Methacholine responsiveness in mice from 2 to 8 wk of age

Elizabeth M. Bozanich,1 Tibor Z. Jánosi,1,2 Rachel A. Collins,1 Cindy Thomrin,1 Debra J. Turner,1 Zoltán Hantos,1,2 and Peter D. Sly1

1Division of Clinical Sciences, Telethon Institute for Child Health Research & Centre for Child Health Research, The University of Western Australia, Perth, Australia; and 2Department of Medical Informatics and Engineering, University of Szeged, Szeged, Hungary

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Bozanich EM, Jánosi TZ, Collins RA, Thomrin C, Turner DJ, Hantos Z, Sly PD. Methacholine responsiveness in mice from 2 to 8 wk of age. J Appl Physiol 103: 542–546, 2007. First published May 10, 2007; doi:10.1152/japplphysiol.01253.2006.—Many chronic human lung diseases have their origin in early childhood, yet most murine models used to study them utilize adult mice. An important component of the asthma phenotype is exaggerated airway responses, frequently modelled by methacholine (MCh) challenge. The present study was undertaken to characterize MCh responses in mice from 2 to 8 wk of age measuring absolute lung volume and volume-corrected respiratory mechanics as outcome variables. Female BALB/c mice aged 2, 3, 4, 6, and 8 wk were studied during cumulative intravenous MCh challenge. Following each MCh dose, absolute lung volume was measured plethysmographically at functional residual volume and during a slow inflation to 20-hPa transrespiratory pressure. Respiratory system impedance was measured continuously during the inflation maneuver and partitioned into airway and constant-phase parenchymal components by model fitting. Volume-corrected (specific) estimates of respiratory mechanics were calculated. Intravenous MCh challenge induced a predominantly airway response with no evidence of airway closure in any age group. No changes in functional residual volume were seen in mice of any age during the MCh challenge. The specific airway resistance MCh dose response curves did not show significant differences between the age groups. The results from the present study do not show systematic differences in MCh responsiveness in mice from 2 to 8 wk of age.

Animal models; respiratory impedance; lung volumes

THE USE OF MICE TO STUDY human lung diseases has increased enormously over recent decades, especially with the advent of transgenic technology. Such studies have led to many new insights into disease mechanisms, genetic susceptibilities, and therapeutic advances. Techniques for measuring lung function have been developed and have increased in sophistication (1, 5, 21). However, the vast majority of murine models of human lung disease involve inducing and studying disease in adult mice with mature lungs. Pediatricians recognize that the infant’s lung is not a scale model of an adult’s lung (8); hence, for animal models to mimic the development of human lung diseases, models that initiate disease in infancy should represent a significant advance.

An important component of human asthma is heightened responsiveness to a variety of environmental stimuli (23). This is frequently demonstrated and categorized using an inhaled methacholine (MCh) challenge (11, 23). MCh challenges also form an important part of the assessment of animal models of allergic inflammation and asthma (21) where both inhaled or intravenous routes of delivery are used (15). Previous studies in other species show conflicting results, with reports of increased responsiveness to constrictor (4, 19), decreased responsiveness (18), or no difference (14) in younger animals compared with adults. However, studies examining the relative sensitivities and responses in mice during the period of rapid growth from 2 to 8 wk of age have not been reported.

In most species, including humans and rodents, changes in respiratory mechanical properties with lung volume (Vl) have been documented (16, 17, 20). The knowledge of the actual operating Vl is particularly important in mice where one might expect derecruitment of the airspaces to occur because of the joint effects of anesthesia, the supine position, and the compliant chest wall of this species (22). Measuring volume-corrected, or -specific, respiratory mechanics is especially important where comparisons are to be made between animals of different ages, sizes, and stages of maturity. We have developed sophisticated techniques for measuring specific respiratory mechanics in mice during the main growth periods from infancy to beyond sexual maturity (3, 9, 12), which include measurements made at functional residual capacity (FRC) and during a slow inflation-deflation maneuver. The combination of these measurements with those of absolute Vl allows a full characterization of the mechanical response to MCh in mice.

