MONASH University

Assessing the role of relaxin as a novel treatment opposing lung fibrosis and airway contraction

Maggie Lam

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Abstract

Introduction: Persistent airway inflammation and remodeling leads to airway hyperresponsiveness in asthma. β-adrenoceptor agonists do not always fully relieve symptoms, and remodelling is not currently targeted by current therapies.

The peptide hormone, relaxin (RLX), a Relaxin Family Peptide Receptor 1 (RXFP1) receptor agonist, relaxes vascular smooth muscle and inhibits fibrosis via increased nitric oxide (NO) and inhibition of TGFβ-mediated signalling (Samuel *et al.*, 2007; Leo *et al.*, 2014).

Critically, chronic RLX treatment reverses fibrosis and AHR in mouse models of allergic airways disease (Royce *et al.*, 2014). Its acute effects on airway contraction have not been explored. The major aims of this thesis further characterized the anti-fibrotic effects of RLX and its potential bronchodilator actions. Relaxation to RLX alone and in combination with β_2-adrenoceptor agonists was assessed in multiple species, and in mouse models of inflammation and/or fibrosis.

Methods and results:

Small airways (<2 mm in humans) are implicated in the pathology of lung diseases. New approaches were developed to prepare viable precision cut lung slices (PCLS) to visualize changes in intrapulmonary airway area. Results showed that airway reactivity was preserved after delayed slicing or cryopreservation.

Further evidence of the inhibitory effects of RLX on remodeling and fibrosis was obtained using the fibroblast collagen gel contraction assay. Contraction by non-asthmatic and asthmatic fibroblasts was increased by TGFβ and inhibited by RLX.

Compelling *in vitro* data established that RLX is a novel dilator of both large and small airways across multiple species from rodent to human. In rat airways, RLX elicited relaxation with greater potency than the β_1/β_2-adrenoceptor agonist isoprenaline. RLX pretreatment also inhibited the development of airway contraction in multiple species. This bronchoprotective response was inhibited by COX and NOS inhibitors, implicating PGE_2 and NO as key mediators. Pilot data showed RLX elicited relaxation in human airways in perfused PCLS, suggestive of dilator mechanisms which are epithelial-independent.

Notably, RLX increased the potency of the clinically used β_2-adrenoceptor agonist salbutamol. In mouse PCLS, where salbutamol only elicits partial relaxation, RLX increased salbutamol efficacy. In guinea pig and human PCLS, where salbutamol is a full agonist, RLX increased salbutamol potency.

The potentiation of salbutamol-mediated relaxation by RLX was assessed in PCLS from models of lung fibrosis (bleomycin), acute inflammation (house dust mite, HDM) and airway fibrosis (TGFβ-overexpression). Bleomycin induced downregulation of RXFP1 receptors and loss of potentiation by RLX. In contrast, salbutamol-mediated relaxation was still increased by RLX following HDM challenge and TGFβ-overexpression, despite evidence of airway inflammation and/or fibrosis.

Conclusions: RLX has potential benefit as an adjunct dual-action anti-fibrotic and dilator therapy. In addition to its potent anti-remodelling actions, RLX elicits acute airway relaxation and bronchoprotection via multiple mechanisms that may vary between species. The potentiation of responsiveness to β-adrenoceptor agonists by RLX in human airways and under asthma-relevant disease conditions has implications for improved relief of symptoms. While the mechanisms underlying these novel actions remain to be fully elucidated, translation of these promising preclinical findings is critical in the development of RLX as a novel therapeutic opposing key aspects of airway pathology in lung diseases.

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Contents

Abbreviations

α-SMA	alpha- smooth muscle actin
AC	adenylate cyclase
ACh	acetylcholine
AHR	airway hyperresponsiveness
AI	airway inflammation
AngII	angiotensin II
AT2R	angiotensin II receptor
ASM	airway smooth muscle
AWR	airway wall remodelling
β_2AR	beta 2 adrenoceptor
BLM	bleomycin
Ca^{2+}	calcium
CaM	calmodulin
cAMP	3',5'-cyclic adenosine monophosphate
CICR	calcium-induced calcium release
cGMP	3',5'-cyclic guanosine monophosphate
COX	cyclooxygenase
CS	cigarette smoke
CTGF	connective tissue growth factor
DAG	diacylglycerol
DEX	dexamethasone
DMSO	dimethyl sulfoxide
EFS	electrical field stimulation
ECM	extracellular matrix
EMT	epithelial-to-mesenchymal transition
EP	prostaglandin E_2 receptor
ET-1	endothelin-1
FEV1	forced expiratory volume in 1 second
FGF	fibroblast growth factor
FRET	fluorescence resonance energy transfer
GC	guanylate cyclase
GPCR	G protein-coupled receptor
GRK	G-coupled receptor kinase
HA	histamine
HDM	house dust mite
IgE	immunoglobulin E
IL	interleukin
ILD	interstitial lung diseases
INDO	indomethacin
IP_3	inositol-1,4,5-phosphate
IPF	idiopathic pulmonary fibrosis
i.n.	intranasal
I/R	ischemia-reperfusion
ISO	isoprenaline
*i.*v.	intravenous
LABA	long acting beta adrenoceptor agonist
L-NAME	N^G-nitro-L-arginine methyl ester
LTD_4	leukotriene D_4
MCh	methacholine
MHC	myosin heavy chain
MLC20	myosin light chain (20 kDa)

MLCK	myosin light chain kinase
MLCP	myosin light chain phosphatase
MMP	matrix metalloproteinase
MYPT1	myosin phosphatase target subunit 1
NANC	non-noradrenergic, non-cholinergic
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
ODQ	1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline
OVA	ovalbumin
NHP	non-human primates
NO	nitric oxide
NOS	nitric oxide synthase
PAR	protease-activated receptor
PCLS	precision cut lung slice
PDE	phosphodiesterase
PDGF	platelet-derived growth factor
PGD_2	prostaglandin D_2
PGE_2	prostaglandin E_2
PGI_2	prostacyclin
PIP_2	phosphatidylinositol 4,5-bisphosphate
PIRF	pirfenidone
PKA	protein kinase A
PKC	protein kinase C
PLA2	phospholipase A2
PLC	phospholipase C
PPARγ	peroxisome proliferator-activated receptor gamma
RGZ	rosiglitazone
RLX	relaxin
ROCK	Rho-associated protein kinase
RXFP1	relaxin family peptide receptor 1
RyR	ryanodine receptor
SABA	short acting beta adrenoceptor agonist
SAMA	short-acting muscarinic antagonist
SALB	salbutamol
5-HT	serotonin
s.c.	subcutaneous
SERCA	sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase
SP	substance P
SR	sarcoplasmic reticulum
SQ22,536	9-(tetrahydro-2-furanyl)-9H-purin-6-amine
TGFβ	transforming growth factor 1
TIMP	tissue inhibitor of metalloproteinases
T_H	T helper cell
TNFα	tumour necrosis factor alpha
TXA_2	thromboxane A_2

Thesis overview

This thesis examines the potential role of relaxin, a human reproductive peptide, as a novel therapeutic opposing fibrosis and airway contraction in asthma. Insights gained from *in vitro* studies in cells, isolated airways and precision cut lung slices (PCLS) are presented, as well as from *in vivo* mouse models of disease, comparing relaxin with current therapeutics.

Chapter 1 is the General Introduction comprising two major sections, both based on review publications (Lam *et al.*, 2018, 2019). The first section outlines current knowledge on airway contraction, mechanisms of altered contraction in asthma, and the limitations of current bronchodilator therapy (Lam *et al.*, 2019). The second section of Chapter 1 describes the known effects of relaxin in lung diseases, including asthma (Lam *et al.*, 2018). While the chronic anti-fibrotic actions of relaxin were clearly established prior to the studies presented in this thesis, there was previously limited information on its potential bronchodilator actions.

Chapter 2 covers general methods and techniques used in successive chapters throughout the thesis.

Chapter 3 is a result chapter which focuses on optimising the PCLS technique to assess small airway reactivity. It serves as secondary methods chapter.

Chapter 4 to 7 are results chapters, which characterise the actions of relaxin:

- to oppose fibrosis in human airway fibroblasts (Chapter 4);
- as a bronchodilator/bronchoprotective agent of airways from naïve mice, rat, guinea, marmoset and human lungs (Chapter 5, mostly published as (Lam *et al.*, 2016);
- to potentiate dilator responses to salbutamol from multiple species (Chapter 6); and
- to potentiate dilator responses to salbutamol in airways from mouse models of disease, using bleomycin treatment, house dust mite challenge and TGFβ-overexpression (Chapter 7)

Chapter 8 is the General Discussion which summarises all results presented in this thesis and how they contribute to our current understanding of the emerging role of relaxin as a novel anti-fibrotic and bronchodilator to oppose lung pathology.

Chapter 1: General Introduction

1.1 Asthma

Chronic respiratory diseases are the third leading cause of death worldwide, accounting for over 3 million deaths globally each year (Lozano *et al.*, 2012). Of these, asthma affects over 2 million Australians and 300 million people on a global scale (World Health Organization, 2013). As of 2010, asthma was ranked 14th in years lived with a disability across all diseases (Gibson *et al.*, 2013) and was responsible for 1 in 250 deaths worldwide annually (AIHW Australian Centre for Asthma Monitoring 2005).

Asthma is characterised by three major characteristics: airway inflammation (AI), airway wall remodelling (AWR) and airway hyperresponsiveness (AHR). The complicated interactions between these characteristics are summarised in Figure 1.

Allergic asthma is the most common phenotype of asthma, and involves T_H2 inflammatory pathways. T_H2 cells migrate to the airway epithelium and secrete cytokines such as interleukin-5 (IL-5) and IL-13 (Robinson *et al.*, 2017). T_H2 cells also recruit eosinophils, basophils and mast cells (Holgate *et al.*, 2015), with eosinophilic inflammation shown to be a risk factor for exacerbations (Zeiger *et al.*, 2014) that correlates with airway obstruction (Horn *et al.*, 1975). In non-allergic or severe asthma phenotypes, inflammation may be driven by T_H1, T_H17 cells and neutrophils. The main T_H17 cytokine, IL-17, is also implicated in causing airway obstruction and AHR (Robinson *et al.*, 2017; Samitas *et al.*, 2017).

Major features of airway remodelling with the potential to alter airway reactivity include increased airway smooth muscle (ASM) and fibrosis. It is being increasingly recognised that the distal lung is a major site both inflammation and remodelling leading to increased airway resistance (Mechiche *et al.*, 2003; Burgel, 2011). Studies using ASM cells, airways and animal models of disease have characterised increased responsiveness to contractile agonists under the influence of airway inflammation. However, relatively few studies have assessed the reactivity of intrapulmonary airways, rather than tracheal or bronchial preparations in asthma (Donovan *et al.*, 2013). Studies using precision cut lung slices (PCLS), in which contraction of intrapulmonary airways can be assessed *in situ*, are proving informative in this regard.
Patients with asthma experience symptoms of breathlessness, coughing and chest-tightness. Current treatments relieve the increased bronchoconstriction associated with asthma and target inflammation to minimise the frequency and severity of these symptoms. However, since bronchodilators and anti-inflammatory glucocorticoids can become less effective with repeated use and in severe disease, new asthma therapies are still required.

1.1.1 Airway inflammation

The role of inflammation in promoting increased airway contraction in chronic lung diseases is well established (Figure 1) and forms the basis for the use of anti-inflammatory treatment with glucocorticoids to reduce the frequency and severity of asthma attacks. Indeed, AHR is evident in short-term animal models of allergic airways disease (AAD) when elevated eosinophils and T_H2 cytokines are present, even when AWR is absent.

1.1.1.1 Mediators

The release of bronchoconstrictors such as histamine and leukotriene D_4 (LTD_4) from mast cells during an allergic response can directly cause airway narrowing in asthma. However, key inflammatory cytokines elevated in the airways have also been shown to indirectly increase airway responsiveness. For example, *in vitro* treatment of isolated human airways with tumour necrosis factor-α (TNF-α) exaggerates contraction to muscarinic agonists, bradykinin and serotonin (5HT) (Sukkar *et al.*, 2001; Chen *et al.*, 2003; Cooper *et al.*, 2009), with increased expression of contractile agonist receptors providing a possible mechanism (Zhang *et al.*, 2004). IL-13 also significantly increases maximal force generation in mouse trachea (Tliba *et al.*, 2003) and small airway contraction in mouse and human PCLS (Cooper *et al.*, 2009; Jiang *et al.*, 2012). Expression of receptors for cysteinyl leukotrienes ($CysLT_1$) on ASM is also increased by the Th2 cytokine IL-13 (Matsumoto *et al.*, 2012). More recently, the T_H17 subset of T cells has been shown to contribute to the development of more severe asthma phenotypes, particularly associated with neutrophilia (Hirose *et al.*, 2017). The T_H17 cytokine IL-17A enhances contractile force generation of mouse tracheal rings and human bronchi (Kudo *et al.*, 2012).

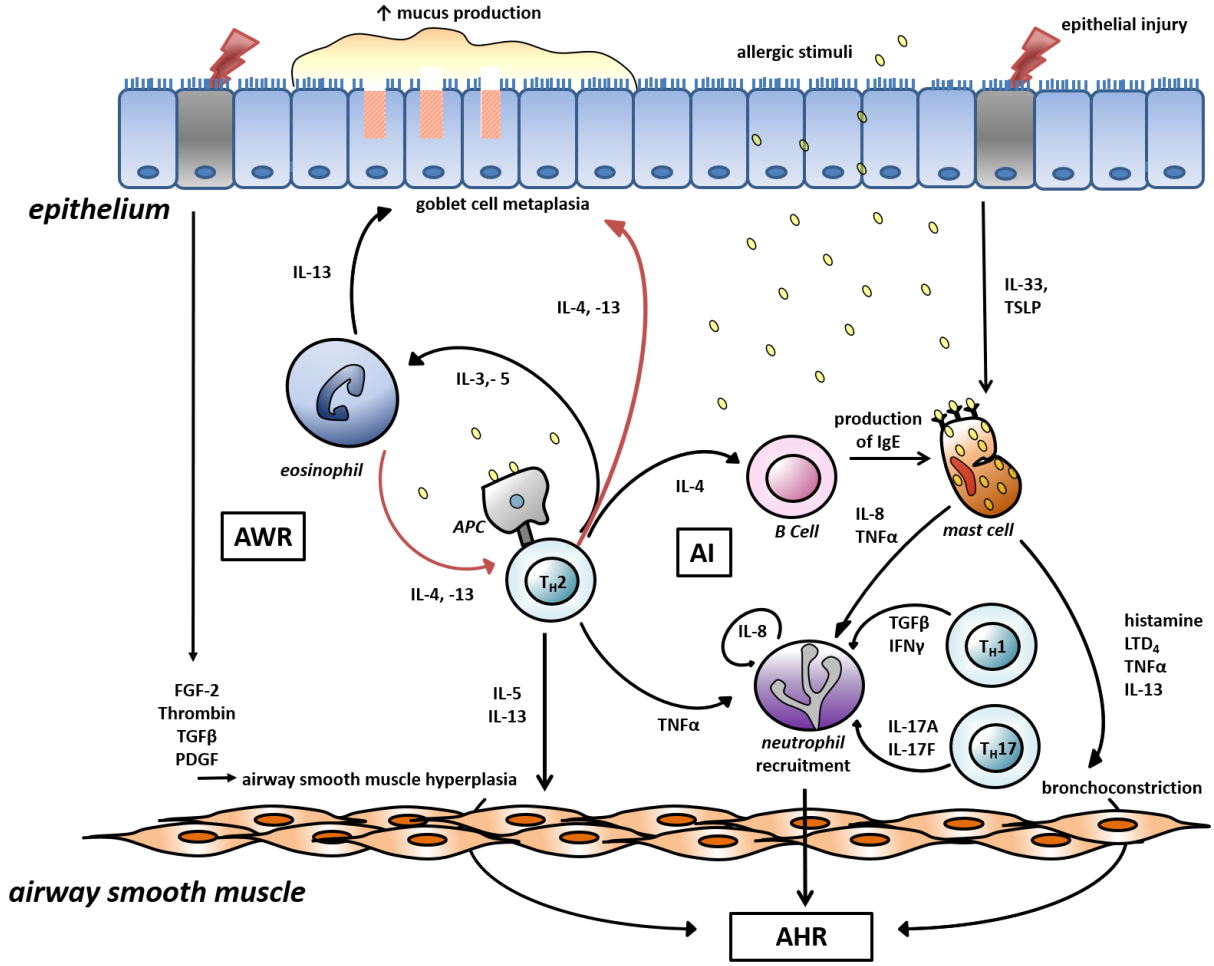


Figure 1. Contribution of airway inflammation and remodelling to AHR. The airway inflammation (AI) cascade can be stimulated by exposure to allergens and epithelial damage, leading to airway wall remodelling (AWR) and airway hyperresponsiveness (AHR). Exposure to normally innocuous substances (pollen, certain foods, some drugs) evokes production of immunoglobulin E (IgE) antibodies which prime mast cells within tissues. Repeated exposure to the same substance or antigen is recognised by the antibodies that activate mast cell degranulation, resulting in the release of preformed mediators including histamine and leukotriene D_4 (LTD_4), which directly induce bronchoconstriction as well as tumour necrosis factor-α (TNFα) and interleukin-13 (IL-13) which indirectly increase airway reactivity. Inflammation also involves the release of multiple cytokines upon activation of T-helper cells, T_H1 and T_H2. T_H2 cytokines including IL-3, -4, -5 and -13 activate mast cells, B cells, eosinophils, neutrophils and airway smooth muscle cells to increase the production of other inflammatory cytokines particularly in allergic asthma. T_H1 cytokines such as interferon-γ (IFNγ) and transforming growth factor-β (TGFβ) activate macrophages and stimulate neutrophil recruitment particularly in neutrophilic asthma. The T_H17 cytokines, IL-17A and IL-17F. can also cause neutrophil recruitment. TGFβ as well as mitogens such as fibroblast growth factor-2 (FGF-2), thrombin and platelet-derived growth factor (PDGF) contribute to fibrosis and airway smooth muscle hyperplasia that characterise AWR. Both increased airway inflammation and airway smooth muscle hyperplasia contribute to the development of AHR.

These *in vitro* findings have been validated in animal models of allergic airways disease, using ovalbumin (OVA) or house dust mite (HDM) to induce asthma-like characteristics. The development of *in vivo* AHR due to allergen sensitization is prevented by targeting specific cytokines using monoclonal antibodies, receptor antagonists or gene deletion. For example, the inhibitory effects of IL-13 antibodies (Wills-Karp *et al.*, 1998), deficiency of IL-17 (Kudo *et al.*, 2012) or a soluble TNF-α antagonist (Renzetti *et al.*, 1996) on AHR have been demonstrated. Administration of IL-13 alone also causes rapid induction of AHR in mice *in vivo,* independently of allergen challenge (Jiang *et al.*, 2012). Notably, TNF-α inhalation increases AHR to methacholine (MCh) challenge in human subjects with mild asthma (Thomas and Heywood, 2002).

1.1.1.2 Cellular sources

The cellular sources of inflammatory mediators in the airways vary with different chronic lung diseases. Eosinophils, mast cells and T_H2 cells are key players in allergic asthma, while T_H1 cells or neutrophils are implicated in more severe asthma. These circulating inflammatory cells and the resident airway epithelium are a rich source of mediators contributing to inflammation. T_H2 cytokines (IL-4, IL-5, IL-13) and others including TNF-α, IL-1, IL-8 and IL-17 are major drivers of airway inflammation in asthma (Holgate *et al.*, 2015) (Figure 1).

ASM itself can respond to these stimuli by producing inflammatory chemokines and cytokines such as eotaxin, IL-1 and IL-8. This may attract inflammatory cells into the airways and amplify the signals they generate (reviewed by Howarth *et al.*, 2004). The release of eotaxin from asthmatic ASM in culture is higher than from non-asthmatic ASM, both under basal conditions and in response to asthma-relevant cytokines (Chan *et al.*, 2006). This observation has been confirmed by increased immunostaining of eotaxin in the ASM layer of asthmatic airways (Ghaffar *et al.*, 1999), consistent with a hypersecretory phenotype of ASM *in vitro* and potentially *in vivo* in human asthma. The numerous synthetic products of ASM and their autocrine and paracrine effects also have the potential to promote inflammation-associated increases in contraction (Howarth *et al.*, 2004).

1.1.2 Airway wall remodelling

The major features of airway remodelling include thickening of the epithelium, airway fibrosis and increased ASM, evident in both large and small airways (Figure 2). These structural changes are not targeted by current pharmacological treatments with either anti-inflammatory drugs used as preventer medication or with bronchodilators used as relievers to oppose airway contraction.

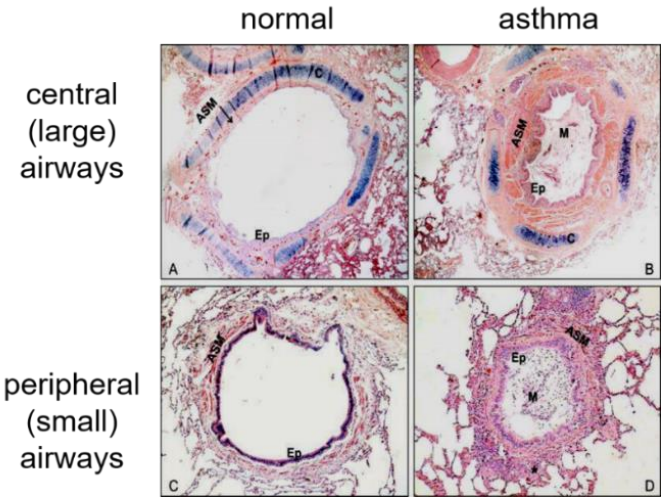


Figure 2. Large and small airways from a non-asthmatic or asthmatic patient. In comparison to normal airways (A and C), airways from a patient with fatal asthma demonstrated (B) mucus plugging within the airway lumen as well as epithelial folding and thickened ASM layer. (D) Small airways also showed widespread inflammation to surrounding peribronchiolar alveoli. Magnification: A, B x25; C, D x100. C= Cartilage; Ep= Epithelium; M= mucus. (*adapted from Mauad et al., 2007)*

1.1.2.1 Role of the epithelium

The airway epithelium provides a physical barrier between the internal and external environment, and may be disrupted in asthma contributing to the development of AHR. Immediately after injury, increased levels of growth factors including TGFβ drive epithelial cell migration and differentiation (epithelial-to-mesenchymal transition, EMT) to promote wound healing. These processes become dysregulated in asthma where TGFβ further promotes epithelial thickening and fibrosis. A key component of airway remodelling is goblet cell meta-hyperplasia and mucus hypersecretion, which occur in response to T_H2 cytokines (IL-4, -5, -9 and -13). Excessive mucus secretion in addition to epithelial thickening can also compromise airflow in asthma.

1.1.2.2 Fibrosis

Airway fibrosis in asthma is caused by the accumulation of ECM proteins, and occurs as a result of aberrant wound healing. In the normal wound healing process, fibroblasts differentiate into activated myofibroblasts and produce ECM proteins including collagen and fibronectin. Myofibroblasts express the contractile protein, alpha smooth muscle (α-SMA) to contribute to fibroblast contraction. Once the injured site is repaired, myofibroblasts undergo apoptosis and allow tissues to retain their normal structure and function. In asthma, myofibroblast apoptosis does not occur or occurs at a much slower rate which results in excessive collagen deposition (Michalik *et al.*, 2018).

ASM and fibroblasts can both produce and respond to the pleiotropic cytokine TGF-β1 to produce ECM proteins (reviewed in Ward and Hirst, 2007), resulting in both subepithelial fibrosis and alterations in the ECM interspersed in the ASM layer. Both TGF-β1 and connective tissue growth factor (CTGF), which is implicated in mediating the effects of TGF-β1, are increased in asthma (Howarth *et al.*, 2004; Johnson *et al.*, 2006; Al-Alawi *et al.*, 2014). The profile of ECM produced by ASM from asthmatics differs from ASM from healthy subjects, namely increases in collagen I, III and V and fibronectin and decreases in collagen IV and elastin (Johnson, 2001).

ECM deposition due to collagen synthesis can be balanced by its degradation by proteinases referred to as matrix metalloproteinases (MMP) (Yaguchi *et al.*, 1998). The activity of MMPs can be inhibited by tissue inhibitors of metalloproteinases (TIMPs) (Razzaque and Taguchi, 2003). TGFβ reduces overall collagen turnover by increasing both collagen deposition and TIMP activity and inhibiting MMP activity, consistent with its profibrotic actions.

The overall consequence of the altered ECM production in asthma is fibrosis and stiffening of airways. This may result in reduced compliance and increased airway resistance (Seow, 2013), leading to significant airway obstruction (James *et al.*, 1989).

1.1.2.3 Role of airway smooth muscle

Increased ASM is a key feature of airway remodelling, and has been implicated as the most important factor contributing to AHR in asthma (Lambert *et al.*, 1993). Based on studies of biopsies from subjects with severe or fatal asthma, the thickness of the ASM layer is related to disease severity rather than duration (Benayoun *et al.*, 2003; James *et al.*, 2009). In addition, thickening of the ASM layer is evident in both large and small airways (Figure 2).

Mitogenic stimuli known to be elevated in asthmatic airways include growth factors such as fibroblast growth factor-2 (FGF-2), inflammatory cell-derived mediators such as thrombin, and contractile agonists such as endothelin-1 (ET-1), thromboxane A_2 (TXA_2) and leukotriene (LTD_4) (Figure 1). Despite convincing evidence of increased ASM in asthma, it has been difficult to establish that ongoing proliferation occurs *in situ*, as airway biopsies from asthmatic subjects with established remodelling show limited staining for proliferative markers in ASM (Benayoun *et al.*, 2003). However, the proliferative response of primary cultured human ASM from those with asthma is increased compared to ASM from those without asthma (Hirst *et al.*, 2004). This increased proliferation may be specific to ASM, since airway myofibroblasts from asthmatic subjects do not show a similar increase *in vitro* (Ward *et al.*, 2008).

Another mechanism that may contribute to the increase in ASM in remodelled airways is migration (Salter *et al.*, 2017). *In vitro* studies show that many mediators implicated in asthma increase ASM migration. These include growth factors such as platelet-derived growth factor (PDGF), lipid metabolites such as cys-LTs and prostaglandin D_2 (PGD_2), and inflammatory T_H2 cytokines such as interleukin-5 (IL-5), IL-4 and IL-13 (Salter *et al.*, 2017). An isolated report has demonstrated increased migration in ASM from asthmatics compared to non-asthmatics (Kanabar *et al.*, 2006).

Notably, the altered ECM within the airways can also promote the production of inflammatory cytokines such as eotaxin from ASM (Chan *et al.*, 2006). *In vitro* studies have also shown that migration of ASM cells grown on membranes coated with collagens III and V and fibronectin is increased compared to cells grown on collagen I, elastin and laminin (Parameswaran *et al.*, 2004). In addition, the proliferation of ASM from non-asthmatics is increased when these cells are grown on an ECM bed produced by asthmatic ASM (Johnson *et al.*, 2004). Thus, altered ECM production by the increased ASM in asthma can act in an autocrine fashion to promote its own contribution to inflammation, airway remodelling and increased contraction.

1.1.3 Airway contraction

Increased airway contraction leads to airway narrowing and symptoms of breathlessness as seen in asthma. Before addressing altered reactivity in asthma, it is important to establish the mediators and mechanisms underlying airway contraction and relaxation.

1.1.3.1 Mediators

Numerous endogenous mediators from a variety of cellular sources regulate airway tone by causing contraction or relaxation (Table 1). These effects are known to vary between species and with airway size. Increased levels of these neurally-derived or circulating mediators can directly alter airway reactivity.

Muscarinic receptor agonists

The endogenous muscarinic receptor agonist *acetylcholine (ACh)* is released from postganglionic nerves and induces contraction via activation of postjunctional M3 receptors (Table 1). This is prevented by atropine and terminated by acetylcholinesterase. M2 autoinhibitory receptors on postganglionic nerve terminals in both central airways and bronchi can inhibit ACh release to oppose contraction (Zaagsma *et al.*, 1997). Exogenous muscarinic receptor agonists such as MCh or carbachol are resistant to breakdown by acetylcholinesterase so are used experimentally to assess AHR.

The degree of contraction to muscarinic agonists and other contractile mediators varies with airway size. In studies measuring isometric force in isolated airways from humans (Finney *et al.*, 1985), and changes in lumen area in mouse PCLS (Martin *et al.*, 1996), small airways were markedly more sensitive to contractile agonists than large airways. Similar heterogeneity was evident in pig airways using an optical imaging technique called anatomical optical coherence tomography, such that narrowing to carbachol increased from proximal to distal airways (Noble *et al.*, 2010). An elegant study comparing contractility to MCh in different generations of the same airway in mouse PCLS (Bai *et al.*, 2007) revealed that airway contraction in response to the same agonist concentration was greater in the middle generation than either the distal or proximal airway. Given the intermediate thickness of the ASM layer in the middle generation, it is clear that additional factors such as receptor density and/or expression of downstream signalling factors are also likely to vary with airway size to regulate airway contraction.

Mast-cell derived mediators

Autacoids released from mast cells such as *histamine* and *serotonin (5HT)* have the potential to contribute to airway contraction as well as inflammation during an allergic response, via activation of G_q-coupled H_1 and $5HT_2$ receptors present on ASM (Table 1).

Mast-cell derived *thrombin* and *trypsin* also exert direct and indirect effects on ASM to regulate airway contraction *in vitro* and *in vivo* via protease-activated receptors (PARs) 1-4 (Table 1). They are also broadly pro-inflammatory and induce proliferation of ASM *in vitro* although these

actions may by receptor-independent (Tran and Stewart, 2003). PARs are a family of G-protein coupled receptors (GPCRs) with an unusual irreversible mechanism of activation. Extracellular proteolytic activation results in cleavage of specific sites in the extracellular domain, leading to formation of a new N-terminus which functions as a tethered ligand. Binding of this ligand to an exposed site in the second trans-membrane loop of the receptor triggers G-protein binding and intracellular signalling. PAR agonists have been shown to mediate relaxation via endogenous PGE_2 production in mouse, rat, guinea pigs and human airways, a mechanism that is at least partly dependent on the presence of a functional epithelium (Cocks *et al.*, 1999; Chambers *et al.*, 2001). Activation of PAR-2 can induce contraction of human airways when endogenous PGE_2 production is inhibited by indomethacin, and can also potentiate contraction to histamine (Chambers *et al.*, 2001).

Arachidonic acid metabolites

A diverse range of metabolites of arachidonic acid modulating airway contraction, relaxation and inflammation are also synthesized by mast cells, as well as by the airway epithelium and ASM itself (reviewed by Clarke *et al.*, 2009) and implicated in AHR. Phospholipase A2 (PLA2) initiates this pathway, releasing arachidonic acid from membrane phospholipids. Subsequent degradation via COX produces the prostanoids, encompassing the prostaglandins PGD_2, PGE_2, $PGF_{2\alpha}$ and PGI_2 (prostacyclin) and thromboxane A_2 (TXA_2). The alternative lipoxygenase (LOX) pathway gives rise to the cys-LTs (Clarke *et al.*, 2009).

Of the lipid mediators, the *cys-LTs, LTC4, LTD4 and LTE4* are the most potent contractile agonists acting via their activation of $CysLT_1$ receptors (Table 1). Initially recognized as the active components of slow-reacting-substance of anaphylaxis (SRS-A), they are at least 200 times more potent than histamine in human airways (Capra *et al.*, 2006) and are markedly more potent in small (<2 mm) than large human airways (Mechiche *et al.*, 2003). Strong contraction to LTB4 is also evident in guinea pig airways mediated via stimulation of BLT1/BLT2 receptors (Clarke *et al.*, 2009), but rodents generally respond weakly or not at all to leukotrienes (Seehase *et al.*, 2011).

Prostanoids can mediate either contraction or relaxation of ASM, or in some instances both (Table 1). PGE_2 and PGI_2 are generally considered to be bronchodilators, while PGD_2, $PGF_{2\alpha}$ and TXA_2 induce varying degrees of bronchoconstriction (Clarke *et al.*, 2009). The response to PGE_2 is mediated via its four EP (EP1-4) receptor subtypes. EP4 receptors mediate potent relaxation to PGE_2 in human airways, via activation of adenylate cyclase (AC) and increased synthesis of cAMP (Benyahia *et al.*, 2012). PGE_2 maintains the tone of the guinea pig trachea

through a balance between activation of contractile EP1 receptors and relaxant EP2 receptors (Säfholm *et al.*, 2013). EP2 receptors have been implicated in PGE_2-mediated relaxation of mouse airways, where the EP1/2 antagonist AH6809, but not the EP4 antagonist L-161982, reduces the dilator potency of PGE_2 (Clarke *et al.*, 2009; Fitzpatrick *et al.*, 2013). In addition, PGE_2 acts via EP3 receptors to decrease the release of contractile neurotransmitters from parasympathetic neurons in guinea pig airways (Spicuzza *et al.*, 1998). In canine bronchial segments, a stable analogue of PGI_2 causes direct relaxation and at higher concentrations, also inhibits ACh release in response to electrical field stimulation (EFS) to reduce cholinergic contraction (Clarke *et al.*, 2009).

PGD_2, $PGF_{2\alpha}$ and TXA_2 mediate airway contraction via non-selective thromboxane receptors (TP). The stable TXA_2 mimetic elicits direct contraction of human and guinea pig airways (Ressmeyer *et al.*, 2006), but its actions in mouse airways are indirectly mediated by increasing M3-mediated bronchoconstriction (Allen *et al.*, 2006). TP-dependent contraction to PGD_2 in guinea pig airways and human bronchial rings is opposed by its actions at prostaglandin D1 receptors (DP1), which mediate relaxation (Clarke *et al.*, 2009). Human and rat airways are markedly more sensitive to the contractile effects of $PGF_{2\alpha}$ via TP than guinea pig airways (Clarke *et al.*, 2009).

Endothelin-1 (ET-1) and nitric oxide (NO)

Endothelin-1 and NO are other endogenous mediators produced by the airway endothelium with opposing effects on airway tone. ET-1 is a 21 amino acid peptide which is increased in asthma and mediates potent contraction. These actions are mediated via actions at ET_A and ET_B receptors expressed on ASM as well as by potentiating cholinergic responses (Goldie *et al.*, 1996; Knott *et al.*, 1996). Contraction to ET-1 is opposed by its dilator responses mediated via activation of ET_B receptors on the airway epithelium that cause the release of NO (Hay, 1999). Age-dependent changes in reactivity to ET-1 have been reported, with more effective contraction in newborn rabbits, and relatively increased relaxation in adult due to ET-1 mediated PGE_2 release (Grunstein *et al.*, 1991). NO also plays a role as a non-noradrenergic, non-cholinergic (NANC) neurotransmitter mediating airway relaxation.

Table 1. Mediators of airway contraction and relaxation. Additional indirect contractile mechanisms are mediated by increased release of ACh from parasympathetic nerves by adenosine, bradykinin, endothelin and prostanoids.

			Mechanism	
Agonist		**Cellular origin**	**Contraction (G_q)**	**Relaxation (G_s)**
Acetylcholine		parasympathetic cholinergic nerves	M_3	M_2 (pre-junctional G_i)
Adenosine			A_1	A_{2b}
Adrenaline		adrenal chromaffin cells		β_2
Bradykinin		mast cells	BK_1	
Ca^{2+}, polycations		extracellular	CaSR	
Endothelin-1		epithelium, endothelium, inflammatory cells	ET_A, ET_B	ET_B (epithelium) via PGE_2 /NO
GABA			$GABA_B$ (G_i)	$GABA_A$
H^+		extracellular pH	OGR1	
Histamine		mast cells, basophils	H_1	H_2
LTB_4, cys-leukotrienes (LTC_4, D_4, E_4)		mast cells, leukocytes, epithelium, platelets	BLT_1/BLT_2, $CysLT_1$	
Neuropeptides CGRP, Neurokinin A, Substance P		sensory nerves	NK_1 (direct); NK_1/NK_2 via prostanoid	
Nitric oxide		parasympathetic cholinergic nerves, epithelium		GC via cGMP
Platelet activating factor		platelets, leukocytes,		
Prostanoids	PGD_2	mast cells, leukocytes, epithelium, ASM	TP	DP_1
	PGE_2		EP_1, EP_3	EP_2, EP_4
	$PGF_{2\alpha}$		TP, EP_1, FP	
	PGI_2			IP_1
	TXA_2		TP	
Serotonin		mast cells (rodent>human)	$5HT_2$	
Thrombin, trypsin		mast cells	PAR_1	$PAR_{2/4}$ via PGE_2
Vasoactive intestinal peptide		parasympathetic cholinergic nerves		$VPAC_2$

1.1.3.2 Mechanisms of airway contraction

Calcium signalling

Contractile mediators act via their respective GPCRs on ASM to induce synthesis of inositol-3-phosphate (IP_3) and the subsequent release of calcium from intracellular stores leads to the development of contraction (Figure 3). Calcium release from the sarcoplasmic reticulum does not occur in a steady static pattern; rather, calcium is released in rhythmic oscillations (reviewed by Jude *et al.*, 2007). Agonist-induced airway contraction involves the production of IP_3, which opens inositol-3-phosphate receptors (IP_3R) enabling calcium release from the endoplasmic reticulum. This efflux of calcium activates IP_3R and ryanodine receptors (RyR) to cause further cyclical release of calcium due to continual release and reuptake of calcium via sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA). Calcium binds to calmodulin dependent kinases which regulate the phosphorylation of transcription factors such as cAMP responsive element binding protein (Jude *et al.*, 2007). In addition, the Ca^{2+}/calmodulin complex activates myosin light-chain kinase (MLCK) which phosphorylates myosin light chain (MLC) to cause smooth muscle contraction (Berair *et al.*, 2013). Myosin light chain phosphatase (MLCP) counteracts this process by dephosphorylating MLC. However, MLCP activity is inhibited by protein kinase C (PKC) and RhoA/Rho Kinase which render it inactive via phosphorylation. Disruptions to these pathways may contribute to ASM hypercontractility.

Calcium sensitivity

Airway contraction can still be maintained even when free intracellular Ca^{2+} is reduced by its reuptake into sarcoplasmic reticulum (SR) Ca^{2+} stores via SERCA or other mechanisms. A parallel pathway, termed Ca^{2+} sensitivity contributes to sustained ASM contraction by regulating the amount of force produced for a given level of free intracellular Ca^{2+} (Figure 3) (Bai *et al.*, 2009; Ressmeyer *et al.*, 2010). Ca^{2+} sensitivity is associated with decreased activity of type-1 protein phosphatase, MLCP, mediated by RhoA/Rho and DAG/PKC/CPI-17 signalling pathways. MLCP normally dephosphorylates MLC to provide constraint on MLCK-induced contraction. Inhibition of MLCP leads to sustained phosphorylation of MLC and associated cross-bridge activity to provide the main driver of the latter phase of a prolonged airway contraction (Pelaia *et al.*, 2008).

RhoA is a member of the Rho family of small GTPases (Seasholtz *et al.*, 2018). It is highly expressed in ASM, present in the cell membrane as an active form bound to GTP (GTP-RhoA) and an inactive cytoplasmic form bound to GDP (GDP-RhoA) (Fukata *et al.*, 2001; Puetz *et al.*, 2009). Agonist binding to receptors coupled to heterotrimeric GTP-binding proteins, G_q and $G_{12/13}$, activates the guanine-exchange protein RhoGEF (DerMardirossian and Bokoch,

2005; Bos *et al.*, 2007). This converts GDP-RhoA to GTP-RhoA to activate Rho-kinase (ROCK) (DerMardirossian and Bokoch, 2005; Bos *et al.*, 2007). The active enzyme then phosphorylates the regulatory subunit (MYPT1) of MLCP to inhibit its enzymatic activity (Matsui *et al.*, 1996). As a consequence, MLC remains in its phosphorylated form and contraction is sustained.

DAG-induced activation of PKC provides an additional mechanism that regulates MLCP activity (Nishizuka, 1992). PKC phosphorylates the PKC-potentiated protein phosphatase 1 inhibitor protein of 17 kDa (CPI-17) (Eto *et al.*, 1997). Phosphorylated CPI-17 has higher affinity for the catalytic subunit of MLCP (PP1c), inhibiting its activity and thereby reducing the dephosphorylation of MLC (Eto *et al.*, 1997; Kitazawa *et al.*, 2000; Sakai, 2005).

A recent study has compared pharmacological responses to carbachol and KCl with physiological responses to electrical field stimulation in bovine trachea to compare the signalling leading to sustained MLC phosphorylation and airway contraction (Gao *et al.*, 2017). Carbachol treatment increases phosphorylation of the MLCP regulatory subunit MYPT1 (via ROCK) and the phosphorylation of CPI-17 (via PKC), while KCl does not induce CPI-17 phosphorylation. In contrast to carbachol, atropine-sensitive EFS does not lead to MYPT1 phosphorylation, except in the presence of the cholinesterase inhibitor neostigmine. The authors suggest that a single stimulus provides sufficient ACh to induce rapid CPI-17 phosphorylation, but that ACh-induced $G_{q12/13}$ signalling leading to ROCK and MYPT1 occurs more slowly, and is thus only activated when the rapid degradation of ACh released by EFS is inhibited (Gao *et al.*, 2017).

The mechanisms underlying Ca^{2+} sensitivity in response to contractile agonists have been explored further by clamping the ASM intracellular Ca^{2+} to a fixed concentration so that contraction occurs in the absence to SR-localized Ca^{2+} oscillations. Two experimental approaches have been used to achieve this. So-called ‘skinned’ airways can be prepared by permeabilizing ASM cell membranes with pore-forming α-toxin or non-ionic detergents such saponin, β-escin, or Triton X-100 (Yoshii *et al.*, 1999; Somlyo and Somlyo, 2003; Pelaia *et al.*, 2008). An alternative pharmacological approach employing caffeine and ryanodine applied to PCLS avoids the potential loss of cell constituents with these chemical treatments (Sanderson *et al.*, 2008). Caffeine activates and opens RyRs by increasing their sensitivity to Ca^{2+}. The addition of ryanodine then maintains the RyR receptor in a partially open state. The emptying of Ca^{2+} from the SR results in the activation of stored-operated Ca^{2+} entry (SOCE), leading to sustained Ca^{2+} influx. It is likely that extrusion of Ca^{2+} via PMCA and NCX are still active under these conditions to limit SOCE. Consequently, the intracellular and extracellular Ca^{2+} levels

are stabilized when the Ca^{2+} movements across the ASM membrane reach a new equilibrium. This protocol allows assessment of responses to graded concentrations of extracellular Ca^{2+}, as well as the contribution of ROCK and PKC signalling to contractile responses.

Y-27632, a specific ROCK inhibitor, reverses contraction to MCh in α-toxin permeabilized rabbit and human airway preparations (Yoshii *et al.*, 1999). In caffeine/ryanodine-treated mouse PCLS, a sustained MCh-induced contraction is reversed by Y27632 or by GF-109203X, a highly selective PKC inhibitor, without changes in intracellular Ca^{2+} (Sanderson *et al.*, 2008). The effects of direct activation of PKC have also been assessed in mouse PCLS exposed to the caffeine/ryanodine treatment (Mukherjee *et al.*, 2013). Phorbol esters elicit strong airway contraction, associated with phosphorylation of CPI-17 and MLC, suggesting that PKC mediates sensitization of the contractile response to Ca^{2+} via MLCP inhibition (Mukherjee *et al.*, 2013).

ASM Ca^{2+} sensitivity varies with age and between species. In chemically skinned tracheal fibres from fetal and suckling pig airways, sensitivity to Ca^{2+} is three- to fourfold higher than airways from older animals (Sparrow and Mitchell, 2017). The Ca^{2+} sensitivity of mouse ASM is Ca^{2+} dependent, whereas the Ca^{2+} sensitivity of rat or human ASM is not (Bai *et al.*, 2009; Ressmeyer *et al.*, 2010). In caffeine/ryanodine treated human PCLS, intracellular Ca^{2+} can be progressively increased by increasing extracellular Ca^{2+}, leading to stepwise increases in sustained contraction of small airways. Airway contraction in these permeabilized human PCLS is increased further by histamine, even though intracellular Ca^{2+} levels are clamped (Bai *et al.*, 2009; Ressmeyer *et al.*, 2010).

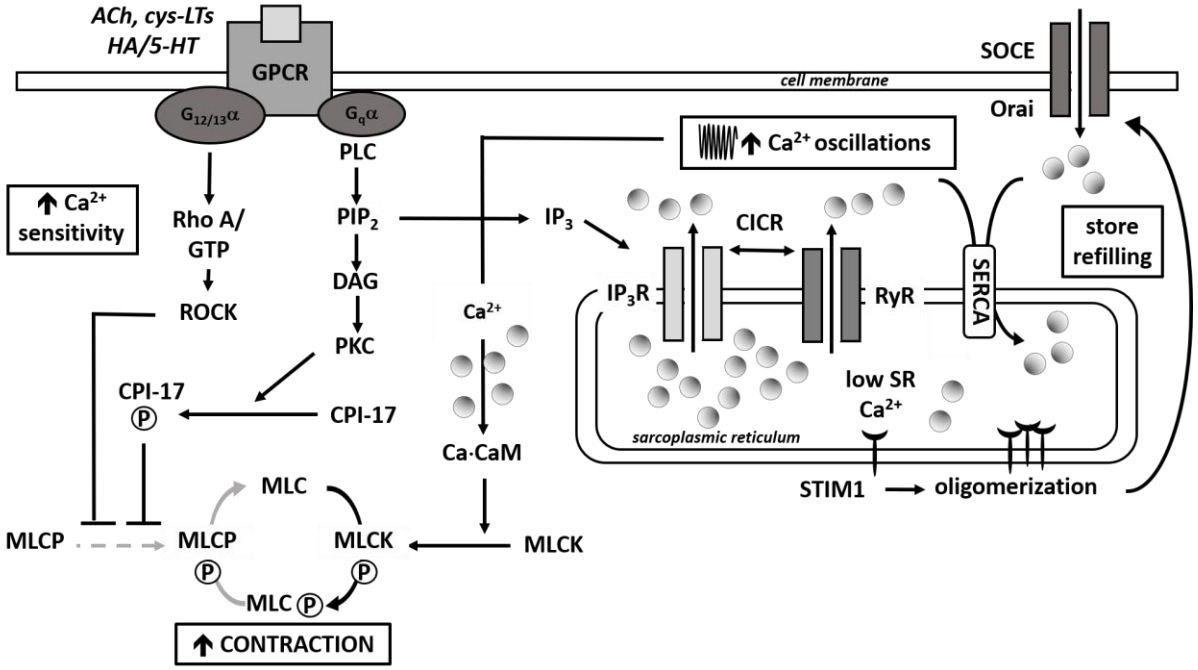


Figure 3. Contractile mechanisms in airway smooth muscle. Receptor binding by contractile agonists such as acetylcholine (ACh), histamine (HA, in human airways), serotonin (5HT, in rodent airways) or cysteinyl-leukotrienes (cys-LTs), initiates a signalling cascade that leads to airway contraction via the parallel pathways of calcium (Ca^{2+}) oscillations and sensitivity. Through coupling to G_q, inositol-1,4,5-trisphosphate (IP_3) is generated from the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by phospholipase C (PLC). IP_3 initiates Ca^{2+} oscillations by activating IP_3 receptors on the sarcoplasmic reticulum (SR) to release Ca^{2+}. SR-localized ryanodine receptors are activated by calcium-induced calcium release (CICR) and by cADP ribose (cADPR, not shown). Ca^{2+} oscillations occur as the Ca^{2+} released by IP_3R and RyR is taken up into SR stores via sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA). Increased Ca^{2+} binds to calmodulin (CaM) to activate myosin light chain kinase (MLCK). This phosphorylates myosin (MLC), leading to the formation of actin-myosin cross-bridges and contraction. The parallel Ca^{2+} sensitivity pathway inhibits the activity of MLC phosphatase (MLCP) to maintain contraction. Agonist binding activates the guanine-exchange protein RhoGEF (not shown) to converts GDP-RhoA (inactive) to GTP-RhoA (active). This activates Rho-kinase (ROCK) to phosphorylate MYPT1, the regulatory subunit of MLCP, to inhibit its enzymatic activity. Additionally, DAG-induced activation of protein kinase C (PKC) phosphorylates the PKC-potentiated protein phosphatase 1 inhibitor protein of 17 kDa (CPI-17) to inhibit MLCP. Sustained phosphorylation of MLC provides the main driver of the latter phase of a prolonged airway contraction. ASM Ca^{2+}stores are replenished via SERCA and by the oligomerization of the SR-localized STIM1, which then associates with Orai in the plasma membrane causing it to form channels, which mediate store-operated Ca^{2+} entry (SOCE).

1.1.4 Airway hyperresponsiveness (AHR)

AHR occurs when airways contract too easily and too much (Woolcock *et al.*, 1998). Increased airway sensitivity is not only a consequence of elevated levels of endogenous mediators but is also due to increased ASM mass and the influence of the inflammatory environment (Figure 4).

Early studies found that lung tissue and bronchi harvested from asthmatic patients released more histamine and were more responsive to house dust mite challenge compared to non-asthmatic tissues (Schild *et al.*, 1951). Critically, asthmatic patients can achieve greater maximal airway narrowing with histamine than non-asthmatic patients. Not only was this achieved at a lower concentration in asthmatic airways but the extent of airway narrowing, as measured by a decline in forced expiratory flow, was greater (Woolcock *et al.*, 1984).

1.1.4.1 Increased airway smooth muscle

The increased bulk of ASM in the airway wall has the potential to exacerbate the influence of multiple factors to further increase ASM contraction. The increase in airway wall thickening may cause ASM shortening which leads to increased luminal narrowing in asthma. In animal models of allergic asthma (using ovalbumin (OVA) or house dust mite (HDM)) or samples from asthmatics, changes in airway responses can be assessed *in vitro* in isolated ASM cells or airway preparations such as rings or PCLS or *in vivo* using invasive plethysmography to measure reactivity, most commonly to MCh.

1.1.4.2 ASM hypercontractility

It remains unclear whether ASM itself has intrinsic and persistent hypercontractility that contributes to AHR. *In vitro*, the contraction of human airways from subjects with asthma is reported to be increased, decreased or not different compared to airways from control subjects (reviewed in Wright *et al.*, 2013). Airway preparations from animal models of allergic airways disease with established *in vivo* AHR also yield conflicting results. For example, MCh-induced contraction of mouse tracheal rings, measured as increased isometric force, is increased following chronic OVA challenge but contraction of small airways in PCLS, measured as reductions in airway area, is reduced in the same model (Donovan *et al.*, 2013). These inconsistent findings may reflect differential contributions of inflammatory and structural influences on contraction. While increased ASM and inflammation may synergise to promote contraction in tracheal rings, interactions of ASM with non-contractile airway wall elements such as fibrosis in the smaller airways within precision cut lung slices (PCLS) may limit

contraction. Nevertheless, compelling *in vitro* and *in vivo* evidence implicates specific inflammatory mediators as major influences on ASM that increase airway contraction.

Additional insights from studies of isolated ASM suggest that shortening velocity of ASM *in vitro* is an additional measure of intrinsic ASM contractility. At the single cell level, the maximum capacity and velocity of shortening of zero loaded single ASM cells can be recorded. The response to electrical field stimulation is significantly increased in ASM from subjects with asthma relative to controls (Ma *et al.*, 2002). Furthermore, using collagen gel contraction assays, gels containing ASM cells from asthmatic donors have greater reductions in area in response to histamine and bradykinin (Matsumoto *et al.*, 2007; Sutcliffe *et al.*, 2012). In explants from agarose-inflated lungs, the rate of airway contraction is also greater in the hyperresponsive Fischer rat than Lewis rats (Wang *et al.*, 1997), while greater velocity and maximum ASM shortening is observed in human bronchi passively sensitized with serum containing high IgE (Mitchell *et al.*, 1994). This intrinsic ASM hypercontractility may also correlate with *in vivo* measurements of airway responsiveness. Higher cell traction forces at baseline and enhanced stiffening (contraction) in response to MCh and histamine, demonstrating that ASM may have an inflammation-independent contraction mechanophenotype in asthma (An *et al.*, 2016).

Several mechanisms underlying these intrinsic differences in ASM contraction have been described. MLCK is expressed at higher levels in biopsies from patients with asthma of increasing severity (Benayoun *et al.*, 2003), and increased in ASM from asthmatics associated with increased shortening velocity (Ma *et al.*, 2002). However, elevated MLCK was not detected in asthmatic ASM in collagen gels where greater contraction to histamine was detected (Matsumoto *et al.*, 2007). Fast MHC isoform and transgelin are also increased in human asthma and in hyperresponsive Fisher rats (Léguillette *et al.*, 2009). Of note, assessment of the rate of actin filament propulsion in purified tracheal myosin from Fischer rats is increased compared to normoresponsive Lewis rats, demonstrating the potential functional significance of increased fast myosin heavy chain (MHC) overexpression. Further studies to define the molecular mechanics of ASM are required to provide further insights into the contribution of intrinsic dynamics of ASM to AHR and asthma.

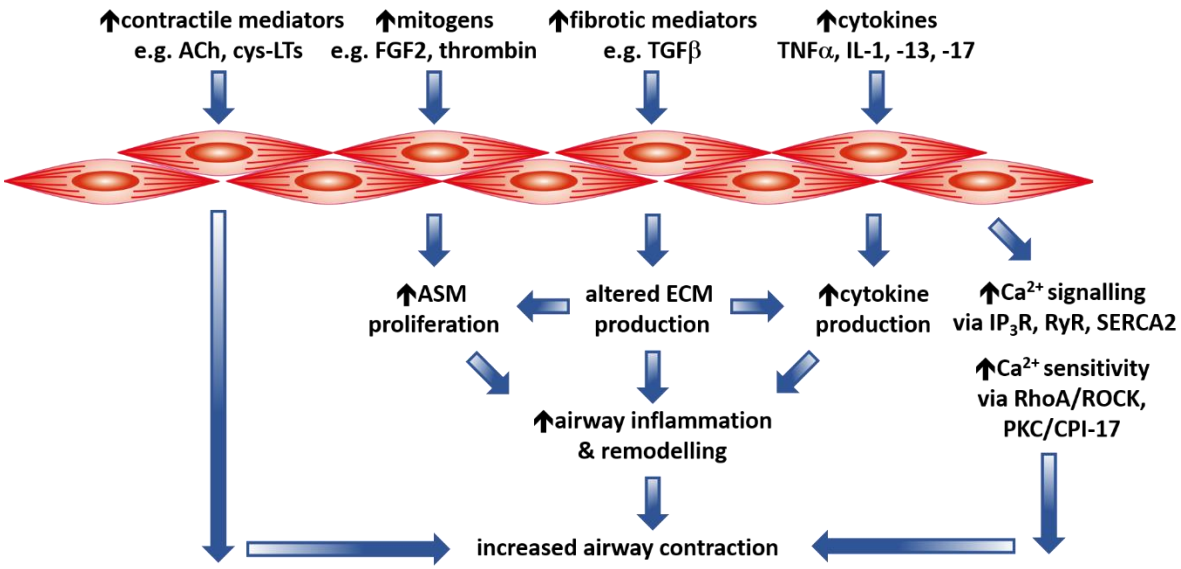


Figure 4. Mechanisms of increased airway contraction. Airway narrowing in chronic lung diseases may be increased as a consequence of multiple influences on ASM. These include elevated levels of contractile agonists acting an increased bulk of ASM in the remodelled airway. ASM can both produce and respond to inflammatory cytokines that increase the expression of receptors and dysregulate major pathways of SR-dependent Ca^{2+} signalling and Ca^{2+} sensitivity leading to contraction. Pro-fibrotic mediators alter the extracellular matrix (ECM) in the airways and promote further ASM proliferation and inflammation.

1.1.4.3 Altered ASM Ca^{2+} signalling

Changes in Ca^{2+} homeostasis and abnormal Ca^{2+} handling by ASM in response to contractile agonists have been identified as key mechanisms underlying increased ASM contraction. It is clear that elevated cytokines can influence the responsiveness of the increased bulk of ASM in the remodelled airway wall, and may regulate multiple aspects of Ca^{2+} signalling to drive increased airway contraction in chronic lung diseases. Studies in ASM and intact airways have assessed the direct effects of inflammatory stimuli as well as disease status (Sukkar *et al.*, 2001; Amrani, 2007; Gao *et al.*, 2010). Changes in SR-dependent Ca^{2+} oscillations and Ca^{2+} sensitivity, as well as in the dynamic properties of ASM are evident, consistent with the increased airway contraction observed *in vivo* (Hunter *et al.*, 2003; Janssen and Killian, 2006).

