Airway basement membrane perimeter in human airways is not a constant; potential implications for airway remodeling in asthma

Brent E. McParland,1 Peter D. Paré,2 Peter R. A. Johnson,1 Carol L. Armour,3 and Judith L. Black1

Departments of 1Pharmacology and 3Pharmacy, University of Sydney, New South Wales 2006, Australia; and 2University of British Columbia, James Hogg iCAPTURE Centre for Cardiovascular and Pulmonary Research, St. Paul’s Hospital, Vancouver, British Columbia, Canada V6Z 1Y6

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McParland, Brent E., Peter D. Paré, Peter R. A. Johnson, Carol L. Armour, and Judith L. Black. Airway basement membrane perimeter in human airways is not a constant; potential implications for airway remodeling in asthma. J Appl Physiol 97: 556–563, 2004. First published April 2, 2004; 10.1152/japplphysiol.00982.2003.—Many studies that demonstrate an increase in airway smooth muscle in asthmatic patients rely on the assumption that bronchial internal perimeter (P_i) or basement membrane perimeter (P_bm) is a constant, i.e., not affected by fixation pressure or the degree of smooth muscle shortening. Because it is the basement membrane that has been purported to be the indistensible structure, this study examines the assumption that P_bm is not affected by fixation pressure. P_bm was determined for the same human airway segment (n = 12) fixed at distending pressures of 0 cmH2O and 21 cmH2O in the absence of smooth muscle tone. P_bm for the segment fixed at 0 cmH2O was determined morphometrically, and the P_bm for the same segment, had the segment been fixed at 21 cmH2O, was predicted from knowing the luminal volume and length of the airway when distended to 21 cmH2O (organ bath-derived P_i). To ensure an accurate transformation of the organ bath-derived P_i value to a morphology-derived P_bm value, had the segment been fixed at 21 cmH2O, the relationship between organ bath-derived P_i and morphology-derived P_bm was determined for five different bronchial segments distended to 21 cmH2O and fixed at 21 cmH2O (r^2 = 0.99, P < 0.0001). Mean P_bm for bronchial segments fixed at 0 cmH2O was 9.4 ± 0.4 mm, whereas mean predicted P_bm, had the segments been fixed at 21 cmH2O, was 14.1 ± 0.5 mm (P < 0.0001). This indicates that P_bm is not a constant when isolated airway segments without smooth muscle tone are fixed distended to 21 cmH2O. The implication of these results is that the increase in smooth muscle mass in asthma may have been overestimated in some previous studies. Therefore, further studies are required to examine the potential artifact using whole lungs with and without abolition of airway smooth muscle tone and/or inflation. bronchial hyperreactivity; pathology; smooth muscle; morphometry

IT IS GENERALLY ACCEPTED that airway smooth muscle (ASM) mass is increased in asthma (2, 3, 6–8, 10, 12, 16, 18, 27–29), and it has been suggested that the increase in smooth muscle mass may play a pivotal role in causing exaggerated airway narrowing, which is a characteristic feature of asthma (19). Early studies showing that ASM mass was increased in asthma were criticized on the basis that the comparisons may have been between airways with different internal diameters (6, 16). In the cardiovascular field, this selection bias was overcome by normalizing the cross-sectional area of vascular wall structures to the internal elastic lamina, which was found to be a constant over a wide range of physiological pressures (4). This observation was also found to be valid for the internal perimeter (P_i) of guinea pig airways (15), and in both guinea pig and human airways P_i appeared constant in the face of substantial ASM shortening, i.e., the airway mucosa folded rather than narrowed concentrically at low distending pressures and during ASM contraction (14). These observations led to several studies in which airway wall-compartment dimensions were normalized to P_i or basement membrane perimeter (P_bm) (2, 3, 7, 8, 16, 18, 27), and the results confirmed previous findings that ASM, submucosal, and adventitial areas are increased in asthma.

In an airway cut in cross section, the P_i follows the luminal surface of the epithelium, and P_bm follows the basement membrane that subtends the epithelium. Therefore, the difference in length for the two measurements depends on the cross-sectional area of the epithelium. In the present study, we reexamined the assumption that P_i and P_bm are constant over a range of distending pressures used to fix human bronchi. Both measurements were chosen because, although it is the basement membrane layer that has been purported to be the indistensible structure in the face of substantial airway narrowing or distension (15), historically it was the P_i that was alleged to be a constant (14). In most previous studies (2, 3, 6–8, 10, 16, 18, 28, 29), comparison of airway dimensions have been made on lungs obtained at postmortem, which for nondiseased lungs were usually fixed by inflating with formalin until the lung was fully distended (inflation pressure of 20–30 cmH2O). Asthmatic lungs, on the other hand, are often fixed without inflation. If P_i and P_bm increase as a result of fixing lungs inflated, then normalized airway wall-compartment areas, including ASM area (WA_m), will appear decreased in nondiseased airways relative to asthmatic airways. In addition, lengthening of the airway during pressure fixation will further reduce the apparent wall area. Even when asthmatic lungs are fixed by inflation, it is likely that increased stiffness of the airways due to a thickened reticular layer, increased extracellular matrix, and increased ASM tone, especially in patients who die in status asthmaticus, would decrease the degree of airway distension relative to that for nondiseased lungs.

