Structure and function of human peripheral airways in obstructive airways disease.

by

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We accept this thesis as conforming to the required standard

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Abstract

Obstructive airways diseases such as Chronic Obstructive Pulmonary Disease (COPD) and asthma are characterized by airflow obstruction, and by structural changes in the airway wall associated with chronic inflammation. The degree to which these changes are related to airflow obstruction and hyperresponsiveness is not completely understood. The aims of the investigations carried out in this thesis were to relate peripheral airway dimensions, in vitro contractile properties, and muscle protein content, to pulmonary function measured before surgery in subjects who had varying degrees of airflow obstruction. The hypothesis was that an increase in airway smooth muscle (ASM) mass and contractility leads to exaggerated airway narrowing and airflow obstruction, and that the increased ASM is accompanied by dedifferentiation of the muscle during airway remodelling. Connective tissue deposition could also take place in the airway wall and lead to increased passive elastance and attenuation of bronchoconstriction.

Airway dimensions of isolated human peripheral airways were measured by morphometry and the passive and active mechanical properties were measured in vitro by myography. The maximal isometric force (Fmax), stress (Fmax/ASM), airway diameter at Lmax (Dmax), maximal isotonic shortening (%Lmax), normalized airway smooth muscle (ASM/Dmax) were determined.
Western blot analysis was performed to characterize the content and distribution of myosin and actin. The smooth muscle phenotype was assessed by the ratio of muscle (SM-MHC) to non-muscle (NM-MHC) myosin, and of α-actin to total actin. Pulmonary function was assessed prior to surgery. Fifteen airways were studied from nonobstructed (NOB), and 15 from obstructed (OB, FEV₁/FVC<70%) patients (62±10 yrs, mean±SD).

Thickening of the smooth muscle, and not the inner or outer wall area, was significantly related to pulmonary function parameters, FEV₁ (forced expiratory volume in 1s of the forced vital capacity), FEV₁/FVC (ratio of FEV₁ to the forced vital capacity), FEF₂₅₋₇₅ (forced expiratory flow at 50% of FVC), DFEV₁ (change in FEV₁ after bronchodilator administration) (p<0.03). There was a significant correlation between Fmax and FEV₁ (%predicted) (r=-0.579, p<0.004), between Fmax and FEV₁/FVC (%) (r=-0.720, p<0.003), and between stress and FEV₁/FVC(%) (-0.611, p<0.002). There was no correlation between isotonic shortening and either measure of pulmonary function. Both force and stress were significantly increased (p<0.05) in OB (Fmax=0.87±0.80 g, stress=76±47 mN/mm²) versus NOB (Fmax=0.42±0.18 g, stress=51± 21 mN/mm²). ASM and ASM/Dmax were both significantly increased in the OB patient group (p<0.05). In addition, OB ASM exhibited decreased relaxation responses to MK886, a leukotriene biosynthesis inhibitor, and significant reduction in the force and shortening contractions when compared to NOB. These changes in contractility were not accompanied by alterations in the content of contractile and noncontractile proteins, or in the content or composition of connective tissue.
surrounding the muscle. These results suggest that obstructive airways disease is associated with an increase in the ability of the ASM to generate force.
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<td>Chronic obstructive pulmonary disease</td>
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<td>AHR</td>
<td>Airway hyperresponsiveness</td>
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<td>OB</td>
<td>obstructed</td>
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<td>NOB</td>
<td>nonobstructed</td>
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<td>FEV$_1$,</td>
<td>forced expiratory volume in 1s of the forced vital capacity</td>
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<td>FVC ,</td>
<td>forced vital capacity</td>
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<td>FEV$_1$/FVC %</td>
<td>forced expiratory volume in 1 s as a percentage of the forced vital capacity</td>
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<td>FEF$_{50}$</td>
<td>forced expiratory flow at 50% of the forced vital capacity</td>
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<td>FEF$_{25-75}$</td>
<td>forced expiratory flow between 25 and 75 % of the forced vital capacity</td>
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<td>TLC</td>
<td>total lung capacity</td>
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<td>FRC</td>
<td>functional residual capacity</td>
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<td>RV</td>
<td>residual volume</td>
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<td>change in FEV$_1$ as a percentage of the predicted FEV$_1$</td>
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<td>length for maximal isometric force</td>
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<td>length at which an airway starts shortening</td>
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<td>Transpulmonary pressure</td>
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<td>airway smooth muscle</td>
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<td>smooth muscle</td>
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<td>internal perimeter</td>
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<td>basement membrane perimeter</td>
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<td>outer muscle perimeter</td>
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<td>outer area</td>
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<td>cartilage area</td>
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<td>extracellular matrix</td>
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1.1 General Introduction.

Obstructive airways diseases such as chronic obstructive pulmonary disease (COPD) and asthma affect a substantial percentage of the population. The prevalence of asthma ranges from 3 to 15% of the population worldwide and is still increasing\textsuperscript{1, 2, 3, 4, 5, 6, 7}. The prevalence of COPD in North America is 10% in the age group 55 to 85 years\textsuperscript{8} and it reflects the smoking history of the population. The economic impact of treating asthma and COPD is substantial in both Canada\textsuperscript{9} and the United States\textsuperscript{10, 11} and underscores the need for a better understanding on the pathophysiology of these diseases.

Obstructive airways disease is characterized by increased airflow obstruction and exaggerated airway narrowing, or hyperresponsiveness.\textsuperscript{12} In addition, there are structural changes in the airway wall associated with a chronic inflammatory process.\textsuperscript{13, 14} The mechanism(s) underlying this disease is unclear. It is known that individuals who have asthma and COPD exhibit changes in their pulmonary function, with varying degrees of airflow obstruction and airway hyperresponsiveness. In addition, there are accompanying structural changes in the airway wall components and parenchyma. COPD is also characterized by
parenchymal changes, loss of lung elastic recoil, and increased airway collapsibility.

There are, however, many gaps in our understanding of the pathophysiology of the airways. First, the structural changes that exist in chronically inflamed peripheral airways have not been fully characterized, particularly with regards to the smooth muscle phenotype and its surrounding extracellular matrix. Second, it is not clear whether functional alterations occur as a result of specific morphological changes. Third, the degree to which structural changes contribute to airflow obstruction and airway hyperresponsiveness has not been formally established.

Detailed structure-function studies are necessary in order to further our understanding of the mechanisms underlying obstructive airways disease and to improve their treatment. There is a need for more information on the contractile properties of peripheral airways, to answer the question whether changes in contractility correlate not only with altered structure but also with the severity of altered pulmonary function. To date most of the structure-function data comes from tissue obtained at post mortem, from biopsies of large central airways, or from surgical specimens.

Structure-function studies of human airways are, however, difficult to carry out due to the difficulty in obtaining samples from human lungs. Biopsy specimens do not provide an adequate amount of tissue to study smooth muscle function in vitro as few samples can be taken and only from the large airways. Autopsy specimens are also of limited value since the viability of the tissue may
be compromised as a result of delays between the time of death and collection of tissue. Furthermore, death from asthma is rare in Canada. The studies in this thesis will be performed using human airways obtained from lobectomy or pneumonectomy specimens from patients who undergo surgery for removal of a solitary lung lesion. The lung tissue is obtained from St. Paul's Hospital, Vancouver, British Columbia. From hospital data collected over the last 20 years, it is known that most of these patients are smokers and have variable degrees of airflow obstruction (COPD), about 10% are non-smokers and only about 4% are asthmatic. Airway hyperresponsiveness has also been established in some of these patients. These studies will therefore focus primarily on COPD.

The purpose of this thesis is to examine the contractile and structural properties of peripheral airways from individuals who have normal lung function and individuals who have airflow obstruction. Function and structure will be characterized in the small, peripheral airways. In addition, pulmonary function measured preoperatively will be correlated with the structure-function data of the isolated airways.
1.2 Outline of the studies.

This thesis is centred on the structural changes present in the peripheral airways of individuals who have obstructive airways disease, including asthma and COPD, and the relationship between these alterations to airflow obstruction and airway hyperresponsiveness.

The general features that characterize asthma and COPD will be described in Chapter 2. Measures of airflow obstruction and airway hyperresponsiveness using pulmonary function tests will be defined.

In Chapter 3 the structural changes found in asthma and COPD will be discussed. First, the normal architecture of the airway wall, including the epithelium, submucosa, smooth muscle, and adventitia, will be reviewed. Second, what is known about the structural and functional alterations in the smooth muscle and surrounding extracellular matrix will be discussed in relation to airflow obstruction and airway hyperresponsiveness, followed by the hypotheses and aims of the studies.

The first study, described in Chapter 4, is an investigation on the relationship between changes in airway dimensions and abnormalities in lung function in patients who have varying degrees of airflow obstruction. The dimensions of peripheral airways obtained from surgical specimens are compared to determine whether alterations in structure are related to pulmonary function measured prior to surgery.
In **Chapter 5** the passive and active mechanical properties of peripheral airways are reported. Measurements of *in vitro* contractility are related to the amount of smooth muscle and to pulmonary function carried out before surgery to examine whether alterations in the intrinsic contractile properties of airway smooth muscle are related to airflow obstruction.

The presence of intrinsic airway smooth muscle tone in normal and chronically inflamed airways, and its possible effect on the active contractile properties of airway smooth muscle, are examined in **Chapter 6**. The isometric force and isotonic shortening are studied in the presence and absence of a leukotriene biosynthesis inhibitor that is known to decrease tone in human airway smooth muscle.

Structural remodelling appears to be central to the development of airway obstruction and hyperresponsiveness. In addition to changes in the mechanical properties of the muscle, phenotypic changes in the smooth muscle or the relative content of the extracellular matrix constituents could be altered as a result of the remodeling process. In **Chapter 7** changes in contractility and airway dimensions are related to alterations in the content of contractile and noncontractile proteins. The distribution of extracellular matrix components (collagen, elastin, proteoglycans and hyaluronan) are also examined. The smooth muscle biochemistry and matrix morphology are ultimately related to the *in vitro* contractility and to airways dimensions and pulmonary function reported in the previous chapters.
In Chapter 8 the studies in this thesis are summarized. The results are discussed in terms of the relationships between structural and functional findings in the peripheral airways, and their potential significance to airflow obstruction and hyperresponsiveness in vivo. The contribution of these alterations to the pathogenesis of airways obstruction are discussed as well as directions for future research.
REFERENCES.


7 Sears MR. Descriptive epidemiology of asthma. Lancet. 350 (Suppl):2:SII1-4, 1997


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Chapter 2

Definitions and terminology

2.1 Asthma

Asthma is defined as a clinical syndrome characterized by increased responsiveness of the tracheobronchial tree to inhaled non-specific irritants, with variable airways obstruction.¹ The major symptoms are paroxysms of dyspnea, wheezing and cough. These symptoms range from mild and almost undetectable to severe and unremitting (status asthmaticus).¹ Variable airflow obstruction can be manifested as spontaneous fluctuations in the severity of obstruction, substantial improvements in the obstruction following bronchodilators or corticosteroids, or increased obstruction caused by drugs or other stimuli.

Asthma can occur at any age but usually starts early in life.² It has been reported in cohort studies that childhood asthma symptoms diminish in approximately one half of asthmatics by the second or third decade of life.³ Up to two-thirds of asthmatic children continue to experience symptoms of asthma in adulthood.⁴ Although many adults may “outgrow” their asthma, after the age of 25 more than half still have the disease.⁵

A high percentage of asthmatic individuals are allergic to inhaled allergens. Allergen provocation can lead to an immediate, or early, asthmatic response, and often a late phase, or delayed response. The allergen can interact
with specific IgE on mast cells and basophils and lead to the release of preformed granule-associated mediators and the formation of membrane-derived substances. In the early phase, the allergic reaction is the result of direct effects by mediators. The late phase occurs 3 to 10 hours after the initial challenge, and is characterized by an intense infiltration of inflammatory cells in the bronchial mucosa. Inhaled antigen first interacts with mast cells in the epithelium, and mediators are released from these mast cells, causing alterations in epithelial permeability and antigen penetration through the mucosa. Antigen can then interact with IgE on tissue mast cells and circulating basophils. The predominant inflammatory cells in the mucosa and alveolar tissue are eosinophils, neutrophils, and T-lymphocytes, which are found in high concentration in peripheral airways.\textsuperscript{6}

The presence of airway inflammation has been found in both children and adults with asthma of different severity, including stable asthma. Indicators of airway inflammation such as hydrogen peroxide in exhaled air are elevated in stable asthmatic children when compared to healthy controls.\textsuperscript{7} A high number of activated eosinophils have been found in bronchial biopsies taken from adults with stable allergic asthma,\textsuperscript{8} and significant correlations between eosinophil numbers and FEV\textsubscript{1} have been reported.\textsuperscript{9} In addition, chronic inflammatory changes have been observed in bronchial biopsies from patients with severe asthma even after treatment with inhaled steroids.\textsuperscript{10}

2.2 Chronic obstructive pulmonary disease (COPD).

Chronic obstructive pulmonary disease, COPD, is a term used to define a
group of diseases that include emphysema and chronic bronchitis. These diseases may be present in addition to bronchial hyperresponsiveness, but the predominant clinical feature in COPD is lower than normal limitation of expiratory airflow. Chronic obstructive pulmonary disease is defined by the American Thoracic Society as a disorder characterized by abnormal tests of expiratory flow that do not change markedly over periods of several months. This definition excludes diseases of the upper airways, bronchiectasis, and cystic fibrosis. The main symptom of COPD is dyspnea, frequently associated with cough, wheezing, excess mucous production, and recurrent respiratory infections.

Characteristically, COPD affects older persons, who have a long history of cigarette use. This disorder does not, however, occur exclusively in smokers. About 20% of all smokers have susceptibility to cigarette smoke and constitute a subgroup of smokers that develops symptoms and COPD in late middle age. Despite other minor or rare risk factors such as alpha-1-antitrypsin deficiency, dusty occupational environments, and childhood respiratory illnesses, the main etiologic factor in COPD is cigarette smoking. Cigarette smoking has been correlated to the accelerated rate of loss of lung function, to the severity of inflammation of small airways, and to bronchial hyperresponsiveness. In healthy non-smoking individuals aged 25 to 35 years, the forced expiratory volume in one second (FEV₁) falls about 20-30 ml/yr. In smokers there is an increased decline in FEV₁ and in COPD patients there is an even larger decline in FEV₁ (~40-80ml/yr). While continued smoking leads to the increased decline in lung function, cessation of smoking can reduce this to a near normal
level of lung function.\textsuperscript{23,24}

In addition to causing airway obstruction, cigarette smoking also causes thickening of the peripheral airways in patients who have COPD\textsuperscript{25} and various pathologic changes such as inflammatory cell infiltration and connective tissue deposition.\textsuperscript{26,27} Much of the work that has been carried out in the last 30 years has been centred on the idea that this inflammatory process in the small conducting airways causes them to narrow leading to the reduction in forced expiratory flow observed in COPD.\textsuperscript{28,29,30,31,32}

The cellular inflammatory response that occurs in the airspaces in COPD, is composed primarily of neutrophils.\textsuperscript{33} It has been demonstrated that smoke in the lungs increases retention of neutrophils in the lung,\textsuperscript{34} that the chemotactic activity of neutrophils is higher in smokers than in controls,\textsuperscript{35} and that the number of neutrophils is higher in heavy smokers.\textsuperscript{36} Neutrophils are thought to cause tissue damage by releasing mediators such as proteases and oxidants.\textsuperscript{37,38} Cigarette smoking is believed to cause emphysema by producing an imbalance in the protease-antiprotease levels in the peripheral lung areas.\textsuperscript{39} This imbalance could be due to an increase in the proteolytic enzymes released by inflammatory cells, or to a decrease in the function of the natural inhibitors of these enzymes.\textsuperscript{40,41,42,43} The excess of proteases relative to antiproteases is believed to degrade extracellular matrix proteins.\textsuperscript{44} Oxidants in cigarette smoke have been implicated in the inactivation of antiproteases, and in having a direct toxic effect on lung structures.\textsuperscript{45}
2.2.1 Emphysema.

This disease has been defined by the American Thoracic Society as abnormal permanent enlargement of the airspaces distal to the terminal bronchioles, accompanied by destruction of their walls, without obvious fibrosis.\textsuperscript{1} There are different types of emphysema depending on the location of the injury. Centriacinar, or centrilobular, emphysema, affects the respiratory bronchioles, in the proximal part of the acinus. It is associated with cigarette smoking and airflow obstruction and results in dilatation of the respiratory bronchioles. In panacinar, or distal, emphysema the entire acinus is involved. This form of emphysema is often associated with alpha-1-antiprotease deficiency.\textsuperscript{46} It has also been described in the lung bases of patients with centrilobular emphysema.\textsuperscript{1} The main structures involved are the distal part of the acinus, alveolar ducts and sacs.\textsuperscript{1} Despite these subdivisions, there is a large amount of overlap, particularly when the emphysema becomes severe. The assessment of the severity of this disease is often done by morphology scores in addition to an index of airflow obstruction such as the FEV\textsubscript{1}.\textsuperscript{47,29,48}

2.2.2 Chronic bronchitis.

Chronic bronchitis is defined by chronic and recurrent excess mucus secretion into the bronchial tree, most days for at least three months of the year for at least two successive years.\textsuperscript{1}
The above definitions of asthma and COPD include a broad range of patients. In reporting studies patient groups should be defined using a comprehensive criteria. For the purpose of this thesis, the patients in this study are characterized based on a detailed questionnaire of respiratory symptoms, smoking history, allergies, and pre-operative medications. Pulmonary function tests are carried out prior to surgery and include assessment of airflow limitation and degree of reversibility of airflow obstruction in response to bronchodilators.

2.3 Markers of abnormal lung function.
Alterations in lung function can be assessed by measurement of airway hyperresponsiveness, as well as by baseline lung function parameters obtained from maximal expiratory flow maneuvers.

2.3.1 Airway hyperresponsiveness (AHR).
Asthma and COPD are characterized by exaggerated airway narrowing. This feature is known as airway hyperresponsiveness and is characterized by an increase in the sensitivity of the tracheobronchial tree to inhaled bronchoconstricting stimuli, an increase in the reactivity (see below), and an increase in the maximal airway narrowing. Airway hyperresponsiveness is defined as an increased tendency of the airways to narrow in response to non-specific stimuli of pharmacological (i.e. histamine, methacholine), chemical (i.e. SO₂), physical (i.e. cold or dry air), or physiological (i.e. exercise) origin.
In humans airway responsiveness is routinely measured by dose-response curves\textsuperscript{54 55} in which dose is the amount of agonist administered and the response is an indirect measure of airway narrowing. Changes in pulmonary function parameters such as forced expiratory volume or flow are measured in response to doubling doses of aerosolized histamine or methacholine and reflect the degree of airway patency. The dose-response curve is plotted on a log-linear scale and the resulting sigmoid shaped curve can be described by its position, its slope, and its maximal-response plateau.

The position of the curve along the x-axis is referred to as the sensitivity. If the FEV\textsubscript{1} is used the position is normally defined as the provocative concentration (PC\textsubscript{20}) or dose (PD\textsubscript{20}) that causes a 20\% fall in FEV\textsubscript{1}. Increased sensitivity is demonstrated by a shift to the left on the dose-response curve.\textsuperscript{55} The slope of the dose-response curve, was proposed by Orehek et al.\textsuperscript{56} to assess what the authors termed the "reactivity" of the tracheobronchial tree. Since there is no standardized method to assess the slope and the techniques reported in the literature vary widely,\textsuperscript{56 57} the slope of the dose-response curve is not routinely measured.

The maximal-response plateau on the dose-response curve reflects the maximal degree of airway narrowing that can be achieved by a subject exposed to a bronchoconstricting stimulus.\textsuperscript{49 58} Asthmatic subjects show a greater fall in FEV\textsubscript{1} in response to increasing doses of inhaled agonist, resulting in a higher maximal-response in mild asthma, and in the absence of a plateau in moderate asthma. Similar results have been reported in COPD.\textsuperscript{59 60}
2.3.2 Airflow obstruction: forced expiration.

Reduced maximal expiratory flow is an important characteristic of asthma and COPD. Measurement of forced flow-volume curves is performed to calculate the forced expiratory volume in 1 s of the forced vital capacity (FEV₁), the forced vital capacity (FVC), the force expiratory flow at 50% of FVC (FEF₅₀), and the forced expiratory flow between 25-75% of FVC (FEF₂₅₋₇₅). This manoeuvre involves maximal effort throughout the procedure. The ratio of FEV₁ to FVC (FEV₁/FVC) is often used as an indicator of airflow obstruction. In this thesis a value of 70% is used as the lower limit of the normal range as defined by 95% confidence intervals for FEV₁/FVC. Flow limitation may result where flow limiting segments are formed. The places where the flow limiting segment develops depend on the airway resistance upstream of the low limiting segment and the lung elastic recoil pressure.

The purpose of these studies is to explore some of the potential mechanisms that underlie airflow obstruction and airway hyperresponsiveness. These mechanisms probably exist at the level of the smooth muscle and the surrounding extracellular matrix. This idea is based on the observation that during pulmonary function tests the airways rapidly constrict in response to the inhaled challenging agent and rapidly dilate in response to bronchodilators. The time course of these events means that the responses must be due to airway smooth muscle shortening.
The fact that normal subjects and some mild asthmatics can reach plateaus means that a maximal degree of airway smooth muscle shortening occurs in vivo without causing asphyxia. Woolcock et al.\textsuperscript{63} and Macklem\textsuperscript{58} proposed that hyperresponsive individuals probably lack a normal mechanism that inhibits excessive airway narrowing. This abnormality could be a breakdown in the interdependence between the parenchyma and the airway wall, a change in the resting length of ASM or an increase in the ASM force.\textsuperscript{58} Although challenge tests will not be done in this thesis, abnormalities in lung function will be assessed by the FEV\textsubscript{1}/FVC and by the reversibility of airflow obstruction in response to bronchodilators. Functional changes at the level of the smooth muscle as well as structural changes in the airways will be correlated to pulmonary function.

Table 2.1 summarizes some of the important features found in obstructive airways disease. The main alterations in lung function have been discussed in this chapter. The structural and functional changes in the airways will be discussed in chapter 3 as well as the possible relationships between these changes in the airways and abnormalities in lung function.
### Table 2.1

**OBSTRUCTIVE AIRWAYS DISEASE CHARACTERISTICS**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Asthma</th>
<th>COPD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Functional-</strong></td>
<td>Starts at any age, usually early in life</td>
<td>Affects older persons who have long history of cigarette use</td>
</tr>
<tr>
<td><strong>Symptoms</strong></td>
<td>Shortness of breath</td>
<td>Shortness of breath</td>
</tr>
<tr>
<td></td>
<td>Episodic cough</td>
<td>Chronic cough, mucous production</td>
</tr>
<tr>
<td></td>
<td>Wheezing</td>
<td>Wheezing</td>
</tr>
<tr>
<td><strong>Functional</strong></td>
<td>Relatively normal baseline lung function (FEV&lt;sub&gt;1&lt;/sub&gt;).</td>
<td>Reduced baseline FEV&lt;sub&gt;1&lt;/sub&gt;.</td>
</tr>
<tr>
<td><strong>Lung function</strong></td>
<td>Substantial reversibility of airflow obstruction in response to bronchodilators</td>
<td>Increased decline in FEV&lt;sub&gt;1&lt;/sub&gt;.</td>
</tr>
<tr>
<td></td>
<td>Marked airway hyperresponsiveness (increased sensitivity and increased maximal responsiveness)</td>
<td>Partial reversibility of airflow obstruction in response to bronchodilators</td>
</tr>
<tr>
<td><strong>Structural</strong></td>
<td>Lumen — mucus plug</td>
<td>Lumen — mucus hypersecretion</td>
</tr>
<tr>
<td></td>
<td>Epithelium — shedding, squamous cell metaplasia</td>
<td>Epithelium — shedding, squamous cell metaplasia</td>
</tr>
<tr>
<td></td>
<td>Connective tissue — deposition in submucosa, adventitia, thickened reticular layer, Airway smooth muscle — increased</td>
<td>Connective tissue — deposition in submucosa and adventitia, Airway smooth muscle — increased</td>
</tr>
<tr>
<td></td>
<td>Inflammation — infiltration by inflammatory cells (eosinophils, neutrophils, T-lymphocytes), blood vessel congestion, edema</td>
<td>Inflammation — infiltration by inflammatory cells (neutrophils), blood vessel congestion, edema Increased mucous gland and goblet cell Parenchymal changes - loss of lung elastic recoil, emphysema</td>
</tr>
</tbody>
</table>

FEV<sub>1</sub> = Forced expiratory volume in 1s of the forced vital capacity.
REFERENCES.


10 Lundgren R, M Soderberg, P Hortedt, R Stenling. Morphological studies of bronchial mucosa from asthmatics before and after ten years of treatment with inhaled steroids. Eur Respir J. 1:883-889, 1988


Prescott E, AM Bjerg, PK Andersen, P Lange, J Vestbo. Gender difference in smoking effects on lung function and risk of hospitalization for COPD: results from a Danish longitudinal population study. Eur Respir J. 10:822-827, 1997


MacNee W. Neutrophil traffic and COPD Eur Respir Rev. 43:124-127, 1997


60 Sterk PJ, EH Bel. The shape of the dose-response curve to inhaled bronchoconstrictor agents in asthma and COPD. Am Rev Respir Dis. 143:1433-7, 1991


3.1 Structure of the airway tree.

The tracheobronchial tree branches out from the trachea down to the peripheral lung in an asymmetrical dichotomous pattern. The composition and integrity of this branching structure, function to effectively move about 10,000-15,000 l of air in and out of the lung each day. The airways must condition the air and render it free of environmental irritants and allergens before it reaches the respiratory portion of the lung.

The conducting airways comprise the trachea, extrapulmonary bronchi, intrapulmonary bronchi, and membranous bronchioles. Their main function is to transport air from the environment to the alveoli for gas exchange, and to humidify, filter, and heat the air to body temperature. It is in the large central airways where most particles and micro-organisms are cleared by mucociliary action. By definition, bronchi are airways that contain cartilage, in contrast to bronchioles that do not. Examination of normal lungs made from casts of the airways reveal that the number of branches from the trachea (generation 0) to the peripheral lung vary depending on the pathway taken. The number of branches can vary from 8 to 24, with a mean of about 13 branches. The first ten
generations contain most bronchi, while most of the distal airways are composed of bronchioles. The total cross-sectional area of the airways expands rapidly beyond the 2 mm airways. In adults the cross-sectional area starts at 2.5 cm$^2$ in the trachea and reaches 12,000 cm$^2$ at the level of the respiratory bronchioles. This large area is required for rapid diffusion of O$_2$ and CO$_2$ into the gas exchanging surface of the lung, the alveoli. Results from these quantitative studies are consistent with direct physiological measurements that indicate the central airways are the major site of resistance to airflow in the normal lung and that airway resistance falls dramatically as the cross-sectional area of the conducting airways increases.\cite{3,4}

In diseases where there is chronic inflammation of the airways, the normal functions of the respiratory system may be altered. The structural changes that are found in chronically inflamed airways may be responsible but how these changes lead to airflow obstruction and airway hyperresponsiveness is not clear. In the next section, the normal structure of the airway wall will be described. The structural changes that occur in asthma and COPD will be discussed as well as how these abnormalities may relate to maximal airflow limitation and bronchial responsiveness.

3.2 Composition of the airway wall.

3.2.1 THE EPITHELium

The respiratory epithelium consists of several layers of cells that provide an interface between the external environment and the underlying structures.
The epithelium of the trachea and major bronchi consists of pseudostratified ciliated columnar cells admixed with goblet cells. Towards the peripheral airways, the epithelium changes to progressively more cuboidal, nonciliated cells and the number of glands within the bronchial wall progressively decreases.\(^5\)

The epithelium has several important functions. First, the epithelium acts as a physical barrier to impede access of foreign and noxious substances into the interstitium. The epithelium effectively achieves this function by having a heterogeneous population of cells involved in mucus secretion, ciliary motility, and ion transport. Goblet cells and submucosal glands produce mucus, which traps inhaled particles and microorganisms and clears them by mucociliary action. Mucus contains defensins, which have recently been shown to have bactericidal activity.\(^6\) Epithelial cells are joined by tight junctions which form a selectively permeable resistive barrier to paracellular movement of ions, macromolecules, and water. The epithelium can prevent leakage of solutes into the airways as well as infiltration of irritant substances into the interstitium.\(^7\)\(^8\) In addition, the epithelium can humidify the air and prevent fluid loss from the airway surface mucosa.

Second, the epithelium functions as an immunological barrier. Airway epithelial cells such as dendritic cells can express major histocompatibility complex (MHC) class I and II molecules.\(^9\)\(^10\)\(^11\) This means that dendritic cells can interact with lymphocytes by presenting antigen and that their ability to express MHC antigens may, in turn, be modulated by the presence of inflammation in their immediate environment.\(^12\)
Third, the epithelium is a metabolic structure. It contains degradative enzymes, including neutral endopeptidase which can break down substance P and neurokinin A released from sensory nerves.\textsuperscript{13, 14} It also contains enzymes for synthesizing arachidonic acid metabolites,\textsuperscript{15} nitric oxide,\textsuperscript{16} nonprostanoid inhibitory factor(s),\textsuperscript{17} endothelin,\textsuperscript{18} cytokines,\textsuperscript{19, 20} and growth factors.\textsuperscript{19, 21} These substances modulate smooth muscle tone by relaxing or inactivating bronchoconstricting substances and neurotransmitters.\textsuperscript{22, 23, 24}

In chronic obstructive airways diseases the respiratory epithelial cells are abnormal and damaged. Histologic observation shows shedding and damage of the epithelium in the airways of individuals who died from asthma, and in airway samples from biopsies of patients with mild asthma.\textsuperscript{25, 26} Similar observations have been reported for COPD.\textsuperscript{27, 28} In disease, the fragile nature of the epithelium is evidenced by widespread areas of exfoliation and by the increased loss of cells seen in the sputum and bronchoalveolar lavage (BAL).\textsuperscript{25, 29} There is epithelial cell proliferation and squamous cell metaplasia showing that a repair process is taking place.\textsuperscript{26}

Damage and loss of the epithelium can have important consequences. Firstly, reduced mucus clearance can result from dysfunction in ciliary motility. The ensuing mucus accumulation can lead to infections by colonization of inhaled bacteria.\textsuperscript{30} In COPD increased mucus has been reported in the airway epithelium.\textsuperscript{31, 32} In asthma the accumulated mucus and shed epithelium have been shown to occlude the airway lumen in severe acute asthma attack\textsuperscript{33} and in patients dying in status asthmaticus.\textsuperscript{34, 31} In addition, the accumulated debris,
plus inflammatory cells and fluid in the lumen, can fill the airway interstices and amplify airway smooth muscle constriction and airflow obstruction.35 36

Secondly, loss of integrity of the physical barrier will allow increased access of allergen or other noxious stimuli to antigen-presenting cells, mast cells, or smooth muscle within the airway wall. In addition, areas of desquamation can expose intraepithelial nerve fibres, which when stimulated may cause reflex bronchoconstriction by releasing tachykinins such as substance P and neurokin A. Similarly, the loss of degradative enzymes such as neutral endopeptidases may reduce the inactivation of these endogenously released contractile neurotransmitters. Thirdly, loss of epithelial derived relaxant factor(s) may result in enhanced smooth muscle contraction to spasmogens.

In both asthma and COPD these interacting functions have a profound effect in increasing the sensitivity of the bronchial smooth muscle and leading to airway hyperresponsiveness.37 38 24 23 39

3.2.2 THE BASEMENT MEMBRANE

Observation with the light microscope shows that the basal aspect of the epithelial cells is attached to a basement membrane. The basement membrane acts as an extracellular scaffold and may be important in determining the folding pattern of the mucosa during smooth muscle shortening.40 41 Transmission electron microscopy of human bronchial mucosa reveals that the so-called basement membrane is composed of two layers.42 The first layer is the basal lamina, or true basement membrane. It is approximately 80 nm thick (not
resolved by light microscopy whose resolution is 200 nm). The main components are type IV collagen, laminin, fibronectin, and proteoglycans. \(^{43}^{44}\)

The second layer is the lamina reticularis, which is found beneath the basal lamina. This is a thicker layer about 8 \(\mu\)m in width and can be seen by light microscopy. \(^{42}\) It consists of a loose arrangement of collagen fibers intermixed with other components of the surrounding connective tissue. \(^{45}^{46}\)

Thickening of the basement membrane has been reported in asthma. \(^{26}^{29}\) and in COPD. \(^{72}^{55}\) It is the lamina reticularis and not the basal lamina that acquires a hyaline appearance and becomes thickened in asthma. The term subepithelial fibrosis has been coined to describe thickening of this layer. \(^{56}\) In immunohistochemical studies of bronchial biopsies, Roche et al. demonstrated increased deposition of collagen types III and V, in addition to fibronectin in the reticular lamina. \(^{56}\) The cause of this increased amount of reticulin is unclear but it has been hypothesized that it may be due to an increase in the synthesis and secretion of collagen by epithelial cells \(^{57}\) or subepithelial myofibroblasts, \(^{49}\) or to reduced degradation of collagen. The number of subepithelial myofibroblasts has been associated with the thickness of the lamina reticularis \(^{49}\) which would implicate these cells in the changes induced in this layer.

Results from modelling studies indicates that thickening, and possibly stiffening, of the basement membrane may increase the load impeding smooth muscle shortening and act as a protective mechanism against airway hyperresponsiveness. \(^{40}\) See next section.

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3.2.3 THE SUBMUCOSA OR LAMINA PROPIA.

This layer comprises the tissue between the basement membrane and the smooth muscle layer. It consists of loose connective tissue, including fibrillar collagen and elastic fibers. It supports many small mucous and serous glands (in trachea, main bronchi, intrapulmonary bronchi) which are connected to excretory ducts that pierce the mucosa and empty their secretions at the epithelial surface. The submucosa is rich in fibroblasts, small nerves, and is permeated by a rich network of blood capillaries. The bronchial arterial, venous and lymphatic vessels form a dense capillary network embedded in this fibrous tissue and make up 4-10% of the inner wall area. The function of the blood vessels is to supply nutrients to the epithelium, to condition the inspired air, and to distribute inflammatory mediators. Another network comprised of elastic fibres has been described in the lamina propria, attached to the basal lamina of the epithelium, smooth muscle cells, and cartilage.

There is substantial evidence that there is increased microvascular permeability and plasma exudation in asthma, as well as an increase in the number and size of bronchial microvessels. It has been hypothesized that vascular engorgement may contribute to increasing airway resistance by thickening of the inner wall and amplifying the effects of smooth muscle shortening as will be explained below.

