Does the length dependency of airway smooth muscle force contribute to airway hyperresponsiveness?

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Lengthening or shortening of ASM from its optimal length (L0) results in an immediate decrease in force; on lengthening, forces drop to about 60% of their original values, and on shortening, forces drop to about 30% of their original values. This reduction in force allows ASM to operate at a longer length, which could increase ACh-induced force by 1.8 – 117.8% (depending on ASM length and ACh concentration) and enhance the increased resistance to airflow by 0.4 – 4,432.8%. In conclusion, elongation of ASM imposed by airway wall remodeling and/or hyperinflation may allow ASM to operate at a longer length and to consequently generate more force and respond to lower concentration of spasmogens. This phenomenon could contribute to airway hyperresponsiveness.

THERE ARE ONLY FEW PUBLISHED studies in which the in situ length (L0 in situ) of the airway smooth muscle (ASM) was used to investigate its contractile properties. More often, ASM preparations are stretched progressively until a further stretch does not increase, or actually reduces, the force generated in response to a given contractile stimulus. This length is then called Lmax or optimal length (L0) and is used to assess the effect of different interventions on contractility.

Lengthening or shortening of ASM from its L0 results in an instantaneous decline of force, which is proportional to the magnitude of the length change (9, 10, 19, 32, 33). However, ASM is endowed with the ability to adapt to new lengths and “regain” some of the force loss caused by length changes (25). This recovery can be full (i.e., the force recovers back to maximal force) within a certain range of lengths and occurs usually within 30 min. This phenomenon is called “length adaptation,” and it relies on the plasticity of both the contractile apparatus (4, 26) and the adhesomes [i.e., the dynamic, multi-protein modules that ensure the physical linkage between the cells within tissues to the extracellular matrix (ECM)] (8, 34). This capacity to adapt suggests that there is a range of L0 values for ASM in which maximal force can be developed.

It is unknown whether the in vivo operating length of ASM is within the range of lengths at which ASM can generate maximal force. The possibility remains that the in situ length of ASM is not within this range of L0 values. This is a major issue owing to the well-defined length dependency of ASM contractility beyond lengths for which length adaptation allows full recovery to maximal force. Together, these considerations raise the important questions: 1) whether the range of L0 values is near L0 in situ, and 2) whether the length increment or decrement which causes force reduction ex vivo is near the physiological range of lengths that ASM experiences in vivo.

The first aim of this study was to describe in detail the “length dependency of ASM force” in response to different contractile stimuli, using L0 in situ as the reference length. Precautions were taken to keep the ASM preparations at L0 in situ. The force generated in response to electrical field stimulation (EFS), as well as to increasing concentrations of either ACh or K+ was investigated at three different lengths (0.7, 1, and 1.3 L0 in situ) in ovine tracheal strips. The force generated by human bronchial rings was also investigated at those three lengths in response to methacholine (MCh). We report that stretching ASM within a physiological range of lengths that can be achieved in vivo increases its force-generating capacity.

The second aim of this study was to predict the consequence of this length dependency of ASM force on airway responsiveness in hyperinflated and remodeled asthmatic lungs. The assumption being that the propensity of asthmatic subjects to hyperinflated [based on increased functional residual capacity (FRC) in such patients], combined with airway remodeling that thickens the airway wall area internal to the ASM layer (thickening of the epithelium and lamina propria), stretch the operating length of ASM. The increased operating length of asthmatic ASM was calculated based on morphological data, assuming that the total cross-sectional area internal to the apical surface of the epithelium of asthmatic airways matches identical-sized airways of nonasthmatic subjects. The change of force caused by this estimated increase of ASM length was...
then calculated based on our experimental results, and Lambert’s computational model was used to predict its effect on ASM shortening, airway narrowing, and resistance to airflow (16). We documented that the length dependency of ASM force can have a significant impact on the degree of airway responsiveness and could contribute to airway hyperresponsiveness (AHR) in some asthmatics.

METHODS

Ovine muscle tissue preparation and preconditioning. Sheep tracheas obtained from a local abattoir were used to perform the experiments in the present study. All experimental procedures were approved by the Animal Care Committee and Biosafety Committee of the University of British Columbia and conformed to the guidelines set out by the Canadian Council on Animal Care. Immediately after the sheep were killed, the tracheas were removed and placed in room temperature Krebs solution (pH 7.4; 118 mM NaCl, 4 mM KCl, 1.2 mM NaH2PO4, 22.5 mM NaHCO3, 2 mM MgSO4, 2 mM CaCl2, and 2 g/l dextrose). After transportation to the laboratory, tracheas were stored at 4°C until further processed.

The day of the experiment, an ~2-cm segment was first cut from the trachea and put into a waxed-floor dissecting bath filled with room temperature Krebs’ solution. Precautions were taken to maintain ASM at its original length throughout the procedure. Specifically, while the tracheal segment was still intact and the trachealis muscle still passively stretched by the preload exerted by the cartilage rings (13), two incisions of the epithelial layer overlaying the posterior membranous sheath were performed (perpendicular to the muscle bundles), and the distance between them was measured with a millimeter ruler under a dissecting microscope. The anterior side (cartilage side) of the tracheal segment was then cut, and the open segment was pinned down on the ventral side (epithelial side) onto the waxed floor to dissect away the adventitia. The segment was then flipped over and pinned down on the dorsal side, making sure that the distance between the two incisions in the epithelium was the same as previously measured. The epithelial layer was removed, and a muscle strip was isolated without cutting the anchoring points on the cartilage at both ends. While still attached to the cartilage, two aluminum clips were attached on both ends of the muscle strip. The distance between the two aluminum clips was measured, and only then was the muscle strip cut distal to the clips at both ends, separating it from the rest of the tracheal segment. The isolated tracheal strip was attached to a servo-controlled lever in between platinum electrodes. The strips were then immersed in an organ bath, which contained an external chamber in which 37°C water circulated, and an inner chamber filled with Krebs solution at all time. Once mounted in the organ bath, the tracheal strip was stretched so that the distance between the two clips was equal to the distance measured before cutting the muscle strip from the tracheal segment.

