The airway smooth muscle response to strain and the relationship with airway hyper-responsiveness

By

Thomas K Ansell BSc (Hon, First class)

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<th>Description</th>
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<tbody>
<tr>
<td>5-HT</td>
<td>5-Hydroxytryptamine</td>
</tr>
<tr>
<td>$\beta_2$-receptors</td>
<td>Adrenergic $\beta_2$-receptors</td>
</tr>
<tr>
<td>$\dot{V}$</td>
<td>Airflow</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>AHR</td>
<td>Airway hyper-responsiveness</td>
</tr>
<tr>
<td>$A_i$</td>
<td>Internal lumen area</td>
</tr>
<tr>
<td>$A_{m_o}$</td>
<td>Outer ASM perimeter</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>$\alpha$OCT</td>
<td>Anatomical optical coherence tomography</td>
</tr>
<tr>
<td>APA</td>
<td>Australian Postgraduate Award</td>
</tr>
<tr>
<td>ASM</td>
<td>Airway smooth muscle</td>
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<tr>
<td>AuPS</td>
<td>Australian Physiological Society</td>
</tr>
<tr>
<td>$B_1$-receptor</td>
<td>Bradykinin 1-receptor</td>
</tr>
<tr>
<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic 3’, 5’ adenosine monophosphate</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic 3’, 5’ guanosine monophosphate</td>
</tr>
<tr>
<td>CCh</td>
<td>Carbachol</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>CPAP</td>
<td>Continuous positive airway pressure</td>
</tr>
<tr>
<td>CPI-17</td>
<td>Protein kinase C-dependent phosphatase inhibitor</td>
</tr>
<tr>
<td>DAG</td>
<td>Di-glyceride</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
</tr>
<tr>
<td>DI</td>
<td>Deep inspiration</td>
</tr>
<tr>
<td>EC_50</td>
<td>Sensitivity</td>
</tr>
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<td>Maximum response</td>
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</tr>
<tr>
<td>eNANC</td>
<td>Excitatory non-cholinergic/non-adrenergic</td>
</tr>
<tr>
<td>ERS</td>
<td>European Respiratory Society</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FEV_1</td>
<td>Volume of forced expiration in the first 1 second</td>
</tr>
<tr>
<td>FOT</td>
<td>Forced oscillation technique</td>
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<tr>
<td>FRC</td>
<td>Functional residual capacity</td>
</tr>
<tr>
<td>FVC</td>
<td>Forced vital capacity</td>
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<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factors</td>
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<tr>
<td>GCS</td>
<td>Glucocortiosteriods</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony stimulating factor</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Name</td>
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<tr>
<td>----------</td>
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<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>H₁-receptor</td>
<td>Histamine 1-receptor</td>
</tr>
<tr>
<td>H₂-receptor</td>
<td>Histamine 2-receptor</td>
</tr>
<tr>
<td>H and E</td>
<td>Haematoxylin and Eosin</td>
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<tr>
<td>IL-1β</td>
<td>Interleukin-1β</td>
</tr>
<tr>
<td>IL-2</td>
<td>Interleukin-2</td>
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<tr>
<td>iNANC</td>
<td>Inhibitory non-cholinergic/non-adrenergic</td>
</tr>
<tr>
<td>IP₃</td>
<td>Inositol triphosphate</td>
</tr>
<tr>
<td>LABA</td>
<td>Long-acting β₂-agonists</td>
</tr>
<tr>
<td>L-NOARG</td>
<td>N-nitro-L-arginine</td>
</tr>
<tr>
<td>Lₒ</td>
<td>Optimum length</td>
</tr>
<tr>
<td>L-T curve</td>
<td>Length-tension curve</td>
</tr>
<tr>
<td>L-Type</td>
<td>Long lasting-type</td>
</tr>
<tr>
<td>M₂-receptor</td>
<td>Muscarinic 2-receptor</td>
</tr>
<tr>
<td>M₃-receptor</td>
<td>Muscarinic 3-receptor</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>MHC</td>
<td>Myosin heavy chain</td>
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<tr>
<td>MLCK</td>
<td>Myosin light chain kinase</td>
</tr>
<tr>
<td>MLCP</td>
<td>Myosin light chain phosphatase</td>
</tr>
<tr>
<td>MRLC</td>
<td>Myosin II regulatory light chain</td>
</tr>
<tr>
<td>MCh</td>
<td>Methacholine</td>
</tr>
<tr>
<td>NA</td>
<td>Noradrenaline</td>
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<tr>
<td>NHMRC</td>
<td>National Health and Medical Research Council</td>
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<tr>
<td>NK1-receptor</td>
<td>Neurokinin 1-receptor</td>
</tr>
<tr>
<td>NK2-receptor</td>
<td>Neurokinin 2-receptor</td>
</tr>
<tr>
<td>NKA</td>
<td>Neurokinin A</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>Pa</td>
<td>Airway lumen pressure</td>
</tr>
<tr>
<td>Pal</td>
<td>Alveoli pressure</td>
</tr>
<tr>
<td>PD2</td>
<td>-log10(EC50)</td>
</tr>
<tr>
<td>PGE2</td>
<td>prostaglandin E2</td>
</tr>
<tr>
<td>PGF2α</td>
<td>prostaglandin F2α</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>P_m</td>
<td>Mouth pressure</td>
</tr>
<tr>
<td>P_mo</td>
<td>Outer ASM perimeter</td>
</tr>
<tr>
<td>P_mot</td>
<td>Trough immediately prior to breathing manoeuvres</td>
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<tr>
<td>P-MC</td>
<td>Pharmo-mechanical coupling</td>
</tr>
<tr>
<td>P_pl</td>
<td>Pleural space pressure</td>
</tr>
<tr>
<td>P_tm</td>
<td>Transmural pressure</td>
</tr>
<tr>
<td>P_tp</td>
<td>Transpulmonary pressure</td>
</tr>
<tr>
<td>R_sw</td>
<td>Resistance to flow through the airways</td>
</tr>
<tr>
<td>ROK</td>
<td>Rho kinase</td>
</tr>
<tr>
<td>RyR-receptor</td>
<td>Ryanodine-receptor</td>
</tr>
<tr>
<td>RV</td>
<td>Residual volume</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SP</td>
<td>Substance P</td>
</tr>
<tr>
<td>TLC</td>
<td>Total lung capacity</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TSANZ</td>
<td>Thoracic Society of Australia and New Zealand</td>
</tr>
<tr>
<td>T-Type</td>
<td>Transient-type</td>
</tr>
</tbody>
</table>
UWA  University of Western Australia

VIP  Vasoactive intestinal peptide

WA₁  Inner wall area
Summary

Airway hyper-responsiveness (AHR, i.e. excessive bronchoconstriction to an inhaled bronchial challenge) is believed to be a major contributor to airflow limitation, a primary characteristic of asthma. The cause(s) of AHR remain unclear but likely involve abnormalities in airway smooth muscle (ASM) and/or airway wall structure/function. More recently, the dynamic mechanical environment of the lung has been identified as an important regulator of airway responsiveness and may be susceptible to inflammatory disease processes, contributing to AHR. In normal healthy individuals, deep inspiration (DI) produces a transient reversal of bronchoconstriction (i.e. bronchodilation). The underlying mechanism by which DI produces bronchodilation is thought to involve stretch-induced relaxation of ASM, due to perturbed cross-bridge binding and/or de-polymerisation of the contractile apparatus. However, the bronchodilatory response to DI is attenuated or abolished in patients with asthma and this may contribute to the development of AHR. The general aim of this thesis is to better understand the ASM response to strain during breathing manoeuvres and the relationship with AHR.

The first aim of this thesis was to determine whether an increase in ASM stress or the accompanying increase in strain mediates the bronchodilatory response to DI. During DI a distending force is applied to ASM, (i.e. mechanical stress) and the muscle is lengthened (i.e. strain), which produces a transient reversal of bronchoconstriction (i.e. bronchodilation). The relative contribution of ASM stress and strain to the
bronchodilatory response to DI is unknown. I used whole porcine bronchial segments in vitro and a servo-controlled syringe pump that applied fixed-transmural pressure ($P_{tm}$) or fixed-volume oscillations, simulating tidal breathing manoeuvres and intermittent DI. I show that ASM strain, rather than stress, is the critical determinant of bronchodilation and unexpectedly, that the rate-of-inflation during DI also impacts on bronchodilation, independent of the magnitudes of either stress or strain.

The second aim of this thesis was to determine whether pharmacological bronchodilators produce part of their physiological action through reduction of airway stiffness that enhances the relaxation produced by oscillatory loads. In patients with asthma, airflow limitation is at least partially reversed by administration of pharmacological bronchodilators, typically $\beta_2$-agonists. In addition to receptor-mediated bronchodilation, the dynamic mechanical environment of the lung itself can produce bronchodilation. I show that the bronchodilatory response to the non-specific $\beta$-agonist, isoprenaline, was greater during simulated breathing manoeuvres, compared with the response under static conditions.

The third aim of this thesis was to determine whether the pro-inflammatory cytokines, tumor necrosis factors (TNF) and/or interleukin-1$\beta$ (IL-1$\beta$), modulate the ASM response to strain and therefore, the bronchodilatory response to DI. In isolated ASM strips/rings in vitro, culture with TNF and/or IL-1$\beta$ increased isometric force production (i.e. a hyper-contractile phenotype) to acetylcholine (ACh), carbachol (CCh), bradykinin and the non-specific G-protein coupled receptor (GPCR) agonist, 5-
hydroxytryptamine (5-HT). Whilst chronic inflammation of the airway wall and the failure of DI to produce bronchodilation are common to asthma, whether TNF and IL-1β modulate the ASM response to strain is unknown. I used a novel methodology of culturing airway segments with TNF and IL-1β. I show that whilst 2 days of culture with TNF and IL-1β induced an increase in the maximum airway narrowing to ACh, it did not modulate the ASM response to strain during DI.

The fourth and final aim of this thesis was to determine, using an intact airway segment preparation, the effect of sustained changes in $P_{tm}$ (and therefore ASM perimeter) on ASM contraction and airway narrowing. A newly found property of ASM, identified in isolated strips, is the capacity to optimise force production following strain, a phenomenon termed ‘length-adaptation’. Despite evidence of length-adaptation in isolated ASM strips, the presence and/or potential role of length-adaptation in situ and therefore, the implications for airway function in health and disease, is uncertain. I used anatomical optical coherence tomography (aOCT) to directly measure muscle length in situ. Whilst I reasoned that prolonged changes in $P_{tm}$ would produce an increase in ASM force production toward pre-strain levels, I show little effect of prolonged changes in $P_{tm}$ on bronchoconstriction.

This thesis highlights the importance of ASM strain in regulating the bronchodilatory response to breathing manoeuvres. I showed that ASM strain, rather than mechanical stress, is the critical determinant of bronchodilation. The implications are that airway wall stiffening observed in patients with asthma is likely to reduce ASM strain
produced by breathing manoeuvres and may contribute to the failure of DI to produce bronchodilation. The greater bronchodilatory response to β2-agonists during breathing manoeuvres are likely explained by the effect of isoprenaline on reducing airway wall stiffness, which increased ASM strain, producing greater bronchodilation. Any intervention that reduces airway wall stiffness is likely to be beneficial in reversing bronchoconstriction by enhancing the bronchodilatory response to breathing manoeuvres. I show that TNF and IL-1β had no effect on the ASM response to strain, suggesting that chronic inflammation of the airway wall does not directly contribute to the failure of DI in patients with asthma. Finally, I showed that sustained changes in P_{tm}, which is the physiological determinant of ASM length, have little effect on airway narrowing. I propose that, under physiological levels of strain, ASM does not undergo contractile apparatus re-arrangement. In conclusion, the studies in this thesis, using intact airway segments, allows the following conclusions to be drawn: Airway wall stiffness is increased in asthma and may limit ASM strain during DI. The loss of that inhibitory effect would favor increased bronchoconstriction and may contribute to the development of AHR. Reducing airway wall stiffening represents a potential second target for novel pharmacological bronchodilators to treat asthma by virtue of reducing ASM stiffness and increasing ASM strain during breathing manoeuvres.
List of publications

The experiments in this thesis were published in peer-reviewed journals in the form of the papers shown below.

Chapter 2 Bronchodilatory response to deep inspiration in bronchial segments: The effects of mechanical stress versus strain


Chapter 3 Pharmacological bronchodilation is partially mediated by reduced airway wall stiffness

I have also published previous experiments in peer-reviewed journals in the form of the papers shown below.


List of abstracts

The experiments in this thesis were presented at conferences in the form of the abstracts shown below.

Chapter 2  Bronchodilatory response to deep inspiration in bronchial segments:  
The effects of mechanical stress versus strain


Chapter 3  Pharmacological bronchodilation is partially mediated by reduced airway wall stiffness

Chapter 4  Do TNF and IL-1β modulate the airway smooth muscle response to strain?


Ansell T. K., Noble P. B., Mitchell H. W., McFawn P. K., Airway narrowing and bronchodilatory response to deep inspiration in cultured and cytokine treated bronchial segments, TSANZ, Western Australian local scientific meeting, 22-23 April 2011.
Chapter 5 Mechanical properties of the airway wall during sustained inflationary and deflationary transmural pressures

Ansell T. K., McFawn P. K., Mitchell H. W., Noble P. B., Airway narrowing, smooth muscle force and wall compliance at low and high mechanical strains, Australian Physiological Society (AuPS), Scientific meeting, 4-7 December 2011.
1.1 Introduction

Airway hyper-responsiveness (AHR, i.e. excessive bronchoconstriction to an inhaled bronchial challenge) is believed to be a major contributor to airflow limitation, a primary characteristic of asthma (99, 173). The cause(s) of AHR remain unclear but likely involve abnormalities in airway smooth muscle (ASM) and/or airway wall structure/function. More recently, the dynamic mechanical environment of the lung has been identified as an important regulator of airway responsiveness and may be susceptible to inflammatory disease processes, contributing to AHR (6). In normal healthy individuals, deep inspiration (DI) produces a transient reversal of bronchoconstriction (i.e. bronchodilation) to a number of different inhaled ASM contractile agonists (53, 73, 117, 137). The underlying mechanism by which DI produces bronchodilation is thought to involve stretch-induced relaxation of ASM (59-61, 68), due to perturbed cross-bridge binding (60, 61) and/or de-polymerisation of the contractile apparatus (66). However, the relative contribution of mechanical stress and strain to the bronchodilatory response to DI is unknown. The bronchodilatory response to DI is attenuated or abolished in patients with asthma (58, 106, 142) and this may contribute to the development of AHR.
1.2 Airway narrowing

Airflow

Airflow ($\dot{V}$) is regulated by the resistance to flow through the airways ($R_{aw}$), which is determined by the following equation:

$$\dot{V} = \frac{P_m - P_{al}}{R_{aw}}$$

Where, $P_m$ is the pressure at the mouth, $P_{al}$ is the pressure in the alveoli and the difference between these pressures determines the respiratory driving pressure. The major determinant of the $R_{aw}$ is airway calibre (99, 173). Any reduction in calibre (i.e. airway narrowing, bronchoconstriction) will increase $R_{aw}$ and decrease airflow for a given respiratory driving pressure (i.e. airflow limitation). Two inter-dependent factors determine airway lumen calibre: the degree of airway smooth muscle (ASM) activation that produces airway narrowing, as well as a complex series of loads arising from the airway wall and lung parenchyma.

Airway smooth muscle activation

Airway smooth muscle contraction produces airway narrowing. A diverse range of agonists, including neurotransmitters and non-neural mediators produce ASM contraction. In health, ASM activation is dominated by extensive para-sympathetic,
cholinergic innervation (16, 19). The cholinergic neurotransmitter, acetylcholine (ACh), is released from post-synaptic cholinergic nerves and binds to muscarinic 3 (M₃)-receptors on the ASM (Figure 1.2.1). On the post-ganglionic nerves, ACh also binds to muscarinic 2 (M₂)-receptors, providing a negative feedback-loop by inhibiting the further release of ACh. In isolated ASM strips (158) and whole bronchial segments (10, 11) \textit{in vitro}, exogenous ACh produces ASM contraction. Although there is relatively little evidence in the literature for the release of excitatory non-cholinergic/non-adrenergic (eNANC) neurotransmitters in humans, several studies have demonstrated the release of neuropeptides, such as tachykinins, in other animals (16, 20, 37, 171). In rodents, substance P (SP) and neurokinin A (NKA) are both released from capsaicin-sensitive C-fibres, binding to neurokinin 1 and 2 (NK₁ and NK₂)-receptors on the ASM, respectively. Despite the lack of evidence for the release of SP in humans, exposure of isolated human ASM strips \textit{in vitro} to exogenous SP produces contraction which is unaffected by the muscarinic-receptor antagonist, atropine (107).
In disease, pro-inflammatory cytokines may trigger the secondary-release of contractile non-neural mediators, such as histamine, bradykinin and the paracrine, prostaglandin \( F_2\alpha \) (PGF\(_2\alpha\)) (17, 20), from numerous cell types, including inflammatory cells (29). Histamine and bradykinin are both released from mast cells, binding to histamine 1 (H\(_1\)) (17, 20) and bradykinin 2 (B\(_2\))-receptors on the ASM (84), respectively. Whilst in normal healthy individuals \textit{in vivo}, inhalation of exogenous bradykinin produces little or no ASM contraction, in patients with asthma, it produces potent ASM contraction (17), suggesting that the expression of B\(_1\)-receptors is also modified by chronic inflammation of the airway wall. However, the mode of action of PGF\(_2\alpha\) is not completely understood.
As well as ASM contraction, a diverse range of neurotransmitters and non-neural mediators also produce relaxation (i.e. reversal of contraction). In health, ASM relaxation is dominated by inhibitory non-cholinergic/non-adrenergic (iNANC) neurotransmitters, such as nitric oxide (NO) and vasoactive intestinal peptide (VIP), although the former is typically thought to be more important. Nitric oxide is co-released from adrenergic nerves, diffusing directly through the ASM cell membrane, binding to guanylyl cyclase, whilst VIP is co-released from cholinergic nerves, binding to VIP-receptors on ASM (however, these appear to only be expressed on ASM in large calibre airways) (16, 171). In whole bronchial segments *in vitro*, the NO donor, sodium nitroprusside, relaxes ASM (10). Whilst there is little or no sympathetic, adrenergic innervation of human ASM (16, 19), adrenergic innervation of ASM has been demonstrated in other animals. In rodents, the adrenergic neurotransmitter, noradrenaline (NA), is released from efferent adrenergic nerves, binding to adrenergic β_2 (β_2)-receptors on ASM. Despite the lack of adrenergic innervation of human ASM, β_2-receptors are expressed. In isolated strips (64) and whole bronchial segments (10) *in vitro*, exogenous β_2-agonists, such as the non-specific β-agonist, isoprenaline, relax ASM. Indeed, exogenous isoprenaline is more potent at relaxing ASM than NO (10). Nitric oxide and other relaxant non-neural mediators, such as the paracrine, prostaglandin E_2 (PGE_2) may also be released from airway epithelial cells. Prostaglandin E_2 appears to be triggered by histamine binding to histamine 2 (H_2)-receptors on epithelial cells (16, 171). In isolated bronchial strips (i.e. intact airway strips) *in vitro*, denuding of the airway epithelium increases contraction to exogenous histamine by ~30 % (92), highlighting the importance of the airway epithelially-released mediators, such as PGE_2, in regulating bronchoconstriction.
**Airway smooth muscle contraction**

Excitation-contraction coupling of ASM is primarily through trimeric G-protein coupled receptor (GPCR) and the activation of G₉/G₁₁ sub-unit pathways, termed ‘pharmo-mechanical coupling’ (P-MC, i.e. independent of cell membrane depolarization) (70). Binding of contractile agonists to GPCR and G₉/G₁₁ sub-units on ASM, such as ACh, activates the phospholipase C (PLC), which produces an increase in inositol triphosphate (IP₃), whereby IP₃ binds to IP₃-receptors on the sarcoplasmic reticulum, resulting in intra-cellular Ca²⁺ release. As well as P-MC, electro-mechanical coupling (E-MC) also produces ASM contraction, although typically thought to be more important at high doses of contractile agonist and in tracheal, compared with ASM (83). Binding of contractile agonists to GPCR and G₁/G₆ sub-units opens Na⁺ channels, depolarising the ASM cell membrane and activating voltage-gated transient-type (T-type), as well as long lasting-type (L-type) Ca²⁺ channels. Extra-cellular Ca²⁺ also binds to Ca²⁺/ryanodine (RyR)-receptors on the sarcoplasmic reticulum, triggering the release of intra-cellular Ca²⁺, a phenomenon termed ‘Ca²⁺-induced-Ca²⁺-release’ (153). More recently, this dogma has been challenged by a study using isolated ASM rings *in vitro*, which showed that ASM contraction to the depolarizing solution, KCl, is at least partially blocked by the RyR-receptor antagonist, ryanodine (52), suggesting that L-type Ca²⁺ channels are mechanically coupled to RyR-receptor channels and are therefore, independent of extra-cellular Ca²⁺.
Contraction of ASM is regulated by the myosin II regulatory light chain (MRLC) (70, 140, 152). Phosphorylation of the MRLC allows for cross-bridge cycling to occur between actin filaments and the myosin heavy chain (MHC) (Figure 1.2.2). The number of cross-bridge attachments and the rate-of-cross-bridge cycling determines the level of ASM force production (154). Intracellular Ca\textsuperscript{2+} binds to calmodulin (CaM) and activates myosin light chain kinase (MLCK), whereby MLCK phosphorylates the MRLC. In contrast, myosin light chain phosphatase (MLCP) dephosphorylates the MRLC, inhibiting ASM contraction. The balance of MLCK/MLCP activity can, therefore, be thought of as regulating ASM contraction. The balance of MLCK/MLCP activity is also modulated by other GPCR and G\textsubscript{q}/G\textsubscript{11} sub-unit pathways, such as rho kinase (ROK), protein kinase C (PKC) and protein kinase C-dependent phosphatase inhibitor (CPI-17), a phenomenon termed ‘Ca\textsuperscript{2+}-sensitization’ (159). Binding of contractile agonists to GPCR and G\textsubscript{q}/G\textsubscript{11} sub-units activates guanine nucleotide exchange factors (GEF), which produces an increase in Rho A, ROK, di-glyceride (DAG) and PKC, as well as DAG, which produces and increase in CPI-17, whereby ROK and CPI-17 both inhibit MLCP activity and therefore, increase phosphorylation of the MRLC. Indeed, the importance of Ca\textsuperscript{2+}-sensitization was highlighted by a study using isolated bronchial rings in vitro, which showed that the ROK inhibitor, Y-27632, reversed ~50 % of the ASM contraction to ACh (42).
Figure 1.2.2. A diagram of the contraction of ASM. Phosphorylation of the myosin regulatory light chain (MRLC) allows for ASM force production. Whilst intracellular Ca\(^{2+}\) binds to calmodulin (CaM) and activates myosin light chain kinase (MLCK), which phosphorylates the MRLC, myosin light chain phosphatase (MLCP) dephosphorylates the MRLC. Figure from Somlyo and Somlyo, 2003 (152).

Activation-relaxation coupling of ASM is also primarily P-MC, through GPCR and G\(_s\) sub-unit pathways. Binding of relaxant agonists to GPRC and G\(_s\) on ASM, such as isoprenaline, activates the adenylyl cyclase, which produces an increase in protein kinase A (PKA) and cyclic 3’, 5’ adenosine monophosphate (cAMP), whereby cAMP inhibits MLCK activity and therefore, decreases phosphorylation of the MRLC (16, 18). Similarly, the diffusing of NO directly through the ASM cell membrane and binding to guanylyl cyclase also activates the cyclic 3’, 5’ guanosine monophosphate (cGMP) pathway, whereby cGMP decreases the phosphorylation of the MRLC (16, 18). However, in a study using isolated ASM cells, it has also been shown that GPCR are directly coupled to maxi-K channels (93), which inhibit cell membrane depolarization and therefore, at least part of the relaxation to β\(_2\)-agonists is dependent on cell membrane depolarization.
Static loads

A complex series of loads determine ASM contractility and oppose airway narrowing. At a static lung volume (e.g. functional residual capacity, FRC), this series of loads can be thought of as the pre-load, which sets the initial airway lumen calibre (and therefore, the length of the muscle) and the after-load, which opposes muscle shortening. In normal healthy individuals, the length (i.e. perimeter) of the ASM is set by pressure difference across the airway wall, termed the ‘transmural pressure’ \( P_{tm} \), which increases and decreases with lung inflation and deflation. The \( P_{tm} \) arises due to the distending forces of the lung parenchyma. The initial length of the ASM determines contractility, which is described by the classic muscle length-tension (L-T) curve. If the ASM length is shifted from optimum \( (L_o) \) down the descending limbs of the L-T curve, the corresponding decrease in the over-lap of cross-bridges in the contractile apparatus decreases ASM force for any degree of activation (however, the effect appears to be somewhat dependent on the mode of contraction (154)). More recently, the dogma that the ASM \( L_o \) is fixed has been challenged by several studies (26, 133) (see 1.4 Airway smooth muscle response to strain).

Transmural pressure is defined using the following equation:

\[
P_{tm} = P_a + \text{Elastic recoil of the alveoli} - P_{al}
\]
Where, $P_a$ is the pressure in the airway lumen. At end expiration and inspiration, with no airflow, $P_a$ and the $P_{al}$ are equal to atmospheric pressure (i.e. 0 cmH$_2$O) and the $P_{tm}$ is dominated by the elastic recoil of alveoli, which arises due to the pressure difference across the alveoli wall. The alveoli are connected to the airway wall through parenchymal attachments, termed ‘airway-parenchymal coupling’. At end expiration, the elastic recoil of the alveoli is equal to the pressure difference across the alveoli wall, termed the ‘transpulmonary pressure’ ($P_{tp}$), which is defined by the following equation:

$$P_{tp} = P_{al} - P_{pl}$$

Where, $P_{pl}$ is the pressure in the pleural space. Assuming the $P_{pl}$ is -5 cmH$_2$O, the $P_a$ and therefore, the $P_{tm}$, are both 5 cmH$_2$O.

Depending on the after-load, the mode of ASM contraction of muscle may be isometric (same length, i.e. against an unmoveable after-load), isotonic (same tension, i.e. against no or a constant after-load) or auxotonic (i.e. against an elastic after-load). During an isometric contraction, the after-load is too great for the ASM to overcome and contraction occurs without muscle shortening but with maximum force production. During an isotonic contraction, the after-load is constant during the contraction and low enough for the muscle to overcome. Once activated, ASM contraction is initially isometric until force production is sufficient to overcome the after-load and the muscle shortens isotonically down the descending limb of the length-compliance curve (109). In vivo, contraction will be auxotonic, as the after-load the muscle must overcome increases as the muscle shortens. That is, ASM contraction must overcome the elastic
after-load, provided by deformation of the airway wall and airway-parenchymal
coupling (see below). Elastic after-loads are those that increase as the muscle shortens,
similar to the force needed to stretch a spring, which increase as the spring is stretched.

In order for ASM to shorten and produce airway narrowing, the muscle must overcome
a series of three after-loads; the constant after-load provided by the \( P_{im} \), the elastic after-
load from deformation of lung tissue and the elastic after-load of the deformation of the
airway wall itself. As described above, \( P_{im} \) at end expiration and inspiration is set by the
estatic recoil of the alveoli. Airway-parenchymal coupling also provides an after-load,
which increases as airway narrowing stretches the lung parenchymal attachments.
Greater airway narrowing result in more parenchymal deformation and therefore, the
after-load increases as the ASM shortens. Increased lung volume also stretches the
parenchymal attachments and is likely to increase the parenchymal after-load in
addition to increasing the isotonic after-load, provided by the increase in \( P_{im} \). As well as
classic L-T effects, any increase in lung volume (i.e. from 5 to 10 cmH2O) will increase
the after-load and is likely to attenuate airway narrowing (109). However, the
importance of the elastic after-load has been brought into question by a study using
whole bronchial segments, which showed no difference in airway narrowing at the same
\( P_{im} \) both \textit{in vivo} and \textit{in vitro} (125).

