Increased narrowing of bronchial segments from immature pigs

H.W. Mitchell, P.K. McFawn, M.P. Sparrow


ABSTRACT: Bronchial narrowing was investigated to determine whether changes in smooth muscle force, described previously in different aged pigs, are associated with differences in airway narrowing. 

The sensitivity of bronchial segments from suckling and young pigs was compared by perfusion through the lumen with a Krebs solution at a pressure of 5 cmH₂O, measuring the reduction in flow in response to carbachol and histamine. Segments of the same internal diameter (l.d. approx. 2.5 mm) from each age were used by selecting proximal segments from suckling and distal segments from young pigs. 

The sensitivity to carbachol or histamine was the same in smooth muscle strips from proximal and distal bronchi in each age. Furthermore, segments from either age had a similar pressure-volume relationship between -10 and 30 cmH₂O. However, concentration-flow curves showed that the airways from sucklings were five times more sensitive than airways from young animals to carbachol (p<0.01) and, less consistently, to histamine (p>0.05), when flow was reduced by 50% of maximum. Flow was abolished by maximum concentrations of carbachol at both ages whereas histamine stopped flow in the young segments and reduced it by 80% in the suckling age group. 

Data indicate a greater sensitivity of bronchial narrowing to carbachol in the intact airway – this is consistent with a greater force production in suckling pig airway smooth muscle. These findings support a postnatal development of airway function, as suggested from clinical observations and provocation studies in humans. 


Receptor-coupled production of force by airway smooth muscle changes progressively during maturation. Tracheal and bronchial preparations from immature guinea-pigs are more sensitive to contractile drugs than the adult [1, 2] but the extent to which these sensitivity changes reflect development of smooth muscle cells is unclear. In the pig, airways smooth muscle at 4 weeks of age develops nearly twice the force to carbachol than smooth muscle from 26 weeks and mature animals [3]. This difference in stress is further increased when force is corrected for the differing amounts of smooth muscle present in the airway wall during development. 

How differences in the contractile response of airways smooth muscle (i.e. in isometric force) from young and old are associated with the narrowing characteristics of the intact airway is uncertain. In human infants the provocative doses of methacholine and histamine required for flow limitation are lower than the equivalent doses in normal adults, but this is partly due to different breathing patterns and drug delivery in infants and children [4, 5]. The responsiveness of the airways from different ages may be affected by differences in the structure and geometry of the airways [6, 7] as well as in the mechanical properties of the smooth muscle itself. Less than half the maximum force developed by smooth muscle of the airway wall seems necessary to effectively close perfused segments of small airways [8]. Structural properties which determine the airway compliance are of particular importance since the compliance helps set the operating length of the smooth muscle and it imposes loads against which muscles have to perform (i.e. shorten). 

We have investigated bronchial narrowing in pig airway segments to determine whether the changes in smooth muscle force, previously described in the suckling and adult, are associated with differences in airway narrowing. The static pressure-volume relationship of the bronchi used was determined to examine muscle load at the different ages. 

Materials and methods 

Segment preparation 

Lungs were obtained from recently slaughtered (<30 min) Largewhite/Landrace-cross pigs. The ages of the
pigs were approximately 4–5 weeks (sucklings) and 26 weeks (young). After packing the lungs in ice they were transported to the laboratory where bronchial segments were dissected as previously described (8). We used segments of the 'stem' bronchus which runs the entire length of the lower lobes. Segments approximately 35 mm long were used for perfusion.

The two ends of each segment were tightly cannulated with tubing whose dimensions were slightly larger than the i.d. of the segment at atmospheric pressure. The segments were then mounted in horizontal chambers which were filled with a Krebs solution (37°C, pH 7.4, gassed with 5% CO₂ in O₂). This Krebs solution was regularly exchanged for fresh throughout the experiment. The lumen of each segment was perfused with Krebs solution under a constant head of pressure (5 cmH₂O) set from the reservoir. The flow-rate through the segment was measured with a differential pressure transducer (type MPX10DP, Motorola Semiconductors, Phoenix, USA) which was set to detect the pressure-drop along a custom built flowhead in a manner similar to that commonly used to record airflow in animal experiments. The flowhead and transducer were previously checked for linearity and were calibrated over the range of flows encountered in an experiment. Flow-rate was monitored on a chart recorder. The resistance was calculated by dividing pressure by flow.

Response to carbachol and histamine

The concentration-response relationship to carbachol and histamine in perfused segments was compared in the same sized airway from suckling and young pigs. Because of the different size of the lungs, airways from different locations of the 'stem' bronchus [8] were used. From the suckling pig a segment from the mid-region (between airway divisions nos. 10–17) was used whilst in the young pig a segment was prepared from the more basal region of the bronchus (divisions nos. 15–22).