The length of the tracheal strip was then corrected for the lack of transpulmonary pressure (Pt), which is present in vivo but absent when the distance between the two epithelial incisions was measured. The tracheal luminal caliber was corrected to a Pt of 5 cmH2O to mimic the L_in situ of the trachealis muscle at FRC. The correction was based on the relationship between Pt and fractional cross-sectional area of the airway lumen described by Lambert and coworkers (17). The percentage change in the perimeter at the middle of the ASM layer caused by this increased luminal area was calculated, taking into account the thickness of the airway wall layers internal to ASM (epithelium and lamina propria), which were assumed to be incompressible. This percentage change in ASM perimeter was then used to stretch the ASM strip. This adjusted length is hereafter called L_in situ and is assumed to be the length at which the ASM operates in vivo at FRC. The setup allowed measurement of isometric force in response to either EFS or different spasmogens dissolved at different concentrations in the Krebs solution. Only tissues with no intrinsic tone were used for these experiments. The ASM was kept at L_in situ during the preconditioning period.

The preconditioning consisted of repeated 9-s EFS at 5-min intervals until the ASM generated a stable maximal isometric force with low resting tension, as has been described in detail previously (2). The 9-s EFS was chosen because it allowed sufficient time for the muscle to reach its isometric force plateau. The stimulator voltage (peak-to-peak) was set at 10 V, which was the voltage required to elicit maximal EFS-induced force. These parameters were kept the same throughout the experiments.

Human muscle tissue preparation and preconditioning. Human lung tissue was obtained from patients undergoing lobectomy or segmentectomy for lung cancer removal. Consent was obtained from every donor, and the use of human lung tissue was approved by the ethical review committee of the Institut Universitaire de Cardiologie et de Pneumologie de Québec. From each lung specimen, one bronchial ring was dissected free of parenchyma and mounted in an organ bath identical to the one used for ovine tracheal strips (see above). The resting force was calculated to simulate a Pt at FRC (i.e., 5 cmH2O). This was accomplished as follows: 1) before mounting the bronchial ring in the organ bath, the internal diameter was measured at zero cmH2O transmural pressure (i.e., atmospheric pressure on both the internal and external side of the airway wall); 2) the airway generation from which the ring was derived was estimated based on its diameter using previous morphometric data of human lungs (30); 3) simple trigonometry was used to find the radius at the middle of the ASM layer, using the known areas of the different layers constituting the airway wall (epithelium, lamina propria, ASM, and adventitia); 4) with this radius and the pressure (5 cmH2O), Laplace’s law (tension = pressure x radius) was used to convert pressure into tension at the middle of ASM layer; 5) the length of the isolated bronchial ring was then used to convert tension (g/cm) into force (mN). The later was the force applied by the lever on the bronchial ring at baseline. At that length, the bronchial ring was said to be at L_in situ.

The preconditioning period was identical to that for the sheep tracheal strips described above, except that the parameters for EFS were different. The duration was set to 20 s, the frequency to 60 Hz, the pulse duration to 2 ms, and the voltage at 20 V. These were determined to be optimal for maximal EFS-induced force in preliminary studies using human bronchial rings with intact epithelium.

Protocol. ASM strips isolated from sheep trachea or human bronchial rings were initially set at the length at which ASM operates in vivo (L_in situ). After the preconditioning period (see above), the force generated by ASM strips in response to EFS was measured at three different lengths, 0.7, 1, and 1.3 L_in situ, before and after length adaptation. Length adaptation is a well-known process that takes ~30 min during which ASM regains some (sometimes all) of its force-generating capacity lost after a length perturbation, which can include elongation, length reduction, a single stretch, or length oscillations (4). The length perturbations made in the present study consist of elongation (from 1 to 1.3 L_in situ) or reduction (from 1 to 0.7 L_in situ). These three lengths were tested in every tissue. The sequence of the length change (elongation or reduction) was randomized. Following length adaptation (once the force generated by EFS no longer increased with subsequent EFS), concentration curves were generated with either ACh or K+ for ovine tracheal strips and with MCh for human bronchial rings.

Computational model. The thickness of the epithelium, lamina propria, ASM layer, and adventitia for different airway sizes from both asthmatic subjects and nonasthmatic subjects was taken from morphological studies performed by James and coworkers (12) and Kuvano and coworkers (14). The thickening of the epithelium, lamina propria, and ASM observed in asthmatic subjects was used to estimate the amount of ASM elongation. The postulate here is that ASM in remodeled asthmatic airways operates at a longer length, which is
based on the assumption either that the thickening of the airway wall in asthmatic subjects occurs outwardly, or that there is hyperinflation to bring the airway luminal area to a caliber similar to that observed in nonasthmatic subjects for airways of identical size. Airway size was based on the perimeter of the basal lamina (11). The percentage of ASM elongation caused by the thickening of the airway wall internal to ASM for the 17 generations (0 to 16) of conducting airways is shown in Table 1.

