Pulmonary function, bronchial reactivity, and epithelial permeability are response phenotypes to ozone and develop differentially in healthy humans

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Que LG, Stiles JV, Sundy JS, Foster WM. Pulmonary function, bronchial reactivity, and epithelial permeability are response phenotypes to ozone and develop differentially in healthy humans. J Appl Physiol 111: 679–687, 2011. First published June 23, 2011; doi:10.1152/japplphysiol.00337.2011.—Effect of laboratory exposure to O₃ (220 ppb) and filtered air (FA) on respiratory physiology were evaluated at two time points (acute and 1 day postexposure) in healthy cohort (n = 138, 18–35 yr, 40% women) comprised mainly of Caucasian (60%) and African American (33.3%) subjects. Randomized exposures had a crossover design and durations of 2.25 h that included rest and treadmill walking. Airway responsiveness (AHR) to methacholine (Mch) and permeability of respiratory epithelium (EI) to Tc99m-DTPA clearance half-time (Tc99m-DTPA clearance half-time). Based on conventional thresholds of response and dichotomous overlap among subjects classified as responsive for respective FEV₁, AHR, and EI endpoints suggests these are separate and independent phenotypes of O₃ exposure. FEV₁: airway reactivity; epithelial integrity; ethnicity

AIRBORNE ENVIRONMENTAL IRRITANTS can exert an initiating role in the exacerbation of disease processes, especially those associated with respiratory health (12, 32, 50). Inhalable irritants include both gas and solid phase air pollutants such as ozone (O₃) and particulate matter (PM). O₃, a highly reactive gas and major component of urban smog, is formed in the presence of sunlight from the interaction of volatile organic compounds with oxides of nitrogen, both of which are generated from multiple sources, i.e., motor vehicle exhaust, industrial emissions, gasoline vapors, and chemical solvents. In addition, photooxidative aging of airborne emission mixtures enhances toxicity of respirable particulates (8, 44).

An edemagenic lung irritant like O₃ can deposit on epithelial membrane surfaces of the airway and also penetrate to the more distal surfaces essential for gas exchange (24). Toxicity of O₃ to respiratory tissues is primarily attributed to generation of reactive oxygen metabolites (ROM) and ozononation of fatty acids present at epithelial cell surfaces and in lung lining fluids (30, 38). In controlled laboratory settings, early (3 h) and late (1-day post) responses of human subjects to O₃ exposure include an infiltration into airway and alveolar surface fluids of serum proteins, inflammatory cells, and mediators (10, 29). The presence of cellular and molecular markers of epithelial membrane injury and the diffusivity of small hydrophilic molecules within submucosal tissues suggests epithelial injury together with soluble cellular and secreted mediators likely contribute to enhancement of physiological and airway functional responses, i.e., airway hyperreactivity (43).

We previously established that exposure of human subjects to moderate concentrations of O₃ can alter the integrity of epithelial surfaces of the lower respiratory tract; although not a consistent observation acutely at the point of exposure, an increase in paracellular permeability of low-molecular weight hydrophilic materials was found to be present at 18–20 h postexposure (17). An alteration of epithelial integrity conveys a risk that xenobiotic and mitogenic substances that are normally contained by barrier defenses at epithelial surfaces may gain access to submucosal tissue elements leading to injury and increases in tissue repair (47). In an additional investigation we determined that airway hyperresponsiveness (AHR) in response to nonspecific bronchoprovocation was also present at ~1-day postexposure, although a range in severity of the airway hyperreactivity was codependent on environmental temperature conditions of the exposure to O₃ (14). Airway hyperresponsiveness is present in all humans with asthma, at least when symptomatic. The long-term effect(s) of increased airway responsiveness are unknown, although it is frequently considered as a risk factor for the development of chronic lung disease. Both of these prior laboratory studies although informative for the delineation of O₃-induced changes in epithelial and airway functions and with statistically significant outcomes, were accomplished in a limited number of subjects.

The objective of the present investigation was to test the hypothesis that O₃-induced changes in epithelial membrane integrity associate with the development of AHR and increased airway sensitivity to the prototypical bronchoprovocation agonist, methacholine. Airway exposure to irritants such as cigarette smoke has been associated with sensitivity to methacholine in habituated smokers (45), and although respiratory epithelial integrity is well known to be susceptible to cigarette smoke (27), the presence of bronchial AHR in at least young smokers appears unrelated to epithelial permeability (46). However, for other inflammatory airway diseases, such as stable asthma, the opposite has been demonstrated, and respiratory membrane permeability correlated with the patient’s
degree of airway AHR (26, 48). Whether barrier integrity of epithelial lining cells known to be ozone labile contributes to and correlates with bronchial reactivity to agonist stimulation of the airway has not been evaluated in humans. To test the hypothesis, we recruited healthy, adult subjects and monitored post-O3 the changes in pulmonary function at early and late (20–24 h) time points; additionally at the later time point dual assessments of epithelial integrity and non-specific AHR as well were accomplished.

