Comparison of in vivo airway responsiveness and in vitro smooth muscle sensitivity to methacholine in man

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ABSTRACT Airway responsiveness to methacholine varies between normal people and is increased in patients with asthma. The importance of airway smooth muscle sensitivity in determining in vivo responsiveness is unknown. We have examined this question by comparing in vivo airway responsiveness with in vitro airway smooth muscle sensitivity to methacholine in 10 patients undergoing thoracic surgery. In vivo responsiveness was determined by administration of inhalations of doubling concentrations of methacholine. Results were expressed as the provocation concentration (PC) causing a decrease in forced expiratory volume in one second of 20% ($PC_{20}FEV_1$), specific airway conductance of 35% ($PC_{35}SGaw$), and maximal expiratory flow at 35% vital capacity, measured for the partial ($\dot{V}_{35(p)}$) and complete ($\dot{V}_{35(c)}$) flow volume curves, of 35% ($PC_{35}\dot{V}_{35(p)}$; $PC_{35}\dot{V}_{35(c)}$). In vitro airway smooth muscle sensitivity was determined from specimens obtained at thoracotomy. Log dose-response curves to methacholine were constructed and the concentration causing a 50% maximum contraction (EC_{50}) was derived. There were differences between patients for both in vivo airway responsiveness and in vitro smooth muscle sensitivity to methacholine. There were no significant relationships between the in vivo and in vitro measurements. The results suggest that factors other than solely the sensitivity of smooth muscle must determine in vivo airway responsiveness to methacholine.

Airway responsiveness to the cholinergic agonist methacholine varies between normal people¹ and is increased in patients with asthma.^{1,2} The reduction in airway calibre induced by this agonist involves the contraction of airway smooth muscle.³ Thus the difference in responsiveness to methacholine in normal and asthmatic subjects may be related to variations between individuals in their airway smooth muscle sensitivity to this agent. Alternatively, the variability between subjects in responsiveness may be a consequence of differences in the neural or humoral control (or both) of airway smooth muscle. Previous studies have found that in vitro airway smooth muscle sensitivity to a cholinergic agonist varies between individuals,^{3,4,5} but the in vitro values were not related to in vivo measurements of responsiveness.

In this study we have compared in vivo airway

responsiveness to methacholine with in vitro sensitivity of preparations of isolated bronchi taken from the same patient at thoracotomy, to determine whether variation in responsiveness to methacholine may be explained by differences in the sensitivity of airway smooth muscle to the drug.

Methods

PATIENTS

Ten patients scheduled to undergo lobectomy or pneumonectomy were studied (table). Eight patients had operable bronchial carcinoma, one had a tuberculoma, and one had a solitary metastasis from a renal clear cell carcinoma. All had been smokers but at the time of study two had stopped smoking more than a year previously. Four patients fulfilled the Medical Research Council's definition of chronic bronchitis.⁶ Only one patient (No 2) gave a history of wheezy dyspnoea and hay fever. Five were atopic as indicated by a weal of 2 mm or more

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in response to prick skin testing with one or more of seven common allergens (house dust; *Der-matophagoides pteronyssinus*; cat; dog; feathers; mixed grass pollen; *Aspergillus fumigatus*), although only three patients had raised specific IgE concentrations. Four patients were on regular drug treatment before surgery (table). In patient 2 no inhaled salbutamol or oral aminophylline was administered within 12 hours of methacholine challenge or surgery. Each patient was premedicated with papaveretum and hyoscine. Anaesthesia was induced with either Althesin or thiopentone sodium and maintained with nitrous oxide and halothane. Various neuromuscular and anaesthetic blocker drugs were given. The trachea was intubated and the lungs were ventilated artificially.

All patients gave informed consent and the experimental protocol was approved by the Western Infirmary ethical committee.

