MECHANICAL PROPERTIES OF ASTHMATIC AIRWAY SMOOTH MUSCLE

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ONLINE SUPPLEMENTARY MATERIAL

MATERIALS AND METHODS

Tissue Preparation and Equilibration: The dissection and preparation of the trachealis smooth muscle for mechanical measurements and of the lung for morphometric studies was accomplished as previously described (1,2). The lungs for this study were donated for research and obtained from the International Institute for the Advancement of Medicine (IIAM: Edison, NJ - http://www.iiam.org/). The lungs were handled using a protocol identical to that used to preserve lungs for transplantation. Briefly, after surgical removal the lungs were flushed with Custodiol HTK solution (Odyssey Pharmaceuticals: East Hanover, NJ) and transported by plane on ice to Vancouver. The transport time was 15-20 h. Immediately on arrival at the hospital, the tracheal tissue was dissected free of the lung and was stored at 4ºC in PSS. The remaining lung was inflated with Cryomatrix (Shandon, Pittsburg, PA) diluted 50% with normal saline, rapidly frozen solid in liquid nitrogen vapor, cut into 2-cm thick transverse slices (10-12/lung), and sampled with a power-driven hole-saw to obtain tissue cores 1.5 cm in diameter and 2 cm long. These were processed for histological examination and stained with hematoxylin and eosin (H&E) for morphometric examination of airway dimensions.

The tracheal dissection was undertaken within a day. Tracheal rings were placed in Ca^{2+}-free Physiological Salt Solution (PSS) to relax the muscle in order to accurately determine the *in situ* resting length of the tracheal smooth muscle (herein referred to as reference length, L_{ref}). Connective tissue and epithelium was carefully removed to isolate a smooth muscle bundle. Muscle strips measuring 1-1.5 mm wide, 0.5 mm thick, and 6 mm long, were fixed on each end with aluminum foil clips and mounted vertically on a force-length transducer. The muscle was then equilibrated at L_{ref} by periodic electrical field stimulation (EFS) at 5-minute intervals for a period of 1.5 h. The EFS consisted of a train of bi-polar sinusoidal waves, 60 Hz and 20 V peak-to-peak amplitude. The parameters for the EFS were chosen to ensure maximal stimulation of the muscle. The contraction is primarily due to the EFS-induced release of acetylcholine from nerves; with atropine we get ≤20% of the force without atropine in our human preparations. On average, it took 8 seconds for the EFS-induced force to reach a plateau. The CysLT1 receptor antagonist montelukast (10^{-6} M) was added to the PSS for all of the experiments to prevent or eliminate intrinsic tone.

Mechanical Measurements: After equilibration, the maximal isometric force produced in response to EFS at L_{ref} was determined (herein called F_{max}). As previously described, the force-length relationship was determined at five different lengths: 0.5, 0.75, 1.0, 1.25, and 1.5 L_{ref} (1). The muscle was allowed to adapt at each length for 20 minutes during which EFS was applied at 5-minute intervals. Force-velocity curves were determined using quick release (quick switch from isometric to isotonic contraction) at five graded loads (between 10 and 100% F_{max}) at L_{ref}. Velocities at different loads were determined at two time points, at the peak of tetanic force, and midway to the peak (1,3). The velocity was determined from the slope of the length trace 100 ms after the quick release during a period of steady-state shortening [see ref 1 for details]. The curves were fit using Hill's hyperbolic equation (4). Maximal isotonic shortening at no load was determined by linear extrapolation from the isotonic EFS contractions against two preloads: 10% and 20% F_{max}. Response to mechanical perturbation was determined by applying a passive (i.e., muscle was in relaxed condition) 10 minute, 0.2 Hz, 30% L_{ref} length oscillation (60% L_{ref} peak-to-peak amplitude – ie 30% lengthening). Force recovery following oscillation was followed for 30 minutes with EFS at 5-minute intervals.

Histology and morphometry of tracheal smooth muscle and lung: At the end of the mechanical measurements the trachealis tissue was fixed at L_{ref} in 10% formalin for histology and processed as previously described (1) (Figure E1). The amount of muscle in the preparations was determined on transverse sections stained with Masson's trichrome and quantified using Image ProPlus 4.5 (MediaCybernetics: Bethesda, MD). Connective tissue embedded within muscle bundles was excluded by color segmentation. The fractions of ASM in the tissue preparation and of connective tissue within the muscle bundles were measured.

The maximal stress produced by the muscle was determined by dividing F_{max} (mN) by the

cross-sectional area (mm^2) of muscle present in the preparation (the resulting units are mN/mm^2 or kilopascals, kPa).