Recurrent Ca^{2+} oscillations in mature ASM become aberrant in severe asthma, with increases in frequency related to disease severity (Sweeney *et al.*, 2014). Furthermore, the Th2-derived cytokine IL-13 increases basal oscillations in human ASM (Matsumoto *et al.*, 2012). Given the contribution of Ca^{2+} to both contractile and synthetic functions of ASM, these changes provide indirect evidence of altered Ca^{2+} signalling as a driver of changes in structure and function of inflamed airways in asthma.

Calcium responses to contractile agonists are increased in isolated ASM treated with cytokines such as TNF-α and IL-13 (Amrani, 2007; Risse *et al.*, 2011). The exaggerated response in IL-13-treated ASM was attributed to enhanced Ca^{2+} release since levels of calponin, smooth muscle α-actin, vinculin, and myosin were unchanged (Risse *et al.*, 2011). ASM from asthmatics also shows exaggerated Ca^{2+} responses relative to ASM from non-asthmatics (Jude *et al.*, 2010; Mahn *et al.*, 2010).

There are multiple sites in the Ca^{2+} mobilization pathways for dysregulation under inflammatory conditions and in disease. To date, there is no evidence that expression of IP_3R itself is increased in human ASM from asthmatics (Mahn *et al.*, 2009; Sweeney *et al.*, 2015) despite its upregulation in response to IL-13 (Kellner *et al.*, 2008). Levels of bradykinin-induced IP_3 and Ca^{2+} responsiveness to photolysis of caged IP_3 are also similar in ASM from non-asthmatic and asthmatic subjects (Sweeney *et al.*, 2015). However, both IP_3 levels and IP_3R-mediated Ca^{2+} release are increased in ASM cells from a rat asthma model (Tao *et al.*, 2000). This is associated with lower expression/activity of IP_3-5 phosphatase, providing a mechanism promoting AHR through increased availability of IP_3, rather than increased IP_3R expression.

RyR activity may be increased in asthma by changes in the receptor itself or in response to increased cADPR via CD38. IL-13 upregulates RyR expression in human ASM (Kellner *et al.*, 2008). FKBP12.6 normally stabilizes RyR in a closed (inactive) state, but this regulatory protein is dissociated in rat ASM treated with asthmatic serum or with the cytokines IL-5, IL-13 or TNF-α, and also in isolated airways from a guinea pig model of OVA-induced AHR (Du *et al.*, 2014). Expression of CD38 in human ASM is increased by TNF-α (Amrani, 2007), and elevated in the airways of sensitized and challenged mice and in ASM from asthmatic compared to non-asthmatic donors (Jain *et al.*, 2008; Jude *et al.*, 2010). The TNF-α-induced increase in contractile responses of tracheal rings from non-sensitised mice is abrogated in mice deficient in CD38 (188). Thus, increased RyR expression, reduced inhibition of its activity or increased activation via CD38 could induce higher ASM Ca^{2+} for the same level of agonist to increase contraction, as recently modelled (Croisier *et al.*, 2015).

Increased SR-dependent Ca^{2+} signalling, via effects on both IP_3R and RyR, is implicated in the pro-contractile effects of IL-13 (Kellner *et al.*, 2008). Treatment of human ASM with IL-13 also further enhances the frequency of LTD4-induced Ca^{2+} oscillations, and the proportion of oscillating ASM cells (Matsumoto *et al.*, 2012). The increases in Ca^{2+} oscillations are suppressed to a similar degree by inhibitors of IP_3R or RyR (Matsumoto *et al.*, 2012). These changes are associated with increased mRNA for CD38 as well as the LTD4 receptor $CysLT_1$,

(Matsumoto *et al.*, 2012). These findings support the integrated contribution of increases in receptor expression and signalling to promote contraction.

Reduced SERCA2-mediated Ca^{2+} reuptake into the ASM SR could also increase airway contraction. IL-13 decreases the expression of SERCA2 in human ASM, associated with increased cytokine release (Kellner *et al.*, 2008). Expression of SERCA2 is lower in both native and cultured ASM from endobronchial biopsies of patients with mild and moderate/severe asthma compared to healthy subjects, and associated with increased ASM proliferation and cytokine release (Mahn *et al.*, 2009). However, the finding of decreased SERCA2 in asthmatic ASM is not universal (Sweeney *et al.*, 2015) and its contribution to increased airway contraction has yet to be assessed. Further studies are required to fully define the contribution of SR-dependent Ca^{2+} signalling to AHR.

Increased Ca^{2+} sensitivity of ASM via RhoA/ROCK and CPI-17 leading to increased contraction has also been demonstrated under inflammatory conditions and in allergen models (reviewed by Sakai *et al.*, 2017). Both TNF-α and IL-13 induce RhoA transcription in human ASM, via STAT6 and NFκB signalling, and increased phosphorylation of the regulatory subunit of MLCP (Hunter *et al.*, 2003). In α-toxin-permeabilized guinea pig airways, the TNF-α-induced increases in the proportion of active GTP-bound RhoA and increased MLC phosphorylation are inhibited by preincubation with the ROCK inhibitor Y27632 (Hunter *et al.*, 2003). IL-17A enhances contraction of mouse tracheal rings and human bronchi through an epithelial-independent effect that is not mediated via TNF-α, but is prevented by Y27632 (Kudo *et al.*, 2012). Repeated allergen challenge also appears to increase Ca^{2+} sensitization via ROCK or CPI-17 associated with AHR (Sakai *et al.*, 2017). These findings remain to be confirmed in ASM and airways from asthmatic subjects.

A recent study suggests that expression of G_q-protein coupled calcium-sensing receptors (CaSR) is increased in the ASM layer of bronchial biopsies from asthmatic subjects and in the airways of allergen-challenge mice (Yarova *et al.*, 2015). These receptors are known to control Ca^{2+} homeostasis by regulating the release of parathyroid hormone from the parathyroid gland via PLC-dependent pathways, but their role in the lungs has no previously been characterised. In addition to elevated levels in asthmatic ASM, CaSR expression is increased in ASM from non-asthmatic subjects by treatment with asthma-relevant cytokines TNF-α and IL-13. Activation of CaSR with polycations elevates intracellular Ca^{2+} in human ASM and increases the contractile response of mouse trachea and airways in PCLS to ACh (Yarova *et al.*, 2015).

This novel pathway may not only contribute to increased ASM responsiveness but may also increase Ca^{2+} sensitivity.

1.1.4.4 Influence of fibrosis

Although fibrosis can contribute to the development of AHR, it is unclear whether it directly influences airway contraction. The influence of airway fibrosis on airway contraction is difficult to define but increased fibrosis has been associated with increased asthma severity and reduced airway distensibility *in vivo* (Boulet *et al.*, 1997; Ward *et al.*, 2001).

TGFβ is the major mediator of fibrosis released during bronchoconstriction (Oenema *et al.*, 2013). TGFβ1 promotes the differentiation of fibroblasts into myofibroblasts, resulting in the stiffening of airways (Thannickal *et al.*, 2003). This suggests that airway responses may be limited by reduced airway distensibility. However, other processes which occur simultaneously including the upregulation of contractile protein expression via Smad protein signalling and dysregulated Ca^{2+} mechanisms can promote airway contraction and contribute to AHR. TGFβ increases the expression of SM22 calponin, α-SMA, MLCK and Ca^{2+} pathway proteins in both human ASM cells (Goldsmith *et al.*, 2006; Gao *et al.*, 2013) and guinea pig PCLS (Oenema *et al.*, 2013).

Interestingly, activation of muscarinic receptors with MCh was shown to augment TGFβ-induced contractile protein expression via the phosphorylation of glycogen synthase kinase-3β and 4E-binding protein 1 (Oenema *et al.*, 2012). In guinea pig PCLS, expression of contractile proteins was enhanced in both peripheral and central airways. This augmented response was blocked when actin polymerisation or TGFβ kinase was inhibited (Oenema *et al.*, 2012). The use of other bronchoconstrictors such as KCl also enhanced the contractile phenotype induced by TGFβ (Oenema *et al.*, 2012). In severe asthma where there is excessive fibrosis, it is unclear which TGFβ-mediated effect will prevail. The extent to which TGFβ regulates airway contractility and stiffness in asthma is yet to be determined.

In vivo airway fibrosis and inflammation was associated with the development of AHR in an allergic asthma mouse model (Royce *et al.*, 2009). Chronic administration of an anti-fibrotic treatment which didn't have any marked effect on airway inflammation inhibited and reversed AHR (Royce *et al.*, 2009). This suggests that fibrosis may directly contribute to the development of AHR.

In addition to potentially increasing airway sensitivity to contractile mediators, fibrosis and its downstream effectors may also limit bronchodilator efficacy. Bronchodilators must overcome the increased airway contraction (functional antagonism) and stiffness associated with fibrosis in asthma.

1.1.5 Current asthma treatments and limitations

Given this background it is clear that airway reactivity is altered in asthma and may be influenced by inflammation and remodelling, including fibrosis. Numerous experimental *in vitro* cell-based and tissue approaches and *in vivo* animal models that have been applied to provide insights into disease-relevant mechanisms leading to increased contraction or impaired relaxation as well as assessment of novel therapeutics compared to current treatments.

Multiple factors contribute to increased airway contraction in chronic lung diseases. Inflammation is targeted by inhaled glucocorticoids, the primary preventer used in the treatment of asthma. Activation of the glucocorticoid receptor results in the increased transcription of anti-inflammatory proteins (Hansbro *et al.*, 2017) and decreased expression of multiple inflammatory genes. However, a small percentage of asthmatics are steroid-resistant while others with severe asthma deteriorate unless maintained on oral steroids (Barnes, 2004). Symptoms of breathlessness are targeted by β_2-adrenoceptor (β_2AR) agonist such as salbutamol, the most commonly used acute reliever treatments for asthma. However, frequent use of salbutamol to overcome increased symptoms in severe disease results in desensitisation and loss of efficacy. Currently available therapies for asthma do not affect airway remodelling or provide a cure for this disease.

1.1.6.1 Targeting inflammation and fibrosis

Corticosteroids are the first line therapy to target inflammation in chronic asthma. The mechanism of action of glucocorticoids can be simplified into two major pathways: transactivation and transrepression (Barnes, 2010). Transactivation involves the increased synthesis of anti-inflammatory proteins such as annexin A1 while transrepression involves the inhibition of pro-inflammatory transcription factors such as NF-κB and activator protein 1 (Barnes, 2010).

While the gene-related mechanisms by which corticosteroids reduce inflammation are well defined, its effects on pro-inflammatory cells and mediators are less clear. Glucocorticoids reduce eosinophil numbers in lung biopsies and BAL fluid from asthmatic patients (Lim *et al.*, 1999; Basyigit *et al.*, 2004; Berry *et al.*, 2007). *In vitro* studies suggest that this may be due to

the steroid-induced apoptosis of eosinophils (Anderson, 1996; Meagher *et al.*, 1996). However, findings in *in vivo* asthma models are less convincing given that eosinophil apoptosis may occur regardless of steroid treatment (Erjefält and Persson, 2000). It is unclear whether glucocorticoids inhibit the generation of Th2 cytokines including IL-4, -5 and -13 which regulate airway eosinophilia. Neutrophils are relatively less sensitive to glucocorticoids (Schleimer, 1990) than eosinophils and this may be attributed to the inhibition of neutrophil apoptosis by steroids (Cox and Austin, 1997; Strickland *et al.*, 2002).

Mast cells are a rich source of mediators which contribute to inflammation and increased airway contraction. In lung biopsies from patients with mild atopic asthma, glucocorticoids were shown to reduce mast cell numbers localised to the airway mucosa (Jeffery *et al.*, 2013). However, the incubation of mast cells from the lung parenchyma with steroids for 12-24 h did not inhibit the release of histamine, PGD_2 or leukotriene C4 (Schleimer *et al.*, 1983).

Although glucocorticoids are widely used to target inflammation, their direct effects on inflammatory cells is variable. Nevertheless, glucocorticoids have been shown to reduce eosinophils and mast cells in the lungs of asthmatic patients.

Current guidelines suggest the use of progressively higher doses of inhaled corticosteroids with increasing severity of asthma symptoms (Table 2) (Global Initiative for Asthma, 2018, 2019). Given the influence of inflammation on airway contraction, it is important to optimise anti-inflammatory therapy, when glucocorticoids are ineffective. Clinical translation of experimental findings implicating specific mediators of inflammation as drivers of AHR has proved challenging, due to the potential redundancy of the multiple inflammatory cells and cytokines impacting on airway reactivity (Figure 1). To date, the use of selective biologics targeting elevated IgE, eosinophilic inflammation by IL-5 and Th2 cytokines including IL-13 has also been shown to be of benefit in selected patients with asthma (Darveaux and Busse, 2015).

Table 2. Recommended options for initial controller treatment in adults with asthma.

(Global Initiative for Asthma, 2018, 2019)

presenting symptoms	preferred initial controllers	other controller options
• need for SABA ≥ twice a month • no waking due to asthma in last month • no risk factors (ie. smoking, low lung function) • no exacerbations in the last year	as needed low dose ICS/LABA	low dose ICS taken whenever SABA is taken
• infrequent asthma symptoms • >1 risk factor • used oral corticosteroids in the last year • been in intensive care for asthma	daily low dose ICS or as-needed low dose ICS/LABA	leukotriene receptor antagonist (LTRA), or low dose ICS taken whenever SABA is taken
• need for SABA between twice a week and twice a month • waking up due to asthma ≥ once in a month	low dose ICS/LABA	medium dose ICS, or low dose ICS/LTRA
• troublesome asthma symptoms most days • waking up due to asthma ≥ once in a week	Medium dose ICS/LABA	high dose ICS, add-on tiotropium or add-on LTRA
• severely uncontrolled asthma, or with an acute exacerbation	high dose ICS/LABA refer for phenotypic assessment ± add-on therapy (ie. tiotropium, anti-IgE, anti-IL4/5R	low dose OCS

ICS= inhaled corticosteroids; LABA= long acting β_2-agonist; LTRA= leukotriene receptor antagonist; OCS= oral corticosteroid; SABA= short-acting β_2-agonist

While glucocorticoids are generally effective at treating inflammation, they do not target fibrosis. Airway fibrosis can be modelled *in vitro* using fibroblasts or ASM seeded in collagen matrices, where cell-mediated contraction can be enhanced by the profibrotic mediator TGF-β1. The glucocorticoid, dexamethasone, was shown to augment collagen gel contraction by bronchial fibroblast and primary HFL-1 fibroblasts (Miki *et al.*, 2000). In addition, budesonide had no effect on TGF-β1-induced human bronchial fibroblast differentiation measured as collagen deposition and changes in MMP and TIMP expression favouring fibrosis (Todorova et al., 2011). Furthermore, it has been demonstrated that ET-1-induced collagen remodelling is resistant to steroid treatment in collagen gels containing human ASM (Bourke *et al.*, 2011).

Some studies in bronchial biopsies from asthmatic patients and in OVA mouse models of AAD demonstrate the ability of steroids to partially inhibit subepithelial fibrosis (Hoshino *et al.*, 1999; Lee *et al.*, 2008), while others show no effect on airway remodelling (Duechs *et al.*, 2014). Critically, treatment with corticosteroids did not decrease production of pro-fibrotic cytokines (TGFβ-1, IL-11 and IL-17) or reduce the deposition of collagen type I and III in the airways of severe asthmatics (Chakir *et al.*, 2003).

1.1.6.2 Targeting airway contraction

Inhibition of airway contraction can be achieved by activating pathways to oppose contractile signalling via Ca^{2+} signalling and sensitivity (Figure 5). Airway relaxation can occur in response to activation of ASM β_2ARs – either by endogenous adrenaline or by synthetic selective short-acting β_2AR agonist (SABA) salbutamol or long-acting β_2AR agonists (LABAs), such as formoterol. Similar pathways are activated downstream of EP_2/EP_4 receptors by prostaglandin E_2, IP receptors by prostacyclin or A2b receptors by adenosine (Billington *et al.*, 2013).

Agonist binding leads to downstream signalling via receptor-coupled G_α subunits of the G_s subfamily of heterotrimeric G proteins. Stimulation of AC activity increases synthesis of cAMP and results in bronchoprotection (preventing the development of contraction) or bronchodilation (reversal of established contraction).

Other agents that increase cAMP and cGMP also share some common mechanisms and target multiple pathways that contribute to airway contraction (Figure 5). Substances that release NO directly activate intracellular GC to increase cGMP and cause airway relaxation but are less efficacious than those that increase cAMP. Phosphodiesterase (PDE) inhibitors such as theophylline decrease cyclic nucleotide breakdown and can enhance responsiveness to β_2AR agonists (Billington *et al.*, 2013).

The short-acting β_2AR agonist (SABA) salbutamol is the most commonly used inhaler medication to provide relief of acute asthma symptoms, while long-acting β_2AR agonists (LABAs), such as formoterol, are only used in combination with glucocorticoids as preventer medication in more severe disease. A clear benefit of these agents is their potential to simultaneously provide functional antagonism to the actions of the numerous pro-contractile agonists, but their efficacy may be limited in severe asthma.

Inhibition of Ca^{2+} signalling and sensitivity

The inhibitory effects of βAR agonists on ACh-induced Ca^{2+} oscillations were first described in isolated ASM (Nuttle and Farley, 1996). Inhibition was also evident in response to other cAMP-elevating agents, namely the stable cell-permeant analogue of cAMP (8-bromo-cAMP), the AC activator forskolin (FSK) and the non-selective PDE inhibitor 3-isobutyl-1-methylxanthine (IBMX) (Nuttle and Farley, 1996; Billington *et al.*, 2013).

Recent evidence is consistent with inhibitory effects of cAMP on Ca^{2+} release from the SR, the major mechanism initiating airway contraction. In mouse and human PCLS, βAR agonists (isoprenaline, non-selective, and salbutamol and fomoterol, both β_2-selective), as well as FSK and 8-bromo-cAMP, simultaneously reduce the amplitude and frequency of Ca^{2+} oscillations within ASM and relax intrapulmonary airways pre-contracted with MCh or histamine (Delmotte and Sanderson, 2008; Sanderson *et al.*, 2008; Ressmeyer *et al.*, 2010). In addition, IP_3-induced Ca^{2+} oscillations are reduced by the elevation in intracellular cAMP induced by FSK (Sanderson *et al.*, 2008).

NOC-5, a NO donor, also decreases 5HT-induced Ca^{2+} oscillations and airway contraction, and inhibits the increase of intracellular Ca^{2+} and contraction in response to photolytic release of IP_3 (Perez-Zoghbi *et al.*, 2010). The effects of NOC-5 are mimicked by the stable cGMP analogue, blocked by the GC inhibitor ODQ, and increased in the presence of zaprinast or vardenafil, two selective inhibitors of cGMP-specific PDE5 (Perez-Zoghbi *et al.*, 2010). These combined findings imply that elevated levels of cAMP and cGMP reduce the sensitivity of the IP_3R to IP_3, resulting in reduced Ca^{2+} release from SR stores and airway relaxation (Sanderson *et al.*, 2008; Perez-Zoghbi *et al.*, 2010).

Agents that increase cAMP can also cause airway relaxation by decreasing Ca^{2+} sensitivity (Billington *et al.*, 2013). In intact (i.e. not chemically permeabilized) guinea pig trachea, isoprenaline and FSK reduce MCh-induced contraction to a greater extent than Ca^{2+} mobilization, implicating inhibition of an additional Ca^{2+}-independent mechanism (Oguma *et al.*, 2006). More direct measures show that βAR agonists reverse carbachol-induced phosphorylation of Rho and ROCK, measured by Western in bovine trachea (Liu *et al.*, 2006) and that isoprenaline also increases MLCP activity, assayed directly using ^{32}P-labeled myosin (Janssen, 2015).

In PCLS following caffeine/ryanodine treatment, increased Ca^{2+} oscillations in response to contractile agonists are abolished, and contraction is due to Ca^{2+} sensitivity alone. Under these

conditions, relaxation to salbutamol, FSK and IBMX at fixed Ca^{2+} concentrations is maintained (Delmotte *et al.*, 2010). Of note, lower concentrations of formoterol are required to relax human airways when contraction is due to ASM Ca^{2+} sensitivity alone than when Ca^{2+} oscillations are also occurring (Delmotte *et al.*, 2010; Ressmeyer *et al.*, 2010), while neither NOC-5 nor a stable cGMP analogue induced relaxation in agonist-contracted airways after caffeine/ryanodine treatment (Perez-Zoghbi *et al.*, 2010). These differences may contribute to the longer duration of action of formoterol, and may contribute to the relatively lower dilator responses to agents that increase cGMP compared to cAMP.

cAMP-dependent hyperpolarization and consequent reduction of Ca^{2+} influx via activation of BK channels has been reported in ASM (Kotlikoff and Kamm, 2003). However, airway relaxation to βAR agonists and NO donors in many species including humans is either unaffected or only partially inhibited in the presence of K^+ channel blockers (Morgan *et al.*, 2014). This suggests that it is the inhibition of Ca^{2+} oscillations and sensitivity, rather than opening of BK channels, that mediates relaxation in the response to these agents.

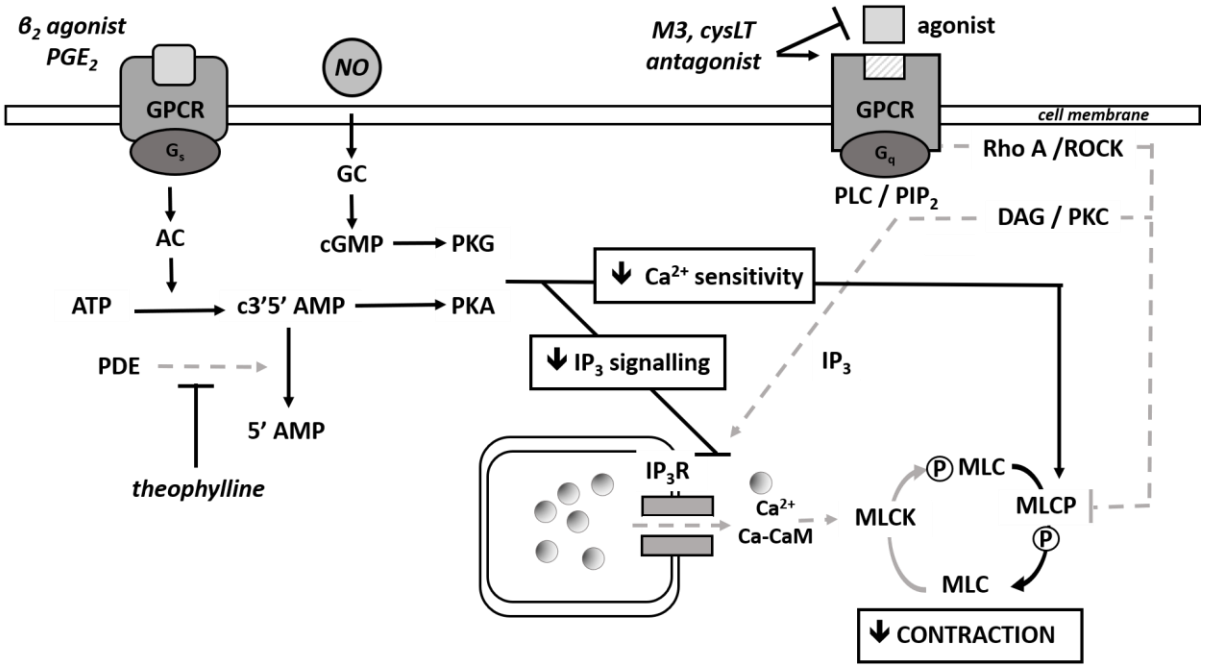


Figure 5. Dilator mechanisms in airway smooth muscle. Airway relaxation can be elicited when bronchodilators bind to G_s-coupled receptors on ASM. Agonists include β_2-adrenoceptor agonists (adrenaline, short- and long-acting β_2-adrenoceptor agonists (SABA, LABA)) and prostaglandin E_2 (PGE_2). Activation of adenylate cyclase (AC) generates c3'5'AMP from intracellular ATP. This can activate the cAMP- dependent protein kinase (protein kinase A, PKA). PKA phosphorylates multiple targets that regulate airway contraction, leading to decreased sensitivity of IP_3R to IP_3 and reduced Ca^{2+} oscillations. Ca^{2+} sensitivity is decreased through increased activity of myosin light chain phosphatase (MLCP). Dephosphorylation of myosin light chain (MLC) reduces formation of actin-myosin cross-bridges and opposes contraction. The breakdown of c3'5'AMP to 5'AMP by phosphodiesterase (PDE) can be inhibited by theophylline to elicit slowly developing airway relaxation and to enhance the actions of β_2-adrenoceptor agonists. An alternative dilator pathway via guanylate cyclase (GC) and cGMP in response to nitric oxide (NO) results in similar downstream signalling to the AC/cAMP pathway. Antagonists of muscarinic receptors for acetylcholine (ACh) or cysteinyl-leukotrienes (cys-LTs), selectively oppose the signalling cascades that lead to airway contraction via the parallel pathways of Ca^{2+} oscillations and sensitivity, but unlike β_2-adrenoceptor agonists, do not actively oppose the actions of other mediators of bronchoconstriction.

Functional antagonism

Mechanistically, the extent of airway relaxation versus contraction is a balance between the actions of bronchodilators versus bronchoconstrictors, known as functional or physiological antagonism. Thus, a highly potent β_2AR agonist that is maximally effective in a partially contracted airway becomes a less potent partial agonist in a maximally contracted airway. This is demonstrated *in vitro* in guinea pig trachea when both the potency and efficacy of salbutamol is reduced at increasing levels of carbachol-induced contraction (Lemoine and Overlack, 1992). The reduced tracheal smooth muscle relaxation correlates with a reduced ability of the drug to stimulate β_2AR-coupled AC, resulting in lower levels of cAMP to oppose contraction. β_2AR agonist-induced cAMP levels are also reduced in ASM from asthmatics, however the underlying mechanism is enhanced degradation rather than decreased synthesis, due to increased expression of PDE4D and hydrolysis of cAMP (Trian *et al.*, 2011).

In the clinical context, more severe asthma leads to greater AHR, increased airway contraction and potential lack of symptom control with standard doses of SABA. While this may be managed by the introduction of anti-inflammatory glucocorticoids to reduce the severity of symptoms, this is not always effective. The more frequent use of higher doses of β_2AR agonists promotes harmful effects in both the short and long term. Acute high doses of inhaled SABA can cause adverse effects if significant systemic absorption occurs. Loss of selectivity as a bronchodilator acting only in the airways occurs in terms of both location and site of action, exemplified by β_1AR-mediated tachycardia and β_2-mediated skeletal muscle tremor (Pera and Penn, 2016).

Homologous receptor desensitization and downregulation

Functional hyporesponsiveness may occur due to decreased expression of β_2ARs on ASM from subjects with asthma, either in response to repeated or chronic exposure to SABA or LABA or due to the effects of inflammatory mediators elevated in the airways.

SABA and LABA treatment can cause homologous desensitization and potentially downregulation of ASM β_2ARs (Billington *et al.*, 2013). Agonist occupation of β_2ARs leads to the binding of β-arrestin, which if prolonged, leads to receptor internalization. Internalized receptors can then be trafficked to lysosomes for degradation, resulting in a reduction in total ASM β_2-AR protein. Of note, siRNA targeting of β-arrestin in human ASM increases signalling downstream of β_2AR activation, while genetic ablation of β-arrestin increases both signalling and relaxation of contracted mouse airways *ex vivo* and *in vivo* (Deshpande *et al.*, 2012; Pera *et al.*, 2015). Although G protein-coupled receptor kinase (GRK) and β-arrestin are expressed

at similar levels in ASM from non-asthmatic and asthmatic subjects, a recent study has shown a defect in the dephosphorylation of endosomal β_2ARs which normally promotes their recycling back to the plasma membrane as resensitized receptors ready for agonist binding (Gupta *et al.*, 2015). Thus, GRK/arrestin-dependent mechanisms leading to desensitization of ASM β_2ARs and reduced resensitization are both likely to contribute to the loss of dilator efficacy with chronic use β_2AR agonists in asthma.

Heterologous receptor desensitization due to inflammation

A significant complication of chronic β_2AR activation was revealed when a prospective trial to introduce LABA in addition to usual therapy, the Salmeterol Multicentre Asthma Research Trial (SMART), was terminated early due to an increase in mortality in the treated group (Nelson *et al.*, 2006). Subsequent meta-analysis suggested that symptomatic relief provided by LABA was masking underlying effect uncontrolled airway inflammation, leading to increased severe and life-threatening asthma exacerbations, as well as asthma-related deaths (Salpeter *et al.*, 2006). As such, LABA are not used as sole therapy in the treatment of asthma, but only in combination with anti-inflammatory glucocorticoids.

The importance of controlling inflammation in asthma is therefore paramount. While inflammatory cytokines and lipid mediators can indirectly and directly increase airway contraction, they can also reduce airway sensitivity to bronchodilators. *In vitro*, both IL-4 and IL-13 impair isoprenaline-induced decreases in ASM cell stiffness (Laporte *et al.*, 2001). Moreover, chronic treatment of mouse tracheal rings with IL-1β or TNF-α attenuates the relaxant effect of β_2AR agonists (Guo *et al.*, 2005), and these cytokines synergize to reduce ASM responsiveness (Shore *et al.*, 1997). The loss of sensitivity to β_2AR agonists after IL-1β or TNF-α is mediated via induction of COX2 (Shore *et al.*, 1997). Subsequent increased PGE_2 production and activation of PKA leads to receptor phosphorylation and heterologous desensitization of β_2AR signalling (Guo *et al.*, 2005; Billington *et al.*, 2013).

Other dilators

The efficacy of SABA and LABA as bronchodilators may be limited in inflamed hyperresponsive airways and it is estimated more than half of asthmatic patients do not receive adequate control with current available treatments (Peters *et al.*, 1998).

The non-specific PDE inhibitor theophylline has potential benefit as bronchodilator, mediating relaxation via preventing the hydrolysis of cAMP and cGMP to their inactive forms. However, it has limited bronchodilator potency and efficacy when used alone compared to β_2-AR agonists, since it only causes the gradual accumulation of second messengers rather than driving their rapid synthesis. The use of theophylline is therefore primarily to enhance responsiveness to β_2AR agonists (Billington *et al.*, 2013) although additional beneficial anti-inflammatory actions have been described (Pera and Penn, 2016).

While pharmacological antagonists of muscarinic receptors, such as tiotropium, and leukotriene receptors such as montelukast, are also used clinically, the selectivity of their actions only provides bronchoprotection against airway contraction to ACh or cys-LTs, respectively. Of note, histamine receptor antagonists are not effective, even in allergic asthma, as they do not oppose the contribution of numerous other mediators to the allergic response leading to airway contraction. Although muscarinic receptor antagonists and leukotriene receptor antagonists are used less frequently than β_2-AR agonists, they are of benefit when vagal tone and cholinergic signalling are increased, or if levels of the potent bronchoconstrictor cys-LTs are elevated as proposed in aspirin-sensitive asthma.

Given the limitations of β_2AR agonists and these other bronchodilators, there are still opportunities for improved therapy to oppose airway contraction in asthma.

1.2 Relaxin

The peptide hormone, relaxin, is a Relaxin Family Peptide Receptor 1 (RXFP1) receptor agonist with cardioprotective effects in acute heart failure as well as established anti-fibrotic actions in several organs, including the lung. The investigation of relaxin as a novel bronchodilator is the major focus of studies outlined in this thesis.

Relaxin is a peptide originally identified as a hormone of pregnancy and primarily produced in the ovaries and placenta of pregnant mammals. It is also produced to a lesser extent in the prostate and testes (Bathgate *et al.*, 2013) of males as well as several non-reproductive organs of both genders including the heart (Dschietzig *et al.*, 2001) and lung (Royce *et al.*, 2009). It is now known that the actions of relaxin are not restricted to reproductive organs but extend to other organs including the heart, kidneys, skin, vasculature and lungs (Bathgate *et al.*, 2013; Royce *et al.*, 2014b; Samuel *et al.*, 2016).

Relaxin has been shown to abrogate fibrosis. Secretion of endogenous relaxin in pregnancy was thought to facilitate parturition by softening the ECM of the cervix and relaxing pelvic ligaments (Hisaw and Zarrow, 1950; Burger and Sherwood, 1995; Timmons *et al.*, 2011). Indeed, treatment of pregnant rats with a neutralizing antibody to relaxin led to more dense collagen bundles in the cervix (Lee *et al.*, 1992), while relaxin-deficient mice demonstrated incomplete cervical ripening and complications during labour that could be prevented by relaxin treatment (Parry *et al.*, 2009). However, a recent Phase II clinical trial showed that intravenous treatment with relaxin to induce labour did not advance the softening of the cervix (Weiss *et al.*, 2016). It has now been suggested that relaxin regulates collagen turnover in pregnancy to maintain vascular compliance and uterine blood flow. Vodstrcil *et al.* (2012) showed that rats treated with a monoclonal antibody against relaxin during late pregnancy developed increased uterine artery wall stiffness. The expression of RXFP1 receptors in the smooth muscle layer of the uterine artery (Vodstrcil *et al.*, 2012a) also implicated relaxin as a potential vasodilator.

In humans, relaxin belongs to a family of seven peptide hormones involved in the activation of G-protein coupled receptors (Bathgate, 2006). This family comprises of three relaxin peptides, termed H1 relaxin, H2 relaxin and H3 relaxin, and four insulin-like peptides (Wilkinson *et al.*, 2005). The recombinant human form of H2 relaxin (the major stored and circulating form of relaxin) has been shown to possess unique combined abilities to reduce fibrosis, inhibit inflammation, and induce smooth muscle relaxation in multiple organ systems. Its therapeutic

potential was confirmed in the 2013 RELAX-AHF (phase III) study, where early worsening of acute heart failure in hospital was reduced in relaxin-treated patients (30 µg/kg/day continuous *i.v.* infusion for 48 h). Following 6 h H2 relaxin treatment, these patients demonstrated reduced dyspnoea, which persisted throughout the next 5 days. Results at 180 days also demonstrated lower cardiovascular-related mortality and fewer adverse effects (Teerlink *et al.*, 2013). Interestingly, these findings were not replicated in a larger follow-up RELAX-AHF2 (phase III) study (Teerlink *et al.*, 2017).

The potential therapeutic application of H2 relaxin, which will simply be referred to as relaxin, to oppose airway inflammation, fibrosis and AHR, as well as to directly elicit airway relaxation will be outlined in this chapter.

1.2.1 Relaxin and RXFP1 expression

Relaxin, a member of the relaxin peptide family, is encoded by the relaxin genes *rln1*, *rln2* and *rln3* and other insulin-like genes in humans. *Rln2* codes for the main circulating form of relaxin, human gene-2 relaxin (relaxin). Relaxin consists of two chains (A and B) linked by disulfide bonds. The B chain contains two arginine residues at positions 13 and 17 required for the binding of relaxin to its cognate G-protein coupled receptor, RXFP1. The expression and distribution of RXFP1 have been thoroughly evaluated in human and rodent cardiac, vascular and pulmonary tissue (Dschietzig *et al.*, 2011; Royce *et al.*, 2013a; Jelinic *et al.*, 2014; Lam *et al.*, 2016).

Potential roles for relaxin/RXFP1 in the regulation of contractility, airway remodelling and fibrosis were suggested by the localization of RXFP1 in neonatal rat cardiac myocytes and endothelial cells combined with relatively high expression in fibroblasts (The Human Protein Atlas; Samuel *et al.*, 2004)**.** In addition, RXFP1 was co-localised with α-smooth muscle actin (α-SMA) in smooth muscle in rat abdominal aorta, vena cava and pulmonary, renal and mesenteric arteries (Jelinic *et al.*, 2014). Furthermore, higher expression of RXFP1 in endothelial cells relative to vascular smooth muscle cells suggested that relaxin may regulate contractile function through endothelial-dependent and/or endothelial-independent mechanisms (Jelinic *et al.*, 2014). Although RXFP1 gene expression was similar in mesenteric arteries of spontaneously hypertensive and normal rats (Drongelen *et al.*, 2013), RXFP1 protein expression was reported to be lower in the atrial myocardium of patients with heart failure compared to non-diseased patients (Dschietzig *et al.*, 2011). Nevertheless, the therapeutic efficacy of relaxin was established in a Phase II trial in this disease context (Teerlink *et al.*, 2013).

In human lung biopsies (Royce *et al.*, 2013a) and mouse lung tissue (Royce *et al.*, 2013b), both relaxin and RXFP1 protein expression have been demonstrated, with co-expression detected in epithelial cells and to a lesser extent, fibroblasts and ASM, suggestive of an autocrine/paracrine mode of hormone action. Decreased expression of relaxin itself was also observed in biopsies from asthmatic patients, and associated with increased thickness of both the epithelial layer and reticular basement membrane (Royce *et al.*, 2013a). RXFP1 was shown to be relatively lower in biopsies from subjects with asthma and in fibroblasts from IPF patients compared to healthy controls (Royce *et al.*, 2013a; Tan *et al.*, 2016). However, this reduction in RXFP1 expression may not limit the therapeutic potential of relaxin in disease, as treatment with relaxin was associated with increased RXFP1 expression in a mouse model of AAD (Royce *et al.*, 2013b). These combined findings provide further impetus for assessing the therapeutic benefit of supplementing a potential lack of endogenous relaxin (Royce *et al.*, 2016). As detailed below, broad beneficial actions of relaxin in validated animal models of acute and chronic lung diseases have now been clearly established.

1.2.2 Anti-inflammatory effects of relaxin

Chronic inflammatory cascades contribute to increased airway contraction and disease progression with worsening symptoms in lung diseases. Although glucocorticoids are commonly used to treat inflammation, they become less effective with increasing disease severity and more prominent fibrosis (Ward and Walters, 2005). This relative insensitivity has been attributed to a variety of intrinsic mechanisms such as increased expression of the inactive glucocorticoid receptor isoform and changes in transcription factor abundance and/or localization (Keenan *et al.*, 2015). However, extrinsic factors, notably changes in ECM composition including increased collagen, have also been implicated in loss of steroid responsiveness (Keenan *et al.*, 2015).

1.2.2.1 Animal models of inflammation

There is increasing evidence that relaxin may elicit acute anti-inflammatory effects in both the lung and other organs. In perfused rat lungs *ex vivo*, ischemia-reperfusion (I/R) injury increased neutrophil accumulation, neutrophil elastase and ET-1 levels (Alexiou *et al.*, 2010, 2013). In the presence of relaxin (5 nM), these pro-inflammatory markers were significantly reduced compared to vehicle-treated I/R rat lungs. Although a direct comparison between relaxin and glucocorticoids has yet to be made in this setting of acute inflammation, *in vivo* pre-treatment with dexamethasone was shown to markedly attenuate I/R-induced oedema, tissue inflammation and inflammatory cytokines (Sun *et al.*, 2009).

Further anti-inflammatory actions of relaxin have been demonstrated in chronic lung disease models. In a cigarette-smoke (CS) model of COPD, guinea pigs were exposed to 8 week CS treatment, followed by daily relaxin (1 or 10 µg/day *s.c.*, or 10 µg/mL/day via aerosol) (Pini *et al.*, 2016). Via either route, relaxin markedly inhibited CS-induced inflammation, reducing pro-inflammatory cytokines, TNFα and IL-1β, in lung tissue whilst increasing levels of the anti-inflammatory cytokine, IL-10. Although the effect of relaxin on the neutrophilia seen in this chronic model was not assessed, Bani and colleagues have shown that porcine relaxin can inhibit the activation of human neutrophils (Masini *et al.*, 2004) as well as the lipopolysaccharide-induced adhesion of neutrophils to coronary endothelial cells in rats (Nistri *et al.*, 2003) (via a nitric oxide (NO)-dependent mechanism; suggesting that relaxin may inhibit neutrophil infiltration and/or activation as part of its anti-inflammatory actions.

In the context of airway inflammation associated with asthma and allergy, relaxin shows promise but its effects have yet to be fully defined. In OVA-sensitized guinea pigs, pretreatment with relaxin (30 µg/kg i.n; twice daily for 4 days before each antigen challenge) inhibited the migration of pro-inflammatory cells into the lung and reduced mast cell degranulation and release of inflammatory mediators after allergen challenge (Bani *et al.*, 1997). However, in an OVA-induced mouse model of chronic AAD, the increase in eosinophils and inflammatory cell infiltration was not affected by relaxin treatment when administered after the onset of established inflammation (Royce *et al.*, 2009, 2013b). Dexamethasone has consistently been shown to reduced levels of both eosinophils and Th2-cytokines including IL-5, -6, -13 and 17A in chronic asthma models (Herbert *et al.*, 2008). These effects of dexamethasone (Patel *et al.*, 2016) or other corticosteroids (Royce *et al.*, 2013b) were not enhanced, but only maintained by relaxin co-administration. Relaxin was more recently found to promote macrophage polarization in the kidney towards a potentially tissue-repairing M2

macrophage phenotype (Chen *et al.*, 2017); and may thus promote similar effects in the lung (Jiang and Zhu, 2016).

1.2.3 Anti-fibrotic effects of relaxin

Fibrosis is a major contributor to both the pathology of chronic respiratory diseases and accumulation of fibrotic tissue leads to loss of sensitivity to therapy. While ECM deposition is critical for normal wound repair and restoration of organ function, dysregulation of the balance of synthesis and degradation of collagen can lead to excessive scarring, reducing compliance and dilator responsiveness in fibrotic airways. There is an unmet need for effective therapeutics that abrogate the increasing contribution of fibrosis to airway remodelling seen in more severe asthma, as this is unresponsive to steroid treatment. Accumulating evidence demonstrates the significant potential of relaxin as a novel anti-fibrotic therapy for chronic lung diseases.

1.2.3.1 Fibroblasts *in vitro*

Relaxin has been shown to limit excessive fibrosis by promoting the degradation of collagen via the increased activation of MMPs such as collagenases and gelatinases. Several studies have characterised the *in vitro* effects of relaxin on dermal, renal and cardiac fibroblasts, showing that relaxin consistently reduces transforming growth factor beta-1 (TGFβ1)-stimulated increases in proliferation, procollagen-α1 mRNA, collagen synthesis and secretion (Unemori and Amento, 1990; Masterson *et al.*, 2004; Samuel *et al.*, 2004). In TGF-β1-stimulated human dermal fibroblasts and primary rat renal myofibroblasts, MMP-1, -2, -9 and -13 respectively were increased with relaxin treatment (Chow *et al.*, 2012). Similar findings have been obtained in human lung fibroblasts, where enhanced collagen and fibronectin expression in response to TGF-β1 was inhibited by relaxin (up to 100 ng/ml, 16.8 nM) via the increased expression of pro-collagenase (MMP-1) (Unemori *et al.*, 1996). These actions of relaxin could inhibit fibrosis in the context of lung diseases.

1.2.3.2 Animal models of fibrotic disease

In animal models, relaxin expression has been shown to be protective against normal age-related progression of lung fibrosis (Samuel *et al.*, 2003). Parenchymal fibrosis, bronchial epithelial thickening and enhanced collagen deposition around pulmonary arterioles were higher in relaxin-deficient mice compared to wild-type mice by 9 months of age (Samuel *et al.*, 2003). This lung phenotype, reminiscent of increased pulmonary fibrosis, resulted in lower peak expiratory flow and lung recoil. In addition, these mice demonstrated airway wall thickening and a focal loss of alveolar structure. With 14 day relaxin treatment (0.5 mg/kg/day, *s.c.*), total collagen content in the lungs of 9- and 12-month old relaxin-deficient mice was reduced to levels exhibited by age-matched wild-type mice (Samuel *et al.*, 2003).

Relaxin has also been demonstrated to have beneficial effects on key histological markers of bronchial remodelling in *in vivo* models of COPD, IPF and asthma. In a chronic model of COPD, daily systemic or aerosol administration of relaxin to guinea pigs abrogated increases in airway smooth muscle thickness and collagen accumulation induced over 8 week exposure to CS (Pini *et al.*, 2016). In contrast, studies in numerous preclinical animal models have shown that the inflammatory and structural changes associated with CS exposure are steroid-insensitive (Stevenson and Belvisi, 2008).

In mouse models utilising bleomycin to induce key features of IPF, pirfenidone was shown to have potential of as an anti-fibrotic agent (Mouratis and Aidinis, 2011), while glucocorticoids reduced oedema but failed to suppress pulmonary fibrosis (Langenbach *et al.*, 2007; Oku *et al.*, 2008). Relaxin-deficient mice had exaggerated fibrosis in response to bleomycin (Huang *et al.*, 2011), while continuous subcutaneous infusion of a RXFP1 receptor agonist, CGEN25009, or treatment with relaxin itself, reduced bleomycin-induced lung fibrosis (Unemori *et al.*, 1996; Pini *et al.*, 2010; Huang *et al.*, 2011). In these studies, relaxin reduced parenchymal collagen deposition, as measured by histological scoring of alveolar fibrosis and hydroxyproline assays of total lung collagen (Unemori *et al.*, 1996; Pini *et al.*, 2010; Huang *et al.*, 2011). A direct comparison of the relative efficacy of relaxin and pirfenidone on reversal on established fibrosis would therefore be of interest in determining their relative therapeutic potential in the treatment of IPF.

In models of AAD, airway remodelling and fibrosis are implicated in the development of AHR. Increases in subepithelial collagen were amplified in relaxin-deficient mice following allergen challenge, with these mice having lower levels of MMP-9 and failing to respond to OVA with

the compensatory increase in MMP-2 seen in wild-type mice. Despite this, AHR was not greater in OVA-challenged relaxin-deficient mice compared to their wild-type counterparts. However, when infused over the second half of a 4 week exposure to OVA, relaxin (1 mg/mL, *s.c.*; 1 µL/h for 2 weeks) reduced airway collagen (Kenyon *et al.*, 2003). Treatment was also able to reverse established OVA-induced remodelling and fibrosis (Royce *et al.*, 2009, 2013b, 2014a). These effects were mediated via the upregulation of MMP-2 and -9 to increase collagen turnover and inhibition of TGF-β1 signal transduction at the level of Smad2 phosphorylation to decrease collagen synthesis (Royce *et al.*, 2009, 2013b, 2014a). When relaxin was administered either during or post-allergen challenge, these anti-fibrotic effects were associated with reduced AHR to MCh (Royce *et al.*, 2009, 2013a, 2014a).

Recent studies have assessed the effects of treatment with the combination of relaxin (0.5 mg/kg/day, *i.n.*) and human bone marrow-derived mesenchymal stem cells (Royce *et al.*, 2015) or human amnion epithelial cells (Royce *et al.*, 2017), administered for two weeks after a chronic allergen challenge. These treatments markedly reduced OVA-induced airway subepithelial collagen and total lung collagen to levels comparable to saline-treated mice, and also reversed established AHR (Royce *et al.*, 2009). The effects of combination treatment with relaxin (0.8 mg/mL *s.c.*; 0.5 µL/h for 14 days) and methylprednisolone (0.3 mg/kg *i.p.*; 3 times per week for 6 weeks) has also been assessed with both drugs administered during chronic allergen challenge (Royce *et al.*, 2013b). In this particular model, glucocorticoid treatment alone did not reduce fibrosis or AHR, but the combined effects of methylprednisolone and relaxin reduced subepithelial collagen thickness to a greater extent than corticosteroid treatment alone. Additionally, the inhibition of AHR induced by relaxin alone was maintained with the combination treatment (Royce *et al.*, 2013b).

Table 3. *In vivo* effects of relaxin in animal models of lung disease

model	species	relaxin dose (daily)	outcomes
cigarette smoke (COPD)	guinea pig [161]	10 µg/day *s.c.* for 8 wks *(with smoke)*	↓ TNF-α, IL-1β ↑ IL-10 ↓ airway smooth muscle ↓ collagen
ovalbumin/AAD (asthma)	mouse [172]	0.5 mg/kg *i.n.* for 14 d *(post-allergen)*	↔ BAL cell count ↑ MMP-2 and MMP-9 ↓ collagen ↓ AHR
bleomycin (IPF)	mouse [162]	100 µg *i.p* for 7 d *(post-bleomycin)*	↓ lung injury (TBARS) ↓ leukocyte infiltration (MPO) ↓ bronchial smooth muscle layer ↓ parenchymal collagen ↓ contractile protein (MLC20)

AHR= airway hyperresponsiveness; BAL=bronchoalveolar lavage; IL= interleukin; *i.n.*= intranasal; MLC20= myosin light chain (20 kDa); MMP= matrix metalloproteinase; MPO= myeloperoxidase; *s.c.*= subcutaneous; TBARS= thiobarbituric acid reactive substances; TNFα= tumour necrosis factor-α

1.2.4 Inhibition of contractility by RLX

1.2.4.1 Fibroblast contractility

Fibrosis is associated with differentiation of fibroblasts to myofibroblasts, characterised by increased expression of contractile proteins as well as greater synthesis of collagen. In addition to its actions in promoting collagen degradation, relaxin (16.8 nM) has been shown to abrogate TGF-β1-stimulated increases in α-SMA expression and contraction of collagen I gel matrices by cardiac and renal fibroblasts (Masterson *et al.*, 2004; Samuel *et al.*, 2004). Both fibroblast foci in lung biopsies from IPF patients and the lungs of mice following bleomycin treatment show increased levels of MLC20, a biomarker of *in vivo* cellular contractility (Huang *et al.*, 2011). *In vitro* treatment with exogenous relaxin (100 nM) prevented both collagen deposition and the increase in MLC20 in fibroblasts from IPF patients, while MLC20 overexpression prevented the protective effects of relaxin. The functional consequences of relaxin-mediated inhibition of MLC20 were confirmed by the reduction in the number of wrinkle-forming fibroblasts. In the same study, an even greater increase in bleomycin-induced MLC20 expression was evident in relaxin-null mice and prevented by *in vivo* treatment with relaxin (0.5 mg/kg/day, *s.c*. for 7 days, 14 days after bleomycin administration) (Huang *et al.*, 2011).

Table 4. *In vitro* effects of relaxin

	[relaxin]	outcomes
Human neutrophils [129]	1-100 nM	↓ neutrophil activation ↑ nitric oxide synthase ↑ nitric oxide generation
Human lung fibroblasts [130]	0.02-16.8 nM	↓ interstitial collagen type I and III ↓ collagen ↑ MMP-2 ↓ contractile protein (MLC20)
Rat perfused lungs *post ischemia-reperfusion* [2,3]	5 nM	↓ neutrophil accumulation ↓ neutrophil elastase ↓ ET-1

ET-1= endothelin-1; MLC20= myosin light chain (20 kDa); MMP= matrix metallopreoteinase

.2.4.2 Airway contractility in vitro

Chronic lung diseases are often associated with increased airway contraction that may not be adequately treated with current bronchodilators (Cazzola *et al.*, 2012). Of note, saline-treated relaxin-deficient mice exhibit AHR even in the absence of allergen challenge (Samuel *et al.*, 2007) and chronic treatment with relaxin has consistently been shown to reverse established AHR in mouse models of AAD (Royce *et al.*, 2009, 2014b), identifying a potential role for relaxin in the regulation of airway contractility as well as fibrosis. Indeed, the expression of RXFP1 in uterine, vascular as well as airway smooth muscle and their associated endothelium and epithelium suggests that relaxin may directly regulate muscle tone.

The vasodilator actions of relaxin (0.1-1000 nM) have been well-established *in vitro*, and include partial relaxation of rat uterine arterial rings precontracted with phenylephrine (Longo *et al.*, 2003). Angiotensin (Ang)-II-induced maximum constriction was greater in small mesenteric arteries from pregnant relaxin-deficient mice compared to matched wild-type mice, further implicating the protective effects of endogenous levels of this hormone (Marshall *et al.*, 2016).

Until recently, only one study had assessed the effects of relaxin on airway contraction. The authors demonstrated that relaxin, tested at a single concentration of 1000 nM, elicited approximately 75% of the maximal relaxation to the non-selective βAR agonist isoprenaline in mouse lung strips precontracted with carbachol (Samuel *et al.*, 2003). This suggests that some of the *in vivo* effects of chronic treatment with relaxin in reducing AHR may be due to direct inhibition of contraction.

1.2.5 Signalling mechanisms of relaxin

1.2.5.1 Receptor-dependence

Although relaxin is thought to mediate its actions via activation of RXFP1, selective RXFP1 antagonists have yet to be used to confirm the receptor dependence underlying its beneficial effects in the lung. However, RXFP1-deficient mice have provided some insights into the mechanism underlying its protective roles.

RXFP1-dependence has been implicated in the potential effects of endogenous relaxin on age-related fibrosis in vivo, since both peribronchial and perivascular fibrosis occurred more rapidly in the lungs of RXFP1-null mice than their age-matched wild-type counterparts (Kamat et al., 2004; Feng et al., 2005; Samuel et al., 2009).

The relaxin analogue B7-33, a single B-chain mimetic of relaxin, has been shown to activate RXFP1, and showed similar anti-fibrotic efficacy to relaxin in a chronic AAD model (Hossain et al., 2016), supporting a key role for RXFP1 in mediating the actions of relaxin. However, gene deletion of RXFP1 did not exacerbate OVA-induced inflammation, with similar increases in serum IgE, and circulating or bronchoalveolar lavage (BAL) cells seen in RXFP1-deficient mice and wild-type mice (Samuel et al., 2009). OVA-treated RXFP1-knockout mice also had comparable levels of myofibroblast differentiation markers, collagen deposition and gelatinase expression compared to OVA-treated wild-type mice. These combined findings suggest that while relaxin may act via RXFP1 in regulating/protecting against collagen deposition and fibrosis to maintain homeostasis, it may act through both RXFP1-dependent and -independent pathways to oppose the fibrosis associated with inflammation in AAD. The role of RXFP1 in the regulation of airway smooth muscle contraction by relaxin remains to be confirmed.

Relaxin may also mediate effects via glucocorticoid receptor (GR) activation. Inhibition of endotoxin-stimulated secretion of cytokines from human macrophages by relaxin was prevented by the GR antagonist RU-486 (Dschietzig et al., 2009a), and associated with GR binding and activation, nuclear translocation and DNA binding (Dschietzig et al., 2009b). However, the protective effects of relaxin in reducing I/R-induced inflammatory changes in rat lungs were not affected by RU-486 (Alexiou et al., 2013). Thus, it appears that relaxin may inhibit inflammation via actions as an agonist at both GR and RXFP1, and therefore has the potential to offer additional benefit over commonly used glucocorticoids acting via GR alone.

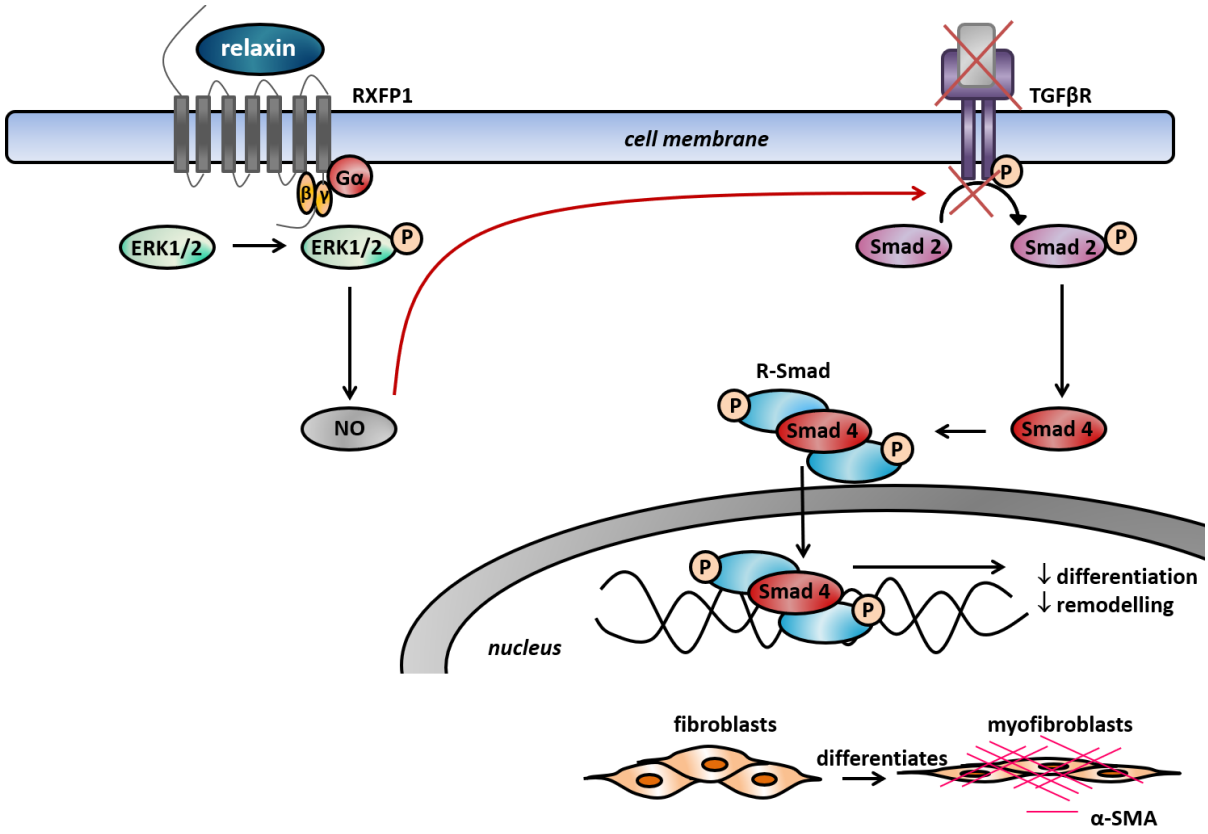
The ant-fibrotic actions of relaxin are also mediated through the activation of the Notch-1 pathway in cardiac fibroblasts. Relaxin was shown to prevent the reduced expression of Notch-1 in cardiac fibroblasts following TGFβ1 stimulation (Sassoli et al., 2013). Additionally, inhibition of Notch-1 signalling potentiated TGFβ-induced myofibroblast differentiation and diminished the effects of relaxin (Sassoli et al., 2013). Although similar results were shown in human umbilical vein endothelial cells (Zhou et al., 2015), no studies thus far have determined the contribution of this signalling pathway to the anti-fibrotic actions of relaxin in the lung.

