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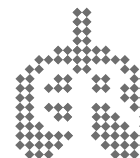

Effects of allergen on airway narrowing dynamics as assessed by lung-slice technique

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ABSTRACT: Asthma is characterised by an excessive airway narrowing in response to a variety of stimuli, called airway hyperresponsiveness (AHR). Previous comparisons between mouse strains have shown that increased velocity of airway narrowing correlates with baseline airway responsiveness. These data prompted the investigation into models of induced AHR to see whether airway narrowing dynamics correlated with *in vivo* responsiveness.

In an attempt to reproduce some of the features of asthma, BALB/c mice were sensitised and subjected to either brief or chronic periods of allergen exposure. Brief exposure involved two challenges with intranasal chicken egg ovalbumin (OVAin). Chronic exposure involved six 2-day periods of OVAin challenges, each separated by 12 days. Control mice received intranasal saline challenges. Outcomes included videomicrometry of lung slices (magnitude and velocity of airway narrowing), *in vivo* respiratory physiology measurements and histological staining with morphometric analysis.

Neither brief nor chronic allergen exposure resulted in greater airway narrowing and increased velocity compared with saline controls. Structural changes in the airway, such as goblet cell hyperplasia, subepithelial fibrosis and increased contractile tissue, were detected in mice chronically challenged with allergen.

In conclusion, increased responsiveness to methacholine following allergen challenge may not be due to an intrinsic change to the smooth muscle *per se*, but rather to other changes in the lung, which ultimately manifest as an increase in respiratory resistance.

KEYWORDS: **Airway smooth muscle, asthma, contraction, lung slices, remodelling**

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Asthma is a chronic inflammatory lung disease characterised by variable airflow obstruction, airway inflammation and airway hyperresponsiveness (AHR). AHR refers to the increased ability of the airways to narrow following exposure to bronchoconstrictor agonists, such as methacholine and histamine, as compared with normal individuals [1]. The pathophysiological mechanisms that contribute to AHR are still unclear, although ongoing airway inflammation and airway remodelling are believed to play major roles, and elevated interleukin (IL)-13 levels seem to be necessary and sufficient for AHR in brief animal models of allergic asthma [2, 3]. It has been suggested that these allergic mediators can act directly on airway smooth muscle (ASM) to increase contractile responses [4]. Airway remodelling describes the structural changes of airway walls observed in asthmatics, and includes ASM hypertrophy and/or hyperplasia, airway fibrosis and increased mucus production.

ASM contraction *in vivo* is neither isometric nor isotonic but rather auxotonic, in which the ASM shortens against an increasing load imposed by the attachments of the surrounding lung parenchyma, parallel elastic elements (*i.e.* extracellular matrix) and mucosal folding. Although it is known that isometric force measurements do not completely reproduce the *in vivo* situation, many studies use isometric force generation as the index of contractile function [5–9]. Many investigators have used isometric force generation as an index of AHR; however, isometric force measurements are currently thought to underestimate differences in airway responsiveness. Recently, airway contraction dynamics, namely maximal narrowing and velocity of narrowing, have been proposed to be more relevant markers of AHR, thus raising the possibility that airway dysfunction may arise from abnormalities within the smooth muscle itself. Previous studies have examined airway smooth muscle dynamics in naïve, nonallergen sensitised dogs [5], rats [10]

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and mice [6, 7, 11] but, to the present authors' knowledge, no other group has used a murine model of allergen-induced AHR to investigate airway contraction dynamics. Previous comparisons between naïve mouse strains have shown that increased velocity of airway narrowing correlates with airway responsiveness [6]. Consequently, the purpose of the present study was to examine the ASM-mediated airway narrowing dynamics and determine whether this could account for increased responsiveness to methacholine *in vivo* in a murine model of allergic asthma.

MATERIALS AND METHODS

Animals

Female BALB/C (wild type) mice were purchased (Harlan Sprague Dawley Inc., Indianapolis, IN, USA). Mice were aged 6–8 week (chronic protocol) or 10–12 week (brief protocol) and housed in environmentally controlled, specific pathogen-free conditions for 1 week prior to and for the duration of the experiments. All experimental procedures were approved by the Animal Research Ethics Board at McMaster University (Hamilton, ON, Canada), and conformed to the National Institute of Health guidelines for experimental use on animals.