The matrix elements of the airway wall have important functions in determining its biomechanical properties. Fibrillar collagens provide the matrix with tensile strength, while elastin provides elastic and resilience properties.
Collagen and elastin are embedded in a matrix of proteoglycans, which form a highly hydrated, gel-like "ground substance". The function of this polysaccharide gel is to resist compressive forces on the matrix. There are also adhesive glycoproteins such as fibronectin and laminin whose function is to help cells attach to the extracellular matrix. Fibronectin promotes the attachment of fibroblasts and various other cells to the matrix, and laminin promotes the attachment of epithelial cells to the basal lamina.67

The mechanical effects of collagen deposition are unknown, but they are likely to depend on the type of collagen being deposited. Collagen types I, III, and V are the fibril-forming collagens, but most collagen fibrils contain more than one collagen type. For instance, type V collagen may be associated with and may regulate the assembly and size of type I collagen fibrils.68 It is the relative proportions of the collagen types that may determine the specific properties of the tissue. Collagen type I is covalently cross-linked to form fibres that are less susceptible to degradation by collagenases than fibres with more type III collagen. Results from immunohistochemical staining have revealed an increase in type III collagen in lung inflammation.

Proteoglycans contribute to the osmotic activity of the tissue and enable the matrix to withstand compressive forces. They consist of protein covalently bound to negatively charged unbranched polysaccharide chains called glycosaminoglycans (GAG). The GAGs are composed of repeating disaccharide units, and one of the two sugar residues in the repeating disaccharide is always an amino sugar which in most cases is sulfated. The presence of sulfate or
carboxyl groups on the sugar residues provide the negative charges and accounts for the osmotic activity (and space filling properties) of proteoglycans and GAGs. The lung contains all of the classes of GAGs. Some of the GAGs identified in the lung include hyaluronan (HA), as well as chondroitin sulphate, and dermatan sulphate. HA synthesis seems to be a generalized early event of acute inflammation, associated with increased fluid content of the matrix, decreased cell adhesion to the matrix and cell migration through the matrix. An increase in synthesis and deposition of HA has been reported in several models of lung inflammation, including bleomycin-induced alveolitis\textsuperscript{69} and in bronchialveolar lavage fluid of human subjects who have adult respiratory distress syndrome (ARDS),\textsuperscript{70} and idiopathic pulmonary fibrosis.\textsuperscript{71}

The increased deposition of connective tissue in the submucosa is well established in both asthma and COPD.\textsuperscript{72, 73, 63, 74, 75} This includes deposition of fibrillar collagen as well as increased deposition of laminins and tenascin.\textsuperscript{76, 77} Increased levels of hyaluronan, fibronectin and laminin products in the bronchoalveolar lavage fluid of asthmatics indicate increased connective tissue turnover\textsuperscript{78} and correlate with the severity of asthma and eosinophil activation.\textsuperscript{79} Similarly, HA and fibronectin have been found to be elevated in the bronchoalveolar lavage fluid of COPD patients,\textsuperscript{80, 81, 82} and HA correlated with the severity of airflow obstruction.\textsuperscript{80, 81, 82}

The increased thickness of the submucosal layer could have important consequences in the function of the airways. First, it may cause amplification of the effects of smooth muscle shortening,\textsuperscript{83} simply by increasing the area of the
tissue internal to the smooth muscle layer, leading to airway narrowing. Second, an increase in the thickness of the submucosal layer could cause the opposite effect and lead to attenuation of airway narrowing because of an increase in the elastic load on ASM. According to Lambert,\textsuperscript{40} thickening of the tissue internal to the smooth muscle, regardless of the mechanical properties of the tissue, will result in an increase in the elastic load opposing smooth muscle shortening during constriction. He postulated that the folding of the mucosal membrane which occurs during ASM contraction and airway narrowing requires energy. Since the bending stiffness of a substance is related to its thickness cubed, the increased thickness of the connective tissue internal to the muscle layer will mean that the muscle will have to generate more force to produce the folds which occur during airway narrowing. These predictions were supported by studies where ASM shortening and lumenal narrowing were inversely related to the number of mucosal folds and to airway submucosal thickness.\textsuperscript{84}

In addition to the increased thickness, the mechanical properties of the connective tissue could be altered during remodelling. An increase in the stiffness of the submucosa could produce the same effect described above, attenuation of airway narrowing. An increase in the amount of collagen, for instance, could increase the tensile stiffness and the resistance to deformation of the airway wall. Alternatively, a decrease in the stiffness of the tissue could occur from reduced cross-linking of collagen, and would favour airway narrowing. At present there are no data documenting changes in the mechanical properties of the connective tissue of the airway wall. Reduced distensibility has been reported
in the airway wall of asthmatic subjects, but this finding was not correlated with the thickness of the subepithelial layer.

3.2.4 THE ADVENTITIA

The adventitia comprises the tissue between the outermost layer of smooth muscle and the surrounding parenchyma. It contains loose bundles of collagen, blood vessels, lymphatics, and nerves. In the peripheral conducting airways, this layer interacts with the surrounding lung parenchyma via alveolar attachments which are distributed along the circumference of the adventitia.

Thickening of this layer has been reported in both asthma and COPD. There are no data, however, documenting changes in the composition of the connective tissue of this layer as a result of chronic inflammation. Thickening of this layer has been attributed to infiltration of inflammatory cells, edema and connective tissue deposition. In addition, there could be an increase in the number or dilatation of the bronchial microvessels in disease. In only one study morphometric values were obtained for the proportion of the adventitia occupied by vessels in asthma (14%) and COPD (7%) versus control (8%). Disease and normal values, however, were not significantly different.

Thickening of this layer may have important functional consequences. Under normal circumstances, during airway smooth muscle contraction, the adventitial tissue is deformed and acts by providing a load that impedes ASM shortening. The balance between smooth muscle shortening and load on the muscle from tethering of the parenchyma could be altered if this layer became

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thickened. This could occur by functional uncoupling of the airway smooth muscle from the series elastic load provided by the surrounding lung parenchyma. Figure 3.1 shows schematically the effect increased adventitial area. An increase in the adventitial layer could lead to lumenal narrowing if either it encroached inward, and/or if uncoupling caused relaxation of the surrounding lung parenchyma in the same way that it occurs during lung deflation. As a result of uncoupling, greater airway narrowing could result if more airway smooth muscle contraction occurred before the surrounding parenchyma was deformed enough to provide the elastic load required to limit smooth muscle shortening.

3.2.5 CARTILAGE

Cartilage is a major component of the airway wall in the bronchi. It is present in all the conducting airways particularly in the trachea and main stem bronchi where it forms horse-shoe shaped structures around the airways. Towards the periphery, cartilage decreases progressively and disappears at the level of airways 1-2 mm in diameter. Cartilage plates of variable shape are found in the walls of the smaller bronchi. Cartilage makes up 25-63% of the total wall in the large airways and 4-10% in the smaller airways. The function of cartilage is to maintain the stability of the airways and to provide them with support and structural integrity, particularly in the trachea and major bronchi.

Cartilage is composed of type II collagen fibers entrapped in a matrix of proteoglycan aggregates. The main proteoglycan of cartilage is aggrecan,
which is a 1-2 MDa molecule containing both chondroitin sulfate and keratan sulfate glycosaminoglycans chains. The glycosaminoglycans are able to form aggregates by binding to hyaluronan (up to 100 proteoglycan molecules can associate with a single hyaluronan molecule). In addition there is a link glycoprotein that interacts with hyaluronan and the core protein of the proteoglycan, and forms a stable ternary structure. Proteoglycans are negatively charged at physiological pH and create an osmotic swelling pressure that is balanced by tension in the collagen matrix.

The mechanical properties of cartilage stem from the ability of its collagen fibers to provide tensile stiffness and to entrap the large aggregates of hydrophilic proteoglycans. The reversible redistribution of proteoglycan-bound water through the matrix under loading is believed to provide cartilage with its resilient properties. There is a fine balance between this redistribution of water and the ability of the proteoglycans to stay in place by the formation of stable aggregates which function to resist compression or shear stress.

There is considerable evidence that there are age-related structural changes in cartilage which occur concomitantly with changes in airway function. Alterations have been reported in collagen content (decrease in hydroxyproline content) and proteoglycan structure (size, charge density, aggregaton properties, and proteolysis in link proteins) with increasing age. These changes are consistent with the progressive increase in resistance to compression during development to maturity, followed by the progressive decline in resistance to compression that takes place thereafter. These structural changes are
accompanied by parallel changes in maximal expiratory flow that occur with increasing age, so that there is a progressive increase with age to maturity, and a gradual decrease after the end of the third decade of life.

While composition and mechanical properties of collagen have been shown to change with age and may contribute to the changes observed in lung function, it is unclear whether these properties are altered in disease. A number of investigators have found atrophy or decreased bronchial cartilage in patients with COPD. Others, however, found no significant changes in the amount of bronchial cartilage in COPD or in asthma. Changes in the composition of cartilage in chronically-inflamed airways were reported in a recent study. Although the total amount of cartilage as a proportion of the airway wall was unchanged in disease, the measured degenerative changes were significant in the cartilage of autopsied lungs of patients with both chronic bronchitis and asthma.

Cartilage is considered to be one of the most important structural components of the airway wall because of its capacity to resist deformation from changes in the transmural pressure, particularly during forced expiration. Cartilage provides an elastic load on the smooth muscle, which acts to impede smooth muscle shortening and regulate airway narrowing. In animal studies proteolytic degradation of cartilage, achieved by using papain in vitro and in vivo, resulted in increased compliance of the trachea, reduced ability to resist negative pressures, and a decrease in maximal expiratory flow in the lung. In vitro experiments performed in canine airway smooth muscle where
cartilage had been completely removed showed that smooth muscle shortening tripled versus preparations where cartilage was left intact.\textsuperscript{104}

Studies in patients with COPD indicate that chronic inflammation of the airways decreases the amount of cartilage and possibly its composition, contributing to airflow obstruction.\textsuperscript{87 93 94 95 106} In contributing to the stiffness of the airway wall, cartilage may ultimately have a role in determining maximal expiratory flow rates in the lung. Some investigators have examined the mechanical properties of cartilage in normal airways,\textsuperscript{90 107} but further studies are necessary to document whether these properties are changed in disease.

3.2.6 AIRWAY SMOOTH MUSCLE

3.2.6 (a) Normal function and structure of airway smooth muscle.

The function of smooth muscle is to regulate airflow in and out of the lung. This is carried out by fine adjustment of smooth muscle tone. Tone functions to maintain structural stability, and prevent airway collapse. In addition, smooth muscle tone contributes to the elastic recoil of the lung\textsuperscript{108} and has been shown to play a role in airway collapsibility in isolated airways of humans\textsuperscript{109 110 111 112} rabbits\textsuperscript{113} and pigs\textsuperscript{114}. Airflow regulation is achieved by modulating the degree of smooth muscle shortening which adjusts airway diameter and ultimately causes changes in airway resistance.\textsuperscript{115}

The anatomical arrangement of the smooth muscle varies depending on its location in the tracheobronchial tree. The smooth muscle is found from the trachea down to the bronchioles.\textsuperscript{116} In the trachea and mainstem bronchi the
smooth muscle is located within the posterior membranous sheath and is arranged transversely at right angles to the long axis of the airway. In the peripheral airways the smooth muscle is arranged helically around the airways and surrounds the entire lumen.\textsuperscript{117, 118}

It has been suggested that the orientation of ASM could be an important determinant of airway narrowing\textsuperscript{36} but there is little information on the precise geometry of the smooth muscle. By using three-dimensional reconstruction of intraparenchymal airways, Ebina et al.\textsuperscript{116} found that ASM is arranged at an angle of 30° with the cross-sectional plane. Using different approaches, other investigators found this angle to be between 13° and 15° in a number of different species.\textsuperscript{119, 120} To the extent that airway smooth muscle is arranged obliquely ASM shortening could be associated with changes in airway length as well as changes in airway calibre. Bates and Martin provided a theoretical analysis of the bronchoconstrictive effects of smooth muscle arrangement within the airway wall.\textsuperscript{121} Their results suggest that the effect on lumen diameter for a given degree of shortening depends on the orientation of the smooth muscle around the airway. These studies support the idea that a small angle with the cross-sectional plane would mean that a greater portion of the force generation would occur in the circumferential direction, and favour airway narrowing rather than airway shortening during smooth muscle contraction. There is no data reporting alterations in the arrangement of ASM in disease.

Morphometric studies of the trachea and central bronchi have shown that in normal subjects approximately 3% -10% of the airway wall consists of smooth
muscle.\textsuperscript{34, 32, 122} Similarly, about 5-10\% of the airway wall is occupied by smooth muscle in membranous airways (diameter 0.3 mm-3 mm)\textsuperscript{63} and 8-17\% in cartilaginous airways (diameter 1.5-11 mm).\textsuperscript{123}

3.2.6 (b) Structural changes in airway smooth muscle.

An increase in the smooth muscle layer has been reported in asthma\textsuperscript{31, 34, 124, 48, 125, 126, 127, 63, 74} and to a lesser extent in COPD.\textsuperscript{31, 128, 63, 72, 127, 73} The increase in the smooth muscle layer could be due to hypertrophy or hyperplasia of the smooth muscle cells, or to an increase in deposition of connective tissue surrounding the smooth muscle cells. There have been few studies looking at the cell number and size in disease but the reported data\textsuperscript{125} suggests that both are increased at least in asthma. Ebina et al.\textsuperscript{48} have shown that there is increased airway smooth muscle in large and small airways of some asthmatics and that these patients appear to be more symptomatic than those in whom the hypertrophy and hyperplasia is localized to the large airways. While in most studies an increase in the smooth muscle layer has been shown, in a recent stereological study, Thomson et al.\textsuperscript{129} found that the connective tissue inside smooth muscle bundles contributes substantially to what may have been considered to be muscle by previous investigators. Thomson et al. measured the cross sectional area of the smooth muscle, the connective tissue within smooth muscle bundles and the extracellular matrix between muscle cells at a higher magnification than has been used by classical morphometric techniques. They used large airways of five asthmatic patients obtained from surgical and autopsy
experiments, compared them to five nonasthmatic smokers, and found that there were no differences between the two groups.

In COPD the amount of smooth muscle has been shown to be increased in small membranous airways, but whether it is increased in the larger cartilaginous airways is unclear.

3.2.6 (c) Functional consequences of increased smooth muscle.

An increase in the amount of airway smooth muscle could have significant functional consequences. If the contractile function of the muscle remained normal, an increase in the smooth muscle layer would have a geometric effect leading to airway narrowing. If in addition to an increase in smooth muscle mass, there was a proportional increase in force generating ability of the muscle, the increased force generation could result in increased shortening because that would increase the ability of the muscle to overcome elastic loads.

3.3 Airway smooth muscle contractility.

3.3.1 Contractile properties of airway smooth muscle.

The normal contractile function has been studied by Ishida et al. who examined 10 main stem bronchial airways from human resected lung specimens and two trachealis muscle specimens obtained at autopsy. In response to electrical field stimulation the smooth muscle shortened by 25% of its initial length and generated about 41 mN/mm² of isometric force at optimal length.
In peripheral airways normal contractile function has been little studied. No data to date has been reported on the complete length tension and length shortening characteristics of human peripheral airway smooth muscle. The contractile properties of smooth muscle have been the subject of several studies. But most of these have focused on the trachea or large airways because such airways are more easily obtained. Relatively little information exists on the function of peripheral airway smooth muscle.

Whether hypertrophy or hyperplasia result in increased tension generating ability of smooth muscle is also unclear. There are no data documenting whether the increased muscle is phenotypically normal or whether there are alterations in the contractile function, the contractile proteins, or the organization of the smooth muscle as a result of chronic inflammation.

3.3.2 Alterations in contractility in asthma and COPD

It is unclear whether the contractile properties of smooth muscle are altered in disease. Several studies have been carried out in vitro to characterize the altered contractile properties of human ASM but few have been able to determine whether alterations in ASM play a role in airway hyperresponsiveness and airflow obstruction.

3.3.2 (a) Force generation

In only three laboratories an increase in the force generation has been demonstrated in asthmatic\textsuperscript{132} \textsuperscript{133} \textsuperscript{134} \textsuperscript{135} or COPD\textsuperscript{136} versus control airway smooth
muscle preparations from resected and autopsy specimens. Other investigators have not been able to show increased force generation in disease.\textsuperscript{137 138 139 140}

These inconsistent findings may be attributable to several methodologic limitations in these studies, such as the use of a small sample size, sampling from different parts of the tracheobronchial tree, and lack of normalization of force. In only one study the amount of muscle within the airway preparation was determined.\textsuperscript{135} This makes it difficult to evaluate whether force was altered in the other studies due to changes in the smooth muscle mass. Another limitation in methodology has been the failure to determine the length at which maximal isometric force occurs (Lmax). If the airways of asthmatics or COPD patients are stiffer than the control airways, then a given load will stretch the tissues to different lengths. That is, a standard load that would stretch a preparation from an asthmatic subject to Lmax, might stretch that from a normal subject beyond Lmax, where less force is generated. Alternatively, if chronically inflamed airways were less stiff, the reverse would be true. According to Bramley and colleagues,\textsuperscript{135} if an asthmatic airway (which has decreased elastance) is stretched to the length used routinely for normal tissues, it will be stretched beyond Lmax and therefore the force will artificially lower than Fmax.

In the study by Bramley et al.\textsuperscript{135} where the amount of smooth muscle was used to normalize force, the resulting stress was significantly greater in asthmatic versus control preparations. This finding suggests that an increase in the smooth muscle mass alone cannot account for the force generated, and that alterations
in the contractile properties of the smooth muscle may be occurring. That is, there could be alterations in the contractility of myocytes, or alternatively, in the myocyte-myocyte interactions, or in the parallel elastic elements exerting a load on the muscle affecting the transmission of force.

3.3.2 (b) Isotonic Shortening

In only two studies isotonic shortening has been evaluated in human airways in asthma\textsuperscript{135} and COPD.\textsuperscript{146} In the asthmatic airways the shortening was about three times greater than that of the controls. In COPD the amount of shortening was not different in comparison with the controls. The results of increased shortening in the asthmatic case support the idea that an increase in smooth muscle shortening determines exaggerated airway narrowing in asthma.\textsuperscript{36}

The factors that determine smooth muscle shortening are those that determine isometric force such as the amount of force generation (which depends on the length of the muscle, and the amount of smooth muscle), as well as the load on the muscle.

The load on the muscle is the load against which the muscle contracts. It is exerted by the viscous and elastic loads produced by the structural components of the airway wall and by the tension provided by the transmural pressure. The loads contributed by the airway wall are provided by the cartilage and connective tissue surrounding the muscle and comprising the parallel elastic elements. The role of the cartilage in impeding smooth muscle shortening has
been discussed in section 3.2.5. A decrease in the elastance of the parallel elastic elements has been hypothesized to contribute to an increase in smooth muscle shortening in asthma.\textsuperscript{135}

The amount of shortening has been shown to be related to the elastic loads provided by the surrounding extracellular matrix. Bramley et al.\textsuperscript{135} showed that the increased shortening observed in one case of asthma was associated with a decrease in the passive elastance of the tissue. The same authors also showed that when the matrix elements were degraded by collagenase, the amount of shortening increased after treatment.\textsuperscript{147}

Another important load is that contributed by the transmural pressure, which is, in the static state, that produced by the transpulmonary pressure, or the static elastic recoil pressure of the lung. The transmural pressure is related to the alveolar attachments to the airway wall. When smooth muscle is stimulated to contract, the forces of interdependence tend to oppose airway smooth muscle shortening, as this happens at fixed lung volume. As smooth muscle shortens it has to deform the surrounding parenchyma, and the radial tension resulting from the stretch applied to the alveolar attachments is believed to provide a significant load which could reduce the amount of shortening and the degree to which the airway can narrow.

An additional load on smooth muscle during shortening could be the radial constraint applied by the extracellular matrix elements. This constraint could act on smooth muscle cells as they shorten, thereby, limiting the amount of shortening by individual cells. This "radial constraint" hypothesis was postulated
by Meiss,\textsuperscript{148} and states that there are tissue-based constraints on radial expansion at short lengths. Such radially-directed forces arise when cells assume extreme configurations countered by forces arising from other cells and from connective tissue attachments. As the muscle shortens under an isotonic load, the cells must assume a more rounded shape as they increase in cross-sectional area, since constant volume is maintained. As the airway smooth muscle shortens, and the circumference of the cells increase, the connective tissue becomes stretched and resists the increase in cell radius. Lateral forces generated by the cells on each other may also become significant, adding to the length-dependent impediment on the contractile apparatus. Shortening continues until the muscle can no longer overcome the axial and radial forces.

Meiss also showed that if canine smooth muscle preparations, which normally shorten by \textasciitilde75\%, are constrained by nonextensible silastic rings shortening is reduced.\textsuperscript{149} The connective tissue content of human airways is higher than that of the dog trachealis and may constrain the ASM and reduce its ability to shorten. See passive tension below.

\textit{In vivo}, shortening is likely to result from a combination of isometric and isotonic contractions. \textit{In vitro} the "isotonic shortening" condition is a contraction against an elastic load. Assuming the stress is distributed evenly over the internal and external parallel elastic component (PEC), the passive tension is initially borne by the PEC within the smooth muscle, and by the PEC external to the muscle made up of collagen and elastin. During "isotonic" contraction of the muscle the internal and external PECs shorten along their passive length-tension
curve such that after maximal contraction, the muscle must generate force to overcome the load borne by the PECs. That is, the muscle has to contract against an elastic load when stimulated in a preloaded "isotonic" fashion.

Even in preparations that have low passive tension such as that observed in canine and porcine tissues,\textsuperscript{150} "isotonic" contraction from Lmax is also associated with the necessity for the smooth muscle to generate some active force. Since the passive tension at Lmax is small in the pig and the dog, the amount of active force that the muscle has to generate during its "isotonic" contraction is small. When the passive tension at Lmax is large, however, such as that observed in human preparations from central airways,\textsuperscript{150} "isotonic" shortening requires the development of a significant increase in force by the muscle. There is no substantial difference between the length at maximal force generation and maximal shortening for the dog and pig because the passive tension at Lmax is small, and the fraction of the wall made up of muscle is large, so that contraction more closely approximates true isotonic contraction. In human preparations from central airways the substantial load constitutes an auxotonic load. Throughout this thesis, the term "isotonic shortening" will refer to a "contraction under constant external load", and will apply to the auxotonic condition.

3.3.2 (c) Velocity of shortening.

The velocity of shortening has not been evaluated in either asthma or COPD. Passively sensitized smooth muscle from human airways was shown to
have an increase in both the velocity and capacity of shortening. Several studies aimed at examining the velocity of shortening have been carried out in animal models of asthma. Airway smooth muscle from allergen-sensitized animals exhibits an increase in the velocity and shortening capacity, but not the isometric force, compared to littermate controls. This increase in shortening velocity has been partially explained by an increase in the amount and total activity of the myosin light chain kinase measured in the same preparations.

3.3.2 (d) Passive tension.

The passive, or resting, tension (or passive elastance) results from stretching the relaxed smooth muscle preparation. Large human airways from morphologically normal lung specimens have been shown to have high passive tension, particularly at the length required for optimal isometric contraction. The high passive tension required to stretch the muscle is believed to be a reflection of the high connective tissue content in the preparations. The observation that there is a relationship between passive tension and connective tissue, and that both of these are negatively related to the degree of smooth muscle shortening has been reported in several species.

There is little evidence that there are alterations in the passive tension in asthma or COPD. Data from the asthmatic airway studied by Bramley et al. indicates that in large airways the passive elastance at optimal length is reduced in asthma compared to control subjects. In addition, there was increased force
and shortening in the asthmatic smooth muscle. These findings suggest that decreased airway elastance of the tissue may facilitate shortening due to the lower load the muscle must overcome in order to shorten. Changes in the passive tension of peripheral airways have not been studied in normal or in chronically inflamed airways.

3.4 Airway smooth muscle phenotype.

3.4.1 Changes in airway smooth muscle phenotype.

Even though there is agreement by most investigators that the amount of airway smooth muscle is increased in both COPD and asthma, it is unknown whether the phenotype of the muscle is altered in these chronic inflammatory disorders. Specifically, there are no studies on whether the increase in force that has been reported can be explained only by an increase in smooth muscle mass. In the study by Bramley et al.\textsuperscript{135} both force and smooth muscle mass were increased. But the stress was still higher in the asthmatic airways suggesting that an increase in the smooth muscle mass alone cannot account for the force generated, and that alterations in the contractile properties of the smooth muscle may be occurring.

In recent studies of smooth muscle cells in culture several investigators have suggested that structural remodelling of the airway wall could be accompanied by a change in the contractile state of the smooth muscle.\textsuperscript{156} In addition, there has been an increasing interest in the cytokines and growth factors which cause smooth muscle to proliferate in vitro.\textsuperscript{157 158 159} but the
relevance of such studies to in vivo smooth muscle hyperplasia and/or hypertrophy is unclear.

3.4.2 Structural and synthetic properties of smooth muscle.

Mature smooth muscle cells normally exist in a quiescent differentiated state with a low rate of proliferation. They have a high content of contractile proteins and few biosynthetic organelles. Their main function is to contract. In contrast, proliferating smooth muscle cells have a low content of contractile proteins, high level of biosynthetic organelles, and may lose the ability to contract. Their function is to produce factors involved in the synthesis and deposition of extracellular matrix (ECM) components. Proliferating smooth muscle cells are implicated in the remodelling process of chronic inflammation because of this ability to synthesize ECM, which contributes to thickening of the airway wall and ultimately leads to airway narrowing.

There is evidence that airway smooth muscle cells can undergo a reversible change in phenotype from a contractile to a synthetic state when stimulated to divide in cell culture. In recent studies of various types of smooth muscle several molecular markers of ASM phenotype have been defined. The cellular content of these markers has been shown to change dramatically during proliferation indicating that smooth muscle cells are capable of phenotypic plasticity which is necessary for growth and repair following injury. Proteins found in the contractile phenotype have been shown to be progressively lost during proliferation when non-muscle (NM) proteins become more abundant.
Halayko et al. studied canine tracheal SM cells in primary cultures to investigate the modulation of ASM from a contractile to a noncontractile phenotype during ASM cell proliferation. Using immunoblot analysis these authors looked at concomitant changes in the expression of cytocontractile and cytoskeletal proteins. They found that when cells became proliferative after 5 days in culture, the content of smooth muscle myosin heavy chain (sm-MHC), sm-α-actin, calponin, and desmin diminished by >75%; in addition, myosin light chain kinase (MLCK), h-caldesmon, and β-tropomyosin also decreased significantly. Conversely, the content of nm-MHC, l-caldesmon, vimentin, and α/β-protein kinase C (PKC) increased. In addition, after the cells reached confluence, the content of sm-MHC and sm-α-actin protein increased, although not to baseline values.

This phenotype plasticity has been observed in vascular smooth muscle cells which undergo a change in phenotype from a contractile to a synthetic state when stimulated to divide in cell culture and in vivo in the atherosclerotic lesion. If a similar change in ASM phenotype occurred in the airway during chronic inflammatory disorders, one would predict decreased contractile function and attenuated airway narrowing, unless the magnitude of the increased muscle mass more than compensated for the decreased contractile function.

Alternatively, episodes of inflammation with proliferation of smooth muscle could be followed by quiescent periods when the hyperplastic muscle may redifferentiate to a contractile phenotype. In a recent study, Ma et al. showed that serum-deprived cultured cells return to a contractile state at post confluence,
but differ from the original; they exhibit a reappearance of SM-MHC and appear contractile but the myosin heavy chain isoform responsible for higher shortening velocity, SM-B, is lost and there is an elevated content of NM-MHC. Some of the contractile cells were found to have greater shortening capacity than the original freshly isolated cells. If similar phenotypic oscillation occurs in vivo in response to repeated episodes of inflammation and repair, the outcome could be the increased contractility and relative irreversibility of hyperresponsiveness found in obstructive airways disease.

The phenotype of ASM in chronically inflamed airways is unknown. It is also unclear whether the contractile properties of ASM are related to the presence of diverse and stable subpopulations of ASM cells which could change in response to inflammatory stimuli, as has been shown in the pulmonary artery, or to the transient phenotypic modulation of a single population of differentiated ASM cells. The phenotype of any one cell could depend on the microenvironment produced by the surrounding cells, soluble factors, and the extracellular matrix.

This thesis will examine the question of whether biochemical alterations are present in the smooth muscle and in the extracellular matrix of chronically inflamed peripheral airways. It will also examine how such alterations may be related to the in vitro function of airway smooth muscle as well as lung function in patients with obstructive airways disease. A study on the mechanisms of airway remodelling by the extracellular matrix is outside the scope of this thesis. Nonetheless, some of the important features regarding remodelling of the ECM
and its role in the modulation of ASM will be discussed in the next section.

3.5 The extracellular matrix (ECM) and airway remodelling in chronic inflammation.

3.5.1 Alterations in the ECM.

In addition to the contractile phenotype of the airway smooth muscle and the elastic loads provided by lung parenchymal recoil and airway cartilage rigidity, the extracellular milieu of the muscle may have an important effect on ASM's capacity to generate force and to shorten. Alterations in the ECM may be involved in the structural remodelling of the airway wall in asthma and COPD, but the nature of this process has not been well studied.

In asthma there is increased matrix deposition in the lamina reticularis and in the submucosa which involves deposition of fibrillar collagen types III and V, and the glycoproteins fibronectin, laminin, and tenascin beneath an apparently normal lamina densa. (See section 3.2. on the Basement membrane and The Submucosa). In addition, the proteoglycan versican and the glycosaminoglycan hyaluronan (HA) have been found to be increased in the submucosa by immunohistochemical staining of airways obtained postmortem from subjects who died from asthma. Proteoglycan content was also found to be increased in mild asthma in airway tissues obtained at biopsy, and was correlated with the degree of airway responsiveness.
Little is known about the alterations that may be present in the airways of COPD patients. Although thickening of the connective tissue surrounding the muscle has been documented in COPD, the nature of this thickening has not been well studied. In only a few studies inflammatory markers such as fibronectin and hyaluronan were found to be elevated in the bronchoalveolar lavage fluid of COPD patients, and in one study, hyaluronan was inversely correlated with pulmonary function.

3.5.2 Functional consequences of structural changes in the ECM.

The potential consequences of connective tissue deposition are exaggerated airway narrowing by amplifying the effect of smooth muscle shortening, if thickening occurs inside the muscle layer, or by uncoupling the muscle from the load provided by the surrounding parenchyma, if thickening occurs outside the muscle layer.

Alternatively, attenuation of airway narrowing could also occur if the elastic loads on the smooth muscle are increased. When ASM shortens it necessarily thickens and if the interstitial matrix in which the muscle cells are imbedded limits that thickening, the resulting radial constraint could oppose the force and decrease the shortening by the muscle. Meiss et al. postulated the "radial constraint" hypothesis (see section 3.2.6), to explain the constraints on expansion of the muscle when it shortens. Lambert et al. proposed that an increase in the thickness of the connective tissue can also affect the load on the
muscle by increasing the stiffness of the tissue, and consequently the load opposing ASM shortening.

Another possibility is that there is proteolysis associated with chronic inflammation in asthma and COPD. In order to test the effect of the extracellular matrix constraint on the smooth muscle, Meiss et al.\textsuperscript{148} lightly digested the extracellular matrix with collagenase and found increased contractility and shortening. These experiments were done in canine trachealis muscle preparation that contains only a small amount of connective tissue and which shortens by about 75% when maximally stimulated. Isolated human smooth muscle cells from large and small airways have also been shown to shorten by this much.\textsuperscript{163} In contrast, intact human airway smooth muscle preparations shorten by only ~20-30%. The content of connective tissue in the airway wall surrounding smooth muscle in human airways is higher than that in dog trachealis.\textsuperscript{120} It is possible that this rich matrix imposes more radial constraint for human ASM in situ and accounts for the lower ASM shortening observed.

Changes in the extracellular matrix could alter the constraint and consequently affect ASM shortening. Reducing this constraint by proteolytic disruption of the extracellular matrix surrounding the ASM cells and bundles could decrease the radial constraint on the muscle and allow increased shortening to occur. In experiments carried out by Bramley et al.\textsuperscript{147} freshly excised human airway smooth muscle preparations were incubated with collagenase and this resulted in increased force and shortening. These findings support the idea that the extracellular matrix by imposing a radial constraint, may
have an important role in limiting ASM shortening in human airways, and that degradation of the matrix in disease could lead to increased shortening and airway narrowing.

### 3.5.3 Modulation of smooth muscle phenotype by the ECM in chronic airways disease.

The relationship between smooth muscle phenotype stability and ECM synthesis has not been studied. As a result of inflammation, this interaction may be a key aspect in the pathogenesis of asthma and COPD. Results from vascular studies\(^{160,161,164,167,168,169,170,171}\) indicate that during the process of injury and repair the ECM contributes to smooth muscle proliferation and structural remodelling.

In the presence of inflammatory stimuli, ASM may undergo loss of contractile elements and altered smooth muscle function towards a myofibroblast-like state. Evidence that fibroblasts may have an important role in airway wall remodelling is indicated by their increased numbers at sites of lung injury and repair in the bronchi of asthmatic patients.\(^{172}\) Although the origin of myofibroblasts is unknown, their morphological features are intermediate between those of fibroblasts and smooth muscle cells.\(^{172}\) Further evidence of fibroblast involvement is provided by the role of proinflammatory cytokines\(^{168}\) in the regulation of ASM cell proliferation and collagen synthesis by fibroblasts.\(^{56,172}\) In addition to growth factors and cytokines, the structural components of the surrounding ECM can also influence phenotype stability of smooth muscle.\(^{160}\)
ECM elements that promote the contractile phenotype are basement membrane components laminin and collagen type IV, whereas those that promote the transition of contractile smooth muscle cells to the synthetic phenotype are ECM components fibronectin and collagen types I and III. Once converted, the synthetic cells can produce cytokines and ECM components which alter the surrounding ECM environment regulating the phenotypic diversity of adjacent smooth muscle cells.

In studies of atherosclerosis it has been demonstrated that it is the synthetic phenotype of smooth muscle that determines the ECM of the lesion. Studies by Hirst et al. have shown that proliferating human smooth muscle cells express immunoreactive fibronectin, laminin, and collagen type III, and that proliferation stimulated by PDGF-BB is enhanced by fibronectin and collagen type I, but not by laminin. This differential response of ASM to ECM proteins also correlates with reduced expression of contractile marker proteins such as SM α-actin and myosin heavy chain by growth-promoting ECM elements such as fibronectin and collagen I.

3.6 Compartments of the airway wall.

In this thesis the nomenclature used to define the different compartments of the airway wall will be that proposed for morphometrical studies. This nomenclature is based on the morphological features seen on histological specimens (Figure 3.2). The airway is divided into two major compartments: an inner wall and an outer wall. The inner wall is that defined by the outer border of
the smooth muscle layer and the lumenal border of the epithelium. It is composed of the epithelium, basement membrane, lamina propria, and smooth muscle. The outer wall is defined by the outer border of the smooth muscle and the outer layer of the adventitia. It is composed of cartilage and the adventitia.

