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**Differential effects of inhaled methacholine on circumferential wall and vascular smooth muscle
of 3rd generation airways in awake sheep**

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Running Head: Differential cholinergic effects on airway smooth muscle

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32 ABSTRACT

33 Evolution and natural selection ensures that specific mechanisms exist for selective airway absorption
34 of inhaled atmospheric molecules. Indeed nebulized cholinergic agonists used in asthma challenge
35 tests may or may not enter the systemic circulation. We examined the hypothesis that inhaled
36 cholinergic agonists have selective access. Six sheep were instrumented under general anaesthesia
37 (intravenous (i.v.) propofol 5 mg.kg⁻¹, 2-3% isoflurane – oxygen) each with pulsed-Doppler blood flow
38 transducers mounted on the single bronchial artery, and sonomicrometer probes mounted on the
39 intrapulmonary 3rd generation lingula lobe bronchus. Continuous measurements were made of
40 bronchial blood flow (Q_{br}) and conductance (C_{br}), bronchial hemi-circumference (CIRC_{br}) and wall
41 thickness (WALL TH_{br}) in recovered, standing, awake sheep. Intravenous methacholine (MCh) 0.125-
42 2.0 µg.kg⁻¹, at highest dose caused a 233% rise in Q_{br} (P< 0.05), and 286% rise in C_{br} (P< 0.05). CIRC_{br}
43 fell to 90% (P< 0.05); WALL TH_{br} did not change. In contrast nebulised MCh 1-32 mg.mL⁻¹ inhaled
44 through a mask at highest dose caused a rise in ventilation, and a rise in Q_{br} proportional to P_a without
45 change in C_{br}. CIRC_{br} fell to 91% (P< 0.05), and WALL TH_{br} did not change. Thus inhaled MCh has
46 access to cholinergic receptors of bronchial circumferential smooth muscle to cause airway lumen narrowing,
47 but effectively not to those of the systemic bronchovascular circulation. It is speculated that the
48 mechanism is selective neuroparacrine inhibition of M3 bronchovascular cholinergic receptors by prostanoids
49 released by intense MCh-activation of epithelial and mucosal cells lining the airway.

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51 **Keywords:** bronchial circulation, neuroparacrine inhibition, sonomicrometry, prostanoids,
52 evolutionary lung protection

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57 INTRODUCTION

58 In recent years clinical practice has challenged the theory that evolution and natural selection
59 prevents absorption of inhaled molecules threatening homeostasis. A specific example is the
60 inhalation of the cholinergic agonist methacholine (MCh) in asthma patients to determine whether
61 airway wall circular smooth muscle (as distinct from the smooth muscle of its own reticulated
62 bronchovascular bed) is hyperreactive in asthma patients (8). In this process it is assumed the inhaled
63 agonist does not compromise the bronchovascular or wider systemic circulation because there are
64 apparently few cardiorespiratory side effects, e.g. hypotension. In addition, there is controversial
65 evidence for possible differential MCh engagement of the airway smooth muscle structures and
66 penetration into the bronchovascular bed. In 1985, two papers (21, 27) used different techniques to
67 measure bronchial blood flow (Q_{br}) in sheep and each showed that inhaled aerosolised MCh caused
68 flow in the bronchovascular bed to increase. By contrast, Barker et al (1) used dimethyl ether uptake
69 to measure blood flow in the trachea, and showed aerosolised MCh had no effect on the mucosal
70 component of tracheal wall blood flow. Later, the same group using colour-coded microspheres to
71 separate changes in bronchial mucosal and deep wall blood flow, confirmed that the nebulised
72 cholinergic agonist acetylcholine (ACh) did not affect bronchial blood flow, but intravenous (i.v.)
73 ACh did, suggesting that intravascular ACh had access to airway bronchovascular cholinergic receptors
74 whereas aerosolised ACh did not. Both routes of application raised lung airflow resistance. In 1998
75 Charan et al (9) confirmed the earlier findings of differential effects on Q_{br} by the two access routes
76 and showed that aerosolised ACh in halothane anaesthetised sheep may at low and high doses mildly
77 constrict or dilate the bronchovascular bed, respectively. Changes in airflow resistance were not
78 recorded. The same group then attempted to show in anaesthetised, open chest sheep that the
79 constrictor effects of aerosolised MCh on the airway circular smooth muscle were indirect via initial
80 absorption into the bronchial circulation, i.e. there was no barrier to penetration of the bed. Low doses
81 of MCh infused into the bronchial artery of sheep provoked airflow resistance effects. The authors
82 concluded initial absorption of MCh into the bronchovascular bed and systemic recirculation may be a
83 prerequisite for airway wall constriction.

84 Taken together, these studies support the interesting postulate that a physiological barrier can
85 exist to prevent penetration of the bronchovascular circulation by inhaled MCh when there is
86 simultaneous cholinceptor-induced constriction of the circular bronchial smooth muscle, but the
87 investigations were inconclusive. If, however, the postulate was confirmed, the data would raise
88 speculation concerning an evolutionary mechanism protecting the mammalian species from inhaled
89 environmental molecules which, if absorbed into the bronchial and wider systemic circulation threaten
90 survival.

