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

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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/jap.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 morphometry-derived P_{bm} value, had the segment been fixed at 21 cmH₂O, the relationship between organ bath-derived P_i and morphometry-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 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

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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 cmH₂O with airways fixed at 0 cmH₂O, 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 P_{bm} at 0 cmH₂O. The present study uniquely addresses this problem by predicting the P_{bm} of the airways fixed at 0 cmH₂O (airway B') had they been fixed at 21 cmH₂O (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 cmH₂O, have been outlined below.

The first step is to measure the volume and length of all segments at 21 cmH₂O 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 cmH₂O and the P_{bm} measured morphometrically (A', Fig. 1) on airways fixed at 21 cmH₂O. This was done to predict the theoretical P_{bm} (B'', Fig. 1), if the airways that were fixed at 0 cmH₂O were fixed at 21 cmH₂O, based on their volumetrically determined P_i at 21 cmH₂O (B, Fig. 1).

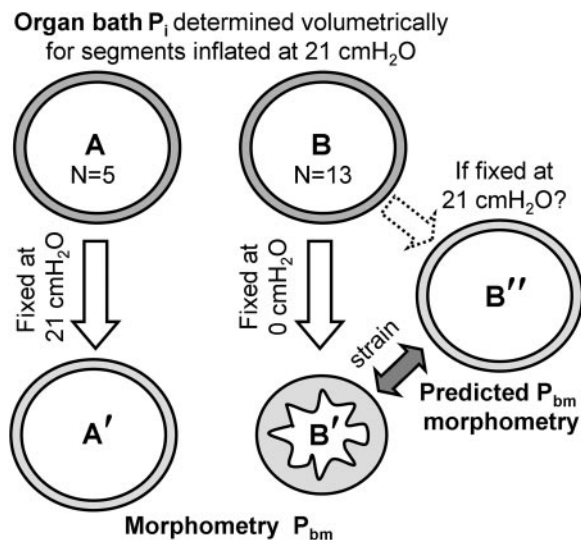


Fig. 1. Flow schematic of the experimental protocol, which shows representative airway cross sections and the critical steps required to determine the predicted basement membrane perimeter (P_{bm}) in airways had they been fixed inflated at 21 cmH₂O (B'') instead of without inflation at 0 cmH₂O (B'). Organ bath internal perimeter (P_i) was determined from knowledge of the luminal volume and length of a bronchial segment inflated to 21 cmH₂O in the organ bath (A and B); morphometry P_{bm} was determined from taking measurements from fixed serial sections for airways fixed inflated at 21 cmH₂O (A') and without inflation at 0 cmH₂O (B'); predicted morphometry P_{bm} (B'') if segments fixed without inflation at 0 cmH₂O were fixed inflated at 21 cmH₂O was determined from knowledge of the relationship between organ bath P_i (A) and morphometry P_{bm} (A') for bronchial segments fixed at 21 cmH₂O. Circumferential strain of the basement membrane was measured as the difference in the predicted morphometry P_{bm} at 21 cmH₂O and measured morphometry P_{bm} at 0 cmH₂O for the same airway. It is important to note that calculated strain is the result of differences in P_{bm} after fixation without inflation at 0 cmH₂O and with inflation at 21 cmH₂O and, therefore, is a measurement of apparent strain, which may not accurately reflect true mechanical strain.

The third step is to compare the predicted P_{bm} at 21 cmH₂O (B'', Fig. 1) to the measured P_{bm} at 0 cmH₂O (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 carbogenated (5% CO₂ in oxygen) Krebs-Henseleit solution [composition (in mM): 118 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 NaH₂PO₄, 25.5 NaHCO₃, 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 cmH₂O 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 cmH₂O. The bronchial segments were bathed in, and perfused by, Krebs-Henseleit solution maintained at 37°C and continually gassed with 5% CO₂ 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 cmH₂O was estimated by measuring the volume of luminal fluid that was expelled to a column by applying a positive pressure (~50 cmH₂O) 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 cmH₂O 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

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

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 cmH₂O) 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 cmH₂O) 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.

The following perimeter measurements were taken: outer airway wall, cartilage, P_{bm} , and P_i . The total wall area (WA_t) 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 WA_m . 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 $\times 400$. 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 (A), the thickness (T) was determined by solving a quadratic expression $T = [P - (P^2 - 16A)^{0.5}]/4$. 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 (5).