Finally, deformation of airway wall structures may resist airway narrowing and
therefore, provide an internal elastic after-load. Starting from the airway lumen and
proceeding through the wall, the layers encountered are the mucosa (which consists of
the airway epithelium, basement membrane and lamina propria), the submucosa (which consists of the ASM and supporting structures), the cartilage plates and a network of connective tissue, termed the ‘adventitia’ (Figure 1.2.3) (13). Mucosal folding resists airway narrowing due to the deformation of the epithelium during any reduction in airway calibre below that present when $P_{tm} = 0 \text{ cmH}_2\text{O}$ (145, 172). Airway narrowing below the calibre present when $P_{tm} = 0 \text{ cmH}_2\text{O}$ produces deformation of the wall with compression, folding or bending of structures such as elastin, collagen and cartilage plates (126).

**Figure 1.2.3.** A diagram of the morphological structures that make up the airway wall. The layers encountered are the mucosa (which consists of the epithelium, basement membrane and lamina propria), the submucosa (which consists of the ASM and supporting structures), the cartilage plates and the adventitia. Figure from Bai and colleagues, 1994 (13).

In addition to force production (i.e. isometric force for muscle shortening), contraction of ASM increases stiffness. Activation stiffens ASM due to both cross-bridge formation
and actin filament polymerisation (7). In whole bronchial segments in vitro, exogenous ACh stiffens the airway wall (11). Once activated, the stiffness of the ASM dominates the compliance curve of the airway wall over the non-contractile structures (124). Similarly, pharmacological bronchodilators reverse ASM stiffness as well as contraction. In whole bronchial segments in vitro, exogenous isoprenaline reverses airway wall stiffness (10).
1.3 Airway hyper-responsiveness

Airway responsiveness (i.e. the level of bronchoconstriction to ASM contractile agonists) is measured by spirometry and the forced oscillation technique (FOT). During spirometry, individuals inspire to total lung capacity (TLC) and forcibly expire to residual volume (RV), allowing the volume of forced expiration in the first 1 second (FEV₁) and the forced vital capacity (FVC, TLC – RV, a measure of lung volume) to be measured. Using the FOT, a constant or variable frequency pressure wave is superimposed over tidal breathing, allowing both $R_{aw}$ and lung elastance to be calculated. Spirometry and FOT are typically conducted prior to and following inhalation of ASM contractile agonists (i.e. a bronchial challenge), allowing the % decrease in FEV₁ or $R_{aw}$ to be calculated.

Airway hyper-responsiveness (i.e. excessive bronchoconstriction to an inhaled bronchial challenge) is a primary characteristic of asthma and is considered a major contributor to airflow limitation (99, 173). Patients with asthma show an increase in both the sensitivity and reactivity (i.e. maximum response) to numerous ‘direct’ ASM contractile agonists, such as MCh (35, 87, 150) and histamine (45, 87, 179). The degree of AHR increases with the severity of reported asthma symptoms (i.e. mild, moderate or severe asthma), whereby patients with severe asthma may not reach a plateau in their bronchial challenge dose-response curve (Figure 1.3.1) (179). More recently, it has been shown that inhalation of numerous ‘indirect’ ASM contractile agonists, such as adenosine-5-monophosphate (49), bradykinin (149), and the osmotic mediator, mannitol
(8), produce a reduction in FEV₁ in asthmatic patients but little or no response in normal healthy individuals. ‘Indirect’ ASM contractile agonists are likely to produce ASM contraction via inflammatory cells, such as mast cells (161).

**Figure 1.3.1.** The bronchial challenge dose-response curve (DRC, % reduction in the volume of the forced expiration in the first 1 second, FEV₁) to histamine (0.001 to 100 µM) in normal healthy individuals (n = 10), as well as patient with mild (n = 2) and moderate (n = 8) asthma. The degree of AHR to histamine increases with the severity of reported asthma symptoms. Figure from Woolcock and colleagues, 1984 (179).

In patients with asthma, airflow limitation is at least partially reversed by administration of pharmacological bronchodilators, typically β₂-agonists. In isolated ASM strips (64) and whole bronchial segments (10) *in vitro*, β₂-agonists relax ASM in a dose-dependent manner and *in vivo*, increasing dose of β₂-agonists produces greater improvement in FEV₁ (21). However, inhaled β₂-agonist therapy is somewhat limited by a decrease in sensitivity in patients with asthma, compared with normal healthy individuals (21).
well as $\beta_2$-agonists, increasing dose of NO also produces greater improvement in FEV$_1$, at least in patients with mild asthma (88).

_Chronic inflammation of the airway wall_

The cause(s) of AHR remain unclear but likely involve abnormalities in ASM and/or airway wall structure/function. These abnormalities may be present from birth (81) and/or may develop due to chronic inflammation of the airway wall present in patients with asthma (75). In disease, pro-inflammatory cytokines are released from numerous cell types, including mast cells (29). Increased levels of the pro-inflammatory cytokines, tumour necrosis factor (TNF), interleukins 1$\beta$, 2, 4, 5 and 6 (IL-1$\beta$, IL-2, IL-4, IL-5 and IL-6) as well as granulocyte macrophage colony stimulating factor (GM-CSF), have been detected in the sputum of patients with symptomatic asthma (29, 31). As previously discussed, pro-inflammatory cytokines may trigger the secondary-release of contractile mediators. Indeed, pharmacological anti-inflammatories, such as glucocorticosteroids (GCS), are potent inhibitors of asthma exacerbations in patients with even severe asthma. Glucocorticosteroids diffuse directly through the ASM cell membrane, binding to GCS-receptors (18). Binding of GCS to GCS-receptors activates nucleic transcription factors, which bind to specific GCS recognition elements, whereby the expression of receptors on the ASM is increased (i.e. $\beta_2$-receptors) or decreased (i.e. $M_3$-receptors). In addition to modifying the expression of receptors on the ASM, binding of GCS to GCS-receptors may also inhibit the transcription of pro-inflammatory cytokines in inflammatory cells.
However, the relationship between AHR and chronic inflammation of the airway wall remains unclear. The degree of AHR does not correlate with the presence of inflammatory cells, such as macrophages, lymphocytes, neutrophils and eosinophils in sputum (35, 47), bronchiolar lavage (47) or airway wall biopsies (47) from asthmatic patients, suggesting that the abnormalities in the ASM and/or airway wall are present, despite a lack of acute inflammation. That is, AHR can persist in the absence of inflammatory cells in the airway wall. Multiple abnormalities in the ASM and/or airway wall are likely to contribute to AHR in patients with asthma (23, 24, 36). Chronic inflammation may alter the proportions of the morphological layers that make up the airway wall, in particular, the ASM layer, termed ‘airway wall re-modelling’ or increase ASM force independent of ASM mass (i.e. hyper-contractility). More recently, the dynamic mechanical environment of the lung has been identified as an important regulator of airway responsiveness and may be susceptible to inflammatory disease processes, contributing to AHR (6).

*Airway wall re-modelling*

There is debate as to whether airway wall re-modelling causes AHR. Airway wall re-modelling may contribute to AHR in patients with asthma by virtue of decreasing the after-load or increasing ASM mass and therefore, force production. Alternatively, thickening of the mucosa may also oppose bronchoconstriction, due to an increase in the after-load provided by mucosal folding (96). Indeed, airway wall re-modelling has been suggested as a mechanism protective of AHR (113). Airway wall re-modelling alters the proportions of the morphological layers that make up the airway wall, in
particular, the ASM layer (97). Several morphometric studies (38, 54-56, 72, 76, 80, 82, 95, 121, 132, 177) have demonstrated that the thickness of the ASM layer, if normalized for airway calibre by perimeter of the basement membrane, is increased in bronchi from patients with asthma or a history of asthma (Figure 1.3.2). The thickness of the ASM layer correlates with the severity of reported asthma symptoms (95, 132).

**Figure 1.3.2.** A transverse image of relaxed bronchi stained with Haematoxylin and Eosin (H and E) from a normal healthy individual (top) and a patient with a history of asthma (bottom). The arrows identify the ASM layer. The thickness of the ASM layer is increased in patients with a history of asthma, compared with normal healthy individuals. Scale bar = 1 mm. Figure from Noble and colleagues, 2013 (121).
The increase in the thickness of the ASM layer may result from hyperplasia (an increase in the number of ASM cells, i.e. ASM cell proliferation) (55, 72, 80, 177), hypertrophy (an increase in the size of ASM cells) (55, 56, 80) and/or an increase in the amount of extra-cellular matrix (80). Two studies (80, 177) have shown that the relative contribution of hyperplasia appears to be greater in moderate and severe asthma (i.e. fatal asthma). A study, using isolated cells in culture, has shown that proliferation is increased in ASM cells from patients with asthma (86). Proliferation is also increased in isolated ASM cells from normal healthy individuals, following culture with TNF (5), suggesting that hyperplasia mediated by inflammatory disease processes. Regardless of the relative proportions of hypertrophy, hyperplasia or extra-cellular matrix, increased ASM mass is likely to result in a greater number of contractile proteins and therefore, increased ASM force (98). However, 2 studies (63, 170), found no difference in isometric force to carbachol (CCh) and histamine, compared with tissue from normal healthy individuals. A more recent study by Noble and colleagues (121), using whole bronchial segments from patients with a history of asthma in vivo, showed that airway narrowing to ACh was increased by ~57 % and correlated with the thickness of the ASM layer, suggesting that an increase in ASM mass contributes to AHR.

In addition to an increase in ASM force, airway wall re-modelling may contribute to AHR in patients with asthma by virtue of reduced elastic after-loads provided by parenchymal attachments and/or the airway wall itself (97). Several studies in patients with asthma in vivo, using static pressure-volume compliance curves (57, 178), high resolution imaging (62) and FOT (32), have shown that lung elastic recoil is reduced at TLC (at least in patients with moderate and severe asthma), compared with normal
healthy individuals. Reduced lung elastic recoil manifests as reduced $P_{tm}$, which favours increased airway narrowing due to a decrease in the after-load.

Finally, airway wall re-modelling could contribute to AHR, independent of an increase in ASM force or decrease in the after-load, by virtue of geometric effects during bronchoconstriction. In addition to ASM layer, airway wall re-modelling increases the thickness of the mucosa (38, 82, 95, 139). For the same magnitude of ASM shortening, thickening of the mucosa is likely to result in greater reduction of airway wall calibre (74, 82, 95, 98, 172).

**Airway smooth muscle hyper-contractility**

There is also debate as to whether chronic inflammation of the airway wall present in patients with asthma induces ASM hyper-contractility and therefore, may cause AHR. In addition to an increase in the thickness of the ASM layer, chronic inflammation of the airway wall may also induce an increase in ASM force independent of ASM mass. It is possible that ASM from patients with asthma exhibits a hyper-contractile phenotype. However, there are relatively few studies and contrasting evidence in the literature, using tissue from patients with asthma. Three studies (14, 30, 50) demonstrated increased isometric force production to ACh, CCh, MCh and histamine, if normalised for ASM mass, using isolated ASM strips *in vitro* from patients with asthma. In contrast, 2 studies (63, 170) demonstrated decreased isometric force production (i.e. a hypo-contractile phenotype) to CCh and histamine, using isolated ASM strips *in vitro* from patients with asthma.
Due to the difficulty in obtaining tissue from asthmatic patients, several studies have used tissue from normal healthy individuals or other animals, exposed to pro-inflammatory cytokines. Although numerous pro-inflammatory cytokines are likely to contribute to the development of AHR to varying degrees, TNF and IL-1β have previously been shown to induce AHR. In isolated ASM strips/rings in vitro, culture with TNF (1, 41, 136, 155) and/or IL-1β (136, 184) increased isometric force production (i.e. a hyper-contractile phenotype) to ACh, CCh, bradykinin and the non-specific GPCR agonist, 5-hydroxytryptamine (5-HT). Pro-inflammatory cytokine-induced ASM hyper-contractility occurs due to an increase in intra-cellular Ca²⁺ release from the sarcoplasmic reticulum (3-5) and Ca²⁺-sensitization, through the GPCR ROK pathway (79).
1.4 Airway smooth muscle response to strain

Deep inspiration

The normal resting breathing rhythm consists of both tidal breathing and intermittent deeper inspirations (deep inspirations, DI) that occur ~6 times/hr (22). In normal healthy individuals in vivo, DI produces a transient reversal of bronchoconstriction (i.e. bronchodilation) to a number of different inspired ASM contractile agonists (53, 58, 73, 117, 137, 142). Bronchodilation lasts for at least ~1 to 2 min but may persist beyond that time (138). A deeper DI produces greater bronchodilation in vivo (53, 137) and in studies simulating DI in whole bronchial segments in vitro (103, 124, 169). Although there is contrasting evidence in the literature, bronchodilation to DI may also, in part, be dependent on the rate-of-inflation. Hida and colleagues (73) demonstrated a greater bronchodilatory response with a faster DI in normal healthy individuals. In contrast, Duggan and colleagues (53) showed no effect of rate-of-inflation in normal healthy individuals, despite a tendency towards a greater bronchodilatory response.

Deep inspiration also transiently attenuates the magnitude of subsequent bronchoconstriction, a phenomenon termed ‘bronchoprotection’ (89, 110, 142, 150). The bronchoprotective effects of DI are typically demonstrated when FEV$_1$ is used to measure bronchoconstriction. Since standard spirometry to measure FEV$_1$ also necessitates taking DI during inspiration to TLC, it has been suggested that the bronchoprotective effects are intertwined with the bronchodilatory response (46, 176). There also appears to be a strong methodological dependency as to whether the bronchoprotective effects of DI are observed. In particular, when FOT is used to
measure bronchoconstriction or resistance, DI does not appear to attenuate subsequent bronchoconstriction in normal healthy individuals (39, 151) or mice (176) *in vivo*. However, the former study in normal healthy individuals (39), showed that DI is effective at preventing airway closure, as reflected in a reduced fall off in FVC or respiratory reactance.

Studies showing the bronchodilatory response and bronchoprotective effects of DI support a role of the normal resting breathing rhythm as an important regulator of airway responsiveness. When normal healthy individuals withheld from taking DI for a period of 10 min prior to (i.e. bronchoprotection) (35) and/or during (i.e. bronchodilation) (35, 40, 91, 116, 150) a bronchial challenge, they developed AHR-like characteristics, whereby the maximum response of the DRC was shifted towards that of the patients with asthma. Furthermore, when DI were recommenced subsequent to the bronchial challenge, the bronchodilatory response to DI was attenuated, similarly to the impaired bronchodilation to DI in patients with asthma (see below) (91, 150). The increased bronchoconstriction in normal healthy individuals to a bronchial challenge in the absence of DI, suggests that DI is an important regulator of airway responsiveness and that the disruption of this mechanism could contribute to AHR.

Both the bronchodilatory response (Figure 1.4.1) (58, 73, 106, 142) and bronchoprotective effects (89, 142, 150) of DI are attenuated or abolished in patients with asthma and this may contribute to the development of AHR. The disruption of the bronchodilatory response to DI in patients with asthma is related to the mode of
bronchoconstriction. Bronchodilation to DI was attenuated when bronchoconstriction was induced by inhaled ASM contractile agonists (i.e. exogenous) (58, 142), whereas in the presence of spontaneous ASM tone (i.e. intrinsic), during an asthma exacerbation, DI enhanced bronchoconstriction (106). In addition, withholding DI in patients with asthma (prior to or during bronchial challenge) has no effect on airway responsiveness (35, 150) and therefore, bronchoprotection to DI does not seem to be present in patients with asthma.

Figure 1.4.1. The bronchodilatory response to deep inspiration (DI, grey bars, % reduction in FEV₁) and no DI (white bars) following bronchoconstriction to inhaled methacholine (MCh) that produced a 20 % reduction in FEV₁ in normal healthy individuals (n=10), as well as patients with mild (n = 14) and moderate to severe asthma (n = 12). The bronchodilatory response to DI is attenuated in patients with mild and moderate to severe asthma. Mean ± SEM. Figure from Scichilone and colleagues, 2001 (142).
The underlying mechanism by which DI produces bronchodilation in normal healthy individuals is determined by the relative hysteresis of the lung parenchymal attachments and the airway wall. When a load is transiently applied to most materials, the length of the material will be greater than before the load was applied, a phenomenon termed ‘hysteresis’. In the lung, if inflated from 5 to 30 cmH₂O Pₐ and then returned back to 5 cmH₂O Pₐ, lung volume is greater than before the inflation, even though the pressure has returned to its original level. If the hysteresis of the airway wall is greater than the hysteresis of the lung parenchyma, the balance of the bronchoconstrictor and bronchodilatory loads is such that airway calibre is increased. In the presence of ASM tone, hysteresis of the airway wall could, on the one hand, be increased by stretch-induced release of an ASM relaxant agonist, such as NO. That is, bronchodilation to DI (where hysteresis of the airway wall is greater than hysteresis of the lung parenchyma) could arise due to pharmacologically induced ASM relaxation. A study by Brown and colleagues (33), using anesthetised and ventilated dogs \textit{in vivo}, showed that the NO blocker, N-nitro-L-arginine (L-NOARG) abolished the bronchodilatory response to DI, suggesting that DI may trigger the release of NO from adrenergic nerves or airway epithelial cells. In contrast, my previous study in whole bronchial segments \textit{in vitro} (10), showed no difference in the ASM relaxation in the presence of L-NOARG, suggesting no stretch-induced release of NO. However, by far the most compelling mechanism to explain the bronchodilatory response or bronchoprotective effects of DI is that ASM stretch directly produces relaxation. The most critical evidence in the literature is that stretch-induced ASM relaxation has been observed in isolated ASM strips, in the absence of the airway epithelium, a source of bronchoactive mediators, as well as innervation and airway wall reflexes (61, 64, 65, 147, 165).
Airway smooth muscle stretch

The bronchodilatory response to DI is thought to involve stretch-induced relaxation of ASM (59-61, 68). During DI, the mechanical stress, which transiently distends the airway wall, also lengthens the ASM (i.e. strain). However, studies showing a role of ‘stretch’ in mediating ASM relaxation, present protocols cannot separate the relative contributions of mechanical stress and strain. In vitro, length-oscillation (i.e. stretch) of isolated ASM strips (61, 64, 65, 147, 165) and oscillation of lumen volume in whole bronchial segments (10, 11) attenuates isometric force production. Increasing amplitude of oscillation produces greater attenuation of ASM force in an amplitude-dependent manner (65, 67, 68). In isolated ASM strips, oscillatory loads sufficient to produce a 4% change in muscle length attenuates ASM force by 50 % (64), suggesting that such oscillatory loads could be an important regulator of airway responsiveness. Similar results have been shown in whole bronchial segments (124) and lung slices (104), which show that increasing inflation produces greater bronchodilation. Studies using isolated ASM strips, whole bronchial segments and even lung slices suffer from two related methodological limitations. Firstly, what is the physiologically relevant magnitude of ASM stretch? Secondly, is the distending force applied to the ASM (i.e. mechanical stress) or change in length (i.e. strain) the critical determinant of ASM relaxation? In order to lengthen the muscle, a greater load is applied, so it is unclear if it is the increase in mechanical stress or the accompanying increases in strain mediates ASM relaxation. Furthermore, the bulk of previous studies using isolated ASM strips (61, 64, 65, 147, 165) or whole bronchial segments (10, 11) have targeted a fixed change in muscle length or lumen volume and applied whatever force or pressure was
needed to achieve that level of stretch, so that it is unclear whether the loads applied are physiological.

During bronchoconstriction, ASM contraction stiffens the airway wall (10, 90, 124) altering the relationship between the mechanical stress and strain. *In vivo*, the magnitude of ASM stretch will depend on both the change in $P_{eq}$ and the stiffness of the airway wall. If the bronchodilatory response to DI depends on ASM strain, as opposed to the mechanical stress, then bronchodilation will also fall with increasing airway wall stiffening. When breathing manoeuvres are simulated in whole segments using a fixed-volume oscillations, analogous to a fixed-length oscillations in isolated ASM strips, DI produces potent bronchodilation (124). However, contraction of ASM stiffens the airway wall (10, 90, 124), so that the $P_{tm}$ required to impose a fixed-volume change increases greatly, reaching 80 cmH$_2$O at the maximum dose of ACh (124). When the imposed $P_{tm}$ is limited to more physiological levels, the effectiveness of simulated breathing movements is greatly reduced (103, 124, 169). Notably, under high levels of ASM activation, even oscillations simulating inflation from FRC at 5 cmH$_2$O to TLC at 30 cmH$_2$O (i.e. a $P_{tm}$ swing during DI of $\Delta$25 cmH$_2$O) are likely to produce negligible ASM strain (120) suggesting that if DI-induced bronchodilation depends on ASM strain then high levels of ASM contraction should abolish DI bronchodilation.

If DI-induced bronchodilation depends on ASM strain not mechanical stress, then any intervention that reduces airway wall stiffness would likely increase the bronchodilatory response to breathing manoeuvres. As previously discussed, as well as producing
bronchodilation, β2-agonists reduce airway wall stiffness (10). Previous studies in isolated ASM strips (64) and whole bronchial segments (10) in vitro, have concluded that oscillatory loads and pharmacologically-induced ASM relaxation do not interact. However, those previous studies used fixed-length or fixed-volume oscillations (i.e. fixed-strain), that are independent of airway wall stiffness. I hypothesise that any intervention that reduces airway wall stiffness is likely to be beneficial in reversing bronchoconstriction by enhancing the bronchodilatory response to breathing manoeuvres as a result of increased ASM strain (i.e. a synergistic relationship) (10). This hypothesis, relies on the assumption that ASM strain as opposed to mechanical stress, is the critical determinant of ASM relaxation.

There are contrasting theories on the cellular mechanism(s) underlying the ASM response to stretch. Stretch-induced ASM relaxation may involve perturbed cross-bridge binding (60, 61) and/or re-modelling (i.e. de-polymerisation and re-polymerisation) of the contractile apparatus (66). Whilst ASM force production requires cross-bridge cycling, maintenance of force requires very low rates of cross-bridge cycling, termed ‘the latch state’. The rate-of-cross-bridge cycling is constantly moving toward the latch state equilibrium (i.e. maintenance of ASM force while there is a low rate of cross-bridge cycling). Fredberg and colleagues (59) have proposed that oscillatory ASM stretch disrupts cross-bridge cycling by physically breaking cross-bridges, termed ‘the perturbed-equilibrium model’. Alternatively, the phenomenon of length-adaption, where the contractile apparatus is re-arranged (see Length-adaptation), has been evoked to explain bronchodilation (165). Constantly changing the muscle length during breathing might start the re-arrangement of the contractile apparatus that
occurs in length-adaptation but never allow the process to finish as length is constantly changing. One ultra-structural imaging study, using isolated ASM, demonstrated that the MHC de-polymerizes in response to stretch (94), providing support to the contractile apparatus re-arrangement hypothesis. Re-arrangement of the contractile apparatus may also explain the bronchoprotective effects of DI, which cannot be explained by perturbed cross-bridge binding, since bronchoprotection refers to protective effects induced prior to the development of tone.

The apparent rate-dependence of a transient inflationary manoeuvre such as DI, is qualitatively similar to the frequency-dependence of isometric force production during length-oscillation of isolated ASM (2, 65, 147, 165). The cellular mechanism(s) underlying the rate-dependence of DI may involve the relative rates of cross-bridge cycling/length-oscillation (i.e. the frequency of oscillation). Assuming that stretch-induced ASM relaxation is caused by the perturbed-equilibrium model, then the rate-of-stretch could alter the number of broken cross-bridges. If the frequency of oscillation is faster than the rate-of-detachment and re-attachment of cross-bridges, then fewer detached cross-bridges can re-attach during the oscillation cycle (69) and so that force production will fall more for a fast than a slow stretch.

*Length-adaptation*

As previously discussed, changes in ASM pre-load and therefore, the initial length of the ASM, determine contractility by shifting the muscle away from $L_o$ (154). A recently discovered property of airway smooth muscle (ASM), identified in isolated muscle
strips, is the capacity to optimise force production following sustained changes in length (26, 133), a phenomenon termed ‘length-adaptation’ (Figure 1.2.4) (15). Length-adaptation manifests as a shift in the active and passive length-tension curve. If the ASM length is shifted from $L_o$ for a sustained period of time (i.e. lengthening or shortening), force production recovers back to that present at $L_o$ following adaptive re-arrangement of the contractile apparatus (118, 143, 166). Re-arrangement of the contractile filaments is hypothesised to occur through changes in the length of actin and/or myosin filaments achieved through de-polymerization of the filaments and re-polymerization into filament adapted to the new length. Changes in filament length may be initiated by mechanosensors (i.e. integrins) that respond to the physical deformation (i.e. strain) of cells and initiate the downstream re-modelling process (69).

Length-adaptation may also, in part, explain the attenuation of ASM force production in response to oscillatory (or transient) changes in ASM length (94, 147, 164, 165) and contribute to the bronchodilatory response to DI. When muscle length is constantly changing, the re-arrangement process may start but never reach completion, as the length keeps changing, resulting in a reduction in the amount of polymerised actin and/or myosin and therefore, facilitate reduced force production.
Despite evidence of length-adaptation in isolated ASM strips, the presence and/or potential role of length-adaptation in situ and therefore, the implications for airway function in health and disease, is uncertain. In the disease, lung hyper-inflation (105, 130) or mechanical ‘uncoupling’ of the lung parenchyma with emphysema and/or reduced elastic recoil pressure (51, 57) may increase or decrease ASM perimeter, respectively. Another possible scenario is that the ASM may be chronically shortened due to persistent levels of basal ASM tone (25). If length-adaptation were present in vivo, such disease-related changes in ASM perimeter may be accompanied by changes in contractile capacity, such that force production could be potentiated at these abnormally increased or decreased perimeters. Indeed, length-adaptation to a short
ASM perimeter has been proposed as a potential mechanism underlying airway hyper-responsiveness (AHR i.e. excessive airway narrowing) in obstructive disease (166).

The failure of deep inspiration to produce bronchodilation and airway hyper-responsiveness in patients with asthma

The mechanism(s) by which DI fails to produce bronchodilation in patients with asthma and the relationship between DI and AHR is unknown. The impaired bronchodilatory response to DI and AHR could be independent characteristics of asthma or the failure of DI to produce bronchodilation could be caused by or be a cause of AHR (119). Airway wall stiffness is increased in asthma (28, 85, 175) and may limit ASM strain during DI. Passive airway wall stiffness could be increased in asthma due to the increased wall thickness produced by remodeling (32, 167). Contraction of ASM also stiffens the airway wall (10, 90, 124), suggesting that the impaired bronchodilation to DI patients with asthma might be a result of AHR. Deep inspiration fails to produce bronchodilation in asthmatic patients, even when asthmatic and normal healthy individuals are matched for changes in bronchoconstriction (i.e. resistance) (183). However, matching for the same level of bronchoconstriction may not mean that normal healthy individuals and patients with asthma have the same level of airway wall stiffness. A recent study by Raqeeb and colleagues (135) showed that not all the increase in ASM stiffness during contraction is due to cross-bridge formation. Consequently, whilst matching of the level of bronchoconstriction is appropriate in such studies, this does not necessarily mean that the level of induced airway wall stiffness is also matched.
Rather than AHR causing impaired bronchodilation to DI it is possible that an altered DI response contributes the development of AHR in patients with asthma. If normal tidal breathing is sufficient to attenuate ASM contraction, then the loss of that inhibitory effect would favor increased bronchoconstriction and may contribute to the development of AHR. Whilst there is debate as to whether smaller tidal oscillations are sufficient to inhibit airway responsiveness (120), standard spirometry necessities taking DI and therefore, the impaired response to DI in patients with asthma would exaggerate the degree of AHR observed during a bronchial challenge.