91 In the present study we examined this hypothesis using a new approach of awake sheep
92 relevant to past cholinceptor activation investigations but eliminating factors which may modify the
93 physiology of any putative protective barrier. We used direct measurement for the first time of
94 changes in high airflow resistance airways under natural conditions of negative pressure ventilation,
95 rather than the indirect approach of measuring pressure-airflow relations under anesthesia with an
96 intubated lung and positive pressure ventilation. The sheep were instrumented under general
97 anaesthesia with the AIDA (Airway Internal Diameter Assessment Instrument) ultrasonic package of
98 transducers (4, 41) designed to measure directly and simultaneously high frequency changes in
99 bronchial blood flow, airway circumference, and wall thickness. The airway dimension transducers
100 were mounted on the subcarinal, intrapulmonary 3rd generation airway of the lingula lobe bronchus.
101 They were placed there to track the effects of MCh on the airway generation where small changes in
102 dimensions provoke the greatest airflow resistance changes in the lung (28). The recovered
103 preparation also avoids modifying effects of anaesthetic agents on airways central and peripheral
104 autonomic control, at rest and during disturbances, and the mechanical and functional airway
105 distortions of positive-pressure ventilation. To observe the stimulus-response effects of externally
106 applied MCh on the airway circular wall and bronchovascular smooth muscle, the sheep were pre-
107 trained to accept a face mask and breathe nebulised MCh. The results were compared in the same
108 sheep with i.v. stimulus-response effects of MCh, to show and compare in the same awake animals
109 the effects of intravascular engagement of cholinceptors on airway circular wall and bronchovascular
110 smooth muscle. The observations confirming a physiological barrier to penetration of inhaled MCh

into the bronchial circulation would be circumferential airway wall shortening, and depending on its magnitude, airway wall thickening, but no change in bronchovascular conductance.

METHODS

Female mixed breed sheep (30-45 kg) were used in these studies carried out on the UC Davis campus. Experimental protocols were reviewed and approved by UC Davis Institutional Animal Care and Use Committee. UC Davis has Animal Welfare Assurance on file with the US Public Health Service.

Surgical preparation:

Anaesthesia was induced using i.v. thiopentone sodium (15 mg.kg⁻¹), or propofol (5 mg.kg⁻¹), and maintained by 2-3% isoflurane-oxygen via endotracheal tube. Six sheep were instrumented (Fig.1) with pulsed-Doppler flow probes mounted on the bronchial artery, and sonomicrometry probes positioned on the 3rd generation lingula lobe bronchus. The former made continuous measurements of bronchial blood flow, and the sonomicrometers continuous measurements of bronchial hemi-circumference (a two-crystal transit time sonomicrometer (TTS)) and bronchial wall thickness (single crystal sonomicrometer, SCS). Positioning TTS and SCS probes on the lingula lobe bronchus was a novel modification to our previous implantation of probes on the left main bronchus (4, 41). The current site and instrumentation permits continuous and direct measurement of the changing dimensions of the airway generation most relevant to resistance to airflow (28). Theoretical aspects of linearity, frequency response and the application of the instrument package were previously described in detail (4, 31, 41). In this study the incision for left thoracotomy was made in either the 4th or 5th intercostal space. On entry through the pleura, the ribs were retracted and the lingula lobe lifted and extended away from the lung hilum to reveal its underlying surface. The upper and lower left lobes were then packed ventrally. A pulmonary vein (PV) is clearly seen on the undersurface of the lingula lobe (Fig. 1) lying superficially under the visceral pleura as it proceeds to enter the pericardium. The lingula lobe bronchus is visible deep to the PV. The surgeon's assistant holds the lingula lobe tip gently between fingers with the aid of a swab,

while the surgeon cauterizes the visceral pleura on each side of the PV. The PV is then lifted undamaged using a smooth-surface Ligaloop[®] (Braun-Dexon, Germany) to allow further blunt dissection on each side of the bronchus. The TTS transducers heads were then buried on either side of the bronchus and sutured in place using 7-0 prolene, followed by tissue-glue for further fixation.

The SCS transducer was positioned approximately 10 mm distal to the TTS transducers to minimise electronic interference. The SCS dual-element transducer had a cellulose tissue strip glued to the back of the transducer head. The four apices of the tissue strip were then sutured onto the bronchus adventitia, before being tissue-glued to ensure acoustic coupling between the transducer and the bronchial wall. The lingula lobe was then returned to its original position in the chest. Following implantation of the TTS and SCS probes, all three lobes of the left lung were packed ventrally to expose the first part of the descending aorta, where the bronchoesophageal trunk and common bronchial artery were defined. The pulsed-Doppler blood flow probes used were 1.5-2.0 mm internal cuff diameter with fine silk sutures threaded through the edges of the Doppler cuff. The selection of the correct cuff size for enclosure but without occlusion of the artery is made on inspection, and the artery diameter is recorded. The probe is placed around the bronchial artery (Fig. 1) and secured by tying the edges of the cuff together (for further details, see (23, 31)). After implantation of the pulsed-Doppler flow probe, all three instruments were tested for recording efficacy with the lung in normal anatomical position. The reliability of the instruments was accepted during this phase if they (i) showed a stable baseline and pulsatile wave forms characteristic of the bronchial artery, airway circumference, and the airway wall when the ventilator was turned off, and (ii) showed the cyclic changes superimposed on this pulsatility when the ventilator was turned on (4). The reliability was further tested when an early experiment found the SCS instrument recorded no apparent change in airway wall thickness during exposure to either inhaled or i.v. MCh, and its validity was questioned. After the experiment, the sheep was briefly anaesthetized and an FG-7 embolectomy balloon catheter (Fogarty, American Edwards) passed a measured distance via the femoral artery. The catheter tip was positioned immediately above the diaphragm. On recovery, inflating the balloon raised upper body blood pressure (Fig. 2) in the standing sheep and recorded, for the first time and as expected, a

baroreflex-induced simultaneous increase in bronchial circumference (34), an SCS recorded bronchial wall thinning, and a pressure-passive change in the bronchial circulation (24), indicating instrument reliability.

