PROLONGED EFFECTS OF AIRWAY EPITHELIAL DENUDATION ON THE MECHANICAL PROPERTIES AND PROLIFERATION OF AIRWAY SMOOTH MUSCLE IN THE GUINEA PIG

by

David A. J. Loewen

B.Sc., The University of British Columbia, 1994

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE in

THE FACULTY OF GRADUATE STUDIES

Department of Experimental Medicine

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

September 1998

© David A. J. Loewen
In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Experimental Medicine

The University of British Columbia
Vancouver, Canada

Date OCT 13, 1998

DE-6 (2/88)
The epithelium serves a multitude of functions in the airway wall. More than just acting as a physical barrier, it secretes chemical mediators which regulate smooth muscle function. Injury to the airway epithelium, and with it a loss of these functions, may contribute to the excessive airway narrowing to contractile stimuli observed in asthmatics. However, the long term effects of epithelial denudation on the mechanical properties or proliferation of airway smooth muscle have not been investigated. We hypothesized that epithelial removal from guinea pig tracheal rings would enhance stress generation, shortening and proliferation of airway smooth muscle over time. Paired epithelium intact (Ep+), and denuded (Ep-) rings were incubated in organ culture for 0, 1, 3, or 7 days, followed by measurement of passive isometric stress at \( L_{\text{max}} \) (\( S_{0} \)), maximum active isometric stress (\( S_{\text{max}} \)), and maximum isotonic shortening (\( \Delta L_{\text{Max}} \)) to acetylcholine stimulation. Contractile dose responses to histamine and carbachol, and relaxant dose responses to isoproterenol, were also determined. In parallel, paired tracheal rings, immunohistochemical staining for 5-bromo-2'-deoxyuridine was used to calculate the proliferation index (PI). Epithelium removal led to a significant increase in \( S_{\text{max}} \), and \( \Delta L_{\text{Max}} \) to acetylcholine in addition to increases in \( S_{\text{max}} \) and sensitivities to histamine measured on 0, 1, and 7, but not on day 3. Some epithelial regeneration of a stratified squamous type was evident in epithelium denuded rings measured on day 3, and thus may account for the lack of increase on day 3. The addition of flurbiprofen, a cyclooxygenase inhibitor, did not mimic the effects of epithelial removal suggesting that other epithelium-derived factors may account for the differences. Epithelial removal did not alter constriction to carbachol or
relaxation to isoproterenol. No differences in the smooth muscle cross-sectional area or PI were detected between Ep+ and Ep- rings regardless of day, suggesting that epithelium removal and regeneration had no significant effect on the proliferation of smooth muscle. These results demonstrate that the enhanced force generation and shortening caused by epithelial removal is not due to smooth muscle hypertrophy or hyperplasia. In addition, the rapid re-epithelialization and time dependent altered smooth muscle mechanics in this thesis establishes a useful model for studying the effects of airway remodeling in vitro.
TABLE OF CONTENTS

ABSTRACT .................................................................................................................. II

TABLE OF CONTENTS .............................................................................................. IV

LIST OF FIGURES .................................................................................................... VI

LIST OF TABLES ....................................................................................................... VII

ACKNOWLEDGMENTS .............................................................................................. VIII

INTRODUCTION ......................................................................................................... 1

I) BACKGROUND / SUMMARY ............................................................................... 1
   A) Asthma .................................................................................................................. 1
   B) Epithelial Injury in Asthma ................................................................................. 2

II) LUNG EPITHELIUM ............................................................................................. 4
   A) The Epithelium as a Part of the Airway Wall: .................................................. 4
   B) Epithelial Cell Types: ....................................................................................... 4
      i) Basal: ............................................................................................................... 5
      ii) Secretory ....................................................................................................... 6
      iii) Ciliated ....................................................................................................... 7
   C) Functions of the Epithelium: ......................................................................... 7
      i) Barrier / Protective: ..................................................................................... 8
      ii) Metabolic Products: .................................................................................... 9
         a) Inflammatory Mediators: ..................................................................... 9
         b) AA Metabolites: ..................................................................................... 11
         c) Other Broncho-active Mediators: ....................................................... 14
         d) Degradative Enzymes: ....................................................................... 16
      iii) Injury and Repair: ..................................................................................... 17

III EFFECTS OF EPITHELIUM DENUDATION ON SMOOTH MUSCLE ........... 21
   A) Effects of Epithelial Denudation on Smooth Muscle Mechanics ................. 21
      i) Normal Smooth Muscle ............................................................................. 21
      ii) Effect of Epithelial Denudation on Smooth Muscle Mechanics .......... 22
   B) Effect of Epithelial Denudation on Smooth Muscle Growth ....................... 24
      i) Increase in Smooth Muscle ...................................................................... 24
      ii) Heterogeneous Population of Smooth Muscle Phenotypes ................. 26
      iii) Epithelial Regulation of Smooth Muscle Growth .................................. 27

IV) THESIS INVESTIGATION ................................................................................... 30

METHODS ............................................................................................................... 32

I) AIM #1: MECHANICAL PROPERTIES OF SMOOTH MUSCLE .................. 32
   A) Experimental Protocol .................................................................................. 32
      i) Organ Culture: ......................................................................................... 34
LIST OF FIGURES

Figure 1: Experimental Design......................................................... 33
Figure 2: Effect of epithelial removal on passive isometric stress.............. 48
Figure 3: Effect of 5 μM flurbiprofen on passive isometric stress. ............ 49
Figure 4: Effect of time in organ culture on passive isometric stress........... 50
Figure 5: Effect of epithelial removal on maximum active isometric stress..... 53
Figure 6: Effect of 5 μM flurbiprofen on maximum active isometric stress..... 54
Figure 7: Effect of time in organ culture on maximum active isometric stress... 55
Figure 8: Effect of epithelial removal on maximum isotonic shortening......... 59
Figure 9: Effect of 5 μM flurbiprofen on maximum isotonic shortening......... 60
Figure 10: Effect of time in organ culture on maximum isotonic shortening.... 61
Figure 11: Sensitivity to histamine. .................................................. 65
Figure 12: Sensitivity to carbachol. .................................................. 69
Figure 13: Sensitivity to isoproterenol. .............................................. 74
Figure 14: Images of epithelium intact airways. .................................... 78
Figure 15: Images of epithelium denuded airways. .................................. 80
Figure 16: Number of epithelial cells.................................................. 83
Figure 17: Proliferation index of epithelium.......................................... 84
Figure 18: Images of airway smooth muscle. ........................................ 87
Figure 19: Proliferation index of airway smooth muscle............................ 88
Figure 20: Total area of airway smooth muscle....................................... 89
LIST OF TABLES

Table 1: Smooth muscle proteins involved in contraction ........................................ 21
Table 2: A selection of airway smooth muscle contractile and relaxant agonists, 
their receptors, and the primary intracellular second messenger signaling 
pathway used ............................................................................................................ 22
Table 3: Maximum contractile responses $S_{max}$ to histamine .................................. 64
Table 4: Sensitivity to histamine .............................................................................. 66
Table 5: Maximum contractile responses $S_{max}$ to carbachol ................................. 68
Table 6: Sensitivity to carbachol ............................................................................. 70
Table 7: Maximum relaxant responses $S_{max}$ to isoproterenol ................................. 73
Table 8: Sensitivity to isoproterenol ...................................................................... 75
Table 9: End of experiment responsiveness ............................................................. 76
Table 10: Qualitative morphometric analysis of epithelium intact tracheal rings .... 77
Table 11: Qualitative morphometric analysis of epithelium denuded tracheal rings .. 79
It was three and a half years ago that I first met Dr.'s Peter Paré, and Bob Schellenberg as I was searching for a graduate supervisor in the Department of Experimental Medicine. Since that time I have had the privilege of working in their laboratories and receiving their tutelage. During the course of my thesis work, both Bob and Peter have been extremely supportive and encouraging, and always made time for me. From talking about bike commuting to discussing good hiking routes, both Bob and Peter have taken an interest in me as a person, as well as a student. I cannot emphasize enough how Bob and Peter have made this project the best educational experience of my life.

I would like to thank both Rick Hegele, and Tony Bai for being members of my supervisory committee. Rick generously allowed me to 'invade' his extra sessions for members of his laboratory to study Robbins Pathologic Basis of Disease. These sessions were always interesting, patriotic (go Canadian research go!), and have sparked my interest in Pathology. Tony has given invaluable input and advice on my project since my first Airways Meeting presentation.

I would also like to thank Jenny Hards for her technical wizardry with paraffin sectioning and BrdU staining, and the countless hours she spent producing incredible sections for me. I would like to thank Randy Thomson, Stuart Greene, and Yulia D-Yachkova for their help with this thesis. Without advice on JB-4 embedding, computer and photographic wizardry, and significant statistical scholarship, I could not have finished this thesis.

I am also grateful to Anabelle Opazo-Saez, Andrew Bramley, and Darryl Knight for showing their interest in me and my project. Annabelle, Andrew and Darryl have all been there for me to encourage me after bad presentations, give advice on how to make stubborn pieces of smooth muscle respond, and bounce crazy and not so crazy ideas off of.

Lastly, I would like to thank my family and closest friends: my roommates Dale, Chris, Ian and Deanna, who have endured my expansionist-like approach to working at home; my sister, Patricia, and Andrea Safertal for hours of proof-reading, and encouraging me to go on; and my Mom who since the beginning has been unconditionally supportive of me and my education.

Anabelle, Andrew, Darryl
INTRODUCTION

I) Background / Summary

A) Asthma

_Dum sprio, spero - while I breathe, I hope_

Asthma is a disease which affects millions of people around the world. Despite increased knowledge of the causes of asthma and improved treatments, asthma prevalence appears to be increasing throughout the world, and particularly in children (CDC, 1995; Burr et al., 1994; Crane et al., 1994; Mellis et al., 1994; Bousquet and Michel, 1991). Copious research has explored the pathophysiology of asthma, yet defining the disease has proven to be difficult due to the variations of symptoms, and a lack of understanding of the causes. Moser (1993), in _Bronchial Asthma_, writes:

"Asthma poses special definition challenges, but seekers after truth about 'what asthma is' ultimately face the reality that there are no absolutes; rather, there are a variety of truths."

The American Thoracic Society Board of Directors adopted the following definition in 1986:

"Asthma is a clinical syndrome characterized by increased responsiveness of the tracheobronchial tree to a variety of stimuli. The major symptoms of asthma are paroxysms of dyspnea, wheezing, and cough, which may vary from mild and almost undetectable to severe and unremitting (status asthmaticus). The primary physiological manifestation of this hyperresponsiveness is variable airways obstruction. This can take the form of spontaneous fluctuations in the severity of obstruction, substantial improvements in the severity of obstruction following bronchodilators or corticosteroids, or increased obstruction caused by drugs or other stimuli. Histologically, patients with fatal asthma have evidence of mucosal edema of the bronchi; infiltration of the bronchial mucosa or submucosa with inflammatory cells, especially eosinophils; and shedding of epithelium and obstruction of peripheral airways with mucus."
Until the development of good bronchial biopsy techniques, pathological observations of asthmatic airways were limited to post-mortem studies. From asthmatic tissue of people who died of status asthmaticus, Dunnill (1960) made several observations. Gross findings included a striking feature of mucous plugs, usually in the smaller bronchi, but occasionally in the main bronchi. Histological examinations showed a dense exudate in the airway lumen containing eosinophils, and epithelial cells, mucosal edema, hypertrophy of the smooth muscle, a thickened basement membrane, and regions of denuded, metaplastic, or single layered epithelium. Although the thickened basement membrane is now known to be a thickened collagen layer beneath the basement membrane (Beasley et al., 1989), and the idea of increased smooth muscle in asthma has been recently challenged (Thomson et al., 1996), Dunnill's observations were robust enough to remain relatively unchallenged for 37 years. A notable addition to these observations is that of inflammation of the mucosa and submucosa, particularly characterized by eosinophilia (Kay, 1996). Results from bronchial biopsies have given insight into airway features of mild asthmatics, which include increased collagen deposition below the basement membrane, and increased numbers of eosinophils with morphologic evidence of activation (Beasley et al., 1989).

B) Epithelial Injury in Asthma

The primary question of this thesis originates from one of the cardinal features of asthma, namely epithelial damage and denudation. In 1962, Naylor reported that 42% of asthmatics and only 3% of non-asthmatics in his study had large numbers of columnar epithelial cell clusters in their sputa. The numbers of these 'Creola bodies' as he named them, increased after asthmatic attacks. From
his work, Naylor suggested that the loss of ciliated columnar epithelium could be of a great magnitude in asthmatics. The contribution that epithelial damage gives to asthma was confirmed by results from studies showing that the degree of epithelial loss correlates with airways sensitivity to inhaled histamine (Beasley et al., 1989), and the degree of overall airway hyperresponsiveness (Jeffrey et al., 1989).

The mechanism by which epithelial damage could affect airways responsiveness has yet to be determined; however, several hypotheses regarding this observation have been made and tested. An ever-increasing body of knowledge highlights the many functions of the epithelium in the airways, including that of a protective barrier, a source of many humoral factors, including arachidonic acid metabolites, and a site of neuropeptide degradation by metabolic enzymes (Spina, 1994). In addition, the epithelium has been likened to the conductor of the inflammatory orchestra in asthma. Loss or changes of these functions of the epithelium due to injury and repair processes would likely affect the airway tissue beneath it, much like endothelial damage and repair helps lead to pathological changes observed in blood vessels in vascular disease (Stewart et al., 1993).

To date, many investigators have explored the effect of epithelial denudation on the \textit{in vitro} pharmacological responses of smooth muscle to various contractile and relaxant stimuli to address the bigger question of how epithelial injury might lead to the symptoms of asthma, and in particular, airway hyperresponsiveness (Hay et al., 1986; Bramley and Piper, 1991; Bai and Prasad, 1994b; Da Silva et al., 1994; Knight et al., 1995a; Figini et al., 1996; Lowenders et al., 1996). These \textit{in vitro} studies have only examined acute effects of epithelial removal and have not evaluated mechanical responses, or structural changes at different time points in the injury/repair process. Evaluation of these aspects forms the basis of this thesis.
II) Lung Epithelium

A) The Epithelium as a Part of the Airway Wall:
The airway wall is made of a variety of tissue and cell types, and its structure can be divided into the mucosa, submucosa, and adventitia (Bai et al. 1994a). The mucosa itself can be subdivided into three further components. The airway epithelium, which varies from ciliated pseudo-stratified columnar in the trachea to simple squamous in the alveoli, rests on a basement membrane made of type IV collagen and laminin (Williams et al., 1995). The lamina propria, made of a network of woven collagen and elastin fibres, and blood vessels comprises the third subdivision. Smooth muscle, sero-mucus glands, loose connective tissue, and other connective tissue cells such as fibroblasts and myofibroblasts make up the submucosa, whereas the C-shaped hyaline cartilage is the major component of the adventitia in the trachea (Bai et al., 1994). In addition, a variety of inflammatory cells, particularly macrophages, dendritic cells, mast cells, eosinophils, neutrophils, and T-cells can be present in the airways particularly in disease states (Howarth et al., 1994; Schon-Hergrad et al., 1991; Serti et al., 1986). A network of sensory nerves innervate the airways extending dendritic processes into the epithelium (Laitinen, 1985).

B) Epithelial Cell Types:
Twelve epithelial cell types have been identified in the mammalian lung (Robbins and Rennard, 1997). The more proximal airways, such as the trachea, and bronchi, contain mostly tall, pseudo-stratified columnar cells, whereas more distal airways, such as the bronchioles, contain low cuboidal shaped cells. Thus, the proportion and size of each epithelial cell type varies along the tracheal-
bronchial tree and between animal species, even though the overall function which
the epithelial layer provides is very similar. It serves as a protective barrier to
foreign substances in the airway lumen, and as a metabolically active participant of
the airway wall (Spina, 1994). Three categories of epithelial cell type have been
suggested from the numerous ultrastructural, functional, and biochemical studies:
basal, secretory, and ciliated (Spina, 1994).

i) Basal:
Most epithelial cells, except the basal cell, are columnar in shape with cell
projections reaching both the basement membrane and airway lumen. Basal cells
are flattened in appearance, have a small cytoplasm to nucleus ratio, and do not
reach the apical surface (Harkema et al., 1991). As the height of the epithelial layer
decreases distally along the bronchial tree, the basal cell number also decreases
(Evans et al., 1990). Once thought to be the major progenitor cell for the epithelium
(it is now believed that secretory cells fulfill this function), basal cells are primarily
responsible for attachment of the pseudo-stratified epithelium to the basement
membrane using hemi-desmosome structures (Evans et al., 1989). Whilst inter-
epithelial cell attachment is facilitated by desmosomes, only basal cells have hemi-
desmosomes which have the necessary extracellular matrix binding sites that can
bind to the basement membrane (Evans et al., 1989). In addition to their progenitor
and structural functions, basal cells are also believed to play a biochemical role in
the airway, as neutral endopeptidase (NEP), 15-lipoxygenase, and recently
Leukemia Inhibitory Factor (LIF), have been localized to basal cells (Spina 1994;
Sigal et al., 1992; Knight et al., 1997).
Secretory cell types are characterized by their spherical membrane bound granules which produce the protective mucous fluid that traps foreign objects in the airway lumen (Harkema et al., 1991). Secretory cells are also now believed to be the major progenitor cells of the epithelium (Spina, 1994; Johnson et al., 1990).

The mucous goblet cell predominates the secretory epithelial population of the upper airways and can be identified by its electron lucent granules, numerous golgi, basally located nucleus, and a granular endoplasmic reticulum (GER) (Spina, 1994). Release of acid mucins from its granules can be increased by many exogenous factors (ie cigarette smoke, sulphur dioxide), and endogenous factors (ie ATP, high or low pH, hypo-osmolality, sensory neuropeptides via vagal nerve activation, platelet activating factor) (Spina, 1994; Harkema et al., 1991). Goblet cell hyperplasia is a common finding in many pathological states such as chronic bronchitis, cystic fibrosis, and bronchial asthma resulting in excess mucous which fills the airways (Harkema et al., 1991).

The Clara cell is the second major type of secretory cell, and is most commonly found in the distal airways. It is characterized by ovoid electron dense granules, and an agranular endoplasmic reticulum in the apical cytoplasm, and GER basally (Harkema et al., 1991). In addition to secretory functions, Clara cells are believed to metabolize xenobiotic compounds using the p450 mono-oxygenase enzyme system (McManus et al., 1987), and may produce antiproteinases, such as secretory leukocyte protease inhibitor (SLPI) (de Water et al., 1986).

Serous cells are similar in structure to Clara cells differing only by slightly larger and more numerous electron dense granules (Spina, 1994). Populating the upper airways, the serous cells, which were first described in specific pathogen free
rats, have been recently identified in the human lung (Rogers et al., 1993). Neuroendocrine cells are the least common secretory cell type, and are characterized by their neurosecretory-like granules containing a dense core (Harkema et al. 1991).

iii) Ciliated
Ciliated cells are the predominant epithelial cell type in the airways, accounting for over 50% of all epithelial cells (Spina, 1994). Ciliated cells arise from either basal or secretory cells and are believed to be terminally differentiated (Ayers and Jeffrey, 1988). However, this belief has been recently challenged by in vivo epithelial regeneration observations by Erjefält et al. (1995). With 200-300 motile cilia per cell, and numerous mitochondria at their apical ends, ciliated cells are responsible for generating directional mucus flow to remove foreign objects from the airways (Harkema et al., 1991). A multitude of factors have been shown to effect ciliary beat frequency (CBF), and thus muco-ciliary clearance, in a variety of experimental models and animal species (Spina, 1994). In summary, LTC4, LTD4, PGE1, PGE2, capsacin, isoproterenol, angiotensin II, endothelin, and bradykinin can increase CBF, while adenosine, eosinophil peroxidase, and major basic protein decrease CBF. It has been speculated that loss of the epithelium, and thus loss of the muco-ciliary system, would hamper the movement of mucus and contribute to the formation of mucous plugs observed in the smaller airways of asthmatic patients.