Sample calculation for determining organ bath P_i , morphometry P_{bm} , and apparent strain. The following is an example of the calculations performed to determine the predicted morphometry-derived P_{bm} for one bronchial segment had it been fixed distended to 21 cmH₂O instead of 0 cmH₂O. Luminal volume (113 μ l) and length (14.2 mm) gave an organ bath-derived P_i of 10.0 mm (Eq. 1). P_{bm} derived from morphometry in the same specimen fixed at 0 cmH₂O was 6.5 mm. The organ bath P_i at 21 cmH₂O was then used to determine a predicted morphometry-derived P_{bm} value if the segment had been fixed at 21 cmH₂O. To make this adjustment, bronchial segments fixed while distended to 21 cmH₂O were used to derive the linear regression equation between the organ bath-derived P_i values at 21 cmH₂O and the morphometry-derived P_{bm} values. The equation for morphometry-derived P_{bm} was $1.37 \cdot \text{organ bath-derived } P_i - 2.3$ ($R^2 = 0.99$). When this equation was used for this example, the P_{bm} , had the segment been fixed at 21 cmH₂O, was equal to $11.4 \text{ mm} = 1.37 \cdot 10.0 \text{ mm} - 2.3 \text{ mm}$. This gives a fixation-induced apparent strain value for P_{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 P_{bm} and strain. The inclusions of these parameters had no significant effect on the final measurement of strain (data not shown).

Examining a possible source for the underestimation of organ bath-derived P_i . The organ bath-derived P_i value underestimated the morphometry-derived P_{bm} value (Fig. 3). The reason for the discrepancy is apparent by observation of the sections fixed at 21 cmH₂O (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 ($_{adj}P_i$) was performed for bronchial segments fixed at 21 cmH₂O using

$$_{adj}P_i = \left(\frac{\text{morphometry } P_{bm}}{P_{Abm}} \right) \text{organ bath } P_i \quad (2)$$

where $\text{morphometry } P_{bm}$ is the morphometry-derived P_{bm} , P_{Abm} is a theoretical perimeter derived from knowing the area enclosed by morphometry P_{bm} if that area was circular, the ratio of $\text{morphometry } P_{bm} / P_{Abm}$ 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 cmH₂O) in the presence of intrinsic ASM tone and after maximal ASM relaxation, which was achieved by adding isoproterenol (100 μ M). Volume at each transmural pressure was determined by applying an external pressure of 50 cmH₂O 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 values for volume, 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_{bm} . Mean and 95% confidence intervals were used for these data. Comparisons of morphometric measurements obtained from bronchi fixed without inflation (≈ 0 cmH₂O) and with inflation (21 cmH₂O) 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 cmH₂O. Least-squares regression analysis based on measured and calculated values from segments fixed at 21 cmH₂O were used to derive the predicted morphometry-derived P_{bm} values at 7 and 21 cmH₂O for airways fixed at 0 cmH₂O. 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 (≈ 0 cmH₂O), 5 were fixed with pressure (21 cmH₂O), and the other 10 (9 patients) were used for pressure-volume curves and were not fixed. Of the 13 bronchial segments fixed at 0 cmH₂O, 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_{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 cmH₂O) 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 cmH₂O (47 ± 6 yr) was not different from those whose segments were fixed at 21 cmH₂O (53 ± 10 yr).

Organ bath-derived luminal volume and P_i . There was no significant difference in luminal volume at 21 cmH₂O [209 μ l (95% confidence interval: 183–237 μ l) vs. 203 μ l (95% confidence interval: 124–300 μ l)], length (1.9 ± 0.1 vs. 2.1 ± 0.2 cm), or the raw organ bath-derived P_i (11.9 ± 0.3 vs. $11.0 \pm$

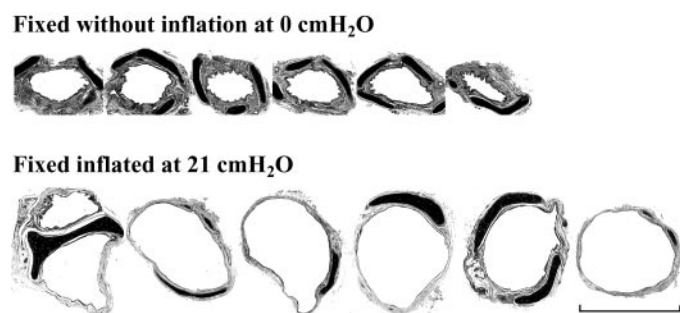


Fig. 2. Serial images from 2 human airway segments that had similar organ bath-derived P_i (only every second serial section shown) but very different morphometry-derived P_{bm} . *Top*: airway was fixed without inflation (0 cmH₂O), which mimics the method of fixation for asthmatic airways. *Bottom*: airway was fixed inflated using a transmural pressure of 21 cmH₂O, which mimics the method of fixation that is commonly used for “normal” airways. The scale (5 mm) for both airways is the same. Note that the luminal area was not a perfect circle for sections from airways fixed at 21 cmH₂O. This distortion may either be caused by compression during sectioning or be due to the presence of bifurcations.