The present study was performed to determine whether MCh responsiveness differed in mice from 2 to 8 wk of age. BALB/c mice were studied because of their prominence in allergy/asthma models. Mice aged 2, 3, 4, 6, and 8 wk of age underwent intravenous MCh challenges, and the absolute Vl and volume-corrected (specific) respiratory mechanics were measured as the outcome variables.

METHODS

Animal preparation. To allow mice to be studied at the exact ages of 4, 6, and 8 wk, age-specific female BALB/c mice were purchased (ARC Murdoch). Timed-pregnant BALB/c mice were also purchased to allow their offspring to be studied at the exact ages of 2 and 3 wk (Table 1). The mice were prepared according to previously published methods (3). All procedures were approved by the TICHR Animal Ethics Committee and conformed to National Health and Medical Research Council of Australia guidelines. Briefly, following induction of anesthesia with an intraperitoneal injection containing xylazine (2 mg/ml, Troy Laboratories) and ketamine (40 mg/ml, Troy Laboratories) at a dose of 0.01 mg/ml mice were tracheostomized and connected to the ventilator (flexiVent, SCIREQ, Montreal, Canada) and...
ventilated (tidal volume = 8 ml/kg; breathing frequency = 450 breaths/min; positive end-expiratory pressure = 2 hPa). Under light microscopy, a saline-filled polyethylene cannula (length = 27 mm; outer diameter = 0.61 mm; Microtube Extrusions) was introduced into the right jugular vein and secured with silk ligature. The cannula was then connected to a syringe pump (Stoelting Syringe Pumps, Wood Dale, IL) and saline infusion was commenced to maintain patency of the line. The mouse was then scaled into a 160-ml custom-made whole body plethysmograph (12) and allowed to stabilize for 5 min. Our protocols do not use muscle paralysis, but the ventilation pattern ensures that mice remain apneic during the measurement period (3, 15). Supplemental doses of anesthesia were given as required (approximately every 40–60 min).

Vl. The technique we have used to measure Vl in apneic mice has previously been described in detail by Janosi et al. (12). Briefly, two small electrodes were placed into the intercostal muscles of the mouse and connected to an electrical stimulator (Grass Instruments, Quincy, MA). Ventilation was paused, the positive end-expiratory pressure was removed with the airway, and plethysmograph was opened to atmosphere to allow the lungs to reach the elastic equilibrium volume at transrespiratory pressure of 0 hPa, defined as FRC. With the plethysmograph closed and the airway occluded, five to eight stimulated breathing efforts were induced over a 10-s period. FRC was then calculated by using Boyle’s principle (6). Vl was then increased by lowering the plethysmograph pressure from 0 to −20 hPa in a quasi-linear fashion during 15–20 s. The increase in Vl from FRC to transrespiratory pressure = 20 hPa (VL20) achieved during the slow deep inflation (sDI) maneuver was determined by integrating the flow into the animal through the wave tube as previously described (12). The inflation phase was followed by a slow passive expiration to transrespiratory pressure = 0 hPa, where the measurement of FRC was repeated in a subgroup of animals.

Respiratory system impedance. Respiratory system impedance (Zrs) was measured using the low-frequency (4–38 Hz) forced oscillation technique and a wave-tube system as described previously (9). A model with airway and constant phase tissue compartments (10) was fitted to Zrs to derive parameters of airway mechanics [airway resistance (Raw) and inertance], tissue mechanics with coefficients of tissue damping (G), elastance (H), and hysteresivity (η = G/H). Although we note that Raw corresponds to the sum of Newtonian resistances in the total respiratory system, we have previously described in detail by Janosi et al. (12). Briefly, two small electrodes were placed into the intercostal muscles of the mouse and connected to an electrical stimulator (Grass Instruments, Quincy, MA). Ventilation was paused, the positive end-expiratory pressure was removed with the airway, and plethysmograph was opened to atmosphere to allow the lungs to reach the elastic equilibrium volume at transrespiratory pressure of 0 hPa, defined as FRC. With the plethysmograph closed and the airway occluded, five to eight stimulated breathing efforts were induced over a 10-s period. FRC was then calculated by using Boyle’s principle (6). Vl was then increased by lowering the plethysmograph pressure from 0 to −20 hPa in a quasi-linear fashion during 15–20 s. The increase in Vl from FRC to transrespiratory pressure = 20 hPa (VL20) achieved during the slow deep inflation (sDI) maneuver was determined by integrating the flow into the animal through the wave tube as previously described (12). The inflation phase was followed by a slow passive expiration to transrespiratory pressure = 0 hPa, where the measurement of FRC was repeated in a subgroup of animals.

