Airway Narrowing Assessed by Anatomical Optical Coherence Tomography In Vitro: Dynamic Airway Wall Morphology and Function

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ABSTRACT

Regulation of airway caliber by lung volume or bronchoconstrictor stimulation is dependent on physiological, structural and mechanical events within the airway wall, including airway smooth muscle (ASM) contraction, deformation of the mucosa and cartilage, and tensioning of elastic matrices linking wall components. Despite close association between events in the airway wall and the resulting airway caliber these have typically been studied separately: the former primarily using histological approaches, the latter with a range of imaging modalities. We describe a new optical technique, anatomical optical coherence tomography (aOCT), which allows changes at the luminal surface (airway caliber) to be temporally related to corresponding dynamic movements within the airway wall. A fiber-optic aOCT probe was inserted into the lumen of isolated, liquid-filled porcine airways. It was used to image the response to ASM contraction induced by neural stimulation and to airway inflation and deflation. Comparisons with histology indicated that aOCT provided high-resolution images of the airway lumen including mucosal folds, the entire inner wall (mucosa and ASM), and partially the cartilaginous outer wall. Airway responses assessed by aOCT revealed several phenomena in ‘live’ airways (i.e., not fixed) previously identified by histological investigations of fixed tissue, including a geometric relationship between ASM shortening and luminal narrowing, and sliding and bending of cartilage plates. It also provided direct evidence for distensibility of the epithelial membrane and anisotropic behavior of the airway wall. Findings suggest that aOCT can be used to relate changes in airway caliber to dynamic events in the wall of airways.
A number of experimental approaches have been adopted to investigate the fundamental processes controlling airway caliber in response to physiological interventions. Principal amongst these are histological examinations that provide detailed morphology of the airway wall and lumen under various evoked physiological conditions (15-17, 20, 32, 35-37, 39). These post-mortem approaches on fixed airways have been used to determine the physiological, structural and mechanical properties of the airway wall that can explain or predict the pre-mortem lung function. For example, the physiological consequences of airway smooth muscle (ASM) activation by agonists, and the consequential deformation of airway wall structures are concepts that have partly arisen out of studies employing histological techniques (17, 22, 31, 37, 49). A variety of imaging modalities have also been used to assess the above mechanisms important to lower and upper airway caliber during evoked activation of ASM. Imaging techniques thus employed include magnetic resonance imaging (2, 11), X-ray imaging (7, 9, 18), quantitative video-bronchoscopy (23, 33) -endoscopy (29, 33), and -microscopy (6, 10). Although imaging approaches have provided a valuable means of recording airway responses in airways *in vivo* or *in vitro*, present techniques do not discriminate the different wall components regulating or initiating the bronchoconstriction that have been pursued in histological investigations.

Despite the impetus to respiratory research provided by the above imaging and histological techniques, the importance of a number of properties in the constricted airway wall that potentially regulate overall airway narrowing and responsiveness remain obscure, largely due to shortcomings of the histological approach. A most obvious deficiency is an inherent assumption that the effect of fixation and tissue processing does not modify, or interact with, the interventions to which a ‘live’ airway (i.e., not fixed) is subjected. Moreover the histological approach can not be used to make repeated interventions in the same live airway,
but instead requires multiple, matched samples leading to reduced statistical power which could explain present inconsistencies in the literature. One such inconsistency is the capacity for the airway epithelium to stretch; some histological based studies report an indistensible membrane (15, 16), whereas others show considerable stretch with lung inflation (26, 34). Whether or not the epithelial membrane distends impacts not only on the mechanical loads present on ASM but also on the use of the internal perimeter of the epithelium (i.e., luminal border) to normalize airway measurements (15). A further uncertainty, as yet largely unexplored by either histological or other techniques, is whether the airway wall behaves uniformly (isotropically) in response to ASM activation. Given the likely differences in elastic moduli of the structural components of the airway wall, including the mucosa and cartilage, deformation (strain) may also vary during airway narrowing. Non uniformity in the elastic properties of the wall could have substantial implications to the mechanical loads opposing ASM shortening and airway narrowing in mid and large airways, particularly in the event of airway inflammation and remodeling in disease.

Anatomical optical coherence tomography (aOCT) is a recently developed imaging modality that is capable of acquiring high-resolution luminal and subsurface images of airway cross sections in real time (3, 48). aOCT is conceptually similar to ultrasound but utilizes near infrared light waves instead of sound waves enabling a far higher resolution than is possible with ultrasound. Like standard OCT which is used clinically as a diagnostic tool (5, 12, 13), aOCT is based on the concept of interferometry but applied over an increased axial scanning range that has allowed the technology to be used for full-circumference measurements of large hollow passages such as airways (3, 48). Most importantly, aOCT has the capacity to discriminate subsurface structures providing an alternative to the histological approach but with the advantage of being able to examine the responses to multiple interventions in a single live airway. aOCT could therefore be used to test concepts about the behavior of the airway
previously based on evidence from fixed tissue post mortem as well as examining dynamic airway structure-function relationships which have not previously been possible.