IN VIVO MEASUREMENTS

Airway resistance and thoracic gas volume were measured in a constant volume body plethysmograph (Fenyves and Gut) by the methods of Du Bois *et al.*⁷ The results were expressed in terms of specific airways conductance (sGaw), which is the reciprocal of airways resistance per litre of thoracic gas volume. The mean of eight values recorded was taken as sGaw. Partial and complete expiratory flow volume (PEFV and CEFV) curves were obtained with a heated pneumotachograph having integration of flow and recorded on an X-Y recorder (Hewlett-Packard 7041A). The flow-volume curves were

obtained in the following manner. After a period of normal tidal breathing each patient expired maximally from end tidal inspiratory volume to residual volume (RV) to obtain the PEFV curve. When RV was reached the patient inspired to total lung capacity (TLC) and expired maximally to RV to obtain the CEFV curve. From the CEFV curve the forced expiratory volume in one second (FEV₁) and forced vital capacity (FVC) were obtained. The volume corresponding to 65% below total lung capacity (TLC) was obtained from the mean FVC of at least five baseline curves. Maximum expiratory flows at this lung volume were measured from the partial ($\dot{V}_{35(p)}$) and complete ($\dot{V}_{35(c)}$) flow volume curves for baseline and subsequent curves—that is, curves were matched at TLC in order to compare flow rates. Body plethysmographic measurements always preceded flow volume recordings.

Routine preoperative pulmonary function tests were performed as follows: spirometry was measured on a water sealed spirometer (Godart); static lung volumes were determined by the helium dilution technique and from spirometric readings; single breath transfer factor for carbon monoxide (TLCO) was determined by the method of Ogilvie *et al.*⁸ Predicted normal values were taken from Cotes.⁹

Methacholine inhalation tests were carried out with a modification of the method described by Cockcroft *et al.*¹⁰ Aerosols were generated with the same Wright nebuliser by air at 50 lb/in² (345 kPa) at a flow rate of 8 l min⁻¹ to give an output of 0.15 ml min⁻¹. Patients wore a nose clip and aerosols were inhaled by tidal breathing through a loose

Table Characteristics of the 10 patients: respiratory function, atopy, smoking, and clinical features

Patient No	Age (y)	Sex	Height (cm)	FEV ₁		VC (%)	RV (%)	TLCO (%)	Atopy	Current smoker	Diagnosis		Medication
				(l)	(%*)						Surgical	other	
1	50	M	170	3.92	123	122	95	61	+	+	s	Chronic bronchitis, HPOA	Indomethacin Dextropropoxyphen Paracetamol
2	43	M	177	1.67	45	64	108	68	+	+	s	Chronic bronchitis, wheezy dyspnoea	Salbutamol (inhaled) Aminophylline (oral) Temazepam Levorphan Metaclopramide Inositol
3	49	F	155	2.18	96	95	148	77	—	+	l	Peripheral vascular disease	
4	63	M	178	2.91	92	98	115	75	+	+	s		
5	64	M	169	2.85	106	133	135	55	+	+	a	Chronic bronchitis	
6	66	F	165	1.64	81	69	100	106	+	+	c	Hypertension	Chlorthalidone
7	61	F	153	1.55	81	97	140	115	—	—	l		
8	62	M	173	2.32	81	88	102	114	—	+	s		
9	56	M	180	2.76	79	94	118	79	—	+	s	Chronic bronchitis	
10	69	M	168	2.82	110	97	60	141	—	—	t		

FEV₁—forced expiratory volume in one second; VC—vital capacity; RV—residual volume; TLCO—transfer factor for carbon monoxide; s—squamous cell carcinoma; l—large cell carcinoma; a—adenocarcinoma; c—clear cell carcinoma; t—tuberculosis; HPOA—hypertrophic pulmonary osteoarthropathy.
*Percentage of predicted normal value.⁹

fitting facemask. Buffered normal saline was inhaled first, followed by doubling concentrations of phosphate buffered methacholine (2–128 mg/ml). Each inhalation was for 2 minutes at 12 minute intervals. Before the first inhalation sGaw and five PEFV and CEFV curves were recorded. From 1.5 minutes after each inhalation responses were measured by sGaw and two PEFV and CEFV curves. Inhalations were continued until the FEV₁ had fallen by 20% or more, or until the maximal concentration had been administered. Log dose-response curves were plotted and results were expressed as the provocation concentration causing a 20% decrease in FEV₁ (PC₂₀FEV₁), a 35% decrease in sGaw (PC₃₅sGaw), and a 35% decrease in $\dot{V}_{35(p)}$ (PC₃₅ $\dot{V}_{35(p)}$) and in one case (No 2) in which the FEV₁ fell by 20% after inhalation of the lowest concentration of methacholine, extrapolated PC values were calculated. The PC₂₀FEV₁ was not measured in patient No 1.

