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THE EFFECTS OF ALLERGEN ON AIRWAY NARROWING DYNAMICS AS ASSESSED BY A LUNG SLICE TECHNIQUE

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Running Head: Effect of allergen on airway narrowing dynamics

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Abstract

Asthma is characterized by airway hyperresponsiveness (AHR): excessive airway narrowing in response to a variety of stimuli. Certain previous comparisons between mouse strains have shown that increased velocity of airway narrowing correlates with *baseline* airway responsiveness. These data prompted the investigation into our own 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 sensitized and subjected to either brief or chronic periods of allergen exposure. Brief exposure involved 2 challenges with intranasal chicken egg ovalbumin (IN OVA). Chronic exposure involved 6 H two-day periods of IN OVA 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.

We found that neither brief nor chronic allergen exposure resulted in greater airway narrowing and increased velocity as compared to 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.

We conclude that 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, ultimately manifesting as an increase in respiratory resistance.

Keywords: airway smooth muscle, asthma, contraction, lung slices, remodeling,

Introduction

Asthma is a chronic inflammatory lung disease which is characterized 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 to normal individuals (1). The pathophysiological mechanisms that contribute to AHR are still unclear, although ongoing airway inflammation and airway remodeling are believed to play major roles, and elevated 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 remodeling 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 now 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 naive, non-allergen sensitized dogs (5), rats (10), and mice (6; 7; 11), but to our knowledge, no other group has used a murine model of allergen-induced AHR to investigate airway contraction dynamics. Certain previous comparisons between naive mouse strains have shown that increased velocity of airway narrowing correlates with airway responsiveness (6). Consequently, the purpose of this 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 from Harlan Sprague Dawley Inc. (Indianapolis, IN). Mice were aged 6-8 wk (chronic protocol) or 10-12 wk (brief protocol) and housed in environmentally controlled, specific pathogen-free conditions for 1 week prior to study, and for the duration of the experiments. All experimental procedures were approved by the Animal Research Ethics Board at McMaster University, and conformed to the NIH guidelines for experimental use on animals.

Sensitization. BALB/c mice were sensitized as described previously by us (12). Briefly, mice received intraperitoneal (IP) injections of ovalbumin (OVA) conjugated to aluminum potassium sulfate on Days 1 and 11, and with intranasal (IN) OVA on Day 11.

Exposure. Sensitized BALB/c mice were subjected to either brief or chronic allergen exposure protocols. The brief protocol involved two 2-day periods of IN OVA (100 μg in 25 μl saline) challenges. The chronic protocol involved six 2-day periods of IN OVA, each separated by 12 days (total of 12 exposures over a 10 week period). Control mice were subjected to the same sensitization protocol but received IN saline exposures. The mice subjected to the brief exposure protocol were studied 24 hours after the final exposure to either allergen or saline, while 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 analyzed using the thin lung slice technique (below).

Solutions. Agarose type VII solution (4% w/v, Sigma Chemical Corp., St. Louis, Mo) was dissolved in distilled water at 60°C, cooled to 37°C, and mixed with 2H 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 euthanized by CO_2 followed by terminal exsanguination. The trachea was exposed and cannulated using a blunt-ended 19G needle, followed by chest wall removal to expose the lungs. The lungs were inflated with approximately 1.2 ml agarose (2% in Hanks' Buffered Saline solution [HBSS]; 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 4°C 1X HBSS and the whole mouse preparation was placed at 4°C for 15 minutes. The lungs were removed and placed in 4°C HBSS for an additional 30 minutes to ensure the complete gelling of the agarose within the lungs. The lungs were separated into individual lobes and bathed in cold HBSS. Slices were cut in 4°C HBSS with a tissue slicer (model EMS-4000), from the right upper lobe approximately 120 μm thick. Lung slices were bathed in room temperature HBSS until all slices were obtained from the lobe.