Multiple randomly sampled H&E stained images of the lung from the same subjects whose tracheas were studied, were digitized using a brightfield scanner at 20x and 40x magnification. The scanned images were loaded into the Aperio Specturm database (Aperio Technologies, Inc.Vista, CA) and examined by an observer (CP) blinded to the clinical status of the subject. All airways that were cut in cross section (a short to long axis ratio of at least 0.6) were included in the analysis. The basement membrane length of the airway was determined by manual tracing. The areas of airway wall compartments for a total of 207 airways from 21 cases were analyzed. Regions of interest were: epithelium, lamina propria, smooth muscle, adventitia, and total airway wall area. Initially, 20 airways were selected at random (10 from non-asthmatics and 10 from asthmatics) in order to determine the most appropriate method for measuring the areas. Using Aperio's Image Scope software, each compartment was traced to give an estimation of the area. Intra-observer error for blinded repeat measurements of the wall area regions were relatively small. The mean errors for measurements were; epithelial area = 2.1% (range of -12% to 21%), smooth muscle area= 2.7% (-17% to 29%), lamina propria = -0.7% (-30% to 14%) adventitia = -6.4% (20% to -27%) and total wall = -4.2% (-12% to 8%). To compare the efficiency of tracing and point counting the same 20 airways were analyzed using point counting in Image Pro Plus. The appropriate number of points to place on the image was also determined. Varying numbers of points (from 2000 to 6000) were placed on each image and % error was calculated as referenced to the traced areas. The variance decreased as a function of the number of points and a protocol with 3551 points per image was selected as most efficient and used to calculate each wall compartment area. The remaining airways were analyzed using point counting at this point density. The fraction of points (out of 3551) that fell on the compartment of interest was multiplied by the total area of the airway to give an estimation of the area of the region of interest.

Statistical Analysis: For the analysis of airway dimensions we compared groups and calculated individual data for comparison with the trachealis muscle physiology. Because there was a variable number of airways and a variable airway size range between subjects, we elected to test for differences in airway dimensions using a linear mixed-effects model which accounted for this variability (5). The model had a fixed effect term for group and random effects terms for lung and the airway within each lung. The model was applied separately to the area measurements of epithelium, lamina propria, smooth muscle, adventitia and total wall all referenced to basement membrane perimeter. A similar analysis was applied to the relationships between the square root of the airway compartment areas and the basement membrane perimeter. The model- derived mean values for the ratio as well as the slopes and intercepts were compared between the asthmatics and non-asthmatics. The software used for this analysis was R (version 2.10.1 - R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org.) A p-value < 0.05 was considered significant. In addition we calculated slopes and intercepts of the square root of airway smooth muscle area versus basement membrane perimeter for each subject. Using this relationship the airway smooth muscle area for an airway with a diameter of 1 mm was calculated for each subject for comparison with the physiological results from their trachealis muscle.

In the analysis of the airway wall dimensions we used the square root of the wall areas for graphical purposes to linearize the relationship with basement membrane perimeter since this allows easier curve fitting. However we used the actual areas of the tissue wall compartments for statistical comparison between groups. This could have been confounded if we were comparing airways of different size since the ratio of airway wall area/Pbm increases as airways get smaller. However since there was no difference in the mean airway size (Pbm) between the asthmatics and normals we feel that this analysis is valid.

Force and length measurements were normalized to F_{max} or L_{ref} respectively and expressed as

fractions of F_{max} and L_{ref}. Velocity of shortening was expressed as ΔL_{ref}/sec. Aggregate data were expressed as mean ± SEM. One and two way ANOVA and regression analyses were accomplished using GraphPad Prism 5 (GraphPad Software, Inc.: La Jolla, CA). $p \leq 0.05$ was considered to be sufficient to reject the null hypothesis.

RESULTS

Trachealis and Intra-parenchymal airway morphology: The mean % ASM in the tissue preparations of the non-asthmatic subjects was 25.5 ± 9.0% while in the asthmatics it was 28.8 ± 8.7% (p=0.53). The mean % connective tissue in muscle bundles in the non-asthmatic subjects was 39.8 ± 5.7% and in the asthmatics it was 31.9 ± 11.2% (p=0.12). The airway wall dimensions of 207 airways from 11 asthmatic and 9 non asthmatic donor lungs were analyzed. (Table 2) The ratios of area to Pbm for the smooth muscle (p<0.001), lamina propria (P =0.013), adventitia (P=0.020), and total wall (P=0.024) were greater in the asthmatic than the non-asthmatic subjects, while the epithelial area was not significant (P=0.053). A similar mixed effect model analysis was done comparing the slopes and intercepts of the square roots of the airway wall compartment areas to Pbm. The results are shown in Table E1 in the online supplement. The analysis showed that the slope of square root of ASM area versus Pbm was steeper (P>0.001) in the asthmatic subjects compared with the non asthmatic subjects. Figure E2 shows the relationship between the square root of smooth muscle area and Pbm for asthmatics and non asthmatics with the fatal and non-fatal asthmatics shown separately. It is apparent that the ASM area is greater at all levels of Pbm in the asthmatic subjects.

Trachealis muscle mechanics: Of the 21 donor lungs used in the study, trachealis muscle mechanics were successfully completed on 6 non-asthmatic and 8 asthmatic lungs (Table 1). The average ages of the non-asthmatics and asthmatics were 25.3±8.1 years and 15.7±2.3 years respectively (t-test: p=0.247). Muscle stress generated with maximal EFS was 152.9±107.7 kPa in the asthmatic and 161.0±50.7 kPa in non-asthmatic preparations (p=0.858 – Main paper figure. 1).

Length-Force Properties and Length Adaptation: Length-force properties were examined by recording force at five different lengths: 0.50, 0.75, 1.00, 1.25, 1.50 L_{ref} (Main paper figure 2A). The direction of the length change was randomly determined initially then repeated in the same order for all the following samples because it simplified the analysis. Immediately following a length change the active force produced by the muscle declined and gradually recovered over the 20-minute period in which the muscle length was held constant and EFS was applied at 5 min intervals (Main paper figure 2B). After each length change to a shorter or longer length than L_{ref}, and the determination of immediate force and adapted force at that length, the muscle was returned to L_{ref} to allow for a period of adaptation. The small decrease in force at L_{ref} which occurred over time (<15%) was corrected for as previously described (1). Except for the asthmatic group at 1.25 L_{ref}, force did not recover to the level of F_{max} at any of the length steps. The changes in passive force following length changes and repeated stimulations are shown in figure 2C in the main paper. The passive force increased dramatically after length changes to longer lengths and decreased immediately following a change to a shorter length. While the passive force at longer lengths declined over the 20-minute period, it remained elevated across both groups when compared to the passive force at L_{ref}.