IN VITRO MEASUREMENTS

Bronchial tissue was obtained immediately after removal at thoracotomy. Similar sized samples of second to sixth order bronchi were dissected from macroscopically normal tissue and then stored overnight at 4°C in well oxygenated Krebs-Henseleit solution of the following composition (mmol/l): NaCl 118, KCl 4.7, MgSO₄·7H₂O 1.2, KH₂PO₄ 1.2, CaCl₂·6H₂O 2.5, NaHCO₃ 25, glucose 11.7. The next day rings of bronchi were dissected out and sectioned to produce transverse strips of tissue that were suspended under a resting tension 1.5–2.0 g in 20 ml organ baths containing Krebs-Henseleit solution maintained at a constant temperature of 37°C. The tissues were allowed to equilibrate for 60 minutes, after which they were washed three times. Changes in tension were recorded isometrically with Grass FT03C forced displacement transducers and displayed on a Grass (model 7) polygraph.

After equilibration the tissue was exposed twice to near maximally effective concentrations of methacholine (10 μmol/l, determined in preliminary experiments) at 30 minute intervals to gauge the magnitude, normality, and reproducibility of the contractions. A cumulative concentration-effect curve was then constructed by adding increasing concentrations of methacholine (from 10 nmol/l to 1 mmol/l) until a maximum response was obtained. Each addition of the drug was made at the peak of effect produced by the preceding concentration. In each experiment the concentrations of methacholine that produced a 20% (EC₂₀) and 50% (EC₅₀) maximal contraction were calculated from the graphically displayed data. Additionally, the maximum tension generated was determined and expressed in g ten-

sion. At least four bronchial strips from each patient were tested and mean values for EC₂₀, EC₅₀, and maximum tension generated determined.

In preliminary experiments no significant difference was found between the sensitivity of bronchial strips taken from second and from sixth order bronchi.

STATISTICAL ANALYSIS

In the comparison of *in vivo* and *in vitro* results correlation coefficients were calculated from linear regression by the method of least squares analysis. PC results were analysed under a natural logarithm transformation. The level of statistical significance was set at $p < 0.05$.

Results

IN VIVO

Airway responsiveness to methacholine varied between individual patients (fig 1). There were significant correlations ($p < 0.05$) between PC₂₀FEV₁ and PC₃₅ $\dot{V}_{35(c)}$ ($r = 0.64$), PC₃₅sGaw and

Fig 1 Airway responsiveness of ten patients to inhaled methacholine, one of whom (○) had symptoms of wheezy dyspnoea. Results were expressed as the provocation concentration (PC) causing a decrease in forced expiratory volume in one second of 20% (PC₂₀FEV₁), specific airway conductance of 35% (PC₃₅sGaw), and maximal expiratory flow at 35% vital capacity, measured from the complete ($\dot{V}_{35(c)}$) and partial ($\dot{V}_{35(p)}$) flow volume curves, of 35% (PC₃₅ $\dot{V}_{35(c)}$, PC₃₅ $\dot{V}_{35(p)}$).

$PC_{35}\dot{V}_{35(p)}$ ($r = 0.65$), and $PC_{35}\dot{V}_{35(p)}$ and $PC_{35}\dot{V}_{35(c)}$ ($r = 0.77$).

Baseline FEV_1 (expressed as a percentage of the predicted normal value) correlated with $PC_{35}\dot{V}_{35(p)}$ ($r = 0.71$; $p < 0.05$) but was not significantly related to the other measurements of in vivo responsiveness ($PC_{20}FEV_1$ ($r = 0.59$); $PC_{35}sGaw$ ($r = 0.32$); $PC_{35}\dot{V}_{35(c)}$ ($r = 0.53$)). There was no significant correlation between baseline $sGaw$ (absolute value) and measurements of in vitro sensitivity.