Carbachol or histamine was introduced through the lumen of the perfused bronchial segments [8]. Increasing concentrations were used until the flow of Krebs solution stopped or until no further response (fall in flow) was obtained despite increasing drug concentration. The flow and resistance after each drug concentration was calculated.

In a separate experiment we measured the sensitivity of smooth muscle from proximal and distal bronchi. EC₅₀ values were obtained from the concentration-response curve to carbachol and histamine in strips of airway wall in an organ bath. Muscle response was measured from isometric force produced as described before [3]. Force responses were normalized to the maximum response in each tissue and the concentration of drug producing half maximum response (EC₅₀) determined.

Static compliance

The static pressure-volume relationship of airways from suckling and young pigs was determined using segments in modified perfusion apparatus. The outflow cannula was closed via a 3-way tap and a calibrated Motorola pressure transducer was connected to a side arm of the perfusion inlet. The volume of the segment was increased by 0.04 ml increments by introducing Krebs solution with a microsyringe through another 3-way tap on the inlet. Pressure was allowed to stabilize (approximately 2–3 min) after each change in volume until the stress relaxation was >80% complete. Three inflationary and deflationary pressure-volume loops between -10 to 30 cmH₂O were recorded in each segment. The curves produced from these loops were always superimposed. The initial volume of the segment with zero transmural pressure was measured by filling the segment with Krebs solution at atmospheric pressure. Change in pressure (from atmospheric) and change in volume divided by initial volume (i.e. strain), were then plotted.

Airway morphometry

At the conclusion of the perfusion experiment, the segment was rapidly frozen in isopentane (at -196°C) at a transmural pressure of 5 cmH₂O. Sections, 15 μm thick, were cut at -18°C on a cryostat. Three transverse sections prepared from proximal, mid and distal regions of the segment were air dried and fixed in methanol for 10 min, then stained with Haematoxylin and Chromotrop 2R. The folded internal margin of the epithelium was traced, using a camera lucida fitting on a microscope, on a graphics tablet connected to an Apple IIE microcomputer. Similarly, the external perimeter of the smooth muscle (which is continuous) was measured. Assuming that the length of the epithelium and the wall area are constant [9] the airway lumen could be reconstructed so that the fully relaxed internal perimeter and hence diameter and the wall area could be calculated [9]. Airway morphometry of the distal ends of the segments is presented because the resistance would be highest at this point.

Solutions and statistics

Data shown are mean±SEM with n=number of animals. The significance of the difference between means was compared using the Student's t-test with p<0.05 considered significant.

Mean pressure-volume curves for each age were drawn from the mean triplicate compliance loops in each segment. Mean slope±SEM at each age group were calculated over the pressure range 0–10 cmH₂O. Differences between slopes were evaluated by analysis of co-variance. For the concentration-resistance relationships for carbachol in suckling and young airway segments a single exponential curve was fitted.
to the geometric means of the resistances obtained at each concentration used. Fitting was done by the method of least squares using computer software (Cricket Graph). The resistance in the flowhead (0.08 cmH$_2$O·ml·1·min$^{-1}$) was subtracted from resistance recorded when a tissue was connected.

Krebs solution had the following composition (in mM): 121 NaCl, 5.4 KCl, 1.2 MgSO$_4$, 1.2 NaH$_2$PO$_4$, 25 NaHCO$_3$, 11.5 glucose and 2.5 CaCl$_2$. The solution was continuously gassed with 95% O$_2$/5% CO$_2$ mixture. The drugs used were carbamylcholine (carbachol, Sigma Chemicals) and histamine phosphate (Sigma Chemicals). These were prepared in Krebs solution on the day of each experiment. All drug solutions were kept on ice.

Results

The effect of age on the responsiveness of perfused segments to carbachol and histamine was studied using preparations approximately 3.5 cm long. The relaxed internal diameters and airway wall areas at the distal part of each segment were the same (table 1). The resting flow rates were 5.8±0.5 and 4.9±0.4 ml·min$^{-1}$ (Ns) respectively.

<table>
<thead>
<tr>
<th>Diameter mm</th>
<th>Wall area mm$^2$</th>
<th>PW</th>
<th>EC$_{50}$ μM Carbachol</th>
<th>EC$_{50}$ μM Histamine</th>
<th>R$_5$ Carbachol</th>
<th>R$_{20}$ Carbachol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suckling</td>
<td>2.68±0.15</td>
<td>1.24±0.09</td>
<td>0.181±0.011</td>
<td>1.7±0.5</td>
<td>11.0±8.9</td>
<td>5.5</td>
</tr>
<tr>
<td>VS</td>
<td>NS</td>
<td>NS</td>
<td>p&lt;0.01</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young</td>
<td>2.59±0.07</td>
<td>1.25±0.03</td>
<td>0.195±0.010</td>
<td>8.5±2.9</td>
<td>63±32</td>
<td>41</td>
</tr>
</tbody>
</table>