The leads of the three probes were passed together through the chest wall and tunnelled subcutaneously and exited at a dorsal position caudal to the left scapula. The probe leads were secured to skin using monofilament suture. Also positioned in the pleural space was a pleural catheter (Pleurocath, PRODIMED, FRANCE), which was passed through the chest-wall and skin, and secured with suture. The pleural catheter was connected to a chest drain system (Portex Ambulatory Chest Drainage System, SIMS PORTEX LIMITED, UK) and left overnight to prevent pneumothorax. With the pleural catheter and probes secured in position, the chest was evacuated and closed. Intravascular catheters were also implanted in the superficial cervical artery and vein at the base of the neck for measurement of arterial and right atrial pressures (5). The emerging catheters were tunnelled subcutaneously caudally and passed together with probe leads from skin through rear portals of an overlying custom-made saddle-pack and coiled securely within. Sheep were allowed to recover for at least one week prior to any further experimental intervention. All experiments were carried out with the sheep standing in a metabolic cage within an air-conditioned laboratory where the ambient temperature range was 19-23 °C and 45-55% humidity. During experiments the variables recorded were aortic pressure (P_a), right atrial pressure (P_v), heart rate (HR), bronchial blood flow (Q_{br}), bronchial hemi-circumference ($CIRC_{br}$) and wall thickness ($WALL\ TH_{br}$). Bronchial blood flow conductance (C_{br}), the reciprocal of flow resistance, was calculated from the formula $C_{br} = Q_{br} \cdot P_a^{-1}$. In this calculation, the downstream bronchial circulation drainage pressures reducing the pressure gradient for flow in the equation were ignored, because the errors involved in so doing would be no more than 5% (24). Intravenous or inhaled methacholine experiments and cholinergic blocking studies for each were performed on each sheep on separate days. Data were recorded continuously and stored on a PowerLab/8SP recording system (AD instruments, Castle Hill, Australia).

Intravenous methacholine dose-response protocol; methscopolamine cholinergic blockade

Intravenous infusions (i.v.) of methacholine chloride (MCh, 0.125, 0.250, 0.5, 1.0, 2.0 $\mu\text{g.kg}^{-1}$ in 5 mL 0.9 % NaCl) were administered over 3s via the right atrial catheter in sheep standing at rest. The responses at each dose were recorded as the mean peak effects (e.g. Fig. 3, left) for each variable. At least ten minutes between infusions was allowed for recorded variables to return to steady-state control levels. Cholinoceptor blockade was induced i.v. by receptor antagonist methscopolamine bromide 270 $\mu\text{g.kg}^{-1}$ in 5 mL 0.9% NaCl. This dose was sufficient to block the P_{a} , Q_{br} , C_{br} and airway dimension changes evoked by i.v. MCh 1.0 and 2.0 $\mu\text{g.kg}^{-1}$ (e.g. Figs. 3, 4). The block was effective within minutes and lasted 2-3.5 hr (23).

Nebulization parameters

A Tyco Oxineb nebuliser was used with a mist generating flow rate of 10 L.min^{-1} ; air pressure 50 psi. These parameters were chosen to generate an individual droplet size of 1-5 μm in diameter suitable for inhalation through a conical face mask. The assumed size was based on nebuliser manufacturer guidelines and comparisons of droplet sizes created by nebulisers from several manufacturers (11, 22, 33). It was estimated (16) that this sized hydrophilic particle under normal nasal breathing conditions would penetrate mainly to upper bronchial airways, and that increased ventilation effort generating higher air flow rates would cause penetration and deposition in the lower bronchial and alveolar regions. These predictions are consistent with the imaging and clearance findings in awake sheep by Gunther et al (18) using $^{99\text{m}}$ technetium-labelled diethylenetriaminepentacetate- tagged particles in normal saline.

Mask effect

Preliminary studies showed that the application of the facemask in itself produced a physiological response (Figs. 5, 6). Experiments were done on all sheep prior to the first inhaled methacholine challenge to determine whether the response to mask alone, i.e. sheep breathing room air through the mask, differed from the response to sheep breathing normal saline vehicle through the mask. Sheep standing at rest breathed room air through the conical facemask for 5 minutes followed by 10 min recovery. They then inhaled nebulized normal saline for 5 minutes, followed by 10 min recovery. It was noted and recorded that peak, steady-state responses to the mask stimulus occurred at

2 min after application, and that one minute after the mask was removed at 5 min, all variables had returned to resting control levels. To define effects of breathing nebulised MCh or ipratropium bromide (IPRA) the mean bronchial airway dimensions and haemodynamic responses at each dose were therefore recorded and analysed using control (pre-mask) data, and data one minute after the mask was removed from the sheep (Figs.5, 6).