IN VITRO

The sensitivity to methacholine of different bronchial strips from individual patients assessed by EC_{50} was highly reproducible ($r = 0.87$). In contrast, there were appreciable differences in the sensitivity to methacholine of bronchial strips prepared from different patients as indicated by the range of EC_{20} and EC_{50} values obtained. The mean (SEM) EC_{20} value from all preparations was 0.41 (0.12) $\mu\text{mol/l}$ (range 0.029 – 0.93 $\mu\text{mol/l}$) and the mean EC_{50} value was 3.9 (1.0) $\mu\text{mol/l}$ (range 0.41 – 8.1 $\mu\text{mol/l}$). The mean maximum tension generated was 1.44 (0.29) g (range 0.37 – 3.59 g).

COMPARISON OF IN VIVO AND IN VITRO RESPONSIVENESS TO METHACHOLINE

There were no significant correlations between any of the measurements of in vivo responsiveness and in vitro smooth muscle sensitivity to methacholine. The lack of relationship is illustrated for $PC_{20}FEV_1$

against EC_{50} (fig 2), for $PC_{35}sGaw$ against EC_{50} (fig 3), and for $PC_{35}\dot{V}_{35(c)}$ and $PC_{35}\dot{V}_{35(p)}$ against EC_{50} (fig 4). Furthermore, there were no significant relationships between in vivo measurements and maximum tension generated.

The patient who had a history of wheezy dyspnoea and the lowest $PC_{20}FEV_1$ (No 2) did not show increased in vitro smooth muscle sensitivity to methacholine (EC_{50} 4.0 $\mu\text{mol/l}$), nor was the maximum tension generated increased (1.41 g).

Discussion

This study demonstrated no significant relationship between measurements of in vivo airway responsiveness to methacholine and in vitro sensitivity of isolated bronchial smooth muscle obtained from the same patients. Previous studies have examined either in vivo human airway responsiveness^{1,2} or in vitro human airway smooth muscle sensitivity to this pharmacological stimulus^{3,5} but not in vivo and in vitro responses in the same subjects.

Several factors might have influenced in vivo and in vitro measurements. Unlike in studies carried out in vitro, the dose of methacholine administered to airway smooth muscle in vivo cannot be determined accurately. It is dependent on the technique of aerosol generation, particle size, and breathing pattern.^{11,12} In this study methacholine was administered via a Wright nebuliser and by the same method in each individual. Thus although the dose reaching the lung was unknown it should have been

Fig 2 Comparison of in vivo airway responsiveness to methacholine expressed as $PC_{20}FEV_1$ and in vitro smooth muscle sensitivity expressed as EC_{50} (mol/l) ($r = -0.35$; $p > 0.05$).

Fig 3 Comparison of in vivo airway responsiveness to methacholine expressed as $PC_{35}sGaw$ and in vitro smooth muscle sensitivity expressed as EC_{50} ($r = -0.03$; $p > 0.05$).

Fig 4 Comparison of *in vivo* airway responsiveness and *in vitro* smooth muscle sensitivity to methacholine. Panel (a) PC₃₅ V_{35(c)} against EC₅₀ ($r = -0.33$; $p > 0.05$). Panel (b) PC₃₅ V_{35(p)} against EC₅₀ ($r = -0.30$; $p > 0.05$).

similar in all patients. The site of deposition of the drug within the airways might also influence the response obtained. The patients we studied were current smokers or ex-smokers and had a degree of small airway disease. A Wright nebuliser produces a diffuse pattern of deposition within the lung,¹² but in individuals with small airway narrowing the aerosol may be deposited to a greater extent in the larger airways. We examined tissue samples from second to sixth order bronchi. *In vitro* results therefore reflected the sensitivity of larger airways. Comparison of EC₅₀ and PC₃₅ sGaw, a measurement of large airway responsiveness,¹³ might have been expected to show the closest relationship if any had existed.

Nevertheless, a relationship between *in vivo* airway responsiveness and *in vitro* sensitivity of more peripheral airway smooth muscle cannot be excluded from this study. In addition, the access of a bronchoconstrictor drug to airway smooth muscle, and thus the response to that drug may be affected by airway permeability.¹⁴ But although airway permeability was found to be increased in smokers¹⁵ no relationship was found between the level of airway permeability and airway responsiveness.¹⁶

It is impossible to produce a full dose-response curve to a bronchoconstrictor agent in man, so the position on the *in vitro* curve at which *in vivo* responses are measured is unclear. If, for example, the *in vivo* measurements fell on the lower non-linear region of the full *in vitro* log dose-response curve, then *in vivo* and *in vitro* measurements might not be correlated even if *in vivo* responsiveness was determined by smooth muscle sensitivity. We found no relationship, however, between *in vivo* responsiveness and a measure of *in vitro* sensitivity taken from the lower part of the dose-response curve (EC₂₀).