The increase of force caused by ASM elongation occurring in any chosen airway generation was then determined based on our experimental data, which (as explained above) was acquired at different ASM lengths (0.7, 1, or 1.3 $L_{\text{in situ}}$) and using different stimuli (EFS, ACh, K$^+$, MCh) at different concentrations (for ACh, K$^+$, and MCh). 0.7 and 1.3 of $L_{\text{in situ}}$ were chosen because they are just beyond the extreme limits of strain that ASM can experience in vivo due to changes in lung volume occurring during breathing maneuvers (6) and postural changes (28).

To convert force into tension, the forces generated in response to $3 \times 10^{-5}$ M of ACh at different lengths were normalized relative to the total force obtained in response to EFS and then converted to stress (kPa or g/cm$^2$), assuming that ASM generates 100 kPa in response to EFS at $L_{\text{in situ}}$ (5). This was the reference point, which was then used to calculate the stress generated by ACh at different concentrations. Knowing the thickness of ASM layer at that airway generation (see Table 1), the stress was then converted to tension (g/cm, i.e., in grams of force per centimeter of airway length).

We then used Lambert’s computational model (16) to estimate the contribution of this increased ASM force to ASM shortening, airway narrowing, and airflow resistance. The model can predict the amount of airway narrowing by superimposing the tension generated by ASM at different lengths in response to a chosen contractile stimulus and the theoretical load impeding ASM shortening at any given airway generation at any chosen Pt. The point of intersection between the two curves represents the point at which ASM would stop shortening (where the load impeding ASM shortening is equal to the tension generated by ASM at that length in response to the chosen contractile stimulus). The theoretical load in this model mimics the physiological load impeding ASM shortening in vivo (18). In the example shown in detail in the present study, the load opposing muscle shortening was calculated for an intraparenchymal conducting airway of the 10th generation in a lung inflated to FRC (Pt of 5 cmH$_2$O). The size and the length of the airways of different airway generations were chosen according to Wiggs and coworkers (30). The load takes into account the passive elastic properties of the airway wall, which were derived from analyses of the relationship between the Pt and the fractional area of the airway lumen (17). The load also takes into account the force of airway-parenchymal interdependence, which is the load imposed by distortion of the lung parenchyma as ASM shortens. The later is calculated from the shear modulus of the lung parenchyma, which was estimated to be 0.7 Pt. (15), and the changes in adventitial diameter during airway narrowing, which is geometrically related to the changes of ASM length during shortening (16). The loads were corrected for changes in airway radius, which occur during ASM shortening, using LaPlace’s law ($T = P \times R$, where T is wall tension, P is pressure, and R is radius) derived for a thin-walled cylinder.

**Statistical analysis.** The force obtained for each ovine tracheal strip was normalized to maximal isometric force, which is the force generated by the strip in response to EFS at $L_{\text{in situ}}$ following the preconditioning period minus the resting tension. Data shown are means ± SE of the total force. The force generated by the human bronchial rings is expressed in absolute values (mN). Two-way ANOVA with repeated measures was performed to evaluate the change of force over a range of concentrations of the contractile agents (ACh, K$^+$, MCh) at different ASM lengths (0.7, 1, or 1.3 $L_{\text{in situ}}$), as well as to evaluate their interaction. These analyses were followed by Bonferroni’s a posteriori test to evaluate the change of force in response to different concentrations of contractile agents between selected pairs of ASM lengths. All graphs and statistical analyses were performed using Prism 4 (GraphPad Software, San Diego, CA), and a $P < 0.05$ was considered sufficient to reject the null hypothesis.

The total force, but not the isolated active force, is presented because it is the total force that contributes to narrowing in our model. In intraparenchymal airways, even when the muscle is fully relaxed, the wall is under tension. This is because the passive components of the airway wall, including the relaxed muscle, are stretched. This passive tension represents an inwardly directed force tending to constrict the airways. In fact, this tension is equal to the force originating from the Pt, but in the opposite direction (the parenchymal recoil is an outwardly directed force that tends to dilate the airways).

The active tension that is generated by the contractile stimuli (EFS, K$^+$, and MCh) at different ASM lengths (0.7, 1, or 1.3 $L_{\text{in situ}}$) were corrected for changes in airway radius, which occur during ASM shortening, using LaPlace’s law ($T = P \times R$, where T is wall tension, P is pressure, and R is radius) derived for a thin-walled cylinder.

**Table 1. Percentage increase of ASM length in remodeled asthmatic airways caused by thickening of the epithelium, lamina propria, and ASM.**