The extent of length adaptation and a comparison between groups is illustrated in Figure 3 in the main paper. To quantify length adaptation, the first contraction after a length change was compared to the last contraction at that length. For both the non-asthmatics and the asthmatics, significant length adaptation occurred (ANOVA: p<0.0001). For the non-

asthmatic subjects Bonferroni post-tests revealed significantly greater force following adaptation at 0.5 L_{ref} ($P<0.05$) and 1.5 L_{ref} ($p<0.001$) but not at 0.75 and 1.25 L_{ref}. The asthmatics demonstrated greater active force following adaptation at 1.25 and 1.50 L_{ref} ($p<0.001$) but not at 0.75 or 0.5 L_{ref}. Before adaptation the active force at 1.50 L_{ref} was significantly lower in asthmatics compared to non-asthmatics ($p=0.05$) and after the adaptation period the asthmatics produced less force than the adapted non-asthmatics at 0.50 L_{ref} ($p<0.01$). Figure 4 in the main paper shows the changes in passive force with changes in length and during the adaptation process. Both the non-asthmatics and asthmatics demonstrated significant passive force adaptation - i.e. the passive force decreased significantly after the period of adaptation among both groups (ANOVA: $p=0.024$ for non-asthmatics and $p=0.018$ for asthmatics). Bonferroni post-tests demonstrate that the passive force at 1.50 L_{ref} for the asthmatics was significantly greater than that of the non-asthmatics before adaptation ($p<0.05$). In addition the passive force at 1.50 L_{ref} was significantly lower in the asthmatics after adaptation than before adaptation ($p<0.05$).

Shortening Velocity: Two sets of force-velocity relationships were determined: one during the early-phase of contraction and one at the peak of tetanic contraction, as previously described (1,3). The force-velocity curves for individual muscle strips varied widely as shown in online Figures E 2A and B. Force-velocity data for each muscle strip was initially fitted with Hill's hyperbolic equation before averaging the curves (Main paper figures 5A and B). There were no differences in the shape or position of the force-velocity relationships between non-asthmatic and asthmatic subjects.

Maximal Isotonic Shortening: Four non-asthmatic and four asthmatic muscle strips were analyzed to determine maximal isotonic shortening (Main paper figure 6). The extent of shortening was recorded at preloads of 10% and 20% of EFS induced F_{max}. Maximal shortening, as determined by extrapolation, to no load was 72.2±4.9% for the non-asthmatics and 70.5±5.6% for asthmatics ($p=0.946$). With no difference between the groups, all muscle strips were combined into one linear regression (central dashed line, Main paper Figure 6) and maximal shortening was calculated as 71.4±3.4% ($p<0.0001$, $r^2=0.708$). In the unloaded condition, the 95% confidence intervals were 64.0-78.8% shortening.

Recovery from Mechanical Perturbation: Active force recovery following a ten-minute length oscillation was followed for 30 minutes in both non-asthmatic and asthmatic tracheal strips (Main paper figure 7). The response to oscillation was significantly different between asthmatics and non-asthmatics ($p<0.0001$ for two way ANOVA with time and group as variables). Initially following oscillation the non-asthmatics produced an average force of 0.63±0.03 F_{max} compared to 0.81±0.04 F_{max} in asthmatics (Bonferroni post-tests: $p<0.01$). The recovery of force was greatest over the first five minutes in the non-asthmatics, however, the average force recovery was below F_{max} at 0.95±0.03 F_{max}. The asthmatics demonstrated slower force recovery but recovered beyond F_{max} after 30-minutes (1.05±0.03 F_{max}). Non-linear regression analysis of the non-asthmatic and asthmatic data demonstrated these differences in recovery rate. The regressions were fit using a one-phase exponential equation. For the non-asthmatics: $force = 0.947 - 0.296e^{-0.139t}$ ($r^2=0.73$); for asthmatics: $force = 1.053 - 0.243e^{-0.073t}$ ($r^2=0.48$) where *force* was relative to F_{max} and t was time in minutes.

Comparison of Tracheal Smooth Muscle Function with Intraparenchymal Smooth Muscle Morphometry: Since ASM area, normalized to Pbm, was the morphologic variable that best separated asthmatic from non-asthmatic airways we calculated the smooth muscle area at a Pbm of 3140 μm (which conforms to an airway with a diameter of 1000 μm) to use as a continuous variable to compare with trachealis muscle function. Although this variable separated asthmatics from non-asthmatics (0.27 +/- 0.15 um2 versus 0.20 +/- 0.09 um2 P = 0.07) there were no significant relationships between this variable and any of the functional variables when we included all subjects in the analysis or when we limited the analysis to asthmatics. Figure E4 shows the relationship of trachealis muscle stress versus the ASM area in an idealized airway with a diameter of 1000 μm. Similar results were obtained with use of

the slope of the relationship between the square root of ASM area and Pbm as the morphological estimate of airway remodeling (data not shown).