In this study, we utilized aOCT with an *in vitro* airway preparation to describe integrated dynamic structural and physiological changes across the airway wall not seen previously in tissue, and by this establishing the potential of aOCT for examining structure-function relationships in airways. Imaging of the airway wall by aOCT allowed us to concurrently record lumen narrowing, ASM shortening and associated changes in the airway wall following ASM contraction to electrical stimulation and lung (airway) inflation and deflation. In order to compare aOCT findings with more commonly reported histologically derived profiles, aOCT scans in live airways were compared with morphometry of the same airways after histological processing. We were also particularly interested whether surface and subsurface imaging by aOCT could be used to further address some unresolved issues. To this end we sought evidence in live airways for stretching of the epithelium and for anisotropic behavior of the airway wall evidenced by movement and distortion of outer and inner components of the airway wall.
METHODS

Animal handling

All animal experiments conformed to the American Physiological Society’s ‘Guiding Principles in the Care and Use of Animals’ and were approved by our institutional ethics and animal care unit. Eight White Landrace pigs (25 ± 1 Kg) were sedated with tiletamine/zolazepam (4.4 mg/Kg im.) and xylazine (2.2 mg/Kg im.) and then exsanguinated under pentobarbitone sodium anesthesia (30 mg/Kg iv.). Lungs were removed and transported on ice to the laboratory for dissection of airways.

Airway preparation

A length of the bronchial tree was dissected from the lower lobe of the right lung, beginning from a lobar bronchus and extending distally ~5-6 cm. All side branches were ligated producing a leak-free airway tube. Airway generation was determined by counting the number of side branches defining the trachea as generation #0. The two ends of the bronchial preparation were cannulated and the preparation was placed horizontally in an organ bath containing gassed (95% O₂, 5% CO₂) Krebs solution (mM: NaCl 121; KCl 5.4; MgSO₄ 1.2; NaHCO₃ 25; sodium morpholinopropane sulphonic acid 5.0; glucose 11.5; and CaCl₂ 2.5) at 37°C. The preparation was stretched to a length shown previously to approximate functional residual capacity in the pig lung, i.e., ~105% of the fully deflated length at 0 cmH₂O (33). Intraluminal pressure was set by the height of a reservoir containing Krebs solution connected at the distal side of the preparation. Unless otherwise stated, intraluminal pressure (and consequently transmural pressure) was 5 cmH₂O.

Anatomical Optical Coherence Tomography

Airway dimensions were measured using aOCT (3, 48). The technique uses interference of two light beams to measure the distance to an object. The imaging system contains a near infrared, low-coherence light source with a center wavelength of 1310 nm. Light from this
source is split into two beams, with one beam (reference beam) kept internal to the system and reflected by a rapidly moving mirror, and a second beam (sample beam) directed through a focusing probe towards the airway wall. When light is reflected from the airway wall, the two beams are recombined within the system and the resulting optical interference signal is recorded by a photodetector. A characteristic signal is detected when the optical path lengths of both light beams are equal to within a distance known as the optical coherence length, 0.03 mm in this case, and the strength of signal is proportional to reflectance of the object. Thus, by modifying the length of the reference beam with the moving mirror, the reflection from the sample can be measured at a particular distance from the focusing probe and by scanning this distance through a predefined range, it is possible to construct a depth scan to the airway lumen and through the airway wall.

The focusing probe was enclosed within a transparent catheter (OD 2.2 mm) and was carefully and gradually inserted into the airway lumen, beginning at the cannula in the proximal end of the airway and advanced down to the internal edge of the distal airway cannula where the probe catheter was locked in position. The airway lumen was sealed by wrapping silicon tape around the catheter at its point of insertion at the proximal airway. Within the catheter, the probe was rotated by a motorized stage at 0.2Hz, acquiring 1250 depth scans per rotation. These scans were then reconstructed to form 2D cross-sectional images of the airway lumen, which were displayed in real time on a computer monitor. The slow probe rotation speed was chosen to provide high resolution and detail of anatomical/subsurface structures (e.g., mucosal folds). The translation of the probe within the airway was precisely controlled by a motorized translation stage.

Unlike previous studies in vivo (3, 25, 48), aOCT recordings were performed in a liquid filled airway preparation. Measurements using aOCT are dependent on the refractive index of the medium, which for Krebs solution was determined to be 1.37. To validate the
measurements in solution, five Krebs solution-filled phantom tubes of known diameters (4-20 mm) were measured using \( \alpha \)OCT. The relationship between actual and measured cross sectional areas of phantom tubes is shown in Fig. 1. Measured cross sectional areas compared well with actual areas as calculated from diameter measurements with electronic calipers (slope of 1) with an average % difference \((\text{actual-measured}/\text{actual}) \times 100\) of \(-1.7 \pm 0.7\%\) (N = 5 measurements).