C) Functions of the Epithelium:
The realization that the epithelium plays more than a protective function in the airways has increased over the last few decades. The vast repertoire of
inflammatory mediators that the epithelium produces, both basally, and upon stimulation, indicate its role in airway inflammation (Rennard et al., 1995). The epithelium is also a major source of arachidonic acid metabolites, which help regulate airway smooth muscle (ASM) tone, epithelial mucous secretion, and inflammation (Holtzman, 1992). Endothelin, nitric oxide (NO), and the putative epithelium derived relaxing factor (EpDRF) also originate from the epithelium, and all influence ASM tone (Howarth et al., 1995; Barnes, 1994a). The epithelium also is a site of metabolic enzymes, such as neutral endopeptidase (NEP), histamine N-methyltransferase, endothelin converting enzyme (ECE), secretory leukocyte protease inhibitor (SLPI), and anti-oxidants which metabolize numerous paracrine humoral factors and leukocyte secretions (Robbins and Rennard, 1997). Lastly, the epithelium also is a source of repair substances and mechanisms which help restore the airways after injury (Erjefält et al., 1995).

i) Barrier / Protective: The mucociliary layer, and the adhesive mechanisms that bind neighbouring epithelial cells, give the epithelium its physical barrier function in the airways (Sparrow et al., 1995; Robbins and Rennard, 1997). Secretions of mucus and fluid from the epithelium, carried on a wave of beating cilia, move trapped airborne particles up and out of the respiratory tract (Borson, 1991). Tight junctions (zonula occludens) located between the apices of neighbouring epithelial cells restrict paracellular diffusion of electrolytes and other molecules (Gumbiner, 1987). Other cell adhesive mechanisms, such as desmosomes, intermediate junctions (zonula adherins), and gap junctions help maintain the structural integrity of the epithelium (Robbins and Rennard, 1997). Epithelium damaged by ozone, viruses, leukocyte granular proteins, and other noxious molecules may actually have sustained
damage to desmosomes and other cell anchoring mechanisms (Montefort et al., 1993). Observations of sloughed, yet intact, epithelium in the sputum of asthmatics (Naylor, 1962) are consistent with this hypothesis. Further evidence that the epithelium has an important barrier function in the airways comes from studies using perfused whole airway segments. Mucosally applied ASM contractile agonists such as acetylcholine, histamine, and KCl, were significantly less effective, and potent than when applied serously (Iriarte et al., 1990). This difference was attenuated when the epithelium was removed (Gao and Vanhoutte, 1994; Sparrow and Mitchell, 1991; Munakata et al., 1989). In these studies, evidence of other epithelial functions affecting the changes in efficacy and sensitivity were ruled out, suggesting that only a physical barrier could account for the difference. In a similar experiment, increased sensitivity to a mucosally applied antigen, ovalbumin, was observed after removal of the epithelium, suggesting epithelial damage in asthmatics may be one mechanism whereby inhaled allergens may lead to an amplification of allergic sensitization (Undem et al., 1988).

ii) Metabolic Products:

a) *Inflammatory Mediators:*

The epithelium can produce a large number of inflammatory mediators suggesting that it may play a key role in the inflammation observed in asthma (Martin et al., 1997; Rennard et al., 1995). Most of the studies exploring epithelium-produced cytokines have used primary cell culture or transformed cell lines. Although there are discrepancies between culture systems used, most of the mediators synthesized by cultured epithelium have been localized to the epithelium by immunohistochemistry (Berkman et al., 1996b; Sousa et al., 1996; Hamid et al., 1993; Marini et al., 1992), or are present in broncho-alveolar lavage fluid from
asthmatics (Alam et al., 1996; Maestrelli et al., 1994; Teran et al., 1994). In cell culture, the epithelium has been shown to produce interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), RANTES, granulocyte macrophage-colony stimulating factor (GM-CSF), granulocyte-colony stimulating factor (G-CSF), transforming growth factor-β (TGF-β), in addition to expressing receptors for IL-1, IL-6, tumor necrosis factor-α (TNF-α), and major histocompatibility complex (MHC) class I and II (Martin et al., 1997; Rennard et al., 1995). The majority of these cytokines, and their receptors are expressed basally in low concentration. However, upon stimulation by various cytokine mixtures, the cultured epithelial cells up-regulate mRNA expression and protein synthesis for these cytokines, and their receptors (Martin et al., 1997). This up-regulation can be inhibited by corticosteroids such as dexamethasone (Amsellem et al., 1996; Bader and Nettesheim, 1996; Berkman et al., 1996a; Takizawa et al., 1996; Gormand et al., 1995). IL-1β, and IL-6 act primarily as T, and B cell activators while IL-8 and RANTES are chemotactic for neutrophils (Barnes, 1994b). GM-CSF is critical for prolonging eosinophil survival in the local environment, but can also activate neutrophils (Barnes, 1994b). Activation of the IL-1 receptor increased GM-CSF production by cultured epithelial cells indicating an autocrine role for IL-1 in the airways (Marini et al., 1992). Due to variations in IL-1 receptor expression in the cultured airways, it was suggested that the IL-1 receptor may only be expressed after epithelial injury or disruption. The IL-6 receptor expressed in cultured epithelium may also be regulated in an autocrine fashion as IL-1, and IL-6 increased its expression (Takizawa et al., 1996).
b) AA Metabolites:
The airway epithelium is capable of producing eicosanoid products several of the enzyme pathways which metabolize arachidonic acid (AA) (Holtzman, 1992). The epithelial AA metabolites then affect airway function through regulation of smooth muscle tone, mucin secretion, inflammation, and neurotransmitter release. Cyclooxygenase (COX), lipoxygenase, and monooxygenase are the three major enzymes which metabolize AA to produce eicosanoids (Holtzman, 1992). COX produces PGG₂/PGH₂, which can be further converted into prostaglandins (PG), and thromboxanes (TX). Lipoxygenase products include 5, 12, and 15 hydroxyeicosatetraenoic acid (HETE), lipoxins, leukotriene B₄, and the slow acting substance of anaphylaxis, now known to be the leukotrienes C₄, D₄, and E₄. Monooxygenase products include the epoxyeicosatetraenoic acids (EET) (Holtzman, 1992).

The conversion of AA to PGH₂, the substrate for PG and TX production, requires two enzyme steps of which COX provides the first. Both steps are provided by one enzyme, prostaglandin endoperoxidase GH synthetase (PGHS), which has been found to be expressed in either a constitutive or inducible form (Walenga et al., 1996). Interestingly, it is the inducible form of PGHS, PGHS-2, and not the constitutive form, PGHS-1, that is expressed in cultured human tracheal and bronchial epithelial cells (Walenga et al., 1996). PGHS-1 is thought to be the 'housekeeping' form of the enzyme due to its constitutive cell expression, while the inducible PGHS-2 form of the enzyme is only expressed after exposure to cytokines, growth factors, or other inflammatory factors (Walenga et al., 1996). It is unknown whether the reversal of the common PGHS expression pattern is normal or a result of injury in cell culture. In addition, expression of PGHS-2 in cultured A549 cells, a
transformed pulmonary epithelial cell line, can be up-regulated by IL-1 and TNF-α. This up-regulation can be inhibited by dexamethasone (Nettesheim and Bader, 1996; Mitchell et al., 1994), suggesting that prostaglandins or thromboxanes may help mediate the inflammatory functions of IL-1 and TNF-α.

The prostaglandins PGE$_2$, PGF$_{2α}$, PGİ$_2$, and the thromboxane TXA$_2$ are all produced by airway epithelium through the PGHS pathway. However, the major prostaglandin found in epithelium in a variety of species, including human, equine, canine, rat, and guinea pig, is PGE$_2$ (Holtzman, 1992). PGE$_2$ is generally described as a broncho-protective mediator due to its relaxant effects on ASM and inhibition of mucous secretion, but it also can cause smooth muscle contraction (Thein and Walters, 1995). Two of the four known PGE$_2$ receptors (EP$_1$, and EP$_2$), have been localized to guinea pig ASM and are likely to mediate the paradoxical effects of PGE$_2$ (Coleman et al., 1994). In most other species, including human, only the EP$_2$ receptor, which mediates smooth muscle relaxation, is present, while the EP$_1$ receptor, which mediates smooth muscle contraction, is only found in the guinea pig (Coleman et al., 1994). Epithelial PGE$_2$ release can be induced by bradykinin (Bramley et al., 1990), β-adrenergic receptor agonists (Liedtke, 1988), and histamine (Knight et al., 1995b), and thus it is thought that in epithelium intact airways PGE$_2$ can regulate smooth muscle contraction by contractile agonists. In addition, PGE$_2$ is thought to inhibit pre-junctional release of acetylcholine by vagal nerve endings in the airway, indicating another mechanism whereby PGE$_2$ regulates smooth muscle contraction (Matsumoto et al., 1996). PGİ$_2$ is also a smooth muscle relaxant, although it too has been recently shown to contract guinea pig bronchi, likely through activation of tachykinin release from afferent nerves (Mapp et al.,
Both PGF$_{2\alpha}$, and TXA$_2$ cause ASM contraction, and both are likely to mediate this effect through the TP prostaglandin receptor (Coleman et al., 1994).

Generally, it is thought that only cells of bone marrow origin, such as the eosinophil, neutrophil, basophil, or monocyte, express the lipoxygenase enzymes. However, 15-lipoxygenase has been immunohistochemically located to basal and ciliated cells of the airway epithelium (Sigal et al., 1992). Indeed, the epithelium is an abundant source of 15-HETE (Salari and Chan-Yeung, 1989), and can produce limited quantities of other lipoxygenase metabolites including a variety of di-HETE's, leukotrienes, and 12-HETE (Hunter et al., 1985). Three lipoxygenase enzymes, 5, 12, and 15-lipoxygenase, can metabolize AA generating 5, 12, and 15-hydroperoxyeicosatetraenoic acids (HPETE), respectively (Holtzman, 1992). 5-HPETE is rapidly converted to leukotriene A$_4$ (LTA$_4$), the precursor to the potent smooth muscle constrictors, LTB$_4$, LTC$_4$, LTD$_4$, and LTE$_4$. Of the leukotrienes, only LTB$_4$ is known to be produced by the airway epithelium, and this has only been observed in the dog (Holtzman, et al. 1983). 15-HPETE is also rapidly converted, but into 15-HETE. While its precise function in the airways is still unknown, 15-HETE has been shown to slowly constrict human ASM perhaps through release of leukotrienes from an unknown tissue source (Schellenberg et al., 1994). In addition, an increase in the amount of 15-lipoxygenase was detected in the bronchial epithelium of asthmatics and people with bronchitis, suggesting that 15-lipoxygenase and its products may play a role in these inflammatory diseases (Shannon et al., 1993). Lastly, the monooxygenase pathway of AA metabolism, mediated by NADPH cytochrome p-450 reductase, is also present in airway epithelial cells (McManus et al., 1987). It produces a variety of EET's, which may
facilitate a portion of the smooth muscle relaxation mediated by the epithelium in *in vitro* pharmacological studies (Gao and Vanhoutte, 1993; Raeburn et al., 1988).

c) Other Broncho-active Mediators:

In addition to the AA metabolites already discussed, the airway epithelium synthesizes other broncho-active mediators such as endothelin, nitric oxide (NO), and the controversial epithelium derived relaxing factor (EpDRF). Since its discovery in 1988, knowledge of endothelin and its biological effects has been rapidly growing. In 1989, Black et al. detected endothelin by fast protein liquid chromatography (FPLC) in cultured epithelial cells from the dog and pig. Since then, endothelin has been found in cultured airway epithelial cells from the human, rabbit, and guinea pig (Mattoli et al., 1990; Rennick et al., 1992; Barnes et al., 1994c) and has been localized by immunohistochemistry to human airway epithelium (Marciniak et al., 1992), particularly in the epithelium from bronchial biopsies from symptomatic asthmatics (Ackerman et al., 1995). Bronchoalveolar lavage fluid (BAL) from non-steroid treated asthmatics has also been found to contain significantly greater amounts of endothelin than that of non-asthmatics (Howarth et al., 1995). Three isoforms of endothelin, endothelin-1 (ET-1), endothelin-2 (ET-2), and endothelin-3 (ET-3), have been characterized of which only ET-1 and ET-3 have been localized to the airway epithelium (Marciniak et al., 1992). Two endothelin receptors, ET<sub>A</sub> and ET<sub>B</sub>, have been identified and endothelin may mediate its potent ASM contractile responses via the ET<sub>B</sub> receptor (Barnes et al., 1994c). The ET<sub>A</sub> receptor has been localized to cultured canine epithelial cells (Ninomiya et al., 1995), and it may be that endothelin acts on the epithelial ET<sub>A</sub> receptor in a paracrine fashion, mediating the release of AA metabolites from the epithelium and thereby modulating endothelin's own effects on the smooth muscle.
and epithelial ciliary beat frequency (CBF) (Wu et al., 1993; Tamaoki et al., 1991). Endothelin is synthesized as a pro-ET or Big ET, and must be metabolized by endothelin converting enzyme (ECE) to its active form (Barnes et al., 1994c). ECE activity and mRNA for ECE has been found in guinea pig airway tissues, giving further evidence that endothelins are locally produced in the epithelium (Noguchi et al., 1991; Shima et al., 1995).

NO is generated by the conversion of L-arginine and oxygen to L-citrulline by the enzyme nitric oxide synthase (NOS)(Howarth et al., 1995). There are two known forms of NOS, a constitutive (cNOS), and inducible (iNOS) form, yet only the inducible form (iNOS) appears to be expressed in the human airway epithelium (Kobzik et al., 1993). Further evidence that NO is produced by the epithelium comes from cell culture studies where human bronchial epithelial cells expressed iNOS mRNA and nitrate, especially in response to a combination of INF-γ, TNF-α, and IL-1β (Robbins et al., 1994b). In addition, iNOS, and not cNOS, has been localized to the epithelium of asthmatic but not control patients (Hamid et al., 1993). Altered levels of NO production may be important in asthma due to NO's bronchodilatory effects, as illustrated by NO induction of bronchodilation in vivo, and smooth muscle relaxation in vitro in the guinea pig (Dupuy et al., 1992; Munakata et al., 1990). Further in vitro evidence suggests that NO is released from the guinea pig epithelium by histamine, bradykinin, and adenosine. The released NO acts either to dampen the effect of contractile agonists, such as histamine (Yan et al., 1994), and bradykinin (Figini et al., 1996), or to mediate the effects of relaxant agonists, such as adenosine (Ali et al., 1997). Indeed, NO was proposed to be a candidate for the EpDRF because it is produced by the epithelium and has relaxant effects on smooth muscle (Munakata et al., 1990). However, experiments using
NOS inhibitors and epithelium removal show NO is not responsible for the EpDRF activity (Munakata et al., 1990). Prostaglandins, and in particular PGE$_2$, have also been shown not to be EpDRF as once suggested, since indomethacin does not fully inhibit epithelium-dependent relaxation of ASM (Tamaoki et al., 1994; Fernandes and Goldie, 1990; Hay et al., 1988). Thus EpDRF remains elusive (Goldie et al., 1993), with some research suggesting it does not exist at all, or that it only plays a minor role in the airway (Strek et al., 1993).

d) Degradative Enzymes:
In addition to being a source of metabolic factors, the epithelium contains enzymes which metabolize various factors. One example is ECE, which was described previously in this chapter. Perhaps the best described metabolic enzyme of the epithelium is NEP, which cleaves substrate peptides on the amino terminus of hydrophobic residues (Borson, 1991). NEP has been localized to the epithelium and in particular, basal cells, by enzymology and immunohistochemical methods (Borson, 1991). Tachykinins such as substance P, and neurokinins A and B, are the best and the most commonly described substrates of NEP. Their inactivation by NEP may explain the leftward shift of dose responses to these peptides in airways treated with NEP inhibitors (Frossard et al., 1989). Other peptides found in the airways such as endothelin, bradykinin, gastrin releasing peptide, cholecystokinin, vasoactive intestinal peptide, and angiotensins I, and II are also degraded by NEP (Vijayaraghavan et al., 1990; Frossard et al., 1990; Borson, 1991). Koga et al., (1992) hypothesized that inhibition of acetylcholinesterase in the epithelium was responsible for the leftward shift in dose responses to acetylcholine in guinea pig tracheal strips. However, their hypothesis contradicts work by Small et al., (1990) which showed no evidence of acetylcholinesterase, or its activity in the guinea pig.
trachea, and its epithelium. Histamine N-methyltransferase (HMT), an histamine degrading enzyme, has also been localized to the guinea pig epithelium by in situ hybridization (Ohrui et al., 1992). Inhibition of the degradation of endogenous, mast cell-released histamine, or exogenously added histamine by an HMT inhibitor, may account for the increased responses to histamine observed both in vivo, and in vitro in the guinea pig (Sekizawa et al., 1993; Ohrui et al., 1992). Inhibition of another histamine-degrading enzyme, diamine oxidase, was also observed to enhance histamine responses of guinea pig perfused trachea (Lindström et al., 1991); however, this work has not since been confirmed (Ohrui et al., 1992; Sekizawa et al., 1993). SLPI, a potent inhibitor of neutrophil elastase, is also found in human bronchial epithelium, particularly in the secretory granules of Clara and goblet cells (de Water et al., 1989; Vogimeier et al., 1991). Expression of SLPI can be increased in cultured human epithelium by incubation with a SLPI substrate, elastase, and corticosteroids (Abbinante-Nissen et al., 1995). Lastly, cultured guinea pig epithelial cells, and a human bronchial cell line both contain anti-oxidant properties suggesting the epithelium may protect the airways from inhaled oxidants (ozone, NO₂), or locally produced oxidants released by inflammatory cells (Cohn et al., 1994; Kinnula et al., 1994).

iii) Injury and Repair:

The airway epithelium has a tremendous capacity to repair itself after injury. In a recent in vivo study, Erjefält et al., (1995) showed that only fifteen minutes after an 800 μm wide patch of tracheal epithelium was removed from anesthetized guinea pigs using a blunt probe, epithelial cells on the denuded margin, including secretory and ciliated cells, began to dedifferentiate, flatten, and migrate over the denuded zone. Proliferation of epithelial cells did not follow until 30 hours after the initial
injury, and after the denuded zone had been completely recovered by a tight, flattened, undifferentiated epithelium. A full differentiated epithelium, complete with the same proportion of ciliated to non-ciliated cells as found in control epithelium, was present by five days. Previous work conducted on hamsters and rats, showed a similar capacity of the epithelium to repair itself (Lane and Gordon, 1974). However, some of these studies had side effects of local bleeding and damage to the basement membrane (Horiba and Fukuda, 1994), which may have had additional effects. Earlier work, also by Erjefält et al., (1994), showed rapid accumulation of a plasma exudate within fifteen minutes after the same epithelium denuding procedure. In the exudate they measured fibrin and fibronectin, both extracellular matrix elements involved in repair mechanisms. In an analogous study of epithelial injury in the rat, expression of fibronectin, integrin α5β1 (a fibronectin receptor), vinculin, and actin (both cytoskeletal proteins associated with integrins) were measured over time as the epithelium recovered after injury (Horiba and Fukuda, 1994). Increased expression of integrin α5β1 and vinculin appeared on the basal surface of regenerating epithelial cells after the appearance of plasma fibronectin, suggesting that the presence of plasma fibronectin may induce their expression. Indeed, fibronectin and two basement membrane components, type IV collagen, and laminin, promote directional migration of cultured bovine bronchial epithelial cells (Shoji et al., 1990; Rickard et al., 1993). Expression of integrin α5β1 could facilitate this movement. In the studies by Erjefält et al., (1994, 1995), and Horiba and Fukuda, (1994), migration of leukocytes into the wound area was also observed. In addition, migration of fibroblasts was observed but only by Horiba and Fukuda, (1994). Perhaps the damage to the BM caused by removing the epithelium induced fibroblasts, which make collagen and other ECM elements, to migrate into
the wound area. In Erjefält's study, no damage to the BM occurred, and therefore no fibroblasts were observed.

Human bronchial epithelial cells in primary culture have also been shown to produce two matrix metalloproteinases (92-kDa, and 72-kDa in size) with known specific activities for the sub-epithelial basal lamina (Yao et al., 1996). Expression of these metalloproteinases was increased with lipopolysaccharide stimulation, leading to the speculation that inflammatory signals received by the airway epithelium may lead to secretion of metalloproteinases, and therefore remodeling of the basal lamina (Yao et al., 1996).

The epithelium itself is a source of substances which aid the injury and repair process, namely TGF-β₁, fibronectin, and a variety of integrins (Aubert et al., 1994; Shoji et al., 1990; Wang et al., 1996). TGF-β₁ has a variety of effects on epithelial cells. Earlier work in cultured rabbit epithelial cells by Jetten et al., (1986), showed TGF-β₁ inhibited cell proliferation and enhanced the development of a squamous phenotype. More recent work also on cultured rabbit epithelial cells confirmed the anti-proliferative effects of TGF-β₁ (Boland et al., 1996). However, a squamous epithelium was not observed. Rather, TGF-β₁ enhanced epithelial cell migration and increased cell outgrowth area that correlated with cell spreading and lack of cell stratification. In addition, cell shape changes were observed with TGF-β₁, consistent with actin cytoskeletal reorganization. Vinculin, an actin binding protein, was observed co-localizing at the end of stress fibres in areas corresponding to focal adhesins. In cultured human bronchial epithelial cells, TGF-β₁, with EGF, upregulated expression of the integrins ανβ₆, and α5β₁, both fibronectin receptors, and α2β₁, a basally expressed collagen and laminin receptor (Wang et al., 1996).
Fibronectin has been shown to be upregulated by TGF-β1 in cultured bovine epithelium (Romberger et al., 1992). TGF-β1 has been shown to stimulate the expression of desmosomal proteins in cultured epithelium (Yoshida et al., 1992). Finally, in cultured bovine bronchial epithelial cells, increased cell density reduced the basally produced TGF-β1 synthesis (Sacco et al., 1992). Thus, it is possible that after epithelial injury, high levels of epithelium-derived TGF-β1 are present to mediate its multitude of repair effects. As the epithelium recovers, the high levels of TGF-β1 would return back to baseline.