Diameter: internal diameter of the relaxed airway at the distal end; Wall area: region enclosed by the luminal surface of the epithelium internally and by the outer boundary of the smooth muscle externally. PW: proportion of wall area to the total relaxed cross-sectional area of wall plus lumen. Data represent the mean±SE of four animals for each age group. Sensitivity is compared on the basis of EC$_{50}$; concentration of carbachol or histamine producing half of the maximum drop in flow rate. Mean EC$_{50}$ was calculated after log transformation of data. NS: not significant. In the two right-hand columns a comparison of the concentrations at which airway resistance (R) equals 5 and 20 cmH$_2$O·ml$^{-1}$·min$^{-1}$, denoted as R$_5$ and R$_{20}$ respectively. Data represents the mean of preparations from four suckling and seven young pigs.

Firstly, the static compliance of the segments was determined from the pressure-volume curves to assess whether the wall properties were comparable. In segments from four suckling and three young pigs the compliance was very similar at both high and low pressures (fig. 1). The mean slopes over the range 0–10 cmH$_2$O were 0.061±0.006 and 0.064±0.008 respectively, in suckling and young pigs (Ns). At a transmural pressure of 5 cmH$_2$O (the pressure used here to construct the concentration-response curves) the volumes were on average 1.45 and 1.55 times the initial volume at atmospheric pressure in the suckling and young airways respectively.

Figure 2 shows that concentration-response curves of bronchial segments from suckling pigs to both carbachol and histamine are displaced to the left of those in young pigs. At 3×10$^{-7}$ M, carbachol reduced flow through the segments from sucklings whereas flow was not significantly reduced in segments from young animals. Higher concentrations of carbachol, 10$^{-3}$ M and 10$^{-4}$ M respectively, virtually abolished flow in both age groups. In five preparations from young pigs flow ceased completely and in two it decreased by >96%. The EC$_{50}$ in the suckling animals was significantly less (p<0.01) indicating a greater sensitivity to carbachol at this age (table 2). With histamine, flow
was also reduced with the curves from sucklings lying to the left of those from the young pigs. This was most noticeable at threshold concentrations of histamine, but the shift was not significant at the EC50 level (table 1). Furthermore the maximum fall in flow was 80±11% in suckling segments, i.e. there was a plateau in the suckling dose-response curve whereas histamine ultimately abolished the flow in the young segments.

![Graph A](image)

![Graph B](image)

**Fig. 2.** - Effect of carbachol (upper panel, A) and histamine (lower panel, B) on flow through the lumen of bronchial segments from the airways of suckling (n=4) and young (n=7) pigs. In each experiment airway segments were exposed to carbachol, then after recovery, to histamine. Horizontal bars show the ±S of the mean EC50 (drug concentration producing half maximum contraction). •: suckling; ••: young.

The difference in sensitivity was more apparent when comparing resistances (fig. 3). The dose of carbachol producing a resistance of 5 cmH2O·m-1·min-1, denoted here as R5, indicated that the bronchial segments from the sucklings were greater than seven times more sensitive than those from the young pigs (table 1). A similar difference in sensitivity was observed at other levels of resistances (e.g. R20).

**Table 2.** - Sensitivity (EC50) of airway strips to carbachol or histamine

<table>
<thead>
<tr>
<th>Bronchus</th>
<th>Carbachol</th>
<th>Histamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proximal</td>
<td>Suckling</td>
<td>0.79±0.12</td>
</tr>
<tr>
<td></td>
<td>Young</td>
<td>0.89±0.12</td>
</tr>
<tr>
<td>Distal</td>
<td>Suckling</td>
<td>0.78±0.12</td>
</tr>
<tr>
<td></td>
<td>Young</td>
<td>1.20±0.20</td>
</tr>
</tbody>
</table>

Sensitivity is shown as the drug concentration (µM) producing half maximum contraction (mean EC50 values obtained on log-transformed data) in 4 airway strips/age from either the proximal or distal bronchus.

The sensitivity of the smooth muscle in isolated strips of the bronchial wall taken from proximal and distal sites of the airways (comparable to those of the segments used) was then determined. Table 2 shows that the EC50 for carbachol and histamine are similar at both sites in the bronchial tree. The concentration-response curves were essentially similar in shape to those described earlier [3].

**Fig. 3.** - Concentration-resistance curves for carbachol from bronchial segments from suckling and young pigs. Resting resistances were not significantly different. An exponential curve for each age group was fitted to the geometric means. At higher concentrations the near complete closure caused resistance to approach infinity •: suckling; ••: young.

