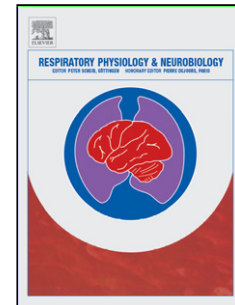


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Mechanical consequences of allergic induced remodeling on mice airway resistance and compressibility

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Highlights

- We developed and evaluated an approach to detect the mechanical consequences of airway remodeling in mice.
- We examined the relationship between maximal tidal expiratory flow and airflow resistance following methacholine-induced bronchoconstriction in mice subjected to acute and chronic antigen challenge protocols.
- The results we obtained show that the technique has promise for providing novel insight on the role of airway remodeling in airway hyperresponsiveness.

Abstract

The effect of remodeling on airway function is uncertain. It may affect airway compressibility during forced expirations differently than airflow resistance, providing a tool for its assessment. The aim of the current study was to compare the effects of acute and chronic antigen challenge on methacholine-induced bronchoconstriction assessed from resistance and maximal tidal expiratory flow. Balb/C mice were sensitized with ovalbumin (OVA) and challenged either daily for three days with intra-nasal OVA or daily for 5 days and three times a week for 5 subsequent weeks. Acute and chronic allergen challenge induced airway hyperresponsiveness (AHR) to methacholine. However the relationship between maximal tidal expiratory flow and resistance during methacholine challenge was different between the two conditions, suggesting that the determinants of AHR are not identical following acute and chronic allergen exposure. We conclude that the contrast of changes in maximal tidal expiratory flow and respiratory resistance during methacholine-induced bronchoconstriction may allow the detection of the mechanical consequences of airway remodeling.

Key Words: Airway remodeling, airway resistance, respiratory resistance, maximal expiratory flow, airway hyperresponsiveness.

1. Introduction

Airway inflammation and smooth muscle are responsible for the clinical features of asthma. With disease progression, remodeling causes airway structural changes that may affect the mechanical properties of the airways and airway responsiveness through geometric effects (Lambert et al., 1993; Wiggs et al. 1990; 1997), changes in tissue biomechanics (Kaczka et al., 1999) and alterations in the interaction between airways and parenchyma (Bates and Lauzon, 2007). Elastic properties of the airways have an important role in determining airflow and are a factor in the explanation of expiratory flow limitation by the tube wave speed theory (Hyatt et al, 1981; Hyatt, 1983). The maximal expiratory flow at a given lung volume is a function of the elastic modulus of the choke point in the airway and extrinsic factors such as lung elastic recoil, the pressure gradient between the alveoli and the choke point as well as upstream airway calibre. Although airway remodeling occurs both in asthma and in COPD, it is not clear exactly how it affects the elastic properties of the airways, due to the difficulty in acquiring *in vivo* dynamic measurements of the airway properties. Discordance between FEV₁ and flow resistance has been shown in several studies in the context of increased airway wall compliance in bronchiectasis (Hellinckx et al., 1998), but less is known in the context of airway remodeling in asthma. The *in vivo* measurement of airway wall compliance could help to clarify the role of remodeling in the dynamic behaviour of airways.

Several approaches have been proposed to detecting the changes in airway distensibility, presumably reflecting the effects of remodeling as well as airway smooth muscle tone. The change in anatomic dead space with lung volume has been proposed as a non-invasive index of airway distensibility or stiffness (Fowler, 1948).

Using this test the airways of subjects with mild asthma have been shown to be stiffer than normal (Wilson et al., 1993) but the measurement by this technique appears to be dependent on lung volume history and ventilation inhomogeneity (Johns et al., 2006). Recently a forced oscillation technique has been used to evaluate distensibility as the linear slope of conductance versus volume between total lung capacity (TLC) and 75% TLC and between 75% TLC and functional residual capacity (FRC), demonstrating a reduction in airway distensibility indices in asthmatic compared to healthy subjects (Browne et al., 2007). These studies measure airway properties during slow inflationary manoeuvres and do not investigate the behaviour of airways during forced expiratory manoeuvres, when airways are compressed and flow limitation occurs. Brackel et al (2000) showed a decrease in central airway compliance in asthmatic subjects during forced expirations, suggesting a “protective” role of remodeling that increases the mechanical load against smooth muscle contraction.

Studying the behaviour of airways at baseline and following contractile agonist challenge under conditions of tidal expiratory flow limitation may help better understand the role of remodeling in AHR. Contraction of the smooth muscle (ASM) surrounding an airway narrows the airway lumen at a rate determined by the force-velocity behaviour of the muscle and the load against which it has to contract. As the muscle contracts the load against which it contracts increases to a point where it matches the maximum force that muscle is capable of generating and the airway cannot narrow further (Lambert et al., 1993). Changes in the elastic properties of airways and parenchyma likely affect this process determining the magnitude of narrowing, but also the behaviour of the airway when it is acting as a flow-limiting

segment. For this reason we developed a method to study airway behaviour under conditions of tidal expiratory flow limitation and to quantify the relation between maximal expiratory flow and resistance in different functional phenotypes of airway pathology using murine models of acute and chronic asthma.