In addition, relaxin has been implicated as an indirect mediator of the angiotensin type 2 receptor (AT2R) pathway to oppose renal fibrosis. Through bioluminescence resonance energy transfer, relaxin was shown to act through heterodimers that could form between RXFP1 and the AT2 receptor to increase pERK1/2 and nNOS expression (Chow et al., 2014). While angiotensin II signalling has not been associated with fibrosis in the airways, further investigation into this pathway may provide insight into the potential role of relaxin in vascular remodelling in pulmonary arterial hypertension via its actions through AT2 receptors.

Another alternative signalling pathway mediating the effects of relaxin has been demonstrated in cells overexpressing (HEK-RXFP1) or naturally expressing (THP-1) RXFP1, whereby antifibrotic effects were mediated through activation of peroxisome-proliferator activated receptorγ (PPARγ) via the cAMP/PKA pathway (Singh and Bennett, 2009; Singh et al., 2015). Although this mechanism for relaxin has yet to be tested in the lung, PPARγ receptor agonists such as rosiglitazone are well-established anti-fibrotic agents in chronic lung diseases (Honda, Marquillies, Capron, & Dombrowicz, 2004; Tan, Dagher, Hutton, & Bourke, 2010) and like relaxin itself, have been shown inhibit the development of AHR and cause direct relaxation of both large and small airways (Honda et al., 2004; Ward et al., 2004; Bourke et al., 2014).

1.2.5.2 Downstream signalling

Multiple mechanisms have been implicated in the actions of relaxin in opposing inflammation, reducing fibrosis and eliciting relaxation. Relaxin has been shown to exert its beneficial effects by inhibiting the downstream signalling pathways of specific drivers of fibrosis (summarised in Figure 6), and by opposing multiple mechanisms driving inflammation or contraction.



(revised from (Chow et al., 2012))

Figure 6. Mechanism of action of relaxin on fibrosis. TGFβ-1 activates its receptor on fibroblasts to cause Smad2 (and Smad3) phosphorylation (the complexing of phosphorylated Smad2 and phosphorylated Smad3 with Smad4; and the translocation of Smad2/3/4 complexes from the cytoplasm to the nucleus) leading to fibroblast differentiation into myofibroblasts. This is evidenced by increased α-smooth muscle actin (α-SMA), excessive collagen production and fibrosis. Relaxin acts on its cognate RXFP1 receptor to activate two subclasses of G-proteins, $G_{\alpha s}$ and $G_{\alpha OB}$ (not shown). Stimulation of the RXFP1 receptor by relaxin then causes ERK1/2 phosphorylation and stimulation of neuronal nitric oxide (NO) synthase (nNOS)-induced NO/sGC/cGMP signalling. NO/sGC/cGMP inhibits Smad2 phosphorylation and increases the expression of matrix metalloproteinases-1, -2 and -9 (MMP-1, -2, -9). These gelatinases help to break down extracellular matrix proteins.

The coupling of RXFP1 activation to ERK1/2/NO/sGC/cGMP signalling has been well characterised, leading to anti-inflammatory, anti-fibrotic and dilator effects (Figure 6). In a model of I/R injury of rat lungs, the inhibition of neutrophilic inflammation by relaxin was

associated with protection against I/R-induced downregulation of eNOS expression and NOS activity. This anti-inflammatory effect was blunted by PD-98059 and wortmannin implicating PI3K and ERK1/2 as downstream mediators of relaxin-induced NO production (Alexiou *et al.*, 2013). Additionally, *in vitro* activation of human neutrophils, induced in response by different proinflammatory agents (N-formylmethionine-leucyl-phenylalanine and phorbol-12-myristate-13-acetate), was inhibited by relaxin. This inhibition was prevented by co-treatment with L-NMMA, an inhibitor of NO production (Masini *et al.*, 2004). Thus, relaxin and associated increases in NO signalling could confer protection following acute lung injury, and may also be of relevance in limiting neutrophil activation in lung diseases such as severe asthma and COPD.

Relaxin may also exert its anti-fibrotic effects through the NO/sGC/cGMP pathway to negate the pro-fibrotic influence on TGF-β1 signalling (Figure 6). In rat renal myofibroblasts (activated fibroblasts), inhibition of TGF-β1-induced collagen synthesis and gelatinase activity by relaxin was prevented by the ERK1/2 inhibitor, PD98059, the NOS inhibitor, L-NAME, and the guanylate cyclase inhibitor, ODQ (Chow *et al.*, 2012; Wang *et al.*, 2016). Similar findings were seen in lung myofibroblasts where relaxin-mediated inhibition of collagen synthesis was cAMP-independent, but associated with increases in NO synthesis and cGMP levels, and prevented by inhibition of NOS (Huang *et al.*, 2011).

In terms of specifically regulating TGF-β1 signalling, relaxin was shown to activate RXFP1 to inhibit TGF-β1-induced phosphorylation and translocation of Smad2 to the nucleus in human renal fibroblasts, in the absence of any direct effects on Smad3, Smad4, Smad7 or ERK/MAPK (Heeg *et al.*, 2005). Consistent with these findings, *in vivo* treatment with relaxin was able to disrupt TGF-β1 signal transduction in a chronic model of AAD (Royce *et al.*, 2014b). Allergen challenge increased TGF-β1, airway collagen deposition and AHR, with subsequent relaxin treatment reducing Smad2 phosphorylation without affecting TGF-β1 expression, leading to partial reversal of the pro-fibrotic actions of TGF-β1 in the lung as well as reduced AHR (Royce *et al.*, 2014a).

The effects of relaxin on smooth muscle tone may be mediated either by direct effects or via increased synthesis of endothelial/epithelial-derived factors to oppose contraction (Figure 2). Evidence from vascular studies implicates endothelial-derived NO and prostaglandins, and subsequent increases in cGMP and cAMP since relaxin-mediated relaxation of rat uterine artery was decreased by L-NAME, ODQ, and the adenylate cyclase inhibitor, SQ22-22 (Longo *et al.*, 2003), while endothelial removal abolished acute relaxin-induced vasodilation of small

rat renal arteries (McGuane *et al.*, 2011). Similar downstream mechanisms to those established in the vascular context have been implicated in the regulation of airway smooth muscle contraction by relaxin. The capacity of relaxin to elicit relaxation via these multiple mechanisms may allow its efficacy to be maintained even if epithelial-mediated signalling is impaired as part of the disease process.

1.3 Aims and hypothesis

The overarching aim of this thesis is to identify relaxin as an improved treatment targeting increased fibrosis and contraction in chronic lung diseases. The current background shows that relaxin may possess these dual actions relevant to asthma.

The anti-fibrotic effects of relaxin have been established in lung fibroblasts from IPF patients, but this has yet to be tested in airway fibroblasts in asthma. Relaxin *in vivo* inhibited the development of parenchymal fibrosis in a bleomycin-injured mouse model. Furthermore, a significant body of evidence supports the efficacy of relaxin in reversing established steroid-resistant airway fibrosis in chronic AAD. Although relaxin has been shown to be an effective vasodilator, only a single study to date has examine and demonstrated its bronchodilator efficacy.

It is critical to elucidate the beneficial effects of relaxin when used alone under conditions where current treatments are ineffective, and to assess its potential for additivity or synergy in combination with suboptimal therapies. Further confirmation of efficacy in validated models of chronic lung diseases relative to current therapy will support clinical translation of relaxin as a novel therapeutic opposing fibrosis and contraction in lung diseases.

The hypothesis of this thesis is that relaxin will oppose fibrosis and airway contraction in and maintain efficacy in disease context. This is will be addressed by the following aims. To:

1. optimise the preparation of precision cut lung slices (PCLS) (Chapter 3) in order to increase their application for later studies (Chapters 5-7)
2. compare the anti-fibrotic actions of relaxin to existing therapies using healthy and asthmatic human fibroblasts in collagen gel contraction assays (Chapter 4)
3. establish the *in vitro* dilator efficacy of relaxin in large and small airways and to define its underlying mechanisms of action (Chapter 5)
4. assess the combined effect of relaxin and βAR agonists across species, including under conditions of reduced responsiveness to and to define the mechanism of potentiation (Chapter 6)
5. determine whether the dilator effects of combined relaxin-salbutamol treatment are maintained in an acute HDM model (representing airway inflammation), a chronic TGFβ-overexpression model (airway fibrosis) and a chronic bleomycin model (lung fibrosis) (Chapter 7)

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Chapter 2: General Methods

The general methods outlined in this chapter have been applied over multiple chapters. These comprise of myograph and organ bath experiments (Chapters 5, 6), the precision cut lung slice (PCLS) technique (Chapters 3, 5-7) and histology and immunohistochemistry (Chapters 3, 5-7).

Additional methods specific to individual chapters and detailed experimental protocols, using the general methods described here, are outlined later in this thesis.

2.1 Sources of animals and human lung tissues

The following animals (aged 6-14 weeks) were obtained from the Monash Animal Research Platform (MARP), Victoria, for use in all experiments:

- naïve Balb/c mice (Chapters 3, 5 and 6), also used for bleomycin model (Chapter 7)
- naïve C57Bl/6 mice used for house dust mite (HDM) and TGFβ-overexpression model (Chapter 7)
- naïve Sprague Dawley rats (Chapter 4)
- naïve Tricolour guinea pigs (chapter 6)

All experimental procedures in animals were approved by the relevant Animal Ethics Committees of Monash University (approval MARP 2014/066, MARP 2018/018 and MARP 2018/027).

Additional tissue samples were obtained as follows:

- naïve marmoset lungs provided by Dr. Yan Wong from the Department of Physiology, Monash University (Chapters 3, 6)
- human lung samples provided by Dr. Glen Westall and Dr. Jade Jaffar, Alfred Hospital (Monash University Human Research Ethics Committee (MUHREC, approval CF16/406) (Chapters 3, 6)
- murine lungs from C57Bl/6 mice subjected to HDM provided by Dr. Maria Sukkar (Chapter 7; Nath *et al.*, 2014)
- murine lungs from transgenic TGFβ-overexpressing mice on a C57Bl/6 mice background provided by Prof. Phil Bardin and Dr. Belinda Thomas (Chapter 7)

2.2 Tissue collection

Mice and rats were humanely euthanized by sodium pentobarbitone overdose (60 mg/mL administered *i.p*. at a volume of 1 mL per 100 g body weight) for preparation of trachea tissue and PCLS. Guinea pigs were gently restrained and stunned by a single blow to the head. Loss of consciousness was confirmed by the lack of withdrawal reflex, followed by immediate exsanguination of the carotid artery.

2.3 Myograph and organ bath experiments

Segments of mouse, rat or guinea pig trachea of similar length were mounted on steel wires in a 5mL myograph (mouse, rat) or 10 mL organ bath (guinea pig) containing Krebs-Henseleit solution. The buffer solution was maintained at 37°C and bubbled with carbogen (95% O_2 with 5% CO_2, pH 7.4; 59 mM NaCl, 2.3 mM KCl, 0.69 M $MgSO_4.7H_20$, 2.5 mM $CaCl_2.6H_2O$, 0.6 mM KH_2PO_4, 10 mM EDTA, 25 mM $NaHCO_3$, and 6 mM glucose). One wire was connected to a force displacement transducer for continuous recording of changes in isometric tension (ΔmN or Δg), using Power Lab Chart Software (ADI Instruments) while the other served as an anchor for tissue preparation.

Tissues were stretched to optimal resting tensions (based on the response to KPSS 123.7 mM K^+) for each species and allowed to equilibrate for 1 hr. Following equilibration, tissues were challenged with potassium physiological salt solution (KPSS) to confirm tissue viability. A supramaximal concentration ACh was also tested to determine maximum airway contraction.

Responses to all drugs, either as a single addition or a part of a cumulative concentration-response curve, were allowed to reach a plateau before the next drug or concentration was added. Contraction responses were normalised to the KPSS or maximal ACh response. Relaxation responses were expressed as % relaxation after pre-contraction, usually with a submaximal concentration of contractile agonist.

2.4 Precision cut lung slice technique (PCLS)

PCLS were prepared with minor modifications from previously published methods (Bourke *et al.*, 2014; Donovan *et al.*, 2014) (Figure 1).

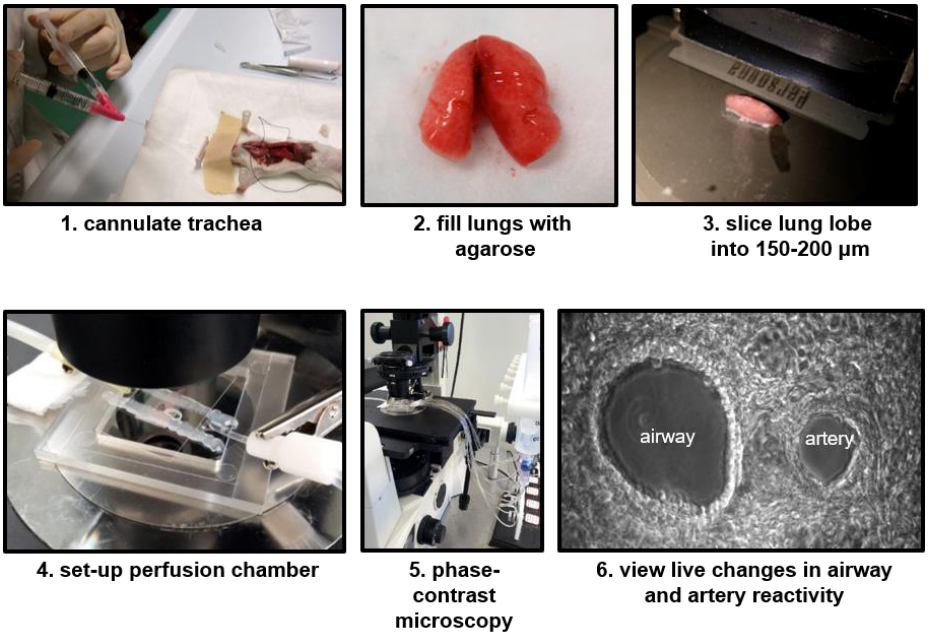


Figure 1. Summary of PCLS preparation and culture. Briefly, mice (1) trachea were cannulated in order to (2) fill lungs with warm agarose. Lungs were cooled at 4°C to provide a firm sample which can be sectioned. Lungs were removed from the chest cavity and a lobe was isolated and (3) slice at 150-200 µm with a vibratome. Following overnight incubation, PCLS were (4) set-up in a customised chamber. Responses to drug perfusions were (5) visualised with phase-contrast microscopy where (6) live changes in airway reactivity were recorded.

2.4.1 Lung slice - preparation

Mouse, rat or marmoset trachea were cannulated with a catheter containing two ports (20 G Intima, Becton Dickinson, VIC, Australia) and lungs were inflated with ~1.2 ml for mouse and ~10 mL agarose gel for rats and marmosets (2% agarose in 1x HBSS at 37°C), followed by a bolus of ~0.5 mL or 3 mL air. Lungs were cooled by bathing in cold HBSS/HEPES and rodents were then kept at 4°C to allow the agarose to solidify, before the lungs were removed. A lobe, most commonly the upper right lobe, was isolated and adhered with cyanocrylate to a mounting plate in a vibratome at a speed setting of 5.5 and frequency setting of 6 (VT Compresstome, Precisionary Instruments, Greenville, NC, US or Leica VT1200S). PCLS of 150-200 µm thickness were prepared and transferred into cell culture plates containing DMEM, supplemented with 1% penicillin-streptomycin and incubated for generally 24 h (37°C, 5% CO_2) prior to experiments as described below.

For human lung samples, one main bronchus was identified and cannulated. Other open-ended airways were clamped in order to prevent agarose from leaking. Lungs were filled with

20-40 mL agarose (depending on the size of the lung sample), followed by a bolus of 5-10 mL air then cooled. Human lungs were sectioned into 200 µm thin slices and cultured overnight as for other species before experiments were conducted.

2.4.2 Lung slice - mounting and microscopy

PCLS were transferred to HBSS/HEPES and mounted individually in custom-made perfusion chambers. To prepare the chamber, a PCLS was on a 45 x 50 mm cover glass. A hole was cut in a piece of nylon mesh over a viable airway (~200 µm diameter) to enable the airway to be visualized and to secure the PCLS during perfusion. Airways were selected based on the presence of an intact layer of epithelial cells with ciliary activity. A line of silicon grease was run along opposing sides the lung slice-nylon mesh preparation. This was covered by an additional 11 x 30 mm coverglass to form an enclosed chamber (approximate volume of 100 µL). Airway viability was confirmed based on responsiveness to a contractile agonist.

Phase-contrast microscopy was used to visualise airways within lung slices through an inverted microscope (Diaphot 300; Nikon) with a 10X objective lens, zoom adapter, reducing lens and camera (CCD camera model TM-62EX; Pulnix, JAI Inc. San Jose, CA). The second set-up consisted of an inverted microscope (Olympus IX50) with external light source (TH4-200), 10X objective lens and camera (CCD camera model BM-141GE, JAI Inc. San Jose, CA).

2.4.3 Lung slice - perfusion experiments

Slices were then perfused at a constant rate (1-3 mL/min) at room temperature through a gravity-fed perfusion system with eight separate channels connected to a manifold with a single outflow needle (Warner Instruments Inc., USA). Each channel was regulated by a valve (LFA, The Lee Company, CT, USA) under manual control from a local switchboard. Separate drug solutions were successively perfused at fixed time intervals depending on the drug under investigation.

2.4.4 Lung slice- image recording and analysis

Changes in airway lumen area were captured in the form of digital images (744 x 572 pixels) in time lapse (0.5 Hz) using image acquisition software (Video Savant; IO Industries, Inc.). Obtained files were converted to TIFF files and analysed using NIH/Scion software (Scion Corporation; download www.scioncorpcom) or ImageJ.

To differentiate the airway lumen from the surrounding parenchyma, a grey scale threshold of 10 was chosen using the VideoSavant software. Changes in airway lumen area were calculated as a percentage of the initial area derived from changes in the raw pixel count. When there was a lack of contrast between the lumen and the surrounding tissue, TIFF files are imported into ImageJ. These images stacks were analysed under an optimal threshold to better distinguish the lumen from the surrounding tissue. If these automated processes were ineffective due to lack of contrast, freehand manual tracing of the airway lumen was conducted where the last 10 frames of each perfusion condition were averaged using ImageJ.

For contraction experiments, raw pixel data for airway lumen area was expressed as % initial lumen area. For relaxation protocols, data was expressed as % relaxation (usually normalized to submaximal precontraction). All data was averaged over 30 frames (the last min) for each perfusion interval or over the last 10 frames with manual analysis.

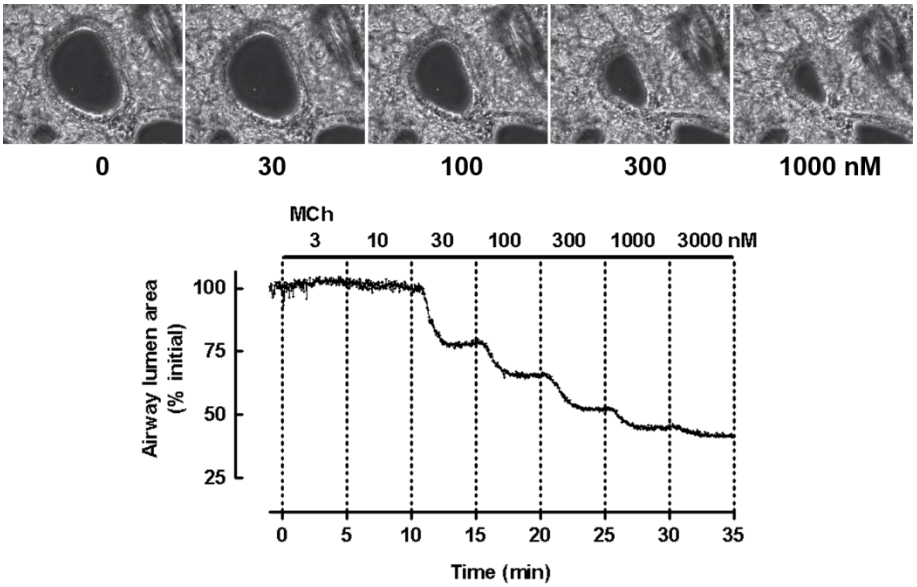


Figure 2. MCh-induced contraction in a small airway of in a mouse PCLS. Representative images were taken towards the end of each 5 min perfusion period. Individual points of the traces generated from each experiment represent data obtained from frames collected at 2s interval (0.5 Hz) and expressed as % initial lumen area relative to time.

2.5 Preparation of lung sections

Unused lung lobes from all experiments were fixed in 10% neutral buffered formaldehyde (Monash Histology Services; Department of Anatomy, Monash University) overnight and routinely processed and embedded in paraffin wax.

Formalin-fixed, paraffin-embedded tissues were serially cut into 4 µm thick sections and placed on SuperFrost charged microscope slides (Mkro Glass, Australia). To assess outcomes of fibrosis, sections were subjected to various histologic stains or immunohistochemistry.

2.5.1 Airway inflammation scoring

Blinded analysis was performed on H&E stained tissue sections to assess inflammation. Slides were washed and dewaxed before being incubated in Mayer's haematoxylin for 5 min, followed by eosin for another 5 min (performed by Monash Histology Platform).
Slides were assessed for airway inflammation where a score ranging from 0-4 was assigned for each airway (Borzone *et al.*, 2001; Nakagome *et al.*, 2006; Royce *et al.*, 2015) as summarized in Table 1.

Table 1. Description of inflammation scores.

score	Airway
0	no detectable inflammation
1	low peribronchial inflammatory cell aggregates; pooled area <0.1 mm^2
2	mild peribronchial inflammatory cell aggregates; pooled area ~0.2 mm^2
3	moderate peribronchial inflammatory cell aggregates; pooled area ~0.3 mm^2
4	widespread peribronchial inflammatory cell aggregates; pooled area ~0.6 mm^2

2.5.2 Airway morphometry

Blinded analysis was performed on Masson's Trichrome stained sections to assess airway structural changes. Dewaxed sections were incubated in pre-warmed Bouin's fixative for 1h and stained with Weigert's iron haematoxylin for 10 min, followed by Biebrich scarlet (stains non-collagen areas red) for another 15 min. Slides were also treated with phosphotungstic acid for 7 min to develop colour. Sections were then incubated in Aniline Blue solution for 15 min (Royce et al., 2013). Aniline Blue binds to collagen which results in the blue pigmentation of collagen fibers (performed by Monash Histology Platform).

Representative photomicrographs generated by Brightfield microscopy were scanned at x40 magnification using ScanScope AT Turbo (Aperio, CA, USA). Airways (5-10) were randomly selected from the tissue and analysed via Aperio ImageScope software. For all experiments, blinded analysis of slides was conducted.

Manual analysis of epithelial and subepithelial thickness relative to the basement membrane length was performed using the following equations:

$$\text{epithelial thickness} = \frac{(\text{basement membrane area- lumen area})}{\text{basement membrane length}}$$

$$\text{sub-epithelial thickness} = \frac{(\text{subepithelial collagen area-basement membrane area})}{\text{basement membrane length}}$$

2.5.3 Immunohistochemistry for RXFP1

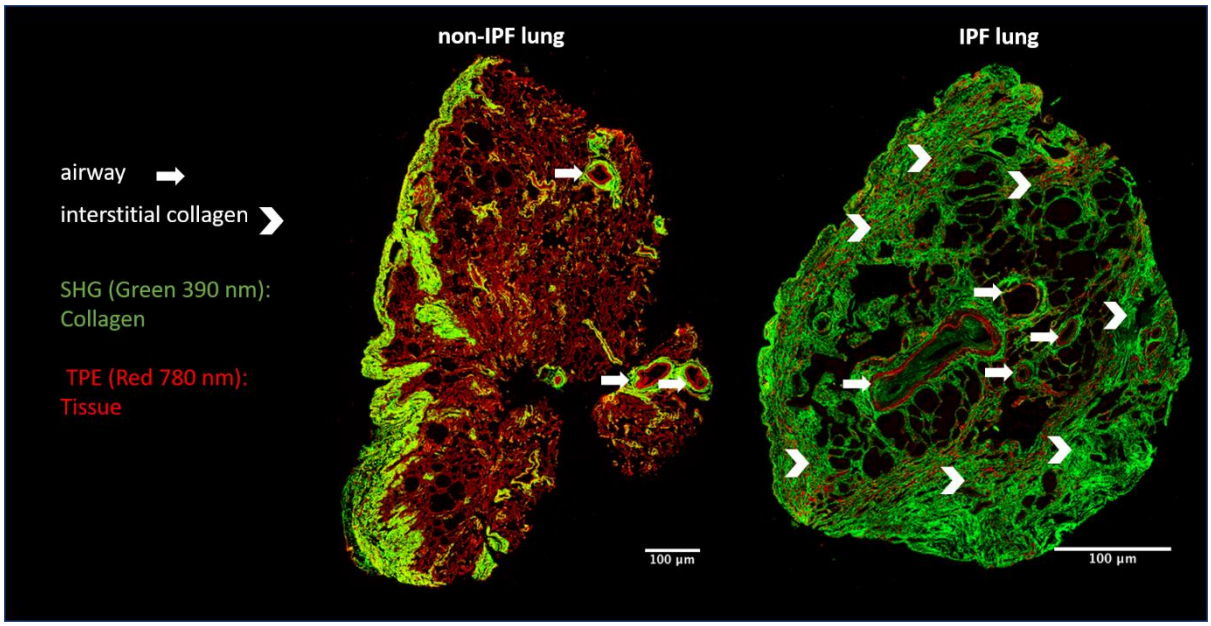
Immunohistochemistry for the RXFP1 receptor was performed. One serial section for each treatment group was used to detect RXFP1 while another section was used as a negative control. Sections were dewaxed twice in xylene and twice in absolute ethanol and rehydrated in 70% ethanol. Sections were then washed with ionized water before being transferred and submerged into 3% hydrogen peroxide to inhibit endogenous peroxidase activity. Slides were washed and placed in pre-heated citrate buffer for antigen retrieval for 5 min and placed in a water bath (room temperature) for cooling (25 min). Each section was circled with a DAKO hydrophobic pen and incubated in 50 µL DAKO diluent to block non-specific protein binding. Immediately after the diluent was removed, 50 µL of a monoclonal mouse antibody of RXFP1 (1:500, Santa Cruz, J2612) was added to each slide except for the negative control slide.

Following overnight incubation in a humidification chamber, sections were washed in Tris buffer to remove excess primary antibody residue. All sections were incubated with the secondary antibody (Horseradish peroxidase (HRP)-conjugated rabbit polyclonal antibody, Santa Cruz) for 1 hr for the detection of the monoclonal RXFP1 antibody. Slides were then rinsed in Tris buffer and quickly incubated in 3-3’ diaminobenzidine (DAB) chromagen (DAKO Denmark, Iostrup, Denmark kits) which interacts with HRP. This caused RXFP1 to be identified by the brown DAB-based staining of receptor localisation. Slides were quenched in distilled water to stop any further reaction. Positive DAB staining was visualized and scanned by Monash Histology Platform for morphometric analysis.

2.5.4 Quantification of collagen by Genesis 200 / Histoindex

Sections from disease models were additionally dewaxed in xylene and dehydrated in ethanol before being placed into the Genesis 200 (Histoindex Inc., Singapore). The Genesis 200 is an automated, high resolution, stain-free imaging system. Collagen area and fibre density were quantified and visualised using Second Harmonic Generation (SHG) with Two-photon Excitation Fluorescence (TPE)- based microscopy. Detection of collagen with SHG is demonstrated by green light emission whilst TPE presents lung tissue in red.

Images generated by the Genesis 200 were assessed by the Fibroindex application which provided morphological analysis of collagen area within the tissue and more specifically around the airways.



Images provided by Cem Erdem, Honours, Monash University

Figure 3. Genesis 200 scanning of donor lung tissue from a patient with idiopathic pulmonary fibrosis (IPF), obtained from the Alfred Hospital Bio-bank. Genesis 200 scanning demonstrates increased airway and interstitial collagen deposition (as depicted by arrows) in an IPF lung compared to a non-IPF lung as shown with the increase in SHG (Green). In the non-IPF lung, TPE (Red) analysis predominates.

2.6 Statistical Analysis

Myograph, organ bath and PCLS

Concentration response curves were fitted with non-linear regression curve fit to derive the EC_{50} and maximum response values. Results were analysed via unpaired t-tests or one-way ANOVA for multiple comparisons between groups as appropriate. Statistical significance was accepted at $P<0.05$. All data analysis was performed using GraphPad Prism v6 and v7 (GraphPad Software, San Diego, CA, USA) and expressed as the mean ± s.e.m.

Histology and morphometry

Statistical analysis was performed using GraphPad v6 and v7 (GraphPad Software Inc., CA, USA) and expressed as the mean ± s.e.m. Data was analysed via unpaired t-test between control and disease groups (Chapter 7). In each case, data was considered significant with $P<0.05$.

2.7 References

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Chapter 3: Validation of new approaches to optimize the PCLS technique to assess small airway reactivity

3.1 Introduction

3.1.1 Assessing *in vitro* airway reactivity in large versus small airways

Understanding airway responsiveness is important to elucidate mechanisms underlying changes in chronic lung diseases such as asthma and to assess current and emerging treatments. The approaches used for *in vitro* studies vary depending on the airway size under investigation, influencing their potential applicability and clinical relevance (Table 1).

The majority of studies examining responses of isolated airways to contractile or dilator agonists have used readily dissected airway smooth muscle strips and intact tracheal or large bronchial rings, measuring changes in force under isometric conditions (reviewed by Wright *et al.*, 2013). However, smaller intrapulmonary airways (defined as <2 mm in humans, <300 µm in mouse) are a major site of inflammation and remodelling in lung diseases in which increased contraction severely restricts airflow (Wagner *et al.*, 1998). Despite their significant contribution to lung disease and importance as potential targets for treatment, small airways remain understudied because of their relative inaccessibility compared to larger airways. The precision cut lung slice (PCLS) technique has been developed to enable *in vitro* assessment of small intrapulmonary airways, allowing responsiveness to added agonists to be visualized as changes in the area of small airways (and adjacent arterioles) (Figure 1) (Sanderson, 2011).

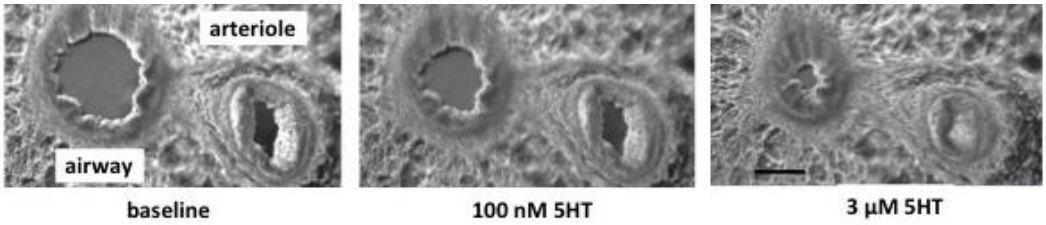

Figure 1. Phase contrast images of rat PCLS. Images depict intrapulmonary airway (left) and arteriole (right) that are differentially contracted to increasing concentrations of serotonin (5HT). In this PCLS, the airway but not the arteriole contracts to 100 nM 5HT, while the arteriole but not the airway is completely occluded at 3 µM 5HT, demonstrating differences in agonist potency and efficacy (scale bar, 200 µm).

To prepare PCLS from whole lungs or wedge preparations to study small airway responses, tissues are inflated via the trachea or accessible airways with warmed liquid agarose. The agarose is pushed through the airways into alveolar spaces to ensure that airways remain patent and subsequently cooled to stiffen the lung tissue for slicing while preserving airway structure (Sanderson, 2011; Morin *et al.*, 2013). While this technique is more complex then

preparing isolated airway rings, it generates a high yield of PCLS from a single lung lobe or sample compared to the limited number of ring preparations that can be dissected from an experimental animal or lung sample.

Unlike isolated ring or strip preparations, airways within PCLS are maintained within a microenvironment of multiple resident cells and remain tethered to the surrounding parenchyma, In PCLS, contraction is measured under auxotonic conditions, whereby ASM shortens against an increasing load imposed by airway attachments to the surrounding lung parenchyma including its extracellular matrix (ECM). These conditions more closely mimic the airways within the lung *in situ* compared to in isolated airway rings. This approach permits the assessment of both the rate and extent of reactivity expressed as changes in airway lumen. In addition, there is the potential to assess the differential reactivity of airways compared to adjacent pulmonary arterioles (Figure 1).

Another potential advantage of PCLS is that capacity to perform confocal imaging of PCLS loaded with Ca^{2+}-sensing dye to allow simultaneous examination of smooth muscle signaling pathways related to contraction. This approach has been applied to both mouse and human PCLS (Sanderson, 2011).

Dilator responses can readily be assessed in both ring preparations and PCLS. Given the evidence of AHR in the distal lung (Wagner *et al.*, 1998) and differences in sensitivity to both contractile and dilator agonists with airway size (Bai *et al.*, 2007; Donovan *et al.*, 2013), it is important to extend assessment of novel dilators to the small airways.

	organ bath	**PCLS**
airway	trachea, bronchi	intrapulmonary airways
diameter	mm	µm
tissues per mouse	1-2 rings	>10 slices/lobe
conditions	isometric	auxotonic
confirmed viability in culture	1-2 days	2-3 days
parenchymal attachment	no	yes
arterioles present	no	yes [27]
calcium imaging	no	yes [25]
contractile responses	**change in force (Δ mN, g)**	**change in area (% initial airway area)**
methacholine	5 mN [11]	40-50% [11,12]
high K^+	10-15 mN [37] sustained	10-20% [2] transient
relaxation responses	**(max % relaxation)**	**(max % relaxation)**
isoprenaline	30% [12]	50-70% [7]
salbutamol	50% [12]	50% [7,11]

Table 1. Comparison of the traditional organ bath studies and the PCLS approach to study mouse airways *in vitro*

3.1.2 Applications and limitations of PCLS

As previously outlined, PCLS can be used for studies of airway contraction and relaxation, offering unique insights into small airway reactivity. The majority of PCLS studies to date have been in mice (Bai and Sanderson, 2006; Bai *et al.*, 2007), with fewer using rats (Lam *et al.*, 2016), guinea pigs (Ressmeyer *et al.*, 2006), marmoset (Seehase *et al.*, 2011) and more recently human tissues (Ressmeyer *et al.*, 2006; Cooper *et al.*, 2009; Koziol-White *et al.*, 2016).

Rodents are easily accessible and convenient to use in models which replicate key features of disease conditions (Moeller *et al.*, 2008; Royce *et al.*, 2014) such as increased reactivity to MCh, used as the gold standard bronchoconstrictor (Royce *et al.*, 2009, 2015). While *in vivo* studies in models have demonstrated changes in whole lung responses, they do not permit specific insights on small airway reactivity. Mouse PCLS have been used to elucidate mechanisms of contraction to MCh including changes in calcium signalling (Bai and Sanderson, 2006).

It is important to consider potential differences between mouse and human airways to define the best setting for assessment of contractile responses. Rodent airways respond to 5HT, but not to histamine nor LTD_4, although all are released by mast cells and mediate the allergic response leading to contraction of human airways (Martin *et al.*, 2000; Perez and Sanderson, 2005; Sanderson, 2011). There is a need to assess airway pharmacology in animals that better resemble the human condition such as the marmoset or other non-human primates (Seehase *et al.*, 2011; Schlepütz *et al.*, 2012). Using PCLS generated from marmosets, maximum airway contraction to methacholine, histamine, LTD4, U46619 and ET-1 was shown to be similar to airway contraction in human PCLS (Seehase *et al.*, 2011). However, preparation of lung slices requires a degree of expertise so that lungs are not under- or over-inflated by agarose. There is a need to ensure that the volume of agarose is corrected for the size of lung samples across all species.

As previously outlined, one advantage of the PCLS technique is the ability to generate many slices per animal or clinical sample. However, it is important to establish whether there is significant variability between reactivity of PCLS obtained from different regions of the same lung. This would exclude the location of the PCLS as a variable when looking **within and between animals** for comparisons of potency of current dilators such as salbutamol (SALB)

with novel dilators, as has previously been performed for rosiglitazone (Bourke *et al.*, 2014) and chloroquine (Tan and Sanderson, 2014).

PCLS can be treated with cytokines elevated in the disease context for additional assessment of altered contraction. Incubation of rat PCLS with a cytokine cocktail of TNFα, IL-1β and IFNγ for up to 4 h was shown to cause time-dependent airway contraction which was attributed to the release of endogenous thromboxane receptor (TXA_2) agonists (Martin *et al.*, 2001). In addition, the effect of incubation with other mediators such as endothelin-1 have yet to be tested in small airways. The endothelin system is activated in inflammation and ET-1 can directly cause acute bronchoconstriction and may contribute to longer term remodelling.

The ability to utilise PCLS requires skill in PCLS preparation as well as access to specialised equipment including a vibratome and phase contrast microscope. Wider applications of the PCLS technique might be achieved if lung tissues could be transported from a lab where the tissues were being collected to another lab where expertise and a PCLS platform is available. It is important to validate whether this collaboration would still result in viable PCLS if the lungs were dissected from the mouse without inflation and shipped in cooled physiological solution such as HBSS, or whether it would be necessary to inflate the lungs with agarose which would therefore require training at site of origin and cool before removal and shipping (Sanderson, 2011).

Even if approaches for transferring lungs are validated, the ability to utilise the full number of PCLS obtained from each lung may still be limited. The dedicated phase contrast microscope required to assess continuous changes in airway area can only assess one PCLS at a time, limiting the number of preparations that can be analysed in a single day. In addition, the viability and preservation of contractile function of small airway preparations in culture has only been clearly established over several days.

Previous studies have systematically assessed the effect of pH, temperature and buffer differences on airway contraction (FitzPatrick, 2011). Studies have shown that PCLS remain viable for up to 3 days in the optimal mixture of media, gas and temperature (Liberati *et al.*, 2010) but that contraction decreased with extended time in culture (Sanderson, 2011). To maximize experimental outputs whilst reducing animal numbers, it is necessary to explore alternative approaches of extending airway viability.

Recent preliminary work suggests that the viability of PCLS could be extended by cryopreservation to maximise its application (Bai *et al.*, 2016). Cryopreservation is used to preserve the structure and function of intact living cells and tissues. Recent advances in the PCLS technique have demonstrated that cryopreservation does not alter PCLS viability (Rosner *et al.*, 2014).

Using the conventional cryopreservative dimethyl sulfoxide (DMSO), PCLS can be frozen, stored, and thawed for study of airway reactivity at extended time points. Previous studies in mouse PCLS have shown that cryopreservation of slices for up to two weeks does not alter airway contraction to MCh (100 nM – 100 µM) or relaxation to the dilator, chloroquine (1 mM, single concentration) (Rosner *et al.*, 2014). In human PCLS, airways were shown to additionally retain immune function and airway reactivity (Bai *et al.*, 2016). Although airways from frozen PCLS maintain their ability to contract to MCh, it important to validate this single finding. Additionally, potential changes in dilator responses have yet to be thoroughly explored.

This background identifies opportunities and approaches that may improve and extend the applicability of the PCLS technique. Demonstrating contractile responses in small airways in mouse, rat, marmoset and human PCLS was undertaken to support the later cross-species assessment of relaxin as a novel dilator and to move beyond airways from naïve mice to experimental models of disease. Additional methodological studies were performed to optimise the conditions under which viable PCLS can be prepared to inform approaches applied in this thesis, or as validation for future studies.

3.1.3 Aims

In the current study, we aimed to:

1. establish conditions to prepare functional PCLS across multiple species (mouse, marmoset, human)
2. assess variability of airway responses between PCLS
    a. from different lung lobes
    b. following *in vitro* cytokine treatment
3. validate conditions for preparation of functional PCLS after
    a. delayed filling of lobes
    b. delayed slicing of lobes
    c. cryopreservation of PCLS

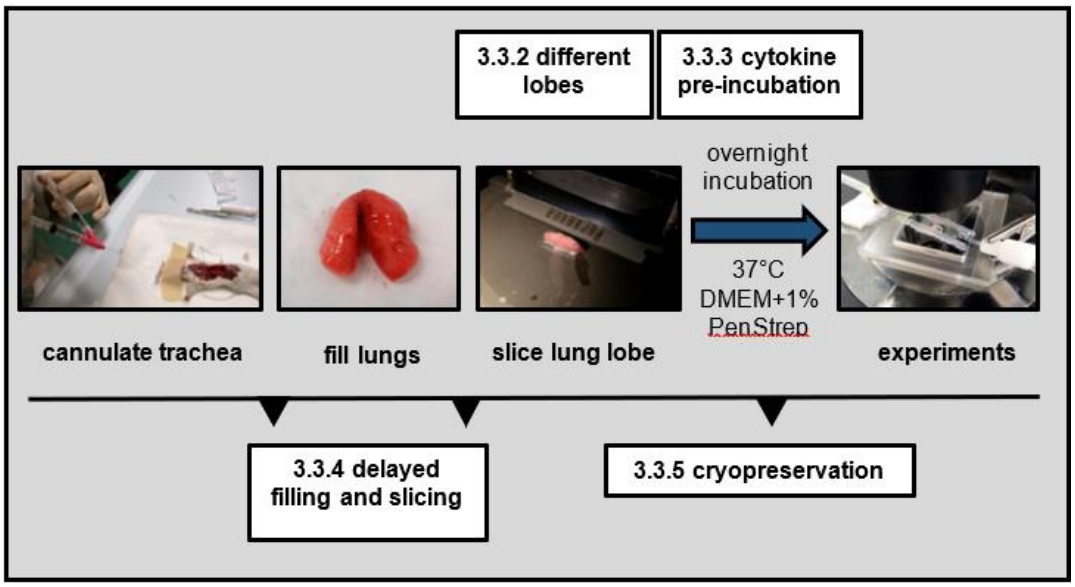

Figure 2. Preparation of PCLS showing variations in experimental approaches assessed in this chapter.

3.2 Methods

3.2.1 Materials and solutions

Methacholine (MCh) and salbutamol (SALB) (both from Sigma-Aldrich, St Louis, MO, USA); endothelin-1 (ET-1, GenScript, NJ, USA); 1x Dulbecco's Modified Eagle Medium (DMEM, 4.5g/L D-glucose and 1g/L D-glucose), 10x Hank's Balanced Salt Solution (HBSS), 1M N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES), penicillin streptomycin (pen strep) (all from Gibco Life Technologies, AU); recombinant mouse TNFα (Sapphire Bioscience). Stock solutions of MCh (10^{-1}M) and SALB (10^{-2}M) were prepared in water.

3.2.2 Animals and ethics

All experimental procedures using mice and rats were approved by a Monash University Animal Ethics Committee (AEC number: MARP2/2014/066), which adheres to the Australia Code of Practice for the Care and Use of Animals for Scientific Purposes. Male Balb/C mice (8 weeks) and rats (6-8 weeks) were housed in the Animal Facility, Monash University and maintained on a fixed 12 h light, 12 h dark lighting schedule, with free access to food and water at all times.

3.2.3 Tissue collection

Mice and rats were humanely euthanized by sodium pentobarbitone overdose (0.6 mg/mL administered *i.p.* at a volume of 1 mL per 100 g body weight) for preparation of PCLS.

Lungs from marmosets (400 g body weight) were kindly provided as salvaged tissues by Dr. Yan Wong, Physiology Department, Monash University.

Human lung samples obtained from donors who passed away from non-related respiratory events were provided by Dr. Glen Westall from the Alfred Hospital (Monash University Human Research Ethics Committee (MUHREC, approval CF16/406).

3.2.4 PCLS preparation

PCLS were prepared with modifications from published methods (Bourke *et al.*, 2014; Donovan *et al.*, 2014) as previously outlined in detail (Chapter 2.5).

Mouse, rat and marmoset trachea were cannulated with a catheter containing two ports (20 G Intima, Becton Dickinson, VIC, Australia) and lungs were inflated with ~1.2 mL, ~1.4 mL or ~10 mL agarose gel respectively (2% agarose in 1xHBSS at 37°C), followed by a bolus of ~0.5 mL, ~1.5 mL or 3 mL air to push agarose into alveolar spaces away from airways. For some mice, lungs were removed following euthanasia, and incubated in cold 1xHBSS (20°C) for 24 h prior to inflation.

For human lung samples, one main bronchus was identified and cannulated. Other open-ended airways were clamped in order to prevent agarose from leaking. Lungs were filled with 20-40 mL agarose (depending on the size of the lung sample), followed by a bolus of 5-10 mL air.

Lungs were cooled by bathing in cold HBSS/HEPES were then kept at 4°C to allow the agarose to solidify. For mice, the superior right lobe was most commonly used to prepare PCLS, but the inferior right, middle right and left lobes were also used for some studies. For some mice, inflated lungs were incubated in cold HBSS for 24 h prior to slicing. For rats and marmosets, the superior right lobe was used for PCLS. For human samples, a portion of inflated lung containing airways was dissected for slicing.

All tissue samples were adhered with cyanocrylate to a mounting plate in a vibratome (Compresstome, Precisionary Instruments, Greenville, NC, US). Multiple PCLS were prepared at 150-200 µm in thickness for mice, rat and marmoset lung lobes and 250-300 µm thickness for human lung samples.

Freshly cut PCLS were then transferred into cell culture plates containing DMEM, supplemented with 1% penicillin-streptomycin and incubated for 24 h (37°C, 5% CO_2) prior to experiments. Some mouse PCLS were incubated overnight with a single concentration of TNFα (10 ng/mL) or ET-1 (5 nM).

Some mouse PCLS were placed in cryovials containing DMEM+10% DMSO (2 slices/mL/vial) and stored in 100% isopropyl alcohol-filed Nalgene Mr. Frosty Freezing Container (Sigma-

Aldrich) and frozen at -80°C (Bai *et al.*, 2016). PCLS were frozen for up to 2 weeks prior to thawing. For experiments, cryovials were thawed rapidly in 37°C water bath. PCLS were transferred into 24-well plates containing fresh DMEM supplemented with antibiotic and cultured for 24 h before commencing experiments.

3.2.5 PCLS protocols

PCLS were transferred to HBSS/HEPES and mounted in custom-made perfusion chambers (~100 µL volume). A viable airway (150-500 µm diameter) was selected from each slice based on the presence of an intact layer of epithelial cells with ciliary activity. PCLS were perfused at a constant rate (~0.5 mL/min) through an eight-channel gravity-fed perfusion system under vacuum.

Airways were perfused with HBSS to establish a baseline area before perfusion with increasing concentrations of MCh (10-3000 nM). For some cryopreserved PCLS, airways were pre-contracted with 300 nM MCh and then perfused with increasing concentrations of the β_2-adrenoceptor agonist, salbutamol (SALB, 10 nM – 1000 µM).

3.2.6 Statistical analysis

All statistical analysis was performed as described in General Methods (Chapter 2.7). Airway contraction was expressed as % initial airway area. Relaxation was normalised to MCh pre-contraction and expressed as % relaxation. Concentration response curves were fitted with non-linear regression curve fit to derive the EC_{50} and maximum response values. Results were analysed via unpaired t-tests or one-way ANOVA for multiple comparisons between groups as appropriate. Statistical significance was accepted at $P<0.05$.

3.3 Results

3.3.1 Airway contraction to MCh is similar in PCLS from multiple species

To establish the conditions required to prepare PCLS across multiple species, lobes from mouse, rat and marmoset lungs and human lung samples were inflated with agarose until firm enough to section (Table 2). To assess whether airways from PCLS were viable following preparation, airways were contracted with MCh. The experimental trace for MCh concentration-response curve in a mouse PCLS shows that a threshold response was seen at 0.01 µM MCh, and that plateau responses were achieved within 5 min at each concentration up to 1 µM MCh which caused maximum contraction (Figure 3A). The potency and maximum contraction to MCh was similar across all species, where airway lumen area was reduced by ~45% on average (Figure 3B, C). The difference in airway contraction to the highest concentration of MCh tested (3 µM) in separate airways within species was more variable in human and marmoset than in mouse or rat.

	mouse	**rat**	**marmoset**	**human**
filling volume (mL)	1.2	10	10	>20
slicing thickness (µm)	150-200	150-200	150-200	250-300
airway diameter (µm)	150-400	200-400	200-500	>300

Table 2. Parameters for preparation of PCLS from mouse, rat, marmoset and human lungs

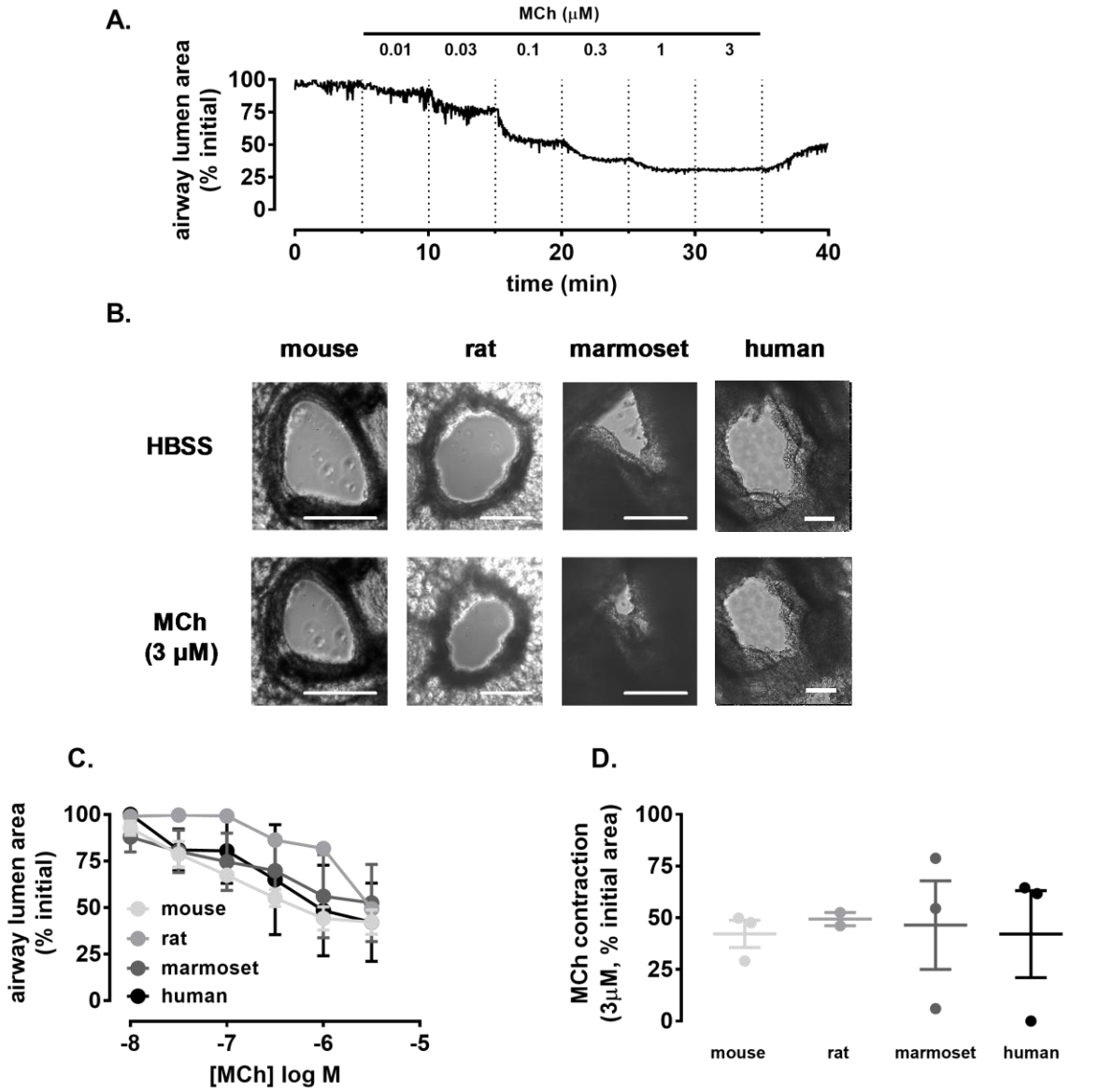

Figure 3. Airway contraction to MCh is similar in mouse, rat, marmoset and human PCLS. PCLS were assessed for changes in intrapulmonary airway area in response to MCh by phase-contrast microscopy. (A) Representative trace depicts MCh-induced contraction of mouse intrapulmonary airways. (B) Representative phase-contrast images are derived from the last frame of HBSS and MCh perfusion for each species. (C) PCLS generated from mouse, marmoset and human lung lobes were perfused with increasing concentrations of MCh (0.01-3 μM) at 5 min intervals. (D) Maximum contraction to the highest tested concentration of MCh (3 μM) is shown. All responses are expressed as % initial airway lumen area (n= 3/group; mean ± s.e.m.). Scale bars represents 200 μm.

3.3.2 Airway contraction to MCh is similar in mouse PCLS prepared from different lung lobes

To assess intra-lung variability in airway contraction between lung lobes, mouse PCLS were prepared from the superior, middle and inferior right and left lung lobes (Figure 4A) and perfused with increasing concentrations of MCh. Airways from each lobe were size-matched (Figure 4B) and contracted to MCh to similar extents (Figure 4C). There was no difference in either maximum contraction (~50-75% reduction in initial airway area, N.S.) or MCh potency (EC_{50} ~10^{-7}M) in all groups (Table 3).