3.7 Hypotheses.

3.7.1 General Hypothesis.

The central hypothesis of this thesis is that **phenotypic changes in the airways of asthmatic and COPD patients, secondary to chronic immunologic inflammation (asthma) and chemically-induced inflammation (COPD), result in structural remodelling of the airways which in turn affects airway function and leads to hyperresponsiveness.** Structural remodelling could involve changes in the muscle or in the extracellular matrix affecting the balance between the force generating ability of the airway smooth muscle and the elastic loads on the muscle. Increased airway narrowing could result from increased force generation by the muscle, decreased load on the muscle or airway wall thickening so that a normal amount of ASM shortening would result in increased airway resistance and excessive airway narrowing.

3.7.2 Specific hypotheses:

1. Airway smooth muscle contractility (isometric force and isotonic shortening) and airway smooth muscle mass are increased in obstructive airways disease, and are related to the severity of airflow obstruction.
2. Intrinsic tone is increased in airway smooth muscle from subjects who have more severe airflow obstruction, is due primarily to leukotriene synthesis, and can influence the degree of shortening and force generation.

3. The increase in smooth muscle mass observed in asthma and COPD is associated with dedifferentiation of the smooth muscle, resulting in a decrease in the content of contractile proteins (myosin and α-actin) and the novel expression of non-contractile proteins (non-muscle myosin and non-muscle actin).

4. There is increased deposition of collagen, proteoglycans, elastin, and total connective tissue content in the airway wall in asthma and COPD.

5. Connective tissue deposition in the airway wall in disease increases the passive elastance of the airway and tends to attenuate excessive bronchoconstriction.

6. Despite increased airway stiffness it is the increase in force and muscle mass that constitutes the most important structural alteration in the airways leading to increased airflow obstruction and hyperresponsiveness.

3.7.3 Specific aims:

1. To characterize the passive and active mechanical properties of peripheral airways from the lungs of normal subjects and from patients with COPD and asthma obtained at surgical resection.

2. To examine intrinsic tone and its effect on ASM contractility in the airway smooth muscle preparations from different patient groups.
3. To use morphometric techniques to quantify differences in the amount of smooth muscle and airway wall compartments in the patient groups.

4. To relate the functional alterations to measurable structural changes (1 and 2 above).

5. To describe and quantify the distribution of connective tissue proteins and extracellular matrix components (collagen, proteoglycans, elastin and hyaluronan) in the airway wall in the patient groups.

6. To use Western blot analysis to determine the distribution and content of myosin and actin isotypes in the ASM from the patient groups.

7. To relate changes in *in vitro* contractility, airway dimensions and protein biochemistry to pulmonary function measured prior to surgery.
Figure 3.1. Effect of increased adventitial area. An increase in the thickness of the adventitia, or outer wall, could uncouple the airway smooth muscle (ASM) from the surrounding parenchymal recoil. A greater ASM shortening and lumenal narrowing could occur before the elastic recoil prevents further shortening. In this example, shortening occurs until the stress in the parenchyma (represented by the springs) prevents further shortening and narrowing at a resistance (R) of 8. After thickening of the outer wall, the stress in the parenchyma is decreased, and greater ASM shortening can occur. (Adapted from Pare PD, TR Bai. Airway remodelling in chronic obstructive pulmonary disease. Eur Respir J. 6:259-263, 1996)
Figure 3.2. Schematic representation of an airway showing measured airway dimensions. Definitions of abbreviations:

- Pi = Internal perimeter
- Pbm = Basement membrane perimeter; Abm = Basement membrane area
- Pmo = outer muscle perimeter; Amo = Internal area
- Po = Outer perimeter; Ao = Outer area
- WAm = Smooth muscle area; WAcart = cartilage area
- WAi = (Amo - Abm) Inner wall area;
- WAo = (Ao - Amo) Outer wall area
- WAt = (Ao - Abm) Total wall area
REFERENCES.


19 Stadnyk AW. Cytokine production by epithelial cells. FASEB J. 8:1041-1047, 1994


28 Chang SC. Microscopic properties of whole mounts and sections of human bronchial epithelium of smokers and nonsmokers. Cancer. 10:1246-1262, 1957


39 Chung KF. Role of inflammation in the hyperreactivity of the airways in asthma. Thorax. 41:657-652, 1986

40 Lambert, R.K. Role of bronchial basement membrane in airway collapse. J


Salvato G. Some histological changes in chronic bronchitis and asthma. Thorax. 23:168-172, 1968


Miller WF. The Lung, 3rd ed. , Charles C. Thomas, Springfield, IL, 1943

Von Yayek H. The Human Lung. Hafner, New York, 1960, pp.139-161


4.1 INTRODUCTION.

Human obstructive airways diseases such as asthma and chronic obstructive pulmonary disease (COPD) are characterized by increased airflow obstruction and exaggerated airway narrowing, or hyperresponsiveness. The mechanism(s) underlying these abnormalities is still unclear. In previous studies from several laboratories it has been shown that airway obstruction is associated with a chronic inflammatory process in both membranous and cartilaginous peripheral airways in both asthma and COPD.

The degree to which inflammatory processes contribute to airflow obstruction and hyperresponsiveness has not formally been established. Several possible explanations have been postulated. Exudation of fluid and cells into the lumen and the airway wall has been shown to increase airway resistance, but since subjects with different degrees of airflow obstruction have similar levels of cellular infiltration, this cannot be the only explanation. The presence of lumenal secretions could also narrow the airways by altering the surface tension of the liquid lining the small airways or by plugging the airways.

Deposition of connective tissue during the repair stage of inflammation
could thicken the airway wall, including the smooth muscle layer, and narrow the
airway. One hypothesis is that exaggerated airway narrowing results from airway
wall thickening acting in series with normal smooth muscle shortening. In support
of this idea James and colleagues\(^2\) reported an increase in the airway wall tissue
internal to the smooth muscle as well as an increase in the smooth muscle layer
in the airways of asthmatic subjects. Subsequently, Bosken and colleagues\(^9\)
demonstrated thickening of the subepithelial connective tissue, and the
adventitial layers in COPD, and Kuwano and colleagues\(^{14}\) found thickening of the
airway wall in asthma and increased smooth muscle mass in COPD. Results
from these studies suggest that airway wall thickening contributes to
exaggerated airway narrowing by amplifying the effect of smooth muscle
shortening, if thickening occurs inside the muscle layer, or by uncoupling the
muscle from the load provided by the surrounding parenchyma, if thickening
occurs outside the muscle layer.

A second hypothesis is that exaggerated airway narrowing is the result of
increased smooth muscle shortening in response to inhaled bronchoconstricting
stimuli. Increased smooth muscle shortening could occur from either increased
smooth muscle mass or from changes in the intrinsic properties of the muscle.
An increase in the amount of smooth muscle would allow greater force
generation and more muscle shortening against the elastic loads provided by the
parenchyma and the stiffness of the airway wall. It is possible that a change in
the balance between the force generating ability of the muscle and the loads on
the muscle could lead to lumenal narrowing and airflow obstruction.
In only a few studies a relationship between abnormalities in lung function and changes in airway dimensions has been reported in subjects who have COPD and asthma. In this study the dimensions of peripheral airways obtained from surgical specimens were compared to determine whether alterations in airway dimensions have an effect on airflow obstruction. In the next chapter, these findings will be correlated with the measurements of contractility carried out on a parallel airway ring resected from the same airway segment to examine whether alterations in the intrinsic contractile properties of airway smooth muscle are related to airflow obstruction.

The aims of this study were to measure airway dimensions of peripheral airways from the lungs of normal and subjects who had COPD and asthma, and to relate the morphological variables to pulmonary function measured prior to surgery. The hypothesis is that an increase in the thickness of the airway wall and in the smooth muscle layer in peripheral airways leads to exaggerated airway narrowing and airflow obstruction. The airway dimensions of peripheral airway rings (ID 2.3±0.8 mm) were measured and compared to pulmonary function parameters of subjects with varying degrees of airflow obstruction.
4.2 METHODS.

4.2.1 Study Population.

Lung tissue was obtained from 30 patients who had a surgical resection of a lung or lobe for a solitary peripheral lung lesion at St. Paul's Hospital. A patient was not included in this study if a lung lesion obstructed a segmental or larger bronchus or if pneumonitis was diagnosed. Subjects were studied with the approval of both the University of British Columbia and St. Paul's Hospital Ethics Committees and after obtaining informed consent of the patients. Subjects were characterized based on a detailed questionnaire of respiratory symptoms, smoking history, and allergies before surgery.

Pulmonary function tests were done prior to surgery and included maximal expiratory flow volume curves. Twenty-eight of the patients had a diagnosis of bronchogenic carcinoma, while the remaining 2 had a diagnosis of carcinoid tumor. Twenty-one of the patients had been life-long smokers, 4 were ex-smokers (had not smoked for 7, 15, 40 and 50 years), and 5 were non-smokers. Only four patients had a past history of allergies.

Pre-operative medications included sedatives (Ativan), laxatives (Docusate sodium), heparin, antibiotics (Cefazolin sodium), analgesics (acetaminophen), and in some cases beta-agonists (Ventolin, Bricaryl). Asthma and COPD were defined on clinical criteria using the definition of the American Thoracic Society. Table 4.1 summarizes the data for the subjects in this study.
4.2.2 Lung Function Studies.

Lung function studies were done within a week prior to surgery. These studies were carried out in the pulmonary function laboratory of St. Paul's Hospital and the methods have been described by Moore et al.\textsuperscript{18} Briefly, the subjects were seated in a volume displacement, pressure-compensated body plethysmograph, with a nose clip in place. Volume was measured with a Krogh water-seated spirometer coupled to a linear displacement transducer (type 300 HR Shaevitz, Pennsauken, NJ). Flow was measured using a Fleisch No. 3 pneumotachometer coupled to a Sanborn 270 differential pressure transducer (Validyne MP 45-28, $\pm$ 3.5 cm H\textsubscript{2}O, Validyne, Northridge, CA). Functional residual capacity (FRC) was determined using Boyle's law technique, and total lung capacity (TLC) was calculated by adding the inspiratory capacity to FRC.

Maximal expiratory flow-volume curve were used to calculate the forced expiratory volume in the 1\textsuperscript{st} second of the forced vital capacity (FEV\textsubscript{1}), the forced vital capacity (FVC), the forced expiratory flow at 50% of FVC (FEF\textsubscript{50}) and the forced expiratory flow between 25-75% of FVC (FEF\textsubscript{25-75}). The FEV\textsubscript{1} was expressed as percent predicted using the equations of Crapo.\textsuperscript{19} The ratio of FEV\textsubscript{1} to FVC (FEV\textsubscript{1}/FVC) was also calculated and was used as an indicator of airflow obstruction. A value of 70% is approximately the lower limit of the normal range as defined by 95 % confidence intervals for FEV\textsubscript{1}/FVC,\textsuperscript{19} and was therefore used to assess the severity of airflow obstruction in the subjects in this study.

Reversibility of bronchial obstruction was expressed as the absolute
change in FEV\(_1\) as a percentage of the predicted FEV\(_1\) (DFEV\(_1\) %pred) and as a percentage of the actual pre-bronchodilator FEV\(_1\) (DFEV\(_1\) %ini). At least two expiratory efforts were carried out and were accepted when the two values for each variable measured were within 5% of each other.

4.2.3 Morphological Studies.

Lung tissues were selected from a macroscopically tumor-free part of the specimen, and immediately transferred to ice-cold sterile Krebs-Henseleit buffer (in mM: 118 NaCl, 4.7 KCl, 2.5 CaCl\(_2\)\(\cdot\)H\(_2\)O, 1.2 MgSO\(_4\)\(\cdot\)7H\(_2\)O, 1.2 kH\(_2\)PO\(_4\), 25 NaHCO\(_3\), and 11.1 glucose). Segments (30 mm long) from subsegmental bronchi were dissected free of blood vessels and surrounding parenchyma. Segments were obtained from each subject and airway rings were cut and randomly selected for studies in morphology, physiology, and biochemistry. The physiology studies will be described in chapters 5 and 6, and the biochemistry studies will be described in chapter 7.

The resected airway rings were fixed in 10% formalin for at least 24 h with no tension. The rings were embedded such that the short axis of the airway was parallel to the cutting surface. The fixed tissues were processed for histology and embedded in paraffin. The paraffin blocks were sectioned at a thickness of 4 \(\mu\)m on a microtome. The entire specimen was sectioned but only every 50\(^{th}\) section (approximately every 100 \(\mu\)m) was mounted on a glass slide. Three slides were selected using systematic random sampling as described by Gundersen and Jensen\(^{20}\) and stained with Aldehyde Fuschin Gomori Trichrome. The maximal
measured area shrinkage factor has been shown to be 10-14% with this method.\textsuperscript{21}

4.2.4 Stereologic Methods.

4.2.4 (a) Measurement of airway wall dimensions.

Cross-sectional areas of the airways were measured at a magnification of 40x-200x (4x-20x objective lens) using a Nikon microscope with a camera lucida (Nikon Labophot; Nikon Canada Inc., Mississauga, ON, Canada), a digitizing board (Summasketch II model MM II 1201; Summagraphics, Seymour, CT), and the Bioquant BQ system software (R&M Biometrics Inc., Nashville, TN) on an IBM compatible personal computer. The camera lucida superimposed the cursorlight of the digitizing board on the microscope image of the airway.

The measurements that were made are shown in figure 4.1. These included the internal perimeter (Pi) defined as the inner border of the epithelium, the basement membrane perimeter (Pbm) defined as the inner border of the basement membrane, the external perimeter (Pmo) defined as the outer border of the muscle, and the outer or adventitial perimeter (Po) defined as the alveolar-airway interface. The amount of cartilage (cart) was measured if present. Where the smooth muscle was discontinuous, the perimeter was interpolated between the ends of the adjacent portions of muscle. In these airways the parenchymal tissue was removed by the dissection procedure, and only some of the parenchymal attachments were left on the airway wall. The computer program calculated the areas enclosed by these perimeters: \(A_i, A_{bm}, A_{mo}, A_o\). The
internal wall area (WA_i) was obtained by subtracting Abm from Amo. The outer wall area (WA_o) was obtained by subtracting Amo from Ao. The total wall area (WA_t) was obtained by subtracting Abm from Ao.

Airway size was determined by the basement membrane perimeter, Pbm. The internal perimeter, Pi, was not used because in some of the airways there was damage and shedding of the epithelium. The use of Pbm is justified by the close correlation between Pbm and Pi (r=0.9). The luminal perimeter has been shown to be relatively uninfluenced by smooth muscle contraction or by the degree of lung inflation.\textsuperscript{22}

The degree of smooth muscle contraction, or airway narrowing, was calculated to assess a possible influence by the level of airflow obstruction. The degree of airway narrowing was determined by calculating the ratio of the lumenal area to the ideal lumenal area. The lumenal area was given by Abm, the area enclosed by Pbm. The ideal lumenal area was obtained from the perimeter measured, Pbm, assuming the airway was a circle with an area given by:

\[
\frac{\text{perimeter}^2}{4\pi}.
\]

There was no difference in the degree of airway narrowing between the airways of obstructed and nonobstructed subjects (24.0 ± 12.6 and 26.4 ± 15.0 % ideal lumenal area respectively).

All measurements were performed by the same observer (AOS). The intra-observer variability was assessed by measuring a set of 6 airways on two different occasions. The inter-observer variability was assessed by having a second observer measure a set of 6 randomly selected airways. The coefficient of variation was less than 10% for intra- and inter-observer measurements.
4.2.4 (b) Measurement of smooth muscle.

The area of smooth muscle was obtained by first measuring the relative area of the airway wall occupied by muscle. Profiles of the smooth muscle were measured in the inner wall by point-counting. Four fields were selected in each slide in a systematic random fashion. Point counting was done at x20 (objective) magnification, using an unbiased counting frame and a grid density that resulted in an intrasubject coefficient of variation of less than 5% for the variable measured. The point grid (13 points x13 points) was superimposed onto a segment of the airway wall, and the number of points falling on smooth muscle were counted.

The relative, or fractional, area of smooth muscle was calculated by dividing the number of points falling on muscle by the total number of points falling on the inner wall. The fraction of smooth muscle in the inner wall area was given by, \( FR_{sm} = \frac{\#\text{points in SM}}{\#\text{points in WA}_i} \). The absolute area was then obtained by multiplying this fraction by the area of the inner wall (WAi) measured by digitization, as described in the previous section. Area of smooth muscle, \( WA_{sm} = \left[\frac{\#\text{points in SM}}{\#\text{points in WA}_i}\right] \times WA_i \). The total wall area (WAi) and the basement membrane perimeter were used as normalizing factors.

4.2.5 Data Analysis.

Data have been expressed as means ± SD unless otherwise stated. Intra- and inter-observer variability were assessed by calculating the difference of the first and second measurement, and expressing it as percentage of the average
of both measurements. To detect errors in the measurements dependent on airway size, the percentage difference was plotted as a function of Pbm.

The relationships between airway wall dimensions (WAi, WAo, WAm) and airway size (Pbm) were analyzed using multiple linear regressions. Differences in these relationships between subjects who had varying degrees of airflow obstruction were assessed by repeated measures analysis of variance (RmANOVA). The airway wall dimensions have been square root transformed to linearize the relationships when plotted against airway size (Pbm). In previous studies\(^9\)\(^14\)\(^15\) relationships between the square root of airway wall measurements and airway size were found to be linear. Transformation of the data to the square root provided regressions of data approximately normally distributed around the regression lines. The intercept was examined for its linear relation to pulmonary function parameters (FEV\(_1\), FEV\(_1\)/FVC, FEF\(_{50}\), FEF\(_{25-75}\), DFEV\(_1\) %pred, and DFEV\(_1\) %ini).

In addition, the inner wall, outer wall and the smooth muscle areas were normalized by the total wall area in order to describe their distribution within the airway wall, for different sized airways. In order to assess differences in pulmonary function, subjects were divided into obstructed and non-obstructed groups based on an FEV\(_1\) of 70%. Differences in FEV\(_1\), FEV\(_1\)/FVC, FEF\(_{50}\), FEF\(_{25-75}\), FRC, RV, DFEV\(_1\)%pred, and DFEV\(_1\)%ini were analyzed using unpaired t-tests. Bonferroni corrections were made for multiple comparisons. P values were considered to be statistically significant when less than 0.05.
4.3 RESULTS.

4.3.1 Lung Function Studies.

Table 4.2 shows the mean lung function values for the subjects in this study. Fifteen subjects had an FEV₁/FVC < 70% and were considered to have airflow obstruction. This group included subjects with mild and severe reduction of maximum expiratory airflow. 9 subjects had an FEV₁/FVC in the range 60-70% and 6 subjects had an FEV₁/FVC of less than 60%. The functional residual capacity, residual volume, and total lung capacity were increased (p<0.05), while the FEF₅₀ and FEF₂₅₋₇₅ were significantly lower (p<0.001) in the obstructed patient group. DFEV₁%pred, and DFEV₁%ini were also increased in the obstructed group (p<0.05). The group that had an FEV₁/FVC > 70% was considered to be non-obstructed. Table 4.3 shows the lung function characteristics for obstructed and nonobstructed subjects.

4.3.2 Stereological Analysis.

4.3.2 (a) Frequency Distribution.

Figure 4.2 shows the frequency distribution for the airways in this study. A total of 30 airways were studied, one from each patient. The mean Pbm value was 7.1 ± 2.5 mm (range 3.1-13.0 mm) which is equivalent to a diameter of 2.3 ± 0.8 mm (range 1.0-4.0 mm). 26 airways were cartilaginous and 4 airways were membranous. There was no relationship between airway size (Pbm) and the intra- or inter-observer variability for any variable.
4.3.2 (b) Mean airway dimensions.

The mean values for the morphometric measurements of WAi, WAo, WAt and WAm are shown in table 4.4. The size of the inner wall area was about one fourth of the outer wall area (WAi=0.77±0.54 versus WAo=3.2 ± 2.4 mm$^2$). The area of smooth muscle was 0.15±0.12 mm$^2$, which constitutes only about 20% of the inner wall area, and only about 5% of the total wall area. The amount of cartilage was approximately 15% of the total wall. The mean Pbm and corresponding diameter are also shown.

4.3.2 (c) Airway dimensions and airway size.

Figure 4.3 shows the inner wall area (√WAi), outer wall area (√WAo), and smooth muscle area (WAm), as a percentage of the total wall area (%WAt), plotted as a function of airway size (Pbm). The largest portion of the airway wall was occupied by the outer wall area across the entire range of airway sizes. There was, however, some variability in this distribution, especially in airways with a Pbm <5mm, where WAo ranged between 40 – 90%, and WAi ranged between 10 – 60% of the total wall area. The percentage of the smooth muscle in the total wall area ranged between 1 and 17%. The mean values for WAi/WAt, WAo/WAt, and WAm/WAt are shown in table 4.4.

4.3.3 Airway wall dimensions and lung function.

Figure 4.4 shows the airway wall dimensions versus airway size (Pbm). There were significantly (p<0.001) linear relationships between Pbm and √WAi,
WAo, and \( \sqrt{WAm} \). The smooth muscle, inner and outer wall areas increased as a function of airway size. The intercepts and slopes of the regressions of \( \sqrt{WAo} \) and \( \sqrt{WAi} \) on Pbm were not related to any measures of lung function (FEV\(_1\), FEV\(_1\)/FVC, FEF\(_{50}\), FEF\(_{25-75}\), DFEV\(_1\)\%pred, DFEV\(_1\)\%ini). The intercept of the regression of \( \sqrt{WAm} \) on Pbm was significantly related to the FEV\(_1\), FEV\(_1\)/FVC, FEF\(_{25-75}\), DFEV\(_1\)\%pred, and DFEV\(_1\)\%ini (p<0.03) but not to the FEF\(_{50}\). Subjects with a thicker smooth muscle layer had more severe reduction of maximum expiratory airflow. There was no relationship between the amount of cartilage and lung function (data not shown). Table 4.5 summarizes the regression equations for muscle, inner and outer wall areas.
4.4 DISCUSSION.

The purpose of this study was to characterize the airway wall dimensions of peripheral airways, and to relate these properties to pulmonary function in subjects who had variable degrees of airflow obstruction. These measurements were also carried out to examine the relationship between morphological changes and alterations in the contractile and biochemical properties of smooth muscle to be discussed in chapters 5 and 6.

4.4.1 Lung Function

An FEV₁/FVC of less than 70% was used to define airflow obstruction. A level of 70% is approximately the lower limit of the normal range for FEV₁/FVC within 95% confidence intervals. Subjects categorized as being obstructed on the basis of FEV₁/FVC also showed hyperinflation and gas trapping with significant increases in FRC and residual volume. Only one of the subjects had any features suggestive of asthma. The measurements of FEV₁/FVC (%), FEV₁ (% predicted), FEF₅₀, FEF₂₅₋₇₅, DFEV₁%pred, and DFEV₁%ini were used as continuous variables to examine the relationship between airflow obstruction and the magnitude of airway smooth muscle, inner, and outer wall areas.

4.4.2 Morphological measures and correlations with in vivo function.

The results showed that the smooth muscle layer, and not the inner or outer wall areas, was thicker in subjects with more severe airflow obstruction.
Thickening of the smooth muscle layer was related to several pulmonary function parameters, the FEV₁/FVC, FEV₁, FEF₂₅₋₇₅, DFEV₁%pred, and DFEV₁%ini. In addition, the amount of airway smooth muscle measured by morphometry was significantly higher over the entire size range of peripheral airways. These findings are consistent with previous reports of increased smooth muscle mass in both asthma and COPD. Kuwano and colleagues found an increase in the smooth muscle layer in membranous airways (D 0.3 – 3 mm) from subjects with COPD and asthma. Bosken et al studied airway dimensions in the airways of lungs from 30 smokers with airflow obstruction (FEV1/FVC<65%) and compared them to the airways dimensions in 30 subjects without airflow obstruction. These authors found a significant increase in the amount of smooth muscle, in addition to an increase in the inner and the outer airway wall of membranous airways. Since only 11% of the airways they studied were cartilaginous they were unable to establish the same relations in cartilaginous airways.

The finding that thickening of the smooth muscle area was related to the degree of airflow obstruction supports the hypothesis in this study that an increase in the smooth muscle mass narrows the airway and results in a reduction of maximal expiratory flow. An increase in muscle mass does not necessarily lead to exaggerated airway narrowing, since the presence of elastic loads provided by the lung recoil and the intrinsic stiffening of the airway wall could act to impede smooth muscle shortening. In the mathematical model developed by Lambert and colleagues, the most important determinant of
airway resistance is the balance between the force generated by the muscle and the elastic loads against which the muscle is working. The force generated by the muscle was assumed to be proportional to the amount of smooth muscle. Increased smooth muscle mass could produce more force, more smooth muscle shortening, and increase baseline resistance and maximal airway resistance. Lambert et al.\textsuperscript{30} used the morphologic data of Kuwano and coworkers, and showed that the increased outer wall, inner wall, and smooth muscle areas of asthma and COPD can explain increases in baseline resistance. They also found that the increase in smooth muscle mass is likely to be the most important alteration responsible for increased airway resistance in response to bronchoconstricting stimuli in asthma and COPD.

The increase in smooth muscle mass could result in exaggerated airway narrowing (increased airway resistance) by causing an overall increase in the inner wall thickness which would amplify the effect of smooth muscle shortening on airway caliber by encroaching into the lumen.\textsuperscript{2} Since the airway wall was not found to be increased as a function of airflow obstruction, the findings in this study do not support amplification of airway resistance as an explanation for excess airway narrowing.

It was surprising that the inner wall area was not significantly thickened in airways from severely obstructed subjects. Previous investigators\textsuperscript{14,9,10} reported significant differences in WAi between control and COPD subjects. In cartilaginous airways Tiddens et al.\textsuperscript{10} examined a wider range of airway sizes (D 1.5 — 11 mm) and found a significant relationship between the inner wall area
and airflow obstruction. But such study involved a much larger number of airways (n=341), many airways from the same patient, and more subjects in the severe airflow obstruction category, possibly reducing the variability in the signal to noise ratio. Bosken and Kuwano reported similar findings in membranous airways. (See table 4.6 for prediction equations).

The outer wall area was not correlated to airflow obstruction in this study. Thickening of the outer wall has been hypothesized to lead to uncoupling of the smooth muscle layer from the parenchymal distending forces, leading to increased airflow obstruction. Macklem et al. suggested that thickening of the outer airway wall "uncouples" the airway smooth muscle from the surrounding parenchyma and allows it to shorten excessively. An increase in the adventitia would lead to a reduction of interdependence between muscle tension and parenchymal recoil decreasing the load on the smooth muscle, allowing greater shortening. Since no excess tissue was detected in the outer wall this idea does not explain the differences in pulmonary function observed in the subjects in this study. Furthermore, since most of the airways in this study were cartilaginous, it is possible that the effect of changes in the outer wall on ASM is reduced by the cartilage plates. This would mean that that the outer wall of cartilaginous airways of this study has a smaller contribution to airway narrowing than membranous airways.
4.4.3 Stereology of airway dimensions.

The wall area compartments, and the amount of smooth muscle were obtained by morphometry of cross-sectional areas of the airways. While the areas of the inner and outer wall were measured by digitization, the area of smooth muscle was estimated by point-counting. On these sections the smooth muscle appears as longitudinal fibers. Simply tracing the area of these fibers would overestimate the amount of muscle present in the airway wall. In one study it was shown that digitization of what some investigators have considered to be smooth muscle area is really interstitial connective tissue. Tiddens et al. used an automated image analysis system that allowed him to threshold the level of colour that differentiated smooth muscle from other structures on trichrome stained slides. He also found that the measurements of smooth muscle had a large variability. In this study point counting the cross sections of the airways minimized this source of error and reduced the variability in the measurement of smooth muscle.

4.4.4 Implications for therapy.

The results from this study suggest that the main problem in this group of patients with COPD is excess force generation by increased muscle mass. Therefore, therapy with bronchodilators may be effective for these patients by reversing the airway narrowing that results from bronchoconstriction. Treatment with bronchodilators has been shown to improve the FEV$_1$. Although markers of inflammation were not studied, it is likely that thickening of the smooth muscle
layer is the result of an inflammatory process in subjects with asthma and COPD. To the extent that this inflammation is reversible, treatment with inhaled and oral corticosteroids could prove to be effective in reducing airflow obstruction in this group of subjects. Glucocorticoids act primarily as anti-inflammatory agents in both asthma\textsuperscript{34} and COPD\textsuperscript{35} and can partially reduce the airway hyperresponsiveness and airflow obstruction present in these conditions.

In summary, this study extends previous results that membranous peripheral airways of subjects who have COPD show increased smooth muscle mass and provides new evidence that there is a similar relationship in cartilaginous airways. Airway remodeling occurs in the inflamed airways of subjects who have COPD and asthma and is associated with airflow limitation and exaggerated airway narrowing. These results suggest that the amount of smooth muscle in peripheral airways may be an important determinant of abnormal airway function in this group of subjects.
Table 4.1

STUDY POPULATION CHARACTERISTICS

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<table>
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<tbody>
<tr>
<td>n</td>
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</tr>
<tr>
<td>Age, yrs</td>
<td>62±10 (40-81)</td>
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<tr>
<td>Sex, female: male</td>
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<tr>
<td>Pack years*</td>
<td>41±23 (1-112)</td>
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<tr>
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<tr>
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<td>Non-smokers, n</td>
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<tr>
<td>Lung/Lobe resected, right, n</td>
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<tr>
<td>Lung/Lobe resected, left, n</td>
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* Pack years= the number of years smoking one pack of cigarettes per day. Age and pack years are expressed as means ± standard deviation, and range. n=number
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<tr>
<th>LUNG FUNCTION CHARACTERISTICS</th>
<th>Mean ± SD</th>
<th>Range</th>
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<tbody>
<tr>
<td>FEVi, %pred</td>
<td>78 ± 20</td>
<td>29-113</td>
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<td>FVC, %pred</td>
<td>88 ± 18</td>
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<td>FEV1/FVC, %</td>
<td>70 ± 11</td>
<td>36-84</td>
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<td>FEF50, %pred</td>
<td>54 ± 36</td>
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<td>FEF25-75, %pred</td>
<td>56 ± 29</td>
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<td>TLC, %pred</td>
<td>105 ± 17</td>
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<td>FRC, %pred</td>
<td>114 ± 29</td>
<td>63-130</td>
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<td>RV, %pred</td>
<td>121 ± 35</td>
<td>50-207</td>
</tr>
<tr>
<td>DFEVi, %pred</td>
<td>5 ± 4</td>
<td>0 - 14</td>
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<tr>
<td>DFEVi, %ini</td>
<td>8 ± 8</td>
<td>0 - 42</td>
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Definition of abbreviations:
FEVi = forced expiratory volume in 1s of the forced vital capacity; FVC = forced vital capacity; FEV1/FVC = forced expiratory volume in 1 s as a percentage of the forced vital capacity; FEF50 = forced expiratory flow at 50% of the forced vital capacity; FEF25-75 = forced expiratory flow between 25 to 75 % of the forced vital capacity; TLC = total lung capacity; FRC = functional residual capacity; RV = residual volume. DFEVi, %pred = change in FEVi as a percentage of the predicted FEVi; DFEVi, %ini = change in FEVi as a percentage of the actual pre-bronchodilator FEVi.
Table 4.3
LUNG FUNCTION CHARACTERISTICS

<table>
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<th>OBSTRUCTED MEAN ± SD</th>
<th>NONOBSTRUCTED MEAN ± SD</th>
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<tr>
<td></td>
<td>n=15</td>
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<td>FEVi, %pred</td>
<td>65 ± 20</td>
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<td>FVC , %pred</td>
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<tr>
<td>TLC , %pred</td>
<td>112 ± 17†</td>
<td>98 ± 14</td>
</tr>
<tr>
<td>FRC , %pred</td>
<td>130 ± 28†</td>
<td>99 ± 21</td>
</tr>
<tr>
<td>RV , %pred</td>
<td>147 ± 27†</td>
<td>99 ± 23</td>
</tr>
<tr>
<td>DFEVi , %pred</td>
<td>6 ± 5†</td>
<td>3 ± 3</td>
</tr>
<tr>
<td>DFEVi , %ini</td>
<td>12 ± 12‡</td>
<td>3 ± 3</td>
</tr>
</tbody>
</table>

Definition of abbreviations: FEVi = forced expiratory volume in 1s of the forced vital capacity; FVC = forced vital capacity; FEVi/FVC = forced expiratory volume in 1 s as a percentage of the forced vital capacity; FEF_{50} = forced expiratory flow at 50% of the forced vital capacity; FEF_{25-75} = forced expiratory flow between 25 and 75 % of the forced vital capacity; TLC = total lung capacity; FRC = functional residual capacity; RV = residual volume. DFEVi %pred = change in FEVi as a percentage of the predicted FEVi; DFEVi %ini = change in FEVi as a percentage of the actual pre-bronchodilator FEVi. FEF_{50} and FEF_{25-75}, were significantly lower (*p<0.001) and TLC, FRC, RV were significantly increased (†p<0.05) in obstructed subjects. DFEVi %pred and DFEVi %ini were higher in the obstructed group (‡p<0.05).
### Table 4.4

**AIRWAY DIMENSIONS**

<table>
<thead>
<tr>
<th>Description</th>
<th>Mean ± SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of airways, N</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Basement membrane perimeter (mm)</td>
<td>7.10 ± 2.50</td>
<td>3.1-13.0</td>
</tr>
<tr>
<td>Diameter (mm)</td>
<td>2.30 ± 0.80</td>
<td>1.0 ± 4.0</td>
</tr>
<tr>
<td>Inner wall area, WAi (mm$^2$)</td>
<td>0.77 ± 0.54</td>
<td>0.14-2.30</td>
</tr>
<tr>
<td>Inner wall area as a fraction of total wall area, WAi/WAt</td>
<td>0.23 ± 0.13</td>
<td>0.10-0.60</td>
</tr>
<tr>
<td>Outer wall area, WAo (mm$^2$)</td>
<td>3.20 ± 2.40</td>
<td>0.31-9.50</td>
</tr>
<tr>
<td>Outer wall area as a fraction of total wall area, WAo/WAt</td>
<td>0.77 ± 0.13</td>
<td>0.49-0.90</td>
</tr>
<tr>
<td>Total wall area, WAt, (mm$^2$)</td>
<td>4.00 ± 2.70</td>
<td>0.60-11.0</td>
</tr>
<tr>
<td>Smooth muscle area, WAm, (mm$^2$)</td>
<td>0.15 ± 0.12</td>
<td>0.03-0.40</td>
</tr>
<tr>
<td>Smooth muscle area as a fraction of total wall area, WAm/WAt</td>
<td>0.05 ± 0.04</td>
<td>0.01-0.17</td>
</tr>
<tr>
<td>Wall Area Cartilage, WAcart (mm$^2$)</td>
<td>0.73 ± 0.60</td>
<td>0.03 - 1.9</td>
</tr>
<tr>
<td>Wall area cartilage as a fraction of total wall area, WAcart/WAt</td>
<td>0.15 ± 0.09</td>
<td>0.02 – 0.40</td>
</tr>
</tbody>
</table>