Incubation media for tissue slices. Lung slices were transferred to Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) with 15 mM 4-(2-hydroxyethyl)-1- piperazine ethanesulfonic acid (HEPES) buffer, L-glutamine, and pyridoxine HCl, supplemented with penicillin-streptomycin, amphotericin B, L-ascorbic acid, transferrin, selenium, and insulin, 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) on the stage of the microscope (Nikon TMD) and viewed at 100x 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 (CCD) camera (CV-252; Nikon, Japan), a frame grabber board and image

acquisition software (Video Savant, IO Industries, London, ON). Frames were captured in time-lapse (except for data shown in Figure 2, this was done at a rate of one frame per 5 seconds) for the allergen experimental protocols, stored in TIF stacks of several hundred frames and analyzed using Scion image analysis software (Scion Corporation, Frederick, MD). In particular, images with 10-bit gray 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), identifying all contiguous white pixels 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), then counting the number of (white) pixels within any object larger than that cut-off value. Given the dramatic difference in brightness between the airway lumen and any object in view (be that parenchymal/wall tissue or "debris"), minor changes in thresholding level would have little or no affect on the absolute measurements made, and would have no affect whatsoever on the relative changes which we report here (*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 1H HBSS in order to obtain a baseline. After baseline images had been recorded, a constant flow of 10^{-6} M ACh was superfused over the lung slice preparation for 5 min; Bergner and Sanderson have shown this concentration of Ach to evoke nearly maximal responses (13). Airway area was measured with respect to time, by pixel summing, with the use of Scion software. The cross-sectional airway area ranged from 17,000 to 44,000 μm^2 (corresponding to a diameter range of 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) 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 (SCIREQ Scientific Respiratory Equipment Inc., Montreal, Canada). Responsiveness to methacholine was measured on the basis of the response of total respiratory system resistance (R_{RS}) to increasing intravenous doses of methacholine (MCh) (n=10 per group). Each mouse was anesthetized with Avertin (2,2,2-Tribromoethanol, Sigma, Canada) via IP injection at a dose of 240 mg/kg and underwent tracheostomy with a blunted 18-gauge needle, then were connected to the FlexiVent computer-controlled small animal ventilator (SCIREQ, Montreal, Canada). Animals were ventilated quasi-sinusoidally (150 breaths/min, 10 ml/kg, IE ratio of 66.7%, and a pressure limit of 30 cmH_2O). A script for the automated collection of data was then initiated, with the PEEP level set at 2 cmH_20 and default ventilation for mice. After the mouse was stabilized on the ventilator, the internal jugular was cannulated using a 25-gauge needle. Paralysis was achieved using pancuronium (0.03 mg/kg intravenously) to prevent respiratory effort during measurement. To provide a constant volume history, data collection was preceded by a 6 sec TLC perturbation (peak amplitude 25 cmH_20). Twenty seconds later the user was prompted to intravenously inject saline then 10, 33, 100, and 330 μg/kg of MCh (ACIC [Can], Brantford, ON, Canada). For each dose, a maximum of thirteen "QuickSnap-150" perturbations (single sinusoidal inspiration/expiration of 0.4 sec duration with a volume amplitude relative to weight of 10 ml/kg) were performed for approximately 45 sec followed 10 sec later by a 6 sec TLC. Each breath was delivered after allowing the mouse to expire passively for 1s against a positive pressure of 2 cmH_2O. Respiratory mechanics were calculated from simultaneous

recording of airway pressure, change in lung volume and airflow into/out of the lungs. Total respiratory system resistance (R_{RS}), and elastance (E_{RS}) were calculated for each breath by using multiple linear regression to fit this data to the following linear single compartment model of the mouse respiratory system.

$$P = (E_{RS} * V) + (R_{RS} * \dot{V}) + P_o$$

where Po is the end alveolar pressure determined by the PEEP applied to the expired line of the ventilator. After the last dose was complete, the mouse was removed from the ventilator for further tissue collection. Heart rate and oxygen saturation were monitored via infrared pulse oxymetry (Biox 3700; Ohmeda, Boulder, CO) 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 by us 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 alpha smooth muscle actin (α-SMA).

Statistical analysis. All data are expressed as mean ± standard error of the mean (SEM). 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 Student's *t*-test. P values less than 0.05 were considered to be significant.

Results

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

To confirm that our allergen exposure protocol is associated with increased cholinergic responsiveness in mice, we measured *in vivo* respiratory function in BALB/c mice following brief or chronic allergen exposure (these mice were separate from the animals used for lung slice experiments). These mice showed significant increases in maximal methacholine-induced total respiratory system resistance (Rrs) compared to corresponding saline controls ($n=10$; $p<0.05$; Figure 1). Identical observations were made in similarly treated mice where respiratory responses to methacholine were measured using a flow interruptor method (12) (Data not shown).

