

Ultrastructure of Human Tracheal Smooth Muscle from Subjects with Asthma and Nonasthmatic Subjects

Standardized Methods for Comparison

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Abstract

A characteristic feature of asthma is exaggerated airway narrowing, termed airway hyper-responsiveness (AHR) due to contraction of airway smooth muscle (ASM). Although smooth muscle (SM)-specific asthma susceptibility genes have been identified, it is not known whether asthmatic ASM is phenotypically different from nonasthmatic ASM in terms of subcellular structure or mechanical function. The present study is the first to systematically quantify, using electron microscopy, the ultrastructure of tracheal SM from subjects with asthma and nonasthmatic subjects. Methodological details concerning tissue sample preparation, ultrastructural quantification, and normalization of isometric force by appropriate morphometric parameters are described. We reasoned that genetic and/or acquired differences in the ultrastructure of asthmatic ASM could be associated with functional changes. We recently reported that asthmatic ASM is better able to maintain and recover active force generation after length oscillations simulating deep inspirations. The present study was designed to seek structural evidence to account for this observation. Contrary to our hypotheses, no significant qualitative or quantitative differences were found in the subcellular

structure of asthmatic versus nonasthmatic tracheal SM. Specifically, there were no differences in average SM cell cross-sectional area; fraction of the cell area occupied by nonfilamentous area; amounts of mitochondria, dense bodies, and dense plaques; myosin and actin filament densities; basal lamina thickness; and the number of microtubules. These results indicate that functional differences in ASM do not necessarily translate into observable structural changes.

Keywords: electron microscopy; quantitative morphometry; force normalization

Clinical Relevance

The present study is the first to systematically quantify, using electron microscopy, the ultrastructure of tracheal smooth muscle from subjects with asthma and nonasthmatic subjects. The standard protocol described in this article may be used as a reference for future studies making similar measurements so that the results can be compared.

Excessive narrowing of airways due to shortening of airway smooth muscle (ASM) is a major contributor to exacerbations of

asthma (1) and is the characteristic feature of airway hyper-responsiveness (AHR). However, questions regarding the role of

ASM in asthma and AHR with respect to whether ASM from subjects with asthma is structurally and functionally different from

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healthy control subjects are still under debate. Part of the controversy stems from the lack of a standardized methodology for quantifying extracellular and intracellular structures and for analysis of functional parameters such as normalization of muscle force. The present study is the first to systematically quantify the ultrastructure of ASM cells from subjects with asthma and nonasthmatic subjects at nanometer resolution. The experiments were designed to detect minute changes in structure that might have functional implications. Although asthma susceptibility genes, including smooth muscle (SM)-specific ADAM33, have been identified (2–6), it is not known if polymorphisms in these genes affect SM cell structure and function. It is well documented that thickening of the airway wall (which is a hallmark of asthma) includes increased ASM mass (7–13), which is also correlated with asthma severity (7, 10, 12), although not necessarily with airway dysfunction (14–17). The increase in ASM mass could, at least in theory, lead to excessive narrowing of the airway (18) if the contractile phenotype of the ASM was maintained during cellular hyperplasia and hypertrophy. However, there is evidence suggesting that the ASM phenotype may also be altered in asthma, taking on a more synthetic and less contractile phenotype (19). The relationships between genetic alterations, airway remodeling, and airway dysfunction in asthma are not entirely clear, and further investigation in this area is needed. It is important that future structural studies be conducted in a methodologically rigorous and uniform manner so that the results can be compared.

A recent study of the same tracheal SM used in this report identified an interesting functional difference between asthmatic and nonasthmatic SM: the former possesses a greater ability to maintain and recover force after length oscillations simulating deep inspiration (20). This suggests that genetic and/or acquired factors may alter tracheal SM to allow it to be more resistant to mechanical perturbation. This functional difference could have important implications for the deficient deep inspiration-induced bronchodilatory and bronchoprotective effects observed in patients with asthma (21–26). The increased resistance to strain observed in the asthmatic ASM may be due to a reinforced cytoskeleton, which could be manifest in ultrastructural changes.

Besides establishing a standard method for quantifying airway tissue structure from electron micrographs, another goal of the present study was to test the hypothesis that genetic and/or acquired alterations could change ASM structure and provide an explanation for the observed functional difference in force maintenance and recovery after length oscillation in asthmatic and nonasthmatic tracheal SM (20). Specifically, we quantified and compared ultrastructural features related to the contractile apparatus and the cytoskeleton (i.e., the abundance of contractile filaments, dense bodies, dense plaques, and microtubules). We also looked for structural evidence for a change between contractile and synthetic phenotypes (i.e., abundance of mitochondria and cell area occupied by synthetic vesicles) and for evidence of infiltration by fibroblasts and inflammatory cells in the tracheal SM bundle.

Materials and Methods

Tissue Preparation and Image Quantification

The study was approved by the University of British Columbia – Providence Health Care Ethics Committee (Certificate #H06–03267). The human lungs were donated for research through the International Institute for the Advancement of Medicine (Edison, NJ). Tracheal and intraparenchymal ASM tissues from the same donors were used in our previous studies to determine mechanical function and morphometric analysis of the muscle (20, 27). Information on subject demographics, clinical details, and cause of death has been provided by Chin and colleagues (20), and the same information is provided here in Table 1 with notes on specific measurements made on the tracheal and intraparenchymal SM in this study. Although all subjects are included, not all subjects were used for all measurements because not all donor lungs were prepared for examination by transmission electron microscopy (TEM). The measurements performed included (1) ultrastructural quantification of the cross-sectional area of tracheal SM and of intracellular organelles and structures using TEM, (2) TEM quantification of nonmuscle cells within or surrounding tracheal SM cell bundles, (3) TEM quantification of

mitochondrial content in intraparenchymal ASM, (4) Hansel's staining for eosinophils in intraparenchymal airways, (5) immunohistological staining for mast cells in intraparenchymal airways, and (6) histological staining for intraparenchymal airway structures. The specific measurements made on tissue samples from each subject are shown in the first column of Table 1.

Tracheal and intraparenchymal SM preparations were fixed for TEM and immunohistology. Multiple random images were taken for each tissue sample, and the image files were coded so the experimenters were “blind” with respect to the tissue origin during image quantification. All tissues were fixed in the relaxed state. Morphometric analysis was performed manually with the help of the software Image Pro Plus. Identification of intra- and extracellular structural elements, such as mitochondria, dense bodies, basal lamina, and myosin filaments, was done manually.

Details of tissue preparation, electron microscopy (EM), immunohistochemistry, and morphometric analysis are presented in the online supplement.

Statistical Analysis

The codes indicating subjects with asthma and nonasthmatic subjects were revealed only after the analysis of each group was finished. For morphometric quantification, a number of images per subject were analyzed (the specific numbers are presented in the RESULTS section). Morphometric measurements on samples from each subject were averaged before the means were averaged for group comparison. For parameters sensitive to airway size, the measurements were normalized to the length of basement membrane perimeter length. Results were expressed in means $\pm$ SD unless specified; n refers to the number of subjects. One-way ANOVA was used to compare the means between groups. The Mann-Whitney test was used for nonparametric comparison, and $P < 0.05$ was considered sufficient to reject the null hypothesis. We also performed a *post hoc* power analysis to determine what percentage difference in individual structural features we would have been able to detect with a power of 0.8, an α of 0.05, the number of samples studied, and the standard deviation of the individual measurements in our subjects.

Table 1. Subject Demographics and Clinical Data

Measurements*	Sex	Age (yr)	Weight (kg)	Height (cm)	Ethnicity	Cause of Death	Patient Medical History	Known Medication	Terminal Medication
Nonasthmatic subjects									
D, E	M	20	86	185	Caucasian	Head trauma	Cigarettes (2/wk); vodka half bottle per week; marijuana weekly	None	Vasopressors
A, B, D, E	F	4	17.5	104	Hispanic	Head trauma	None	None	Dopamine
A, B, D, E	M	22	112	193	Caucasian	Head trauma	Occasional marijuana (once a year)	None	Vasopressors
D, E	F	63	77	159	Caucasian	Gastrointestinal bleed	Hypertension for 2 years	Fentanyl, Vicodin, Ambien, Valium, Lexapro, unknown antihypertensive	Dopamine, insulin
D, E	M	14	50	165	Caucasian	Head trauma	None	None	Nitroprusside
A, B, C, D, E	F	19	87	165	Caucasian	Gastrointestinal bleed	Hypertension (2 yr)	Unknown antihypertensive	Dopamine
A, B, D, E	F	20	72.2	165	Caucasian	Head trauma	Smoker (<1 PPD for 2 yr); marijuana smoked (unknown frequency)	Pain medications and inhalants	Vasopressors
C, D, E	M	24	81.8	175	Hispanic	Head trauma	Alcohol on weekends since age 19	None	Dopamine, vasopressin, heparin
D, E	M	12	48.1	160	Caucasian	Head trauma	None	Multivitamins with iron	Vasopressin, heparin
A, B, C, D, E	F	42	90	178	Caucasian	Head trauma	Hypertension	Claritin	Vasopressin, heparin, crystalloids, mannitol
A, B, D	M	5	N/A	N/A	Caucasian	Unknown	N/A	None	None
D, E	F	47	82	167.5	N/A	Cardiovascular accident	N/A	N/A	N/A
D, E	M	47	105	167.5	N/A	CNS tumor	N/A	N/A	N/A
Subjects with asthma									
D, E	F	8	30.5	117	Hispanic	Asthma attack	Asthmatic, diagnosed at age 3	Albuterol, Singulair, ranitidine	Methylprednisone, azithromycin, ceftriaxone, vasopressin, dobutamine, levothyroxine, norepinephrine, mannitol
A, B, D, E	M	11	65.3	152.5	Caucasian	Head trauma	Asthmatic, diagnosed at age 6	None	N/A
D, E	F	21	55	170	Caucasian	Drug overdose (acetaminophen)	Asthma; cervical cancer free for 1 yr; smoker (2 PPD for 6 yr)	Sulfa, aspirin, albuterol, Advair, and nickle	N/A

(Continued)

Table 1. (Continued)

Measurements*	Sex	Age (yr)	Weight (kg)	Height (cm)	Ethnicity	Cause of Death	Patient Medical History	Known Medication	Terminal Medication
D, E	F	15	56	162.5	Caucasian	Anoxia, probable asthma attack	Environmental allergies and asthma; smoked cigarettes <1 PPD for 1 year; marijuana 2 times a week	Singulair, Advair, Claritin, Flovent, albuterol, Ritalin, Fluoxetine, sodium, Cetrizine, methylphenidate, Solumedrol albuterol, prednisone	N/A
A, B, C, D	M	11	69	170	Caucasian	Anoxia, probable asthma attack	Asthmatic, diagnosed at age 2	Albuterol	Dopamine and vasopressors
D, E	F	26	91.6	168	Caucasian	Anoxia, probable asthma attack	Asthmatic, diagnosed in childhood; seizure in 5th grade; frequent urinary tract infection; cigarettes 1 pack per week since age 16; hard liquor, heavy drinker	Albuterol, Advair, lorazepam, Xanax	Dopamine and vasopressors
C, D, E	M	10	43	157.5	Caucasian	Asthma attack	Asthmatic, diagnosed at age 4	Advair, Allegra, albuterol	Dopamine, heparin, insulin, fentanyl
A, B, D, E	M	25	103	185	Hispanic	Suicide	Asthmatic, diagnosed at age 11, cigarettes 2 PPD for 12 years; marijuana 6 times per day for 12 years	Albuterol, Advair, naproxen, tracdodone	Dopamine
A, B, D, E	F	15	67	177.8	Caucasian	Asthma attack	Asthma, diagnosed at birth	Prednisone, albuterol	Dopamine, dextrose
A, B, C, D, E	M	23	52.6	152.4	Hispanic	Asthma attack	Seizures related to asthma	N/A	N/A
A, B, D, E	M	10	15	149	African American	Asthma attack	Asthmatic, diagnosed at age 1	Concerta, Lexapro	Vasopressin
C, D		19	70.5	175	Caucasian	Asthma attack	Asthmatic, diagnosed at age 13	β -agonist	N/A

Definition of abbreviations: CNS, central nervous system; F, female; M, male; N/A, not applicable; PPD, packs of cigarettes per day.