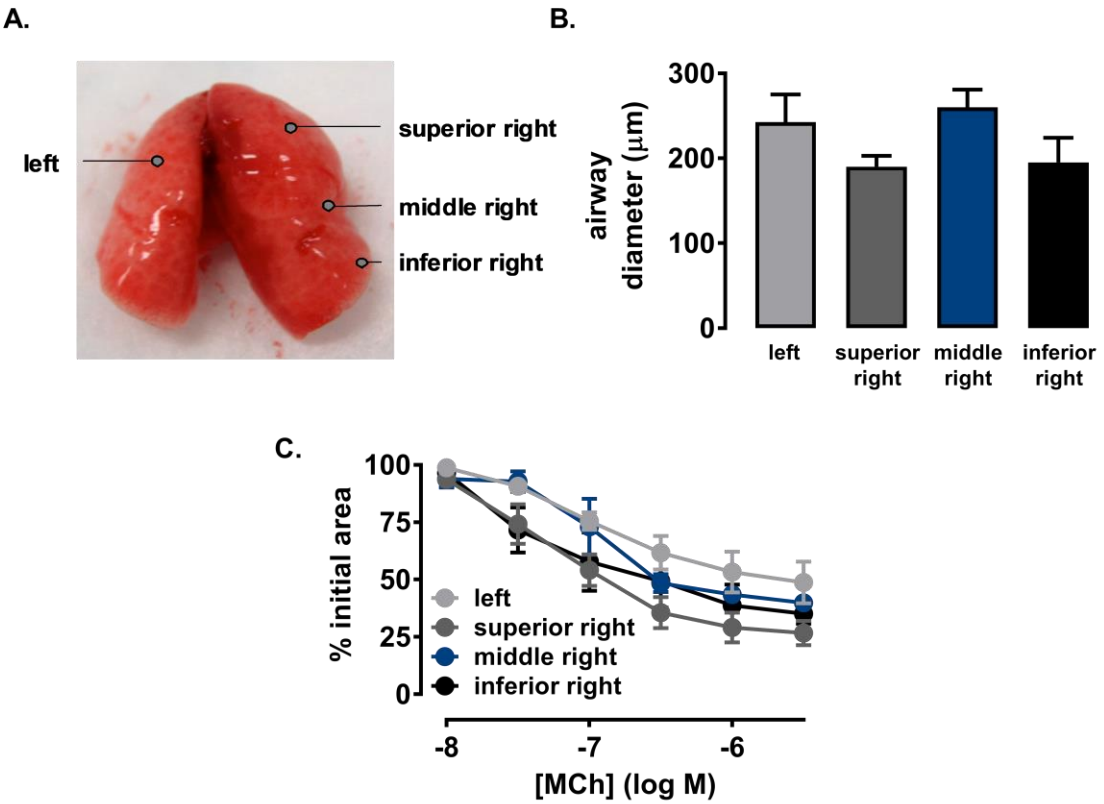

Figure 4. Airway contraction to MCh is similar in mouse PCLS from different lung lobes. Mouse PCLS generated from the superior, middle and inferior right and left lobes from (A) agarose-inflated mouse lungs were perfused with increasing concentrations of MCh (0.01-0.03 μM) at 5 min intervals. (B) Comparison of airway diameters in PCLS from different lobes were used to (C) assess contraction. All responses are expressed as a % initial airway lumen area (n= 3-4/group; mean ± s.e.m.).

lobe	pEC_{50}	max contraction (% reduction in area)	n
left	7.0 ± 0.1	52.5 ± 10.1	4
superior right	7.1 ± 0.2	76.7 ± 6.3	4
middle right	7.0 ± 0.2	64.7 ± 0.6	3
inferior right	7.1 ± 0.5	67.5 ± 4.6	4

Table 3. Comparison of potency and maximum contraction to MCh between airways from different mouse lung lobes.

3.3.3 Airway contraction to MCh in mouse PCLS is increased by pre-incubation with TNF-α

To demonstrate cytokine-induced changes in airway reactivity, mouse PCLS were incubated overnight with TNFα and assessed for responsiveness to MCh. In the absence of treatment, the maximum airway contraction to MCh, expressed as a reduction in airway area, was ~70% (Figure 5A, B). Whilst MCh potency was unchanged (pEC_{50}: control 6.5 ± 0.8; +TNFα 6.8 ± 0.4), airway contraction to MCh was markedly increased following TNFα pre-incubation compared to contraction in control airways with complete airway closure (Figure 5B and C, P<0.001). In contrast, overnight incubation with the contractile agonist ET-1 completely suppressed subsequent MCh-induced contraction (Figure 5).

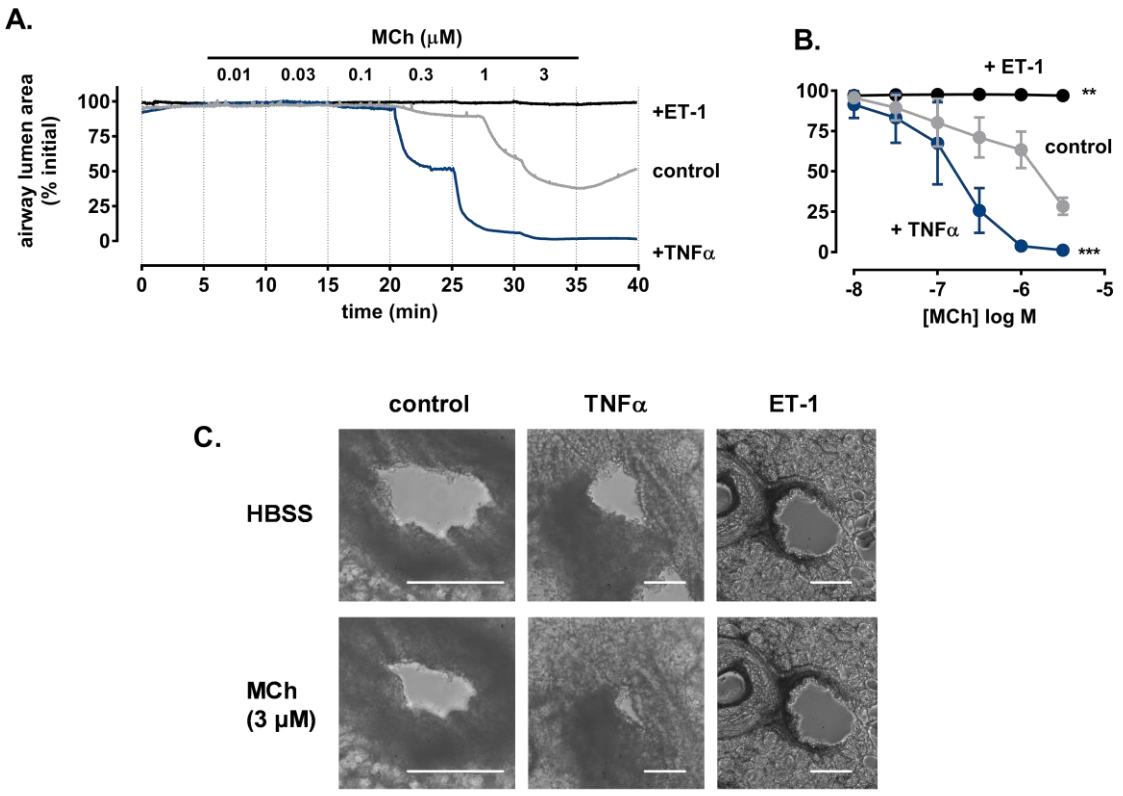

Figure 5. Airway contraction to MCh in mouse PCLS is increased by pre-incubation with TNFα but prevented by ET-1. Mouse PCLS were incubated overnight in the absence or presence of TNFα (10 ng/mL) or ET-1 (5 nM). (A) Representative traces illustrate the effect of pre-incubation on MCh-induced contraction in PCLS. (B) PCLS were perfused with increasing concentrations of MCh (0.01 - 3 μM). (C) Representative phase-contrast images of airways derived from the last frame of HBSS and MCh (3 μM) perfusion following pre-incubation with TNFα and ET-1 (magnification of x4). Representative image in control slices are acquired at a magnification of x10. All responses are expressed as a % initial airway lumen area (n= 3/group; mean ± s.e.m.). Scale bars represent 250 μm.

3.3.4 Airway contraction to MCh in mouse PCLS is maintained despite delayed filling or slicing

To validate alternative conditions for the preparation of functional airways, PCLS were prepared from dissected lungs stored in cold HBSS for 24 h post-dissection prior to inflation with agarose (Figure 6, delayed filling). Concentration-dependent airway contraction to MCh was maintained compared to PCLS prepared using the standard protocol as shown by images and combined data (Figure 6A, B). Neither MCh potency (pEC_{50}: no delay 6.9 ± 0.2, 24 h delay 6.7 ± 0.3) nor maximum airway contraction was altered (% initial area: no delay 36.1 ± 4.8, 24 h delay 36.5 ± 10.5) compared to PCLS prepared from lungs inflated *in situ* immediately after euthanasia and cut within 24 h (Figure 6B).

The effect of delayed slicing of inflated lungs on airway reactivity was also assessed using PCLS sectioned at 1-, 8- and 24- h post-fill (Figure 7, delayed slicing). Airway contraction to MCh was maintained across all slicing time points where potency (Table 4) and maximum contraction to MCh were unchanged (Table 4, Figure 7).

There was no increase in variance in the contraction to MCh between routinely sliced PCLS and PCLS produced following delayed filling or slicing.

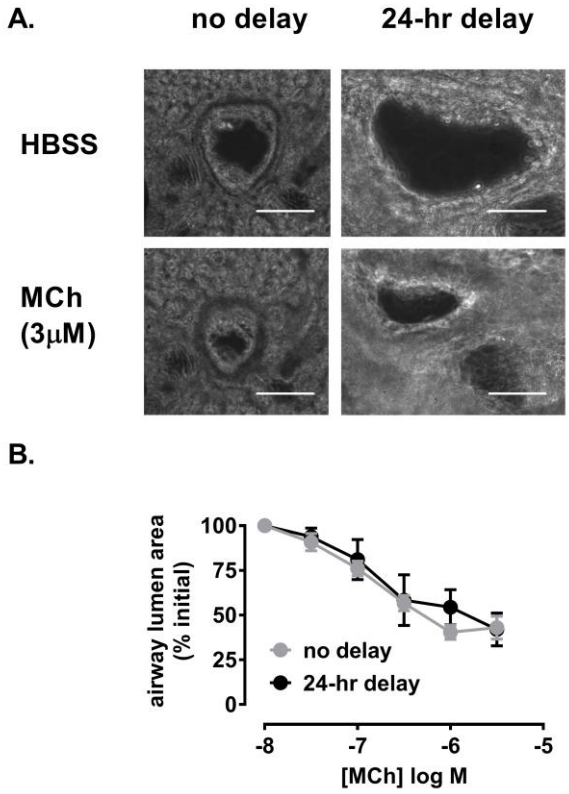

Figure 6. Airway contraction to MCh in mouse PCLS is maintained despite delayed filling. PCLS were prepared using standard conditions in which lungs were inflated with agarose *in situ* immediately post mortem (no delay) and from dissected lungs stored in HBSS at 4°C for 24-h prior to instillation of agarose and slicing (24-h delay) (A) Representative phase-contrast images of airways derived from the last frame of HBSS and MCh (3 μM) perfusion in no delay and 24 h delay groups (B) Grouped data shows airway contraction to increasing MCh. All responses are expressed as a % initial airway lumen area (n= 4/group; mean ± s.e.m.). Scale bars represent 150 μm.

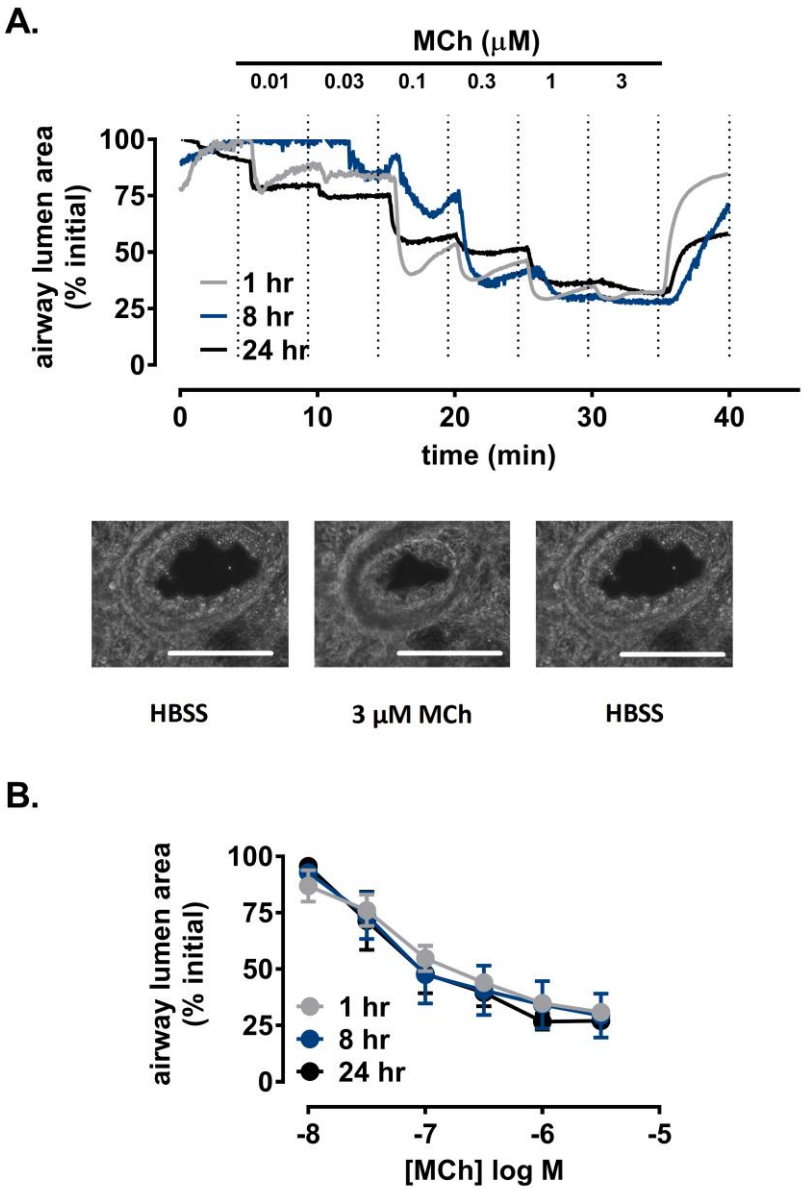

Figure 7. Airway contraction to MCh in mouse PCLS is maintained despite delayed slicing. PCLS were prepared from inflated mouse lungs stored in HBSS at 4°C for 1-, 8- or 24-h. (A) Representative traces from PCLS prepared following delayed slicing by 1-, 8- and 24-h show contraction of individual airways to increasing MCh concentrations. Representative phase-contrast images were derived from the last frame of HBSS and MCh (0.3 μM) perfusion from 1 h delayed slicing. (B) Grouped data shows airway contraction to MCh. All responses are expressed as a % initial airway lumen area (n= 4/group; mean ± s.e.m.). Scale bar represents 200 μm.

delaying before slicing	pEC_{50}	max contraction (% reduction in area)
1 h	7.1 ± 0.2	70.4 ± 2.1
8 h	7.5 ± 0.3	70.7 ± 9.2
24 h	7.4 ± 0.4	75.1 ± 0.4

Table 4. Comparison of potency and maximum contraction to MCh between airways from lobes sliced at 1-, 8- and 24-h post fill.

3.3.5 Airway contraction to MCh and relaxation to salbutamol is maintained following cryopreservation of mouse PCLS

To assess the the effect of cryopreservation on airway reactivity, PCLS were frozen for up to two weeks before experiments were conducted. Comparison of control (never frozen) and cryopreserved (frozen and thawed) slices demonstrated no apparent differences in airway or artery morphology under phase contrast microscopy, (Figure 8A, 9A) or contraction to MCh (Figure 9B).

Concentration-dependent contraction of airways to MCh was evident in control and cryopreserved slices where there was no significant differences between groups in MCh potency (EC50 ~ 10^{-7}M) or fitted maximum contraction (% reduction in airway area ~60%).

Dilator effects of SALB were compared in intrapulmonary airways from control and cryopreserved PCLS. Phase-contrast images depict an adjacent airway and artery in a mouse PCLS. Arteries are smaller in size and are inclined to collapse without gelatin or heparin instillation to clear blood from arteries (Paddenberg *et al.*, 2006). Unlike the airway, and as expected, the artery neither contracted to MCh nor relaxed to SALB.

There was no difference between control and frozen slices in the level of pre-contraction to MCh (not shown). In MCh-pre-contracted airways, SALB elicited ~50% relaxation in both control and frozen airways with similar potency ($P>0.05$ for both parameters, Figure 9).

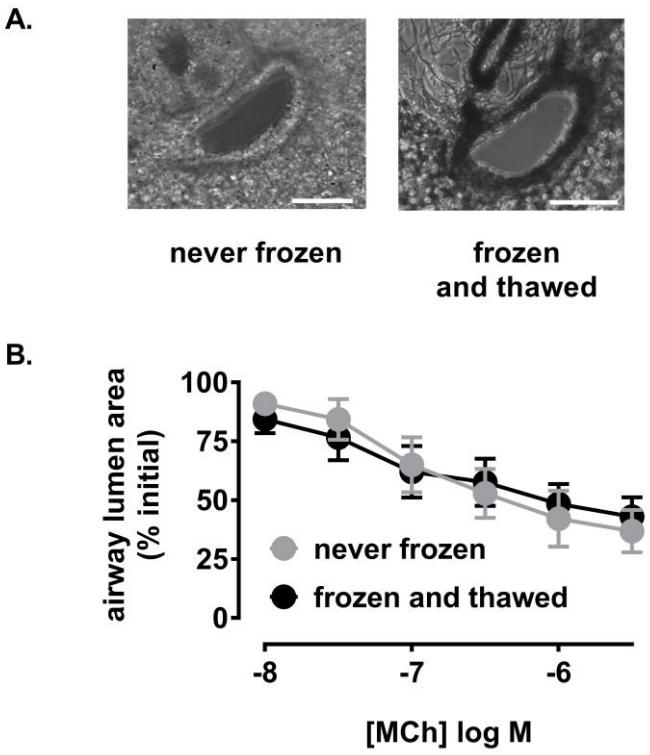

Figure 8. Airway contraction to MCh is maintained following cryopreservation of PCLS. Airway contraction to MCh was assessed in mouse PCLS that were freshly prepared (control) or thawed after cryopreservation for up to 2 weeks (frozen). (A) Representative phase-contrast images show the morphology of a single airway from a control and frozen PCLS. (B) Control and cryopreserved PCLS were contracted to increasing concentrations of MCh (0.01-3 µM). All responses are expressed as a % initial airway lumen area (n=6/group; mean ± s.e.m.). Scale bars represents 150 µm.

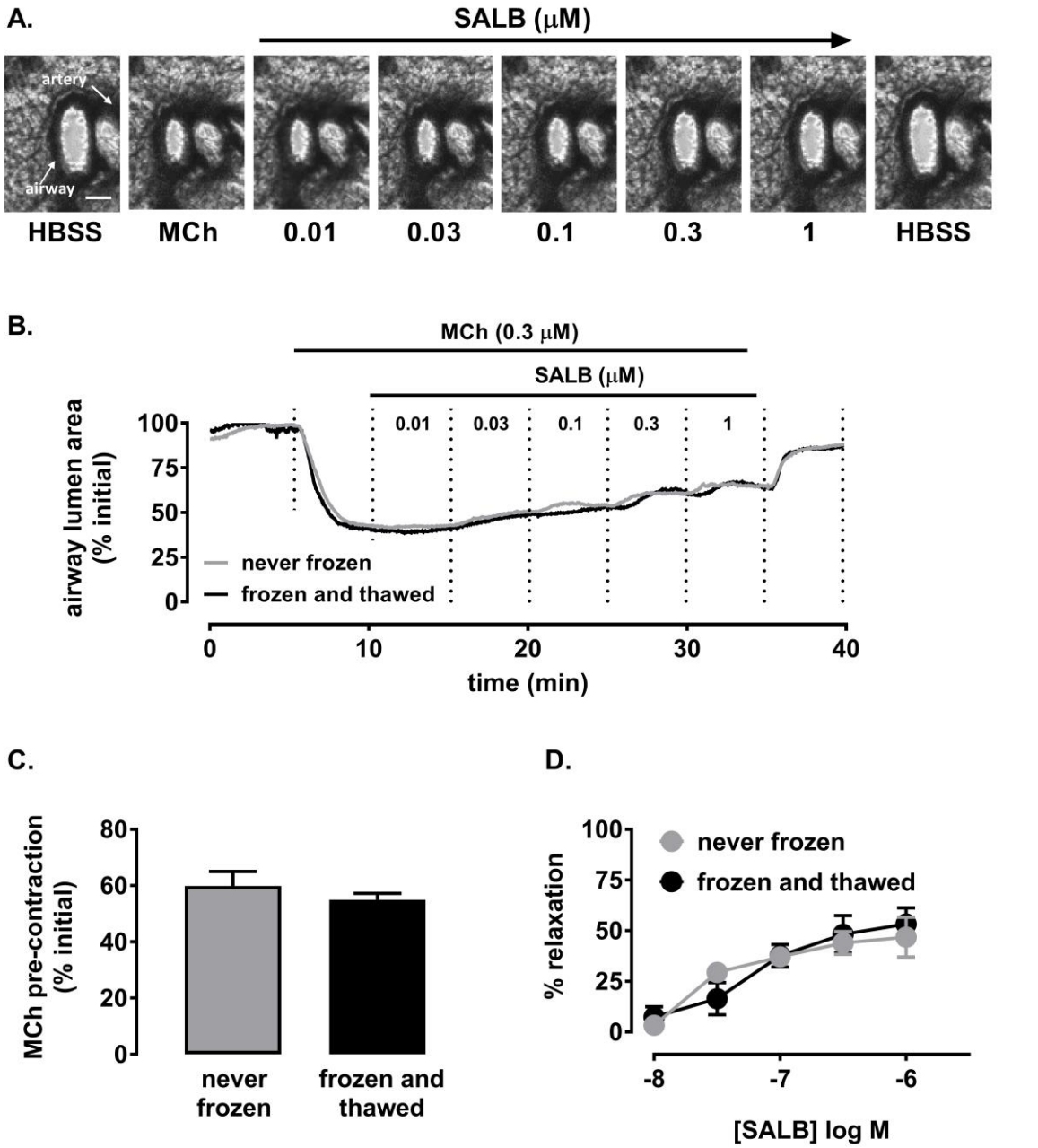

Figure 9. Airway relaxation to SALB is maintained following cryopreservation of PCLS. Airway relaxation to salbutamol (SALB) was assessed in mouse PCLS that were freshly prepared (control) or thawed after cryopreservation for up to 2 weeks (frozen). (A) Representative phase-contrast images were derived from the last frame of each concentration of SALB perfusion. (B) Representative traces from never frozen and frozen and thawed PCLS demonstrate individual airways pre-contracted with MCh then relaxing in response to increasing SALB concentrations. Airways were (C) pre-contracted with 0.3 μM MCh prior to measurement of (D) relaxation responses to SALB (0.01– 0.3 μM). All responses are expressed as a % initial airway lumen area (n= 3/group; mean ± s.e.m). Scale bar represents 160 μm.

3.4 Discussion

In the present study, we have extended the standard protocols used for the PCLS technique, by exploring species differences, alternative methods for preparation of PCLS as well as the effects of *ex vivo* cytokine incubation on airway reactivity.

Due to their accessibility and convenience, mice are the most commonly used species to assess airway reactivity both *in vivo* and *in vitro*, including in PCLS. However, there is a need to validate conditions to prepare PCLS from other species whose airway responses may more closely resemble those of human airways. In addition, it is necessary to confirm the consistency of findings within species to support the validity of any effects of interventions, such as changes in airway responsiveness with *ex vivo* treatment of PCLS with cytokines, or comparisons between different agonists. Despite the potential advantage of generating multiple PCLS from each lobe or lung sample to increase experimental outputs using PCLS, a major limitation of this approach is the requirement for specialised equipment and the time-consuming nature of each experiment. Therefore, there was a need to investigate procedures to extend the applicability and viability of PCLS.

In summary, we have demonstrated that MCh-induced contraction of airways from marmoset and human PCLS is similar to mouse and rat PCLS. There was also no difference in contraction to MCh between different lung lobes from mice, but pre-incubation of PCLS with TNFα markedly increased MCh-induced contraction. Despite delays in filling or slicing of lungs, airways in PCLS from mice maintained their sensitivity and maximal contraction in response to MCh. Similarly, airways from PCLS that were frozen for up to 2 weeks retained their ability to contract to MCh as well as relax to SALB. These findings suggest that contraction to MCh is preserved across species, between lung lobes, and despite delays in preparation or cryopreservation. However, contraction to MCh can be increased following incubation with an inflammatory cytokine known to be elevated in chronic lung diseases.

Marked differences in airway pharmacology and physiology are well established across species, so it is critical to determine species-specific responses in order to identify the most clinically relevant models for airway studies. Although readily available, it has been shown that rodent airways do not respond to histamine and leukotrienes as opposed to human airways which are sensitive to these contractile mediators (Seehase *et al.*, 2011). In contrast, serotonin is a potent bronchoconstrictor in rodent airways but not in human airways(Seehase *et al.*,

2011). Due to the relatively close phylogenetic relationship to humans, marmosets and other non-human primates (NHPs) are commonly used for studies in the fields of toxicology, infectious diseases and neuroscience. Fewer studies have assessed changes in airway reactivity in marmosets or NHPs, partly due to ethical constraints as well as the higher costs associated with breeding and husbandry. Obtaining human lung tissue is also challenging due to the rigorous requirements for ethics approval to obtain post-mortem tissue. Fortunately, lung tissue from marmosets was available for salvage for this study, while several unused donor lungs were provided from the local lung transplant program. This meant that the current study was able to compare the preparation and intrapulmonary airway contraction of PCLS across mouse, rat, marmoset and human airways, species that are all known to contract in response to the common agonist MCh. In all cases, the use of the PCLS technique enabled multiple slices from each lung sample to be obtained to maximise experimental outputs.

The preparation of PCLS from mice, rats and marmosets was relatively straightforward as the trachea could be readily cannulated for inflation of the lung with agarose. It was critical that lungs were firm enough to slice but not so over-inflated that airway architecture would be damaged or the physical presence of excess agarose in airways could oppose airway contraction. In addition, the temperature at which agarose was instilled into lungs was important in the preparation of patent lung slices. If the agarose temperature is too high, it could damage the airway epithelium. On the other hand, if the agarose temperature is too low, it would solidify before reaching the distal lung or become trapped in the lining of the airway. This may be shown by the loss of beating cilia as evidence of damage to the epithelium. The relatively small size of the lungs and the volumes required to inflate them meant that these potential obstacles to obtaining viable PCLS were not generally encountered.

However, major difficulties arose during the initial preparation of human PCLS. Human lung tissue was provided as a surgical resection sample which only consisted of a partial lobe. This meant that the lung sample lacked an intact pleural margin and agarose leaked from the multiple open-ended airways during inflation. Previous studies suggest that cannulating multiple small airways of the lung sample while clamping others can limit agarose leakage (Ressmeyer *et al.*, 2010). Whilst performing the current experiments, it was clear that this technique did not entirely prevent the outflow of agarose. To address this, lobes were also directly injected with agarose with a fine gauge needle to inflate regions of the lungs to yield viable airways.

Similar contractile responses to MCh in mouse, rat, marmoset and human intrapulmonary airways were obtained. This finding differs from a single study which showed that mouse airways are more responsive to MCh than marmoset or human airways (Seehase *et al.*, 2011). To date, the sample size of our study was very limited for marmoset and human PCLS, and the extent of airway contraction to MCh were quite variable in these species. These differences may be due to relative inexperience in preparation of human and marmoset airways. Another factor may relate to comparisons between equal sized airways from different species, as these are likely to be associated with different airway generations. Within a species, it is appropriate to use similar sized airways for comparative experiments (Bai *et al.*, 2007). However, across multiple species, the use of matched airway sizes may not be appropriate. Given that human samples are difficult to obtain and human PCLS are difficult to prepare, it is likely that further characterisation of responses will continue to be required in other larger species.

PCLS are traditionally produced from the same lung lobe to reduce a potential source of variability across all experiments. From a single mouse lobe, up to 10 slices containing airways of the appropriate size can readily be generated using a vibratome. It is therefore important to assess whether there are potential differences in airway reactivity between lobes to further maximise potential experimental outputs. Airways from mouse PCLS demonstrated similar contraction to MCh, regardless of the lobe they were generated from. The airways tested from each lobe were matched for size as previous studies have shown a correlation between airway size or generation with airway reactivity (Chitano *et al.*, 1985; Bai *et al.*, 2007). In particular, a previous study showed that distal and proximal mouse airways displayed less contraction than intermediate sized airways (Bai et al., 2007, AJRCMB). Furthermore, in both mouse and rat, small airways have been shown to be more responsive to contractile mediators than larger airways (Wohlsen *et al.*, 2003; Donovan *et al.*, 2013). Therefore, it is appropriate to compare airways that are matched for size, regardless of which lobe they are from.

Having confirmed consistent findings for MCh-induced contraction between size-matched airways in mouse PCLS, we then investigated whether reactivity could be altered by *in vitro* exposure to mediators implicated in chronic lung diseases namely TNFα and ET-1. TNFα and ET-1 levels are increased in asthma (Xu and Zhong, 1999; Berry *et al.*, 2007) where TNFα contributes significantly to the progression of disease by driving inflammation which can lead to the development of airway hyperresponsiveness. Using these stimuli, we showed that airway contraction to MCh is increased following overnight incubation with TNFα but not ET-1.

The increased contraction to MCh with TNFα pre-incubation is consistent with previous studies in which TNFα treatment enhanced contraction to carbachol in mouse trachea (Chen *et al.*, 2003) and 5HT in guinea pig trachea (Makwana *et al.*, 2012). These effects were attributed to increased expression of their receptors (Hakonarson *et al.*, 1996), although the expression of the M3 receptor that mediates contraction to MCh was not assessed in the current study. It is unknown whether the concentration of ET-1 for pre-incubation reduced tissue viability. High dose ET-1 may have caused irreversible contraction so that airways were unable to further contract as the contraction to ET-1 itself cannot readily be reversed (FitzPatrick, 2011). Irrespective of the mechanism, it is clear that PCLS incubation with the inflammatory cytokine TNFα has the potential to mimic key changes in reactivity associated with disease.

To perform PCLS experiments requires expertise in lung inflation and slicing tehcniques**,** as well as specialised equipment including a phase-contrast microscope to record experiments. This limits the broad applicability of the technique as well as the number of protocols that can be performed in one day. Another factor is the limited period of viability of PCLS in culture, such that the multiple PCLS available may not all be able to be utilised. Several alternative approaches were assessed to attempt to overcome these challenges.

Firstly, the current study demonstrated the effects of delayed filling on airway contraction in mouse PCLS. Introducing a period of time between dissection and filling would allow for the transfer and delivery of lung samples between the point of collection and a site where there is capacity to inflate and slice the lungs. Contraction to MCh was unaffected by delayed agarose injection and slicing for up to 24 h suggesting that storage of uninflated lungs in cold buffer did not reduce airway viability and that the time for PCLS preparation could potentially be delayed by a day. This finding has important implications for human lung samples which are notoriously difficult to obtain. It is now feasible that unused donor lungs could be stored in appropriate preservation solution for at least several hours following removal from a deceased body prior to collection and preparation of viable PCLS.

Secondly, the impact of delaying slicing on airway responses in mouse PCLS was explored. We proposed that a lab with the appropriate expertise could prepared agarose-inflated lungs for transfer to our site, which possesses the equipment to prepare PCLS and conduct subsequent experiments. Airways from PCLS cut up to 24 h post-inflation retained their ability to contract to MCh. Of relevance to this thesis, the delayed slicing procedure was then applied successfully to a mouse model conducted interstate, whereby inflated lungs were shipped to our laboratory for slicing and analysis (Refer to Chapter 7- HDM model).

Although these delayed filling and slicing approaches have the potential to increase productivity in the use of multiple PCLS by extending airway viability by a few more days, validation of functional airway responses after cryopreservation has the potential to extend airway function for much longer. The final approach taken in the current study confirmed a single previous study (Rosner *et al.*, 2014) by demonstrating that contraction of mouse airways to MCh was comparable in PCLS that had never been frozen with those that had been frozen for up to 2 weeks. Although there were differences in the maximum contraction to MCh between multiple studies using frozen PCLS, this may be due to the differences in reactivity between Balb/C mice used in this study compared to C57BL/6 mice used by others (Kudo *et al.*, 2012; Rosner *et al.*, 2014). Systematic analysis of responses did report a large variation in airway contractile responses to MCh in frozen PCLS prepared from the same animal and experimental conditions (Rosner *et al.*, 2014). A previous study has shown that ovine lung slices can be maintained for up to 1 month in culture to evaluate the effect of viruses for gene therapy (Rosales Gerpe *et al.*, 2018). Whether airway reactivity is maintained is unknown. Future studies should assess the effect of prolonged storage of lung slices on airway reactivity with the goal to establish a human PCLS biobank to overcome the limitations associated with accessibility and availability of human lung samples.

Although airway contraction has been systematically assessed in cryopreserved PCLS, investigation of its effects on airway relaxation are limited. In one study, the maximally effective concentration of the novel dilator chloroquine (1 mM) elicited relaxation of airways pre-contracted to MCh in mouse PCLS after cryopreservation (Rosner *et al.*, 2014). The effect of cryopreservation on airway relaxation was also analysed in human PCLS pre-contracted to histamine, whereby concentration-dependent relaxation to the long-acting β_2-adrenoceptor agonist, formoterol was maintained (Bai *et al.*, 2016). Surprisingly, no studies have assessed the effect of cryopreservation on relaxation to salbutamol, considered the gold standard bronchodilator treatment for asthma. Our findings show concentration-dependent relaxation to SALB was maintained following cryopreservation with no changes in maximum or potency in mouse PCLS. These findings indicate that cryopreserved PCLS can be used to compare the effect of novel dilator treatments to current therapies and to also assess their potential underlying mechanisms.

Additional techniques to preserve PCLS viability for periods up to several months in culture are progressively being developed, involving supplementation of media with various factors supporting growth such as insulin (Bai *et al.*, 2018). Recent assessment of fibrosis in lung

slices was performed with TGFβ-pre-incubation. Sections of human lung explants cultured with TGFβ for a week demonstrated increased collagen levels and α-SMA expression (Roach *et al.*, 2018). Similarly, healthy human PCLS that were pre-incubated with a "fibrosis cocktail", consisting of TGFβ, TNFα, lysophosphatidic acid (LPA) and PDGF-BB, exhibited increased collagen and α-SMA as well as alveolar thickening (Alsafadi *et al.*, 2017). Other studies have measured the release of inflammatory mediators such as TNFα in mice and marmoset PCLS (Seehase *et al.*, 2011; Donovan *et al.*, 2015). Future studies in which PCLS are treated with exogenous mediators of fibrosis and/or inflammation may allow for the assessment of airway reactivity in disease context as a prelude to *in vivo* animal models.

Table 5. Recommendations for preparation of viable PCLS

Recommendations	References
Incubate overnight after cutting	Perez and Sanderson, 2005
Use PCLS for up to 3 days	Sanderson, 2011
Use PCLS for up to 7 days	Bai *et al.*, 2018
Generate PCLS after delayed filling	Figure 6
Generate PCLS after delayed slicing	Figure 7
Cryopreservation of PCLS for up to 2 weeks	Rosner *et al.*, 2014

In conclusion, we have established improvements in the preparation of PCLS with the potential to extend airway viability and maximise experimental outcomes from the high yield of slices that can be generated from the lungs of multiple species, including human. It was possible to generate functional PCLS across all species, as applied to subsequent chapters (Chapter 5, 6 and 7). We have showed that the transfer of uninflated or inflated lung samples for the assessment of airway reactivity in PCLS is feasible, which could potentially facilitate numerous collaborations between respiratory researchers in different locations. Further studies are required to validate that PCLS viability can be extended from weeks to months, either by cryopreservation or by improved culture conditions. Another key step will be to establish increased access to human tissues to gain additional expertise with this more challenging preparation. Recommendations for the preparation of viable PCLS have been summarised in Table 5. Findings from this chapter underpin the application of PCLS throughout subsequent Chapters to investigate the effect of novel treatments targeting the small airways as well as exploring their underlying mechanism.

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Chapter 4: Relaxin inhibits remodelling of collagen gels by human airway fibroblasts

4.1 Introduction

Lung fibrosis represents an aspect of the dysregulated wound healing response to chronic lung injury and is a key feature of asthma and idiopathic pulmonary fibrosis (IPF) (Nakamura *et al.*, 2015; Samuel *et al.*, 2016). Lung fibrosis, characterized by the excessive deposition of extracellular matrix (ECM) proteins including collagen, fibronectin and elastin, manifests as the scarring and stiffening of lung tissue. This can lead to reduced airway and lung compliance and contributes to poor responsiveness to current pharmacological therapies (Samuel *et al.*, 2016). A major driver of fibrosis is transforming growth factor beta 1 (TGFβ1) which can be produced by the damaged epithelium, leukocytes or fibroblasts in both asthma and IPF.

TGFβ1-stimulated proliferation and differentiation of fibroblasts into myofibroblasts is associated with increased collagen synthesis and also increased expression of the contractile protein, alpha smooth muscle actin (α-SMA). Differentiated fibroblasts can therefore affect both the composition and structure of the surrounding ECM. Whilst studying cells in 2-dimensional cultures may provide for assessment of ECM production, 3-dimensional collagen gels seeded with fibroblasts facilitates the study of cell behaviour in an environment more closely resembling *in vivo* conditions (Helary *et al.*, 2006). When embedded in floating type I collagen gels, fibroblasts can remodel the surrounding collagen network by causing gels to become more closely packed (Helary *et al.*, 2006). This gel “contraction” in the presence of fibroblasts alone is dependent on the activity of collagen degrading matrix metalloproteinases (MMPs) and may be further increased by multiple pro-fibrotic mediators, including TGFβ1 (Kondo *et al.*, 2004; Montesano *et al.*, 1988). The collagen gel assay can therefore provide a functional model of wound healing and tissue scarring in which cell-mediated reorganisation of the matrix via interactions between fibroblasts and the ECM leads to collagen remodelling.

The effectiveness of glucocorticoids for the treatment of asthma may be altered under conditions of increased fibrosis and remodelling. Glucocorticoids, such as dexamethasone, reduce inflammation and inhibit fibroblast proliferation (Stewart *et al.*, 1995), but these beneficial effects become impaired in the presence of increased nonfibrillar type 1 collagen (Bonacci *et al.*, 2006) which is increased in the ECM in asthmatic airways. In clinical trials, glucocorticoid treatment did not significantly improve the primary (force vital capacity- a measurement of lung function) or secondary (dyspnoea scores and image changes on high-resolution tomography) end points in adult patients with pulmonary fibrosis (Hoyles et al., 2006). The lack of efficacy of glucocorticoids to oppose fibrosis is consistent with results from

gel contraction assays. In collagen gels seeded with human fetal lung fibroblasts, glucocorticoid treatment augmented collagen gel contraction (Wen *et al.*, 2001) and did not reduce contraction of gels seeded with human ASM (Bourke *et al.*, 2011).

Pirfenidone is used in the treatment of IPF patients but has not been tested for antifibrotic efficacy in asthma (King *et al.*, 2014; Noble *et al.*, 2011). Its mechanism of action, as defined *in vitro*, involves the inhibition of TGFβ1-induced expression of α-SMA and pro-collagen I (Conte *et al.*, 2014) as well as collagen types I and III fibril formation (Hall et al., 2018, Knuppel et al., 2017). These antifibrotic effects have been observed in both silicosis and allograft murine models of pulmonary fibrosis in which pirfenidone reduced levels of hydroxyproline, a major component of collagen, and also total lung collagen respectively (Feng *et al.*, 2010; Liu *et al.*, 2005). Despite these beneficial experimental effects, pirfenidone does not effectively reverse established fibrosis in IPF and only slows the inevitable progression of this disease.

Relaxin (RLX) is a dimeric peptide hormone with well-documented anti-fibrotic actions. Upon binding to its cognate receptor relaxin family peptide receptor 1 (RXFP1), RLX has been shown to inhibit and even reverse the development of fibrosis by reducing the proliferation and differentiation of fibroblasts into myofibroblasts as well as myofibroblast contractility *in vitro* (Huang *et al.*, 2011) and total collagen deposition mediated by TGFβ1 *in vivo* (Royce *et al.*, 2014). RLX has also been shown to promote collagen degradation by increasing MMPs and decreasing TIMPs (tissue inhibitor of metalloproteinases) in a chronic model of allergic airways disease (Royce *et al.*, 2014; Royce *et al.*, 2009). Of relevance, human lung fibroblasts that were treated with RLX demonstrated a reduction in both collagen type I and III expression whilst promoting the activity of MMP-1 and -2 (Unemori *et al.*, 1996).

The absence of effective treatments to oppose fibrosis in asthma supports the need to compare the efficacy of RLX and pirfenidone relative to glucocorticoids on fibroblast-ECM remodelling.

4.1.1 Aims

The aims of this study were to:

1. optimise cell density and viability for fibroblast collagen gel experiments
2. compare the effect of RLX and pirfenidone relative to glucocorticoid treatment on collagen gel contraction mediated by non-asthmatic and asthmatic fibroblasts

4.2 Methods

4.2.1 Materials and solutions

Dexamethasone (from Sigma-Aldrich, St Louis, MO, USA); pirfenidone (Tocris Bioscience, Bristol, UK); antibiotic-antimycotic, 1x Dulbecco's Modified Eagle Medium (1g/L D-glucose), heat activated fetal bovine serum (FBS), minimum essential media (MEM), penicillin streptomycin (pen strep), trypsin (all from Gibco Life Technologies, AU); human recombinant TGFβ1 (R&D systems); recombinant human gene-2 relaxin (RLX, kindly provided by Corthera Inc., San Mateo, CA, USA; a subsidiary of Novartis AG, Basel, Switzerland).

Stock solution of RLX (10^{-2}M) was prepared in 20 mM sodium acetate buffer, pH 5.0. Pirfenidone (10^{-1}M) was prepared in 100 mM HCl and dexamethasone (10^{-3}M) was prepared in PBS. TGFβ1 (20µg/mL) was prepared in 4 mM HCl with 0.1% BSA.

4.2.2 Cell culture

Fetal lung fibroblasts (HFL-1) were used to validate cell density requirements for collagen gel experiments. HFL-1 were obtained from the ATCC (Manassas, VA, USA) at passage 10 (CCL-153™). Non-asthmatic and asthmatic primary lung fibroblasts (PLF) were generated from normal bronchial tissue, collected from 5 subjects after lobectomy for operable lung cancer (non-asthmatic: PLF-11, -12, -46; asthmatic: PLF-121 (COPD and asthmatic patient, FEV1 26%) and PLF-135 (asthmatic patient, FEV1 80%)). PLF were kindly provided by Prof. Phillip Bardin and Dr. Belinda Thomas (Hudson Institute of Medical Research, AU). Cells were maintained at 37°C in 5% CO_2 air in Dulbecco's Modified Eagles Medium (DMEM, for fetal lung fibroblasts) or Minimum Essential Media (MEM, for primary lung fibroblasts) (supplemented with 2 mM in L-glutamine, 10% vol/vol fetal bovine serum (FBS) and 100 U/mL-1 penicillin G). Cells were split at 1:4 ratio per week and grown to confluence in 250 mL flasks for use between passages 11-14 for HFL-1 and 5-6 for PLF. The viability of cells was recorded following serum-deprivation and prior to collagen gels experiments. Flasks of cells which were <70% viable were not used for experiments.

4.2.3 Preparation of collagen gels

Rat tail tendon collagen was prepared as previously described (Bourke *et al.*, 2011). Collagen bundles from rat tails were dissected under aseptic conditions and extracted in 0.5 M acetic acid at 4°C and allowed to cool for 24 h. This solution was centrifuged at 10,000 rpm at 4°C for 2 h over 3 days to separate the collagen solution from the collagen fibres (fibrillar type I

collagen). The collagen solution was dialysed in water to remove any excess acetic acid and stored at 20°C. One rat tail provided 10-20 mL of collagen solution.

Confluent fibroblasts were serum-deprived for 72 h in FBS-free media to stop cell growth. Cells were trypsinized (0.5% trypsin-EDTA), collected and centrifuged at 10,000 rpm for 5 mins. Cells were then resuspended at 0.25-0.5x10^6 cells/mL in 4X DMEM and combined with the collagen solution (1 part cells:3 part collagen) at 4°C. The cell-collagen mixture was transferred into 24-well culture plates (500 µL/gel). Once set, gels were dislodged into 6-well culture plates with 1 mL 1xDMEM and suspended in a total volume of 2 mL 1X DMEM at 37°C for up to 72 h.

4.2.4 Collagen gel experiments

The density required for cell-mediated gel contraction was optimised by seeding gels with cells at 0.25x10^6, 0.5x10^6 and 1x10^6 cells/mL. Gels were further treated with different concentrations of TGFβ1 (40, 100, 400 pM) to determine the optimal TGFβ1 concentration for additional contraction of gels.

Gels were treated at t=0 with TGFβ1 in the absence and presence of RLX (100 nM), pirfenidone (500 µM) or dexamethasone (100 nM) or left untreated apart from the addition of vehicle(s) (20 mM sodium acetate buffer, 100 µM HCl). as control Images were captured at t=0, 1, 2, 4, 24, 48 and 72 h.

4.2.5 Analysis and statistical tests

Initial gel areas were constrained to the diameter of a single well within a 24-well plate. Areas of gels at all time points were manually measured with Image J and expressed as % of well area in a 6-well plate. This was then normalised to gel area at t=0 and expressed as gel contraction (% initial area). Values of *n* represented the average of duplicate gels, either for each separate experiment for HFL-1 or from different PLF cultures. All data are expressed as the mean ± s.e.m unless otherwise indicated.

Results for responsiveness of HFL-1 to TGF-β1 were analysed using a non-parametric unpaired Student's *t*-test. Responses of PLF to treatment in the presence and absence of TGFβ1 were analysed with a one-way ANOVA and multiple comparisons test. Statistical significance was accepted at $P<0.05$. All graphs and statistical tests were generated using *GraphPad Prism 7* software.

4.3 Results

4.3.1 Optimisation of collagen gel experiments

To assess whether HFL-1 could mediate collagen remodelling, gels were prepared in the absence and presence of cells seeded at different densities. There was little change in gel area over 48 h in unseeded collagen gels. At cell densities ranging from 0.25 $x10^6$-$1x10^6$ cells/mL, there was a reduction in collagen gel area. This reduction was consistent between replicates ($P<0.05$ vs 0 cell density) (Figure 1A). A time-course was then conducted, comparing cell-free gels with gels seeded at a density of $0.5x10^6$ cells/mL as a submaximal response. Although there were no differences in areas up to 24 h, HFL-1-mediated contraction was evident at later time-points, reaching a reduction in gel area of up to 17.5% at 72 h ($P<0.05$ vs no cells at 72 h) (Figure 1B).

An optimum concentration of TGFβ1 to induce HFL-1-mediated collagen remodelling beyond cell-mediated effects was then defined to enable the assessment of treatments. Pre-incubation of gels with TGFβ1 at 40-400 pM resulted in a concentration-dependent reduction in gel area at 24 hr but contraction to similar extents (% gel reduction CTRL: 14.1±1.5; 400 pM TGFβ: 29.4 ± 1.1, $P<0.001$) was seen by 72 h irrespective of the TGFβ1 concentration tested (Figure 2). The greatest reduction in gel area occurred with 400 pM at 24 h.

The effect of cell viability on collagen remodelling was tested. In gels seeded at the same density, but with a lower percentage of viable cells, there was little reduction in gel area either in the absence or presence of TGFβ1 (Figure 3). HFL-1-mediated contraction of 20% was further increased by TGFβ1 treatment when cell viability was higher (Figure 3).

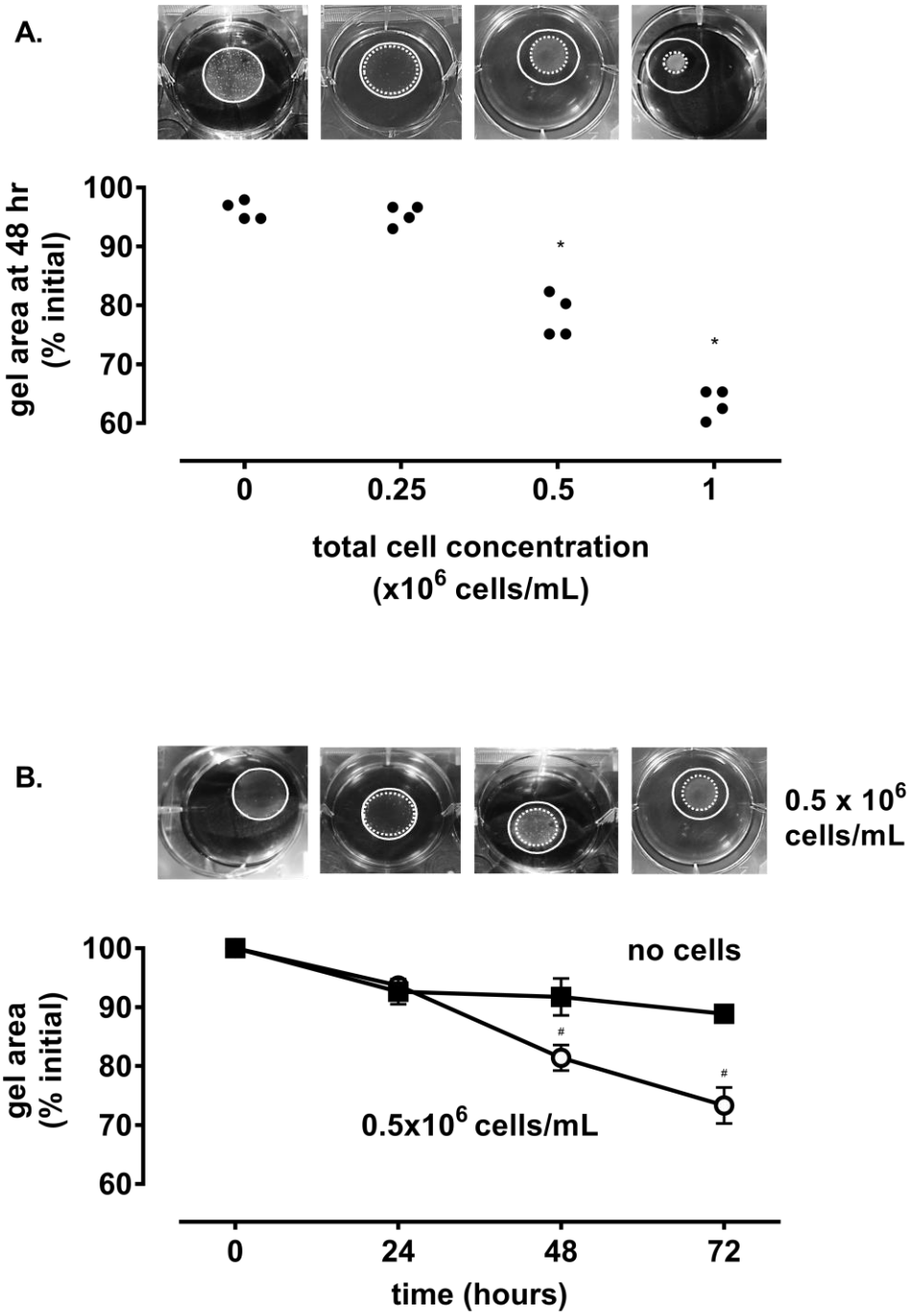

Figure 1. Collagen gel contraction is cell density- and time- dependent. The effects of (A) cell density at 48 h and (B) time on collagen gel area were assessed in gels seeded with fetal lung fibroblasts (HFL-1). Results are expressed as % initial area. Data shows replicates from n=4 cell passages. *P<0.001 vs 0 cell concentration at 72 h. Panel B shows replicates from different cell passages averaged from duplicates within passage. Representative images demonstrate changes in gel area where white circle= initial gel area, white dotted circle= gel area at (A) different densities and (B) different times. #P<0.05 vs no cells at 48 and 72 h.

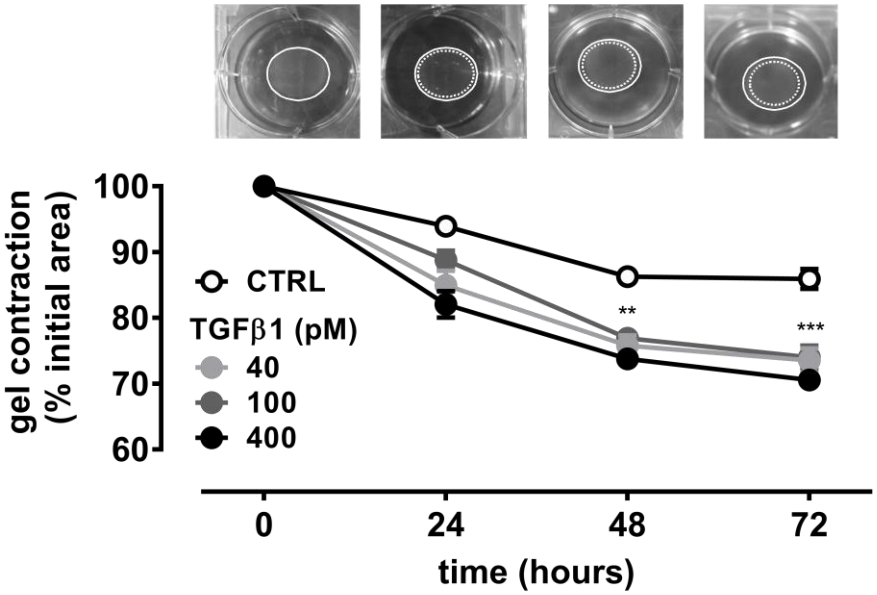

Figure 2. Collagen gel contraction is increased by TGFβ1. Collagen gel area is reduced with TGFβ1 pre-incubation in gels seeded with human fetal lung fibroblasts (HFL-1) at $0.5x10^6$ cells/mL. Gel areas show effect of fibroblast density over 72 h. Data shows duplicates from a n=3 cell passage. Representative images demonstrate changes in gel area where white circle= initial gel area, white dotted circle= gel area at different times. $^{**}P<0.01$, $^{***}P<0.001$ vs CTRL at respective time points.

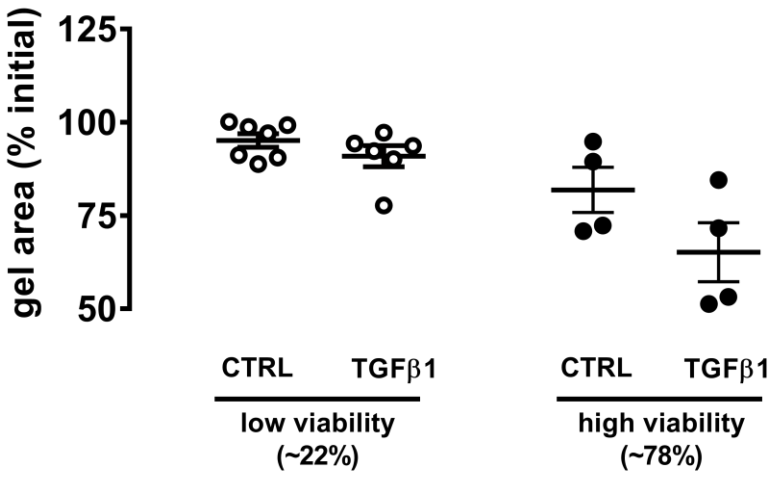

Figure 3. Collagen gel contraction to TGFβ1 is dependent on cell viability. The effect of cell viability in the presence and absence of TGFβ1 (40 pM) at 72 h were assessed on collagen gels seeded with human fetal lung fibroblasts (HFL-1). Results are expressed as % initial area. Data show replicates from cell passages averaged from n=3 passages.

4.3.2 Cell-mediated contraction by primary lung fibroblasts is increased in asthma

The effect of treatment on fibroblast-mediated contraction was then assessed. To determine whether fibroblast-mediated gel contraction varied with disease, non-asthmatic and asthmatic lung fibroblasts were seeded into collagen gel matrices. In the absence of treatment, contraction of collagen gels occurred in a time-dependent manner where there was a noticeble reduction in area at 24 h. Non-asthmatic PLF-1 gels demonstrated a maximum reduction of 24.8 ± 1.8% in initial gel area in comparison to gels seeded with asthmatic firoblasts which contracted by 39.7% of its initial area (Figure 4).

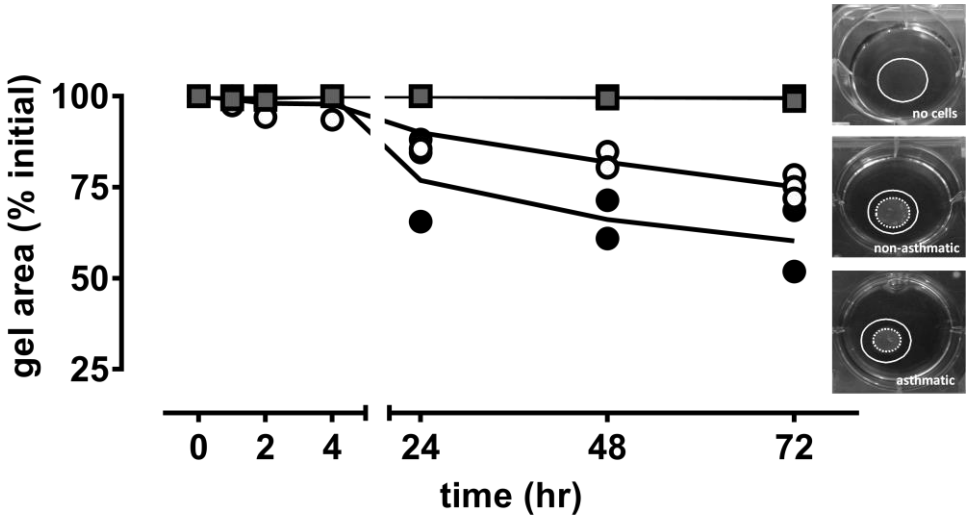

Figure 4. Cell-mediated contraction is increased in collagen gels seeded with fibroblasts from asthmatic sufferers. Gels were prepared in the absence (squares) or presence of primary lung fibroblasts (PLF-1) from non-asthmatic (open circles) or asthmatic (closed circles) subjects, and areas measured over 72 h. Representative images show effect of cell-mediated contraction at 72 h where white circle= initial gel area, white dotted circle= gel area. Results are expressed as % initial area. Data is averaged from duplicate gels within the same passage of fibroblasts from a given culture, then averaged across cultures (n= 3 non-asthmatic, n= 2 asthmatic).