30 airways were studied, from a total of 30 patients (one airway per patient). The diameter was calculated from $P_{bm} + \pi$, based on the assumption that the airway is a circle with perimeter $P_{bm}$.
Table 4.5
AIRWAY DIMENSIONS AS A FUNCTION OF AIRWAY SIZE AND COVARIABLES

<table>
<thead>
<tr>
<th>Equation</th>
<th>p Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\sqrt{\text{WAm}} = 0.048(Pbm) + 0.25 - 0.0030(\text{FEV}_1/\text{FVC}))</td>
<td>0.014</td>
</tr>
<tr>
<td>(\sqrt{\text{WAm}} = 0.044(Pbm) + 0.18 - 0.0020(\text{FEV}_1))</td>
<td>0.015</td>
</tr>
<tr>
<td>(\sqrt{\text{WAm}} = 0.046(Pbm) + 0.07 - 0.0008(\text{FEF}_{50}))</td>
<td>NS</td>
</tr>
<tr>
<td>(\sqrt{\text{WAm}} = 0.046(Pbm) + 0.11 - 0.0014(\text{FEF}_{25-75}))</td>
<td>0.005</td>
</tr>
<tr>
<td>(\sqrt{\text{WAm}} = 0.045(Pbm) - 0.002 + 0.0080(\text{DFEV}_1%\text{pred}))</td>
<td>0.021</td>
</tr>
<tr>
<td>(\sqrt{\text{WAm}} = 0.046(Pbm) - 0.002 + 0.0040(\text{DFEV}_1%\text{ini}))</td>
<td>0.016</td>
</tr>
<tr>
<td>(\sqrt{\text{WAI}} = 0.100(Pbm) + 0.27 - 0.0021(\text{FEV}_1/\text{FVC}))</td>
<td>NS</td>
</tr>
<tr>
<td>(\sqrt{\text{WAI}} = 0.097(Pbm) + 0.25 - 0.0014(\text{FEV}_1))</td>
<td>NS</td>
</tr>
<tr>
<td>(\sqrt{\text{WAI}} = 0.098(Pbm) + 0.16 - 0.0005(\text{FEF}_{50}))</td>
<td>NS</td>
</tr>
<tr>
<td>(\sqrt{\text{WAI}} = 0.098(Pbm) + 0.20 - 0.0010(\text{FEF}_{25-75}))</td>
<td>NS</td>
</tr>
<tr>
<td>(\sqrt{\text{WAI}} = 0.098(Pbm) + 0.07 + 0.0140(\text{DFEV}_1%\text{pred}))</td>
<td>NS</td>
</tr>
<tr>
<td>(\sqrt{\text{WAI}} = 0.099(Pbm) + 0.09 + 0.0060(\text{DFEV}_1%\text{ini}))</td>
<td>NS</td>
</tr>
<tr>
<td>(\sqrt{\text{WAo}} = 0.21(Pbm) + 0.75 - 0.0080(\text{FEV}_1/\text{FVC}))</td>
<td>NS</td>
</tr>
<tr>
<td>(\sqrt{\text{WAo}} = 0.21(Pbm) + 0.05 - 0.0020(\text{FEV}_1))</td>
<td>NS</td>
</tr>
<tr>
<td>(\sqrt{\text{WAo}} = 0.21(Pbm) + 0.18 - 0.0010(\text{FEF}_{50}))</td>
<td>NS</td>
</tr>
<tr>
<td>(\sqrt{\text{WAo}} = 0.21(Pbm) + 0.14 - 0.0010(\text{FEF}_{25-75}))</td>
<td>NS</td>
</tr>
<tr>
<td>(\sqrt{\text{WAi}} = 0.21(Pbm) + 0.07 + 0.0360(\text{DFEV}_1%\text{pred}))</td>
<td>NS</td>
</tr>
<tr>
<td>(\sqrt{\text{WAi}} = 0.21(Pbm) + 0.10 + 0.0160(\text{DFEV}_1%\text{ini}))</td>
<td>NS</td>
</tr>
</tbody>
</table>

Definition of abbreviations:
- \(\sqrt{\text{WAm}}\)=area of smooth muscle (mm\(^2\)); \(\sqrt{\text{WAI}}\)=inner wall area (mm\(^2\)); \(\sqrt{\text{WAo}}\)=outer wall area (mm\(^2\)); Pbm=basement membrane perimeter (mm), is used as an indicator of airway size.
- FEV\(_1\)=force expiratory volume in 1 s of the forced vital capacity; FEV\(_1)/\text{FVC}=\text{forced expiratory volume in 1 s as a percentage of the forced vital capacity}; \text{FEF}_{50}=\text{forced expiratory flow at 50}\%\text{ of the forced vital capacity}; \text{FEF}_{25-75}=\text{forced expiratory flow between 25 and 75}\%\text{ of the forced vital capacity}
- *p value refers to the significance effect of the lung function covariable.
Table 4.6

COMPARISON OF PREDICTION EQUATIONS FOR AIRWAY DIMENSIONS

<table>
<thead>
<tr>
<th></th>
<th>√WAm vs Pbm</th>
<th>√WAi vs Pbm</th>
<th>√WAo vs Pbm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Slope (intercept)</td>
<td>Slope (intercept)</td>
<td>Slope (intercept)</td>
</tr>
<tr>
<td>Opazo Saez et al.*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (FEV₁/FVC=80%)</td>
<td>0.048 (0.010)</td>
<td>0.100 (0.10)</td>
<td>0.210 (0.11)</td>
</tr>
<tr>
<td>COPD (FEV₁/FVC=60%)</td>
<td>0.048 (0.070)</td>
<td>0.100 (0.14)</td>
<td>0.210 (0.27)</td>
</tr>
<tr>
<td>Tiddens et al.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (FEV₁/FVC=80%)</td>
<td>0.020 (0.230)</td>
<td>0.069 (0.194)</td>
<td>0.240 (0.160)</td>
</tr>
<tr>
<td>COPD (FEV₁/FVC=40%)</td>
<td>0.020 (0.230)</td>
<td>0.069 (0.384)</td>
<td>0.240 (0.160)</td>
</tr>
<tr>
<td>Bosken el al.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (FEV₁/FVC&gt;75%)</td>
<td>0.040 (0.050)</td>
<td>0.096 (0.107)</td>
<td>0.093 (0.099)</td>
</tr>
<tr>
<td>COPD (FEV₁/FVC&lt;65%)</td>
<td>0.045 (0.056)</td>
<td>0.101 (0.142)</td>
<td>0.099 (0.148)</td>
</tr>
<tr>
<td>Kuwano et al.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (FEV₁/FVC=80%)</td>
<td>0.020 (0.056)</td>
<td>0.057 (0.083)</td>
<td>0.079 (0.116)</td>
</tr>
<tr>
<td>COPD (FEV₁/FVC=65%)</td>
<td>0.035 (0.016)</td>
<td>0.065 (0.057)</td>
<td>0.086 (0.084)</td>
</tr>
<tr>
<td>Asthma (FEV₁/FVC=?</td>
<td>0.063 (0.001)</td>
<td>0.119 (0.008)</td>
<td>0.137 (0.085)</td>
</tr>
</tbody>
</table>

Definitions of abbreviations:
√WAm= smooth muscle area (mm²); √WAi=inner wall area (mm²); √WAo=outer wall area (mm²); Pbm=basement membrane perimeter (mm). FEV₁/FVC=forced expiratory volume in 1 s as a percentage of the forced vital capacity.

*Opazo Saez et al. is the present study.
Figure 4.1. Airway dimensions.
Definitions of abbreviations:
Pi = Internal perimeter
Pbm = Basement membrane perimeter; Abm = Basement membrane area
Pmo = outer muscle perimeter; Amo = Internal area
Po = Outer perimeter; Ao = Outer area
WAm = Smooth muscle area; WAcart = cartilage area
WAi = (Amo - Abm) Inner wall area
WAo= (Ao - Amo) Outer wall area
WAt = (Ao- Abm) Total wall area
Figure 4.2. Frequency distribution of peripheral airway size (n=30), from 30 patients studied, classified according to the basement membrane perimeter.
Figure 4.3. A. Airway smooth muscle (WAm) as a percentage of the total wall area (WAt) versus basement membrane perimeter (Pbm). B. Inner wall area (WAi) as a percentage of WAt versus Pbm. C. Outer wall area (WAo) as a percentage of WAt versus Pbm.
Figure 4.4 Airway dimensions versus airway size, and lung function as covariables. A. Airway smooth muscle area (WAm) vs basement membrane perimeter (Pbm). The dashed regression line represents patients with an FEV₁/FVC = 80%; the solid line represents an FEV₁/FVC = 60%. B. Inner wall area (WAi). C. Outer wall area (WAo). 30 airways from 30 patients were studied. Note the square root transformed y-axis.
REFERENCES.


26 Heard BE, S Hossain. Hyperplasia of bronchial muscle in asthma. J Pathol 110:319-331, 1973


35 Weir DC, PS Burge. Effects of high dose inhaled beclomethasone dipropionate, 750 μg and 1500 μg twice daily, and 40 mg per day oral prednisolone on lung function, symptoms, and bronchial hyperresponsiveness inpatients with non-asthmatic chronic airflow obstruction. Thorax. 48:309-316, 1993
Chapter 5

Peripheral airway smooth muscle contractility and airflow obstruction.

5.1 INTRODUCTION.

Increased airway narrowing in response to non-specific stimuli is a characteristic feature of human obstructive airways diseases such as asthma and chronic obstructive pulmonary disease (COPD). The pathophysiological changes leading to this hyperresponsiveness and airflow obstruction are still unknown. Several mechanisms have been postulated to explain this abnormality, including alterations in the neuro-humoral control of airway smooth muscle (ASM), increased sensitivity of ASM, increased mucosal permeability, increased mucosal secretions, and mechanical factors related to remodelling of the airways. In spite of the potential contributions of some of these mechanisms, the reversibility and rapid onset of airway narrowing indicate that airway smooth muscle contraction is central to the pathophysiology underlying exaggerated airway narrowing.

To determine whether abnormalities in ASM play a role in obstructive airways disease, a number of studies have been carried out to examine in vitro contractility of isolated human airway smooth muscle from patients who have varying degrees of airflow obstruction and airway responsiveness. While in most
studies a relationship between in vitro and in vivo responsiveness has not been shown,\textsuperscript{12, 13, 14, 15, 16, 17, 18} in a few increased contractility has been found in chronic obstructive pulmonary disease (COPD),\textsuperscript{19} and asthma.\textsuperscript{20, 21, 22, 23} These inconsistent findings may be attributable to several problems in study design, such as the use of a small sample size, sampling from different parts of the tracheobronchial tree, lack of normalization of force, and absence of measurements of isotonic shortening.

The aims of this study were to characterize the passive and active mechanical properties of peripheral airways from the lungs of normal subjects and patients who had COPD and asthma, and to relate in vitro contractility to pulmonary function measured before surgery. In the present study both isometric force generation and isotonic shortening were measured in peripheral airway rings (2.5±0.8 SD mm) from patients who had varying degrees of airflow obstruction. The hypothesis is that altered ASM contractility and airway smooth muscle mass contribute to the pathogenesis of obstructive airways disease.
5.2 METHODS.

5.2.1 Study Population.

Lung tissue was obtained from 30 patients who had a surgical resection of a lung or lobe for a solitary peripheral lung lesion at St. Paul's Hospital (Vancouver, BC, Canada). A patient was not included in this study if a lung lesion obstructed a segmental or larger bronchus or if pneumonitis was diagnosed. Subjects were studied with the approval of both the University of British Columbia and St. Paul's Hospital Ethics Committees and after obtaining informed consent from the subjects. Patients were characterized on the basis of a detailed questionnaire concerning respiratory symptoms, smoking history, and allergies before surgery.

Pulmonary function tests were done before surgery and included maximal expiratory flow volume curves. Twenty-eight of the patients had a diagnosis of bronchogenic carcinoma, while the remaining 2 had a diagnosis of carcinoid tumor. Twenty-one of the patients had been life-long smokers, 4 were ex-smokers (had not smoked for 7, 15, 40 and 50 years), and 5 were non-smokers. Only four patients had a past history of allergies.

Pre-operative medications included sedatives (lorazepam, Ativan), laxatives (docusate sodium), heparin, antibiotics (cefazolin sodium), analgesics (acetaminophen), and in some cases β-agonists (albuterol [Ventolin], terbutaline [Bricaryl]). Preliminary studies showed no differences in in vitro contractility as a result of the various medications received while in hospital. Asthma and COPD
were defined on the basis of clinical criteria using the definition of the American Thoracic Society.\textsuperscript{24} Table 5.1 summarizes the data for the patients in this study.

5.2.2 Lung Function Studies.

Lung function studies were done within the week before surgery. Methods have been previously described.\textsuperscript{25} Briefly, the subjects were seated in a volume displacement, pressure-compensated body plethysmograph, with a nose clip in place. Volume was measured with a Krogh water-seated spirometer coupled to a linear displacement transducer (type 300 HR; Shaevitz, Pennsauken, NJ). Flow was measured with a Fleisch No. 3 pneumotachometer coupled to a Sanborn 270 differential pressure transducer (MP 45-28, ± 3.5 cm H\textsubscript{2}O, Validyne, Northridge, CA). Functional residual capacity (FRC) was determined by the Boyle's law technique, and total lung capacity (TLC) was calculated by adding the inspiratory capacity to FRC.

Maximal expiratory flow-volume curves were used to calculate the forced expiratory volume in 1s of the forced vital capacity (FEV\textsubscript{1}), the forced vital capacity (FVC), the forced expiratory flow at 50\% of FVC (FEF\textsubscript{50}) and the forced expiratory flow between 25-75\% of FVC (FEF\textsubscript{25-75}). The FEV\textsubscript{1} was expressed as percentage predicted using the equations of Crapo and co-workers\textsuperscript{26} The ratio of FEV\textsubscript{1} to FVC (FEV\textsubscript{1}/FVC) was also calculated and was used as an indicator of airflow obstruction. A value of 70\% is approximately the lower limit of the normal range as defined by 95 \% confidence intervals for FEV\textsubscript{1}/FVC,\textsuperscript{26} and was therefore used to categorize the subjects in this study. At least two expiratory
efforts were carried out and were accepted when the two values for each variable measured were within 5% of each other.

5.2.3 In vitro studies.

Specimens were selected from a macroscopically tumor-free part of the specimen, and immediately transferred to ice-cold sterile Krebs-Henseleit buffer (in mM: 118 NaCl, 4.7 KCl, 2.5 CaCl₂H₂O, 1.2 MgSO₄7H₂O, 1.2 kH₂PO₄, 25 NaHCO₃, and 11.1 glucose). Segments (30 mm long) from subsegmental bronchi were dissected free of blood vessels and surrounding parenchyma (figure 5.1). Segments were obtained from each subject and one airway ring was randomly selected for studies in physiology. Tissues were stored overnight in a large volume of cooled (4°C) Krebs buffer that was previously aerated with carbogen (95% O₂-5% CO₂). This procedure has been shown to wash out drug residues and any substances liberated during the dissection.²⁷ ²⁸ ²⁹ The pH of the solution ranged between 7.2-7.3. Viability of responses after storage was tested on a number of the airways by eliciting a near maximal contraction with acetylcholine (10⁻⁵ M) the day of thoracotomy, and on the next day at the beginning and at the end of the experiment. The responses to cholinergic agonist remained unchanged for up to 48 hrs, and were reproducible throughout the experiment (data not shown) which is consistent with previous findings.²⁷

In vitro studies were carried out the day after thoracotomy to characterize the length-tension curves for each airway ring as previously described.³⁰ Passive tension, isotonic shortening, and isometric force generation were measured in
vitro by a servo-controlled myograph (figure 5.2). The motor arm of this apparatus could be fixed to achieve an isometric contraction or maintain constant force allowing an isotonic contraction (G325D pen motor [General Scanning, Watertown, MA]; Kulite BG-10 force transducer [Durham Instruments, Pickering, Ontario, Canada]). The frequency response was 100 Hz and the resolution was 0.02 g for force and 0.01 mm for length measurements.

5.2.4 Protocol.

Each airway ring was placed in an organ bath that contained fresh Krebs-Henseleit buffer that was replaced continuously, and bubbled with 95%O₂-5%CO₂ at 37°C. The airway was mounted between a force transducer and a motor arm and allowed to equilibrate at zero resting tension for 1 h. The weight of the tissue was incorporated into a balancing current in the electronic circuit in order to achieve zero tension. The initial length of the airway ring was measured with an optical micrometer to an accuracy of 0.01 mm. All subsequent changes in length were referenced to this value so that changes in length could be calculated.

Each airway ring was slowly stretched three times by loads that were comparable to optimal length determined during preliminary experiments, and then was allowed to equilibrate in the organ bath with no load for 1 h. After equilibration, passive, isometric, and isotonic length force relationships were obtained. Contractions were elicited by acetylcholine (10⁻⁵M). Measurement of isometric force was first performed at lengths below the level at which passive
tension was first detected. A small preload was then applied to the ring to stretch it, and the length was recorded once it stabilized. An isometric contraction with this preload was performed by maintaining the airway length constant while stimulating and measuring the change in force. Once the force returned to baseline (same preload), an isotonic contraction was elicited and the length change measured.

Complete isometric and isotonic length-force curves were obtained by serially increasing the smooth muscle length to 10-20% more than the length at which maximal force occurred. For each length, an isometric contraction was followed by an isotonic contraction. From the isometric force-length curve, Lmax was determined as the length at which maximal active isometric force (Fmax) occurred. In the rare instances where the maximal force at two lengths was similar, the shorter of the two lengths was chosen. Lmax was used to calculate the diameter at Lmax (Dmax) by assuming that, the airway perimeter was twice the measured length at Fmax [diameter=2xlength/π].

5.2.5 Morphological Studies.

5.2.5 (a) Tissue preparation and airway sampling.

After completion of the isometric and isotonic length-force measurements, each airway ring was removed from the organ bath and fixed in a solution of 2.5% (vol/vol) glutaraldehyde (Polysciences, Warrington, PA) in 0.1 M sodium cacodylate buffer (Polysciences) at the tension that stretched each ring to Lmax. The tissue specimens were dehydrated through a series of graded ethanols and
embedded in glycol methacrylate plastic (JB-4, Polysciences). The maximal measured area shrinkage factor has been shown to be less than 4% with this method.  

The airway rings were oriented within the JB-4 blocks such that cross-sectional profiles of the smooth muscle fibers could be measured within the axial sections of the airway wall. JB-4 blocks were sectioned at a thickness of 2 μm on a microtome (JB-4 type Sorvall microtome; Ivan Sorvall, Newtown, CT), using glass knives prepared on an LKB knife maker (LKB knife maker type 7801B; LKB-Produkter AB, Stockholm, Sweden). The entire specimen was sectioned but only every 50th section (approximately every 100 μm) was mounted on a glass slide and stained with an aqueous solution of 1% (wt/vol) toluidine blue O (C.I. 52040; Sigma, St. Louis, MO) and 1% (wt/vol) sodium borate at a pH 8.7. Three slides were selected by a systematic random method as described by Gundersen and Jensen.  

5.2.5 (b) Stereologic Methods.  

Cross-sectional areas of the smooth muscle and the total amount of tissue in the airway wall were measured at a magnification of x200 (x20 objective lens), using a Nikon microscope with a camera lucida (Nikon Labophot; Nikon Canada, Mississauga, ON, Canada), a digitizing board (Summasketch II model MM II 1201; Summagraphics, Seymour, CT), and the Bioquant BQ system software (R&M Biometrics Inc., Nashville, TN) on an IBM compatible personal computer.
Figure 5.3 shows a tissue section that was digitized by tracing around the muscle bundles. The total tissue area was measured by tracing around the outer border of the entire tissue. The intra- and inter-observer coefficient of variation was less than 5% for each variable measured. Measurements were made on three slides and a mean value was obtained for each variable. The fraction of the airway wall made up of muscle was calculated by dividing the smooth muscle area by the total area of tissue in the airway wall (WA).

5.2.6 Data Analysis.

Data have been expressed as means ± SD unless otherwise stated. Force and length measurements were standardized respectively and expressed as %Fmax and %Lmax. The muscle stress was calculated by dividing force values by the cross-sectional areas of muscle and expressed as mN/mm². This method of force normalization was recommended by Stephens and co-workers for comparison of muscle strips from the same or different species. Although a more precise denominator would be to measure the total number of myosin filaments parallel to the force vector, by densitometry of myosin heavy chain bands or by quantitative immunohistochemistry of transverse sections, this method was not applied as it did not aid the specific aims of this study. The area of smooth muscle was normalized by the airway diameter measured at Lmax (Dmax), and by the total wall area (WA).

The amount of isotonic shortening was analyzed in three ways: i) by dividing the shortening by the length from which the preparation started.
shortening, and expressing it as per cent initial length, %Li; ii) by dividing shortening by Lmax, and expressing it as %Lmax; iii) by calculating shortening at the operating length of the muscle at FRC (see the next section).

Force, stress and shortening were related to the FEV₁, and to FEV₁/FVC values by Pearson correlations. Differences between obstructed and nonobstructed groups in force, stress, shortening, smooth muscle (ASM, ASM/Dmax, ASM/WA), FEV₁%pред, FEV₁/FVC%, FEF50%pред, FEF25-75%pред, FRC %пред and RV %пред were analyzed using unpaired t-tests. Bonferroni corrections were made for multiple comparisons. P values were considered to be statistically significant when less than 0.05.

5.2.7 Transpulmonary pressure at Lmax (Ptp).

To relate the \textit{in vitro} mechanics to the operating length tension curves of peripheral airways \textit{in vivo}, the "transpulmonary" pressures were calculated for each length of the \textit{in vitro} length tension curves. The transpulmonary pressure at Lmax, the length at FRC, passive tension at FRC, and shortening at FRC were derived. FRC was assumed to occur at a transpulmonary pressure of 5 cm H₂O.

The basic assumption made in estimating these variables is that in the absence of smooth muscle contraction the interstitial pressure surrounding the airways \textit{in vivo} is equal to the pleural pressure. The transpulmonary pressure at Lmax was calculated using the LaPlace equation, \( P = T/r \), where \( T \) is the wall tension obtained from the passive tension at Lmax in mN per mm of airway width, and \( r \) is the airway radius at Lmax, calculated from the measured airway.
perimeter. The airway wall thickness was $1/10^{th}$ to $1/20^{th}$ of the airway diameter. The LaPlace equation is valid only for thin-walled shells and it is therefore valid for the airways in this study.
5.3 RESULTS.

5.3.1 Lung Function Studies.

Table 5.2 shows the mean lung function values for the patients in this study. Fifteen patients had an FEV₁/FVC < 70% and were considered to have airflow obstruction. The functional residual capacity, residual volume, and total lung capacity were increased (p<0.05), while the FEF₅₀ and FEF₂₅₋₇₅ were significantly lower (p<0.001) in the obstructed patients.

5.3.2 In vitro Studies.

Figure 5.4 shows the cumulative frequency distribution of the diameter at Lmax, Dmax, for the airways studied. Thirty airways with a mean diameter of 2.5 mm at Lmax were used for the in vitro studies. 15 airway rings were obtained from obstructed patients, and 15 from nonobstructed patients. Both groups had similar sized airways, with most airways having a diameter between 2 and 3 mm (at Lmax). There was no difference in Dmax between the two patient groups.

The passive, isometric and isotonic length-force relationships from one airway ring from the nonobstructed group are shown in figure 5.5. Total force, as percentage of the maximal active isometric force, Fmax, is plotted as a function of length, expressed as percentage of Lmax. The active isometric force was obtained by subtracting the passive tension from the total force. The active isometric force increased until a peak occurred at Lmax, beyond which increments in length produced no further increases in active force. The passive
tension increased as the ring was stretched and was substantial at Lmax. Peripheral airway smooth muscle was able to develop force at lengths as low as 35% Lmax (data not shown). Preloaded isotonic shortening has also been plotted and is indicated at Lmax by an arrow. On average, maximal shortening occurred at ~80% Lmax, and was negligible near 40% Lmax.

The mean values for maximal isometric force, passive tension at Lmax, maximal isotonic shortening, and length for maximal shortening are shown in table 5.3 for obstructed (OB, n=15) and nonobstructed (NOB, n=15) patients. Maximal isometric force and stress were significantly higher in the obstructed group (p<0.05), but there was no difference in the passive tension between obstructed and nonobstructed patients. In both patient groups there was large variability in the passive tension at Lmax, ranging between values of 30 to near 400% Fmax.

In addition, there was no difference in the maximal isotonic shortening between the two patient groups, whether it was normalized by the initial length from which the airway preparation started shortening, Li, (29.7±15.1% Li, OB; 22.4±12.7% Li, NOB), or by the length at maximal isometric force, Lmax (23.5±10.6% Lmax, OB; 18.7±10.3% Lmax, NOB). The length at which maximal shortening occurred was below Lmax and was similar between the two patient groups. When normalized by Lmax, the values for maximal shortening occurred at a length about 10% higher than when normalized by Li.

There was a significant correlation between force and shortening (%Li) (r=0.389, p<0.05), force and shortening (%Lmax) (r=0.442, p<0.05), stress and
shortening (%Li) ($r=0.488$, $p<0.02$), stress and shortening (%Lmax) ($r=0.538$, $p<0.01$) (figures 5.6.A-D).

5.3.3 Correlations between *in vivo* and *in vitro* function.

Figures 5.7.A-D show the relationships between force and stress, and two measures of pulmonary function, the FEV$_1$ (%pred) and FEV$_1$/FVC (%). There was a negative correlation between force and the FEV$_1$ (%pred) ($r=-0.579$, $p<0.004$), and force and the FEV$_1$/FVC (%) ($r=-0.720$, $p<0.003$). Stress was also negatively correlated with the FEV$_1$/FVC (%) ($r=-0.611$, $p<0.002$), but was not correlated with the FEV$_1$ (%pred). Shortening was not correlated with either measure of pulmonary function (figures 5.8A-D).

5.3.4 Stereological Analysis.

The mean values obtained from morphometry are shown in table 5.4. Results for the amount of smooth muscle, the normalized smooth muscle (ASM/Dmax), and the total airway wall area are shown for airways from obstructed and nonobstructed patients. Both the amount of ASM and ASM/Dmax were significantly increased in the obstructed group ($p<0.05$ and $p<0.01$ respectively). Although there was no difference in the total airway wall cross-sectional area between the two patient groups, the fraction of the total airway wall made up of muscle was significantly higher ($p<0.02$) in airways from obstructed patients.

Figure 5.9 shows photomicrographs of transverse sections of the airway
rings from one nonobstructed and one obstructed patient at three different magnifications (x2, x6.3, and x20 objective lens). A thicker band of muscle can be seen in the obstructed case.

5.3.5 Transpulmonary pressure at Lmax (Ptp).

Table 5.5 shows the values calculated for Ptp at Lmax. It also shows the values for the length at an FRC value assumed to be 5 cmH$_2$O, the passive tension at FRC, and the shortening at FRC. Ptp at Lmax was significantly higher in the obstructed group (p<0.05), while airway length and passive tension at FRC were significantly reduced in the obstructed group (p<0.05). There was no difference in the amount of isometric force or isotonic shortening at FRC between the patient groups. Figure 5.10 shows significant correlations between Ptp at Lmax and FEV$_1$/FVC (%) ($r$=-0.390, p<0.05), and between passive tension at FRC and FEV$_1$/FVC (%) ($r$=0.412, p<0.02).
5.4 DISCUSSION.

5.4.1 Lung Function.

The purpose of this study was to characterize the mechanical properties of peripheral airways, and to relate these properties to pulmonary function in patients who had variable airflow obstruction, predominantly secondary to chronic cigarette smoking. The patients were categorized into an obstructed and nonobstructed group based on the ratio of FEV₁ to FVC. An FEV₁/FVC of less than 70% was used to define airflow obstruction. Measurements of FEV₁/FVC (%) and FEV₁ (% predicted) were also used as continuous variables to examine their relationships to airway smooth muscle force, stress and shortening. As expected, patients categorized as being obstructed on the basis of FEV₁/FVC also showed hyperinflation and gas trapping with significant increases in FRC and residual volume. Only one of the patients had any clinical features suggestive of asthma.

5.4.2 In vitro function and correlations with in vivo function.

Maximal isometric force was significantly higher in obstructed patients. In addition, when the force was normalized by the amount of smooth muscle, the values of stress developed by the smooth muscle were still significantly higher in the obstructed individuals, indicating that the increase in force is, at least in part, due to increased ASM contractility and not due simply to increased ASM mass. Since a supra-maximal concentration of acetylcholine was used as the agonist, a
change in sensitivity cannot explain these results. An increase in muscle stress could be due to a fundamental change in the contractile apparatus of the muscle or to diminished activity of a mechanism(s) that normally attenuates muscle stress during maximal stimulation.

A fundamental change in the contractile properties could be caused by intracellular reorganization of the contractile apparatus resulting in an increase in the number of myosin filaments or in the number of contractile units in parallel. Changes in the molecular mechanism underlying altered contractility could occur at the level of the enzymes involved in smooth muscle contraction. An increase in the amount and activity of myosin light chain kinase, and in the activity of myosin ATPase, have been found in sensitized canine airway smooth muscle but only in association with an increase in the velocity and capacity of shortening, and not force generation. Alternatively, there could be more myosin and actin per unit cross-sectional area of muscle or an alteration in the expression pattern of myosin isoforms.

Increased stress could also be due to loss, in the tissue of obstructed patients, of an inhibitory factor or factors such as relaxant prostanoids or acetylcholinesterase normally produced by airway epithelial cells. Altered contractility could also result from the loss of radial constraint associated with alterations in the extracellular matrix surrounding muscle cells. The design of this study does not allow a conclusion as to which of these mechanisms led to increased ASM stress in the obstructed group. Possible alterations in the biochemical properties of ASM will be examined in chapter 7.
In addition to studying airflow obstruction as a categorical variable the relationships between ASM force, stress, and shortening were examined in relation to the severity of pulmonary dysfunction. The results of these analyses support the categorical analysis in that maximal isometric force was significantly related to both FEVi/FVC (%) and FEV1 (% pred) and FEVi/FVC (%) was significantly related to maximal isometric stress.

The observation that both FEV1/FVC (%) and FEV1 (% pred) are significantly related to maximal force but that the significance of the relationship with FEV1 (% pred) is lost when maximal stress is used as the dependent variable suggests that, in part, the relationship between muscle force and airflow obstruction is due to a significant increase in the amount of muscle in the airways of obstructed subjects. This finding is consistent with previous reports of increased smooth muscle mass in both asthma39 40 41 and COPD.42 42 However, the fact that a significant relationship still remains between stress and FEV1/FVC (%) supports the contention that there is also a change in the ASM contractile function in patients who have airflow obstruction.

The relationships between force and FEV1/FVC (%) and force and FEV1 (% pred) appear to be heavily influenced by a single individual who had the most severe airflow obstruction and an airway in which the muscle made considerable isometric force. The large amount of force generated by the airway from this individual is consistent with the fact that this airway had approximately twice the amount of smooth muscle as the mean of the rest of the group. Even if this subject is removed from the study, all significant correlations remain.
Nonetheless, it should be noted that this was the individual with the most severe obstructive lung disease in the obstructed group and was the only patient with an FEV₁/FVC (%) less than 50% included in this study. This censoring in the data to patients with relatively mild chronic airflow obstruction is related to the fact that patients with severe airflow obstruction are not selected for surgery.

This is the first study in which a significant relationship has been demonstrated between ASM force and stress and lung function. In previous studies relations between in vitro ASM function and in vivo lung function or measures of airway responsiveness have been found inconsistently and the varied results may have been related to deficiencies in the design of the studies.

In several of the positive studies the responses from only one patient with asthma have been compared with a group of control patients. With the exception of two studies, the data reported to date has only included measurements of isometric force, and not isotonic shortening, which may be a more appropriate in vitro indicator of the potential for airway narrowing in vivo. In addition, there are few studies in which the measurements of force have been converted to stress by correction for muscle cross-sectional area. Only Ishida and co-workers and Bramley and colleagues measured both the force and smooth muscle cross sectional area and calculated the stress generated by the muscle. In the study by Ishida and co-workers only main stem airways from 10 subjects were studied and comparisons between obstructed and nonobstructed individuals were not done. In this study both the isometric force and isotonic shortening were measured, and the force was normalized by the measured
amount of smooth muscle. We did not, however, make measurements of velocity of shortening, which may be also be altered in chronically inflamed airways.\textsuperscript{45}

Maximal isotonic shortening was not significantly different between obstructed and nonobstructed subjects. This was surprising in light of the observation that there were positive correlations between ASM force and stress and the amount of shortening. An increase in force without a significant increase in shortening must be related to differences in the loads acting on the smooth muscle. Although not significant, the passive tension at Lmax (expressed as \%Fmax), tended to be lower in the obstructed individuals, and this ~20% reduction in passive preload could have influenced the subsequent isotonic shortening. Maximal unloaded shortening was not measured; the preload required to stretch the muscle to Lmax remained constant during shortening and constituted an elastic, or auxotonic load.

An additional load on smooth muscle during shortening has been described by Meiss,\textsuperscript{38} who showed that if canine smooth muscle preparations, which normally shorten ~75%, are constrained by nonextensible silastic rings shortening is reduced. The connective tissue content of human peripheral airways is much higher than the relatively pure smooth muscle of canine trachealis and it is possible that this connective tissue radially constrains the ASM and reduces its ability to shorten.

The high passive tension required to stretch the muscle to Lmax is a reflection of this high connective tissue content and the resultant radial constraint
could explain the relatively lesser shortening in the human tissue as well as the failure to find a significant difference in maximal shortening despite differences in force and stress between obstructed and nonobstructed individuals. It is also possible that the small number of patients may have contributed to the large variability in the signal to noise ratio of the measured shortening.

5.4.3 Stereology of smooth muscle.

To calculate smooth muscle stress the cross-sectional area of the smooth muscle was obtained by morphometry. Thomson and co-workers\(^4^6\) showed that with morphometry some of what is usually considered smooth muscle area is really interstitial connective tissue. They found that this overestimation of muscle area was greatest when thick (>5 \(\mu\)m) sections of longitudinally arranged smooth muscle bundles were examined. Although thin sections (2 \(\mu\)m) were used in this study and the morphometric analysis was performed on cross sections of muscle to minimize this source of error, higher magnification would likely have allowed more precise quantification of muscle and thus larger values for stress. There is no reason to believe that this artifact would have been different in obstructed versus nonobstructed individuals.