Validation of experimental technique

Elsewhere (15), we have validated several technical aspects of the lung slice technique used here (extent of inflation; concentration of agarose; wide range of airway sizes). In order to evaluate the rate of data acquisition which was optimal for estimating contraction velocities, we made a single recording at 10 frames per second (fps), then produced from that several other videos with lower frame rates by selecting the appropriate frames (*e.g.*, every 10^{th} frame to obtain an effective video rate of 1 fps): the latter were then analyzed for velocity of constriction.

Figure 2A shows a segment of the 10 fps recording of the constrictor response to ACh, while the estimations of instantaneous velocities made from a 120 second portion of that video (beginning approximately 30 seconds prior to cholinergic stimulation) are given in the top tracing in Fig. 2B (note the different time-scales in Fig. 2A and Fig. 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 (previously described by Sanderson *et al*. (13)); other possible sources of "noise" (much less than 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 2nd frame considered), as well as slower effective video rates down to 0.1 fps (only every 100th frame). It can be seen that 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 approximately 0.33 Hz as well as the peak velocity of Ach-evoked contraction *per se*. Reducing the effective 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, we chose 0.2 fps as the optimal recording rate.

Airway narrowing dynamics after brief or chronic allergen exposure.

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. We used 10^{-6} M ACh to assess responsiveness, since other groups (11; 16) and our own group (15) have shown this concentration to be essentially maximally effective; we felt a full concentration-response relationship was unnecessary, since Fig. 1 did not indicate there to be a change in sensitivity.

The maximal narrowing of the airways in response to 10^{-6} M ACh did not differ significantly between BALB/c brief allergen-exposed mice and saline control mice (Figure 3A),

nor did the peak velocity of that narrowing differ (Figure 3B). Similarly, after chronic allergen exposure, neither the maximal narrowing of the airways (Figure 4A) nor the velocity of that narrowing (Figure 4B) in response to 10^{-6} M ACh 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 remodeling in BALB/c mice following chronic allergen exposure, paraffin-embedded sections were stained and assessed by morphometric analysis as we have described previously (13). There were significant increases in the amount of mucin-containing, periodic acid Schiff positive goblet cells (Figures 5A and 6A), subepithelial collagen deposition (Figures 5B and 6B), and alpha smooth muscle actin staining (Figures 5C and 6C) 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, we provide evidence that allergen-induced increased responsiveness to methacholine assessed *in vivo* is not associated with increased airway narrowing velocity or magnitude of narrowing measured in isolated lung tissues using an *ex-vivo* lung slice technique.

Experimental models of allergen exposure have been used for well over a decade in the study of asthma and the pathophysiological changes associated with it. Previously, our laboratory has characterized a murine model of brief allergen exposure resulting in transient AHR. Although this model has been investigated for its airway responsiveness, airway inflammation via bronchoalveolar lavage (BAL) and tissue sections, to our knowledge there are no data addressing whether airway contraction dynamics are altered following brief allergen exposure.

Our results here 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 Th2 cytokines interleukin (IL)-4, IL-5, and IL-13 have been previously reported in this and other brief models of allergen exposure (12; 17; 18): those previous findings led us to hypothesize 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 this model. Indeed, it is known that in vitro exposure of airway smooth muscle to IL-13 can increase specific (carabchol) and non-specific (KCl) contractions (4). It is possible in the present study that any inflammatory mediators which were present in vivo are easily lost during the overnight incubation (as well as by superfusion during the experiment per se). 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 this study is that we did not compare relaxant responses in these two groups of animals.