2. Materials and methods

2.1 Animals.

Six to eight week old, female Balb/c mice were purchased from Charles River, Canada. Mice were housed in a conventional animal facility under a 12 hours light/dark cycle with free access to food and water. A McGill University Institutional Animal Care Committee approved experimental procedures.

2.2 Sensitization and Challenge.

Mice were sensitized with two intraperitoneal (ip) injections of 10 µg ovalbumin (OVA) in 1 mg aluminium hydroxide at days 0 and 10. Animals were randomly assigned to 4 experimental groups: Acute OVA (n=8), acute PBS (n=8), chronic OVA (n=7), chronic PBS (n=8).

2.3 Acute challenge.

Starting at day 17, mice were challenged once a day for 3 days in a custom-built device to constrain and create a nose-only exposure to an ultrasonic nebulisation (Ultra-Neb 100, DeVilbiss Health Care; Somerset, Pa) of 5% OVA in PBS or PBS alone for 30 minutes. Respiratory mechanics were studied 24 hours after the third challenge.

2.4 Chronic challenge.

Starting at day 19, mice were challenged once a day for 5 days in a custom-built device to constrain and expose the nose to an ultrasonic nebulisation of 5% OVA in PBS or PBS alone for 30 minutes and then they received 3 challenges a week for 5 weeks until day 57. Respiratory mechanics were studied 24 hours after the last challenge.

2.5 Experimental Setup.

On the day of the experiment mice were anaesthetized with xylazine hydrochloride (10 mg/kg, ip) followed by sodium pentobarbital (32 mg/kg, ip). Once anaesthesia was achieved, mice were tracheostomized with an 18 gauge cannula (1.3 cm, typical resistance of 0.2 cmH₂O.s/mL; Brico Medical Supplies Inc., Dayton, NJ, USA), connected to a computer controlled small animal ventilator (*flexiVent FX* operated by *flexiWare* v7.0.2 software; SCIREQ Inc., Montreal, QC, Canada) and ventilated with a tidal volume of 10 mL/kg at a frequency 150 breaths/min in a quasi-sinusoidal fashion with a positive end-expiratory pressure (PEEP) of 3 cmH₂O. Before initiating a sequence of baseline measurements, the respiratory system was inflated to a transrespiratory pressure of 30 cmH₂O over a period of 3 seconds and held at that pressure for the same time period in order to recruit any atelectatic areas of lung and to standardize lung volume history. A muscle relaxant, pancuronium chloride (0.8 mg/kg, ip), was then administered and the animal was allowed to stabilise for approximately 2 minutes.

The mechanical properties of the respiratory system were assessed using single frequency (2.5 Hz; Snapshot-150, 1.2 s) and broadband forced oscillation signals (1-20.5 Hz; Quick Prime-3, 3 s) as well as a quasi-static, step-wise, pressure-controlled

pressure-volume loop (PVs-P, 16 s) (McGovern et al., 2013). The linear single-compartment model was used to evaluate total respiratory resistance (R_{rs}) and elastance (E_{rs}) while the constant phase model allowed the partitioning of the responses within the lungs (Hantos et al., 1992). This latter model fits the input impedance as a function of frequency in the equation: $Z_{rs}(f) = R_N + j * 2\pi f * I_{aw} + (G - j * H) / (2\pi f)^\alpha$, where Z is input impedance and expresses the combined effects of resistance, compliance, and inertance as a function of frequency (f); R_N is Newtonian “airway” resistance; I_{aw} is airway inertance and is dominated by the mass of gas in the central airways; and impedance of tissue is accounted for by G (tissue damping) and H (tissue elastance). G is closely related to peripheral airway and tissue resistance and reflects energy dissipation in the lung tissues, j is an imaginary number, H is tissue elastance and reflects energy storage in the tissues, α is $2/\pi \tan^{-1}(H/G)$, and f is respiratory frequency (Hantos et al., 1992). The deflation limb of the PV loop was fit to the Salazar-Knowles equation to obtain static compliance (C_{st}), an estimate of the inspiratory capacity (A) and the shape constant k (Salazar and Knowles, 1964).