4.3.3 Cell-mediated contraction of collagen gels is not inhibited by relaxin, dexamethasone nor pirfenidone

There was no attenuation of contraction by any treatment in either non-asthmatic or asthmatic fibroblasts (Figure 5). However, areas were generally reduced to a greater extent in the presence of dexamethasone for gels seeded with non-asthmatic fibroblasts (Figure 5C) but not asthmatic fibroblasts (Figure 5D).

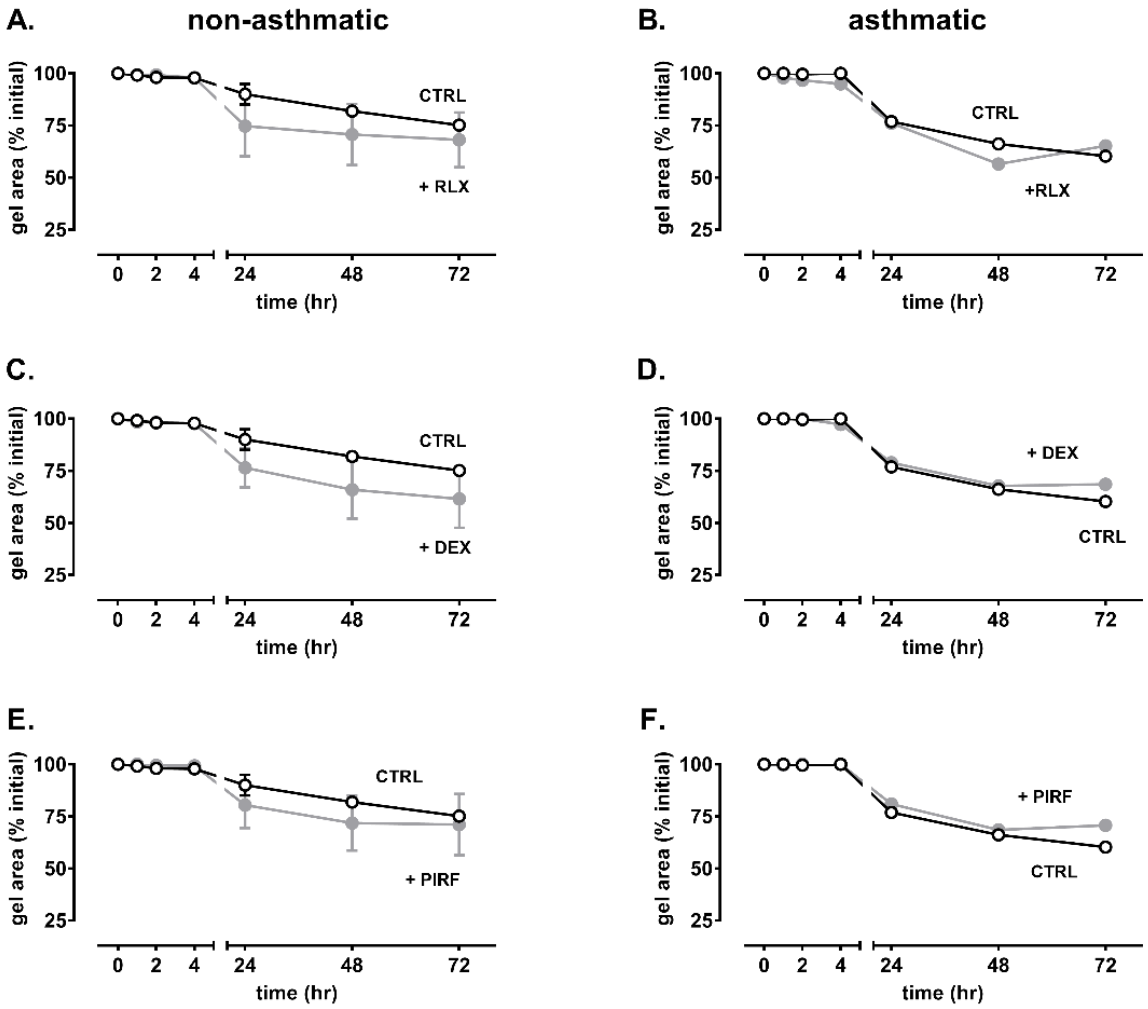

Figure 5. Cell-mediated collagen gel contraction by non-asthmatic and asthmatic fibroblasts is not inhibited by relaxin, dexamethasone or pirfenidone. The effect of (A, B) RLX (0.1 µM), (C, D) dexamethasone (DEX, 0.1 µM) and (E, F) pirfenidone (PIRF, 500 µM) on collagen gel contraction over 72 h were assessed in gels seeded with (A, C, E) non-asthmatic and (B, D, F) asthmatic fibroblasts. Results are expressed as % initial area (duplicates for n=3 non-asthmatic; n=2 asthmatic).

4.3.4 TGFβ1 increases collagen gel contraction by non-asthmatic and asthmatic fibroblasts

To assess whether the effect of TGFβ1 on gel contraction varied with disease, non-asthmatic and asthmatic lung fibroblasts were seeded into collagen gel matrices. TGFβ1 incubation caused a further ~25% decrease reduction in gel area in both non-asthmatic and asthmatic fibroblasts (Figure 6). A marked reduction in gel area relative to untreated gels was evident at 24 hr which persisted until 72 h with final reductions in area of ~50% in gels with non-asthmatic fibroblasts and and ~ 75% with asthmatic fibroblasts (Figure 6).

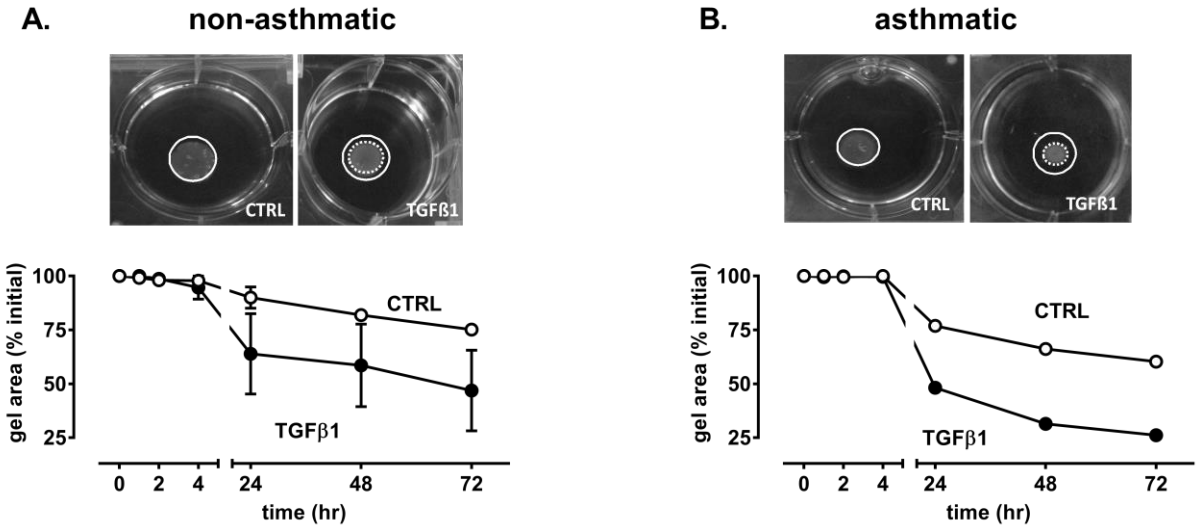

Figure 6. TGFβ1-induced contraction is increased in collagen gel seeded with non-asthmatic and asthmatic fibroblasts. The effect of TGFβ1 (40 pM) on collagen gel contraction over 72 h were assessed in gels seeded with (A) non-asthmatic and (B) asthmatic fibroblasts. Representative mages depict changes in gel area following TGFβ1 treatment at 72 h where white circle= initial gel area, white dotted circle= gel area. Results are expressed as % initial area (duplicates for n=3 non-asthmatic; n=2 asthmatic).

4.3.5 TGFβ1-induced contraction in gels seeded with asthmatic fibroblasts is inhibited by relaxin but not dexamethasone or pirfenidone

In the presence of RLX (0.1 μM), TGFβ1-mediated contraction of collagen gels was reduced at 72 h in non-asthmatic PLF-1 gels (Figure 7A, B). In gels seeded with asthmatic PLF-1, RLX inhibited contraction as early as 24 h and the extent of contraction at 72 h was similar to cell-mediated contraction in the absence of TGFβ1 (Figure 7B).

When tested at the same concentration as RLX, dexamethasone was unable to overcome TGFβ1-mediated contraction of gels seeded with non-asthmatic or asthmatic PLF-1 (Figure 7C, D).

Pirfenidone was tested at a 5000-fold higher concentration (500 μM) than RLX. Pirfenidone only inhibited contraction of gels seeded with non-asthmatic PLF-1 and only at early time points (Figure 7E). Pirfenidone did not inhibit the increased TGFβ1-mediated contraction of gels seeded with asthmatic fibroblasts.

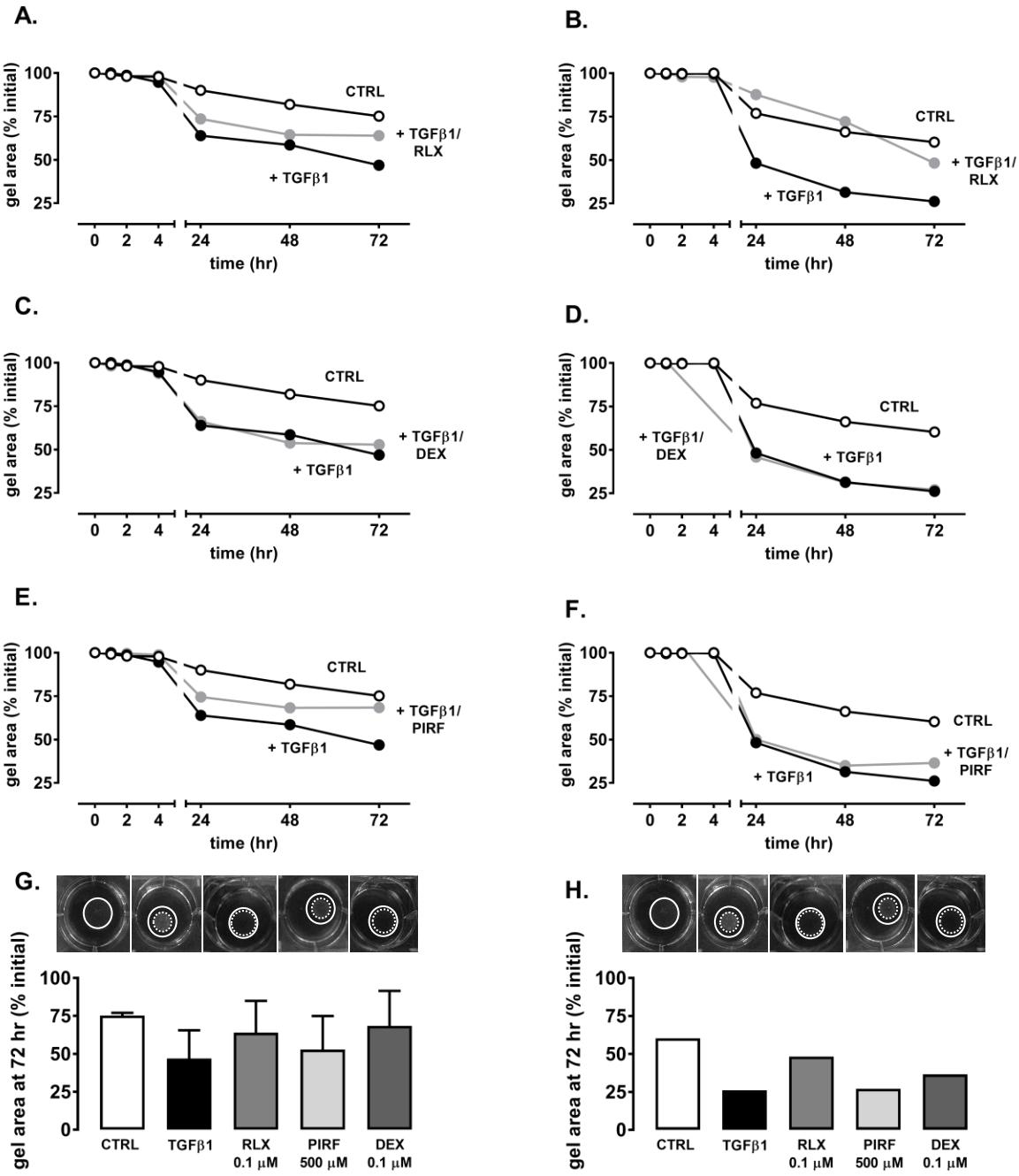

Figure 7. TGFβ1-induced contraction in gels seeded with asthmatic fibroblasts is inhibited by relaxin but not dexamethasone or pirfenidone The effect of (A, B) RLX (0.1 μM), (C, D) dexamethasone (DEX, 0.1 μM) and (E, F) pirfenidone (PIRF, 500 μM) on collagen gel contraction in the presence of TGFβ1 (40 pM) over 72 h were assessed in gels seeded with (A, C, E) non-athmatic and (B, D, F) asthmatic fibroblasts. Grouped data and representative images demonstrate changes in gel area at 72 h (G, H) Representative images demonstrate changes in gel area where white circle= initial gel area, white dotted circle= gel area. Results are expressed as % initial area. Data in time-dependent curves is presented without error bars for clarity (duplicates for n= 3 non-asthmatic; n= 2 asthmatic).

4.4 Discussion

Airway fibrosis is a hallmark of airway wall remodelling in asthma that is resistant to treatment with anti-inflammatory glucocorticoids (Hansbro *et al.*, 2017). In the present study, lung fibroblasts were cultured in three-dimensional collagen gels to assess gel contraction as a functional response consistent with tissue remodelling and fibrosis in disease. Studies using HFL-1 validated the importance of cell density and viability on fibroblast-mediated contraction as well as the capacity of TGFβ1 to further reduce gel area. In gels containing primary fibroblasts, the increase in contraction in gels seeded with asthmatic compared to non-asthmatic cells was further increased by TGFβ1 treatment. Preliminary studies comparing RLX, dexamethasone and pirfenidone revealed differential effects on both cell- and TGFβ1-mediated contraction. None of the treatments decreased cell contraction in gels seeded with either non-asthmatic or asthmatic fibroblasts. However, only RLX appeared to completely prevent TGFβ1-mediated collagen remodelling by asthmatic fibroblasts.

The collagen gel contraction model is used to study wound healing and remodelling by fibroblasts. Optimisation of cell density in this model is critical for further assessment of stimulated responses. Collagen gel contraction using the HLF-1 cell line was shown to be cell density- and time- dependent which is consistent with previous literature in HLF-1 and human bronchial epithelial cells (Mio *et al.*, 1998; Horie *et al.*, 2014) and healthy airway smooth muscle cells (Bourke *et al.*, 2011). This reduction in area may be caused by the generation of tension by fibroblasts onto ECM resulting in strong cell-matrix adhesion (Roy *et al.*, 1999) or by collagen degradation. However, HFL-1 produce low levels of MMPs and high levels of TIMPs which likely results in the inhibition of collagen degradation (Liu *et al.*, 2006). Therefore, the reduction in gel area is unlikely due to collagen degradation. In addition, in gels seeded with ASM, there was no change in hydroxyproline, a major component of collagen in contracted gels, and collagen gel wet but not dry weights were reduced (Bourke *et al.*, 2011). Changes in collagen amount can be assessed by fluorescence resonance energy transfer (FRET) for procollagen or qPCR for collagen mRNA as a measure of new collagen synthesis. This reflects cellular reorganisation of the surrounding collagen fibrils which results in a more tightly packed matrix.

It is important to assess the optimal cell density required to generate sufficient tractional force to remodel or condense collagen fibrils. The proportion of dead cells could potentially reduce the number of total cells below the minimum number required for effective gel contraction. In

addition, the physical presence of non-viable cells may prevent the interactions between viable fibroblasts and the collagen matrix required for matrix remodelling. Moreover, non-viable cells release factors associated with their cell death, potentially interfering with the pathways necessary for successful gel contraction. One such response to cell death is the increased release of the inflammatory mediator PGE_2 (Hangai *et al.*, 2016). Of relevance to this study, reduced contraction of PLF-seeded collagen gels was associated with elevated PGE_2 levels (Skold *et al.*, 1999). The addition of exogenous PGE_2 suppressed the contraction of collagen gels which was reversed with indomethacin treatment (Skold *et al.*, 1999). It remains to be confirmed whether elevated PGE_2 contributed to the reduction in gel area seen in the current study when fibroblast viability was low.

Whilst cell-mediated gel contraction may represent a normal wound healing process, further contraction induced by TGFβ1 may mimic the aberrant wound healing response in asthma (Lehtonen *et al.*, 2016). Previous studies using either a fibroblast cell line or primary fibroblasts have shown that the addition of increasing concentrations of TGFβ1 increases both the rate and extent of collagen gel contraction (Kobayashi *et al.*, 2005; Lehtonen *et al.*, 2016). In contrast, the range of concentrations tested in the current study only caused concentration-dependent contraction at 24 h, and similar reductions in gel areas at 72 h. Although this suggests that sensitivity to TGFβ1 may vary with the type or source of fibroblast used, the selected TGFβ1 concentration for studies in primary cells represented a submaximal stimulus that could be influenced by asthma status or regulated by drug treatment.

While the collagen gel model has been established as a unique functional assay reflecting the potential contribution of myofibroblast contractility to wound healing and airway remodelling, this study represents the first demonstration of increased contraction by asthmatic fibroblasts. Previous *in vitro* studies have shown that proliferation of fibroblasts from non-asthmatic and asthmatic patients was similar but stimulation with IL-1 caused a greater increase in IL-8 and GM-CSF release from asthmatic cells (Ward *et al.*, 2008). In the context of fibrosis, matrix production in the absence of TGFβ1 stimulation by asthmatic fibroblasts was greater than non-asthmatic fibroblasts as evidenced by increased total collagen (Nihlberg *et al.*, 2010) and procollagen I synthesis (Todorova *et al.*, 2011). The increase in gel-mediated contraction by asthmatic fibroblasts seen here may be due to increased MMP activity. The capacity of cells to tether to surrounding collagen fibrils is dependent on MMPs which are not only involved in collagen breakdown but also invade interstitial type I collagen barriers to contribute to remodelling (Rowe *et al.*, 2011). Several MMPs including MMP-1, -2, and -9 are detectable in asthmatic airways, and are associated with asthma pathophysiology in *in vitro* and *in vivo*

studies (Dolhnikoff *et al.*, 2009; Hacha *et al.*, 2012) where they regulate ECM turnover. Whether MMPs are key regulators in the increased collagen remodelling by asthmatic fibroblasts in the current study could be assessed using MMP inhibitors.

Additionally, increased collagen gel contraction by asthmatic fibroblasts may be due to the greater upregulation of α-SMA. In the absence of TGFβ1, post-mortem lung sections from asthmatics demonstrated increased α-SMA positive myofibroblasts in the lung parenchyma (Boser *et al.*, 2017). An *in vitro* study showed that contraction of collagen gels was greater in lung fibroblasts from allergen (ovalbumin, OVA)- challenged mice compared to contraction by fibroblasts from control mice (Sugiura *et al.*, 2007). This was related to increased release of TGFβ and expression of α-SMA by OVA-fibroblasts in the absence of exogenous stimuli (Sugiura *et al.*, 2007). This suggests that endogenous mediators released by asthmatic or asthmatic-like fibroblasts may contribute to collagen gel contraction.

The upregulation of the pleiotropic cytokine TGFβ1 in asthma has been implicated in the fibroblast proliferation and differentiation that leads to airway fibrosis. In the current study, TGFβ1 increased contraction of gels seeded with both non-asthmatic and asthmatic fibroblasts consistent with its profibrotic action. Mechanisms involved in TGFβ1-mediated contraction of collagen gels have been assessed in embryo fibroblasts derived from Smad3 null mice (Liu *et al.*, 2003). These fibroblasts lost the capacity to induce collagen gel contraction following overnight TGFβ1 exposure which suggests TGFβ1-mediated gel contraction is dependent on Smad signalling (Liu *et al.*, 2003). Increased phosphorylation of Smad2/3 is also evident in the lungs of OVA-treated mice which also exhibit elevated TGFβ1 levels (Chen *et al.*, 2011). In bronchial biopsies, greater expression of pSmad2 in asthmatic patients compared to non-asthmatic patients (Sagara *et al.*, 2002) also suggests that TGFβ1-mediated Smad signalling contributes to the development of airway remodelling in asthma.

The additional increase in fibroblast contraction in response to TGFβ1 has also been attributed to the increased expression of the contractile protein, α-SMA, associated with the transition of fibroblasts into the more fibrotic myofibroblasts (Makinde *et al.*, 2007). It remains to be determined whether the TGFβ1-induced increase in α-SMA is greater in asthmatic than non-asthmatic fibroblasts. It appears less likely that the greater reduction in gel area is due to TGFβ1-induced proliferation. Even though we have shown that cell density determines the extent of gel contraction, it is unlikely that cell number would be significantly increased within 24 h, and as previously reported, the proliferative responses of non-asthmatic and asthmatic

fibroblasts are similar (Ward *et al.*, 2008). Further characterisation of the mechanisms involved in TGFβ1-mediated contraction may provide insights into altered signalling in disease.

Treatments that specifically target fibrosis in lung disease have limited efficacy. The current study compared the anti-remodelling effects of RLX, pirfenidone and dexamethasone on cell-mediated and TGFβ1-induced gel contraction (summarised in Table 1). RLX and pirfenidone are putative antifibrotics while dexamethasone is a glucocorticoid which reduces inflammation. Glucocorticoids are used to treat inflammation in a range of chronic lung diseases where fibrosis is a hallmark.

drug treatment	**fibroblast alone**		**+TGFβ1**	
	non-asthmatic	**asthmatic**	**non-asthmatic**	**asthmatic**
no treatment	↑	↑↑	↑↑	↑↑↑
+RLX	–	–	↓	↓
+dexamethasone	↑	–	–	–
+pirfenidone	–	–	↓	–

Table 1. Effect of TGFβ1 and drug treatments on collagen gel contraction by non-asthmatic and asthmatic fibroblasts. In the absence of drug treatment, arrows indicate the extent of gel contraction relative to non-asthmatic fibroblasts with asthma status and TGFβ1 treatment. For drug treatments, arrows indicate change in contraction relative to the same cell type without or with TGFβ1. ↑ = further contraction, ↓ = inhibition, – = no change.

An intermediate concentration of RLX was selected based on a study in human lung fibroblasts which showed that 16.8 nM was required to inhibit TGFβ1-induced collagen expression while 166.6 nM was shown to reduce collagen content and the expression of pro-fibrotic markers (Unemori *et al.*, 1996). Since pirfenidone at 500 µM reduced expression of pro-fibrotic genes including α-SMA and COL3A1 in human IPF fibroblasts, this concentration was selected for experiments in the current study (Lehtonen *et al.*, 2016; Knüppel *et al.*, 2017). The effective concentration of dexamethasone required to alter procollagen I and TGFβ1-induced α-SMA expression and reduce proliferation of HLF-1 varied across different studies, ranging from 5 nM-100 µM (Brenner *et al.*, 2001; Gu *et al.*, 2004). As such, the concentration of dexamethasone used in the current study was matched to the selected concentration of RLX to allow for the direct comparison of their potential efficacy.

Fibroblast-mediated gel contraction in the absence of TGFβ1 was not affected by RLX or pirfenidone. This is consistent with their proposed mechanisms of action which reduce TGFβ1-mediated pro-fibrotic responses. Moreover, only dexamethasone promoted rather than inhibited fibroblast-mediated gel contraction. The observed increase in contraction to dexamethasone alone may be due to inhibition of endogenous PGE_2 production and increased F-actin content (Skold *et al.*, 1999; Miki *et al.*, 2000). Our current findings suggest that glucocorticoid treatment could worsen fibrosis by promoting ECM remodelling in non-asthmatic and asthmatic lungs.

In the current study, gels containing non-asthmatic and asthmatic PLF-1 were treated with TGFβ1 and assessed for changes in gel area. Dexamethasone did not inhibit the additional TGFβ1-mediated contraction of gels seeded with non-asthmatic or asthmatic fibroblasts which was expected given its lack of anti-fibrotic efficacy.

Pirfenidone was able to inhibit contraction of non-asthmatic PLF-1 gels but was unable to overcome contraction to TGFβ1 in asthmatic PLF-1 gels. The anti-fibrotic actions of pirfenidone have been thoroughly characterised in human nasal polyp-derived fibroblasts (Shin *et al.*, 2015) and lung stromal fibroblasts (Lehtonen *et al.*, 2016). Pirfenidone was shown to reduce TGFβ1 induced proliferation, and α-SMA expression by downregulating the Smad2/3 signalling pathway implicated in the development of fibrosis. This suggests that the drivers of fibrosis may vary between organs, affecting their sensitivity to pirfenidone treatment. Indeed, the differential effects of pirfenidone in targeting fibrosis in IPF suggests that the fibrotic processes may even vary within the lung depending on the disease (the parenchyma in IPF vs the airway in asthma). Of significance to this study, pirfenidone also inhibited the contraction of collagen gels seeded with keloid lung fibroblasts to TGFβ1 suggesting that the antifibrotic properties of pirfenidone were maintained in the disease context (Saito *et al.*, 2012). Given these past findings, it was somewhat surprising that pirfenidone was unable to inhibit TGFβ1-mediated gel contraction of asthmatic fibroblasts. While it is possible that the concentration of pirfenidone used may have not been high enough to overcome the increased collagen remodelling by these diseased cells. asthmatic fibroblast, the current study does not support pirfenidone as an anti-fibrotic treatment for asthma.

In contrast to dexamethasone and pirfenidone, RLX inhibited the increased contraction to TGFβ1 in both non-asthmatic and asthmatic collagen gels. In non-asthmatic fibroblasts, RLX appeared to inhibit collagen gel contraction with greater potency than pirfenidone, since RLX exerted an equal inhibitory effect at a concentration 5000-fold lower than that of pirfenidone.

Unlike pirfenidone, the efficacy of RLX was maintained in asthmatic fibroblasts. The anti-fibrotic effects of RLX are mainly mediated through the inhibition of TGFβ1-signalling and associated downstream phosphorylation of Smad2/3 proteins (Chow *et al.*, 2012). It has been proposed that in rat renal fibroblasts, RLX acts on the RXFP1 receptor to induce the phosphorylation of ERK1/2 which results in the generation of NO. NO inhibits Smad2 phosphorylation and subsequently, increases MMP expression (Chow *et al.*, 2012). Therefore, inhibition of Smad protein phosphorylation by RLX may underly its inhibition of the contraction of collagen gels. In addition, RLX treatment decreases α-SMA expression which indicates that the differentiation of fibroblasts into myofibroblasts is inhibited. Because α-SMA contributes to the increased contractility of asthmatic fibroblasts, it is expected that the contraction of collagen gels seeded with these fibroblasts is suppressed by RLX.

Collagen content was not measured in this study due to difficulties in detecting the differences in synthesised collagen and the collagen gel itself. Despite this limitation, RLX has been shown to reduce collagen synthesis in non-asthmatic lung fibroblasts (Unemori *et al.*, 1996). Collagen degrading MMPs are also increased with RLX treatment (Chow *et al.*, 2012). Remodelling of collagen gels seeded with fibroblasts may be dependent on MMP activity to separate cells from their matrix connects (Phillips *et al.*, 2003). As such, RLX may oppose gel remodelling by regulating MMP activity. However, it is unknown whether these findings are consistent in collagen gels seeded with asthmatic fibroblasts. RLX may also inhibit gel contraction via the release of PGE_2 (Lin *et al.*, 2017).

Although the collagen gel assay permits the assessment of treatment on the progression of remodelling, it does not readily allow assessment of reversibility. Evidence from a mouse model of allergic airways disease showed that RLX can both inhibit and reverse established fibrosis (Royce *et al.*, 2009). The next logical step would be to test the effect of the RLX in *in vitro* preparations, particularly from human tissue. A study has demonstrated the effect of TGFβ1 on human IPF lung slices where gene expression of fibrotic markers as well as secreted collagen I were shown to increase (Alsafadi *et al.*, 2017). This *in vitro* model would allow for the assessment of RLX-mediated reversal of TGFβ1-induced effects. Chronic treatment with RLX on human IPF fibroblasts, which demonstrate higher TGFβ1 gene expression and activity (Tatler *et al.*, 2016), may additionally support its role in reversing fibrosis.

The current study provides further evidence of the antifibrotic potential of RLX in the context of asthma. Preliminary data suggests that RLX opposes collagen gel contraction and therefore

reduces collagen remodelling. Validation of the collagen gel model allowed for the assessment of differences between non-asthmatic and asthmatic fibroblasts following treatment showed only RLX had inhibitory effects in both. These promising findings support the role of RLX as a potent anti-fibrotic treatment in asthma where there is excessive collagen remodelling.

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Chapter 5: Relaxin elicits bronchodilation and bronchoprotection in multiple species

5.1 Introduction

(This Chapter has mostly been published in Lam et al., 2016. The published paper was reformatted to maintain consistency across all chapters in this thesis.)

Asthma is a chronic inflammatory disease of the airways, affecting 300 million world-wide (Masoli *et al.*, 2004). Recruitment of inflammatory cells and stimulation of resident structural cells in asthma promotes epithelial damage, goblet cell metaplasia, fibrosis and the accumulation of airway smooth muscle (ASM) (Mauad *et al.*, 2007). These inflammatory and structural changes contribute to the development of airway hyperresponsiveness (AHR) (Laprise *et al.*, 1999), characterized by excessive bronchoconstriction to allergic and non-allergic stimuli (Yeganeh *et al.*, 2013).

While treatment with the β_2-adrenoceptor (β_2-AR) agonist salbutamol (SALB) generally reverses asthma symptoms, dilator responses in many patients may be limited by factors such as frequent use and/or viral infection leading to tolerance and receptor desensitization (Duechs *et al.*, 2014; Spina, 2014). β_2-AR agonists have also been shown to have relatively lower efficacy in small airways in the distal lung, where increased inflammation and AHR contributes to asthma severity (Donovan *et al.*, 2013; Bourke *et al.*, 2014). Thus, it is important to identify novel agents to reverse or inhibit the development of contraction to act as bronchodilators or bronchoprotective agents in both large and small airways.

Relaxin (RLX) is the recombinant drug-based version of the major stored and circulating form of the human gene-2 relaxin hormone, which mediates its physiological actions via activation of Relaxin Family Peptide Receptor 1 (RXFP1) (Samuel *et al.*, 2007). Although RLX is generally associated with vascular changes in pregnancy and childbirth, its recent clinical assessment for acute heart failure was associated with beneficial effects on hemodynamics and reduced mortality (Teerlink *et al.*, 2013, 2017). These findings were consistent with previous findings of RLX-enhanced vasorelaxation in isolated rodent small renal and mesenteric arteries (McGuane *et al.*, 2011; Leo *et al.*, 2014) and human gluteal and subcutaneous resistance arteries (Fisher *et al.*, 2002; McGuane *et al.*, 2011). RLX has also been shown to influence gastrointestinal motility in mice via the generation of nitric oxide (NO) from an intact epithelial layer (Baccari *et al.*, 2013).

Of relevance to asthma, chronic *in vivo* administration of RLX inhibited fibrosis and the development of AHR in a mouse model of ovalbumin-induced allergic airways disease (AAD)

mimicking key features of asthma (Royce *et al.*, 2009, 2013). Although RLX is able to exert protective effects in the lung, and has also been identified as a safe and efficacious relaxant of vascular and uterine smooth muscle (Bani *et al.*, 1998; Tan *et al.*, 1998), its acute effects on airway contraction have yet to be characterized. Critically, the potential of RLX as a bronchodilator have not been assessed in human preparations, where validation would further support its clinical translation for asthma.

Another potential novel bronchodilator of interest is rosiglitazone (RGZ), originally identified as a potent agonist of peroxisome proliferator activated receptor γ (PPARγ). RGZ has recently been shown to elicit acute airway relaxation independently of PPARγ activation in mouse precision cut lung slices (PCLS) (Donovan *et al.*, 2014) and mouse trachea (Donovan *et al.*, 2015). Furthermore, RGZ was more efficacious than β_2-AR agonists in mouse airways, albeit at lower potency (Donovan *et al.*, 2014). Like RLX, RGZ has been shown to inhibit fibrosis and the development of AHR in mouse models of allergic AAD (Honda *et al.*, 2004; Ward *et al.*, 2004; Donovan *et al.*, 2012).

Given this background, it is hypothesised that RLX would both reverse established airway contraction and inhibit the development of contraction. We also proposed that RLX may have potential additive effects with RGZ and SALB, since these other dilators mediate airway relaxation via different mechanisms.

5.1.1 Aims

The aims of this study were to

1. assess the potential bronchodilator and bronchoprotective effects of RLX
2. compare the effects of RLX with the PPARγ agonist RGZ and the β-AR agonists salbutamol (SALB) and isoprenaline (ISO)
3. assess the efficacy of RLX in airways of different sizes and from different species

5.2 Methods

5.2.1 Materials and solutions

Acetylcholine (ACh), methacholine (MCh), substance P, isoprenaline (ISO), salbutamol (SALB) and Nω-nitro-L-arginine methyl ester (L-NAME) (all from Sigma-Aldrich, St Louis, MO, USA); endothelin-1 (ET-1, GenScript, NJ, USA); indomethacin and rosiglitazone (RGZ) (both from Cayman Chemical, Ann Arbor, MI, USA); recombinant human gene-2 relaxin (RLX, kindly provided by Corthera Inc., San Mateo, CA, USA; a subsidiary of Novartis AG, Basel, Switzerland).

5.2.2 Animals and tissue collection

As described in General Methods: Chapter 2.1 and 2.2.

5.2.3 Myograph and organ bath experiments

Trachea were quickly dissected and mounted in Krebs-Henseleit buffer solution for experiments using myograph for mouse or rat trachea (Danish MyoTechnology, Arthuus Denmark, 5 mL bath) or standard organ baths for guinea pig trachea (10 mL bath) as described in General Methods (Chapter 2.4). Tissues were set to optimum resting tension for maximum development of contraction to potassium physiological salt solution (KPSS; 123.7 mM K^+). A maximal contraction to ACh (30 μM) was also obtained in all tissues.

To assess airway relaxation, cumulative additions of MCh were made to establish a submaximal contraction (50-70% of ACh maximum). Concentration-response curves were then constructed to RLX, RGZ, ISO or SALB. Alternatively, selected concentrations of RLX (0.1 μM), RGZ (10, 100 μM) or ISO (1, 10 μM) were added alone or in combination.

To assess potential inhibition of airway contraction, trachea were pre-incubated with selected concentrations of RLX (0.1 μM), RGZ (100 μM) or ISO (10 μM) for 30 min prior to the construction of cumulative MCh or ET-1 concentration-response curves.
The potential contribution of the accumulation of epithelial-derived factors to inhibition of airway contraction was assessed in trachea pre-incubated in the absence or presence of RLX (0.1 μM) for 2 h. The bath solution was then either left or replaced before concentration-response curves were generated to ET-1.

To assess epithelial-dependence, the epithelium was removed by gentle agitation of the lumen using a wooden toothpick and confirmed by loss of relaxation to the epithelial-dependent dilator substance P (1 µM) and H&E staining of tracheal sections after experiments. Substance P is a neuropeptide which is commonly used to confirm the loss of the epithelium (Fine et al., 1989).

The contribution of NO and cyclooxygenase (COX)-derived mediators was assessed using the NO synthase (NOS) inhibitor, L-NAME (100 µM) or COX inhibitor, indomethacin (3 µM) added during the RLX pre-incubation period before ET-1 curves. Finally, to assess the contribution of cAMP and cGMP signaling, SQ22536 (10 µM) and/or ODQ (1 µM), inhibitors of adenylate cyclase and guanylate cyclase respectively, were added to both intact and denuded trachea before a submaximal contraction to MCh was established for assessment of relaxation to RLX (0.1 µM).

5.2.4 Preparation of precision cut lung slices (PCLS)

PCLS were prepared as previously described and detailed in General Methods (section 2.5). Mice and rats were euthanized as described previously and dissected to expose the heart and trachea. Trachea were cannulated with a catheter containing two ports (20 G Intima, Becton Dickinson, VIC, Australia) and lungs were inflated with ~1.2 or 10 mL agarose gel (2% in 1x HBSS at 37°C), followed by a bolus of ~0.5 or 3 mL air. Lungs were cooled by bathing in cold HBSS/HEPES and kept at 4°C to allow the agarose to solidify, before the lungs were removed. The upper right lobe was isolated and adhered with cyanoacrylate to a mounting plate in a vibratome (Compresstome, Precisionary Instruments, Greenville, NC, US). PCLS of 200 µm thickness were prepared and transferred into cell culture plates containing DMEM, supplemented with 1% penicillin-streptomycin and incubated for 24 h (37°C, 5% CO_2) prior to experiments.

For human lung samples, one main bronchus was identified and cannulated. Other open-ended airways were clamped in order to prevent agarose from leaking. Lungs were filled with 20-40 mL agarose (depending on the size of the lung sample), followed by a bolus of 5-10 mL air. Human lungs were sectioned into 200 µm thin slices as for rodent lungs and incubated overnight before experiments were conducted.

PCLS were transferred to HBSS/HEPES and mounted in custom-made perfusion chambers (~100 µL volume). A viable airway (~200 µm diameter) was selected from each slice based

on the presence of an intact layer of epithelial cells with ciliary activity. PCLS were perfused at a constant rate (~0.5 mL/min) through an eight-channel gravity-fed perfusion system under vacuum.

Slices were initially perfused with MCh (0.3 μM) to establish a submaximal pre-contraction, then perfused with MCh in combination with RLX (0.1 μM) or a β-AR agonist (ISO 1 μM or SALB 10 μM). For rat PCLS only, perfusion was then stopped to permit assessment of airway responses to MCh in the presence of dilator under static conditions.

5.2.5 Analysis of airway contraction

Phase contrast microscopy was conducted using an inverted microscope (Nikon Ti-U, NY, USA) to observe drug-induced airway changes, employing 10X objective lens, zoom adapter, reducing lens and camera (CCD camera model TM-62EX; Pulnix, Takex, Japan). Changes in airway lumen area were captured as digital images (744 x 572 pixels) in time lapse (0.5 Hz) using image acquisition software (Video Savant; IO Industries, Inc. Ontario, Canada). Obtained files were converted to TIFF files and analyzed using NIH/Scion software (Scion Corporation; download www.scioncorpcom) or ImageJ.

5.2.6 Immunohistochemistry

Lungs were dissected whole and fixed in neutral buffered formalin before being embedded in paraffin wax. Sections (5 μm) were cut and mounted on SuperFrost PLUS slides (Menzel-Gläser, Braunschweig, Germany). Immunohistochemistry was performed as Jelinic *et al* (2014). Lung sections were incubated overnight at 4°C with 3 μg/ml rat RXFP1 antiserum (#107, raised against amino acid residues 107 – 119 of the rat RXFP1 protein) or pre-immune serum (rabbit IgG). Immunoreactivity was detected using the MACH2™ system (Biocare Medical, Concord, CA, USA) and 3, 3' diaminobenzidine (DAB) as the chromagen substrate (Vector Laboratories, Burlingame, CA, USA). The rat RXFP1 antibody used has previously been shown to be selective, with no cross-reactivity to another relaxin-family peptide receptor (RXFP2) (Jelinic *et al.*, 2014).

5.2.7 Statistical analysis

For trachea, contraction was normalized to the maximum ACh contraction for MCh or the KPSS response for ET-1. For airways in PCLS, contraction was expressed as % initial airway area. Relaxation was expressed as % of the MCh submaximal pre-contraction. All data are expressed as the mean±s.e.m. Non-linear regression of concentration-response curves was

performed using GraphPad Prism v6 (GraphPad Software, San Diego, CA, USA) to obtain fitted maxima and pEC_{50} values where possible. Results were analyzed via t-tests or one-way ANOVA for multiple comparisons between groups as appropriate; where statistical significance was accepted at $P<0.05$.

5.3 Results

5.3.1 RLX, RGZ and ISO elicit relaxation of rat trachea

Dilator effects of RLX, RGZ, and ISO in rat trachea were compared after pre-contraction to similar submaximal levels with MCh (300 nM), measuring changes in isometric force in a static organ bath (5 mL volume; Figure 1A). When added at 5–10 min intervals, RLX did not cause any relaxation up to 0.1 µM, the highest concentration available (Figure 1B). In contrast, RGZ and ISO induced concentration-dependent relaxation (fitted maximum % relaxation: ISO 45.6 ± 5.3; RGZ 138.1 ± 30.0; $P<0.05$). When compared to RGZ, ISO was more potent (pEC_{50}: ISO 8.0 ± 0.3; RGZ 4.7 ± 0.2; $P<0.001$), but only RGZ elicited complete relaxation (Figure 1A).

When the highest concentrations of each drug tested were allowed to remain in contact with the pre-contracted trachea, marked relaxation to RLX, as well as to RGZ and ISO, became evident. Representative traces show the effects of addition of RLX (0.1 µM), RGZ (100 µM), and ISO (1 µM) relative to a time control showing maintained contraction to MCh alone over 200 min (Figure 1B). Partial relaxation to RLX occurred at slower rate than either RGZ or ISO, which caused near complete and partial tracheal relaxation within approximately 50 and 5 min respectively (Figures 1B, C). Notably, the extent of relaxation to 0.1 µM RLX (% relaxation: 52 ± 15%) was comparable to the maximal ISO-induced airway relaxation (50 ± 9%).

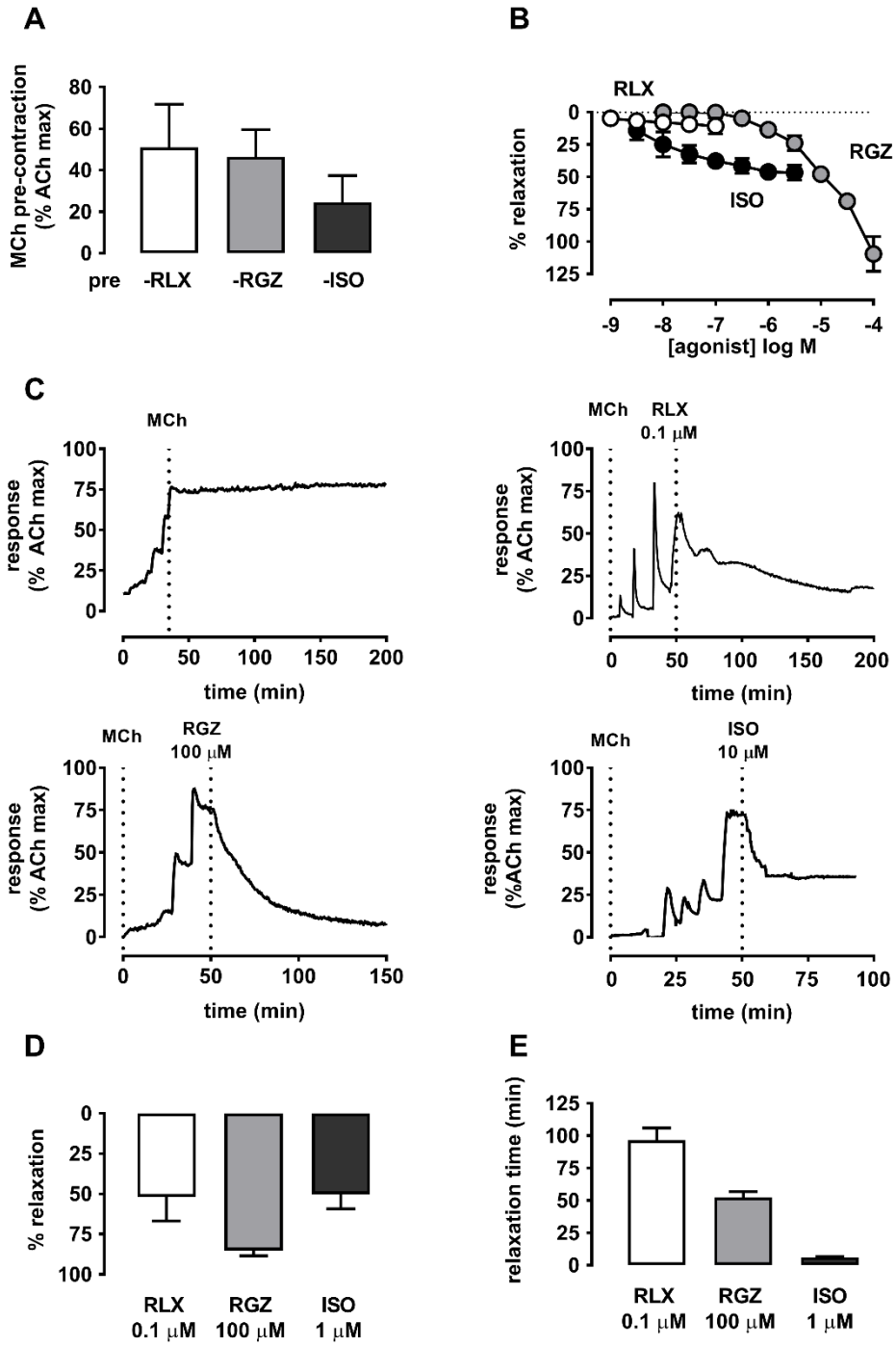


Figure 1. RLX, RGZ and ISO elicit relaxation of rat trachea. Rat trachea were mounted under isometric conditions for optimum development of contraction. Trachea were (A) precontracted with MCh, prior to preparation of (B) concentration-response curves to RLX, RGZ or ISO. (C) Representative traces show relaxation to RLX, RGZ and ISO. (D) Relaxation responses and (E) time to maximum relaxation to single additions of RLX (0.1 μM), RGZ (100 μM) or ISO (1 μM) were also assessed. Extent of relaxation is expressed as % of submaximal MCh pre-contraction, with relaxation time expressed in min (mean ± s.e.m. for n= 4 per group).

5.3.2 RLX potentiates tracheal relaxation to RGZ and β-adrenoceptor agonists

Effective single dilator concentrations of RLX, RGZ, and ISO were also tested in combination in rat trachea (Figure 2). Combining RLX (0.1 µM) with a submaximal concentration of RGZ (10 µM) did not induce greater relaxation compared to RGZ alone, while near-maximal relaxation to RGZ (100 µM) was maintained in the presence of RLX (Figure 2A). However, the addition of RLX markedly increased the rate of relaxation to both concentrations of RGZ tested ($P<0.001$, $P<0.01$ vs. RGZ alone, Figure 2B). In contrast, the addition of RLX resulted in greater relaxation to ISO (0.1, 10 µM) ($P<0.05$ vs. ISO alone), increasing the efficacy of both the submaximal and maximally effective concentrations of ISO (Figure 2C). The rapid rate of ISO-mediated relaxation was not further increased by RLX (Figure 2D).

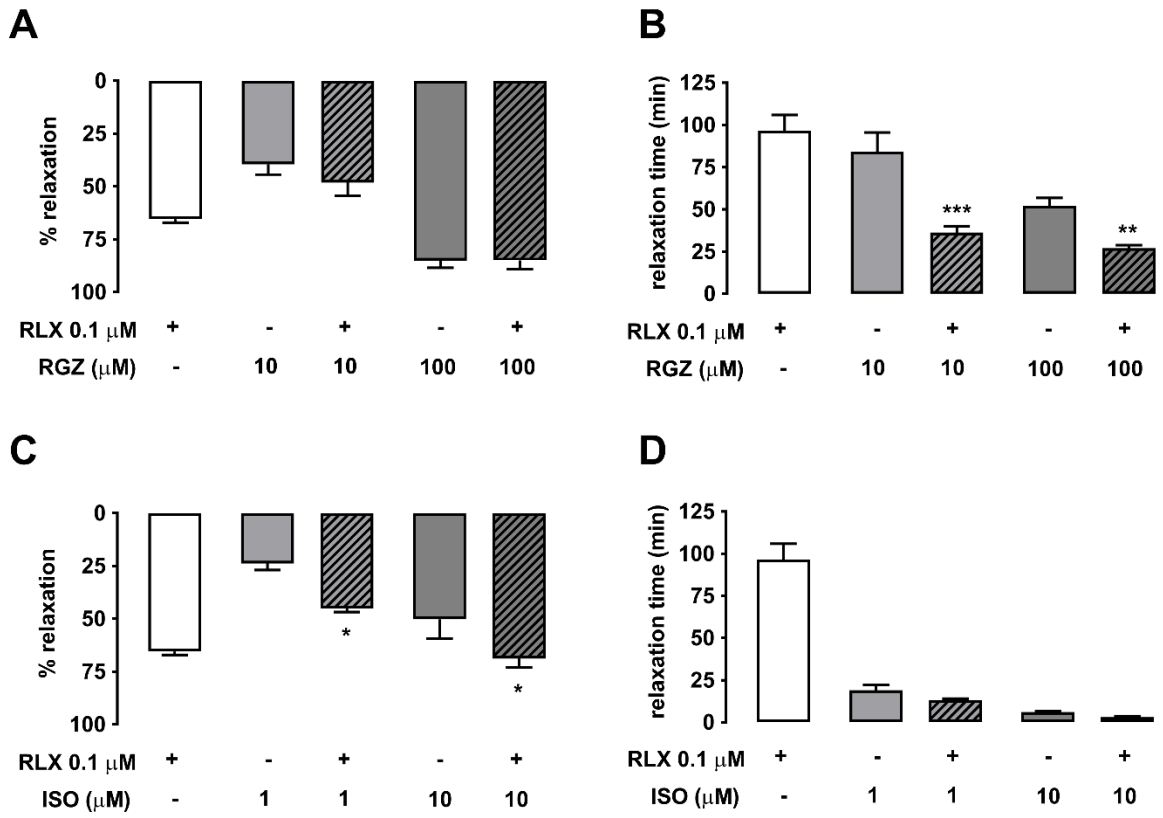


Figure 2. RLX potentiates relaxation to RGZ and ISO in rat trachea. Rat trachea were precontracted with MCh, prior to measurement of (A, C) relaxation responses and (B, D) time to relaxation for RLX (0.1 µM), RGZ (10, 100 µM) and ISO (0.1, 1 µM) alone and in combination. Extent of relaxation is expressed as % submaximal MCh pre-contraction, with relaxation time expressed in min (mean ± s.e.m for n= 4-5 per group). *P<0.05, **P<0.01, ***P<0.001 vs. the matched concentration of RGZ or ISO alone.

5.3.3 RLX, RGZ and ISO are differentially bronchoprotective

The bronchoprotective effects of RLX, RGZ, or ISO in inhibiting the development of contraction were then compared in rat, mouse and guinea pig trachea. Following 30 min pre-incubation with maximally effective dilator concentrations of RLX, RGZ or ISO, concentration-dependent contraction was established. Full and partial curves were obtained for MCh and the much slower-acting contractile agonist, ET-1, over the concentration ranges tested (Figure 3, 4, 5).

Although, the maximum contraction to MCh was reduced by 25% with either RLX (0.1 µM) (Figure 3A) or ISO (1 µM) in rat trachea (Figure 3C), this did not reach significance (Figure 3D). ISO but not RLX inhibited MCh contraction by 83% in mouse trachea (Figure 4A, 4C), but neither were effective in guinea pig trachea (Figure 5A, 5C). In contrast, pre-incubation with an effective dilator concentration of RGZ (100 µM) for 30 min inhibited the development of contractile responses to MCh by 90% in both rat (Figure 4B) and mouse trachea (Figure 5B) but not in guinea pig trachea (Figure 6B).

RLX, RGZ, and ISO all significantly inhibited contraction to ET-1 in rat and mouse trachea (Figures 3E–H, 4E-H). When these drugs were compared at the same concentration (0.1 µM), RLX reduced contraction to 10^{-7} M ET-1 by 50% in both species (Figures 3E, 4E) while at 0.1 µM RGZ was only inhibitory in mouse trachea (Figure 4F). In guinea pig trachea, only ISO inhibited the development of ET-1 contraction where both RLX and RGZ were ineffective (Figure 5E-H).

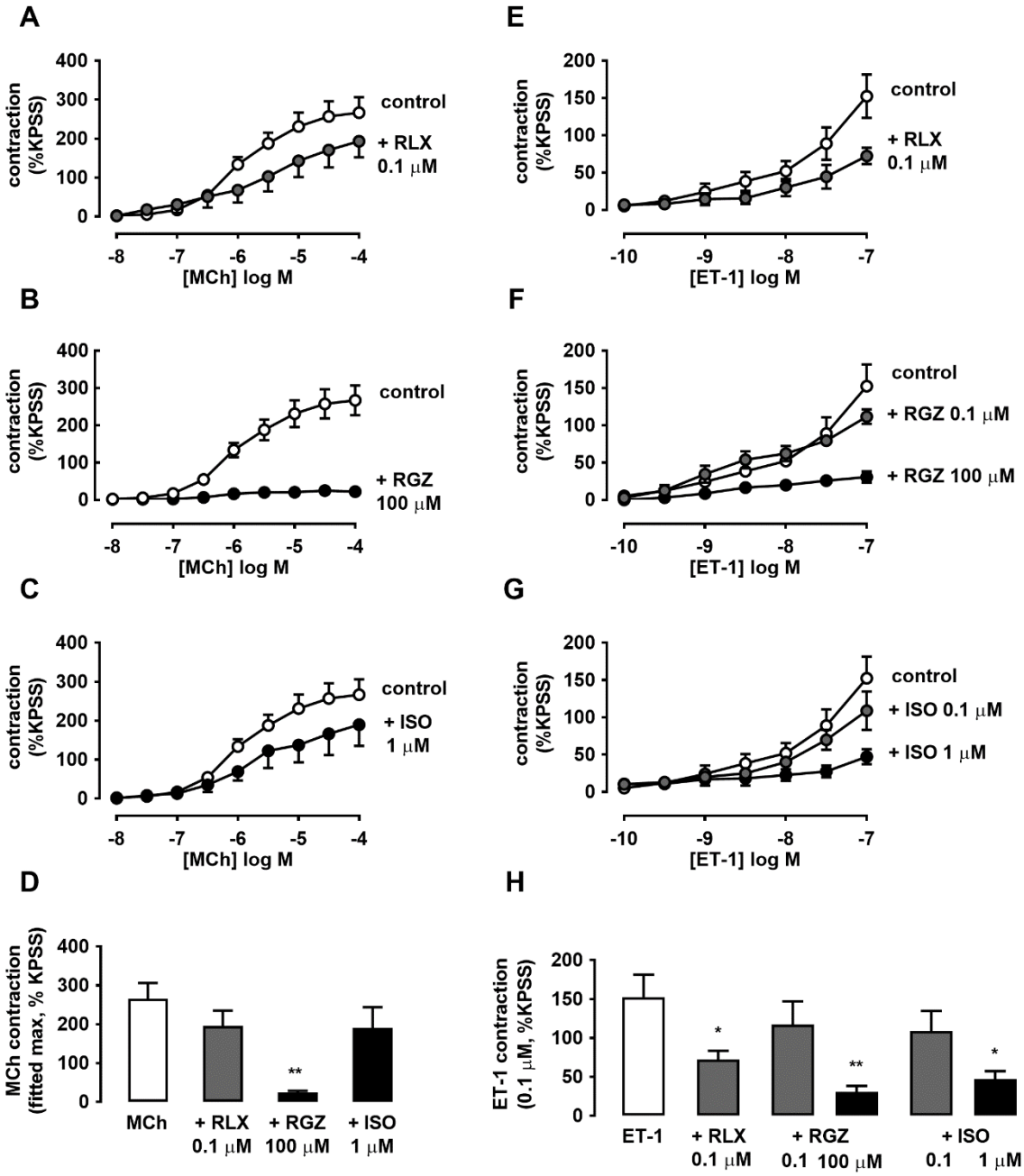


Figure 3. RLX, RGZ and ISO differentially inhibit the development of rat tracheal contraction. The effects of pretreatment with RLX, RGZ and ISO (30 min) on the development of contraction to MCh (left panels) and ET-1 (right panels) were assessed, testing (A, E) RLX (0.1 μM), (B, F) RGZ (0.1, 100 μM) and (C, G) ISO (0.1,1 μM). For clarity, the same control data is shown on panels A-C for MCh and panels E-G for ET-1. (D) Maximum contraction to MCh determined from fitted concentration-responses curves using GraphPad Prism. (H) Contraction to the highest tested concentration of ET-1 (0.1 μM). All responses are expressed as % KPSS standard contraction (mean±s.e.m, n=4-5 per group). *P<0.05, **P<0.01 vs MCh or ET-1 alone.