**METHODS**

Subject recruitment and characteristics. Healthy women and men were recruited by advertisement to participate in the research. Subjects within the normal range of body mass index and without evidence of pulmonary disease or respiratory symptoms suggestive of asthma (cough, wheezing, dyspnea, and chest tightness) as determined by clinical history and physical examination were selected for enrollment. Subject ethnicity was based on self-reported selection. Atopic status of the volunteers was determined by skin test to 10 aeroallergens common to North Carolina region. Subject exclusions: a recent (within 4 wk) respiratory infection, current or prior smoking history, evidence of pulmonary disease or respiratory symptoms suggestive of asthma (cough, wheezing, dyspnea, and chest tightness), and greater than 10% fall in forced expiratory volume in 1 s (FEV1) after 10 min of walking on a treadmill were excluded from participating in the protocol. Table 1 lists ethnicity and sex distributions of the subjects (n = 138) recruited to the protocol and means ± SD for age, BMI, vital capacity (VFC), FEV1, and the FEV1/FVC ratio for female and male subjects at enrollment. Overall, the majority of the subjects were of Caucasian (60.0%) or African American (33.3%) ancestry and 39.8% were women; pulmonary function values for all subjects were within predicted normal range for age, sex, and body habitus. BMIs (kg/m²) were within range of normal and similar for women and men, with means of 22.82 ± 0.28 SD, and 24.05 ± 0.24 SD. Of the 138 subjects recruited to the study, 121 were skin tested (within 4 wk) respiratory infection, current or prior smoking history, and included a conditioned air ventilation system; the air supply was HEPA-filtered, dried, and conditioned to a selected range of temperatures (20–23°C) and relative humidity (45–55%). Exposures had durations of 135 min and a subject’s physical activity level during exposure alternated between 15-min periods of rest (sitting) and treadmill walking. The activity period was to mimic an individual performing mild physical activity under ambient conditions. To obtain a consistent delivery of O3 to similar areas of the respiratory tract during activity periods, the degree of walking necessary to raise minute ventilation to ~6–8× forced vital capacity (FVC, l/min) was established during history assessment and screening procedures, and thus this amount of minute ventilation was the targeted ventilation used during activity periods of the exposures (14). For exposure to O3 the dose was controlled to within ±10 ppb of 220 ppb air and was monitored continuously for O3 (Tetodyne, Advanced Pollution Instrumentation, model 400E, San Diego, CA). O3 was generated from 100% O2 source by cold plasma corona discharge (Ozotech, Yreka, CA) and mixed with filtered air before addition to the chamber. Minute ventilation, frequency of ventilation, pulse rate, and transcutaneous O2-sat were monitored periodically during the exposures. During summer months in the Triangle area of NC the ambient level of O3 may peak in excess of 120 ppb (daily readouts of air quality for Durham County and RTP community of NC are available by internet from EPA), and therefore to avoid secondary exposures, protocols (filtered air or O3) were postponed 10–14 days during periods when community O3 levels consistently peaked above 120 ppb.

Bronchoprovocation methods. Subjects were pretrained to follow a visual display guide and regulate their inspiratory flow rates (<0.5 l/s) during inhalation of saline (0.9% NaCl) aerosol, followed by increasing doses of methacholine diluted in 0.9% NaCl. Methacholine chloride (Mch, provocholine, Metapharm, Coral Springs, FL) solutions were made fresh for each test challenge. The Mch aerosol, generated by jet nebulization and powered from a filtered air supply at 35 psi, was inhaled with a nose-clip in place, during mouth breathing to inspiratory capacity with a dosimeter (Spiru-elektro 2, Respiratory Care Center, Finland) to control onset and duration of nebulization. At the end of the aerosol inhalation breath, a 5-s breath-hold was instituted, before a slow exhalation to rest lung position. The starting aerosol dose level of Mch was 0.025 mg/ml and then increased on each subsequent aerosol dose to a maximal level of 25 mg/ml. After the fifth aerosol inhalation at each Mch dose, subjects relaxed for 2 min and then had their spirometric response measured for each cumulative Mch dose inhaled. Challenge procedures were halted if FEV1 fell by 20% or more from the maximum FEV1 that was recorded at baseline (following 0.9% NaCl) or during the challenge or when the maximum Mch dose was delivered. Based on advantages of analysis method recommended by O’Connor and colleagues (36) and its sensitivity to quantitate AHR in a general population by Schwartz et. al. (41), we characterized Mch responsiveness by calculating the dose-response slope for each subject. To avoid negative slopes, the absolute decline in FEV1 was defined as the difference between the maximum FEV1 over all Mch levels tested and FEV1, at the last level measured, irrespective of the dose level at which the maximum FEV1 occurred (41). Thus the percentage decline was computed using the maximum FEV1 as the reference value; and the dose–response slope was then defined as the ratio between the percentage decline in FEV1 and the total cumulative dose of Mch (mg inhaled from the nebulizer). A doubling of the slope of the Mch dose–response curve after ozone compared with after filtered air exposure was used as criteria to define a subject as ozone responsive for this endpoint (AHR to Mch).