DISCUSSION

A strength of the present study is the well preserved tissue from asthmatic and non-asthmatic individuals. Some previous studies have been done on airway tissue recovered at autopsy while others have examined specimens obtained at surgery. The tissue used in this study came from the lungs of asthmatic patients and age-matched control subjects obtained through the International Institute for the Advancement of Medicine. This non-profit organization obtains consent to use tissue for research from the families of patients prior to their death and handles the organs as they would be handled for organ transplantation

ASM Stress: There is little evidence to support the hypothesis that asthmatic ASM produces more force when adjusted for the cross-sectional area of muscle. However few studies have measured force generated in asthmatics and far fewer have measured the area of the smooth muscle in order to normalize for the size of the muscle strip. Our data suggests that asthmatic ASM does not produce more stress (force per unit muscle area) than non-asthmatic ASM. However there was wide variation of stress produced in each group and a post-hoc power analysis determined that ~38 donors per group would be required to detect a 10% difference in mean stress between groups given this variation. While statistical significance may be possible with a larger sample size it is not certain whether this level of difference in stress production would be physiologically relevant

We used the response to electrical field stimulation as F_{max} in these studies. We have found that maximal EFS produces ~75-80% of the force achieved using high concentrations of acetylcholine. True maximal stress would have been better determined by stimulating with 10^{-3} M Ach. However we doubt that a different result would have been found using Ach.

The Length-Force Relationship: Using *in situ* length as reference length (L_{ref}), both the asthmatics and the non-asthmatics had similar length-force relationships. Although the asthmatic ASM seemed to produce less force at the shorter and longer lengths than the non-asthmatics these differences were not significant. However following length adaptation, which was exhibited in both groups, the asthmatic ASM produced less force than the non-asthmatic ASM at the shortest length we examined (0.50 L_{ref}). This observation suggests that asthmatic ASM is not more capable of length adaptation to short lengths than non-asthmatic ASM.

Shortening velocity: Another hypothesis that has been suggested to explain airway hyperresponsiveness is increased shortening velocity. This hypothesis has been supported by the findings of Jiang et al. (6), in which sensitized canine bronchial smooth muscle was found to shorten faster than non-sensitized muscle and contained more myosin light chain kinase (MLCK). These results were further supported by a later study employing isolated ASM cells from asthmatics obtained by bronchial biopsy (7). The unloaded asthmatic bronchial smooth muscle cells were found to shorten faster and more extensively than non-asthmatic smooth muscle and contain more MLCK mRNA. Mitchell et al (8) also found that passively sensitized human ASM strips shortened faster than paired non-sensitized strips. In another study, sensitized human airways were found to have greater MLCK and myosin heavy chain (MHC) content (9).

Another potential explanation of increased shortening velocity could be a change in the predominant myosin isoform in asthma. Leguillette et al (10) found increased expression of mRNA for the fast myosin heavy chain isoform, transgelin, and myosin light chain kinase in the bronchial biopsies of patients with asthma. Immunohistochemistry also demonstrated the expression of these genes.

Despite these reports, our data suggests that a difference in shortening velocity is not an important contributor to AHR. A limitation of this conclusion is that we were technically unable to measure shortening velocity at very low loads. Using our apparatus we were not able to determine velocities at forces below 2 mN which was approximately 5% F_{max} . The study of larger strips of ASM may circumvent this limitation; ie F_{max} would be large enough to avoid loads below 2 mN while working in the 5% F_{max} range.

How do we reconcile our results with previous studies? It is likely that the differences seen between the results of Ma *et al.* (7) and our own are methodological. In that study, single smooth muscle cells were isolated from bronchial biopsy samples and stimulated to contract under no load. Importantly, the cells were not held at a given length but assumed to be relaxed in the absence of a stimulus. Also, all of the contractions were recorded at room temperature. Our measurements were made at 37˚C and the intact muscle strips were held at *in situ* length before shortening. The isolated cells only shortened 25-40% from initial length (6) which is similar to the fractional shortening reported in previous studies of isolated human ASM (11,12) in which the muscle length was maintained at L_{max}, while our preparations, which where conditioned at *in situ* length consistently shortened ~ 70% if unloaded. Ammit *et al.* (8) found increases in both MLCK and MHC in sensitized human ASM tissue. However none of their subjects had asthma and they did not measure the mechanical properties of the tissue.

Response to Mechanical Perturbation: The different response to strain in the asthmatic ASM strips is the most intriguing aspect of this study (Fig. 7). It is well known that asthmatics' airways respond differently than those of non-asthmatics to the stretch which accompanies deep inspiration (13-17). *In vivo* the effects of deep inspiration can be assessed by taking big breaths before or after the administration of a bronchonstricting stimulus. When applied after bronchoconstriction, DI produces a bronchodilating effect while taken before the administration of a constrictor it causes a broncho-protective effect, i.e., less constriction of the bronchi when stimulated. Asthmatics may have a reduced bronchodilating effect of deep inspiration, especially during spontaneous attacks of asthma (18), but more consistently show a defective broncho-protective effect such that prior deep inspiration fails to attenuate subsequent constriction (19-201.