To assess airway function during tidal expiratory flow limitation a series of ten tidal breaths was generated with each breath having progressively higher imposed expiratory flows while the pattern of inspiratory flow remained unchanged (Figure 1A). The progressive increase in the expiratory flow was created by negative pressure, through a computer-controlled faster retrieval of the ventilator piston. For a 20 g mouse, the inspiratory flow generated was constant at 1.67 mL/s and the expiratory flows were respectively: 1, 2, 3, 5, 7, 8, 10, 12, 15 and 17 mL/s. During the entire duration of the perturbation (2 s), the resulting pressure and volume changes were recorded at the airway opening at a sampling rate of 1024 Hz and the airway

opening tidal expiratory flow (F_{aw}) was derived from the gas compression-corrected volume signal (V_{tr}) directly in the system operating software (Figure 1B, C). Peak tidal expiratory flow (Figure 1D) was determined during each of the ten tidal breaths with progressively increasing expiratory flow. A plateau in the peak tidal expiratory flow during the manoeuvre despite an increase in the driving pressure generated by the ventilator piston was considered as expiratory flow limitation and was chosen as the maximal flow.

The impact of imposed progressive negative pressure tidal expirations on respiratory mechanics parameters was assessed under baseline conditions. It was evaluated for the constant phase model parameters (R_N , G , H) by calculating the absolute difference (Δ) in the outcome value obtained from two measurements taken immediately before and after an imposed series of ten tidal breaths with increasingly negative pressure expirations.

Airway responsiveness was assessed at increasing methacholine (Sigma Chemical, St. Louis, MO) concentrations (3.125 -100 mg/mL) delivered as an aerosol using a small (2-4 μ m) particle size Aeroneb Lab nebulizer (Aerogen Inc., Galway, Ireland) that was synchronized with inspiration and operated at 50% duty cycle for a 10 second period during which the ventilation profile remained unchanged. Measurements were then taken over a period of 3-5 minutes starting immediately after the end of nebulization. Over that time period, signals were recorded in a closely spaced manner (5-6 s apart) using an automated sequence which included, in order, a single frequency forced oscillation signal (2.5 Hz; Snapshot-150, 1.2 s), a broadband frequency forced oscillation signal (1-20.5 Hz; Quick Prime-3, 3 s) and an assessment

of airway function during tidal expiratory flow limitation by a series of ten tidal breaths having progressively higher imposed expiratory flows (2 s). The sequence of measurement signals was repeated every 17 seconds. This provided for each subject a total of 12-15 experimental data points for each measurement signal per methacholine challenge. At the end of each data collection, the respiratory system was inflated to 30 cmH₂O to restore airway patency before the next aerosol challenge. Peak responses were determined at each challenge for each subject and for each of the respiratory mechanics parameters. These values were expressed as a percentage of baseline. Linearized concentration-response curves were constructed, as previously reported, by plotting response values as their natural logarithm against the concentration of methacholine (Walker et al., 2012, Truchetti et al., 2014). The slope of the best straight line fitting the data was obtained by linear regression analysis.

2.6 Maximal tidal expiratory flow and respiratory resistance during methacholine-induced bronchoconstriction.

For each measurement sequence, maximum tidal expiratory flow (F_{aw}) was plotted against the corresponding respiratory system resistance (R_{rs}). This was done for all subjects and measurement sequences were taken following methacholine aerosol challenges over similar ranges of resistance values across experimental groups. To estimate the association, the experimental data were fit to a single phase exponential decay model that included random effects to account for the correlation between measurements made on the same mouse. The model was estimated via adaptive Gaussian Hermite quadrature. Four parameters were extracted from that model and used for group comparisons, namely Y_0 : the maximum flow at the intercept with the Y axis or, in other words, at a hypothetical resistance of zero where the main

determinant of expiratory flow is the elastic properties of the airways and of the respiratory system; K: the rate of decline in maximal tidal expiratory flow as the resistance increases with methacholine-induced bronchoconstriction; plateau: the value of the maximal tidal expiratory flow as it approaches an asymptote which, in a physiological context, reflects the flow at the point of equilibrium between the tone generated by the methacholine contraction and the load against which the airway smooth muscle contracted, and span: the difference between Y_0 and plateau. The same analysis was repeated using maximal tidal expiratory flow and R_N estimated from the constant phase model.

2.7 Bronchoalveolar lavage, histology and morphometry.

Following *in vivo* assessment of lung and airway mechanics, chest and abdominal cavities were opened, the inferior vena cava was sectioned, and 5 mL of sterile PBS were injected in the right ventricle. Lungs were then instilled with 0.6 mL of sterile 10% FBS followed by two separate 1mL instillations to collect BAL. From the recovered cell pellet, total live and dead cells were counted using trypan blue exclusion. Cytospin slides were prepared using a cytocentrifuge (Shandon, Pittsburgh, PA) and stained with Diff-Quick stain (Jorgensen Labs Inc., Loveland, CO). Differential cell counts, including epithelial cells, were determined on 300 cells per slide.