Sensitisation

BALB/c mice were sensitised as described previously [12]. Briefly, mice received intraperitoneal (IP) injections of ovalbumin (OVA) with aluminum potassium sulfate on days 1 and 11, and with intranasal OVA (OVA_{in}) on day 11.

Exposure

Sensitised BALB/c mice were subjected to either brief or chronic allergen exposure protocols. The brief protocol involved two 2-day periods of OVA_{in} (100 μg in 25 μL saline) challenges. The chronic protocol involved six 2-day periods of OVA_{in}, each separated by 12 days (total of 12 exposures over a 10-week period). Control mice were subjected to the same sensitisation protocol but received intranasal saline exposures. Mice subjected to the brief exposure protocol were studied 24 h after the final exposure to either allergen or saline and chronic mice were studied 4 weeks after the final exposure to either allergen or saline. Separate groups of mice (10 saline control, 10 allergen-exposed) were studied in each protocol (brief *versus* control) and analysed using the thin lung-slice technique.

Solutions

Agarose type-VII solution (4% (weight/volume); Sigma Chemical Corp., St Louis, MO, USA) was dissolved in distilled water at 60°C, cooled to 37°C and mixed with 2 × concentration Hanks' Balanced Salt Solution (HBSS) to give a 2% agarose-HBSS solution at 37°C.

Preparation of lung slices

Lung slices were prepared as previously described in mice [13] with slight modifications. Briefly, mice were euthanised by CO_2 followed by terminal exsanguination. The trachea was exposed and cannulated using a blunt-ended 19-gauge needle, followed by chest wall removal to expose the lungs. The lungs were inflated with ~1.2 mL 2% agarose-HBSS solution at 37°C. To clear the airway lumen, 0.1–0.2 mL of air was injected to flush the agarose-HBSS solution out of the airways into the alveolar tissue. The lungs were rinsed with 1 × concentration HBSS at 4°C and the whole mouse preparation was placed at 4°C for 15 min. The lungs were removed and placed in HBSS at 4°C for an additional 30 min to ensure the complete gelling of the agarose within the lungs. The lungs were separated into individual lobes and bathed in cold HBSS. Using a tissue slicer (model EMS-4000; Electron Microscope Sciences, Fort Washington, PA, USA), ~120 μm thick slices from the right upper lobe were cut in HBBS at 4°C.

Incubation media for tissue slices

Lung slices were transferred to Dulbecco's Modified Eagle's Medium (Invitrogen, Carlsbad, CA, USA) with 15 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid buffer, L-glutamine and pyridoxine HCl, supplemented with penicillin-streptomycin, amphotericin B, L-ascorbic acid, transferrin, selenium and insulin, and then incubated overnight at 37°C.

Image acquisition

Lung slices were placed between two glass cover slips and held in position by a piece of nylon mesh (CMN-300-B; Small Parts, Miami Lakes, FL, USA) on the stage of the microscope (Nikon TMD, Nikon Canada Inc., Mississauga, ON, Canada) and viewed at 100 × magnification. Lung slices were selected for study only if: 1) the airway of interest was free of agarose; 2) beating of cilia was observed; and 3) the epithelium of the airway was intact. Phase contrast images were recorded with a digital charge-coupled device camera (CV-252; Nikon), a frame-grabber board and image acquisition software (Video Savant; IO Industries, London, ON, USA). Frames were captured in time-lapse (except for data used to test the effect of frame rate, which was performed at a rate of 1 frame every 5 s) for the allergen experimental protocols, stored in tagged image format (.tif) stacks of several hundred frames and analysed using Scion image analysis software (Scion Corporation, Frederick, MD, USA). In particular, images with 10-bit grey scale resolution were converted to binary by defining all pixels greater than a given brightness to white (*e.g.* the background seen through the lumen), and all those less intense to black (*e.g.* tissue). Additionally, all contiguous white pixels were identified as objects/particles, excluding any bounded object smaller than a given size (defined by the user to be much smaller than the airway lumen area, but much larger than any passing debris) and then the number of (white) pixels within any object larger than the cut-off value were counted. Given the dramatic difference in brightness between the airway lumen and any object in view (parenchymal/wall tissue or debris), minor changes in threshold level would have little or no effect on the absolute measurements made, and would have no effect whatsoever on the relative changes reported herein (*i.e.* the changes in diameter occurring from start to finish in a given recording).