Fibronectin can be synthesized by human bronchial cultured epithelial cells, and its expression can be regulated by endothelin (Marini et al., 1996), TNF-α and INF-γ (Härkönen et al., 1995), insulin (Romberger et al., 1995), and TGF-β1 (Linnala et al., 1995). However, fibronectin produced by the epithelium is not thought to be as critical in epithelial repair as plasma derived fibronectin. In in vivo studies, immunohistochemical localization of epithelium derived fibronectin lags the influx of plasma fibronectin by eight hours, by which time the epithelium has covered the denuded zone (Erjefält et al., 1994; Erjefält et al., 1995).
III Effects of Epithelium Denudation on Smooth Muscle

A) Effects of Epithelial Denudation on Smooth Muscle Mechanics

i) Normal Smooth Muscle

As in skeletal muscle, interactions between two proteins, actin and myosin, are primarily responsible for force generation in smooth muscle. However, a number of other contractile, regulatory, and cytoskeletal proteins also play a role in smooth muscle contractions, and particularly in maintaining a sustained contractile response (see table 1) (Rasmussen et al., 1987). In fact, it is the ability to sustain a contraction over time while oxygen consumption, and intracellular calcium (Ca\(^{++}\)) simultaneously decrease, that is unique to smooth muscle. The latch-bridge hypothesis developed to explain this smooth muscle phenomenon has been recently reviewed (Stephens et al., 1992; Rasmussen et al., 1987).

Table 1: Smooth muscle proteins involved in contraction (Rasmussen et al., 1987)

<table>
<thead>
<tr>
<th>Contractile</th>
<th>Regulatory</th>
<th>Cytoskeletal</th>
</tr>
</thead>
<tbody>
<tr>
<td>actin</td>
<td>myosin light chain</td>
<td>intermediate filaments:</td>
</tr>
<tr>
<td>myosin: heavy chain</td>
<td>kinase (MLCK)</td>
<td>desmin</td>
</tr>
<tr>
<td>light chains</td>
<td>calmodulin</td>
<td>vimentin</td>
</tr>
<tr>
<td></td>
<td>caldesmon</td>
<td>α-actinin</td>
</tr>
<tr>
<td></td>
<td>calponin</td>
<td>vinculin</td>
</tr>
<tr>
<td></td>
<td>tropomyocin</td>
<td></td>
</tr>
</tbody>
</table>

Unlike skeletal muscle, whose contraction is dependent upon membrane depolarization, smooth muscle contractions are stimulated by neural, and humoral signals. In general, influx of extracellular calcium causes muscle contraction whether it is stimulated via membrane depolarization and opening of voltage-gated calcium channels, or changes in intracellular second messenger pathways. Generally, contractile agonists, such as histamine, acetylcholine, endothelin,
leukotrienes, and thromboxane, stimulate smooth muscle contraction by binding to their receptors, thereby activating the membrane associated phospholipase C (PLC), which liberates inositol 1,4,5-trisphosphate (IP$_3$), and diacylglycerol (DAG) (see table 2). IP$_3$ initiates the release of calcium from intracellular stores (ie sacroplasmic reticulum) resulting in the increase in [Ca$^{++}$], necessary for muscle contraction (Rasmussen et al., 1987). Activation of adenylate cyclase (AC) by smooth muscle relaxant agonists such as isoproterenol, and prostaglandin E$_2$ binding to their respective receptors, results in an increase in cAMP, which relaxes smooth muscle by decreasing Ca$^{++}$.

**Table 2:** A selection of airway smooth muscle contractile and relaxant agonists, their receptors, and the primary intracellular second messenger signaling pathway used.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>SM Receptor</th>
<th>2$^{nd}$ Messenger Pathway</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Contractile</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscarinic: ie carbachol, acetylcholine</td>
<td>M$_3$</td>
<td>PLC, ↑ IP$_3$, ↑ [Ca$^{++}$]</td>
<td>(Barnes, 1993; Morrison et al., 1992)</td>
</tr>
<tr>
<td>Histamine</td>
<td>H$_1$</td>
<td>PLC, ↑ IP$_3$, ↑ [Ca$^{++}$]</td>
<td>(Barnes, 1987)</td>
</tr>
<tr>
<td>Endothelin</td>
<td>ET$_B$</td>
<td>PLC, ↑ IP$_3$, ↑ DAG</td>
<td>(Barnes, 1994c)</td>
</tr>
<tr>
<td>Tachykinins: ie substance P, NKA, NKB</td>
<td>NK1, NK2</td>
<td>PLC, ↑ IP$_3$, ↑ [Ca$^{++}$]</td>
<td>(Ellis, 1995)</td>
</tr>
<tr>
<td>Thromboxane</td>
<td>TP</td>
<td>PLC, ↑ IP$_3$, ↑ [Ca$^{++}$]</td>
<td>(Coleman et al., 1994)</td>
</tr>
<tr>
<td>Prostaglandin F$_{2a}$</td>
<td>FP</td>
<td>PLC, ↑ IP$_3$, ↑ [Ca$^{++}$]</td>
<td>(Coleman et al., 1994)</td>
</tr>
<tr>
<td>Leukotrienes C$_4$, D$_4$, E$_4$</td>
<td>LT1, LT2</td>
<td>NA</td>
<td>(Jonsson, 1998)</td>
</tr>
<tr>
<td><strong>Relaxant</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>$\beta_2$ adrenergic</td>
<td>AC, ↑ cAMP, ↓ [Ca$^{++}$]</td>
<td>(Torphy, 1994)</td>
</tr>
<tr>
<td>Prostaglandin E$_2$</td>
<td>EP$_2$</td>
<td>AC, ↑ cAMP, ↓ [Ca$^{++}$]</td>
<td>(Coleman et al., 1994)</td>
</tr>
</tbody>
</table>

**ii) Effect of Epithelial Denudation on Smooth Muscle Mechanics**

A multitude of research has pursued the effect of epithelial removal on the pharmacological responses of airway smooth muscle to a variety of spasmogens
and bronchodilators in a variety of animal species including cow (Sadeghi-Hashjin et al., 1996), dog (Gao and Vanhoutte, 1994; Stuart-Smith and Vanhoutte, 1987), human (Knight et al., 1995a; Bai and Prasad, 1994; Bramley and Piper, 1991), pig (Stuart-Smith and Vanhoutte, 1988), rabbit (Loenders et al., 1996), and guinea pig (Ninomiya et al., 1996; Da Silva et al., 1994; Kamikawa, 1993; Strek et al., 1993; Preuss et al., 1992; Morrison and Vanhoutte, 1992; Braunstein et al., 1988; Lundblad and Persson, 1988; Goldie et al., 1986; Hay et al., 1986; Holroyde, 1986). The net result indicates epithelial removal enhances airway smooth muscle responses, but as stated by many of these authors, this epithelium-based enhancement is dependent upon the species studied, the tissue preparation, and the spasmogens and bronchodilators employed.

Work conducted with guinea pig trachealis unanimously showed that epithelium removal increased the sensitivity of airway preparations to histamine (Kamikawa, 1993; Preuss et al., 1992; Braunstein et al., 1988; Goldie et al., 1986; Hay et al., 1986; Holroyde et al., 1986). However, the maximum responses to histamine determined in these studies were not consistent, with some showing an increase (Kamikawa et al., 1993; Braunstein et al., 1988; Hay et al., 1986), whilst others showed no change (Preuss et al., 1992; Goldie et al., 1986; Holroyde 1986). The results showing the effect of epithelial removal on responses to acetylcholine and other pharmacological agonists were equally inconsistent. Epithelial removal has been shown to have no effect on the response of guinea pig trachealis to acetylcholine (Strek et al., 1993; Goldie et al., 1986), or cause an increase in sensitivity to acetylcholine (Lundblad and Persson, 1988, Farmer et al., 1986; Holroyde, 1986). Increases (Lundblad and Persson, 1988; Farmer et al., 1986; Holroyde, 1986) or no change (Goldie et al., 1986) in the sensitivities to
isoproterenol have been observed whilst maximal relaxant responses have been shown to both increase (Goldie et al., 1986), and decrease (Lundblad and Persson, 1988) with epithelial removal.

Although the variations in the results may be partially accounted for by species differences and differences in tissue preparations (strips, rings, spirals, and chains have been used), it is likely that the main reason for the inconsistencies described above is the lack of proper in vitro preconditioning. In other words, None of these previous studies have determined and then used $L_{\text{max}}$ when conducting their pharmacological responses. The maximum isometric response that a tissue can produce is produced at a specific muscle length referred to as $L_{\text{max}}$ (Stephens et al., 1969). Determining $L_{\text{max}}$ and performing experiments at this length are essential for obtaining accurate isometric pharmacological responses (Mitchell et al., 1991).

In the aforementioned studies, guinea pig trachealis tissues have been set at preloads varying from 0.5 g to 2 g tension. In some studies, these preloads have been determined by preliminary work and then used for all subsequent experiments. However, the $L_{\text{max}}$ for each tissue is unique, and thus should be determined for every tissue (Stephens et al., 1969). The aforementioned studies have also failed to explore the effect of epithelial removal on other mechanical properties such as the passive tension at $L_{\text{max}}$, and isotonic shortening.

B) Effect of Epithelial Denudation on Smooth Muscle Growth

i) Increase in Smooth Muscle

One of the pathological findings described by Huber and Koessler (1922) regarding asthma was an increased thickness of the airway smooth muscle layer. Since then, many investigators have confirmed this early finding. In 1973, Hossain
reported that both the volume of smooth muscle, and the number of smooth muscle nuclei were increased by three times in the bronchi of five asthmatics compared with control bronchi, thereby suggesting the increased smooth muscle volume was due to hyperplasia. Others have used various morphometric analyses to determine smooth muscle thickness is increased in asthmatics (Saetta et al., 1991; Kuwano et al., 1993). Ebina et al. (1993) determined two patterns of increased smooth muscle in asthmatic airways, which he named type I and type II. Type I asthmatic lungs had primarily smooth muscle hyperplasia, found only in the large bronchi, while type II asthmatic lungs had smooth muscle hypertrophy, in both the small and large bronchi. In contrast, two recent reports suggest that either the increase in smooth muscle observed in asthma may be overestimated (Perry et al., 1994), or that there is no increase at all (Thomson et al., 1996). Thomson et al. (1996) were surprised by their findings; however, they concluded that earlier studies had overestimated smooth muscle areas by using thick light microscopy sections, and analysis procedures using too low a magnification to clearly determine smooth muscle areas. Regardless of whether smooth muscle undergoes hypertrophy or hyperplasia in asthma, or is not increased at all, the question of how an increase in ASM thickness could lead to excessive airway narrowing was addressed by James et al. (1989). In their study, James et al. (1989) used a formula to calculate the airway resistance at various states of smooth muscle shortening of airways from asthmatics and controls. They found that the increased airway wall thickness of asthmatics would result in severe airways resistance at the same degree of smooth muscle shortening that resulted in minor airways resistance in controls. Thus, the putative increase in smooth muscle of asthmatic airways would cause an increase in airway resistance by virtue of its bulk alone, without having any increases in contractility.
ii) Heterogeneous Population of Smooth Muscle Phenotypes

To further explore the mechanisms of ASM growth, Halayko and Stephens (1994) hypothesized that there was a heterogeneous population of smooth muscle phenotypes throughout the airways, of which two phenotypes would predominate: a contractile phenotype or a proliferative/synthetic phenotype. In their recent work, Halayko et al. (1996) showed that freshly isolated canine tracheal smooth muscle cells differed in their contractile protein profile when compared to the same cells after four to seven days in primary culture. The fresh, contractile tracheal smooth muscle cells were relatively rich in contractile proteins such as smooth muscle myosin heavy chain (MHC), smooth muscle α-actin, calponin, desmin, β-tropomyosin, myosin light chain kinase (MLCK), and h-caldesmon, whereas the cells in primary culture had reduced contractile protein expression and increased expression of non-muscle MHC, l-caldesmon, protein kinase C, and CD44. These results not only substantiate the original hypothesis of Halayko and Stephens (1994), but also suggest that smooth muscle cells may be able to shift from one phenotype to another. Halayko et al. (1997b) showed that canine tracheal smooth muscle is composed of two different phenotypes, as determined by flow cytometry, immunoblotting, and primary culture attachment efficiency. Type A smooth muscle cells were spheroid, had less smooth muscle MHC content, and had >60% attachment efficiency in culture. Type B cells were elongated, were higher in smooth muscle MHC, and had a <10% attachment efficiency in culture. In asthma, an increase in the synthetic or proliferative phenotype of smooth muscle may account for the increase in smooth muscle that has been observed.
iii) Epithelial Regulation of Smooth Muscle Growth

In their review, Halayko and Stephens (1994) likened the epithelial damage observed in asthma to endothelial damage in vascular disease which causes remodeling and thickening of the vascular wall. In concert with this idea, Stewart et al. (1993) suggested that loss of the epithelium, and subsequent epithelial regeneration would alter the balance of epithelial derived, smooth muscle mitogenic signals, resulting in smooth muscle proliferation. In primary culture of cow, dog, sheep, guinea pig, and human ASM, a variety of inflammatory mediators, cytokines, smooth muscle spasmogens, and other humoral factors have been shown to cause smooth muscle proliferative activity. These factors include endothelin, epidermal growth factor, histamine, insulin-like growth factors, IL-1β, IL-6, leukotriene-D₄, mast cells tryptase, platelet-derived growth factor (PDGF), serotonin, substance-P, thrombin, and thromboxane (Noveral et al., 1995; Noveral et al., 1994; and reviewed by Knox, 1994).

The epithelium is a source of six smooth muscle mitogenic modulators; endothelin, thromboxane, IL-1β, IL-6, prostaglandin E₂, and transforming growth factor-β (TGF-β). In primary culture of rabbit tracheal smooth muscle cells, incubation with endothelin caused an increase in cell count in a time and dose dependent fashion (Noveral et al., 1992a). A proliferative response of cultured tracheal smooth muscle cells to endothelin was also observed in the guinea pig (Stewart et al., 1994), sheep (Glassberg et al., 1994), and human where the endothelin effects were mediated by binding to its ETₐ receptor on the smooth muscle (Panettieri et al., 1996). In cultured ovine smooth muscle, the proliferative effects of endothelin were mediated by both ETₐ and ETₐ receptor types, although a ETₐ receptor antagonist provided greater inhibition to this effect than a ETₐ
receptor antagonist (Carratu et al., 1997). Interestingly, Noveral et al. (1992a) showed that the proliferative response to endothelin was inhibited by indomethacin, and a thromboxane receptor (TP) antagonist. Further work by Noveral et al. (1992b) showed that thromboxane A₂ mimetics caused cultured rabbit tracheal smooth muscle cells to increase in cell count, confirming that at least in the rabbit, endothelin mediates some of its mitogenic effects through autocrine release and action of thromboxane. In the later study, thromboxane mimetics were also shown to stimulate the release of another autocrine mitogenic agent, identified as leukotriene D₄ (Noveral et al., 1992b).

The inflammatory mediators IL-1β, and IL-6, are also mitogenic for airway smooth muscle. Incubation of guinea pig airway smooth muscle with either IL-1β, or IL-6, resulted in an increase in [³H]-thymidine incorporation into the cells, and an increase in the number of cells as determined by a hemacytometer (De et al., 1995). In addition to the hyperplastic effects, IL-1β, and IL-6 also caused hypertrophy of the cultured airway smooth muscle cells. Curiously, IL-1β has also been shown to increase expression of COX-2, and one of its prostanoid products, PGE₂, in human cultured airway smooth muscle (Belvisi et al., 1997). Thus, IL-1β could potentially help to modulate airway smooth muscle growth through this pathway.

Whilst endothelin and thromboxane cause ASM proliferation in culture, PGE₂, and TGF-β primarily inhibit ASM proliferation, although this is not always the case. Incubation of serum-stimulated smooth muscle cells from the guinea pig trachea, with PGE₂ resulted in a decrease in [³H]-thymidine incorporation into those cells (Florio et al., 1994). This anti-proliferative effect of PGE₂ may be mediated by an increase in intracellular cAMP, as in separate experiments PGE₂ caused a 30 times
increase in cAMP, and increases in cAMP were correlated with a decrease in $[^3\text{H}]$-thymidine incorporation into the cultured smooth muscle cells. Interestingly, low concentrations of PGE$_2$ caused a slight increase in $[^3\text{H}]$-thymidine incorporation in cultured human ASM cells (Johnson et al., 1995). Johnson et al. (1995) suggested that the proliferative effect of PGE$_2$ may be mediated by the TP receptor, the same receptor used by thromboxane to cause proliferation. However, higher concentrations of PGE$_2$ did result in a decrease in $[^3\text{H}]$-thymidine incorporation of PDGF stimulated cells (Johnson et al., 1995).

Incubation of growth induced cultured human ASM cells with TGF-β resulted in a decrease in $[^3\text{H}]$-thymidine incorporation and cell count number (Cohen et al., 1997). However, this anti-proliferative effect was abolished when the cultured smooth muscle cells were incubated with TGF-β 24 hours after thrombin stimulation. In bovine tracheal smooth muscle cells, a bimodal proliferative effect of TGF-β was also observed (Black et al., 1996). After 24 hours of incubation with TGF-β, $[^3\text{H}]$-thymidine incorporation into smooth muscle cells was reduced, although cell count numbers did not change. After 48 hours incubation with TGF-β, $[^3\text{H}]$-thymidine incorporation was increased as were cell count numbers by 72 hours incubation. It was suggested that the proliferative effects of TGF-β were mediated by autocrine release and action of another growth factor, such as PDGF (Black et al., 1996). Although the differences in the proliferative actions of TGF-β may be contributed to by variations in incubation times, mitogenic agents used, and animal species, the role of TGF-β in regulation of smooth muscle proliferation is less clear than the other factors described.
IV) Thesis Investigation

As discussed, the airway epithelium is a metabolic ‘megastore’ and contributes to the physiology, and pathophysiology of the airway wall through its products, and other functions. Epithelial denudation is also a common finding in both mild and fatal asthma. Therefore, it comes as no surprise that much effort has been spent investigating the role that airway epithelial denudation plays in regulating airway smooth muscle function.

It may seem redundant to continue to study the effects of airway epithelial denudation on airway smooth muscle mechanics, when a plethora of investigators have previously addressed this problem. However, most of the airway smooth muscle function studies have investigated the effect of epithelial denudation entirely on the pharmacological responses of the smooth muscle (i.e. maximal responses, and sensitivities), and only immediately after removing the epithelium. To our knowledge, no previous work has adequately addressed how epithelium removal affects full, isometric and isotonic length-tension properties of the smooth muscle, or how epithelium removal may affect smooth muscle mechanics over time. In light of the fact that epithelial injury and repair may play a role in airway wall remodeling, no previous work has been able to address how epithelial denudation, and subsequent repair may affect smooth muscle proliferation. This thesis is based on addressing these problems.
Hypothesis:

Removal of the epithelium from guinea pig tracheal rings will increase the force generation, shortening, and proliferation of the underlying airway smooth muscle over time in organ culture.

Aim #1:

Investigate the effect of epithelial removal on the smooth muscle mechanical parameters of maximal active isometric stress, passive isometric stress at the same length, maximal isotonic shortening, and the smooth muscle pharmacological responses to histamine, carbachol, and isoproterenol.

Aim #2

Investigate the effect of epithelial removal on the proliferation index of smooth muscle using the thymidine nucleotide analogue, 5'-bromo-2'-deoxyuridine.
METHODS

I) Aim #1: Mechanical Properties of Smooth Muscle

A) Experimental Protocol

To evaluate the effects of epithelial removal over time, I employed an organ culture technique previously developed in our laboratory. Airway rings have been successfully maintained in this organ culture system for up to seven days, and in two species: rabbit and guinea pig (McKay et al., 1995; Knight et al., 1997).

Tracheas were obtained from female Hartley guinea pigs (Charles River, St. Constant, Que.) 350-500g in weight, euthanized with intra-peritoneal injections of sodium pentobarbital (Euthanyl Forte; MTC Pharmaceuticals, Cambridge, Ont.). Tracheas were removed from each animal and placed in ice-cold, sterile Liebowitz L-15 media (GIBCO BRL, Burlington, Ont.) supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin (GIBCO BRL). Extraneous blood vessels, and connective tissue were removed from each trachea using aseptic technique, and 2mm from each cut end was removed.