*Measurement A: Electron microscopy (EM) quantification of intracellular structure in tracheal smooth muscle (SM); samples from six subjects with asthma and six nonasthmatic subjects were used. Measurement B: EM quantification of nonmuscle cells within or surrounding tracheal SM cell bundles; samples from six subjects with asthma and six nonasthmatic subjects were used. Measurement C: EM quantification of cellular content of mitochondria in intraparenchymal airway SM; samples from four patients with asthma and three nonasthmatic subjects were used. Measurement D: Hansel's staining for eosinophils in intraparenchymal airways; all samples from subjects with asthma and nonasthmatic subjects were used. Measurement E: Immunostaining for mast cells in intraparenchymal airways; samples from 10 subjects with asthma and 12 nonasthmatic subjects were used.

Results

Evidence for Airway Inflammation and Remodeling in Asthmatic Lungs

Before examining the ultrastructural details of tracheal SM from asthmatic and nonasthmatic donor lungs, we tested whether the typical morphological features of airway inflammation and remodeling were present in the lungs of donors with asthma. In our previous study (20), the areas of ASM, lamina propria, and adventitia were significantly larger in the intraparenchymal airways of the subjects with asthma compared with nonasthmatic subjects. Because only a subset of donor tissues from the previous study had tissue fixed for TEM, we re-examined the evidence for airway remodeling in this subset of donor tissues. Tissue samples from six asthmatic donors (out of the 12 used in our previous studies) and six from nonasthmatic donors (out of the nine used in our previous studies) were used in the present study for ultrastructural quantification. We used intraparenchymal tissue samples from the same five (out of six; one sample was omitted due to poor tissue fixation) donors with asthma and six (out of six) nonasthmatic donors for re-examination of airway structural remodeling. The results show that the intraparenchymal airway wall thickness (normalized to airway perimeter length) was significantly greater in the asthmatic group ($P < 0.05$) (Table 2). SM area, collagen area, and basement membrane thickness were also greater but did not reach statistical significance at the 0.05 level, likely due to the small sample size (Table 2).

In the present study, we also quantified the abundance of mast cells and eosinophils in intraparenchymal airways surrounding the SM cell bundles. The number of mast cells

surrounding asthmatic airways was significantly higher than that in nonasthmatic airways (Figure 1A). Figures 1B and 1C show cell counts for eosinophils surrounding intraparenchymal airways. Although no significant difference was found for asthmatic and nonasthmatic groups (Figure 1B), when the subgroup of patients with fatal asthma ($n = 4$) was compared with the nonasthmatic group ($n = 5$), a significant difference was found (Figure 1C). There was no noticeable difference in the pattern of association of mast cells and eosinophils with epithelial cells, SM cells, fibroblasts, or other components of the airway wall in the three groups (nonasthmatic, all asthmatic, and fatal asthmatic). The present results confirm and extend our previous finding (20) showing airway remodeling and inflammatory cell infiltration in the intraparenchymal airways of the same asthmatic lungs from which tracheal SM were used for ultrastructural analysis in the present study.

Ultrastructure of Tracheal SM

Figure 2 shows an example of a tracheal SM cell cross-section containing dense plaques, dense bodies, microtubules, mitochondria, sarcoplasmic reticulum, myosin (thick) filaments, and actin (thin) filaments. Because there is a large amount of actin filaments present in a cell, only selected areas (Figure 2A, *yellow circles*) that satisfied a set of criteria (described briefly in the figure legend and in more detail in the online supplement) were used in quantifying the actin filament density. Other cell structural elements were quantified per cell cross-section or cell perimeter. These ultrastructural quantifications would allow us to detect cell phenotype changes, such as an increase in synthetic activity reflected in the increase of cell fraction of sarcoplasmic reticulum, Golgi apparatus, and synthetic vesicles or an increase in cellular metabolism reflected in

the increase in cell fraction of mitochondria. The quantification would also allow us to determine if any changes in the structure of the contractile apparatus (abundance of contractile filaments, dense bodies, and dense plaques) could explain changes (or the lack thereof) in contractility.

Hypertrophy of SM cells sometimes is accompanied by an increase in the cell cross-sectional area. The average cross-sectional area of individual tracheal SM cells was not significantly different between nonasthmatic subjects (6.95 ± 1.92 SD μm^2) and subjects with asthma (7.19 ± 1.67 SD μm^2 ; $P > 0.05$) (Figure 3A). The percent area of cell containing mitochondria in nonasthmatic tracheal SM ($2.42 \pm 0.54\%$) was also not significantly different from that in asthmatic tracheal SM ($2.36 \pm 0.37\%$; $P > 0.05$) (Figure 3B). Furthermore, the number of mitochondria per cell cross-section (3.5 ± 1.5) and the area of mitochondria in cell cross-sectional area (0.044 ± 0.011 μm^2) in nonasthmatic tracheal SM was not significantly different compared with asthmatic tracheal SM (3.75 ± 1.17 and 0.046 ± 0.011 μm^2 , respectively; $P > 0.05$) (Figures 3C and 3D). Comparison of the numbers of organelles in two-dimensional cell sections could be affected by the size of the organelles; the more meaningful comparison is made between the areas occupied by the organelles in cell sections from asthmatic and nonasthmatic tissue samples. The thickness of the basal lamina surrounding nonasthmatic tracheal SM was on average 0.090 ± 0.026 μm , whereas it was 0.097 ± 0.034 μm ($P > 0.05$) (Figure 3E) for asthmatic tracheal SM. The number of microtubules per cell cross-section in nonasthmatic tracheal SM (5.96 ± 4.18) was not significantly different from that in asthmatic tracheal SM (4.74 ± 1.62) (Figure 3F). The percent area of

Table 2. Measurements of Airway Remodeling in Asthmatic Intraparenchymal Airways Compared with Nonasthmatic Intraparenchymal Airways

Measurements	Nonasthmatic ($n = 6$)	Asthmatic ($n = 5$)	P Value [†]
Smooth muscle area/ P_i , mm	$0.0102 \pm 0.0039^*$	0.0184 ± 0.0083	0.06
Epithelium area/ P_i , mm	0.0213 ± 0.0060	0.0313 ± 0.0147	0.16
Wall area/ P_i , mm	0.122 ± 0.065	0.249 ± 0.042	0.005
Collagen area/ P_i , mm	0.0495 ± 0.0433	0.101 ± 0.033	0.06
Basement membrane thickness, mm	3.87 ± 3.15	7.07 ± 2.09	0.09

Definition of abbreviation: P_i , internal airway perimeter.

*Values are mean $\pm$ SD.

[†] $P < 0.05$ denotes significance by Student's t test.

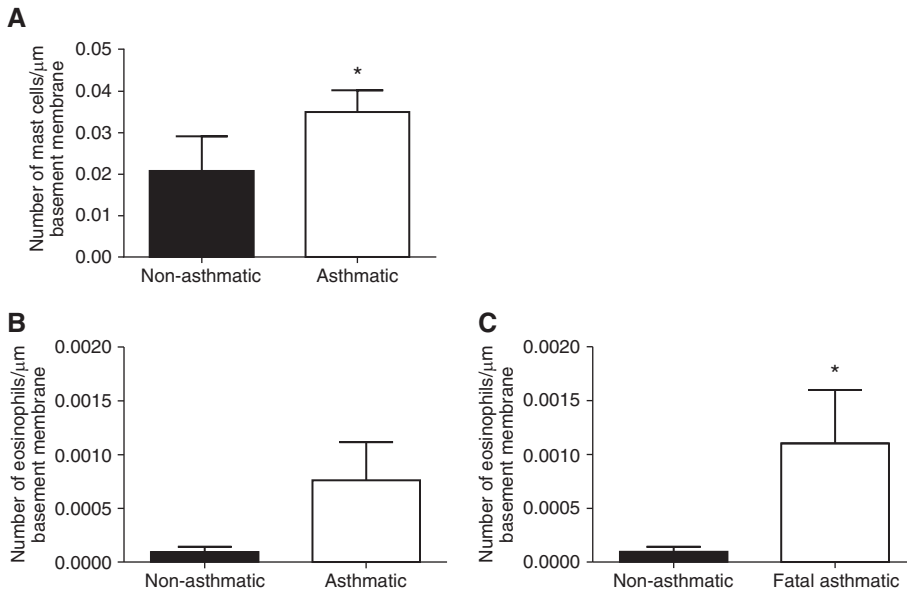


Figure 1. (A) The number of mast cells per micrometer of basement membrane length was significantly more in asthmatic than in nonasthmatic airways ($P < 0.05$; t test). The data were obtained from 10 nonasthmatic subjects and 12 subjects with asthma. Five images per subject were included in the analysis. (B) Number of eosinophils per micrometer of basement membrane length in nonasthmatic subjects and all subjects with asthma. (C) Number of eosinophils per micrometer of basement membrane length in nonasthmatic subjects and the eight subjects with fatal asthma. * $P < 0.05$ by Mann-Whitney test. Data represent the total number of eosinophils per image divided by the total basement membrane length of airways analyzed. Data were obtained from 12 nonasthmatic subjects and 12 subjects with asthma; within the asthmatic subjects, eight were fatal (death due to asthma) and four were nonfatal.

dense bodies in nonasthmatic tracheal SM was $3.0 \pm 0.82\%$, whereas the area of dense bodies in asthmatic tracheal SM was $3.0 \pm 1.1\%$ ($P > 0.05$) (Figure 3G). The percent of the cell membrane length containing dense plaques in nonasthmatic tracheal SM was $38.4 \pm 2.6\%$ and was not significantly different from asthmatic tracheal SM, in which $39.9 \pm 3.4\%$ of the cell membrane length was occupied by dense plaques ($P > 0.05$) (Figure 3H).