**Discussion**

We compared airway narrowing in suckling and young animals using airways of the same lumen and wall dimensions. The importance of starting diameter and the wall thickness on narrowing has been shown hypothetically for both blood vessels [10] and airways [6, 7, 11]. Consequently, the bronchial segments used in the present study were prepared from different, though overlapping, regions of the 'stem' bronchus in the two ages of pigs. Thus, both physical and structural properties of the airway segments used were very similar. It is improbable that airway narrowing
described here in suckling and young were biased by differences in responsiveness of the smooth muscle at proximal and distal regions of the bronchus because the EC_{50} values for the two drugs used showed no topographical variation (this study and [3]).

Narrowing in airway segments from suckling pigs occurred at lower concentrations of carbachol than in the young. Previous studies using strips of airway wall [1-3, 12] have determined smooth muscle force in isolation — i.e. without consideration of factors which influence airway geometry. In vivo experiments on bronchial responsiveness in animals have yielded varied results [2, 13]. Studies in human young suggest that the bronchi are responsive to histamine and cholinomimetics [14, 15]. In our experiments the thresholds to carbachol and to histamine in the suckling were lower while the slope remained the same in preparations from both age groups. Increasing the wall/lumen ratio in tubular structures could increase the slope, but not the threshold, of the dose-response curve [6, 7, 10]. How the accessibility of the smooth muscle, through the epithelium, contributed to airway sensitivity [16] in the two ages of bronchi was not investigated. Airway narrowing in the present experiments occurred without the limiting influence of parenchymal tethering. Differences in sensitivity in vitro could be increased or decreased in vivo by the prevailing lung elastic recoil. Elastic recoil may be less in the neonate pig [17] in which case the sensitivity difference described here may be even larger in intact lungs.

Although the airway walls were geometrically similar, the smooth muscle in the suckling airway develops more stress to the drugs used [3] and, therefore, greater shortening, at equivalent levels of stimulus and equivalent airway wall loads. Airway smooth muscle shortening is related to elastic and cartilaginous loads [18-20]. The static deflationary compliance of the young and suckling airways used in our study were the same, as previously described in rabbit airways in vitro [13]. Since the compliance and the diameter of the segments from the suckling and young pigs was not different we assumed that the operating length of the smooth muscle within the airway wall was the same but whether it was at its optimal length (L_{o}) at 5 cmH_{2}O in each case is uncertain.

The greater threshold sensitivity of airway narrowing to carbachol in the suckling may be closely related to greater force produced at this age [3]. Maturation in Ca^{2+}-regulated smooth muscle force occurs at the level of the contractile machinery because it is evident in chemically skinned tracheal smooth muscle. In intact smooth muscle the maximum muscle response to carbachol was approximately doubled, but there was no change in the EC_{50} so that the slopes of the dose-response curves at the two ages were different. In the present study a difference in maximum response (i.e. airways resistance) to carbachol was not seen between segments from suckling and young animals — an observation compatible with the suggestion that airway closure occurs before maximum force development by the smooth muscle [8]. For this reason, and also because of differences in the surfaces exposed to the drugs, the EC_{50} values for flow reduction in perfused segments and for force production in smooth muscle strips for either of the age groups are not directly comparable. However, we have shown that there is a considerable shift in threshold or sensitivity to carbachol in the perfused preparations of airway from suckling compared with the young. As noted above, in strips of bronchial and bronchiolar wall, the EC_{50} for carbachol was unchanged with age. Present and previous results from isolated strips are compatible if functional airway narrowing is seen as involving only low levels of smooth muscle contraction.

With histamine there also tended to be a decrease in the threshold sensitivity in the suckling animals but the maximum response (i.e. decrease in flow-rate) was less in the suckling segments than in the young because high doses of histamine failed to close-off the airway to the perfusate. Strips of suckling bronchiolar wall develop relatively little maximum stress — only some 2.5 mN/mm² tissue to histamine compared with approximately 4 mN/mm² in the young bronchiol [3]. Since histamine closed-off the perfused segments in the latter i.e. the young pigs, it might be inferred that a wall stress approaching 4 mN/mm² is needed to close-off the liquid-perfused airway. This is substantially less than the maximum active stress of 7 mN/mm² previously described in young pig bronchiolar strips [3] or the 20 mN/mm² circumferential stress calculated in canine bronchial segments stimulated with high concentrations of acetylcholine (22).

Our finding that the sensitivity of airway narrowing is greater in the immature animal provides support for the postnatal development of airway responsiveness which was previously suggested from clinical history [16] and from provocation studies in humans [14, 15].

Acknowledgements: The excellent technical assistance of K.E. Willet is acknowledged. This research was supported by the Australian Research Council and the National Health and Medical Research Council of Australia. P.K. McFawn was supported by the Sudden Infant Death Syndrome Foundation of Western Australia.

References