Table 1. **Subject characteristics**

<table>
<thead>
<tr>
<th>Ethnicity</th>
<th>Women</th>
<th>Men</th>
</tr>
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<tbody>
<tr>
<td>Subject number</td>
<td>55</td>
<td>83</td>
</tr>
<tr>
<td>Caucasian</td>
<td>28</td>
<td>55</td>
</tr>
<tr>
<td>African American</td>
<td>24</td>
<td>22</td>
</tr>
<tr>
<td>Hispanic</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Asian</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Age, yr</td>
<td>23.05 ± 3.86</td>
<td>22.25 ± 4.10</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>22.82 ± 2.07</td>
<td>24.05 ± 2.19</td>
</tr>
<tr>
<td>FEV₁ (l/s) @ baseline</td>
<td>3.10 ± 0.44</td>
<td>4.43 ± 0.73</td>
</tr>
<tr>
<td>FVC (l) @ baseline</td>
<td>3.64 ± 0.52</td>
<td>5.42 ± 0.91</td>
</tr>
<tr>
<td>FEV₁/FVC</td>
<td>0.86 ± 0.07</td>
<td>0.82 ± 0.09</td>
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</table>

Values are means ± SD. BMI, body mass index; FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity.
Epithelial integrity methods. Lung clearance of the hydrophilic radionuclide, $^{99m}$Tc-diethylentriamine pentaacetic acid ($^{99m}$Tc-DTPA, MW = 490 Daltons; Draxis Pharmaceutical, Quebec, Canada) through paracellular pathways following aerosol deposition onto epithelial surfaces was measured non-invasively by scintigraphy with a γ-camera (Picker, Dyna 5). We used these methods previously in both animal models and humans (15, 17), and for human subjects, imaging is acquired from the posterior aspect in an erect, sitting position. $^{99m}$Tc-DTPA on occasion is sampled prior to and postaerosolization to certify stability of the radiolabel during the measurement by chromatographic analysis (6). Prior to inhalation of $^{99m}$Tc-DTPA a ventilation scan is acquired with $^{133}$Xenon gas and rebreathing circuit (Pulmonmex, Biode, NY) to obtain a steady-state image of regional lung volume at functional residual capacity (16). The ventilation image is subsequently used to correct for tissue attenuation and to precisely outline ventilated lung regions for analysis of deposition and lung clearance kinetics of $^{99m}$Tc-DTPA. The $^{99m}$Tc-DTPA aerosol, generated by jet nebulization technique (Ultravent, Mallinkrodt, St. Louis, MO) and energized with a filtered air supply at 35 psig, is delivered at ambient pressure as an aqueous droplet (equivalent aerodynamic diameter of 1.2 μm and a σg > 1.2). To facilitate preferential deposition onto epithelial surfaces of the airway, $^{99m}$Tc-DTPA aerosol is inhaled with an aerosol dosimeter (Spira-elektra 2, Respiratory Care Center, Finland) and mouth breathing in a seated position using a controlled inspiratory flow rate (17, 18). Analysis methods to characterize the regional clearance kinetics of $^{99m}$Tc-DTPA from the lung have been developed previously and retention levels of $^{99m}$Tc-DTPA are decay corrected and graphs are constructed of lung clearance vs. time and indexed as a half-time (T1/2) of clearance using a linear regression analysis (assumes a mono-exponential clearance process) (17). Analysis techniques based on the ventilation scan with Xe133 evaluated the penetration and distribution of the $^{99m}$Tc-DTPA droplets onto the distal airway. A number of factors that may influence the clearance of small soluble molecules through paracellular pathways of alveolar epithelia, i.e., surface area, lung volume, position of the lung with respect to gravity, etc., are less influential to clearance of these molecules from the epithelial surfaces of the distal airway. Clearance of soluble markers from the peripheral airway by mucociliary clearance has not been problematic previously or in the present study using the methods described above for aerosol delivery and clearance assessment of $^{99m}$Tc-DTPA.