In vitro *measurement of airway narrowing*

The lung slice was superfused for 5 min with 1 × concentration HBSS in order to obtain a baseline. After baseline images had been recorded, a constant flow of 1 μM acetylcholine was superfused over the lung-slice preparation for 5 min. BERGNER and SANDERSON [13] have shown this concentration of acetylcholine to evoke nearly maximal responses. Airway area was measured with respect to time by pixel summing, using

the Scion software (Scion Corperation). The cross-sectional airway area ranged 17,000–44,000 μm^2 (corresponding to a diameter range 145–235 μm). Airway narrowing was expressed as the percentage decrease in airway area in comparison to the initial airway area measurement. The peak velocity of airway narrowing was calculated using a simple mathematical algorithm (SigmaPlot; Systat Software Inc., Point Richmond, CA, USA), which numerically differentiates the values of area with respect to time (calculates the slopes between every point in the lumen area *versus* time plots).

In vivo *measurement of responsiveness to methacholine*

Respiratory physiology measurements were obtained using the flexiVent™ rodent ventilator system (Scientific Respiratory Equipment Inc., Montreal, Canada). Responsiveness to methacholine was measured on the basis of the response of total respiratory system resistance (RRS) to increasing intravenous doses of methacholine (n=10 per group). Each mouse was anaesthetised with Avertin (2,2,2-tribromoethanol; Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) *via* IP injection at a dose of 240 $mg \cdot kg^{-1}$ and underwent tracheostomy with a blunted 18-gauge needle, then were connected to the flexiVent™ computer-controlled small animal ventilator (Scientific Respiratory Equipment Inc.). Animals were ventilated quasi-sinusoidally (150 $breaths \cdot min^{-1}$, 10 $mL \cdot kg^{-1}$, inspiratory/expiratory time ratio of 66.7% and pressure limit of 30 cmH_2O). A script for the automated collection of data was then initiated, with the positive end-expiratory pressure (PEEP) level set at 2 cmH_2O and default ventilation for mice. After the mouse was stabilised on the ventilator, the internal jugular was cannulated using a 25-gauge needle. Paralysis was achieved using pancuronium (0.03 $mg \cdot kg^{-1}$ administered intravenously) to prevent respiratory effort during measurement. To provide a constant volume history, data collection was preceded by a 6-s total lung capacity perturbation (peak amplitude 25 cmH_2O). After 20 s, saline was injected intravenously followed by 10, 33, 100 and 330 $\mu g \cdot kg^{-1}$ methacholine (ACIC, Brantford, ON, Canada). For each dose, a maximum of 13 ''QuickSnap-150'' perturbations (single sinusoidal inspiration/expiration ratio of 0.4 s duration with a volume amplitude relative to weight of 10 $mL \cdot kg^{-1}$) were performed for ~45 s, followed 10 s later by a 6-s TLC. Each breath was delivered after allowing the mouse to expire passively for 1 s against a positive pressure of 2 cmH_2O. Respiratory mechanics (RRS and elastance resistance (ERS)) were calculated from simultaneous recording of airway pressure, change in lung volume and airflow in or out of the lungs, using multiple linear regression to fit these data to the following linear single compartment model of the mouse respiratory system:

$$P=(E_{RS} \times V)+(R_{RS} \times V')+P_o \quad (1)$$

where P is pressure, P_o is the end alveolar pressure determined by the PEEP applied to the expired line of the ventilator, V is volume and V' is flow. After the last dose was complete, the mouse was removed from the ventilator for further tissue collection. Cardiac frequency and oxygen saturation were monitored *via* infrared pulse oxymetry (Biox 3700; Ohmeda, Boulder, CO, USA) using a standard ear probe placed over the proximal portion of the mouse's hind limb.