Inhaled methacholine via conical facemask; ipratropium cholinceptor blockade

Sheep standing at rest inhaled saline vehicle and each of six consecutive and increasing concentrations of nebulized MCh (1, 2, 4, 8, 16, 32 mg. mL⁻¹) each in 3 mL of 0.9% NaCl for 5 minutes followed by at least 10 minutes recovery, i.e. recovery time was increased after inhalation of 8, 16 and 32 mg.mL⁻¹ methacholine to allow recorded variables to return to resting control levels. These concentrations were equivalent to those used by Charan et al (1998) in sheep twice the weight of those used in the present study. These doses were tolerated by all sheep. At the highest doses there was some nasal snuffling. Cholinceptor blockade for these experiments was induced by nebulized IPRA, 0.6 mg.mL⁻¹ in 3 mL 0.9% NaCl inhaled over 5 min. Preliminary studies with inhaled IPRA using human recommended doses for blocking cholinceptor activity at rest showed, by contrast with i.v. methscopolamine, minimal effects on resting airway wall and vascular smooth muscle tone. For this reason, the dose of inhaled IPRA used for the sheep were elevated to three times those recommended for man. The clinical guidelines for man indicated that peak blocking effects would occur 35 min after the total dose of inhaled IPRA. This period was adopted before testing first, the magnitude of the IPRA block followed by the magnitude of the IPRA plus i.v. methscopolamine block to the largest (challenge) dose of inhaled agonist MCh (32 mg.mL⁻¹).

Statistics

Mean data points describing the responses in sheep were read during a steady-state control period, at the time-point of largest deviation in bronchial blood flow and airway dimensions, and in some cases recovery. The responses were analysed using one-way analysis of variance with repeated measures (RM ANOVA). Post-hoc Student-Newman-Keuls tests were then used to compare mean controls and responses at specific doses. Dose-related data was graphed as the absolute mean change

from control and analysed as linear or non-linear functions using GraphPad Prism v.5.04 (GraphPad Software Inc.). Statistical significance was accepted at $P < 0.05$.

RESULTS

Resting hemodynamics and bronchial dimensions

The resting data within 6 sheep immediately prior to each of the 4 experimental protocols studied are shown in Table 1, i.e. control prior to i.v. methacholine challenge; control prior to inhaled methacholine challenge; control prior to mask application and room air breathing; control prior to mask application and sheep breathing nebulised normal saline. Within each variable, there were no significant differences between any of the control periods. The data confirm good stability across time for the preparation.

Effects of intravenous methacholine on airway dimensions and hemodynamics, and of intravenous methscopolamine

A record of the time-course of events following i.v. infusion of MCh $1.0 \mu\text{g.kg}^{-1}$ and its effective blockade by methscopolamine is shown in Fig.3.

The average dose-response curves are shown in Fig. 4. At the lowest doses no fall in bronchial flow and conductance was ever seen. The maximal response to methacholine occurred at $2.0 \mu\text{g.kg}^{-1}$. P_a fell to 72% of resting control ($P < 0.001$); HR rose to 156% ($P < 0.001$); Q_{br} and C_{br} rose to 233% ($P < 0.05$) and 286% ($P < 0.05$) respectively; $CIRC_{br}$ fell to 90% of resting control ($P < 0.001$); WALL TH_{br} did not change, neither the slope and intercept for the regression line differed from zero ($P > 0.6$).

Methscopolamine block in itself caused on average a change in resting values. There was a mild rise in P_a , in HR (to 141%), Q_{br} (to 117%), C_{br} (110%) and in $CIRC_{br}$ (105%; all $P \leq 0.05$), but not in WALL TH_{br} , indicating as expected significant resting cholinoreceptor tone. Thereafter, i.v. MCh dose-response effects were completely blocked.

Mask effects and response to inhaled methacholine on airway dimensions and hemodynamics, and to inhaled ipratropium

A record for the immediate mask effects is shown in Fig. 5. Respiratory rate fell from 18 breaths /min to 12/min, there was a rise in P_a , and a fall in HR. Q_{br} and C_{br} fell to 74% and 58%, respectively. $CIRC_{br}$ fell to 90% of resting control but WALL TH_{br} did not change. The average peak effects for breathing air or normal saline (Fig.6) were similar; for the latter $15P_a$ rose to 106% of resting control ($P < 0.05$); HR fell to 92% ($P < 0.05$), Q_{br} , C_{br} and WALL TH_{br} did not change, while $CIRC_{br}$ fell to 95% of resting control ($P < 0.01$).

After 2.4 min with 'mask ON' (Fig. 5), the effects of inhaling 16 mg. mL^{-1} nebulized agonist MCh prevailed. The initial fall in mean bronchial blood flow and conductance never intensified with time and dose; both flow and conductance began to rise, and the amplitude of the phasic $CIRC_{br}$ trace became larger. These changes occurred concomitantly with increasing amplitude swings of up to 40 mmHg in right atrial pressure (P_v), reflecting large intrathoracic pressure and tidal volume changes with ventilation. At one minute after the mask was removed ('mask OFF') the amplitude of both the $CIRC_{br}$ and P_v phasic traces were still large and in a steady-state, but as the mean P_v trace returned to pre-mask baseline, the mean $CIRC_{br}$ trace remained at 90 % below the pre-mask baseline.