In order to minimize the intra-animal variation, an experimental design utilizing paired tracheal rings for each intervention was developed according to the following protocol (see figure 1). Tracheas from each animal were cut in half with one half having its epithelium removed by gently rolling a sterile strand of orthodontic floss (Super Floss; Oral B, Mississauga, Ont.) through the lumen. To compensate for any bias in the experiments between removing the epithelium from either the proximal or distal ends of the trachea, half of the tracheas had their epithelium removed from each end. From each tracheal half, six, 2-3 mm wide rings
Figure 1: Experimental Design

were cut, generating a total of 12 tracheal rings from each animal, six referred to as epithelium intact, Ep+, and six referred to as epithelium denuded, Ep-. Morphological analysis confirmed the epithelium was removed effectively, leaving only a few scattered basal cells (see results). Three Ep+ and three Ep- rings were kept for immediate use, while the remaining rings were placed in organ culture for a specified period of time of either 1, 3, or 7 days. These time points were chosen in order to effectively study the effect of time in culture for up to one week without redundancy. Of the six tracheal rings available for a given days' experiments, four rings (two Ep+, and two Ep-) were converted into tracheal strips for studying smooth muscle mechanics, and two rings (one Ep+, and one Ep-) were immediately fixed in formalin for use in determining the smooth muscle proliferation index.
i) Organ Culture:

The organ culture methodology used was previously established by McKay et al. (1995), but will be reviewed here. Tracheal rings were placed in separate 35 mm culture dishes (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ) with approximately 2 mL CMRL media (CMRL-1066 1X without L-glutamine; GIBCO BRL) supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 1 μg/mL insulin, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1μg/mL amphotericin B (all GIBCO BRL). The culture dishes were placed in a sealed chamber (Bellco, Vineland, NJ) which was subsequently purged with a gas mixture containing 5% CO₂, 45% O₂, 50% N₂ (PRAXAIR, Mississauga, Ont.), and incubated at 37°C on a rocker platform (Bellco) at 10 cycles per minute. The rocking motion allowed an air-liquid interface (ALI) in the lumen of each tracheal ring in their culture dishes which was necessary to maintain healthy ciliated epithelial cells (Gray et al., 1996). The chamber was re-gassed every 24 hours, and the CMRL supplemented media replaced every 48 hours. Tracheal rings were harvested after 1, 3, or 7 days (n=10 tracheas per time point) in organ culture after which they would be harvested for either smooth muscle mechanic studies or immunohistochemical studies.

ii) Smooth Muscle Mechanics

a) Length-Tension Relationship:

On a predetermined day, four tracheal rings, two Ep+ and two Ep-, either freshly prepared (day 0 controls), or harvested from organ culture (day 1, 3, or 7), were tested for smooth muscle mechanics. Each of the four rings were cut through the cartilage directly across from the smooth muscle and mounted as strips in four water jacket baths containing 37°C Krebs-Henseleit solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄.7H₂O, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, 11.1
mM glucose) (all Krebs-Henseleit reagents supplied by BDH, Toronto, Ont.) and bubbled continuously with 5\% CO₂, 95\% O₂ to maintain a pH of 7.4. Each tracheal strip was suspended using two clips attached at each smooth muscle-cartilage interface to ensure only the mechanics of the smooth muscle, and not cartilage, were studied. One clip was hooked onto a fixed point, while the other clip was hooked onto the force transducer arm of a servo-controlled myograph (Raytech Instruments, Vancouver, B.C.).

Once mounted in the organ baths, the following equilibration protocol was used. Tracheal strips were gently stretched from 0 to 1 g tension three times in 0.2 g increments, and left at a near 0 g resting tension for 1 hour, with rinsings of fresh Krebs-Henseleit every 15 minutes. After the equilibration period, an isometric, isotonic length-tension relationship for each tracheal strip was determined. Although the responses of the smooth muscle in each strip was not truly isotonic, but rather auxotonic, the overall response of the strip as a whole was referred to as isotonic. An optical micrometer was used to verify the absolute length of the smooth muscle throughout each experiment. Beginning at a length that gave a passive resting tension of near 0 g, each strip was stimulated with 10⁻⁵ M acetylcholine (Sigma, Oakville, Ont.), and allowed to contract isometrically, with the response recorded until the maximum response was reached (approximately five minutes). The baths were rinsed three times in ten minutes, and the strips were allowed to relax to their pre-stimulated baseline tone. Next, each strip was set at the passive tension of the previous isometric response, allowed to equilibrate for ten minutes, stimulated with 10⁻⁵ M acetylcholine, and allowed to contract isotonically against the set tension. The response was recorded until a maximum was reached (approximately five minutes), followed by the identical rinsing regiment used for the isometric response.
Each strip was then set to a new length and the isometric and isotonic responses were repeated at each new length until a maximum isometric response was reached. The length at which each strip produced a maximal isometric response is referred to as $L_{\text{max}}$. In general, the strips were lengthened stepwise until passive tensions of near 0 g, 0.25 g, 0.50 g, 0.75 g, 1.0 g, and 1.25 g were reached. Isometric force values were normalized to stress in kg/cm$^2$ (see below), and isotonic responses were expressed as the amount shortened as a percentage of the initial length ($L_i$).

b) Dose Responses to Carbachol, Histamine, and Isoproterenol

After a full length-tension relationship to acetylcholine was determined for each muscle strip, the strips were set to $L_{\text{max}}$, and allowed to equilibrate for 30 minutes, with a single rinse after 15 minutes. A full, cumulative dose response curve to either carbachol ($10^{-9}$ M to $10^{-4}$ M) (Sigma), or histamine ($10^{-9}$ M to $10^{-4}$ M) (Sigma) was then performed on all four strips. The entire response was recorded. After the maximal response, the following recovery protocol was used. Strips were rinsed three times in ten minutes, then allowed to rest for one hour, with frequent rinsings (every 15 minutes). This protocol allowed the muscle strips to relax fully to their pre-stimulated resting tension. If a dose response to carbachol was performed, then a second dose response to isoproterenol ($10^{-9}$ M to $10^{-5}$ M) (Sigma) was executed. These strips were pre-contracted with an EC$_{70}$ dose of carbachol (determined from the preceding carbachol dose response), and when a contractile plateau was reached the cumulative dose response to isoproterenol was performed, with the full response recorded on the computer. The strips were allowed to relax
using the same recovery protocol used after dose responses to carbachol and histamine.

c) Testing Responsiveness at the End:

In some experiments, the strips were allowed to return to baseline following either the histamine or isoproterenol dose responses, and given a final stimulation of $10^{-5}$ M acetylcholine to ensure the tissues were equally responsive as they were at the beginning of the experiment.

d) Effect of Cyclooxygenase Products:

Since cyclooxygenase (COX) products have been shown to increase the baseline resting tension in the guinea pig trachealis (Braunstein et al., 1988), Flurbiprofen (Sigma), an inhibitor of both COX-1 and COX-2 (Meade et al., 1993), was added to one epi (+) bath and one epi(-) bath for the duration of the mechanics experiments. After each rinsing, flurbiprofen was added to the selected baths to ensure a final bath concentration of 5 μM.

e) Calculation of Cross Sectional Area of Smooth Muscle:

To standardize the isometric responses, the cross sectional area of smooth muscle from each muscle strip was determined using established methodology from our laboratory (Thomson et al., 1996, Opazo-Saez and Paré, 1994) which will be reviewed here. After the final mechanical response (see above), the strips were fixed for 24 hours in 10% buffered neutral formalin (Fisher Scientific, Fairlawn, NJ). While being fixed, each strip was suspended in a 20 mL glass jar using suture thread and a weight which corresponded its passive tension at $L_{\text{max}}$. The fixed strips
were then dehydrated in a graded ethanol series, and embedded in glycol methacrylate plastic (JB-4; Polysciences Inc.). The strips were orientated within the JB-4 block such that cross sectional profiles of smooth muscle could be seen within the axial sections of the airway wall. 2 μm thin sections were made every 80 μm of block depth on a JB-4 microtome (JB-4 Type Sorvall Microtome; Ivan Sorvall Inc., Newtown, CT), until the smooth muscle in each tissue was exhaustively sectioned. Sections were mounted on glass slides and numbered. Five sections were selected in a systematic random fashion, from the slide set of each tissue. The sections were stained with an aqueous solution of 1% (wt/vol) toluidine blue-O (C.I. 52040; Sigma) and 1% (wt/vol) sodium borate (BDH) at pH 7.4, and then coverslipped. The smooth muscle cross sectional area and the length of the basement membrane were determined for each selected tissue section at a magnification of X200, using a Nikon microscope with a camera lucida (Nikon Labophot; Nikon Canada Inc., Mississauga, Ont.), a digitizing pad (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 arithmetic mean of the areas and lengths from the five sections of each tissue were determined and used to estimate the smooth muscle area and basement membrane length for each tissue. All isometric force values determined from the muscle mechanics of each strip were normalized by their own smooth muscle cross sectional area yielding stress values expressed as kg force generated per cm² of smooth muscle area (kg/cm²).
B) **Statistical Analysis**

Passive isometric stress at $L_{\text{max}}$, maximum active isometric stress, and maximum isometric responses to histamine, and carbachol were expressed in units of kg/cm$^2$. The maximum isometric relaxation response to isoproterenol was expressed as a percentage of the maximum contractile response to carbachol. Maximum isotonic shortening was expressed as the absolute length of muscle shortening ($\Delta L$) as a percentage of the initial length of muscle before stimulation ($L_i$). The sensitivities of tracheal strips to histamine, carbachol, and isoproterenol were expressed as pD$_2$ values (-Log EC50 or the concentration required to give a 50% maximal response) and calculated using equation (1).

$$pD_2 = 0.25 + \log_{10}\left(\frac{\text{dose of } Y}{\text{max. response}}\right) - \frac{\sum \text{responses as } \% \text{ of max.}}{200}$$

All values are presented as arithmetic mean ± standard error of the mean (SE), unless otherwise noted.

Paired student's t-tests were used to evaluate differences in overall responses regardless of day, between epithelium intact (Ep+), and epithelium denuded (Ep-) tracheal strips, and between strips with and without flurbiprofen. Interactions between the effect of epithelium removal, addition of flurbiprofen, and time in culture (day X with its day 0 control) were evaluated using a randomized block design analysis of variance (ANOVA). This approach allowed the determination of the significance of epithelium, flurbiprofen, and time effects, as well as their interaction, whilst accounting for the paired ring design. Log$_{10}$ and square root data transformations were used where necessary in order to stabilize variances, and satisfy the homogeneity of variances assumption for the ANOVA. Bonferroni
adjustments were applied post-hoc to p-values to compensate for multiple comparisons. A p-value of <0.05 was considered to be significant.

II) Aim #2: Proliferative Index for Epithelium and SM

A) Methodological Background

i) Use of 5'-bromo-2'-deoxyuridine

To test the hypothesis that epithelium removal would alter guinea pig airway smooth muscle proliferation over time in organ culture, 5'-bromo-2'-deoxyuridine (BrdU) was used as an indicator of proliferation. BrdU, a thymidine nucleotide analogue, is a tissue culture agent used to estimate the proliferation of cultured tissues, including airway epithelium, and airway smooth muscle (Kim et al., 1998; Brown et al., 1995; White et al., 1995). Cells in tissues culture supplemented with BrdU will incorporate the thymidine analogue into their replicating DNA during the 'S' phase of the cell cycle (Alberts et al., 1994). Presuming that once a cell has begun 'S' phase it will proceed continuously, without stopping, until 'M' phase, or mitosis, histologic detection of any 'S' phase marker such as BrdU in a cell, will indicate that that cell underwent cell division.

At the onset of this study, two potential problems with using BrdU were identified. In most tissue culture experiments in which BrdU was used to indicate cell proliferation, the range of concentrations used was variable (from 10 μM to 10 mM) (Kim et al., 1998; Brown et al., 1995; White et al., 1995; Pugh et al., 1995; Yamamoto and Yamamoto, 1994) as were the times when the BrdU was added into the tissues culture media. To address the latter problem of when to add BrdU into our organ culture system, it was decided that the BrdU should be present throughout the experiment, as it was unknown when the cells (in particular, smooth muscle) in
the cultured guinea pig tracheal rings would divide. A set of preliminary experiments were performed to address the first problem of how much BrdU to add into the culture media. In these experiments, a concentration of 0.3 mM BrdU in the culture media was determined to be optimal.

ii) Calculation of Proliferative Index

To calculate an unbiased estimate of the proliferation index (PI) for both smooth muscle, and epithelium from cultured tracheal rings, the area fractions (AF) of cells that stained positive for BrdU incorporation, and the area fraction of all cells were needed as described in equation (2).

\[
PI = \frac{AF \text{ of BrdU positive cells}}{AF \text{ of all cells}} \tag{2}
\]

To estimate both area fractions, a stereologic technique described by Gundersen et al. (1988) was used. The AF of a target tissue can be estimated by superimposing a systematic grid over an image of the target tissue, and counting both the number of grid points that hit the target tissue, and the total number of grid points that hit the image. The AF can be calculated as in equation (3).

\[
AF = \frac{\text{number of target tissue hits}}{\text{total number of hits}} \tag{3}
\]

In this experiment, the target tissue was BrdU positive nuclei, and so equation (3) can be rewritten as equation (4).

\[
AF \text{ of BrdU positive cells} = \frac{\text{number of hits on BrdU positive nuclei}}{\text{total number of hits in area}} \tag{4}
\]

The AF of all cells can also be estimated in a similar fashion, as in equation (5).

\[
AF \text{ of all cells} = \frac{\text{number of hits on all nuclei}}{\text{total number of hits in area}} \tag{5}
\]

Equations (4), and (5) can be substituted into equation (2) to yield equation (6).
Equation (6) can be simplified into equation (7).

\[
PI = \frac{\text{number of hits on BrdU positive nuclei}}{\text{number of hits on all nuclei}}
\]

To ensure an adequate number of nuclei were counted to estimate the PI, the following protocol was used, and also involved systematic random sampling. Each tracheal ring set aside for the proliferation study (described above) was fixed, sectioned, and stained as described below. Two consecutive sections from each ring were taken from the centre of each ring to remove any interference caused by the two cut edges. Both sections were used in the estimation of the smooth muscle and epithelium PI's for that particular animal. A nomogram from Gundersen et al. (1987) was used to estimate the total number of epithelium and smooth muscle cells needed to be counted in order to reduce the estimate of the coefficient of error (CE) to 0.05. The necessary number of epithelial cells to count was 55, and the necessary number of smooth muscle cells to count was 25.

B) **Experimental Protocol**

On a predetermined day, the two tracheal rings from each animal, one Ep+, and one Ep-, either freshly prepared (day 0 controls), or harvested from organ culture (day 1, 3, or 7), were fixed in 10% buffered neutral formalin for 24 hours. Rings harvested from organ culture had 0.3 mM 5'-bromo-2'-deoxyuridine (BrdU; Sigma) supplemented in their organ culture media. After fixation, rings were dehydrated and embedded in paraffin (St. Paul's Hospital Histological Services,
Vancouver). Two, 4.0 μm sections were made from the centre of each embedded ring in a cross sectional plane such that smooth muscle bundles were longitudinal in view. A microtome cutter ("820" Microtome; Spencer) was used for sectioning. Sections were mounted on silane coated slides (Marienfeld, Germany) and baked at 37°C overnight to ensure adequate adhesion of the section to the slide.

i) BrdU Immunohistochemistry

Sections were stained immunohistochemically for BrdU incorporation into cell nuclei using a previously established APAAP methodology (Bicknell et al., 1994) that will be reviewed here. Paraffin was removed from the sections using two 10 minute washes of xylene (Fisher Scientific), rehydrated in graded ethanol washes from 100% to 70%, and finally, rinsed twice with distilled water. At 37°C, sections were digested for 10 minutes in a 0.4% pepsin (p-7000; Sigma) solution acidified to a pH of 2.5, then rinsed with distilled water, and finally, treated with 2 N HCl for 1 hour to denature DNA. Three, 10 minute washes with 0.1 M borate buffer, pH 8.5, were used to neutralize the acid and were followed by three, 10 minute washes with 0.1% Tween 20 in 50 mM Tris Cl, 150 mM NaCl, pH 7.6 (Tris Buffered Saline, or TBS). Sections were then incubated with 5% normal rabbit serum for 15 minutes. Next, sections were incubated with 2 μg/mL mouse anti-BrdU antibody (DAKO Laboratories, Copenhagen, Denmark) prepared with 1% bovine serum albumin in TBS at room temperature for 1 hour in an humidified chamber. Two, five minute washes with 0.1% Tween 20 in TBS followed. Nonimmune mouse IgG (DAKO Laboratories) at 2 μg/mL was used as a negative control. The secondary antibody, rabbit-antimouse IgG antibody (DAKO Laboratories), was then applied in a 1:20 dilution for 30 minutes, followed by two more five minute washes with 0.1% Tween 20 in TBS. Sections were incubated for 30 minutes with a 1:50 dilution of a mouse
monoclonal alkaline phosphatase anti-alkaline phosphatase (APAAP) complex (DAKO Laboratories) followed by a final 2X five minute wash with 0.1% Tween 20 in TBS. The alkaline phosphatase was developed for 20 minutes in 100 mL TBS at pH 8.7, after the addition of a mixture of 0.5 mL of 4% sodium nitrite, 0.2 mL of 5% fuschin (Merck, Rahway, New Jersey) in 2 M HCl, and 50 mg naphthol-AS-B1 phophate (Sigma) dissolved in 0.6 mL N,N-dimethylformahide (Fisher Scientific). Endogenous alkaline phosphatase was blocked by the addition of 17.5 mg levamisole (Sigma) to the colour reaction. Light counterstaining with Meyer's hematoxylin (C.I. 75290; Sigma) for 60 seconds followed to visualize non-BrdU stained nuclei. All sections were then permanently coverslipped.

ii) Stereology

The slides to be used for imaging were randomized and coded. Images for analysis from each slide were projected onto a 15” colour video monitor (CT-1331YC, Panasonic, Osaka, Japan) using a colour video camera (Pulnix TMC-7, Sunnyvale, CA.) mounted onto a Nikon light microscope with a 40 times Nikon apochromatic objective lens. A clear piece of overhead acetate was then superimposed onto the colour monitor screen. This piece of acetate had a systematic grid printed onto it, and the acetate filled the entire surface of the monitor screen. The number of points on the grid was established to yield a count of at least 55 epithelial cells and 25 smooth muscle cells per animal. The total number of BrdU positive nuclei and the number of all cell nuclei were estimated by counting the nuclei upon which the grid points hit.
C) Statistical Analysis:
The total number of epithelial cells counted per airway are presented as the arithmetic mean of the raw number ± SE. Likewise, the PI's for the epithelium and smooth muscle as calculated above are presented as the arithmetic mean ± SE. The amount of smooth muscle in each airway is expressed as the area of smooth muscle in mm$^2$ (ASM) corrected for by the length of basement membrane in mm (LBM). The resulting ASM/LBM (mm) values are presented as arithmetic mean ± SE.

Paired student’s t-tests were used to evaluate the effect of time in culture (day X versus day 0) on the total cell count for both Ep+ and Ep- rings. The changes in total cell number from day 0 to day X were evaluated using a split-plot design ANOVA. The effects of epithelium removal, and time in culture on the PI's for epithelium, and smooth muscle were also determined using a split-plot design ANOVA. A square root transformation was used on PI values to stabilize the variances. Differences in the total amount of smooth muscle were determined using paired student’s t-tests. Bonferroni adjustments were applied post-hoc to p-values to compensate for multiple comparisons. A p-value of <0.05 was considered to be significant.
RESULTS

I) Aim #1: Mechanical Properties of Smooth Muscle

A) Passive Isometric Stress at $L_{\text{max}}$

i) Effect of Epithelial Removal

Overall, removal of the epithelium from guinea pig tracheal strips had no significant effect on passive isometric stress ($S_0$) measured at $L_{\text{max}}$ (see figure 2). However, when the $S_0$ results were compared within day groups (i.e. results specific to a particular day such as day 0 only, day 1 only etc.), there were some significant differences between the $S_0$ measured from epithelium intact and epithelium denuded strips.

In tracheal strips from day 0 (n=27), the $S_0$ of epithelium intact preparations was not significantly different from the $S_0$ of epithelium denuded preparations (see figure 2A). Following the addition of flurbiprofen, there was still no significant difference between the $S_0$ of epithelium intact or denuded preparations. There was a significant difference in $S_0$ ($p<0.04$) between epithelium intact strips, and epithelium denuded strips ($E_{p+} = 1.01\pm0.30 \text{ kg/cm}^2$ versus $E_{p-} = 0.65\pm0.13 \text{ kg/cm}^2$) on day 1 (n=9), regardless of the addition of flurbiprofen ($E_{p+} = 0.80\pm0.14 \text{ kg/cm}^2$ versus $E_{p-} = 0.35\pm0.09 \text{ kg/cm}^2$) (see figure 2B). Although the $S_0$ of epithelium intact, and epithelium denuded tracheal strips on day 3 (n=8) ($E_{p+} = 1.63\pm0.30 \text{ kg/cm}^2$ versus $E_{p-} = 1.19\pm0.13 \text{ kg/cm}^2$) showed a trend towards being significant, on statistical analysis no such differences were found, even after the addition of flurbiprofen ($E_{p+} = 1.52\pm0.36 \text{ kg/cm}^2$ versus $E_{p-} = 0.90\pm0.20 \text{ kg/cm}^2$) (see figure 2C). Furthermore, no changes in $S_0$ due to epithelial removal were detected in tissues on
day 7 (n=10), and again the addition of flurbiprofen had no significant effect on the $S_0$ measured in either epithelium intact or denuded tracheal strips (see figure 2D).

ii) Effect of the Addition of Flurbiprofen

In contrast to the overall lack of effect of epithelial removal on $S_0$, the addition of flurbiprofen resulted in a significantly lower $S_0$ measured at $L_{max}$ than tracheal strips without flurbiprofen ($p<0.003$) (see figure 3). Neither the removal of epithelium from tracheal strips, nor the amount of time each tracheal ring was incubated in organ culture altered this significant flurbiprofen effect. Although only tissues from day 0 shared this effect when results were analyzed within day groups, tissues from days 1, and 3 showed a trend towards having a significant flurbiprofen effect on $S_0$ measurements.