Criteria for severity of ozone responses (FEV1, PD20 Mch) and $^{99m}$Tc-DTPA T1/2). We and others have demonstrated that the functional and biologic responses to ozone in laboratory studies are diverse, with healthy subjects demonstrating mild to high sensitivity to ozone (4, 37, 45). Physiological responses have generally been found to follow dose-response relationships with threshold concentrations leading to more robust responses (23, 42). For the present study we used a dichotomous classification to rank subjects as responsive to ozone based on exposure-induced changes in FEV1, epithelial permeability to $^{99m}$Tc-DTPA, and bronchial responsiveness to Mch. Thus we considered a subject to be FEV1 responsive, if immediately after exposure to ozone the subject had an acute pre-post change in FEV1 of ≥15%. This convention is consistent with prior studies. For example others have classified subjects as O3 “sensitive” who had a ≥10% decrease in the FEV1 acutely following exposure to 200 ppb (4) or a fall of 15% or greater for more effective concentrations (≥400 ppb) (37). Although less is known whether response thresholds also exist a full day after exposure for ozone-induced alterations in epithelial integrity and/or responsiveness to Mch, by our own convention we set changes in permeability (≥15% decrease in the T1/2) or sensitivity to Mch (doubling in the slope of the Mch dose-response curve) as criteria to identify ozone-responsive subjects for these response phenotypes.

Statistical measures. To ensure controlled exposure conditions of temperature, relative humidity, and ozone concentration, continuous read-outs of conditions permitted readjustment whenever necessary. For assessment of MV during exercise activity periods, expired minute volume was measured and recorded directly for 1–2 min by mouth breathing with a dry gas meter. Standard statistical methods were used to determine the mean (±SE) of response endpoints. For basic comparisons between responses to FA and O3, e.g., to determine the significance of differences in $^{99m}$Tc-DTPA lung clearance, dose-response slope to Mch challenge, and FEV1 and FVC, Snedcor’s F test and two-tailed Student’s t-test were used to determine the equality of variances and the significance of the differences in response to treatment (FA vs. O3). Linear regression analyses (method of least squares) was used to test for I) associations between FEV1 and FVC outcomes measured either acutely after FA or ozone or at ~1 day postexposure and 2) possible correlations between response elements (FEV1, Mch dose response slope, and T1/2 permeability). For intercomparisons of group data based on ethnicity or sex for a measured endpoint (FEV1, FVC, Mch dose response slope, T1/2) an unpaired t-test was applied to determine if differences between groupings or sex were significant.

RESULTS

Measures of airway functional response to ozone. By design, minute ventilations (MV) during the treadmill activity periods of each exposure treatment (FA and O3) were targeted for a given individual and based upon lung volume (see METHODS). The MV achieved by the subjects during activity periods did not differ significantly between FA and O3 exposures; the means for female subjects were 22.47 (±3.78 SD) and 23.88 (±4.72 SD) l/min, and for the male subjects, means of 31.49 (±6.28 SD) and 32.14 (±6.14 SD) l/min for FA and O3 exposures, respectively. In general, and as reported previously, during exposures and when minute ventilation is increased by treadmill walking, subjects reduce the depth of the tidal volume inspired with a compensatory increase in the frequency of respiration (19). The mean changes in pulmonary function of the subjects measured immediately after exposure to O3 and FA are presented in Fig. 1. FEV1 and FVC responses were calculated as the percent change from the functional values observed pre-exposure. Immediately following exposure to O3, the mean changes in pulmonary function of the subjects measured immediately after exposure to O3 and FA are presented in Fig. 1. FEV1 and FVC responses were calculated as the percent change from the functional values observed pre-exposure.
reductions in functional indices of the FEV₁ and FVC were observed with mean decreases of 8.80% (±0.79 SE) and 7.18% (±0.64 SE), respectively. These impairments, as shown in Fig. 1, were significant compared with function following exposure to FA. Additionally, when remeasured at ~1-day postexposure, FEV₁ and FVC after ozone, although in recovery, were still slightly reduced, e.g., mean decreases of 2.31% (±0.35 SE) and 1.78% (±0.31 SE), respectively from the pre-exposure values. Although these changes at ~24 h after ozone were improved, the difference was significant compared with function 24 h after FA exposure. When compared in total (men and women together), the mean FEV₁ and FVC responses to ozone of the two largest ethnicity groups (African American and Caucasian) did not differ significantly; however, when analyzed separately between sex FEV₁ response of African American men (mean decrease of 16.79%) to ozone was more robust than observed for either black women (decrease of 6.18%) or Caucasian men (7.94% fall from pre-exposure FEV₁), and these differences were significant, $P \leq 0.003$. For comparison, the differences between sex and ethnic subject groupings with respect to the acute FEV₁ and FVC mean responses to O₃ are presented in Table 2. As shown above in Fig. 1, there were no substantive effects either acutely or at 1-day postexposure from exposure to FA on FEV₁ or FVC and thus response data to FA are not included in Table 2. When the functional responses to ozone for all subjects are viewed together, FEV₁ was found to range from no change, to considerably robust responses; this is demonstrated in a frequency response histogram, presented in Fig. 2. A range in sensitivity to ozone based on the decrement in FEV₁ was observed at the early time point, acutely and at end exposure. For example, and based on a threshold of a $\geq$15% decrease in FEV₁, 28 of the 138 subjects (20.3%) exhibited this level or greater of impairment of FEV₁ function. The pre/post changes in FEV₁ after ozone, both acutely and after ~24 h, were associated with the respective and corresponding pre/post changes in FVC; these relationships are presented in Fig. 3. A and B, and, when fit with a least squares linear regression, were found to be highly correlated with correlation coefficients ($r^2$) $>0.70$.