In addition to the brief allergen exposure model described above, chronic allergen exposure of the actively sensitized BALB/c mouse is another well-established model that includes several pathological features associated with the asthmatic airway. In our model of chronic exposure to allergen, AHR persists for at least 8 weeks following the final allergen exposure, well beyond the resolution of acute inflammatory events (14). Structural changes in the airway, often termed airway remodeling, may be partly responsible for the sustained AHR *in vivo*: we hypothesized that these changes would also manifest as changes in airway dynamics in lung slices *in vitro*. In the current study, we observed increased indices of airway remodeling in mice four weeks following chronic allergen exposure. Specifically, we observed increased deposition of subepithelial collagen, increased contractile tissue, and goblet cell metaplasia in the first generation airway in the left lobe. These findings of the current study are consistent with previous studies from our laboratory (14) and with other reports in the literature (17; 19). Interestingly, following chronic allergen exposure, we did not observe a significant increase in either maximum narrowing or peak velocity, despite the fact that BALB/c mice had demonstrated increased indices of airway remodeling. A possible explanation could be the intranasal allergen delivery system may not distribute the allergen to the small airways (> 200 μm in diameter) which were used in the *in vitro* studies; however, we have previously shown structural changes are present in the first generation airway of the left lobe as well as 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 we used to examine allergen-induced changes in maximal narrowing and velocity may not contribute to *in vivo* assessed airway dysfunction, 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) 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 our laboratory may be indicative of resistance changes in the large airways as well, although we have not yet tested this hypothesis. 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 and coworkers (21) in a rat model of prolonged allergen exposure. In that study, Palmans *et al.* (21) observed an increase in collagen deposition, which was accompanied by a decrease in AHR after 4 wk of continuous allergen exposure and even more so after 12 wk exposure. The authors 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 (21). 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 this paper, our 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 non-airway events may play a significant role. It is therefore possible that one of the reasons for our failing to observe agreement between *in vivo* and *ex vivo* responsiveness changes after allergen exposure was that a significant component of the allergen-induced change in the *in vivo* measurement was mediated through factors other than true changes in airway diameter, including changes in lung parenchymal resistance (23), or increased heterogeneity of airway diameter (24), without actual changes in airway resistance. While we have not performed detailed analysis of precisely where the increase in methacholine induced respiratory system resistance occurs after allergen, we feel that the data presented here may indicate that the increased responsiveness to methacholine may not simply be due to increased narrowing of the airway.

In conclusion, we have used an *ex vivo* lung slice technique to assess cholinergic-induced airway contraction dynamics following allergen exposure. Although we were able to detect increased responsiveness to methacholine *in vivo*, no differences in airways contraction dynamics could be shown following brief or chronic exposure to allergen within the BALB/c mouse strain. "AHR" observed in mice *in vivo* are likely a result of factors other than intrinsic changes in smooth muscle dynamics, and reflect changes in airways other than those observed using our thin lung slice technique.

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Figure Legends

Figure 1. Physiology measurements in BALB/c mice during increasing doses of MCh. A) Airway function measured in mice 24 hours after brief exposure to saline (solid circles) or OVA (open circles). B) Airway responsiveness measured in mice 4 weeks after chronic exposure to saline (solid circles) or OVA (open circles). Values given are mean ± SEM; 10 mice per group. * Indicates p value less than 0.05 compared with corresponding control animals.

Figure 2 Effect of varying rate of data acquisition (video frame rate) on the estimation of velocity of airway narrowing. The response to Ach (10^{-5} M) was recorded at 10 frames per second **(A)**. A 120 second long segment of this recording, beginning approximately 30 seconds prior to onset of cholinergic stimulation, was extracted and reduced to different frame rates by omitting frames (see Methods). These were then analyzed for velocity of airway narrowing **(B)**.

Figure 3 Airway narrowing dynamics following the brief allergen exposure protocol. A) Maximum airway narrowing in response to ACh (10^{-6} M) in lung slices following allergen exposure. No significant differences were found between the allergen exposed mice as compared to the saline control mice. B) Peak velocity of airway narrowing in response to ACh (10^{-6} M) in murine lung slices following allergen exposure. No significant differences were found between the allergen exposed mice as compared to the saline control mice. Values given are mean ± SEM.