Following BAL, lungs were harvested and the right lung was fixed with an intratracheal perfusion of 10% buffered formalin at a pressure of 25 cmH₂O for 30 minutes. Following fixation lungs were bisected into superior and inferior portions, embedded in paraffin and 5µm sections were studied. The slides were examined by

light microscopy (Olympus microscope BX51/BX61; Carson Group, Markham, ON, Canada) and images captured and analysed with commercial software (Image-Pro Plus; Media Cybernetics, Silver Spring, MD, USA).

Smooth muscle content was determined in tissue sections by immunostaining for smooth muscle α -actin (α -SMA). Sections were stained with a mouse anti α -SMA monoclonal antibody (clone 1A4; Sigma-Aldrich Co.) and a biotinylated horse anti-mouse IgG, rat adsorbed (Vector Laboratories). The signal was detected with Vector[®] Red. ASM mass was measured by tracing the ASM bundles on digital images. The total area of the ASM bundles was then corrected for airway size using the square of the basal membrane perimeter. We measured an average of 6 airways per animal randomly chosen and judged to be sectioned transversely based on a ratio of the long to short axis < 2 . Collagen content was evaluated using Picrosirius red staining. Two blinded observers scored each airway simultaneously using a scale from 0 to 4 depending on the collagen content around the airway. Airways that received a score with more than 1 point of difference between the two observers were not included. The score for each airway was calculated as the average of the two scores. Goblet cell hyperplasia was assessed using periodic acid Schiff (PAS) (Sigma Chemical). To quantify the extent of goblet cell differentiation PAS positive cells were counted and normalized by the basal membrane perimeter.

2.8 Statistical Analysis.

The analysis of respiratory mechanics parameters and maximal tidal expiratory flow obtained under baseline conditions was performed by a single factor analysis of variance (ANOVA) followed by a Tukey post hoc test using the natural logarithm of

each experimental parameter (GraphPad Prism version6; GraphPad Software, San Diego, USA). Similarly, the impact of imposed progressive negative pressure tidal expirations on respiratory mechanics parameters was analysed using the natural logarithm of the individual absolute difference (Δ) calculated from before and after the imposed series of tidal breaths. The analysis of the methacholine concentration-response curves was performed using the inverse of the slope of the linearized natural logarithm-transformed concentration-response curve as well as the natural logarithm of the maximal response at the highest methacholine challenge concentration. Differences in the intercept of maximal tidal expiratory flow-resistance curve with the y axis, the rate of decay of the exponential function the plateau values and the span were analysed using *a priori* contrasts with correction for correlation between measurements on the same animal (SPSS statistical software, IBM). BAL cell counts and histology scores were analysed using a single factor ANOVA and the logarithm transform of the data followed by a Tukey post hoc test. A p value < 0.05 was considered significant. Data are expressed as means $\pm$ standard error (SE).

3. Results

3.1 Baseline respiratory system mechanics and tidal expiratory flow.

Under baseline conditions, prior to provocation with methacholine, respiratory resistance (R_{rs}), airway resistance (R_N) and maximal tidal peak expiratory flows (F_{aw}) were not statistically different between all experimental groups (Figure 2A, 2B and 2C, respectively). Statistical differences in E_{rs} (Figure 2D) and H (Figure 2E) were however noted between the mice undergoing the acute protocol and those subjected to the chronic one, which were also older at the time of assessment. In addition, there was a small but statistically significant difference in G between the acute PBS and OVA challenged animals (Figure 2F). There was no such difference between the

chronic PBS and OVA challenged mice (Figure 2F). PV curves of PBS exposed animals overlapped with those of OVA challenged mice for both the acute and the chronic OVA protocols (Figure 3A, 3B). Consistent with the differences seen in E_{rs} and H between acute and chronic protocols and the age of the respective groups at the time of assessment, there was an upward shift in the PV curves of the mice subjected to the chronic protocol relative to the mice undergoing the acute one (Figure 3A, 3B). The upward shift in the PV curves of mice subjected to the chronic protocol (Figure 3B) was associated with increases in parameter A , derived from the Salazar-Knowles equation fitting the deflation arm of the PV curves, which estimates the inspiratory capacity (Figure 3C) and in C_{st} (Figure 3D) (Salazar and Knowles, 1964). However, the lack of change in the dimensionless and volume-independent shape constant k , describing the curvature of the curves, confirms a lack of change in the intrinsic elastic properties of the respiratory system at baseline among experimental groups (Figure 3E) (Carbonara and Eidelman, 2005, Salazar and Knowles, 1964). Finally, there was no significant difference in the area between the inflation and deflation limb of the PV curves between experimental groups (Figure 3F).