In this study we applied a length oscillation of 60% peak to peak which means a 30% lengthening strain. Accounting for the compliance of the apparatus, this translates into a ~25% lengthening of the smooth muscle preparation. This value of strain was selected because it resulted in significant force loss and decreased myosin thick filament density in previous studies (22). However this is more strain than is experienced by most of the ASM in the lung during a deep inspiration. If the airways dilate isotropically with the lung parenchyma the circumferential strain is about 20% with an inhalation from FRC to TLC since FRC is about 50% TLC (linear dimensions will change as the cube root of lung volume). Since most airways are less distensible than the lung the strain that most of the smooth muscle experiences is even less than 20%. Thus the strain we applied is more than is experienced physiologically by the trachealis muscle. However in our experiments we are using the trachealis as a "model" of airway smooth muscle and have no reason to believe that it's response to strain is different than that of smaller intraparenchymal airways which experience a strain closer to what we used. In a previous study we found that the decrease in force after oscillation was linearly related to the amplitude of oscillation. Thus it would be anticipated that the effect size, but not the observed difference between asthmatics and non-asthmatics, would be influenced by a lesser strain.

We also applied a longer period of length oscillation than is used *in vivo* to detect bronchoprotection. Again this was chosen based on a previous protocol which was designed to maximize the effect of length oscillation on subsequent force development. However we

found that, although the number of oscillations influences the response, the amplitude is the most important parameter in terms of force decrease post oscillation (22).

The difference in the response to DI in asthmatic subjects *in vivo* has been variously attributed to a failure of DI to translate into the same ASM strain in stiffened asthmatic airways or to an intrinsic difference in ASM mechanics. *In vivo*, deep inspiration applies a stress to the smooth muscle and the resultant strain is dependent on the stiffness of the airway wall as well as the elastic recoil of the lung (i.e. the same volume change produces less strain if lung recoil is reduced as it is in emphysema). Brown et al (23) used CT to estimate the strain (airway dilatation) produced by DI in normal and mild-moderate asthmatic subjects and found no difference. They suggested the different response to strain in asthma was at the basis of the defective response to DI. Our results support the contention that there is an intrinsic difference in smooth muscle behavior in asthmatic tissue. We applied a length oscillation rather than a force oscillation. While this is non-physiological, since strain is a dependent variable *in vivo*, it allows us to suggest that differences in the muscle response to strain, rather than attenuated strain due to stiff airways, is at the basis of the differential response.

However a similar strain experienced by an airway strip *in vitro* or by an airway *in vivo* may result in a different strain being applied to the contractile apparatus of the airway. An airway strip or circumferentially stretched airway can be simplistically thought of as a contractile element in series with an elastic element. The relative strains experienced by the contractile and series elastic elements can be different for any overall strain. There are ample data suggesting that the relaxed airways of asthmatic subjects are stiffer than those of normal subjects (23-27). Although in one recent study Williamson et al (28) showed that the central airway area-pressure curve was left-shifted in asthma, their results also showed that the maximal achievable airway caliber was less than in normal. The second significant difference we observed between the asthmatic subjects' and non-asthmatic subjects' trachealis mechanics was in their passive stiffness (Main paper figure 4). This difference cannot be attributed to an increased connective tissue content in the ASM preparations from the asthmatic subjects. We dissected the posterior membranous sheath of the trachea in an attempt to obtain as pure a smooth muscle sample as possible and there was no difference in the % ASM in the cross sectional area of the preparations between asthmatics and non-asthmatics; (28.8 +/- 8.7% versus 25.5 +/- 9.0 % respectively – P =0.53). An increased matrix content within ASM bundles of asthmatics has previously been reported (29,30) but once again we did not find a difference; 39.8 +/- 5.7% versus 31.9 +/- 11.2 % in asthmatic and non-asthmatic subjects respectively (p=0.12).

Thus another possibility to explain our results and by extrapolation those seen *in vivo* , is that the mechanical properties of the matrix or of the smooth muscle cells themselves are different in asthma such that more of the strain is born by series elastic elements. This would result in less strain of the contractile units for a given strain of the strip or airway and thus less disruption of the contractile apparatus and dissolution of myosin filaments. Whatever the mechanism, the finding of a different response to strain in asthmatic and non-asthmatic ASM represents an important finding and supports the growing evidence that the dynamic response of the airway is a critical factor determining airway hyperresponsiveness.

In conclusion, this study is the first to systematically examine the complete array of mechanical properties of ASM from asthmatic and non-asthmatic individuals. While the sample size was relatively small and the variability of response was wide we found no substantial difference in the stress produced by the asthmatic muscle preparations; indeed at lengths longer and shorter than Lref maximal stress tended to be somewhat less in asthmatic airway smooth muscle preparations. There was no difference in the velocity or extent of shortening.

On the other hand, the results show, for the first time, that there is a difference in the ASM response to stretch in asthma. Following simulated deep inspiration there was less attenuation

in ASM force in the asthmatic tissue. These results suggest that there is an intrinsic difference in the ASM's response to strain in asthma. The increased passive stiffness of the asthmatic preparations may, in part, contribute to this difference, which is supported by *in vivo* data showing less airway dilation induced by a DI in asthmatics (31). Another possibility is that the myosin thick filaments in asthmatic ASM are less prone to dissolution and rearrangement.

It is remarkable that after many decades of investigation we are not closer to understanding the role of smooth muscle in airway hyperresponsiveness despite its central importance in the pathophysiology of asthma. The results of this study coupled with the consistent *in vivo* observation of reduced bronchoprotection induced by DI suggest that we may finally be making progress.