Therefore, the objective of this study was to investigate the hypothesis that, in human isolated airway segments, without smooth muscle tone, the P_bm is increased if airways are fixed inflated at 21 cmH2O compared with airways fixed without inflation (~0 cmH2O). To test this hypothesis, we studied
human bronchial segments predominantly from nonasthmatic subjects obtained at the time of surgical resection.

MATERIALS AND METHODS

Summary of the theoretical basis used for experimental methodology. It is not possible to obtain a measurement of $P_{bm}$ at two different fixation pressures for the same airway. One way around this problem would be to compare the $P_{bm}$ of airways fixed at 21 cmH$_2$O with airways fixed at 0 cmH$_2$O, which are represented by airways A’ and B’, respectively, in Fig. 1. Such a study, however, could then be criticized on the basis that the comparison between the two groups was made between airways that could have had different

In order to overcome this limitation, the present study uniquely addresses this problem by predicting the theoretical $P_{bm}$ of the airways fixed at 0 cmH$_2$O (airway B’), had they been fixed at 21 cmH$_2$O (airway B”). Due to the complexity of the methodology used, the three critical steps required to determine the predicted $P_{bm}$, had the segments been fixed at 21 cmH$_2$O, have been outlined below.

The first step is to measure the volume and length of all segments at 21 cmH$_2$O in an organ bath and calculate the average $P_i$ of the preparations (A and B, Fig. 1) assuming the luminal geometry to be a cylinder.

The second step is to establish the relationship between the $P_i$ calculated from the volumetric measurement (A, Fig. 1) at 21 cmH$_2$O and the $P_{bm}$ measured morphometrically (A’, Fig. 1) on airways fixed at 21 cmH$_2$O. This was done to predict the theoretical $P_{bm}$ (B’, Fig. 1), if the airways that were fixed at 0 cmH$_2$O were fixed at 21 cmH$_2$O, based on their volumetrically determined $P_i$ at 21 cmH$_2$O (B, Fig. 1).

The third step is to compare the predicted $P_{bm}$ at 21 cmH$_2$O (B’, Fig. 1) to the measured $P_{bm}$ at 0 cmH$_2$O (B’, Fig. 1). The difference in fixation length has been reported as apparent strain.

Lung collection and preparation of human bronchial segments. Human lung was obtained from patients undergoing surgical resection for either carcinoma or lung transplantation. Collection of lung and use of lung specimens was approved by the Human Ethics Committee at the University of Sydney. Hospital pathologists examined all surgical specimens macroscopically to ensure that the bronchial segments that were studied were not invaded by carcinoma. Tissue was immediately transported to the laboratory in ice-cold carbogased (5% CO$_2$ in oxygen) Krebs-Henseleit solution [composition (in mM): 118 NaCl, 4.7 KCl, 2.5 CaCl$_2$, 1.2 MgSO$_4$, 1.2 NaH$_2$PO$_4$, 25.5 NaHCO$_3$, 11.1 d-glucose]. For measurement of $P_{bm}$ and $P_i$, a total of 18 bronchial segments from 14 patients with a variety of disease conditions were prepared using a method described for canine lung (22). Briefly, bronchi were dissected free of the surrounding parenchyma, and bifurcations were tied off as close to the main stem bronchus as possible to produce a bronchial segment (1.4–2.4 cm long, 3–6 mm mean internal diameter at 7 cmH$_2$O transmural pressure) that was “fluid tight” throughout its length. Bronchial segments were attached to organ bath adapters, placed into a 50-ml horizontal organ bath (Respiratory Research Group, Sydney, Australia), stretched to 120% of resting axial length to minimize further lengthwise stretching, and set at a transmural distending pressure of 7 cmH$_2$O. The bronchial segments were bathed in, and perfused by, Krebs-Henseleit solution maintained at 37°C and continually gassed with 5% CO$_2$ in oxygen to maintain a pH of 7.35. Segments were equilibrated for 90 min, during which the bathing fluid on the inside and outside of the bronchial segment was exchanged at intervals of 15 min.