5.4.4 Transpulmonary pressure at Lmax.

The estimated transpulmonary pressure at Lmax was higher in the airways from obstructed individuals, while the length of the muscle preparation at "FRC" was lower. The basic assumption made in estimating the transmural
pressure at Lmax and the length at FRC is that in the absence of smooth muscle contraction *in vivo* the peribronchial interstitial pressure is equal to pleural pressure (i.e. the parenchymal attachments on the airway do not exert additional stretch in the absence of muscle shortening). This assumption has been validated by Sasaki and colleagues.\(^47\) The shorter length of the smooth muscle at FRC suggests that the airways of the obstructed group are stiffer and that more passive tension is required to stretch these airways to the length at which maximal isometric forces are developed.

In summary, the peripheral airway smooth muscle of patients who have COPD showed increased contractility in addition to increased volume. Airway remodeling occurs in the inflamed airways of patients who have COPD and asthma and is associated with airway hyperresponsiveness. In COPD the emphasis has been placed on loss of lung recoil and fibrosis of the small airways. These results suggest that altered peripheral airway smooth muscle function may be an important determinant of abnormal airway function in this group of patients.
Table 5.1
STUDY POPULATION CHARACTERISTICS

<table>
<thead>
<tr>
<th>PATIENT CHARACTERISTICS*</th>
<th>OBSTRUCTED n=15</th>
<th>NONOBSTRUCTED n=15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yrs</td>
<td>62±8</td>
<td>62±12</td>
</tr>
<tr>
<td>Sex, female:male</td>
<td>6:9</td>
<td>6:9</td>
</tr>
<tr>
<td>Pack years*</td>
<td>54±23</td>
<td>30±15</td>
</tr>
<tr>
<td>Current smokers, n</td>
<td>13</td>
<td>8</td>
</tr>
<tr>
<td>Ex-smokers, n</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Non-smokers, n</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Lung resected</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right Lung, n</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>Left Lung, n</td>
<td>6</td>
<td>9</td>
</tr>
</tbody>
</table>

* Pack years = the number of years smoking one pack of cigarettes per day. Age and pack years are expressed as means ± standard deviation. n=number
**Table 5.2**

LUNG FUNCTION CHARACTERISTICS*  

<table>
<thead>
<tr>
<th></th>
<th>OBSTRUCTED</th>
<th></th>
<th>NONOBSTRUCTED</th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MEAN ± SD</td>
<td>n=15</td>
<td>MEAN ± SD</td>
<td>n=15</td>
</tr>
<tr>
<td>FEV1, %pred</td>
<td>65 ± 20</td>
<td>90 ± 10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FVC, %pred</td>
<td>84 ± 22</td>
<td>93 ± 12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FEV1/FVC, %</td>
<td>60 ± 8</td>
<td>77 ± 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FEF50, %pred</td>
<td>30 ± 12*</td>
<td>80 ± 36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FEF25-75, %pred</td>
<td>32 ± 12*</td>
<td>81 ± 19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLC, %pred</td>
<td>112 ± 17†</td>
<td>98 ± 14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FRC, %pred</td>
<td>130 ± 28†</td>
<td>99 ± 21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RV, %pred</td>
<td>147 ± 27†</td>
<td>99 ± 23</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Definition of abbreviations: FEV1 = forced expiratory volume in 1s of the forced vital capacity; FVC = forced vital capacity; FEV1/FVC = forced expiratory volume in 1 s as a percentage of the forced vital capacity; FEF50 = forced expiratory flow at 50% of the forced vital capacity; FEF25-75 = forced expiratory flow between 25 and 75% of the forced vital capacity; TLC = total lung capacity; FRC = functional residual capacity; RV = residual volume. FEF50 and FEF25-75 were significantly lower (*p<0.001) and TLC, FRC, RV were significantly increased (†p<0.05) in obstructed subjects.
<table>
<thead>
<tr>
<th></th>
<th>OBSTRUCTED n=15</th>
<th>NONOBSTRUCTED n=15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximal isometric force, Fmax, g</td>
<td>0.87 ± 0.80 *</td>
<td>0.42 ± 0.18</td>
</tr>
<tr>
<td>Maximal isometric stress, mN/mm²</td>
<td>76 ± 47 †</td>
<td>51 ± 21</td>
</tr>
<tr>
<td>Passive tension at Lmax, g</td>
<td>0.61 ± 0.26</td>
<td>0.50 ± 0.44</td>
</tr>
<tr>
<td>Passive tension at Lmax, %Fmax</td>
<td>109.8 ± 88.8</td>
<td>133.8 ± 105.5</td>
</tr>
<tr>
<td>Maximal isotonic shortening, %Li (in mm)</td>
<td>29.7 ± 15.1</td>
<td>22.4 ± 12.7</td>
</tr>
<tr>
<td>Maximal isotonic shortening, %Lmax (in mm)</td>
<td>23.5 ± 10.6</td>
<td>18.7 ± 10.3</td>
</tr>
<tr>
<td>Length at which maximal shortening %Li, occurs, %Lmax</td>
<td>77.7 ± 13.2</td>
<td>82.4 ± 17.1</td>
</tr>
<tr>
<td>Maximal isotonic shortening, %Lmax (in mm)</td>
<td>23.5 ± 10.6</td>
<td>18.7 ± 10.3</td>
</tr>
<tr>
<td>Length at which maximal shortening, %Lmax occurs, %Lmax</td>
<td>87.5 ± 11.3</td>
<td>89.4 ± 12.6</td>
</tr>
<tr>
<td>Isotonic shortening at Lmax, %Lmax=%Li (in mm)</td>
<td>17.8 ± 9.6</td>
<td>12.7 ± 8.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Definition of abbreviations: Fmax=maximal isometric force, Lmax= length at which maximal isometric force occurs, Li= initial length for muscle shortening. Values represent means ± SD.
*Fmax was significantly higher in the obstructed patient group (p<0.05).
†Maximal isometric stress was significantly increased in the obstructed group (p<0.05)
Table 5.4

STEREOLOGICAL VALUES

<table>
<thead>
<tr>
<th></th>
<th>OBSTRUCTED</th>
<th>NONOBSTRUCTED</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MEAN ± SD</td>
<td>MEAN ± SD</td>
</tr>
<tr>
<td></td>
<td>(N=15)</td>
<td>(n=15)</td>
</tr>
<tr>
<td>Dmax, mm</td>
<td>2.4 ± 0.7</td>
<td>2.8 ± 1.0</td>
</tr>
<tr>
<td>ASM, mm(^2)</td>
<td>0.11 ± 0.04*</td>
<td>0.09 ± 0.03</td>
</tr>
<tr>
<td>ASM/Dmax, mm(^2)/mm</td>
<td>0.05 ± 0.01†</td>
<td>0.04 ± 0.03</td>
</tr>
<tr>
<td>WA, mm(^2)</td>
<td>2.23 ± 1.12</td>
<td>2.43 ± 0.64</td>
</tr>
<tr>
<td>ASM/WA, mm(^2)/mm(^2)</td>
<td>0.06 ± 0.03‡</td>
<td>0.04 ± 0.02</td>
</tr>
</tbody>
</table>

Definition of abbreviations: Dmax = airway diameter at Lmax; ASM = airway smooth muscle; ASM/Dmax = normalized airway smooth muscle; WA = airway wall area; ASM/WA = airway smooth muscle as a proportion of the airway wall.

* ASM was significantly increased in the obstructed group (\(p < 0.05\)).
† ASM/Dmax was significantly higher in the obstructed group (\(p < 0.01\)).
‡ ASM/WA was significantly increased in the obstructed group (\(p < 0.02\)).
### Table 5.5
PRESSURE-LENGTH PARAMETERS

<table>
<thead>
<tr>
<th></th>
<th>OBSTRUCTED MEAN ± SD n=15</th>
<th>NONOBSTRUCTED MEAN ± SD n=15</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ptp at Lmax</strong>, cmH₂O</td>
<td>20.0±11.0 *</td>
<td>12.7±9.0</td>
</tr>
<tr>
<td><strong>Length at FRC, %Lmax</strong></td>
<td>70.1±17.6 †</td>
<td>80.8±12.7</td>
</tr>
<tr>
<td><strong>Passive Tension at FRC, %Fmax</strong></td>
<td>26.0±28.0 ‡</td>
<td>51.1±35.4</td>
</tr>
<tr>
<td><strong>Isometric force at FRC, g</strong></td>
<td>0.35±0.28</td>
<td>0.20±0.13</td>
</tr>
<tr>
<td><strong>Isometric force at FRC, %Fmax</strong></td>
<td>48.0±28.0</td>
<td>50.0±31.0</td>
</tr>
<tr>
<td><strong>Isotonic shortening at FRC, mm</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>%Li</td>
<td>0.56±0.41</td>
<td>0.68±0.45</td>
</tr>
<tr>
<td>%Lmax</td>
<td>21.8±15.6</td>
<td>18.3±12.7</td>
</tr>
<tr>
<td></td>
<td>14.6±10.2</td>
<td>17.3±11.2</td>
</tr>
</tbody>
</table>

Definition of abbreviations:

FRC=functional residual capacity; Ptp=transpulmonary pressure at Lmax.

*Ptp was significantly increased in the obstructed patient group (p<0.05).
†Length at FRC was significantly reduced in the obstructed patient group (p<0.05).
‡Passive tension at FRC was significantly lower in the obstructed group (p<0.01).
Figure 5.1. Dissected airway preparation. This specimen was obtained from macroscopically tumor-free lung tissue of a patient who had surgical resection of a lobe for removal of bronchogenic carcinoma. Subsegmental airways are shown after removal of blood vessels and surrounding parenchyma.
Figure 5.2. Experimental set-up. An airway ring was mounted in an organ bath between a force transducer and a motor arm. Contractions were elicited by acetylcholine (10^{-5} M). Isometric force and isotonic shortening were measured in vitro by a servo-controlled myograph. The motor arm of this apparatus could be fixed to achieve an isometric contraction, or it could be allowed to move to maintain constant force and achieve an isotonic contraction. The organ bath contained Krebs-Henseleit buffer that was replaced continuously and bubbled with 95%O_2-5%CO_2 at 37° C.
Figure 5.3. Transverse-section of an airway stained with toluidine blue O. The airway smooth muscle (SM) was measured at a magnification of x200 (x20 objective). SM in the tissue section was digitized by tracing around the muscle bundles. The total connective tissue (CT) in the airway wall was measured by tracing around the outer border of the airway preparation at a lower magnification.
Figure 5.4. The cumulative frequency distribution for the peripheral airways used in this study (n=30) are shown classified according to their airway diameter at $L_{\text{max}}$, $D_{\text{max}}$. Airways from obstructed (n=15) and nonobstructed (n=15) subjects were studied. The distribution of airways on the basis of size was similar in both groups.
Figure 5.5 Representative length-force relationships for a peripheral airway ring are shown. The force, expressed as a percentage of the maximal active isometric force (Fmax), has been plotted versus the length of the airway preparation, expressed as a percentage of Lmax, the length at which Fmax occurs. The plot shows the passive tension (empty circles), the active isometric force (filled circles), the total force (filled squares), and the active isotonic shortening (empty triangles). The active isometric force (total force - passive tension) increases at each length as the airway is stretched until it reaches a maximal value. Beyond that point, further increases in length result in a decrease in isometric force. Isotonic shortening at Lmax is shown by an arrow.
Figure 5.6. Correlations between shortening and force. (A) Shortening as a percentage of the length from which the airway preparation started shortening, Li, has been plotted versus maximal isometric force in grams (g), r = 0.389, p<0.05. (B) Shortening % Li versus maximal isometric stress (mN/mm²), r = 0.488, p<0.02. (C) Shortening as a percentage of the length for optimal isometric force, Lmax, versus force (g), r=0.442, p<0.05. (D) Shortening % Lmax versus stress (mN/mm²), r=0.538, p<0.01. N= 30 airways from obstructed and nonobstructed individuals.
Figure 5.7. Maximal isometric force and stress versus pulmonary function. (A) Force (g) versus FEV\textsubscript{1} (%pred), $r = -0.579$, $p < 0.004$. B) Stress (mN/mm\textsuperscript{2}) versus FEV\textsubscript{1} (%pred), $r = -0.328$, p-NS. (C) Force (g) versus FEV\textsubscript{i}/FVC (%), $r = -0.720$, $P < 0.003$. (D) Stress (mN/mm\textsuperscript{2}) versus FEV\textsubscript{i}/FVC (%), $r = -0.611$, $p < 0.002$, for all airways ($n=30$) from obstructed and nonobstructed individuals. When the subject with extreme values is removed the correlations change as follows: force versus FEV\textsubscript{1}, $r = -0.423$, $p<0.04$; stress versus FEV\textsubscript{1}, $r = -0.158$, p-NS; force versus FEV\textsubscript{i}/FVC, $r = -0.554$, $p<0.004$; stress versus FEV\textsubscript{i}/FVC, $r = -0.484$, $p<0.02$. 

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Figure 5.8. Maximal isotonic shortening versus pulmonary function. (A) Shortening as a percentage of the length from which the airway preparation started shortening, $L_i$, has been plotted versus $FEV_1$ (%pred), $r=-0.230$, p-NS. (B) Shortening as a percentage of the length for optimal isometric force, $L_{max}$, versus $FEV_1$ (%pred), $r=-0.318$, p-NS. (C) Shortening as a percentage of $L_i$ versus $FEV_1/FVC$ (%), $r=-0.217$, p-NS. (D) Shortening as a percentage of $L_{max}$ versus $FEV_1/FVC$ (%), $r=-0.247$, p-NS, for all airways ($n=30$) from obstructed and nonobstructed individuals.
Figure 5.9. Photomicrographs of transverse sections taken from airway rings of an obstructed and a nonobstructed subject. Magnifications shown are x2 (objective) (A, B), x6.3 (C,D) and x20 (E,F). Airway smooth muscle (SM) was increased in the obstructed case (shown by arrows at x2 and x6.3). CT=connective tissue; EP=epithelium.
Figure 5.10. To relate in vitro mechanics to the operating length of the airways in vivo, the "transpulmonary" pressure (Ptp) at Lmax were estimated from the length-tension curves using the Laplace equation (P=T/r, where P=Ppt; T=the wall tension obtained from the passive tension at Lmax in mN per mm of airway width; r=radius at Lmax). The passive tension at FRC, assumed to be 5 cmH₂O, was also calculated. The assumption made in estimating these variables is that in the absence of smooth muscle contraction the interstitial pressure surrounding the airways in vivo is equal to the pleural pressure. (A) Transpulmonary pressure (Ptp) at Lmax versus FEV₁/FVC (%), r=-0.390, p<0.05. (B) Passive tension at FRC (%Fmax) versus FEV₁/FVC (%), r=0.412, p<0.02. N =30 airways.
REFERENCES.


7 Persson CG. Plasma exudation in tracheobronchial and nasal airways: a mucosal defence mechanism becomes pathogenic in asthma and rhinitis. Eur Respir J Suppl. 12:652s-656s, 1990


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Chapter 6

Peripheral airway smooth muscle tone and contractility.

6.1 INTRODUCTION.

In the previous chapter the passive and active mechanical properties of airway smooth muscle (ASM) were examined in vitro in tissues obtained from the lungs of subjects who had varying degrees of airflow obstruction. Complete length-force relationships were performed by stretching the airway preparations until the maximal force, $F_{\text{max}}$, was achieved at an optimal length known as $L_{\text{max}}$. As the tissue was stretched the passive, or resting, tension increased gradually. In the previous chapter, the passive tension was used to define a combination of the passive properties of the tissue and the active "intrinsic tone" of smooth muscle. In this chapter, the resting tension is defined as the passive tension plus the active "intrinsic tone" of airway smooth muscle. The nature and amount of the intrinsic tone has not been systematically studied. Its role in influencing the isometric force generation and isotonic shortening is the focus of this study.

The capacity of ASM to generate intrinsic tone varies among species. Excised strips of the posterior membrane of the trachea from the guinea pig$^{1\ 2\ 3}$ and central$^{4\ 5\ 6}$ and peripheral airways$^{7\ 8}$ of the human have been shown to
develop a high degree of intrinsic tone. In contrast little tone has been reported in isolated tracheal smooth muscle from the dog ⁹ ¹⁰ ¹¹ ¹² and pig. ¹³ In the guinea pig intrinsic tone is contributed by prostanoids. ¹⁴ The role of prostanoids in human ASM tone is unclear. In some studies indomethacin had little or no effect on intrinsic tone, ¹⁵ ¹⁶ ¹⁷ increased tone, ¹⁷ ¹⁸ or reduced tone ¹⁹ ²⁰ The role of leukotrienes in maintaining tone is well established in human isolated airways. Several investigators have reported that intrinsic tone is inhibited by a 5-lipoxygenase inhibitor ¹⁹ and by the cysteinyl-leukotriene antagonist FPL 55712. ¹⁵ ¹⁹ FPL 55712 has also been shown to possess phosphodiesterase activity, making its mechanism of action less specific. Other more potent leukotriene antagonists devoid of phosphodiesterase activity ²¹ ²² have been shown to have bronchodilator activity in vivo. ²³ ²⁴ ²⁵ In a recent study, Ellis and Undem ⁷ found that in isolated human peripheral airways, intrinsic tone was largely due to the continual production and release of cysteinyl-leukotrienes, and, to a lesser extent, histamine. In their study intrinsic tone averaged more than 50% of the available tone in fresh tissues. None of these studies, however, systematically quantified alterations in contractile function after reducing intrinsic tone.

The purpose of this study was to assess whether intrinsic airway smooth muscle tone had an effect on the active mechanical properties of peripheral ASM. In the present study the passive tension, the isometric force generation and the isotonic shortening were measured in peripheral airway rings (2.5±0.8 SD mm internal diameter) from subjects who had varying degrees of airflow obstruction. These contractile responses were measured before and after
addition of MK886, a leukotriene (LT) biosynthesis inhibitor that acts by blocking the binding of the 5-lipoxygenase-activating protein (FLAP). The hypothesis is that airways from subjects who have more severe airflow obstruction will exhibit an increase in intrinsic tone, that intrinsic tone will be decreased by MK886 in airways from all subjects but that this decrease will be altered in disease, and that a reduction in tone will result in increased shortening and altered ASM force.
6.2 METHODS.

6.2.1 Patients.

Lung tissue was obtained from 10 patients who had a surgical resection of a lung or lobe for a solitary peripheral lung lesion at St. Paul's Hospital (Vancouver, BC, Canada). Pulmonary function tests were done before surgery and included maximal expiratory flow volume curves. All of the subjects had a diagnosis of bronchogenic carcinoma. Nine of the patients had been life-long smokers, and only one was an ex-smoker (had not smoked for 40 years). Pre-operative medications included sedatives (lorazepam, Ativan), laxatives (docusate sodium), heparin, antibiotics (cefaizolin sodium), analgesics (acetaminophen), and in some cases β-agonists (albuterol [Ventolin], terbutaline [Bricaryl]). Preliminary studies showed no differences in in vitro contractility as a result of the various medications received while in hospital. Asthma and COPD were defined on the basis of clinical criteria using the definitions of the American Thoracic Society. Only one of the subjects had any features suggestive of asthma. Table 6.1 summarizes the data for the patients in this study.

Pulmonary function studies were done within 1 week before surgery and included the forced expiratory volume in 1s of the forced vital capacity (FEV₁), the forced vital capacity (FVC), the forced expiratory flow at 50% of FVC (FEF₅₀), the forced expiratory flow between 25-75% of FVC (FEF₂₅₋₇₅), the functional residual capacity (FRC), and the total lung capacity (TLC). The FEV₁ was expressed as percentage predicted using the equations of Crapo and co-
The ratio of FEV\textsubscript{1} to FVC (FEV\textsubscript{1}/FVC) was also calculated and was used as an indicator of airflow obstruction. A value of 70\% is approximately the lower limit of the normal range as defined by 95 \% confidence intervals for FEV\textsubscript{1}/FVC\textsuperscript{29} and was therefore used to categorize the subjects in this study. A value of less or greater than 70\% meant that subjects were "obstructed" or "nonobstructed" respectively.

6.2.2 In vitro studies.

Macroscopically tumor-free human lung tissue was obtained from surgical specimens and immediately transferred to ice-cold sterile Krebs-Henseleit buffer (in mM: 118 NaCl, 4.7 KCl, 2.5 CaCl\textsubscript{2}H\textsubscript{2}O, 1.2 MgSO\textsubscript{4}7H\textsubscript{2}O, 1.2 kH\textsubscript{2}PO\textsubscript{4}, 25 NaHCO\textsubscript{3}, and 11.1 glucose). Airway rings were obtained from third-and fourth-order bronchi and were dissected free of blood vessels and surrounding parenchyma. Tissues were stored overnight in a large volume of cooled (4\(^\circ\)C) Krebs buffer that was previously aerated with carbogen (95\% O\textsubscript{2}-5\% CO\textsubscript{2}). This procedure has been shown to wash out drug residues and any substances liberated during the dissection.\textsuperscript{30} The pH of the solution ranged between 7.2-7.3. Viability of responses after storage was tested on a number of the airways by eliciting a near maximal contraction with acetylcholine (10\textsuperscript{-5} M) the day of thoracotomy, and on the next day at the beginning and at the end of the experiment. The responses to cholinergic agonist remained unchanged up to 48 hrs, and were reproducible throughout the experiment (data not shown) which is consistent with previous findings.\textsuperscript{30}
In vitro studies were carried out the day after thoracotomy to characterize the length-tension curves for each airway ring. Passive tension, isotonic shortening, and isometric force generation were measured in vitro by a servo-controlled myograph. The motor arm of this apparatus could be fixed to achieve an isometric contraction or maintain constant force allowing an isotonic contraction (G325D pen motor [General Scanning, Watertown, MA]; Kulite BG-10 force transducer [Durham Instruments, Pickering, Ontario, Canada]). The frequency response was 100 Hz and the resolution was 0.02 g for force and 0.01 mm for length measurements.

6.2.3 Protocol.

Each airway ring was placed in an organ bath that contained fresh Krebs-Henseleit buffer that was replaced continuously, and bubbled with 95%O₂-5%CO₂ at 37°C. The airway was mounted between a force transducer and a motor arm and allowed to equilibrate at zero resting tension for 1 h. The weight of the tissue was incorporated into a balancing current in the electronic circuit in order to achieve zero tension. The initial length of the muscle was measured with an optical micrometer to an accuracy of 0.01 mm. All subsequent changes in length were referenced to this value so that changes in length could be calculated.

Each airway ring was slowly stretched three times by loads that determined optimal length during preliminary experiments, and then was allowed to equilibrate in the organ bath with no load for 1 h. After equilibration, passive,
isometric, and isotonic length-force relationships were obtained. Contractions were elicited by acetylcholine \((10^{-5}\text{M})\). Measurement of isometric force was first performed at lengths below the level at which passive tension was first detected. A small preload was then applied to the ring to stretch it, and the length was recorded once it stabilized. An isometric contraction with this preload was performed by maintaining the airway length constant while stimulating and measuring the change in force. Once the force returned to baseline (same preload), an isotonic contraction was elicited and the length change measured. Complete isometric and isotonic length-force curves were obtained by serially increasing the smooth muscle length to 10-20% more than the length at which maximal force occurred. For each length, an isometric contraction was followed by an isotonic contraction. From the isometric force-length curve, \(L_{\text{max}}\) was determined as the length at which maximal isometric force (\(F_{\text{max}}\)) occurred. In the rare instances where the maximal force at two lengths was similar, the shorter of the two lengths was chosen. \(L_{\text{max}}\) was used to calculate the diameter at \(L_{\text{max}}\) (\(D_{\text{max}}\)) by assuming that in the stretched ring, the airway perimeter was twice the measured length at \(F_{\text{max}}\) [\(\text{diameter} = 2 \times \text{length}/\pi\)]. Contractile responses at \(L_{\text{max}}\) were obtained by subtracting the passive from the total tension.

### 6.2.4 Measurement of intrinsic tone and contractility.

To assess the contribution of intrinsic tone to the passive length-tension curve and, in addition, to test its effect on contractility, the FLAP (5-lipoxigenase activating protein) inhibitor MK886 \((10^{-5}\text{M})\) (Merk-Frosst, Canada, Que, Canada)
was added as a single concentration (10⁻⁵ M) and the entire protocol was repeated as described above. The airway ring was stretched by stepwise increments and once the length was stable, MK886 was added to the bath and the new resting tension was recorded. Acetylcholine (10⁻⁵ M) was added to the bath and an isometric contraction was performed. The tissue was then washed and allowed to equilibrate to the preload measured before MK886 was added. MK886 was again added at the same length (and preload) and an isotonic contraction was elicited by acetylcholine (10⁻⁵ M).

The relaxation to MK886 (designated a in figure 1) was calculated as the percent of maximal relaxation in response to 10 mM theophylline (designated b in figure 1) which was added at the end of the experiment as a single concentration. In preliminary experiments adding 1 mM isoproterenol to the preparation after theophylline caused no further relaxation. This relaxation was therefore considered to be maximal relaxation, and a measure of the total intrinsic tone of the airway preparations. Also, in preliminary experiments, addition of 1 μM indomethacin did not alter baseline tone. The maximal available tone (designated c in figure 6.1) was taken as the difference between the isometric force after addition of acetylcholine (10⁻⁵ M) and the passive tension after addition of theophylline (10⁻² M). The total amount of intrinsic tone, as well as the active isometric force developed in each preparation were expressed as a percentage of this maximal tone.

After completion of the in vitro studies, each airway ring was fixed at Lmax in 2.5% glutaraldehyde (Polysciences, Warrington, PA) in 0.1 M sodium
cacodylate buffer (Polysciences), dehydrated in ethanol, and embedded in glycol methacrylate plastic (JB-4; Polysciences). 2 μm thick sections were cut and stained with an aqueous solution of 1% (wt/vol) toluidine blue O (C.I.52040; Sigma, St. Louis, MO) and 1% (wt/vol) sodium borate at pH 8.7. Cross-sectional areas of smooth muscle were measured at a magnification of x200 (x20 objective lens), using a Nikon microscope with a camera lucida (Nikon Labophot; Nikon Canada, Mississauga, ON, Canada), a digitizing board (Summasketch II model MM II 1201; Summagraphics, Seymour, CT), and the Bioquant BQ system software (R&M Biometrics Inc., Nashville, TN) on an IBM compatible personal computer. The intra- and inter-observer coefficient of variation was less than 5% for each variable measured. Measurements were made on three slides and a mean value was obtained for each variable.

6.2.5 Data Analysis.

Data have been expressed as means ± SD unless otherwise stated. Force and length measurements were standardized and expressed as %Fmax and %Lmax respectively. The muscle stress at Lmax was calculated by dividing force values by the cross-sectional areas of muscle measured by morphometry, and was expressed as mN/mm². The amount of isotonic shortening was analyzed in two ways: i) by dividing the shortening by the length from which the preparation started shortening, and expressing it as per cent initial length, %Li; and ii) by dividing shortening by Lmax, and expressing it as %Lmax.

Comparisons of passive tension, force, and shortening before and after

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treatment at different lengths were made using a one-way repeated measures analysis of variance. P values were considered to be statistically significant when less than 0.05.

6.2.6 Drugs.

The following drugs were used: MK886 (3-[1-(4-chlorobenzyl)-3-t-butyl-thio-t-isopropyl-indol-2-yl]-2,2-dimethylpropanoic acid) was a gift from Merck Frosst Canada (Dorval, Quebec). Acetylcholine, indomethacin, isoproterenol, and theophylline were obtained from Sigma Chemical (St. Louis, MO). Indomethacin and MK886 were dissolved in absolute ethanol. The other drugs were dissolved in Kreb’s solution.
6.3 RESULTS.

6.3.1 Clinical Data.

Table 6.2 shows the mean lung function values for the patients in this study. Five patients had an FEV₁/FVC < 70% and were considered to have airflow obstruction. The functional residual capacity, and residual volume were increased (p<0.05), and the FEF₅₀ and FEF₂₅-₇₅ were significantly lower (p<0.05) in the obstructed patients.

Ten airways with a mean diameter of 2.8 mm at Lmax were used for the in vitro studies. 5 airway rings were obtained from obstructed (OB) patients, and 5 from nonobstructed (NOB) patients. Both groups had similar sized airways. There was no difference in the diameter at Lmax between the two patient groups.

6.3.2 Quantification of intrinsic tone.

The mean values for passive tension, expressed as %Fmax, in obstructed and nonobstructed groups before and after addition of MK886, the 5-lipoxygenase-activating protein (FLAP) inhibitor, are shown in table 6.3A. The passive tension at Lmax in tissues stored overnight averaged 108%Fmax in NOB and 134%Fmax in OB. After addition of MK886 the passive tension was significantly (p<0.05) reduced in the airways of NOB at lengths between 70 to 105 %Lmax, but there was no change in the airways of obstructed subjects.

Figure 6.2 shows the change in intrinsic tone expressed as a percentage
of the maximal relaxation in response to theophylline. Changes in tone in response to MK886 have been plotted as a function of the length of the airway preparations, expressed as a percentage of Lmax. Addition of MK886 effectively relaxed the airways from the nonobstructed group at 70, 80, 90, 100 and 105 %Lmax (p<0.05) but had little or no effect in those of the obstructed group. Significant (p<0.05) differences in relaxations to MK886 occurred at lengths 70 to 105 %Lmax between OB and NOB. Relaxations ranged between approximately 30 to 90% in NOB and between 1 to 50% in OB airways.

The mean values for passive tension after addition of theophylline are shown in table 6.3B. Theophylline significantly relaxed the airways at all lengths in the obstructed group (p<0.05), and at lengths greater than 70 %Lmax in the nonobstructed group (p<0.05). The magnitude of the changes produced by adding theophylline was the same in OB compared to NOB.

The total amount of intrinsic tone expressed as a percentage of the maximal available tone is shown in table 6.4. Intrinsic tone was obtained from the difference in the passive tension before and after addition of theophylline. The maximal available tone was calculated from the difference between the isometric force elicited by acetylcholine and the passive tension after addition of theophylline. The intrinsic tone was similar at lengths between 70% and 100%Lmax, where it ranged between 38 and 42% in OB, and between 38 to 53% in NOB. There was no difference in the levels of intrinsic tone between the airways of obstructed and nonobstructed patients.
6.3.3 Effect of alterations in tone on active isometric force and isotonic shortening.

The mean absolute values of active isometric force expressed as a percentage of Fmax before and after addition of MK886 are shown in table 6.5 for airways from obstructed and nonobstructed groups. There was no difference in the absolute force generated by airways after adding MK886 within each group.

The changes in active isometric force in airways from obstructed and nonobstructed individuals after addition MK886 are shown in Figure 6.3. The change in isometric force, as percentage of the maximal available tone, is plotted as a function of length, expressed as a percentage of Lmax. The maximal available tone was determined as described above. Addition of MK886 resulted in enhancement of the isometric force at all lengths in NOB airways when compared to the OB airways, with increases ranging between 2 to 20%. In OB airways isometric force was lower after MK886 was added, and the decreases in force ranged between 2 to 20%. These changes in isometric force were significantly different between the two groups at 90, 105, and 110%Lmax.

The mean values of active isotonic shortening before and after addition of MK886 are shown in table 6.6 for airways from obstructed and nonobstructed groups. Values are expressed as a percentage of Li (table 6.6A) and as percentage of Lmax (table 6.6B). There was a significant decrease in the amount of shortening in airways from OB at 60 and 70 %Lmax after adding MK886 (p<0.05). In contrast, in NOB, there was a significant increase in shortening at
100, 105, and 110 %Lmax (p<0.05).

The changes in isotonic shortening after addition of MK886 are shown in figure 6.4. The effect of MK886 is represented by the difference between values measured before and after addition of the drug. Isotonic shortening normalized by the initial length from which the airway preparation started shortening, Li, is shown in figure 6.4A, while shortening normalized by the length at maximal isometric force, Lmax, is plotted in figure 6.4B. In both cases isotonic shortening is plotted as a function of the length, expressed as a percentage of Lmax. These relationships are shown for airways from obstructed and nonobstructed groups. The effect of MK886 on isotonic shortening was significantly different at 60 to 110 %Lmax when expressed as %Li (p<0.05), and at all lengths except for 90%Lmax when expressed as %Lmax.

Table 6.7 summarizes the mean values of force, stress, and shortening at Lmax before and after addition of MK886. Shortening at Lmax, expressed as %Li or %Lmax, was significantly increased in the presence of MK886 in airways from NOB (p<0.05). In OB airways the shortening appeared to be reduced but the changes were not significant. There were no significant alterations in the amount of isometric force or stress generated after addition of MK886 in OB or NOB and there was no shift in Lmax as a result of MK886.
6.4 DISCUSSION.

The purpose of this study was to quantify the level of intrinsic tone in human peripheral airways, to examine the contribution of leukotrienes to intrinsic tone by using a leukotriene biosynthesis inhibitor, and to test whether alterations in intrinsic tone have an effect on the active contractile properties of airways in patients who have varying degrees of airflow obstruction. The patients were categorized into an obstructed and nonobstructed group based on the ratio of \( \text{FEV}_1 \) to FVC. An \( \text{FEV}_1/\text{FVC} \) of less than 70% was used to define airflow obstruction. As expected, patients categorized as being obstructed on the basis of \( \text{FEV}_1/\text{FVC} \) also showed hyperinflation and gas trapping with significant increases in FRC and residual volume.

6.4.1 Quantification of intrinsic tone.

The results of this study demonstrated the presence of intrinsic tone in isolated peripheral airways that were stored overnight after surgical resection. Intrinsic tone was examined in airways from obstructed and nonobstructed individuals. In both groups the amount of intrinsic tone averaged approximately 40% of the maximum available active tension at lengths that ranged between 60 to 100\%L_{max}. These results are consistent with those of previous studies demonstrating the existence of intrinsic tone in isolated human bronchi. \(^4615317\)

Results from the nonobstructed group, indicate that intrinsic tone was due primarily to cysteinyl-leukotriene receptor stimulation with no contribution by
prostaglandins. In this study, the 5-LO activating protein inhibitor MK886 was used to inhibit tone. MK886 effectively reduced tone by up to 90% of the maximal relaxation elicited by theophylline in the airways from nonobstructed patients. This finding indicates that in this group of airways most of the tone was due to production of cysteinyl leukotrienes. In support of this observation, Ellis and Undem\textsuperscript{7} found that human bronchi, either freshly isolated or stored overnight, exhibited intrinsic tone that was also mediated by cystenyl leukotrienes. Other investigators have also found evidence that implicates these agents in the production and maintenance of tone.\textsuperscript{19 15 21 22 24} Although the role of prostaglandins is unclear, in most studies the inhibition of prostaglandin by indomethacin did not alter the level of intrinsic tone.\textsuperscript{16 15}

MK886 had no effect on intrinsic tone of airways from obstructed patients. The amount of relaxation varied between only 1 and 50% of that elicited by theophylline. It is possible that MK886 may not have been as effective in this group of airways because the tone was due to cysteinyl leukotrienes that are already in the tissue before addition of drug. It has been shown that peptidoleukotrienes are continuously being synthesized in the airway tissues\textsuperscript{7 19} and conceivably if a greater level was present in the airways of obstructed patients, that could result in a smaller relaxation to the FLAP inhibitor. The use of a leukotriene receptor antagonist could have blocked the effect of leukotrienes present in the tissues. In humans most of the actions of the cysteinyl leukotrienes are mediated by the CysLt1 receptor, including contraction of human airway smooth muscle, chemotaxis, and increased vascular permeability.
CysLT1-receptor antagonists include zafirlukast and MK-571.