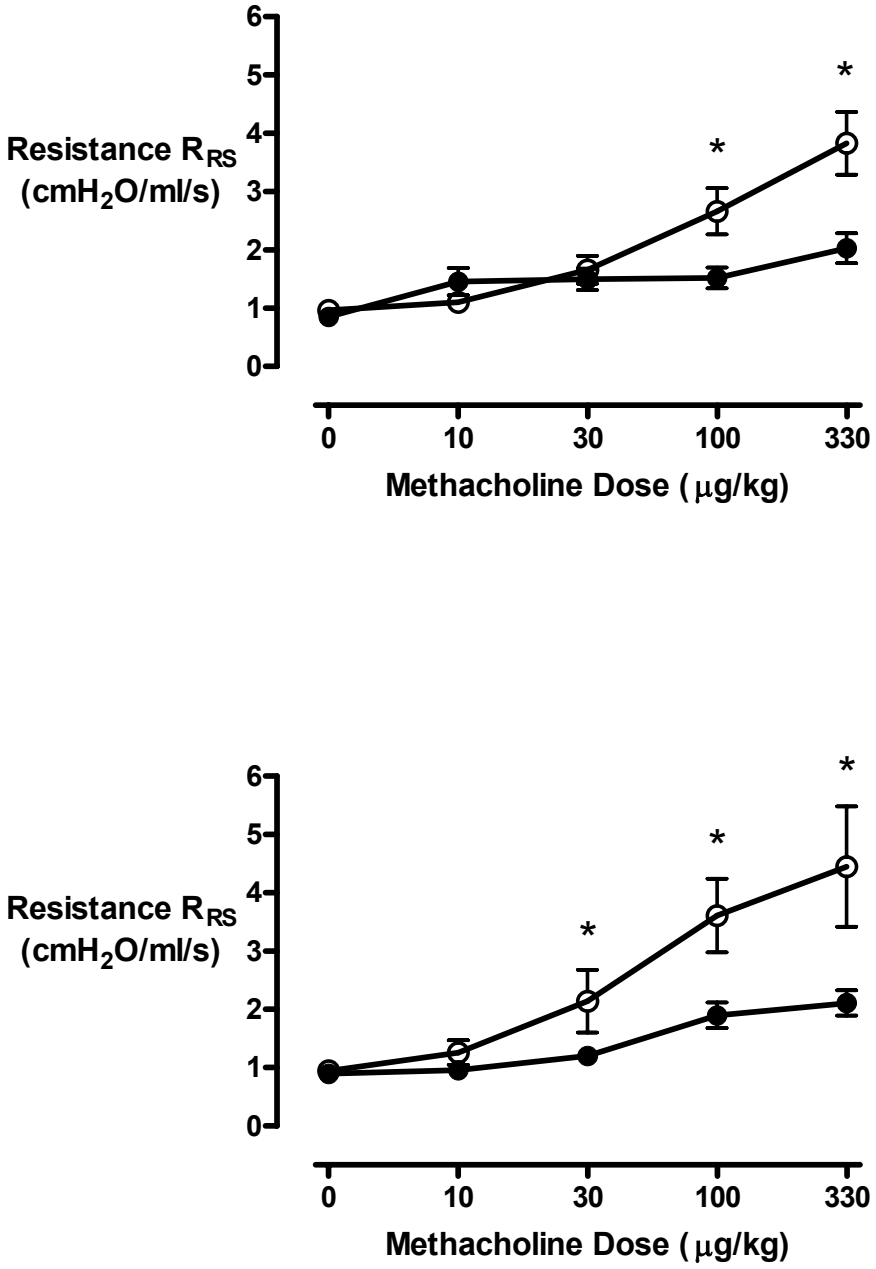
Figure 4 Airway narrowing dynamics following the chronic allergen exposure protocol. A) Maximum airway narrowing in response to ACh (10^{-6} M) in lung slices following allergen

exposure. No significant differences were found between the allergen exposed mice as compared to the saline control mice. B) Peak velocity of airway narrowing in response to ACh (10^{-6} M) in murine lung slices following allergen exposure. No significant differences were found between the allergen exposed mice as compared to the saline control mice. Values given are mean ± SEM.

Figure 5 Morphometric changes in airways of mice following chronic exposure to saline or allergen. Staining as assessed using morphometry for mucin-containing goblet cells (PAS) in the epithelium, collagen (PSR) and contractile elements (α-SMA) in the 20 μm region beneath the epithelium. Data are expressed as number of goblet cells per millimeter basement membrane length (A) or as a percentage of the region of interest that was positively stained (B and C). Values given are mean ± SEM. * Indicates p value less than 0.05 compared with corresponding control animals.

Figure 6 Histological sections of airway wall from chronically exposed mice. Representative staining for PAS positive goblet cells (Panels A-B), collagen deposition (PSR, viewed using polarized light microscopy) (Panels C-D), and contractile elements (α-SMA) (Panels E-F) in the airways of mice after chronic exposure to OVA (Panels A, C, E), or saline (Panels B, D, F).