**Experimental protocol**

Airway preparations were allowed 1 hour to equilibrate to organ bath conditions before experimentation, during which the lumen and adventitia of the segment was regularly flushed with fresh Krebs solution. Tissue viability was confirmed by observing airway contractions to acetylcholine (ACh; \(10^{-3}\) M) followed by a recovery period of at least 1 hour.

After the equilibration period, a single airway generation was chosen that was at least 1 cm proximal to the distal cannula and which was also free of side branches. This region typically corresponded to generation \#7 (mode value) and it was at this point that all \( \alpha \)OCT recordings were conducted. Two protocols were used to assess airway changes during: (i) an acute period of airway narrowing; and (ii) different inflationary and deflationary pressures. Airway narrowing was induced by electrical field stimulation (EFS) of cholinergic nerve endings. Stimulation parameters were selected based on preliminary experiments that showed maximum narrowing for pulses of amplitude 60V, 5-ms pulse width and 30 Hz frequency. EFS was maintained until a plateau in lumen narrowing was reached (~25 sec). \( \alpha \)OCT recordings were then continued until full airway relaxation had occurred, which was typically ~40 sec after the end of EFS. For measurements at different airway pressures, \( \alpha \)OCT scans were performed sequentially at intraluminal pressures of +5 cmH\(_2\)O (baseline pressure), -5 cmH\(_2\)O and +25 cmH\(_2\)O. An intraluminal pressure of -5 cmH\(_2\)O induced a compressive force on the airway whilst inflation to 25 cmH\(_2\)O increased the distending force on the airway wall simulating
conditions present at total lung capacity. Recordings were made after a static period of 5 min at each respective pressure.

**Histology**

To characterize our findings with \(a\)OCT, we compared images of \(a\)OCT derived airways with morphometry of the same airways after histological processing. After experimentation relaxed airway preparations were fixed in the organ bath in formaldehyde solution (4% vol/vol in Krebs solution) applied to both the adventitial and luminal bathing solutions. After 12 hours *in situ* fixation the \(a\)OCT scan was repeated at the same location as for the pre-fixation scan. The refractive index of the formaldehyde solution was measured and was determined to be the same as normal Krebs solution i.e. 1.37. Airways were then removed from the bath and transferred to a fresh fixative solution for at least 24 hr prior to processing. The region of airway processed was the same location at which \(a\)OCT recordings were performed, determined by airway generation and also the distance from cannula inserts. The tissue was cryo-protected by soaking for one hour at 4\(^0\)C in each of 10%, 25%, 50% and 75% vol/vol Tissue Tek embedding media in PBS, followed by 24 hr in 100% embedding media. Tissue was then transferred into an aluminum foil mold containing fresh 100% embedding media before being rapidly frozen in liquid nitrogen-cooled isopentane. Frozen sections (10 \(\mu\)m) were cut onto positively charged slides, air dried, and stained using a Servio Stain kit (Royal Perth Hospital) before mounting with Depex mounting media. Mounted slides were viewed on an Olympus BH-2 microscope equipped with an Olympus SPlan FL-1 1x objective lens, and images captured with a Sony DFW-SX900 video camera and Fire-i 1.21 software (Unibrain). Scale information was calculated by capturing images of a 1 mm graticule at identical microscope and software settings.
Analysis and statistics

Several morphological indices of the airway wall (4) were determined from images acquired by aOCT across each of the different conditions (contraction, inflation and deflation) and also from matched histological images. These included internal epithelial perimeter (\( P_i \)) (i.e., the luminal border) and the associated area enclosed by that border (\( A_i \)), the perimeter of the outer border of the muscle layer (\( P_{mo} \)) and the area enclosed (\( A_{mo} \)), inner airway wall area and wall thickness. Inner wall area and wall thickness were determined as follows:

\[
\text{Inner wall area} = A_{mo} - A_i
\]

\[
\text{Wall thickness} = \frac{\sqrt{A_{mo}} - \sqrt{A_i}}{\sqrt{\pi}}
\]

Thickness, as calculated, is an average across the entire wall and assumes circularity. For aOCT recordings airway dimensions were measured using a custom designed program (developed using the C++ software language), while Image J software (NIH, USA) was used for histological images. Areas and perimeters were determined by manual tracing. In our previous study this approach returned inter and intra observer variabilities of < 2% (50). Notably, automated techniques are available to reduce analysis time. For larger studies, appropriate methods may be adapted from HRCT (19) or through the use of algorithms such as active appearance models (30).