Organ bath derived luminal volume and calculated $P_i$. After the equilibration period, isoproterenol (100 μM, Sigma Chemical, St. Louis, MO) was added to the organ bath to fully relax bronchial segments (9). After complete relaxation, the luminal volume of the bronchial segment at transmural pressures of 7 and 21 cmH$_2$O was estimated by measuring the volume of luminal fluid that was expelled to a column by applying a positive pressure (~50 cmH$_2$O) to the outside of the bronchial segment (23). In the absence of ASM tone, the transverse cross-sectional profiles of the bronchial segment at 7 and 21 cmH$_2$O were assumed to be represented by a circle. The average $P_i$ was calculated from knowledge of the length (L; in cm) and volume (V; in ml) of the segment (Eq. 1). The length of the preparation was measured with a Vernier caliper as the distance between the ends of the two adapters, which only included the compressible section of the airway segment

\[
V (\text{cylinder}) = \frac{LP_i^2}{4\pi}, \text{ solving for } P_i = 2\sqrt{\frac{V}{L}} \tag{1}
\]

Morphometry measurements. On completion of the organ bath estimate of $P_i$, 13 tissues from 9 patients were fixed without inflation by placing the tissues directly into neutral-buffered, 10% formalin (transmural pressure ~ 0 cmH$_2$O) for not less than 48 h before tissue processing. These preparations were also allowed to assume an unstressed length (no axial strain). Five bronchial segments from five patients were fixed by inflation (21 cmH$_2$O) at a length of 120% unstressed length (axial strain). The fixed bronchial segments were embedded in paraffin wax, 6-μm cross sections were cut serially at 0.8-mm intervals down the entire length, and sections were expanded by floating on water at 45°C before adhering to slides. Sections were stained with Gomori elastin trichrome stain (Probing and Structure, Queensland, Australia). All nomenclature for the airway dimensions and areas were as proposed by Bai et al. (1). Measurements of perimeter were taken for all sections, whereas four sections equally spaced down the length of the segment were used for measuring $W_{Am}$, epithelial layer thickness, and subepithelial layer thickness.

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The following perimeter measurements were taken: outer airway wall, cartilage, \( P_{\text{bm}} \), and \( P_i \). The total wall area (\( W_{\text{A}} \)) was derived by subtracting the area subtended by \( P_i \) from the area subtended by outer airway wall perimeter. Point counting was used to estimate the mean \( W_{\text{A}} \). Epithelial layer thickness and subepithelial layer thickness were quantified by tracing the entire perimeter of the epithelial layer from images captured at a magnification of \( \times400 \). For each airway section, three fields of view were chosen, which were based on clock positions 4, 9, and 12. By knowing the perimeter (\( P \)) and the area (\( \Lambda \)), the thickness (\( T \)) was determined by solving a quadratic expression \( T = [P - \pi P^2 - 16\Lambda]^{1/2}/4 \). Epithelial wall area was derived by multiplying the average thickness for each section by the \( P_i \). Measurements of length and area were multiplied by estimated shrinkage factors of 1.11 and 1.21, respectively.

Sample calculation for determining organ bath \( P_i \), morphometry \( P_{\text{bm}} \), and apparent strain. The following is an example of the calculations performed to determine the predicted morphometry-derived \( P_{\text{bm}} \) for one bronchial segment had it been fixed distended to 21 cmH\(_2\)O instead of 0 cmH\(_2\)O. Luminal volume (113 \( \mu \)l) and length (14.2 mm) gave an organ bath-derived \( P_i \) of 10.0 mm (Eq. 1). \( P_{\text{bm}} \) derived from morphometry in the same specimen fixed at 0 cmH\(_2\)O was 6.5 mm. The organ bath \( P_i \) at 21 cmH\(_2\)O was then used to determine a predicted morphometry-derived \( P_{\text{bm}} \) value if the segment had been fixed at 21 cmH\(_2\)O. To make this adjustment, bronchial segments fixed while distended to 21 cmH\(_2\)O were used to derive the linear regression equation between the organ bath-derived \( P_i \) values at 21 cmH\(_2\)O and the morphometry-derived \( P_{\text{bm}} \) values. The equation for morphometry-derived \( P_{\text{bm}} \) was 1.37-organ bath-derived \( P_i \) - 2.3 (\( R^2 = 0.99 \)). When this equation was used for this example, the \( P_{\text{bm}} \) with the segment been fixed at 21 cmH\(_2\)O, was equal to 11.4 mm = 1.37 -10.0 mm - 2.3 mm. This gives a fixation-induced apparent strain value for \( P_{\text{bm}} \) equal to 0.75. - (11.4 - 6.5)/6.5. In additional analyses, both the contribution to the luminal volume made by bifurcations down the length of the airway segment and the effect of cone geometry rather than cylinder geometry were factored into the measurement of mean \( W_{\text{A}} \) and strain. The inclusions of these parameters had no significant effect on the final measurement of strain (data not shown).