Figure 1


Resistance R_{RS} (cmH_2O/ml/s)
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Methacholine Dose (μg/kg)
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100
330
Methacholine Dose (μg/kg)

Figure 2

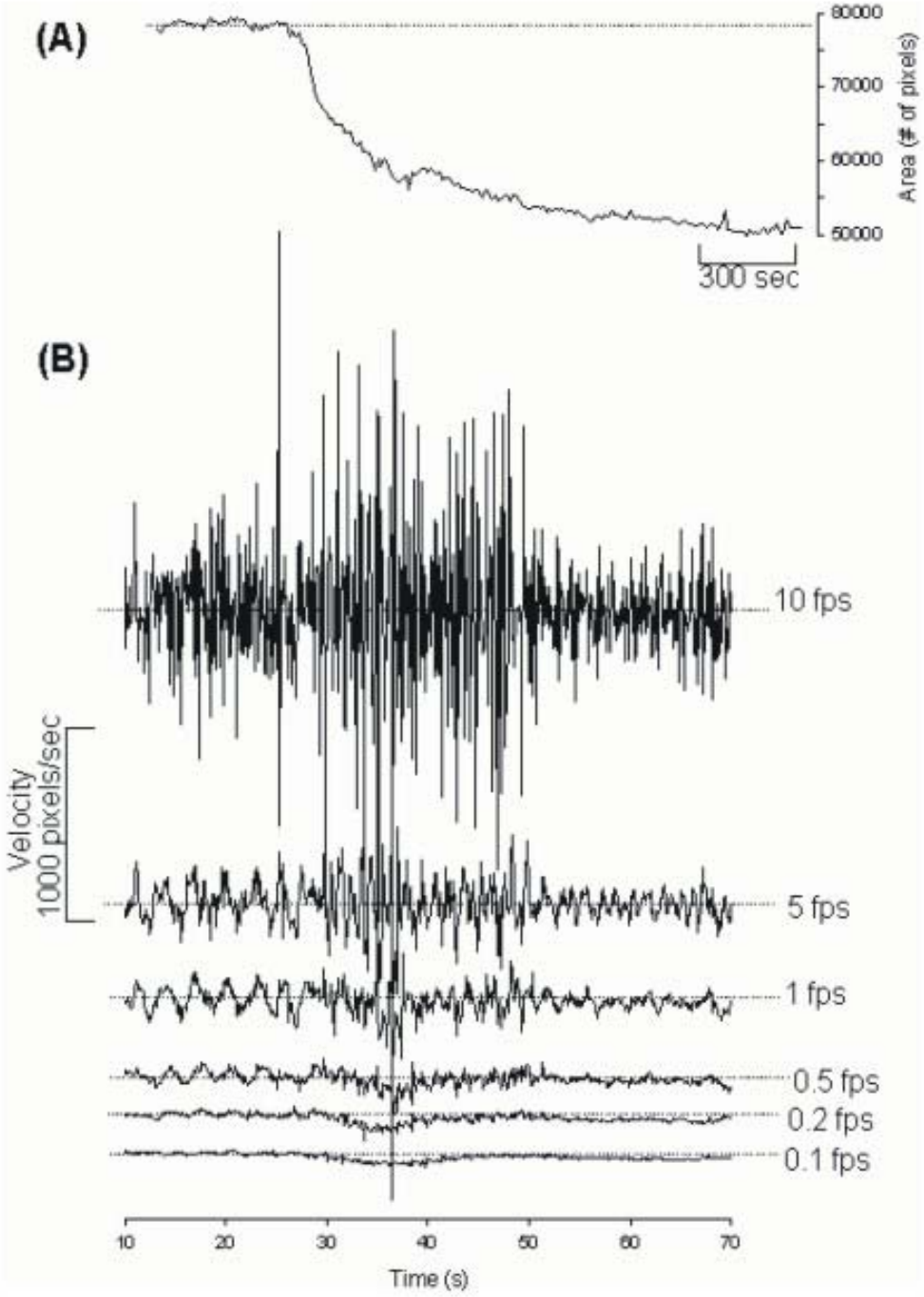
(A)
80000
70000
60000
50000
Area (# of pixels)
300 sec
(B)
10 fps
5 fps
1 fps
0.5 fps
0.2 fps
0.1 fps
Velocity
1000 pixels/sec
10
20
30
40
50
60
70
Time (s)