A final consideration is whether the impaired bronchodilatory response to DI can be explained independently of airway wall stiffening. Two studies (34, 78) have demonstrated that while distension of the airway during the DI itself was similar between normal healthy individuals and patients with asthma, following DI, bronchodilation was only observed in the normal healthy individuals, whilst there was little change or bronchoconstriction was observed in the patients with asthma. These findings suggest that it is not the magnitude of the stretch applied to the ASM but the ASM response to stretch that is altered in patients with asthma. It is possible that in asthma there is a phenotypic change in the ASM response to stretch and/or stress during DI. In a study by Chin and colleagues (43), there was no difference in the mechanical properties of ASM from normal healthy individuals and patients with asthmatic patients, other than the ASM response to a stretch. The cause of the altered ASM response to stretch in tissue from patients with asthma is uncertain. Whilst culture of ASM strip with TNF (1, 41, 136, 155) and/or IL-1β (136, 184), is known to induce a hyper-contractile phenotype whether TNF and IL-1β modulate the ASM response to stretch is unknown.
1.5 Aims and organisation

The general aim of this thesis is to better understand the ASM response to strain during breathing manoeuvres and the relationship with AHR. This thesis used an established intact bronchial segment model. My laboratory has previously modelled tidal breathing and DI manoeuvres in both animal (124, 169) and human (122) whole bronchial segments, including those from patients with asthma (121). I chose to use porcine airways to study the effects of ASM strain in response to DI and the resultant bronchodilation (Figure 1.5.1). My laboratory has used porcine airways previously to assess the role of DI amplitude and to demonstrate the importance of airway wall stiffness to this response, particularly during ASM contraction (124). Many of these findings have been replicated recently in studies utilising human tissue (104, 121). These findings suggest that the porcine airway behaves similarly to the human airway, although some species differences exist (120).
In Chapter 2 of this thesis, the first aim was to determine whether an increase in ASM stress or the accompanying increase in strain mediates the bronchodilatory response to DI. During DI a distending force is applied to ASM, (i.e. stress) and the muscle is lengthened (i.e. strain), which produces a transient reversal of bronchoconstriction (i.e. bronchodilation). The relative contribution of ASM stress and strain to the bronchodilatory response to DI is unknown. I used whole porcine bronchial segments in vitro and a servo-controlled syringe pump that applied fixed-transmural pressure (P_{tm}) or fixed-volume oscillations, simulating tidal breathing manoeuvres and intermittent DI. I hypothesise that ASM strain, rather than stress, will be the critical determinant of bronchodilation to DI.
In Chapter 3 of this thesis, the second aim was to determine whether $\beta_2$-agonists exert a secondary bronchodilator effect by virtue of reducing airway wall stiffness and therefore, increasing ASM strain produced by breathing manoeuvres. In patients with asthma, airflow limitation is at least partially reversible by administration of pharmacological bronchodilators, typically $\beta_2$-agonists. In addition to receptor-mediated bronchodilation, the dynamic mechanical environment of the lung itself can produce bronchodilation. My hypothesis that ASM strain, rather than stress, will be the critical determinant of bronchodilation to DI, has implications for bronchodilatory response to $\beta_2$-agonists. Any intervention that reduces airway wall stiffness is likely to be beneficial in reversing bronchoconstriction by enhancing the bronchodilatory response to breathing manoeuvres. Whilst contraction of ASM stiffens the airway wall (10, 90, 124), $\beta_2$-agonists reduce airway wall stiffness in a dose-dependent manner (10). My findings support an important role of pharmacological bronchodilators in mediating the bronchodilatory response to breathing manoeuvres by virtue of reduced airway wall stiffness, likely to be of clinical significance.

In Chapter 4 of this thesis, the third aim was to determine whether the pro-inflammatory cytokines, TNF and/or IL-1$\beta$, modulate the ASM response to strain and therefore, the bronchodilatory response to DI. In vitro, culture with TNF (1, 41, 136, 155) and/or IL-1$\beta$ (136, 184) increases isometric force production in isolated ASM strips/rings (i.e. a hyper-contractile phenotype), suggesting that TNF and IL-1$\beta$-induced hyper-contractility may contribute to AHR. Whilst chronic inflammation of the airway wall and the failure of DI to produce to bronchodilation are common to asthma, whether
TNF and IL-1β modulate the ASM response to strain is unknown. I used a novel methodology of culturing airway segments with TNF and IL-1β. I hypothesised that TNF and IL-1β would decrease ASM relaxation to any magnitude of strain during DI and that this would attenuate the resulting bronchodilatory response.

In Chapter 5 of this thesis, the fourth and final aim was to determine, using an intact airway segment preparation, the *in situ* effect of sustained changes in $P_{tm}$ (and therefore ASM perimeter) on ASM contraction and airway narrowing. A newly found property of ASM, identified in isolated strips, is the capacity to optimise force production following strain, a phenomenon termed ‘length-adaptation’ (26, 143). Despite evidence of length-adaptation in isolated ASM strips, the presence and/or potential role of length-adaptation *in situ* and therefore, the implications for airway function in health and disease, is uncertain. I reasoned that if length-adaptation is important to normal airway function then I would see adaptation (a time-dependent increase in contractile response) to the physiological determinant of airway ASM perimeter, $P_{tm}$.

In Chapter 6 of this thesis, I further discuss the findings of Chapters 2 to 5 in order to address the general aim of this thesis, to better understand the ASM response to strain during breathing manoeuvres and the relationship with AHR.
Chapter 2 Bronchodilatory response to deep inspiration in bronchial segments: The effects of mechanical stress versus strain

2.1 Introduction

In normal healthy individuals in vivo, deep inspiration (DI) produces a transient reversal of bronchoconstriction (i.e. bronchodilation) to a number of different inspired airway smooth muscle (ASM) contractile agonists (53, 58, 73, 117, 137, 142). The underlying mechanism by which DI produces bronchodilation in normal healthy individuals thought to involve stretch-induced relaxation of ASM (59-61, 68) due to perturbed cross-bridge binding (60, 61) and/or de-polymerisation of the contractile apparatus (66). Since the bronchodilatory response to DI is attenuated or abolished in patients with asthma (58, 73, 106, 142), the underlying mechanism is of considerable interest.

In vitro, length-oscillation (i.e. stretch) in isolated ASM strips (61, 65, 147, 165) and oscillation of lumen volume in intact airways (i.e. whole bronchial segments) (10, 11) attenuates force. Increasing amplitude of oscillation produces greater attenuation of ASM force in an amplitude-dependent manner (65, 67, 68). Similarly, in normal healthy individuals in vivo, a deeper depth of DI produces greater bronchodilation (53, 137). The amplitude-dependence of bronchodilation has typically been explained by increased ASM stretch (61, 124, 147). However, a DI manoeuvre results in both a distending force being applied to ASM (i.e. mechanical stress), as well as a change in length (i.e.
strain). The relative contribution of ASM mechanical stress and strain to the bronchodilatory response to DI is unknown.

In order to better understand the role of ASM mechanical stress, as opposed to strain, in mediating the bronchodilatory response to DI, I manipulated the relationship between ASM mechanical stress and strain during oscillation. The relationship was altered by activation of ASM, which stiffens the airway wall (10, 90, 124), or by changing the rate-of-inflation during DI, which utilises the viscous properties of the intact airway. Firstly, since the stiffness of the airway wall is governed by the level of ASM contraction (7), the magnitude of ASM strain produced by fixed-transmural pressure ($P_{tm}$) oscillations will fall with increasing dose of contractile agonist. Conversely, if the airway lumen is oscillated by a fixed-volume, the mechanical stress produced by oscillation will increase during contraction (124). The viscous and inertial properties of the intact airway provide a second method to alter the relationship between ASM mechanical stress and strain that is independent of the dose of contractile agonist (and therefore, independent of the level of ASM contraction). A slower rate-of-inflation for a given $P_{tm}$ (i.e. a fixed-$P_{tm}$ DI) will result in greater ASM strain than a faster manoeuvre by reducing the viscous and inertial load on the airway wall. Conversely, a faster rate-of-inflation for a given volume (i.e. a fixed-volume DI) will result in greater mechanical stress than a slower manoeuvre.

The aim of the present study was to determine whether an increase in ASM mechanical stress or the accompanying increase in strain mediates the bronchodilatory response to DI. I hypothesised that strain, rather than mechanical stress, is the critical determinant
of bronchodilation. I used whole porcine bronchial segments *in vitro*, that were contracted to acetylcholine (ACh) under static (i.e. no oscillation) conditions or during tidal breathing with intermittent DI manoeuvres. A servo-controlled syringe pump was used to simulate breathing manoeuvres. I predicted that fixed-$P_{\text{tm}}$ DI would produce significantly less strain with increasing ASM contraction and therefore, less bronchodilation. In comparison, fixed-volume DI, whilst accompanied by greater mechanical stress, would not produce greater bronchodilation. Under conditions where the rate-of-inflation was varied, I predicted that bronchodilation would be determined by the magnitude of ASM strain, rather than mechanical stress.
2.2 Methods

Animal handling

All animal experiments conformed to institutional ethics and animal care unit regulations (Animal Ethics Committee, University of Western Australia (UWA), Crawley, WA, Australia). Male White Landrace pigs, ~35 kg, were initially sedated with tiletamine-zolazepam (4.4 mg/kg⁻¹ I.M.) and xylazine (2.2 mg/kg⁻¹ I.M.) and then exsanguinated under sodium pentobarbitone anaesthesia (30 mg/kg⁻¹ I.V.). The lungs were immediately removed and transported on ice to the laboratory.

Airway segment preparation

Airway segments were dissected from the main stem bronchus of the left or right lower lobe within ~60 min of being removed. All side branches were ligated with surgical silk and a ~20 mm long airway segment was cannulated at both ends, as previous described (10, 11). The mode generation was 18 at the distal and 12 at the proximal end (where the generation of the trachea = 0), with an internal diameter of ~2 mm at the distal and ~3 mm at the proximal end. Following cannulation, the airway was mounted horizontally in an organ bath containing gassed (95 % O₂ and 5 % CO₂) Krebs solution (121 mM NaCl, 5.4 mM KCl, 1.2 mM MgSO₄, 25 mM NaHCO₃, 5 mM sodium morpholinopropane sulfonic acid, 11.5 mM glucose and 2.5 mM CaCl₂; pH 7.3) at 37 °C. The length of the segment was stretched to 105 % of its length in the fully deflated lung, shown previously to approximate the length at functional residual capacity (FRC) (125).
The proximal end of the airway lumen was connected to a reservoir filled with Krebs solution, the height of which set the initial $P_{\text{tm}}$ (5 cmH$_2$O) and which was used to flush the lumen with Krebs solution between experiments. The distal end of the airway was connected to a liquid filled syringe pump. The syringe pump was capable of simulating breathing manoeuvres in 1-of-2 ways: fixed-$P_{\text{tm}}$ oscillations or fixed-volume oscillations (see below). All protocols were performed in a closed system, created by closure of a tap between the airway and the Krebs solution reservoir. The system was leak free with negligible compliance (0.0113 µL/cmH$_2$O with a ~7.0 mL system volume).

*Airway narrowing and fixed-$P_{\text{tm}}$ oscillations*

A custom-built servo-controlled syringe pump and pressure transducer were used to measure airway narrowing and to apply fixed-$P_{\text{tm}}$ oscillations (i.e. tidal breathing and DI manoeuvres) (121, 122). Airways were connected to a 1 mL glass syringe driven by a feedback-controlled servomotor (model M540; McLennan Servo Supplies, Surrey, U.K.) and motor controller (Shane De Catania, Perth, WA, Australia). Transmural pressure was measured *via* a calibrated pressure transducer (model MLT0380/D; ADInstruments, Bella Vista, NSW, Australia) with feedback to the servomotor. Using this approach, $P_{\text{tm}}$ was set to the desired level (i.e. static or oscillatory, see *Experimental protocols*) and ASM activation resulted in a decrease in lumen volume (i.e. airway narrowing, Figure 2.2.1A). Changes in airway luminal volume (i.e. airway narrowing and fixed-$P_{\text{tm}}$ oscillations) were measured *via* a calibrated displacement transducer (model HEDS-5540#A06; RS Components, Smithfield, NSW, Australia) that measured the rotation of the syringe motor. Both pressure and volume displacement were recorded
by a PowerLab data-acquisition system (model 4/30; ADInstruments) and displayed on a computer monitor.

**Figure 2.2.1.** An example trace of a cumulative dose-response curve (DRC) to acetylcholine (ACh, $10^{-7}$ to $3x10^{-3}$ M, *arrows*, text labels shown only for whole log doses) using fixed-transmural pressure ($P_{tm}$, A) and fixed-volume (B) oscillations. At the time scale shown, individual oscillations are not visible but appear as a thick line, the thickness of which indicates the magnitude of the $P_{tm}$ and volume oscillations. In response to ACh, lumen volume reduced during fixed-$P_{tm}$ oscillations and $P_{tm}$ increased during fixed-volume oscillations, in a dose-dependent manner. Dose-response curves were performed under static conditions (trace not shown) and during tidal breathing with intermittent deep inspiration (DI) manoeuvres.
Active pressure and fixed-volume oscillations

In a separate group of airways, measurements of ASM force and fixed-volume oscillations were applied using the same syringe pump oscillator described above but using the displacement transducer and not pressure transducer as the feedback control to the servomotor. Using this approach, lumen volume does not decrease in response to ASM activation but instead results in an increase in $P_{tm}$ (i.e. active pressure) that represents ASM force production (Figure 2.2.1B). For comparisons with experiments that used fixed-$P_{tm}$ oscillations, the volume of oscillation (i.e. tidal breathing and DI manoeuvres) was that which produced the same $P_{tm}$ in the relaxed state (i.e. prior to the administration of the contractile agonist), unless otherwise stated and was fixed thereafter (see Experimental protocols).

Experimental protocols

After dissection and mounting, airways were initially equilibrated to organ bath conditions for ~60 min under a static $P_{tm}$ of 5 cmH$_2$O, approximating the mechanical environment present at FRC, in vivo. The Krebs solution in the organ bath and lumen was replaced every 10 min to remove the effects of metabolites and bronchoactive mediators released from the epithelium. Viability of the tissue was subsequently confirmed through stimulation with ACh (10$^{-4}$ M) added to the organ bath followed by a 30 min washout and recovery period at 5 cmH$_2$O $P_{tm}$. Three different protocols were followed (see below). In protocol 1, the amplitude-dependence of the bronchodilatory response to DI was established, whilst protocols 2 and 3 were designed to determine the
relative contribution of ASM mechanical stress and strain to the bronchodilatory response to DI.

**Protocol 1: The amplitude-dependence of the bronchodilatory response to DI**

Airways were subsequently narrowed to a single, moderate dose of ACh (10^{-5} M) and subjected to 5 fixed-P_{tm} DI from sub-maximal to supra-maximal P_{tm} (20, 30, 40, 50 and 60 cmH_{2}O P_{tm}). For these experiments, DI manoeuvres comprised of a 2 s inflation, a 2 s hold at the peak of inflation and a 2 s deflation (a 6 s manoeuvre). Deep inspirations were applied amidst a background of tidal breathing (Δ5 cmH_{2}O at 0.25 Hz) and the bronchodilatory response allowed to fully recover before application of the next DI manoeuvre (typically ~1 min).

**Protocols 2 and 3: The contributions of ASM mechanical stress and strain to the bronchodilatory response to DI**

The relative contributions of ASM mechanical stress and strain to the bronchodilatory response to DI were assessed by manipulating the strain produced by a fixed-P_{tm} DI, or the mechanical stress produced by a fixed-volume DI. In protocol 2, the ASM mechanical stress and strain relationship was altered by activation of ASM with ACh, which increases airway wall stiffness (7). With increasing airway wall stiffness, the magnitude of ASM strain (i.e. the ΔDI volume) to a fixed-P_{tm} change decreases, whilst the magnitude of mechanical stress (i.e. the ΔDI P_{tm}) to a fixed-volume change increases in a dose-dependent manner. Full dose-response curves (DRC) were constructed to ACh (10^{-7} to 3x 10^{-3} M) under both static (5 cmH_{2}O P_{tm}) and oscillatory
conditions in a randomised order. The oscillatory protocol comprised tidal breathing ($\Delta 5$ cmH$_2$O at 0.25 Hz) and intermittent DI manoeuvres ($\Delta 25$ cmH$_2$O, a 6 s manoeuvre, described above) applied once contraction at each dose of ACh had plateaued. For the fixed-volume approach, the volume changes used were adjusted for each airway so that tidal breathing was $\Delta 5$ cmH$_2$O and DI manoeuvres were $\Delta 25$ cmH$_2$O in the relaxed state. Experiments conducted using the fixed-$P_{tm}$ or fixed-volume approaches were performed in separate groups of airways.

Whilst protocol 2 allowed us to assess the relative contributions of ASM mechanical stress and strain on the bronchodilatory response to DI, increasing ACh dose and therefore, ASM activation, may have independent effects (related to the biology of the cell, i.e. re-modelling of the contractile apparatus) beyond that of a simple change in airway wall stiffness. To address this possibility, in protocol 3, the relationship between ASM mechanical stress and strain was not altered by increasing dose of contractile agonist but by varying the rate-of-inflation during DI. Due to the visco-elastic properties of the intact airway, slower DI produce greater strain (i.e. greater $\Delta$DI volume) during a fixed-$P_{tm}$ oscillation than faster DI. Conversely, for a fixed-volume oscillation, slower DI produce less mechanical stress (i.e. less $\Delta$DI $P_{tm}$) than faster DI of the same volume. Airways were contracted to a single, moderate dose of ACh ($10^{-5}$ M) and subjected to DI ($\Delta 25$ cmH$_2$O) of 3 different rates in a randomised order: a slow DI comprising a 5 s inflation and deflation (a 10 s manoeuvre, i.e. no hold at the peak of inflation), a moderate DI comprising a 2 s inflation and deflation (a 4 s manoeuvre) and a fast DI comprising a 1 s inflation and deflation (a 2 s manoeuvre). Deep inspirations were applied amidst a background of tidal oscillations ($\Delta 5$ cmH$_2$O at 0.25 Hz) and the bronchodilatory response allowed to fully recover before application of the next DI
manoeuvre. For fixed-volume oscillations, the tidal and DI volumes used were adjusted for each airway to match the volume measured during fixed-$P_{tm}$ tidal breathing and in the moderate rate DI manoeuvre under contracted conditions, respectively.

**Morphometry**

Morphometric analyses were carried out to estimate the magnitude of ASM strain produced by DI manoeuvres. Following experimentation, airways were removed from the organ bath and fixed in 4% formaldehyde solution under atmospheric pressure (i.e. 0 cmH$_2$O $P_{tm}$). Distal and proximal regions of the airway segment were processed into paraffin blocks. Transverse airway sections were cut at a thickness of 5 μm and stained with haematoxylin and eosin (H and E). Inner wall area ($A_{wi}$) was calculated from the area enclosed by the outer ASM perimeter ($A_{mo}$) - the area enclosed by the internal lumen area ($A_i$) (13) using ImageJ (version 1.45j, National Institutes of Health, MD, U.S.A.). Measurements at distal and proximal locations were averaged and corrected for horizontal stretch (105% of its length in the fully deflated lung), which reduces the cross sectional area of the wall, assuming tissue volume is constant. The calculated inner wall area was also corrected for tissue shrinkage that occurs during histological processing. In a recent study from my laboratory using human bronchi, shrinkage was estimated to be ~15% following processing (121).

**Analysis and statistics**

Lumen volume (i.e. prior to the administration of ACh) was measured by the volume that could be withdrawn until closure in the relaxed airway at 5 cmH$_2$O $P_{tm}$ (67). Airway
narrowing to ACh (for the fixed-P_m approach) was expressed as % lumen volume (where 100 % airway narrowing indicates airway closure). As described above, morphometry allowed the outer ASM perimeter (P_{mo}) to be calculated using the following equation:

\[ P_{mo} = \sqrt{\frac{4 \times \pi \times (W_A + \frac{\text{Lumen Volume}}{\text{Airway Length}})}} \]

Where, lumen volume is volume of the lumen at the trough of the pressure cycle at the time of measurement and airway length is the length of the airway segment mounted in the organ bath. The equation assumes \( W_A \) is constant at all \( P_m \), that \( P_{mo} \) is circular and that the lumen is cylindrical. Active pressure to ACh (for the fixed-volume approach) was expressed as \( \Delta P_m \). Comparisons between static and oscillatory conditions were made at troughs of the oscillation cycle (volume or pressure, depending on the approach used). Dose-response curves had variable slope sigmoidal curves fitted to individual airways. Sensitivity (PD_{2} = \log_{10}(EC_{50})) to ACh was calculated for individual airways under static and oscillatory conditions. During fixed-P_m oscillations, ASM strain was calculated using the following equation:

\[ \text{ASM Strain} = \frac{\Delta D_l}{\text{Pre Dl}} \]
Where, $\Delta DI$ is the trough to peak change in $P_{mo}$ during DI and Pre DI is the $P_{mo}$ immediately prior to DI. During fixed-volume oscillations, mechanical stress was defined as the $\Delta DI$ $P_{tm}$. The bronchodilatory response to DI was defined as % reversal of contraction to ACh using the equation (124):

$$\text{Bronchodilation} = \frac{\text{Pre DI} - \text{Post DI}}{\text{Pre DI}} \times 100$$

Where, for the fixed-$P_{tm}$ approach, Pre DI and Post DI are the airway narrowing immediately prior to and immediately following DI, respectively. For the fixed-volume approach, Pre DI and Post DI are the active pressure immediately prior to and following DI, respectively. Therefore, 100 % reversal indicates that the post DI airway narrowing or active pressure returned to pre-contraction values (i.e. full reversal of the response to ACh). Comparisons of the bronchodilatory response were not possible at low doses of ACh ($\leq 3 \times 10^{-6}$ M), which produced little to no contraction. Scatter plots of bronchodilation to DI against either ASM strain or stress had linear lines-of-best-fit fitted to individual airways. The intercept, slope and Pearson’s correlation coefficient ($r$) were calculated for individual airways. Following DI, the bronchodilatory response was measured for a further 1 min and 1-phase exponential decay curves fitted to individual airways. The decay constant ($k$) following DI was calculated for individual airways and was used to assess the kinetics of airway re-narrowing following DI.

Specific compliance of the airway wall was calculated from the $\Delta$volume in relation to the $\Delta P_{tm}$ during the inflationary limb of the tidal oscillation cycle using the equation:
Specific Compliance = \frac{\Delta \text{Tidal Volume}}{\Delta \text{Tidal P}_{\text{tm}} \times \text{Lumen Volume}}

Where \( \Delta \text{tidal volume} \) and \( \Delta \text{tidal P}_{\text{tm}} \) are the trough to peak changes in volume and pressure during tidal oscillation and lumen volume is volume of the lumen at the trough of the pressure cycle at the time of measurement.

Differences between groups were analysed using 1-way repeat measures analysis of variance (ANOVA) and Newman-Keuls post hoc test, unless otherwise stated below. Airway narrowing and active pressure DRC were analysed using 2-way repeat measures ANOVA and Newman-Keuls post hoc test with dose of ACh and the condition (i.e. static or oscillatory) as the repeat measures variables. Sensitivity to ACh under static and oscillatory conditions was analysed using paired t-tests. Dose-response curves of bronchodilation to DI were analysed using 2-way ANOVA and Newman-Keuls post hoc test. Data analysis and statistical tests were performed using Statistica (version 8.0; StatSoft, Tulsa, OK, U.S.A.) and GraphPad Prism (version 5.0d; GraphPad Software, La Jolla, CA, U.S.A.) Data are presented as means ± standard error of the mean (SEM), where \( n \) = number of animals.
2.3 Results

Protocol 1: The amplitude-dependence of the bronchodilatory response to DI

Airways produced 35.4 ± 7.4 % narrowing in response to a single, moderate dose of ACh (10^{-5} M). I observed a strong amplitude-dependence to the bronchodilatory response to DI (Figure 2.3.1A). Bronchodilation increased from 18.5 ± 3.7 % to 50.6 ± 7.1 % reversal of narrowing when DI amplitude increased from 20 to 60 cmH\textsubscript{2}O P\textsubscript{tm} (i.e. Δ15 to Δ55 cmH\textsubscript{2}O). Airways rapidly re-narrowed following DI and the decay constant (average \(k\) of 0.280 ± 0.0246 s\(^{-1}\)) was independent of amplitude (Figure 2.3.1B). As expected, increasing amplitudes of DI also produced greater ASM strain (Figure 2.3.2). Deep inspiration to 20 cmH\textsubscript{2}O P\textsubscript{tm} produced 0.08 ± 0.01 ASM strain (i.e. an 8 % increase in ASM perimeter), which increased to 0.26 ± 0.02 (i.e. an 26 % increase in ASM perimeter) with DI to 60 cmH\textsubscript{2}O P\textsubscript{tm}.
Figure 2.3.1. The effect of amplitude of fixed-$P_{in}$ DI (5 to peak $P_{in}$ of 20 to 60 cmH$_2$O $P_{in}$) on bronchodilation (% reversal, A) and the time-course of re-narrowing following DI (B) in airways narrowed to a moderate dose of ACh ($10^{-5}$ M). Bronchodilation to DI increased with increasing amplitude ($p<0.001$). There was rapid re-narrowing following DI and bronchodilatory responses were typically reversed in <1 min. The decay constant ($k$) following DI was independent of DI amplitude. $n = 6$. Mean ± standard error of the mean (SEM).

Figure 2.3.2. Airway smooth muscle (ASM) strain produced by increasing amplitudes of fixed-$P_{in}$ DI (5 to peak $P_{in}$ of 20 to 60 cmH$_2$O $P_{in}$) in airways narrowed to a moderate dose of ACh ($10^{-5}$ M). Increasing amplitude of fixed-$P_{in}$ DI produced greater ASM strain ($p<0.001$). $n = 6$. Mean ± SEM.
In protocol 2, the relationship between ASM mechanical stress and strain was altered by increasing airway wall stiffness using cumulative-doses of ACh (10^{-7} to 3 \times 10^{-3} M). Specific compliance of the airway wall fell from 0.0086 \pm 0.0009 \text{cmH}_2\text{O}^{-1} in the relaxed state, to 0.0029 \pm 0.0001 \text{cmH}_2\text{O}^{-1} at the maximal dose of ACh (Figure 2.3.3). Scatter plots of bronchodilation against either ASM strain produced by fixed-$P_m$ DI (Figure 2.3.4A) or mechanical stress produced by fixed-volume DI (Figure 2.3.4B) had linear lines-of-best-fit fitted to individual airways. Bronchodilation was positively correlated with ASM strain. The average slope and intercept of the lines fitted between bronchodilation and ASM strain was 814.1 \pm 206.3 (i.e. \sim 8 \% bronchodilation per 1 \% ASM strain) and 0.026 \pm 0.005 (i.e. only ASM strains greater than \sim 3 \% produced bronchodilation), respectively. Unexpectedly, bronchodilation and mechanical stress were negatively correlated with an average slope and intercept of -0.34 \pm 0.08 \text{cmH}_2\text{O}^{-1} and 505.3 \pm 202.3, respectively. Pearson’s correlation coefficients for individual airways are shown in Table 2.3.1.
Figure 2.3.3. Specific compliance of the airway wall (cmH₂O⁻¹) in the relaxed state and in airways narrowed to ACh (10⁻⁷ to 3x 10⁻³ M). Airway walls stiffened strongly in response to ACh (p<0.001). n = 6. Mean ± SEM.

