continuously via a sampling port, and O₃ concentration was measured by an O₃ chemiluminescent analyzer (Model 49; Thermo Electron Instruments, Hopkinton, MA) which was calibrated by an ultraviolet photometric O₃ calibrator (Model 49PS; Thermo Electron Instruments). Except for those mice in which measurements of $\dot{V}_E$ during O₃ exposure were made, during exposure, mice were placed in individual wire mesh cages within the chamber and were awake during exposure.

Whole Body Plethysmography

Mice were placed awake, unrestrained, and uninstrumented in a whole body plethysmograph (Buxco, Troy, NY). A constant bias flow was provided through the system in order to prevent buildup of CO₂. 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. These shape changes can be quantified by the algorithm for enhanced pause (Penh), described by others (29–31). The algorithm for Penh is

$$\text{Penh} = \frac{T_e - T_r}{T_r} \times \frac{\text{PEP}}{\text{PIP}}$$

T_E is expiratory time. (Note that T_E includes any end-expiratory pause or apnea that may occur.) PIP and PEP are peak inspiratory and peak expiratory pressures. The total area under the box pressure versus 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, T_r). During methacholine challenge, Penh has been demonstrated empirically to correlate with pulmonary resistance (29–32), to be markedly reduced by bronchodilators, and is consequently believed to represent airway narrowing. Methacholine-induced changes in Penh are not substantively altered by tracheostomy and consequently are believed to represent changes in the lower airways, rather than the nose and pharynx (30, 33).

Dose–response curves to inhaled aerosolized methacholine were obtained as follows. Aerosols of saline and then of methacholine chloride dissolved in saline increasing in half log intervals from 1 mg/ml to 300 mg/ml were delivered to the chamber for 1 min. Because the peak response to methacholine occurred between 3 and 7 min after the exposure, the average Penh 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.

Measurement of Ventilation during O₃ Exposure

Although whole body plethysmography is useful in assessing Penh and inferring AHR, it is inaccurate for measurements of tidal volume in any situation in which there may be bronchospasm. Therefore, to measure ventilation during O₃ exposure, mice were placed in a Plexiglas restraining tube that served as a head-out flow plethysmograph, as previously described (34). The tube was fitted with a rubber gasket designed to fit snugly around the animal's neck. Once the animal was in the tube, a large piston fitted with a rubber O-ring was moved into place behind it, restraining movement. Air displaced at the body surface as the animal breathed passed across a pneumotach attached to a differential pressure transducer. The front end of the tube was inserted into a port in the O₃ exposure chamber, and the animals were exposed nose only. The flow signal from the pneumotach was fed to a personal computer and analyzed using software (Buxco) that allowed for breath-by-breath measurements of minute ventilation ($\dot{V}_E$), tidal volume (V_T), breathing frequency (f), and end-expiratory pause (EEP).

Bronchoalveolar Lavage

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 1200 rpm at 4°C for 10 min. Cell pellets were resuspended in saline, and the total number of cells counted with a hemocytometer. Aliquots of cells were also centrifuged onto glass slides at 800 rpm for 5 min (Cytospin2; 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. Blood cells were counted in a hemocytometer and differential counts were performed on 200 cells from Leukostat-stained blood smears.

Statistics

Within a given group of animals, changes in Penh induced by methacholine pre-O₃ and post-O₃ were assessed by paired *t* tests. The dose of methacholine required to cause an increase in Penh of 2 units above the baseline value ($EC_2\text{Penh}$) was calculated by log-linear interpolation between the two doses bounding the point at which a 2 unit increase occurred. Differences in $EC_2\text{Penh}$ and BAL and blood cells between wild-type and TNFR-deficient animals were assessed by unpaired *t* tests.