3.2 *Impact of progressive negative pressure expirations on baseline respiratory mechanics.*

The absolute change in the constant phase model parameters (R_N , G , H) before and after the imposition of a series of ten progressive negative pressure expirations is presented in Figure 4. Overall, airway resistance (R_N) varied on average under baseline conditions from 0.25 ± 0.01 cmH₂O.s/mL before to 0.26 ± 0.01 cmH₂O.s/mL (n=30) immediately after 10 imposed breaths with progressively higher negative pressure expirations. Parameters G and H changed respectively from 3.22 ± 0.07 to

3.29 ± 0.07 cmH₂O/mL (n=30) and from 16.25 ± 0.36 to 16.76 ± 0.42 cmH₂O/mL (n=30) under similar conditions. The absolute change for any of these parameters was not statistically significant between the different experimental groups (Figure 4).

3.3 Airway hyperresponsiveness.

Ovalbumin challenge induced changes in airway responsiveness to methacholine. Both the acute and the chronic OVA challenged mice displayed increases in the slopes of the linearized concentration-response curves of multiple respiratory mechanics parameters compared to their respective PBS-challenged controls (Figure 5). The slope increase between acute or chronic OVA challenged mice relative to their respective PBS control groups was significant for R_{rs} , E_{rs} , G , and H (Figure 5A, B, D, E). A significant slope increase was also noted for R_N between the acute OVA and acute PBS challenged groups (Figure 5C). The chronically OVA challenged mice did not display a change in the slope of the R_N linearized concentration-response curve relative to their respective PBS controls. However, the linearized mean concentration-response curve in that group was shifted upwards relative to its PBS control group (Figure 5C). The magnitude of change in airway responsiveness at the highest methacholine challenge concentration was significantly higher in chronic compared to acute OVA exposed animals for parameter E_{rs} and H (Figure 5B, 5E).

3.4 Maximal tidal expiratory flow and respiratory resistance during methacholine-induced bronchoconstriction.

The relationships between maximal tidal airway expiratory flow (F_{aw}) and respiratory system resistance (R_{rs}) or Newtonian resistance (R_N) are shown in Figure 6, panels A and B respectively. As expected, maximal tidal expiratory flows fell as resistance rose

and the relationship could be described by a single phase exponential function. Table 1 reports the parameters derived from the curve fitting of maximal tidal expiratory flow against R_{rs} or R_N (Figure 6) and the p values for contrasts are provided in Table 2. Whether R_{rs} or R_N was considered, maximum flow at the intercept with the Y axis (Y_0) was reduced following chronic OVA exposure compared to its respective control group and was the lowest relative to all other experimental conditions (Figure 6; Table 1 and 2). No statistical difference was found in Y_0 for R_{rs} between acute OVA challenged mice and their controls (Figure 6A) but the two groups differed significantly when the relationship was expressed against R_N (Figure 6B). The maximal tidal expiratory flow at the plateau, as well as the rate of decline in maximal flow with increasing resistance, was not statistically different between experimental groups when R_{rs} was considered (Table 1 and 2). When R_N was taken into account, the mean plateau value reached in the acute PBS group was significantly lower than the one from the chronic PBS group but the rates of decline in maximal flow with increasing resistance were equivalent (Table 1 and 2). The span (Y_0 - plateau) was significantly reduced following acute and chronic OVA exposure compared to their respective control groups and changed statistically between the acute and chronic OVA groups for both R_{rs} and R_N (Table 1 and 2).

3.5 *Bronchoalveolar lavage fluid inflammatory cells and histology.*

The total cell count in bronchoalveolar lavage fluid was higher in both acute and chronic OVA challenged mice versus the respective controls. Acute OVA exposed mice showed an increase in eosinophils and a decrease in lymphocyte number compared to the chronic OVA-exposed animals (Figure 7). PAS positive goblet cells were significantly increased in the OVA-exposed animals compared to their

respective controls, and in the chronic OVA-exposed animals the number of positive cells corrected for the basal membrane perimeter was significantly higher than in the acute OVA-exposed group. Picrosirius red staining showed an increase in collagen content in the chronic OVA-exposed animals compared to controls and acute OVA-exposed animals. No difference in airway smooth muscle mass was detected (Figure 8).

4. Discussion

In the current study, we compared the increases in respiratory system and airway resistances evoked by methacholine to the reduction in maximal tidal expiratory flow to obtain insights into the possible differences resulting from acute and chronic allergen exposures. The application of an imposed series of ten tidal breaths with increasing negative pressure expirations was used to study murine airway behaviour under conditions of induced tidal expiratory flow limitation. The approach was derived from the work of Valta et al. (1994), where a brief negative pressure was applied at the airway opening of mechanically ventilated patients during expiration in order to detect expiratory flow limitation. As described by these authors (Valta et al., 1994), in the absence of flow limitation, expiratory flow increased following the application of a negative pressure at the airway opening as a result of a greater expiratory pressure gradient, but remained unchanged in conditions where expiratory flow limitation was present. Hence, the present methodology aimed at gradually increasing the expiratory pressure gradient to a point where the maximal tidal expiratory flow reached a plateau. This novel active approach to probe for lung function abnormalities in mice was also applied in the presence of contractile agonist challenges for an evaluation of airway behaviour under conditions of greater functional impairment, which was concurrently recorded using the forced oscillation