Fig. 7 shows that the average dose-response curves for inhaling nebulised methacholine. The threshold for change for all parameters was 8 $mg.mL^{-1}$. Maximum plateau effects occurred at doses from 16-32 $mg.mL^{-1}$. No fall in bronchial flow or conductance was ever seen at low doses, and no rise in these variables was observed at higher doses. At the plateau effect, P_a rose to 113% ($P = 0.03$) of normal saline control, HR to 131% ($P = 0.001$) and $CIRC_{br}$ fell to 91% ($P < 0.001$), the same level as with i.v. methacholine. Q_{br} rose to 125% ($P = 0.06$). C_{br} and WALL TH_{br} did not change from inhaling saline levels, the slope and intercept for each regression relationship did not differ from zero ($P > 0.55$).

At rest, inhalation of the cholinceptor antagonist IPRA did not change P_a , but HR rose by 120% ($P = 0.05$), and changes in Q_{br} , C_{br} and $CIRC_{br}$ were not significant. But the addition of i.v. methscopolamine to IPRA caused a significant rise in Q_{br} (117%), C_{br} (110%), $CIRC_{br}$ (105%) and in HR (140%; all changes $P \leq 0.05$). WALL TH_{br} did not change. These data confirm cholinceptor tone in the airway.

At rest in 5 sheep, inhalation of the cholinceptor agonist MCh 32 mg.mL⁻¹ caused CIRC_{br} to fall to 90% of resting (see above), but after inhaled IPRA in the same sheep a minor fall was still present to 94 % and significant; Rest-IPRA ($P_{\text{diff}} < 0.01$). Addition of i.v. methscopolamine abolished the remnant effect.

DISCUSSION

In awake sheep inhaled or intravenous MCh evokes striking differences in effect on airway smooth muscle structures. By either route MCh causes constriction of the 3rd generation bronchus. However, i.v. MCh results in a dose-dependent rise in both bronchial blood flow and conductance, whereas inhaled MCh causes no change in bronchovascular conductance because bronchial blood flow and aortic pressure rise together. Neither inhaled nor i.v. methacholine at any dose cause a change to bronchial wall thickness.

The use of awake sheep has advantages for confirming or otherwise previous findings in anaesthetised mammals and for defining new physiological events under normal conditions. One advantage is preserving negative pressure ventilation and avoiding positive-pressure ventilation in seeking the natural mechanical and reflex effects and time-course of MCh-induced changes in the airway wall. A second is preservation of resting and reflex neurohumoral transmission against the physiological confounding effects of anaesthetic agents, e.g. barbiturates block autonomic activity both centrally (29) and peripherally (13, 17), while chloralose (6) and fentanyl (26) enhance sympathetic and parasympathetic autonomic activity, respectively. Moreover, halothane and related halogenated ethers uncouple smooth muscle-endothelial electrotonic transmission in vascular beds to an unknown extent in anaesthetised experimental animals (2, 3, 12). Thus in the absence of these modifying factors at rest the smooth muscle of both airway wall and its vascular supply is tonically constricted by cholinceptor activity, in agreement with previous studies in awake animals (15, 23). It would be expected therefore, that the experimental superimposition of an inhaled cholinceptor agonist would intensify these effects, and the resultant disturbances create secondary reflex effects. It

follows that analysis of the final net response is usefully translatable to awake man for understanding mechanisms of cholinergic control in aerosol MCh challenge procedures.

The need to deliver MCh mist by mask rather than by mouthpiece in provocation testing may modify the effects of inhaled MCh. However, any untoward effects of breathing by mask were separated from the analysis of MCh-evoked changes by control studies. The data provide new information about trigeminal sensory reflex control of the lower airways from the face. While we did not analyse the sensory-autonomic events underlying the response, the initial slowing of respiratory rate, small rise in aortic pressure and bradycardia are identical to the events following trigeminal nerve stimulation in awake mammals including man (38, 39). They are also reversible, and are opposite to any defence-alarm reaction (19). The continuing response following inhalation of MCh by mask is therefore the integrated direct and secondary reflex effects of MCh on the airways, including behaviour. It has been noted for many years that trigeminal stimulation is also accompanied by variable airway changes (42), but this is the first demonstration of a reproducible mask-reflex constriction of the intrapulmonary bronchi. The effect occurs without change in the bronchial circulation, or in wall thickness. These findings are relevant to mask application in man, e.g. when used in provocation testing, or in paediatric and adult anaesthesia, particularly if inflammation has sensitised the lower airways.

The current studies using direct measurement of bronchial dimensions extend the findings of Scuri et al (35), and Charan et al (10), who found that cholinergic agonists by either inhaled or intravascular routes caused a rise in whole lung airflow resistance. In the current study both inhaled and i.v. MCh cause dose-related airway wall circular smooth muscle constriction, and the plateau effects at the highest doses indicate that maximum circumferential shortening of the bronchus by each route is the same at 10%. The inhaled effects are partly blocked by inhaled ipratropium and completely blocked by added i.v. methscopolamine, while the i.v. effects are completely blocked by i.v. methscopolamine, confirming they are cholinergic induced. This indicates that the cholinergic agonist has good access to airway wall smooth muscle from both exogenous and endogenous routes. Also, the incomplete cholinergic block by inhaled ipratropium in the large

doses used shows ipratropium is relatively limited for cholinergic antagonism when used by the inhaled route in the awake state.