Table 1. Morphometry-derived, organ bath-derived, and predicted results for P_{bm} and P_i at 21 cmH₂O from each bronchial segment fixed at 21 cmH₂O

Patient No.	P_i 21 O-Bath	P_i 21 Morph	P_{bm} 21 Morph	P_{bm} 21 Predicted	%Diff
1	13.7	16.2	16.4	16.5	0.7
2	9.5	10.7	10.8	10.8	-0.2
3	10.8	12.5	12.7	12.6	-0.7
4	11.5	13.5	13.6	13.4	-1.2
5	9.4	10.4	10.5	10.7	1.6
Mean	11.0	12.7	12.8	12.8	0.03
SE	0.8	1.1	1.1	1.1	0.49

Organ bath (O-Bath) results were derived from measurements of luminal volume and length of the airway in the organ bath at 21 cmH₂O, and morphometry (Morph) results were obtained by taking measurements from serial sections of airways fixed inflated at 21 cmH₂O. O-Bath results (P_i 21 O-Bath) were fitted to the linear regression equation derived from the plot of O-Bath-derived internal perimeter (P_i) vs. morphometry-derived basement membrane perimeter (P_{bm}) to give predicted P_{bm} at 21 cmH₂O. Percent difference (%Diff) was calculated as the percent difference between P_{bm} 21 Morph and P_{bm} 21 Predicted.

0.8 mm) between the bronchial segments fixed at 0 and 21 cmH₂O, respectively. Individual values for organ bath-derived P_i are shown in Tables 1 and 2.

Morphometry-derived P_{bm} and P_i . The appearance of sections from bronchial segments fixed at 21 cmH₂O was very different from those fixed at 0 cmH₂O (Fig. 2). Even in the absence of smooth muscle tone, mucosal folding was apparent in sections from bronchial segments fixed at 0 cmH₂O but not in those fixed at 21 cmH₂O, and the airway wall appeared much thicker in segments fixed at 0 cmH₂O. The luminal area within the cross sections of airways fixed at 21 cmH₂O was not always a perfect circle, as was assumed. After tissue processing, the measured length of bronchial segments fixed at 21 cmH₂O was 41% greater than that of segments fixed at 0 cmH₂O. Given that there was no shrinkage of axial length for segments fixed at 21 cmH₂O, tissues fixed at 0 cmH₂O were ~85% of the length before fixation, i.e., the length before the segments were mounted into the organ bath.

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} [$P_{bm} = 1.37 \cdot \text{organ-bath } P_i \text{ (in mm)} - 2.3$; $R^2 = 0.997$, $P < 0.0001$; 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 $52 \pm 6\%$, 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 $13 \pm 3\%$ ($n = 4$ 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

Table 2. Morphometry-derived, O-Bath derived, and predicted values for P_{bm} and P_i at 0, 7, and 21 cmH₂O from each bronchial segment fixed at 0 cmH₂O

Patient No.	P_i 0 Morph	P_{bm} 0 Morph	P_i 7 O-Bath	P_i 7 Predicted	P_{bm} 7 Predicted	Strain 7	P_i 21 O-Bath	P_i 21 Predicted	P_{bm} 21 Predicted	Strain 21
6a	10.5	10.7	9.6	10.7	10.9	0.01	13.2	15.7	15.8	0.47
6b	11.3	11.6	9.9	11.0	11.3	-0.03	13.3	15.8	16.0	0.38
7	9.8	10.0	10.6	12.0	12.2	0.22	12.5	14.7	14.8	0.48
8	10.2	10.5	8.4	8.9	9.3	-0.12	11.1	12.6	12.9	0.23
9a	9.3	9.5	9.2	10.1	10.3	0.08	10.7	12.1	12.3	0.29
9b	8.5	8.9	9.0	9.8	10.1	0.14	10.4	11.7	11.9	0.35
10	9.3	9.5	9.9	11.1	11.3	0.19	11.7	13.6	13.8	0.45
11	9.6	9.8	11.5	13.4	13.5	0.38	13.0	15.4	15.5	0.58
12a	6.3	6.5	8.5	9.3	9.4	0.46	10.0	11.4	11.4	0.75
12b	7.3	7.4	10.8	12.4	12.5	0.68	12.6	14.9	15.0	1.02
13a	8.6	8.9	9.1	10.0	10.2	0.15	11.8	13.8	13.9	0.57
13b	9.2	9.4	10.2	11.6	11.8	0.25	12.7	15.0	15.1	0.61
Mean	9.2	9.4	9.7	10.9*	11.1*	0.20*	11.9*	13.9*	14.1*	0.52*
SE	0.4	0.4	0.5	0.4	0.7	0.06	0.3	0.5	0.5	0.06