Graphical presentation and statistical analyses of data were performed using Graphpad Prism (v4.03, GraphPad Software, CA, USA). The effect of ASM contraction or inflation/deflation on each of the morphological indices described above was analyzed by repeated measures one-way ANOVAs and Newman-Keuls Post hoc tests, or where appropriate Student’s paired t-test.
Comparisons between histological measurements and OCT before and after fixation were also made by repeated measures one-way ANOVAs. Linear relationships between variables were determined by Pearson’s correlation analysis. Unless otherwise stated, data are presented as means ± standard error where n equals the number of animals or airway segments. A p-value < 0.05 was considered statistically significant.
RESULTS

General features of \( \alpha \)OCT derived images and comparison with histology

Fig. 2 shows that \( \alpha \)OCT scans provide a well defined view of the luminal surface of the airway including shallow mucosal folds. The inner airway wall (comprising the mucosa and ASM, which were not separately distinguished), and some surrounding cartilaginous plates were also clearly evident. Whilst cartilage plates could be identified in each of the airways tested, they were not always visible around the entire circumference of the airway and as such outer airway wall area could not be measured. Since the internal epithelial perimeter (luminal border, i.e., \( P_i \)) and the outer margin of ASM (\( P_{mo} \)) were clearly defined, other morphometric parameters could be determined including inner wall area and thickness. A comparison between \( \alpha \)OCT and histologically derived airway morphometry is presented in Table 1. The regions of the airway used to produce \( \alpha \)OCT and histological images were closely matched, although not identical, as described in the discussion. Histology-derived measurements tended to be less than the pre-fixed \( \alpha \)OCT measurements, including \( P_i \), which was ~8% lower by histology. This difference was not attributed to shrinkage of tissue during fixation because \( \alpha \)OCT measurements in the same fresh and formaldehyde fixed airways were the same (Table 1.)

Airway wall and lumen narrowing to electrical field stimulation

\( \alpha \)OCT was used to measure airway narrowing and ASM shortening to EFS and also to assess dynamic changes occurring within the airway wall during bronchoconstriction. Example cross-sectional images from one airway before and during contraction to EFS are shown in Fig. 3A. EFS produced a 57.7 ± 4.1 % decrease in luminal cross sectional area (\( A_i \)) which was reversed ~40 sec after the cessation of EFS. Changes in airway luminal area in response to EFS are shown in Fig. 3B. Since the outer margin of ASM could be identified, ASM shortening in response to EFS could be determined from the proportional change in \( P_{mo} \)
in the relaxed then contracted airway. Mean ASM shortening in response to EFS in 6 airways was 27.9 ± 2.9 %. We plotted the relationship between % ASM shortening and % lumen narrowing for the 6 airways studied. For this plot airway narrowing was expressed as the % decrease in airway diameter derived from measurements of A_i assuming circularity (Fig. 3C). As expected, the magnitude of ASM shortening was strongly associated with the magnitude of luminal narrowing (r = 0.95, P < 0.01).

In the example shown in Fig. 3A mucosal folds were present in both the relaxed and EFS contracted airway. The depth of the interstices of the folds was difficult to detect during ASM contraction, as the sides of folds were closely apposed. Difficulty in detecting fold interstices likely explain an apparent decrease in P_i with ASM contraction (P < 0.001, N = 6, data not shown) which we therefore attribute to underestimation of epithelial length. Difficulty in detecting folds during contraction may also explain why we did not observe a deepening of folds during airway narrowing. During contraction the inner airway wall became thicker (Figs 3A/4A), which is a geometric consequence of the encroachment of the ASM layer. Despite the considerable inner airway wall thickening with contraction, there was also a modest (~6-7%) but statistically significant decrease in inner airway wall area (Fig. 4B) indicating that wall area was not conserved. The above changes to wall dimensions were transient and fully reversed with ASM relaxation.

Another striking feature seen when comparing images of a relaxed then contracted airway was the movement of cartilage plates associated with ASM contraction (Fig. 3A). In the relaxed airway the plates overlap to some extent, however, during ASM contraction, the same plates were seen to slide past each other producing greater overlapping. There was also evidence that the tips and body of cartilage plates flexed during bronchoconstriction so that the angle of curvature was increased.
Note that a faint ‘echo’ of the lumen wall is visible in Fig. 2 and Fig. 3A. This imaging artifact was observed in some scans, and was due to an additional reflection present in the aOCT reference arm during some studies. However, the artifact was well separated from the airway wall and did not confound analysis of airway structures. Subsequent optimization of the scanner’s optics removed this artifact in later studies.

Although the entire thickness of the outer wall was unable to be imaged, the inner layers of cartilage and their spatial relation to the ASM were recorded. A frequently observed response of the airway during ASM contraction was an increase in the thickness of the region between the inner airway wall and adjacent cartilage plates (Fig. 5A). To assess thickening of that region, the distance from the outer margin of ASM to the inner margin of cartilage was measured in relaxed airways and at the peak of ASM contraction to EFS. For the analysis, the 2D image of the airway cross section was subdivided into four equal quadrants, and quadrants where cartilage was clearly visible in each condition (i.e. baseline/contracted/relaxed) were analyzed. Multiple measurements were performed at each region and these were then averaged. Using the above criteria a total of 15 quadrants from six airways were analyzed and 13 of those showed thickening of the space between the inner airway wall and cartilage (Fig. 5B), which was reversed after ASM relaxation.