<table>
<thead>
<tr>
<th>Airway Generations</th>
<th>Luminal Area at FRC</th>
<th>Epithelial Area</th>
<th>Lamina Propria Area</th>
<th>ASM Thickness</th>
<th>Ratio of Lumen to ASM Perimeters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Asthmatic</td>
<td>Normal</td>
<td>Asthmatic</td>
<td>Normal</td>
</tr>
<tr>
<td>0</td>
<td>247.38</td>
<td>9.03</td>
<td>14.89</td>
<td>9.81</td>
<td>34.15</td>
</tr>
<tr>
<td>1</td>
<td>113.85</td>
<td>4.30</td>
<td>7.14</td>
<td>4.61</td>
<td>15.76</td>
</tr>
<tr>
<td>2</td>
<td>50.65</td>
<td>2.16</td>
<td>3.63</td>
<td>2.28</td>
<td>7.60</td>
</tr>
<tr>
<td>3</td>
<td>22.64</td>
<td>1.10</td>
<td>1.87</td>
<td>1.13</td>
<td>3.63</td>
</tr>
<tr>
<td>4</td>
<td>13.59</td>
<td>0.74</td>
<td>1.27</td>
<td>0.75</td>
<td>2.34</td>
</tr>
<tr>
<td>5</td>
<td>8.26</td>
<td>0.50</td>
<td>0.86</td>
<td>0.49</td>
<td>1.48</td>
</tr>
<tr>
<td>6</td>
<td>5.15</td>
<td>0.34</td>
<td>0.60</td>
<td>0.33</td>
<td>0.96</td>
</tr>
<tr>
<td>7</td>
<td>3.33</td>
<td>0.25</td>
<td>0.44</td>
<td>0.23</td>
<td>0.63</td>
</tr>
<tr>
<td>8</td>
<td>2.22</td>
<td>0.18</td>
<td>0.33</td>
<td>0.17</td>
<td>0.43</td>
</tr>
<tr>
<td>9</td>
<td>1.51</td>
<td>0.14</td>
<td>0.26</td>
<td>0.12</td>
<td>0.30</td>
</tr>
<tr>
<td>10</td>
<td>1.06</td>
<td>0.11</td>
<td>0.20</td>
<td>0.09</td>
<td>0.21</td>
</tr>
<tr>
<td>11</td>
<td>0.77</td>
<td>0.09</td>
<td>0.17</td>
<td>0.07</td>
<td>0.14</td>
</tr>
<tr>
<td>12</td>
<td>0.58</td>
<td>0.07</td>
<td>0.14</td>
<td>0.06</td>
<td>0.12</td>
</tr>
<tr>
<td>13</td>
<td>0.44</td>
<td>0.06</td>
<td>0.12</td>
<td>0.05</td>
<td>0.09</td>
</tr>
<tr>
<td>14</td>
<td>0.35</td>
<td>0.05</td>
<td>0.11</td>
<td>0.04</td>
<td>0.07</td>
</tr>
<tr>
<td>15</td>
<td>0.28</td>
<td>0.05</td>
<td>0.10</td>
<td>0.04</td>
<td>0.06</td>
</tr>
<tr>
<td>16</td>
<td>0.23</td>
<td>0.04</td>
<td>0.09</td>
<td>0.03</td>
<td>0.05</td>
</tr>
</tbody>
</table>

All the values are areas in mm$^2$, except the thickness of airway smooth muscle (ASM) in mm. The ratio of lumen to ASM perimeters is the ratio of the perimeter at the apical surface of the epithelium on the perimeter at the middle of the ASM layer. FRC, functional residual capacity.
Length Dependency of ASM Force in AHR • Lee-Gosselin A et al.

ACh, K\(^+\), or MCh) is added to the passive tension of the airway wall, so that passive and active tensions of the airway wall are working in parallel. Obviously, the passive tension is progressively lost when the passive components of the airway wall are unstretched during narrowing of the airways. In fact, the passive tension of the wall eventually becomes 0. At 0, the passive elements of the airway wall transitions from being in a state of tension to a state of compression. Passed this point, these passive components become an additional load impeding ASM shortening. All of that is taken into account in the model. Therefore, it is the total tension, not just the active force, that drives airway constriction. That is the reason why we are showing the total force.

RESULTS

The force generated by ASM at three different lengths before and after length adaptation is shown in Fig. 1. Longer ASM generated more force than shorter ASM in response to the same stimulus (EFS) (P < 0.0001). For EFS, the length effect was confined to the change from 0.7 to 1.0 L\(_{\text{in situ}}\); there was no difference between 1 and 1.3 L\(_{\text{in situ}}\). Also apparent in Fig. 1 is the effect of length adaptation (increase of force over time) at 0.7 and 1, but not at 1.3, L\(_{\text{in situ}}\). Notice that the total force is shown here, not just the EFS-induced force, which has usually been reported in prior studies documenting length adaptation. The total force is EFS-induced force plus the passive resting tension. When EFS-induced force is plotted instead of total force, one can observe a decrease following elongation that recovered over time, as observed at shorter length (0.7 L\(_{\text{in situ}}\)) and as reported previously (4). However, during the length adaptation process (at 1.3 L\(_{\text{in situ}}\), EFS-induced force increased while the resting tension decreased, so that the total force did not change over time following elongation.

Following length adaptation, a concentration-response curve was constructed at every length with either ACh or K\(^+\). The results are presented in Fig. 2 for ACh in A and for K\(^+\) in B. As expected, the force generated by ASM was related to the concentration of either ACh or K\(^+\) used. More surprisingly, the length dependency of ASM force in response to ACh or K\(^+\) was distinct from the one in response to EFS. While EFS-induced force plateaued at 1 L\(_{\text{in situ}}\) (Fig. 1), the force in response to ACh or K\(^+\) increased as the length increased. The effect of length was significant (P < 0.0001) for both ACh and K\(^+\). Longer ASM (1.3 L\(_{\text{in situ}}\)) generated more force in response to the same stimuli than shorter ASM (0.7 L\(_{\text{in situ}}\)) (P ≤ 0.05; starting at 3 \times 10^{-7} \text{ M} for ACh and at 23 mM for K\(^+\)). The difference between 1 and 1.3 L\(_{\text{in situ}}\) was not statistically significant. The sensitivity also increased as a function of lengthening (EC\(_{50}\) values were 5.8 \times 10^{-5}, 6.0 \times 10^{-6}, and 1.8 \times 10^{-6} \text{ M} for ACh and 36.4, 31.4, and 24.1 mM for K\(^+\)) at 0.7, 1, and 1.3 L\(_{\text{in situ}}\), respectively). Not shown in these figures is that the maximal force generated by both ACh and K\(^+\) was greater than the force generated by EFS.