Baseline airway calibre or tone or both may be an important determinant of airway responsiveness,¹⁷ because resistance is inversely proportional to the fourth power of the radius when flow is laminar. In this study, only starting FEV₁ correlated significantly with PC₃₅ V_{35(p)} suggesting that the variation in responsiveness to methacholine between patients was not due only to differences in baseline airway calibre. Possibly small but important differences in baseline airway calibre or tone may not have been detected by the methods used, or differences between subjects in the distribution of resistance within the airways might have influenced *in vivo* measurements of responsiveness.

Variations in upper airway calibre may also effect airway resistance. Higenbottam¹⁸ demonstrated that aerosolised histamine produced a variable reduction in laryngeal calibre with an associated fall in FEV₁. If, as he proposed, this was due to stimulation of vagal sensory endings methacholine would not be expected to produce a similar effect.¹⁹

In normal subjects inhalation to total lung capacity can reduce the effect of induced bronchoconstriction.²⁰ This would affect FEV₁ and V_{35(c)} but not sGaw or V_{35(p)} measurements. As none of these measurements correlated with *in vitro* sensitivity it is unlikely that this bronchodilatation is an important factor in the lack of relationship between *in vivo* and *in vitro* measurements.

To minimise the effect of preoperative and perioperative drugs on the response of smooth muscle to methacholine, all tissues were washed twice on removal, stored for 16 hours in Krebs-

Henseleit solution, and then washed again both before and after suspension in the organ bath. The effects of atropine, a drug pharmacologically similar to hyoscine, can be removed from in vitro preparations within minutes by washing.^{21 22} Brink *et al*⁴ have shown no difference in the sensitivities of fresh and stored human airway smooth muscle preparations.

We also found no significant relationship between in vivo responsiveness to methacholine and the maximum tension generated by bronchial strips from each patient. Maximum tension may be related to the quantity of smooth muscle present in each bronchial strip which ideally should be measured but this is technically difficult. In an attempt to correct for variations in the amount of tissue and hence smooth muscle present, the maximum force generated was corrected for the wet weight of tissue in each strip. This was compared with the indices of in vivo responsiveness but no relationship was found.

This study has considered only one agonist, methacholine, which may be acting on muscarinic receptors on airway smooth muscle,³ submucosal glands,²³ presynaptic receptors on sympathetic nerves,²⁴ and mast cells.²⁵ The bronchoconstrictor response may therefore be due to mechanisms other than solely a direct effect on airway smooth muscle.

Patients selected for this study were due to undergo thoracic surgery. Most had bronchial carcinoma and were current smokers or ex-smokers and only one showed in vivo hyperresponsiveness, with a PC₂₀FEV₁ below 8 mg/ml.² In these patients our findings suggest that smooth muscle sensitivity alone does not determine in vivo airway responsiveness to methacholine.

The cause of airway hyperresponsiveness in asthma is unknown. It could be due to an abnormality in airway smooth muscle or to the neural or humoral control of the airways (or both).²⁶⁻²⁸ In this study only one patient had some features of asthma. Although we were able to study him only over a short period and were unable to assess the airway response to a "trial of steroids," he had symptoms of wheezy dyspnoea, showed a 14% increase in FEV₁ after bronchodilator, had Curschmann's spirals in his sputum, and had an increased response to inhaled methacholine. The airway smooth muscle strips from this patient, however, were not hypersensitive to methacholine. This finding, if confirmed in other patients with definite asthma, would indicate that airway hyperresponsiveness is not attributable to an increased sensitivity to methacholine at the receptor level (as indicated by the EC₅₀ value). In support of this finding with methacholine, Dahlen and coworkers²⁹ recently reported that the response of bronchial strips to histamine, prostaglandin F_{2α}

and leukotriene D₄ were similar in normal and asthmatic subjects.

Addendum

Since our original submission of this paper, Vincenc and coworkers³⁰ have reported that they found no relationship between in vivo and in vitro responses to histamine in a group of non-asthmatic patients undergoing thoracic surgery.

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