On day 0 (n=27), $S_0$ values for epithelium intact strips with flurbiprofen (0.74±0.09 kg/cm$^2$) were significantly lower ($p<0.001$) than epithelium intact strips without flurbiprofen (1.08±0.12 kg/cm$^2$) (see figure 3A). The removal of epithelium from paired strips did not alter the significant flurbiprofen effect (with $F = 0.68±0.11$ kg/cm$^2$; no $F = 1.24±0.18$ kg/cm$^2$). Although epithelium intact strips with flurbiprofen on day 1 (n=9) were not significantly different from their paired strips without flurbiprofen (with $F = 0.80±0.14$ kg/cm$^2$ versus no $F = 1.01±0.30$ kg/cm$^2$), they showed a trend towards being significant (see figure 3B). In tissues from the same day, differences between epithelium denuded strips with (0.35±0.09 kg/cm$^2$), and without (0.65±0.13 kg/cm$^2$) flurbiprofen did not reach statistical significance, but showed a trend towards being significant. A similar pattern in $S_0$ values as seen in tissues from day 1 was observed for $S_0$ results from day 3 (n=8) (see figure 3C). $S_0$ values from epithelium intact strips with, and without flurbiprofen were not significantly different (with $F = 1.52±0.36$ kg/cm$^2$ versus no $F = 1.63±0.30$ kg/cm$^2$),
Figure 2: Effect of epithelial removal on passive isometric stress. Passive isometric stress was measured at $L_{\text{max}}$ from guinea pig tracheal smooth muscle strips suspended in organ baths. Ep+ (Epithelium intact), Ep- (Epithelium denuded). 5 μM flurbiprofen was present in two organ baths (Ep+,F; Ep-,F). A. day 0. B. After 1 day in organ culture. C. After 3 days in organ culture. D. After 7 days in organ culture. * $p<0.04$. 
Figure 3: Effect of 5 μM Flurbiprofen on passive isometric stress. Passive isometric stress was measured at $L_{\text{max}}$ from guinea pig tracheal smooth muscle strips suspended in organ baths. 5 μM flurbiprofen was present in two organ baths (Ep+,F; Ep-,F). Ep+ (Epithelium intact). Ep- (Epithelium denuded). A. day 0. B. After 1 day in organ culture. C. After 3 days in organ culture. D. After 7 days in organ culture. * p<0.001.
Figure 4: Effect of time in organ culture on passive isometric stress. Passive isometric stress was measured at $l_{max}$ from guinea pig tracheal smooth muscle strips suspended in organ baths. Ep+ (Epithelium intact), Ep- (Epithelium denuded). 5 μM flurbiprofen was present in two organ baths (Ep+,F; Ep-,F). A. After 1 day in organ culture with day 0 controls. B. After 3 days in organ culture with day 0 controls. C. After 7 days in organ culture with day 0 controls. * p=0.004. † p=0.02.
as epithelium denuded strips with, and without flurbiprofen (with $F = 0.90 \pm 0.20$ kg/cm$^2$ versus no $F = 1.19 \pm 0.13$ kg/cm$^2$) were not significantly different. In tracheal strips from day 7 ($n=10$), neither epithelium intact strips with or without flurbiprofen (with $F = 1.05 \pm 0.19$ epi (+); no $F = 0.86 \pm 0.15$ kg/cm$^2$), nor epithelium denuded strips with or without flurbiprofen (with $F = 0.69 \pm 0.08$ kg/cm$^2$; no $F = 1.11 \pm 0.17$ kg/cm$^2$) showed significantly different $S_0$ measurements (see figure 3D).

iii) Time in Culture

Of the tracheal strips harvested after one day in tissue culture, only epithelium denuded strips, including those with and without the addition of flurbiprofen, produced a significantly different ($p=0.004$) $S_0$ at $L_{\text{max}}$ when compared to their day 0 controls (see figure 4A). In contrast, all tracheal smooth muscle strips on day 3, regardless of epithelium status (intact versus denuded), or the addition of flurbiprofen, produced an overall increase in $S_0$ ($p<0.02$) when compared to their day 0 controls (see figure 4B). On day 7, there was no significant difference in $S_0$ compared with their day 0 controls (see figure 4C)

B) Maximal Active Isometric Stress

i) Effect of Epithelial Removal

Overall, removal of epithelium from guinea pig tracheal strips resulted in a statistically significant increase ($p<0.003$) in maximal active isometric stress ($S_{\text{max}}$) (see figure 5). When comparisons were made within day groups, only tracheal strips from day 3 failed to show a difference in $S_{\text{max}}$ between epithelium intact and epithelium denuded preparations.

On day 0 ($n=27$), epithelium denuded tracheal strips generated statistically significant higher $S_{\text{max}}$ values ($p<0.03$) than epithelium intact tracheal strips ($\text{Ep}+ = 2.37 \pm 0.19$ kg/cm$^2$ versus $\text{Ep}- = 2.87 \pm 0.20$ kg/cm$^2$) (see figure 5A). The addition of
flurbiprofen did not alter the significant effect of epithelial removal on $S_{\text{max}}$ (Ep+ = $2.51\pm0.18$ kg/cm$^2$ versus Ep- = $2.88\pm0.17$ kg/cm$^2$). In tracheal strips from day 1 (n=9), epithelium denuded strips produced a significantly higher $S_{\text{max}}$ ($p<0.01$) than strips with their epithelium intact counterpart (Ep+ = $2.49\pm0.25$ kg/cm$^2$ versus Ep- = $3.52\pm0.26$ kg/cm$^2$) (see figure 5B). However, in paired strips which had the addition of flurbiprofen, no significant differences existed between epithelium intact and denuded preparations (Ep+ = $3.42\pm0.67$ kg/cm$^2$ versus Ep- = $3.48\pm0.40$ kg/cm$^2$). There were no significant differences between the $S_{\text{max}}$ produced by epithelium intact and epithelium denuded tracheal strips on day 3 (n=8) (see figure 5C). In tracheal strips with flurbiprofen added, there was still no significant difference between the $S_{\text{max}}$ produced by epithelium intact and epithelium denuded preparations. On day 7 (n=10), the epithelial effect returned (see figure 5D). The $S_{\text{max}}$ produced by epithelium intact tracheal strips was significantly lower than the $S_{\text{max}}$ produced by epithelium denuded tracheal strips (Ep+ = $1.64\pm0.27$ kg/cm$^2$ versus Ep- = $2.28\pm0.21$ kg/cm$^2$). Again, the addition of flurbiprofen did not change the epithelial effect (Ep+ = $2.12\pm0.34$ kg/cm$^2$ versus Ep- = $2.76\pm0.33$ kg/cm$^2$).

ii) Effect of the Addition of Flurbiprofen

Unlike the overall significant difference observed between the $S_{\text{max}}$ produced by epithelium intact and epithelium denuded tracheal strips, a similar comparison between tracheal strips with and without flurbiprofen produced no significant difference (see figure 6). However, when $S_{\text{max}}$ values were compared within day groups, some differences were found.

On day 0 (n=27), differences between the $S_{\text{max}}$ produced by tracheal strips with and without flurbiprofen, whether they had intact or denuded epithelium were not significant (see figure 6A). On day 1 (n=9), there was a significant difference
Figure 5: Effect of epithelial removal on maximum active isometric stress. Maximum isometric stress was generated by guinea pig tracheal smooth muscle strips suspended in organ baths upon stimulation with $10^{-5}$ M acetylcholine. Ep+ (Epithelium intact). Ep- (Epithelium denuded). 5 μM flurbiprofen was present in two organ baths (Ep+,F; Ep-,F). A. Day 0. B. After 1 day in organ culture. C. After 3 days in organ culture. D. After 7 days in organ culture. * p<0.03. † p<0.02. ‡ p<0.005
**Figure 6:** Effect of 5 μM flurbiprofen on maximum active isometric stress. Maximum isometric stress was generated by guinea pig tracheal smooth muscle strips suspended in organ baths upon stimulation with 10^{-5} M acetylcholine. 5 μM flurbiprofen was present in two organ baths (Ep+,F; Ep-,F). Ep+ (Epithelium intact). Ep- (Epithelium denuded). A. Day 0. B. After 1 day in organ culture. C. After 3 days in organ culture. D. After 7 days in organ culture. † p<0.02. * p=0.02
**Figure 7: Effect of time in organ culture on maximum active isometric stress**

Maximum isometric stress was generated by guinea pig tracheal smooth muscle strips suspended in organ baths upon stimulation with $10^{-5}$ M acetylcholine. Ep+ (Epithelium intact). Ep- (Epithelium denuded). 5 μM flurbiprofen was present in two organ baths (Ep+,F; Ep-,F). A. After 1 day in organ culture with day 0 controls. B. After 3 days in organ culture with day 0 controls. C. After 7 days in organ culture with day 0 controls. † p=0.01
(p<0.006) between the $S_{\text{max}}$ produced by epithelium intact tracheal strips with and without flurbiprofen (with $F = 3.42\pm0.67$ kg/cm$^2$ versus no $F = 2.49\pm0.25$ kg/cm$^2$), yet no such difference was evident in epithelium denuded strips (see figure 6B). In epithelium intact tissues on day 3 (n=8), there was a statistically significant increase (p<0.02) in the $S_{\text{max}}$ produced by tracheal strips without flurbiprofen as compared to tracheal strips with flurbiprofen (with $F = 2.59\pm0.21$ kg/cm$^2$ versus no $F = 1.83\pm0.30$ kg/cm$^2$) (see figure 6C). Again, this increase in $S_{\text{max}}$ was also observed between epithelium denuded tracheal strips with and without flurbiprofen (with $F = 1.78\pm0.29$ kg/cm$^2$ versus no $F = 2.66\pm0.29$ kg/cm$^2$). In tracheal strips from day 7 there were no significant differences between tissues with and without flurbiprofen, regardless if the epithelium was intact or denuded (see figure 6D).

iii) Time in Culture

When $S_{\text{max}}$ values from all tracheal strips on day 1 were compared with their paired, day 0 controls, no statistically significant differences were found (see figure 7A). Likewise, $S_{\text{max}}$ produced by tracheal strips on day 3 were not statistically significant from their day 0 controls (see figure 7B). However, the $S_{\text{max}}$ produced by tracheal strips on day 7 were all statistically lower (p=0.01) than their day 0 controls (see figure 7C).

C) Maximal Isotonic Shortening

i) Effect of Epithelial Removal

Removal of the epithelium from guinea pig tracheal strips caused an overall significant increase (p<1x10$^{-6}$) in the maximal isotonic shortening ($\Delta L_{\text{Max}}$) produced by those tissues when compared against their epithelium intact control tissues (see figure 8). Neither the addition of flurbiprofen nor the time each tracheal strip spent in organ culture altered this significant effect. When the results were compared
within day groups, only tracheal strips on day 3 did not produce this significant epithelial effect (see figure 8).

On day 0 (n=27), epithelium denuded tracheal strips produced a significantly higher \( \Delta L_{\text{max}} \) (p<0.001) than epithelium intact strips (Ep+ = 39.5±2.8% versus Ep- = 48.2±2.6%) (see figure 8A). With the addition of flurbiprofen, there were no alterations in this significant epithelial effect (Ep+ = 49.5±2.8% versus Ep- = 56.7±2.2%). In tracheal strips on day 1 (n=9), epithelium denuded tissues also produced a significantly greater \( \Delta L_{\text{max}} \) (p=0.006) than their epithelium intact counterparts (Ep+ = 39.9±5.7% versus Ep- = 60.2±3.7%) (see figure 8B). Again, the addition of flurbiprofen did not change the significant difference between the \( \Delta L_{\text{max}} \) generated by epithelium intact and epithelium denuded tracheal strips (Ep+ = 53.8±4.9% versus Ep- = 64.8±7.1%). In contrast to the results from days 0, and 1, epithelium denuded tracheal strips on day 3 (n=8), did not produce a greater \( \Delta L_{\text{max}} \) than epithelium intact strips, either with or without the addition of flurbiprofen (see figure 8C). However, in tracheal strips on day 7 (n=10), the tracheal strips with denuded epithelium again produced a significantly greater \( \Delta L_{\text{max}} \) (p<0.001) than their epithelium intact controls (Ep+ = 31.5±4.8% versus Ep- = 46.8±4.5%) (figure 8D). This significant epithelial effect was not attenuated in tissues which had the addition of flurbiprofen (Ep+ = 38.8±4.8% versus Ep- = 53.0±3.0%).

ii) Effect of the Addition of Flurbiprofen

The addition of flurbiprofen to tracheal strips caused an overall significant increase (p<0.00003) in the \( \Delta L_{\text{max}} \) produced by these strips (see figure 9). This increase in \( \Delta L_{\text{max}} \) occurred in both epithelium intact and epithelium denuded preparations, and was not affected by the incubation time in organ culture. Although day group analysis on tissues from days 1 and 3 did not show this significant
flurbiprofen effect, tissues from these days showed a trend towards significance between tracheal strips with and without flurbiprofen.

On day 0, epithelium intact tracheal strips with flurbiprofen produced a significantly greater $\Delta L_{\text{Max}}$ ($p<0.001$) than epithelium intact strips without flurbiprofen (with F = 49.5±2.8% versus no F = 39.5±2.8%) (figure 9A). The $\Delta L_{\text{Max}}$ measured in epithelium denuded strips with flurbiprofen was also significantly greater ($p<0.001$) than in epithelium denuded strips without flurbiprofen (with F = 56.7±2.2% versus no F = 48.2±2.6%). In tracheal strips from days 1, and 3, there were no significant differences in the $\Delta L_{\text{Max}}$ measured in epithelium intact tissues with or without flurbiprofen (see figures 9B, and 9C). A significant difference also did not occur between epithelium denuded tissues with and without flurbiprofen. However, the $\Delta L_{\text{Max}}$ produced by tissues with flurbiprofen showed a trends towards being significantly greater than their counterparts without flurbiprofen. On day 7 (n=10), the $\Delta L_{\text{Max}}$ produced by epithelium intact tissues with flurbiprofen were significantly greater ($p=0.02$) than the $\Delta L_{\text{Max}}$ produced by epithelium intact tissues without flurbiprofen (with F = 31.5±2.5% versus Ep- = 38.8±4.8%) (see figure 9D). This significant difference was repeated when the $\Delta L_{\text{Max}}$ produced by epithelium denuded tissues with and without flurbiprofen were compared (with F = 46.8±4.5% versus no F = 53.0±3.0%).

iii) Time in Culture

All guinea pig tracheal strips after 1 day in organ culture (n=9) produced a significantly greater $\Delta L_{\text{Max}}$ ($p=0.007$) than their day 0 control strips (see figure 10A). No such difference occurred between the $\Delta L_{\text{Max}}$ values produced between day 3
Figure 8: Effect of epithelial removal on maximum isotonic shortening. Maximum isotonic shortening was generated by guinea pig tracheal smooth muscle strips suspended in organ baths upon stimulation with $10^{-5}$ M acetylcholine. Ep+ (Epithelium intact). Ep− (Epithelium denuded). 5 µM flurbiprofen was present in two organ baths (Ep+,F; Ep−,F). A. Day 0. B. After 1 day in organ culture. C. After 3 days in organ culture. D. After 7 days in organ culture. *p<0.001. †p=0.006. ‡p<0.001.
Figure 9: Effect of $10^{-5}$ M Flurbiprofen on maximum isotonic shortening. Maximum isotonic shortening was generated by guinea pig tracheal smooth muscle strips suspended in organ baths upon stimulation with $10^{-5}$ M acetylcholine. 5 μM flurbiprofen was present in two organ baths (Ep+, F; Ep-, F). Ep+ (Epithelium intact). Ep- (Epithelium denuded).  A. Day 0.  B. After 1 day in organ culture.  C. After 3 days in organ culture.  D. After 7 days in organ culture. * p<0.001. † p=0.02.
Figure 10: Effect of time in organ culture on maximum isotonic shortening. Maximum isotonic shortening was generated by guinea pig tracheal smooth muscle strips suspended in organ baths upon stimulation with 10^{-5} M acetylcholine. Ep+ (Epithelium intact), Ep- (Epithelium denuded). 5 µM flurbiprofen was present in two organ baths (Ep+,F; Ep-,F). A. After 1 day in organ culture with day 0 controls. B. After 3 days in organ culture with day 0 controls. C. After 7 days in organ culture with day 0 controls. * p=0.007. † p<0.001.
tracheal strips and their day 0 control strips (see figure 10B). Epithelium intact tissues on day 7 (both with and without flurbiprofen), were not only significantly different (p<0.001) than their day 0 epithelium intact control tissues, but were also significantly different (p<0.001) from all epithelium denuded strips regardless of day (see figure 10C). Day 7 epithelium denuded tracheal strips were not different from their day 0 controls.

D) Pharmacological Response to Histamine

i) Maximal Contractile Responses (S\text{max})

Histamine (10^{-9} M to 10^{-4} M) induced concentration-dependent, and cumulative isometric contractions in guinea pig tracheal strips (see figure 11). Overall, neither epithelial removal, nor the addition of flurbiprofen had a significant effect on the maximal isometric contractile response (S\text{max}) to histamine produced by guinea pig tracheal strips. When S\text{max} values to histamine were compared within day groups, there were some significant epithelial and flurbiprofen effects. However, there was no pattern to these significant effects.

On day 0 (n=16), epithelium denuded tissues without flurbiprofen produced significantly greater S\text{max} values to histamine than either epithelium intact tissues without flurbiprofen (p<0.02), or epithelium denuded tissues with flurbiprofen (p<0.02) (see table 3A). In tracheal strips without flurbiprofen on day 1 (n=5), the S\text{max} of epithelium denuded tissues was significantly higher than epithelium intact tissues (p<0.005). In addition, the S\text{max} of epithelium intact tissues with flurbiprofen were significantly greater (p<0.003) than epithelium intact tissues without flurbiprofen (see table 3A). On day 3 (n=4), neither epithelial removal, nor the addition of flurbiprofen had any significant effect on the S\text{max} of tracheal strips to
histamine. Epithelium denuded tracheal strips on day 7 (n=5), both with and without flurbiprofen produced significantly greater (p<0.05) $S_{\text{max}}$ to histamine than their paired epithelium intact tissues (see table 3A). When each day group from either days 1,3, or 7 were compared with their respective day 0 controls, no significant difference in $S_{\text{max}}$ to histamine was evident (see table 3B).

ii) Sensitivities

Overall, the sensitivity of tracheal strips to histamine was significantly increased (p<0.002) in epithelium denuded tissues when compared to epithelium intact tissues (see table 4). However, an overall significant increase in sensitivity to histamine was not evident when results from tissues with and without flurbiprofen were compared (see table 4). When sensitivities to histamine were analyzed within day groups, only tracheal strips on day 3 showed no significant difference between epithelium intact and denuded tissues (see figure 11, and table 4). Again, when compared within day groups, there were no significant differences in sensitivity to histamine between tracheal strips with and without flurbiprofen (see figure 11, and table 4).

On day 0 (n=16), epithelium denuded tracheal strips were significantly more sensitive (p<0.001) to histamine than epithelium intact tracheal strips (see table 4A). This significant difference was not altered with the addition of flurbiprofen. In tracheal strips from day 1 (n=5), epithelium denuded tissues again were significantly more sensitive (p<0.001) than epithelium intact tissues, with the addition of flurbiprofen having no additional effect (see table 4A). On day 3 (n=4), epithelial removal had no significant impact on the sensitivity of tracheal strips to histamine (see table 4A). However, epithelium denuded strips on day 7 (n=5) were
Table 3: Maximum contractile responses ($S_{\text{max}}$) to histamine. Effect of epithelial removal, and addition of 5 μM Flurbiprofen on the maximum isometric stress generation of guinea pig tracheal smooth muscle to histamine dose responses ($10^{-3}$ M to $10^{-4}$ M). Strips were suspended in organ baths. A. Comparing histamine $S_{\text{max}}$ from day 0, day 1, day 3, and day 7. B. Investigating the additional effect of time in organ culture on histamine $S_{\text{max}}$ (presented with day 0 controls). Values are expressed as $S_{\text{max}}$ means ± SEM. Ep+ (Epithelium intact). Ep- (Epithelium denuded). Flurb (Flurbiprofen).

A. | kg/cm$^2$ | Ep+ | Ep- | Ep+ with Flurb | Ep- with Flurb |
--- | --- | --- | --- | --- |
| day 0 (n=16) | 2.86±0.35 | 3.96±0.41$^*$ | 2.86±0.31 | 2.90±0.23 |
| day 1 (n=5) | 3.60±0.20 | 4.49±0.56$^†$ | 4.92±1.18$‡$ | 4.31±0.47 |
| day 3 (n=4) | 1.62±0.55 | 2.58±0.51 | 3.07±0.47 | 2.94±0.37 |
| day 7 (n=5) | 2.40±0.70 | 3.32±0.37$≈$ | 2.75±0.69 | 3.95±0.78$≈$ |

$^*$ p<0.02 compared with Ep+. $^†$ p<0.005 compared with Ep+. $‡$ p<0.03 compared with Ep+ $≈$ p<0.05 compared with Ep+, and Ep+ with Flurb.