MCh challenge. Doubling doses (6–48 μg·min⁻¹·kg⁻¹) of β-methacholine chloride (Sigma-Aldrich) were delivered for 5 min by constant infusion via the jugular vein cannula. We have previously established, by measuring Zrs each minute (data not shown), that a steady-state constriction is achieved by 5 min and verify this in each animal by monitoring Pr during mechanical ventilation. FRC was measured, and a single sDI maneuver was performed with the infusion continuing to run.

Statistical analysis. Differences in FRC before and after an sDI maneuver performed at baseline and the maximum MCh dose in a subgroup of mice (3 and 4 mice from the 3- and 8-wk group, respectively) were determined using paired t-tests. MCh responsiveness, calculated as the concentration causing a 200% increase (EC200) in Raw, was assessed by one-way ANOVA. Differences in FRC with age, changes during MCh challenge, and dose-response curves for Raw, G, H, and η were tested by two-way repeated-measures ANOVA followed by the Holm-Sidak method for all pairwise multiple comparison procedures. All statistical analyses were performed using SigmaStat software (version 3.2, SPSS Science).

RESULTS

Mouse characteristics and baseline lung function, measured at FRC, are shown in Table 1. On group mean data, progressive increases are seen in body weight and in FRC with age and progressive decreases seen in the absolute values of Raw, G, and H. The value of η shows little change across the age groups.

Vl. during MCh challenge. No systematic changes were seen in FRC during MCh challenge in any age group (P = 0.78; Fig. 1). FRC measured at the beginning and end of an inflation maneuver in mice aged 3 and 8 wk showed that Vl was not altered by the maneuver either under control conditions (3 wk: 0.12 ± 0.02 vs. 0.11 ± 0.03 ml, P = 0.27; 8 wk: 0.23 ± 0.05 vs. 0.22 ± 0.03, P = 0.90) or following the highest dose of MCh (3 wk: 0.11 ± 0.005 vs. 0.10 ± 0.02, P = 0.78; 8 wk: 0.22 ± 0.04 vs. 0.22 ± 0.04, P = 0.88).

MCh concentration-response curves at FRC. MCh concentration-response curves, with respiratory mechanical parameters expressed in absolute terms, are shown in Fig. 2. These curves show that some degree of heterogenous constriction occurs, as evidenced by increases in G accompanied by those in Raw but not H. These changes result in an increase in η with MCh dose fairly uniformly in all age groups.

Raw normalized by actual Vl. Specific Raw (sRaw) was normalized by the absolute Vl at which it was measured [FRC (Fig. 3A) and VL20 (Fig. 3B)]. At FRC, sRaw is the highest in 2-wk-old mice, both at baseline and after each concentration of MCh. The other age groups all start at a similar baseline sRaw. The 3- and 4-wk-old animals show increased MCh responses

Table 1. Mouse characteristics and baseline lung function, measured at end expiration with a transrespiratory pressure of 0 hPa