Sensitivity to Mch challenge. The results for responsiveness to Mch of the subjects are shown in Fig. 4, where the mean values for slopes of the Mch dose-response curves measured at the 1-day post ozone and FA time points are presented for 134 of the subjects (4 subjects who demonstrated extreme sensitivity to Mch in the steepness of their dose-response curves, and based on statistical evaluation were considered outliers to the normal distribution of values, were not included in the

Table 2. Acute airway function changes post ozone

<table>
<thead>
<tr>
<th>Ethnicity Grouping</th>
<th>FEV₁ Change (% of PreValue)</th>
<th>FVC Change (% of PreValue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>African American</td>
<td>16.79 ± 3.04*</td>
<td>-12.63 ± 2.43†</td>
</tr>
<tr>
<td>Male (n = 22)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>African American</td>
<td>-6.18 ± 1.51</td>
<td>-6.00 ± 1.52</td>
</tr>
<tr>
<td>Female (n = 24)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>-7.94 ± 1.00</td>
<td>-6.00 ± 0.67</td>
</tr>
<tr>
<td>Male (n = 55)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>-8.32 ± 1.36</td>
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</tr>
<tr>
<td>Female (n = 28)</td>
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<td></td>
</tr>
<tr>
<td>Others (n = 28)</td>
<td>-3.88 ± 1.64</td>
<td>-4.55 ± 1.49</td>
</tr>
<tr>
<td>Male (n = 6)</td>
<td>-1.33 ± 0.67</td>
<td>-4.17 ± 0.44</td>
</tr>
<tr>
<td>Others (n = 3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female (n = 3)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. Others, subjects from Asian or Hispanic ethnicities.

* $P < 0.01$, † $P < 0.001$, significantly different vs. changes in airway function of other Ethnicity groups.

Fig. 2. Frequency distribution of subjects with change in FEV₁ after ozone. Acute FEV₁ response, calculated as percent change from pre-exposure value, of subjects ($n = 138$) exposed to O₃. Filled bars represent number of subjects with $\geq$15% decrease in FEV₁.

Fig. 3. Relationship between change in FEV₁, to change in FVC after ozone. A: acute change in FEV₁ and FVC, presented as % change from pre-exposure value, are correlated; B: change in FEV₁ and FVC at ~24 h post ozone are correlated. For graphs each point represents data for a single subject ($n = 138$); solid line: data fit to a linear regression with goodness of fit $r^2 = 0.70$, $P < 0.001$. 
analyses for calculation and comparison of mean responses). Mean values for the dose-response slopes (decrease of FEV$_1$ − %/mg Mch) were 14.15%/mg (±1.82 SE) after FA compared with 22.61%/mg (±3.85 SE) after ozone, and this difference was significant, $P < 0.003$. For each subject the value of the Mch dose-response slope after O$_3$ was divided by the slope after FA; this ratio was used to generate a frequency response histogram shown in Fig. 5 and demonstrates the distribution of O$_3$-responsive subjects. For example, the subjects with a ratio value that was $\geq 2$, i.e., a doubling of the slope of the dose-response curve after ozone compared with FA, were considered to be responders, i.e., susceptible to O$_3$-induced airway hyperresponsiveness. Overall, of the 134 subjects evaluated, after ozone 31 subjects (23.1%) had changes in sensitivity to Mch at or above this threshold (equal to or more than a doubling of the slope of the Mch dose-response curve). Mean values for the slope of the Mch dose-response curve were similar between ethnic and/or sex comparisons, and significant differences were not observed between groupings (data not shown).