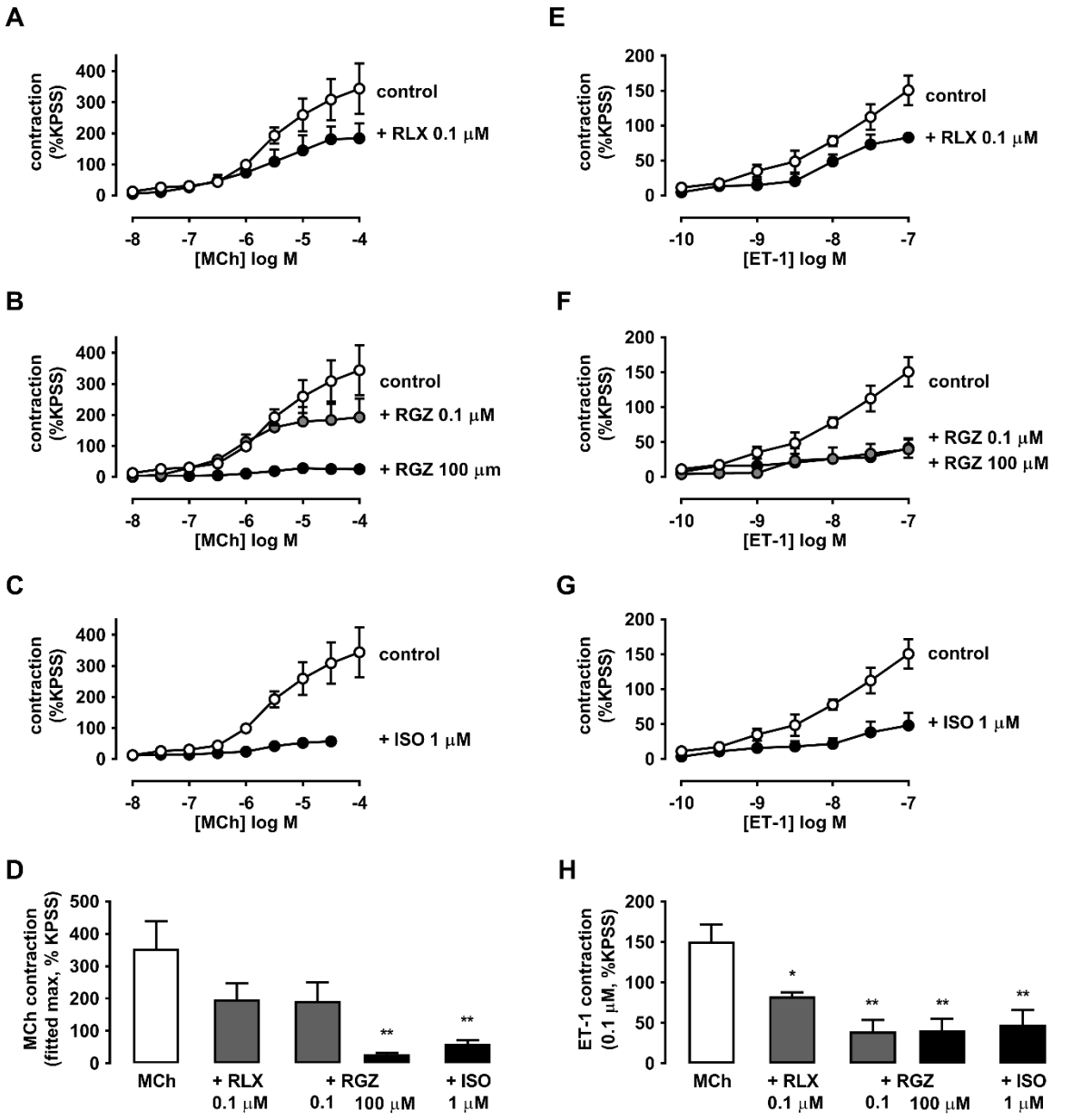


Figure 4. RLX, RGZ and ISO inhibit the development of mouse tracheal contraction. The effects of pretreatment with RLX, RGZ and ISO (30 min) on the development of contraction to MCh (left panels) and ET-1 (right panels) were assessed, testing (A, E) RLX (0.1 μM), (B, F) RGZ (0.1, 100 μM) and (C, G) ISO (0.1,1 μM). (D) Maximum contraction to MCh determined from fitted concentration-responses curves using GraphPad Prism. (H) Contraction to the highest tested concentration of ET-1 (0.1 μM). All responses are expressed as % KPSS standard contraction (mean±s.e.m, n= 4 per group). *P<0.05, **P<0.01 vs MCh or ET-1 alone.

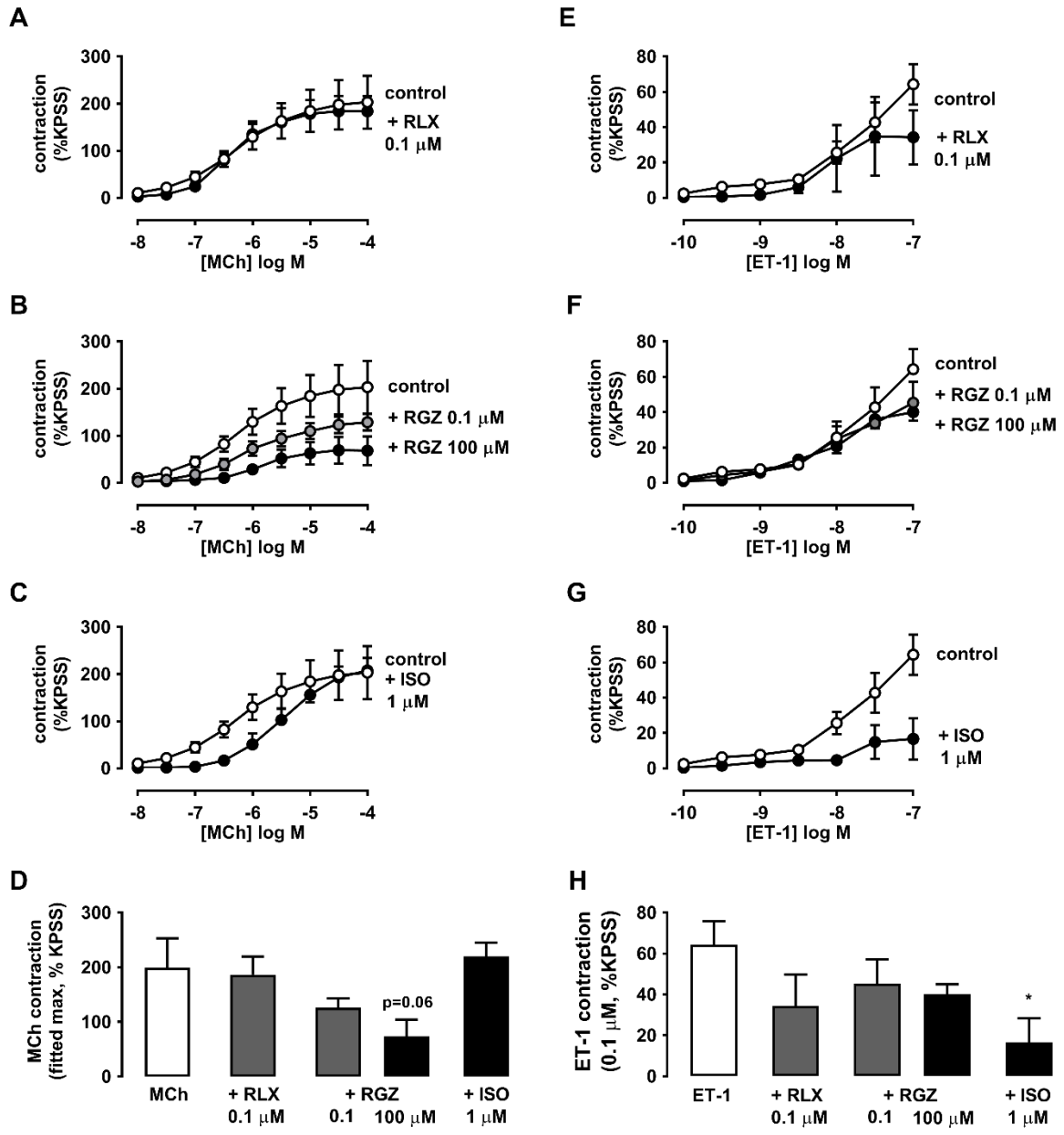


Figure 5. RLX, RGZ and ISO differentially inhibit the development of guinea pig tracheal contraction. The effects of pretreatment with RLX, RGZ and ISO (30 min) on the development of contraction to MCh (left panels) and ET-1 (right panels) were assessed, testing (A, E) RLX (0.1 μM), (B, F) RGZ (0.1, 100 μM) and (C, G) ISO (0.1,1 μM). (D) Maximum contraction to MCh determined from fitted concentration-responses curves using GraphPad Prism. (H) Contraction to the highest tested concentration of ET-1 (0.1 μM). All responses are expressed as % KPSS standard contraction (mean±s.e.m, n=4 per group). *P<0.05, **P<0.01 vs MCh or ET-1 alone.

5.3.4 RLX-mediated inhibition of tracheal contraction is increased with time and dependent on released factors

The effect of increasing RLX preincubation time on the inhibition of development of ET-1 contraction by RLX was assessed in rat trachea (Figure 6A). Pre-incubation with RLX for 120 min completely inhibited the development of ET-1 contraction ($P<0.01$ vs. control), while 30 min preincubation only partially inhibited contraction.

Rat trachea were also preincubated in the absence or presence of RLX, and contraction to ET-1 assessed after the bathing solution was replaced with buffer without RLX (washout) to remove any endogenous mediators released by RLX. In the absence of RLX pre-incubation, ET-1 mediated contraction after washout was lower than without washout (Figure 6B washout compared to Figure 6A control). When pre- treatment with RLX was followed by washout, there was no inhibition of contraction to ET-1 (Figure 6B).

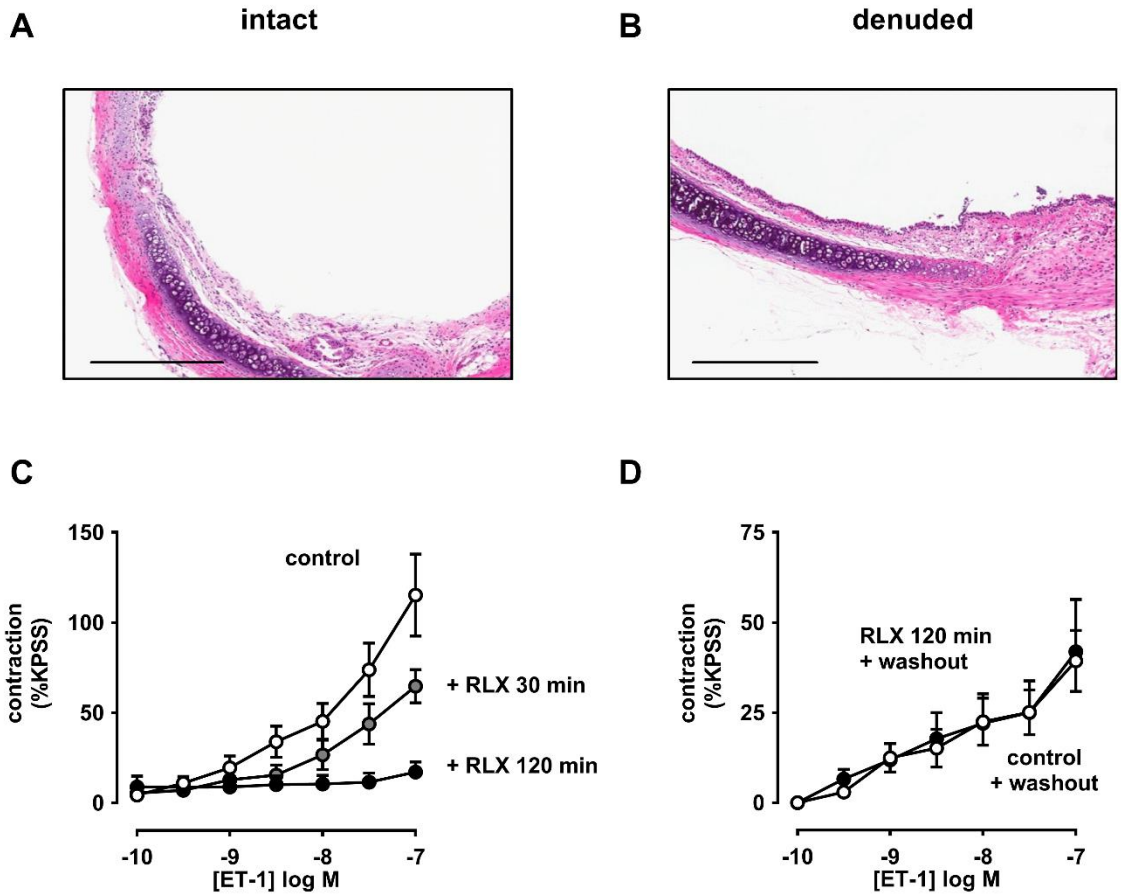


Figure 6. RLX-mediated bronchoprotection is increased with time and dependent on released factors in rat trachea. ET-1-induced contractions were assessed (A) in the absence or presence of RLX (0.1 μM) following pre-treatment with for 30 min and 120 min and (B) in the absence of RLX following 120 min in the absence or presence of RLX (0.1 μM) followed by replacement of the bathing solution. All responses are expressed as % KPSS standard contraction (mean ± s.e.m, n= 4-7 per group).

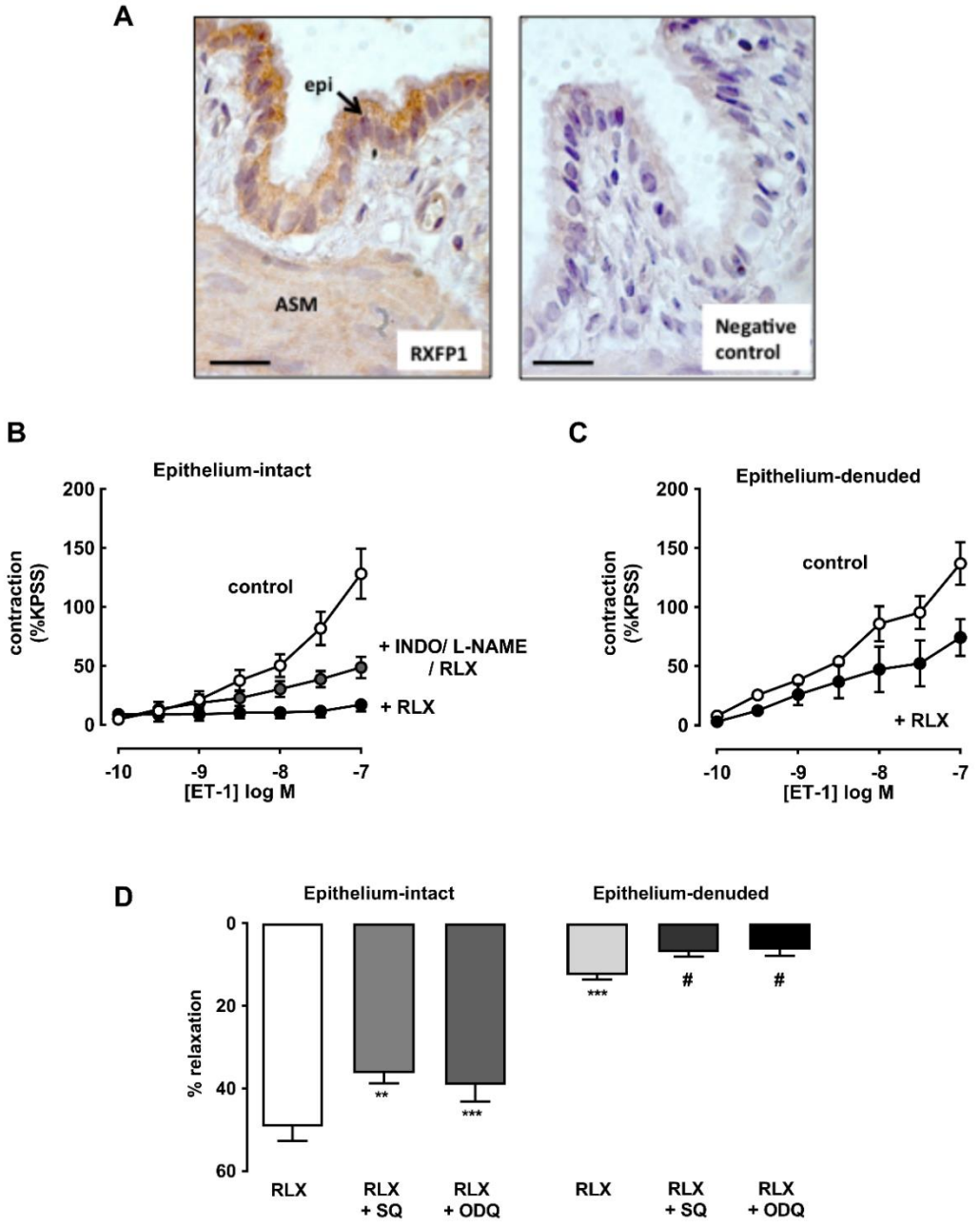
5.3.5 RLX acts via epithelial-dependent and -independent mechanisms

To explore potential sites of action of RLX, expression of its cognate receptor RXFP1 was determined in rat lungs by immunohistochemistry. RXFP1 was localized in rat airways, with a greater intensity of staining detected in the nuclei and cytoplasm of the airway epithelium than smooth muscle (Figure 7A).

To explore potential mediators of inhibition of contraction by RLX, responses to ET-1 were determined in intact rat trachea after 120 min preincubation with RLX in the absence or presence of indomethacin (COX inhibitor) and/or L-NAME (NOS inhibitor). Contraction to 100 nM ET-1 (125 ± 24% KPSS) was almost completely abolished in the presence of RLX (17 ± 6% KPSS). Neither inhibitor alone significantly reduced this inhibitory effect of RLX (data not shown). However, the marked inhibition of contraction to ET-1 by RLX was reduced in the presence of the combination of indomethacin and L-NAME (56 ± 7% KPSS, $P<0.01$ cf. ET-1 + RLX alone) (Figure 7B).

The tracheal epithelium was then removed in order to assess epithelial-dependence. Epithelial denudation was confirmed by conventional haematoxylin staining of formalin-fixed sections (data not shown) and reduced responsiveness to substance P (% relaxation: intact 92.4 ± 2.7%; denuded 10.1 ± 4.0, $P<0.01$). In contrast to results obtained in intact trachea (control vs RLX, Figure 7A), preincubation with RLX for 120 min only partially inhibited contraction to ET-1 following epithelial removal (Figure 7C).

The effects of epithelial removal on relaxation to RLX following pre-contraction with MCh was also assessed (Figure 7D) as well as potential roles for cAMP and cGMP using SQ22536 and ODQ. The level of MCh pre-contraction was similar in epithelial-intact and epithelial-denuded trachea (ΔmN: intact 2.4±0.1, denuded 2.2±0.7, NS). Although relaxation to RLX (0.1 µM) was reduced by 40% with epithelial removal, a small but significant reversal of the contraction to MCh was still evident (Figure 7D). Inhibition of adenylate cyclase and guanylate cyclase, with SQ22536 and ODQ respectively, reduced relaxation to RLX in both intact and epithelial-denuded trachea.



(RXFP1 stained sections provided by Dr. Maria Jelinic and A/Prof. Laura Parry)

Figure 7. RLX acts via epithelial-dependent and –independent mechanisms in rat trachea. RXFP1 protein was localized in rat lungs by immunohistochemistry, and responses to RLX in epithelial-intact and -denuded trachea were compared in the absence and presence of signalling inhibitors. (A) Immunoreactive RXFP1 is predominantly localized in airway epithelium (epi) and smooth muscle (ASM). Contraction to ET-1 in (B) intact trachea under control conditions, and with RLX (0.1 µM) in the absence and presence of INDO (3 µM) or L-NAME (100 µM) and (C) in denuded trachea in the absence and presence of RLX (0.1 µM). Responses to ET-1 are expressed as a % of KPSS contraction. (D) Responses to RLX (0.1 µM) in intact and denuded trachea in the absence or presence of SQ22536 (10 µM) or ODQ (1 µM), expressed as % relaxation of MCh pre-contraction. Data is expressed as mean±s.e.m. (n= 4-8 per group). **P<0.01, ***P<0.001, vs RLX in intact trachea, #P<0.05 vs RLX in denuded trachea. Scale bar= 20 µm.

5.3.6 RLX elicits relaxation of rat and human but not mouse intrapulmonary airways in lung slices

Having established dilator responses to RLX in rat trachea, additional studies used PCLS from rat, mouse and human lungs to determine whether efficacy was also evident in small airways.

Using rat PCLS, the effect of RLX (0.1 μM) on intrapulmonary airway contraction was assessed, measuring changes in airway area under perfused and static conditions in a small volume customized chamber (~100 μL volume).

Representative traces and sequential images of the same airway in PCLS show RLX perfusion over rat lung slices following MCh pre-contraction (Figure 8A). In rat PCLS, RLX rapidly and partially reversed the MCh- induced reduction in airway area (Figure 8A), with greater relaxation achieved under static than perfused conditions (% relaxation: perfused 7±3%, static 64±8%, $P<0.001$; Figure 8A). In contrast, relaxation to both RGZ and ISO was similar under perfused and static conditions, causing near- complete and partial relaxation respectively, similar to that seen in trachea (Figure 8B).

Perfusion with RLX in mouse lung slices (Figure 9) did not cause airway relaxation but was not tested under static conditions as shown by the representative trace and PCLS images.

However, in human PCLS, RLX-mediated relaxation (Figure 10A) was comparable to SALB-mediated relaxation (Figure 10B) of intrapulmonary airways with both dilators delivered under perfused conditions.

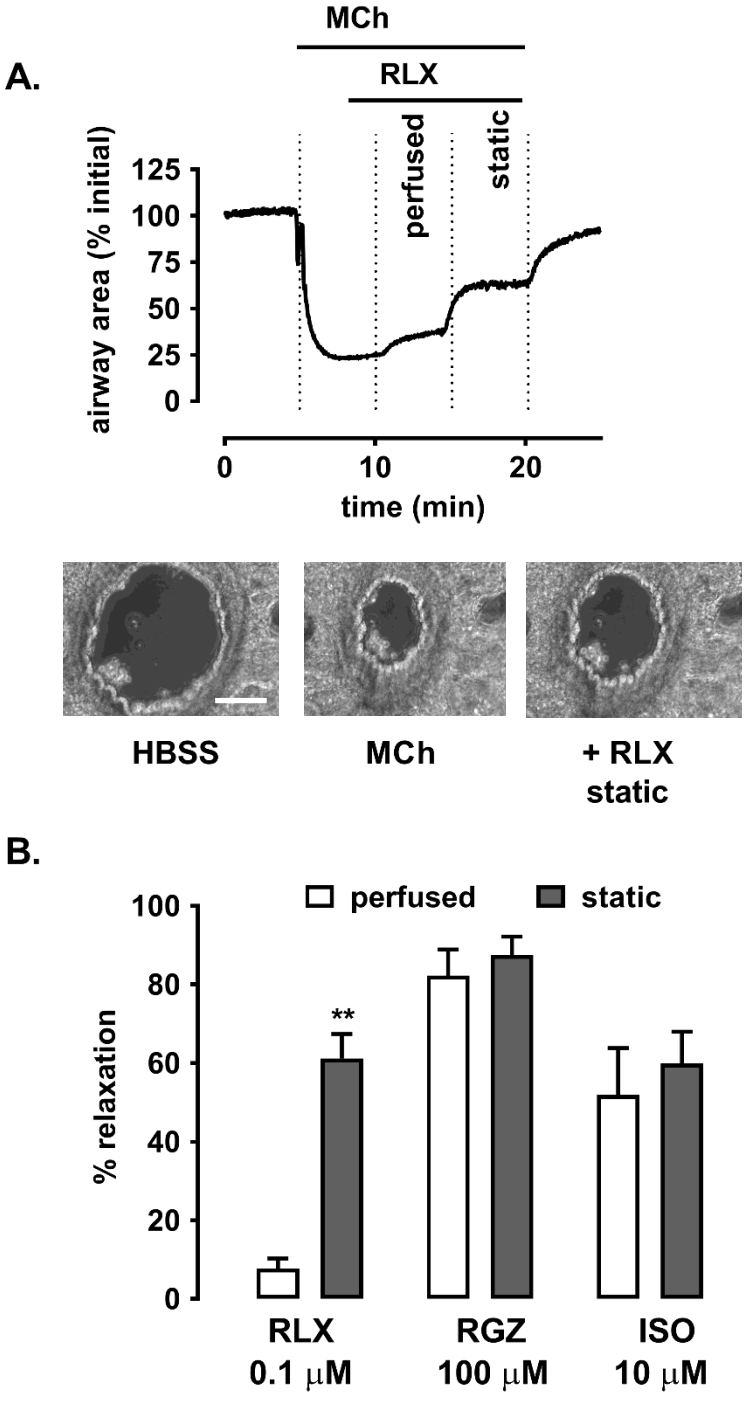


Figure 8. RLX elicits relaxation of rat intrapulmonary airways in PCLS. Rat lung slices were mounted in a customised chamber for assessment of changes in intrapulmonary airway area by phase-contrast microscopy. Airways from rats were pre-contracted with MCh (0.3 µM) in the presence of RLX (RLX, 0.1 µM). (A) Representative time-course and phase-contrast images of the last frame of each condition. Traces show airway area values in pixels determined from gray-scale analysis of images using Video-Savant. (B) Grouped data shows relaxation by RLX, RGZ (100 µM) and ISO (10 µM) under perfused and static conditions. Data is expressed as % relaxation of MCh pre-contraction (mean±s.e.m, n= 4 per group). **P<0.01 vs. relaxation to RLX under perfused conditions. Scale bar represents 100 µm.

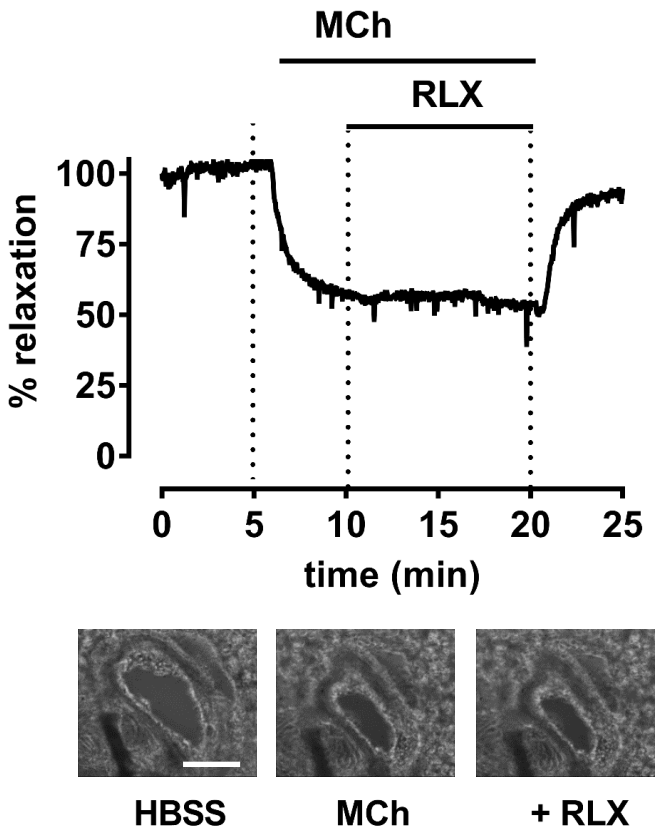


Figure 9. RLX does not elicit relaxation of mouse intrapulmonary airways in PCLS. An airway from mouse PCLS were pre-contracted with MCh (0.3 µM) in prior to the addition of RLX (0.1 µM) under perfused conditions. A representative trace shows area values in pixels determined from gray-scale analysis of images using Video-Savant. Scale bar represents 160 µm.

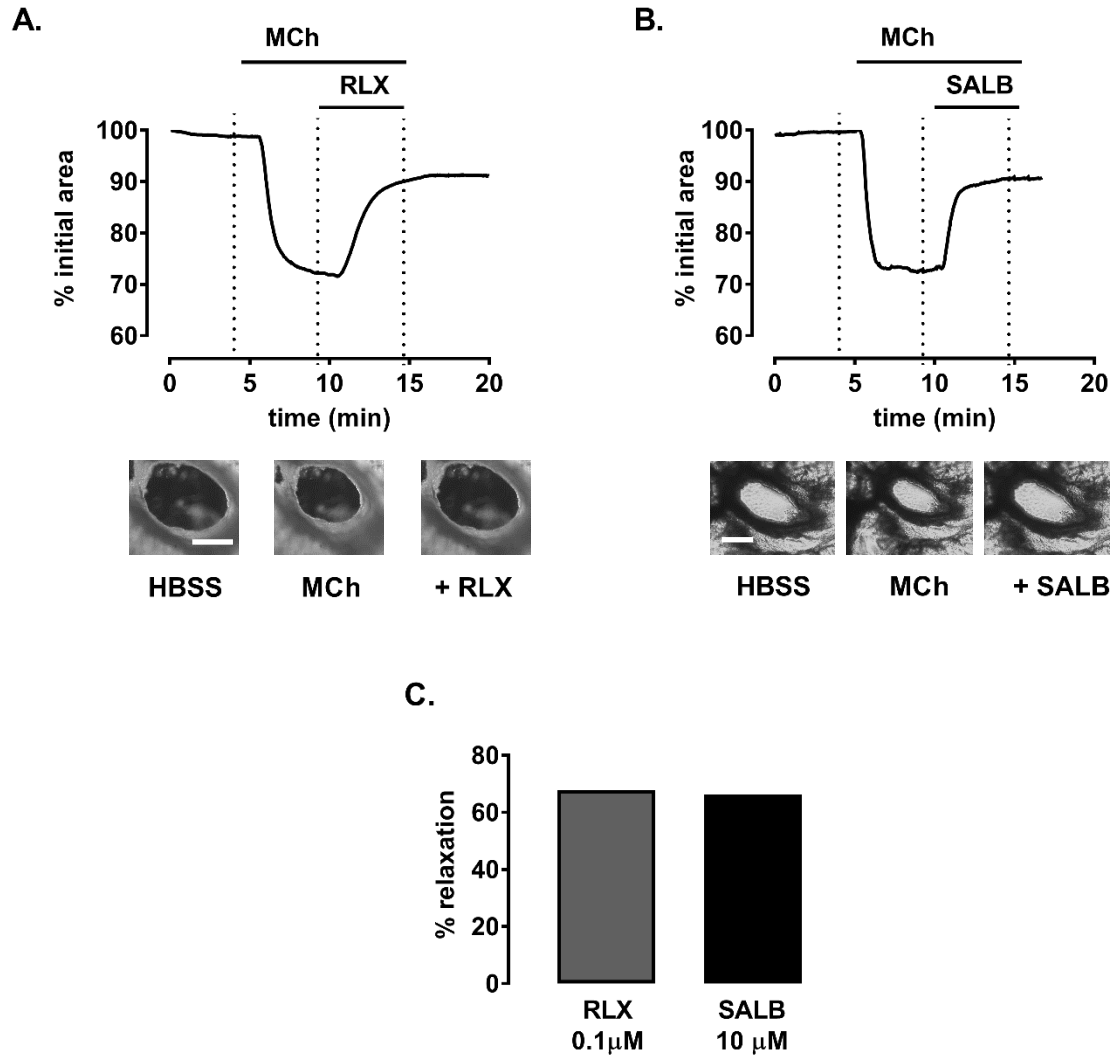


Figure 10. RLX elicits relaxation of human intrapulmonary airways in PCLS. Airways from human PCLS were pre-contracted with MCh (0.3 µM) prior to the addition of (A) RLX (0.1 µM) or (B) SALB (10 µM). Representative traces show area values in pixels determined from gray-scale analysis of images using Video-Savant. (C) Grouped data shows relaxation by RLX or SALB (10 µM) under perfused conditions. Data is expressed as % relaxation of MCh pre-contraction (n= 1/group). Scale bar represents 200 µm.

5.4 Discussion

This chapter provides the first evidence that RLX is an effective bronchodilator agent in multiple species and is able to inhibit the development of contraction to ET-1. Compared to the β-AR agonist ISO and the novel dilator RGZ, RLX induced slower relaxation in precontracted rat trachea, with more rapid relaxation elicited in intrapulmonary airways in rat PCLS. Perfusion with RLX also elicited complete relaxation of small airways in human PCLS. The actions of RLX appeared to be mediated by the release of endogenous factors (NO, prostaglandin E_2 (PGE_2)), but were only partially epithelial- dependent. In addition, RLX potentiated the extent and rate of ISO- and RGZ-mediated relaxation respectively in rat trachea. These data suggest that RLX may target alternative dilator mechanisms to β_2-AR agonists and offer potential benefit in combination treatments to overcome excessive bronchoconstriction in asthma.

Bronchodilators are the mainstay of pharmacologic therapy for relief of asthma symptoms. However, there is an unmet medical need for more effective treatment under conditions of reduced responsiveness to β-AR agonists. Identifying novel bronchodilators that target alternative mechanisms may offer additional benefit during a severe asthma attack or following β-AR desensitization (Cazzola *et al.*, 2012).

To date, the acute effects of RLX in the regulation of airway contraction have not been examined. Relaxin is a pregnancy-related hormone, but has also been shown to elicit relaxation of rat renal and mesenteric arteries (McGuane *et al.*, 2011; Leo *et al.*, 2014). In the context of the lung, chronic treatment with RLX protected against the development of airway remodelling, reversed established fibrosis and reduced AHR in chronic AAD models (Royce *et al.*, 2009, 2013).

The majority of studies presented here were performed using rat airways. In this study, we assessed potential dilator effects of RLX in comparison to the non-selective β-AR agonist ISO and the selective β_2-AR agonist SALB. We also compared RLX with the PPARγ agonist RGZ, which was recently shown to exert acute relaxation in mouse trachea and small airways (Bourke *et al.*, 2014; Donovan *et al.*, 2014). We utilized MCh, the gold standard to induce airway contraction both *in vitro* and *in vivo* (Donovan *et al.*, 2013), and ET-1, which is upregulated in asthma (Trakada *et al.*, 2000) and contributes to both airway contraction and fibrosis (Ahmedat *et al.*, 2013).

RLX was shown to be an effective but slowly acting dilator in trachea, reversing an established MCh-induced contraction. RLX also showed bronchodilator efficacy in rat PCLS, causing rapid relaxation that was increased under static conditions relative to perfused conditions. These differences informed later studies of the mechanism of action of RLX. Of note, the efficacy of the highest concentration of RLX tested was similar to the maximal response to ISO, but this partial relaxation to RLX in both large and small airways was achieved at a 10-fold lower concentration than the β-AR agonist. RGZ was confirmed as a maximally effective dilator of rat airways, with potency in the high µM range consistent with previous data obtained in mice (Donovan *et al.*, 2015). Future studies will apply higher concentrations of RLX than those available for this study to see if complete relaxation can be achieved with greater efficacy than β-AR agonists which failed to cause complete relaxation in rat trachea.

In assessing bronchoprotection, RLX pre-incubation (0.1 µM) did not inhibit the development of contraction to MCh in rat trachea. Only RGZ at a 1000-fold higher concentration was able to inhibit MCh-induced contraction. In contrast, pre-treatment with RLX inhibited the development of contraction to the potent bronchoconstrictor ET-1. Similar inhibition was seen with effective dilator concentrations of ISO and RGZ (1 and 100 µM respectively), but only RLX was effective when comparisons were made at the same concentration and pre-incubation time (0.1 µM, 30 min). Although RLX-mediated inhibition of contraction appears to be dependent on its ability to prevent contraction induced by different contractile agonists, these findings support a greater bronchoprotective action of RLX, in addition to its dilator actions, both evident at lower concentrations than ISO and RGZ.

Despite only slowly developing dilator responses, we have confirmed a robust bronchoprotective action of RLX in rat trachea. We extended these experiments to other species including the mouse and guinea pig. Mice trachea and PCLS provide opportunities to explore the effect of treatment in lung disease models which are more accessible. Similar to humans, guinea pig trachea possess a high density of β_2-ARs which allows for the assessment of functional antagonism (Lemoine and Overlack, 1992).

To identify its site of action, we showed that RXFP1, the cognate receptor for RLX, was expressed in both airway epithelium and smooth muscle where the mechanism underlying dilation and protection were then explored. Inhibition of airway contraction and relaxation to RLX was reduced, but not abolished, by epithelial removal. This is in contrast to acute vascular relaxation to RLX that was abolished by endothelial removal alone (McGuane *et al.*, 2011) despite localization of RXFP1 in both endothelial and vascular smooth muscle cells (Novak *et*

al., 2006; Jelinic *et al.*, 2014). In the absence of a commercially available RXFP1 antagonist to confirm receptor- dependence, RLX appears to be regulating airway contraction via actions on both epithelium and ASM. Further assessment when RXFP1 expression is reduced or abolished, either by using siRNA strategies *ex vivo* or using airway preparations from a knockout model, should be considered to confirm this mechanism of action.

In exploring the mechanisms underlying the effects of RLX, it was notable that RLX-mediated relaxation was more rapid in small airways in rat PCLS than rat trachea, occurring within minutes under static conditions. In addition, RLX-mediated inhibition of rat tracheal contraction was almost completely abolished with longer pre-treatment, and prevented when the bathing solution was removed prior to assessing contraction. Potentially higher levels of endogenous mediators of relaxation released in response to RLX would be present in the relatively smaller chamber used for the lung slice studies or with longer time of exposure. While it remains to be confirmed whether the increased rate and extent of relaxation to RGZ and ISO respectively seen in trachea when in combination with RLX is also evident when tested in PCLS, these combined findings are consistent with the requirement for the release and accumulation of endogenous factors for relaxation in response to RLX.

The potential identity and origin of these endogenous mediators were then explored. In intact rat trachea, inhibition of contraction by RLX was reduced in the presence of the combination of the NOS inhibitor L-NAME or the COX inhibitor indomethacin, and also by epithelial removal. This implicates both PGE_2 and NO, released from the epithelium, as endogenous mediators contributing to the effects of RLX. The ASM is an additional source of PGE_2 which may regulate proliferation and cytokine release (Holgate *et al.*, 2003). Since inhibitory effects of RLX on contraction were still evident in epithelial- denuded tissues, RLX may also act via RXFP1 on ASM to increase levels of ASM-derived mediators such as PGE_2 and/or mediate direct effects on ASM to oppose contraction. In recent studies, RLX was shown to increase prostacyclin release to exert relaxation in vascular tissue (Jelinic *et al.*, 2014; Leo *et al.*, 2014; Sarwar *et al.*, 2016). This further suggests that prostaglandins are key contributors to RLX-mediated relaxation.

Other signalling pathways that may mediate RLX-induced bronchodilation were explored. The guanylate cyclase inhibitor, ODQ and the adenylate cyclase inhibitor SQ22536, in both intact and epithelial-denuded trachea, reduced relaxation to RLX. These findings are also consistent with contributions of NO and PGE_2 released from epithelium and/or ASM in response to RLX, as these mediators activate guanylate cyclase (GC) and adenylate cyclase (AC) respectively in intact tissue to oppose ASM contraction. However, RLX itself has been shown to directly

increase cAMP and activate protein kinase A (PKA) in both transfected HEK cells expressing RXFP1 (Halls *et al.*, 2007) and bronchial epithelial cells to stimulate migration and ciliary beat frequency (Wyatt *et al.*, 2002). Overall, the mechanisms driving relaxation appear to involve indirect effects of RLX mediated by NO and PGE_2, and direct effects of RLX on both epithelial cells and ASM.

Characterization of the mechanisms and relative efficacy of novel dilators is necessary to support studies of their potential benefit in combination with existing therapy with β-AR agonists. We have previously shown that RGZ reversed MCh- induced contractions in mouse trachea and lung slices through PPARγ- and epithelial-independent mechanisms involving the attenuation of Ca2+ oscillations (Bourke *et al.*, 2014; Donovan *et al.*, 2015). We assessed the effects of RLX in combination with RGZ, ISO and SALB, to determine whether the mechanism of action of RLX, newly identified here, would increase the extent or rate of relaxation to RGZ or the β-AR agonists.

Although the combination of RGZ and RLX was not more effective at eliciting relaxation in rat trachea, the response was markedly more rapid than either treatment alone. In addition to its epithelial-independent mechanisms, RGZ has also been shown to inhibit PGE_2 breakdown (Henry *et al.*, 2005), so the PGE_2-dependent pathways contributing to RLX-mediated relaxation may have been further enhanced in this particular combination.

The effects of RLX in combination with ISO were tested in rat trachea, where relaxation is mediated via both β_1- and β_2-ARs and ISO elicits rapid but incomplete relaxation. Under these conditions, RLX increased ISO- mediated relaxation at a similar rate compared to ISO alone, despite the slow relaxation seen with RLX alone. Thus, although the rapid accumulation of cAMP in response to ISO may be maximal, the additional ability of RLX to generate cGMP (via NO) may contribute to greater relaxation to β-AR agonists when their efficacy is limited as in rat trachea.

Although RLX elicited relaxation in small airways from rat PCLS, there was no evidence of relaxation in mouse airways. Rather than performing experiments under static conditions, mice PCLS were only perfused with RLX which may have contributed to this negative finding. In contrast, perfusion with RLX in human PCLS caused complete relaxation, even under perfused conditions. The effects of RLX on the epithelium and ASM may differ between species due to differences in structure (Patra, 1986; Gomes *et al.*, 2000) or receptor expression which is yet to be quantified. In addition, airways of matched size from different species represent different airway generations. Even though airways are of equal size, PCLS

from mouse airways are produced from generations 7-10 (Bai *et al.*, 2007) while PCLS from humans are produced from generations 12-16 (Ressmeyer *et al.*, 2006). Differences in airway generation within species has been shown to influence airway contraction whereby contraction was greater in middle generation airways compared to distal or proximal generations (Bai *et al.*, 2007). Therefore, these differences in airway generation may contribute to the differences in airway responses to RLX seen between species.

RLX therefore offers intriguing possibilities as an alternative or additional therapy for asthma. Phase 3 clinical trials have been conducted for the use of RLX to treat acute heart failure, where its safety and efficacy have been established (RELAX- AHF2 trial, Teerlink *et al.*, 2017). RLX has previously been demonstrated to oppose the development of airway remodelling and AHR, and shown to have greater anti-fibrotic effects when used in combination with prednisolone in an experimental model of AAD (Royce *et al.*, 2013). Here we demonstrate further potential benefits of RLX, mediating bronchodilator actions by mechanisms that differ from and potentially enhance responses to β-AR agonists. It would therefore be of interest to assess both its bronchodilator efficacy and potential anti-inflammatory actions in this disease context to support its further preclinical evaluation. Future studies should define the therapeutic potential of RLX as an add-on reliever medication for asthma, particularly when β-AR responsiveness is limited.

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Chapter 6: Relaxin potentiates salbutamol-mediated airway relaxation in multiple species

6.1 Introduction

Asthma is a heterogenous disease that is characterised by airway hyperresponsiveness associated with inflammation and remodelling. Although, treatment with short-acting β_2-AR agonists (SABA) alone to oppose bronchoconstriction is restricted to only a small proportion of patients, current guidelines now recommend the use of daily low dose inhaled corticosteroids in combination with long acting β_2-AR (LABA) (Global Initiative for Asthma, 2019). However, the efficacy of SABA such as salbutamol (SALB) in patients on these preventer medications may still be inadequate to fully relieve symptoms and may be progressively reduced with increasing disease severity, when airway remodelling and/or persistent exacerbations lead to greater levels of bronchoconstriction. Frequent use of high doses of SABA under these circumstances can also lead to β_2-AR desensitisation, and further loss of both potency and efficacy (Deshpande and Penn, 2006; J.K.L. *et al.*, 2011; Pera and Penn, 2016).

Treatment with LABA alone, as a therapeutic strategy to elicit sustained bronchodilation in asthma, is now precluded due to its use being associated with increased mortality, potentially as a consequence of the LABA masking persistent inflammation (Nelson *et al.*, 2006; Salpeter *et al.*, 2006). Even when used in combination with glucocorticoids, LABA are only still able target the same signalling pathways utilised by SABA, with activation of β_2-ARs, and are dependent on the increased synthesis of cAMP to mediate relaxation.

It is clear that despite reducing inflammation, glucocorticoids are ineffective at reversing established airway hyperresponsiveness or the eventual loss of lung function due to structural changes including fibrosis. While the use of glucocorticoids can oppose inflammation to reduce the frequency and severity of asthma attacks, a significant percentage of asthmatics have uncontrolled asthma, even with the addition of long-acting β_2-AR agonists (LABA) (Peters *et al.*, 1998).

Other add-on dilator therapies are also limited in their capacity to oppose excessive bronchoconstriction. While the PDE inhibitor theophylline offers the potential for synergistic actions with SABA by inhibiting the breakdown of newly synthesised cAMP, it has a narrow therapeutic index and potential off target effects that limit its clinical application (Nicholson *et al.*, 1995; Billington *et al.*, 2007). Both short- and long-acting muscarinic antagonists (SAMA) have been demonstrated to be of benefit in COPD when used in combination with SABA or LABA (Bateman *et al.*, 2013). However, these agents only oppose contraction mediated by

the endogenous muscarinic agonist ACh but not to other contractile mediators implicated in asthma such as histamine.

There remains an unmet need for improved treatments that oppose airway contraction in asthma. Relaxin (RLX) is a potential therapeutic candidate, due to its acute dilator properties combined with its chronic anti-fibrotic actions (Chapter 4, 5; Lam *et al.*, 2018). Not only does RLX mediate airway relaxation in multiple species, but studies in rat airways show that its mechanism of action differs from β_2-AR agonists and that it potentiates isoprenaline (ISO)-mediated relaxation. To date, the only studies of RLX in combination with other asthma therapies have been limited to chronic studies performed in models of allergic airways disease, assessing effects on fibrosis and remodelling and associated AHR (Royce *et al.*, 2013, 2015). In these studies, chronic treatment with RLX has been combined with the glucocorticoid, methylprednisolone and/or mesenchymal stem cells (MSCs) (Royce *et al.*, 2013, 2015). Of note, subepithelial fibrosis was reduced by treatment with RLX alone, and further reduced, when combined with methylprednisolone or MSCs (Royce *et al.*, 2013). However, the improvement in AHR with chronic RLX treatment alone was not increased with the addition of methylprednisolone or MSCs (Royce *et al.*, 2015).

The acute effects of RLX in combination with SABA to oppose airway contraction remain to be clearly defined. Previous chapters demonstrated the potent bronchodilator actions of RLX in addition to its anti-fibrotic properties. The capacity of RLX to elicit bronchodilation and bronchoprotection (Chapter 5) coupled with its ability to inhibit fibroblast-mediated collagen contraction (Chapter 4) suggests it may be a novel drug candidate for asthma treatment. Of particular interest is its potential role in enhancing relaxation to β-AR agonists. Alone, RLX and the β-AR agonist, ISO, each elicited partial relaxation in rat trachea. When combined, single concentrations of RLX (0.1 µM) and ISO (1 µM) caused complete relaxation. There is a need to further explore the mechanisms underlying this additive dilator response, and to systematically define the effect of RLX on β-AR agonist potency, with increased focus on the clinically relevant dilator SALB.

To date, the combined effects of RLX and ISO have only been assessed in large airways where responses were measured as a change in isometric force (Chapter 5). Small airways are a relatively under-investigated site of severe inflammation and remodelling as well as increased resistance (Wagner *et al.*, 1998), and their reactivity can be assessed using precision cut lung slices (PCLS) where responses can be visualised as changes in airway area. While treatment with β-AR agonists alone has been shown to elicit relaxation of

intrapulmonary airways from PCLS (Delmotte and Sanderson, 2008; Fitzpatrick *et al.*, 2013), it is yet to be determined whether additive effects of RLX relaxation on ISO-mediated relaxation in large airways are maintained or potentiated when assessing RLX and SALB in the distal lung.

The underlying mechanisms mediating the enhanced dilator response to β-AR agonists in the presence of RLX are also yet to be explored. RLX-mediated relaxation occurs through both epithelial-dependent pathways via the generation of NO and PGE_2 and direct effects on smooth muscle Chapter 5. Airway relaxation by SALB occurs independently of the epithelium where binding of SALB to β-ARs on the airway smooth muscle activates cAMP-dependent pathways resulting in bronchodilation. The combined epithelial -dependent and -independent effects of RLX may contribute to its ability to augment relaxation response to β-AR agonists.

MCh-induced contraction is driven by increases in both Ca^{2+} oscillations and sensitivity in airway smooth muscle as detailed in the General Introduction (Chapter 1.1.3.2, Perez and Sanderson, 2005). Both pathways are inhibited by SALB (Delmotte and Sanderson, 2008) but whether RLX enhances SALB-mediated inhibition of oscillations or sensitivity needs to be examined, particularly since both pathways are implicated in the increased contractile responses seen in asthma (Savineau and Marthan, 1997; Sweeney *et al.*, 2014; Sakai *et al.*, 2017).

To assess inhibition of contraction due to Ca^{2+} sensitivity alone, PCLS can be treated with caffeine to empty intracellular Ca^{2+} stores and with the ryanodine receptor (RyR) agonist, ryanodine, to clamp Ca^{2+} and abolish subsequent oscillations to contractile agonists (Sanderson *et al.*, 2008). The subsequent contraction due to increased Ca^{2+} sensitivity alone is inhibited by SALB (Delmotte and Sanderson, 2008). Therefore, assessing whether RLX still potentiates SALB-mediated relaxation in caffeine/ryanodine-treated PCLS may elucidate one mechanism underlying its effects.

Phorbol esters such as phorbol 12,13-dibutyrate (PdBu) can elicit contraction by direct activation of protein kinase C (PKC) in airway smooth muscle (ASM). Upon activation, PKC induces CPI-17 phosphorylation which leads to the inhibition of myosin light chain phosphatase (MLCP), to oppose relaxation (Sakai *et al.*, 2017). Assessment of dilator responses to SALB alone and in combination with RLX in PdBu-contracted airways will allow for further insights into the mechanism of potentiation of relaxation by RLX.

To date, the additive effects of RLX have only been assessed in rodent large airways. Confirmation of RLX efficacy in smaller airways and in other more clinically relevant species provides a pathway to support translation of these findings for treatment of asthma. Unlike humans, the branching pattern of airways in rodents is monopodial with a lack of respiratory bronchioles, and ISO is a more potent dilator than SALB, reflecting the contribution of both β_1 and β_2-ARs to airway relaxation (Henry *et al.*, 1990).

In contrast, guinea pig airways express high levels of β_2-ARs, and have similar potency (~10^{-7}M) and efficacy (100% relaxation) to human airways (Källström *et al.*, 1994; Ressmeyer *et al.*, 2006). Guinea pig airways are also susceptible to functional antagonism, with reduced efficacy and potency of SALB with increasing contraction, mimicking the reduced responsiveness to SALB in severe asthma (Lemoine and Overlack, 1992).

The dichotomous branching of marmoset airways as well as the abundance of respiratory bronchioles are similar to humans (Plopper and Hyde, 2008) making the marmoset a potentially more relevant species for preclinical testing of novel treatments. To date, studies have only compared airway contraction but not relaxation between marmoset and human airways. Findings indicate that the maximum airway contraction to MCh was similar between marmosets and human, albeit at varying concentrations. MCh was 5.4-fold less potent in human airways compared to marmoset airways, most likely due to differences in receptor density (Seehase *et al.*, 2011). Whether the bronchodilator actions of SALB are similar, and whether RLX potentiates this response in both species is yet to be assessed. Consistent findings of SALB and RLX efficacy in marmoset and human airways may suggest that this species offers a better option to validate responses in rodents or guinea pigs, as well as overcome the limited access to human tissue.

This background provides the impetus for the current study, where systematic examination of the potential additive effects of RLX on β-AR agonist-mediated relaxation was undertaken. We hypothesised that SALB-mediated relaxation would be potentiated by RLX and that this effect would be maintained across species and under conditions of reduced β_2-AR-mediated relaxation.

6.1.1 Aims

The experimental aims were to:

1. determine the capacity of RLX to potentiate the effects of SALB across species and under conditions where dilator efficacy of SALB is compromised
2. assess potential mechanisms underlying potentiation of β-AR-mediated relaxation by RLX

6.2 Methods

6.2.1 Materials and solutions

Methacholine (MCh), isoprenaline (ISO), salbutamol (SALB), Nω-nitro-L-arginine methyl ester (L-NAME), caffeine and phorbol 12, 13-dibutyrate (PdBu) (all from Sigma-Aldrich, St Louis, MO, USA); indomethacin (Cayman Chemical, Ann Arbor, MI, USA); recombinant human gene-2 relaxin (RLX, kindly provided by Corthera Inc., San Mateo, CA, USA; a subsidiary of Novartis AG, Basel, Switzerland); ryanodine (Calbiochem, Australia); RXFP1 antibody (Santa Cruz, California).

6.2.2 Animals and tissue collection

As per General Methods: Chapter 2.1 and 2.2.

6.2.3 Myograph and organ bath experiments

As described previously (Chapter 2.4), rat and guinea pig trachea were quickly dissected and mounted in Krebs-Henseleit buffer solution for experiments using myograph for rat trachea (5 ml bath) or standard organ baths for guinea pig trachea (10 ml bath). Tissues were set to optimum resting tension for maximum development of contraction to potassium physiological salt solution (KPSS; 123.7 mM K^+) for subsequent measurement of changes in isometric force to constrictors and dilators. A maximal contraction to ACh (30 μM) was also obtained in all tissues.

In protocols specific to this chapter, airway relaxation was assessed after cumulative additions of MCh were made to establish a submaximal contraction (50-70% of ACh maximum). Concentration-response curves were then constructed to ISO or SALB in the absence and presence of RLX (100 nM, 10 min pre-incubation).

To assess the role of the epithelium in mediating relaxation, the epithelium was removed by gentle agitation of the lumen using a wooden toothpick in guinea pig trachea.

6.2.4 Precision cut lung slice (PCLS) experiments

As described previously (Chapter 2.5), PCLS were prepared with minor modifications from previously published methods (Bourke *et al.*, 2014; Donovan *et al.*, 2014). Mouse, rat and

marmoset trachea were cannulated with a catheter containing two ports (20 G Intima, Becton Dickinson, VIC, Australia) and lungs were inflated with ~1.2 ml for mouse and ~10 mL agarose gel for rats and marmosets (2% agarose in 1x HBSS at 37°C), followed by a bolus of ~0.5 mL or 3 mL air. Lungs were cooled by bathing in cold HBSS/HEPES and the rats were then kept at 4°C to allow the agarose to solidify, before the lungs were removed. The upper right lobe was isolated and adhered with cyanocrylate to a mounting plate in a vibratome (Compresstome, Precisionary Instruments, Greenville, NC, US). PCLS of 200 µm thickness were prepared and transferred into cell culture plates containing DMEM, supplemented with 1% penicillin-streptomycin and incubated for 24 h (37°C, 5% CO_2) prior to experiments.

For human lung samples, one main bronchus was identified and cannulated. Other open-ended airways were clamped in order to prevent agarose from leaking. Lungs were filled with 20-40 mL agarose (depending on the size of the lung sample), followed by a bolus of 5-10 mL air then cooled. Human lungs were sectioned into 200 µm thin slices and cultured overnight as for other species before experiments were conducted.