Lung histology and morphometry

The lungs were processed as described in detail previously [14]. Following agar infusion, the left lobe was fixed in formalin for 24 h, after which the left lung was cut in half and imbedded in paraffin. Transverse sections (3-μm thick) were cut and assessed with the following stains: periodic acid Schiff, Picro-Sirius red, and α- smooth muscle actin (SMA).

Statistical analysis

All data are expressed as mean±SEM and n refers to the number of animals used. Only one airway from one lung slice was used from each animal. Statistical comparisons between groups were carried out using an unpaired t-test and p-values <0.05 were considered to be significant.

RESULTS

Brief or chronic allergen exposure increases methacholine responsiveness in BALB/c mice

In order to confirm that the allergen exposure protocol was associated with increased cholinergic responsiveness in mice, *in vivo* respiratory function was measured in BALB/c mice following brief or chronic allergen exposure (these mice were separated from the animals used for lung-slice experiments). These mice showed significant increases in maximal methacholine-induced RRS compared with corresponding saline controls (n=10; $p<0.05$; fig. 1). Identical observations were made in similarly treated mice, where respiratory responses to methacholine were measured using a flow interruptor method (data not shown) [12].

Validation of experimental technique

Several technical aspects of the lung-slice technique used herein have been validated: extent of inflation; concentration of agarose; and wide range of airway sizes [15]. In order to evaluate the optimal rate of data acquisition for estimating contraction velocities, a single recording at 10 frames per second (fps) was performed and divided into several other videos with lower frame rates by selecting appropriate frames (*e.g.* every 10th frame to obtain an effective video rate of 1 fps). The latter were then analysed for velocity of constriction.

Figure 2a shows a segment of the 10-fps recording of the constrictor response to acetylcholine and the estimations of instantaneous velocities made from a 120-s portion of that video (beginning ~30 s prior to cholinergic stimulation) are given in the top tracing in figure 2b. The noise in the latter is primarily produced by minor changes in airway lumen area from one frame to the next as the muscle twitches, a phenomenon previously described by BERGNER and SANDERSON [13]. Other possible sources of noise (<5%) may pertain to debris drifting through the field of view and/or subtle changes in illumination. This noise might hamper any accurate determination of peak velocity of airway closure.

Figure 2b also shows the estimations of velocity obtained at an effective video rate of 5 fps (only every other frame included), as well as slower effective video rates down to 0.1 fps (only every 100th frame). Decreasing the video rate to 1 or 0.5 fps substantially reduces the inherent noise in the velocity estimations, revealing an underlying oscillation with a frequency of ~0.33 Hz, as well as the peak velocity of acetylcholine-evoked contraction *per se*. Reducing the effective

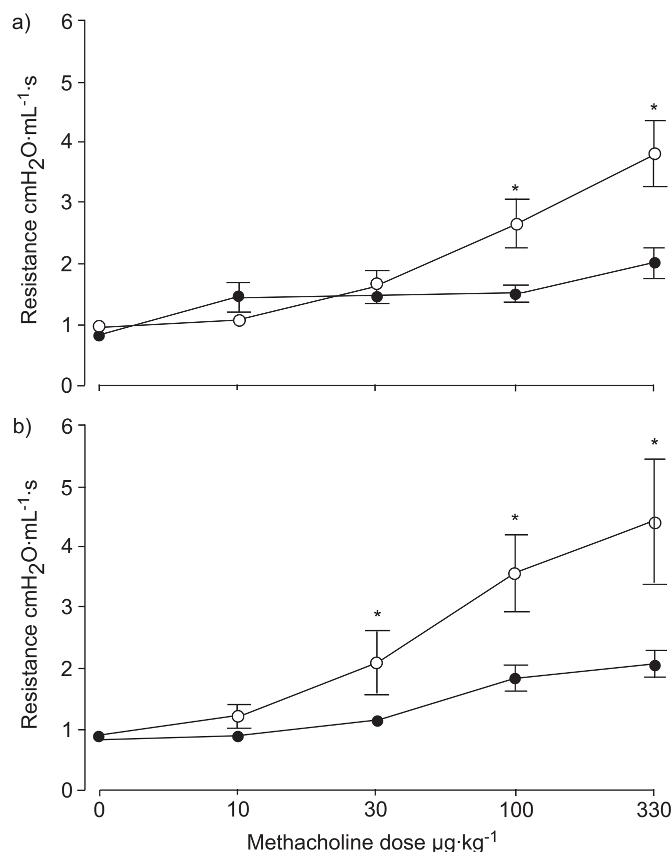

FIGURE 1. Physiology measurements in BALB/c mice during increasing doses of methacholine a) 24 h after brief exposure to saline (●) or ovalbumin (OVA; ○) or b) 4 weeks after chronic exposure to saline (●) or OVA (○). Data are presented as mean±SEM. n=10 per group. *: $p<0.05$ compared with corresponding control animals.