Thick (myosin) filament density in nonasthmatic tracheal SM was 37.8 ± 4.8 filaments/ μm^2 , and in asthmatic tracheal SM it was 34.3 ± 9.6 filaments/ μm^2 (Figure 3I). Thin (actin) filament density in nonasthmatic tracheal SM was $1,600 \pm 98$ filaments/ μm^2 , and in asthmatic tracheal SM it was $1,492 \pm 129$ filaments/ μm^2 (Figure 3J). In both cases, the thick and thin filament densities were not significantly different ($P > 0.05$). The filament density measured from randomly selected muscle cell transverse sections has been shown to be an index of filament content in the cell (28). The nonfilamentous area (i.e., the area occupied by sarcoplasmic reticulum, Golgi apparatus, and other synthetic vesicles) as a percentage of the whole cell area in

nonasthmatic tracheal SM was 13.7 ± 3.4 , which was not significantly different ($P > 0.05$) from the area measured in asthmatic tracheal SM (17.9 ± 8.5) (Figure 3K).

Nonmuscle Cells and Infiltration of Inflammatory Cells in the Tracheal SM Cell Bundle

Inflammatory cell infiltration in the tracheal SM cell bundles appeared to be limited to mast cells and macrophages. No eosinophils or dendritic cells were found in the tracheal SM cell bundles in the nonasthmatic and asthmatic groups.

Fibroblasts were commonly observed within or near the tracheal SM cell bundles. They were always surrounded by collagen and elastin fibers. In nonasthmatic tracheal SM bundles, the ratio of fibroblasts to tracheal SM cells was 0.093 ± 0.059 , whereas in asthmatic tracheal SM bundles, the ratio was 0.058 ± 0.024 ($P > 0.05$) (Figure 4A). Mast cells were observed in tracheal SM cell bundles in three of six nonasthmatic subjects and in five of six subjects with asthma. However, the ratio of mast cells to SM cells was not significantly different ($P > 0.05$) between nonasthmatic subjects (0.0035 ± 0.0061) and subjects

with asthma (0.0033 ± 0.0029) (Figure 4B). Macrophages were observed in three of six nonasthmatic subjects and in one of six subjects with asthma; however, the ratio of macrophages to tracheal SM cells was not significantly different ($P > 0.05$) between the nonasthmatic (0.0037 ± 0.0047) and asthmatic groups (0.0031 ± 0.0073) (Figure 4C). Finally, the ratio of nerve bundles to tracheal SM cells from the nonasthmatic group (0.032 ± 0.017) was not different from the asthmatic group (0.027 ± 0.024 ; $P > 0.05$) (Figure 4D).

Quantification of Cell Area and Mitochondrial Content in Intraparenchymal ASM

The intraparenchymal airways were the sites where we found evidence for inflammation and airway remodeling (Figure 1). Here we examined the SM cellular content of mitochondria to determine if the inflammatory airway environment was associated with an increase in mitochondrial content.

In the intraparenchymal airway, the SM cell cross-sectional area was not significantly different between nonasthmatic subjects ($16.1 \pm 4.3 \mu\text{m}^2$) and subjects with asthma ($15.2 \pm 9.6 \mu\text{m}^2$) ($P > 0.05$) (Figure 5A). In addition, the mitochondrial area as a percentage of the total cell area in nonasthmatic intraparenchymal SM ($4.8 \pm 1.9\%$) was not significantly different compared with that in the asthmatic counterpart ($3.6 \pm 0.9\%$; $P > 0.05$) (Figure 5B). The number of mitochondria in nonasthmatic intraparenchymal SM (7.7 ± 1.5) was not significantly different compared with that in asthmatic SM (7.3 ± 3.3 ; $P > 0.05$) (Figure 5C). Finally, the area of the mitochondria in nonasthmatic intraparenchymal SM ($0.096 \pm 0.029 \mu\text{m}^2$) was not significantly different compared with that in asthmatic SM ($0.067 \pm 0.025 \mu\text{m}^2$; $P > 0.05$) (Figure 5D). The mitochondrial area as a percentage of total cell cross-sectional area is a better reflection of the mitochondrial content in the cell because it is independent of the size and number of the mitochondria. The intraparenchymal SM tissue was fixed while it was embedded in lung parenchyma; the cross-sections of the muscle cells were cut at random angles. As a result, the average cross-sectional area of the intraparenchymal SM appears to be larger than that of the tracheal SM where the

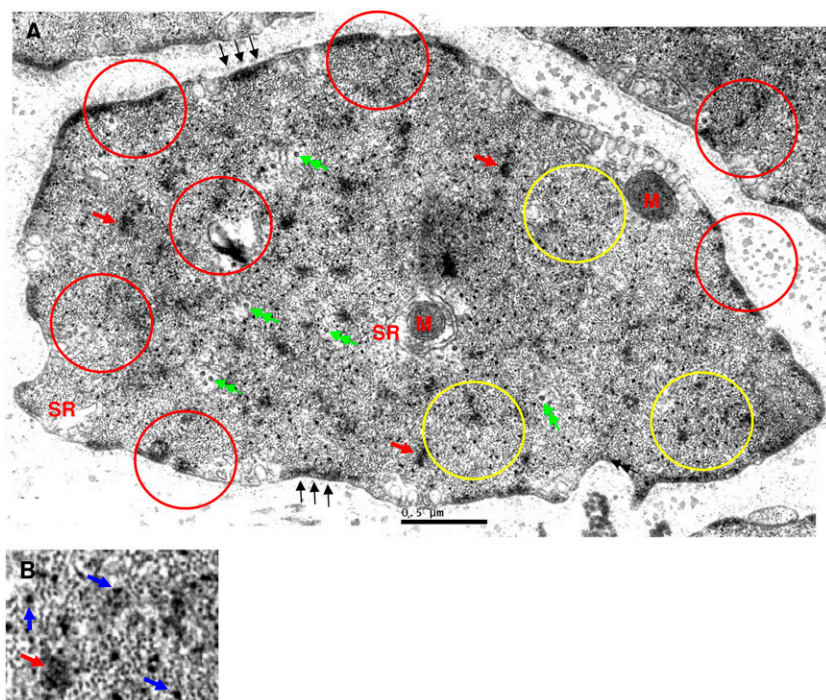


Figure 2. Electron micrograph of a transverse section of tracheal smooth muscle from a nonasthmatic subject. (A) A complete cell cross-section from two merged micrographs. Parallel black arrows point to dense plaques, red arrows indicate dense bodies, and double arrows point to examples of microtubules. M, mitochondria; SR, sarcoplasmic reticulum. Ten circles of $0.1832 \mu\text{m}^2$ were randomly placed by computer on each micrograph, and only circles (yellow) that fell on cytoplasmic areas predominantly containing thick and thin filaments (and occasionally dense bodies) were used for the counting of actin thin filaments. Scale bar represents $0.5 \mu\text{m}$. (B) An enlarged area ($3\times$ higher magnification than the image in A) from within the lower-right yellow circle. Red arrow points to a dense body; blue arrows point to myosin thick filaments. Actin filaments can be seen as small round dots surrounding myosin filaments.

strips were fixed in a known orientation and the cross sections were cut perpendicular to the long axes of the cells.

The lack of significant differences in structural features in the comparison of asthmatic and nonasthmatic tissue could be due to an insufficient number of samples. To determine the percentage change in each measured parameter we would have been able to detect given our sample size and the variance between subjects (standard deviation of all subjects), we performed a *post hoc* analysis for the comparisons made in Figures 3–5. Table E20 in the online supplement lists, for each measured parameter, the lower limit of the percentage difference that the study is powered to detect. Power analyses for some specific comparisons relevant to our hypotheses will be discussed.

Discussion

In this study, quantitative comparisons were made of the extracellular and subcellular

structures of tracheal SM between subjects with asthma and nonasthmatic subjects using EM. Due to the lack of a standard approach in characterizing ASM functional and structural properties, comparison of results from different studies from different laboratories is often difficult and is sometimes impossible. The protocol established in the present study may be used as a reference for future studies. Some details of quantitative EM morphometry are described in the main text of the article, but the bulk of the description is presented in the online supplement.

The most significant findings of this study are that functional differences between asthmatic and nonasthmatic ASM may not have a structural correlate and that intrinsic genetic alterations associated with asthma do not manifest as structural changes in tracheal SM cells. No structural basis for the resistance to force decline and enhanced force recovery after length oscillation in asthmatic trachealis (20) could be found. This suggests that whatever

changes have occurred in the properties of asthmatic SM, mechanical or otherwise, do not manifest as structural alterations that can be measured with quantitative EM. This does not mean that there are no structural changes. Subtle changes, such as attachment of actin filaments to dense bodies, cannot be detected with our current methodology. Other nonstructural changes, such as up- or down-regulation and over- or underexpression of certain enzymes and the presence or absence of inflammatory mediators, will also be “invisible” in this study.

The difference in force decline after length oscillation seen between asthmatic and nonasthmatic tracheal SM by Chin and colleagues (20) was 15 to 20%. If a structurally reinforced cytoskeleton in asthmatic SM is responsible for the reduced force decline, we might expect to see comparable changes in the abundance of cytoskeletal structures such as actin filaments, dense bodies, and dense plaques. The power analysis (Table E20) indicates that for abundance of actin filaments and dense plaques where we could detect a difference of 10% or greater (power = 0.8; $\alpha = 0.05$), we only saw 3 and 4% differences in our measurements (Figures 3H and 3J).