RESULTS

Airway responsiveness to inhaled aerosolized methacholine was measured before and after exposure to O₃ (0.5 ppm for 3 h) in wild-type mice and in mice genetically deficient in both the p55 and p75 TNFR. Prior to O₃ exposure, there was no significant difference in airway responsiveness between wild-type mice and p55/p75 TNFR $-/-$ mice, except at the very highest concentration of methacholine where the p55/p75 TNFR $-/-$ mice had a statistically greater response than wild types ($p < 0.05$). Exposure to O₃ caused an increase in airway responsiveness to methacholine in wild-type mice as indicated by the leftward shift in the dose–response curve (Figure 1, *left*). O₃ also caused a significant increase in airway responsiveness in p55/p75 TNFR $-/-$ mice (Figure 1, *right*), but the magnitude of the change induced by O₃ was not as great. In particular, wild-type mice showed significant differences pre- and post-O₃ at concentrations as low as 3 mg/ml, whereas in the p55/p75 TNFR $-/-$ mice, significant effects of O₃ were not observed until a methacholine concentration of 10 mg/ml. Even at that concentration, the magnitude of the change induced by O₃ was significantly less than in the wild-type mice ($p < 0.05$). Overall, O₃ caused a 15-fold decrease in the concentration of methacholine required to cause an increase in Penh of 2 units ($EC_2\text{Penh}$) in wild-type mice, but only a 5-fold decrease in

Figure 1. Changes in Penh (above saline values) induced by inhaled aerosolized methacholine prior to (closed symbols) and 3 h after cessation of exposure to O₃ (0.5 ppm, 3 h) (open symbols) in wild-type (C57BL/6) mice (*left*) and double receptor-deficient (p55/p75 TNFR $-/-$) mice (*right*). Results are mean $\pm$ SE of data from six mice in each group. * $p < 0.05$ compared with pre-O₃.

Figure 2. Changes in Penh (above saline values) induced by inhaled aerosolized methacholine prior to (closed symbols) and 3 h after cessation of exposure to O₃ (0.5 ppm, 3 h) (open symbols) in wild-type (C57BL/6) mice (left) and p75 receptor-deficient (p75 TNFR ^{-/-}) mice (right). Results are mean $\pm$ SE of data from six mice in each group. * p < 0.05 compared with pre-O₃.

EC₂Penh in p55/p75 TNFR ^{-/-} mice. Similar results were obtained in p5 TNFR ^{-/-} mice (Figure 2). In contrast, O₃-induced AHR was not reduced in p55 TNFR ^{-/-} mice compared with wild-type control mice (Figure 3). Note that because the various groups of receptor-deficient animals were studied at different times, and because we observed differences in O₃-induced AHR across cohorts of wild-type mice studied at different times, each group of mutant mice was compared with a cohort of wild-type mice exposed to O₃ at the same time in the same exposure chambers.

The dose of O₃ delivered to the lungs is the product of O₃ concentration, exposure time, and $\dot{V}_E$ (35–37). To ensure that differences in O₃-induced airway hyperresponsiveness between wild-type and knockout mice were not simply the result of differences in O₃ dose arising from differences in $\dot{V}_E$, we measured $\dot{V}_E$ during O₃ exposure in wild-type and TNF double receptor knockout mice (Figure 4). Prior to O₃ exposure $\dot{V}_E$ averaged 2.8 ± 0.21 ml/min/g in wild-type and 2.5 ± 0.21 ml/min/g in p55/p75 TNFR ^{-/-} mice (NS). In wild-type mice, O₃ exposure caused a marked decrease in $\dot{V}_E$. The decrease in $\dot{V}_E$ was significant (p < 0.01) within 30 min of the onset of O₃ exposure and remained so throughout the remainder of the O₃ exposure period. $\dot{V}_E$ also decreased significantly during O₃ exposure of TNF double receptor knockout mice, but the magnitude of the response was much less. Overall, after 3 h of O₃ exposure, $\dot{V}_E$ decreased by $64 \pm 4\%$ in wild-type, but only $24 \pm 5\%$ in TNF receptor-deficient mice (p < 0.01). Thus, integrated over the 3-h exposure, the net inhaled dose of O₃ was slightly greater in the TNF receptor-deficient mice. In wild-type mice, the decrease in $\dot{V}_E$ was the result of changes in both tidal volume (V_T), which decreased $33 \pm 1\%$ over the course of the 3 h O₃ exposure, and frequency, which decreased by

Figure 3. Changes in Penh (above saline values) induced by inhaled aerosolized methacholine prior to (closed symbols) and 3 h after cessation of exposure to O₃ (0.5 ppm, 3 h) (open symbols) in wild-type (C57BL/6) mice (left) and p55 receptor-deficient (p55 TNFR ^{-/-}) mice (right) obtained from Jackson Labs (28). Results are mean $\pm$ SE of data from four mice in each group. * p < 0.05 compared with pre-O₃.