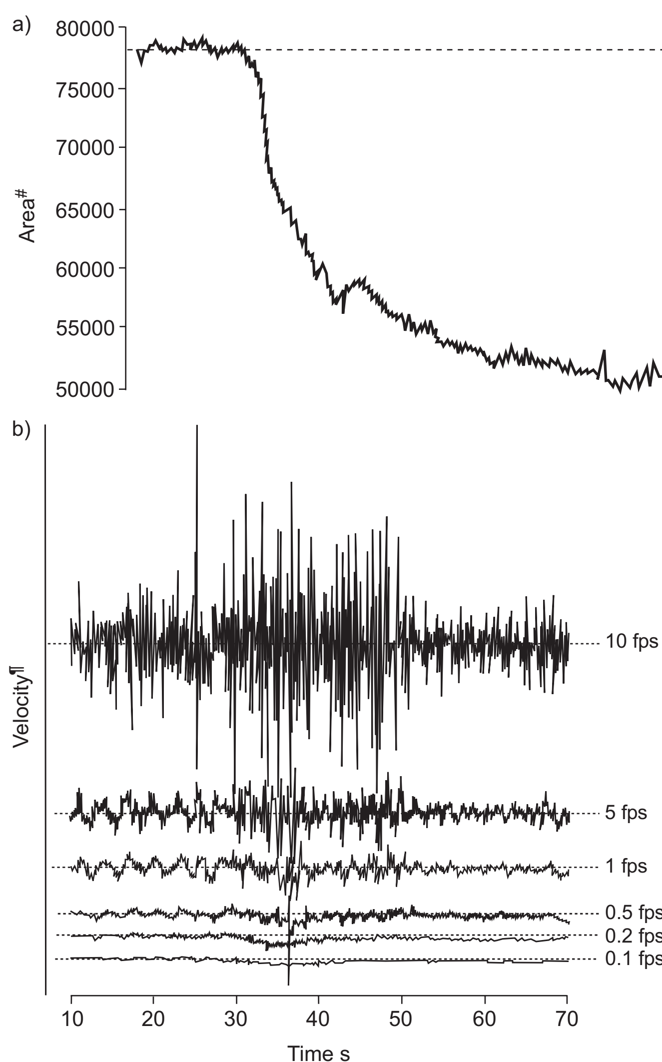

FIGURE 2. Effect of rate of data acquisition (video frame rate) on the estimation of velocity of airway narrowing. a) The response to acetylcholine (10^{-5} M) was recorded at 10 frames per second (fps). b) A 120-s segment of this recording, beginning ~30 s prior to onset of cholinergic stimulation, was extracted and reduced to different frame rates, which were subsequently analysed for velocity of airway narrowing. #: measured as number of pixels; ¶: 1,000 pixels·s^{-1}.

video rate even further to 0.1 fps removes the oscillations, leaving only the moment-by-moment estimation of contraction velocity. Based on this analysis, 0.2 fps was chosen as the optimal recording rate.

Airway narrowing dynamics after brief or chronic allergen exposure

In order to investigate whether the allergen-induced increased methacholine responsiveness was accompanied by greater velocity and/or magnitude of cholinergic-induced airway narrowing, lung slices were obtained from BALB/c allergen-exposed animals and control animals. Acetylcholine was used at 1 µM to assess responsiveness, this concentration has been shown to be essentially maximally effective [11, 15, 16] and, as figure 1 did not indicate there to be a change in sensitivity, a full concentration–response relationship was unnecessary.