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Table E 1

	Non-Asthmatic				Asthmatic			
		Mean	SE	95% CI		Mean	SE	95% CI
Smooth muscle area	I	0.0161	0.0149	(-0.0150 0.0472)	I	0.0094	0.0116	(-0.0148 0.0336)
	S	0.0434	0.0050	(0.0336 0.0533)	S	0.0643	0.0040	(0.0565 0.0721)
Epithelial area	I	0.0327	0.0160	(-0.0007 0.0661)	I	0.0739	0.0121	(0.0486 0.0991)
	S	0.0720	0.0072	(0.0577 0.0863)	S	0.0715	0.0061	(0.0594 0.0836)
Lamina propria area	I	0.0202	0.0176	(-0.0167 0.0571)	I	0.0648	0.0130	(0.0376 0.0920)
	S	0.1074	0.0062	(0.0952 0.1197)	S	0.1115	0.0048	(0.1020 0.1210)
Adventitial area	I	-0.0690	0.0521	(-0.1780 0.0400)	I	-0.0110	0.0423	(-0.0997 0.0776)
	S	0.2057	0.0247	(0.1569 0.2545)	S	0.2220	0.0218	(0.1790 0.2651)
Total area	I	0.2223	0.0499	(0.1178 0.3268)	I	0.3075	0.0400	(0.2238 0.3912)
	S	0.2475	0.0228	(0.2024 0.2925)	S	0.2669	0.0199	(0.2276 0.3061)

I = intercept, S = slope. SE = standard error 95% CI = 95% confidence intervals

FIGURES:

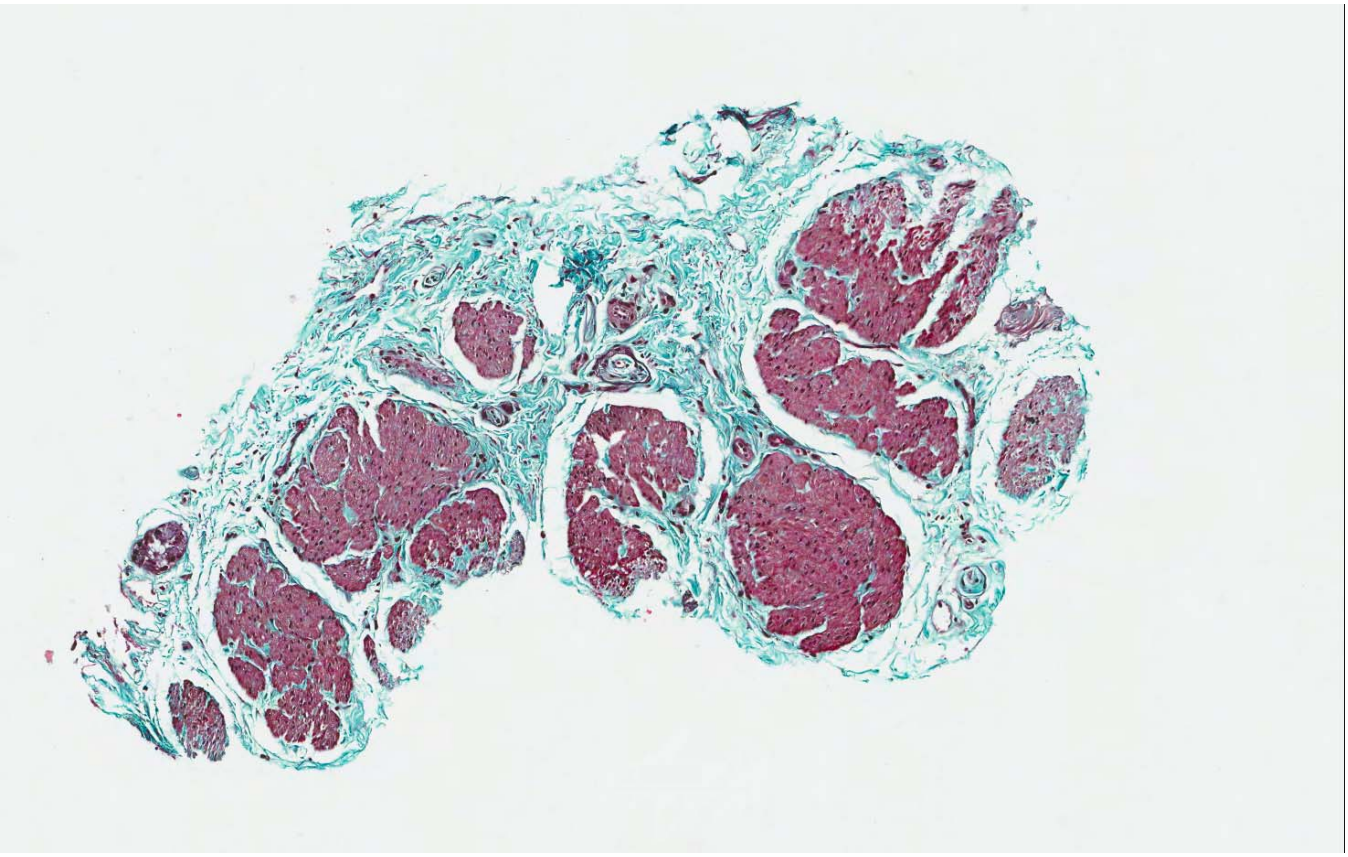

Figure E1. A Masson's Trichrome stained cross section of a trachealis muscle strip used for *in vitro* mechanics. The cross-sectional area of ASM was determined using color segmentation to separate it from the non-muscle tissue in the preparations. The smooth muscle is red, connective tissue blue, and nuclei black. Color segmentation was used to exclude the blue connective tissue within muscle bundles. Image width = 1.14 mm.