<table>
<thead>
<tr>
<th>Age, wk</th>
<th>N</th>
<th>Body Weight, g</th>
<th>FRC, ml</th>
<th>Raw, hPa·s·ml⁻¹</th>
<th>G, hPa/ml</th>
<th>H, hPa/ml</th>
<th>η</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>4</td>
<td>7.1 ± 0.49</td>
<td>0.13 ± 0.03</td>
<td>1.41 ± 0.48</td>
<td>31 ± 2</td>
<td>125 ± 19</td>
<td>0.26 ± 0.02</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>11.3 ± 1.52</td>
<td>0.15 ± 0.03</td>
<td>0.75 ± 0.29</td>
<td>22 ± 0</td>
<td>96 ± 20</td>
<td>0.23 ± 0.02</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>14.7 ± 1.34</td>
<td>0.18 ± 0.03</td>
<td>0.68 ± 0.84</td>
<td>20 ± 3</td>
<td>86 ± 13</td>
<td>0.24 ± 0.02</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>16.0 ± 0.78</td>
<td>0.24 ± 0.03</td>
<td>0.42 ± 0.13</td>
<td>11 ± 1</td>
<td>50 ± 2</td>
<td>0.22 ± 0.02</td>
</tr>
<tr>
<td>8</td>
<td>5</td>
<td>18.5 ± 0.39</td>
<td>0.29 ± 0.02</td>
<td>0.80 ± 0.49</td>
<td>9 ± 1</td>
<td>39 ± 7</td>
<td>0.23 ± 0.03</td>
</tr>
</tbody>
</table>

Data are means ± SE. FRC, functional residual capacity; Raw, airway resistance; G, tissue damping; H, tissue elastance; η, tissue hysteresivity. *P value for difference across the age groups by ANOVA.
compared with the other groups, reaching a similar $s_{Raw}$ to the 2 wk following the highest MCh concentration. There are no obvious differences in MCh responses between the 6- and 8-wk-old mice. At high VL, the dose-response relationships exhibit milder age differences and dose dependences (Fig. 3B).

The sensitivity to MCh, expressed as the EC$_{200}$ $s_{Raw}$, shows a similar trend in group mean data, being 279 ± 85 g/kg in 2-wk-old animals, 239 ± 31 g/kg in 3-wk-old animals, 173 ± 63 g/kg in 4-wk-old animals, 220 ± 62 g/kg in 6-wk-old animals, and 252 ± 51 g/kg in 8-wk-old animals. The EC$_{200}$ $s_{Raw}$ was not significantly increased in any age group compared with the 8-wk-old animals ($P > 0.2$ for all comparisons).

DISCUSSION

The data from the present study represent a comprehensive assessment of MCh responsiveness in mice from 2 to 8 wk and show that, when the VL at which respiratory mechanics is measured is taken into account, there are no differences in responses across this age range in mice with normal lungs.

A concern with interpreting the results of MCh challenges in many studies in small animals is knowing whether the changes in respiratory mechanics measured are due to constriction of airway smooth muscle in conducting airways, changes in the mechanical properties of the pulmonary parenchyma, changes in VL, airway closure, or combinations or some of these phenomena (13, 15, 24). These concerns are particularly relevant when studying mice as young as the ones we are reporting in the present study. One may expect that younger mice may be more prone to heterogenous airway constriction, progressive atelectasis, or airway closure during the MCh challenge. The ability to measure absolute VL simultaneously, i.e., within the same data epoch, with measurements of respiratory mechanics provides an opportunity to determine whether changes in VL contribute to the changes in mechanics. In the present study, we are confident that the changes we report in $s_{Raw}$ are due to airway smooth muscle constriction, with little or no contribution from atelectasis or airway closure for several reasons. We do not observe changes in FRC measured immediately before the sDI maneuver following any dose of MCh (Fig. 1). Furthermore, the lack of increase in $H$ during the MCh challenge (Fig. 2) argues against either progressive atelectasis or airway closure, since either would result in effectively smaller and stiffer lungs. In addition, the lack of a change in FRC when measured immediately before and after an sDI maneuver in either younger (3 wk old) or the oldest mice suggests that the preinflation VL has not been changed by the MCh infusion, i.e., there were no closed airspaces recruited by the sDI maneuver. Similarly, the unchanged FRC and $H$ both indicate that there was no change in intrinsic stiffness of the lung parenchyma. This is consistent with previous studies in rats (2) and mice (7, 24).