Airway inflation and deflation

A number of morphological features of the airway lumen and wall were imaged before and after deflation to an intraluminal pressure of -5 cmH_2O or inflation to +25 cmH_2O (N = 5). Deflation reduced luminal area ($A_l$) from 33.7 ± 4.7 mm$^2$ at +5 cmH_2O to 18.2 ± 2.9 mm$^2$ ($P < 0.001$) whereas inflation increased area to 44.9 ± 6.4 mm$^2$ ($P < 0.01$).

Deflation to -5 cmH_2O produced changes in the inner airway wall that largely mimicked ASM contraction. Inner wall thickness was increased (Fig. 6A, $P < 0.001$) and there was a trend for a decrease in wall area (Fig. 6B), although unlike EFS, this was not statistically
significant. Deflation also produced a thickening or separation of the region between the ASM and cartilage, which was observed in 10 out of 12 quadrants sampled across five airways (Fig. 6C). In comparison, airway inflation significantly reduced inner airway wall thickness (Fig. 6A, P < 0.01), but had no effect on inner airway wall area (Fig. 6B) and did not produce any change in thickness of the region between ASM and cartilage (Fig. 6C).

Lastly, changes in internal epithelial perimeter (luminal border, i.e., P_i) by inflation were assessed. Airway inflation from 5 to 25 cmH_2O significantly increased P_i from baseline by 13.6 ± 1.4 % (P < 0.001) (Fig. 7). Measurement of P_i in response to deflation to -5 cmH_2O was not undertaken because, as mentioned previously, the P_i in the interstices of a closely inwardly folded membrane could not be resolved by the present aOCT system.
DISCUSSION

Functionally, the most relevant conformational change in response to ASM activation and shortening is narrowing of the airway lumen, which increases the resistance to airflow. However, airway narrowing also involves movement and deformation of airway wall structures, including thickening and folding of the mucosal membrane (15-17, 49) and stress and strain of other wall components such as elastin, collagen and cartilage (27, 28, 38, 43). Airway luminal narrowing is strongly dependent on these dynamic processes and, as such, there is a need to assess events associated with ASM activation not only at the luminal surface but within the wall itself. We report a new application of a recently developed technology that, for the first time, allows changes occurring at the luminal surface to be related to corresponding dynamic structural changes occurring within the airway wall. Our results demonstrate the capacity for aOCT to measure airway luminal dimensions as well as wall morphology in live airway preparations (i.e., not fixed) and importantly in the same airway under several different physiological conditions, a suitable approach for examining dynamic structure-function relationships in airways.

In the present study, we used aOCT to measure changes occurring in both the lumen and wall in response to ASM contraction, inflation and deflation. The imaging resolution of the airway lumen by aOCT was sufficient to detect the presence of mucosal folds, which are not reported with other imaging techniques (8). The use of aOCT for measurements of luminal dimensions has been validated in previous studies using phantom tubes of known diameters and comparing aOCT measurements to those obtained with X-ray CT showing good agreement (3). Unlike previous studies that have measured the dimensions of air filled tubes including upper and lower airway passages (3, 25, 48), measurements in our in vitro airway preparation were made in Krebs solution, which has different refractive properties than air (i.e. refractive index of 1.37 for Krebs solution compared to 1.0 for air). After correcting for the refractive index of Krebs
solution, we show here that aOCT also accurately resolves luminal dimensions of liquid filled phantom tubes (Fig. 1). The use of a liquid medium would negate surface tension effects normally present in vivo. While such forces will be very small in these large diameter airways, more local effects may impact on certain parts of the airway microenvironment such as cilia and possibly mucosal folds.

To establish which airway wall structures and features were identified by aOCT, images were compared to histology obtained from a similar region of the same airway. In addition to the detection of mucosal folds present at the luminal surface, aOCT visualized the entire inner airway wall including the mucosa and ASM (without distinguishing between the two), and also partially visualized the outer airway wall as shown by images of cartilage plates. However not all cartilage plates were visible around the entire circumference of the airway wall, which is likely related to a loss of signal strength when imaging deeper into the airway wall, a limitation inherent to OCT. In general, there were good correlations between structures identified by aOCT and histology, although the images were not identical (Fig. 2). It is likely that the regions assessed by aOCT and histology were not precisely the same due to the different slice thicknesses of the two techniques. Specifically, the minimal width of the light beam itself (138 µm at the beam waist) results in a difference of this amount between the regions investigated. Further, there may have also been an angular offset in the plane in which aOCT images were acquired compared to histology due to flexing of airway segments during histological embedding.