Radioisotopic measures of epithelial permeability. The clearance rate of hydrophilic $^{99m}$Tc-DTPA radiomarker from the lung’s epithelial surfaces at 20 h postexposure was also observed to be enhanced (more permeable epithelial surface) by exposure to O$_3$. Mean results for T1/2 clearance times (time for 50% of the initial radioisotopic lung burden deposited to be cleared from the lung) are presented in Fig. 6, and although the difference in the mean values for clearance between FA and O$_3$ were small, by paired analysis the values differed significantly, with a smaller mean value for T1/2 after ozone, i.e., a faster clearance rate. However, mean permeability (T1/2) responses to ozone between ethnicity or sex groupings were not found to differ significantly. Similar to the divergence in sensitivity to ozone for the functional indices of FEV$_1$ and slope of Mch dose-response curve, epithelial permeability was also observed to separate subjects into responder and nonresponder categories. A frequency response histogram for permeability is presented in Fig. 7 and demonstrates the range of O$_3$-induced changes in the T1/2 clearance index. Three of the 138 subjects entered into the protocol were not evaluated with the radioisotopic technique for personal or technical reasons. A threshold change representing an equal or greater than 15% decrease in the T1/2 for $^{99m}$Tc-DTPA after O$_3$ compared with the corresponding T1/2 of the subject after FA was used to classify a responder to O$_3$. 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subject as an ozone responder. Thirty-five (25.9%) of the 135 subjects evaluated met or exceeded this threshold.

**Interaction of response elements.** Consistent with earlier controlled laboratory studies and exposure to O₃ (22), atopic status was not found to be influential for altering sensitivity to ozone for any of the three endpoints that were measured. With respect to interaction between endpoints, some overlap was found for those subjects who demonstrated an O₃-induced increase in sensitivity to Mch, as well as a loss of epithelial membrane integrity, but this appeared to be a random occurrence. Also the intensity of a given responder’s sensitivity to O₃ did not appear to be related for these two response elements; for example, the responders who demonstrated the largest ozone-induced decreases in the T1/2 clearance time, did not demonstrate an equally severe change in the slope of the Mch dose-response curve. We also analyzed for an interaction between epithelial integrity and/or Mch sensitivity with ozone-induced changes in FEV₁ for those subjects who met or exceeded the threshold for an acute change (decrement of 15% or greater in FEV₁). Likewise a direct relationship was not found between acute changes in FEV₁ and either of the other two response elements (epithelial permeability or Mch sensitivity) evaluated at the 20 h time point. Presented in Table 3 are the results of regression analyses to determine if there was an association between ozone-induced changes in epithelial permeability and development of Mch AHR, and there also was an association between the acute changes in FEV₁ measured acutely post-ozone and either of the response elements, epithelial permeability or Mch AHR. None of the response elements were shown to demonstrate a linear correlation with either of the other responses ($r^2 = 0.02$, $P > 0.10$). Thus each response element appears to be a separable phenotype and independent of each other. A Venn diagram in Fig. 8 graphically demonstrates the independence of the three response elements.

**DISCUSSION**

Our results represent the largest single cohort study to date to evaluate airway and respiratory epithelial responses to ozone in young healthy adult subjects in a controlled laboratory setting. The experimental design of the protocol had a straightforward rationale to determine if two known airway responses that occur temporarily at near identical time points postexposure to ozone, were functionally associated. The concept that an injured and hyper-permeable epithelium facilitates and enables access of agonist stimuli to submucosal tissues is supported by studies in airway diseases of a chronic inflammatory nature, such as asthma where a close relationship has been shown between airway epithelial permeability and airway hyperresponsiveness (26, 48). Although it was not an initial goal, the sample size of our study population ($n = 138$) permitted us with confidence to strengthen prevailing dogma that similar to spirometric changes induced by ozone wherein subjects exhibit low to high responsiveness (4, 49), a dichotomous range in subject susceptibility to ozone is likewise demonstrated for AHR and epithelial permeability phenotypes.