Another possibility is that intrinsic tone in airways from patients with airflow obstruction may be due more predominantly to histamine. Intrinsic tone has been shown to be mediated by histamine in airways from patients undergoing lung resection and from organ donors. Ellis et al.\textsuperscript{7} found that the histamine antagonist pyrilamine inhibited intrinsic tone by 30% when added to peripheral airways (3-12 mm inner diameter) that had been previously treated with a leukotriene biosynthesis inhibitor. Creese and Temple\textsuperscript{35} reported that mepyramine inhibited intrinsic tone in large and small bronchi. In this study most of the intrinsic tone was reduced by leukotriene biosynthesis inhibitor, so selective histamine antagonists were not used. In airways from nonobstructed subjects tone was reduced by more than 90% of the maximal relaxation obtained by theophylline after addition of the leukotriene biosynthesis inhibitor, and any contribution by histamine would be small. The design of this study does not allow a conclusion as to what mechanism mediates intrinsic tone in the obstructed group. If more histamine was being produced, sensitive assays might yield a greater spontaneous release of this mediator.\textsuperscript{36}

Few investigators have systematically quantified the level of tone in isolated human airways.\textsuperscript{6,7,19,15} In this study relaxations in intrinsic tone were expressed as a percentage of the maximal inhibitory response to theophylline determined at the end of the experiment. Theophylline reduced tone in both obstructed and nonobstructed airways at nearly all lengths. Only at 60\%L_{\text{max}} the reduction in tone was not significant in airways from the nonobstructed
group. This could have been due to the large variability in the signal. The variation in relaxation was not due to a lack of intrinsic tone because theophylline always produced relaxation.

The total amount of intrinsic tone expressed at the maximum available tone to acetylcholine was similar in both groups of airways. The assumption made was that theophylline completely relaxed the ASM preparation and that acetylcholine was able to contract the ASM to a near maximal value at the concentration applied. Greater stimulation may have resulted from addition of carbachol or BaCl₂, but it was assumed that stimulation by acetylcholine would have similar characteristics in all human tissues at least within each group. In addition, acetylcholine was chosen in order to replicate the contractile responses of the airway tissue measured in chapter 5 so that comparisons could be made when looking at alterations in tone in similar airway preparations.

6.4.2 Effect of alterations in tone on active isometric force.

In order to test the effect of alterations in tone on isometric force, responses were measured before and after addition of the FLAP inhibitor, MK886. Although MK886 had no effect on the force generated within each group, there was a differential response to the biosynthesis inhibitor and this response was significantly different between OB and NOB. The NOB tissue exhibited a trend toward an increase in isometric force, while in the OB tissue force appeared to be depressed but within each group the effect of the drug was not significant. This was not surprising in the case of NOB airways because
addition of the leukotriene synthesis inhibitor was expected to reduce intrinsic tone and result in more 'available tone' for active force generation. In contrast, the high level of tone in the OB tissues may have prevented the generation of more isometric force. Contractile agonists, such as histamine and cysteinyl leukotrienes, may decrease\textsuperscript{37 38 39} or increase\textsuperscript{40} the potency and efficacy of a second contractile agonist, such as methacholine, depending on the concentration of agonist. Alternatively, the lack of a clear signal could simply be due to the small number of patients in this study.

6.4.3 Effect of alterations in tone on active isotonic shortening.

The presence of the FLAP inhibitor MK886 resulted in increased isotonic shortening in the airways from NOB patients at 100 to 110 \%Lmax. In contrast, airways from OB patients exhibited reduced shortening at 60 to 80 \%Lmax, and no further changes were observed at longer lengths. The effect of MK886 on shortening appears to be similar to that observed for isometric force generation. Like for the force, enhanced shortening in airways from the NOB group could be due to reduced intrinsic tone and more 'available tone' for muscle contraction. In airways from OB tissues the high level of tone, which did not change with MK886, would limit shortening.

Since the airways from OB subjects did not have a significantly different amount of intrinsic tone, it is also possible that factors other than intrinsic tone may be implicated in the depressed shortening observed after addition of MK886.
Mechanical factors leading to reduced shortening could be involved. These are related to high passive tension due to stiffer airways or high connective tissue content. The functional consequences of these structural changes have been discussed in chapters 3, 4, and 5. Connective tissue could radially constrain the ASM and reduce its ability to shorten in this group of patients.

The relationship between the amount of connective tissue and contractility will be studied in chapter 7.
Table 6.1

STUDY POPULATION CHARACTERISTICS

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* Pack years= the number of years smoking one pack of cigarettes per day. 
Age and pack years are expressed as means ± standard deviation. n=number
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<td>FRC, %pred</td>
<td>113 ± 18*</td>
<td>96 ± 23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RV, %pred</td>
<td>139 ± 24*</td>
<td>90 ± 31</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Definition of abbreviations: FEV₁ = forced expiratory volume in 1 s of the forced vital capacity; FVC = forced vital capacity; FEV₁/FVC = forced expiratory volume in 1 s as a percentage of the forced vital capacity; FEF₅₀ = forced expiratory flow at 50% of the forced vital capacity; FEF₂₅-₇₅ = forced expiratory flow between 25 and 75% of the forced vital capacity; TLC = total lung capacity; FRC = forced vital capacity; RV = residual volume. FRC and RV were significantly higher (p<0.05), and FEF₅₀ and FEF₂₅-₇₅ %pred were lower (p<0.05) in the obstructed subjects.
Table 6.3

A. INTRINSIC TONE, %Fmax: EFFECT OF MK886

<table>
<thead>
<tr>
<th>%Lmax</th>
<th>OBSTRUCTED</th>
<th>NONOBSTRUCTED</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-drug</td>
<td>MK886</td>
</tr>
<tr>
<td>60</td>
<td>23.0 ± 23.0</td>
<td>15.0 ± 15.0</td>
</tr>
<tr>
<td>70</td>
<td>28.4 ± 34.0</td>
<td>21.0 ± 26.0</td>
</tr>
<tr>
<td>80</td>
<td>44.0 ± 34.0</td>
<td>40.0 ± 33.4</td>
</tr>
<tr>
<td>90</td>
<td>71.0 ± 56.0</td>
<td>81.0 ± 56.0</td>
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<tr>
<td>100</td>
<td>133.8 ± 118.0</td>
<td>120.4 ± 124.0</td>
</tr>
<tr>
<td>105</td>
<td>180.0 ± 116.0</td>
<td>159.0 ± 134.4</td>
</tr>
<tr>
<td>110</td>
<td>221.0 ± 110.0</td>
<td>182.0 ± 140.0</td>
</tr>
</tbody>
</table>

B. INTRINSIC TONE, %Fmax: EFFECT THEOPHYLLINE

<table>
<thead>
<tr>
<th>%Lmax</th>
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<th>NONOBSTRUCTED</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-drug</td>
<td>Theophylline</td>
</tr>
<tr>
<td>60</td>
<td>23.0 ± 23.0</td>
<td>2.0 ± 1.3*</td>
</tr>
<tr>
<td>70</td>
<td>28.4 ± 34.0</td>
<td>2.6 ± 2.5*</td>
</tr>
<tr>
<td>80</td>
<td>44.0 ± 34.0</td>
<td>9.0 ± 6.3*</td>
</tr>
<tr>
<td>90</td>
<td>71.0 ± 56.0</td>
<td>20.4 ± 20.0*</td>
</tr>
<tr>
<td>100</td>
<td>133.8 ± 118.0</td>
<td>38.4 ± 31.5*</td>
</tr>
<tr>
<td>105</td>
<td>180.0 ± 116.0</td>
<td>57.4 ± 45.0*</td>
</tr>
<tr>
<td>110</td>
<td>221.0 ± 110.0</td>
<td>84.0 ± 57.0*</td>
</tr>
</tbody>
</table>

Definition of abbreviations: Fmax = maximal isometric force, Lmax = length at which maximal isometric force occurs. *In table 3A the passive tension was significantly reduced by MK886 in the nonobstructed at 70% to 105%Lmax (p<0.05). †Table 3B shows the effect of theophylline. The passive tension was significantly lower in the nonobstructed group at 70% to 110%Lmax and the obstructed group at 60 to 110%Lmax (p<0.05). Values represent means ± SD. N=5 airways from 5 subjects per group.
### Table 6.4

**INTRINSIC TONE AS A %MAXIMAL AVAILABLE TONE**

<table>
<thead>
<tr>
<th>%Lmax</th>
<th>OBSTRUCTED N=5</th>
<th>NONOBSTRUCTED N=5</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>22.0 ± 17.0</td>
<td>27.0 ± 27.0</td>
</tr>
<tr>
<td>70</td>
<td>42.0 ± 17.0</td>
<td>38.0 ± 18.0</td>
</tr>
<tr>
<td>80</td>
<td>41.0 ± 15.0</td>
<td>40.0 ± 15.0</td>
</tr>
<tr>
<td>90</td>
<td>40.0 ± 11.0</td>
<td>53.0 ± 29.0</td>
</tr>
<tr>
<td>100</td>
<td>38.0 ± 11.0</td>
<td>42.0 ± 19.0</td>
</tr>
<tr>
<td>105</td>
<td>45.0 ± 9.0</td>
<td>56.0 ± 30.0</td>
</tr>
<tr>
<td>110</td>
<td>56 ± 11.0</td>
<td>66.0 ± 13.0</td>
</tr>
</tbody>
</table>

Definition of abbreviations: Lmax = length at which maximal isometric force occurs. All values of intrinsic tone are expressed as %maximal available tone, calculated from the difference between the isometric force after addition of acetylcholine (10⁻⁵M) and the resting tension after addition of theophylline (10⁻²M). There was no difference in the total amount of intrinsic tone as a percentage of the maximal available tone between airways from obstructed and nonobstructed individuals. Values represent means ± SD.
Table 6.5

EFFECT OF MK886 ON ACTIVE ISOMETRIC FORCE, %Fmax

<table>
<thead>
<tr>
<th></th>
<th>OBSTRUCTED N=5</th>
<th></th>
<th>NONOBSTRUCTED N=5</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-drug</td>
<td>MK886</td>
<td>Pre-drug</td>
<td>MK886</td>
</tr>
<tr>
<td>60</td>
<td>26.0 ± 26.1</td>
<td>20.0 ± 19.7</td>
<td>20.4 ± 21.0</td>
<td>24.4 ± 15.0</td>
</tr>
<tr>
<td>70</td>
<td>37.0 ± 34.2</td>
<td>25.0 ± 25.0</td>
<td>37.4 ± 18.0</td>
<td>43.4 ± 12.0</td>
</tr>
<tr>
<td>80</td>
<td>51.0 ± 32.5</td>
<td>44.6 ± 32.3</td>
<td>56.2 ± 20.2</td>
<td>61.6 ± 11.1</td>
</tr>
<tr>
<td>90</td>
<td>67.6 ± 33.0</td>
<td>47.0 ± 22.0</td>
<td>76.0 ± 15.0</td>
<td>80.0 ± 16.2</td>
</tr>
<tr>
<td>100</td>
<td>100.0 ± 0.0</td>
<td>62.4 ± 47.0</td>
<td>100.0 ± 0.0</td>
<td>111.0 ± 37.7</td>
</tr>
<tr>
<td>105</td>
<td>83.4 ± 11.3</td>
<td>56.0 ± 46.0</td>
<td>85.0 ± 12.2</td>
<td>109.4 ± 41.0</td>
</tr>
<tr>
<td>110</td>
<td>58.0 ± 8.4</td>
<td>37.0 ± 34.6</td>
<td>69.0 ± 23.0</td>
<td>99.0 ± 47.6</td>
</tr>
</tbody>
</table>

Definition of abbreviations: Fmax = maximal isometric force, Lmax = length at which maximal isometric force occurs. There was no difference in the isometric force after addition of MK886 in airways from obstructed or nonobstructed individuals. Values represent means ± SD.
Table 6.6

A. EFFECT OF MK886 ON ACTIVE ISOTONIC SHORTENING, %Li

<table>
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</thead>
<tbody>
<tr>
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<td>Pre-drug</td>
<td>MK886</td>
<td>Pre-drug</td>
<td>MK886</td>
</tr>
<tr>
<td>60</td>
<td>44.3 ± 4.0</td>
<td>25.0 ± 5.0*</td>
<td>8.3 ± 11.0</td>
<td>24.0 ± 20.0</td>
</tr>
<tr>
<td>70</td>
<td>43.0 ± 10.0</td>
<td>21.0 ± 9.8*</td>
<td>16.0 ± 11.0</td>
<td>27.0 ± 9.0</td>
</tr>
<tr>
<td>80</td>
<td>37.0 ± 5.7</td>
<td>25.7 ± 10.0</td>
<td>19.4 ± 9.0</td>
<td>27.0 ± 6.0</td>
</tr>
<tr>
<td>90</td>
<td>24.3 ± 12.5</td>
<td>21.0 ± 16.1</td>
<td>21.0 ± 6.4</td>
<td>28.2 ± 10.3</td>
</tr>
<tr>
<td>100</td>
<td>20.2 ± 7.0</td>
<td>16.0 ± 10.3</td>
<td>14.4 ± 5.3</td>
<td>24.0 ± 8.3†</td>
</tr>
<tr>
<td>105</td>
<td>16.0 ± 5.8</td>
<td>11.0 ± 6.8</td>
<td>9.0 ± 3.2</td>
<td>20.0 ± 6.0†</td>
</tr>
<tr>
<td>110</td>
<td>13.0 ± 5.8</td>
<td>9.0 ± 7.0</td>
<td>5.0 ± 2.5</td>
<td>12.0 ± 7.1†</td>
</tr>
</tbody>
</table>

B. EFFECT OF MK886 ON ACTIVE ISOTONIC SHORTENING, %Lmax

<table>
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<th>NONOBSTRUCTED</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Pre-drug</td>
<td>MK886</td>
<td>Pre-drug</td>
<td>MK886</td>
</tr>
<tr>
<td>60</td>
<td>27.3 ± 3.0</td>
<td>15.0 ± 3.0*</td>
<td>7.3 ± 7.8</td>
<td>14.0 ± 11.3</td>
</tr>
<tr>
<td>70</td>
<td>31.0 ± 6.8</td>
<td>14.0 ± 7.0*</td>
<td>12.0 ± 8.6</td>
<td>15.8 ± 9.6</td>
</tr>
<tr>
<td>80</td>
<td>30.0 ± 4.6</td>
<td>19.3 ± 6.7*</td>
<td>15.6 ± 7.0</td>
<td>21.0 ± 5.4</td>
</tr>
<tr>
<td>90</td>
<td>21.2 ± 11.0</td>
<td>18.3 ± 14.0</td>
<td>19.0 ± 5.5</td>
<td>25.0 ± 8.7</td>
</tr>
<tr>
<td>100</td>
<td>20.2 ± 7.0</td>
<td>16.0 ± 10.3</td>
<td>14.4 ± 5.3</td>
<td>24.0 ± 8.3†</td>
</tr>
<tr>
<td>105</td>
<td>18.8 ± 4.0</td>
<td>11.8 ± 7.0</td>
<td>9.8 ± 4.1</td>
<td>18.4 ± 7.8†</td>
</tr>
<tr>
<td>110</td>
<td>14.5 ± 7.0</td>
<td>11.0 ± 8.0</td>
<td>5.0 ± 3.1</td>
<td>12.2 ± 7.8†</td>
</tr>
</tbody>
</table>

Definition of abbreviations: Fmax = maximal isometric force, Lmax = length at which maximal isometric force occurs. Li = initial length.

*†Table 5A. Isotonic shortening, % Li, was significantly increased after addition of MK886 in airways from nonobstructed (NOB, n=5) at lengths 100 to 105 %Lmax (p<0.05), and decreased in obstructed (OB, n=5) at 60 to 70 %Lmax.

*†Table 5B. Isotonic shortening, Lmax, was significantly increased after addition of MK886 in airways from NOB (p<0.05) at lengths 100 to 105 %Lmax (p<0.05), and decreased in OB at 60 to 80 %Lmax. Values represent means ± SD.
Table 6.7
EFFECT OF MK886 ON CONTRACTILITY AT Lmax

<table>
<thead>
<tr>
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<th>NONOBSTRUCTED</th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-drug</td>
<td>MK886</td>
<td>Pre-drug</td>
<td>MK886</td>
</tr>
<tr>
<td>Fmax, g</td>
<td>0.53 ± 0.43</td>
<td>0.33 ± 0.30</td>
<td>0.40 ± 0.20</td>
<td>0.44 ± 0.30</td>
</tr>
<tr>
<td>Max. stress</td>
<td>60 ± 47</td>
<td>37 ± 23</td>
<td>53 ± 25</td>
<td>63 ± 33</td>
</tr>
<tr>
<td>&lt;L at Lmax, %Li</td>
<td>20.2 ± 7.0</td>
<td>16.0 ± 10.3</td>
<td>14.4 ± 5.3</td>
<td>24.0 ± 8.3*</td>
</tr>
<tr>
<td>Lmax, %</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>102</td>
</tr>
</tbody>
</table>

Definition of abbreviations: Fmax = maximal isometric force, Lmax = length at which maximal isometric force occurs, <L at Lmax = isotonic shortening at Lmax, Li= initial length for muscle shortening.

*Shortening at Lmax, %Li was significantly increased in airways from nonobstructed individuals after addition of MK886 (p<0.05). There was no shift in Lmax after MK886. Values represent means ± SD.
Figure 6.1. Representative tracing of relaxation of intrinsic tone by MK886.

- **a**: relaxation to MK886
- **b**: maximal relaxation to theophylline (intrinsic tone)
- **c**: maximal contraction to acetylcholine.
Figure 6.2. Relaxations of intrinsic tone by MK886, 5-LO activating protein inhibitor, are shown in isolated peripheral airways. The change in intrinsic tone in response to MK886, expressed as a percentage of the maximal relaxation in response to theophylline, has been plotted versus the length of the airway preparation, expressed as a percentage of the length at which Fmax occurs, Lmax. Relaxations of airways from the nonobstructed (NOB) group are shown by closed squares, and in the obstructed (OB) group they are indicated by open squares. MK886 resulted in a significant difference in intrinsic tone at 70%, 80%, 90%, 100 and 105 %Lmax between OB and NOB (*p<0.05). Values are expressed as the mean ± SEM; n = 5 in NOB, n=5 in OB.
Figure 6.3. The change in active isometric force expressed as a percentage of the maximal available tone, has been plotted versus the length of the airway preparation, expressed as a percentage of the length at which Fmax occurs. The maximal available tone was determined from the difference between the isometric force elicited by acetylcholine and the passive tension after addition of theophylline. The relationships are shown for nonobstructed (NOB, n=5) and obstructed (OB, n=5) patient groups, after treatment with the 5-LO activating protein inhibitor, MK886. The effect of MK886 is shown in NOB by closed triangles and in OB by open triangles. Addition of MK886 resulted in a significant difference in the isometric force between OB and NOB at 90, 105, and 110 %Lmax (*p<0.05). Values represent means ± SEM.
Figure 6.4. The change in isotonic shortening after addition of MK886 is plotted versus the length of the airway preparation, expressed as a percentage of Lmax. Relationships are shown for airways from nonobstructed (NOB, n=5) and obstructed (OB, n=5) patients. The effect of MK886 is shown in NOB by closed squares, and in OB by open squares. A. Shortening as a percentage of the length from which the airway started shortening, Li. Addition of MK886 resulted in a significant difference in isotonic shortening at 60, 70, 80, 90, 100, 105, and 110 %Lmax between OB and NOB (*p<0.05). B. Shortening as a percentage of Lmax. There was a significant difference at all lengths except at 90 %Lmax (†p<0.05). Values are expressed as means ± SEM.
REFERENCES


12 Okazawa M, K Ishida, J Road, RR Schellenberg PD Paré. In vivo and in vitro correlation of trachealis muscle contraction in dogs. J. Appl. Physiol. 73:1486-1493, 1992


Ariens EJ, AM Simonis. A molecular basis for drug action: the interaction of one or more drugs with different receptors. J Pharm Pharmacol. 16:289-312, 1984


40 Gerthoffer WT. Agonist synergism in airway smooth muscle contraction. Pharmacol Exp Ther. 278:800-807, 1996
AIRWAY WALL REMODELLING IN PERIPHERAL AIRWAYS.

7.1 INTRODUCTION.

Obstructive airways diseases such as Chronic Obstructive Pulmonary Disease (COPD) and asthma are characterized by airflow obstruction, and by structural changes in the airway wall associated with inflammation.\(^1\) There is inflammatory cell infiltration,\(^2\) \(^3\) \(^4\) \(^5\) \(^6\) increased airway smooth muscle mass\(^7\) \(^8\) \(^9\) \(^10\) \(^11\) \(^12\) \(^13\) and abnormal thickening of the "basement membrane" due to increased deposition of extracellular matrix (ECM).\(^14\) \(^15\) This structural remodelling appears to be central to the development of poorly reversible airway obstruction and hyperresponsiveness. It is not clear, however, whether there are phenotypic changes in the smooth muscle or whether the mechanical properties or the relative content of the different constituents of the extracellular matrix are altered as a result of the remodelling process.

An increase in the force generation has been reported in isolated airway smooth muscle from patients who have asthma\(^16\) \(^17\) \(^18\) \(^19\) and COPD,\(^20\) but it is unknown whether the changes in contractile function simply result from an increase in smooth muscle mass, or from alterations in the phenotype of the muscle. Results from studies of smooth muscle cells in culture indicate that
structural remodelling of the airway wall could be associated with the contractile state of the smooth muscle. In recent studies of various types of smooth muscle, several molecular markers of ASM phenotype have been determined (Table 7.1). The cellular content of these markers has been shown to change dramatically during proliferation suggesting that smooth muscle is capable of phenotypic plasticity. The contractile phenotype has been shown to contain smooth muscle myosin heavy chain (SM-MHC), SM-α-actin, calponin, desmin, h-caldesmon, myosin light chain kinase (MLCK), and β-tropomyosin, all of which are progressively lost during proliferation while non-muscle (NM) myosins and actins (β-, γ-), l-caldesmon, vimentin, protein kinase C, and CD44 become more abundant. If a similar phenotypic change occurred with ASM hyperplasia during remodelling in chronic airways inflammation the expected result would be reduced contractile function and attenuated airway narrowing, unless the magnitude of the increased muscle mass more than compensated for the decreased force generation.

Alternatively, temporal changes in the expression and content of proteins that form the contractile apparatus could occur in vivo following recurrent episodes of inflammation and repair. A phase of inflammation with proliferation of smooth muscle could be followed by periods of recovery when such cells might redifferentiate to a contractile phenotype. In support of this idea, Halayko et al. found that serum-deprived cultured cells returned to a contractile state post confluence although they exhibited an increased content of NM-MHC. In addition some of the contractile cells were found to have a greater shortening
capacity than the original freshly isolated cells. The higher content of non-contractile proteins and hypercontractile smooth muscle cells in chronically inflamed airways could explain the increased contractility and the relative irreversibility of hyperresponsiveness found in obstructive airways diseases.

Proliferating smooth muscle cells could also be implicated in the remodelling process by their ability to synthesize ECM, causing thickening of the airway wall. Exaggerated airway narrowing could result from thickening of the tissue internal to the muscle layer, which could amplify the effect of smooth muscle shortening, or from thickening of the tissue external to the muscle layer, which could uncouple the muscle from the load provided by the surrounding parenchyma. The nature of this thickening, however, is not well understood. In a number of studies increased deposition of fibrillar collagen, and glycoproteins (fibronectin, laminin, tenascin) has been reported in asthma. Relatively little is known about changes in other ECM components such as proteoglycans. In two studies immunohistochemical staining revealed an increased deposition of versican and hyaluronan in asthma. Alterations in the composition of the ECM have not been well documented in COPD. In a few studies examination of inflammatory markers has shown an increase in fibronectin and hyaluronan in the bronchoalveolar lavage fluid of COPD patients.

The purpose of this study was to test the hypothesis that an increase in smooth muscle mass is associated with dedifferentiation of the smooth muscle, resulting in a decrease in the ratio of contractile to non-contractile proteins, and that these changes are accompanied by an increase in the deposition of
collagen and proteoglycans in the ECM. The aims of this study were to quantify the ratio of muscle (SM-MHC) to non-muscle (NM-MHC) myosin, and of \( \alpha \)-actin to total actin, and to examine the distribution of extracellular matrix components (collagen, proteoglycans and hyaluronan) in the peripheral airways of subjects who had varying degrees of airflow obstruction. Measurements of collagen and a connective tissue component that included proteoglycans and elastin were carried out by morphometry in aldehyde-fuchsin Gomori trichrome stained airways rings. Localization of hyaluronan was performed in airway rings adjacent to those used for the morphological studies. Western blotting was performed in protein homogenates extracted from additional rings in the same airways. The results of the muscle biochemistry and matrix morphometry were related to the \textit{in vitro} contractility of airway smooth muscle, to airway dimensions, and to pulmonary function measured prior to surgery.
7.2 METHODS.

7.2.1 Patients.

Airway tissue was obtained from 30 patients who had surgical resection of a lung or lobe for a peripheral pulmonary nodule at St. Paul's hospital. A patient was not included in this study if a lung lesion obstructed a segmental or larger bronchus or if pneumonitis was diagnosed before or after surgery. Subjects were studied with the approval of both the University of British Columbia and St. Paul's Hospital Ethics Committees and after obtaining informed consent of the subjects. Patients were characterized based on a detailed questionnaire of respiratory symptoms, smoking history, and allergies before surgery.

Pulmonary function tests were done prior to surgery and included the forced expiratory volume in 1s of the forced vital capacity (FEV₁), the forced vital capacity (FVC), and lung volumes. The ratio of FEV₁ to FVC (FEV₁/FVC) was used as an indicator of the severity of airflow obstruction. A value of 70% is approximately the lower limit of the normal range as defined by 95 % confidence intervals for FEV₁/FVC, and was therefore used to categorize the subjects in this study. Reversibility of bronchial obstruction was expressed as the absolute change in FEV₁ as a percentage of the predicted FEV₁ (DFEV₁ %pred) and as a percentage of the actual pre-bronchodilator FEV₁ (DFEV₁ %ini).

Table 7.2 summarizes the demographic data for the subjects in this study. Twenty-eight of the subjects had a diagnosis of bronchogenic carcinoma, and 2 had a diagnosis of carcinoid tumor. Only one patient had a history suggestive of
asthma and four had a past history of allergies. Twenty-one of the patients had been life-long smokers, 4 were ex-smokers. Pre-operative medications included sedatives (Ativan), laxatives (Docusate sodium), heparin, antibiotics (Cefazolin sodium), analgesics (acetaminophen), and in some cases beta-agonists (Ventolin, Bricaryl). Asthma and COPD were defined on clinical criteria using the definition of the American Thoracic Society.  

7.2.2 Tissue preparation.

Lung tissues were selected from a macroscopically tumor-free part of the lung and immediately placed on ice. Peripheral airways from subsegmental bronchi were isolated and dissected free of blood vessels and surrounding parenchyma. Segments were cut into airway rings and randomly selected for studies of physiology, morphology and biochemistry. The morphology studies are described in chapter 4 and the physiology studies in chapter 5. Serial sections from the airway ring processed for the morphology studies of chapter 4 were used for the immunohistochemistry studies described in this chapter. Four to six airway rings adjacent to those used for morphology and physiology were used for the biochemical studies.

7.2.3 Quantification of smooth muscle proteins.

7.2.3 (a) Protein extraction.

Freshly excised airway tissue from obstructed (n=10) and nonobstructed (n=7) was used for analysis of contractile and noncontractile protein. Tissue was
frozen in liquid nitrogen and stored at -70°C. When all airway samples were collected, the tissues were taken out of the freezer, thawed and weighed. Airway tissues were cut into small pieces (1x1mm), frozen in liquid nitrogen, and homogenized using a tissue grinder (VARI-MIX III, Caulk, Dentsply, Milford, Del.) for 30 s at high speed in extraction buffer (10 μl/mg original wet wt of tissue) which contained tris(hydroxymethyl)aminomethane (Tris, 62.5 mM, pH 6.8), 11.25% glycerol, 2% sodium dodecyl sulfate (SDS), 5% 2-mercaptoethanol, and 0.0013% bromophenol Blue. To ensure that the samples were fully solubilized and denatured, they were heated in a 95°C water bath for 12 min with periodic agitation every 2-3 min. Extracts were centrifuged at 15,000 rpm for 10 min, the supernatant was recovered in a fresh tube and the pellet was discarded. The samples were then stored at -70°C until used for electrophoresis.

7.2.3 (b) BSA protein assay.

The protein contents of all samples were determined using the Bio-Rad protein assay, which is based on the method of Bradford. This assay is used to determine the concentration of soluble protein based on the differential color change of a dye in response to various concentrations of protein. It involves adding an acidic dye to protein solution and measuring the absorbance at 595 nm with a spectrophotometer.

The protein assay is based on the observation that the maximum absorbance for an acidic solution of Coomassie brilliant blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. This shift occurs once the
anionic form of the dye becomes stabilized by hydrophobic and ionic interactions with the protein. The dye reacts mainly with arginine residues. The protein concentration is obtained by comparison to a standard curve.

7.2.3 (c) Electrophoresis.

The proteins in the samples were size fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) on 8 x 10 cm minigels, using the method developed by Laemmli. Myosin heavy chain isoforms were separated by 4% and actin isoforms by 10% polyacrylamide separating gels, with 3% stacking gels. An equal amount of protein was loaded in each lane to be compared. In preliminary experiments sample loads were optimized for best separation and visualization and found to be 2 $\mu$g/lane for $\alpha$- and total actin, 4 $\mu$g for SM-MHC, and 20 $\mu$g for NM-MHC.

The running tank buffer contained 25 mM Tris, 192mM glycine, and 0.1%SDS (pH 8.3). Electrophoresis was conducted using a constant voltage of 200 V for approximately 1h. Gels were either fixed immediately and stained with 0.1% Coomassie Blue R250 in 40% ethanol-10% acetic acid (30 min), and were used for immunoblotting studies to confirm identification of myosin heavy-chain or actin bands. Stained gels were later destained with 40% ethanol-10% acetic acid and stored.

All samples were processed at the same time for separation and identification of each contractile protein. One sample was loaded on all mini-gels to control for variability among different gels.
7.2.3 (d) Western Blotting.

Proteins separated by SDS-PAGE were electroblotted to PDVF paper under buffer solution (pH 8.3) containing 25 mM Tris, 192 mM glycine, and 20% methanol. The transfer was carried out at a constant current of ~600 mAmps, 15°C for 2 h.

7.2.3 (d-i) SM-MHC and NM-MHC.

PVDF sheets were blocked overnight at 4°C in 10 mM tris-buffered saline (pH 7.4), 0.1% Tween 20 (TBS-T) containing 3% nonfat dried milk. After three washes in TBS-T, the PVDF sheets were incubated with a primary antibody, either monoclonal mouse hSM-V anti smooth muscle myosin (Sigma Immunochemicals, St Louis, MO) diluted 1:10,000, or polyclonal anti non muscle myosin (Biogenesis) diluted 1:1,000 in 1% milk in TBS-T for 1 h at room temperature (Sigma).

The sheets were then washed 3 times in 1% milk in TBS-T and incubated for 30 min at room temperature with a secondary antibody labeled with horseradish-peroxidase, either goat anti-mouse directed against monoclonal mouse hSM-V anti SM-MHC, or goat anti-rabbit directed against polyclonal anti NM-MHC (Sigma). Both secondary antibodies were diluted 1: 2,000 in 1% milk in TBS-T.

Blots were washed in TBS-T and dipped into luminol substrate solution for detection of specific proteins by chemiluminescence (Amersham).
Chemilumigrams were developed on Hyperfilm-ECL (Amersham). The exposure times ranged between 5 s to 1 min.

7.2.3 (d-ii) Sm-α-actin and total actin.

The same protocol was used for immunoblotting the actin proteins except that the blocking solution contained 2% bovine serum albumin (BSA) instead of milk. The primary antibody used was either monoclonal mouse 1A4 anti smooth muscle α-actin (Sigma) diluted 1:2,000, or polyclonal A-2066 anti smooth muscle total actin (Sigma) diluted 1:500, both in 2%BSA in TBS-T for 2 h at room temperature. The secondary antibody labeled with horseradish-peroxidase was either goat anti-mouse (α-actin) diluted 1:4,000, or goat anti-rabbit (total actin) diluted 1: 2,000 in 2% BSA in TBS (Sigma).

7.2.4 Quantification of myosin and actin bands by densitometry.

The relative content of myosin and actin in the tissue samples was determined by quantitative densitometry using an LKB Ultrascan XL laser densitometer. The bandwidths were scanned by consecutive parallel and adjacent passes that were 800 μm each. Data for each scan were captured using LKB 2400 Gel Scan XL software. The area under each absorbency peak, corresponding to a specific protein band was quantified using Gaussian fit integration from a common baseline. This area was collected in arbitrary units (absorbance units x mm), normalized by the amount of homogenate protein
loaded per gel, and expressed as a ratio of $\alpha$-actin:total actin and SM-MHC:NM-MHC, instead of a specific amount of protein.

All values obtained were normalized to an internal control to allow comparison between samples. For quantitative estimation of proteins using SDS-PAGE gels and Western blots, errors may originate from differences in gel characteristics, transfer conditions, antibody quality, or exposure time of chemilumigrams. To minimize the contribution of such errors, all samples were processed simultaneously, and an internal control sample was run on all gels to normalize for variability of the signal from gel to gel. The densitometry value for the control sample from each blot was used as a correction factor. In the case of actin two control samples were loaded in each gel and the mean of the two densitometry values was used as the control value.

Densitometric values were also normalized by the original fresh wet weight of the tissue, and by the smooth muscle fraction in the original tissue sample (see below).

7.2.5 Measurement of smooth muscle.

Smooth muscle was measured by point counting as described in chapter 4. Freshly isolated airways were fixed in 10% formalin with no tension, embedded in paraffin, and sectioned perpendicularly to the long axis of the airway. 4µm thick sections were stained with aldehyde fuchsin Gomorl trichrome. This stain allows identification of the smooth muscle by a red color. Point counting was carried out at x20 (objective) magnification, using an unbiased
that provided an intrasubject coefficient of variation of less than 5% for the variable measured. The grid was superimposed on non-overlapping microscope fields. Measurements were made in four fields selected in each slide in a systematic random fashion.