Some PCLS were thawed from storage (as described in Chapter 3.2.4) for experiments. Other slices were generated from lungs which were sliced 24 h post fill (Chapter 3.3.4).

PCLS were transferred to HBSS/HEPES and mounted in custom-made perfusion chambers (~100 µL volume). A viable airway (~200 µm diameter) was selected from each slice based on the presence of an intact layer of epithelial cells with ciliary activity. PCLS were perfused at a constant rate (~0.5 mL/min) through an eight-channel gravity-fed perfusion system under vacuum.

Phase contrast microscopy was conducted using an inverted microscope (Nikon Ti-U, Melville, NY, USA) to observe drug- induced airway changes in PCLS, employing 10X objective lens, zoom adapter, reducing lens and camera (CCD camera model TM- 62EX; Pulnix, Takex, Japan). Changes in airway lumen area were captured as digital images (744 × 572 pixels) in time lapse (0.5 Hz) using image acquisition software (Video Savant; IO Industries, Inc., London, ON, Canada). Obtained files were converted to TIFF files and analyzed using NIH/Scion software (Scion Corporation; download www.scioncorpcom) and ImageJ.

In protocols specific to this chapter, PCLS were initially perfused with MCh (0.3 µM) to establish a submaximal pre-contraction, then perfused with MCh in combination with

increasing concentrations of SALB in the absence or presence of RLX (0.1 µM, 10 min pre-incubation).

In mouse PCLS only, the contribution of NO and cyclooxygenase (COX)-derived mediators was assessed using the NO synthase (NOS) inhibitor, L-NAME (100 µM) and/or the COX inhibitor, indomethacin (3 µM) which were added prior to the addition of MCh in combination with SALB and RLX. In other mouse PCLS, airways were pre-contracted to PdBu (1 µM, Mukherjee *et al.*, 2013) prior to the measurement of relaxation responses in the absence and presence of RLX.

Separate mouse PCLS were treated with 20 mM caffeine and 50 µM ryanodine to abolish ASM intracellular Ca^{2+} oscillations whereby subsequent airway contraction and relaxation is due to changes in Ca^{2+} sensitivity alone (Perez and Sanderson, 2005; Croisier *et al.*, 2015).

6.2.5 Immunohistochemistry

As described previously (Chapter 2.6), immunohistochemistry for the RXFP1 receptor was performed. Formalin-fixed tissues from mice, marmoset and human lungs were washed in 70% ethanol before being processed, paraffin embedded and cut into 4 µm sections. Immunohistochemistry was performed using a monoclonal mouse antibody to RXFP1 (Santa Cruz, J2612, 1:500). Horseradish peroxidase-conjugated rabbit polyclonal antibody was used as the secondary antibody (Santa Cruz). Detection of the receptor in mice was by biotinylation of the primary antibody using the animal research kit (DAKO Denmark, Glostrup, Denmark kits) with 3-3' diaminobenzidine (DAB) as the chromagen substrate.

6.2.6 Statistical analysis

Airway relaxation responses were normalized to % of the MCh submaximal pre-contraction. All data are expressed as the mean ± s.e.m. Non-linear regression of concentration-response curves was performed to obtain fitted maxima and pEC_{50} values where possible. When not possible, estimated maxima were compared via an unpaired t-test. Results were analyzed via t-test or one-way ANOVA for multiple comparisons between groups as appropriate; where statistical significance was accepted at $P<0.05$. All data analysis was performed using GraphPad Prism v6 (GraphPad Software, San Diego, CA, USA).

6.3 Results

6.3.1 RLX potentiates tracheal relaxation to β-AR agonists in rat and guinea pig trachea

The combined effects of RLX and β-AR agonists were explored. In rat trachea, where activation of both β_1 and β_2 ARs contributes to relaxation (Henry *et al.*, 1990), the non-selective β_1 / β_2 AR agonist ISO elicited ~75% relaxation to oppose a submaximal pre-contraction to MCh (Figure 1A). In separate airways with a similar level of pre-contraction, there was no evidence of relaxation with RLX pre-treatment over 10 min prior to the addition of ISO. Despite the apparent leftward shift in the concentration-response curve to ISO in the presence of RLX, there was no significant increase in ISO potency (P= 0.11) or efficacy (P= 0.08) (Figure 1A).

Further studies were performed in guinea pig trachea (Figure 1B, 1C). As previously shown in rat trachea, RLX alone did not cause rapid relaxation of guinea pig trachea (data not shown). Consistent with the predominant role of β_2-AR in guinea pig airways (O'Donnell and Wanstall, 1979; Tanaka *et al.*, 2004), the selective β_2-AR agonist SALB was more potent than the β_1/β_2-AR agonist ISO (pEC_{50}: SALB 7.4 ± 0.3, ISO 6.6 ± 0.2, Figures 1B, C), with both dilators causing relaxation beyond the baseline tone prior to MCh. Pre-incubation with RLX resulted in a 27.1-fold increase in ISO potency (P<0.001, n= 4, Figure 1B) with a similar leftward shift seen for SALB in the presence of RLX (P <0.05, n= 3, Figure 1C).

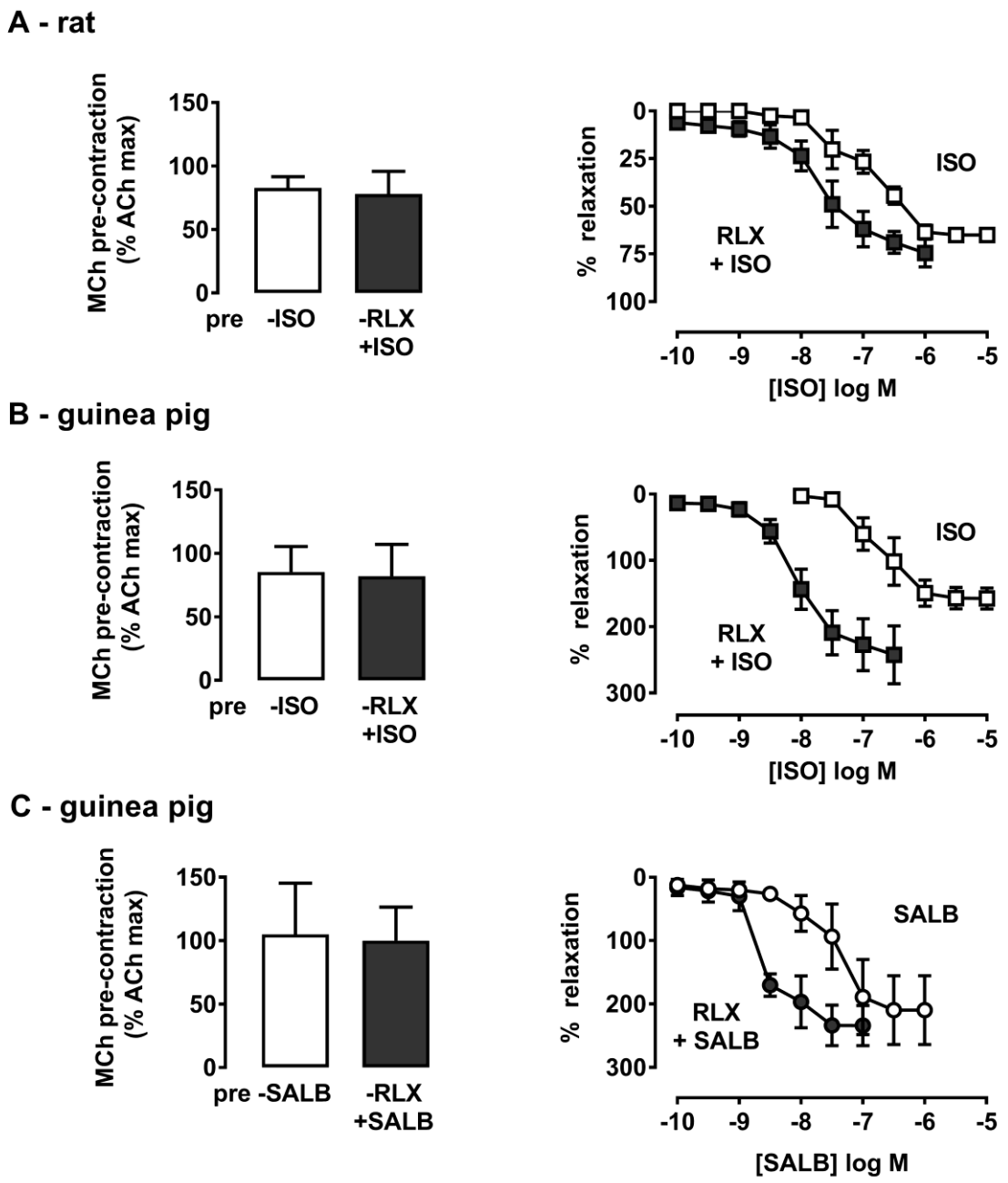

Figure 1. RLX potentiates tracheal relaxation to β-AR agonists. Rat and guinea pig trachea were precontracted with graded concentrations of MCh to achieve matched contraction between groups (A, B, C left panels). Relaxation responses were then assessed in response to ISO in the absence and presence of RLX (0.1 µM) in (A) rat and (B) guinea pig trachea or SALB in (C) guinea pig trachea. Pre-contraction is expressed relative to a maximal contraction to ACh (30 µM). Relaxation is expressed as % of the submaximal MCh pre-contraction (mean ± s.em. for n= 4-5 per group).

6.3.2 RLX potentiates SALB-mediated relaxation to oppose increased contraction in guinea pig trachea

The potentiation of SALB-mediated relaxation by RLX was then explored in guinea pig trachea under conditions where increased pre-contraction is known to limit the efficacy of SALB (Lemoine and Overlack, 1992). Relaxation responses following submaximal (low) or maximal (high) MCh pre-contraction were established (% ACh max: low 45.9 ± 8.0; high 132.6 ± 31.6) (Figure 2A, 3A). Concentration-dependent relaxation to SALB was markedly reduced in maximally contracted trachea (maximum % relaxation: post-low contraction 213 ± 44.6; post-high contraction 69.9 ± 6.0, $P<0.05$) (Figure 2B). There was no significant loss of potency (Table 1).

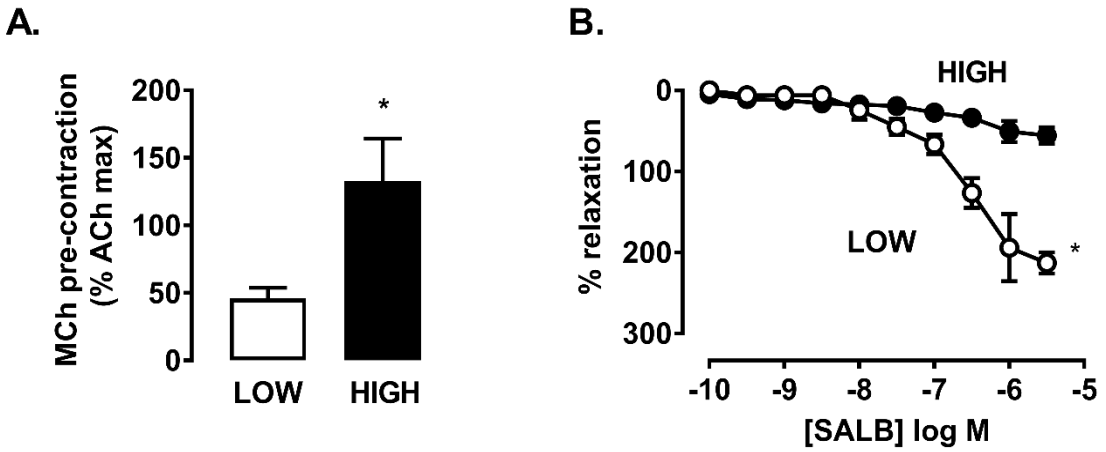

Figure 2. SALB-mediated relaxation is reduced in maximally contracted guinea pig trachea. Guinea pig trachea were (A) pre-contracted with MCh to obtain a submaximal (low) or maximal (high) contraction, prior to measurement of (B) relaxation responses to SALB (0.1 nM-10 µM). Pre-contraction is expressed relative to a maximal contraction to ACh (30 µM). Relaxation is expressed as % of MCh pre-contraction (mean ± s.e.m. n= 4 per group).

Under these conditions where relaxation to SALB was impaired, the ability of RLX to potentiate SALB-mediated relaxation was measured again (Figure 3). With matched submaximal MCh pre-contraction (Figure 3A), pre-incubation with RLX caused a 11.2-fold increase in SALB potency with no change in efficacy (Figure 3B, Table 1). In maximally contracted trachea (Figure 3A), a similar leftward shift was seen for SALB in the presence of RLX compared to SALB alone (Figure 3C, Table 1). RLX also caused a significant increase in relaxation to SALB but full relaxation was still not achieved (maximum % relaxation SALB: 69 ± 6; +RLX: 85 ± 4, Figure 3C).

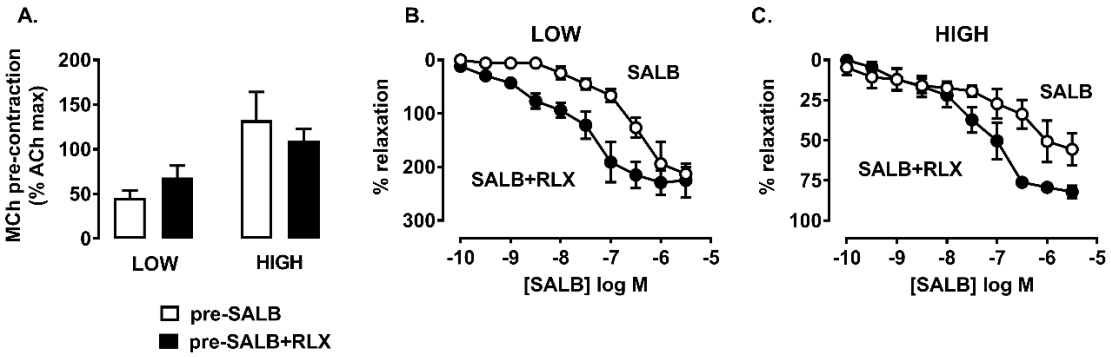

Figure 3. Potentiation of SALB-mediated relaxation by RLX is maintained in maximally contracted guinea pig trachea. Guinea pig trachea were (A) pre-contracted with MCh to obtain a submaximal (LOW) or maximal (HIGH) contraction, prior to measurement of (B) relaxation responses to SALB (0.1 nM-10 µM) in the absence or (C) presence of RLX (0.1 µM). Pre-contraction is expressed relative to a maximal contraction to ACh (30 µM). Relaxation is expressed as % of MCh pre-contraction (mean ± s.e.m. n= 3-4 per group). Data for SALB alone is reproduced from Figure 2, note different scales for relaxation responses after low vs high pre-contraction.

Table 1. The effect of level of pre-contraction on dilator efficacy and potency of salbutamol (SALB) in the absence and presence of relaxin (RLX, 0.1 µM) in guinea pig trachea

	LOW		HIGH	
	% relaxation	pEC_{50}	% relaxation	pEC_{50}
SALB	217 ± 44	6.7 ± 0.1	69 ± 6†	6.2 ± 0.5
SALB + RLX	234 ± 27	7.6 ± 0.2*	85 ± 4*	7.3 ± 0.1*

Maxima and pEC_{50} values were obtained from fitted curves to dilators after submaximal (LOW) or maximal (HIGH) precontraction and expressed as mean ± s.e.m. (n= 3-4 per group), $^{*}P<0.05$ compared to SALB alone, $^{\dagger}P<0.05$ compared to LOW.

6.3.3 RLX may potentiates SALB-mediated relaxation via epithelial-dependent mechanisms in guinea pig trachea

The underlying mechanisms of RLX-mediated potentiation of airway relaxation responses to SALB were investigated in intact and epithelial-denuded guinea pig trachea.

In intact trachea, pre-incubation with RLX caused a 11-fold leftward shift of the SALB curve ($P<0.01$) but did not affect maximum relaxation ($P= 0.36$) (Figure 4B, Table 2). Epithelial denudation was confirmed by the loss of a dilator response to Substance P (% relaxation: intact 94.2 ± 2%; denuded 15.4 ± 6%). The level of MCh pre-contraction was similar in intact and epithelial-denuded trachea (Figure 4C). However, in epithelial-denuded trachea, the trend toward increased SALB potency and maximum relaxation in the presence of RLX did not reach significance (pEC_{50} values SALB 7.4 ± 0.3; SALB + RLX 8.1 ± 0.2, $P= 0.08$, Figure 4D, Table 2).

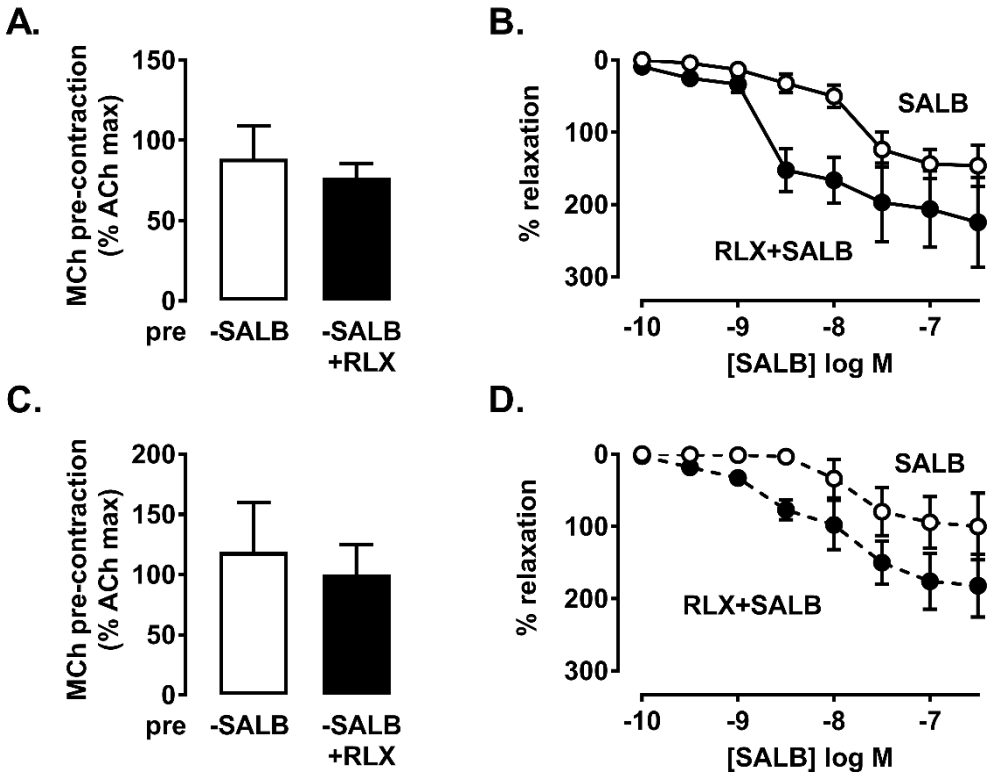

Figure 4. Potentiation of SALB-mediated relaxation by RLX may be attenuated in epithelial-denuded guinea pig trachea. Guinea pig trachea were (A, C) pre-contracted with MCh, prior to measurement of relaxation responses in the absence or presence of RLX (0.1 µM) in (B) epithelial-intact and (D) -denuded trachea. Pre-contraction is expressed relative to a maximal contraction to ACh (30 µM). Relaxation is expressed as % of MCh pre-contraction (mean ± s.e.m. n= 4 per group).

Table 2. The effect of epithelial removal on dilator efficacy and potency of salbutamol (SALB) in the absence and presence of relaxin (RLX, 0.1 µM) in guinea pig trachea

	INTACT		DENUDED	
	% relaxation	pEC_{50}	% relaxation	pEC_{50}
SALB	159 ± 23	7.9 ± 0.1	121 ± 32	7.4 ± 0.3
SALB + RLX	208 ± 43	8.7 ± 0.1**	219 ± 41	8.1 ± 0.2

Maxima and pEC_{50} values were obtained from fitted curves to dilators in intact and epithelial-denuded airways after submaximal MCh contraction and expressed as mean ± s.e.m. (n= 3-4 per group), **P<0.01 compared to SALB alone.

6.3.4 RLX potentiates SALB-mediated relaxation in mouse airways in PCLS

PCLS were used to assess the bronchodilator potential of RLX in combination with SALB in mouse intrapulmonary airways. Representative traces show changes in lumen area in airways pre-contracted to MCh prior to the addition of increasing concentrations of SALB in the absence and presence of RLX (0.1 µM) (Figure 5A, B, D). The extent of MCh pre-contraction was variable between the two groups (Figure 5C). Before the addition of SALB alone, airways were contracted to ~50% (Figure 5A) as opposed to before the addition of RLX where airways were almost fully contracted (Figure 5D). Perfusion with RLX alone for 5 min did not cause relaxation. Despite the disparity in levels of pre-contraction, only partial relaxation was elicited by SALB alone, while complete relaxation to SALB occurred over the same concentration range in the presence of RLX (% relaxation: 10 µM SALB 57 ± 3.5; +RLX 96.7 ± 3.3, P<0.001 Figure 4C).

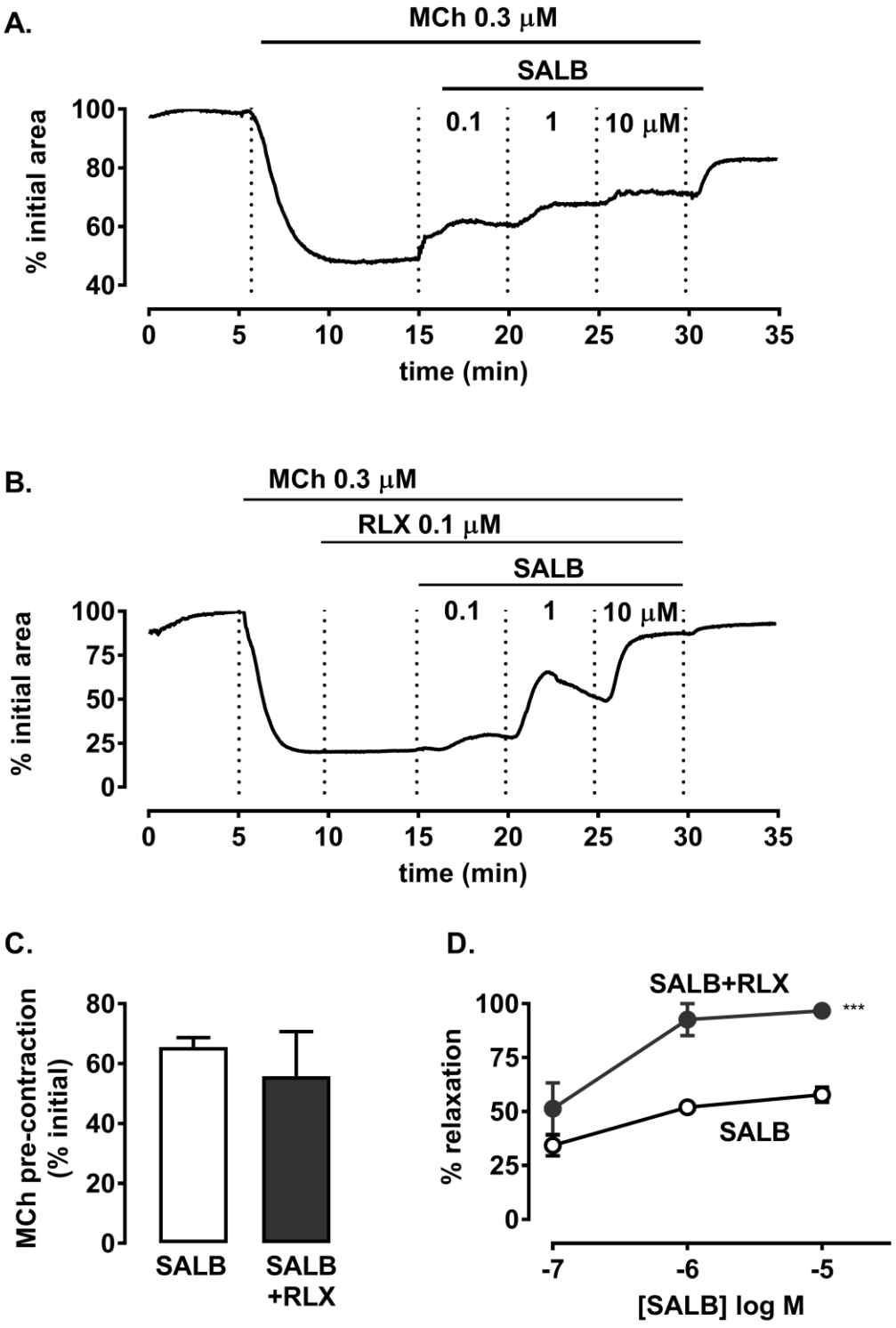

Figure 5. RLX potentiates SALB-mediated relaxation in mouse airways in PCLS. Responses to SALB in the absence and presence of RLX in PCLS were compared in mouse PCLS. Representative traces show airway pre-contraction with MCh prior to measurement of relaxation responses to SALB in the (A) absence and (B) presence of RLX (0.1 μM). (C) Grouped data for relaxation to SALB is expressed as % of MCh pre-contraction (mean ± s.e.m. n= 3-4 per group). ***P<0.001 vs SALB alone estimated maxima.

6.3.5 Potentiation of SALB-mediated relaxation by RLX in mouse airways in PCLS is blocked by L-NAME and INDO

The contribution of epithelial-derived mediators to the potentiation of SALB-mediated relaxation by RLX was assessed in mouse airways. Since the epithelium cannot be mechanically removed in PCLS, responses were measured in the absence or presence of the NOS inhibitor L-NAME and the COX inhibitor indomethacin (INDO) to limit the synthesis of epithelial-derived relaxing factors NO and PGE_2 respectively.

Pre-contraction to MCh was not altered by L-NAME and INDO pre-treatment (Figure 6A). The increased relaxation to 10 µM SALB in the presence of RLX was suppressed in the presence of L-NAME and INDO (Figure 6B, % relaxation: SALB+RLX 57.9 ± 1.9; +L-NAME/INDO 36.3 ± 7.5, P= 0.06).

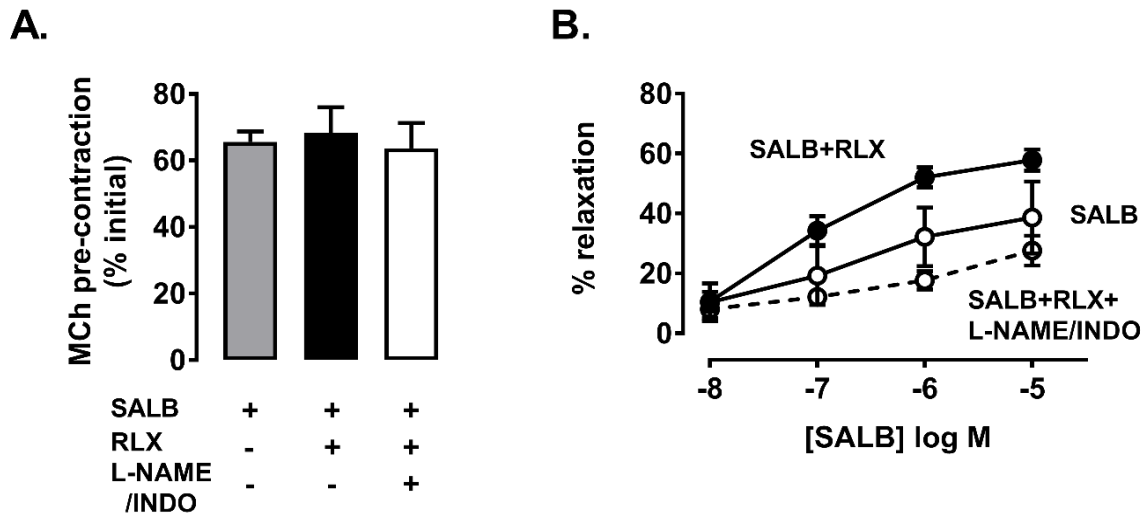

Figure 6. Potentiation of SALB-mediated relaxation by RLX in mouse airways in PCLS is blocked by L-NAME and INDO. Airways in mouse PCLS were (A) pre-contracted with MCh, prior to measurement of (B) relaxation responses in the absence or presence of RLX (0.1 µM) and INDO (3 µM) and L-NAME (100 µM). Extent of relaxation is expressed as % of MCh pre-contraction (mean±s.e.m. n= 4 per group).

6.3.6 RLX potentiates SALB-mediated relaxation by opposing protein kinase C (PKC) signalling in mouse airways in PCLS

The ability of RLX to potentiate SALB-mediated relaxation was assessed in mouse PCLS when contraction was elicited by PdBu, a PKC activator. PdBu reduced airway area by ~25% with concentration-dependent partial relaxation to SALB achieved over the concentration range tested (Figure 7B). Relaxation to SALB was increased in the presence of RLX, appearing to reach a maximum of almost 60% at 10-fold lower concentrations than under control conditions (Figure 7B).

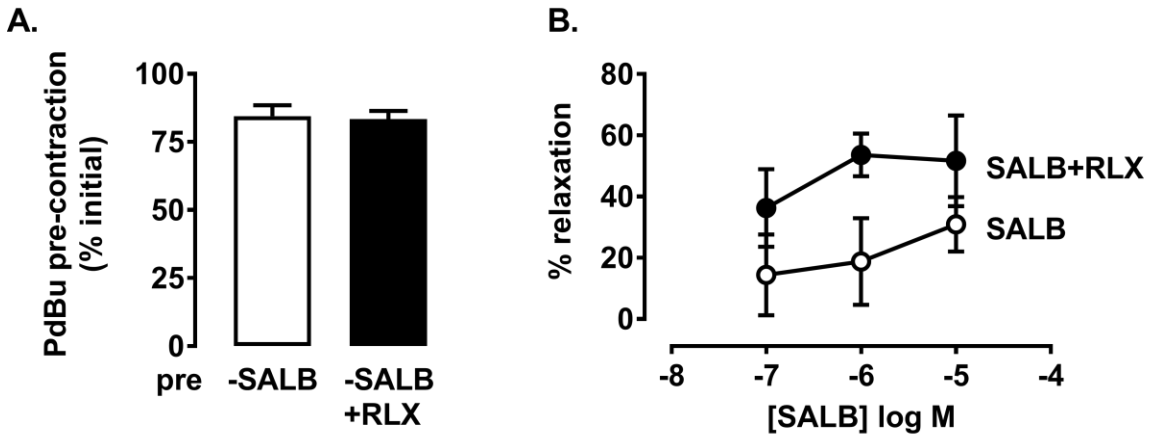

Figure 7. RLX potentiates relaxation to SALB in mouse airways pre-contracted with PdBu. Airways in mouse PCLS were (A) pre-contracted with PdBu (1 µM), prior to measurement of (B) relaxation responses in the absence and presence of RLX. Relaxation is expressed as % of PdBu pre-contraction (mean ± s.e.m. n= 3 per group).

6.3.7 RLX does not potentiate SALB-mediated relaxation by opposing increased calcium sensitivity in mouse airways in PCLS

The potentiation of relaxation to SALB by RLX was also determined in PCLS treated with caffeine/ryanodine to determine if RLX could increase SALB-mediated inhibition of contraction due to increased calcium sensitivity alone.

Representative traces of the full protocol show control responses to MCh, followed by SALB in the absence or presence of RLX (Figure 8A, B) and matched responses after caffeine/ryanodine treatment (Figure 8C, D). The initial contraction in response to caffeine is due to emptying of internal Ca^{2+} stores. Co-treatment with ryanodine then locks ryanodine receptors on the sarcoplasmic reticulum in an open state so that re-exposure to caffeine fails to yield a response. As a consequence, MCh-induced Ca^{2+} oscillations are abolished and subsequent contraction to MCh is due to increased Ca^{2+} sensitivity alone (Croisier *et al.*, 2015).

Contraction to MCh after caffeine/ryanodine was maintained (Figure 8, 9A) and the partial relaxation to SALB was observed was also similar to untreated slices (Figure 8A). Although RLX potentiated SALB-mediated relaxation in untreated slices (Figure 9B), the response to SALB alone was impaired when tested in combination with RLX after caffeine/ryanodine treatment (Figure 9C).

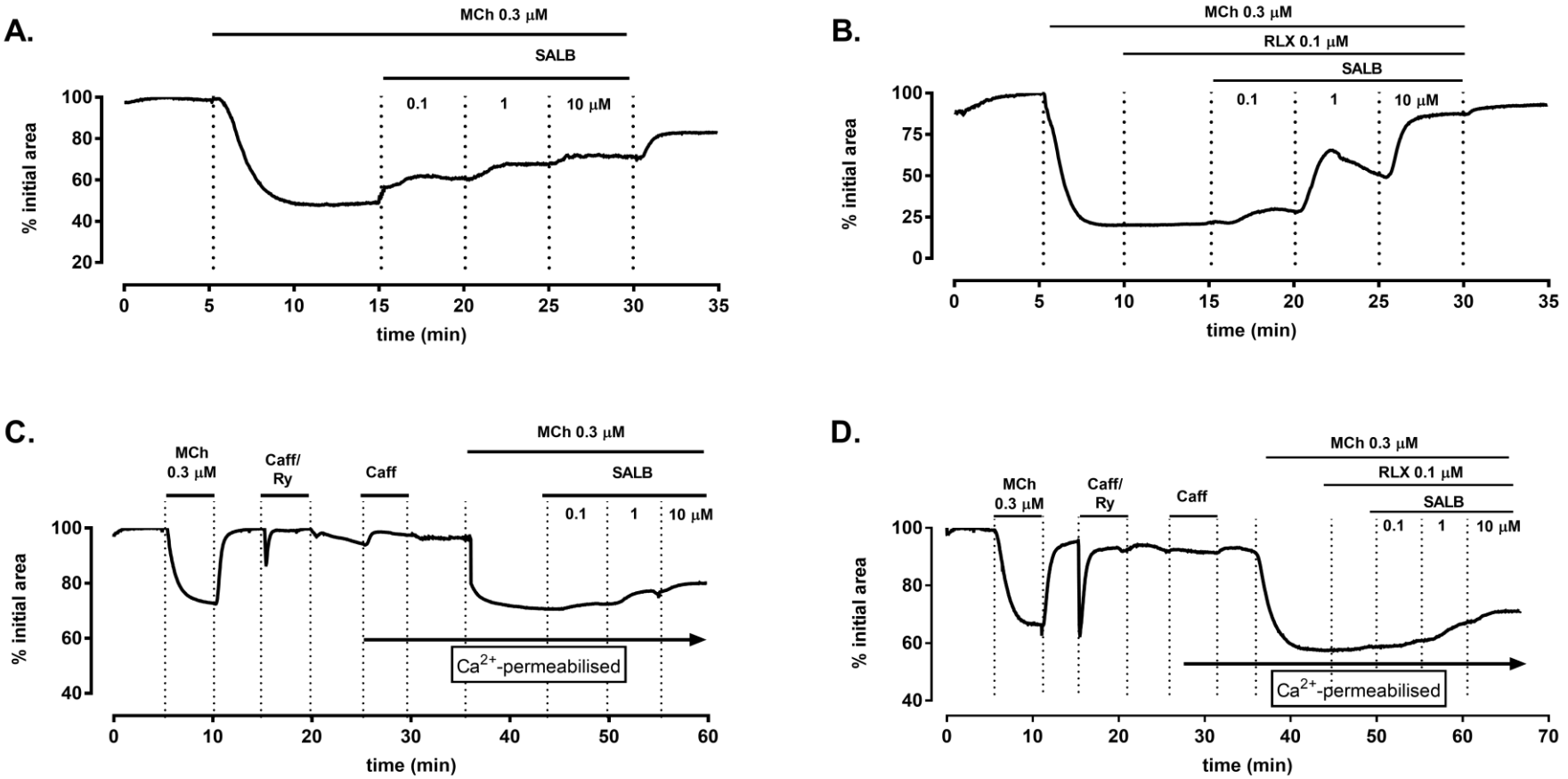

Figure 8. Relaxin does not potentiate SALB-mediated relaxation by inhibiting calcium sensitivity (traces) in mouse small airways. Representative traces of relaxation to SALB in the (A) absence and (B) presence of RLX in mouse airways in PCLS following (C, D) caffeine and ryanodine (Caff/Ry) treatment. Figures 8A and B are reproduced from Figure 5A and B for comparison.

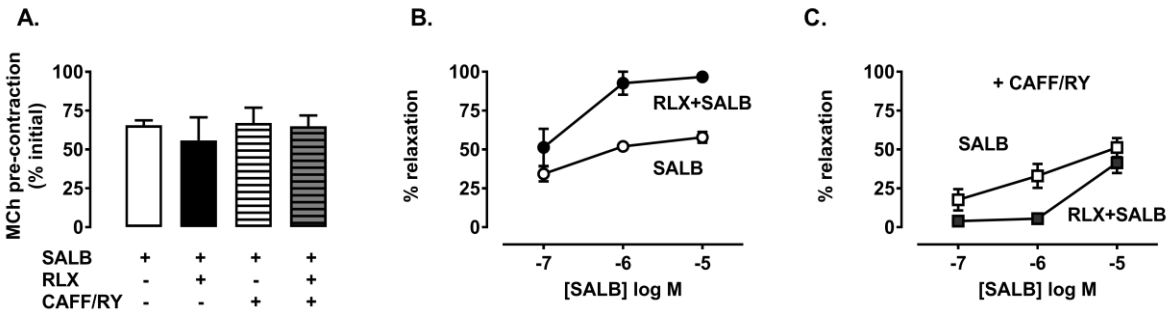

Figure 9. Relaxin does not potentiate SALB-mediated relaxation when airway contraction is due to increased calcium sensitivity in mouse PCLS. Airways in mouse PCLS were (A) pre-contracted to MCh, prior to measurement of relaxation responses to SALB in the absence and (B) presence of RLX following (C) Caff/Ry treatment. Relaxation is expressed as % of MCh pre-contraction (mean ± s.e.m. n= 4 per group). Data in Figure 9B is reproduced from Figure 5C for comparison.

6.3.6 RLX may potentiate airway relaxation to SALB in human and marmoset airways in PCLS

Potentiation of SALB-mediated relaxation by RLX was then explored in small airways using PCLS prepared from 4 marmoset lungs and a single human lung (Figure 10, 11). Receptor expression for RXFP1 was initially confirmed by immunohistochemistry. RXFP1 was present in airways from both species, with similar staining detected in the epithelium and airway smooth muscle (Figure 11A, B).

Pre-contraction of marmoset and human lung slices were matched (Figure 10A) prior to the addition of increasing concentrations of SALB. When tested over the same concentration range, SALB elicited < 50% relaxation of marmoset airways but complete relaxation in a single human airway (Figure 10B).

Airways from lung slices were perfused with RLX for 10 min prior to the addition of SALB. In marmoset airways, there was no evidence of relaxation with RLX alone, and only a modest leftward shift in the SALB curve with no apparent increase in maximum relaxation (Figure 11C). In a single pilot study using human PCLS, RLX alone elicited ~15% relaxation (shown as increased response at the lowest SALB concentration) (Figure 11D). In the presence of RLX, SALB potency was increased by ~3-fold (Figure 11D).

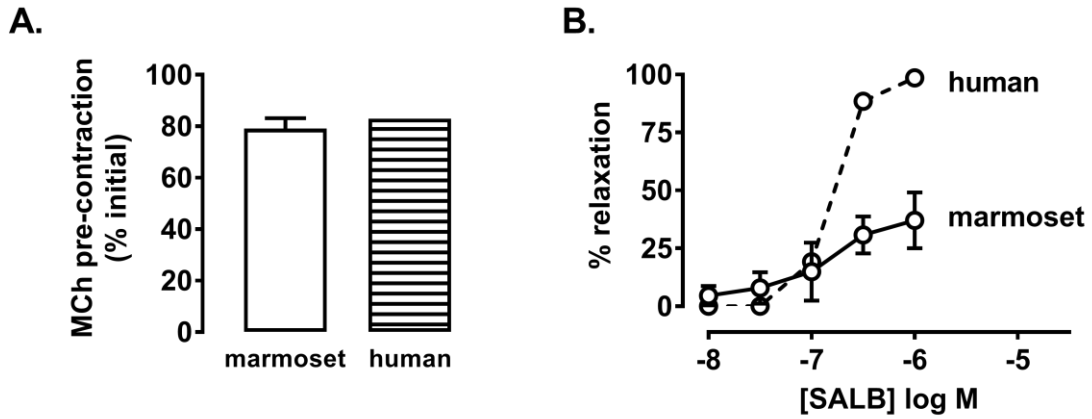

Figure 10. SALB-mediated relaxation is greater in human than marmoset airways in PCLS. Responses to SALB were compared in PCLS from both species. Airways were (C) pre-contracted with MCh, prior to measurement of (D) relaxation responses in both species. Relaxation is expressed as % of MCh pre-contraction (mean ± s.e.m, n= 4 for marmoset, n= 1 for human).

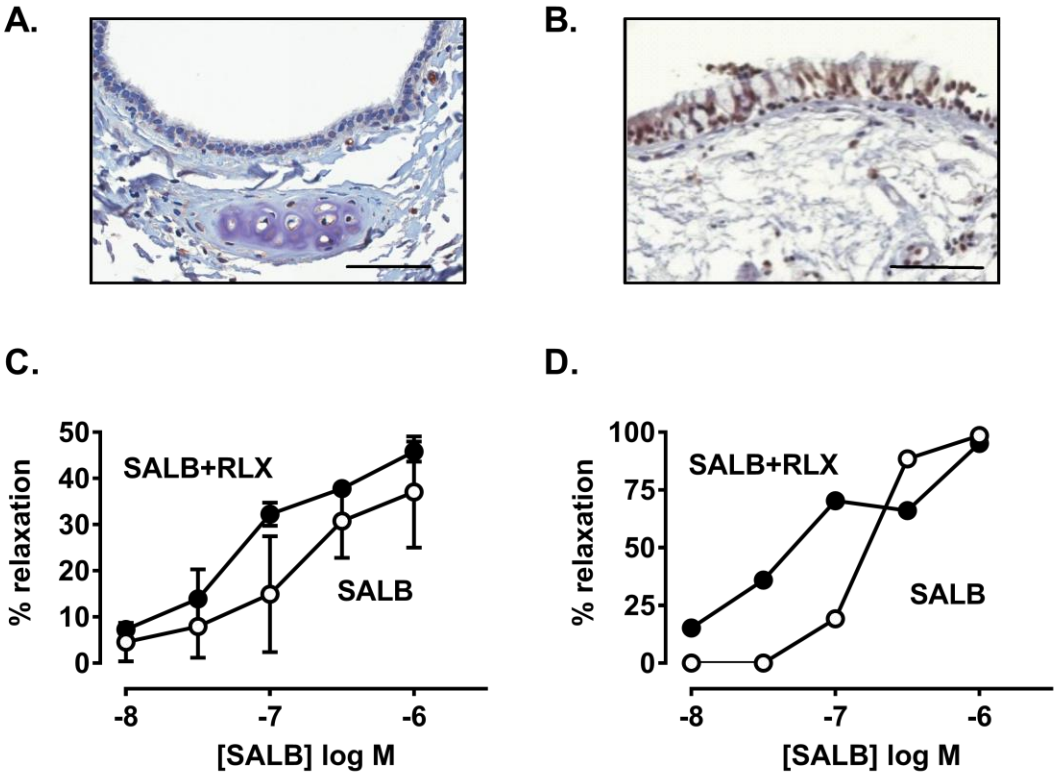

Figure 11. RLX potentiates SALB-mediated relaxation in human but not marmoset airways in PCLS. RXFP1 protein was identified in the lumen of airways from (A) marmoset and (B) human lungs by immunohistochemistry. Responses to SALB in the absence and presence of RLX in PCLS were compared across both species. Airways were pre-contracted with MCh, prior to measurement of relaxation responses in both species and in the absence or presence of RLX (0.1 µM) in (C) marmoset and (D) human PCLS. Relaxation is expressed as % of MCh pre-contraction (mean ± s.e.m. n= 1 human, n= 4 marmoset). Scale bar represents 100 µm.

6.4 Discussion

There is a clear unmet need for improved bronchodilator therapy for patients with poorly controlled asthma, as the requirement for repeated use of SABA such as SALB in severe disease may result in receptor desensitisation and loss of efficacy (Davis *et al.*, 1980). Despite the clear benefits of add-on combination treatment with glucocorticoids and LABA to oppose both airway inflammation and increased contraction, additional dilators such as theophylline are still required when symptoms remain uncontrolled (Chung and Adcock, 2004). However, this additional dilator therapy may be of limited benefit because of the overlapping signalling pathways of SABA, LABA and theophylline that lead to relaxation. Assessment of novel dilator combinations is therefore important, particularly in the small distal airways which are more sensitive to contractile agonists (Burgel, 2011). In the current study, the effects of RLX on dilator responses to the β_2-AR agonist SALB were assessed in multiple species.

Overall, RLX increased the potency of both ISO and SALB in rat and guinea pig trachea whilst maximum relaxation to SALB was increased in the presence of RLX in mouse airways. Preliminary results also suggested that RLX increased the potency of SALB in marmoset and human airways. Based on studies in guinea pig and mouse airways, these actions of RLX appeared to be mediated through release of the epithelial-derived relaxing factors NO and prostaglandin E_2 (PGE_2), as well as by direct regulation of ASM signalling pathways to oppose contraction. These findings suggest that RLX may elicit its effects through alternate mechanisms to SABA resulting in additive or synergistic dilator responses.

Relaxin potentiates salbutamol-mediated airway relaxation in multiple species

In vitro studies assessing reactivity have used airways from rodents through to humans, with the choice based on factors ranging from convenience to clinical relevance. Although we and others have shown that contraction of trachea and intrapulmonary airways to MCh was similar in mouse, rat, marmoset and human airways (Chapter 3, Seehase *et al.*, 2011; Schlepütz *et al.*, 2012) relaxation responses to SALB alone were variable in both potency and efficacy, attributable to the previously reported differences in β_2-AR expression between species (Zaagsma *et al.*, 1983).

Rodent trachea and lungs exhibit mixed populations of β_1- and β_2-ARs in the epithelium and airway smooth muscle, leading to greater relaxation to ISO than SALB. The predominant subtype in both guinea pig and human lungs is the β_2-AR (Zaagsma *et al.*, 1983), consistent with the robust relaxation to SALB seen here in both species in the current study. Somewhat

surprisingly, the efficacy of SALB in marmoset lungs was much lower than human airways, despite both species being primates. Informed by these differences, as well as tissue availability, the exploration of the mechanisms underlying the regulation of β_2-AR-mediated relaxation by RLX focussed on rodent airways to support future translation to preclinical models of lung disease, and guinea pig airways to provide a potentially more relevant comparator with human airways.

The previous finding in rat trachea which showed that RLX elicits slow airway relaxation and potentiates relaxation to a single concentration of ISO (Chapter 5) was extended and confirmed, since concentration-response curves for ISO were left-shifted, indicating increased ISO potency in the presence of RLX. The effects of RLX on both ISO and the more clinically relevant SALB were then assessed in guinea-pig trachea. The greater potency and efficacy of SALB than ISO in guinea pig trachea was consistent with the relatively higher β_2-adrenoreceptor expression in this species. Relaxation beyond the initially established basal tone in the absence of RLX suggests that the β-AR agonists may be opposing the effect of accumulating contractile mediators released from the tissue over the course of the experiment. Of note, RLX markedly increased the potency of both ISO and SALB in guinea pig trachea, confirming that its ability to augment relaxation was not specific to opposing activation of β_1- or β_2- ARs.

It was important to test the potentiation of relaxation by RLX under conditions where SALB efficacy is limited by functional antagonism (Lemoine and Overlack, 1992). This was established experimentally here in maximally contracted guinea pig trachea, where the efficacy of SALB was markedly reduced. Under these conditions, which mimic a more severe acute asthma attack, RLX was still able to increase SALB potency and partially restored efficacy. While these findings are promising, it remains to be determined whether higher concentrations of RLX would be able to fully restore the dilator response to SALB, to the levels seen in the partially contracted airways.

Because small airways play an integral role in asthma pathophysiology, it was hoped to extend the findings in guinea pig trachea to intrapulmonary airways of the same species. However, this aim was not achieved, as patent airways could not be obtained for assessment due to irreversible airway spasm occurring during preparation of guinea pig PCLS. It has been demonstrated that this spasm could be overcome by administering a high dose of ISO to guinea pigs prior to euthanasia (Oenema *et al.*, 2013) but this approach could not be used here due to potential desensitisation of β-ARs.

Mouse PCLS have been commonly used to assess small airway reactivity, but SALB only elicits partial relaxation in this species (Cazzola *et al.*, 2012; Donovan *et al.*, 2015). However, this relatively poor efficacy compared to guinea pig and human airways provided scope to demonstrate any add-on effect of RLX to SALB-mediated relaxation. In mouse PCLS, perfusion with RLX alone did not elicit relaxation, but complete relaxation to SALB was now evident in the presence of RLX, demonstrating synergy between RLX and SALB in mediating airway relaxation.

These combined studies in rat and guinea pig trachea and mouse PCLS showed that RLX was able to increase the potency and/or efficacy of β-adrenoreceptor agonists in both large and small airways. The implication of these findings is that in the presence of RLX, a lower dose of SALB would be required to achieve the same level of relaxation as a higher dose alone. As a consequence, RLX may provide protection against the β_2-AR desensitisation occurring with frequent use of high dose SALB. This could be confirmed experimentally by comparing airway relaxation in PCLS exposed to repeated high doses of SALB known to induced desensitisation, with relaxation to repeated lower doses of SALB in the presence of RLX. The protection conferred by RLX would be confirmed in a final test with high dose SALB alone, where the response to SALB would only be maintained in the airway treated with RLX.

Despite these promising findings, it is important to note that even though relaxation was augmented by RLX, full relaxation to SALB was not achieved in guinea pig trachea under conditions of maximal contraction. Even though a lower dose of SALB may be more effective in the presence of RLX, there is still a need to minimise the severity of asthma exacerbations with preventers to ensure full relief of symptoms.

Mechanisms underlying the potentiation of airway relaxation by relaxin

The underlying mechanisms involved in the potentiation of SALB-mediated relaxation by RLX were then explored. Activation of β_2-ARs expressed on ASM by SALB stimulates adenylyl cyclase to increase the production of cAMP. Increased cAMP has been shown to inhibit MCh-induced Ca^{2+} sensitivity and oscillations via protein kinase A, resulting in less IP_3R activation and Ca^{2+} release from the sarcoplasmic reticulum (Bai and Sanderson, 2006). The RXFP1 receptor is expressed on the airway smooth muscle and the epithelium. Direct and indirect activation of RXFP1 with RLX involves both the adenylyl cyclase/cAMP and guanylyl cyclase/cGMP/NO pathways leading to relaxation (Chapter 5), while the effect of RLX on Ca^{2+} signalling has yet to be assessed. Although the mechanisms of each treatment alone have

been defined to some extent, there is still a need to investigate the potential pathways involved in mediating the additivity or synergy between RLX and SALB.

To explore these potential mechanisms, guinea pig trachea and mouse PCLS were used to assess the requirement for an intact epithelium and release of associated epithelial-derived relaxing factors. The advantage of using guinea pig trachea is that it is convenient for the physical removal of the epithelium for these mechanistic studies. The similar trend for increased relaxation to SALB in the presence of RLX in both intact and denuded tracheal preparations suggests that the potentiation of relaxation may be independent of epithelial-derived relaxing factors. In support of this, potentiation by RLX in mouse airways persisted under conditions where both SALB and RLX were continuously perfused over the PCLS, such that these factors could not readily accumulate in close proximity to the airway. However, there was also clear evidence of loss of potentiation by RLX in mouse airways in the presence of L-NAME and INDO, consistent with the inhibition of epithelial-dependent dilator actions of RLX in rat trachea (Chapter 5). These discrepancies with species and airway size remain to be resolved to determine which factor is more important (NO, PGE_2 or both). The effect of adenylate and guanylate cyclase inhibitors on the potentiation of relaxation by RLX also remains to be tested (as per Chapter 5).

To date, MCh was the only contractile agonist used for studies examining SALB and RLX in mouse PCLS. We therefore determined whether potentiation by RLX was maintained when SALB is opposing contraction to the phorbol ester PdBu. MCh causes contraction via muscarinic receptors to activate the phosphoinositide cascade, leading to the release of Ca^{2+} from internal sarcoplasmic reticulum stores (Janssen and Killian, 2006). Contraction with MCh also occurs via multiple Rho/ROCK-dependent mechanisms that enhance Ca^{2+} sensitivity, including inhibition of MLCP (Janssen and Killian, 2006). In contrast, contraction with PdBu is predominantly driven by direct activation of protein kinase C (PKC) to induce the phosphorylation of CPI-17, resulting in the inhibition of MLCP, which in turn opposes relaxation. PdBu was used in the current study since it induces contraction at a much faster rate than other PKC activators such as phorbol myristate acetate (Mukherjee *et al*., 2013).

Relaxation to SALB alone in PdBu-precontracted airways was less than in MCh-precontracted airways. Although RLX potentiated relaxation to SALB in airways precontracted to either PdBu or MCh, complete relaxation was only achieved in airways pre-contracted with MCh. From these findings, it appears that SALB may be less effective when contraction is due to PKC activation alone, rather than the multiple mechanisms activated by MCh. In addition, the

combination of SALB and RLX may not be able to fully oppose the inhibition of MLCP downstream of PKC that contributes to Ca^{2+} sensitivity.

An alternative way of examining the mechanism of potentiation by RLX was employed, in which relaxation to SALB in the absence and presence of RLX was assessed in PCLS treated with caffeine/ryanodine. This treatment abolishes MCh-induced Ca^{2+} oscillations so that contraction is due to increases in Ca^{2+} sensitivity alone. In the present study, the partial relaxation to SALB was maintained in these so-called “model slices” as previously published (Delmotte and Sanderson, 2008). However, there was a clear loss of potentiation of SALB-mediated relaxation by RLX. This is consistent with the relatively reduced potentiation by RLX in PdBu-contracted airways, and may be a limiting factor for the benefit of RLX as an add-on therapy. Although increased Ca^{2+} sensitivity has yet to be confirmed in asthma, it has been reported following repeated allergen challenge in mouse models (Sakai et al., 2017). It will be of interest to assess RLX alone and in combination with SALB under these conditions which mimic key features of human asthma.

Because the additivity or synergy of RLX with SALB may be dependent on common cAMP/PKA pathways, it can be hypothesised that RLX improves the coupling of β-AR agonists to increase cAMP production or increases the sensitivity of other pathways involved in β-AR-mediated relaxation. Although RLX may protect against β-desensitisation, stimulation of β-ARs could also in turn increase sensitivity to RLX. A recent study in cardiomyocytes has reported that activation of β_1, but not β_2-ARs, significantly inhibited RXFP1 expression which suggests that potential cross-desensitisation by β_2-AR agonists is unlikely (Moore *et al.*, 2014). As such, there is a possibility that β-AR agonists may increase tissue responsiveness to RLX that remains to be explored.

Ideally, these findings in rat, mouse and guinea pig airways would be translated to human airways, and to human asthma. The phylogenetic similarities between marmosets and humans supported the use of the former as a relevant animal model for clinical translation. However, despite the close evolutionary relationship, findings indicate differences in the relaxation of airways between species that were well-matched in terms of level of pre-contraction. However, potentiation of relaxation by RLX in human, but not marmoset, airways was consistent with the greater expression of RXFP1 in human airways. Preliminary immunohistochemical analysis of both rat (Chapter 5) and human airways showed stronger staining for RXFP1 in the airway epithelium than the smooth muscle layer. Although quantitative comparison was not performed, the intensity of RXFP1 staining in marmoset

airways appeared less than rat or human airways. These findings warrant further investigation, along with functional studies utilising an RXFP1 antagonist, as the RXFP1 dependence of the effects of RLX remains to be confirmed. There are no studies to date that have compared the effect of dilators in humans and marmosets despite their close relationship. Given that the responses elicited by guinea pig trachea more closely resemble the responses in human airways, it appears that changes in human airway reactivity could be better modelled in guinea pig airways.

RLX offers promise as an add-on dilator therapy in combination with SALB, due to its potentiation of β-adrenoreceptor-mediated airway relaxation in both large and small airways from multiple species, including human. In these preliminary experiments, human PCLS were treated with RLX under perfused conditions which suggests that relaxation to RLX (shown in Chapter 5) and potentiation of SALB-mediated relaxation by RLX in human airways may not be dependent on the production of epithelial-derived NO and/or metabolites of the COX pathway. In the absence of readily available human diseased airways for further studies, the results obtained here in PCLS from naïve mice provide a prelude and impetus for confirmation of the benefits of RLX and SALB in disease models. Future studies may be able to utilise the improved preservation techniques currently under development to maximise the use of any precious clinical samples that become available to support the potential application of RLX to oppose contraction and preserve β-AR function in the treatment of asthma.