Figure 3

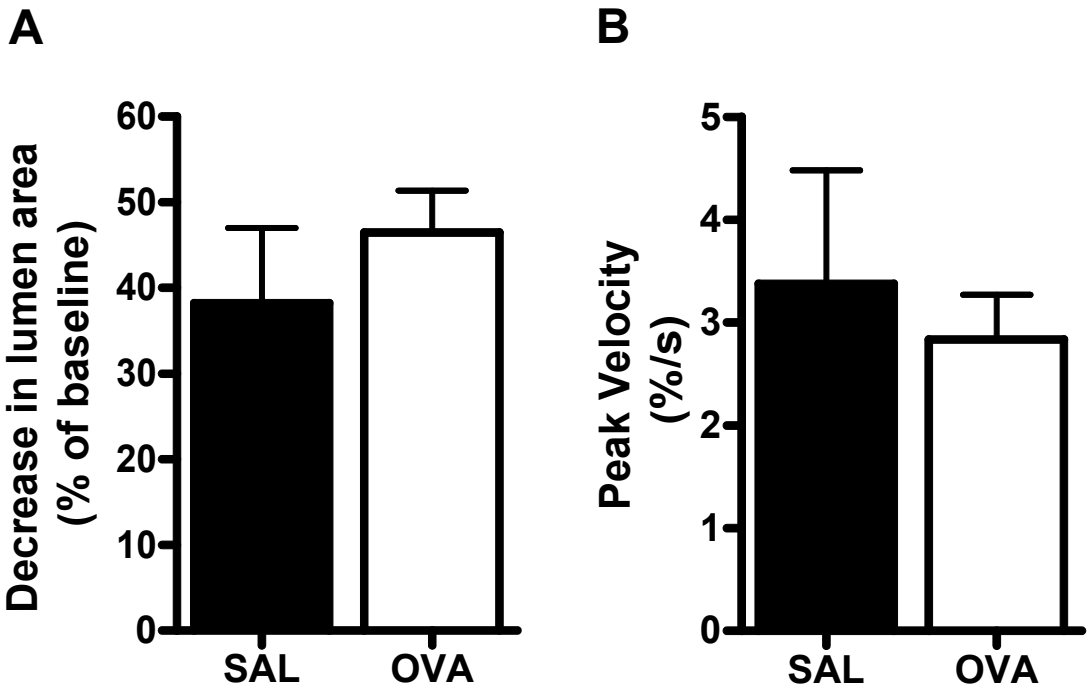
A
Decrease in lumen area
(% of baseline)
60
50
40
30
20
10
0
SAL
OVA
B
Peak Velocity
(%/s)
5
4
3
2
1
0
SAL
OVA

Figure 4

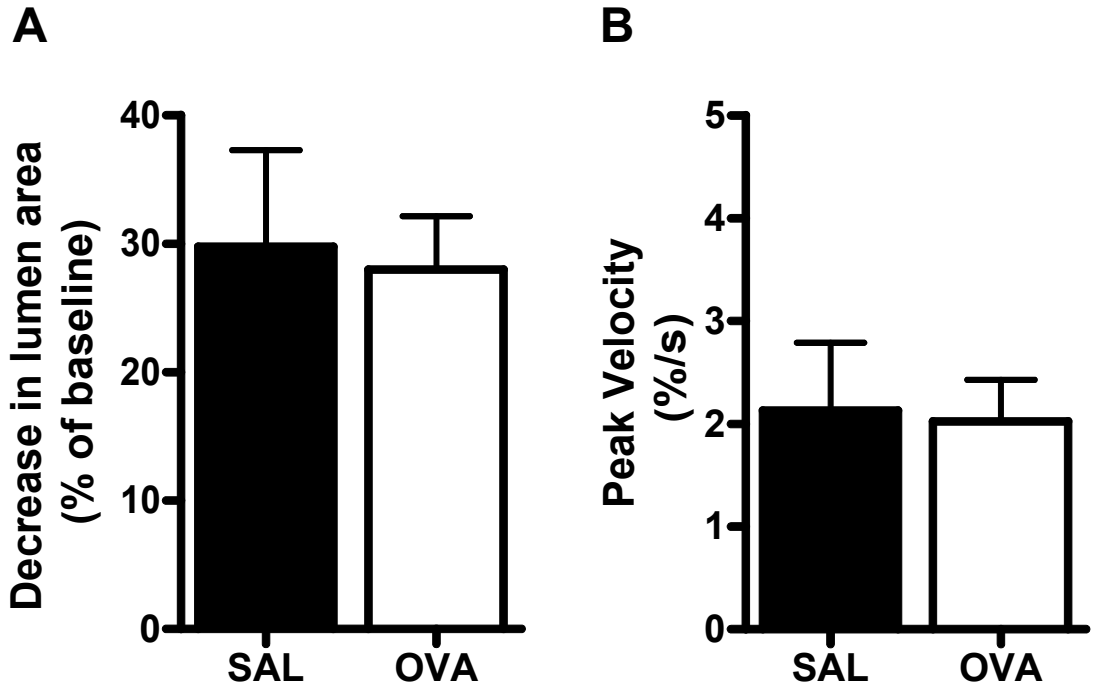
A
Decrease in lumen area
(% of baseline)
40
30
20
10
0
SAL
OVA
B
Peak Velocity
(%/s)
5
4
3
2
1
0
SAL
OVA