Figure 4. Changes in minute ventilation ($\dot{V}_E$) during a 3-h O₃ exposure (1 ppm) expressed as a percentage of baseline values measured in the 20-min period prior to initiation of exposure in wild-type mice and p55/p75 TNFR ^{-/-} mice. Results are mean $\pm$ SE of data from five mice in each group. * p < 0.05 compared with wild type.

about $40 \pm 5\%$. In wild-type mice, the change in breathing frequency during O₃ exposure was largely the result of the addition of a pause at the end of expiration (Figure 5). In TNFR knockout mice exposed to O₃ at the same time, there was only a $10 \pm 7\%$ decrease in tidal volume and an $18 \pm 8\%$ decrease in breathing frequency (p < 0.05 compared with wild types). Furthermore, O₃ caused only minor changes in end-expiratory pause in these mice (Figure 5).

Similar experiments were performed in p55 ^{-/-} and p75 ^{-/-} mice (Figures 6 and 7). (Note that these mice were studied at a different time from the double receptor knockouts.) Because we observed differences in O₃-induced changes in $\dot{V}_E$ across cohorts of wild-type mice studied at different times, each group of mutant mice was compared with wild-type mice that were exposed to O₃ at the same time in the same exposure chambers. Baseline $\dot{V}_E$ normalized for body weight was not different in wild-type, p55 ^{-/-}, or p75 ^{-/-} mice (2.39 ± 0.14 , 2.54 ± 0.13 , and 2.26 ± 0.11 ml/min/g, respectively). Three hours of exposure to O₃ (1 ppm) (Figure 6) caused a substantive and significant decrease in $\dot{V}_E$ that was not different in wild-type and p55 ^{-/-} mice. However, in p75 ^{-/-} mice, 3 h exposure to O₃ failed to cause any significant change in $\dot{V}_E$. Thus, the effect on O₃-induced changes in $\dot{V}_E$ observed in the double receptor knockout mice (Figure 4) is primarily caused by the absence of the p75 rather than the p55 receptor. Compared to wild types, the change in EEP induced by O₃ was significantly reduced in both p75 ^{-/-} and p55 ^{-/-} mice (Figure 7).

In initial experiments in C57BL/6 mice, we did not observe an increase in BAL PMN at either 3 or 21 h after exposure to 0.5 ppm O₃, but we did see an increase in PMN by 21 h but not 3 h after exposure to 2 ppm O₃. Consequently, comparisons of BAL PMN between wild-type and TNF receptor knockout mice were performed with this latter exposure regimen. In air-

Figure 5. End-expiratory pause (EEP) measured during a 3-h O₃ exposure (1 ppm) in wild-type and p55/p75 TNFR ^{-/-} mice. Results are mean $\pm$ SE of data from five mice in each group. O₃ exposure was terminated at $t = 180$ min. * p < 0.05 compared with p55/p75 TNFR ^{-/-}.

Figure 6. Changes in minute ventilation (V_E) during a 3-h O_3 exposure (1 ppm) expressed as a percentage of baseline values measured in the 20-min period prior to initiation of exposure in wild-type, p55 TNFR $^{-/-}$ and p75 TNFR $^{-/-}$ mice. Results are mean $\pm$ SE of data from six mice in each group. * $p < 0.05$ compared with wild-type and p75 $^{-/-}$ groups.

exposed mice, there was no difference in either total BAL cells or in BAL PMN between wild-type mice and mice deficient in both TNF receptors. PMN accounted for less than 1% of the total BAL cells (Figure 8, *top panel*). Surprisingly, 21 h after O_3 exposure, total BAL cells were actually higher ($p < 0.05$) in TNF receptor-deficient mice than in wild types (Figure 8, *lower panel*). Numbers of PMN were not different between the groups, nor was there any difference in the number of PMN expressed as a percentage of total cells in wild-type versus TNF receptor-deficient mice (33% versus 29%, respectively) although in both groups, PMN were markedly increased compared with air-exposed mice. To determine if differences in total BAL cells between wild-type and TNFR-deficient mice were due to differences in circulating leukocyte numbers, we also measured circulating total white blood cells (2.6 ± 0.5 versus 4.1 ± 0.9 cells $\times 10^6$ /ml blood) and total blood PMN (0.46 ± 0.05 and 0.57 ± 0.15 PMN $\times 10^6$ /ml blood) in wild-type and TNF double receptor knockout mice, respectively. The differences were not significant.