Examination of a possible source for the underestimation of organ bath-derived \( P_i \). The organ bath-derived \( P_i \) value underestimated the morphometry-derived \( P_{\text{bm}} \) value (Fig. 3). The reason for the discrepancy is apparent by observation of the sections fixed at 21 cmH\(_2\)O (Fig. 2): the cross-sectional areas of the airway lumen are not perfectly circular. To compensate for this, a post hoc adjustment of the organ bath-derived \( P_i \) was performed for bronchial segments fixed at 21 cmH\(_2\)O using

\[
adjP_i = \frac{\text{morphometry}_{P_{\text{bm}}}}{P_{\text{bm}}} P_i
\]

where \( \text{morphometry}_{P_{\text{bm}}} \) is the morphometry-derived \( P_{\text{bm}} \). \( P_{\text{bm}} \) is a theoretical perimeter derived from knowing the area enclosed by \( \text{morphometry}_{P_{\text{bm}}} \), if that area was circular, the ratio of \( \text{morphometry}_{P_{\text{bm}}} / P_{\text{bm}} \) represents the circularity factor, and \( \text{organ bath}_{P_i} \) is the organ bath-derived \( P_i \).

Pressure-volume curves with and without smooth muscle tone. To test for a possible effect of intrinsic ASM tone on \( P_i \), 10 additional segments from 9 different patients were studied. Pressure-volume points were obtained (0, 3, 7, 11, 16, and 21 cmH\(_2\)O) in the presence of intrinsic ASM tone and after maximal ASM relaxation, which was achieved by adding isoproterenol (100 \( \mu \)M). Volume at each transmural pressure was determined by applying an external pressure of 50 cmH\(_2\)O to the outside of the bronchial segment as described above. If length is assumed to be a constant with respect to inflation, then the relative volumes for volumes as a percent of maximum, would be the same as cross-sectional area. This is possible because the airway segments were lengthened by 20% (axially strained) of the unstressed length before inflation.

Analysis of data. Unless otherwise stated, nontransformed data are expressed as means \( \pm \) SE. Area data were transformed by taking the square root so as to establish a linear relationship with \( P_{\text{bm}} \) and 95% confidence intervals were used for these data. Comparisons of morphometric measurements obtained from bronchi fixed without inflation (\( \approx 0 \) cmH\(_2\)O) and with inflation (21 cmH\(_2\)O) were performed using ANOVA and Fisher’s protected least-squares difference test to detect significant differences, defined as \( P \leq 0.05 \). Paired t-tests were used to compare morphometry and predicted \( P_i \) in specimens fixed at 0 and 21 cmH\(_2\)O. Least-squares regression analysis based on measured and calculated values from segments fixed at 21 cmH\(_2\)O were used to derive the predicted morphometry-derived \( P_{\text{bm}} \) values at 7 and 21 cmH\(_2\)O for airways fixed at 0 cmH\(_2\)O. The program Microsoft Excel 2002 was used for data management, and all statistics were performed using Statview (version 5.01).

**RESULTS**

**Patient data.** Twenty-eight bronchial segments were used in this study. Of these, 13 were fixed by passive perfusion (\( \approx 0 \) cmH\(_2\)O), 5 were fixed with pressure (21 cmH\(_2\)O), and the other 10 (9 patients) were used for pressure-volume curves and were not fixed. Of the 13 bronchial segments fixed at 0 cmH\(_2\)O, 8 paired segments were obtained from four patients and the remaining 5 were from five different patients. One of the 13 tissues studied was considered to be an outlier, because the degree of \( P_{\text{bm}} \) strain (132%) was \( >2 \) standard deviations from the mean, suggesting the possibility of a measurement error. Therefore, to minimize bias, this tissue was removed from the experimental analysis. Bronchial segments fixed with pressure (21 cmH\(_2\)O) were all from different patients. The bronchial segments were obtained from patients who had a range of conditions and operative procedures. The mean patient age for patients whose bronchial segments were fixed at 0 cmH\(_2\)O (47 \( \pm \) 6 yr) was not different from those whose segments were fixed at 21 cmH\(_2\)O (53 \( \pm \) 10 yr).

**Organ bath-derived luminal volume and \( P_i \).** There was no significant difference in luminal volume at 21 cmH\(_2\)O [209 \( \mu \)l (95% confidence interval: 183-237 \( \mu \)l) vs. 203 \( \mu \)l (95% confidence interval: 124-300 \( \mu \)l)], length (1.9 \( \pm \) 0.1 vs. 2.1 \( \pm \) 0.2 cm), or the raw organ bath-derived \( P_i \) (11.9 \( \pm \) 0.3 vs. 11.0 \( \pm \) 0.2 cm)
Results for individual segments fixed at 21 and 0 cmH₂O are shown in Tables 1 and 2, respectively, and the correlations between organ bath-derived \( P_i \) and morphometry-derived \( P_{bm} \) are shown in Fig. 3. Morphometry-derived \( P_{bm} \) was significantly longer (12.8 ± 1.1 mm) for segments fixed at 21 cmH₂O (\( n = 5 \) segments) than segments fixed without pressure (9.4 ± 0.4 mm, \( P < 0.01 \); \( n = 12 \) segments from 8 patients).