The maximal narrowing of the airways in response to 1 µM acetylcholine did not differ significantly between BALB/c brief allergen-exposed mice and saline control mice (fig. 3a), nor did the peak velocity of that narrowing differ (fig. 3b). Similarly, after chronic allergen exposure, neither the maximal narrowing of the airways (fig. 3c) nor the velocity of that narrowing (fig. 3d) in response to 1 µM acetylcholine were significantly different in allergen-exposed and saline control BALB/c mice.

Allergen-induced changes in airway wall structure

In order to examine indices of airway remodelling in BALB/c mice following chronic allergen exposure, paraffin-embedded sections were stained and assessed by morphometric analysis as described previously [13]. There were significant increases in the amount of mucin-containing, periodic acid-Schiff positive goblet cells (fig. 4a), subepithelial collagen deposition (fig. 4b), and α-SMA staining (fig. 4c) in the airways of chronic allergen-exposed mice, compared with saline control mice ($p<0.05$).

DISCUSSION

Using two established models of allergen-induced AHR, the present authors provide evidence that allergen-induced increased responsiveness to methacholine assessed *in vivo* is not associated with increased airway narrowing velocity or

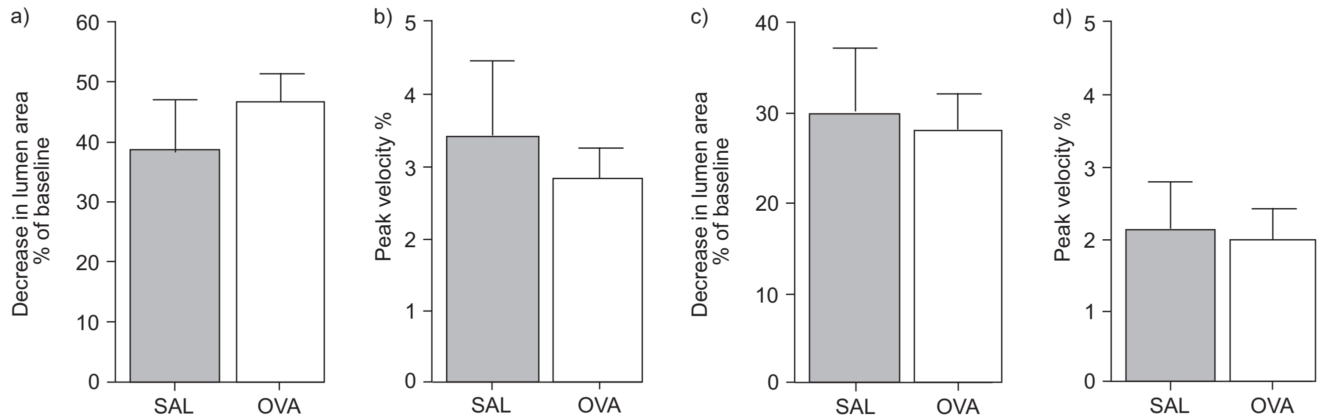

FIGURE 3. Airway narrowing dynamics following a, b) brief allergen exposure and c, d) chronic allergen exposure. a, c) Maximum airway narrowing in response to acetylcholine (1 μM) in lung slices following allergen exposure. No significant differences were found between the allergen-exposed mice compared with the saline control mice. b, d) Peak velocity of airway narrowing in response to acetylcholine (1 μM) in murine lung slices following allergen exposure. No significant differences were found between the allergen-exposed mice compared with the saline control mice. Data are presented as mean±SEM. SAL: mice exposed to saline; OVA: mice exposed to ovalbumin.

magnitude of narrowing measured in isolated lung tissues using an *ex vivo* lung-slice technique.

In recent years, experimental models of allergen exposure have been used in the study of asthma and the pathophysiological changes associated with it. The present authors' laboratory had characterised a murine model of brief allergen exposure resulting in transient AHR [12]. Although this model has been investigated for its airway responsiveness, airway inflammation *via* bronchoalveolar lavage (BAL) and tissue sections, to the authors' knowledge there are no data addressing whether or not airway contraction dynamics are altered following brief allergen exposure.