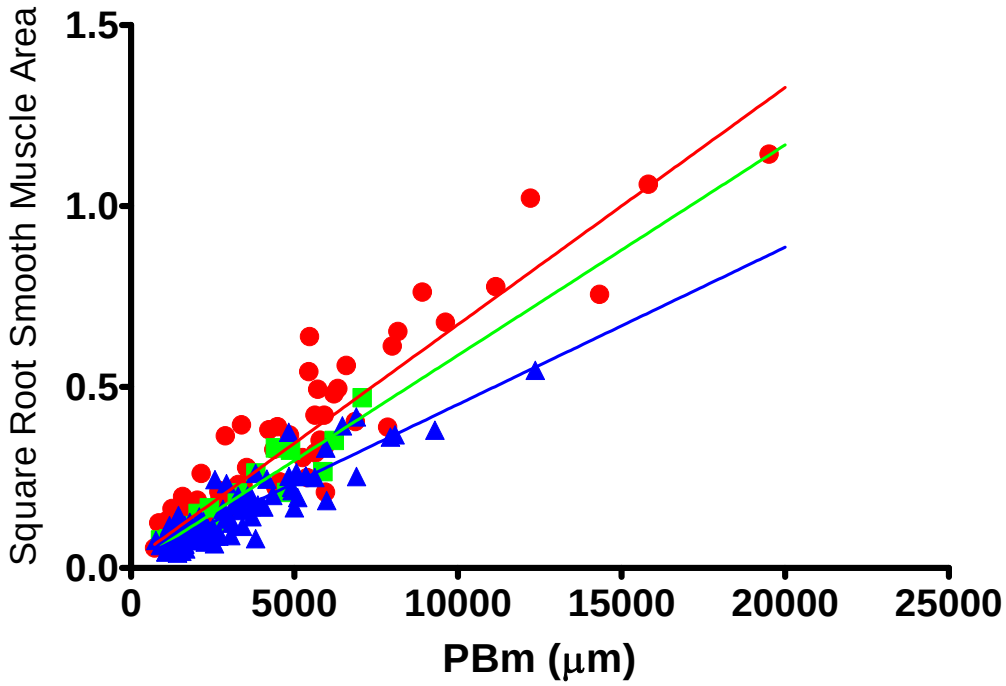

Figure E2. Smooth muscle area versus Pbm in fatal and non-fatal asthmatics and non-asthmatics. The square root of airway smooth muscle area is plotted against airway basement membrane perimeter (Pbm) in millimeters. Non-fatal asthmatics are indicated with green symbols , fatal asthmatics with red symbols and non-asthmatics with blue symbols. Asthmatics have greater smooth muscle area at all levels of Pbm and the data points for non-fatal asthmatics are closer to those of fatal asthmatics than to non-asthmatics.