The maximum excitatory cholinergic effects of 10% circumferential shortening evoked at the high airflow resistance airways by either inhaled or vascular route appear relatively small. While there may be restraint (25) to further shortening by airway wall cartilage, at this site a 10% shortening calculates using the Poiseuille equation as a considerable rise of 150% in airflow resistance, all other factors in the equation remaining constant. The contraction of the airways is likely associated with a decrease in pulmonary compliance (43), and this effect together with the rise in airflow resistance will evoke a significant increase in respiratory work load. It is therefore not surprising the awake sheep react to raise tidal volume, as indicated by the marked negative swings in central venous pressure, as the dose rises above 8 mg.mL⁻¹ to (a maximum of) 32 mg.mL⁻¹. It is likely that the rise in aortic pressure and heart rate observed are secondary to the mechanical and reflex events arising from an expected rise in cardiac output, and in sensory input from the muscles of breathing and sensitised pulmonary stretch receptors (43).

The rise in arterial pressure following inhaled MCh might be expected to evoke secondary baroreflex effects. However, the simultaneous rise in heart rate rather than bradycardia suggests as the animal increases ventilation the lung inflation reflex dominates the baroreflex (20) and possibly any reflex vagal effects on airway structures. The pressure-passive nature of the bronchovascular response is consistent with recent data in awake sheep (40) and awake dogs (24) concerning the absence of baroreflex control of the bronchovascular bed. When upper body blood pressure rises, the systemic baroreflex does not functionally engage the tonic autonomic constrictor pathways of the bronchovascular bed, and bronchial blood flow rises pressure-passively without change in vascular conductance (24). In the case of baroreflex control of airway wall circumferential smooth muscle, the rise in aortic pressure might be expected to dilate and thin the airway (see Fig. 2, and (34)) by withdrawal of tonic vagal constrictor control, but the airway wall constricts (Fig. 7). Therefore, the results suggest that following epithelial absorption of inhaled MCh, circumferential shortening of the airway wall is caused by a dominant, direct and local MCh excitatory cholinergic engagement from

the interstitial space, and that this process has no effect on the bronchovascular bed. It follows that bronchovascular penetration of MCh and its systemic recirculation is not a significant player in the observed airway wall contraction.

We have not used specific cholinceptor antagonists to identify which cholinceptors are involved in the mechanisms of airway circular wall contraction, and the i.v. vascular smooth muscle dilator effects. However, the dominant functional types for each site have been identified (32). Both the circular smooth muscle of the airway wall and vascular bed are dominated by M3 excitatory receptors, and the M2 receptor plays a role in the neural presynaptic inhibition of M3 effects. Engagement of smooth muscle cell membrane M3-cholinceptors evokes depolarisation and activates Ca^{2+} entry across the cell membrane, and Ca^{2+} release from sarcoplasmic reticulum via intracellular PLC-CD38 pathways. The rise in Ca^{2+} results in phosphorylation of myosin light chain and subsequent contraction of the circular wall smooth muscle (14, 30). On the other hand, the mechanisms underlying the absence of a response in bronchovascular smooth muscle to inhaled MCh are probably unique.

The finding of differential engagement of the bronchovascular bed by the different access routes also extends studies in anaesthetised sheep (9, 10, 35). It has been suggested (10) that airway wall contraction following inhalation of MCh may not be by direct action across the airway wall, but by indirect mechanisms following initial penetration of the mucosal bronchovascular bed by MCh followed by systemic recirculation. In this view the contraction of the bronchial wall is actioned centrifugally from the bronchovascular bed. If this were so, in the light of the dilator sensitivity of the bed to MCh demonstrated in the same animals, a dose-related rise in bronchovascular conductance should occur as the airway wall contracts, and it does not. It could be argued that airway circular wall contraction is primary to inhaled MCh epithelial absorption, and that the absence of bronchovascular conductance change is a zero sum effect, e.g. at low doses of a limited vascular wall penetration causing direct cholinceptor-induced vasoconstriction (35), offset by either a more complete direct penetration at higher doses to immediately engage the endothelium and evoke a balanced conductance effect (35), or, penetration to the bronchovascular bed, systemic recirculation, and indirectly via

endothelium evoke a balanced conductance effect (10). This would seem unlikely, as the data points across the entire dose-response range of inhaled MCh concentrations show no hint of vascular contraction or dilatation e.g. at low and high concentrations, respectively, taking into account scrutiny of the time-course of conductance change for each dose given, and the variance calculations between dose. In addition, as the airway wall contracts, there is a dose-related rise in aortic pressure, rather than an expected fall if MCh was circulating. And while bronchial blood flow rises, the response is pressure-passive and not due to a conductance rise. This contrasts with the dose-related rise in bronchial flow and conductance evoked from the smallest i.v. doses of circulating MCh. The data therefore suggests an alternative explanation. The airway circumferential wall contraction is a direct effect of exogenous MCh engaging excitatory M3 cholinceptors of the bronchial wall smooth muscle, and engagement of vascular smooth muscle M3 cholinceptors simply does not occur, rather than reflect a zero sum effect.