We previously reported (20) that there was no difference in the isometric stress generated by asthmatic and nonasthmatic tracheal SM preparations (the same preparations used in the present study). Values for stress are obtained by normalizing isometric force by the cross-sectional area of the tissue occupied by muscle cells, excluding the extracellular matrix (20). Because isometric stress associated with active contraction in a muscle cell is regarded as a reflection of the efficacy of myosin interaction with actin filaments, the absence of a difference in isometric stress was interpreted (20) as no difference in the properties of the contractile proteins in asthma. There are three pieces of information missing in the previous study that could potentially change that conclusion: (1) the percentage of nonmuscle cells within the tracheal SM preparations was not known, (2) the percent area within the muscle cell cross-sections occupied by contractile filaments (i.e., total muscle cell area minus the areas occupied by organelles like nuclei, mitochondria, sarcoplasmic reticulum, and Golgi apparatus) was not determined, and (3) the number density of the myosin and actin filaments in the areas containing contractile filaments was not determined. The present

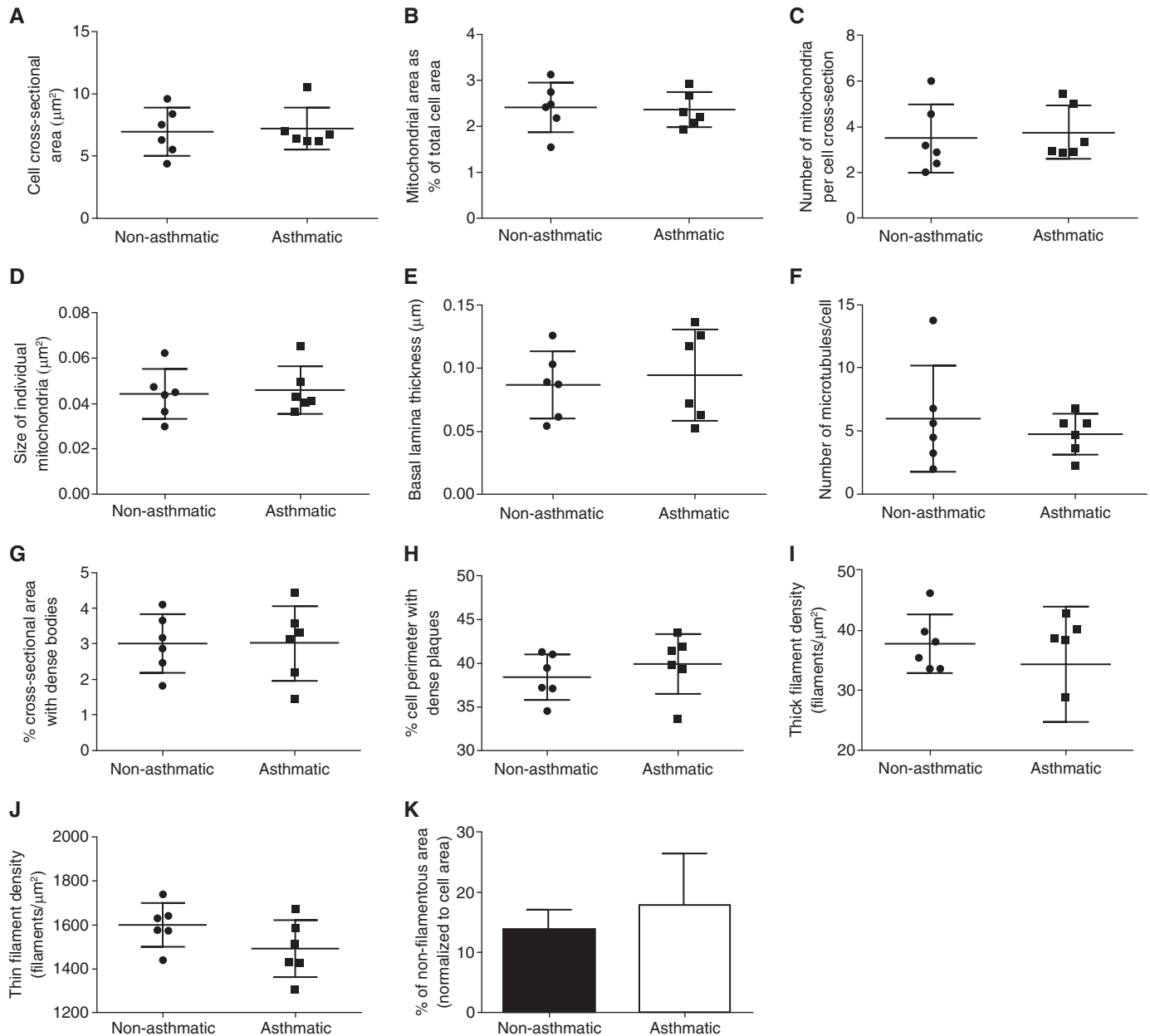


Figure 3. Quantification and comparison of cell cross-sectional areas, mitochondrial contents, basal lamina thickness, and microtubule counts in nonasthmatic and asthmatic tracheal smooth muscle (SM) cells. (A) Tracheal SM cell cross-sectional area. (B) Mitochondrial cross-sectional area as a percentage of total cell cross-sectional area. (C) Number of mitochondria per cell cross-sectional area. (D) Area of individual mitochondrial cross-sections. (E) Thickness of basal laminae. (F) Number of microtubules per cell cross-section. (G) Percentage of cell cross-sectional area occupied by dense bodies. (H) Percentage of cell perimeter occupied by dense plaques. (I) Myosin (thick) filament density. (J) Actin (thin) filament density. (K) Percentage of cell cross-sectional area occupied by organelles such as nuclei, mitochondria, sarcoplasmic reticulum, and Golgi apparatus. No statistically significant differences were found for comparisons within each panel. Each data point represents one subject with average measurements from 10 to 15 images. The within-subject coefficient of variation is presented in the online supplement.

study provides the missing information as described below. The number of nonmuscle cells within the tracheal SM bundles was very small and was not different in asthmatic compared with nonasthmatic tracheal SM preparations (Figure 4). The filamentous area (i.e., total cell area [Figure 3A] minus the

nonfilamentous area [Figure 3K]) was not different between the two groups. Finally, the thick and thin filament densities (Figures 3I and 3J) were not different between the two groups. This means that if the comparison of isometric stress was based on normalization by filamentous area and

contractile filament densities rather than total cell area (as Chin and colleagues [20] did), we would have arrived at the same conclusion that the ability for actomyosin interaction to generate force is not altered in asthma. It is therefore highly unlikely that there is a genetic modification of the

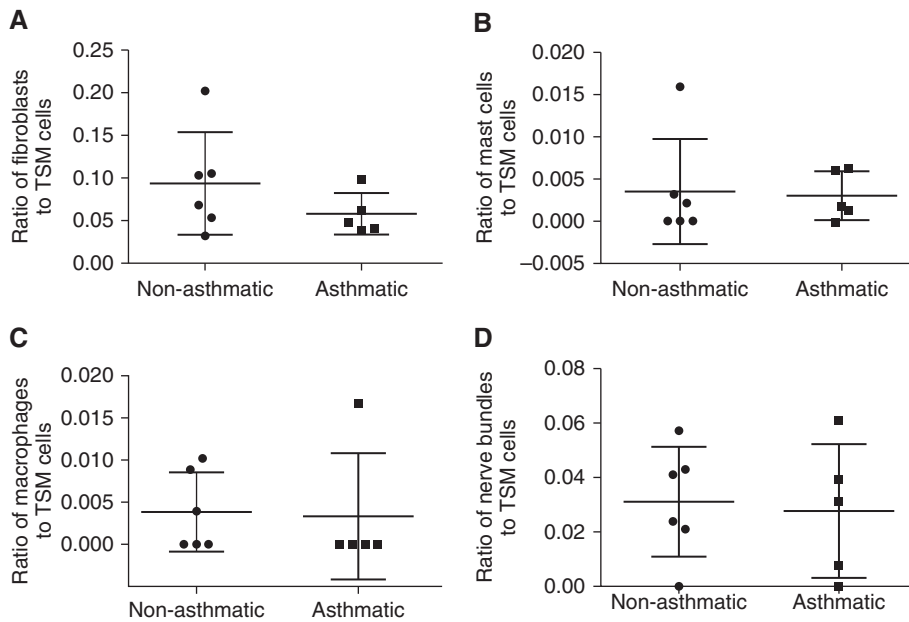


Figure 4. Quantification and comparison of the amount of nonmuscle cells within bundles of asthmatic and nonasthmatic tracheal SM. No significant difference was found for any comparison. Each data point represents one subject with an average of measurements from five images per subject. The within-subject coefficient of variation is presented in the online supplement. TSM, tracheal smooth muscle.

contractile proteins in the trachealis by the disease.

The force-producing elements in SM are the contractile filaments. The true stress,

reflecting the ability of myosin cross-bridges to interact with actin filaments to produce force, should therefore be based on the cell cross-sectional area occupied by the contractile

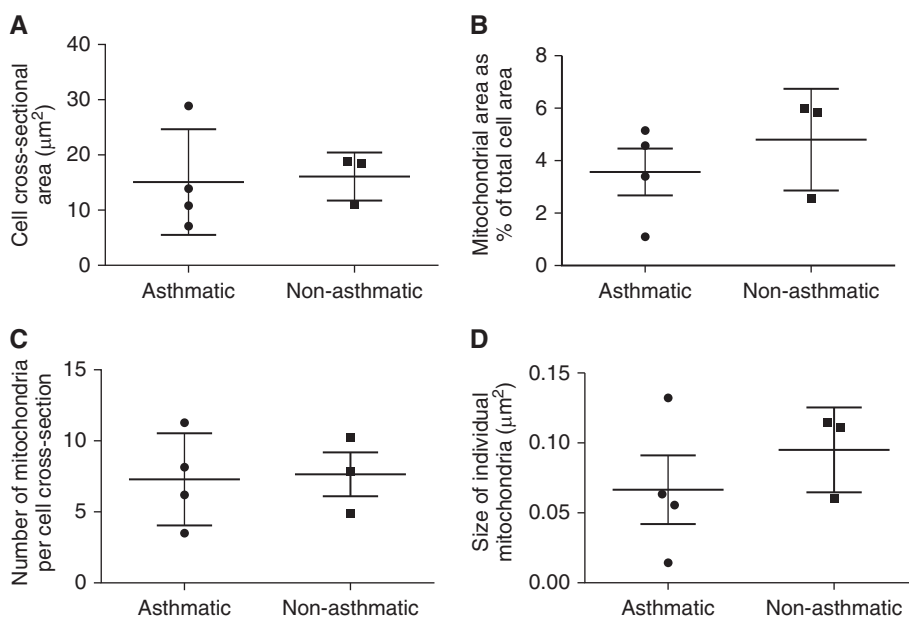


Figure 5. Quantification and comparison of mitochondrial contents in intraparenchymal SM cells from subjects with asthma and nonasthmatic subjects. The cell sections were cut at random angles. (A) Intraparenchymal SM cell area in random cross-sections. (B) Mitochondrial area as a percentage of the total cell area. (C) Number of mitochondrial cross-sections per cell area. (D) Area of individual mitochondrial cross-sections. No statistically significant difference was found for any comparison. Each data point represents one subject with an average of measurements from 10 to 15 images per subject. The within-subject coefficient of variation is presented in the online supplement.

filaments with known densities of the thick and thin filaments. The present results therefore confirm our previous conclusion that the efficacy of actomyosin interaction is not altered in asthmatic tracheal SM in terms of force generation. If force normalization is based on the total cell cross-sectional area, then a shift from a contractile phenotype to a synthetic phenotype would result in a decrease in stress because of an increase in the cell area occupied by non-force-producing synthetic vesicles. The present study did not find such a shift.