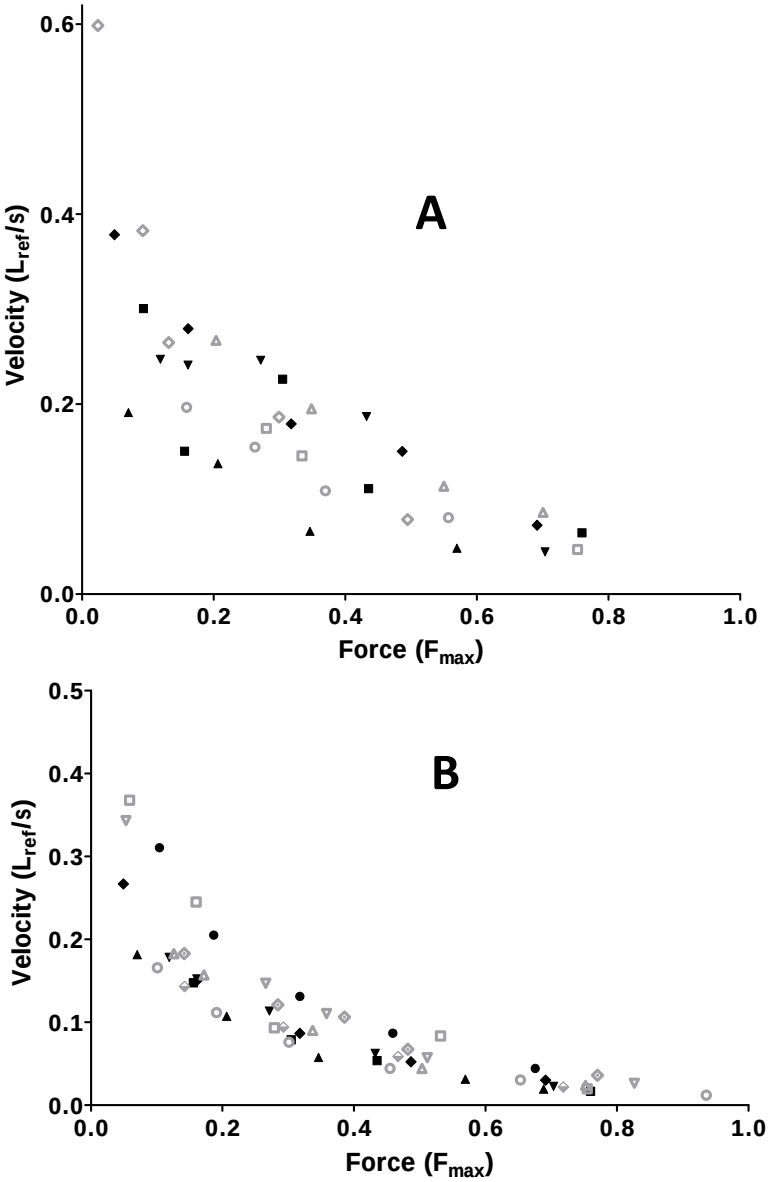

Figure E3 A and B Figure E3 A. Isotonic shortening velocity, individual data points at the early release. Isotonic shortening velocity was measured at different loads at the early phase of contraction. The loads were determined as a percentage of F_{max}. Individual velocity data points recorded at different loads are shown. Each different symbol represents a unique muscle strip. Non-asthmatics are represented as black solid symbols and asthmatics as gray open symbols. Data points for each individual were fitted with Hill's hyperbolic equation and the subsequent curves were averaged to generate curves shown in Figure 5a in main paper. n=4 for each group. **B. Isotonic shortening velocity, individual data points at the late release.** Isotonic shortening velocity was measured at different loads at the late phase of contraction. The loads were determined as a percentage of F_{max}. Individual velocity data points recorded at different loads are shown. Each different symbol represents a unique muscle strip. Non-asthmatics are represented as black solid symbols and asthmatics as gray open symbols. Data points for each individual were fitted with Hill's hyperbolic equation and the subsequent curves were averaged to generate Fig. 5b in main paper (n=5 for the non-asthmatics and n=6 for the asthmatics).

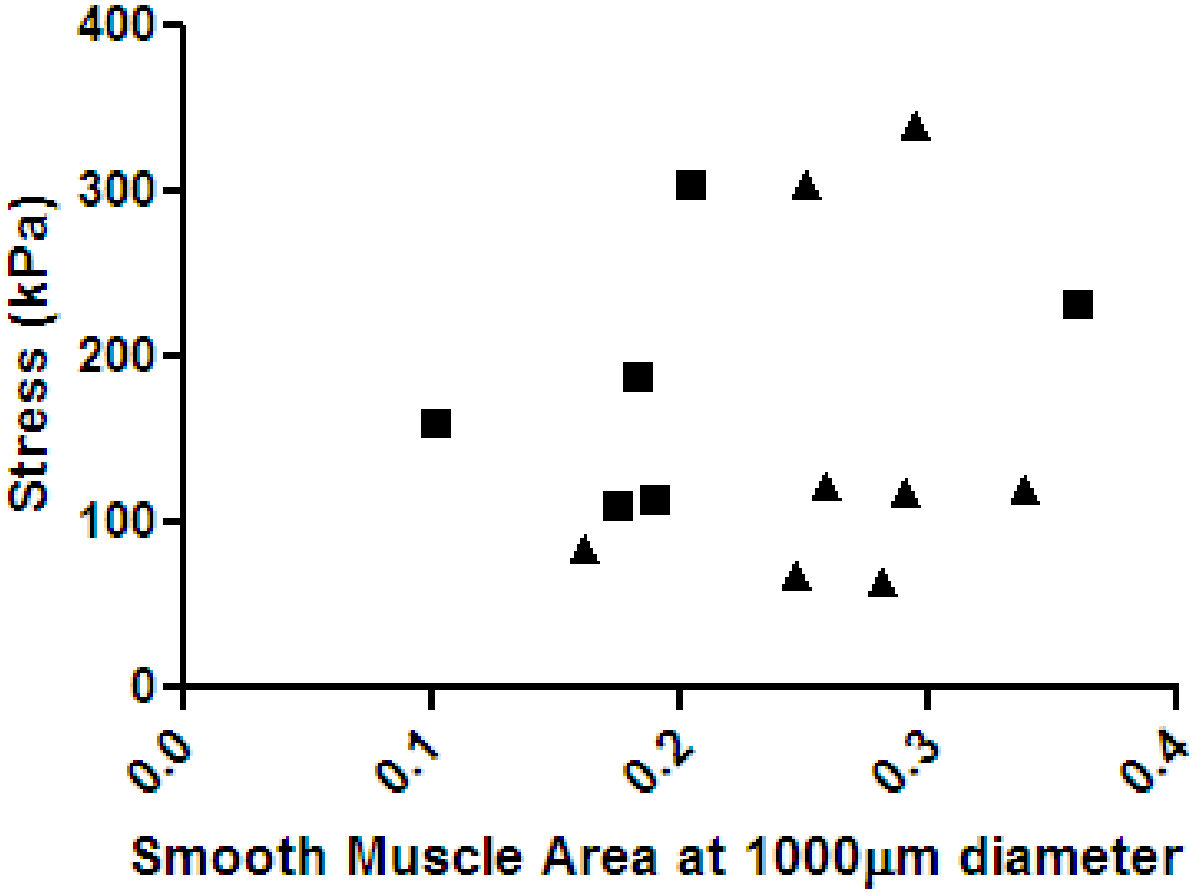

Figure E4. Area of smooth muscle in airways with an internal diameter of 1000 µm plotted against the stress produced by the trachealis muscle in the same patients.