Although our data do not substantiate airway closure in any phase of the experiment, we do have some evidence to support...
a mild to moderate degree of heterogenous airway narrowing, especially in the younger mice, as indicated by the mild increases in G with increasing doses of MCh that were not paralleled by increases in H (Fig. 3). Indeed, the disproportionate increase in G may result from “Pendelluft” between the differentially constricted peripheral regions and not from changes in the intrinsic tissue viscoelasticity. Lutchen et al. (13) and Petak et al. (15) used gases of differing kinematic viscosities in rats and observed no change in the tissue parameters in baseline conditions, whereas during bronchoconstriction the elevations in Raw were accompanied by those in G in a viscosity-dependent manner and no changes in H. Although these studies were conducted in rats, it does not seem too great a leap to suggest that a similar phenomenon may exist in mice.

The fact that we have used intravenous rather than aerosol challenges is likely to have some impact on the data we present here. Petak et al. (15) have previously shown that intravenous MCh challenge produces a more homogenous constriction of conducting airways in rats than does aerosol challenge. In addition, the mice we studied had normal lungs in that they were not sensitized and challenged with allergen. The relatively pure airway constriction we report in the present study is consistent with the data reported by others in nonsensitized and challenge mice; for example, Wagers et al. (24) showed that there were no MCh-induced increases in H in BALB/c or AJ mice under similar circumstances. Aerosol challenges are often considered to be preferable because they more closely mimic the usual human situation. However, we could not use aerosol challenges for technical reasons: any aerosol condensation in the wave tube attachments would destroy the integrity of the measurements. Nevertheless, we must caution against applying the results we present here directly to studies using aerosol MCh challenges.

Overall, the results of the present study show only minor developmental changes in MCh responses in mice. The mice aged 4 and 6 wk appeared to be more responsive to MCh than the youngest or oldest mice, although the differences in EC200 Raw or EC200 sRaw did not reach statistical significance. There does not appear to be any developmental changes in volume dependence of MCh responses, as expressed by the relationship between the values of Raw obtained at both FRC and elevated Vt. and MCh dose (Fig. 3). Indeed, although the increases in sRaw with MCh dose were milder at higher Vt., the dose-dependence relationships became more uniform for the different age groups. We do need to stress that the animals we studied all had normal lungs, and the results of studies using disease models may be different from those we present here.

Previous studies in the literature examining age dependence of constrictor responses have not reported consistent results. Young open-chested piglets have been reported to be more responsive to intravenous histamine at 2–4 days and 2–3 wk of age, especially in the distal airways, than when studied at 10 wk of age (4). Similarly, rabbits are more responsive to intravenous MCh when studied at 1 and 2 mo of age than when studied at 6 mo of age (19). In contrast, the airway reactivity to both aerosolized histamine and carbachol, but not to citric acid, increased progressively with age in lambs studies at 1, 3, 5, and 7 mo and as adults (age not defined) (18). This increased responsiveness was seen if dynamic compliance was used as the outcome variable but not if resistance of the lung was used (18). Murphy et al. (14) measured airway responsiveness in neonatal (4–7 day old) or adolescent (age not defined) guinea pigs and reported no differences in responsiveness to MCh or capsaicin but reduced responsiveness to dry gas hyperpnea. The results of the present study show no substantial differences in responsiveness to intravenous MCh in mice from 2 to 8 wk.

Overall, the results of the present study show only minor developmental changes in MCh responses in mice. The mice aged 4 and 6 wk appeared to be more responsive to MCh than the youngest or oldest mice, although the differences in EC200 Raw or EC200 sRaw did not reach statistical significance. There does not appear to be any developmental changes in volume dependence of MCh responses, as expressed by the relationship between the values of Raw obtained at both FRC and elevated Vt. and MCh dose (Fig. 3). Indeed, although the increases in sRaw with MCh dose were milder at higher Vt., the dose-dependence relationships became more uniform for the different age groups. We do need to stress that the animals we studied all had normal lungs, and the results of studies using disease models may be different from those we present here.
of age. Taken together, these results demonstrate that one cannot extrapolate the results of studies measuring changes in airway responsiveness from one species to another or from one stimulus to another.

In conclusion, the data from the present study show that there are no major changes in MCh responsiveness when $sRaw$ is used as the outcome variable during the period of rapid growth and development in mice from 2 to 8 wk of age.

GRANTS

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