B. | kg/cm$^2$ | Ep+ | Ep- | Ep+ with Flurb | Ep- with Flurb |
--- | --- | --- | --- | --- |
| day 0, (n=5) | 2.68±0.56 | 4.53±0.49 | 3.94±0.16 | 3.23±0.42 |
| day 1 | 3.60±0.20 | 4.49±0.56 | 4.92±1.18 | 4.31±0.47 |
| day 0, (n=4) | 2.51±0.23 | 3.59±0.47 | 2.16±0.43 | 2.25±0.12 |
| day 3 | 1.62±0.55 | 2.58±0.51 | 3.07±0.47 | 2.94±0.37 |
| day 0, (n=5) | 3.54±0.99 | 3.91±1.16 | 2.63±0.64 | 3.46±0.41 |
| day 7 | 2.40±0.70 | 3.32±0.37 | 2.75±0.69 | 3.95±0.78 |
Figure 11: Sensitivity to Histamine. Effect of epithelial removal, and the addition of flurbiprofen on the sensitivity of guinea pig tracheal strips to histamine cumulative isometric dose responses (10^{-9} M to 10^{-4} M). Strips were suspended in organ baths. Ep+ (Epithelium intact). Ep- (Epithelium denuded). 5 μM flurbiprofen was present in two organ baths (Ep+,F; Ep-,F). A. Day 0. B. After 1 day in organ culture. C. After 3 days in organ culture. D. After 7 days in organ culture. Data points may obscure error bars.
**Table 4: Sensitivity to histamine.** Effect of epithelial removal, and addition of 5 μM Flurbiprofen on the sensitivity of guinea pig tracheal smooth muscle to histamine dose responses (10⁻⁹ M to 10⁻⁴ M). Strips were suspended in organ baths. A. Comparing sensitivities to histamine from day 0, day 1, day 3, and day 7. B. Investigating the additional effect of time in organ culture on sensitivities to histamine (presented with day 0 controls). Sensitivities are expressed as means of pD2 values ± SEM. Ep+ (Epithelium intact). Ep- (Epithelium denuded). Flurb (Flurbiprofen).

### A.

<table>
<thead>
<tr>
<th>pD2</th>
<th>Ep+</th>
<th>Ep-</th>
<th>Ep+ with Flurb</th>
<th>Ep- with Flurb</th>
</tr>
</thead>
<tbody>
<tr>
<td>day 0</td>
<td>-6.07±0.08</td>
<td>-6.27±0.08*</td>
<td>-6.06±0.07</td>
<td>-6.35±0.08*</td>
</tr>
<tr>
<td>(n=16)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 1</td>
<td>-6.10±0.20</td>
<td>-6.41±0.28†</td>
<td>-6.17±0.11</td>
<td>-6.70±0.22†</td>
</tr>
<tr>
<td>(n=5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 3</td>
<td>-6.51±0.30</td>
<td>-6.39±0.09</td>
<td>-6.56±0.07</td>
<td>-6.41±0.21</td>
</tr>
<tr>
<td>(n=4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 7</td>
<td>-6.21±0.06</td>
<td>-6.46±0.09‡</td>
<td>-6.23±0.05</td>
<td>-6.46±0.10‡</td>
</tr>
<tr>
<td>(n=5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* p<0.001 compared with Ep+, and Ep+ with Flurb.
† p<0.001 compared with Ep+, and Ep+ with Flurb.
‡ p<0.02 compared with Ep+, and Ep+ with Flurb.

### B.

<table>
<thead>
<tr>
<th>pD2</th>
<th>Ep+</th>
<th>Ep-</th>
<th>Ep+ with Flurb</th>
<th>Ep- with Flurb</th>
</tr>
</thead>
<tbody>
<tr>
<td>day 0</td>
<td>-6.05±0.13</td>
<td>-6.21±0.10</td>
<td>-5.92±0.14</td>
<td>-6.61±0.13</td>
</tr>
<tr>
<td>(n=5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 1</td>
<td>-6.10±0.20</td>
<td>-6.41±0.28</td>
<td>-6.17±0.11</td>
<td>-6.70±0.22</td>
</tr>
<tr>
<td>day 0</td>
<td>-6.07±0.18</td>
<td>-6.49±0.13</td>
<td>-6.20±0.11</td>
<td>6.27±0.06</td>
</tr>
<tr>
<td>(n=4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 3</td>
<td>-6.51±0.30</td>
<td>-6.39±0.09</td>
<td>-6.56±0.07</td>
<td>-6.41±0.21</td>
</tr>
<tr>
<td>day 0</td>
<td>-6.10±0.06</td>
<td>-6.06±0.09</td>
<td>-6.01±0.08</td>
<td>-6.20±0.15</td>
</tr>
<tr>
<td>(n=5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 7</td>
<td>-6.21±0.06*</td>
<td>-6.46±0.09*</td>
<td>-6.23±0.05*</td>
<td>-6.46±0.10*</td>
</tr>
</tbody>
</table>

* p<0.001 compared with day 0 controls.
significantly more sensitive \( (p<0.02) \) to histamine than epithelium intact tracheal strips (see table 4A).

When day results were analyzed with their respective day 0 control tissues, only tracheal strips from day 7 showed a greater sensitivity to histamine than their paired day 0 tracheal strips (see table 4B).

E) **Pharmacological Response to Carbachol**

i) Maximal Contractile Responses \( (S_{\text{max}}) \)

Carbachol \( (10^{-9} \text{ M to } 10^{-4} \text{ M}) \) induced concentration-dependent and cumulative isometric contractions in guinea pig tracheal strips (see figure 12). Overall, neither epithelial removal, nor the addition of flurbiprofen had a significant effect on the maximum isometric contractile response \( (S_{\text{max}}) \) to carbachol produced by guinea pig tracheal strips (see table 5). When compared within day groups, only epithelium denuded tracheal strips produced a significantly greater \( (p=0.05) \) \( S_{\text{max}} \) to carbachol than their epithelium intact pairs (see table 5A). No other statistically significant differences were evident either within day groups (see table 5A), or when tissues were compared with their respective day 0 control tissues (see table 5B).

ii) Sensitivities

There were no overall significant differences evident in guinea pig tracheal strips sensitivities to carbachol due either to epithelial removal or the addition of flurbiprofen. However, when compared within day groups, some significant differences in sensitivities to carbachol were detected (see table 6). Specifically, in tracheal strips from day 1 \( (n=4) \), epithelium denuded tissues with the addition of flurbiprofen were significantly more sensitive to carbachol than both epithelium intact tissues with flurbiprofen \( (p<0.02) \), and epithelium denuded tissues without flurbiprofen \( (p<0.04) \) (see table 6A). On day 7 \( (n=5) \), epithelium denuded tracheal
Table 5: Maximum contractile responses ($S_{\text{max}}$) to carbachol. Effect of epithelial removal, and addition of 5 μM Flurbiprofen on the maximum isometric stress generation of guinea pig tracheal smooth muscle to carbachol dose responses ($10^{-9}$ M to $10^{-4}$ M). Strips were suspended in organ baths. A. Comparing carbachol $S_{\text{max}}$ from day 0, day 1, day 3, and day 7. B. Investigating the additional effect of time in organ culture on carbachol $S_{\text{max}}$ (presented with day 0 controls). Values are expressed as $S_{\text{max}}$ means ± SEM. Ep+ (Epithelium intact). Ep- (Epithelium denuded). Flurb (Flurbiprofen).

A.  

<table>
<thead>
<tr>
<th>kg/cm²</th>
<th>Ep+</th>
<th>Ep-</th>
<th>Ep+ with Flurb</th>
<th>Ep- with Flurb</th>
</tr>
</thead>
<tbody>
<tr>
<td>day 0 (n=13)</td>
<td>3.52±0.34</td>
<td>3.86±0.37</td>
<td>3.91±0.29</td>
<td>3.83±0.37</td>
</tr>
<tr>
<td>day 1 (n=4)</td>
<td>2.63±0.33</td>
<td>3.99±0.35*</td>
<td>2.59±0.47</td>
<td>3.81±0.9*</td>
</tr>
<tr>
<td>day 3 (n=4)</td>
<td>3.57±0.45</td>
<td>3.58±1.06</td>
<td>3.60±0.10</td>
<td>4.44±0.43</td>
</tr>
<tr>
<td>day 7 (n=5)</td>
<td>2.77±0.50</td>
<td>3.08±0.27</td>
<td>3.64±0.59</td>
<td>3.38±0.15</td>
</tr>
</tbody>
</table>

* p=0.05 compared with Ep+, and Ep+ with Flurb.

B.  

<table>
<thead>
<tr>
<th>kg/cm²</th>
<th>Ep+</th>
<th>Ep-</th>
<th>Ep+ with Flurb</th>
<th>Ep- with Flurb</th>
</tr>
</thead>
<tbody>
<tr>
<td>day 0, (n=4)</td>
<td>2.77±0.91</td>
<td>4.04±0.33</td>
<td>3.97±0.15</td>
<td>3.84±0.75</td>
</tr>
<tr>
<td>day 1</td>
<td>2.63±0.33</td>
<td>3.99±0.35</td>
<td>2.59±0.47</td>
<td>3.81±0.9</td>
</tr>
<tr>
<td>day 0, (n=4)</td>
<td>3.60±0.55</td>
<td>3.68±0.76</td>
<td>3.53±0.41</td>
<td>3.92±0.35</td>
</tr>
<tr>
<td>day 3</td>
<td>3.57±0.45</td>
<td>3.58±1.06</td>
<td>3.69±0.10</td>
<td>4.44±0.43</td>
</tr>
<tr>
<td>day 0, (n=5)</td>
<td>4.00±0.40</td>
<td>3.85±0.83</td>
<td>4.13±0.72</td>
<td>3.76±0.81</td>
</tr>
<tr>
<td>day 7</td>
<td>2.77±0.50</td>
<td>3.08±0.27</td>
<td>3.64±0.59</td>
<td>3.38±0.15</td>
</tr>
</tbody>
</table>
**Figure 12:** Sensitivity to Carbachol. Effect of epithelial removal and the addition of flurbiprofen on the sensitivity of guinea pig tracheal strips to carbachol cumulative isometric dose responses (10^{-9} \text{ M} \text{ to} 10^{-4} \text{ M}). Strips were suspended in organ baths. Ep+ (Epithelium intact). Ep- (Epithelium denuded). 5 \mu M flurbiprofen was present in two organ baths (Ep+,F; Ep-,F). A. Day 0. B. After 1 day in organ culture. C. After 3 days in organ culture. D. After 7 days in organ culture. Data points may obscure error bars.
Table 6: Sensitivity to carbachol. Effect of epithelial removal, and addition of 5 μM Flurbiprofen on the sensitivity of guinea pig tracheal smooth muscle to carbachol dose responses (10^{-9} M to 10^{-4} M). Strips were suspended in organ baths. A. Comparing sensitivities to carbachol from day 0, day 1, day 3, and day 7. B. Investigating the additional effect of time in organ culture on sensitivities to carbachol (presented with day 0 controls). Sensitivities are expressed as means of pD2 values ± SEM. Ep+ (Epithelium intact). Ep- (Epithelium denuded). Flurb (Flurbiprofen).

### A.

<table>
<thead>
<tr>
<th>pD2</th>
<th>Ep+</th>
<th>Ep-</th>
<th>Ep+ with Flurb</th>
<th>Ep- with Flurb</th>
</tr>
</thead>
<tbody>
<tr>
<td>day 0 (n=13)</td>
<td>-6.73±0.07</td>
<td>-6.64±0.08</td>
<td>-6.74±0.08</td>
<td>-6.77±0.06</td>
</tr>
<tr>
<td>day 1 (n=4)</td>
<td>-6.45±0.08</td>
<td>-6.52±0.10</td>
<td>-6.57±0.06</td>
<td>-6.68±0.08*†</td>
</tr>
<tr>
<td>day 3 (n=4)</td>
<td>-6.59±0.05</td>
<td>-6.61±0.14</td>
<td>-6.57±0.06</td>
<td>-6.67±0.04</td>
</tr>
<tr>
<td>day 7 (n=5)</td>
<td>-6.49±0.07</td>
<td>-6.50±0.07‡</td>
<td>-6.45±0.07</td>
<td>-6.59±0.05‡</td>
</tr>
</tbody>
</table>

* p<0.04 compared with Ep-. † p<0.02 compared with Ep+ with Flurb.
‡ p<0.01 compared with Ep+, and Ep+ with Flurb.

### B.

<table>
<thead>
<tr>
<th>pD2</th>
<th>Ep+</th>
<th>Ep-</th>
<th>Ep+ with Flurb</th>
<th>Ep- with Flurb</th>
</tr>
</thead>
<tbody>
<tr>
<td>day 0, (n=4)</td>
<td>-6.56±0.08</td>
<td>-6.39±0.07</td>
<td>-6.43±0.03</td>
<td>-6.59±0.04</td>
</tr>
<tr>
<td>day 1</td>
<td>-6.45±0.08</td>
<td>-6.52±0.10</td>
<td>-6.57±0.06</td>
<td>-6.68±0.08</td>
</tr>
<tr>
<td>day 0, (n=4)</td>
<td>-6.62±0.04</td>
<td>-6.63±0.13</td>
<td>-6.77±0.13</td>
<td>-6.77±0.10</td>
</tr>
<tr>
<td>day 3</td>
<td>-6.59±0.05</td>
<td>-6.61±0.14</td>
<td>-6.57±0.06</td>
<td>-6.67±0.04</td>
</tr>
<tr>
<td>day 0, (n=5)</td>
<td>-6.98±0.08</td>
<td>-6.84±0.10</td>
<td>-6.96±0.09</td>
<td>-6.91±0.08</td>
</tr>
<tr>
<td>day 7</td>
<td>-6.49±0.07*</td>
<td>-6.50±0.07*</td>
<td>-6.45±0.07*</td>
<td>-6.59±0.05*</td>
</tr>
</tbody>
</table>

* p<0.001 compared with day 0 controls.
strips were also significantly more sensitive (p<0.01) to carbachol than their paired epithelium intact tracheal strips, regardless of the addition of flurbiprofen (see table 6A). No significant differences in sensitivity to carbachol were evident in tracheal strips from day 0 (n=13), or day 3 (n=4) (see table 6A).

When carbachol sensitivities from days 1, 3, or 7 were compared with their respective day 0 controls, only tracheal strips on day 7 were significantly different (p<0.001) in their sensitivities to carbachol (see table 6B).

**F) Pharmacological Response to Isoproterenol**

i) Maximal Relaxant Responses

Isoproterenol (10\(^{-6}\) M to 10\(^{-4}\) M) produced concentration-dependent, and cumulative isometric relaxations in guinea pig tracheal strips pre-contracted with carbachol (see methods) (see figure 13). Although epithelial removal had no overall significant effect on the maximal relaxant response to isoproterenol, the addition of flurbiprofen resulted in an overall significant decrease (p<0.003) in the maximal relaxant response. The removal of epithelium did not alter this significant flurbiprofen effect.

When the maximal relaxant responses to isoproterenol were compared within day groups, neither tracheal strips from day 0 (n=13), nor day 3 (n=4) showed evidence for a significant epithelial or flurbiprofen effect (see table 7A). On day 1 (n=4), epithelium denuded tracheal strips with the addition of flurbiprofen produced a significantly lower (p<0.04) relaxant response to isoproterenol than their paired epithelium denuded strips without flurbiprofen (see table 7A). There was no significant difference between the relaxant response in epithelium intact tissues with and without flurbiprofen. The addition of flurbiprofen to tracheal strips on day 7
(n=5) caused a significant lowering (p<0.01) in the relaxant response to isoproterenol in both epithelium intact and epithelium denuded tissues (see table 7A).

When the relaxant responses to isoproterenol of tracheal strips from days 1, 3, and 7 were compared with the relaxant responses of strips from their day 0 control tissues, no significant differences were evident (see table 7B).

ii) Sensitivities
Overall, epithelial removal from tracheal strips had a statistically significant (p<0.0002) effect on the sensitivities of these tissues to isoproterenol (see table 8). However, the addition of flurbiprofen had no overall significant effect on the sensitivity of tracheal strips to isoproterenol. When the sensitivities to isoproterenol were compared within day groups, significant differences due to either epithelial removal or the addition of flurbiprofen were not evident on day 1 (n=4), or day 3 (n=4) (see table 8A). Removal of epithelium from tracheal strips on day 0 (n=13) resulted in a significant increase (p<0.001) in the sensitivity to isoproterenol (see table 8A). This significant effect was not altered by the addition of flurbiprofen. Likewise, epithelium denuded tracheal strips, both with and without flurbiprofen, were significantly more sensitive (p<0.02) to isoproterenol than their paired epithelium intact tissues (see table 8A).

When relaxant sensitivities to isoproterenol from days 1, 3, and 7 were compared with their day 0 control tissues, only tissues on day 3 were significantly different (p=0.002) from their day 0 controls (see table 8B).
Table 7: Maximum relaxant responses ($S_{max}$) to isoproterenol. Effect of epithelial removal, and addition of 5 μM Flurbiprofen on the maximum isometric relaxation of guinea pig tracheal smooth muscle to isoproterenol dose responses. Strips were suspended in organ baths and pre-contracted with carbachol. A. Comparing isoproterenol $S_{max}$ from day 0, day 1, day 3, and day 7. B. Investigating the additional effect of time in organ culture on isoproterenol $S_{max}$ (presented with day 0 controls). Values are expressed as means ± SEM. Ep+ (Epithelium intact). Ep- (Epithelium denuded). Flurb (Flurbiprofen).

### A.

<table>
<thead>
<tr>
<th>% carb max</th>
<th>Ep+</th>
<th>Ep-</th>
<th>Ep+ with Flurb</th>
<th>Ep- with Flurb</th>
</tr>
</thead>
<tbody>
<tr>
<td>day 0</td>
<td>77.9±8.4</td>
<td>73.3±3.9</td>
<td>64.9±6.1</td>
<td>73.9±2.5</td>
</tr>
<tr>
<td>(n=13)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 1</td>
<td>75.0±10.3</td>
<td>84.6±5.6</td>
<td>77.4±6.7</td>
<td>66.6±12.3*</td>
</tr>
<tr>
<td>(n=4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 3</td>
<td>80.1±6.5</td>
<td>94.2±11.3</td>
<td>80.8±2.1</td>
<td>73.3±2.0</td>
</tr>
<tr>
<td>(n=4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 7</td>
<td>77.8±3.5</td>
<td>74.4±5.5</td>
<td>66.4±1.3†</td>
<td>63.8±3.2†</td>
</tr>
<tr>
<td>(n=5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* p<0.04 compared with Ep-. † p=0.01 compared with Ep+, and Ep-.

### B.

<table>
<thead>
<tr>
<th>% carb max</th>
<th>Ep+</th>
<th>Ep-</th>
<th>Ep+ with Flurb</th>
<th>Ep- with Flurb</th>
</tr>
</thead>
<tbody>
<tr>
<td>day 0, (n=4)</td>
<td>59.2±16.6</td>
<td>79.8±3.1</td>
<td>52.3±13.5</td>
<td>73.3±1.7</td>
</tr>
<tr>
<td>day 1</td>
<td>75.0±10.3</td>
<td>84.6±5.6</td>
<td>77.4±6.7</td>
<td>66.6±12.3</td>
</tr>
<tr>
<td>day 3</td>
<td>88.7±20.5</td>
<td>67.8±10.6</td>
<td>68.1±14.3</td>
<td>83.5±6.6</td>
</tr>
<tr>
<td>day 0, (n=5)</td>
<td>80.1±6.5</td>
<td>94.2±11.3</td>
<td>80.8±2.1</td>
<td>73.3±2.0</td>
</tr>
<tr>
<td>day 7</td>
<td>16.3±3.2</td>
<td>72.5±5.4</td>
<td>72.3±2.5</td>
<td>68.7±3.5</td>
</tr>
<tr>
<td></td>
<td>77.8±3.5</td>
<td>74.4±5.5</td>
<td>39.8±16.3</td>
<td>63.8±3.2</td>
</tr>
</tbody>
</table>
Figure 13: Sensitivity to Isoproterenol. Effect of epithelial removal and the addition of flurbiprofen on the sensitivity of guinea pig tracheal strips to isoproterenol cumulative relaxant dose responses (10^-9 M to 10^-4 M). Strips were suspended in organ baths, and pre-contracted with carbachol. Ep+ (Epithelium intact). Ep- (Epithelium denuded). 5 μM flurbiprofen was present in two organ baths (Ep+,F; Ep-,F). A. Day 0. B. After 1 day in organ culture. C. After 3 days in organ culture. D. After 7 days in organ culture. Data points may obscure error bars.
Table 8: Sensitivity to isoproterenol. Effect of epithelial removal, and addition of 5 μM Flurbiprofen on the sensitivity of guinea pig tracheal smooth muscle to isoproterenol dose responses (10^{-9} M to 10^{-4} M). Strips were suspended in organ baths and pre-contracted with carbachol. A. Comparing sensitivities to isoproterenol from day 0, day 1, day 3, and day 7. B. Investigating the additional effect of time in organ culture on sensitivities to isoproterenol (presented with day 0 controls). Sensitivities are expressed as means of pD$_2$ values ± SEM. Ep+ (Epithelium intact). Ep- (Epithelium denuded). Flurb (Flurbiprofen).