DISCUSSION

Our results indicate that O_3 -induced AHR is reduced in mice genetically deficient in TNF receptors. Taken together with data in the literature indicating that O_3 induces TNF- α expression in alveolar macrophages and lung epithelial cells (3, 4, 18), and that exogenous TNF- α is capable of inducing AHR in rats and humans (22, 23), these data support the hypothesis that TNF- α contributes to O_3 -induced AHR in mice. In contrast, we did not find any evidence for a role for TNF in the PMN migration induced by acute (2 ppm, 3 h) O_3 exposure: the numbers of PMN in the BAL were not significantly different in wild type compared to p55/p75 TNFR $^{-/-}$ mice.

Figure 7. End-expiratory pause (EEP) measured during a 3-h O_3 exposure (1 ppm) in wild-type, p55 $^{-/-}$, and p75 $^{-/-}$ deficient mice. Results are mean $\pm$ SE of data from six mice in each group. O_3 exposure was terminated at $t = 180$ min. * $p < 0.05$ compared with p55 $^{-/-}$ and p75 $^{-/-}$ groups.

Figure 8. Bronchoalveolar lavage (BAL) cells harvested from wild-type and p55/75 TNFR $^{-/-}$ mice after air exposure (*top panel*) or 21 h after cessation of exposure to O_3 (2 ppm, 3 h) (*lower panel*). Results are mean $\pm$ SE of data from five or six mice in each group. * $p < 0.05$ compared with wild type.

In contrast to the results presented here (Figure 8), Kleeberger and colleagues (19) reported that a TNF- α antibody significantly reduces PMN migration induced by a longer term O_3 exposure (0.3 ppm, 48 h), data consistent with the results of genetic studies that identify a locus on mouse chromosome 17 close to the TNF- α gene that confers susceptibility to O_3 -induced inflammation. A preliminary report by the same group indicates that BAL PMN are also reduced in p55 and p75 TNFR $^{-/-}$ compared to wild-type mice following the same type of O_3 exposure (0.3 ppm, 48 h) (38). The most likely explanation for the difference between the results of Kleeberger and colleagues and those reported here, which indicate no role for TNF in O_3 -induced PMN migration, is the nature of the O_3 exposure (0.3 ppm for 48 h versus 2 ppm for 3 h). In support of this argument, Young and coworkers (20) showed no effect of an anti-TNF antibody on PMN influx into BAL fluid when rats were exposed acutely to O_3 (0.8 ppm, 3 h). Taken together, the data suggest a different mechanistic basis for the PMN migration induced by acute and more long term O_3 exposure or by exposures to low versus higher O_3 concentrations. In particular, TNF appears to be important following longer term exposure to low O_3 concentrations, whereas other factors are important during acute exposure to higher concentrations. Other potential candidates are PAF and the chemokines CINC and MIP-1, each of which have been postulated to be important in PMN migration following acute exposure to O_3 in rats or mice (1, 2, 39). Differences in the mechanism of PMN migration induced by acute compared to the longer term O_3 exposure are consistent with other observations of Kleeberger and coworkers who reported that the genetic factors that confer susceptibility to PMN migration under the two exposure conditions are not necessarily the same (40).

The biological activities of TNF are mediated by its binding to two structurally related, but functionally distinct receptors: TNFR1 or p55 and TNFR2 or p75. Based on the fact that the majority of TNF-dependent inflammatory functions have been ascribed to p55 (26), we expected to find that the p55 TNFR was the receptor important for O_3 -induced AHR. Instead, our results indicated reduced O_3 -induced AHR in p75 $^{-/-}$ but not in p55 $^{-/-}$ mice (Figures 2 and 3). Similarly, the ventilatory response to O_3 was reduced in p75 $^{-/-}$ but not in p55 $^{-/-}$ mice (Figures 4 and 6). Such findings are not without precedent, as p75 has also been shown to mediate some TNF effects (41–45), as well as to share others with p55 (27). For example, p75 $^{-/-}$ mice but not p55 $^{-/-}$ mice are protected from experimental cerebral malaria, perhaps as a result of p75-dependent ICAM-1 upregulation by TNF- α in cerebral microvessels (44). The p75, but not the p55 TNF receptor also

appears to be important in the migration of epidermal Langerhans cells (43). p75 is also the dominant TNFR on T cells, and mediates apoptosis of T cells in response to TNF (41).