0, 7, and 21 denote transmural pressures (in cmH₂O). Letters a and b denote different organ baths when >1 segment was studied from an individual. Units are in mm. Morph results were obtained by taking measurements from serial sections. Predicted values were derived from O-Bath-derived P_i values at 21 cmH₂O that were transformed using the best-fit linear regression equation for segments that were fixed at 21 cmH₂O (see Table 1 and Fig. 3). Strain values are also shown, and a negative strain indicates a P_{bm} less than the morphometry-derived P_{bm} , which we have interpreted as the result of mucosal folding. *Significantly different ($P < 0.001$, paired t -tests) from values obtained at 0 cmH₂O.

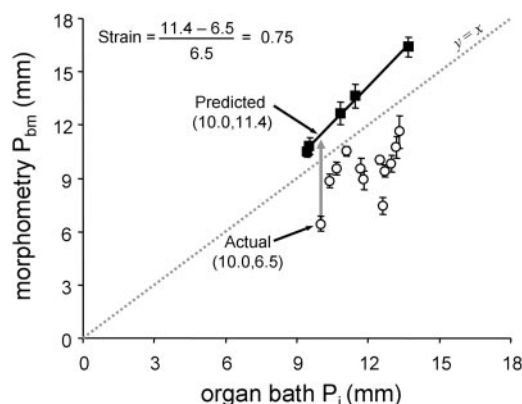


Fig. 3. Scatter plots showing the relationship between organ bath-derived P_i and morphometry-derived P_{bm} for individual segments fixed at 0 cmH₂O ($\circ$; $n = 12$ airways from 8 patients) and 21 cmH₂O ($\blacksquare$; $n = 5$ airways from 5 patients). The line of unity (broken line) is shown for reference, and the linear regression line (solid line) is shown for segments fixed at 21 cmH₂O without an adjustment for noncircularity [$r = 0.99$, $P < 0.0001$; morphometry $P_{bm} = 1.37(\text{organ bath } P_i) - 2.3$]. Also shown, for an individual airway segment fixed at 0 cmH₂O, is an example of how the organ bath-derived P_i value was converted to a morphometry-derived P_{bm} if the airway segment had been fixed at 21 cmH₂O. For the example shown, basement membrane strain at 21 cmH₂O was estimated to be 75%.

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 $\sqrt{WA_m}$ divided by P_{bm} for airways fixed at 0 and 21 cmH₂O. Because the length of the preparations fixed at 0 cmH₂O was $\sim 70\%$ 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 WA_m ($P < 0.05$; 1-tail t -test assuming

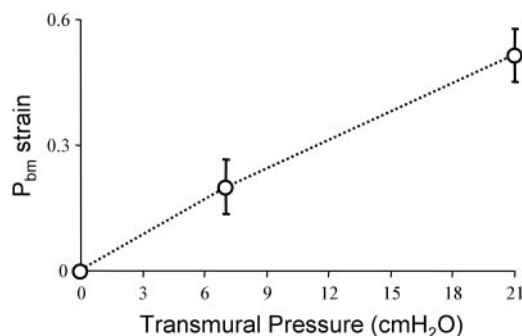


Fig. 4. Mean apparent strain ($n = 12$ airways from 8 patients) for P_{bm} after fixation at pressures of 0, 7, and 21 cmH₂O. The apparent basement membrane strain if the tissues were fixed at 7 and 21 cmH₂O was determined from predicted P_{bm} values (Table 2) based on the best-fit equation of organ bath-derived P_i at 21 cmH₂O vs. morphometry-derived P_{bm} for bronchial segments fixed at 21 cmH₂O (see Fig. 3). Basement membrane strain was significant if tissues were fixed at 7 and 21 cmH₂O, and the relationship between pressure and strain was linear over the pressure range studied (broken line).