<table>
<thead>
<tr>
<th>pD$_2$</th>
<th>Ep +</th>
<th>Ep -</th>
<th>Ep + with Flurb</th>
<th>Ep - with Flurb</th>
</tr>
</thead>
<tbody>
<tr>
<td>day 0</td>
<td>-6.87±0.10</td>
<td>-7.25±0.08*</td>
<td>-6.83±0.07</td>
<td>-7.17±0.08*</td>
</tr>
<tr>
<td>(n=13)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 1</td>
<td>-7.02±0.23</td>
<td>-7.13±0.11</td>
<td>-7.15±0.18</td>
<td>-7.04±0.11</td>
</tr>
<tr>
<td>(n=4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 3</td>
<td>-6.65±0.42</td>
<td>-6.44±0.38</td>
<td>-6.76±0.29</td>
<td>-6.89±0.22</td>
</tr>
<tr>
<td>(n=4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 7</td>
<td>-6.67±0.08</td>
<td>-6.98±0.13*</td>
<td>-6.86±0.15</td>
<td>-6.98±0.13*</td>
</tr>
<tr>
<td>(n=5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* p<0.001 compared with Ep+, and Ep+ with Flurb. † p<0.02 compared with Ep+, and Ep+ with Flurb.

<table>
<thead>
<tr>
<th>pD$_2$</th>
<th>Ep +</th>
<th>Ep -</th>
<th>Ep + with Flurb</th>
<th>Ep - with Flurb</th>
</tr>
</thead>
<tbody>
<tr>
<td>day 0</td>
<td>-6.88±0.36</td>
<td>-7.26±0.08</td>
<td>-6.86±0.17</td>
<td>-7.42±0.05</td>
</tr>
<tr>
<td>(n=4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 1</td>
<td>-7.02±0.23</td>
<td>-7.13±0.11</td>
<td>-7.15±0.18</td>
<td>-7.04±0.11</td>
</tr>
<tr>
<td>day 0</td>
<td>-6.94±0.09</td>
<td>-7.28±0.26</td>
<td>-7.04±0.04</td>
<td>-7.11±0.05</td>
</tr>
<tr>
<td>(n=4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 3</td>
<td>-6.65±0.42*</td>
<td>-6.44±0.38*</td>
<td>-6.76±0.29*</td>
<td>-6.89±0.22*</td>
</tr>
<tr>
<td>day 0</td>
<td>-6.81±0.18</td>
<td>-7.13±0.05</td>
<td>-6.68±0.07</td>
<td>-6.92±0.08</td>
</tr>
<tr>
<td>(n=5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 7</td>
<td>-6.67±0.08</td>
<td>-6.98±0.13</td>
<td>-6.86±0.15</td>
<td>-6.98±0.13</td>
</tr>
</tbody>
</table>

* p=0.002 compared with day 0 controls.
G) End of Experiment Responsiveness

To test the responsiveness of guinea pig tracheal strips at the end of an experiment (i.e. after all mechanics had been performed including dose responses), tissues were once again stimulated with $10^5$ M acetylcholine. The end of experiment responses (EER) were all on average above 85% of the initial maximum isometric stress generated at the beginning of each experiment (see table 9). The average EER values ranged from a low of 85.0±2.7% in day 1 tracheal strips, to a high of 106.0±2.5% in tracheal strips from day 7 (n=12) (see table 9).

**Table 9: End of experiment responsiveness.** Responsiveness of guinea pig smooth muscle tracheal strips at $L_{\text{max}}$ to $10^5$ M acetylcholine at the end of each experiment. Results are were calculated as a percent of the initial maximum isometric stress generated, and expressed as means ± SE.

<table>
<thead>
<tr>
<th>Day</th>
<th>End of Experiment Responsiveness (% of maximum isometric stress at $L_{\text{max}}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>87.4±2.7</td>
</tr>
<tr>
<td>(n=81)</td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>85.0±2.7</td>
</tr>
<tr>
<td>(n=35)</td>
<td></td>
</tr>
<tr>
<td>Day 3</td>
<td>85.4±3.6</td>
</tr>
<tr>
<td>(n=26)</td>
<td></td>
</tr>
<tr>
<td>Day 7</td>
<td>106.0±2.5</td>
</tr>
<tr>
<td>(n=12)</td>
<td></td>
</tr>
</tbody>
</table>
II) Aim #2: Proliferative Index for Epithelium and Smooth Muscle

A) Qualitative Analysis of Epithelium

Overall, the epithelium from guinea pig tracheal rings remained intact, and maintained a healthy pseudo-stratified columnar ciliated appearance after 1, 3, or 7 days in organ culture (see figure 14, and table 10). Before rings were harvested on their respective days, a selection were viewed under a phase-contrast light microscope. Using this technique, rings viewed after 1, 3, or 7 days in organ culture were observed to have actively beating cilia. Although epithelium from some rings harvested after 3 days had a more stratified squamous appearance, and epithelium from some rings harvested after 7 days appeared denuded or stratified squamous in appearance, the majority of the epithelium from these days maintained an intact, pseudo-stratified columnar ciliated appearance (see figure 14, and table 10).

Table 10: Qualitative morphometric analysis of epithelium intact tracheal rings. Tracheal rings from each animal, and each day (0, 1, 3, or 7) were given descriptors for their epithelium status (either intact, denuded or mix), and for the type of epithelium present. pscc = pseudo-stratified columnar ciliated. strsq = stratified squamous. ssq = simple squamous.

<table>
<thead>
<tr>
<th>animal #</th>
<th>Day 0 status</th>
<th>Day 0 type</th>
<th>Day 1 status</th>
<th>Day 1 type</th>
<th>Day 3 status</th>
<th>Day 3 type</th>
<th>Day 7 status</th>
<th>Day 7 type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>intact</td>
<td>pscc</td>
<td>intact</td>
<td>pscc</td>
<td>intact</td>
<td>strsq</td>
<td>intact</td>
<td>pscc</td>
</tr>
<tr>
<td>2</td>
<td>intact</td>
<td>pscc</td>
<td>mix</td>
<td>mix</td>
<td>mix</td>
<td>strsq</td>
<td>mix</td>
<td>strsq</td>
</tr>
<tr>
<td>3</td>
<td>intact</td>
<td>pscc</td>
<td>intact</td>
<td>pscc</td>
<td>intact</td>
<td>pscc</td>
<td>intact</td>
<td>pscc</td>
</tr>
<tr>
<td>4</td>
<td>intact</td>
<td>pscc</td>
<td>intact</td>
<td>pscc</td>
<td>intact</td>
<td>pscc</td>
<td>intact</td>
<td>pscc</td>
</tr>
<tr>
<td>5</td>
<td>intact</td>
<td>pscc</td>
<td>intact</td>
<td>pscc</td>
<td>intact</td>
<td>pscc</td>
<td>intact</td>
<td>pscc</td>
</tr>
<tr>
<td>6</td>
<td>intact</td>
<td>pscc</td>
<td>intact</td>
<td>pscc</td>
<td>intact</td>
<td>pscc</td>
<td>denud ssq</td>
<td>mix</td>
</tr>
<tr>
<td>7</td>
<td>intact</td>
<td>pscc</td>
<td>intact</td>
<td>pscc</td>
<td>intact</td>
<td>pscc</td>
<td>intact</td>
<td>mix</td>
</tr>
<tr>
<td>8</td>
<td>intact</td>
<td>pscc</td>
<td>mix</td>
<td>strsq</td>
<td>mix</td>
<td>strsq</td>
<td>mix</td>
<td>strsq</td>
</tr>
<tr>
<td>9</td>
<td>intact</td>
<td>pscc</td>
<td>intact</td>
<td>pscc</td>
<td>intact</td>
<td>pscc</td>
<td>intact</td>
<td>pscc</td>
</tr>
<tr>
<td>10</td>
<td>intact</td>
<td>pscc</td>
<td>intact</td>
<td>pscc</td>
<td>intact</td>
<td>pscc</td>
<td>intact</td>
<td>pscc</td>
</tr>
</tbody>
</table>
Figure 14: Images of epithelium intact airways. Images of airways with intact epithelium taken from guinea pig tracheal rings after 0 (A), 1 (B), 3 (C), or 7 (D) days in organ culture. Rings were incubated in culture media supplemented with 0.3 mM BrdU, and subsequent tissue sections stained for BrdU (red nuclei) using APAAP technique, and counterstained with Meyer's hematoxylin (blue nuclei). Scale bar is 50 μM in length.
As seen in figure 15A, and table 11, epithelial removal on day 0 for each guinea pig tracheal ring was successful, leaving only a few scattered cuboidal, or squamous type cells, and without apparent damage to the tissue beneath. After one day in organ culture, tracheal rings with their epithelium initially removed on day 0, showed little change from their day 0 status (see figure 15B). Most rings had denuded epithelium with only simple/scattered cuboidal or squamous type cells (see Table 11). However, after 3 days in organ culture tracheal rings initially denuded of their epithelium on day 0 showed tremendous change (see figure 15C). With the exception of two rings, all showed large patches of regenerated epithelium, maintaining a denuded simple/scattered squamous appearance (see table 11). The majority of rings after 3 days had regenerating epithelium with a stratified squamous appearance. No cilia were evident (see table 11). Tracheal rings harvested after 7

Table 11: Qualitative morphometric analysis of epithelium denuded tracheal rings. Tracheal rings from each animal, and each day (0, 1, 3, or 7) were given descriptors for their epithelium status (either denuded or mix), and for the type of epithelium present. scub = simple/scattered cuboidal. ssq = simple/scattered squamous. strsq = stratified squamous.

<table>
<thead>
<tr>
<th>animal</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>status</td>
<td>desc.</td>
<td>status</td>
<td>desc.</td>
</tr>
<tr>
<td>#</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>denud</td>
<td>scub</td>
<td>denud</td>
<td>ssq</td>
</tr>
<tr>
<td>2</td>
<td>denud</td>
<td>scub</td>
<td>denud</td>
<td>scub</td>
</tr>
<tr>
<td>3</td>
<td>denud</td>
<td>ssq</td>
<td>denud</td>
<td>ssq</td>
</tr>
<tr>
<td>4</td>
<td>denud</td>
<td>scub</td>
<td>denud</td>
<td>ssq</td>
</tr>
<tr>
<td>5</td>
<td>denud</td>
<td>scub</td>
<td>denud</td>
<td>scub</td>
</tr>
<tr>
<td>6</td>
<td>denud</td>
<td>ssq</td>
<td>denud</td>
<td>scub</td>
</tr>
<tr>
<td>7</td>
<td>denud</td>
<td>scub</td>
<td>denud</td>
<td>ssq</td>
</tr>
<tr>
<td>8</td>
<td>denud</td>
<td>scub</td>
<td>denud</td>
<td>ssq</td>
</tr>
<tr>
<td>9</td>
<td>denud</td>
<td>scub</td>
<td>denud</td>
<td>ssq</td>
</tr>
<tr>
<td>10</td>
<td>denud</td>
<td>scub</td>
<td>mix</td>
<td>strsq</td>
</tr>
</tbody>
</table>
Figure 15: Images of epithelium denuded airways. Images of airways denuded of epithelium taken from guinea pig tracheal rings after 0 (A), 1 (B), 3 (C), or 7 (D) days in organ culture. Rings were incubated in culture media supplemented with 0.3 mM BrdU, and subsequent tissue sections stained for BrdU (red nuclei) using APAAP technique, and counterstained with meyer's hematoxylin (blue nuclei). Scale bar is 50 μM in length.
days in organ culture failed to reproduce the dramatic changes as seen in rings from day 3 (see figure 15D, and table 11). The majority of rings maintained a denuded appearance, with only a simple mono-layer of squamous type epithelium covering the basement membrane (see figure 15D). Some rings from day 7 did show similar changes as seen in day 3 rings, with large patches of regenerating epithelium with a stratified squamous appearance (see table 11).

B) Quantitative Analysis of Epithelium

i) Total Number of Cells

The total number of epithelial cells were counted from eight high power fields in each tracheal ring incubated for 0, 1, 3, or 7 days in organ culture. On day 0 (n=28), the total number of epithelial cells in epithelium intact rings was 226±13 compared with 29±3 in epithelium denuded rings, of which none had stained positive for nuclear BrdU incorporation (see figure 16). After 1 day in organ culture (n=10), the total number of epithelium in epithelium intact rings had not significantly changed compared to its day 0 control rings (day 0 258±27 versus day 1 197±11) (see figure 16). Likewise, the total number of epithelial cells in epithelium denuded rings on day 1 had not significantly changed compared to its day 0 control rings (day 0 33±6 versus day 1 19±5). At this time, epithelium in both epithelium intact and epithelium denuded rings began to stain positive for nuclear BrdU incorporation. Again, no change in the total number of epithelial cells was evident when results from day 3 (n=8) rings were compared with their day 0 controls (day 0 199±18 versus day 3 184±25), though an increasing number were staining positive for BrdU incorporation into cell nuclei (see figure 16). However, in epithelium denuded rings
from day 3, the total number of epithelium was significantly greater \((p<0.006)\) in day 3 rings than from day 0 control rings (day 0 29±6 versus day 3 110±14), of which the majority were stained positive for nuclear BrdU incorporation (see figure 16). In epithelium intact tracheal rings harvested after 7 days \((n=10)\), the total number of epithelial cells had significantly declined \((p<0.005)\) from the total number of cells in day 0 control rings (day 0 221±16 versus day 7 123±17) (see figure 16) even though the morphologic character of the epithelium had changed little (see table 10). In epithelium denuded tracheal rings from day 7, the total number of cells on day 7 was significantly greater \((p<0.01)\) than the number of cells in day 0 controls (see figure 16). Most epithelial cells from day 7 rings stained positive for nuclear BrdU incorporation (see figure 16).

Of the changes in epithelial cells counts that occurred over time in epithelium denuded rings, both the change that occurred between day 3 and its day 0 control, and day 7 and its day 0 control, were significantly different \((p=0.001)\) than the changes that occurred between day 1 and its day 0 control.

ii) Proliferation Index

The proliferation index (PI) of each tracheal ring was calculated using the number of BrdU positive epithelial cells and the total number of epithelial cells. The PI for epithelium from both epithelium intact and denuded rings on day 0 \((n=28)\) was 0. Within all three day groups of day 1 \((n=10)\), day 3 \((n=8)\), and day 7 \((n=10)\), the PI of epithelium in epithelium denuded rings was significantly greater \((p<0.001)\) than the PI of epithelium in epithelium intact rings (see figure 17). In addition, the epithelium PI significantly increased \((p<0.001)\) from day 1 to day 3, and from day 1 to day 7 (see figure 17). No differences in the epithelium PI between day 3, and day 7 were evident (see figure 17).
Figure 16: Number of epithelial cells. The total number of epithelial cells (Total), both BrDU negative, and BrDU positive (BrDU +) were counted in each airway from tracheal rings incubated for 0, 1, 3, or 7 days in organ culture. Values are presented as means ±SE. Ep+ (epithelium intact). Ep- (epithelium denuded). All statistics on this figure refer to the total number of cells only. * p<0.005 compared with day 0 control. † p<0.006 compared with day 0 control. ‡ p<0.01 compared with day 0 control.
Figure 17: Proliferation index of epithelium. The proliferation index of epithelium was measured from tracheal rings which had been incubated in organ culture for 1, 3 or 7 days. Ep+ (epithelium intact rings). Ep- (epithelium denuded rings). Data points may obscure error bars. * p<0.001 compared with Ep+. † p<0.001 compared with day 3, and day 7.
C) Qualitative Analysis of Smooth Muscle

Overall, there was no apparent change in smooth muscle morphology from both epithelium intact and epithelium denuded rings, and after 0, 1, 3, or 7 days in organ culture (see figure 18). Smooth muscle cells in all views were elongated, and had cigar shaped nuclei, of which very few stained positive for nuclear BrdU incorporation (see figure 18).

D) Quantitative Analysis of Smooth Muscle

i) Proliferation Index

The PI of smooth muscle cells in each tracheal ring were calculated from the number of BrdU positive cells, and the total number of cells counted in four high power fields per ring. Overall, the PIs of smooth muscle (ranging from 1.0±0.4% to 4.2±1.5%) were dramatically lower than those measured in epithelium (ranging from 7.8±2.8% to 97.2±1.7%). There were no significant differences between the smooth muscle PI calculated in epithelium intact or epithelium denuded rings after any length of incubation in organ culture (see figure 19). Even though the smooth muscle PI values were very low, the smooth muscle PI values calculated from tracheal rings on day 1 were significantly lower (p<0.05) than those calculated on either days 3, or 7 (see figure 19). No difference was evident between smooth muscle PI values on days 3, or 7 (see figure 19).

ii) Amount of Smooth Muscle

To evaluate if there were changes in smooth muscle bundle size with epithelium denudation, or with time in organ culture, the cross sectional area of smooth muscle in tracheal strips was corrected by the length of basement membrane in each strip (ASM/LBM ratio). The ASM/LBM ratio in epithelium denuded strips after 1 day in culture was significantly higher (p<0.02) than the
ASM/LBM ratio in day 0 control strips (see figure 20). In spite of this difference, there was no significant difference in the ASM/LBM ratio between epithelium intact and epithelium denuded strips on day 3. No differences in the ASM/LBM ratio were evident between epithelium denuded strips on day 1, and day 0 (see figure 20). Epithelium denuded strips after 7 days in culture had a significantly higher (p<0.05) ASM/LBM ratio than strips from day 0 (see figure 20). Likewise, epithelium intact strips after 7 days in organ culture had a significantly higher (p<0.05) ASM/LBM ratio than strips on day 0. When the ASM/LBM ratios of epithelium intact and denuded strips were compared from day 7 tissues, no significant differences were detected.
Figure 18: Images of airway smooth muscle. Images of airway smooth muscle in longitudinal section taken from guinea pig tracheal rings after 0 (A), 1 (B), 3 (C), or 7 (D) days in organ culture. Rings were incubated in culture media supplemented with 0.3 mM BrdU, and subsequent tissue sections stained for BrdU (red nuclei) using APAAP technique, and counterstained with meyer's hematoxylin (blue nuclei). Scale bar is 50 μM in length.
Figure 19: Proliferation index of smooth muscle. The proliferation index of smooth muscle was measured from tracheal rings which had been incubated in organ culture for 1, 3 or 7 days. Ep+ (epithelium intact rings). Ep- (epithelium denuded rings). * p<0.05 compared with day 7.
Figure 20: **Total area of smooth muscle.** The total area of smooth muscle from tracheal strips incubated in organ culture for 1 (A), 3 (B), or 7 (C) days was expressed as a fraction of the basement membrane length. Ep+ (epithelium intact). Ep- (epithelium denuded). * p<0.02. † p<0.05. ‡ p<0.05.
DISCUSSION

The results of this thesis show that our organ culture system can be used to maintain guinea pig tracheal rings in a viable and functional capacity for up to 7 days, allowing the study of the effect of epithelial removal on smooth muscle mechanics and proliferation. This represents the first evaluation in any species of epithelial removal on the detailed mechanical properties of airway smooth muscle including the passive isometric stress at $L_{\text{max}}$ ($S_0$), maximum active isometric stress ($S_{\text{max}}$), and maximum isotonic shortening ($\Delta L_{\text{max}}$). Unlike previous studies assessing force generation to pharmacological agonists, this study determined the optimal length ($L_{\text{max}}$) for individual tissues when conducting pharmacological responses. Detailed morphology and stereology, which have not been employed in previous studies, were used to determine epithelial status and smooth muscle proliferation. Lastly, this thesis is the first to investigate the effect of longer term epithelial removal on both the mechanical properties and proliferation of airway smooth muscle. Such longer term effects of epithelial removal are more akin to the *in vivo* pathophysiology of epithelial denudation observed in asthma.

It was critical for the purposes of this thesis to establish the effectiveness of the technique used to remove the epithelium. As seen in figure 15 and as described in table 11, the epithelium was almost entirely removed from day 0 control epithelium denuded (Ep-) rings, with only a few scattered, simple cuboidal cells remaining on the basement membrane. The substantial drop in the total number of epithelial cells from epithelium intact (Ep+) rings to Ep- rings (226±13 to 19±3 respectively) emphasizes that the epithelial removal was effective. In addition, there was no visible evidence of physical injury to the lamina propria or submucosa due to epithelial removal.
It was important to demonstrate that the mechanical properties of the smooth muscle and the integrity of the epithelium in control tracheal rings could be maintained regardless of the length of incubation time in organ culture. Although some decreases in smooth muscle function were detected in tracheal strips from day 3 and day 7 when compared with their respective day 0 controls, these decreases did not occur universally in every mechanical parameter that was measured. Whereas a decrease in the $S_{\text{max}}$ was measured in day 7 rings when compared with their day 0 controls, a decrease in $\Delta L_{\text{Max}}$ was only measured in Ep+ day 7 rings and not Ep- day 7 rings when compared with their day 0 controls. No overall changes in the $S_{\text{max}}$ to histamine or carbachol occurred between day 7 rings and their day 0 controls. The sensitivity to carbachol decreased after 7 days in organ culture, yet the sensitivity to histamine increased with the same length of incubation time, and no changes in the sensitivity to isoproterenol were observed. These results cannot be explained by a decrease in smooth muscle function as a result of lengthy incubation times in organ culture.