Table 2.3.1. Pearson’s correlation coefficients for scatter plots of bronchodilation against either ASM strain (airway 1 to 6) or mechanical stress (airway 7 to 12).

<table>
<thead>
<tr>
<th>Airway</th>
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<th>6</th>
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<tr>
<td>ASM Strain</td>
<td>r</td>
<td>p value</td>
<td>0.83</td>
<td>0.0415</td>
<td>0.91</td>
<td>0.0122</td>
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</tr>
<tr>
<td>Stress</td>
<td>r</td>
<td>p value</td>
<td>-0.42</td>
<td>0.4087</td>
<td>-0.96</td>
<td>0.0025</td>
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Bronchodilation is the % reversal in airway narrowing or active pressure following DI, ASM strain is the change in ASM perimeter produced by fixed-Pₘ₀ DI and mechanical stress is the change in Pₘ₀ produced by fixed-volume DI.
The effect of ACh dose on the bronchodilatory response to DI differed between experiments where $P_{tm}$ change was held fixed, compared with fixed-volume change. Bronchodilation to fixed-$P_{tm}$ DI fell substantially with increasing ACh dose from 54.5 ± 12.0 % reversal of narrowing at $10^{-5}$ M ACh (the lowest dose which produced

**Figure 2.3.4.** Scatter plots of bronchodilation (% Reversal) against ASM strain (A) produced by fixed-$P_{tm}$ DI or mechanical stress ($\Delta cmH_2O$, B) produced by fixed-volume DI. Plots comprise 6 measurements per airway in both A and B. Linear lines-of-best fit were fitted to individual airways and the lines in both A and B were drawn using the average intercept and average slope. Bronchodilation was positively correlated with ASM strain and negatively correlated with mechanical stress. $n = 6$. 
contraction) to 0.80 ± 0.33 % reversal at 3 x 10⁻³ M (p<0.001, Newman Keuls post hoc test against 10⁻⁵ M). Whilst bronchodilation to fixed-volume DI also fell with increasing dose, this was far less pronounced. Fixed-volume DI produced 88.9 ± 7.6 % reversal of active pressure at 10⁻⁵ M ACh, which fell to 67.8 ± 5.1 % reversal at 3 x 10⁻³ M (p<0.01, Newman Keuls post hoc test against 10⁻⁵ M).

The greater dependence (i.e. steeper slope) of the bronchodilatory response on strain, rather than mechanical stress, of oscillation and the pronounced stiffening to ACh meant that attenuation of contraction was much weaker using the fixed-Pₜₚₚ approach, compared with the fixed-volume approach. During fixed-Pₜₚₚ oscillations, airways narrowed strongly in response to ACh in a dose-dependent manner, producing a maximum of 92.8 ± 3.5 % narrowing under static conditions, which fell to 81.8 ± 6.9 % under oscillatory conditions (Figure 2.3.5A). In comparison to experiments that used fixed-Pₜₚₚ oscillations, contraction was strongly attenuated during fixed-volume oscillations. Airways produced a maximum of 81.9 ± 4.8 cmH₂O active pressure under static conditions, which fell to 16.4 ± 4.3 cmH₂O under oscillatory conditions (Figure 2.3.5B). Interestingly, the PD₂ (i.e. sensitivity) to ACh under oscillatory conditions for both airway narrowing and active pressure was less than that for specific compliance, indicating that changes in specific compliance were more sensitive to stimulation with ACh than the observed bronchoconstrictor response (Table 2.3.2).
Figure 2.3.5. Cumulative DRC to ACh (10^{-7} to 3 \times 10^{-3} \text{ M}) using fixed-\(P_{\text{m}}\) (% Lumen Volume, A) and fixed-volume (\(\Delta c_{\text{mH}}\text{O}\), B) oscillations. Oscillatory conditions attenuated contraction weakly using the fixed-\(P_{\text{m}}\) approach (\(p<0.05\)) and strongly using the fixed-volume approach (\(p<0.001\)). 
\(n=6\). Mean ± SEM.
Table 2.3.2. PD₂ to ACh (10⁻⁷ to 3x 10⁻³ M) for airway narrowing, active pressure and specific compliance.

<table>
<thead>
<tr>
<th>Fixed-Pₚₚm Approach</th>
<th>Airway Narrowing</th>
<th>Specific Compliance</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>4.39 ± 0.06</td>
<td>5.22 ± 0.10</td>
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</table>

<table>
<thead>
<tr>
<th>Fixed-Volume Approach</th>
<th>Active Pressure</th>
<th>Specific Compliance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.05 ± 0.13</td>
<td>5.35 ± 0.12</td>
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</tbody>
</table>

Changes in specific compliance with ACh were more sensitive than airway narrowing using the fixed-Pₚₚm approach (p<0.001) and active pressure using the fixed-volume approach (p<0.001). n = 6. Mean ± SEM.

Finally, in protocol 3, the relationship between ASM mechanical stress and strain was altered by changing the rate-of-inflation during DI at single, moderate dose of ACh (10⁻⁵ M). As predicted, the ASM strain produced by fixed-Pₚₚm DI varied in a rate-dependent manner. Taking a slow DI produced 0.13 ± 0.01 ASM strain, which fell to 0.11 ± 0.01 with a fast DI (Figure 2.3.6A). Despite producing less ASM strain than a slow DI, a fast or moderate DI produced a greater bronchodilatory response than a slow DI (Figure 2.3.6B). Interestingly, despite less ASM strain, fixed-volume DI produced greater mechanical stress than the fixed-Pₚₚm DI (i.e. greater than 30 cmH₂O Pₚₚm). This is likely explained by the observation that ASM contraction resulted in greater airway wall stiffening using the fixed-volume (0.019 ± 0.0001 cmH₂O⁻¹) than the fixed-Pₚₚm approaches (0.029 ± 0.0006 cmH₂O⁻¹, p<0.05).
The effect of rate-of-inflation during DI on bronchodilation. ASM strain (A), mechanical stress (ΔcmH₂O, C) and bronchodilation (% Reversal, B and D) to fixed-Ptm (black bars) and fixed-volume (grey bars) DI, in airways contacted to a single, moderate dose of ACh (10⁻⁵ M). Slow fixed-Ptm DI produced greater ASM strain than moderate (p<0.001) and fast (p<0.001) DI but less bronchodilation than moderate (p<0.01) and fast (p<0.01) DI. Slow fixed-volume DI produced less mechanical stress than moderate (p<0.001) and fast (p<0.001) DI but despite identical ASM strains, fast fixed-volume DI produced greater bronchodilation than moderate (p<0.001) and slow (p<0.001) DI. n = 6. Mean ± SEM.

The mechanical stress produced by a fixed-volume DI also varied in a rate-dependent manner. Taking a slow DI produced 37.2 ± 1.5 cmH₂O mechanical stress, which increased to 49.0 ± 2.2 cmH₂O with a fast DI (Figure 2.3.6C). Despite identical ASM strains, fast DI and moderate DI also produced a greater bronchodilatory response than a slow DI (Figure 2.3.6D).
2.4 Discussion

The present study determined the relative contributions of ASM mechanical stress and strain to the bronchodilatory response to DI using an intact airway preparation. I manipulated the relationship between ASM mechanical stress and strain by either increasing dose of ACh or varying the rate-of-inflation during DI. I show that ASM strain, rather than mechanical stress, is the critical determinant of bronchodilation and somewhat unexpectedly, that the rate-of-inflation during DI also impacts on bronchodilation, independent of the magnitudes of either mechanical stress or strain.

In protocol 1, I confirmed the findings of previous studies (53, 137) that the bronchodilatory response to DI is, indeed, amplitude-dependent and that increasing amplitude of DI produced greater bronchodilation. I induced a moderate level of airway narrowing (~35 % lumen volume), which I calculate, using the computational model of Lambert and colleagues (99), would produce a ~47 % increase in resistance and therefore, a physiologically relevant reduction in airflow. The amplitudes of DI ranged from sub-maximal manoeuvres (peak $P_{tm}$ of 20 cmH$_2$O $P_{tm}$), maximal DI (30 cmH$_2$O $P_{tm}$) and those greater than expected to occur in vivo (60 cmH$_2$O $P_{tm}$). Importantly, with increasing amplitude of DI, mechanical stress, the accompanying strain and the rate-of-inflation during DI all increased.

To assess the independent effects of ASM mechanical stress and strain on the bronchodilatory response to DI in protocol 2, I compared the effect of increasing doses of
ACh on the magnitudes of bronchodilation to fixed-$P_{tm}$ or fixed-volume DI. A known effect of contractile agonists, such as ACh, is an increase in airway wall stiffness (7), which decreases the ASM strain to a fixed-$P_{tm}$ DI and increases the mechanical stress to a fixed-volume DI. This allowed us to plot relationships separately between strain and bronchodilation and mechanical stress and bronchodilation. I show that the bronchodilatory response to DI and the magnitude of strain were positively correlated, whilst there was a negative correlation between bronchodilation and mechanical stress (discussed below). These data suggest that strain (i.e. length-change), rather than the distending force being applied to the ASM (i.e. mechanical stress) underlies relaxation following DI. The intercept of the linear line-of-best fit between strain and bronchodilation suggests that only ASM strain greater than ~3 % produce bronchodilation. This finding is comparable to a previous study from my laboratory (124), where only ASM strain exceeding ~1 % produced bronchodilation, although in this study bronchodilation referred to reversal of active pressure, as opposed to airway narrowing as in the present study. The cellular mechanism(s) underlying bronchodilation to DI may involve perturbed cross-bridge binding (60, 61) and/or de-polymerisation of the contractile apparatus (66). Physical breaking of ASM cross-bridges could, theoretically, result from either mechanical stress or strain on the ASM fibres, although I suggest it is the latter. Alternatively, re-modelling of the contractile apparatus is thought to involve mechanosensors (i.e. integrins) that respond to the physical deformation (i.e. strain) of cells and initiate the downstream re-modelling process (69). My finding that ASM strain rather than mechanical stress, underlies bronchodilation is consistent with cellular mecanostransduction.
Two recent studies have examined the effect of ASM mechanical stress and strain on contraction. Lavoie and colleagues (104) assessed the behaviour of human lung slices under oscillatory conditions and concluded that reversal of bronchoconstriction was dependent on strain, but did not measure mechanical stress. In contrast, Pascoe and colleagues (129) demonstrated attenuation of ASM force development despite negligible strain using ovine tracheal strips, implicating a role of mechanical stress. In their study, when constant oscillations of $\Delta 20$ cmH$_2$O were introduced prior to ASM contraction, oscillations attenuated ASM force at high doses of contractile agonist despite <1 % strain. I suggest that this finding reflects the ability of strain to inhibit ASM contraction (i.e. bronchoprotection) rather than reversing existing contraction (i.e. bronchodilation), which is the subject of the present study. The apparent disparity between the present findings and those by Pascoe and colleagues may, therefore, relate to the different underlying mechanisms governing bronchodilation and bronchoprotection and their relative sensitivity to ASM mechanical stress and strain.

Whilst bronchodilation was positively correlated with strain, paradoxically, I observed a negative correlation between bronchodilation and ASM mechanical stress. Since high mechanical stress occurred when ASM stiffness was also high (i.e. at high doses of ACh) it is possible that the stiffness of the airway wall approached that of the system and consequently a lesser proportion of the volume during DI was taken up by airway. The resulting decrease in strain would favour a reduction in bronchodilation. However, the compliance of the organ bath set-up is low (relaxed airway/organ bath system absolute compliance ratio of ~219) and even under conditions of maximal airway wall stiffness
(ratio of ~34), the volume taken up by the system was negligible. I believe a more likely explanation for reduced bronchodilation with greater mechanical stress is that there is an independent effect of dose (i.e. the level of pharmacologically induced ASM activation) on the bronchodilatory response to DI. Since increased mechanical stress occurred at high doses of ACh, the negative correlation between bronchodilation to DI and mechanical stress could indicate that high doses of ACh inhibit the bronchodilatory response to DI directly. Previous studies with increasing levels of pharmacologically induced ASM activation have produced conflicting results on the relaxant response to strain. A study by Shen and colleagues (148) showed no effect of ACh dose on the relaxant response to length-oscillation using isolated canine ASM strips. Whilst in vivo, Scichilone and colleagues (141) showed greater bronchodilation to DI with increasing dose of methacholine in normal healthy individuals. Importantly, if as suggested in the present study, the degree of contractile activation negatively impacts the bronchodilatory response, then I would expect that this mechanism would also affect the observed relationship between strain and bronchodilation. The implication is that low strain/high doses of contractile agonist may together explain the fall off in bronchodilation. However I predict that the strain will be by far the dominant effect, since at high doses of contractile agonist, bronchodilation was pronounced to fixed-volume DI (and therefore, high ASM strain) despite maximal contractile activation.

My findings that the magnitude of strain, rather than mechanical stress, determines the bronchodilatory response to DI, have implications for the role of DI in the regulation of airway responsiveness and therefore, airway hyper-responsiveness. Under high levels of
ASM contraction the airway wall stiffens, so that the strain on the ASM becomes negligible and therefore, the bronchodilatory response to DI is attenuated. Further, the present study also showed that sensitivity to ACh was significantly greater for stiffness than for narrowing (i.e. airways stiffen prior to narrowing as the dose of contractile agonist increases). The apparent disconnect between airway wall stiffening and narrowing (or active pressure) raises the possibility that the capacity for an airway to stiffen in addition to its ability to narrow may regulate bronchodilation to DI in health and disease. I suggest that the airways of patients with asthma will not only narrow more at low doses of ACh, compared with normal healthy individuals but will also be stiffer. *In vivo*, studies of DI-induced bronchodilation have typically matched for changes in airway narrowing (i.e. resistance) with contractile agonist (183) between normal healthy individuals and patients with asthma (46). The disconnect between airway narrowing and stiffening raises the possibility that, even if matched for the same level of narrowing by using low doses of contractile agonist in patients with asthma airways may reach a greater level of stiffness and the ASM strained less during DI. Increased airway stiffening separate from airway narrowing may contribute to the reduced bronchodilatory response to DI in patients with asthma.

In order to further assess the relative effects of ASM mechanical stress and strain on the bronchodilatory response to DI, I adopted an additional protocol (protocol 3). In this protocol, the relationship between ASM mechanical stress and strain during DI was not altered by dose of contractile agonist but by varying the rate-of-inflation during DI. By changing the rate-of-inflation, the viscous and inertial loads of the airway will determine
the resulting strain produced by a fixed-\(P_{\text{m}}\) DI and the mechanical stress produced by a fixed-volume DI. I show that when the pressure change for a DI is held fixed, faster DI produce less strain than a slower DI, whilst when the volume change of DI is held fixed; faster DI produce greater mechanical stress than slower DI. Unexpectedly, irrespective of whether the DI was administered using the fixed-\(P_{\text{m}}\) or fixed-volume approach, the faster DI produced greater bronchodilation. This finding suggests a rate-of-change-dependent component of the bronchodilatory response to DI that is independent of the magnitudes of either mechanical stress or strain.

Previous studies, *in vivo*, have examined the importance of the rate-of-inflation during DI. Hida and colleagues (73) demonstrated a greater bronchodilatory response with a faster DI in normal healthy individuals. In contrast, Duggan and colleagues (53) showed no effect of rate-of-inflation, in normal healthy individuals, although the data suggests a trend. It is possible that, *in vivo*, the pressure swing (i.e. mechanical stress) and therefore, strain on the airway wall is greater during a faster DI. I suggest that, separate from the magnitudes of mechanical stress or strain, the rate-of-change of ASM stretch during DI also determines the bronchodilatory response. Importantly, the rate-of-change dependence of DI is preserved in patients with asthma (73), suggesting that an abnormality in this mechanism does not contribute to the reduced bronchodilatory response to DI in patients with asthma. The rate-dependence of a transient inflationary manoeuvre such as DI is qualitatively similar to the frequency-dependence of isometric force production during length-oscillation of isolated ASM (2, 65, 147, 165) and *in vivo*, the response to mechanical ventilation (146). The cellular mechanism(s) may involve the relative rates of cross-bridge cycling and
length-oscillation (i.e. the frequency of oscillation). If the frequency of oscillation is faster than the rate of detachment and re-attachment of cross-bridges, then fewer detached cross-bridges can re-attach during the oscillation cycle (69).

The present study also confirms previous findings in the literature that the modulating effects of breathing are substantially less when modelled using fixed-$P_{tm}$ oscillations than fixed-volume oscillations in whole bronchial segments (67, 101, 102, 120, 122, 124). Fixed-$P_{tm}$ DI are likely to be the more physiological approach (assuming breathing movements are ultimately pressure limited), where the amplitude of DI and the stiffness of the airway wall determine the magnitude of strain on the ASM. In contrast, fixed-volume DI (a less physiological scenario, analogous to length-oscillation in ASM strip studies), which are not dependent on airway wall stiffness, are potent at attenuating force production, even at maximal levels of ASM activation. Importantly, low volume respiratory movements such as tidal breathing may not necessarily be pressure limited during bronchoconstrictor challenge, as pressures may rise to overcome the greater impedance of the respiratory system. The true in vivo scenario may then exist somewhere between the fixed-$P_{tm}$ and fixed-volume scenarios. Nonetheless, the introduction of fixed-$P_{tm}$ oscillations in vitro (124) do bring into the question the importance of the dynamic mechanical environment of the lung in the regulation of airway responsiveness.

Several methodological aspects of the study also require discussion. Firstly, I chose to use porcine airways to study the effects of ASM mechanical stress and strain in response to DI
and the resultant bronchodilation. My laboratory has used porcine airways previously to assess the role of DI amplitude and to demonstrate the importance of airway wall stiffness to this response, particularly during ASM contraction (124). Many of these findings have been replicated recently in studies utilising human tissue (104, 121). These findings suggest that the porcine airway behaves similarly to the human airway, although some species differences exist (120). Certainly, the porcine airway is highly cartilaginous, which likely increases wall stiffness (126). I am now able to compare the dynamic specific compliance of the porcine airway to similarly sized human airways using the same recording system (122). In the relaxed state, the porcine airway is ~3-fold stiffer. Further, the increase in stiffness during ASM activation is substantial in porcine airways (with correlates with greater narrowing in vitro compared with humans), which I suggest is likely to further inhibit the bronchodilatory effect of DI. Finally, the system I use to measure airway narrowing is not a direct measure of calibre. Previous studies in my laboratory that directly measured luminal calibre by video endoscopy reported only a ~49 % narrowing to a maximal dose of ACh (115) which is considerably less than that reported here (~93 % narrowing). One possible explanation is that my measurement of the relaxed lumen volume at the point of collapse underestimates the total volume contained within the bronchial segment. This is possible since only one region along the length of the airway would need to collapse for the airway to be deemed closed. However, whilst this may affect the level of airway narrowing reported, it does not affect the response to DI observed between protocols and therefore, the conclusions drawn from the study.
In conclusion, the present study demonstrates that the bronchodilatory response to DI is indeed amplitude-dependent and that ASM strain, rather than mechanical stress, is the critical determinant of bronchodilation. I also identified a rate-of-change-dependent component to the response to DI that is independent of the magnitudes of either mechanical stress or strain.
Chapter 3 Pharmacological bronchodilation is partially mediated by reduced airway wall stiffness

3.1 Introduction

Bronchoconstriction is initiated by agonist interaction with G-protein coupled receptors (GPCR) (16), which through an intracellular cascade, phosphorylates myosin regulatory light chain (MRLC) and facilitates actin and myosin binding and airway smooth muscle (ASM) contraction. Airway hyper-responsiveness (AHR, i.e. excessive bronchoconstriction to an inhaled bronchial challenge) is a primary characteristic of asthma and is considered a major contributor to airflow limitation (99, 173). In patients with asthma, airflow limitation is at least partially reversed by administration of pharmacological bronchodilators, typically adrenergic \( \beta_2 \) (\( \beta_2 \))-agonists. Agonist binding of \( \beta_2 \)-receptors on airway smooth muscle (ASM) activates adenylyl cyclase, which produces an increase in cyclic 3’, 5’ adenosine monophosphate (cAMP) and de-phosphorylation of the myosin regulatory light chain (MRLC) (18). In isolated ASM strips (64) and whole bronchial segments (10) \textit{in vitro}, \( \beta_2 \)-agonists relax ASM in a dose-dependent manner and \textit{in vivo}, increasing dose of \( \beta_2 \)-agonists produces greater improvement in the forced expiratory volume in the first 1 second (FEV\(_1\)) (21).

In addition to receptor-mediated bronchodilation, the dynamic mechanical environment of the lung can produce bronchodilation. In normal healthy individuals \textit{in vivo}, deep
inspiration (DI) produces a transient reversal of bronchoconstriction (i.e. bronchodilation) to a number of different inspired ASM contractile agonists (53, 58, 73, 117, 137, 142). In Chapter 2 of this thesis, I show that ASM strain, rather than mechanical stress, is the critical determinant of bronchodilation in intact airway segments, suggesting that the underlying mechanism by which DI produces bronchodilation involves direct strain-induced relaxation of ASM, due to perturbed cross-bridge binding (60, 61) and/or de-polymerisation of the contractile filaments (66). As the magnitude of strain applied to the ASM is increased with deeper depth of inspiration, there is increasing bronchodilation (9, 104, 137). Lesser inspirations, such as normal tidal breathing, may also produce some bronchodilation, but which are strongly dependent on the stiffness of the airway wall, as a stiffer airway wall will reduce ASM strain and therefore bronchodilation (71, 103).

Airway wall stiffness is clearly dependent on the structural composition of the airway (126). However, it is also clear that a major contributor to stiffness is the degree of tension present in the ASM (90, 162). Muscle contraction markedly increases stiffness (77, 124), whilst ASM relaxants markedly reduce airway stiffness (10, 77). Given the importance of airway wall stiffness to the bronchodilator efficacy of breathing manoeuvres discussed above, it has been mooted that some proportion of the bronchodilation produced by ASM relaxants, including β-agonists, is attributable to their effect on airway stiffness (10). That is, pharmacological bronchodilators should be expected to enhance the effectiveness of breathing manoeuvres at producing bronchodilation.
The effects of pharmacological bronchodilators and dynamic ASM strain have been compared previously in whole bronchial segments subjected to fixed-volume oscillation (10) and in isolated ASM strips using fixed-length oscillation (64). I showed that bronchodilation produced by the combined effect of pharmacological bronchodilators (including isoprenaline) and ASM strain (i.e. volume oscillation) was not greater than bronchodilation to either alone, suggesting that the pharmacological and physiological mechanisms producing bronchodilation were not synergistic but separate (10). Similar conclusions were reached in the earlier study on isolated ASM strips subject to length oscillation (64). However, because the ASM strain was held fixed in my former study and in the study by Gump and colleagues, any effect of isoprenaline-induced airway wall softening on ASM strain and bronchodilation could not be identified.

I have now built on previous work in my laboratory (10) to determine if there is indeed an enhancement of oscillation induced bronchodilation by $\beta_2$-agonists due to changes in airway wall stiffness. Porcine whole bronchial segments in vitro were contracted to carbachol (CCh) and relaxed to the non-specific $\beta$-agonist, isoprenaline, under static conditions or during simulated breathing manoeuvres. An important adaptation of previous approaches (10) was to simulate breathing manoeuvres by oscillating airway wall transmural pressure ($P_{tm}$), as occurs in lungs in vivo under physiological conditions. Under these conditions the magnitude of ASM strain produced by simulated breathing manoeuvres will be dependent on airway wall stiffness, allowing $\beta$-agonists to modify oscillatory induced bronchodilation through its effects on airway wall stiffness. Present results show the previously postulated synergism between isoprenaline and ASM strain.
regimens, which was not detected by earlier fixed-ASM strain protocols. My findings support an important role of pharmacological bronchodilators in mediating the bronchodilatory response to breathing manoeuvres by reducing airway wall stiffness.
3.2 Methods

Animal handling

All animal experiments conformed to institutional ethics and animal care unit regulations (Animal Ethics Committee, University of Western Australia (UWA), Crawley, WA, Australia). Male White Landrace pigs, ~35 kg, were initially sedated with tiletamine-zolazepam (4.4 mg/kg⁻¹ I.M.) and xylazine (2.2 mg/kg⁻¹ I.M.) and then exsanguinated under sodium pentobarbitone anaesthesia (30 mg/kg⁻¹ I.V.). The lungs were immediately removed and transported on ice to the laboratory.

Airway segment preparation

Airway segments were dissected from the main stem bronchus of the left or right lower lobe within ~60 min of being removed. All side branches were ligated with surgical silk and a ~28 mm long airway segment was cannulated at both ends, as previous described (10, 11). The mode generation was 17 at the distal and 11 at the proximal end (where the generation of the trachea = 0), with an internal diameter of ~2 mm at the distal and ~3 mm at the proximal end. Following cannulation, the airway was mounted horizontally in an organ bath containing gassed (95 % O₂ and 5 % CO₂) Krebs solution (121 mM NaCl, 5.4 mM KCl, 1.2 mM MgSO₄, 25 mM NaHCO₃, 5 mM sodium morpholinopropane sulfonic acid, 11.5 mM glucose and 2.5 mM CaCl₂; pH 7.3) at 37 °C. The length of the segment was stretched to 105 % of its length in the fully deflated lung, shown previously to approximate the length at functional residual capacity (FRC) (125).
The proximal end of the airway lumen was connected to a reservoir filled with Krebs solution, the height of which set the initial $P_{tm}$ (5 cmH$_2$O) and which was used to flush the lumen with Krebs solution between experiments. The distal end of the airway was connected to a liquid-filled syringe pump. The syringe pump was capable of simulating breathing manoeuvres in 1-of-2 ways: fixed-$P_{tm}$ oscillations or fixed-volume oscillations (see below). All protocols were performed in a closed system, created by closure of a tap between the airway and the Krebs solution reservoir. The system was leak free with negligible compliance (0.0113 µL/cmH$_2$O with a ~7.0 mL system volume).