Our current findings confirm and expand on our prior results on the potential for exposure to O₃ to impair epithelial integrity and to increase nonspecific bronchial airway hyperresponsiveness to Mch (14, 17). Furthermore we demonstrate in the present investigation that these responses to O₃ do not occur uniformly across all subjects, and we identify the presence of both resistant and ozone-sensitive subjects. Moreover, and although it is an attractive hypothesis that desquamation and epithelial injury may lead to heightened airways responsiveness, our results do not support a direct relationship between O₃-induced changes in epithelial integrity and the development of airway responsiveness to Mch. These findings were unexpected as we had initially hypothesized that ozone-induced impairment of integrity would lead to an increased permeability of hydrophilic molecules, such as Mch, and contribute to and/or correlate with a change in absorption or availability and increase airway responsiveness to Mch.

**Table 3. Regression analyses for association between phenotypes**

<table>
<thead>
<tr>
<th>Sample Size</th>
<th>Relationship</th>
<th>$r^2$</th>
<th>$P$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$n = 131$</td>
<td>EP ratio (T1/2 O3/FA) = -0.0171 Mch ratio (O3/FA) + 1.00</td>
<td>0.0204</td>
<td>0.103</td>
</tr>
<tr>
<td>$n = 134$</td>
<td>Mch ratio (O3/FA) = -0.0115 FEV₁ change post O3 + 1.55</td>
<td>0.0048</td>
<td>0.427</td>
</tr>
<tr>
<td>$n = 135$</td>
<td>EP ratio (T1/2 O3/FA) = -0.0005 FEV₁ change post O3 + 0.96</td>
<td>0.0007</td>
<td>0.753</td>
</tr>
</tbody>
</table>

EP Ratio (T1/2 O3/FA) represents the ratio of T1/2 permeability values following O₃ and FA exposures, respectively; Mch ratio (O3/FA) represents the ratio of slope for Mch dose-response curves following O₃ and FA exposures, respectively; and FEV₁ change post O₃ represents the change in the FEV₁ assessed immediately postexposure and expressed as % change from the pre-exposure value of FEV₁. $r^2$ is square of the correlation coefficient. The sample sizes vary slightly due to several subjects, as described in the RESULTS, and were not evaluated for either Mch dose-response sensitivity or for the epithelial permeability response.

![Fig. 8. Relationship between subjects classified with responder phenotypes (FEV₁, Mch sensitivity, and epithelial permeability) to O₃. The percentage of subjects, at or above response thresholds (see METHODS) to O₃ for a specific end point, are indicated in the Venn diagram for each end point element (FEV₁, AHR, or Epith Perm). Number in brackets represents the number of subjects classified as O₃ responders for two or more of the indicated end points.](http://jap.physiology.org/)
The overlap among given individual subjects to present as a responder for one, two, or even three response elements (FEV1, epithelial permeability, and airway hyperresponsiveness) within the cohort was limited, as demonstrated in Fig. 8, and thus overall each response element appears to be an independent response. Given the relatively complex physiologies surrounding each of these functional and epithelial responses, this suggests that more than a single host factor is likely responsible for modulating susceptibility to O3. Individual airway responses to ozone, for example the FEV1 and/or AHR, have been shown by us and others to be highly repeatable if the subject is restudied under identical exposure conditions (13, 14, 34). Thus these response elements demonstrate high levels of reproducibility for periods of time that may extend over a period of at least 12 mo. With respect to epithelial integrity and inherent sensitivity to ozone within a given subject, the T1/2 values for a subset of the present subjects evaluated on two separate study days with ozone are presented in Fig. 9. Similar to FEV1 and AHR responses to ozone, at least for healthy subjects, ozone-induced changes in epithelial permeability appear constant and reproducible. We have interpreted this to indicate that each of these response elements (FEV1, AHR, and epithelial integrity) are indeed valid physiological phenotypes to characterize airway vulnerability to ozone, and as shown by us and others, sensitivity to ozone is relatively constant over time in young healthy adults.

Increased ambient levels of O3 have been found to be associated with adverse health effects in both healthy individuals and “sensitive” groups, including asthmatics (32, 33, 39). Furthermore laboratory studies have shown only subsets of normal individuals, estimated at 30 to 50% of the population, experience measurable adverse effects after O3, including even just short-term exposures to O3 levels of ~0.10 to 0.20 ppm (EPA 8 h standard for “moderate” exposure is 0.065 to 0.084 ppm; 3, 9, 34). This has led to the supposition that host genetic factors are likely contributing to differences in O3 sensitivity.