The present results demonstrate that neither maximum narrowing nor peak velocity differed between allergen-exposed mice and saline control animals. The finding that no differences were detected between groups was surprising, given that an increase in BAL type-2 T-helper cytokines IL-4, -5 and -13 have been previously reported in this and other brief models of allergen exposure [12, 17, 18]. Those previous findings led to the hypothesis that the inflammatory mediators present in the airways could affect the dynamic properties of the airway contraction, thus providing a possible mechanism to explain the *in vivo* AHR observed in the model. Indeed, it is known that *in vitro* exposure of airway smooth muscle to IL-13 can increase specific (carbachol) and nonspecific (KCl) contractions [4]. In the present study, it is possible that any inflammatory mediators that were present *in vivo*, are easily lost during the overnight incubation, as well as by superfusion during the experiment itself. Further experiments are warranted to address whether altered levels of cytokines are present in the lung slice tissue prior to and following allergen challenge. Another limitation of the present study is that relaxant responses were not compared in these two groups of animals.

In addition to the brief allergen exposure model described, chronic allergen exposure of the actively sensitised BALB/c mouse is another well-established model that includes several pathological features associated with the asthmatic airway. In the present model of chronic exposure to allergen, AHR persists for ≥8 weeks following the final allergen exposure, well beyond the resolution of acute inflammatory events [14]. Structural changes in the airway, often termed airway remodelling, may be partly responsible for the sustained AHR *in vivo*. The present hypothesis was that these changes would also become manifest in airway dynamics in lung slices *in vitro*. In the present study, increased indices of airway remodelling in mice were observed 4 weeks after chronic allergen exposure. Specifically, increased deposition of sub-epithelial collagen, increased contractile tissue and goblet cell metaplasia were observed in the first generation airway in the left lobe. The present findings are consistent with previous studies by ELLIS *et al.* [14] and with other reports in the literature [17, 19]. Interestingly, following chronic allergen exposure, a significant increase in either maximum narrowing or peak velocity was not observed, despite the fact that BALB/c mice had demonstrated increased indices of airway remodelling. A possible explanation could be that the intranasal allergen delivery system may not distribute the allergen to the small airways (>200 μm in diameter) used in the *in vitro* studies. However, the present authors have previously shown that structural changes are present in the first generation airway of the left lobe, as well as the smaller airways of the right lobe, suggesting that these changes are found throughout the entire bronchial tree [14]. Other models of chronic allergen have not determined the extent of structural changes in the lower airways [17].

An alternative explanation for the negative findings with *ex vivo* assessment of airway function could be that the small airways used to examine allergen-induced changes in maximal narrowing and velocity may not contribute to airway dysfunction assessed *in vivo*, despite having exhibited structural changes from repeated exposure to allergen. Indeed, the allergen-induced AHR observed *in vivo* may be indicative of changes in resistance from the larger airways as opposed to the smaller airways. It has been noted previously by SAPIENZA *et al.* [20]