The length dependency of ASM force was confirmed in human bronchial rings in response to MCh (Fig. 3): 10^{-3} \text{ M} did not increase bronchial force above what was obtained with 10^{-4} \text{ M}, indicating that a plateau was attained. The effects of length (P = 0.002), concentration (P = 0.02), as well as their interaction (P < 0.0001) were significant. The same results were obtained when only MCh-induced force (i.e., total force minus the resting force) was analyzed. The variability was mainly attributed to the fact that the bronchial force generated by ACh or K\(^+\) is smaller than that generated by EFS.
chial rings were of different size. Notably, the force generated by MCh was very weak at 0.7 \( L_{\text{in situ}} \), even at the highest concentrations.

Figure 4 demonstrates how we went on to estimate the gain of force due to thinning of ASM in asthma. Figure 4A illustrates airways from the 10th generation inflated to FRC from either a normal or an asthmatic subject. In this example, increasing the thickness of the layers internal to ASM by 93.0% and ASM layer by 211.3% increases the perimeter at the middle of ASM layer (so ASM length) by 9.4%. The estimated ASM elongation for every airway generation is shown in Table 1. The greater ratio of lumen to ASM perimeters shown in Table 1 is also an indication that ASM elongation is present in asthma. Figure 4B demonstrates this effect at any given luminal area for an airway of the 10th generation. The lines are curvilinear because of the nonlinear relationship between area (\( A \)) and perimeter (\( P \)) [\( A = \pi(P/2\pi)^2 \)]. At the same luminal area, the length of asthmatic ASM is shifted up, as indicated by the arrows. Notice that the vertical distance between the two curves increases during luminal narrowing, indicating the effect of the gain of force due to ASM elongation increases during airway narrowing. Figure 4C illustrates the relationship between ASM force and length in response to a given concentration of ACh (here \( 3 \times 10^{-5} \) M). These data are the same as those presented in Fig. 2A. Notice that, at that concentration, the slope was different between 0.7 to 1 \( L_{\text{in situ}} \) vs. 1 to 1.3 \( L_{\text{in situ}} \). Assuming that normal and asthmatic ASM generates the same amount of force at the same length, our results indicate that a 9.4% increase of asthmatic ASM length at FRC (Fig. 2B) would increase ASM force by 14.8% in response to \( 3 \times 10^{-5} \) M of ACh. Notice that the gain of force observed in the asthmatic subject is exclusively dictated by ASM elongation, not by the increased mass of ASM. The effect of ASM enlargement is discussed below. The gain of force at every luminal area is presented in Fig. 4D. The asthmatic curve is shifted to the left. The implication is that, for any given luminal area, the force generated by ASM is greater in the asthmatic airway because it operates at a longer length. The inflection point in both curves is due to the fact that we only measured ASM force at three different lengths, together with the fact that we obtained different slopes between 0.7 to 1 \( L_{\text{in situ}} \) vs. 1 to 1.3 \( L_{\text{in situ}} \) at that concentration of ACh (Fig. 4C).

One can envision two different extreme scenarios by which the area internal to the epithelium can be identical between normal and asthmatic. The first one is outward airway wall remodeling with no change of lung inflation, and the second one is inward airway remodeling combined with lung hyperinflation.

The results of the first scenario are presented in Fig. 5. Figure 5A shows both the tension generated by ASM at \( 3 \times 10^{-5} \) M of ACh and the loads impeding ASM shortening in a normal and a remodeled asthmatic airway of the 10th generation. The third tension curve on top of Fig. 5A represents the gain of force that would be due to elongation combined with increased ASM mass, as seen in remodeled asthmatic airways (providing that the enlarged ASM generates the same amount of stress). As seen, the increased mass predominates over the other effects reported here and will not be discussed further since it is well described that it has the strongest potential effect on airway responsiveness (16, 21).

The loads (curvy dashed lines) presented in Fig. 5A are the theoretical loads impeding ASM shortening in normal and remodeled asthmatic airways (solid black and gray lines, respectively). These loads are derived from Lambert’s analyses (16) (see METHODS). It was previously established that the load is decreased in asthmatic subjects because of thickening of the adventitia (16). In this example of the 10th generation airway stimulated with \( 3 \times 10^{-5} \) M of ACh, the gain of force caused by ASM elongation due to outward remodeling would decrease airway luminal area by 15% (relative to FRC) compared with ASM not elongated and subjected to the same load (left-pointing arrow). With the decreased load experienced by asthmatic ASM due to remodeling, narrowing would be amplified. The narrowing would be further potentiated if the gain of force due to ASM enlargement is considered. A histogram of those results is shown in Fig. 5B. From left to right, one can thus appreciate the value for unstimulated ASM at FRC (shaded bar) and the additive effects of the following: 1) ACh stimulation (open bar); 2) the gain of force due to ASM elongation (solid bar); 3) the geometrical effect of remodeling (vertical striped bar); and 4) the gain of force due to ASM enlargement (diagonal hatched bar). The attendant ASM shortening and increased resistance to airflow in those different conditions are shown in Fig. 5, C and D, respectively.