The fractional content of smooth muscle was calculated by dividing the number of points falling on muscle by the total number of points falling on the inner wall area (WAi) defined as the area between the outer border of the muscle layer and the epithelial basement membrane. This estimate allowed calculation of protein content derived from muscle of different airways (i.e: total ASM per sample = ASM fraction x weight of airway tissue). It is assumed that shrinkage errors were relatively uniform among the tissues allowing valid comparisons.

Absolute inner wall area and total wall area (WAi) were measured by digitization (see chapter 4). Cross sections of the airways were measured at a magnification of 4x-20x (objective) using a Nikon microscope with a camera lucida (Nikon Labophot; Nikon Canada Inc., Mississauga, ON), a digitizing board (Summasketch II model MM II 1201, Summagraphics, Seymour, CT), and the Bioquant BQ system software (R&M Biometrics Inc., Nashville, TN) on an IBM compatible personal computer. The basement membrane perimeter (Pbm) was measured to assess airway size, instead of the internal perimeter, Pi which could not be measured in all of the airways due to loss of epithelium in some airways. Pi had a close correlation with Pbm, and has been shown to be relatively unaffected by smooth muscle contraction or lung inflation.38
7.2.6 Measurement extracellular matrix (ECM) components.

Extracellular matrix components were also measured by point counting aldehyde fuchsin Gomori trichrome-stained 4μm airway sections from the obstructed (n=15) and nonobstructed (n=15) patient groups. This stain effectively differentiates collagen fibers, which appear green, from proteoglycans and elastin, which appear purple. The distribution and content of these fibers was measured by point-counting using the same method described above for smooth muscle.

Measurements of ECM components were made only in the inner wall area (WAi) defined by the outer border of the smooth muscle since this is the area where most inflammatory changes have been identified by previous studies of chronically inflamed airways in COPD and asthma. The fractional area of collagen was calculated by dividing the number of points falling on collagen by the total number of points falling on the inner wall. The fraction of collagen in the inner wall area was given by \( FR_{collagen} = \frac{\# \text{points in collagen}}{\# \text{points in WAi}} \). The absolute area was then obtained by multiplying this fraction by the area of the inner wall measured by digitization. The area of collagen was thus given by \( WA_{collagen} = FR_{collagen} \times WAi \). The same procedure was used to determine the amount of elastin-proteoglycan fibers, and the amount of other connective tissue not identified by the stain.
7.2.7 Hyaluronan localization.

Airways from obstructed (n=7) and from nonobstructed (n=7) patient groups were used for localization of hyaluronan (HA). Although immunostaining was not used, the term immunohistochemistry was applied because the method is similar in principle to that described for localization of proteins, except that instead of using an antibody, a protein that binds to HA with high affinity was used. Freshly isolated airways were fixed in 10% formalin and embedded in paraffin. 4 μm serial sections were cut and collected on xylene-coated slides, dried and then prepared for localization of hyaluronan.

Slides were incubated in 10% non-immune rabbit serum for 1 hour to block non-specific binding. Next, the slides were incubated with a link protein. This link protein, referred to here as B*HABP, specifically binds to HA, and was used in place of an antibody, using a modification of a technique previously described. Sample slides were incubated in 10% B*HABP for 4 h, and washed 3 times with TBS. Bound B*HABP was detected using streptavidin-alkaline phosphatase (DAKO). This detection system is based on the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique. The streptavidin-alkaline phosphatase, diluted 1:100 in 2% BSA in TBS, was applied for 2 h at room temperature.

The color reaction was developed following the instructions of the supplier (Dakkopatts). The enzyme Naphthol As-Biphosphatase (Sigma) was dissolved in dimethylformamide (DMF), and added to a solution containing 4% NaN₃, 5% new fuchsin (Merck, Rahway, NJ), 50mM Tris buffer (pH 8.7) and 1mM levamisole, to
generate a red product. Levamisole was used to block endogenous alkaline phosphatases. The slides were incubated with this solution for 20 min and then diluted with TBS. The sections were counterstained with Mayer's hematoxylin (Merck), dehydrated, and coverslipped. The distribution and content of hyaluronan was assessed qualitatively by microscopic observation.

Negative controls included (i) preincubation of biotinylated HABP with hyaluronan, (ii) treatment of tissue sections with hyaluronidase (100 µg/µl, type IX hyaluronidase) before localization, and (iii) omission of HABP and incubation with 2%BSA in TBS, in order to demonstrate the specificity of the localization for tissue hyaluronan.

7.2.8 Statistical Analysis.

All measurements of protein contents obtained by laser densitometry were completed in triplicate from each sample. Correction factors for densitometry values were calculated for each antibody using a control sample (#399) run in each gel. For SM-MHC the mean value for the control sample from four gels was 0.956. A correction factor was calculated for each blot by dividing this mean value by the densitometry value of the control sample in that blot. In blot I the SM-MHC value for #399 was 0.31, and the correction factor was obtained by $0.956 \div 0.31 = 3.064$. Every densitometry value in blot I was then multiplied by 3.064. The same procedure was repeated for blots SM-MHC II, III, and IV, as well as for all blots obtained for NM-MHC, α- and total actin. The correction factors for SM-MHC, NM-MHC, α-actin, and total actin were respectively: blot I =
Correlations between smooth muscle phenotype (SM-MHC, NM-MHC, SM-α-actin, total actin), contractility (force, stress, shortening), and lung function (FEV₁, FEV₁/FVC, DFEV₁ %pred, DFEV₁ %ini) were carried out in the subset of 16 subjects whose airways were processed for Western blot analysis. Contractility measurements were performed in chapter 5 and a subset of those was used in this chapter. Morphometrical data including the total wall area (WAt), the inner wall area (WAi), the basement membrane perimeter (Pbm), and the smooth muscle areas were also used to normalize the data.

The relationships between extracellular matrix components, ECM, (collagen, elastin-proteoglycans, other connective tissue) and airway size (Pbm) were analyzed using multiple linear regressions. Differences in these relationships between subjects who had varying degrees of airflow obstruction were assessed by repeated measures analysis of variance (RmANOVA). The areas of ECM components were square root transformed to linearize the relationships when plotted against airway size (Pbm). In previous studies⁴² ⁴³ relationships between the square root of airway wall measurements and airway size were found to be linear. Transformation of the data to the square root provided regressions of data approximately normally distributed around the regression lines.

The intercept was examined for its linear relation to lung function parameters (FEV₁, FEV₁/FVC, DFEV₁ (%pred) and DFEV₁ (%ini)). The area of
ECM components was also expressed as a percentage of the total wall area in order to describe their distribution within the airway wall, for airways of different size. To assess differences in pulmonary function, subjects were divided into obstructed and nonobstructed groups based on an FEV₁/FVC of 70%. Differences in FEV₁, FEV₁/FVC, DFEV₁ (%pred) and DFEV₁ (%ini) were analyzed using unpaired t-tests. Differences in collagen, elastin-proteoglycans, airway smooth muscle, airway size, actin and myosin proteins between obstructed and nonobstructed patient groups were examined using unpaired t-tests. All values are expressed as means ± SD. Statistical significance was accepted at p<0.05.
7.3 RESULTS.

7.3.1 Clinical Data.

The clinical data from the 30 subjects in this study are summarized in table 7.2. Fifteen subjects had an FEV$_1$/FVC<70% and were considered to have airflow obstruction. The obstructed group had the same mean age as the nonobstructed group, but smoked more and had lower FEV$_1$ and FEV$_1$/FVC ratios. Only one patient in the obstructed group had symptoms suggestive of asthma. This patient was a 59 year old female with a history of bronchospasm from childhood, an FEV$_1$ of 39%pred, positive skin tests to a number of allergens, and a smoking history of 16 pack years. Airways from a smaller group of subjects were used for electrophoresis and Western blot analysis. Both obstructed (n=10) and nonobstructed (n=7) subsets had mean pulmonary function and anthropometric data similar to the larger group (table 7.3).

7.3.2 Distribution of airway size.

Figure 7.1 shows the frequency distribution for the airways in this study. 15 airways were obtained from obstructed and 15 from nonobstructed subjects. Airway size was determined by the basement membrane perimeter (Pbm) (See morphometry section, CH4). There was no difference in airway size between the two patient groups (obstructed = 6.9±2.8 mm; nonobstructed = 7.3±2.1 mm).
7.3.3 Contractile and non-contractile proteins in peripheral airways.

7.3.3 (a) Myosin.

Immunoblotting with MHC-specific monoclonal antibody demonstrated the identity of the SM-MHC at 200-204 kDa in isolated human peripheral airways. Figure 7.2 shows the radiograms of blots that were processed simultaneously with samples from obstructed (n=10) and nonobstructed (7) subjects. Immunoblotting revealed no staining in bands other than the 200-204 kDa range. The resolution was sufficient for complete separation of any degradation products. In some of the lanes it was possible to differentiate the two MHC isoforms at 204 (SM1) and 200 (SM2) kDa, but in most the resolution was not sufficient to allow measurement of the individual MHC isoforms. The measurement of the myosin bands, therefore, reflects the sum of the 200 and 204 kDa myosin isoforms. The sample that was obtained from the asthmatic patient did not exhibit a signal for smooth muscle or non-muscle myosin on Western blotting or for total protein on Coomassie blue-stained membranes. The protein in that sample may have degraded below a level of detection for the myosin antibodies or for Coomassie blue, so this sample was excluded from the analysis.

The non-muscle heavy chain was labeled by the non-muscle myosin antibody, and was not recognized by the smooth muscle-specific myosin antibody. NM-MHC was identified as a single lane at 196-198 kDa (Figure 7.2) and migrated with mobility slightly greater than that of SM-MHC.

Table 7.4 summarizes the mean densitometry values obtained from
chemilumigrams for SM- and NM-MHC. The amount of myosin measured by densitometry was normalized by the homogenate protein loaded onto the SDS-PAGE gels, as well as by mg of total protein in the original tissue. The normalized values for SM-MHC were significantly decreased in the obstructed group (p<0.05), but the content of NM-MHC did not differ among the two patient groups. In addition, the total amount of myosin was not different between the two groups.

The total myosin normalized by the total amount of airway smooth muscle in the original tissue sample is also shown in table 7.4. The content of smooth muscle in the tissue sample was calculated by multiplying the smooth muscle areas determined from morphometry (table 7.6) by the wet weight of the original tissues used for protein extraction and Western blotting. The amount of SM-MHC relative to total muscle content was significantly lower (p<0.05) in the obstructed group, but no difference was found in the total NM-MHC normalized by total muscle content.

The ratio of contractile (SM) to non-contractile (NM-MHC) proteins was calculated to assess relative changes in muscle proteins in chronically inflamed airways. This was carried out because differences in specific affinities of the antibodies used negate comparisons of absolute content of two different proteins in the airway samples. There was no difference between the ratio of SM to NM-MHC between obstructed and nonobstructed groups.
7.3.3 (b) Actin.

The profile of SM-α- and total actin proteins in isolated intact human airways is shown in figure 7.3. Prominent bands were visible at 43 kDa for both α-actin and total actin using SDS-PAGE and Coomassie blue staining. The mean densitometry values are shown in table 7.5. The amount of actin was also normalized by the homogenate protein loaded onto the SDS-PAGE gels, as well as by mg of total protein in the original tissue. SM-α-actin/ug was significantly reduced in the obstructed patient group (p<0.05), but there was no difference when values were normalized by mg total protein. In addition, there was no difference in the amount of total actin/ug or total actin/mg total protein between the two groups.

The ratio of α- to total actin was approximately the same in obstructed (1.3±0.6) and nonobstructed (1.5±1.5) groups.

7.3.3 (c) Ratio of actin to myosin.

Figure 7.4 shows linear relationships between SM-α-actin and SM-MHC. This relationship was present whether myosin and actin were normalized by the homogenate protein loaded onto gels (figure 7.4A) or by the total amount of total protein in the airway tissue processed for Western blotting (figure 7.4B).

7.3.4. Amount of airway smooth muscle (ASM).

The amount of ASM measured by morphometry is shown in table 7.6 for obstructed and nonobstructed subjects. The area of ASM is expressed as a
percentage of the inner wall area (WAi), was significantly increased in airways from the obstructed subjects. The values for the airways corresponding to the subset used for protein extraction and Western blotting (OB n=10; NOB n=7) were similar to those shown. In that group, except that the area in OB (0.20 ± 0.13 mm²) was twice that of NOB (0.09 ± 0.07 mm²) airways (p<0.05). The total amount of ASM in the original used for protein extraction was tissues was calculated by multiplying by the wet weight and used for normalization of SM and NM-MHC and actin (see table 7.4 and 7.5 respectively). These total ASM values were not different between OB and NOB because some of the NOB tissues were larger.

7.3.5 Correlations between airway smooth muscle (ASM) phenotype, ASM contractility, and lung function.

7.3.5 (a) In vitro contractility and lung function.

In airways from the 16 subjects used for the biochemical analysis, the maximal isometric force was significantly related to the FEV₁/FVC(%) (r=-0.77, p<0.001), to the FEV₁ (%pred) (r=-0.57, p<0.02), and to DFEV₁ (%ini) (r=0.744, p<0.001), but not to DFEV₁ (%pred). The stress was related to the FEV₁/FVC (%) (r=-0.75, p<0.001) but not to the FEV₁ (%pred) or DFEV₁ (%pred or %ini). The shortening capacity of the muscle was related only to the FEV₁ %pred (r=-0.63, p<0.01) (table 7.7 summarizes the results for FEV₁ (%pred) and FEV₁/FVC(%)).
7.3.5  (b) *In vitro* contractility and ASM phenotype.

There was no correlation between the ratio of contractile to non-contractile myosin (SM-MHC/NM-MHC) and maximal force, stress, or shortening. There was no correlation between the ratio of contractile to non-contractile actin (SM-α-actin/total actin) and maximal force, stress, or shortening.

7.3.5 (c) ASM phenotype and lung function.

There was no relationship between the ratio of contractile to non-contractile myosin (SM-MHC/NM-MHC) or actin (SM-α-actin/total actin) and any measure of pulmonary function (FEV<sub>1</sub>/FVC(%), FEV<sub>1</sub> (%pred), DFEV<sub>1</sub> (%ini), DFEV<sub>1</sub> (%pred)).

7.3.6 Distribution and content of extracellular matrix components.

7.3.6 (a) Measurement of collagen and elastin-proteoglycans.

Table 7.8 shows the mean cross sectional areas for collagen, elastin-proteoglycans, and other connective tissue measured in the inner wall. There was no difference in the amount of any of these ECM components between obstructed and nonobstructed individuals.

Collagen only occupied 6% of the total wall area in both patient groups. The elastin-proteoglycan tissue components made up a similar fraction of the total wall area (7% obstructed, 4% nonobstructed). The category for other connective tissue refers to tissue fibers that did not stain green or purple by the aldehyde fuchsin Gomori trichrome stain. This component occupied 4% of the
total wall area in both groups studied. The inner wall area constituted about 20% of the total wall area. Of the inner wall, approximately 65% was connective tissue, 20% was smooth muscle, and the remaining 15% was a mixture of blood vessels, lymphatic ducts, and empty space. The components of the outer wall were not measured in this study. Figure 7.5 shows the distribution of the various connective tissue components throughout the airway wall.

7.3.6 (b) Distribution of ECM components as a function of airway size (Pbm).

Figure 7.6 shows collagen, elastin-proteoglycans, and other connective tissue as a percentage of the total wall area. The areas of the various connective tissues, have been plotted as a function of airway size (Pbm). Collagen varied across airways of different size, especially in airways with a Pbm 5mm, where collagen ranged between 2 to 20% of WAt. Similarly, the elastin-proteoglycan (EL-PG) fraction varied considerably in airways with a Pbm 5mm (range 2 to 22%WAt). The amount of other connective tissue varied between 1 and 10% of the total wall area as a function of Pbm. The ratio of elastin-proteoglycan to collagen ranged between 0.2 to 2.0 and was not related to Pbm.

7.3.7 ECM components and lung function.

Figure 7.7 shows the areas of the ECM components as a function of airway size (Pbm). There were significantly (0.001) linear relationships between Pbm and √collagen, √elastin-proteoglycans, and √other-connective-tissue. Each of these ECM components increased as a function of airway size. The effect of
the lung function parameters, the FEV₁, FEV₁/FVC, DFEV₁(%pred) and DFEV₁ (%ini), on the intercept of the regression lines between each ECM component and Pbm was examined. The intercepts and slopes of the regressions of √collagen, √elastin-proteoglycans, and √other-connective-tissue were not related to any measure of lung function.

7.3.8 ECM components and in vitro contractility.

There was no relationship between any of the ECM components (collagen, elastin-proteoglycans, other-connective-tissue), normalized by WAt, and the in vitro contractility parameters (maximal force, stress, shortening).

7.3.9 Hyaluronan distribution.

Hyaluronan localization was identified in the airways of both obstructed (n=7) and nonobstructed individuals (n=7). Figure 7.8 shows that hyaluronan was localized in the submucosal layer (between the smooth muscle and the epithelial layer), and between and around the smooth muscle bundles. Microscopic observation indicated no apparent differences in the intensity of the distribution and staining of hyaluronan between obstructed and nonobstructed individuals.

Negative controls confirmed specific detection for hyaluronan, as only staining of the tissue by the antiserum gave results that were distinct from the control experiments (results not shown).
7.4 DISCUSSION.

7.4.1 Study population and lung function.

The purpose of this study was to examine alterations in the phenotype of the smooth muscle and the extracellular matrix within the airway wall of peripheral airways associated with changes in airflow obstruction. The subjects in this study were mostly smokers who required resection for a peripheral coin lesion. Subjects were categorized into obstructed and nonobstructed groups based on an FEV$_1$/FVC of less than 70%. The pulmonary function parameters including both FEV$_1$/FVC and FEV$_1$ were also used as continuous variables to investigate their relationships to the content of ASM proteins determined by densitometry of Western blots, the amount of collagen, elastin, and proteoglycans measured by morphometry, and the degree of in vitro contractility.

7.4.2 Smooth muscle phenotype.

An important feature of normal airway smooth muscle is its high content of thick and thin myofilaments, which form the contractile apparatus of the smooth muscle. The studies in this thesis demonstrated that both SM-MHC and actin are present in human peripheral airway smooth muscle. The smooth muscle phenotype was assessed by using specific antibodies to the muscle specific variants of contractile (SM-$\alpha$-actin, SM-myosin), and non-contractile (NM-myosin) proteins. An antibody to both muscle and non-muscle actin was used to quantify both contractile and non-contractile actins and was used to normalize the relative
changes in α-actin. The pattern of expression of myosin or actin in human airways has so far been characterized in only a few studies. In previous reports, the presence of SM-MHC, NM-MHC, and actin isoforms has been demonstrated in a variety of mammalian species using airway smooth muscle from intact tissue, airway smooth muscle cells (SMC) in culture, and vascular smooth muscle.

The separate expression of SM1 (204 kDa) and SM2 (200 kDa) SM-MHC isoforms was not characterized in this thesis. Their presence was detected in many of the blots, but the signals were not resolved clearly enough for measurement with densitometry on sufficient samples to warrant separate measurements. It is unclear whether the ratio of SM1 to SM2 is an important marker of airways remodelling. There is evidence that the SM2 isoform disappears more rapidly than the SM1 isoform in primary cultures of vascular SMC, and is only present in fully developed airways in the developing lung. It is possible that the relative content of SM1 to SM2 may be important in identifying the fully differentiated smooth muscle and assessing phenotypic remodelling. SM1 and SM2 have been demonstrated in intact human and canine airway smooth muscle, but no differences have been found in their ratio between normal and asthmatic subjects, or between control and sensitized canine tracheal smooth muscle.

The hypothesis tested in this study was that the increased smooth muscle mass observed in both COPD and asthma is associated with dedifferentiation of the smooth muscle, resulting in a decrease in the ratio of contractile to non-
contractile proteins. The results of this study do not support this hypothesis as the ratios of contractile to noncontractile proteins were not different between obstructed and nonobstructed groups. The amount of smooth muscle myosin heavy chain per ug of homogenate protein, per mg total protein, and per ASM tissue content, were found to significantly reduced in subjects with airflow obstruction. The changes in muscle myosin, however, occurred without concomitant changes in the nonmuscle proteins. The results for α-actin were rather inconsistent in that the amount α-actin per ug of homogenate protein was reduced, but not when normalized by mg total protein or by the amount of ASM. Thus, not only the myosin but also the ratio of α-actin to total was similar between the two patient groups. These findings indicate that while some changes appear to be occurring in the muscle's contractile apparatus, phenotypic remodelling defined by a shift in the ratios is not associated with airways inflammation in this group of subjects.

The observation that SM-MHC was reduced in airways from subjects who had airflow obstruction is consistent with the phenotypic remodelling reported in primary cultures of airway and vascular smooth muscle cells. These changes could occur following inflammation and repair and, like in cell culture, could result in a shift in the muscle from a contractile to a non-contractile phenotype. Halayko et al. found that in cultured airway smooth muscle cells the content of SM-MHC protein decreased rapidly before the onset of proliferation of smooth muscle cells with a concomitant increase in NM-MHC protein.

It was expected that a reduction in SM-MHC would be accompanied by an
increase in NM-MHC. The level of non contractile myosin was in fact slightly reduced, but was not significantly different between airways of obstructed and nonobstructed subjects. It is possible that changes in nonmuscle myosin may be occurring but were simply undetected by the methods used in this study. There are also several sources of variability that may have prevented identification of a clear signal in the results. These include the small number of samples, the blot to blot variation in densitometry measures, and the procedure of protein extraction. Despite these potential sources of error the ratio of contractile to noncontractile proteins should have been unaffected by them (as both numerator and denominator would have been influenced in a similar manner).

Changes in muscle but not nonmuscle myosin suggests that there may be differential expression of these two isoforms. These proteins may be under different regulatory control in vivo and the mechanisms regulating SM-MHC content during dedifferentiation may be different from those for NM-MHC. It has been postulated that in culture, loss of SM-MHC is dependent on time in culture,\(^58\) whereas an increase in NM-MHC is related to the passage of cells through mitosis.\(^59\) At postconfluence, the increase in NM-MHC is believed to be the result of an increase in protein half-life at first M phase, which subsequently remains high in culture. It is possible that in vivo NM-MHC accumulation may be lower and even undetectable once SMC cell division has taken place.

Immunoblot analysis revealed that the content of SM-\(\alpha\)-actin was present in all human peripheral airway smooth muscle. Actin is a major contractile and structural protein of smooth muscle. It is a globular protein that can polymerize to
form thin filaments of actin. Six isoforms of actin have been identified and three of these, α, β and γ have been described in tracheal smooth muscle in a ratio of 1.8:1:2. It is unknown where the relative content of these isoforms is altered in obstructive airways disease. SM-α-actin predominates in tissues that require high degree of tonic activity such as airway and vascular smooth muscle, although it is also found in other cell types such as fibroblasts, and several non-muscle cell types. γ-actin is found in tissues characterized by more phasic contractility such as intestinal smooth muscle. β-actin is noncontractile and, relative to the other actins, constitutes a significant component of the cytoplasm of smooth muscle cells.

Hence, the ratio of contractile to total actin was expected to be a sensitive indicator of phenotype shift in airway smooth muscle. Although the amount SM-α-actin per ug homogenate protein was significantly reduced in airways from the obstructed subjects, this change was not significantly different when normalized by total protein, indicating this decrease could have been related to a reduction in total protein in the original samples. The absence of changes in SM-α-actin was surprising, particularly since SM-α-actin was positively correlated with SM-MHC. In addition, the results for actin in intact tissues were expected to parallel the changes in the pattern of expression of α-actin in cultured SMC. Halayko et al. found that both the protein and the mRNA levels decreased by >70% as the SMC became proliferative in culture, using immunoblot and Northern analysis of SM-α-actin content from freshly dissociated and cultured tracheal smooth muscle cells. In the same study, this decrease in α-actin was accompanied by an
increase in $\beta, \gamma$ isoactin transcripts. Similar findings were reported by Fatigati and Murphy\textsuperscript{60} who used two-dimensional gel protein electrophoresis in swine aortic SMC and found that the content SM-$\alpha$-actin decreased with time in primary culture, while that of $\beta$- and $\gamma$-isoactin increased.

The total actin content identified by Coomassie blue staining and measured by densitometry of Western blots was not different between obstructed and nonobstructed patient groups, and the ratio of $\alpha$- to total actin was approximately the same in both. This finding was surprising since a decrease in $\alpha$-actin was expected to be accompanied by a parallel increase in the non-contractile isoforms $\beta$ and $\gamma$. An increase in the nonmuscle actins \textit{in vivo} would suggest a shift toward the expression of genes involved in basic cellular functions such as proliferation, synthesis and secretion in the presence of inflammation.

7.4.3 Relationship between airway smooth muscle phenotype, contractility, and lung function.

The interaction of myosin and actin filaments determine the contractile properties of smooth muscle.\textsuperscript{62} It is accepted that smooth muscle contraction, like skeletal and cardiac muscle, involves the sliding of thick and thin filaments, utilizing the energy supplied by the hydrolysis of ATP by the activity of myosin ATPase.\textsuperscript{63} Changes in the fractional content of these proteins could give rise to differences in the association between actin and myosin at the crossbridge and or the latch bridge level. In this study the differential expression of these proteins
was examined in relation to differences in the contractile function of the tissues, and to the degree of airflow obstruction.

The maximal contractile force was significantly correlated to the FEV1/FVC and the FEV1, the stress was significantly related to the FEV1/FVC and DFEV1, and the shortening capacity of the muscle was correlated to the FEV1. The increase in force is partially due to the increased ASM contractility and not due simply to increased ASM mass. The changes in contractility, however, were not related to the content of SM myosin or SM-α-actin, as the ratio of muscle to non-muscle proteins was unchanged. These results indicate that the increase in force and smooth muscle mass associated with airflow dysfunction in COPD is not explained by a fundamental change in the contractile apparatus. This idea is supported by the fact that normalization of isometric force by the total SM-MHC content in each airway ring still resulted in a significant increase in contractility in the obstructed group. It was expected that reduced expression at least in myosin, would result in reduced contractility. The decrease at the protein level, however, may have been masked by the greater increase in smooth muscle mass.

Alterations in smooth muscle contractility could also lie at the level of regulatory mechanisms. Previous studies have reported an increase in the content of myosin light chain kinase (MLCK) in sensitized canine airway smooth muscle in association with an increase in the velocity and capacity of shortening but not force generation. MLCK has a well established function in activating actomyosin ATPase activity by phosphorylation of the regulatory myosin light
chain thereby initiating crossbridge cycling and contraction. The study of MLCK, and other regulatory proteins such as the 17- and 20-kDa myosin light chains, the thin-filament associated proteins calponin and caldesmon, or the intermediate filament associated proteins desmin and vimentin, is outside the scope of this thesis.

7.4.4 ECM remodelling.

The distribution of extracellular matrix components was examined in the wall internal to the smooth muscle in peripheral airways of subjects who had varying degrees of airflow obstruction. The hypothesis tested was that phenotypic remodelling of the airway wall involves an increase in the deposition of collagen and proteoglycans in the ECM, and that these changes are related to airway narrowing. The results in this study revealed that the content of the connective tissue components measured in the ECM of the inner wall area were not related to the degree of airway smooth muscle contractility or to the level of airflow obstruction.

The stain used for identification and morphometry of the connective tissue was an aldehyde fuchsin Gomori trichrome that allows visualization of smooth muscle, collagen, and two tissue components that stain purple, elastin and proteoglycans. This stain was chosen because it clearly differentiates collagen from other connective tissues, although it does not allow for differentiation between elastin and proteoglycans. The tissue components staining “purple” appeared throughout the airway wall, but were primarily concentrated in the
submucosa and around the smooth muscle. This distribution is consistent with the staining pattern found in human airways, for proteoglycans, including versican, biglycan, decorin, and hyaluronan. The distribution in the various ECM components appeared to be similar in airways from obstructed and nonobstructed groups.

The results reported here for airways from COPD patients are different from those found in severe asthma. Roberts et al. reported that the matrix of thickened airway walls in tissue obtained postmortem from subjects who died from severe asthma stained intensely for versican and hyaluronan, particularly between the epithelium and the smooth muscle. Increased deposition of proteoglycans has also been shown in mild asthma. Huang et al. found an increase in lumican, biglycan, and versican in tissue obtained from lung biopsies of asthmatics, and reported that the overall content of proteoglycans measured by morphometry was correlated with airway responsiveness.

It is unclear whether changes in the content of elastin contribute to remodelling of the ECM in the airways. Current information only documents destruction of elastic fibers in the alveolar structures of emphysematous lungs. If a similar process was occurring in chronically inflamed peripheral airways, the content of elastin could be reduced. In this study it was not possible to determine the exact proportion of elastin from the stain used.

The distribution and content of hyaluronan in peripheral airways was also examined in this study. Specific staining for hyaluronan revealed that it was localized in the submucosal layer, between and around the smooth muscle
bundles. Microscopic observation, however, revealed no apparent differences in the distribution and intensity of the staining between obstructed and nonobstructed individuals. This finding was consistent with the results obtained for the elastin-proteoglycan connective tissue component. These results, however, are not supported by reports that the content of HA is increased in the bronchoalveolar lavage of COPD patients,\textsuperscript{29} \textsuperscript{30} \textsuperscript{31} and that HA is correlated with the FEV1/FVC.\textsuperscript{29}

Another ECM component measured in this study was collagen. In this study the collagen content of the peripheral airways was examined in order to determine whether alterations in its content or distribution in the airway wall are occurring in chronically inflamed airways. Morphometrical analysis revealed no differences in the amount of collagen in the airways of obstructed and nonobstructed subjects. Different collagen types were not characterized, however, and it is possible that while the overall collagen content remained unchanged, differences in the relative abundance of collagen fibrils could occur. For instance, Roche et al.\textsuperscript{14} demonstrated that in asthma the thickened subepithelial basement membrane is composed of increased deposition of collagen types III and V, which appear to be more densely packed than normal. Thickening of this layer and increased fibril density could stiffen this layer, provide it with greater tensile strength and make it more resistant to compression. The potential consequences of these changes, including alterations in the mechanical properties of the matrix are discussed below.
7.4.5 Functional consequences of ECM remodelling.

The morphometrical analysis of airway dimensions revealed an increase in the smooth muscle layer but no differences in the inner wall area between obstructed and nonobstructed groups. These results suggest that in obstructed subjects airway narrowing does not result from a thickened inner wall that would amplify the effect of smooth muscle shortening on airway caliber by encroaching into the lumen.

In this study no alterations were found in the content of collagen or the connective tissue that contained proteoglycans in the airways of obstructed subjects. In addition, there was no correlation between the ECM components and any measure of pulmonary function, indicating that airway narrowing cannot be explained by changes in the composition of the matrix in this group of subjects. Some investigators have speculated that increased proteoglycan deposition in the submucosa could lead to increased tissue turgor and increased resistance to deformation. Similarly, it has been postulated that alterations in the composition of collagen fibrils could result in stiffening of the airway, making it resistant to compression and tension, leading to attenuation of airway narrowing. Although no changes were found in the content of elastin in COPD patients, elastolytic changes in the ECM have been associated with reduced elastance and increased smooth muscle shortening in tissues obtained from asthmatic subjects.

The extent to which airway narrowing occurs will ultimately depend on the balance between the force generating ability of the muscle and the elastic loads.
on the muscle provided by the parenchyma and the stiffness of the airway wall.