How active force is normalized by cross-sectional area can be influenced by what compartments are included in the area. If the cross-sectional area contains both the muscle area and the area of extracellular matrix, an increase in the normalized force (i.e., stress) could stem from an increase in force generated by the muscle cells, or it could be due to a larger proportion of the tissue cross-sectional area occupied by muscle cell relative to extracellular matrix. If the extracellular matrix area is subtracted from the total cross-sectional area so that the force is normalized to just the muscle area, an increase in stress could stem from a greater efficacy in actomyosin interaction (e.g., a greater degree of activation), or it could be due to a larger proportion of the muscle cross-sectional area being occupied by contractile filaments relative to that occupied by organelles. Only when the force is normalized by the cross-sectional area occupied by the contractile filaments can one interpret stress in terms of intrinsic muscle contractility. Nonmuscle cells, such as fibroblasts, mast cells, and other immune cells, are often found within a muscle cell bundle. The area occupied by nonmuscle cells is another variable that needs to be considered in force normalization.

Although there is debate about whether the contractile properties of intraparenchymal SM from subjects with asthma differs from nonasthmatic subjects, there is agreement that cultured ASM cells derived from subjects with asthma possess a more proliferative and synthetic phenotype compared with ASM cells derived from nonasthmatic subjects (29–31). This phenotypic change could be due to intrinsic or environmental factors. If tracheal and intraparenchymal SMs share the same intrinsic properties and environment, one would expect to see some sign of increased proliferative and synthetic activities in asthmatic tracheal SM

because the increased SM mass seen in intraparenchymal airways of these same subjects is almost certainly due to cell growth and proliferation with enhanced protein synthesis. The present results (Figures 3B and 3K) showing no evidence of transformation to a synthetic phenotype in asthmatic tracheal SM suggest that the tissue environment within which SM cells reside could be the determining factor leading to phenotypic changes. One limitation of the present study is that we did not assess the overall inflammatory status of the tracheas. We only examined infiltration of immune cells into the trachealis bundle; the presence of inflammatory cells in other tracheal compartments was not assessed in this study. However, one could speculate that the lack of local inflammation in the intratrachealis microenvironment may be enough to protect this tissue from the cytokine and growth factor-induced changes. Another limitation is that our assessment was based on quantifiable structural changes; other changes, such as the presence of inflammatory mediators, would not be detected in our assessment. Other possible explanations for the discrepancy between our observation in asthmatic tracheal SM and that in cultured asthmatic ASM include differences in epigenetic profiles for the cell populations studied or the fact that cultured ASM cells behave differently compared with the same cells *in situ*. This possibility is not farfetched, as is evidenced by the example of the striking phenotypic plasticity that can be observed in cultured ASM after serum starvation (32).

In a previous study mitochondrial mass was found to be increased (by ~2-fold) in bronchial SM cells from subjects with asthma (31), and mitochondrial biogenesis was thought to be a cause for the cell proliferation and airway remodeling. In our tracheal SM samples we found no evidence of enhanced mitochondrial biogenesis (Figure 3). Surprisingly, when we examined the mitochondrial mass in intraparenchymal

SM cells from airways where there was evidence of inflammation (increased mast cells and eosinophils surrounding the airways [Figure 1]) and airway remodeling (Table 2), there was no evidence for enhanced mitochondrial biogenesis (Figure 5B). It is possible that our sample size may not be large enough to show a difference. Power analysis (Table E20) indicates that we could detect a difference of 40%, but we cannot exclude a possibility of a difference of <40%. The reason for the discrepancy with the previous finding (31) of a large change in mitochondrial mass in subjects with asthma is unclear.

Although the thickness of basement membrane has been shown to be greater in central airways from subjects with asthma (33), the thickness of basal lamina (made up of mainly laminin) surrounding individual ASM cells has not been quantified in asthmatic and nonasthmatic airways. Here we report that there is no difference in the thickness of basal lamina surrounding tracheal SM cells in tracheas from subjects with asthma and nonasthmatic subjects (Figure 3E). The results suggest that the synthesis of laminin is not altered in asthmatic tracheal SM.

The number of microtubules is expected to increase in cells with enhanced synthetic activity because microtubules are involved in the intracellular transport of synthetic vesicles and organelles as well as cell division. The present finding of a lack of difference in the number of microtubules in asthmatic and nonasthmatic tracheal SM is consistent with the finding of no change in the mitochondrial mass (Figure 3B) and synthetic vesicles (Figure 3K); that is, they all indicate that there is no transformation in asthmatic tracheal SM from a contractile to a synthetic phenotype. This conclusion is further corroborated by the data shown in Figure 3, which reveal no change in the ultrastructure of the contractile filament domain within the tracheal SM cells, as

reflected in the number and distribution of dense bodies and dense plaques and in the densities of the myosin and actin filaments.

Despite the signs of airway remodeling in the intraparenchymal asthmatic airways, the number of macrophages and mast cells was not increased within the asthmatic tracheal SM cell bundles (Figure 4). Due to the observational nature of the present study, the functional and activation status of the cells could not be assessed. Eosinophils were found in the intraparenchymal airways of subjects with asthma (particularly those with fatal asthma), but no eosinophils were found in the asthmatic or nonasthmatic tracheal SM cell bundles in the present study. This is surprising because accumulation of these inflammatory cells is regarded as a defining feature of allergic asthma in humans (34). However, there is also evidence that eosinophilia is not associated with every asthma case (35).

A more asthma-relevant ASM to study may be the smaller intraparenchymal ASM. Due to resource limitations we were not able to carry out a parallel study of bronchial SM with tracheal SM. It is possible that the local tissue environment, including inflammatory status and oscillatory stresses and strains, could lead to quantifiable changes in ultrastructure in asthmatic bronchial SM.

Conclusions

Genetic and/or acquired alterations associated with asthma do not lead to detectable changes in the ultrastructure of tracheal SM. No structural basis could be found to explain the enhanced ability of asthmatic tracheal SM to resist oscillation-induced force decline and to facilitate force recovery after oscillation. No evidence of a large (>40%) increase in mitochondrial mass or phenotype change was found in asthmatic trachealis. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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Ultrastructure of human tracheal smooth muscle from asthmatic and non-asthmatic subjects: Standardized methods for comparison

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Online Supplemental Materials

Details of tissue preparations

The whole lungs were obtained as previously described (Chin et al, 2010; 2012). Briefly, after surgical removal the lungs were flushed with Custodiol HTK solution (Odyssey Pharmaceuticals, East Hanover, NJ, USA) and transported on ice by airplane. The time between harvesting the organ and arrival at UBC/St Paul's Hospital was 15–20 hours.

The tracheal and bronchial smooth muscle preparations have been previously described (Chin *et al.* 2012). Briefly, the tissues were kept at 4°C in physiological saline solution (PSS or Krebs' solution) before use. The PSS contained 118 mM NaCl, 4 mM KC, 1.2 mM NaH₂PO₄, 22.5 mM NaHCO₃, 2 mM CaCl₂ and 2 g/l dextrose at pH 7.4. For tracheal smooth muscle preparations, the *in situ* length of the muscle was used as a reference length (L_{ref}). Connective tissue and epithelium were carefully removed to isolate a smooth muscle bundle. Muscle strips of ~6 mm long, 1.5-2 mm wide, and 0.3 mm thick were attached on both ends with aluminum foil clips and mounted vertically on a force-length transducer (Model 300B, Dual-Mode Lever Arm Systems, Aurora Scientific Inc.). The muscle strip was immersed in muscle bath containing PSS aerated with a gas mixture of 95% O₂ and 5% CO₂ at 37°C. During the equilibration period, the muscle strip was activated every 5 minutes with 8-second electric field stimulation (EFS). Some strips exhibited spontaneous contractions. Montelukast (10⁻⁶ M) was added to PSS in all experiments which effectively eliminated the spontaneous contraction without affecting active force induced by EFS. Equilibration was considered complete when stimulations produced a stable maximal isometric force (F_{max}) at L_{ref} with low (<3% of F_{max}) resting tension. The same tracheal smooth muscle preparations were used for mechanics studies published recently (Chin et al, 2012). The muscle tissues were fixed for electron microscopy after completion of mechanical measurements. Intra-parenchymal airways were dissected out for tissue fixation but not mechanics studies.

Electron microscopy (EM)

After equilibration, the muscle was fixed in a Karnovsky's fixative for electron microscopy. Primary fixation for 15 min was done while the muscle preparations were still attached to the apparatus that measured muscle force. The fixing solution contained: 1% paraformaldehyde, 2.5% glutaraldehyde, and 2% tannic acid in 0.1 M sodium cacodylate buffer. The fixing solution was pre-warmed to 37°C. After primary fixation, the muscle strip was removed from the apparatus and cut into small blocks ($\sim 2 \times \sim 0.5 \times \sim 0.2$ mm in dimension) in cold fixation buffer and kept in the same fixative for 2 hours in an ice bucket on a shaker. During the secondary fixation the tissue blocks were first transferred to 1% osmium buffer for 1.5 hours at ice temperature on the shaker, followed by three washes with 1.25% NaHCO₃ aqueous solution and then distilled water (10 minutes per wash). The tissue was stained with 1% uranyl acetate, dehydrated with increasing concentrations of ethanol (50%, 70%, 80%, 90%, 95%, 100%) and then propylene oxide. After dehydration, small tissue pieces were embedded in resin (TAAB 812 mix). The resin blocks were sectioned with a diamond knife. Transverse sections were cut at 60 nm thickness. The sections were further stained with 1% uranyl acetate and lead citrate. Images of transverse sections of muscle cells were obtained with an electron microscope (Philips/FEI Tecnai 12 TEM) equipped with a digital camera (Gatan 792) at a magnification of 37,000X. To capture the whole cross-section of a single cell it often required taking multiple images of a single cell and merging them together with the help of Adobe Photoshop.

Immunohistochemistry

Formalin-fixed, paraffin embedded (FFPE) sections 4 μ m thick were deparaffinized in CitriSolv (Fisher Scientific) using graded series of alcohol. Sections were then autoclaved (bench top Tuttnauer 2340M autoclave) on a 22-minute cycle in TRIS-EDTA buffer, pH9, and were then left to cool for 20 minutes. All the IHC staining was carried out on a Dako Autostainer Universal Staining

System and all incubations throughout the staining run were carried out at room temperature. After cooling, the slides were loaded onto the Autostainer and incubated in Background Sniper (BS966: Biocare Medical; Concord, CA, USA) for 15 minutes to help reduce non-specific binding. The sections were incubated with the primary antibodies. For mast cell labeling, CD117/c-Kit (rabbit polyclonal: ThermoFisher Scientific RB-1518-PABX) was used at 200µg/ml for 60 minutes. Detection was performed using the MACH4 Universal AP staining kit (Biocare Medical). Slides were covered with MACH4 Universal AP Polymer (M4U536) for 10 minutes each. Finally, the sections were incubated with Vulcan Fast Red as chromagen (Biocare, FR805) for 12 minutes and then counterstained with diluted Gill's III Hematoxylin (SurgiPath) for 2 minutes. Primary antibodies were diluted using pH 7.6 Tris buffer with 1% BSA (bovine serum albumin) and the sections were washed with pH 7.6 Tris buffer with 0.05% Tween-20 in between incubations. As negative control, normal rabbit serum was used at the same concentration as the primary antibody. For staining of eosinophils, FFPE sections were treated with standard Hansel's stain following standard staining procedures.