These analyses were done for every airway generation at \( 3 \times 10^{-5} \) M of ACh. The results are presented in Fig. 5E. The gain of force due to ASM elongation has the greatest effect on airway resistance to airflow in airway generations 10 and 11. For those generations, the small gain of force due to ASM elongation actually led to near airway closure, which explains the huge increase in resistance.

The analyses done for every generation were then done for every ACh concentrations presented in Fig. 2A (not shown). Airway closure occurs at more peripheral airway generations at low ACh concentrations and moves progressively to larger airways as ACh concentration is increased. When all of the airways of every generation were combined in series and in parallel in a modeled dichotomous airway tree (and assuming
that the resistance can be calculated based on Poiseuille equation for laminar flow), it was predicted that the increased force induced by increasing the operating length of ASM will enhance the ACh-induced increase of tracheobronchial tree resistance to airflow. The concentration-response curve is presented in Fig. 5.

The results of the second scenario are presented in Fig. 6. Here, a combination of inward remodeling and hyperinflation would increase the operating length of ASM to the same extent as in the first scenario. However, the increase of PL will also increase the load impeding ASM shortening. The loads and the stress generated by ASM at 3 x 10^-5 M of ACh in this example 3 x 10^-5 M of ACh. The force generated by EFS at the FRC L_{in situ} is also indicated for reference. D: the force generated by ACh (in this example 3 x 10^-5 M) at any given luminal area for both normal and asthmatic airways. The luminal areas in a normal airway at FRC and total lung capacity (TLC) are again shown for references. The leftward-pointing arrows starting from FRC and TLC indicate the points on the two curves (normal and asthmatic) where ASM is at the same actual length.

The resistance can be calculated based on Poiseuille equation for laminar flow. The resistance can be calculated based on Poiseuille equation for laminar flow, it was predicted that the increased force induced by increasing the operating length of ASM will enhance the ACh-induced increase of tracheobronchial tree resistance to airflow. The concentration-response curve is presented in Fig. 5F.

The results of the second scenario are presented in Fig. 6. Here, a combination of inward remodeling and hyperinflation would increase the operating length of ASM to the same extent as in the first scenario. However, the increase of Pt will also increase the load impeding ASM shortening. The loads and the stress generated by ASM at 3 x 10^-5 M of ACh for an airway of the 10th generation are shown in Fig. 6A. While inward remodeling encroaches upon the lumen, increasing the Pt protects against narrowing. However, it is important to realize that, for any given load, the elongated ASM can cause more narrowing. A histogram of those results is shown in Fig. 6B. From left to right, one can thus appreciate the value for unstimulated ASM at FRC (shaded bars) and the additive effects of the following: 1) ACh stimulation (open bar); and 2) the gain of force due to ASM elongation (solid bar). This is shown for both normal and asthmatic airway geometry at both 5 and 9 cmH_2O of Pt.

The attendant ASM shortening and increased resistance to airflow in those different conditions are shown in Fig. 6, C and D, respectively.

These analyses were done for every airway generation (at 3 x 10^-5 M of ACh). The results are presented in Fig. 6E. The Pt that would be required in asthmatic airways to renormalize luminal area was different for every generation. The required Pt was not calculated for generations 0–2 because it would need a Pt higher than 30 cmH_2O. We also encountered a problem with smaller airways (generation 11 and higher). In those airways, inward remodeling almost completely filled the airway lumen. Consequently, the pressure-area curves predicted that, during bronchoconstriction, the area of the lumen would reach negative values before transitioning from the point of tension to compression (at a Pt of 0), which is impossible. So in those generations, we converted the “remaining” remodeling (the part that did not fit into the lumen at a Pt of 0) to outward remodeling, as done for the first scenario. We were also unable to calculate the airway resistance to airflow of the entire tracheobronchial tree. This is because the Pt required to renormalize the cross-sectional area would need to be the...
same in every generation for the airways of the entire lung to be considered together.

DISCUSSION

Our results represent incremental progress on observations made in previous studies (8, 9, 19, 32, 33), showing that the force generated in response to a given contractile stimulus is dependent on ASM length. Importantly, instead of setting the length of ASM at $L_o$ (i.e., the length at which ASM generates maximal force), as was previously done, we studied ASM at its in situ FRC length. By changing the operating length of the ASM within the physiological range produced by breathing maneuvers (6) or postural changes (28), we demonstrated that longer ASM generates greater force in response to contractile
stimuli. The implication is that ASM neither operates at peak length on the bell-shaped curve that describes the relationship between length and force, nor within the range of lengths near $L_o$ in which a plateau of force can be observed following length adaptation (4). Instead, ASM length in vivo seems to operate on the ascending limb of this bell-shaped curve, implying that anything that stretches the airway wall in vivo will concomitantly increase ASM force.