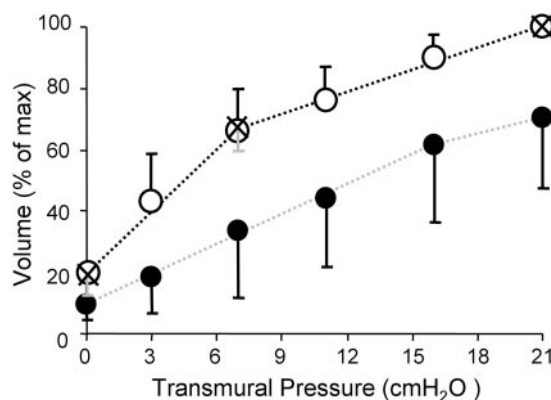


Fig. 5. Mean $\pm$ SD 3-point pressure-volume (P-V) curve (crosses, broken black line) derived for the tissues used for morphometry ($n = 12$ segments from 8 patients) and a more complete curve ($\circ$, broken black line) obtained from 10 isoproterenol-relaxed tissues (9 patients). There was no significant difference between these curves. Also shown is the P-V relationship ($\pm$ SD) in the presence of intrinsic airway smooth muscle tone ($\bullet$, broken gray line), which is significantly different from the P-V relationship in the absence of smooth muscle tone at all pressures. The large standard deviations indicate intrinsic smooth muscle tone is highly variable between different tissues. Note the inflexion point on the P-V curve generated from airways without smooth muscle tone occurs at ~ 7 cmH₂O. This point indicates a change in tissue stiffness, which we have interpreted as the point where the mucosal folds have flattened out. In the presence of smooth muscle tone, the inflexion point appears to occur at a much greater transmurial pressure.

WA_m must necessarily decrease when the airway is axially strained). There was also significantly more muscle for segments fixed at 0 cmH₂O than at 21 cmH₂O when muscle area was expressed as $\sqrt{WA_m}/P_{bm}$. However, WA_t was not increased for airway segments fixed at 0 cmH₂O, suggesting that there was a change in compartment volume with length change, i.e., the total volume of the wall decreased with a decrease in segment length. A consequence of this was that, when WA_m was expressed as a percent of WA_t , there was a significantly higher value at 0 cmH₂O.

Based on these data, we calculated the magnitude of the potential error in the estimation of ASM volume between normal and asthmatic airways if we surmise that the asthmatic airways behaved like noninflated airways and the normal airways behaved as the inflated airways. If, in the present study, we had assumed that P_{bm} was a constant, then $\sqrt{WA_m}$ in the airways fixed at 0 cmH₂O would be 1.71 times greater than in the airways fixed at 21 cmH₂O. If this value is converted to WA_m , then the increase would be 2.9 (1.7²)-fold.

Table 3. Mean values for wall area of bronchial wall compartments in segments fixed at 0 and 21 cmH₂O

Compartment	21 cmH ₂ O	0 cmH ₂ O	P Value	Fold Difference
WA_e	0.24 (0.17–0.33)	0.37 (0.30–0.45)	0.045	1.55
WA_m	0.19 (0.11–0.29)	0.27 (0.21–0.33)	0.05*	1.42
WA_t	7.3 (5.4–9.5)	6.0 (5.2–6.9)	0.17	0.82
$\%WA_m/WA_t$	2.7 ± 0.3	5.1 ± 0.7	0.002	1.88
$\sqrt{WA_m}/P_{bm}$	0.034 ± 0.004	0.058 ± 0.004	0.003	1.71

WA_e , epithelial wall area; WA_m , airway smooth muscle wall area; WA_t , total wall area. *Significantly different by one-tail t -test. Values are means with either 95% confidence intervals, shown in parenthesis, or $\pm$ SE. Cross-sectional area for the epithelial layer was determined by multiplying the thickness of the respective layers by the measured P_{bm} .

DISCUSSION

The results of this study indicate that airway P_i 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 cmH₂O [$\approx$ total lung capacity (TLC)] compared with airways fixed without inflation at 0 cmH₂O. After tissue processing, the bronchial segments fixed inflated (21 cmH₂O) were ~ 1.41 times longer and the cross-sectional WA_m was correspondingly $\sim 41\%$ less than segments that were fixed at 0 cmH₂O 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 WA_m for segments fixed at 0 cmH₂O.