Morphometric Analysis

Sampling and analysis were carried out in a “blinded” manner. The codes indicating asthmatic and non-asthmatic subjects were revealed only after the analysis of each group was finished. Randomly chosen electron micrographs of muscle cell cross-sections per subject were analyzed for various morphometric parameters. The results from the measurement for each subject were averaged first before the means were averaged and plotted. The raw data from individual images (before averaging) are provided along with the coefficients of variation in Tables 1-19. The individual cells were selected from low magnification images to conceal structural details from the experimenters, only after a cell was selected would a high resolution image of the cell be taken. The “randomness” in the selection was facilitated by choosing cells more or less evenly distributed over the whole image

containing tens to hundreds of cells. Specialized image-analysis software (Image Pro-Plus 6.0, MediaCybernetics, Bethesda, MD, USA) was used to help in the morphometric analysis. The area and perimeter of each smooth muscle cell was obtained by manual tracing. The area fractions of mitochondria and dense bodies were obtained by dividing the area of each cell occupied by mitochondria and dense bodies, respectively, by the total area of the cell. The length fraction of dense plaques was calculated by dividing the length of the cell membrane occupied dense plaques by the cell perimeter. Because overestimation of the number of larger particles (or cells) (relative to smaller ones) occurs in randomly selected 2-dimensional images (Hsia et al, 2010), we restricted our comparison of cell abundance to the same cell type (e.g., mast cells vs. mast cells) between tissues from asthmatic and non-asthmatic subjects.

Quantification of the thick and thin filaments.

The quantification of the thick and thin filaments in cross-section was performed by marking and keeping track of the number of filaments counted (tag-point counting). For thin filaments, the Image Pro-Plus 4.0 program randomly placed 10 circles with an area of $0.1832 \mu\text{m}^2$ on each micrograph. Circles that fell on the areas occupied by nucleus, organelles, plasma membrane, or extracellular space were excluded from the study; only the circles that fell on cytoplasmic areas with mostly thick and thin filaments, and occasionally dense bodies, were counted. The area occupied by dense bodies was not included in the calculation of filament density. Of the 10 circles generated by the computer, ~3-4 of them met the criteria. The thin filaments were counted by the tag-point quantification method in each of the non-excluded circles placed in the cytoplasmic areas on the cells. According to standard morphological methods, the filaments that fell on the edge of the circles were counted as half. The total numbers of thin filaments counted were divided by the area of the circle to obtain the thin filament density. For average thick filament density, because the number of thick

filaments per cross section is relatively small, the total number of thick filaments within a cell cross section was counted and divided by the cytoplasmic area of that cell cross section, excluding organelles and nucleus.

Quantification of inflammatory cells

Five low-magnification electron micrographs of tracheal SM bundles were analyzed from each subject. Tracheal SM bundles were randomly selected from each EM section. Non-muscle cells which were completely within the tracheal SM bundle or at its periphery (within 15 μm from the SM cell membrane) were identified, including fibroblasts, mast cells, macrophages, and nerve cells. The ratio of each type of non-muscle cell to the total number of tracheal SM cells in the electron micrograph was calculated.

Quantification of basal lamina thickness

Each tracheal SM cell is surrounded by an external lamina. To determine the thickness of this lamina, we calculated the mean thickness from 5 measurements (spread out approximately evenly along the entire cell perimeter) per cell from electron micrographs of 10-15 tracheal SM cells in each subject.

Quantification of mast cell density

As for the morphometric measurements in EM section, sampling and analysis of immunostained light microscopy sections were carried out in a “blinded” manner. The codes indicating asthmatic and non-asthmatic subjects were revealed only after the analysis of each group was finished. Immunostained mast cells surrounding each airway were identified and counted. Mast cell counts were expressed as the number of cells/ μm basement membrane. The data were obtained from 10 non-

asthmatic and 12 asthmatic subjects. Five images per subject were included in the analysis. For each subject the total number of mast cells divided by the total perimeter length was obtained from each image and the values for all images analyzed were averaged before the mean was include in the group average.

Quantification of eosinophils

The 24 samples (12 asthmatic and 12 non-asthmatic) were blinded to the investigator. The total number of eosinophils found within the walls of all airways per image were normalized to the cumulative basement membrane length of all the airways in the image and expressed as the number of cells/ μm basement membrane. Data were plotted as all asthmatics (fatal and non-fatal) vs. non-asthmatics or only fatal asthmatics vs. non-asthmatics.

Quantification of airway remodeling

The measurements were carried out in a “blinded” manner as described above. Sections measuring 10 μm in thickness were cut from frozen lungs (5 asthmatic and 6 non-asthmatic). 20 sections were cut per tissue sample and every 5th section (1, 5, 10, 15 and 20) was taken for staining with hematoxylin and eosin. The remaining 15 sections were used for various other experiments unrelated to this study. Once stained, slides were cover slipped and digitally scanned using the Aperio® system so that they could be analyzed using Image Pro Plus® software. Airway measurements for airway SM area, epithelial area and wall area were made using a point counting method, where a grid of approximately 4000 points was overlaid onto each airway of interest and the points falling on the area of interest were counted. The measurements for each airway were averaged across all the sections for each subject and then normalized to the internal perimeter (P_i) of the airway. The P_i was measured by tracing along the inside of the epithelium. An additional two sections were

cut from each lung to stain with Masson's trichrome and Picrosirius red for basement membrane and collagen quantification, respectively. To quantify the basement membrane thickness, a random series of line segments was placed over each image and the thickness of the basement membrane was measured at the points where line segments cross over the basement membrane. Thickness measurements were made perpendicular to the epithelium. A minimum of 40 measurements were made for each airway. To analyze the amount of collagen in each airway, images were taken under polarized light where the picrosirius red stain shows birefringence. The collagen shows up red on a black background and using color segmentation, the amount of collagen per airway was quantified.

Non-muscle cells and infiltration of inflammatory cells in the tracheal SM cell bundle

Figures S1A-S1D show examples of non-muscle cells (mast cells, fibroblasts, nerve cells, and macrophages) found in the tracheal SM cell bundles. The number of these non-muscle cells was determined to provide information on the micro-environment surrounding the tracheal SM cells, i.e., the extent of inflammatory cell infiltration, innervation, and synthesis of extracellular matrix proteins.

Cross-sectional areas used for force normalization

Force generated by a muscle is proportional to the muscle's cross-sectional area (perpendicular to the axis of force transmission) which in turn is proportional to the number of contractile units (e.g., sarcomeres in striated muscle) in parallel found in each cross-section. After normalization, the stress (force divided by cross-sectional area) can be used as an index of contractility of the contractile units. For muscle tissue experiments the muscle cross-section is often used for force normalization. Rationale for this practice is based on the assumption that the tissue cross-sectional area is proportional to the number of force-generating contractile units acting in parallel. The rationale is only valid for an ideal muscle preparation that is entirely made up of muscle cells and that the cross-sections of the muscle cells are entirely occupied by contractile filaments. In real preparations, because the

extracellular matrix usually accounts for a significant fraction of the muscle tissue cross-sectional area, the extracellular matrix area needs to be measured and taken into account in force normalization. Because non-muscle cells like mast cells and fibroblasts do not contribute to force generation, the fraction of area occupied by these cells need to be subtracted from the “muscle cross-sectional area”. This is important especially when healthy and diseased muscle tissues are compared because the infiltration into the muscle cell bundle by non-muscle cells may differ significantly in the healthy and diseased states. If the fraction of the muscle cell area occupied by contractile filaments is altered by a change in cell phenotype, for example from a contractile phenotype to a synthetic phenotype, it is important that the cell area be further divided into contractile filament area and non-contractile filament (organelles) area so that the force can be normalized to just the filament area. The stress values obtained in this way would allow the investigators to interpret their data in terms of changes in the intrinsic properties of the contractile units, excluding the effect of phenotype change.

A

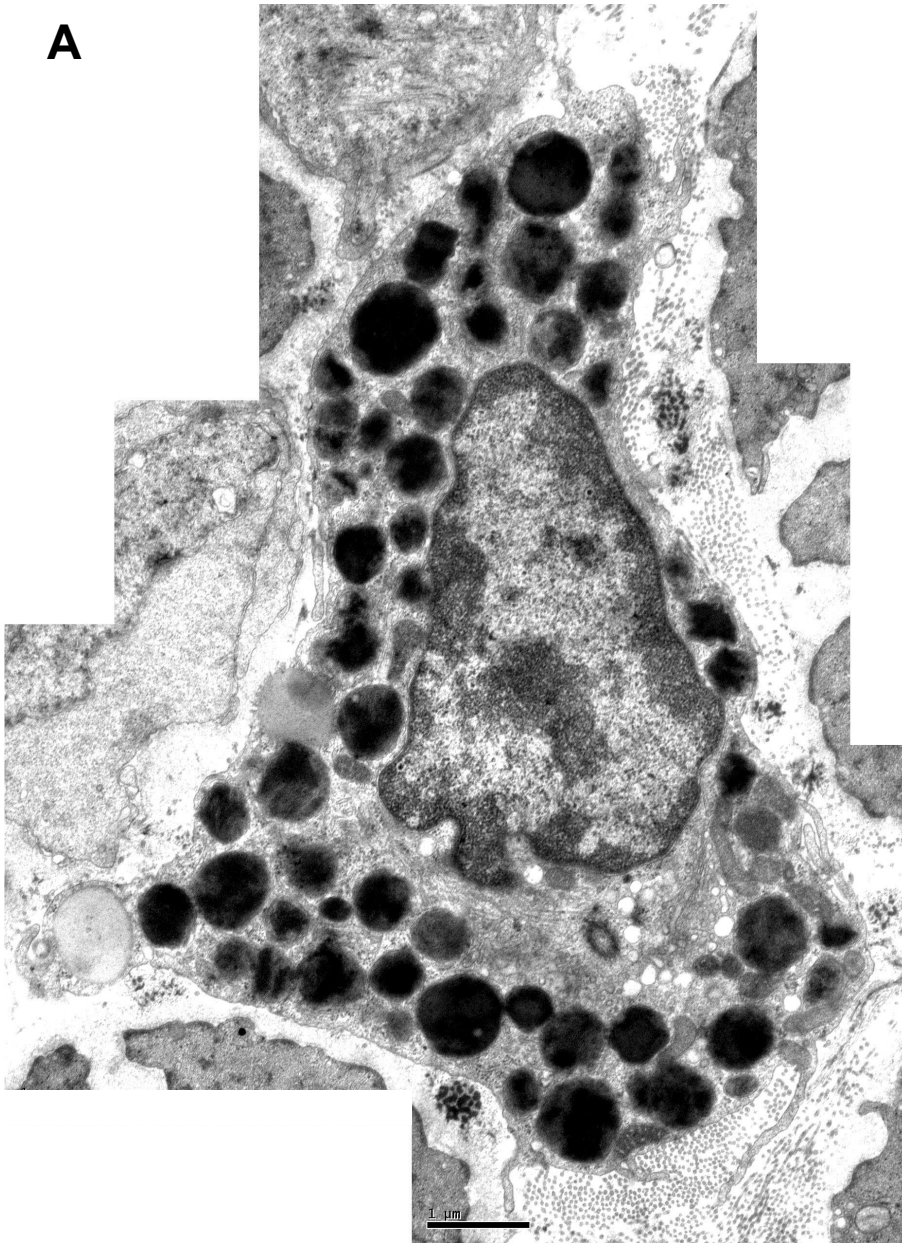


Figure S1A: A mast cell with a nucleus and numerous dark-staining granules, surrounded by smooth muscle cells.