*Simulation of breathing manoeuvres*

A custom-built servo-controlled syringe pump and pressure transducer were used to measure airway narrowing and to apply fixed-$P_{tm}$ oscillations (i.e. breathing manoeuvres), as previously described (121, 122). Airways were connected to a 1 mL glass syringe driven by a feedback-controlled servomotor and motor controller and $P_{tm}$ was measured *via* a calibrated pressure transducer with feedback to the servomotor. Changes in airway luminal volume (i.e. airway narrowing and fixed-$P_{tm}$ oscillations) were measured *via* a calibrated displacement transducer that measured the rotation of the syringe motor. Using this approach, $P_{tm}$ was set to the desired level (i.e. static or oscillatory, see *Experimental protocols*) and ASM activation resulted in a decrease in lumen volume (i.e. airway narrowing, Figure 3.2.1A). Measurement of ASM force and fixed-volume oscillations were applied using the same syringe pump oscillator described above but using the displacement transducer and not pressure transducer as the feedback control to the servomotor. Using this approach, lumen volume does not decrease in response to ASM activation but instead
results in an increase in $P_{tm}$ (i.e. active pressure) that represents ASM force production (Figure 3.2.1B). For comparisons with protocols that used fixed-$P_{tm}$ oscillations, the volume of oscillation (i.e. breathing manoeuvres) was that which produced the same trough to peak change in $P_{tm}$ in the contracted state (i.e. at the peak of contraction following the administration of the contractile agonist) and was fixed thereafter (see *Experimental protocols*).
Figure 3.2.1. An example trace of a cumulative dose-response curve (DRC) to isoprenaline ($10^{-7}$ to $3 \times 10^{-5}$ M, arrows, text labels shown only for whole log doses) using fixed-transmural pressure ($P_{tm}$, A) and fixed-volume (B) oscillations in airways contracted to carbachol (CCh, $10^{-6}$ M). At the time scale shown, individual oscillations are not visible but appear as a thick line, the thickness of which indicates the magnitude of the $P_{tm}$ and volume oscillations. In response to isoprenaline, lumen volume increased during fixed-$P_{tm}$ oscillations and $P_{tm}$ decreased during fixed-volume oscillations, in a dose-dependent manner. Dose-response curves were performed under static conditions (trace not shown) and during continuous large tidal breathing manoeuvres.
**Experimental protocols**

After dissection and mounting, airways were initially equilibrated to organ bath conditions for ~60 min under a static $P_{\text{tm}}$ of 5 cmH$_2$O, approximating the mechanical environment present at FRC, *in vivo*. The Krebs solution in the organ bath and lumen was replaced every 10 min to remove the effects of metabolites and bronchoactive mediators released from the epithelium. Viability of the tissue was subsequently confirmed through stimulation with acetylcholine (ACh, $10^{-4}$ to $10^{-3}$ M) added to the organ bath followed by a 30 min washout and recovery period at 5 cmH$_2$O $P_{\text{tm}}$. Airways were subsequently contracted to a single dose of CCh ($10^{-6}$ M) under both static (5 cmH$_2$O $P_{\text{tm}}$) and oscillatory conditions in a randomised order. For the fixed-$P_{\text{tm}}$ approach, the oscillatory protocol comprised continuous large breathing manoeuvres ($\Delta 15$ cmH$_2$O at 0.25Hz). For the fixed-volume approach, the volume changes used were adjusted for each airway so that breathing manoeuvres were $\Delta 15$ cmH$_2$O in the contracted state. The initial volume change needed to produce a $\Delta 15$ cmH$_2$O after contraction was approximated from previously published experiments (124). After the contraction plateaued, the oscillation volume was adjusted (if needed) to give a $\Delta 15$ cmH$_2$O pressure swing. Tissues were oscillated for 6 min before contraction to CCh. Once contraction to CCh had plateaued, full DRC were constructed to the non-specific $\beta$-agonist, isoprenaline ($10^{-7}$ to $3 \times 10^{-5}$ M). Experiments conducted using the fixed-$P_{\text{tm}}$ or fixed-volume approaches were performed in separate groups of airways.

**Morphometry**

Morphometric analyses were carried out to estimate the magnitude of ASM strain produced by breathing manoeuvres. Following experimentation, airways were removed from the
organ bath and fixed in 4 % formaldehyde solution under atmospheric pressure (i.e. 0 cmH₂O Pₘᵦ). Distal and proximal regions of the airway segment were processed into paraffin blocks. Transverse airway sections were cut at a thickness of 5 μm and stained with haematoxylin and eosin (H and E). Inner wall area (WAᵢ) was calculated from the area enclosed by the outer ASM perimeter (Aᵢₘₒ) minus the area enclosed by the internal area (Aᵢ) (13) using ImageJ (version 1.45j, National Institutes of Health, MD, U.S.A.). Measurements at distal and proximal locations were averaged and corrected for horizontal stretch (105 % of its length in the fully deflated lung), which reduces the cross sectional area of the wall, assuming tissue volume is constant. The calculated inner wall area was also corrected for tissue shrinkage that occurs during histological processing (121).

**Analysis and statistics**

Lumen volume (i.e. prior to the administration of CCh) was measured by the volume that could be withdrawn until closure in the relaxed airway at 5 cmH₂O Pₘᵦ (67). Airway narrowing to CCh (for the fixed-Pₘᵦ approach) was expressed as % lumen volume (where 100 % airway narrowing indicates airway closure). As described above, morphometry allowed the outer ASM perimeter (Pᵢₘₒ) to be calculated using the following equation:

\[
Pᵢₘₒ = \sqrt{4 \times \pi \times (WAᵢ + \frac{\text{Lumen Volume}}{\text{Airway Length}})}
\]

Where, lumen volume is volume of the lumen at the trough of the pressure cycle at the time of measurement and airway length is the length of the airway segment mounted in the organ bath. The equation assumes WAᵢ is constant at all Pₘᵦ, that Pᵢₘₒ is circular and that the
lumen is cylindrical. Active pressure to CCh (for the fixed-volume approach) was expressed as $\Delta P_{tm}$. Comparisons between static and oscillatory conditions were made at troughs of the oscillation cycle (volume or pressure, depending on the approach used). The response to isoprenaline was also expressed as % of the response to CCh (i.e. % contracted). Dose-response curves expressed as % contracted had variable slope sigmoidal curves fitted to individual airways. Sensitivity ($\text{PD}_2 = -\log_{10}(\text{EC}_{50})$) to isoprenaline were calculated for individual airways under static and oscillatory conditions. During fixed-$P_{tm}$ oscillations, ASM strain was calculated as $\Delta P_{mo}/P_{mot}$, where $\Delta P_{mo}$ is the trough to peak change in $P_{mo}$ during the breathing manoeuvre and $P_{mot}$ is the trough immediately prior to the breathing manoeuvres.

Specific compliance of the airway wall was calculated from the $\Delta$volume in relation to the $\Delta P_{tm}$ during the inflationary limb of the tidal oscillation cycle using the equation:

$$\text{Specific Compliance} = \frac{\Delta\text{Volume}}{\Delta P_{tm} \times \text{Lumen Volume}}$$

Where $\Delta$volume and $\Delta P_{tm}$ are the trough to peak changes in volume and pressure during the breathing manoeuvre and lumen volume is volume of the lumen at the trough of the pressure cycle at the time of measurement.

Differences between groups were analysed using 2-way repeat measures analysis of variance (ANOVA) and Newman-Keuls post hoc test with dose of isoprenaline and the condition (i.e. either static or oscillatory) as the repeat measures variables, unless otherwise
stated below. The response to CCh under static and oscillatory conditions and the sensitivity to isoprenaline under static and oscillatory conditions was analysed using paired t-tests. Data analysis and statistical tests were performed using Statistica (version 8.0; StatSoft, Tulsa, OK, U.S.A.) and GraphPad Prism (version 5.0d; GraphPad Software, La Jolla, CA, U.S.A.) Data are presented as means ± standard error of the mean (SEM), where \( n \) = number of animals.
3.3 Results

Under static conditions, CCh (10⁻⁶ M) produced 59.2 ± 4.9 % narrowing (Figure 3.3.1A) and 46.2 ± 2.5 cmH₂O active pressure (Figure 3.3.1B). Isoprenaline (10⁻⁷ to 3x 10⁻⁵ M) reversed airway narrowing and active pressure in a dose-dependent manner. At the maximum dose of isoprenaline, airway narrowing fell to 39.1 ± 3.4 % narrowing and active pressure fell strongly to 2.6 ± 1.8 cmH₂O under static conditions. Interestingly, when expressed as % of the response to CCh, the maximum reversal of active pressure with isoprenaline was greater than the maximum reversal airway narrowing (p<0.001).
Airways also stiffened strongly in response to CCh (p<0.001). Specific compliance of the airway wall fell from 0.0126 ± 0.0013 cmH₂O⁻¹ in the relaxed state, to 0.0037 ± 0.0003 cmH₂O⁻¹ following CCh for the fixed-Pₚₜm approach. Similarly, for the fixed-volume approach, specific compliance fell from 0.0099 ± 0.0011 cmH₂O⁻¹ in the relaxed state, to 0.0014 ± 0.0002 cmH₂O⁻¹ following CCh. Isoprenaline reduced airway stiffness in a dose-dependent manner for both the fixed-Pₚₜm and fixed-volume approaches (Figure 3.3.2).
Specific compliance increased to $0.0111 \pm 0.0011 \text{cmH}_2\text{O}^{-1}$ and $0.0065 \pm 0.0007$ for the fixed-$P_{tm}$ and fixed-volume approaches, respectively.

**Figure 3.3.2.** Specific compliance of the airway wall ($\text{cmH}_2\text{O}^{-1}$) in response to isoprenaline ($10^{-7}$ to $3 \times 10^{-5}$ M) in airways contracted to CCh ($10^{-6}$ M, left of the axis break). Isoprenaline reduced airway wall stiffness in a dose-dependent manner for both the fixed-$P_{tm}$ ($p<0.001$) and fixed-volume ($p<0.001$) approaches. Airways were stiffer for the fixed-volume, compared with the fixed-$P_{tm}$, approach ($p<0.001$). $n = 6$. Mean $\pm$ SEM.

The magnitudes of oscillations (i.e. $\Delta P_{tm}$ or $\Delta$volume, depending on the approach) used were chosen so that contraction prior to the administration of isoprenaline was not substantially attenuated. Airway narrowing and active pressure fell to $47.7 \pm 5.3$ % (Figure 3.3.3A) and $39.4 \pm 3.7$ cmH$_2$O (Figure 3.3.3B) (compared to 59% and 46 cmH$_2$O, see above) during fixed-$P_{tm}$ and fixed-volume oscillations, respectively. There was no difference in PD$_2$ (i.e. sensitivity) to isoprenaline under static, compared with oscillatory, conditions (Table 3.3.1).
Figure 3.3.3. Airway narrowing (% Lumen Volume, A) and active pressure (ΔcmH₂O, B) to CCh (10⁻⁶ M) under static (back) and oscillatory (grey) conditions. Fixed-$P_{tm}$ oscillations attenuated airway narrowing (p<0.05). There was also a tendency towards a reduction in active pressure with fixed-volume oscillation, however, this did not reach statistical significance (p=0.06). n = 6. Mean ± SEM.
Table 3.3.1. PD₂ to isoprenaline (10⁻⁷ to 3 x 10⁻⁵ M) under static and oscillatory conditions.

<table>
<thead>
<tr>
<th></th>
<th>Static</th>
<th>Oscillatory</th>
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</thead>
<tbody>
<tr>
<td>Fixed-Pₜm Approach</td>
<td>Airway Narrowing</td>
<td>Airway Narrowing</td>
</tr>
<tr>
<td></td>
<td>5.60 ± 0.11</td>
<td>6.00 ± 0.07</td>
</tr>
<tr>
<td>Fixed-Volume Approach</td>
<td>Active Pressure</td>
<td>Active Pressure</td>
</tr>
<tr>
<td></td>
<td>6.26 ± 0.14</td>
<td>7.78 ± 1.53</td>
</tr>
</tbody>
</table>

There was no difference in the sensitivity to isoprenaline for airway narrowing or active pressure under static, compared with oscillatory, conditions. n = 6. Mean ± SEM.

By comparing the bronchodilatory response to isoprenaline under static and oscillatory conditions, I sought to determine whether β₂-agonists exerted a secondary bronchodilator effect by virtue of reducing airway wall stiffness and therefore, enhancing the bronchodilatory response to breathing manoeuvres. My results demonstrate that the bronchodilatory response to isoprenaline was greater during fixed-Pₜm oscillations, compared with the response under static conditions (Figure 3.3.4A). At maximal dose of isoprenaline, airway narrowing fell to 10.4 ± 2.6 % (i.e. a ~82 % reversal from contracted) during breathing manoeuvres but to only 39.1 ± 3.4 % (i.e. a ~35 % reversal from contracted) under static conditions. The greater bronchodilatory response to isoprenaline during fixed-Pₜm oscillations is likely explained by the effect of isoprenaline on reducing airway wall stiffness, which increased ASM strain (Figure 3.3.4B), producing greater bronchodilation. Airway smooth muscle strain produced by fixed-Pₜm oscillations increased
from 0.03 ± 0.003 (i.e. a 3 % increase in ASM perimeter) to 0.08 ± 0.007 (i.e. a 8 % increase in ASM perimeter) to isoprenaline in a dose-dependent manner.
In contrast to the experiments where $P_m$ oscillations were held fixed, under conditions where fixed-volume oscillations were applied, ASM strain was constant for each airway at $0.01 \pm 0.002$ (i.e. a 1% increase in ASM perimeter) and therefore, independent of changes in airway wall compliance produced by isoprenaline. Consequently, there was no difference in the response to isoprenaline under static, compared with oscillatory, conditions (Figure 3.3.5). At maximal dose of isoprenaline, active pressure fell to 2.6

**Figure 3.3.4.** Cumulative DRC to isoprenaline ($10^{-7}$ to $3 \times 10^{-5}$ M) under static conditions and using fixed-$P_m$ oscillations (% Contracted, A) and airway smooth muscle (ASM) strain (B) produced by fixed-$P_m$ oscillations in airways contracted to CCh ($10^{-6}$ M, left of the axis break in B). The bronchodilatory response to isoprenaline was greater during fixed-$P_m$ oscillations, compared with the response under static conditions ($p<0.001$). Airway smooth muscle strain produced by fixed-$P_m$ oscillations increased in a dose-dependent manner ($p<0.001$). Cumulative DRC under static conditions in A are re-drawn from Figure 3.3.1A for comparison. $n = 6$. Mean ± SEM.
cmH₂O (i.e. a ~97 % reversal from contracted) during breathing manoeuvres and 2.3
cmH₂O (i.e. a ~98 % reversal from contracted) under static conditions.

Figure 3.3.5. Cumulative DRC to isoprenaline (10⁻⁷ to 3x 10⁻⁵ M) under static conditions and using fixed-volume oscillations (% Contracted) in airway contracted to CCh (10⁻⁶ M). There was no difference in the response to isoprenaline under static, compared with oscillatory, conditions. n = 6. Mean ± SEM.
3.4 Discussion

The present study determined whether pharmacological bronchodilators produce part of their physiological action through reduction of airway stiffness that enhances the relaxation produced by oscillatory loads. I show that the bronchodilatory response to isoprenaline was greater during simulated breathing manoeuvres, compared with the response under static conditions. I propose that the greater bronchodilatory response to isoprenaline during breathing manoeuvres is explained by the effect of isoprenaline on reducing airway wall stiffness, which increased ASM strain, producing greater bronchodilation.

In the present study, I used my established intact bronchial segment model. My laboratory has previously modelled tidal breathing and DI manoeuvres in both animal (9, 124, 169) and human (122) bronchial segments, including those from subjects with reported asthma (121). The aforementioned studies simulate breathing manoeuvres by varying airway \( P_{tm} \). In the present study, the applied fixed-\( P_{tm} \) oscillations modelled breathing manoeuvres (\( \Delta 15 \text{ cmH}_2\text{O} \)), larger than normal tidal breathing but less than a DI (\( \Delta 25 \text{ cmH}_2\text{O} \)). I assume that during bronchoconstriction, \textit{in vivo}, \( P_{tm} \) would increase above that occurring with normal tidal breathing in order to overcome the greater resistance of the respiratory system and to maintain minute ventilation. I induced ~59 % airway narrowing, which we calculate, assuming laminar flow, to be produce a substantial five to six-fold increase in airway resistance.

In order to establish a hypothesised synergy of isoprenaline and oscillation, I compared the bronchodilatory response to isoprenaline under static conditions and during
breathing manoeuvres simulated by oscillating \( P_{tm} \). Isoprenaline produced greater bronchodilation (i.e. reversal of airway narrowing) during fixed-\( P_{tm} \) oscillations, with increasing separation from the static control with increasing dose of isoprenaline, suggesting a synergistic relationship. Since, in Chapter 2 of this thesis, I show that the bronchodilatory response to breathing manoeuvres is dependent on ASM strain and therefore, airway wall stiffness my findings are likely explained by the effect of isoprenaline on reducing airway wall stiffness, which increased ASM strain. As discussed below, synergism was only revealed when oscillations of fixed-\( P_{tm} \) were used, whereas fixed-volume oscillations did not alter the response to isoprenaline.

Studies from my laboratory (10) and others (64) have previously examined the combined effect of pharmacological bronchodilators when the strain applied to the ASM (length-change in isolated ASM strips or lumen volume in airway segments) is held fixed. The principal conclusions drawn from these studies was that ASM length/volume oscillation and pharmacological bronchodilators act via separate pathways. That is, while both oscillation and pharmacological bronchodilators produced ASM relaxation, one did not affect the other. However, in my previous study (10) we proposed one caveat: that pharmacological bronchodilators through their actions on reducing airway stiffness could theoretically maximise the strain-induced relaxation of ASM force, which I have now demonstrated by administering isoprenaline under fixed-\( P_{tm} \) conditions. To further examine the mechanism underlying the greater bronchodilatory response to isoprenaline during simulated breathing manoeuvres, I again compared the relaxant response to isoprenaline during fixed-volume oscillations. Under conditions where fixed-volume oscillations were applied, and ASM strain was constant and therefore independent of changes in airway wall compliance produced by
isoprenaline, there was no synergism between oscillatory and pharmacological pathways. This finding supports my proposal that the greater bronchodilatory response to isoprenaline during fixed-\(P_{m}\) oscillations (i.e. synergism) was mediated by reduced airway wall stiffness.

The increased drug efficacy during \(P_{m}\) oscillation is unlikely to be unique to isoprenaline, nor \(\beta_2\)-agonists in general, rather, any pharmacological bronchodilator that reduces airway wall stiffness is predicted to undergo a similar synergy. I have previously shown in whole bronchial segments (10) \textit{in vitro}, that nitric oxide (NO) also reverses ASM stiffness, and ASM cell stiffness is reduced in culture by numerous bronchodilator agonists such as isoproterenol, prostaglandin E\(_2\) and forskolin amongst others (77). The synergy between pharmacological bronchodilation and breathing stresses is considered ‘mechanical’ through its dependence on ASM stiffness.

With the exception of the specific \(\beta_2\)-agonist, such as salbutamol in 1962 (48) and long-acting \(\beta_2\)-agonists (LABA), such as salmeterol in 1988 (160), pharmacological bronchodilators have remained largely unchanged for 60 years. Not all patients with asthma respond well to current bronchodilator therapy (168). My finding that, at maximal dose, about half of the pharmacological bronchodilator effect is mediated by reduced airway wall stiffness has clinical implications for the treatment of asthma. Reducing airway wall stiffness represents a potential target for novel pharmacological agents (27, 135, 144). Drug design models that consider the agonist's effects on the reversal of both ASM force and stiffness should lead to more effective pharmacological intervention.
To further examine the mechanism underlying the greater bronchodilatory response to isoprenaline during simulated breathing manoeuvres, I also compared the relaxant response to isoprenaline during fixed-volume oscillations, as in my previous study (10). Under conditions where fixed-volume oscillations were applied, ASM strain was constant and therefore, independent of changes in airway wall compliance produced by isoprenaline. My results showed that there was no difference in the relaxant response to isoprenaline under static, compared with oscillatory conditions, which is also consistent with previous studies in isolated ASM strips in vitro (64). That is, when ASM strain produced by oscillations was fixed (i.e. independent of airway wall stiffness), isoprenaline was no more effective at reversing ASM force than under static conditions. This finding supports my conclusion that the greater bronchodilatory response to isoprenaline during fixed-\(P_{tm}\) oscillations was mediated by reduced airway wall stiffness.

Airway wall compliance and ASM strain were somewhat greater during fixed-\(P_{tm}\), compared with fixed-volume, oscillations. There are several possible explanations. Since the airway narrows in the fixed-\(P_{tm}\) approach, the airway wall may operate at a more compliant region of the pressure-volume curve. In the relaxed state, the porcine airway is most compliant below 5 cmH\(_2\)O \(P_{tm}\), before stiffening again at \(-5\) cmH\(_2\)O (126). In comparison, the airway does not narrow in the fixed-volume approach and therefore, may operate at a comparatively stiffer region of the pressure volume curve. This explanation is not entirely sufficient, as there was a tendency for lower specific compliance during fixed-volume oscillations in the relaxed state. An alternative
explanation is that the initial higher pressure swings in the fixed-Pₘₑ protocol facilitated greater wall compliance. The volume oscillations were chosen such that pressure swings following the administration of CCh matched the $\Delta 15$ cmH$_2$O in the fixed-Pₘₑ protocol, which meant that in the relaxed state pressure, pressure swings accompanying fixed-volume oscillations were considerably less (<4 cmH$_2$O). The reduction in airway wall stiffness produced by isoprenaline also differed between the fixed-Pₘₑ and fixed-volume approaches, where the increase in compliance was greater during fixed-Pₘₑ oscillations, which may be explained by further ‘softening’ of the airway wall due to greater ASM strain.

The amplitudes of fixed-Pₘₑ and fixed-volume oscillations in the present study were chosen such that bronchoconstriction was not substantially attenuated by the breathing manoeuvres alone. Fixed-Pₘₑ oscillations only modestly attenuated airway narrowing (~81 % of the response under static conditions), and there was a non-significant tendency towards reduced active pressure during volume oscillation (~85 % of the response under static conditions). During oscillations, the compliance of the airway wall determined the magnitude of ASM strain. Prior to the administration of isoprenaline, ASM strain during fixed-volume oscillations was ~1 %, compared with ~3 % during fixed-Pₘₑ oscillation, due to the difference in compliance between the fixed-Pₘₑ and fixed-volume approaches. Somewhat serendipitously, previous studies including those from my own laboratory and Chapter 2 of this thesis suggest that strain between 1 to 3% are necessary to affect the contractile apparatus (61, 71, 124). Therefore, we are confident that the amplitudes of fixed-Pₘₑ and fixed-volume oscillations in the present study were sufficient to examine the bronchodilatory response to isoprenaline and ASM strain.
Several other interesting and potentially important aspects of this study require discussion. During fixed-volume oscillations, active pressure was completely reversed by high doses of isoprenaline, however, airway wall stiffness had not returned to levels present in the relaxed state. Previous studies in isolated ASM strips in vitro (128) and in Chapter 2 of this thesis, have shown that, in response to contractile agonists, ASM stiffens prior to generating active force. The use of a sub-maximal dose of CCh in the present study likely produced a proportionally greater increase in airway wall stiffness than ASM contraction (i.e. airway narrowing or active pressure). The observation that isoprenaline was not able to completely reverse airway wall stiffening, compared with active force, may be due to the fact that there was more airway wall stiffening to reverse (note that the highest isoprenaline dose was chosen to produce maximum reversal of active force and airway narrowing, rather than airway wall stiffness). A similar disconnect between stiffness and airway narrowing during fixed-$P_{tm}$ oscillations was not observed, which may also be explained by further ‘softening’ of the airway wall due to greater ASM strain. Nonetheless, a scenario in which airway wall stiffening occurs prior to narrowing means that, during exacerbation of asthma, the bronchodilatory response to breathing manoeuvres becomes less effective early in the process.

Finally, my results showed that under static conditions, reversal of active pressure by isoprenaline was greater than the corresponding reversal of airway narrowing. As there was no differences in the dose of CCh or isoprenaline between protocols, I assume that cell signalling was comparable. However, ASM mechanics will be different at the level of the contractile apparatus, since ASM shortens in the fixed-$P_{tm}$ approach whilst in the
fixed-volume approach the muscle contracts isometrically (i.e. no shortening). Airway smooth muscle is responsive to length-change, a phenomenon termed ‘length-adaptation’ (26, 143) and it has been suggested that prolonged ASM shortening facilitates greater contraction (114). In the present study, in the experiments where I measured airway narrowing, length-adaptation may have occurred, producing a reduced bronchodilatory response to isoprenaline. Whilst the present study cannot provide any further explanation as to why the bronchodilatory response to isoprenaline may be less effective in the experiments where we measured airway narrowing, the implication of these findings are that drug design models which measure ASM force, rather than airway narrowing may overestimate the effectiveness of pharmacological bronchodilators. A disconnect between ASM force and airway narrowing may also, at least in part, explain the discrepancies between experiments in isolated ASM strips and \textit{in vivo} responses to breathing manoeuvres (108).

In conclusion, the present study found that the bronchodilatory response to isoprenaline was greater during breathing manoeuvres, compared with the response under static conditions. To my knowledge, this is the first time that a secondary effect of a pharmacological bronchodilator has been experimentally shown and which is likely to be of clinical significance. The implications for the treatment of asthma are that reducing airway wall stiffness represents a potential second target for novel pharmacological agents.
Chapter 4 Do TNF and IL-1β modulate the airway smooth muscle response to strain?

4.1 Introduction

Airway hyper-responsiveness (AHR, i.e. excessive bronchoconstriction to an inhaled bronchial challenge) is a primary characteristic of asthma and is considered a major contributor to airflow limitation (99, 173). The cause(s) of AHR remain unclear but likely involve abnormalities in airway smooth muscle (ASM) structure/function following chronic inflammation of the airway wall present in patients with asthma (75). Increased levels of pro-inflammatory cytokines, such as tumour necrosis factor (TNF) and interleukin-1β (IL-1β), which are released from numerous cell types, including mast cells (29), have been detected in the sputum of patients with symptomatic asthma (29, 31). Although numerous pro-inflammatory cytokines are likely to contribute to the development of AHR to varying degrees, TNF and IL-1β have previously been shown to induce AHR. In isolated ASM strips/rings \textit{in vitro}, culture with TNF (1, 41, 136, 155) and/or IL-1β (136, 184) increased isometric force production (i.e. a hyper-contractile phenotype) to acetylcholine (ACh), carbachol (CCh), bradykinin and the non-specific G-protein coupled receptor (GPCR) agonist, 5-hydroxytryptamine (5-HT).

More recently, the dynamic mechanical environment of the lung has been identified as an important regulator of airway responsiveness and may be susceptible to inflammatory disease processes, contributing to AHR (6). In normal healthy individuals \textit{in vivo}, deep inspiration (DI) produces a transient (i.e. for ~1 to 2 min) reversal of
bronchoconstriction (i.e. bronchodilation) to a number of different inspired ASM contractile agonists (53, 58, 73, 117, 137, 142). In Chapter 2 of this thesis, I show that ASM strain, rather than mechanical stress, is the critical determinant of bronchodilation in intact airway segments, suggesting that the underlying mechanism by which DI produces bronchodilation involves strain-induced relaxation of ASM, due to perturbed cross-bridge binding (60, 61) and/or de-polymerisation of the contractile apparatus (66). However, the bronchodilatory response to DI is attenuated or abolished in patients with asthma (58, 73, 106, 142) and may also contribute to AHR. Whilst chronic inflammation of the airway wall and the failure of DI to produce bronchodilation are both common to asthma, whether inflammation and in particular, pro-inflammatory cytokines, modulate the ASM response to strain during DI is unknown.

The aim of the present study was to determine whether the pro-inflammatory cytokines, TNF and IL-1β, modulate the ASM response to strain and therefore, the bronchodilatory response to DI. I hypothesised that 2 days of culture with TNF and IL-1β would decrease ASM relaxation to any magnitude of strain during DI and that this would attenuate the bronchodilatory response. I used whole porcine bronchial segments in vitro, that were cultured in medium containing TNF and IL-1β. Bronchial segments were then contracted to ACh under static (i.e. no oscillation) conditions or during tidal breathing with intermittent DI manoeuvres. A servo-controlled syringe pump was used to simulate breathing manoeuvres.
4.2 Methods

Animal handling

All animal experiments conformed to institutional ethics and animal care unit regulations (Animal Ethics Committee, University of Western Australia (UWA), Crawley, WA, Australia). Male White Landrace pigs, ~35 kg, were initially sedated with tiletamine-zolazepam (4.4 mg/kg\(^{-1}\) I.M.) and xylazine (2.2 mg/kg\(^{-1}\) I.M.) and then exsanguinated under sodium pentobarbitone anaesthesia (30 mg/kg\(^{-1}\) I.V.). The lungs were immediately removed and transported on ice to the laboratory.