For bronchial segments fixed at 21 cmH₂O in the organ bath, \( P_i \) (Eq. 1) was highly correlated with the morphometry \( P_{bm} \) \([P_{bm} = 1.37\times\text{organ-bath } P_i, (\text{in mm}) - 2.3; R^2 = 0.997, P < 0.0001; \text{Fig. 3}]\). By incorporating the mean circularity factor (1.16 ± 0.02; range: 1.10–1.25) the difference between organ-bath \( P_i \) and morphometry \( P_i \) was largely accounted for (\( P_{bm} = 1.00P_i; R^2 = 0.96 \)). A paired \( t \)-test indicated no significant difference between morphometry-derived \( P_i \) (12.7 ± 1.1 mm) and organ bath-derived \( P_i \) that was corrected for deviations in cross-sectional circularity (corrected \( P_i \): 12.8 ± 1.1 mm). Because morphometry \( P_{bm} \) and organ-bath \( P_i \) at 21 cmH₂O were highly correlated (\( R^2 = 0.99 \)), organ-bath \( P_i \) for bronchial segments fixed at 0 cmH₂O could be accurately converted to give predicted \( P_{bm} \) morphometry values if the segments had been fixed at 21 cmH₂O (Table 2). Predicted values for \( P_{bm} \) and \( P_i \) were determined at 7 and 21 cmH₂O. It was assumed that the deviation from circularity was the same at 7 and 21 cmH₂O. The average apparent strain of the basement membrane for airways fixed at 7 and 21 cmH₂O are shown in Fig. 4, and the relationship between pressure and strain was linear over the pressure range studied. The mean \( P_{bm} \) strain at 7 and 21 cmH₂O was 20 ± 6 and 52 ± 6%, respectively (Fig. 4). The mean between-patient coefficient of variation for strain at 21 cmH₂O was 42% (\( n = 8 \) patients), and the mean within-patient coefficient of variation was 13 ± 3% (\( n = 4 \) pairs of bronchi from 4 patients).

The mean three-point pressure-volume curve derived for the tissues used for morphometry was not significantly different from the more complete curve obtained from 10 different isoproterenol-relaxed tissues (Fig. 5). It is apparent that the pressure-volume relationship of the relaxed preparations is biphasic: a steeper relatively linear slope between 0 and 7 cmH₂O.
cmH2O and a separate flatter relatively linear relationship between 7 and 21 cmH2O. The inflexion point on the pressure-volume curve was determined to occur at ~7 cmH2O. The pressure-volume curve in the presence of intrinsic ASM tone is relatively linear over the entire range of pressures, and the mean segment volume at 21 cmH2O is approximately equal to the volume of the relaxed preparations at 7 cmH2O.

Quantification of bronchial wall compartments. Table 3 shows the airway wall compartment areas (mm2) and the √WAm divided by Pb m for airways fixed at 0 and 21 cmH2O. Because the length of the preparations fixed at 0 cmH2O was ~70% of the organ-bath length when stretched axially to 120% of the unstressed length, one would have predicted a 43% increase in the area of the wall components if wall volume was conserved. This result is what was observed for epithelial wall area (P < 0.05) and WAm (P < 0.05; 1-tail t-test assuming

<table>
<thead>
<tr>
<th>Compartment</th>
<th>21 cmH2O</th>
<th>0 cmH2O</th>
<th>P Value</th>
<th>Fold Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>WAe</td>
<td>0.24 (0.17–0.33)</td>
<td>0.37 (0.30–0.45)</td>
<td>0.045</td>
<td>1.55</td>
</tr>
<tr>
<td>WAm</td>
<td>0.19 (0.11–0.29)</td>
<td>0.27 (0.21–0.33)</td>
<td>0.05*</td>
<td>1.42</td>
</tr>
<tr>
<td>WaI21</td>
<td>7.3 (5.4–9.5)</td>
<td>6.0 (5.2–6.9)</td>
<td>0.17</td>
<td>0.82</td>
</tr>
<tr>
<td>%WAm/WAe</td>
<td>2.7 ± 0.3</td>
<td>5.1 ± 0.7</td>
<td>0.002</td>
<td>1.88</td>
</tr>
<tr>
<td>√WAm/Pbm</td>
<td>0.034 ± 0.004</td>
<td>0.058 ± 0.004</td>
<td>0.003</td>
<td>1.71</td>
</tr>
</tbody>
</table>

WAe, epithelial wall area; WAm, airway smooth muscle wall area; WA1, total wall area. *Significantly different by one-tail t-test. Values are means with either 95% confidence intervals, shown in parenthesis, or ± SE. Cross-sectional area for the epithelial layer was determined by multiplying the thickness of the respective layers by the measured Pb m.
DISCUSSION

The results of this study indicate that airway $P_t$ and $P_{bm}$ do not remain constant for human in vitro bronchial segments in which the smooth muscle has been relaxed with isoproterenol. $P_{bm}$ was observed to be $\sim 52\%$ greater in length for airways fixed inflated at 21 cmH$_2$O [=$\text{total lung capacity (TLC)}$] compared with airways fixed without inflation at 0 cmH$_2$O. After tissue processing, the bronchial segments fixed inflated (21 cmH$_2$O) were $\sim 1.41$ times longer and the cross-sectional $W_{Am}$ was correspondingly $\sim 41\%$ less than segments that were fixed at 0 cmH$_2$O without lengthwise stretching. By combining the effect of fixation pressure on $P_{bm}$ and the effect of lengthwise stretching on cross-sectional area, a processing artifact induced an apparent 2.9-fold increase in $P_{bm}$-normalized $W_{Am}$ for segments fixed at 0 cmH$_2$O.