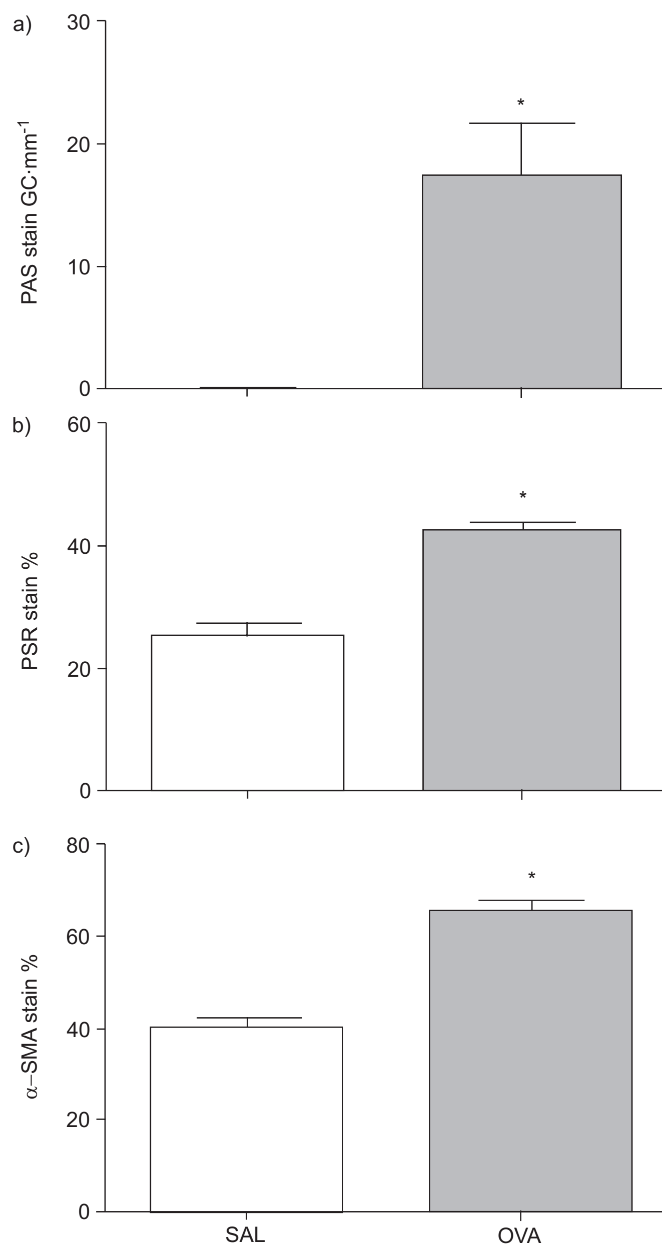

FIGURE 4. Morphometric changes in airways of mice following chronic exposure to saline (SAL) or ovalbumin allergen (OVA). Staining as assessed using morphometry for: a) mucin-containing, periodic acid-Schiff (PAS) positive goblet cells (GC) per millimetre basement membrane length in the epithelium measured using PAS stain; b) collagen measured as percentage Picrosirius red (PSR)-stained; and c) contractile elements measured as percentage α-smooth muscle actin (SMA) stained in the 20 μm region beneath the epithelium. Data are presented as mean ± SEM. *: $p<0.05$ compared with corresponding control animals.

that changes in pulmonary resistance after methacholine inhalation in rats were largely due to changes in the large airway level, not the small airways. Therefore, the reported AHR in mice exposed to allergen in the present authors' laboratory may be indicative of resistance changes in the large airways as well, although this hypothesis has not yet been tested. A third possibility is that the increased collagen deposition may be protective against exaggerated airway narrowing. This theory is supported by observations reported by PALMANS *et al.* [21] in a rat model of prolonged allergen exposure. In that study, an increase in collagen deposition accompanied by a decrease in AHR was observed after 4 weeks of continuous allergen exposure followed by a further increase after 12 weeks exposure. PALMANS *et al.* [21] reasoned that the increase in collagen could stiffen the airway wall, thus reducing the amount of airway narrowing for any given amount of ASM shortening. Furthermore, increased collagen deposited in and around the smooth muscle may also interfere with smooth muscle contraction and, subsequently, lead to a decrease in AHR [22].

In the present study, the *in vivo* measurement of responsiveness to methacholine is that of the entire respiratory system, which may reflect compartments other than the airway lumen diameter, including lung parenchyma. As such, the term airway responsiveness applied to these measurements may be inaccurate, as nonairway events may play a significant role. Therefore, it is possible that one of the reasons for any observed agreement between *in vivo* and *ex vivo* responsiveness changes after allergen exposure, was that a significant component of the allergen-induced change for the *in vivo* measurement was mediated through factors other than true changes in airway diameter. These may include changes in lung parenchymal resistance [23] or increased heterogeneity of airway diameter [24], without actual changes in airway resistance. While the present authors have not performed detailed analyses of precisely where the increase in methacholine induced RRS occurs after allergen, the data presented herein may indicate that the increased responsiveness to methacholine may not simply be due to increased narrowing of the airway.

In conclusion, an *ex vivo* lung-slice technique to assess cholinergic-induced airway contraction dynamics following allergen exposure was used. Although increased responsiveness to methacholine was detected *in vivo*, no differences in airways contraction dynamics could be shown following brief or chronic exposure to allergen within the BALB/c mouse strain. Airway hyperresponsiveness observed in mice *in vivo* is likely to be a result of factors other than intrinsic changes in smooth muscle dynamics and reflects changes in airways other than those observed using the present thin lung-slice technique.

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