Airway segment preparation

Airway segments were dissected from the main stem bronchus of the left or right lower lobe within ~60 min of being removed. All side branches were ligated with surgical silk and a ~26 mm long airway segment was cannulated at both ends, as previous described (10, 11). The mode generation was 18 at the distal and 12 at the proximal end (where the generation of the trachea = 0), with an internal diameter of ~2 mm at the distal and ~3 mm at the proximal end. Following cannulation, the airway was mounted horizontally in an organ bath containing gassed (95 % O\(_2\) and 5 % CO\(_2\)) Krebs solution (121 mM NaCl, 5.4 mM KCl, 1.2 mM MgSO\(_4\), 25 mM NaHCO\(_3\), 5 mM sodium morpholinopropane sulfonic acid, 11.5 mM glucose and 2.5 mM CaCl\(_2\); pH 7.3) at 37 \(\degree\)C. The length of the segment was stretched to 105 % of its length in the fully deflated lung, shown previously to approximate the length at functional residual capacity (FRC) (125).
The proximal end of the airway lumen was connected to a reservoir filled with Krebs solution, the height of which set the initial transmural pressure ($P_{tm}$, 5 cmH$_2$O) and which was used to flush the lumen with Krebs solution between experiments. The distal end of the airway was connected to a liquid filled syringe pump. The syringe pump was capable of simulating breathing manoeuvres in 1-of-2 ways: fixed-$P_{tm}$ oscillations or fixed-volume oscillations (see below). All protocols were performed in a closed system, created by closure of a tap between the airway and the Krebs solution reservoir. The system was leak free with negligible compliance (0.0113 µL/cmH$_2$O with a ~7.0 mL system volume).

**Airway segment culture**

Prior to culturing, cannulated intact bronchial segments were washed in bovine serum albumin containing 1 % penicillin/streptomycin, 0.5 % gentamycin and 1 % Amphotericin B under sterile conditions. Bronchi were cultured in Dulbecco’s modified Eagle medium (DMEM) containing 1 % penicillin/streptomycin, 0.5 % gentamycin, 1 % L-glutamine and 2 % foetal bovine serum (FBS) at 37 °C with 5 % CO$_2$, 5 % humidity. In the TNF+IL-1β treatment group (see Experimental protocols), the culture medium also contained TNF (100 ng/mL) and IL-1β (20 ng/mL). The culture medium was replaced every 24 h to remove the effect of metabolites produced by the tissue.

**Simulation of breathing manoeuvres**

A custom-built servo-controlled syringe pump and pressure transducer were used to measure airway narrowing and to apply fixed-$P_{tm}$ oscillations (i.e. tidal breathing and DI
manoeuvres), as previously described (121, 122). Airways were connected to a 1 mL glass syringe driven by a feedback-controlled servomotor and motor controller. Transmural pressure was measured via a calibrated pressure transducer with feedback to a servomotor and changes in airway luminal volume (i.e. airway narrowing and fixed-$P_{tm}$ oscillations) were measured via a calibrated displacement transducer that measured the rotation of the syringe motor. Using this approach, $P_{tm}$ was set to the desired level (i.e. static or oscillatory, see *Experimental protocols*) and ASM activation resulted in a decrease in lumen volume (i.e. airway narrowing, Figure 4.2.1). In a separate group of airways, measurement of ASM force and fixed-volume oscillations were applied using the same syringe pump oscillator described above but using the displacement transducer and not pressure transducer as the feedback control to the servomotor. Using this approach, lumen volume does not decrease in response to ASM activation but instead results in an increase in $P_{tm}$ (i.e. active pressure) that represents ASM force production.
Figure 4.2.1. An example trace of a cumulative dose-response curve (DRC) to acetylcholine (ACh, $10^{-7}$ to $3 \times 10^{-3}$ M, arrows, text labels shown only for whole log doses) using fixed-transmural pressure ($P_{tm}$) oscillations with the control and the tumour necrosis factor (TNF, 100 ng/mL)/interleukin-$\beta$ (IL-$\beta$, 20 ng/mL) treatment groups. At the time scale shown, individual oscillations are not visible but appear as a thick line, the thickness of which indicates the magnitude of the $P_{tm}$ and volume oscillations. In response to ACh, lumen volume reduced during fixed-$P_{tm}$ oscillations in a dose-dependent manner. Dose-response curves were performed under static conditions (trace not shown) and during tidal breathing with intermittent deep inspiration (DI) manoeuvres.

Experimental protocols

After dissection and mounting, airways were initially equilibrated to organ bath conditions for ~60 min under a static $P_{tm}$ of 5 cmH$_2$O, approximating the mechanical environment present at functional residual capacity, in vivo. The Krebs solution in the organ bath and lumen was replaced every 10 min to remove the effects of metabolites and bronchoactive mediators released from the epithelium. Viability of the tissue was subsequently confirmed through stimulation with ACh ($10^{-4}$ M) added to the organ bath followed by a 30 min washout and recovery period at 5 cmH$_2$O $P_{tm}$. Two different protocols were followed (see below). In protocol 1, the viability of culturing airway
segments was established, whilst protocol 2 was designed to determine whether TNF and IL-1β modulate the ASM response to strain during DI.

**Protocol 1: The viability of culturing airway segments**

To assess the viability of culturing whole bronchial segments, airways were studied fresh (i.e. within ~1 hr of being dissected and prior to culture) and on day 2 of culture without pro-inflammatory cytokines. Full dose-response curves (DRC) were constructed to ACh (10^{-7} to 3x 10^{-3} M) under static (5 cmH_{2}O P_{tm}) conditions. Small P_{tm} oscillations, simulating tidal breathing (\Delta 5 cmH_{2}O at 0.25 Hz), were also applied to calculate the specific compliance of the airway wall prior to administration of ACh (i.e. in the relaxed state).

**Protocol 2: Whether TNF and IL-1β modulate the ASM response to strain during deep inspiration**

To determine whether TNF and IL-1β modulate the ASM response to strain during DI, airways were studied on day 2 of culture with the control treatment (left or right lower lobe) or the TNF+IL-1β treatment (alternate lower lobe, see Airway segment culture). Full DRC were constructed to ACh (10^{-7} to 3x 10^{-3} M) under both static (5 cmH_{2}O P_{tm}) and oscillatory conditions in a randomised order. The oscillatory protocol comprised tidal breathing (\Delta 5 cmH_{2}O at 0.25 Hz) and intermittent DI manoeuvres (\Delta 25 cmH_{2}O, a 2 s inflation, a 2 s hold at the peak of inflation and a 2 s deflation, a 6 s manoeuvre) applied once contraction at each dose of ACh had plateaued. To assess whether TNF and IL-1β increased ASM force, in a separate group of airways, full DRC were
constructed to ACh (10^{-7} to 3 \times 10^{-3} \text{ M}) under static conditions measuring active pressure.

*Morphometry*

Morphometric analyses were carried out to estimate the magnitude of ASM strain produced by breathing manoeuvres. Following experimentation, airways were removed from the organ bath and fixed in 4 \% formaldehyde solution under atmospheric pressure (i.e. 0 \text{ cmH}_2\text{O P}_\text{tm}). Distal and proximal regions of the airway segment were processed into paraffin blocks. Transverse airway sections were cut at a thickness of 5 \mu m and stained with haematoxylin and eosin (H and E). Inner wall area (WA_i) was calculated from the area enclosed by the outer ASM perimeter (A_{mo}) minus the area enclosed by the internal area (A_i) (13) using ImageJ (version 1.45j, National Institutes of Health, MD, U.S.A.). Measurements at distal and proximal locations were averaged and corrected for horizontal stretch (105 \% of its length in the fully deflated lung), which reduces the cross sectional area of the wall, assuming tissue volume is constant. The calculated inner wall area was also corrected for tissue shrinkage that occurs during histological processing (121).

*Analysis and statistics*

Lumen volume was measured by the volume that could be withdrawn until closure in the relaxed airway at 5 \text{ cmH}_2\text{O P}_\text{tm} (67). Airway narrowing to ACh (for the fixed-\text{P}_\text{tm} approach) was expressed as \% lumen volume (where 100 \% airway narrowing indicates
airway closure). As described above, morphometry allowed the outer ASM perimeter (P_{mo}) to be calculated using the following equation:

\[
P_{mo} = \sqrt{4 \times \pi \times (WA_l + \frac{\text{Lumen Volume}}{\text{Airway Length}})}
\]

Where, lumen volume is volume of the lumen at the trough of the pressure cycle at the time of measurement and airway length is the length of the airway segment mounted in the organ bath. The equation assumes WA_l is constant at all P_{im}, that P_{mo} is circular and that the lumen is cylindrical. Active pressure to ACh (for the fixed-volume approach) was expressed as ΔP_{im}. Comparisons between static and oscillatory conditions were made at troughs of the pressure oscillation cycle. Dose-response curves had variable slope sigmoidal curves fitted to individual airways. The maximum response (E_{max}) and sensitivity (PD_2 = -\log_{10}(EC_{50})) to ACh was calculated for individual airways under static and oscillatory conditions. During fixed-P_{im} oscillations, ASM strain was calculated using the following equation:

\[
\text{ASM Strain} = \frac{\Delta DI}{\text{Pre DI}}
\]

Where, ΔDI is the trough to peak change in P_{mo} during DI and Pre DI is the P_{mo} immediately prior to DI. The bronchodilatory response to DI was defined as % reversal of contraction to ACh using the equation (124):
Bronchodilation = \frac{\text{Pre DI} - \text{Post DI}}{\text{Pre DI}} \times 100

Where, Pre DI and Post DI are the airway narrowing immediately prior to and immediately following DI, respectively. Therefore, 100% reversal indicates that the post DI airway narrowing or active pressure returned to pre-contraction values (i.e. full reversal of the response to ACh). Comparisons of the bronchodilatory response were not possible at low doses of ACh (≤3x 10⁻⁶ M), which produced little to no contraction. Scatter plots of bronchodilation to DI against ASM strain had linear lines-of-best-fit fitted to individual airways. The intercept, slope and Pearson’s correlation coefficient (r) were calculated for individual airways.

Specific compliance of the airway wall was calculated from the Δvolume in relation to the ΔPₜₘ during the inflationary limb of the tidal oscillation cycle using the equation:

\text{Specific Compliance} = \frac{\Delta \text{Tidal Volume}}{\Delta \text{Tidal P}_{\text{tm}} \times \text{Lumen Volume}}

Where Δtidal volume and Δtidal Pₜₘ are the trough to peak changes in volume and pressure during tidal oscillation and lumen volume is volume of the lumen at the trough of the pressure cycle at the time of measurement.
Differences between groups were analysed using paired t-tests, unless otherwise stated below. Dose-response curves of bronchodilation to DI and specific compliance of the airway wall were analysed using 2-way analysis of variance (ANOVA) and Newman-Keuls post hoc test, with dose of ACh and the treatment group (i.e. control or TNF+IL-1β) as repeat measures variables. Data analysis and statistical tests were performed using Statistica (version 8.0; StatSoft, Tulsa, OK, U.S.A.) and GraphPad Prism (version 5.0d; GraphPad Software, La Jolla, CA, U.S.A.) Data are presented as means ± standard error of the mean (SEM), where \( n \) = number of animals.
4.3 Results

Protocol 1: The viability of culturing airway segments

Airways narrowed strongly in response to ACh (10^{-7} to 3 \times 10^{-3} M) in a dose-dependent manner under static conditions. However, there was no difference in the E_{max} (i.e. maximum response, fresh; 100.0 ± 0.0 % narrowing, i.e. airway closure in all airways, day 2; 66.8 ± 14.5 % narrowing, Figure 4.3.1A) or PD_{2} (i.e. sensitivity, fresh; 4.21 ± 0.17, day 2; 4.37 ± 0.17, Figure 4.3.1B) to ACh between day 2 of culture and fresh (i.e. prior to culture). There was also no difference in specific compliance of the airway wall after 2 days of culture (0.017 ± 0.0048 cm\text{H}_2\text{O}^{-1}), compared with fresh (0.010 ± 0.0024 cm\text{H}_2\text{O}^{-1}).

Figure 4.3.1. The effect of culture on maximum response (E_{max}, % lumen volume, A) and sensitivity (PD_{2}, B) to ACh under static conditions. There was a tendency towards a reduction in maximum response following 2 days of culture, however, this did not reach statistical significance (p=0.11, analysed using a 1-sample t-test against a hypothetical mean of 100.0). There was no difference in sensitivity to ACh prior to and after 2 days of culture. n = 6. Mean ± standard error of the mean (SEM).
Histological examination revealed denuding of the airway epithelium following culture, compared with typical observations (i.e. fresh) from my laboratory. The denuding of the airway epithelium is not expected to influence bronchoconstriction to ACh applied to the serosal surface or the bronchodilatory response to DI. In preliminary experiments, I found no difference in bronchodilation to DI with L-NOARG (10^-4 M, 31.2 ± 12.2 % and 35.9 ± 12.7 % reversal, n = 3) or following manual denuding of the airway epithelium (79.6 ± 10.4 % and 45.9 ± 4.6 % reversal, n = 3) in airways contracted to a single dose of ACh (10^-5 M).

Protocol 2: Whether TNF and IL-1β modulate the ASM response to strain during deep inspiration

There was no difference in lumen volume, internal perimeter (P_i), thickness of the ASM layer or specific compliance of the airway wall in the relaxed state between the TNF+IL-1β and control treatment groups (Table 4.3.1). Two days of culture with TNF and IL-1β increased airway narrowing to ACh (10^-7 to 3x 10^-3 M) under static conditions (Figure 4.3.2A). Maximum response to ACh increased from 77.7 ± 7.8 % narrowing in the control treatment group, to 95.3 ± 2.1 % narrowing in the TNF+IL-1β treatment group. There was no difference in sensitivity to ACh between the TNF+IL-1β (4.94 ± 0.12) and the control treatment groups (5.54 ± 0.22).
Table 4.3.1. Lumen volume, internal perimeter (Pi), area of the ASM layer and specific compliance of the airway wall in the control and the TNF+IL-1β treatment groups.

<table>
<thead>
<tr>
<th>Control Treatment Group</th>
<th>Lumen Volume (μL)</th>
<th>Pi (mm)</th>
<th>ASM layer (mm²/mm)</th>
<th>Specific Compliance (cmH₂O⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>171.3 ± 9.5</td>
<td>9.13 ± 0.65</td>
<td>0.0835 ± 0.0062</td>
<td>0.0129 ± 0.0011</td>
</tr>
<tr>
<td>TNF+IL-1β Treatment Group</td>
<td>Lumen Volume (μL)</td>
<td>Pi (mm)</td>
<td>ASM layer (mm²/mm)</td>
<td>Specific Compliance (cmH₂O⁻¹)</td>
</tr>
<tr>
<td></td>
<td>190.0 ± 24.6</td>
<td>9.70 ± 0.28</td>
<td>0.0786 ± 0.0027</td>
<td>0.0185 ± 0.0034</td>
</tr>
</tbody>
</table>

There was no difference in lumen volume, Pi, thickness of the ASM layer or specific compliance of the airway wall in the relaxed state between treatment groups. n = 6. Mean ± SEM.
In a separate group of airways, 2 days of culture with TNF and IL-1β induced greater active pressure (i.e. ASM force) to ACh (10^{-7} to 3 \times 10^{-3} M) under static conditions (Figure 4.3.2B). Maximum response to ACh increased from 51.4 \pm 11.1 \text{ cmH}_2\text{O} in the control treatment group, to 72.2 \pm 6.0 \text{ cmH}_2\text{O} in the TNF+IL-1β treatment group. Similarly to airway narrowing, there was no difference in active pressure sensitivity to ACh between the TNF+IL-1β (4.96 \pm 0.26) and the control treatment groups (4.95 \pm 0.26).
Airways stiffened strongly in response to ACh, with specific compliance of the airway wall falling from 0.0185 ± 0.0034 cmH\textsubscript{2}O\textsuperscript{-1} in the relaxed state, to 0.0050 ± 0.0010 cmH\textsubscript{2}O\textsuperscript{-1} at the maximum dose of ACh in the TNF+IL-1β treatment group and from 0.0129 ± 0.0011 cmH\textsubscript{2}O\textsuperscript{-1} to 0.0057 ± 0.0011 cmH\textsubscript{2}O\textsuperscript{-1} in the control treatment group (Figure 4.3.3A). When expressed as the change in specific compliance from the relaxed state, 2 days of culture with TNF and IL-1β induced an increase in airway wall stiffening to ACh. The decrease in specific compliance to ACh was Δ-0.0071 ± 0.0012 cmH\textsubscript{2}O\textsuperscript{-1} in the control treatment group, and Δ-0.0135 ± 0.0030 cmH\textsubscript{2}O in the TNF+IL-1β treatment group (Figure 4.3.3B).
Figure 4.3.3. Specific compliance of the airway wall (cmH\(_2\)O\(^{-1}\)), A) in the relaxed state and in airways narrowed to ACh (10\(^{-7}\) to 3\(\times\) 10\(^{-3}\) M), as well as the change in specific compliance from the relaxed state (\(\Delta\)cmH\(_2\)O\(^{-1}\)), B) in the control and the TNF+IL-1\(\beta\) treatment groups. Airways stiffened strongly in response to ACh in both treatment groups (control; \(p<0.001\), TNF+IL-1\(\beta\); \(p<0.001\)). There was no difference in airway wall stiffening to ACh between treatment groups. When expressed as the change in specific compliance from the relaxed state, culture with TNF and IL-1\(\beta\) induced an increase in airway wall stiffening to ACh (\(p<0.05\)). \(n=6\). Mean ± SEM.

The aim of the present study was to determine whether the pro-inflammatory cytokines, TNF and IL-1\(\beta\), modulate the ASM response to strain and therefore, the bronchodilatory response to DI. Two days of culture with TNF and IL-1\(\beta\) did not modulate the ASM response to strain during DI. Scatter plots of bronchodilation against the ASM strain produced by DI had linear lines-of-best-fit fitted to individual airways (Figure 4.3.4A). As in Chapter 2 of this thesis, bronchodilation was positively
correlated with ASM strain for all airways in both treatment groups (Table 4.3.2). However, there was no difference in the average intercept (TNF+IL-1β; 0.032 ± 0.007, control; 0.029 ± 0.011, i.e. only ASM strains greater than ~3 % produced bronchodilation) or slope (TNF+IL-1β; 284.8 ± 83.5, control; 262.6 ± 61.4, i.e. ~3 % bronchodilation per 1 % ASM strain) of the lines fitted between bronchodilation and ASM strain. Bronchodilation to DI fell substantially with increasing dose of ACh in both treatment groups (Figure 4.3.4B). There was also no difference in the bronchodilatory response to DI between the TNF+IL-1β and control treatment groups at comparable doses of ACh. In the control treatment group, DI produced 16.7 ± 3.7 % reversal of narrowing at 10^-5 M ACh (the lowest dose which produced contraction), which fell to 2.0 ± 1.0 % reversal of narrowing at 3x 10^-3 M ACh. Similarly in the TNF+IL-1β treatment group, DI produced 20.4 ± 4.3 % reversal of narrowing at 10^-5 M ACh, which fell to 3.5 ± 2.1 % reversal of narrowing at 3x 10^-3 M ACh.
Figure 4.3.4. Scatter plots of bronchodilation (% Reversal) against ASM strain (A) produced by DI and bronchodilation to DI (% Reversal, B) in airways narrowed to ACh (10^{-7} to 3x10^{-3} M) in the control and the TNF+IL-1β treatment groups. Plots comprise 6 measurements per airway in A. Linear lines-of-best fit were fitted to individual airways and the lines were drawn using the average intercept and average slope. Bronchodilation was positively correlated with ASM strain in both treatment groups. There was no difference in the average intercept and slope between treatment groups. Bronchodilation to DI fell substantially with increasing dose of ACh in both treatment groups (control; p<0.001, TNF+IL-1β p<0.001). There was no difference in bronchodilation to DI in both treatment groups at comparable doses of ACh. n = 6. Mean ± SEM in B.
Table 4.3.2. Slope, intercept and Pearson’s correlation coefficients for scatter plots of bronchodilation against ASM strain.

<table>
<thead>
<tr>
<th>Airway</th>
<th>1 Left</th>
<th>2 Right</th>
<th>3 Left</th>
<th>4 Right</th>
<th>5 Left</th>
<th>6 Right</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Treatment Group</td>
<td>Slope</td>
<td>Intercept</td>
<td>r</td>
<td>p value</td>
<td>Slope</td>
<td>Intercept</td>
</tr>
<tr>
<td></td>
<td>258.9</td>
<td>0.024</td>
<td>0.98</td>
<td>0.0006</td>
<td>609.6</td>
<td>0.056</td>
</tr>
<tr>
<td>TNF+IL-1β Treatment Group</td>
<td>Slope</td>
<td>Intercept</td>
<td>r</td>
<td>p value</td>
<td>Slope</td>
<td>Intercept</td>
</tr>
<tr>
<td></td>
<td>128.9</td>
<td>0.018</td>
<td>0.99</td>
<td>0.0002</td>
<td>188.9</td>
<td>0.074</td>
</tr>
</tbody>
</table>

Bronchodilation is the % reversal in airway narrowing following DI and ASM strain is the change in ASM length-change produced by DI in the control (airway 1 to 6, left or right lower lobe) and the TNF+IL-1β treatment groups (alternate lower lobe).

Despite no difference in the bronchodilatory response to individual DI (i.e. % reversal), the attenuation of airway responsiveness (i.e. % narrowing) during tidal breathing and DI manoeuvres differed between treatment groups. In the control treatment group, there was no difference between maximum response under oscillatory (67.8 ± 9.7 % narrowing) and static conditions (Figure 4.3.5A). In contrast, in the TNF+IL-1β treatment group, oscillatory conditions attenuated the maximum response, which fell to 70.5 ± 6.0 % narrowing under oscillatory conditions (compared with static conditions;
95.7 ± 2.2 % narrowing, p<0.05, Figure 4.3.5B). There was no difference in sensitivity to ACh (TNF+IL-1β; 4.83 ± 0.12, control; 4.79 ± 0.17) during oscillations.

**Figure 4.3.5.** Cumulative DRC to ACh (10⁻⁷ to 3x 10⁻³ M) under static and oscillatory conditions for the control (A, % Lumen Volume) and TNF+IL-1β (B) treatment groups. Dose-response curves under static conditions are redrawn from Figure 3A for comparison. There was no difference in airway narrowing under static and oscillatory conditions in the control treatment group. Oscillatory conditions attenuated airway narrowing to ACh, compared with static conditions, in the TNF+IL-1β treatment group. n = 6. Mean ± SEM.
4.4 Discussion

The present study determined whether the pro-inflammatory cytokines, TNF and IL-1β, modulate the ASM response to strain and therefore, the bronchodilatory response to DI, using an intact airway preparation. Whilst 2 days of culture with TNF and IL-1β, induced an increase in the maximum airway narrowing to ACh, it did not modulate the ASM response to strain during DI.

In protocol 2, I confirmed the findings of previous studies *in vitro* (1, 41, 136, 155, 184), that culture with TNF and IL-1β induced an increase in maximum isometric ASM force, which in the present study, was reflected by an increase in maximum active pressure to ACh. I found no difference in the area of the ASM layer, suggesting that the increase in maximum ASM force is due to an increase in ASM contractility and not ASM hypertrophy or hyperplasia. Although I found an increase in the maximum response, I found no difference in the sensitivity, which is consistent with the bulk of studies *in vitro*, using ASM strips and/or rings (1, 41, 155). I now extend previous findings to show that the increase in maximum ASM force is sufficient to increase maximum airway narrowing by ~23 % lumen volume, following 2 days of culture with TNF and IL-1β. Airway narrowing is the most physiologically relevant measurement, whereby the ASM shortens in the presence of after-loads that arise due to the $P_{tm}$. To my knowledge, no previous studies *in vitro*, have shown an increase in airway narrowing, as opposed to isometric ASM force, following culture with pro-inflammatory cytokines. Whilst the increase in airway narrowing is likely explained by increased ASM force, there was a tendency towards a decrease in the airway wall stiffness in the relaxed state following culture with TNF and IL-1β, which may favour
increased airway narrowing by virtue of decreasing the ASM after-load. Interleukin-1β induced disruption of elastin fibres in the alveolar wall in mice in vitro (100) and it is possible that IL-1β may also disrupt elastin in the airway wall, increasing compliance.

The ASM force hyper-contractility following culture with TNF and IL-1β (~40 % increase in maximum active pressure to ACh) was somewhat greater than that reported by previous studies, where those authors found an ~8 to ~30 % increase in maximum isometric force to ACh (41, 136, 155). Differences in hyper-contractility may reflect the relative concentrations of TNF (100 ng/mL) and IL-1β (20 ng/ mL) in the present study, compared to previous studies, which used 10 to 100 ng/ mL and/or 10 to 25 ng/ mL, respectively. Regardless of the difference in presence and/or concentrations of TNF and IL-1β, the focus of the present study was on how these pro-inflammatory cytokines modulate the ASM response to strain during DI. That the concentrations used in the present study were sufficient to induce ASM force hyper-contractility, suggests that these were appropriate conditions to study the ASM response to strain during DI.

The aim of the present study was to determine whether the pro-inflammatory cytokines, TNF and IL-1β, modulate the ASM response to strain and therefore, the bronchodilatory response to DI. Previous studies examining airway responsiveness in vitro following pro-inflammatory cytokines have done so under static conditions (i.e. in the absence of oscillatory ASM strain associated with breathing manoeuvres) (1, 41, 136, 155, 184). I found that 2 days of culture with TNF and IL-1β did not modulate the ASM response to strain during DI. The cellular mechanism(s) underlying bronchodilation to DI may involve perturbed cross-bridge binding (60, 61) and/or re-
modelling (i.e. de-polymerisation and re-polymerisation) of the contractile apparatus (66). In particular, re-modelling of the contractile apparatus could be vulnerable to modification by pro-inflammatory cytokines. My finding that 2 days of culture with TNF and IL-1β did not modulate the ASM response to strain during DI suggests that the failure of DI to produce bronchodilation in patients with asthma is unlikely to be due to changes in the ASM induced by the pro-inflammatory cytokines present. It is possible that other pro-inflammatory cytokines, such as interleukins 2, 4, 5 and 6 (IL-2, IL-4, IL-5 and IL-6), as well as granulocyte macrophage colony stimulating factor (GM-CSF) modulate the ASM response to strain during DI. However, as discussed, TNF and IL-1β were sufficient to induce ASM force hyper-contractility, demonstrating that contractile pathways of ASM are susceptible to these pro-inflammatory cytokines. Alternatively, the failure of DI in patients with asthma may be due to a decrease in the magnitude of the ASM strain, rather than a decreased ASM response to strain during DI.

In Chapter 2 of this thesis, I show that the bronchodilatory response to DI is dependent on ASM strain, using the same intact airway preparation and methodology used in the present study. The bronchodilatory response to DI and the magnitude of ASM strain were positively correlated in both treatment groups. Culture with TNF and IL-1β had no effect on either the intercept or slope of the linear line-of-best fit between bronchodilation and ASM strain. In both treatment groups, only ASM strain greater than ~3 % produced bronchodilation, and above this critical level of strain, DI produced ~3 % bronchodilation per ~1 % increase in ASM strain. In the diseased state (e.g., excessive airway wall stiffening), a reduction in the magnitude of ASM strain produced by DI below 3% could abolish the bronchodilatory response to DI, without necessarily modulating the relationship between ASM strain and bronchodilation.
Importantly, the present study used fixed-$P_{tm}$ DI, where the amplitude of DI and the stiffness of the airway wall determine the magnitude of ASM strain. As in Chapter 2 of this thesis, under high levels of ASM contraction the airway wall stiffens, so that the magnitude of ASM strain becomes negligible and therefore, the bronchodilatory response to DI is attenuated. In both treatment groups, bronchodilation to fixed $P_{tm}$ DI fell substantially with increasing doses of ACh. Despite greater bronchoconstriction (airway narrowing and active pressure) to ACh following culture with TNF and IL-$1\beta$, I found no difference in the airway wall stiffness (at least in the contracted state) between the TNF+IL-$1\beta$ and control treatment groups. Greater ASM force production in the TNF and IL-$1\beta$ treatment group suggests more active cross-bridges, which should produce an increase in the airway wall stiffness and a decrease the magnitude of ASM strain at the same dose of ACh. However, I found no difference in airway wall stiffness or the bronchodilatory response to DI between control and TNF+IL-$1\beta$ treatment groups at any dose of ACh.