Postmortem lungs from asthmatics who die in status asthmaticus are often fixed without inflating the lung ($\sim 0$ cmH$_2$O) (2, 6–8, 16, 27). On the other hand, nonasthmatic lungs obtained postmortem are usually fixed by inflating the lung (20–30 cmH$_2$O). In some studies, asthmatic lungs are also fixed inflated (3, 28), but in these lungs severe mucous plugging and increased airway stiffness (high passive tone) may reduce the degree of bronchial distension.

In the present study, bronchial segments fixed without inflation ($\sim 0$ cmH$_2$O) simulated the fixation process usually used for fixing constricted asthmatic lungs postmortem, i.e., by submersion perfusion. Bronchial segments fixed inflated (21 cmH$_2$O) simulated the fixation process often used for inflating and fixing nonasthmatic lungs postmortem. Inflating the lung postmortem not only results in changes in airway diameter but also airway length. Hughes et al. (13) demonstrated that airway length and diameter increase proportionally when canine lung is inflated with air. Specifically, airway diameter increased $\sim 67\%$ and airway length by $60\%$ when lungs were inflated from 0 to 30 cmH$_2$O with air. Interestingly, canine airways dissected from the lung only extend $15\%$ beyond their resting length when inflation pressure is increased from 0 to 30 cmH$_2$O (20), which indicates that parenchymal inflation causes axial distension of the bronchi. In the present study, due to the fact that the airways were tethered at either end, bronchial segments were not able to increase in length without bowing outward in response to an increase in pressure, and the change in length caused by bowing of the tissue could not be easily measured. Therefore, to simulate the effect of inflation on airway length and to minimize bowing of the tissues, all tissues were prestretched axially to $120\%$ of their nonstretched length before volumetric measurements and fixation at 21 cmH$_2$O. The tissues fixed at 0 cmH$_2$O transmural pressure were not stretched axially during fixation. The difference in postfixation lengths of the tissues, which were stretched, was not 20 but $41\%$ because the tissues fixed without lengthwise distension were able to shrink lengthways during the fixation process, whereas tissues that were stretched were fixed while tethered at each end, which prevented lengthwise shrinkage. This difference in length is likely to be similar to the difference that would be expected if the length of airways were compared in noninflated and inflated postmortem lungs.

The calculations of the possible errors in the estimation of ASM percent that are made in this paper are based on the assumption that these values were similar in the airways fixed at 0 and 21 cmH$_2$O. Clearly, some of the differences could be due to real differences in the two groups rather than the artifact of fixation. However, we believe this is unlikely, because the starting volumes, lengths, and organ bath-derived $P_t$ at 21 cmH$_2$O for segments fixed at 0 and 21 cmH$_2$O were not significantly different from each other. Cross-sectional wall areas of the smooth muscle and epithelium were both $\sim 41\%$ greater in the airways that were fixed without lengthwise stretching, which corresponds to the $\sim 41\%$ difference in lengths after fixation. Unexpectedly, total wall cross-sectional area was not $41\%$ less in the stretched airways but approximately the same as that found in airways fixed without lengthwise stretching. The lack of change is puzzling and means that total wall volume is greater at longer length. Because the calculated epithelial and ASM volumes did not change as a function of length, the change in volume with length was due solely to volume changes in the adventitial and submucosal compartments, which contain loose connective tissue. One explanation could be that the interstitial pressure in the tissue changed as a function of length in which it became more negative with stretch and vice versa. Therefore, when the preparations fixed at 0 cmH$_2$O were allowed to shorten, the increase in interstitial pressure could have expelled water.

Whatever the mechanism, the change in $W_{Am}$, without a change in $W_{Am}$, caused a significant change in $W_{Am}$ expressed as a percent of $W_{Am}$. If such an effect also occurs in lungs fixed with formalin, it could explain the finding of increased ASM percentage, which has been reported in asthmatic lungs by some authors (6, 28, 29). Therefore, the artifact associated with fixation of asthmatic and normal lungs at different pressures can contribute to an apparent increase in $W_{Am}$ not only when normalized to basement membrane length, but also when expressed as a percent of the airway wall.