The limitation on how closely we could match the airway region used to compare aOCT to histology may have also contributed to slight differences in quantified airway wall dimensions. Results indicated a tendency for all aOCT-derived measurements to be slightly greater than histology-derived measurements. It is possible that there was some tissue shrinkage following histological processing, although this can not be due to fixation per se.
since airway wall measurements were not different in formaldehyde-filled airways. Some of the differences could reflect a small overestimation of wall dimensions by OCT due to the refractive properties of the airway wall. The refractive index of the heterogeneous airway wall is unknown, and though strongly influenced by water based cellular structures, other wall components such as collagen and elastin also contribute. Studies which determine absolute wall dimensions by OCT should therefore consider the impact of the refractive properties of the airway wall.

The utility of OCT in visualizing dynamic changes in the airway wall associated with airway lumen narrowing is demonstrated by the identification of several events in the live airway that have been observed in previous histological investigations. These include the thickening of the inner airway wall in response to ASM contraction (15, 16), the presence of mucosal folds in both the relaxed and contracted airways (15, 16) and the sliding and bending of cartilage plates accompanying bronchoconstriction (27, 38). Further, by assessing changes in the outer perimeter of the inner airway wall (i.e. Pmo) we were able to compute ASM shortening and construct a geometrical relationship between ASM shortening and luminal narrowing, which to our knowledge is the first such relationship derived from dynamic measurements in a live airway preparation. As identified in previous studies proportional changes in luminal narrowing are greater than ASM shortening (27). Given that many of the above observations have, to some degree, shaped our understanding of mechanism(s) and dynamics of airway narrowing (17, 22, 31, 37, 49), OCT represents a potential step forward by permitting repeated measurements to be obtained in a live, dynamically changing environment under a variety of different experimental conditions.

An important morphological parameter obtained from the application of OCT to airway wall dynamics is internal epithelial perimeter (luminal border), i.e., Pi. The mucosal epithelial membrane is regarded as a significant load bearing element within the airway wall due to an
apparent resistance to stretch and compression, and for this reason $P_i$ has also been used as a universal index of airway size (15, 16). A secondary aim of the present study was to look for evidence of distension of the epithelial membrane (as assessed from the luminal epithelial perimeter, i.e., $P_i$) and to resolve contradictions between previous histological and physiological studies (14-16, 26, 34). The present study is the first to record directly the effects of physiological inflationary pressures on epithelial perimeter in the same live airway preparation. Results indicated that $P_i$ increased by ~14% after inflation from 5 to 25 cmH$_2$O supporting a previous histological based study in this species, which suggested $P_i$ could increase between 10-25% (34). Together, these findings suggest that expansion of the airway lumen with lung inflation is due in part to distention of the epithelium in addition to known accommodation produced by widening of mucosal folds (16, 36). Importantly, a distensible epithelial membrane also brings in to question the use of $P_i$ as an index of airway size and this should be considered by studies that normalize morphological measurements to $P_i$.

In addition to revealing the capacity of the epithelium to stretch, dynamic imaging of the airway wall using aOCT produced several other novel findings. One of these was a modest (~6-7%) but statistically significant decrease in inner wall area with ASM contraction, which was insufficient to prevent the considerable inner wall thickening. Previous histological measurements of inner wall area in relaxed and contracted airways suggested conservation of inner wall area (15, 16, 29), whereas studies of dog airways in vivo report a net decrease in wall area as assessed by HRCT (7). In the latter study by Brown et al., the decrease in wall area was attributed to a systematic error in measurement, which is less likely with aOCT due to a higher radial resolution. However at locations where the inner and outer wall is poorly delineated and cartilage is not visible, aOCT could underestimate wall area in the contracted airway as a result of signal attenuation by the thickened airway wall (N.B., this only effects subsurface measurement). Alternatively, a physiological explanation for a reduction in wall
area could be evoked. As noted by Sasaki et al. (41), ASM contraction may conceivably increase inner wall interstitial pressure (21) promoting an outward fluid shift that reduces area. There appears to be no direct evidence to indicate this possibility, nor indeed is data currently available to estimate fluid kinetics through smooth muscle produced by physiological driving pressures.

Another novel finding was the anisotropic nature of the outer wall’s response to ASM contraction. The sliding and bending of cartilage plates during ASM contraction suggests considerable independence of movement between the different airway wall structures, in particular between the cartilage plates and the inner airway wall. This was evident from measurements of the distance from the inner airway wall to the cartilage which revealed thickening of this region and/or a separation of the inner wall from the cartilage during ASM contraction and deflation. We propose two possible explanations for these findings. First, thickening of this space could be a geometric consequence of a reduced circumference, similar to inner wall thickening with ASM contraction. Secondly, a physical dissociation between the inner airway wall and surrounding cartilage plates may have occurred. There is sound histological and physiological evidence, primarily in pig airways (27-29) for uncoupling between the inner and outer parts of the airway wall caused by ASM contraction. The phenomenon is not restricted to porcine airways however and has been noted in other species including humans (47) and dogs (45). While it is still unclear as to how this uncoupling process might be facilitated, it may involve distention of a fiber-elastic matrix deposited between the inner and outer airway walls (not detected here by aOCT) (28). Such airway wall anisotropy impacts on mechanical loads that restrict and regulate ASM shortening in healthy airways and which could be modified in disease with implications for airway responsiveness. Specifically, stretchable tethers within and between different wall compartments and curling/bending of
cartilage plates represent mural load bearing elements (27, 28, 38, 43) and these in turn influence external parenchymal elastic after-loads developed by distortion of lung tissue (42).