The results from this study indicate that changes in the ECM are not prominent in chronically inflamed peripheral airways, are not related to in vitro contractility, and do not contribute to the severity of airflow obstruction in this group of subjects. Other factors such as increased smooth muscle mass must have a greater impact in causing airway narrowing.
Table 7.1

PHENOTYPIC MARKERS IN AIRWAY SMOOTH MUSCLE

<table>
<thead>
<tr>
<th>Contractile phenotype</th>
<th>Proliferative phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM-myosin heavy chain (SM1,SM2)</td>
<td>NM-myosin heavy chain</td>
</tr>
<tr>
<td>ASM-α-Actin</td>
<td>NM (β- γ-) actin</td>
</tr>
<tr>
<td>Desmin</td>
<td>Vimentin</td>
</tr>
<tr>
<td>h-Caldesmon</td>
<td>l-Caldesmon</td>
</tr>
<tr>
<td>MLCK</td>
<td>PKC</td>
</tr>
<tr>
<td>Tropomyosin</td>
<td>CD44</td>
</tr>
<tr>
<td>Calponin</td>
<td></td>
</tr>
</tbody>
</table>

Markers of airway smooth muscle phenotype. The content of these proteins has been shown to change during proliferation of smooth muscle cells in culture, suggesting that smooth muscle is capable of phenotypic plasticity. As a result of inflammation a phenotypic change could be associated with progressive loss of contractile proteins while the non-muscle proteins would become more abundant.
### Table 7.2

CLINICAL DATA

<table>
<thead>
<tr>
<th>PATIENT CHARACTERISTICS</th>
<th>OBSTRUCTED n=15</th>
<th>NONOBSTRUCTED n=15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yrs</td>
<td>62±8</td>
<td>62±12</td>
</tr>
<tr>
<td>Sex, female: male</td>
<td>6:9</td>
<td>6:9</td>
</tr>
<tr>
<td>Current smokers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pack years</td>
<td>54±23</td>
<td>30±15</td>
</tr>
<tr>
<td>lung resected, right, n: left, n</td>
<td>9:6</td>
<td>6:9</td>
</tr>
<tr>
<td>FEV₁, %pred</td>
<td>65 ± 20</td>
<td>90 ± 10</td>
</tr>
<tr>
<td>FVC , %pred</td>
<td>84 ± 22</td>
<td>93 ± 12</td>
</tr>
<tr>
<td>FEV₁/FVC , %</td>
<td>60 ± 8</td>
<td>77 ± 4</td>
</tr>
<tr>
<td>TLC , %pred</td>
<td>112 ± 17*</td>
<td>98 ± 14</td>
</tr>
<tr>
<td>DFEV₁, %pred</td>
<td>6 ± 5†</td>
<td>3 ± 3</td>
</tr>
<tr>
<td>DFEV₁, %ini</td>
<td>12 ± 12†</td>
<td>3 ± 3</td>
</tr>
</tbody>
</table>

Pack years= the number of years smoking one pack of cigarettes per day. 
n=number. FEV₁= forced expiratory volume in 1s of the forced vital capacity; 
FVC= forced vital capacity; FEV₁/FVC= forced expiratory volume in 1 s as a 
percentage of the forced vital capacity; FEF₅₀= forced expiratory flow at 50% of 
the forced vital capacity; FEF₂₅-₇₅= forced expiratory flow between 25 to 75 % of 
the forced vital capacity; TLC= total lung capacity; DFEV₁,%pred=change in 
FEV₁ as a percentage of the predicted FEV₁; DFEV₁,%ini=change in FEV₁ as a 
percentage of the actual pre-bronchodilator FEV₁ after inhalation of salbutamol. 
TLC was significantly increased (*p<0.05) in the obstructed group, as was 
DFEV₁,%pred and DFEV₁ %ini (†p<0.05)
Table 7.3

CLINICAL DATA FOR SUBJECTS USED IN WESTERN BLOT ANALYSIS

<table>
<thead>
<tr>
<th>PATIENT CHARACTERISTICS</th>
<th>OBSTRUCTED n=10</th>
<th>NONOBSTRUCTED n=7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yrs</td>
<td>62±9</td>
<td>64±10</td>
</tr>
<tr>
<td>Sex, female:male</td>
<td>4:6</td>
<td>2:5</td>
</tr>
<tr>
<td>Current smokers</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Pack years†</td>
<td>54±29</td>
<td>32±12</td>
</tr>
<tr>
<td>Lung resected, right, n: left, n</td>
<td>6:4</td>
<td>3:4</td>
</tr>
<tr>
<td>FEV₁, %pred</td>
<td>63 ± 20</td>
<td>90 ± 13</td>
</tr>
<tr>
<td>FVC , %pred</td>
<td>84 ± 27</td>
<td>92 ± 15</td>
</tr>
<tr>
<td>FEV₁/FVC , %</td>
<td>60 ± 9</td>
<td>77 ± 4</td>
</tr>
<tr>
<td>TLC , %pred</td>
<td>111 ± 20</td>
<td>99 ± 14</td>
</tr>
<tr>
<td>DFEV₁, %pred</td>
<td>7 ± 5</td>
<td>3 ± 3</td>
</tr>
<tr>
<td>DFEV₁, %ini</td>
<td>13 ± 12</td>
<td>4 ± 3</td>
</tr>
</tbody>
</table>

† Pack years = the number of years smoking one pack of cigarettes per day. n=number. FEV₁ = forced expiratory volume in 1s of the forced vital capacity; FVC= forced vital capacity; FEV₁/FVC= forced expiratory volume in 1 s as a percentage of the forced vital capacity; FEF₅₀= forced expiratory flow at 50% of the forced vital capacity; FEF₂₅-₇₅= forced expiratory flow between 25 to 75 % of the forced vital capacity; TLC= total lung capacity; DFEV₁,%pred=change in FEV₁ as a percentage of the predicted FEV₁; DFEV₁,%ini=change in FEV₁ as a percentage of the actual pre-bronchodilator FEV₁ after inhalation of salbutamol.
Table 7.4

MYOSIN DETERMINED BY WESTERN BLOT ANALYSIS

<table>
<thead>
<tr>
<th></th>
<th>OB MEAN ± SD</th>
<th>NOB MEAN ± SD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Airway diameter (mm)</td>
<td>2.6±0.9</td>
<td>2.3±0.7</td>
<td>NS</td>
</tr>
<tr>
<td>Total protein (mg)</td>
<td>2.6 ± 1.2</td>
<td>2.8 ± 2.0</td>
<td>NS</td>
</tr>
<tr>
<td>Total SM-MHC (mg)</td>
<td>0.70 ± 0.70</td>
<td>2.0 ± 1.5</td>
<td>NS</td>
</tr>
<tr>
<td>SM-MHC / μg</td>
<td>0.02 ± 0.02*</td>
<td>0.04 ± 0.03</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>SM-MHC /mg total protein</td>
<td>0.15 ± 0.15*</td>
<td>0.40 ± 0.40</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Total SM-MHC / total ASM</td>
<td>0.23±0.30*</td>
<td>0.44±0.30</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Total NM-MHC (mg)</td>
<td>1.5 ± 1.2</td>
<td>2.8 ± 2.8</td>
<td>NS</td>
</tr>
<tr>
<td>NM-MHC / μg</td>
<td>0.03 ± 0.03</td>
<td>0.05 ± 0.03</td>
<td>NS</td>
</tr>
<tr>
<td>NM-MHC /mg total protein</td>
<td>0.30 ± 0.30</td>
<td>0.43 ± 0.40</td>
<td>NS</td>
</tr>
<tr>
<td>Total NM-MHC / total ASM</td>
<td>0.50±0.30</td>
<td>0.70±0.30</td>
<td>NS</td>
</tr>
<tr>
<td>SM-MHC:NM-MHC</td>
<td>0.60 ± 0.60</td>
<td>0.90 ± 0.40</td>
<td>NS</td>
</tr>
</tbody>
</table>

Mean densitometry results for myosin in airways obtained from obstructed (OB, n=9) and nonobstructed (NOB, n=7) patient groups. Values for smooth muscle (SM) and non-muscle (NM) myosin are expressed in arbitrary units normalized by the amount of crude homogenate protein (μg) that was loaded onto gels for Western blot analysis. Values were also normalized by mg total protein, and by the amount of airway smooth muscle (ASM) in the original tissue sample:

Total SM-MHC = SM-MHC x total protein extracted (same calculation was carried out for NM-MHC).

Total ASM = ASM/WAt x wet tissue weight (OB=3.2 ± 1.2, NOB=4.0 ± 3.5 mg).

Note that these values show there is no difference in the amount of muscle in the original tissue extracted. In fact, the actual amount of muscle normalized by weight and airway size was significantly higher in the OB subjects. See table 7.6. The weight of the tissues from NOB airways were higher.

*SM-MHC was significantly reduced in airways from OB patients. The ratio of SM to NM myosin, used as a marker of phenotypic change, was not significantly different between OB and NOB.
<table>
<thead>
<tr>
<th></th>
<th>OB MEAN ± SD</th>
<th>NOB MEAN ± SD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Airway diameter (mm)</td>
<td>2.6±0.9</td>
<td>2.3±0.7</td>
<td>NS</td>
</tr>
<tr>
<td>Total protein (mg)</td>
<td>2.6 ± 1.2</td>
<td>2.8 ± 2.0</td>
<td>NS</td>
</tr>
<tr>
<td>Total SM-α-actin (mg)</td>
<td>2.1 ± 1.2</td>
<td>3.3 ± 2.5</td>
<td>NS</td>
</tr>
<tr>
<td>SM-α-actin / µg</td>
<td>0.44 ± 0.10*</td>
<td>0.60 ± 0.10</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>SM-α-actin /mg total protein</td>
<td>0.50 ± 0.30</td>
<td>0.60 ± 0.30</td>
<td>NS</td>
</tr>
<tr>
<td>Total SM-α-actin / total ASM</td>
<td>0.80 ± 0.50</td>
<td>0.90 ± 0.50</td>
<td>NS</td>
</tr>
<tr>
<td>Total actin /µg</td>
<td>0.42 ± 0.20</td>
<td>0.63 ± 0.30</td>
<td>NS</td>
</tr>
<tr>
<td>Total actin /mg total protein</td>
<td>0.44 ± 0.30</td>
<td>0.60 ± 0.45</td>
<td>NS</td>
</tr>
<tr>
<td>SM-α-actin: Total actin</td>
<td>1.30 ± 0.60</td>
<td>1.50 ± 1.50</td>
<td>NS</td>
</tr>
</tbody>
</table>

Mean densitometry results for actin in airways obtained from obstructed (OB, n=9) and nonobstructed (NOB, n=7) patient groups. Values for smooth muscle (SM) α-actin and total actin are expressed in arbitrary units normalized by the amount of crude homogenate protein (µg) that was loaded onto gels for Western blot analysis. Values for SM α-actin were also normalized by mg total protein and by the amount of airway smooth muscle in the original tissue sample. Total actin, which refers to both contractile and noncontractile isoforms, was normalized by mg total protein.

Total SM-α-actin = SM-α-actin × total protein extracted
Total ASM = ASM/WAt × wet tissue weight (OB=3.2 ± 1.2, NOB=4.0 ± 3.5 mg).

*SM-α-actin was significantly reduced in airways from OB patients, but the difference was lost when normalized by the total protein and by the total amount of ASM.

The ratio of SM-α-actin to total actin, used as marker of phenotypic change, was not significantly different between OB and NOB.
Table 7.6

ASM IN THE INNER WALL

<table>
<thead>
<tr>
<th></th>
<th>OB Mean ± SD</th>
<th>NOB Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basement membrane perimeter, Pbm (mm)</td>
<td>6.9 ± 2.8</td>
<td>7.3 ± 2.1</td>
</tr>
<tr>
<td>Inner wall area, WAi (mm²)</td>
<td>0.76 ± 0.5</td>
<td>0.80 ± 0.6</td>
</tr>
<tr>
<td>Total wall area, WAt (mm²)</td>
<td>4.0 ± 3.0</td>
<td>4.0 ± 2.0</td>
</tr>
<tr>
<td>Airway smooth muscle area (mm²)</td>
<td>0.16 ± 0.12</td>
<td>0.12 ± 0.11</td>
</tr>
<tr>
<td>Airway smooth muscle area %WAi</td>
<td>21.0 ± 5.0*</td>
<td>15.0 ± 3.0</td>
</tr>
<tr>
<td>Airway smooth muscle area %WAt</td>
<td>6.0 ± 4.0</td>
<td>3.0 ± 3.0</td>
</tr>
<tr>
<td>Airway smooth muscle area /Pbm</td>
<td>2.2 ± 1.0*</td>
<td>1.6 ± 1.0</td>
</tr>
</tbody>
</table>

The amount of airway smooth muscle (ASM) measured in airways from obstructed (OB=15) and nonobstructed (NOB=15) subjects. The area of smooth muscle was measured by morphometry in the inner wall (WAi) of peripheral airways. Values are expressed as mm², and as a percentage of the inner wall area, the total wall area (WAt). Values were also normalized by the basement membrane perimeter (Pbm).

*The amount of ASM expressed as a percentage of the WAi, and normalized by Pbm were significantly higher in the OB group (p<0.05).

The values for smooth muscle in airways from the subset of subjects used for biochemistry (OB, n=10; NOB, n=7) were similar to those shown above, but the area of smooth muscle was significantly higher in the obstructed group (i.e.: ASM area in OB=0.20 ± 0.13 versus NOB=0.09 ± 0.07 mm²) (p<0.05).
Table 7.7

CORRELATIONS BETWEEN LUNG FUNCTION AND IN VITRO CONTRACTILITY

<table>
<thead>
<tr>
<th></th>
<th>FEV₁ (%pred)</th>
<th>FEV₁/FVC(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r value</td>
<td>p&lt;</td>
</tr>
<tr>
<td>Maximal isometric force, Fmax, g</td>
<td>-0.572</td>
<td>0.02</td>
</tr>
<tr>
<td>Maximal isometric stress, mN/mm²</td>
<td>-0.151</td>
<td>NS</td>
</tr>
<tr>
<td>Maximal isotonic shortening, % Li</td>
<td>-0.625</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Correlations between pulmonary function and in vitro contractility are shown for a total of 16 subjects. Correlation coefficients and the corresponding p values are shown. FEV₁ = forced expiratory volume in 1 s of the forced vital capacity. FEV₁/FVC = forced expiratory volume in 1 s as a percentage of the forced vital capacity (FVC). In vitro contractility parameters were obtained from complete length tension curves measured by myography (see chapter 5). Maximal isometric force (Fmax) and isotonic shortening (% initial length, Li) were obtained in response to ACh (10⁻⁵ M). The stress is Fmax normalized by the cross-sectional area of smooth muscle measured by morphometry.
Table 7.8

ECM COMPONENTS IN THE INNER WALL

<table>
<thead>
<tr>
<th>Component</th>
<th>OB Mean ± SD</th>
<th>NOB Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basement membrane perimeter, Pbm (mm)</td>
<td>6.9 ± 2.8</td>
<td>7.3 ± 2.1</td>
</tr>
<tr>
<td>Inner wall area, WAi (mm²)</td>
<td>0.76 ± 0.5</td>
<td>0.80 ± 0.6</td>
</tr>
<tr>
<td>Total wall area, WAt (mm²)</td>
<td>4.0 ± 3.0</td>
<td>4.0 ± 2.0</td>
</tr>
<tr>
<td>Wall area collagen (mm²)</td>
<td>0.16 ± 0.11</td>
<td>0.25 ± 0.26</td>
</tr>
<tr>
<td>Wall area collagen %WAi</td>
<td>22.0 ± 6.0</td>
<td>28.0 ± 8.0</td>
</tr>
<tr>
<td>Wall area collagen %WAt</td>
<td>6.0 ± 5.0</td>
<td>6.0 ± 4.0</td>
</tr>
<tr>
<td>Wall area collagen /Pbm</td>
<td>0.02 ± 0.01</td>
<td>0.03 ± 0.02</td>
</tr>
<tr>
<td>Wall area elastin-PG (mm²)</td>
<td>0.18 ± 0.11</td>
<td>0.15 ± 0.08</td>
</tr>
<tr>
<td>Wall area elastin-PG %WAi</td>
<td>25.0 ± 7.0</td>
<td>23.0 ± 8.0</td>
</tr>
<tr>
<td>Wall area elastin-PG %WAt</td>
<td>7.0 ± 5.0</td>
<td>4.0 ± 2.0</td>
</tr>
<tr>
<td>Wall area elastin-PG /Pbm</td>
<td>0.02 ± 0.01</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>Wall area other CT (mm²)</td>
<td>0.14 ± 0.13</td>
<td>0.15 ± 0.13</td>
</tr>
<tr>
<td>Wall area other CT %WAi</td>
<td>17.0 ± 6.0</td>
<td>18.0 ± 7.0</td>
</tr>
<tr>
<td>Wall area other CT %WAt</td>
<td>4.0 ± 3.0</td>
<td>4.0 ± 2.0</td>
</tr>
<tr>
<td>Wall area other CT /Pbm</td>
<td>0.02 ± 0.01</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>Wall area all CT (mm²)</td>
<td>0.48 ± 0.30</td>
<td>0.55 ± 0.44</td>
</tr>
<tr>
<td>Wall area all CT %WAi</td>
<td>64.0 ± 7.0</td>
<td>69.0 ± 4.0</td>
</tr>
<tr>
<td>Wall area all CT %WAt</td>
<td>17.0 ± 12.0</td>
<td>14.0 ± 7.0</td>
</tr>
<tr>
<td>Wall area all CT /Pbm</td>
<td>0.07 ± 0.01</td>
<td>0.07 ± 0.01</td>
</tr>
</tbody>
</table>

OB= Obstructed patient group; NOB= Nonobstructed patient group. 15 airways were studied in each group. Components of the extracellular matrix (ECM) measured within the inner wall area (WAi), internal to the smooth muscle layer. PG=proteoglycans; CT=connective tissue; Other-CT=other connective tissue. Areas were obtained by morphometry. Values are expressed as mm², and as a percentage of the inner wall area, the total wall area (WAt). Values were also normalized by the basement membrane perimeter (Pbm).
Figure 7.1. Frequency distribution of peripheral airway size for obstructed (n=15) and nonobstructed (n=15) patient groups.
Figure 7.2. Profile of smooth muscle (SM) and non-muscle (NM) MHC obtained by Western blot analysis of smooth muscle from peripheral airways of obstructed (n=7) and non-obstructed patients (n=10). Sample 2434 was used as an internal control. A molecular weight standard (MWS) was loaded in all gels but was not biotinylated and not detected by chemiluminescence.
Figure 7.3. Profile of smooth muscle (SM) α-actin and total actin obtained by Western blot analysis of tissue from peripheral airways of obstructed (n=10) and nonobstructed patients (n=7). Sample 399 was used as an internal control. A molecular weight standard (MWS) was loaded in all gels but was not biotinylated and not detected by chemiluminescence.
Figure 7.4. Correlations between SM-α(alpha)-actin and SM-MHC in airways from obstructed (n=9) and nonobstructed (n=7) patients. A. SM-α-actin versus SM-MHC, normalized by the amount of protein loaded onto gels for Western blot analysis. B. SM-α-actin versus SM-MHC normalized by the total protein extracted from each original tissue sample.
Figure 7.5 Photomicrographs of airway cross-sections stained with an aldehyde fuchsin Gomori trichrome stain from an obstructed and a nonobstructed subject. This stain effectively differentiates collagen fibers, which appear green, from elastin and proteoglycans which appear purple. Collagen, proteoglycans and elastin are distributed throughout the airway wall. Magnifications are shown at x2 (A,B) x10 (C,D), and x20 (objective lens) (E,F).
Figure 7.6. Connective tissue components of the inner wall area, expressed as a percentage of the total wall area (WAt), versus the basement membrane perimeter (Pbm). A. Collagen. B. Connective tissue composed of elastin and proteoglycans (EL-PG). C. Other connective tissue (CT). N=30 airways.
Figure 7.7. Wall area (WA) connective tissue (CT) components versus basement membrane perimeter (Pbm). A. Elastin and proteoglycans (EL-PG). B. Collagen (coll). C. Other (oth-CT). N=30. Note square root transformed y-axes. There were significantly linear relationships between the ECM components and Pbm, but the intercept and slopes of the regressions were not related to pulmonary function.
Figure 7.8. Photomicrographs of airway cross-sections showing localization of hyaluronan. Hyaluronan was found in the submucosal layer (between the smooth muscle and the epithelial layer), and between and around the smooth muscle bundles. There were no apparent differences in the intensity of the distribution and staining of hyaluronan between obstructed and non-obstructed individuals. (The airway of the obstructed subject was split in the fixation process).
REFERENCES.


19 Bramley AM, RJ.Thomson, CR Roberts, RR Schellenberg. Hypothesis: Excessive bronchoconstriction in asthma is due to decreased elastance. Eur J Respir Dis. 7:337-341, 1994


23 Hirst SJ. Airway smooth muscle cell culture: Application to studies of airway wall remodelling and phenotype plasticity in asthma. Eur Resp J. 9:808-820, 1996


240


40 Ripellino JA, MM Klinger, RU Morgolis. The hyaluronic acid binding region as a specific probe for the localization of hyaluronic acid in tissue section. J Histochem Cytochem. 33:1060-66, 1985


45 Chamley-Campbell JH, GR Campbell, and R Ross. The smooth muscle cell in culture. Physiol Rev. 59:1-61, 1979


Chapter 8

Summary of results and general discussion.

Human obstructive airways diseases such as asthma and chronic obstructive pulmonary disease (COPD) are characterized by increased airflow obstruction and exaggerated airway narrowing, or hyperresponsiveness. In addition, there are structural changes in the airway wall associated with chronic inflammation in both asthma\textsuperscript{1,2,3,4} and COPD\textsuperscript{5,6,7,8,9}. The degree to which these changes are related to airflow obstruction and hyperresponsiveness is not completely understood.

In the studies carried out in this thesis the structural and functional changes of peripheral airways obtained from human lungs of subjects who had varying degrees of airflow obstruction were examined. The patients were categorized into an obstructed (OB) and nonobstructed (NOB) group based on the ratio of FEV\textsubscript{1} to FVC. An FEV\textsubscript{1}/FVC of less than 70% was used to define airflow obstruction. Measurements of FEV\textsubscript{1}/FVC (%) and FEV\textsubscript{1} (% predicted) were also used as continuous variables to examine their relationships to airway smooth muscle force, stress and shortening. The majority of the airways were cartilaginous and ranged in size between 2 and 3 mm in diameter. Airways from individuals who had more severe airflow obstruction had increased smooth muscle mass and increased \textit{in vitro} force generating ability. In addition
ASM exhibited abnormal intrinsic tone that had an effect on the ability of the airways to generate force and to shorten. These changes in contractility were not accompanied by alterations in the content of contractile and noncontractile proteins, or by the content of connective tissue surrounding the muscle. These structure function data, in addition to the findings of other investigators, provide a basis for further examination of the role of ASM in the pathophysiology of obstructive airways disease.

It has been reported in a few studies that abnormalities in lung function are related to changes in airway dimensions in subjects who have COPD and asthma. In Chapter 4, measurements were made to determine whether alterations in airway dimensions have an effect on airflow obstruction. Thickening of the smooth muscle layer was related to several pulmonary function parameters, including the FEV₁, FEV₁/FVC, FEF₂₅₋₇₅ and the change in FEV₁%pred after inhalation of bronchodilator. The finding that thickening of the smooth muscle area was related to the degree of airflow obstruction supports the hypothesis in this study that an increase in smooth muscle mass in peripheral airways leads to exaggerated airway narrowing and airflow obstruction.

The role of ASM mass in airflow obstruction is supported by the mathematical models of Lambert et al. 1993 and Wiggs 1990. Results from these studies indicate that much of the resistance to airflow is due to contraction of smooth muscle in small (ID <1-3 mm) peripheral airways, and that increased airway resistance can result from thickening of the subepithelial connective tissue, the smooth muscle, and the adventitial layers. Of these, the
increase in smooth muscle mass is likely to be the most important alteration responsible for increased airway resistance in response to bronchoconstricting stimuli in asthma and COPD.\textsuperscript{15}

An increase in muscle mass would not necessarily lead to exaggerated airway narrowing, since the presence of elastic loads provided by the lung recoil and the intrinsic stiffening of the airway wall could act to impede smooth muscle shortening. Airway resistance is likely determined by the balance between the force generated by the muscle and the elastic loads against which the muscle is working. Increased smooth muscle mass could produce more force, more smooth muscle shortening, and increase baseline resistance and maximal airway resistance. Alternatively, decreased load on the muscle or airway wall thickening (even in the presence of normal ASM shortening) could also lead to excessive airway narrowing.

The relationship between airflow obstruction and abnormalities in ASM contractility were explored in Chapter 5. The aims of the studies were to characterize the passive and active mechanical properties of peripheral airways from the lungs of normal subjects and patients who had COPD and asthma, and to relate \textit{in vitro} contractility to pulmonary function measured before surgery. The findings support the hypothesis that altered ASM contractility and airway smooth muscle mass contribute to the pathogenesis of obstructive airways disease. Significant correlations were found between the amount of force generated and several lung function parameters (FEV\textsubscript{1} and FEV\textsubscript{1}/FVC). In addition, when the force was normalized by the amount of smooth muscle, the stress was still
significantly higher in obstructed subjects, indicating that the increase in force is not only due to an increase in ASM mass but also to an increase in contractility.

These relationships between *in vitro* ASM contractility and airflow obstruction were carried out based on the concept that airway narrowing results from a balance of static forces which sets airway length and ultimately airway caliber. In contrast with this idea, it is recognized that tidal breathing is a dynamic event, and with each tidal breath the smooth muscle is stretched. One possible consequence of stretching and then releasing the constricted smooth muscle is that force generation may be reduced,\textsuperscript{16, 17} due to intracellular remodelling of the contractile apparatus,\textsuperscript{18} or due to changes in its insertion within the cytoskeleton\textsuperscript{19} during inspiration. In addition, stretching of the constricted muscle could allow actin and myosin filaments to slip relative to each other and reverse the mechanism that produced muscle contraction before the stretch.\textsuperscript{20}

At present it is unknown whether constricted ASM from chronically-inflamed airways responds to stretch by a reduction in its capacity to generate force. In some studies the effect of the deep inspiration on the reversal of bronchoconstriction has been assessed.\textsuperscript{21} Skloot and colleagues\textsuperscript{21} found that a deep inspiration substantially reduced the bronchoconstrictor responsiveness induced by methacholine inhalation in normal human subjects, but not in asthmatic subjects. This differential response by asthmatics suggests the possibility that alterations exist in chronically inflamed airways either in the smooth muscle, a contention supported by the results of this thesis, or on the
loads on the muscle such as those provided by the parenchyma. The studies in this thesis were not designed to assess the consequences of the dynamic changes on ASM force generation *in vitro*.

Despite changes in force, the maximal isotonic shortening was not altered in airways from subjects who had greater airflow obstruction. This was surprising in light of the observation that there were positive correlations between stress and shortening. An increase in force without a significant increase in shortening is probably related to differences in the loads acting on the smooth muscle. Although not significant, the passive tension at Lmax (expressed as %Fmax), tended to be lower in the obstructed individuals by ~20%, and this reduction in passive preload could have influenced the subsequent isotonic shortening. Since preloaded shortening was measured, the preload required to stretch the muscle to Lmax remained during shortening and constituted an elastic, or auxotonic load.

An additional load on smooth muscle is that provided by the radial constraint of the connective tissue surrounding the muscle. Meiss\textsuperscript{22} showed that if canine smooth muscle preparations, which normally shorten ~75%, are constrained by nonextensible silastic rings shortening is reduced. The connective tissue content of human peripheral airways is much higher than the relatively pure smooth muscle of canine trachealis and it is possible that this connective tissue radially constrains the ASM and reduces its ability to shorten. The amount of connective tissue surrounding individual myocytes was not measured in this thesis. It is possible that differences exist between individuals who have different
degrees of airflow obstruction. Nonetheless, the high passive tension required to stretch the muscle to Lmax is a reflection of the high connective tissue content of the human airways and the resultant radial constraint could explain the relatively lesser shortening in the human tissue as well as the failure to find a significant difference in maximal shortening despite differences in force and stress between obstructed and nonobstructed individuals.

The nature of the resting tension, which consists of the passive tension plus the active "intrinsic tone" of airway smooth muscle, was investigated in Chapter 6. Specifically, the focus of the studies was to quantify the amount of intrinsic tone and to assess its role in influencing the isometric force generation and isotonic shortening. The results of this study demonstrated that intrinsic tone was present in isolated peripheral airways, and that in nonobstructed subjects tone was due primarily to cysteinyl-leukotriene receptor stimulation with no contribution by prostaglandins. These results are consistent with those of previous studies demonstrating the existence of intrinsic tone in isolated human bronchi. The hypothesis that intrinsic tone would be altered in airways from obstructed subjects, and that these changes would affect ASM force and shortening, was supported by the results. MK886, a leukotriene biosynthesis inhibitor, had no effect on intrinsic tone in the obstructed group and ASM contractile responses were depressed in contrast to the nonobstructed subjects. Enhanced force and shortening in airways from the NOB group could be due reduced intrinsic tone and more 'available tone' for muscle contraction. In airways from OB tissues the high level of tone, which did not change with
MK886, would limit shortening, and could be part of a protective mechanism to prevent excessive airway narrowing.

Alterations in the functional properties of ASM could also be due to a fundamental change in the contractile apparatus. In the investigations carried out in Chapter 7, alterations in the phenotype of the smooth muscle were examined in the airway wall of peripheral airways associated with changes in airflow obstruction. The purpose of this study was to test the hypothesis that an increase in smooth muscle mass is associated with dedifferentiation of the smooth muscle, resulting in a decrease in the ratio of contractile to non-contractile proteins. The smooth muscle phenotype was assessed by the ratio of muscle (SM-MHC) to non-muscle (NM-MHC) myosin, and of $\alpha$-actin to total actin. In studies of smooth muscle cells in culture structural remodelling of the airway wall has been associated with the contractile state of the smooth muscle and several molecular markers of ASM phenotype have been determined (Table 7.1). The cellular content of these markers has been shown to change dramatically during proliferation suggesting that smooth muscle is capable of phenotypic plasticity. The results of this study do not support the hypothesis tested as neither ratio was different between obstructed and nonobstructed subjects. Although SM-MHC was significantly reduced in subjects with airflow obstruction, the ratio of SM to NM-MHC was not altered. Similarly, the ratio of $\alpha$-actin to total was similar between the two patient groups. These findings indicate that while some changes appear to be occurring in the muscle's contractile apparatus, ASM phenotypic remodelling defined by a shift in the
ratios is not associated with increased ASM mass, or increased contractility in this group of subjects.

The content and distribution of extracellular matrix components were also examined in Chapter 7. The hypothesis tested was that phenotypic remodelling of the airway wall involves an increase in the deposition of collagen and proteoglycans in the ECM, and that these changes are related to airway narrowing. The results in this study revealed that the content of the connective tissue components measured in the ECM of the inner wall area were not related to the degree of airway smooth muscle contractility or to the level of airflow obstruction. These results are similar to those reported in Chapter 4 in that the total inner and outer wall areas were not significantly thickened in airways from severely obstructed subjects.

The lack of a difference in airway wall thickening between OB and NOB subjects was surprising since deposition of connective tissue during the repair stage of inflammation could thicken the airway wall, including the smooth muscle layer, and narrow the airway. It has been suggested that exaggerated airway narrowing results from airway wall thickening acting in series with normal smooth muscle shortening. According to this postulate, airway wall thickening contributes to exaggerated airway narrowing by amplifying the effect of smooth muscle shortening, if thickening occurs inside the muscle layer, or by uncoupling the muscle from the load provided by the surrounding parenchyma, if thickening occurs outside the muscle layer. Furthermore, the amount of connective tissue was not related to the passive elastance of the airways, which was hypothesized
to attenuate excessive bronchoconstriction.

In summary, this study extends previous results that membranous peripheral airways of subjects who have COPD show increased smooth muscle mass and provides new evidence that there is a similar relationship in cartilaginous airways. This is the first study in which a significant relationship has been demonstrated between ASM force and stress and lung function. In this study both the isometric force and isotonic shortening were measured, and the force was normalized by the measured amount of smooth muscle. Airway remodeling occurs in the inflamed airways of subjects who have COPD and asthma and is associated with airflow limitation and exaggerated airway narrowing. These results suggest that the smooth muscle in peripheral airways may be an important determinant of abnormal airway function in this group of subjects.

Directions for future research.

The exact mechanism relating the function and structure of airway smooth muscle to airflow obstruction and airway hyperresponsiveness in obstructive airways disease is still incompletely understood. The results from this study suggest that the main problem in this group of patients with COPD is excess force generation due to increased contractility and increased smooth muscle mass. Further studies must be carried out to determine whether the amount of shortening is also related to airway narrowing as shortening may be a more appropriate *in vitro* indicator of the potential for airway narrowing *in vivo*. In
addition, further examination of the mechanisms affecting contractility is necessary. Changes in the contractile properties could be caused by intracellular reorganization of the contractile apparatus resulting in an increase in the number of myosin filaments or in the number of contractile units in parallel. Structure function studies using electron microscopy could reveal changes at the cellular level.

The findings in this study that the main abnormality related to airflow obstruction is at the level of smooth muscle suggests that therapy with drugs that target the muscle should continue to be developed. Bronchodilators have shown to be effective for these patients by reversing the airway narrowing and improving the FEV₁. Although markers of inflammation were not studied, it is likely that thickening of the smooth muscle layer is the result of an inflammatory process in subjects with asthma and COPD. To the extent that this inflammation is reversible, treatment with inhaled and oral corticosteroids could prove to be effective in reducing airflow obstruction in this group of subjects.
REFERENCES.


3 Carroll N, J Elliot, A Morton, and A James. The structure of large and small airways in nonfatal and fatal asthma. Am Rev Respir Dis. 147:405-410, 1993


30 Hirst SJ. Airway smooth muscle cell culture: Application to studies of airway wall remodelling and phenotype plasticity in asthma. Eur Resp J. 9:808-820, 1996

BIBLIOGRAPHY


Ariens EJ, AM Simons. A molecular basis for drug action: the interaction of one or more drugs with different receptors. J Pharm Pharmacol. 16:289-312, 1984


Berend N, JL Wright, WM Thurlbeck, GE Marlin, AJ Woolcock. Small airways


Carroll N, C Cooke, AL James. Bronchial blood vessel dimensions in asthma.


Chamley-Campbell JH, GR Campbell, and R Ross. The smooth muscle cell in culture. Physiol Rev. 59:1-61, 1979

Chang SC. Microscopic properties of whole mounts and sections of human bronchial epithelium of smokers and nonsmokers. Cancer. 10:1246-1262, 1957


Chetta A, A Foresi, M Del Donno, G Bertorelli, A Pesci, D Olivier Airways remodelling is a distinctive feature of asthma and is related to severity of disease. Chest. 111:852-857, 1997

Chung KF. Role of inflammation in the hyperreactivity of the airways in asthma. Thorax. 41:657-652, 1986


Cosio M, KA Hale, DE Niewoehner. Morphologic and morphometric effects of


Devalia JL, H Bayram, C Rusznak, M Calderon, RJ Sapsford, MA Abdelaziz, J


Enarson DA, SC Newman, RL Fan and C Macarthur. Chronic airways obstruction


Gerthoffer WT. Agonist synergism in airway smooth muscle contraction.


Pharmacol Exp Ther. 278:800-807, 1996


266
Hirst SJ. Airway smooth muscle cell culture: Application to studies of airway wall remodelling and phenotype plasticity in asthma. Eur Resp J. 9:808-820, 1996


267


Lei M, H Ghezzo, MF Chen, DH Eidelman. Airway smooth muscle orientation in


Lundgren R, M Soderberg, P Hortedt, R Stenling. Morphological studies of bronchial mucosa from asthmatics before and after ten years of treatment with inhaled steroids. Eur Respir J. 1:883-889, 1988


MacNee W. Neutrophil traffic and COPD Eur Respir Rev. 43:124-127, 1997


McCormack GS, RH Moreno, JC Hogg, PD Paré. Lung mechanics in papain-


272


Ohashi Y, S Motojima, T Fukuda, S Makino. Airway hyperresponsiveness,
increased intracellular spaces of bronchial epithelium, and increased infiltration of eosinophils and lymphocytes in bronchial mucosa in asthma [see comments]. Am Rev Respir Dis. 145:1469-1476, 1992

Okazawa M, K Ishida, J Road, RR Schellenberg PD Paré. In vivo and in vitro correlation of trachealis muscle contraction in dogs. J. Appl. Physiol. 73:1486-1493, 1992


Owens GK, A Loeb, D Gordon, MM Thompson. Expression of smooth muscle-


Prescott E, AM Bjerg, PK Andersen, P Lange, J Vestbo. Gender difference in smoking effects on lung function and risk of hospitalization for COPD: results from a Danish longitudinal population study. Eur Respir J. 10:822-827, 1997


Ripellino JA, MM Klinger, RU Morgolis. The hyaluronic acid binding region as a specific probe for the localization of hyaluronic acid in tissue section. J Histochem Cytochem. 33:1060-66, 1985


276


Salvato G. Some histological changes in chronic bronchitis and asthma. Thorax. 23:168-172, 1968


Stadnyk AW. Cytokine production by epithelial cells. FASEB J. 8:1041-1047,1994


Sterk PJ, and EH Bel. The shape of the dose-response curve to inhaled bronchoconstrictor agents in asthma and COPD. Am Rev Respir Dis. 143:1433-7, 1991

Stewart AG, PR Tomlinson, J Wilson. Airway wall remodelling in asthma: a


Von Yayek H. The Human Lung. Hafner, New York, 1960, pp.139-161


Weir DC, PS Burge. Effects of high dose inhaled beclomethasone dipropionate, 750 µg and 1500 µg twice daily, and 40 mg per day oral prednisolone on lung function, symptoms, and bronchial hyperresponsiveness inpatients with non-asthmatic chronic airflow obstruction. Thorax. 48:309-316, 1993