In the present study, $P_{bm}$ was 1.36-fold greater in airways fixed inflated (21 cmH$_2$O; $n = 5$) compared with airways fixed without inflation (0 cmH$_2$O; $n = 12$). This finding is consistent with the work of Okazawa et al. (24). These authors examined cross sections of rabbit airways fixed at a variety of transmural pressures ($\sim 4$ to 10 cmH$_2$O) in a relaxed state and after maximal activation with nebulized and intravenous carbachol. They found that the reduction in lumen area of the relaxed airways between 10 and 2 cmH$_2$O occurred with little mucosal folding, suggesting that there was strain of the basement membrane between these distending pressures. It was possible that this difference in $P_{bm}$ found in the present study may have been attributed, in part, to a bias in tissue selection due to the small sample size and lack of controlled randomization. This bias was overcome in the present study by effectively measuring $P_{bm}$ in the same airway fixed with and without inflation. It is of course not possible to make a direct measurement of $P_{bm}$ obtained from the same tissue fixed at two different inflation pressures. This problem was addressed by comparing actual morphometric measurements of $P_{bm}$ in an airway fixed without inflation with predicted measurements of $P_{bm}$ if the same airway segment had been fixed inflated. We were able to predict the $P_{bm}$ if the airway had been fixed at 21 cmH$_2$O by knowledge of length and volume of the same airway distended to 21 cmH$_2$O in an organ bath (organ-bath $P_t$). Preliminary experiments demonstrated that organ bath-derived $P_t$ at 21 cmH$_2$O was strongly predictive of morphometry-derived $P_{bm}$ in airways fixed inflated at 21 cmH$_2$O ($r^2 = \ldots$).
of $P_{bm}$ from the same airway were compared, predicted $P_{bm}$ if
the airway had been fixed distended at 21 cmH$_2$O was ~1.5-
fold greater than morphometry $P_{bm}$ in the airway fixed without
inflation (0 cmH$_2$O). This ~1.5-fold increase in $P_{bm}$ is consis-
tent with the 1.36-fold difference in $P_{bm}$ of airways fixed
inflated ($n = 5$) compared with airways fixed without inflation
($n = 12$). In this study, the fold difference in $P_{bm}$ was referred
to as apparent strain, since it was the result of a difference in
length after fixation. The apparent strain does not necessarily
equate to pure mechanical strain of the basement membrane
when inflating the tissue to 21 cmH$_2$O. Both greater shrinkage
and an underestimation of $P_{bm}$ in airways fixed without infla-

This finding that $P_{bm}$ is not a constant in the present study is
counter to the results of James et al. (15). The authors
concluded that airway $P_i$ is a constant, because the relationship
between $W_A$ and $P_i$ of guinea pig airways from lungs fixed at
25, 50, and 100% of TLC and also at 25% TLC with broncho-
constriction remained nearly constant. This result suggested
that $P_i$ could be used to normalize and compare airway wall
areas in asthmatic airways fixed at postmortem without pres-
sure and nonasthmatic airways fixed with pressure. James et al.
suggested that the $P_i$ and $P_{bm}$ of guinea-pig airways remained
constant when the lungs were inflated to TLC, because the
mucosal folds flatten out as the airways distended in response
to pressure (15). The results from the present study do not
necessarily contradict those of James et al. The $P_i$ in guinea pig
trachea was ~25% longer in lungs fixed at TLC compared with
the lungs fixed after bronchoconstriction. It is likely that this
difference was not regarded as being significant, because no
consistent difference was observed further down the airway
tree. Furthermore, they filled lungs to the desired lung volume
as a percent of predicted TLC, and in fluid-filled lungs the
pressures required to reach TLC could be much less than the
20–30 cmH$_2$O that is used to fix the lung postmortem. Another
difference between the studies is that airways in the present
study were inflated after ablation of endogenous smooth mus-
cle tone. Guinea pig lungs are known to have substantial
intrinsic ASM tone after resection (25, 26), and this in com-

In the absence of tidal fluctuations in airway wall
stress, human in vitro airways also develop considerable in-
trinsinc smooth muscle tone (9, 17, 21, 30–32). The shape of
the pressure-volume curve for the human airways we studied,
in the presence of smooth muscle tone, indicated that basement
membrane strain may be minimal, or absent, even when dis-
tended to a transmural pressure of 21 cmH$_2$O (i.e., volume at
21 cmH$_2$O with tone was similar to 7 cmH$_2$O without tone).

The potential significance of the findings of this study is
apparent if we briefly review the pivotal studies that have
reported increased ASM mass in asthma. In the earlier studies
of Huber and Koeslser (12), Dunnill et al. (6), Hossain and
Heard (11), Takizawa and Thurlbeck (29), Hossain (10),
and Sobonya (28), $W_{A_m}$ was found to be increased, on average,
by 2.2 ± 0.7-fold ($n = 6$ studies). These early investigators did
not normalize the amount of ASM to $P_i$ or $P_{bm}$, and they were,
therefore, possibly comparing different-sized airways that were
not lengthened in the case of the asthmatic lungs but were
stretched lengthwise in the control lungs.