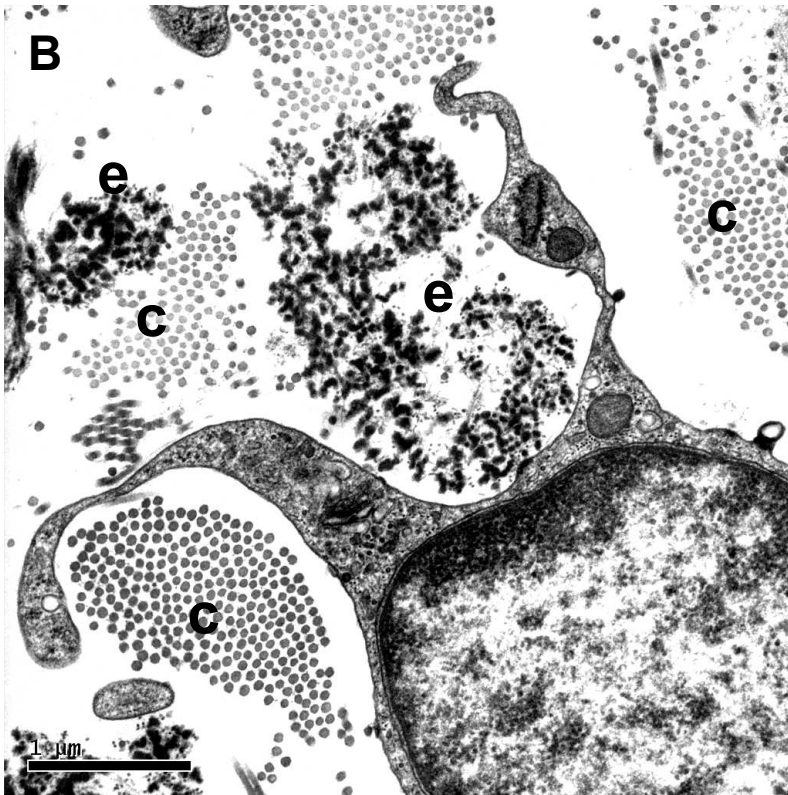


Figure S1B: Part of a fibroblast surrounded by dark-staining amorphous elastin (e) and collagen fibers (c) with round cross-sections.



Figure S1C: A nerve bundle surrounded by collagen fibers. Note that nerve and muscle cells are enveloped by basal laminae (parallel arrows outline parts of the envelope), whereas inflammatory cells and fibroblasts are not.

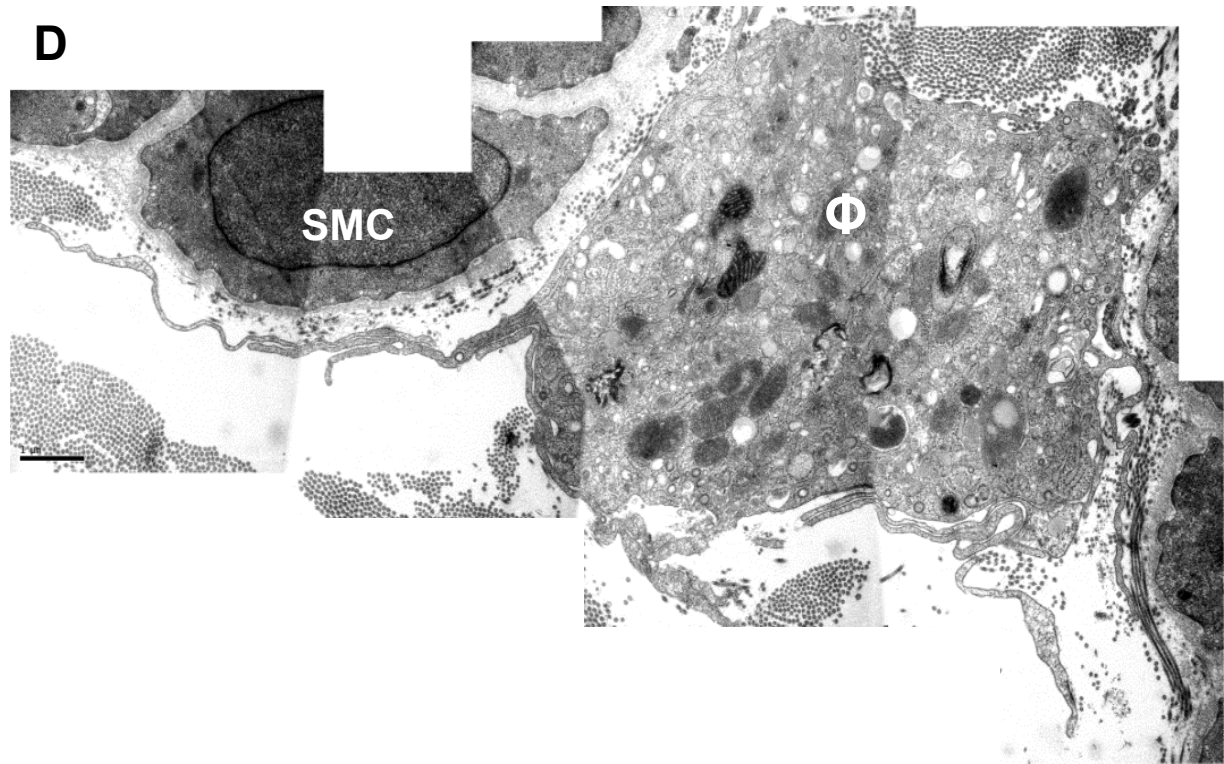


Figure S1D: A macrophage (Φ) near a SM cell and surrounded by collagen fibers. Scale bars in all images: 1 μm .

Table 1: Data points for cell area of tracheal smooth muscle cells in non-asthmatic and asthmatic patients (Fig. 3A in main text)

Patient	Mean (μm^2)	Standard Deviation	Coefficient of variation
Non-asthmatic	7.53	3.22	0.43
Non-asthmatic	6.19	3.98	0.64
Non-asthmatic	5.52	2.83	0.51
Non-asthmatic	9.59	2.22	0.23
Non-asthmatic	8.40	2.46	0.29
Non-asthmatic	4.38	2.04	0.46
Asthmatic	6.43	2.45	0.38
Asthmatic	7.01	3.09	0.44
Asthmatic	6.23	1.41	0.23
Asthmatic	6.72	2.12	0.31
Asthmatic	6.18	2.03	0.33
Asthmatic	10.55	3.85	0.36

Table 2: Data points for mitochondrial area as a percentage of tracheal smooth muscle cell area in non-asthmatic and asthmatic patients (Fig. 3B in main text)

Patient	Mean (% of total cell area)	Standard Deviation	Coefficient of variation
Non-asthmatic	1.53	1.05	0.68
Non-asthmatic	2.19	2.55	1.17
Non-asthmatic	2.74	1.21	0.44
Non-asthmatic	2.42	2.05	0.85
Non-asthmatic	3.13	1.52	0.49
Non-asthmatic	2.48	2.87	1.16
Asthmatic	2.10	1.63	0.78
Asthmatic	1.94	1.34	0.69
Asthmatic	2.21	2.28	1.03
Asthmatic	2.93	2.10	0.72
Asthmatic	2.32	1.40	0.61
Asthmatic	2.68	1.84	0.69

Table 3: Data points for number of mitochondria per tracheal smooth muscle cell cross-section in non-asthmatic and asthmatic patients (Fig. 3C in main text)

Patient	Mean (mitochondria/cross-section)	Standard Deviation	Coefficient of variation
Non-asthmatic	2.90	2.08	0.72
Non-asthmatic	2.00	2.45	1.22
Non-asthmatic	3.17	1.54	0.49
Non-asthmatic	4.56	3.28	0.72
Non-asthmatic	6.00	3.23	0.54
Non-asthmatic	2.40	2.12	0.88
Asthmatic	2.89	2.78	0.96
Asthmatic	2.95	2.39	0.81
Asthmatic	2.88	3.14	1.09

Asthmatic	5.46	4.43	0.81
Asthmatic	3.35	2.08	0.62
Asthmatic	5.00	4.51	0.90

Table 4: Data points for size of individual mitochondria in tracheal smooth muscle cells in non-asthmatic and asthmatic patients (Fig. 3D in main text)

Patient	Mean (μm^2)	Standard Deviation	Coefficient of variation
Non-asthmatic	0.04	0.02	0.63
Non-asthmatic	0.06	0.06	1.01
Non-asthmatic	0.04	0.01	0.26
Non-asthmatic	0.05	0.01	0.23
Non-asthmatic	0.05	0.01	0.31
Non-asthmatic	0.03	0.02	0.64
Asthmatic	0.05	0.04	0.74
Asthmatic	0.04	0.02	0.48
Asthmatic	0.04	0.04	0.87
Asthmatic	0.04	0.02	0.46
Asthmatic	0.04	0.02	0.37
Asthmatic	0.07	0.06	0.95

Table 5: Data points for basal lamina thickness in tracheal smooth muscle cells in non-asthmatic and asthmatic patients (Fig. 3E in main text)

Patient	Mean (μm)	Standard Deviation	Coefficient of variation
Non-asthmatic	0.13	0.07	0.55
Non-asthmatic	0.09	0.04	0.48
Non-asthmatic	0.09	0.03	0.37
Non-asthmatic	0.11	0.06	0.60
Non-asthmatic	0.06	0.03	0.44
Non-asthmatic	0.06	0.03	0.48
Asthmatic	0.13	0.05	0.37
Asthmatic	0.13	0.10	0.74
Asthmatic	0.11	0.04	0.39
Asthmatic	0.08	0.03	0.46
Asthmatic	0.06	0.03	0.49
Asthmatic	0.05	0.02	0.29

Table 6: Data points for number of microtubules per tracheal smooth muscle cells in non-asthmatic and asthmatic patients (Fig. 3F in main text)