The discrepancy between increased ASM force in the TNF and IL-$1\beta$ treatment group and lack of difference in airway wall stiffness may be explained by the disconnect between ASM force and airway wall stiffness. A recent study by Raqeeb and colleagues (135) showed that ASM stiffening is due to a rho kinase (ROK) dependent pathway, that is independent of cross-bridge formation (i.e. ASM stiffening is not just a result of cross-bridge cycling between actin filaments/myosin heavy chain). A disconnect between ASM force and stiffening is also suggested in Chapter 2 of this thesis, where I show that the sensitivity to ACh was greater for stiffness than for narrowing (i.e.
airways stiffen prior to narrowing as the dose of contractile agonist increases). Therefore, it is feasible that TNF and/or IL-1β could alter bronchoconstriction without effecting wall stiffness.

On the other hand, while I found no difference in airway wall stiffening to ACh between the TNF+IL-1β and control treatment groups at any dose of ACh, when expressed as the change in specific compliance from the relaxed state, culture with TNF and IL-1β induced an increase in airway wall stiffening. An increase in stiffening would be expected if the stiffness of the airway wall is governed by the level of ASM activation (7), even with no disconnect between ASM force and stiffness. The increase in airway wall stiffening in the TNF+IL-1β treatment group without any significant difference in specific compliance at any dose of ACh, is most likely due to the substantial variability of specific compliance in the relaxed state in the TNF and IL-1β treatment group. That is, 2 days of culture with TNF and IL-1β could have increased relaxed specific compliance, but the effect was too variable to reach statistical significance. Similarly, it is possible that the greater ASM force production in the TNF+IL-1β treatment group did produce some increase in airway wall stiffness and attenuate the bronchodilatory response to DI but the change was too small to reach statistical significance. However, there was no trend towards a modulated ASM response to strain during DI following 2 days of culture with TNF and IL-1β.

Unexpectedly, in contrast to the lack of an effect on the response to individual DI, I found a difference in the attenuation of airway responsiveness during tidal breathing manoeuvres and intermittent DI between treatment groups. Whilst there was no
difference between static and oscillatory conditions in the control treatment group, oscillations attenuated the maximum response to ACh by ~35% in the TNF+IL-1β treatment group. One possible explanation is that culture with TNF and IL-1β increased the bronchoprotective effect of tidal breathing and intermittent DI. However, this explanation is not entirely satisfactory. Firstly, previous studies have failed to find any evidence of bronchoprotection in whole bronchial segments (71, 123). Secondly, it seems unlikely that the TNF and/or IL-1β could increase (or induce, in whole bronchial segments) bronchoprotection, since the bronchoprotective effects of DI are abolished in patients with even mild asthma (89, 134). An alternative explanation is that the induction of bronchoprotection following 2 days of culture with TNF and IL-1β may be due to a change in the shape of the ASM length-tension curve or the compliance curve of the airway wall. The Ptm sets the initial length of the ASM and therefore, the position of the ASM on it’s classic length-tension curve. Under static conditions, the Ptm was a constant 5 cmH2O, compared with oscillatory conditions where the average Ptm during tidal breathing was 7.5 cmH2O (i.e. average Ptm of 5 to 10 cmH2O). If the optimum length (Lo) occurs at 5 cmH2O (112), the attenuation of ASM force during oscillations may be explained by the change in the average length of the ASM away from the optimal length. Typically, such a small change in Ptm would have little or no effect on ASM contraction, due to the shape of the length-tension curve (112) and for porcine bronchi specifically, maximum active pressure occurs when resting Ptm is anywhere in the range from 5 to 10 cmH2O (156). It is possible that 2 days of culture with TNF and IL-1β induced a change in the shape of either the ASM length-tension curve or the compliance curve of the airway wall, whereby at 7.5 cmH2O Ptm, the ASM was on the descending limb of it’s classic length-tension curve.
In protocol 1, I assessed the viability of culturing whole bronchial segments. Whilst culturing relatively large segments of tissue may have impaired the supply of nutrients or the removal of metabolites produced by the tissue, I show whole bronchial segments remain viable following 2 days of culture. Of interest, I found a difference in the ASM response to strain during DI following 2 days of culture, compared with fresh airway segments in Chapter 2 of this thesis (Figure 4.4.1). Although I showed no difference in the intercept (i.e. only ASM strains greater than ~3 % produced bronchodilation), increasing ASM strain produced less bronchodilation in cultured, compared with fresh airway segments (i.e. the slope of the lines fitted between bronchodilation and ASM strain was steeper in fresh airway segments). In Chapter 2 of this thesis, using fresh airway segments and the same methodology, DI produced ~8 % bronchodilation per 1 % ASM strain, which fell to 3 % bronchodilation per 1 % ASM strain following culture in the present study. As both these studies were conducted using porcine airway segments, using the same methodology and recording system, this difference is likely to be due to culture itself.
Figure 4.4.1. The effect of culture on strain-induced bronchodilation. Linear lines-of-best fit were fitted to individual airways and drawn using the average intercept and average slope from scatter plots of bronchodilation (% Reversal) against ASM strain produced by fixed-$P_{\text{aw}}$ DI. Airways from the control and TNF+IL-1$\beta$ treatment group in the present study are compared with fresh airway segments re-drawn from Chapter 2 of this thesis. Whilst there was no difference between the control and TNF+IL-1$\beta$ treatment groups, both treatment groups had a reduced ASM response to strain during DI, compared with fresh airway segments. The average slope was greater in the fresh airway segments than the control treatment ($p<0.05$) and TNF+IL-1$\beta$ ($p<0.05$) treatment groups. $n = 6$. 

![Graph showing bronchodilation vs. ASM strain for different conditions.](image-url)
It remains unclear how the ASM response to strain during DI may have been altered by the process of culture itself (i.e. without inflammatory cytokines). Denuding of the airway epithelium following culture raises the possibility that the release of epithelial-derived mediators contributes to the bronchodilatory response to DI. Brown and Mitzner (33) showed bronchodilation to DI was abolished by the nitric oxide (NO) synthase blocker, N-nitro-L-arginine (L-NOARG), in mechanically ventilated dogs \textit{in vivo}. However, preliminary studies showed no difference in DI response with the NO synthase blockade, N-nitro-L-arginine (L-NOARG), or manual denudation of the epithelium, suggesting that neither NO nor epithelial denuding modulate the ASM response to strain during DI. Alternatively, the lack of oscillatory strain during culture (i.e. atmospheric pressure for 2 days) is a non-physiological scenario and may have induced a change in the intrinsic ASM response to strain during DI. A recently discovered property of ASM, identified in isolated muscle strips, is the capacity to optimise force production following sustained changes in length, a phenomenon termed ‘length-adaptation’ (26, 143). However, in \textbf{Chapter 5} of this thesis, I show little effect of prolonged changes in $P_{tm}$ on bronchoconstriction and I suggest the possibility that length-adaptation occurred is unlikely.

There was also a tendency towards reduced maximum response to ACh following 2 days of culture, which is consistent with studies \textit{in vitro}, using ASM strips/rings (1, 184), whereby isometric force decreased following 2 days of culture. The decrease in contractile response in culture is therefore not specific to culturing airway segments. Importantly, the decrease in maximum response to ACh was a constant between the TNF+IL-1β and control treatment groups and therefore, unlikely to affect my finding that culture with TNF and IL-1β induced hyper-contraction of ACh.
In conclusion, whilst 2 days of culture with TNF and IL-1β induced an increase in the maximum airway narrowing to ACh, it did not modulate the ASM response to strain during DI. The failure of DI to produce bronchodilation in patients with asthma may therefore, not involve a direct effect of pro-inflammatory cytokines.
Chapter 5 Mechanical properties of the airway wall during sustained inflationary and deflationary transmural pressures

5.1 Introduction

A recently discovered property of airway smooth muscle (ASM), identified in isolated muscle strips, is the capacity to optimise force production following sustained changes in length (26, 133), a phenomenon termed ‘length-adaptation’ (15). Length-adaptation manifests as a shift in the active and passive length-tension curve. If the ASM length is shifted from its relative optimum (L_o) for a sustained period of time (i.e. lengthening or shortening), there is a recovery in force production, following adaptive re-arrangement of the contractile apparatus (118, 143, 166). Length-adaptation may also, in part, explain the attenuation of ASM force production in response to oscillatory (or transient) changes in ASM length (94, 147, 164, 165).

Despite evidence of length-adaptation in isolated ASM strips, the presence and/or potential role of length-adaptation in situ and therefore, the implications for airway function in health and disease, is uncertain. In the normal healthy individual, the length (i.e. perimeter) of the ASM is set by the transmural pressure (P_{tm}, i.e. the pressure difference across the airway wall), which increases and decreases with lung inflation and deflation. Airway P_{tm} arises due to the distending forces of the lung parenchyma. However, in obstructive airway disease, lung parenchymal tethering forces are expected to change, and with it, P_{tm} and the dependent ASM perimeter. For instance, in asthma and chronic obstructive pulmonary disease (COPD), lung hyper-inflation favours an
increase in ASM perimeter (105, 130). In contrast, mechanical ‘uncoupling’ of the lung parenchyma with emphysema and/or reduced elastic recoil pressure is likely to shorten ASM perimeter (51, 57). Another possible scenario is that the ASM may be chronically shortened due to persistent levels of basal ASM tone (25). If length-adaptation were present \textit{in vivo}, such disease-related changes in ASM perimeter may be accompanied by changes in contractile capacity, such that force production could be potentiated at these abnormally increased or decreased perimeters. Indeed, length-adaptation to a short ASM perimeter has been proposed as a potential mechanism underlying airway hyper-responsiveness (AHR i.e. excessive bronchoconstriction to an inhaled bronchial challenge) in obstructive disease (166).

The aim of the present study was to determine, using an intact airway segment preparation, the \textit{in situ} effect of sustained changes in $P_{tm}$ (and therefore ASM perimeter) on ASM contraction and airway narrowing. I reasoned that if length-adaptation is important to normal airway function then I would see adaptation (a time-dependent increase in contractile response) to the physiological determinant of airway ASM perimeter, $P_{tm}$. Outcomes included airway narrowing, ASM tension, compliance and an \textit{in situ} measure of ASM perimeter, the latter made possible through the use of anatomical optical coherence tomography (aOCT) (127).
5.2 Methods

Animal handling

All animal experiments conformed to institutional ethics and animal care unit regulations (Animal Ethics Committee, University of Western Australia (UWA), Crawley, WA, Australia). Male White Landrace pigs, ~35 kg, were initially sedated with tiletamine-zolazepam (4.4 mg/kg \(I.M.\)) and xylazine (2.2 mg/kg \(I.M.\)) and then exsanguinated under sodium pentobarbitone anaesthesia (30 mg/kg \(I.V.\)). The lungs were immediately removed and transported on ice to the laboratory.

Airway segment preparation

Airway segments were dissected from the main stem bronchus of the left or right lower lobe within ~1 hour of being removed. All side branches were ligated with surgical silk and an airway segment was cannulated at both ends, as previously described (10, 11). Following cannulation, the airway was mounted horizontally in an organ bath containing gassed (95 % O\(_2\) and 5 % CO\(_2\)) Krebs solution (121 mM NaCl, 5.4 mM KCl, 1.2 mM MgSO\(_4\), 25 mM NaHCO\(_3\), 5 mM sodium morpholinopropane sulfonic acid, 11.5 mM glucose and 2.5 mM CaCl\(_2\); pH 7.3) at 37 °C. The horizontal length of the segment was stretched to 105 % of its length in the fully deflated lung, shown previously to approximate the length at functional residual capacity (FRC) (125). One end of the airway lumen was connected to a reservoir filled with Krebs solution, the height of which set the initial P\(_{im}\) (5 cmH\(_2\)O) and which was used to flush the lumen with Krebs solution. Depending on the measurement outcome (see below), the opposite end of the airway was either connected to a liquid filled syringe pump, or used to insert
an aOCT probe to provide an *in situ* measure of ASM perimeter (127) (see below), which was then sealed in place using a latex membrane.

**Experimental protocol**

Whilst the time-course of length-adaptation may extend for days, it is substantially accelerated by regular stimulation and contraction of the ASM. In studies where the ASM was stimulated every 5 min, adaptation was evident within a period of ~30 min (43, 44, 94, 133, 165). Therefore, in my study, airways were stimulated to contract (see below) every 5 min prior to and during inflationary or deflationary P\textsubscript{tm} (~50 min).

Airway segments were initially equilibrated to a P\textsubscript{tm} of 5 cmH\textsubscript{2}O to simulate FRC in the normal healthy lung. The equilibration period (at 5 cmH\textsubscript{2}O P\textsubscript{tm}) of 1 hr began immediately after the airway was mounted in the organ bath. The Krebs solution in the organ bath and airway lumen was replaced every 10 min throughout experiments to remove metabolites and any bronchoactive mediators which might be released from the epithelium. Viability of the tissue was subsequently confirmed through stimulation with acetylcholine (ACh, 10\textsuperscript{-4} to 10\textsuperscript{-3} M) added to the organ bath followed by a 30 min washout and recovery period at 5 cmH\textsubscript{2}O P\textsubscript{tm}.

To first establish a baseline contractile response, airways were contracted to parasympathetic nerve activation every five minutes at 5 cmH\textsubscript{2}O P\textsubscript{tm} (Figure 5.2.1). Nerve activation was induced to EFS via platinum electrodes encircling the airway segment and connected to a stimulator (Grass Medical Instrument Stimulator model
Stimulation (30 Hz, 3 ms, 60 V) was maintained for 20-30 s or until a plateau in contractile response (lumen narrowing or tension/active pressure) was reached.

**Figure 5.2.1.** A diagram of the experimental protocol used. Contraction (i.e. airway lumen narrowing or active pressure) was induced to electrical field stimulation (EFS) every 5 minutes. A baseline contractile response was first established at 5 cmH₂O Pₜₜ, followed by an ‘adaptation protocol’, comprising either a rapid hydrostatically-driven inflation to 25 cmH₂O transmural pressure (Pₜₜ) or deflation to -5 cmH₂O Pₜₜ. During the adaptation protocol, EFS was recommenced again at 5 minute intervals for the next 50 mins. Airways were returned to 5 cmH₂O Pₜₜ and EFS was recommenced every 5 minutes until a further 10 stimulations had been recorded.

Once a baseline contractile response was established (~3 stimulations), airways were exposed to an ‘adaptation protocol’, comprising either a rapid hydrostatically-driven inflation to 25 cmH₂O Pₜₜ or deflation to -5 cmH₂O Pₜₜ. Two minutes was allowed for viscoelastic effects to reduce at the new Pₜₜ before EFS was recommenced again at 5
minute intervals for the next 50 mins (i.e. 10 stimulations). The $P_{tm}$ was then returned to 5 cmH$_2$O, and following a similar two minute pause, EFS was recommenced every 5 minutes until a further 10 stimulations had been recorded.

Three experiments were performed using the above protocol: (Experiment 1) airway narrowing was measured from a change in lumen volume during low amplitude $P_{tm}$ oscillations, which also allowed compliance to be measured from $\Delta$volume / $\Delta$pressure; (Experiment 2) airway narrowing was measured from the change in lumen cross section area ($A_i$) and in situ ASM perimeter (outer muscle perimeter, $P_{mo}$) (127) were measured by aOCT under static (i.e. no oscillations) conditions and; (Experiment 3) the active pressure generated by ASM was measured from the increase in lumen pressure under isovolumic conditions. The different recording systems necessitated some variance in airway segment dimensions. Airway diameters, lengths and generations for Experiments 1 to 3 are shown in Table 5.2.1.

### Table 5.2.1. Airway segment dimensions.

<table>
<thead>
<tr>
<th>Generation (Mode)</th>
<th>Diameter (mm)</th>
<th>Length (mm)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Distal</td>
<td>Proximal</td>
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<tr>
<td>Experiment 1</td>
<td>18</td>
<td>12</td>
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<tr>
<td>Experiment 2</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>Experiment 3</td>
<td>16</td>
<td>11</td>
</tr>
</tbody>
</table>

Generation are shown, where the generation of the trachea = 0. Diameters were determined by gently inserting steel rods of known diameter. Length refers to the final bath length, not including the cannula. $n$ = 6. Mean ± standard error of the mean (SEM).
Experiment 1: Airway narrowing and compliance under oscillatory conditions

A custom-built servo-controlled syringe pump and pressure transducer was used to set Ptm, apply fixed-Ptm oscillations and measure airway narrowing (% decrease in lumen volume) to EFS, as previously described (121, 122). Airways were connected to a 1 mL glass syringe driven by a feedback-controlled servomotor and motor controller. Transmural pressure was measured via a calibrated pressure transducer with feedback to a servomotor and changes in airway lumen volume were measured via a calibrated displacement transducer that measured the rotation of the syringe motor. Absolute lumen volume, prior to ASM activation, was determined from the volume that could be withdrawn until airway closure (67). Small pressure oscillations were applied (Δ5 cmH2O) above the target Ptm. That is, when the Ptm under study was: 5 cmH2O, pressure was cycled from 5 to 10 cmH2O; 25 cmH2O, pressure was cycled from 25 to 30 cmH2O; and –5 cmH2O, pressure was cycled from -5 to 0 cmH2O. The resulting pressure and volume oscillations allowed compliance to be calculated:

$$\text{Specific Compliance} = \frac{\Delta \text{Tidal Volume}}{\Delta \text{Tidal Ptm} \times \text{Lumen Volume}}$$

Where Δvolume and ΔPtm are the trough to peak changes in volume and pressure during the breathing manoeuvre and lumen volume is volume of the lumen at the trough of the pressure cycle at the time of measurement. Airway wall stiffening refers to the decrease in compliance produced by EFS.
Experiment 2: Airway narrowing and ASM perimeter under static conditions

Airway lumen cross sectional area was measured by aOCT, as previously described (12). For the present study, the aOCT probe was encased in a transparent catheter (O.D. 2.2 mm), which required a larger airway segment than in experiments 1 and 3 to accommodate the probe and surrounding catheter. During aOCT imaging, broadband near infrared light is emitted from an optical probe. The same probe is used simultaneously to detect reflections of light from the Krebs-tissue interface of the lumen that allows the distance to the luminal surface to be determined by low-coherence interferometry. By rotating the probe within the catheter, a 2D axial (i.e. radial B) image of the airway may be reconstructed (Figure 5.2.2). Importantly, the capacity for aOCT to measure $P_{mo}$ has been previously demonstrated (127), since the light beam penetrates into the tissue and is scattered back from the ASM layer. Airway measurements using aOCT are calibrated to account for the refractive index of the medium (1.37 for Krebs solution). The aOCT probe was rotated at ~0.8Hz, acquiring quantitative images of $A_i$ and $P_{mo}$, which were recorded and displayed in real time on a computer monitor. $P_{tm}$ at all times was set by the height of the attached pressure column.
Figure 5.2.2. An example cross-sectional image of a porcine airway recorded by anatomical optical coherence tomography (aOCT) at 5 cmH\(_2\)O \(P_m\) before (A) and after (B) contraction to EFS. The image identifies the airway epithelium (AE) and the area contained therein \(A_i\), as well as the outer border of the ASM band (ASM) allowing measurement of \(P_{mo}\). Inner wall area \(WA_i\) corresponds to the area of the dark band surrounding the lumen. Cartilage plates (CP) and the catheter containing the optical probe (PB) are also identifiable.
Experiment 3: Active pressure and ASM tension under static conditions

Isovolumic contractions were measured by closure of a tap located between the airway and the pressure column. Under isovolumic conditions, ASM contraction results in an increase in lumen pressure (i.e. active pressure) that represents ASM tension production, providing a close comparison to experiments examining adaptive properties in isolated ASM strip studies (66, 94, 133, 164-166). Other than during the measurement of ASM contraction, the tap remained open and \( P_{tm} \) was set by the height of the pressure column, as in Experiment 2. Active pressure was subsequently expressed as active tension (Nm\(^{-1}\)):

\[
Tension = Active\ Pressure \times \frac{P_{mo}}{2 \times \pi}
\]

The \( P_{mo} \) used to calculate tension was the mean \( P_{mo} \) at the same time point measured in Experiment 2 using aOCT. Airways used for Experiment 2 were larger than those used for Experiment 3, so the calculated tension is an overestimation. However, we expect the relative changes in \( P_{mo} \), produced by inflation to 25 cmH\(_2\)O \( P_{tm} \) and deflation to -5 cmH\(_2\)O \( P_{tm} \), would be expected to be similar for both experiments.

Analysis and statistics

Airway narrowing in response to EFS was expressed as % lumen volume or \( A_i \) (where 100 % airway narrowing indicates airway closure). Comparisons of airway narrowing during pressure oscillations (Experiment 1) were made at the trough of the pressure
cycle (i.e., 5, -5 or 25 cmH₂O). In Experiment 2, morphological indices (i.e. Ai and Pmo) were determined using custom-designed quantification software developed in the C++ language, which allowed manual measurement of airway dimensions from the aOCT datasets. Inner wall area (WAi) was also calculated by subtracting Ai from the area enclosed by the Pmo (i.e., Amo). Active pressure to EFS (Experiment 3) or active tension was expressed as the Δ increase in pressure/tension.

Baseline contractile responses prior to the adaptation protocol were averaged (~3 measurements) for comparisons with contractile responses during and after the adaptation protocol. The immediate effect of rapid changes in Ptm was analysed using paired t-tests. The effect of sustained changes in Ptm during the adaptation protocol was analysed by 1-way analysis of variance (ANOVA) and Dunnet’s post hoc test. Data analysis and statistical tests were performed using Statistica (version 8.0; StatSoft, Tulsa, OK, U.S.A.) and GraphPad Prism (version 5.0d; GraphPad Software, La Jolla, CA, U.S.A.) Data are presented as means ± standard error of the mean (SEM), where n = number of animals.
5.3 Results

Rapid hydro-statically driven inflation to 25 cmH₂O or deflation to -5 cmH₂O produced immediate and sustained changes in relaxed airway dimensions (i.e. volume and Aᵢ) and in the level of the contractile response to EFS (airway narrowing, airway wall stiffening and ASM tension), discussed below.

Experiment 1: Airway narrowing and compliance under oscillatory conditions

Inflation to 25 cmH₂O Pᵢm immediately increased relaxed airway volume by 38.7 ± 2.8 % (Figure 5.3.1A), whilst deflation to -5 cmH₂O Pᵢm decreased relaxed airway volume by 32.0 ± 4.4 % (Figure 5.3.1B). During the sustained Pᵢm change, relaxed airway volume continued to increase in inflated airway and decrease in deflated airways. Upon return to 5 cmH₂O Pᵢm, after 50 min, the volume of airways remained somewhat larger in airways previously inflated to 25 cmH₂O (p<0.001) and smaller in airways previously deflated to –5 cmH₂O Pᵢm (p<0.001).
At baseline (i.e. 5 cmH₂O Pₚₐₜₜ), EFS produced 41.1 ± 4.4 % and 38.7 ± 7.7 % airway narrowing in airways subsequently inflated to 25 cmH₂O and deflated to -5 cmH₂O, respectively (Figure 5.3.1C). During the adaptation protocol, airway narrowing to EFS
was immediately reduced, compared with baseline levels by both inflation and deflation. At 25 cmH\(_2\)O P\(_{tm}\), EFS produced 32.4 ± 3.4 % airway narrowing, while at -5 cmH\(_2\)O P\(_{tm}\), airway narrowing to EFS was 30.5 ± 6.2 %. Airway narrowing to EFS continued to gradually decrease during the adaptation protocol. When normalised to the level of airway narrowing at baseline, the reduction in airway narrowing was greater in airways deflated to -5 cmH\(_2\)O P\(_{tm}\), compared with the airways inflated to 25 cmH\(_2\)O P\(_{tm}\) (p<0.001). Upon return to 5 cmH\(_2\)O P\(_{tm}\), airway narrowing to EFS immediately returned to baseline levels in the airways previously inflated to 25 cmH\(_2\)O P\(_{tm}\), whilst for airways previously deflated to -5 cmH\(_2\)O P\(_{tm}\) narrowing returned to baseline levels after 10 min.

Pressure oscillations and the corresponding volume perturbations allowed the calculation of specific compliance of the airway wall in the relaxed and contracted (i.e. during airway narrowing to EFS) states. At baseline, the specific compliance of relaxed bronchi was 0.0076 ± 0.0010 cmH\(_2\)O\(^{-1}\) and 0.0095 ± 0.0016 cmH\(_2\)O\(^{-1}\) in airways prior to inflation (Figure 5.3.2B), respectively. The compliance of the airway wall was reduced at 25 cmH\(_2\)O P\(_{tm}\) and increased at -5 cmH\(_2\)O P\(_{tm}\). Compliance continued to increase during sustained deflation to -5 cmH\(_2\)O P\(_{tm}\) (p<0.001), whilst sustained inflation at 25 cmH\(_2\)O P\(_{tm}\) produced a statistically significant, albeit small, decrease in compliance (p<0.001). Upon return to 5 cmH\(_2\)O P\(_{tm}\), compliance returned to levels before the adaptation protocol after ~5 min in airways inflated to 25 cmH\(_2\)O P\(_{tm}\) and after ~35 min in airways deflated to -5 cmH\(_2\)O P\(_{tm}\).
At baseline, EFS produced airway stiffening (i.e. a reduction in specific compliance during contraction). However, with inflation to 25 cmH\textsubscript{2}O P\textsubscript{tm}, EFS no longer produced any measureable airway stiffening, that is specific compliance was the same for relaxed and contracted bronchi after inflation. In comparison, airway stiffening to EFS was greater after deflation to -5 cmH\textsubscript{2}O (p<0.001). Stiffening to EFS was not altered during

Figure 5.3.2. Specific compliance of the airway wall at baseline (5 cmH\textsubscript{2}O P\textsubscript{tm}) and during the adaptation protocol (either inflation to 25 cmH\textsubscript{2}O P\textsubscript{tm}, A, or deflation to -5 cmH\textsubscript{2}O P\textsubscript{tm}, B, dashed line). Specific compliance is shown as ‘relaxed’ and ‘contracted’ which refers to before and after activation by EFS respectively. In the relaxed state, specific compliance was reduced with inflation to 25 cmH\textsubscript{2}O (p<0.001) and increased with deflation to -5 cmH\textsubscript{2}O (p<0.05). Stiffening to EFS (decrease in compliance) increased after deflation to -5 cmH\textsubscript{2}O (p<0.001). There was no measurable stiffening to EFS at 25 cmH\textsubscript{2}O P\textsubscript{tm}. n = 6. Mean ± SEM.
sustained deflation at -5 cmH\textsubscript{2}O P\textsubscript{tm}, and upon return to 5 cmH\textsubscript{2}O P\textsubscript{tm}, airway stiffening was the same as that at baseline.