The results of all the studies in which $W_{A_m}$ has been
normalized to $P_i$ or $P_{bm}$ have shown a significant increase in
ASM in airways from asthmatic compared with nonasthmatic
lungs (2, 3, 7, 8, 16, 18, 27). Furthermore, airways from
asthmatic subjects who died in status asthmaticus appear to
have more ASM than airways from asthmatic subjects who
died of other causes. It is normally hypothesized that the
greater $W_{A_m}$ in the group with fatal asthma is a marker of
severity and contributed to their increased risk of dying from
asthma. This makes sense, because it has been proposed that
“for a given maximal muscle stress, greater muscle thickness
allows the development of greater tension and thus more
contraction of the lumen” (19). However, the results presented
in this paper offer an alternative interpretation of these
findings. Three studies, those by James et al. (16), Carroll et al. (3),
and Saetta et al. (27), should be reviewed to examine the
hypothesis that an increase in $W_{A_m}$ normalized to $P_i$ could
potentially be the result of an artifact caused by tissue fixation
procedures. All three studies normalized $W_{A_m}$ using $P_i$. James et al.
grouped airways by $P_i$ into different size ranges: $P_i < 2$ mm,
$P_i$ (membranous) ≥ 2 mm, $P_i$ (cartilaginous) < 10 mm, and $P_i
≥ 10$ mm, and the corresponding ratios of $W_{A_m}$ for asthma
(mostly fatal asthma) vs. nonasthma were ~1.1, 3.0, 4.8, and
3.4 ($P < 0.05$ for the latter 3 values). The nonasthmatic lungs
were inflated fixed with pressures of ≥20 cmH$_2$O, but asth-
matic lungs were fixed by simple immersion. Thus both arti-
facts (change in airway length and $P_{bm}$ strain) could have
contributed to the increase in $W_{A_m}$ in asthmatic subjects.
Carroll et al. inflation fixed all lungs with pressures ≥20
cmH$_2$O, grouped airways by $P_i$ into different size ranges $[P_i <
2, 2 ≤ P_i < 4, 4 ≤ P_i < 10, 10 ≤ P_i < 18, \text{ and } P_i > 18 \text{ (in}
nm)]$, and subdivided asthmatic patients into fatal and nonfatal
asthma groups. The corresponding ratios of $W_{A_m}$ were 1.2,
1.3,* 3.3,* 2.8,* and 2.3* for fatal asthma to nonasthma and
1.2, 1.6,* 1.5, 1.4, and 1.5 for nonfatal asthma to nonasthma
(*$P < 0.05$ (3)). If inflation fixation with pressures of ≥20
cmH$_2$O resulted in comparatively similar lung volumes for
each of the study groups, then only the artifact of $P_{bm}$ strain
could have contributed to the differences in $W_{A_m}$ that was
observed. The smaller increase in $W_{A_m}$ in the airways from the
nonfatal asthma group could be explained on the basis of the
$P_{bm}$ strain being greater than in the fatal asthma airways.
In contrast, greater ASM tone or increased nonmuscle tissue
stiffness could have caused decreased $P_{bm}$ strain in the airways
from the fatal asthma group, which would result in an apparent
increase in $W_{A_m}$.

An artifact of fixation cannot explain all of the reports of
increased ASM in asthmatic compared with nonasthmatic sub-
jects. Saetta et al. (27) compared the area enclosed by ASM
bundle periimeters in small airways ($P_i$ of ~3 mm) from
asthmatic and nonasthmatic tissues and found a 2.3-fold in-
crease in asthmatic compared with the nonasthmatic tissue.
Lungs from both groups were fixed without pressure perfusion,
and the mean $P_i$ of the airways compared were similar, which
means that the increase in $W_{A_m}$ observed cannot be attributed
to differences in fixation pressure or stretched length of the airways.

Despite the studies that show an increase in ASM mass normalized to $P_{0.1}$, the data presented in this study suggest that the increase in ASM volume in asthma may have been overestimated in some studies. The importance of this observation relates to our understanding of the potential causes of excessive airway narrowing and hyperresponsiveness in asthma and indirectly to our efforts to develop new therapeutic strategies for asthma. If the amount of ASM is really approximately three times greater than normal in asthma and if the phenotype of the muscle is preserved despite hyperplasia and/or hypertrophy, then ASM mass per se is likely to be a very important contributor to airway hyperresponsiveness (19). If, on the other hand, ASM is only increased 1.5-fold, then a change in ASM phenotype and/or other structural and functional changes in the airways may need to be invoked to explain airway hyperresponsiveness. Future studies are required to compare ASM amount in asthmatic and nonasthmatic airways that have not been inflated, do not exhibit intrinsic smooth muscle tone, and have been fixed and processed using a method that causes minimal changes in tissue volume.

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