In control Ep+ tracheal rings, the epithelium maintained an intact pseudostratified columnar ciliated morphology in all but six rings. In five of these rings, the epithelium was still intact but had a mixed pseudostratified columnar and squamous, or stratified squamous appearance. On average, the total number of epithelial cells in Ep+ rings after 7 days in culture was less than the total number of cells in Ep+ rings on days 0, 1 or 3. In Ep- rings harvested on day 7, the total number of epithelial cells was significantly greater than on day 0, but was less than the number of cells counted in day 3 Ep- rings. These minor changes in the morphology of the epithelium and epithelial cell count may be due to the presence of TGF-β which is found in serum-containing culture media. TGF-β has been shown to
inhibit airway epithelial proliferation and induce a squamous type morphology (reviewed by Gruenert et al., 1995).

A) Mechanical Properties

Although epithelial removal did not have a clear effect on $S_0$ at $L_{\text{max}}$, it did cause significant increases of both maximal force generation, and shortening. Immediate removal of the epithelium from tracheal strips on day 0 resulted in an increase in $S_{\text{max}}$, and $\Delta L_{\text{Max}}$ when compared to tracheal strips with intact epithelium. On days 1 and 7, an increase in $S_{\text{max}}$, and $\Delta L_{\text{Max}}$ in epithelium denuded tracheal strips was also observed. However, in tracheal strips measured on day 3, no differences were observed between epithelium intact and epithelium denuded tissues.

A possible explanation for the lack of an epithelium effect in day 3 tissues is that with epithelial regeneration, $S_{\text{max}}$, and $\Delta L_{\text{Max}}$ values return to control values. Morphological analysis revealed that the Ep- tracheal rings on days 0, and 1 were denuded of their epithelium, with only a few scattered cuboidal or squamous type cells visible on the basement membrane. In contrast, most epithelium denuded tissues from day 3 had a stratified squamous type epithelium covering the entire basal lamina. Since paired epithelium denuded rings on day 0 showed a denuded epithelium, the only plausible explanation is that the epithelium observed on day 3 had regenerated from the residual cuboidal or squamous cells left after the initial epithelial removal. In addition, the positive staining for BrdU incorporation into the nuclei of nearly 100% of the epithelium in originally epithelium denuded tracheal rings from day 3 confirmed that the epithelium had regenerated. Although this newly regenerated epithelium was not a pseudo-stratified columnar ciliated type, its presence appeared to be sufficient to allow $S_{\text{max}}$ and $\Delta L_{\text{Max}}$ to return to control
values. In Ep- tracheal rings harvested after 7 days, the epithelium had also regenerated, but not to the same extent as it had in tissues from day 3. Both the total number of epithelial cells and the number of tissues with a stratified squamous appearance were less in day 7 Ep- rings than in day 3 Ep- rings. It may be this difference in epithelial regeneration that accounts for the return in significant differences of the $S_{\text{max}}$ and $\Delta L_{\text{max}}$ between Ep+ and Ep- tissues on day 7.

Alternatively, long term changes in epithelium denuded tissues may have occurred by day 7 and account for the increase in $S_{\text{max}}$ and $\Delta L_{\text{max}}$. The differences in $S_{\text{max}}$ and $\Delta L_{\text{max}}$ between Ep+, and Ep- tissues on days 0 and 1 may be explained by the immediate loss of the epithelium. The return to baseline of these mechanical parameters by day 3 may be explained by epithelial regeneration. The increase in $S_{\text{max}}$ and $\Delta L_{\text{max}}$ in Ep- tissues on day 7 may have been due to the long term effects of epithelial removal rather than a decrease in number of epithelial cells. One of the hallmarks of asthma is a chronic injury to the epithelium and subsequent regeneration. If the increase in $S_{\text{max}}$ and $\Delta L_{\text{max}}$ in Ep- strips on day 7 is due to the long term consequences of epithelial removal, then this is consistent with the idea that chronic epithelial denudation and regeneration in asthma may lead to long term changes in the airway wall resulting in an increase in force and shortening.

The increases in both $S_{\text{max}}$ and $\Delta L_{\text{max}}$ do not come as a surprise. As discussed earlier in this thesis, the epithelium is known to be a source of many factors which can influence smooth muscle tone, particularly prostaglandins. Previous authors of in vitro studies have suggested that epithelial removal eliminates tone-generating substances, allowing the smooth muscle to be in a more relaxed state (Braunstein et al. 1988). In addition, epithelial removal eliminates
smooth muscle relaxants such as PGE$_2$ which would normally reduce a contractile response. Thus, epithelial removal could have two possible effects on an isometric, smooth muscle contractile response. Initially, when the muscle is stimulated to contract it would begin in a more relaxed state, and thus generate more active tension. Once the muscle contraction was underway, the lack of epithelial-derived relaxant factors would allow the muscle to contract to a greater extent. Similar effects due to epithelial removal would result in greater isotonic shortening responses from the muscle.

What came as somewhat of a surprise, was the lack of effect of epithelial removal on $S_0$. Consistent with Braunstein et al. (1988), one would expect removal of the epithelium would eliminate tone-generating substances and thereby reduce $S_0$. This did not occur, and hence it may be speculated that other tissues in the airway wall may affect $S_0$, possibly through release of chemical mediators. Since epithelial removal did not lower $S_0$, the increase in $S_{\text{max}}$ and $\Delta L_{\text{max}}$ must have come from an ability to generate more force and shortening with the same $S_0$. This increase may be due to removal of an epithelium-derived relaxing factor, such as PGE2, nitric oxide, or some other yet discovered relaxing factor.

In the guinea pig trachea cycloxygenase (COX) products, especially PGE$_2$ are known to be major epithelium-derived factors that effect the contractile state of the smooth muscle (Holtzman, 1992). As a result, parallel smooth muscle experiments were repeated in paired tracheal rings in the presence of flurbiprofen, a COX-1 and COX-2 inhibitor. It was expected that addition of flurbiprofen would mimic the effect of epithelial removal (Hay et al., 1986). However, the addition of flurbiprofen failed to consistently produce the same increase in $S_{\text{max}}$, $\Delta L_{\text{Max}}$, and other smooth muscle mechanical parameters, as did removal of the epithelium.
This failure of flurbiprofen to mimic epithelial removal cannot be explained by the use of too low a concentration, or a lack of potency of flurbiprofen, since flurbiprofen did produce its own significant effects.

One of the significant effects due to flurbiprofen was a decrease in $S_0$, a decrease that was not observed with epithelial removal. As suggested, other tissues in the airway wall may be a source of COX products which could affect $S_0$. Subsequently one could expect that this decrease in $S_0$ with flurbiprofen could be followed by an increase in $S_{\text{max}}$ with the addition of flurbiprofen. This was not observed. These results are consistent with the epithelium releasing a non-cyclooxygenase relaxant factor, whose effects would mask the potential increase in $S_{\text{max}}$ due to a decrease in $S_0$.

**B) Pharmacological Responses**

Epithelial removal produced very similar effects on the dose responses to histamine as it did on $S_{\text{max}}$ and $\Delta L_{\text{max}}$ to acetylcholine. On days 0, 1, and 7, Ep-tracheal strips produced significantly greater $S_{\text{max}}$'s and showed significantly greater sensitivity to histamine than Ep+ tracheal strips. Again, Ep+, and Ep- tracheal strips on day 3 failed to show differences, and this may be explained by the regeneration of the epithelium as discussed above. The day 0 results are in agreement with many previous studies which demonstrated both increases in $S_{\text{max}}$ (Kamikawa 1993; Hay et al., 1986) and increases in sensitivity (Kamikawa 1993; Preuss et al., 1992; Goldie et al., 1986; Hay et al., 1986) to histamine in Ep- tissues. The authors of these studies concluded that an epithelium-derived relaxing factor was removed with epithelial denudation allowing histamine to produce a stronger contraction. The identity of this epithelium-derived relaxing factor has not been discovered despite copious research. Histamine responses in the guinea pig have been shown to be...
modulated by epithelium-derived PGE$_2$ (Braunstein et al., 1988), and nitric oxide (Yan et al., 1994) released upon stimulation with histamine. However, other research suggests that the epithelium-derived relaxing factor cannot be either of these factors (Fernandes and Goldie, 1990; Munakata et al., 1990; Hay et al., 1988). Since flurbiprofen did not mimic the effects of epithelial removal on the responses to histamine, the results of this study corroborate the latter argument that the response of guinea pig trachealis to histamine is modulated by an epithelium-derived relaxant factor that is unlikely to be a prostaglandin.

Neither epithelial removal nor the addition of flurbiprofen, produced consistent significant effects on either the $S_{max}$ or sensitivities of guinea-pig tracheal strips to carbachol. Because the sample size for these responses was limited, it is difficult to conclude that neither of the interventions had an effect. However, our results are consistent with previous work, which demonstrated that the responses of guinea pig trachealis to carbachol were not altered by epithelial removal (Morrison et al., 1992; Small et al., 1990; Goldie et al., 1986). Since carbachol and acetylcholine utilize the same smooth muscle receptor (namely the M$_3$ receptor) (Morrison et al., 1992), one might expect the two contractile agonists to produce similar responses. Yet as our results indicate and other have shown, only acetic, but not carbamic acid choline esters are influenced by epithelial removal (Morrison et al., 1992; Small et al., 1990; Goldie et al., 1986). To account for this difference in response to the two choline esters, Koga et al. (1992) claimed that the epithelium serves as a source of acetylcholinesterase, which would degrade acetylcholine but not carbachol. However, work by Small et al. (1990) contradicts this finding, suggesting the difference cannot be accounted for by an epithelium based acetylcholinesterase activity.
Like the results of the carbachol dose responses, neither epithelial removal nor the addition of flurbiprofen had consistent effects on the relaxant responses of tracheal strips to isoproterenol pre-contracted with carbachol. The increase in sensitivity to isoproterenol observed in Ep- rings on day 0 is consistent with the results of previous studies (Lundblad et al., 1988; Farmer et al., 1986; Holroyde, 1986), and may be explained by the loss of extraneuronal uptake of isoproterenol into the epithelium (Farmer et al., 1986). An increase in sensitivity to isoproterenol was also observed in Ep- rings harvested after day 7. It is unknown why the same increase in sensitivity was not also observed in Ep- rings from day 1, if the epithelium was indeed a site of extraneuronal uptake of isoproterenol, since morphometric analysis showed the epithelium was still denuded in Ep- rings from day 1. Again, the sample size for these responses was limited, and as a result it is difficult to draw any conclusions about the effect of epithelial removal and the addition of flurbiprofen on the dose responses to isoproterenol.

One difficulty that was encountered when comparing the results of this thesis with previously published work was the lack of consistent determination, and use of $L_{\text{max}}$ when conducting pharmacological experiments. Mitchell et al. (1991) established the necessity of determining $L_{\text{max}}$, and then setting tissues to this length to ensure accurate pharmacological responses to contractile or relaxant agonists. The $L_{\text{max}}$ of all smooth muscle strips in this study were determined individually and employed during pharmacological responses to histamine, carbachol and isoproterenol.

C) Smooth Muscle Proliferation

The second aim of this thesis was to investigate if epithelial removal would alter smooth muscle proliferation. To this end, guinea pig tracheal rings were
incubated in organ culture in the presence of the nucleotide analogue, BrdU, and the uptake of BrdU into cell nuclei was determined by immunohistochemistry. Significant proliferation of the epithelium was detected in rings originally denuded of their epithelium, but regardless of epithelial status, very little airway smooth muscle proliferation occurred. No staining for BrdU was detected in control sections, either from day 0 rings or IgG controls ruling out the possibility that the staining was due to non-specific nuclear incorporation or non-specific staining.

Since the airway epithelium has a high regenerative capacity, it came as no surprise that epithelial proliferation was detected in our experiments, both in Ep+ rings and Ep- rings. In Ep- rings, the epithelial proliferation index (PI) appeared to plateau near 100% by day 3, suggesting that by this time all epithelial cells at one point during their time in culture, underwent cell division. This observation also supports the idea that the epithelial cells present in Ep- rings on days 3 and 7 were daughter cells of the residual cells left after the denudation procedure. In Ep+ rings, the PI appeared to plateau near 40%, also by day 3. Positive BrdU staining occurred in epithelium nuclei close to the basement membrane; presumably because they were basal or secretory cells acting as progenitor cells. The relatively high PI for the epithelium in Ep+ rings at day 3 suggests that in this culture system, the epithelial cells were actively proliferating.

Results of the total number of epithelial cells support the conclusions made from the PI results. In Ep- rings, very few cells were present in day 0 or day 1 rings, yet by day 3, there was a significant increase in the number of cells counted in each ring, most of which stained positive for BrdU. Presumably, a large degree of epithelial proliferation took place between these two time points. In rings from day
7, the total number of epithelial cells in each ring was still greater than those counted on day 0 or day 1, but they were not as numerous as those on day 3.

It was expected that epithelial denudation, and subsequent epithelial regeneration in tracheal rings maintained in our organ culture system would induce proliferation of the smooth muscle in the underlying submucosa. In their *in vivo* guinea pig experiments, Erjefält et al. (1995) documented an increase in the proliferative activity of cells within the submucosa beneath zones of recently repaired epithelium. It was hypothesized that these cells may have been smooth muscle cells stimulated to proliferate by the active epithelial repair process occurring in close proximity. As discussed earlier, Stewart et al., (1993) suggested that epithelial removal and subsequent repair would alter the profile of epithelium-derived smooth muscle mitogen factors. Additional research by Halayko et al. (1997a, 1996) supports the idea of two different phenotypes of smooth muscle cells, one which is contractile, and one which is capable of proliferation. In contrast to the expectation, no differences in the smooth muscle PI were observed between tracheal rings with and without epithelium regardless of the number of days in organ culture.

Ebina et al. (1993) reported both hyperplasia, and hypertrophy in the airway smooth muscle of asthmatic lungs. To determine if epithelial removal had caused an increase in smooth muscle size instead of number, the area of smooth muscle in Ep+ and Ep- rings was calculated. An increase in the smooth muscle area (corrected for by length of basement membrane) was detected in Ep- tissues after 1 and 7 days in organ culture when compared to their respective day 0 controls. The increase in muscle area cannot be accounted for by removal of the epithelium as the same increase was observed in Ep+ rings. The lack of change in smooth
muscle area in day 3 tissues rules out any effect of increasing time in organ culture alone causing an increase in smooth muscle area.

Several conclusions can be made from these results showing a lack of smooth muscle growth. It could be that rings were not held in organ culture long enough to allow the smooth muscle time to proliferate. However, the smooth muscle PI seemed to have plateaued near 4% by day 3, regardless of epithelial status. If further proliferation was to occur after day 7, and especially in Ep- rings, then one would not have expected to see a plateau in the PI as was observed. Alternatively, the balance of smooth muscle mitogens secreted by the epithelium, such as endothelin or thromboxane, may have not been present, or may have been too weak in this culture system to have an effect. The regenerated epithelium may have also been secreting enough growth inhibitory factors, such as TGF-β, to counteract the proliferative effects of potential mitogens. It may be that regeneration of a fully differentiated pseudo-stratified columnar epithelium is needed to produce the mitogenic signal necessary to effectively stimulate the smooth muscle to proliferate. In Ep- rings from days 3 and 7, only a simple or stratified squamous epithelium had regenerated. In addition, other tissues in the tracheal rings may have had an inhibitory effect on smooth muscle proliferation. Heparin, a constituent of normal extracellular matrix (ECM), has been shown to inhibit DNA synthesis in cultured airway smooth muscle (Johnson et al., 1995). Proliferation of cytokine stimulated cultured airway smooth muscle was inhibited when grown in culture dishes seeded with various ECM components, such as fibronectin, laminin and type I collagen (Hirst and Twort, 1997).

Whilst it has been shown that epithelial products can alter the proliferative activity of cultured airway smooth muscle, it is unknown if such changes occur in
As described earlier, Halayko et al. (1996) showed that smooth muscle cells in primary culture rapidly lose their contractile proteins and are replaced by non-contractile proteins. This change in cell phenotype away from a contractile type to a more synthetic type in primary culture may exaggerate the proliferative effects of epithelial products. Our findings suggest that smooth muscle proliferation in the normal airway milieu is tightly regulated and that isolated smooth muscle cells do not represent the \textit{in situ} situation.

Lastly, but more importantly, these results support previous work conducted in our laboratory challenging the importance of smooth muscle growth in asthma (Thomson et al., 1996; Bramley et al. 1994). Thomson et al. (1996) and Bramley et al. (1994) both failed to detect an increase in the smooth muscle in the airways of asthmatic lungs using detailed stereologic techniques. They argued that changes in the mechanical properties of the muscle, or unloading of the muscle due to ECM changes, were more important in determining airway narrowing than an increase in muscle. Consistent with this hypothesis, our results have shown that the long term increase in force generation and shortening in epithelium denuded tracheal strips cannot be explained by an increase in the amount of muscle in those strips.

There is a possibility that the cells that did stain positive for nuclear incorporation of BrdU may not have been smooth muscle cells. No specific smooth muscle identifying markers, such as smooth muscle MHC, smooth muscle \(\alpha\)-actin, and \(h\)-caldesmon (Halayko et al., 1997a) were used to positively identify the smooth muscle cells counted in each ring. As a result, some of the cells which stained positive for BrdU may have been fibroblasts or myofibroblasts within the smooth muscle bundle. Regardless of what cell type in the smooth muscle bundle stained positive, there was very little proliferative activity.
There is also a possibility that the BrdU detected in these cells was incorporated into mitochondrial DNA (mtDNA), or that the cell became polyploidic. BrdU has been used to study the replication of mtDNA in HeLa cells (Davis and Clayton, 1996). In their study, Davis and Clayton (1996) showed that BrdU labeled mtDNA first appeared in the perinuclear mitochondrial organelles, and then radiated out into the cell's complement of mitochondrial organelles. In our study, BrdU labeling of cell nuclei was mostly homogeneous, clearly demarcating the nucleus and its contents, especially in the smooth muscle cells. Thus, it is unlikely that BrdU staining in this study represented mtDNA because of the homogeneous pattern of nuclear staining.

Polyploidy occurs when a nucleus fails to divide after mitosis resulting in a cell with two times or greater the normal diploid DNA content. De et al. (1995) have reported polyploidy in guinea pig cultured airway smooth muscle in response to IL-1β and IL-6, yet it is unknown if polyploidy of airway smooth muscle occurs in vivo since it has been shown in vascular smooth muscle from hypertensive vessels (van Neck et al., 1992; Black et al. 1988a; Black et al. 1988b), and various smooth muscle cancers (Jeffers et al., 1996; Chou et al., 1996; Dorman et al. 1990).

**SUMMARY**

This thesis provides evidence that airway smooth muscle force and shortening are increased with not only acute epithelial removal, but epithelial removal for up to 7 days. In addition, these results suggest that epithelial regeneration may return smooth muscle mechanics to normal. Longer-term effects may still be present, suggesting that repeated epithelial injury as occurs in asthma may result in persistent, enhanced airway smooth muscle force generation and
shortening. Although epithelial removal had no effect on smooth muscle hyperplasia or hypertrophy, the protocol developed in this thesis would be useful in investigating the long term effects of epithelial removal on additional aspects airway wall remodelling.
REFERENCES


Naylor, B. 1962. The shedding of the mucosa of the bronchial tree in asthma. 
*Thorax* 17:69-72.

Nettesheim, P., and Bader, T. 1996. Tumor necrosis factor α stimulates 
arachidonic acid metabolisms and mucus production in rat tracheal epithelial cell 

Ninomiya, H., Uchida, Y., Endo, T., Ohtsuka, M., Nomura, A., Saotome, M., and 
Hasegawa, S. 1996. The effects of calcitonin gene-related peptide on tracheal 

Specific binding of endothelin-1 to canine tracheal epithelial cells in culture. *Am. 

Noguchi, K., Fukuroda, T., Ikeno, Y, Hirose, H., Tsukada, Y., Nishikibe, M., Ikemoto, 
F., Matsuyama, K, and Yano, M. 1991. Local formation and degradation of 
179:830-835.

Noveral, J.P., and Grunstein, M.M. 1992b. Role and mechanism of thromboxane-
induced proliferation of cultured airway smooth muscle cells. *Am. J. Physiol.* 
263:L555-L561.


like growth factor axis in airway smooth muscle cells. *Am. J. Physiol.* 267:L761-
L765.

1992a. Role of endothelin-1 in regulating proliferation of cultured rabbit airway 

Ohrui, T., Yamauchi, K., Sekizawa, K., Ohkawara, Y., Maeyama, K., Sasaki, M., 
Histamine N-methyltransferase controls the contractile response of guinea pig 

shortening and isometric tension generation in rabbit trachealis. *J. Appl. Physiol.* 
77:1638-1643.

1996. Endothelin-1-induced potentiation of human smooth muscle proliferation: 