\textit{Experiment 2: Airway narrowing and ASM perimeter under static conditions}

In response to sustained inflation and deflation, changes in relaxed A\textsubscript{i}, measured using aOCT under static conditions, mirrored those observed for lumen volume in Experiment 1. Inflation to 25 cmH\textsubscript{2}O P\textsubscript{tm} immediately increased A\textsubscript{i} by 24.0 ± 2.1 %, whilst deflation to -5 cmH\textsubscript{2}O P\textsubscript{tm} decreased A\textsubscript{i} by 43.3 ± 3.80 % (Figure 5.3.3A). The corresponding change in P\textsubscript{mo} (i.e. ASM perimeter) was 10.6 ± 1.3 % for inflation, and 22.4 ± 2.2 % decrease during deflation (Figure 5.3.3B). Both A\textsubscript{i} and P\textsubscript{mo} continued to increase during sustained inflation to 25 cmH\textsubscript{2}O (A\textsubscript{i}; p<0.001, P\textsubscript{mo}; p<0.001) and decrease during sustained deflation to – 5 cmH\textsubscript{2}O (A\textsubscript{i}; p<0.001, P\textsubscript{mo}; p<0.001). Upon return to 5 cmH\textsubscript{2}O P\textsubscript{tm}, after 50 min, A\textsubscript{i} and P\textsubscript{mo} were greater in airways inflated to 25 cmH\textsubscript{2}O compared with baseline. In airways deflated to -5 cmH\textsubscript{2}O A\textsubscript{i} and P\textsubscript{mo} were both returned to that measured at baseline after 30 min. WA\textsubscript{i}, was unaffected by a short term or sustained change in P\textsubscript{tm}. 


Figure 5.3.3. $A_i$ (mm$^2$, A) and $P_{mo}$ (mm, B) measured by $aOCT$ at baseline (5 cmH$_2$O $P_{tm}$) and during the adaptation protocol (either inflation to 25 cmH$_2$O $P_{tm}$ or deflation to -5 cmH$_2$O $P_{tm}$, dashed line). $A_i$ increased with rapid inflation ($p<0.001$) and decreased with deflation ($p<0.001$) and then continued to either increase ($p<0.05$) or decrease ($p<0.05$), respectively, during the period of imposed $P_{tm}$ change. $P_{mo}$ increased with inflation ($p<0.001$) and decreased with deflation ($p<0.001$) and with sustained $P_{tm}$, there were statistically significant, albeit small, increases ($p<0.001$) and decreases ($p<0.001$) in $P_{mo}$ respectively. $n=5$. Mean ± SEM.

At baseline, airway narrowing to EFS measured using $aOCT$ was 64.1 ± 3.2 % decrease in $A_i$ and 46.0 ± 5.3 % decrease in $A_i$ in airways subsequently inflated and deflated, respectively ($P<0.001$), despite appropriate anatomical matching and experimental conditioning (Figure 5.3.4A). Similarly to Experiment 1, airway narrowing and ASM shortening to EFS (Figure 5.3.4B) were immediately reduced at 25 cmH$_2$O and -5 cmH$_2$O $P_{tm}$, and continued to gradually decrease, particularly at both -5 cmH$_2$O. The decrease in airway narrowing ($p<0.001$) and muscle shortening ($p<0.001$) was more
pronounced at -5 cmH₂O, compared with 25 cmH₂O, when normalized to baseline levels. Once a Pₘₑ of 5 cmH₂O was reinstated, airway narrowing and muscle shortening to EFS returned immediately to baseline levels in airways previously inflated to 25 cmH₂O, while airways previously deflated to -5 cmH₂O took 15 min for narrowing to recover to baseline.

**Figure 5.3.4.** Airway narrowing (% A_i, A) and muscle shortening (% P_mo, B) to EFS measured by αOCT at baseline (5 cmH₂O Pₘₑ) and during the adaptation protocol (either inflation to 25 cmH₂O Pₘₑ or deflation to -5 cmH₂O Pₘₑ, dashed line). Airway narrowing was immediately reduced in both groups of airways (inflation; p<0.001, deflation; p<0.001) and then remained stable at 25 cmH₂O Pₘₑ while continuing to decrease at -5 cmH₂O (p<0.001). Similar changes were observed with ASM shortening. n = 5. Mean ± SEM.

**Experiment 3: Active pressure and ASM tension under static conditions**

Sustained inflationary and deflationary changes in Pₘₑ had a similar effect on active pressure (Figure 5.3.5A) and ASM tension (Figure 5.3.5B) to that observed for airway
narrowing. There was an immediate decrease in active pressure and tension at both 25 cmH₂O and -5 cmH₂O, as well as continued gradual decrease in active pressure and tension at -5 cmH₂O P_{tm}. Upon return to 5 cmH₂O, active pressure and tension returned immediately to levels before the adaptation protocol in airways inflated to 25 cmH₂O, whilst it took 10 min for previously deflated airways to return to baseline. There was no evidence for an increase in active pressure or tension during the adaptive protocol (i.e., 25 or -5 cmH₂O).

**Figure 5.3.5.** Active pressure (ΔcmH₂O, A) and tension (Nm⁻¹, B) to EFS at baseline (5 cmH₂O P_{tm}) and during the adaptation protocol (either inflation to 25 cmH₂O P_{tm} or deflation to -5 cmH₂O P_{tm}, dashed line). Active pressure was immediately reduced in both groups of airways (inflation; p<0.001, deflation; p<0.001) and then remained stable at 25 cmH₂O, but continued to decrease at -5 cmH₂O (p<0.001). Similar changes were observed with tension. n = 5. Mean ± SEM.
5.4 Discussion

The present study examined adaptive properties of *in situ* ASM in response to sustained physiological changes in $P_{tm}$. Sustained inflationary and deflationary $P_{tm}$ were applied to induce lengthening and shortening, respectively, of the ASM perimeter. Airway calibre, wall compliance and ASM tension were all immediately modified by the rapid change in $P_{tm}$. However, in the presence of regular contractile activation sustained periods of increased or decreased $P_{tm}$ failed to induce changes in airway behaviour suggestive of ASM length-adaptation.

As discussed, in the obstructive lung disease, there are several likely scenarios whereby the ASM perimeter may be shortened (i.e. parenchymal uncoupling) or lengthened (i.e. hyper-inflation) and the present study set about examining the potential implications of these changes in ASM perimeter on airway function.

*Experimental design and rationale*

Investigating the applicability of findings in isolated ASM strip preparations to integrated lung function is most necessary as they do not always translate as might be expected. For instance, force inhibition in response to length-oscillation is well demonstrated in isolated ASM strips but seems to be of lesser importance when the ASM is studied *in situ* (102, 124). Disparities between behaviour observed in airway or muscle preparations and that seen in situ are well recognised (108). In the present study, I assessed whether the ASM length-adaptation demonstrated in isolated ASM strips (43,
44, 94, 133, 165) is expressed in whole bronchial segments and, if so, its role in regulating airway responsiveness.

Three different experimental approaches were used to assess adaptation to a sustained change in $P_{tm}$. In Experiment 1, I measured airway narrowing during oscillatory pressure perturbation, which allowed us to simultaneously measure specific compliance as well as airway narrowing, replicating in vivo conditions where airways are under oscillatory tidal $P_{tm}$. A constantly changing ASM length due to breathing is one of the many differences between isolated ASM strips in vitro and the lung in vivo, which could disrupt the length-adaptation process. In Experiment 2, I measured airway narrowing under static conditions using aOCT. Using static conditions for Experiment 2 allowed us to determine if tidal oscillations inhibited length-adaptation. Whilst aOCT required the use of somewhat larger airways than the other approaches (to accommodate the physical dimensions of the probe), it enabled measurement of ASM perimeter in situ, which can be related back to previous studies using isolated ASM (43, 44, 66, 133, 166). In Experiment 3, I measured active pressure, an index of ASM tension, as previous studies which identified length-adaptation used measurements of muscle tension not shortening. By measuring tension, I can rule out any effects from ASM after-loads masking the presence of length-adaptation. Active pressure was converted to tension from corresponding ASM perimeter measurements determined in Experiment 2. As the airways used for Experiment 2 were larger than Experiment 3, I have overestimated the ASM perimeter and my calculated levels of ASM tension may overestimate tension. However, this has no impact on the relative change in ASM tension in response to sustained $P_{tm}$, which is the focus of the present study.
Airway narrowing, airway smooth muscle contraction and \( P_{tm} \)

For isolated airway behaviour to match that expected under ASM length-adaptation, a change in \( P_{tm} \) from the reference state at 5 cmH\(_2\)O, roughly equivalent to FRC, should be accompanied by both an immediate decrease in the level of airway narrowing produced by EFS and then, a gradual increase in airway narrowing back towards that present prior to the \( P_{tm} \) change. As expected, there was an immediate decrease in airway narrowing with a change in \( P_{tm} \). The decrease in airway narrowing is due to the change in ASM pre-load (the classic L-T relationship), which in the pig airway is sub-optimal for force production away from a \( P_{tm} \) of \( \sim 5 \) cmH\(_2\)O (112, 124). Airway narrowing may also be inhibited due to greater after-load, following a shift to a stiffer region of the airway pressure-volume curve. Since the magnitude of the reduction in ASM tension (Experiment 3), in response to a change in \( P_{tm} \), broadly matched the decrease in airway narrowing under static conditions (Experiment 2), the immediate inhibition of airway narrowing is attributed predominantly to a change in ASM pre-load and not after-load effects.

Following a change in \( P_{tm} \), there was no recovery in airway narrowing (either under static or oscillatory conditions) or ASM shortening. In fact, airway narrowing and ASM shortening tended to further decrease with a sustained period at \(-5 \) cmH\(_2\)O, and to some extent at 25 cmH\(_2\)O. The lack of recovery in airway narrowing or shortening argues against ASM length-adaptation being important at the level of the intact airway. Length-adaptation in isolated ASM is, for the most part, assessed by measuring force production (66, 94, 133, 157, 163), rather than ASM shortening (164). Such approaches...
differ from Experiments 1 and 2, whereby ASM shortening occurred and produced narrowing of the airway lumen. However, these findings cannot be explained by a discrepancy between force and shortening, since in Experiment 3, there was also no evidence of length-adaptation when contraction was assessed by measuring active pressure and ASM tension.

Length changes produced by $P_{tm}$ may have been too small for any adaptive response to be identified. Under static conditions (Experiment 2), inflation to 25 cmH$_2$O produced an $\sim$11 % increase in $P_{mo}$ and a $\sim$22 % decrease after deflation to $-5$ cmH$_2$O, measured by $a$OCT. This level of ASM perimeter change is at the lower end of the spectrum when compared to studies using isolated ASM, where reported length-changes that produce length-adaptation are in the range of $\sim$30 to 70 % (43, 44, 66, 133, 166). I would argue that the range of $P_{tm}$ used in the present study encompassed the physiological range. Inflation to 25 cmH$_2$O is at the plateau of the airway-pressure volume relationship (i.e., near maximal inflation), whilst $-5$ cmH$_2$O sits at the inflection point of the airway pressure-volume curve, below which the airway becomes considerably stiffer. Therefore, changes in ASM perimeter with inflation or deflation, beyond that observed in the present study, are expected to be small.

The inclusion of pressure oscillations (Experiment 1) during the measurement of airway narrowing cannot explain a failure to identify length-adaptation, since results were qualitatively similar under static conditions (Experiment 2). Pressure oscillations at $\Delta 5$ cmH$_2$O are a reasonable approximation of tidal oscillations and are not expected to have a major impact on airway narrowing. In whole bronchial segments, tidal oscillations are
at or below the threshold required to produce bronchodilation (102, 124), and whilst I had some concern that oscillations could offset adaptation, in isolated ASM preparation tidal stresses do not prevent force adaptation (128).

There are two potential methodological explanations for the apparent lack of ASM adaptive response to P_{tm} \textit{in situ}. As established in experiment 2, for a constant P_{tm}, there was observable (minor) creep in ASM perimeter during the adaption protocol. It is certainly feasible that this ‘moving target’ may have offset the ASM adaptive response. Flushing of the airway lumen and the resulting changes in P_{tm} may have similarly disrupted ASM adaptive processes. I nonetheless conclude, that in comparison to the immediate effects of a shift in P_{tm}, the proposed physiological driver of ASM length, adaption to a change in ASM length \textit{in situ} is of much lesser importance to airway function, at least within the context of the short-term but sustained exposure period studied.

An unexpected finding was the gradual decrease in airway narrowing with a sustained P_{tm} change, particularly at -5 cmH_{2}O, which is in strong contrast to what is predicted to occur with ASM length-adaptation. The decrease in airway narrowing was not an issue of viability, which may occur with repeated simulation of ASM and nerve endings, since airway narrowing was seen to recover when P_{tm} was returned to 5 cmH_{2}O. The decrease in airway narrowing may be related to the corresponding change in airway size (volume or A_{i}); specifically, a gradual increase in airway size at 25 cmH_{2}O P_{tm} and a decrease in airway size at -5 cmH_{2}O. The change in airway size theoretically represents a shift to a stiffer region of the pressure-volume curve, above and below 25 cmH_{2}O and
–5 cmH₂O, respectively (126, 174). Whilst wall stiffening is expected to limit airway narrowing, there was a similar gradual decrease in ASM tension, which would not be affected by wall compliance. A final possibility is that the change in ASM tension could occur with a corresponding change in ASM pre-load. Whilst there was a statistically significant change in Pmo with sustained inflation or deflation, the magnitude of this effect was trivially small making any movement along the ASM L-T curve similarly trivial. The precise mechanism for the decrease in airway narrowing during the sustained Pim change remains unclear. This phenomena may inhibit contraction of passively narrowed bronchi in vivo and could be beneficial by limiting bronchoconstriction in patients with chronic airway narrowing from loss of lung elastic recoil or parenchymal attachments following emphysema.

Implications for bronchoprotective and bronchodilatory effects of deep inspiration

In normal healthy individuals in vivo, deep inspiration (DI) produces a transient reversal of bronchoconstriction (i.e. bronchodilation) (53, 73, 117, 137) and may also transiently reduce the magnitude of subsequent bronchoconstriction, a phenomenon termed ‘bronchoprotection’ (89, 110, 142, 150). Length-adaptation theoretically contributes to both bronchoprotective and bronchodilatory effects of DI (163). However, bronchoprotective effects of DI are not seen using airway segment preparations (71, 123) wherein contractile responses are examined before and after large dynamic Pim simulating DI. The present lack of evidence for ASM length-adaption in situ is consistent with the failure of simulated DI to induce bronchoprotection in airway segments. The implication of these observations is that bronchoprotective effects of DI in vivo may instead arise from mechanism(s) other than length-adaptation, notably
enhanced bronchodilatory effects to DI when bronchoconstriction is assessed by the volume of forced expiration in the first 1 second (FEV$_1$), and/or prevention of airway closure (39, 176). With respect to the bronchodilatory effects elicited by DI in airway segments (122, 169), my findings favour a mechanism(s) other than length-adaptation, such as cross bridge perturbation (60, 61).

**Duration of length-adaptation**

The duration allowed for ASM length-adaptation to occur has varied between studies. Early studies on isolated ASM preparations held ASM length fixed for days to weeks (118, 166) demonstrating large shifts in the ASM passive and active length tension curves. However, the process of length-adaptation is accelerated by regular ASM activation and can occur after only a ~30 min period (43, 44, 94, 133, 165). Therefore, I expect that the ~50 minutes duration of altered P$_{tm}$, with regular ASM activation every five minutes, used in the present study, should be sufficient for length-adaptation to occur.

Several previous studies have examined the effect of chronic exposure to increased P$_{tm}$ on airway function, all of which show chronic inflation suppresses airway narrowing (157, 180-182). Indeed, continuous positive airway pressure (CPAP) has been suggested as a novel treatment for asthma (131). *In vitro*, whole rabbit bronchial segments were cultured for 48 hours with and without a positive inflationary pressure which reduced the maximum isometric force produced by the inflated airways (157). In ferrets *in vivo*, CPAP for 2 to 3 weeks reduced airway responsiveness studied at 5 cmH$_2$O (181). Further, the effects of CPAP persisted for at least 24 hours (180). In a
recent study by Xue and colleagues (182), CPAP for only 2 hours was sufficient to alter airway responsiveness in mice.

The evidence to date suggests that the beneficial reduction in airway responsiveness brought about by periodic CPAP are not mediated by ASM length-adaptation. Indeed in the above study in ferrets (181) there was reduced force production in isolated ASM strips even when all tissues were studied at their relative ‘optimum lengths’. Instead of length-adaptation (a shift in the length-tension curve), CPAP appears to reduce ASM contractility by inhibiting myosin regulatory light chain phosphorylation suggesting suppressed excitation–contraction coupling, rather than length-adaptation (181). A separate study by McClean and colleagues (111) showed no change in myosin light chain kinase following a reduction in FRC for 4 weeks, suggesting that the effect may be specific to CPAP therapy.

Airway compliance and $P_{tm}$

Changes in $P_{tm}$ brought about predictable changes in passive (relaxed) specific airway compliance. Assessment of compliance between 25 and 30 cmH$_2$O ($\Delta$5 cmH$_2$O) represents a stiffer region of the pressure-volume curve; whereas, compliance between -5 to 0 cmH$_2$O is at the linear and relatively compliant region of the pressure-volume curve (126, 174). Of more interest was the effect of $P_{tm}$ on airway wall compliance after ASM contraction to EFS. Stiffening of the ASM and airway wall with contractile activation is well established. Compared with the reference $P_{tm}$ of 5 cmH$_2$O, where ASM contraction produced considerable airway stiffening, at 25 cmH$_2$O there was no measureable stiffening with ASM contraction. On the other hand, at -5 cmH$_2$O $P_{tm}$,
stiffening with ASM contraction increased. These observations likely reflect the load bearing elements in the airway wall at different $P_{tm}$. At the plateau of the pressure-volume curve (i.e. at 25 cmH$_2$O), the airway is passively stiff and the addition of ASM contraction is of no consequence. At lower pressures the passive components of the wall are highly compliant, so the compliance of contracted ASM dominates the airway compliance. These data are consistent with findings in normal healthy individuals in vivo, which suggest ASM contraction reduces airway compliance at low but not at high lung volumes (90).

Airway wall stiffening is of potential relevance in a number of airway diseases, including asthma. Various mechanisms can contribute to airway wall stiffening and include re-modelling of the cell structural and/or contractile apparatus (135, 167) and ASM activation (7). In view of the present findings, I propose that lung hyperinflation is another likely contributor to airway wall stiffening, with a potency that at least matches ASM contractile activation at lower lung volumes.

Conclusion

The present study showed that, whilst evoked airway narrowing is regulated by $P_{tm}$, mechanisms other than ASM length-adaption dominate, particularly pre-load effects (i.e., classical length-tension, L-T properties). An unexpected observation was that airway narrowing decreased with a sustained $P_{tm}$ change, and that this change was reversible. The apparent physiological limit on ASM contraction and airway narrowing during a sustained $P_{tm}$ change may be protective and the mechanism requires further investigation.
Chapter 6 General discussion

This thesis highlights the importance of airway smooth muscle (ASM) strain in regulating the bronchodilatory response to breathing manoeuvres. In Chapter 2 of this thesis, I show that ASM strain, rather than mechanical stress, is the critical determinant of bronchodilation in intact airway segments, suggesting that the underlying mechanism by which deep inspiration (DI) produces bronchodilation involves strain-induced relaxation of ASM. Since the bronchodilatory response is dependant on ASM strain and therefore, airway wall stiffness, there are several implications.

Firstly, airway wall stiffening is likely to reduce ASM strain produced by breathing manoeuvres and may contribute to the failure of DI to produce bronchodilation. Airway wall stiffening can also be evoked to explain the fall off in bronchodilation to DI over the dose-response curve (DRC) to acetylcholine (ACh). Airway wall stiffness is increased in asthma (28, 85, 175) and may limit ASM strain during DI. Passive airway wall stiffness could be increased in asthma due to the increased wall thickness produced by remodeling (32, 167). Contraction of ASM also stiffens the airway wall (10, 90, 124), suggesting that the impaired bronchodilation to DI patients with asthma might be a result of AHR. Deep inspiration is most effective at reversing bronchoconstriction at low doses of ASM contractile agonists and therefore, low airway wall stiffness. Excessive airway wall stiffening, either through ASM airway wall re-modelling or ASM contraction may contribute to the failure of DI in patients with asthma.
Secondly, any intervention that reduces airway wall stiffness is likely to be beneficial in reversing bronchoconstriction by enhancing the bronchodilatory response to breathing manoeuvres, such as DI. In Chapter 3 of this thesis, I show that bronchodilation to the non-specific β₂-agonist, isoprenaline, was greater during simulated breathing manoeuvres, compared with the response under static conditions, in intact airway segments. I propose that the greater bronchodilatory response during breathing manoeuvres is explained by the effect of isoprenaline on reducing airway wall stiffness, which increased ASM strain, producing greater bronchodilation. At maximal dose, at least half of the effect of isoprenaline is mediated by reduced airway wall stiffness, suggesting that a significant proportion of the response to pharmacological bronchodilators in vivo, is due to their ability to enhance the bronchodilation to breathing manoeuvres. Two previous studies found no interaction between length-oscillation (64) or oscillation of lumen volume (10) and pharmacological bronchodilators. However, those studies used fixed-length or fixed-volume oscillations that are independent of ASM stiffness (i.e. fixed-strain). In contrast, in the present study, changes in wall stiffness during ASM contraction and relaxation determines the ASM strain produced by fixed-transmural pressure (Pₘ) oscillations.

Of potential importance is that the DRC was significantly greater for stiffness than for narrowing (i.e. airways stiffen prior to narrowing as the dose of contractile agonist increases). The disconnect that I showed between airway narrowing (or ASM force) and airway wall stiffening could explain the findings that DI fails to produce bronchodilation in asthmatic patients, even when asthmatic and normal healthy individuals are matched for changes in bronchoconstriction (i.e. resistance) (183). At the same level of bronchoconstriction, patients with asthma may reach a greater level of
airway stiffness than normal healthy individuals and therefore, the magnitude of strain applied to ASM during DI is less. If matched for a 25% decrease in the volume of forced expiration in the first 1 second (FEV₁, considered a high level of bronchoconstriction in a normal healthy individual), moderate asthmatic patients are likely to require ~3 log doses less agonist than normal healthy individuals (179). Assuming that there is a theoretical half-log increase in the sensitivity of stiffness, compared with airway narrowing (similar to that shown Chapter 2 of this thesis), the same level of bronchoconstriction between groups may result in more than double the increase in airway stiffness in moderate asthmatic patients, compared with normal healthy individuals (Figure 6.1.1). The separation between the normal healthy individuals and moderate asthmatic patients comes about since the normal healthy individuals are approaching the plateau of the stiffness dose-response curve whereas the asthmatic is still on the steep region of the curve. The significance of increased airway stiffness is that the bronchodilatory response to DI may be attenuated or abolished at comparable levels of bronchoconstriction, due to greater airway stiffening in the patients with asthma and therefore, a decrease in ASM strain during DI. While not methodologically feasible, it would be interesting to determine how the bronchodilatory response to DI differs in asthma if the level of airway stiffening rather than airway flow reduction was matched between groups.
Figure 6.1.1. A theoretical model to show how normal healthy individuals and moderate asthmatic patients may not be matched for airway wall stiffness, even if matched for the same level of bronchoconstriction. The bronchial challenge dose-response curve (DRC, % decrease in FEV₁, solid lines) is re-drawn from Woolcock and colleagues, 1984 (179). Curves of airway wall stiffening (% maximum response in normal healthy individuals, dashed lines) are drawn using the same curves as FEV₁ but with a theoretical half-log increase in the sensitivity to histamine. The disconnect between airway narrowing and airway wall stiffening means that even if matched for the % decrease in FEV₁, airway stiffness may not be matched. If matched for a 25 % decrease in FEV₁, bronchoconstriction will be at point A and airway wall stiffness will be at point B in the normal healthy individuals. In contrast bronchoconstriction will be at point C and airway wall stiffness will be at point D in the moderate asthmatic patients.

Chronic inflammation of the airway wall and the failure of DI to produce bronchodilation are both common to asthma. The cause(s) of airway hyper-responsiveness (AHR) remain unclear but likely involve abnormalities in ASM structure/function. The pro-inflammatory cytokines, tumour necrosis factor (TNF) and interleukin-1β (IL-1β) are known to induce ASM hyper-contractility. In Chapter 4 of this thesis, I showed that TNF and IL-1β do not modulate the ASM response to strain during DI, suggesting that chronic inflammation of the airway wall does not directly
contribute to the failure of DI in patients with asthma. It is possible that pro-inflammatory cytokines induce excessive airway wall stiffening by virtue of altering the contractile phenotype of the ASM. Indeed, the airway narrowing maximum response was increased following 2 days of culture with TNF and IL-1β, which extends previous findings of increased ASM force in isolated ASM strips (1, 41, 136, 155, 184). The relative change in airway stiffening was also increased in the pro-inflammatory cytokine treated airways but there was no difference in the ASM response to strain.

My findings in Chapter 4 of this thesis do provide proof of concept that the response of ASM to strain is modifiable, not by pro-inflammatory cytokines but by the process of culturing. When compared with fresh airway segments, the sensitivity to ASM strain was reduced in both treatment groups. The mechanism(s) that caused the decrease in sensitivity to ASM strain following culture remain unclear. It may be due to hypoxia of the ASM or build-up of waste metabolites or the change in the mechanical environment. During culture, the airways were at atmospheric pressure (i.e. 0 cmH₂O P<sub>tm</sub>) and in the absence of simulating breathing manoeuvres.

Sustained periods of ASM strain have been suggested to cause length-adaptation of ASM, whereby force production recovers back to that present at optimum length (L<sub>o</sub>) following adaptive re-arrangement of the contractile apparatus (118, 143, 166). Length-adaptation is one of the hypothesised mechanisms through which oscillatory ASM strain may attenuate ASM force production (94, 147, 164, 165), as a constantly changing length would start the adaptation process but prevent it reaching completion. Length-adaptation to a short ASM perimeter has been proposed as a potential
mechanism underlying AHR in obstructive disease (166). In Chapter 5 of this thesis, I showed that sustained changes in $P_{tm}$, which is the physiological determinant of ASM length, have little effect on airway narrowing. Failure to find length-adaptation in whole bronchial segments could be related to the magnitude of ASM strain required to induce adaptation. The $P_{tm}$ tested in this study covers the physiological range and larger passive changes in ASM strain are unlikely to occur in vivo. One assumption of this thesis is that the porcine airway wall is a model of the human airway. It is important to acknowledge that the stiffness of porcine airway wall is greater than human airways, and for this reason cannot confirm that adaptation is not a property of the human airway wall.

Length-adaptation theoretically contributes to both bronchoprotective and bronchodilatory effects of DI (163). Re-arrangement is hypothesised to occur through changes in the length of actin and/or myosin filament achieved through depolymerization of the filaments and re-polymerization into filament adapted to the new length. The failure to find length-adaptation in whole bronchial segments is consistent with the known behaviour of intact airway segments, in which attempts to find bronchoprotection have also been unsuccessful (71, 123). I propose that, under physiological levels of strain, ASM does not undergo contractile apparatus re-arrangement. The implications are that bronchoprotection in vivo, is not due to a mechanism occurring at the level of the airway wall and consequently changes in airway wall structure/function do not contribute to reduce bronchoprotection in asthma.
In conclusion, the studies in this thesis, using intact airway segments, allows the following conclusions to be drawn. Airway wall stiffness is increased in asthma (28, 85, 175) and may limit ASM strain during DI. The loss of that inhibitory effect would favor increased bronchoconstriction and may contribute to the development of AHR. Reducing airway wall stiffening represents a potential second target for novel pharmacological bronchodilators to treat asthma (27, 135, 144) by virtue of reducing ASM stiffness and increasing ASM strain during breathing manoeuvres.
References


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