The airway wall contraction and bronchovascular dilatation evoked when i.v. delivered MCh traverses outwards from the blood vessel lumen probably reflects the relative efficacy of M3-induced contraction of the airway circumferential smooth muscle, and of MCh-induced relaxation of the vascular wall by hyperpolarising factor and NO endothelial release (44). In contrast, the mechanism for airway contraction when inhaled MCh traverses inwards from the airway lumen but without bronchovascular conductance change, is unknown. The phenomenon may reflect the single or combined action of several mechanisms. First, we did not refine the current analysis by measuring MCh concentrations in the airway interstitial space or in plasma. However, if it is assumed that in the process of provoking a maximal 10% contraction of the airway circumference by either route, the threshold and plateau interstitial MCh concentrations are the same, the dose-response curves suggest that the effective threshold is reached by transient, low circulating MCh concentrations in the vascular route relative to the inhaled route. This suggests in turn that a selective and substantial net biomechanical barrier to inhaled MCh may exist in the airway lining. Second, there may be differential local concentrations of acetylcholinesterase (36) in the airway wall which cope with the inhaled MCh 'saturation' of epithelial/mucosal/wall tissues. Finally, it is of interest to consider that

the proposed phylogenetically older non-neuronal cholinergic system (7, 32, 36) may be interacting with the more recent evolutionary neuronal cholinergic system. The non-neuronal system in the airway has been analysed in detail in rat, guinea pig, and man (36). Inhaled MCh activates epithelial and mucosal M3-cholinoceptors to release prostaglandins (7), especially PGE₂, to inhibit excitatory M3-cholinoceptors on airway wall circumferential smooth muscle. The effect is blocked by indomethacin (7). The process may be reinforced by MCh activation of airway parasympathetic nerve prejunctional inhibitory M2-cholinoceptors to form a neuroparacrine inhibition of bronchial blood vessel walls and thus centripetal transmission to the endothelial cell layer (7, 32). Whether the mechanism preventing MCh itself penetrating through to the bronchovascular bed is related, remains unknown.

The finding of no change in airway wall thickness over the full dose-response range of either inhaled or i.v. MCh may at first seem surprising. The possibility of artifactual recording is ruled out, because the SCS shows the pulsatile wave form typical of the resting bronchial wall in all sheep. Moreover, in a trial of expected SCS capacity in the current study, the instrument recorded thinning of the wall as expected when airway circumference increased during selective activation of the systemic baroreflex. It is likely, therefore, that the maintenance in wall thickness during the maximal 10% narrowing reflects the homeostatic balance struck between normal mechanical and physiological mechanisms (e.g. lymphatic drainage) evoked during negative pressure ventilation in the awake animal. It also shows that the possible obstructive mechanical effects of transcapillary efflux secondary to bronchovascular dilatation in the mucosa play no role in the rise in airflow resistance.

It is concluded that, at the level of the high airflow resistance bronchi in awake sheep, aerosolised MCh can engage excitatory cholinoceptors on airway circular smooth muscle by penetrating the airway epithelium to cause a maximal circumferential shortening of 10%. However, despite penetration of the epithelium to cause wall contraction, inhaled MCh does not activate cholinoceptors of the bronchovascular smooth muscle, or penetrate to cause changes in the systemic circulation. The mechanism for the lack of engagement of the bronchovascular bed when inhaled MCh selectively constricts the airway circular smooth muscle is not known. It is speculated that these

studies demonstrate the expression and function of non-neuronal and neuronal cholinergic system interactions (37) in the evolutionary protection against the effects of inhaled cholinceptor-like agonist molecules.

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Figure Legends

Figure 1. Surgical preparation of 3rd generation bronchus in sheep showing location of transit-time sonomicrometer (TTS) and single-crystal sonomicrometer (SCS) transducers on underside of lingula lobe bronchus, and pulsed Doppler transducer on bronchial artery. These measured simultaneously and continuously bronchial internal hemi-circumference, airway wall thickness and bronchial blood flow, respectively. For technical details, see Methods.

Figure 2. Original record from a 39 kg standing ewe showing for the first time the simultaneous effects of raising upper body blood pressure (whole organism baroreflex) on bronchial dimensions and blood flow in an awake mammal. Blood pressure was raised by inflating a balloon-tipped catheter (see Methods) placed in the lower thoracic aorta. Note the increase in bronchial circumference and simultaneous thinning of the bronchial wall, and a pressure-passive rise in bronchial blood flow. The record shows the capacity of the single-crystal sonomicrometer to record expected changes in bronchial wall when, in the same sheep, no change was recorded after inhaling or i.v. methacholine.

Figure 3. Original record from 45 kg awake ewe showing effects of i.v. injection of methacholine (MCh) before and after methscopolamine blockade. Left panel: phasic traces at slow paper speed of response to i.v. MCh $1.0 \mu\text{g.kg}^{-1}$ with integrator mean running through phasic trace. Right panel: Response to same dose i.v. MCh after i.v. methscopolamine. P_a , aortic pressure; Q_{br} , bronchial blood flow; C_{br} , bronchial blood flow conductance; $CIRC_{br}$, bronchial hemi-circumference; $WALL TH_{br}$, bronchial wall thickness; HR, heart rate. Vertical line denotes point of MCh intervention.