technique. This association of methodologies within a single device also allowed us to characterize the impact the imposition of a series of ten tidal breaths with increasing negative pressure expirations had on baseline respiratory mechanics parameters. The results we obtained showed that the technique was able to generate a progressive increase in the maximal tidal expiratory flow, to cause it to reach a plateau, and to detect a different level of functional impairment following methacholine-induced bronchoconstriction. In addition, it induced no significant changes in respiratory mechanics *per se* thus indicating that this innovative method of assessing lung function has promise for providing novel insight into the role of airway remodeling in AHR.

A number of studies have addressed airway properties in asthma, demonstrating a reduction in distensibility by measuring the changes in anatomical dead space at different lung volumes or the volume dependence of airway resistance measured by forced oscillation (Brown et al., 2007, Johns et al., 2000, 2006, Wilson et al., 1993). The distensibility of excised human asthmatic airway has also been described to be reduced (Khan et al., 2010). In the present study, we assessed airway calibre prior to and during induced bronchoconstriction using measurements of resistance by forced oscillation. In addition we evaluated airway calibre and airway stiffness from maximal tidal expiratory flows. We reasoned that an alteration in the relationship between maximum tidal expiratory flow and resistance for any given degree of bronchoconstriction would reflect changes in airway stiffness. We employed repeated exposure to allergen that has been reported to cause airway remodeling and consequently likely to alter airway properties and we compared the results to those obtained with a short-term allergen exposure. Our method detected significant

differences between the two experimental conditions in the presence of methacholine, which further decreased the maximal tidal expiratory flows, but not under baseline conditions. Compared to their respective control groups, both the acute and the chronic allergen exposure resulted in AHR but only the latter altered the relationship between resistance and maximum tidal expiratory flow over a similar range of resistance values. These findings support the argument that the determinants of AHR mediated by acute and chronic allergen exposure in mice are not identical. Our results appear to disagree with previous data in humans indicating an increase in the stiffness of the airways but these prior publications assessed stiffness during slow inflation manoeuvres that likely do not reflect the dynamic properties of the airways during forced lung deflation.

The differences observed between acute and chronic allergen exposure are presumably a function of the morphological changes in the airways resulting from the prolonged allergen exposure. We observed significantly greater airway responsiveness to methacholine as well as a significant increase in airway collagen deposition and in goblet cell numbers in the chronic allergen-exposed group. However, we failed to detect any increase in airway smooth muscle despite the similarity in our chronic protocol to that previously published (Kearley et al., 2011). This discrepancy could stem from technical differences in the generation of the model and the outcome assessment levels. In comparison to that latter study, we observed in the bronchoalveolar lavage fluid of chronic OVA challenged mice a lower total inflammatory cell count, reduced proportions of eosinophils and neutrophils, and increased proportions of macrophages and lymphocytes. These differences, possibly related to differences in the exposure systems used to deliver the OVA challenges

(nose-only versus whole body exposure system), could potentially be related to the failure of the chronic protocol to induce the previously described airway smooth muscle thickening (Kearley et al., 2011). In addition, there were significant differences in the methods used to quantify smooth muscle; we have assessed the size corrected airway smooth muscle area whereas Kearley et al (2011) calculated airway smooth muscle thickness. The latter measurement is sensitive to airway size and bronchoconstriction.

In spite of a lack of demonstrable increase in airway smooth muscle in the chronic OVA mice, which constitute a limitation of this model, we posit that changes in airway matrix account for the altered dynamic properties of the airways since it seems improbable that changes in goblet cell numbers could alter the airway stiffness. While there was an increase in subepithelial collagen, the more striking change was on the adventitial side of the airway, external to the smooth muscle. Perhaps uncoupling of the airways and parenchyma resulted from the increase matrix protein deposition in this location, accounting for the increased compressibility of the airways during tidal forced expiration. A finite element analysis has demonstrated that subepithelial collagen would be expected to reduce the number of mucosal folds and would promote closure of the structure, suggesting that subepithelial collagen in the current experiments may indeed have altered airway properties in a manner consistent with model predictions (Hrousis et al., 2002, Wiggs et al., 1997). While changes in goblet cell numbers may not have altered the airway stiffness, an exaggerated quantity of mucus in the airways of the chronic OVA mice could possibly have further contributed to airway closure. No direct measurements of mucus secretion were recorded in the present study yet, no significant changes in the rate of decline in

maximal tidal expiratory flow as well as in the plateau were noted, and that whether R_{rs} or R_N was considered in the analysis of the maximal tidal expiratory flow – resistance relationship. Out of the two types of resistances considered in the analysis, more parameters significantly distinguished the chronic OVA group from the acute OVA one when the maximal tidal expiratory flow – resistance relationship was expressed against R_{rs} (Y_0 , span). Since this parameter reflects the overall resistance of the respiratory system which includes the resistance of the conducting airways as well as the resistive properties of the lung tissues, this suggests that the mechanical consequences of remodeling involve effects at the level of the small peripheral airways.