There is a suggestion that a component of the O3-induced responsiveness acutely at a point immediately postexposure is vagally mediated since premedication with a cholinergic antagonist reverses the development of responsiveness in vivo (49). However, in the present study we found the African American male subjects had robust spirometric responses to ozone in the acute post-ozone period demonstrated by FEV1 decrements significantly exceeding changes observed in the African American women and Caucasian men. For the other phenotypic responses to ozone, epithelial permeability and Mch sensitivity, sensitivity to ozone was independent of either ethnicity or sex effects. Our results for decreases in FEV1 after ozone in African American males are consistent with an earlier study in which reductions in FEV1 after ozone also tended to be more severe in African American men (42). Others have also demonstrated that O3-induced decrements in FEV1 are not predicted nor associated with the degree of Mch responsiveness, for example at baseline (2). Based upon recent investigations in humans, the acute, ozone-induced inhibition of the ability to inspire that leads to a decrement in FVC is proposed to be reflex in origin and results from afferent vagal stimulation mediated by bronchial C-fibers in the smaller distal and peripheral airways (37, 40). We demonstrated previously that FRC and/or thoracic gas volume (Vtg) do not change following exposure to ozone (16, 18), and thus a decrease in FVC would be expected to limit elastic recoil and contribute mechanically to a reduction in FEV1. Therefore acutely after ozone exposure, the impairment in pulmonary function and significant association between spirometric FVC and FEV1 variables (Fig. 3A), was not a surprising finding. However, after 20 h of recovery and a much milder stage of airflow obstruction, a close relationship for these two variables is sustained and is a unique observation (Fig. 3B). Correlation of these variables at the later time point is not likely a result of residual reflex afferent stimulation and may reflect delayed mucosal inflammation and tissue edema formation leading to changes in viscoelastic properties and mild airflow obstruction (10).

Previously, we clearly demonstrated that airways may become hyperresponsive at ~20 h postexposure to O3, compared with exposure to FA. A difference in our present approach is that we used FEV1 index to gauge development of AHR, whereas previously we relied on airway conductance (sGaw) acquired by body plethysmography (14). Thus, in the earlier study, Mch sensitivity was more representative of changes in large airway caliber, and in the present study, the changes in airflow conductivity would be applicable to Mch sensitivity of both large and smaller lung airways. A mechanism for the increase in responsiveness has not been established, although as shown by us and others, respectively, neither ozone-induced decrements in spirometric function (14) nor inflammation (monitored by inflammatory cell influx and cytokine profiles of bronchoalveolar lavage fluids)(4) correlate with airway responsiveness. Human bronchial airways evaluated ex vivo respond to oxidative stimuli with hyperreactivity, although this response was disassociated with epithelial permeability (25). There is a suggestion that a component of the O3-induced responsiveness in vivo is vagally mediated since premedication with a cholinergic antagonist reverses the development of responsiveness acutely at a point immediately postexposure (5). Neuronal and reflex activity have been proposed, and in a
recent animal model study we demonstrated that at 20 h post ozone the release of neuroendocrine peptides in airway tissues leads to the development of AHR (51). An additional model study (20) supports the postulate that prejunctional muscarinic M2 receptors (auto-receptors that normally function to limit neurotransmitter release and ablate reflex-induced AHR) on the efferent postganglionic parasympathetic nerves that innervate the airway are impaired by inflammatory cell infiltration post ozone (20).

We found the subjects to be differentially susceptible to O₃-induced changes in epithelial integrity. This is consistent with invasive techniques and collection of bronchoalveolar lavage and localized anesthesia where the presence of increased vascular proteins such as serum albumin and cellular markers of inflammation within epithelial surface fluids procured from small airway and alveolar units also are suggestive that epithelial permeability changes do not occur uniformly, and rather subsets of responder and non-responding subjects are observed (4). Although exercise is a physiological factor known to lead to increases in lung clearance of ⁹⁹ᵐTc-DTPA, epithelial permeability changes are recognized to occur acutely and within 30 min of the end of an exercise period (35). Due to the time difference (we assessed epithelial integrity at 1-day after treadmill activity periods), and the reduced level of physical activity of our protocol design (treadmill walking, rather than maximal exercise levels), it is unlikely that exercise and intermittent increases in MV during the activity periods of the exposure were influential to our observations. Additionally, the levels of activity and MV were by design at equivalent levels during FA and O₃ exposures. There is no obvious mechanism to explain O₃-induced changes in permeability, although reactive oxygen metabolites generated at the interface of O₃ with the respiratory epithelium and interaction with cellular tight-junction proteins is an attractive one (10, 52).

With respect to longevity of a more permeable epithelium, we demonstrated that thresholds for vulnerability to ozone exist at time points at least 1 day postexposure, and the susceptibility to develop AHR and/or increases in epithelial permeability is induced at significant levels (23–24%) within young adult-aged, healthy subjects.

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