We do not know exactly how TNF acts to induce AHR following O₃ exposure. O₃ causes epithelial cell damage, and O₃-induced changes in tracheal permeability have been shown to be attenuated by anti-TNF- α antibodies (20). It is possible that these changes contribute to AHR by increasing the flux of inhaled methacholine across the epithelial barrier (20). Although TNF has been shown to act directly on airway smooth muscle cells and increase their influx of calcium in response to contractile agonists, we believe that such an action of TNF is unlikely to contribute to the effects described here as it is mediated by p55 and not p75 receptors (46), whereas the role of TNF in O₃-induced AHR appears to involve p75 (Figure 2) but not p55 (Figure 3).

The inhaled dose of O₃ is the product of O₃ concentration, exposure time, and ventilation (35–37). Thus, differences in $\dot{V}_E$ between wild-type and TNFR-deficient mice during O₃ exposure would result in differences in the inhaled dose of O₃. To investigate the possibility that differences in the inhaled dose of O₃ might account for differences in the ability of O₃ to induce AHR in wild-type and TNFR-deficient mice, we measured $\dot{V}_E$ during O₃ exposure in wild-type, p55 TNFR $^{-/-}$, p75 TNFR $^{-/-}$, and p55/p75 TNFR $^{-/-}$ mice. Our results indicate that O₃ causes a substantive decrease in $\dot{V}_E$ in wild-type and p55 $^{-/-}$ mice, but smaller decreases in $\dot{V}_E$ in p75 $^{-/-}$ and p55/p75 $^{-/-}$ mice (Figures 4 and 6). The net effect of these differences is that the p75 $^{-/-}$ and p55/p75 $^{-/-}$ mice actually received a *greater* inhaled dose of O₃ than the wild-type mice. Although the inhaled dose of O₃ and the dose delivered to the tissues might not necessarily be the same, these results suggest that differences in dose are likely not responsible for differences in O₃-induced AHR observed in these animals (Figures 1 and 2). However, it is possible that differences in $\dot{V}_E$ during O₃ exposure might have influenced our PMN data. If O₃-induced PMN migration does indeed require TNF, then the absence of any difference in BAL PMNs between p55/p75 TNF $^{-/-}$ and wild-type mice (Figure 8) could be the result of the *greater* inhaled dose of O₃ in the p55/p75 TNF $^{-/-}$ mice obscuring such an effect.

In rats, the decrease in $\dot{V}_E$ that occurs during O₃ exposure (34) appears to be driven by a decrease in metabolic rate, as oxygen consumption and heart rate decrease (47, 48), but arterial PCO₂ is relatively unchanged with O₃ (49). It is likely that this decrease in metabolic rate arises at least in part from a regulated decrease in core body temperature, because the latter decreases 2–3° C during O₃ exposure in rats (47), and as much as 6° C during O₃ exposure in C57BL/6 mice (50). Our results, which indicate a greater decrease in $\dot{V}_E$ during O₃ exposure in wild-type than in p55/p75 TNF $^{-/-}$ or p75 $^{-/-}$ mice, are consistent with the hypothesis that TNF accounts for at least part of the O₃-induced decrease in metabolism. These results are consistent with the observations of other investigators (25) who demonstrated that TNF is a cryogenic or antipyretic factor and acts to attenuate LPS-induced fever. Alternatively, there may be less airway injury in TNFR-deficient mice, resulting in reduced formation of other mediators involved in these metabolic events. The role of TNF in these events is likely to be mediated through the p75 TNF receptor, since O₃-induced changes in $\dot{V}_E$ were reduced in p75 TNF $^{-/-}$ (Figure 4) but not in p55 TNF $^{-/-}$ mice (Figure 6). It is also likely that the changes in EEP induced by O₃ have a different mechanistic basis than the changes in $\dot{V}_E$ induced by O₃, because changes in EEP were significantly attenuated in both p75 $^{-/-}$ and p55 $^{-/-}$ mice (Figure 7), whereas the changes in $\dot{V}_E$ were only attenuated in p75 $^{-/-}$ mice (Figure 6).

In summary, our results indicate that at the O₃ concentrations examined, TNF is important for the airway hyperresponsiveness, but not the PMN migration induced by acute O₃ exposure in mice and that this effect of TNF appears to involve activation of the p75 TNFR. Whether TNF also contributes to exacerbations of asthma that occur during periods of high ambient ozone remains to be determined.

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