There are several areas of respiratory physiology that could be immediately advanced through the use of aOCT. Most significant of these is the regulation of airway narrowing by dynamic mechanical load. It is now well documented that the magnitude of ASM contraction and airway narrowing is suppressed by dynamic load produced by breathing and it is speculated that interruption of the pathways involved could promote airway hyperresponsiveness in obstructive disease (1). Whilst the airway response to dynamic load can be largely explained by ASM plasticity (1), it is unclear whether non-muscle structures also contribute, e.g., does distortion/movement of wall components seen in the present study regulate airway caliber directly or indirectly by modifying ASM responses? Indeed such questions could be addressed through use of aOCT. The rotation frequency of the probe used in the present study was relatively low (0.2 Hz) and, therefore, insufficient to adequately capture more dynamic measurements, such as airway changes accompanying breathing (~0.25 Hz). However, greater rotation speeds are possible and comparable systems have demonstrated speeds up to 30 Hz (46). For regular motion such as breathing, it is also possible to apply respiratory gating techniques (24) to remove motion artefact. There is typically a trade-off between rotation speed and transverse resolution – faster rotation speeds require greater sample rates or greater interpolation. For the current experimental set-up, a slow rotation rate was chosen to maximise image resolution, allowing visualisation of features such as mucosal folds.

A further application of aOCT is to extend the type of structure-function investigations described in the present study to the 3-dimensional architecture of the airway wall. The scanning ‘pullback’ modality of aOCT, whereby the probe is moved slowly along the length of the airway while simultaneous acquiring cross sectional images, allows a large region of the airway to be investigated essentially in 3D. This feature of aOCT has been explored in our
recent studies (25, 50), and we further demonstrate it here by reconstructing a 3D profile of a relaxed airway (Fig. 8) in which the proximal and distal locations of the airway are apparent as well as associated side branches. Future studies could use \(\nu\)OCT to examine regional or anatomical changes in airway function such as whether airway narrowing or compliance varies along the airway wall (proximal to distal) or indeed more locally, such as at branching points where there are changes in both the orientation of ASM and the amount and arrangement of cartilage within the wall (40, 44).

The present study demonstrates the utility of \(\nu\)OCT in measuring airway narrowing and, importantly, in tracking dynamic changes in airway structures of live airways, without the need for fixative, marking or contrast-enhancement agents. In combination, these features provide a powerful tool for studying structure-function relationships in airways, as confirmed by the novel findings reported here, including direct evidence for epithelial distension with inflation and anisotropic behavior of the live airway wall during constriction.
REFERENCES


Table 1. Airway dimensions measured by aOCT and morphometry

<table>
<thead>
<tr>
<th></th>
<th>aOCT (Prior to fixation)</th>
<th>aOCT (Post fixation)</th>
<th>Morphometry</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A_{mo}$ (mm$^2$)</td>
<td>38.9 ± 5.6*</td>
<td>40.4 ± 5.7**</td>
<td>34.0 ± 4.3</td>
</tr>
<tr>
<td>$A_i$ (mm$^2$)</td>
<td>34.4 ± 5.1*</td>
<td>35.6 ± 5.2*</td>
<td>29.8 ± 3.8</td>
</tr>
<tr>
<td>Inner wall area (mm$^2$)</td>
<td>4.5 ± 0.5</td>
<td>4.8 ± 0.5</td>
<td>4.2 ± 0.5</td>
</tr>
<tr>
<td>$P_{mo}$ (mm)</td>
<td>22.4 ± 1.6**</td>
<td>22.7 ± 1.6**</td>
<td>20.9 ± 1.6</td>
</tr>
<tr>
<td>$P_i$ (mm)</td>
<td>22.5 ± 1.7*</td>
<td>22.4 ± 1.6**</td>
<td>20.8 ± 1.4</td>
</tr>
<tr>
<td>Inner WT (mm)</td>
<td>0.21 ± 0.01</td>
<td>0.22 ± 0.01</td>
<td>0.21 ± 0.01</td>
</tr>
</tbody>
</table>

Data are mean ± SE airway dimensions for different airway wall compartments as measured by anatomical optical coherence tomography (aOCT) or morphometry. Measurements by aOCT were performed immediately prior to fixation and after ~12 hrs of fixation. $A_{mo}$, area enclosed by the outer border of airway smooth muscle; $A_i$, area enclosed by the inner epithelial lining (luminal border) of the airway; inner airway wall area; $P_{mo}$, perimeter of outer border of airway smooth muscle; $P_i$, internal epithelial perimeter (luminal border); Inner WT, thickness of inner airway wall. Dimensions measured by the histological approach tended to be less than that by aOCT either prior to or post fixation. There was no difference between airway dimensions measured by aOCT immediately prior to fixation compared to that measured after fixation. N = 5, *P < 0.05, **P < 0.01, compared to morphometry. *(Repeat measures one-way ANOVAs)*
FIGURE LEGENDS

**Figure 1.**
Comparison between the cross sectional area (CSA, mm$^2$) of phantom tubes measured by anatomical optical coherence tomography (aOCT) to actual CSA. Tubes were filled with Krebs solution for measurements. Measured CSA was strongly correlated with actual CSA.