Postmortem lungs from asthmatics who die in status asthmaticus are often fixed without inflating the lung (≈ 0 cmH₂O) (2, 6–8, 16, 27). On the other hand, nonasthmatic lungs obtained postmortem are usually fixed by inflating the lung (20–30 cmH₂O). 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 (≈ 0 cmH₂O) simulated the fixation process usually used for fixing constricted asthmatic lungs postmortem, i.e., by submersion perfusion. Bronchial segments fixed inflated (21 cmH₂O) 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 cmH₂O 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 cmH₂O (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 cmH₂O. The tissues fixed at 0 cmH₂O 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 cmH₂O. 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_i at 21 cmH₂O for segments fixed at 0 and 21 cmH₂O 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 cmH₂O were allowed to shorten, the increase in interstitial pressure could have expelled water.

Whatever the mechanism, the change in WA_e without a change in WA_m , caused a significant change in WA_m expressed as a percent of WA_e . 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 WA_m 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 cmH₂O; $n = 5$) compared with airways fixed without inflation (0 cmH₂O; $n = 12$). 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 transpulmonary pressures (-4 to 10 cmH₂O) 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 cmH₂O 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 cmH₂O by knowledge of length and volume of the same airway distended to 21 cmH₂O in an organ bath (organ-bath P_i). Preliminary experiments demonstrated that organ bath-derived P_i at 21 cmH₂O was strongly predictive of morphometry-derived P_{bm} in airways fixed inflated at 21 cmH₂O ($r^2 =$

0.997, $n = 5$; Fig. 3). When predicted and morphometry values of P_{bm} from the same airway were compared, predicted P_{bm} if the airway had been fixed distended at 21 cmH₂O was ~ 1.5 -fold greater than morphometry P_{bm} in the airway fixed without inflation (0 cmH₂O). This ~ 1.5 -fold increase in P_{bm} is consistent 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 cmH₂O. Both greater shrinkage and an underestimation of P_{bm} in airways fixed without inflation would result in an increase in apparent strain. However, it must be kept in mind that the objective of this study was to investigate whether differences in fixation pressure would affect the measurement of P_{bm} and not whether inflating an airway can cause mechanical strain of the basement membrane.

This finding that P_{bm} is not a constant in the present study is contrary to the results of James et al. (15). The authors concluded that airway P_i is a constant, because the relationship between WA_t and P_i of guinea pig airways from lungs fixed at 25, 50, and 100% of TLC and also at 25% TLC with bronchoconstriction 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 pressure 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 $\sim 25\%$ 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 cmH₂O 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 muscle tone. Guinea pig lungs are known to have substantial intrinsic ASM tone after resection (25, 26), and this in combination with a lower distending pressure could have contributed to the failure to find a change in the P_i of guinea pig airways. In the absence of tidal fluctuations in airway wall stress, human in vitro airways also develop considerable intrinsic 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 distended to a transmural pressure of 21 cmH₂O (i.e., volume at 21 cmH₂O with tone was similar to 7 cmH₂O 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 Koessler (12), Dunnill et al. (6), Hossain and Heard (11), Takizawa and Thurlbeck (29), Hossain (10), and Sobonya (28), WA_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 WA_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 WA_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 WA_m normalized to P_i could potentially be the result of an artifact caused by tissue fixation procedures.

All three studies normalized WA_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 $P_i \geq 10$ mm, and the corresponding ratios of WA_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 inflation fixed with pressures of >20 cmH₂O, but asthmatic lungs were fixed by simple immersion. Thus both artifacts (change in airway length and P_{bm} strain) could have contributed to the increase in WA_m in asthmatic subjects. Carroll et al. inflation fixed all lungs with pressures >20 cmH₂O, grouped airways by P_i into different size ranges [$P_i < 2$, $2 \leq P_i < 4$, $4 \leq P_i < 10$, $10 \leq P_i < 18$, and $P_i > 18$ (in mm)], and subdivided asthmatic patients into fatal and nonfatal asthma groups. The corresponding ratios of WA_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 cmH₂O 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 WA_m that was observed. The smaller increase in WA_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 WA_m .

An artifact of fixation cannot explain all of the reports of increased ASM in asthmatic compared with nonasthmatic subjects. Saetta et al. (27) compared the area enclosed by ASM bundle perimeters in small airways (P_i of ~ 3 mm) from asthmatic and nonasthmatic tissues and found a 2.3-fold increase 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 WA_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_i , 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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