Figure 5

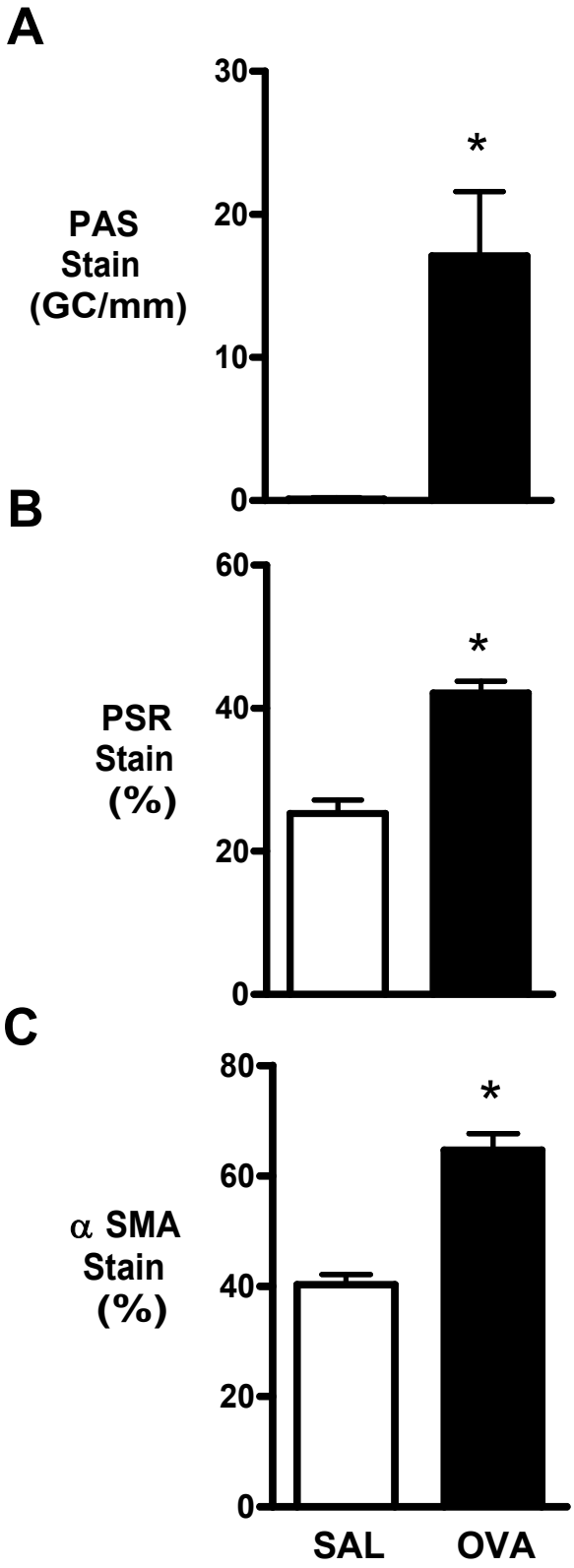
A
PAS
Stain
(GC/mm)
30
20
10
0
*
B
PSR
Stain
(%)
60
40
20
0
*
C
α SMA
Stain
(%)
80
60
40
20
0
*
SAL
OVA

Figure 6