Our data do not support a role for collagen deposition as a protective factor against excessive airway narrowing as has been previously postulated (Wiggs et al., 1990, 1997). A lack of changes in the intrinsic elastic properties of the respiratory system supports the conclusion that differences detected between acute and chronic OVA treatments during conditions of functional impairment induced by tidal expiratory flow limitation are not attributable to altered lung parenchymal properties but rather to differences in airway wall compliance since the differences were studied over similar resistance ranges and therefore airway calibres. Similar conclusions were reached whether the analysis was based on R_{rs} or R_N . However when R_N was used, the method seemed more susceptible to age- or lung volume-related subject variations, as observable by the significant difference in the plateau value between the acute and chronic PBS groups. These conclusions were reached using mice models of allergen-induced airway hyperresponsiveness and remodeling which, as every preclinical model, present limitations. In addition, the validity of the allergic mouse model at

predicting clinical outcomes in human asthma studies has been challenged in the past (Stevenson et al., 2013). These shortcomings, such as the limited amount of induced pathological changes, the use of a single mouse strain and allergen or, more generally, the anatomical and physiological species differences between mice and human, make it difficult to truly assess the relevance of the conclusions reached in this study to human asthma. However, while care should be taken not to over-interpret these results until further validation is provided, it should also be noted that strategies are being proposed to increase the translational value of preclinical murine models. These include steps directed at standardizing lung function measurement techniques as well as introducing novel methods of assessing or reporting lung function, to which the present study adds (Stevenson et al., 2013, Walker et al., 2012, Lundblad 2012, Robichaud et al., 2015).

In conclusion a comparison of maximal tidal expiratory flow created by the application of increasing negative pressure to the airway opening of the mouse during progressive methacholine-induced bronchoconstriction to airflow resistance appears to distinguish changes to airway properties associated with remodeling. This methodology may be useful for the characterization of the mechanical consequences of remodeling in small rodents as well as for the assessment of therapeutics directed against remodeling.

5. Acknowledgement

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6. Disclosures

MN, KS, AB, TMcG and JGM declare no conflicts of interest. AR, LF and TFS were employed at the time of the study by SCIREQ Scientific Respiratory Equipment Inc., a commercial entity involved in a subject area related to the content of this article, and TFS also owned stock.

7. Figure Captions

Figure 1. Illustrative traces.

Illustrative traces from a sham control PBS-exposed mouse (22.5 g) subjected to a series of ten tidal breaths of constant inspiratory flow with progressive negative pressure expirations during tidal breathing generated by accelerating the rate of withdrawal of the ventilator piston. Panel A: inspiratory and expiratory gas compression-corrected volume changes (V_{tr}). Panel B, C: Tidal airway flow (F_{aw})-volume loop during an imposed series of ten tidal breaths with increasing expiratory flows following an aerosol challenge of saline (B) or 100 mg/mL methacholine (Mch) (C); TV: tidal volume. Panel D: Peak expiratory tidal airway flows (F_{aw}) obtained at each of the ten imposed tidal breaths at baseline and following an aerosol challenge of saline or 100 mg/mL methacholine.

Figure 2. Baseline conditions.

Total respiratory system resistance (R_{rs}), elastance (E_{rs}), constant phase model parameters (R_N , G and H) and maximal tidal expiratory flow (F_{aw}) under baseline conditions. Data are expressed as means $\pm$ SE. * $p < 0.05$.

Figure 3. Static elastic properties of the respiratory system.

Pressure-volume (PV) loops for acute OVA, chronic OVA and the corresponding controls, acute PBS and chronic PBS are shown in panel A and B. Parameter A from the exponential function used to fit the deflation limb of each PV loop (Salazar and Knowles, 1964), which provides an estimate of the inspiratory capacity, is shown in panel C. The shape constant k is shown in panel D. Static compliance (C_{st}) is shown

in panel E and the area between the PV loop inflation and deflation limbs is shown in panel F. Data are expressed as means $\pm$ SE. * $p < 0.05$.

Figure 4. Impact of tidal breaths with progressively increasing expiratory flow on respiratory mechanics.