We also demonstrated that this effect does not rely on G protein-coupled receptor activation, since both the sensitivity and the maximal force generated by increasing concentration of $K^+$ was also length dependent (the later causes ASM contraction by plasmalemma depolarization). The magnitude of this length dependency of ASM force is not small. A 30% change of muscle length (length reduction or elongation) changes the concentration of ACh required to generate a given response by approximately one log. So for a shorter (0.7 $L_{in situ}$) ASM to generate the same force as a muscle set at $L_{in situ}$, an ~10-fold higher concentration of ACh is required, and for a longer (1.3 $L_{in situ}$) muscle to generate the same force as a muscle set at $L_{in situ}$, an ~10-fold lower concentration of ACh is sufficient. In addition, both increased reactivity (maximal response achieved) and increased sensitivity (responsive to lower concentrations) were observed at longer ASM length, which genuinely mimics the pathognomonic features of AHR in asthma (31). There are obviously many other muscle (1) and nonmuscle (3) factors that can contribute to AHR in asthma. However, our results suggest that increasing the operating length of ASM could be another factor contributing to AHR in some asthmatic individuals, especially in those with outward remodeling (3) factors that can contribute to AHR in asthma. The bell-shaped curve that defines the relationship between ASM length and ASM force has been well characterized, and most studies investigating the mechanical properties of ASM have studied the muscle preparations at $L_{o}$. This raises the important question of whether the $L_{o}$ is the same or near the $L_{in situ}$; i.e., the length at which the ASM operates when it encircles the airways. By taking many precautions to maintain $L_{in situ}$, our results demonstrated that the $L_{in situ}$ of ASM is substantially shorter than the $L_{o}$. This observation also raises a second important question of whether the amount of ASM stretch that is required to reach the descending limb of the bell-shaped curve is anywhere near the physiological range of stretch that ASM can experience in vivo. In this regard, our results suggested that the eventual stretch causing force reduction ex vivo may not be physiologically attainable. In fact, our results showed that, within the range of lengths at which ASM operates in situ, which fluctuate because of breathing maneuvers and postural changes, the force generated by ASM increases when its length increases. Our results are consistent with the work of Gunst and Stropp (9), who studied bronchial airways from dogs, in which the maximal circumferential tension generated in response to isovolumetric contractions elicited by ACh occurred at near-maximal bronchial volume. The length dependency of ASM force was confirmed in human bronchial rings in response to MCh. Together, it suggests that a lot of studies measuring ASM contractility have overestimated the length of ASM.

$EFS$ vs. $ACh$. Our results clearly demonstrated that the force generated by ACh and K$^+$ increases with incremental changes of length no matter the concentration used. Although the force generated by EFS was also greater at $L_{in situ}$ compared with 0.7 $L_{in situ}$ a plateau was observed between $L_{in situ}$ and 1.3 $L_{in situ}$. This increase of EFS-induced force followed by a plateau was previously documented in human bronchi during incremental augmentation of resting tension (29). It is unclear why there was a difference in the length dependency of ASM force between EFS vs. ACh and K$^+$. The possibility remains that the length dependency of EFS-induced force is different from that of ACh and K$^+$-induced force because the EFS parameters (frequency, voltage, and pulse duration) were only optimized at $L_{in situ}$ in our experiments, and they may have been suboptimal at the other tested lengths.

Molecular mechanisms. The molecular mechanisms responsible for the length dependency of ASM force, both of the increased maximal response and sensitivity, are not completely understood. Yoo and coworkers (32) have shown, using bovine tracheal smooth muscle preparations, that phosphatidylinositol turnover, intracellular Ca$^{2+}$ concentration, and regulatory myosin light chain (rMLC) phosphorylation in response to muscarinic activation were decreased at shorter ASM lengths. Mehta and coworkers (19) also reported a decrease in intracellular Ca$^{2+}$ and rMLC phosphorylation at shorter lengths in canine tracheal muscle preparations in response to both a muscarinic agonist and a high K$^+$ solution. Yoon and coworkers (33) subsequently confirmed the involvement of rMLC phosphorylation in bovine tracheal muscle preparations and also showed that this phenomenon is insensitive to changes in temperature and seemed to be independent of actin filaments.

Length-dependent rearrangement of the contractile apparatus and the cytoskeletal machinery that ensures transmission of force from the contractile apparatus to the surrounding environment can also be involved. The work documenting the phenomenon of length adaptation stipulates that there is an $L_{o}$ (or a range of $L_{o}$ values) at which maximal
overlap between actin and myosin filaments can occur (reviewed in Refs. 4, 26). Other work on the multiprotein cytoskeletal structure that ensures the physical linkage between the contractile apparatus and the ECM (called adhesomes) also suggests that, although dynamically regulated to be optimized at different lengths, maximal mechanotransduction can only occur within a range of ASM lengths (8, 34). Our results clearly demonstrate that the $L_{\text{in situ}}$ that we used in our experiments is shorter than the range of $L_o$ values in which ASM can generate maximal force. Therefore, it is very likely that the length dependency of ASM force observed in the present study is due, in large extent, to
a progressively stronger physical linkage between the contractile apparatus and the ECM, in addition to a progressively greater parallel arrangement of myosin and actin filaments as ASM lengthens.