**Figure 2.**
An example cross sectional image of an airway recorded by aOCT (left) and a histological section from a similar anatomical location of the same airway (right). The image acquired by aOCT identified the entire inner airway wall as defined by the area enclosed by the luminal border of the airway epithelium (AE) and smooth muscle (SM). The inner airway wall is therefore represented by the thick black band surrounding the airway lumen. Also identified were shallow mucosal folds (MF) and cartilage plates (CP). The probe catheter (PR) inserted into the airway for recordings is distinctly visible in the lumen of the airway.

**Figure 3.** (A) Example cross section of an airway recorded by aOCT before stimulation (Baseline), after contraction to electrical field stimulation (EFS, Contracted), and once airways had relaxed after removal of the stimulus (Relaxed). Apparent in the figure is pronounced luminal narrowing, thickening of the inner airway wall as indicated by the black inner band, and also sliding of cartilage plates. Arrows mark the position of a cartilage plate (tip to tip) under each condition. The images show that in the contracted state the plate had slid underneath a surrounding plate. The plate is also seen to bend or curl inward at the point of attachment to the inner airway wall. (B) Luminal area ($A_i$, mm$^2$) measured in six airways across each condition, baseline, contracted and relaxed. (Repeat measures One-way ANOVA) (C) Relationship between airway lumen narrowing and airway smooth muscle (ASM) shortening for the same six airways shown in B. Airway narrowing was the % decrease in lumen diameter calculated from $A_i$ assuming circularity, and ASM shortening the % decrease in $P_{mo}$. (Pearson’s correlation analysis)

**Figure 4.** (A) Inner airway wall thickness (mm) and (B) inner airway wall area (mm$^2$) measured by aOCT. Wall measurements were made before contraction (Baseline), during contraction induced by electrical field stimulation (EFS) (Contracted) and after subsequent relaxation after removal of the stimulus (Relaxed). EFS produced an increase in wall...
thickness and a modest decrease in wall area, which was reversed in each case after cessation
of EFS. N = 6. (Repeat measures One-way ANOVAs)

Figure 5. (A) Example aOCT images indicating an increase in the space between cartilage
and inner wall margins after contraction induced by electrical field stimulation (EFS). Arrows
indicate wall separation in the contracted airway in comparison to that immediately prior to
contraction (Baseline). The cartilage plate (CP), luminal border of the airway epithelium
(AE), and the probe catheter (PR) just visible in the contracted image, are labeled. (B)
Distance between cartilage and inner airway wall margins (mm). Measurements were made
before contraction (Baseline), during contraction to EFS (Contracted) and after subsequent
relaxation after removal of the stimulus (Relaxed). Mean data are 15 quadrants from six
airways. Contraction to EFS produced transient thickening of the region between the inner
airway wall and cartilage, which was reversed after ASM relaxation. (Repeat measures one-
way ANOVA)

Figure 6. The effect of intraluminal pressure on (A) Inner airway wall thickness (mm) and
(B) Inner airway wall area (mm²) measured by aOCT. Wall measurements were made at +5
cmH₂O, -5 cmH₂O and +25 cmH₂O. Wall thickness increased with deflation to -5 cmH₂O and
decreased with inflation to +25 cmH₂O. Intraluminal pressure had no effect on wall area. N =
5. (C) Effect of intraluminal pressure on the thickness (mm) of the space between cartilage
and inner airway wall. Thickening of the region between the inner airway wall and cartilage
occurred after deflation to -5 cmH₂O, but there was no change after inflation to +25 cmH₂O.
N = 10 quadrants from five airways. (Repeat measures One-way ANOVAs)

Figure 7. The effect of inflation on internal epithelial perimeter (luminal border, or ‘Pᵢ’, mm)
as measured by aOCT. Inflation from +5 to +25 cmH₂O increased Pᵢ by 13.6 ± 1.34 %. 
(Paired t-test)

Figure 8. A 3D volume rendering of a porcine bronchus lumen acquired by anatomical
optical coherence tomography. The proximal and distal ends of the bronchus are evident as
well as branching sites of daughter airways.
$r = 1$

Slope = 1
$P < 0.001$

$P$ ($mm$)

$+5$ cmH$_2$O

$+25$ cmH$_2$O