Figure 4. Average dose-response relationship evoked by intravenous injections of methacholine (MCh; $0.125\text{-}2.0 \mu\text{g.kg}^{-1}$) before (solid circles) and after methscopolamine cholinceptor blockade (open circles) in six awake sheep. Ordinate represents mean change from zero effect = saline vehicle control values (see also Table 1) at each dose. P_a , aortic pressure; $CIRC_{br}$, bronchial hemi-circumference; $WALL TH_{br}$, bronchial wall thickness; HR, heart rate; Q_{br} , bronchial blood flow; C_{br} , bronchial blood flow conductance. Error bars at left of data represent 2SE of the mean difference between any two dose-responses calculated by analysis of variance. This provides a visual approximation of the size of the difference required for significance at $P = 0.05$. *denotes $P < 0.05$, for significant departure from control. The small vertical tick on resting control values = negligible effect of saline vehicle control.

Figure 5. Original record from 45 kg ewe breathing nebulised methacholine (MCh) at 16 mg.ml^{-1} for 5 min through a face mask, showing effects on aortic pressure (P_a), right atrial pressure (P_v), bronchial blood flow (Q_{br}) bronchial blood flow conductance (C_{br}), bronchial hemi-circumference ($CIRC_{br}$), bronchial wall thickness ($WALL TH_{br}$), and heart rate (HR). Nebulised MCh was administered at 'mask ON' and finished at 'mask OFF'. Data for MCh effects documented 1 min after 'mask off'. Traces at fast paper speed appear at the beginning and end of the record, and show contrasting dynamic features before, and after MCh inhalation.

Figure 6. Comparison of the absolute effects in six sheep breathing room air ('mask') via facemask, and breathing nebulised 0.9% normal saline ('mask + NS') via facemask. The period between vertical lines represents the 5 min period when the mask was on. Data points were taken prior to mask (Table 1), at the peak response (2 min et seq.) of mask effect, and at 1 min after removing the mask. Notation for $\pm$ S.E. on left of data as in Figure 4. * denotes $P < 0.05$, between control and peak effects, and between control and recovery points.

Figure 7. Dose-response relationships for inhaled, nebulised methacholine (MCh), 1- 32 mg.mL⁻¹, before (solid circles) and after methscopolamine cholinceptor blockade (open circles) in six awake sheep. Plotted values are absolute mean change from pre-mask control (Table 1) at 1 min after mask removal. P_a, aortic pressure; CIRC_{br}, bronchial hemi-circumference; WALL TH_{br}, bronchial wall thickness; HR, heart rate; Q_{br}, bronchial blood flow; and C_{br}, bronchial blood flow conductance. Error bars at left of data above and below resting values in each panel, represent 2SE of the mean difference between any two dose-responses calculated by analysis of variance. This provides a visual approximation of the size of the difference required for significance at $P = 0.05$. * denotes $P < 0.05$, for change from control at each MCh dose.

650 Table 1: Resting hemodynamics and bronchial dimensions in 6 awake sheep.

	P_a (mmHg)	P_v (mmHg)	Q_{br} (ml.s ⁻¹)	C_{br} (mL.s ⁻¹ . mmHg ⁻¹ x100)	$CIRC_{br}$ (mm)	$WALL\ TH_{br}$ (mm)	HR (min ⁻¹)
C1	96 ± 3.3	2 ± 0.6	0.9 ± 0.31	1.0 ± 0.35	8.7 ± 0.63	1.14 ± 0.071	83 ± 8.1
C2	95 ± 3.1	2 ± 0.5	0.7 ± 0.17	0.8 ± 0.19	8.6 ± 0.67	1.14 ± 0.082	86 ± 10.0
C3	95 ± 2.0	2 ± 0.7	0.9 ± 0.18	1.0 ± 0.19	8.8 ± 0.45	1.23 ± 0.105	87 ± 3.8
C4	95 ± 2.1	2 ± 0.5	0.9 ± 0.19	1.0 ± 0.18	9.0 ± 0.45	1.22 ± 0.103	87 ± 4.3

651 Values are mean ± SE. Data points were taken at mean steady state points prior to i.v. and inhaled
652 intervention. C1 = control prior to i.v. methacholine challenges; C2 = control prior to inhaled
653 methacholine challenges. C3 = control prior to mask application and room air breathing; C4 = control
654 prior to mask application and sheep breathing nebulised normal saline. P_a , aortic pressure; P_v , right
655 atrial pressure; Q_{br} , bronchial blood flow; C_{br} , bronchial blood flow conductance; $CIRC_{br}$, bronchial
656 hemi-circumference; $WALL\ TH_{br}$, bronchial wall thickness; HR, heart rate; C= control.
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Fig.1: McLeod et al, 2012

Fig.2: McLeod et al, 2012

Fig.3: McLeod et al, 2012

Fig.4: McLeod et al, 2012

Fig.5: McLeod et al, 2012

Fig.6: McLeod et al, 2012

Fig.7: McLeod et al, 2012