**Computational analyses.** The potential detrimental consequence of greater ASM force at longer length for airway responsiveness is clear. The modeling analyses presented here suggest that the gain of force caused by increasing the operating length of ASM can greatly influence the degree of ASM shortening and, concomitantly, airway narrowing and the resistance to airflow. Two extreme scenarios were presented. Each of the modeled scenario confirms the same cross-section area for the lumen, but has very different implications in terms of ASM shortening, airway narrowing, and airway resistance to airflow, because they affect the load impeding ASM shortening differently. Therefore, varying mechanisms by which ASM is elongated influence the degree of ASM shortening (discussed further below). It is also understood that the extent by which the area internal to the epithelium of asthmatic airways is corrected for to match the one observed in identical-sized airways of nonasthmatic airways can also vary. For example, inward remodeling may occur without reaching a degree of hyperinflation that will normalize luminal area. This, by itself, would increase baseline resistance to airflow and contribute to AHR. However, any level of outward remodeling or any attempt to normalize baseline airway resistance by hyperinflation will also contribute to AHR by increasing the operating length of ASM. The length dependency of ASM force can thus contribute to AHR to a different extent in different individuals. The important thing to realize is that, whenever ASM is elongated, no matter the magnitude, it will be detrimental.

The level of airway responsiveness is dictated by a plethora of factors. Every factor may influence airway responsiveness either positively or negatively, depending on the direction of the changes. A gain of force will certainly have a negative consequence. However, by how much a gain of force will affect ASM shortening is hard to infer, owing to the nonlinear relationship between the load and ASM tension (see Figs. 5A and 6A). In addition, the relationships between ASM shortening, airway narrowing, and airway resistance to airflow are also not linear. Airway remodeling is extensive in asthma and may affect airway responsiveness for any given Pt at any given level of ASM stress (force/cross-sectional area). For example, thickening of the adventitia “decouples” (geometrically) ASM from the increasing parenchymal load that occurs during bronchoconstriction. Another important variable is the thickening of ASM layer, which would increase the amount of tension generated per unit airway length (providing that the enlarged ASM can generate the same amount of stress) (16). As demonstrated in Fig. 5A, the effect of ASM enlargement is greater than the gain of force due to ASM elongation. Also, because of geometrical factors, and assuming that the mechanical properties of the airway wall remain the same (22), an increased amount of material inside the ASM layer would increase airway narrowing for any given degree of ASM shortening (23). The goal of the present analyses was to quantify the effect of the increased force induced by ASM elongation on airway narrowing and resistance to airflow, independently of the other factors that are known to affect airway responsiveness. This is obviously only possible with computational analyses. In vivo, all of these lung alterations will affect airway responsiveness simultaneously and are likely to act synergistically in the generation of AHR.

As a general statement, one can say that outward remodeling would have the worse adverse effect on airway responsiveness. It will be very important to determine whether inward or outward remodeling occurs in asthmatic subjects. Based on our analyses, another general statement can be that hyperinflation reduces airway responsiveness. This is because the gain of load due to hyperinflation exceeded the gain of force caused by ASM elongation. However, even in this scenario, it is important to understand that the length dependency of ASM force is still detrimental. This is because the responsiveness will still be greater than in another scenario where the same gain of load would not be accompanied by a gain of ASM force. It might be more fair to conclude that the length dependency of ASM force mitigates the protective effect of hyperinflation. Together, it highlights again the intricate interplay that exists between the diverse muscle and nonmuscle factors that can influence airway responsiveness (3).

**Limitations of the model.** The static nature of the model (i.e., fixed Pt) ignores the potentially strong influence of cyclical stresses on ASM contractility (6, 7), to which the airway wall is subjected in vivo. However, our laboratory (24) has previously shown, using the same system, that once ASM is activated by ACh, the cyclical strain that simulates the swings in airway wall tension caused by tidal breathing is small and does not affect ASM force significantly.

The isometric contractions used to quantify ASM tension at different lengths are also an important limitation of our analysis. We have used the regression line describing the relationship between isometric tension and ASM length to estimate the amount of shortening that would occur while ASM operated against a physiological load. The isometric stress generated by the muscle at the points of intersection between these lines may not necessarily represent the load where the length of the muscle would stop shortening following the same degree of muscle activation under a physiological load. The other limitations and assumptions of Lambert’s model have been discussed in detail elsewhere (16, 18).
Another limitation is the use of ovine tracheal strips as a model to study the effect of strain (30% length elongation or reduction) on ASM force. It is understood that the behavior of ovine ASM within the trachea may not necessarily be identical to the behavior of ASM within human bronchi. Having said that, it was convincingly demonstrated that the stress generated by the trachea from muscle was not significantly different from that generated by either large or small bronchi (9). It was also demonstrated that the structural and mechanical properties of human ASM are similar to those of other species (5). We used data of sheep for our modeling analyses because the quantitative accuracy of the strain-stress data obtained in tracheal strips is better than in bronchial rings. This is because tracheal bundles are mainly pure ASM (27), in line with the orientation of our lever system; whereas the orientation of ASM within a bronchial ring is not well defined. The stress-strain relationship within a bronchial ring is further confounded by its circular form, as well as by many nonmuscle structures. It will also be interesting to determine whether the length dependency of ASM force is different between asthmatic subjects and nonasthmatic subjects.

Conclusion. Our results and computational analyses suggest that another ASM contractile property, namely the length dependency of ASM force, can be added to the list of factors that can seriously alter airway responsiveness and, perhaps, contribute to AHR in asthmatic subjects.

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Disclosures

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Author Contributions

Author contributions: A.L.-G. and Y.B. analyzed data; A.L.-G. and Y.B. prepared figures; A.L.-G., C.D.P., C.C., C.L.F., J.-Y.S., and Y.B. contributed to AHR in asthmatic subjects. No conflicts of interest, financial or otherwise, are declared by the author(s).

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