The absolute change (Δ) in airway resistance (R_N , panel A), tissue damping (G , panel B) and tissue elastance (H , panel C) caused by the imposition of a series of ten tidal breaths with increasing expiratory flows is shown for each mouse under baseline conditions and grouped according to the experimental protocol. The progressive increase in expiratory flow was created by negative pressure through a computer-controlled faster retrieval of the ventilator piston. Data are expressed as means $\pm$ SE.

Figure 5. Methacholine responsiveness.

Peak respiratory system resistance (R_{rs}), elastance (E_{rs}) and constant phase model parameters (R_N , G and H) following increasing methacholine challenges. Results are presented as the natural logarithm of the percentage of change over baseline and expressed as mean $\pm$ SE. * $p < 0.05$ OVA exposed versus PBS controls; # $p < 0.05$ acute OVA exposed versus chronic OVA exposed.

Figure 6. Relationship between maximal expiratory flow and resistance.

Maximal tidal expiratory airway flow (F_{aw}) is expressed against respiratory resistance (R_{rs} ; panel A) and Newtonian resistance (R_N ; panel B) for acute and chronic OVA and PBS exposed animals. Individual F_{aw} values are reported over equivalent ranges of resistances following methacholine challenge. Baseline resistance values (R_{rs} 0.36 –

0.68 cmH₂O.s/mL; R_N : 0.17 – 0.42 cmH₂O.s/mL) were excluded from the analysis and a single exponential function was fit to the data for all experimental groups.

Figure 7. Inflammatory cells

Inflammatory cell numbers in bronchoalveolar lavage fluid quantified by hemacytometer for total cells counts and for differential cell counts by cytopins stained with DiffQuik. Data are expressed in absolute numbers (A) as well as a percentage of total cells (B). Means $\pm$ SE. * $p < 0.05$.

Figure 8. Histology quantification results

Histology quantification results for goblet cell (A), collagen (B) and airway smooth muscle (C). Illustrative images (x100 magnification) of the three corresponding histochemical and immunohistochemical stains for the acute (panel D, E, F) and chronic OVA exposed animals (G, H, I).

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9. Tables

Table 1. Parameter estimates extracted from the single phase exponential function describing the relationship between maximal tidal expiratory flow and R_{rs} or R_N .

R_{rs}	Acute PBS	Acute OVA	Chronic PBS	Chronic OVA
Y_0	14.15	12.99	13.70	12.14
(mL/s)	(13.46, 14.85)	(12.30, 13.68)	(13.01, 14.40)	(11.29, 13.00)
Plateau	3.94	4.22	4.04	4.13
(mL/s)	(3.24, 4.63)	(3.53, 4.91)	(3.37, 4.71)	(3.34, 4.93)
K	-0.55	-0.48	-0.52	-0.53
(mL/cm H ₂ O.s)	(-0.64, -0.46)	(-0.56, -0.39)	(-0.60, -0.44)	(-0.65, -0.41)
Span	9.77	8.77	10.12	8.01
(mL/s)	(9.41, 10.12)	(8.38, 9.16)	(9.76, 10.47)	(7.55, 8.48)
R_N				
Y_0	15.12	12.62	13.86	11.71
(mL/s)	(140.2, 16.23)	(11.67, 13.57)	(13.00, 14.72)	(10.53, 12.89)
Plateau	3.09	2.92	4.49	3.92
(mL/s)	(2.07, 4.11)	(1.80, 4.05)	(3.85, 5.11)	(3.17, 4.67)
K	-0.94	-0.87	-1.22	-1.10
(mL/cm H ₂ O.s)	(-1.15, -0.73)	(-1.11, -0.62)	(-1.45, -1.00)	(-1.41, -0.78)
Span	10.77	9.70	10.64	7.79
(mL/s)	(10.19, 11.35)	(9.02, 10.38)	(9.89, 11.39)	(7.01, 8.58)

Results are presented as means (and 95% confidence intervals). Y_0 : maximum flow at the intercept with the Y axis; K: the rate of decline in expiratory flow; span: Y_0 - plateau.

Table 2. P values for relevant contrasts:

R_{rs}	Y_0	Plateau	K	Span
Chronic PBS vs Chronic OVA	<0.01	0.86	0.92	<0.01
Acute PBS vs Acute OVA (normalized relative to their own controls)	0.08	0.79	0.39	<0.01
Acute OVA vs Chronic OVA	0.13	0.87	0.49	0.02
Acute PBS vs Chronic PBS	0.35	0.83	0.62	0.16
R_N				
Chronic PBS vs Chronic OVA	<0.01	0.25	0.50	<0.01
Acute PBS vs Acute OVA (normalized relative to their own controls)	0.04	0.65	0.83	0.02
Acute OVA vs Chronic OVA	0.23	0.14	0.25	<0.01
Acute PBS vs Chronic PBS	0.07	0.02	0.07	0.79