Patient	Mean (microtubules/cell)	Standard Deviation	Coefficient of variation
Non-asthmatic	1.94	1.39	0.72
Non-asthmatic	3.20	2.30	0.72
Non-asthmatic	4.47	2.18	0.49
Non-asthmatic	6.78	3.70	0.55
Non-asthmatic	13.75	7.94	0.58
Non-asthmatic	5.60	5.04	0.90
Asthmatic	5.56	3.55	0.64
Asthmatic	6.79	4.22	0.62
Asthmatic	2.25	1.98	0.88
Asthmatic	3.59	2.68	0.75
Asthmatic	5.57	2.87	0.52

Asthmatic	4.67	2.67	0.57
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Table 7: Data points for percentage of tracheal smooth muscle cell area containing dense bodies in non-asthmatic and asthmatic patients (Fig. 3G in main text)

Patient	Mean (%)	Standard Deviation	Coefficient of variation
Non-asthmatic	4.10	0.76	0.19
Non-asthmatic	1.82	0.86	0.47
Non-asthmatic	3.17	1.53	0.48
Non-asthmatic	2.46	0.79	0.32
Non-asthmatic	2.86	0.83	0.29
Non-asthmatic	3.66	2.27	0.62
Asthmatic	4.43	1.96	0.44
Asthmatic	3.31	1.70	0.51
Asthmatic	2.19	1.12	0.51
Asthmatic	1.45	0.90	0.62
Asthmatic	3.12	1.59	0.51
Asthmatic	3.57	1.44	0.40

Table 8: Data points for percentage of tracheal smooth muscle cell perimeter containing dense plaques in non-asthmatic and asthmatic patients (Fig. 3H in main text)

Patient	Mean (%)	Standard Deviation	Coefficient of variation
Non-asthmatic	41.28	11.16	0.27
Non-asthmatic	40.97	10.01	0.24
Non-asthmatic	37.21	9.84	0.26
Non-asthmatic	34.55	7.30	0.21
Non-asthmatic	39.38	8.37	0.21
Non-asthmatic	37.11	9.12	0.25
Asthmatic	39.79	9.22	0.23
Asthmatic	43.45	11.04	0.25
Asthmatic	39.29	6.65	0.17
Asthmatic	33.61	8.15	0.24
Asthmatic	41.95	8.41	0.20
Asthmatic	41.44	6.80	0.16

Table 9: Data points for thick filament density in tracheal smooth muscle cells in non-asthmatic and asthmatic patients (Fig. 3I in main text)

Patient	Mean (filaments/ μm^2)	Standard Deviation	Coefficient of variation
Non-asthmatic	33.62	9.76	0.29
Non-asthmatic	33.58	10.35	0.31
Non-asthmatic	35.38	9.81	0.28
Non-asthmatic	39.77	7.26	0.18
Non-asthmatic	38.15	6.20	0.16
Non-asthmatic	46.20	12.83	0.28
Asthmatic	38.43	13.71	0.36
Asthmatic	38.53	8.10	0.21
Asthmatic	42.66	12.21	0.29
Asthmatic	40.17	9.16	0.23
Asthmatic	28.86	7.96	0.28

Asthmatic	17.27	6.14	0.36
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Table 10: Data points for thin filament density in tracheal smooth muscle cells in non-asthmatic and asthmatic patients (Fig. 3J in main text)

Patient	Mean (filaments/ μm^2)	Standard Deviation	Coefficient of variation
Non-asthmatic	1441.00	252.20	0.18
Non-asthmatic	1574.00	286.00	0.18
Non-asthmatic	1630.00	182.40	0.11
Non-asthmatic	1642.00	331.60	0.20
Non-asthmatic	1576.00	212.80	0.14
Non-asthmatic	1739.00	347.00	0.20
Asthmatic	1674.00	203.90	0.12
Asthmatic	1516.00	124.10	0.08
Asthmatic	1588.00	225.00	0.14
Asthmatic	1430.00	276.00	0.19
Asthmatic	1434.00	253.50	0.18
Asthmatic	1635.00	167.40	0.10

Table 11: Data points for percentage of non-filamentous area in tracheal smooth muscle cells in non-asthmatic and asthmatic patients (Fig. 3K in main text)

Patient	Mean (%)	Standard Deviation	Coefficient of variation
Non-asthmatic	9.90	11.69	1.18
Non-asthmatic	15.46	9.90	0.64
Non-asthmatic	10.66	5.96	0.56
Non-asthmatic	18.92	14.88	0.79
Asthmatic	9.02	6.22	0.69
Asthmatic	14.56	12.44	0.85
Asthmatic	14.57	19.40	1.33
Asthmatic	23.92	16.52	0.69
Asthmatic	32.00	25.57	0.80
Asthmatic	13.27	9.92	0.75

Table 12: Data points for ratio of fibroblasts to tracheal smooth muscle cells in non-asthmatic and asthmatic patients (Fig. 5A in main text)

Patient	Mean	Standard Deviation	Coefficient of variation
Non-asthmatic	0.03	0.02	0.81
Non-asthmatic	0.05	0.05	0.88
Non-asthmatic	0.07	0.03	0.44
Non-asthmatic	0.11	0.04	0.40
Non-asthmatic	0.14	0.05	0.33
Non-asthmatic	0.21	0.12	0.55
Asthmatic	0.04	0.03	0.81
Asthmatic	0.06	0.05	0.93
Asthmatic	0.06	0.02	0.37
Asthmatic	0.07	0.03	0.50
Asthmatic	0.10	0.07	0.69

Table 13: Data points for ratio of mast cells to tracheal smooth muscle cells in non-asthmatic and asthmatic patients (Fig. 5B in main text)

Patient	Mean	Standard Deviation	Coefficient of variation
Non-asthmatic	0.0000	0.0000	-
Non-asthmatic	0.0000	0.0000	-
Non-asthmatic	0.0000	0.0000	-
Non-asthmatic	0.0028	0.0027	0.96
Non-asthmatic	0.0014	0.0013	0.91
Asthmatic	0.0000	0.0000	-
Asthmatic	0.0024	0.0020	0.84
Asthmatic	0.0027	0.0032	1.19
Asthmatic	0.0063	0.0041	0.64
Asthmatic	0.0061	0.0032	0.53

Table 14: Data points for ratio of macrophages to tracheal smooth muscle cells in non-asthmatic and asthmatic patients (Fig. 5C in main text)

Patient	Mean	Standard Deviation	Coefficient of variation
Non-asthmatic	0.0000	0.0000	-
Non-asthmatic	0.0000	0.0000	-
Non-asthmatic	0.0000	0.0000	-
Non-asthmatic	0.0092	0.0057	0.62
Non-asthmatic	0.0104	0.0081	0.77
Asthmatic	0.0000	0.0000	-
Asthmatic	0.0000	0.0000	-
Asthmatic	0.0000	0.0000	-
Asthmatic	0.0000	0.0000	-
Asthmatic	0.0164	0.0222	1.35

Table 15: Data points for ratio of nerve bundles to tracheal smooth muscle cells in non-asthmatic and asthmatic patients (Fig. 5D in main text)

Patient	Mean	Standard Deviation	Coefficient of variation
Non-asthmatic	0.0000	0.0000	-
Non-asthmatic	0.0223	0.0189	0.84
Non-asthmatic	0.0219	0.0185	0.84
Non-asthmatic	0.0411	0.0268	0.65
Non-asthmatic	0.0392	0.0286	0.72
Non-asthmatic	0.0590	0.0474	0.80
Asthmatic	0.0000	0.0000	-
Asthmatic	0.0090	0.0054	0.60
Asthmatic	0.0355	0.0419	1.17
Asthmatic	0.0418	0.0304	0.72
Asthmatic	0.0635	0.0258	0.40

Table 16: Data points for cell area of intra-parenchymal airway smooth muscle cells in non-asthmatic and asthmatic patients (Fig. 6A in main text)

Patient	Mean (μm^2)	Standard Deviation	Coefficient of variation
Non-asthmatic	11.18	2.90	0.26
Non-asthmatic	18.72	8.14	0.43

Non-asthmatic	18.45	15.26	0.83
Asthmatic	10.68	14.55	1.36
Asthmatic	7.15	7.05	0.99
Asthmatic	28.87	22.18	0.77
Asthmatic	13.88	16.93	1.22

Table 17: Data points for mitochondrial area as a percentage of intra-parenchymal airway smooth muscle cells in non-asthmatic and asthmatic patients (Fig. 6B in main text)

Patient	Mean (μm^2)	Standard Deviation	Coefficient of variation
Non-asthmatic	0.06	0.05	0.87
Non-asthmatic	0.06	0.05	0.94
Non-asthmatic	0.03	0.02	0.73
Asthmatic	0.05	0.04	0.77
Asthmatic	0.02	0.02	1.14
Asthmatic	0.04	0.03	0.88
Asthmatic	0.06	0.04	0.71

Table 18: Data points for number of mitochondria per bronchial smooth muscle cell in non-asthmatic and asthmatic patients (Fig. 6C in main text)

Patient	Mean (μm^2)	Standard Deviation	Coefficient of variation
Non-asthmatic	4.89	3.59	0.73
Non-asthmatic	10.21	8.85	0.87
Non-asthmatic	7.83	6.56	0.84
Asthmatic	8.10	4.80	0.59
Asthmatic	3.50	5.93	1.69
Asthmatic	6.21	4.98	0.80
Asthmatic	11.28	13.19	1.17

Table 19: Data points for size of individual mitochondria in bronchial smooth muscle cells in non-asthmatic and asthmatic patients (Fig. 6D in main text)

Patient	Mean (μm^2)	Standard Deviation	Coefficient of variation
Non-asthmatic	0.18	0.19	1.09
Non-asthmatic	0.12	0.06	0.56
Non-asthmatic	0.06	0.04	0.64
Asthmatic	0.06	0.04	0.70
Asthmatic	0.01	0.01	0.92
Asthmatic	0.13	0.08	0.60
Asthmatic	0.06	0.02	0.31

Table 20: Power analysis of % difference detectable with power=0.8 and $\alpha=0.05$

Figure containing data	% Difference detectable	% Difference measured
Fig. 3A (TSM cell area)	30%	4%
Fig. 3B (TSM mito. area)	20%	2%
Fig. 3C (TSM mito. #)	40%	7%
Fig. 3D (TSM mito. size)	30%	4%
Fig. 3E (TSM basal lamina)	40%	4%
Fig. 3F (TSM microtubule)	60%	20%

Fig. 3G (TSM dense bodies)	40%	No difference
Fig. 3H (TSM dense plaques)	10%	4%
Fig. 3I (TSM myosin filaments)	20%	9%
Fig. 3J (TSM actin filaments)	10%	3%
Fig. 3K (TSM non-filamentous area)	60%	30%
Fig. 4A (fibroblasts)	60%	35%
Fig. 4B (mast cells)	450%	417%
Fig. 4C (macrophages)	>1000%	16%
Fig. 4D (nerve bundles)	80%	2%
Fig. 5A (BSM cell area)	50%	6%
Fig. 5B (BSM mito. Area)	40%	15%
Fig. 5C (BSM mito. #)	60%	5%
Fig. 5D (BSM mito. size)	60%	46%

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