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Chapter 7: Assessing relaxin in mouse models of lung disease

7.1 Introduction

The progressive loss of airway function in chronic lung diseases is commonly associated with persistent inflammation, as well as structural changes in the proximal and distal airways and the surrounding parenchyma. Asthma and idiopathic pulmonary fibrosis (IPF) have distinct disease phenotypes with variable contributions of inflammation and fibrosis, limited to the airways in the former and predominantly affecting the parenchyma in the latter.

Asthma affects over 10% of the population and is clinically defined by the presence of reversible airflow obstruction, measured by spirometry as ≥12% improvement in the forced expiratory volume in 1 sec (FEV1) after inhaled salbutamol (SALB). However, in severe asthma or during exacerbations, average post-bronchodilator FEV1 measurements are often less than 100% of predicted value. While this may reflect a contribution of irreversible obstruction, it may also be due to a loss of sensitivity to β_2-AR agonists that leads to persistent symptoms that could potentially be overcome with add-on therapy.

IPF is an interstitial lung disease with an incidence rate of 3-9 cases per 100,000 per year for Europe and North America from 1968-2012 (Hutchinson *et al.*, 2015). Although the prevalence for IPF is much lower than for asthma, the consequences of the disease are more damaging. A meta-analysis of studies over a 17 year period (2000-2017) revealed a median survival rate of 3.2 years despite many patients having only mild-to-moderate IPF (Ryerson and Kolb, 2018). This debilitating disease is characterised by the progressive destruction of lung tissue involving the loss of alveolar structure and parenchymal fibrosis, which leads to symptoms of shortness of breath (dyspnoea), dry cough and fatigue. In IPF, elevated levels of transforming growth factor beta-1 (TGFβ1) contribute to the differentiation of fibroblasts into myofibroblasts. The increased synthesis of interstitial extracellular matrix (ECM) components including collagen I and III by these differentiated cells is a major driver of disease pathology.

There are limited treatment options for IPF. Pharmacological intervention with steroids and immunotherapies are largely ineffective as they only target inflammation which is a secondary contributor to IPF (Raghu *et al.*, 2011). Pirfenidone and nintedanib are newer therapies that both target the pro-fibrotic actions mediated by TGFβ. However, these agents can only slow but not prevent the progression of disease (Kreuter *et al.*, 2015). Allograft lung transplantation remains the only potentially curative treatment option for IPF; however, finding an appropriate donor is difficult, and in some instances, patients can develop chronic lung allograft dysfunction (Fang *et al.*, 2011).

Given the poor prognosis of IPF, it is important to manage symptoms to improve quality of life for these patients. Oxygen therapy is the major treatment used to reduce breathlessness and fatigue. Although IPF is considered a restrictive rather than obstructive disease, it has recently been shown that approximately one in ten patients with IPF also has physiological evidence of reversible airflow limitation (Assayag *et al.*, 2015). Asthma is also a common comorbidity in IPF (Collard *et al.*, 2012), so improved bronchodilator therapy may be of benefit in patients with either or both conditions.

To date, the influence of inflammation and/or fibrosis in mouse models of asthma and IPF on responsiveness to dilator therapies has not been fully defined. Having established that SALB-mediated small airway relaxation is potentiated by RLX (Chapter 6), it is of particular interest to determine whether the response to this novel dilator therapy is maintained in disease relevant mouse models. Additional antifibrotic actions of RLX have previously been demonstrated in chronic models of allergic airways disease (AAD) mimicking key features of asthma, and bleomycin models that display features of IPF (Unemori *et al.*, 1996; Royce *et al.*, 2009) as well as in fibroblasts from subjects with asthma (Chapter 3). Confirmation that RLX also opposes airway contraction in disease models would support future clinical assessment of its novel dual actions, namely to elicit acute airway relaxation alone and/or in combination with SALB as well as to reverse fibrosis with chronic treatment in established disease.

Key features of asthma can be induced in mice by house dust mite (HDM), a naturally occurring allergen that is more relevant to human asthma than commonly used ovalbumin (OVA). Levels of mast cells, eosinophils and neutrophils were noticeably higher in the HDM model compared to other allergy models using OVA (DiGiovanni *et al.*, 2009). Interestingly, even without inducing airway remodelling, acute HDM sensitisation induced greater *in vivo* AHR to MCh compared to an acute OVA model (DiGiovanni *et al.*, 2009). An acute HDM model therefore provides a setting to assess dilator responsiveness to SALB and its potentiation by RLX in inflamed airways in the absence of fibrosis.

The most widely used models of IPF utilise bleomycin, a chemotherapeutic agent that also elicits lung fibrosis (Moeller *et al.*, 2008). In mice, intratracheal instillation of bleomycin caused diffuse fibrosis throughout the parenchyma in addition to airway fibrosis (Unemori *et al.*, 1996; Huang *et al.*, 2011) whilst lung segments from bleomycin-treated sheep displayed increased tissue density and collagen deposition (Organ *et al.*, 2014). Previous studies in bleomycin models have shown that chronic treatment with RLX prevents the development of fibrosis by

inhibiting TGFβ1-mediated collagen deposition and alveolar thickening while increasing levels of collagen-degrading matrix metalloproteinases (Unemori *et al.*, 1996; Pini *et al.*, 2010; Huang *et al.*, 2011).

In terms of lung function, bleomycin exposure is known to reduce lung compliance in mice (Binks *et al.*, 2017) but *in vivo* changes in airway resistance to MCh are not routinely assessed. A single study showed increased maximum contraction to the muscarinic agonist, carbachol in isolated trachea from bleomycin-treated rats (Barrio *et al.*, 2006). To date, *in vitro* dilator responses of airways from a bleomycin model have not been examined.

The pathological structural changes in both IPF and BLM models extend to the distal lung and the surrounding parenchyma, with the potential to impact on small airway reactivity. The application of the precision cut lung slice (PCLS) technique to the BLM model provides a unique approach to assess the reactivity of fibrotic small airways still surrounded by a fibrotic parenchyma. It remains to be determined whether small airway responses to constrictors are increased, and whether the potentiation of SALB-mediated relaxation by RLX is maintained in this IPF relevant setting.

TGFβ1 is a pleiotropic cytokine upregulated in both IPF and asthma (Koćwin *et al.*, 2016), implicated in both parenchymal and airway fibrosis. Normal wound healing processes are regulated by TGFβ1. However, excessive TGFβ1 can also lead to cell damage, apoptosis and airway remodelling. TGFβ1 induces the differentiation of fibroblasts into myofibroblasts that express the contractile protein, α-smooth muscle (α-SMA) and promotes collagen deposition around the airways. TGFβ1 also increases α-SMA expression in airway smooth muscle (ASM) (Slats *et al.*, 2008). Although stiffening of airways has the potential to decrease lung compliance and oppose contraction, the increased level of α-SMA in ASM may also contribute to increased airway contraction (Ojiaku *et al.*, 2017). It is unknown whether one of these effects dominates in disease, and how elevated levels of TGFβ1 could affect airway reactivity overall.

Although the effect of *in vivo* administration of exogenous TGFβ1 on airway reactivity has not been assessed, OVA-induced increases in TGFβ1 levels were associated with airway fibrosis and the development of AHR (Royce *et al.*, 2014). In another chronic OVA model, neutralization of TGFβ1 with a specific antibody enhanced, rather than inhibited, OVA-induced AHR despite suppressing fibrosis (Alcorn *et al.*, 2007). These conflicting results are difficult to interpret given the additional influence of allergen-induced chronic inflammation in these

models, but provide evidence of potential interactions between TGFβ1 levels, fibrosis and airway contractility.

To address the specific role of TGFβ1 on airway structure and responsiveness, a transgenic mouse model has been developed resulting in localised overexpression of TGFβ1 to the airways (Lee *et al.*, 2004). This system is based on the tetracycline-controlled transcriptional suppressor and the reverse tetracycline transactivator. Upon oral doxycycline (dox) administration, the transcriptional suppressor is released which allows the Clara cell-specific promoter to induce gene transcription of bioactive TGFβ1 (Lee *et al.*, 2004; Lee, 2006). While airway fibrosis has been clearly established in this TGFβ1-overexpression model (Lee, 2006), assessment of airway reactivity either *in vivo* or *in vitro* has yet to be assessed. Studies of PCLS from these mice may provide insights into the complex interplay between fibrosis and airway reactivity, as well as define the potential for the combination treatment of RLX and SALB to be of benefit in opposing airway contraction in fibrotic airways.

The differences in inflammation and fibrosis in asthma and IPF are likely to influence airway reactivity and responses to dilator treatment. Previous chapters have demonstrated the therapeutic actions of the peptide hormone relaxin (RLX) as an anti-fibrotic (Chapter 4), AND as a novel dilator (Chapter 5) with additional benefit in combination with the β_2-AR agonist, SALB (Chapter 6). The finding that partial relaxation to SALB was potentiated in the presence of RLX is of particular significance. While it suggests that the use of RLX as an add-on therapy may reduce the need for high doses of SALB and protect against the development of tolerance, it is important to test this in disease context.

In this study, three distinct models of lung diseases were applied, namely the bleomycin model of fibrosis (airway and parenchymal fibrosis); an acute HDM model of AAD (inflammation); and TGFβ1-overexpression model (airway fibrosis). We hypothesised that the potentiation of SALB-mediated relaxation by RLX would be maintained in disease, irrespective of the differential influences of fibrosis and inflammation.

7.1.1 Aims

The experimental aims were to:

1. assess the inflammatory and structural changes induced in a mouse model of asthma (house dust mite, HDM), airway (TGFβ1-overexpression) and parenchymal fibrosis (bleomycin, BLM)
2. assess *in vivo* lung function and small airway reactivity to MCh in these models
3. determine whether potentiation of relaxation by RLX is maintained under disease conditions

7.2 Methods

7.2.1 Materials and solutions

Doxycycline hyclate, methacholine (MCh) and salbutamol (SALB) (all from Sigma-Aldrich St Louis, MO, USA); recombinant human gene-2 relaxin (rhRLX, kindly provided by Corthera Inc., San Mateo, CA, USA; a subsidiary of Novartis AG, Basel, Switzerland); RXFP1 antibody from Santa Cruz Biotechnology (cat# sc-50328); house dust mite (HDM) from Citeq biologics (Groningen, Netherlands), bleomycin (BLM) from Sapphire Bioscience.

7.2.2 Mice and tissue collection

All procedures were approved by a Monash University Animal Ethics Committee (AEC number: MARP2/2018/027 (HDM), MARP/2017/015 (BLEO) and MMCB201/38 (TGFβ1)) and a University of Technology Sydney (UTS) Animal Care and Ethics Committee (ACEC number: ETH16-0979), which adheres to the Australia Code of Practice for the Care and Use of Animals for Scientific Purposes. Balb/C and C57Bl/6 mice were housed in the Animal Facility, Monash University and maintained on a fixed 12 h light, 12 h dark lighting schedule, with free access to food and water at all times.

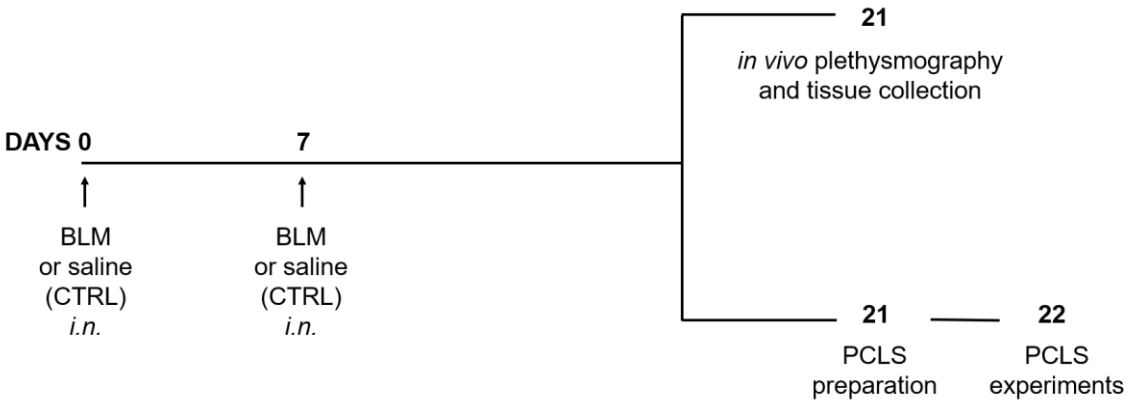
Mice were humanely euthanized by cardiac puncture under anaesthesia with ketamine (200 µg/g) and xylazine (10 µg/g) after plethysmography for collection of lung tissue or by sodium pentobarbitone overdose (60 mg/mL administered *i.p*. at a volume of 1 mL per 100 g body weight) for preparation of PCLS.

7.2.3 Mouse models of lung disease

7.2.3.1 Model 1- Bleomycin model

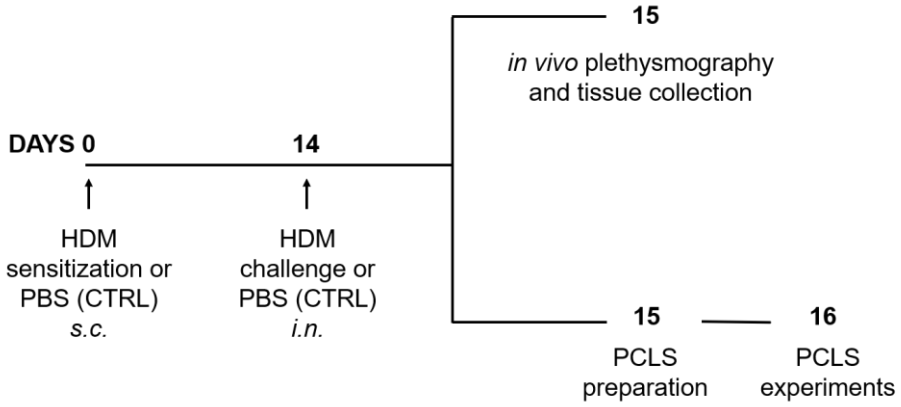
Bleomycin (BLM) is a chemotherapeutic antibiotic, produced by the bacterium “Streptomyces verticillus” (Adamson, 1976), and is the standard agent for induction of experimental pulmonary fibrosis (Moeller *et al.*, 2008).

Female 6-8 week old Balb/C mice were treated with BLM *i.n.* (6 mg/kg, 50 µL) or equivalent volumes of 0.9% saline (CTRL) on days 0 and 7. Mice were sacrificed 14 days after treatment with BLM or saline (21 days total) (Moodley *et al.*, 2009, 2010). Lung function was assessed by invasive plethysmography on day 21, before lung tissue was removed for histology. In separate mice, lungs were isolated for the preparation for PCLS.



7.2.3.2 Model 2 - House dust mite model (HDM)

For the acute AAD model, female 6-8 week old C57Bl/6 mice were administered HDM (1 mg/mL) on day 0 and challenged with 20 µl HDM *i.n* on day 14 (Nath *et al.*, 2014), while PBS control (CTRL) mice received equivalent volumes of PBS over the same period. Lung function was assessed by invasive plethysmography on day 16, before bronchoalveolar lavage fluid (BALF) fluid and lung tissue was collected. In separate mice, lungs were isolated for the preparation for PCLS.



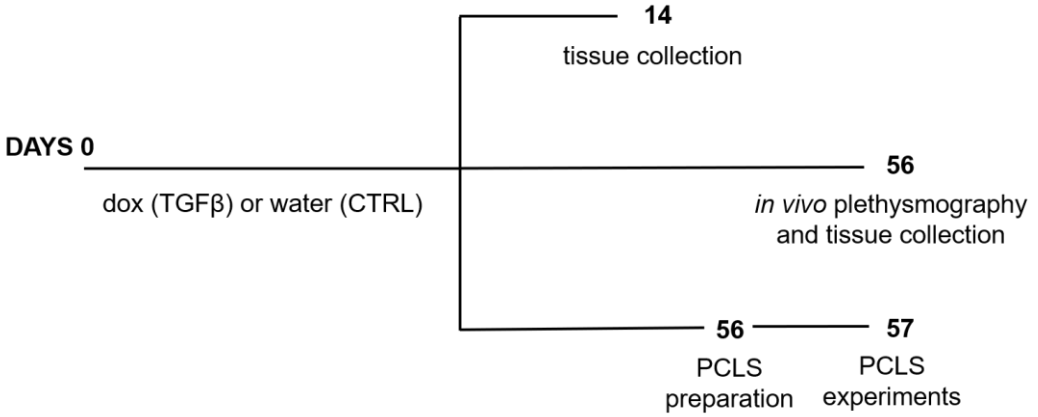
7.2.3.3 Model 3 – TGFβ1 overexpression model

CC10-rtTa-tTS-TGF-β1 mice were generated as previously reported by Lee *et al.* (2004). To induce localized airway fibrosis, male and female 8-14 week old transgene^{+} mice on a C57Bl/6 background were administered doxycycline (0.25 mg/mL) in drinking water to induce TGFβ1 overexpression (TGFβ1). Mice were administered normal drinking water as control (CTRL) (Lee *et al.*, 2004).

Firstly, the effect of doxycycline administration for 2 weeks and withdrawal over 48 h on serum and BALF-TGFβ1 levels was explored.

Lung tissues were collected from mice after 4 and 8 weeks from TGFβ1 and CTRL mice, with Masson's Trichrome staining used to assess changes in collagen. Lung sections produced from 8-week mice were analysed using the to the Genesis 200 (Histoindex) and assessed for interstitial lung collagen and localized airway interstitial collagen.

Separate mice were either administered doxycycline or normal drinking water for 8 weeks, prior to *in vivo* assessment of lung function and tissue collection or *ex vivo* assessment of airway reactivity in PCLS.



7.2.4 Histology

Formalin-fixed lung tissue from mice were routinely processed, paraffin embedded and cut into 4 µm sections. Serial sections were stained with haematoxylin and eosin (H&E, Amber Scientific, Midvale, WA, Australia) to assess inflammation and with Masson's trichrome stain or Histoindex scan to assess collagen deposition (Royce *et al.*, 2013; Chapter 2.5.2).

7.2.5 Assessing inflammation

7.2.5.1 BALF and serum collection

A total of ~1 mL of BALF was collected from right lung as described (Lee *et al.*, 2004) for measurements of total cell counts (HDM and TGFβ1-overexpression model), differential cell counts (HDM model) and TGFβ1 levels (TGFβ1-overexpression model). Serum was prepared from blood and collected from the vena cava for TGFβ1 assays (Model 3).

7.2.5.2 Total and differential cell counts

Lungs were lavaged twice with 0.5 mL sterile 1xHBSS. The collected fluid was spun at 3000 rpm for 10 min at 4°C. Cell pellets were resuspended in sterile HBSS for total and differential cell counts. To perform differential cell counts, cells were spun on glass slides using a Cytospin 4 Cytocentrifuge (Thermo Fisher Scientific) and stained with Diff-Quik®. A total of 200 cells were counted.

7.2.5.3 H&E

Airway inflammation was assessed with H&E staining as detailed in 2.6.1. Slides stained with H&E were assessed for inflammation where a score ranging from 0-4 was assigned for each section (Table 1) (Borzone *et al.*, 2001; Nakagome *et al.*, 2006; Royce *et al.*, 2015).

Table 1. Description of inflammatory scores.

score	lung (model 1- BLM)	airway (model 2- HDM, Model 3 -TGFβ1)
0	no detectable inflammation	no detectable inflammation
1	low interstitial and peribronchial inflammatory cell aggregates	low peribronchial inflammatory cell aggregates; pooled area <0.1 mm^2
2	mild interstitial and peribronchial inflammatory cell aggregates; no obvious change to lung architecture	mild peribronchial inflammatory cell aggregates; pooled area ~0.2 mm^2
3	moderate interstitial and peribronchial inflammatory cell aggregates; diffuse and focal alveolar inflammation; thickening of the alveolar septae	moderate peribronchial inflammatory cell aggregates; pooled area ~0.3 mm^2
4	widespread peribronchial inflammatory cell aggregates; diffuse and focal alveolar inflammation; enlargement or fragmentation of alveolar septa	widespread peribronchial inflammatory cell aggregates; pooled area ~0.6 mm^2

7.2.5.4 Toluidine Blue

Mast cell number in airway sections from Model 2 was assessed with Toluidine blue staining. The cytoplasm of mast cells contains granules which react with Toluidine blue to stain mast cells red-purple (metachromatic staining) and the background blue (orthochromatic staining). Following dewaxing, slides were incubated in Toluidine blue working solution for 2-3 min. All slides were rinsed with alcohol to dehydrate the tissue and prepare the slide for cover-slipping.

7.2.6 Assessing fibrosis and remodeling

7.2.6.1 Masson's Trichrome

Sections from Models 1 and 3 were assessed for collagen levels with Masson's Trichrome as detailed in Chapter 2.6.2.

7.2.6.2 Genesis 200 / Histoindex

Sections from Models 2 and 3 were dewaxed and placed into the Genesis 200 (Histoindex Inc) as described in Chapter 2.6.4. Images generated by the Genesis 200 were assessed by the Fibroindex application which morphological analysis of interstitial collagen area within the tissue and more specifically around the airways.

7.2.7 Detection of RXFP1 with IHC

Immunohistochemistry for the RXFP1 receptor was performed in lung sections from all models using a monoclonal mouse antibody to RXFP1 (Santa Cruz, J2612, 1:500) as detailed in Chapter 2.6.3.

7.2.8 ELISA for serum TGFβ1 levels

To assess TGFβ1 levels, BALF and serum were collected from 2 week TGFβ1 and CTRL mice in Model 3. The 'Human/Mouse TGF beta 1 ELISA Ready-SET-Go! (2nd Generation)' kit was prepared to determine a standard curve for TGFβ1 (15.625-1000 pg/ml). ELISA was performed as per manufacturer's instructions, using 3-5 samples per time point. BALF was diluted to 1/5 and serum to 1/100 with ELISA diluent. Anti-Human/Mouse TGFβ1 purified capture antibody and anti-Human/Mouse TGFβ1 biotin detection antibodies were used to determine concentrations of TGFβ1.

7.2.9 Invasive Plethysmography

Mice were anaesthetized with a mixture of ketamine and xylazine (200 µg/g ketamine, 10 µg/g xylazine, *i.p.*). Following tracheotomy, mice were placed in a plethysmograph chamber (Buxco Research Systems, Wilmington, NC, UA). Baseline values for airway resistance and compliance were established in all models before nebulization with MCh in Models 2 and 3. Mice were nebulized with increasing concentrations of MCh (3.125, 6.25, 12.5, 50 mg/mL) in 2 min intervals. Changes in airway resistance was calculated by subtracting the baseline resistance value from the maximal resistance value induced by each dose (Hartney and Robichaud, 2013).

7.2.10 Precision cut lung slices (PCLS)

The PCLS technique has been described in detail in Chapter 2.5 and Chapter 3. Briefly, lungs were filled with agarose in order to provide a firm sample which can be sectioned by a vibratome into thin slices. These lung slices were incubated overnight and used on the following day for perfusion experiments. In this chapter, MCh (10-3000 nM) and SALB (10-

1000 nM) (in the presence and absence of RLX (100 nM)) concentration response curves were constructed in all models. Some PCLS were thawed from storage (as described in Chapter 3.2.4) for experiments. Other slices were generated from lungs which were sliced 24 h post fill (Chapter 3.3.4).

7.2.11 Analysis

Concentration response curves were fitted with non-linear regression curve fit to derive the EC_{50} and maximum response values. Results were analysed via t-tests or one-way ANOVA for multiple comparisons between groups as appropriate. Statistical significance was accepted at $P<0.05$.

7.3 Results

7.3.1 Bleomycin induces inflammation and airway remodelling

The effect of BLM on inflammation and remodelling were assessed on lung sections stained with H&E and Masson's trichrome respectively. Within two weeks, BLM caused diffuse peribronchial inflammation that extended throughout the parenchyma (Figure 1A). Blinded assessment of sections stained with H&E indicated a 2.2-fold increase in inflammation scores in BLM-injured mice compared to CTRL mice ($P<0.001$, Figure 1B).

BLM did not affect epithelial thickness (Fig 2A-C) but induced severe fibrosis by increasing collagen deposition in both the subepithelial basement membrane regions of the airway wall (Figure 2A, B, D) and surrounding parenchyma (Figure 2A, B, D). Morphometric analysis of multiple airways in lung sections from BLM mice demonstrated an average 40% increase in subepithelial collagen thickness ($P<0.001$ vs CTRL). More than 85% of the multiple individual airways assessed in sections from BLM-treated mice had greater subepithelial collagen deposition than the average control airway (CTRL: 9.8±0.7 µm subepithelial thickness, Figure 2C), while a 51% increase in overall interstitial fibrosis was also evident in the BLM lung sections ($P<0.01$ vs CTRL).

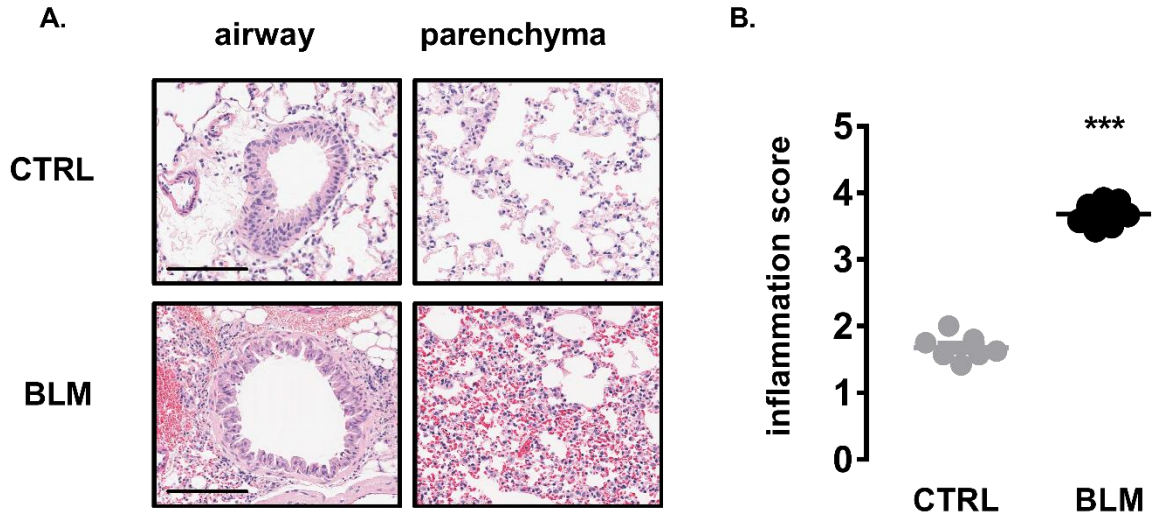


Figure 1. BLM induces diffuse inflammation around the airway and parenchyma. (A) Lung sections from control (CTRL) and bleomycin (BLM) mice were stained with H&E to demonstrate the extent of airway wall and parenchymal inflammatory cell infiltration. Scale bar= 50 µm. (B) Sections (5 per mouse) were scored based on the number and density of inflammatory aggregates on a scale of 0 (no inflammation) - 4 (severe inflammation). Data is expressed as mean±s.e.m. for n= 7 per group. ***P<0.01 vs CTRL.

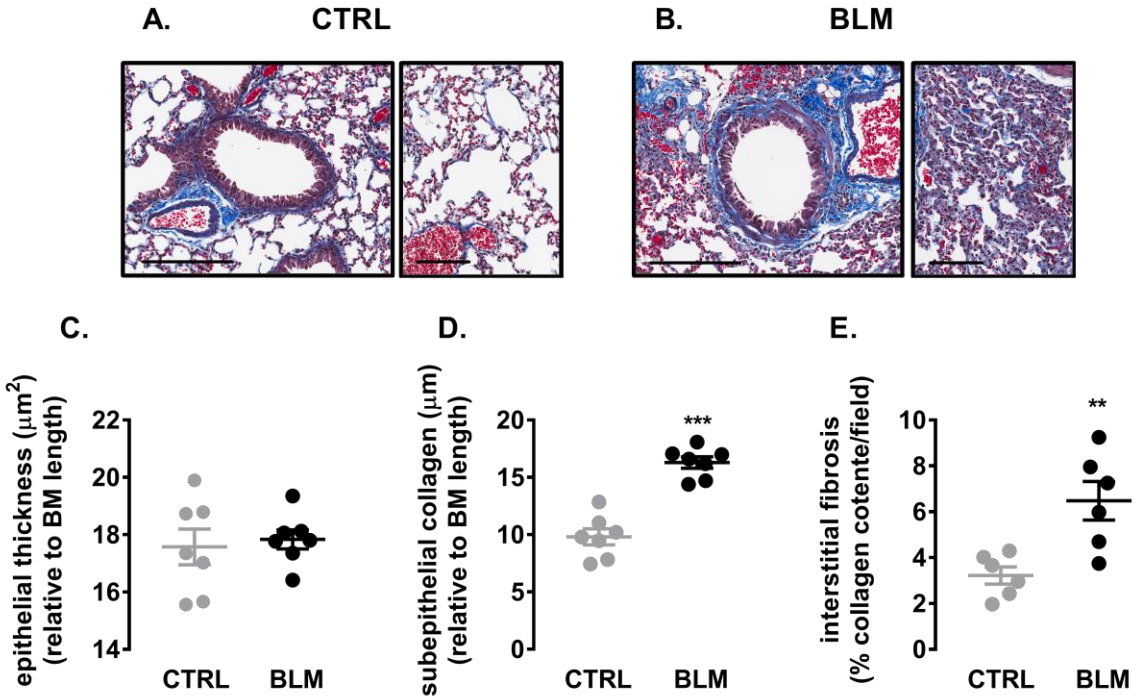
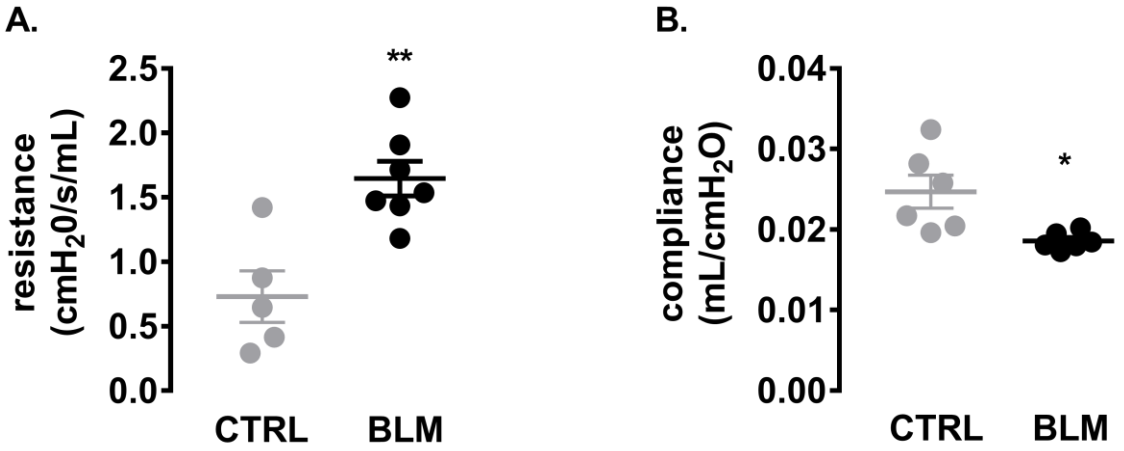


Figure 2. BLM does not alter epithelial thickness but increases subepithelial collagen deposition and interstitial fibrosis. Lung sections from (A) control (CTRL) and (B) bleomycin (BLM) mice were stained with Masson's trichrome to demonstrate the extent of airway wall remodelling. Scale bar= 50 μm. Analysis of sections provided measurements for (C) epithelial and (D) subepithelial thickness (μm) and (E) interstitial fibrosis (μm^2 relative to basement membrane length) averaged from 5-8 airways per section. Data is expressed as mean±s.e.m. for n= 7 per group. $^{**}P<0.01$, $^{***}P<0.001$ vs CTRL.

7.3.2 Bleomycin increases baseline resistance *in vivo* but does not affect MCh contraction *in vitro*

To determine whether these structural changes caused by BLM resulted in differences in lung function, *in vivo* measurements via invasive whole-body plethysmography and *ex vivo* measurements using the PCLS technique were performed. Baseline airway resistance was 128% higher (P<0.01 vs CTRL, Figure 3A) in BLM mice compared to CTRL mice whereas baseline compliance was 25% lower (P<0.05 vs CTRL, Figure 3B).

Reactivity to MCh was not assessed *in vivo*, but contractile responses were assessed in PCLS *ex vivo* as shown in the representative traces (Figure 4A). The diameters of airways examined were similar in both groups, and ranged from 214-251 µm (Figure 4B). The potency of MCh in small airways was unaltered following BLM exposure, but there was a trend for decreased maximum response (% reduction in airway area: CTRL 76.4±5.7, BLM 53.7±9.5, P=0.07, Figure 4A, C).



(data provided by Vivian Mao and Dr. Simon Royce, Monash University)

Figure 3. BLM increases *in vivo* baseline resistance and compliance. Airway (A) resistance and (B) compliance in control (CTRL) and bleomycin (BLM) mice were assessed via invasive plethysmography. Reponses are expressed as resistance or compliance at baseline. Data is expressed as mean±s.e.m. for n= 7 per group. *P<0.05, **P<0.01 vs CTRL.

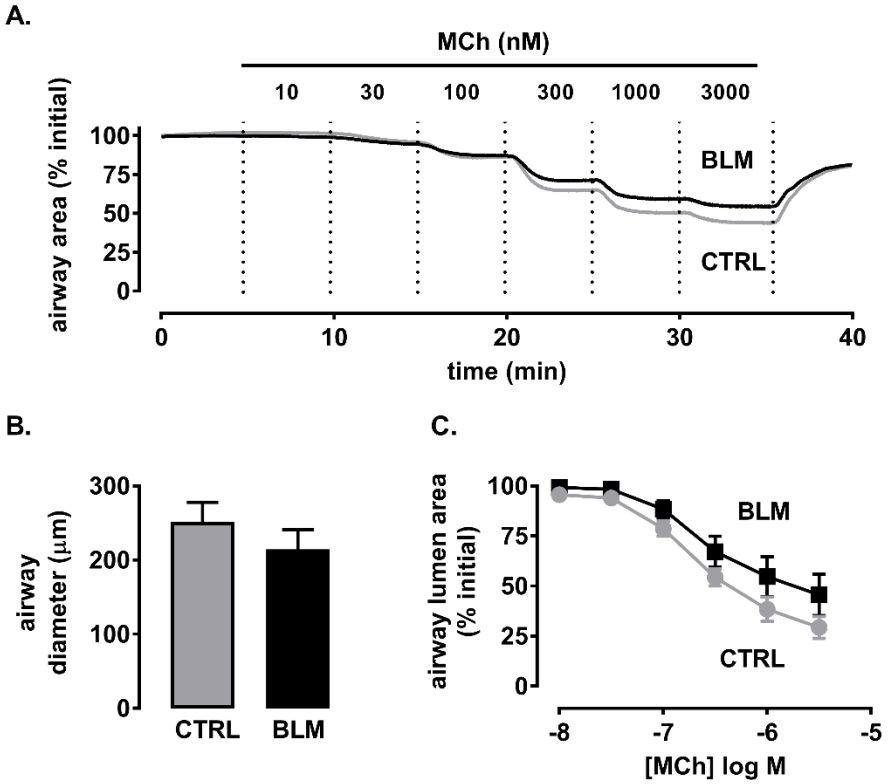


Figure 4. BLM did not alter contraction to MCh in small intrapulmonary mouse airways. Mouse lung slices were mounted in a customised chamber for assessment of changes in intrapulmonary airway area by phase-contrast microscopy. Airways were contracted to increasing concentrations of MCh (0.01-3 μM). (A) Representative trace shows airway area values in pixels determined from gray-scale analysis of images using ImageJ. (B) Airway diameter was noted prior to (C) perfusion with MCh. Data is expressed as % initial airway area (mean±s.e.m. for n= 6-7 per group).

7.3.3 SALB-mediated relaxation is maintained but not potentiated by RLX following bleomycin exposure

Dilator effects of SALB in small intrapulmonary airways from CTRL and BLM-treated mice were compared after pre-contraction to submaximal levels with MCh (0.3 µM). Representative images show marked airway contraction to MCh and partial relaxation to SALB in both groups (Figure 5A). The submaximal contraction to MCh was similar in both groups (Figure 5B). SALB elicited relaxation of size-matched small airways to similar extents in both CTRL and BLM mice (Figure 5C). There was no difference in potency or maximum relaxation to SALB (pEC_{50}: CTRL 6.7 ± 0.4, BLM 6.5±0.4; max % relaxation: CTRL 43.5 ± 7.2, BLM 66.4 ± 7.8).

The combined effects of RLX and SALB on airway relaxation were then assessed in each group. In airways from both CTRL and BLM-treated mice, RLX (0.1 µM) alone did not cause relaxation (not shown). Co-treatment with RLX resulted in increased relaxation to SALB with no change in potency in CTRL airways ($P<0.001$ vs SALB alone, Figure 6A). In contrast, the partial relaxation to SALB in BLM airways was not potentiated by RLX (Figure 6B).

Immunohistochemistry was used to examine the effect of BLM on RXFP1 receptor expression. Staining for RXFP1 was evident in the airway smooth muscle and epithelial layers in CTRL airways. Following allergen challenge, this was reduced by ~80% ($P<0.001$, Figure 7).

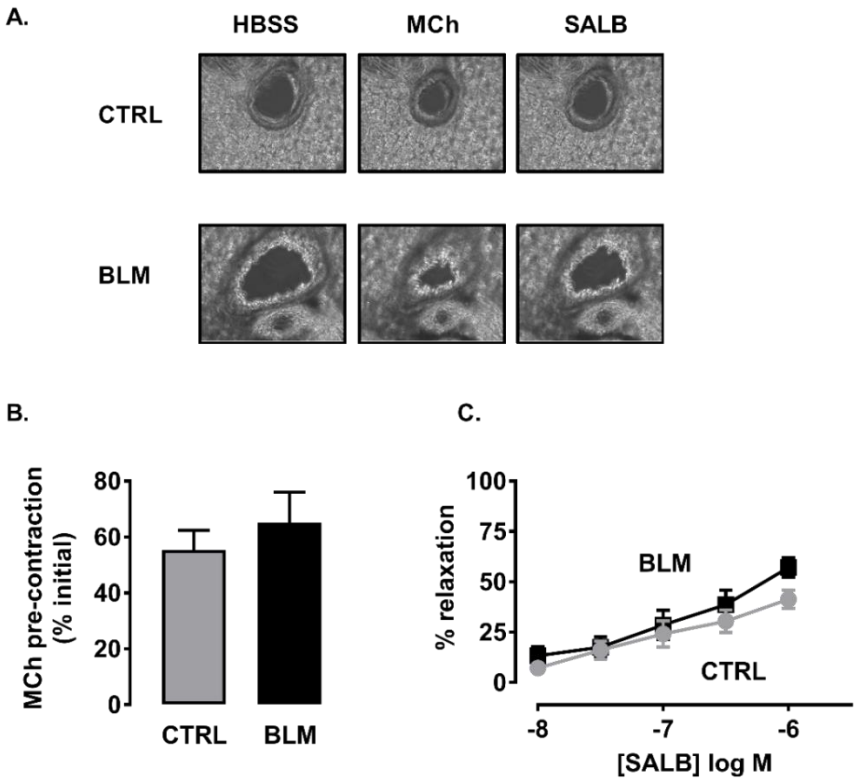


Figure 5. BLM does not alter SALB-mediated relaxation in small intrapulmonary mouse airways. Mouse lung slices were assessed for changes in intrapulmonary airway area by phase-contrast microscopy. (A) Representative phase-contrast images are derived from the last frame depicting MCh contraction (0.3 µM) and SALB relaxation (1 µM). (B) Airways were pre-contracted to MCh (0.3 µM) prior to (C) relaxation with increasing concentrations of SALB. Data is expressed as % initial airway area (mean ± s.e.m. for n= 6-7 per group).

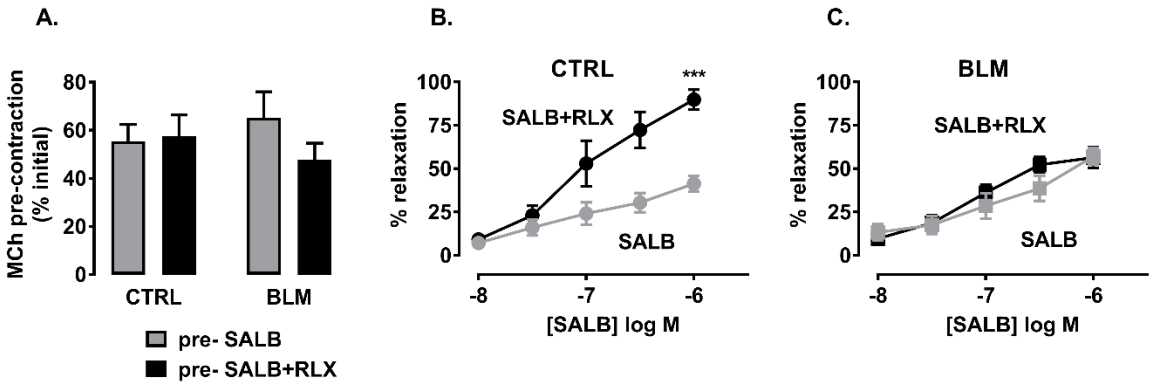


Figure 6. Potentiation of SALB-mediated relaxation by RLX is attenuated following BLM exposure. Mouse lung slices were assessed for changes in intrapulmonary airway area by phase-contrast microscopy. (A) Airways were pre-contracted to MCh (0.3 µM) prior to relaxation with SALB in the absence or presence of RLX (0.1 µM) in (B) control (CTRL) or (C) bleomycin (BLM) mice. Data is expressed as % initial airway area (mean ± s.e.m. for n= 6-7 per group). ***P<0.0001 vs SALB alone.

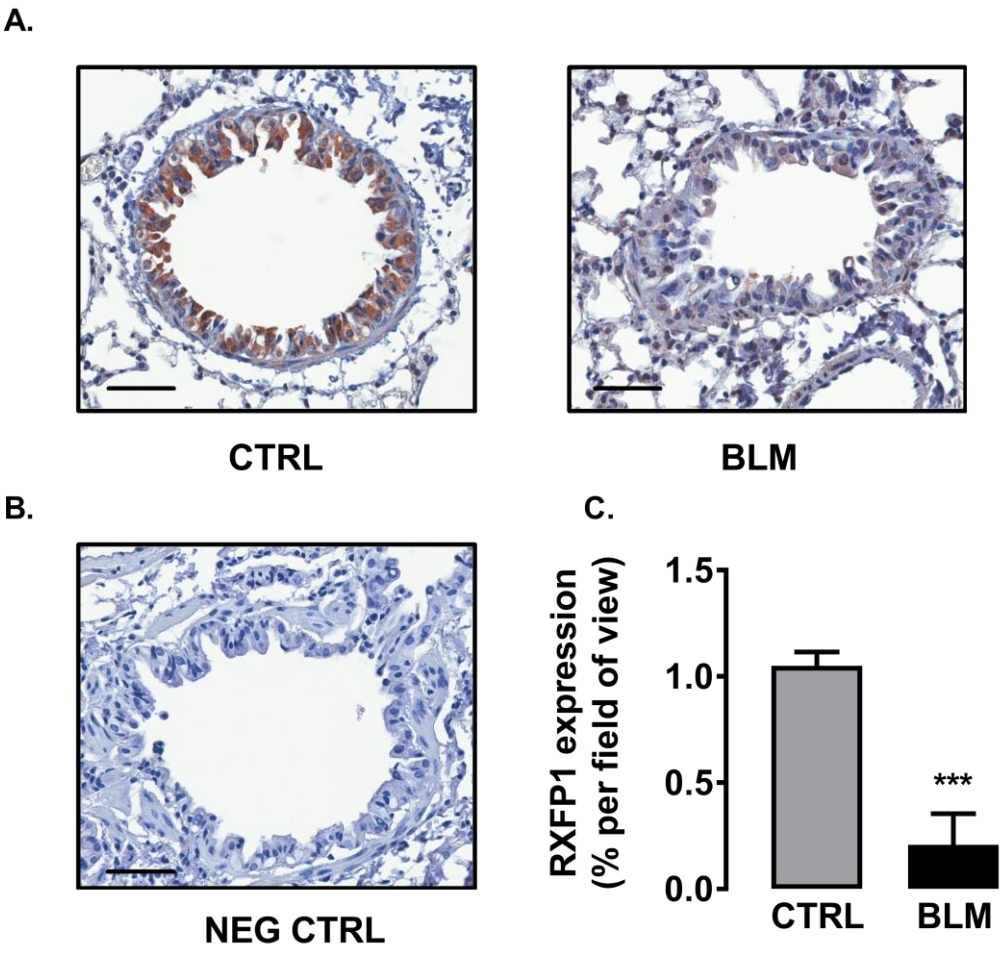
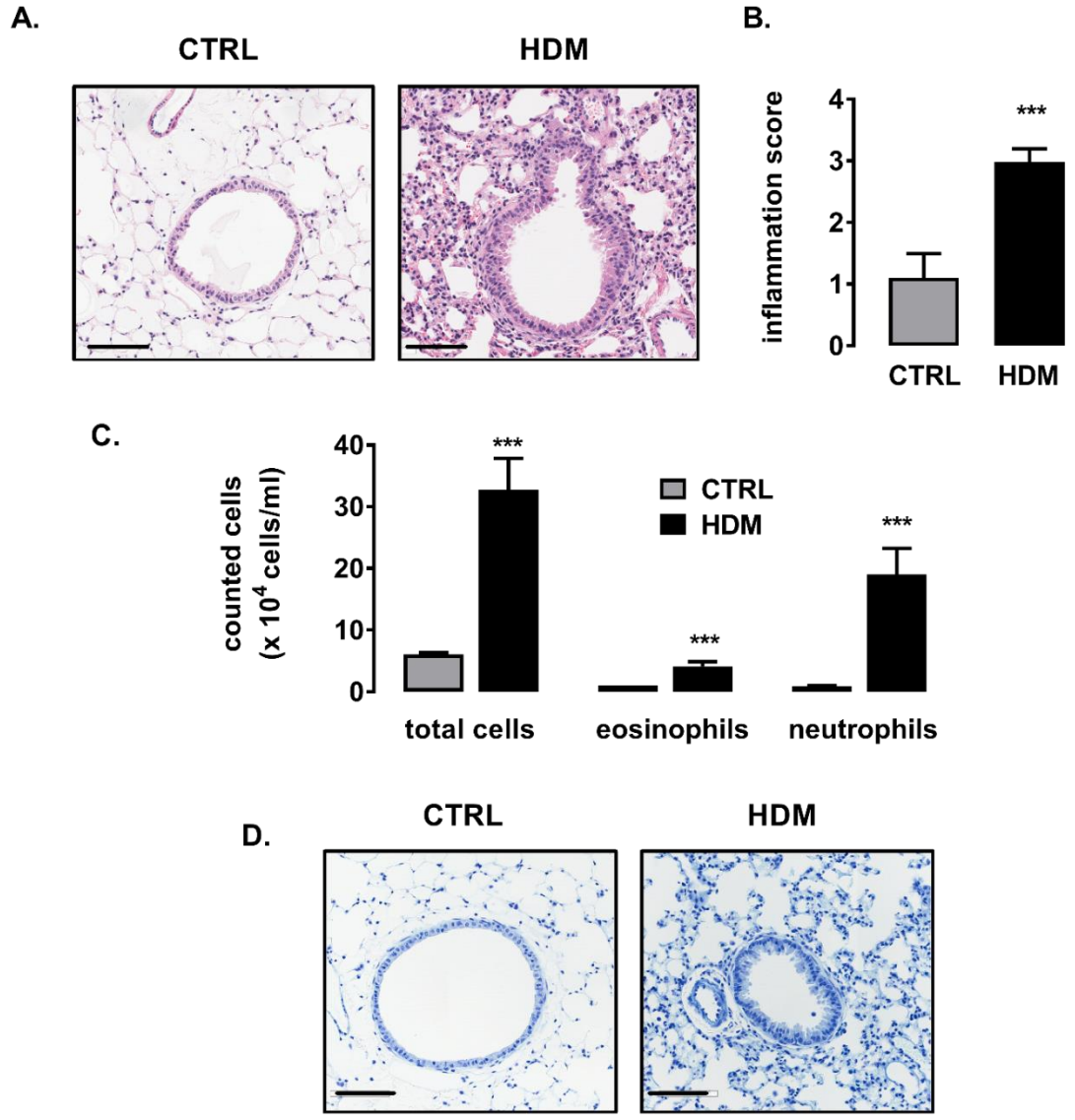


Figure 7. BLM decreases RXFP1 expression in mouse airways. (A) RXFP1 protein was localised in airways of control (CTRL) and bleomycin (BLM) mice by immunohistochemistry. Scale bar= 50 µm. Grouped data shows the levels of RXFP1 expression as % per field of view for each group. Data is expressed as mean ± s.e.m. for n=3-7 per group averaged from multiple fields of view per section.

7.3.4 Acute house dust mite challenge induces airway inflammation but not fibrosis

The effects of acute HDM exposure were assessed via H&E and toluidine blue staining for inflammation; and via Genesis 200 analysis for remodelling and fibrosis. Inflammation induced by HDM was evident in and around the airways (Figure 8A). Inflammation was scored under blinded conditions and showed a 2.6-fold increase following HDM exposure ($P<0.001$, Figure 8B). The type and number of cells involved were assessed in collected BALF (Figure 8C). HDM challenge increased total cell number 6-fold with a greater increase in neutrophils than eosinophils (cells x 10^4 cells/ml: neutrophils: CTRL ≤ 1 vs HDM 19.0±4.3; eosinophils: CTRL ≤ 1 vs 4.1±0.8; $P<0.0001$) (Figure 8C). However, there was no evidence of increased mast cell numbers in the distal airways (Figure 8D) or trachea (not shown) with HDM exposure.

Acute HDM challenge did not cause airway fibrosis as assessed by Genesis 200 scanning and Fibroindex analysis. Collagen distribution and density was similar in CTRL and HDM mice where it contributed to ~15% of total lung area, with no evidence of increased collagen deposition around airways (Figure 9).



(differential cell count data provided by Raj Venkata Allam and Dr. Maria Sukkar, UTS)

Figure 8. HDM exposure increases small airway inflammation. (A) Lung sections from control (CTRL) and house dust mite (HDM) mice were stained with H&E to demonstrate the extent of airway inflammation. Scale bar= 100 µm. (B) Sections (5 per mouse) were scored based on the number and density of inflammatory aggregates on a scale of 0 (no inflammation)-4 (severe inflammation). (C) Bronchoalveolar lavage fluid (BALF) was assessed for differential cell counts (D) Sections were also stained with toluidine blue for mast cell count. Data is expressed as mean±s.e.m. for n= 7-11 per group. ***P<0.001 vs control.

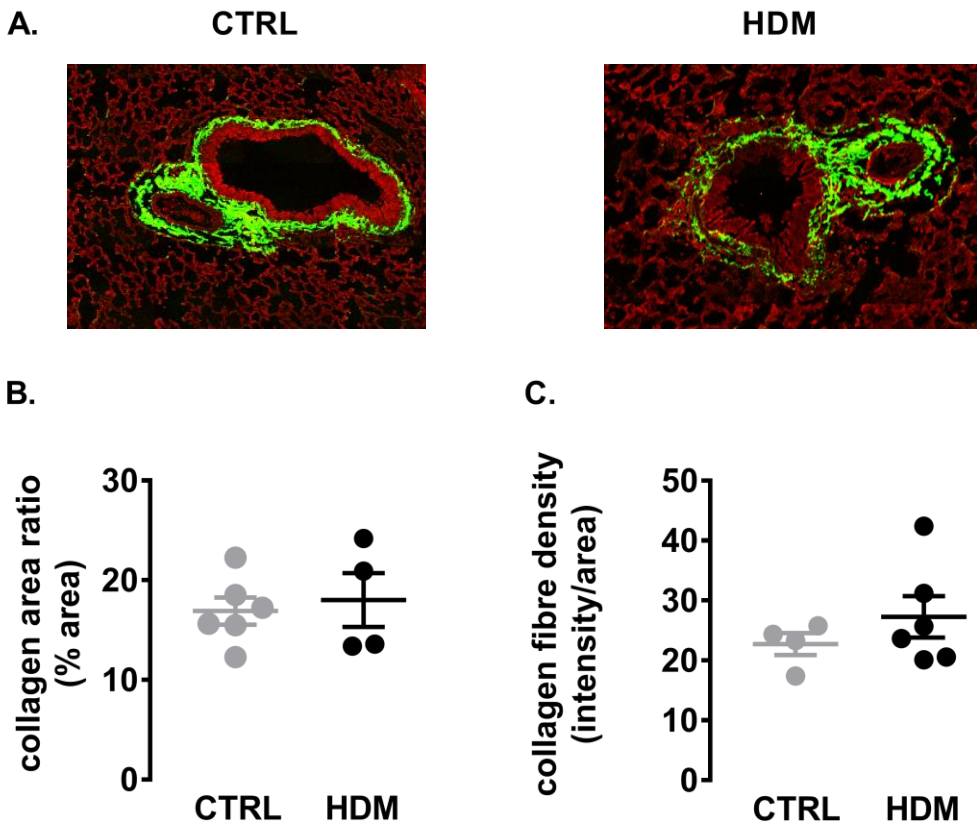
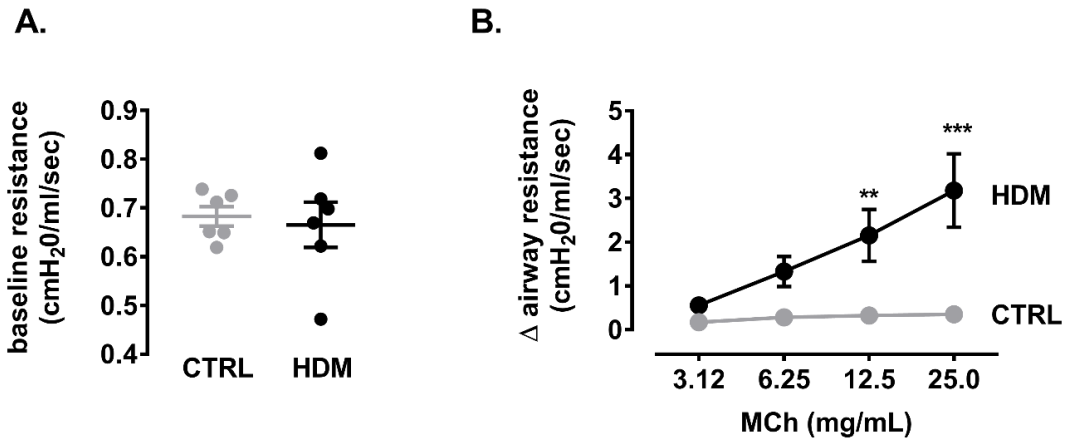


Figure 9. HDM exposure does not alter airway collagen deposition in the lung. (A) Representative sections of airways from mouse lungs challenged with house dust mite (HDM) were scanned with the Histoindex, using second-harmonic generation and two-photon excited fluorescence, and analysed using Fibroindex software to detect collagen accumulation. (B) Total lung collagen area ratio and (C) collagen fibre density were measured. Data is expressed as mean ± s.e.m for n= 4-6 per group.

7.3.5 House dust mite challenge increases airway responsiveness to MCh *in vivo* but not *ex vivo*

Lung function was measured with invasive plethysmography. There was no difference in airway resistance between CTRL and HDM groups at baseline (Figure 10A). At the highest concentration of MCh tested, HDM-challenged mice demonstrated a 9.1-fold increase in airway resistance compared to baseline, while there was little response to MCh in CTRL mice ($P<0.001$, Figure 10B).

To determine the effect of HDM challenge on small airway reactivity, airways from PCLS were contracted with increasing concentrations of MCh. Whilst AHR to MCh was evident *in vivo* after allergen challenge, *ex vivo* assessment of small airway reactivity did not reveal differences in contractile responses to MCh. A similar maximum reduction in airway area of ~50% was seen in both groups with no change in MCh potency (Figure 11A, C).



(data provided by Raj Venkata Allam and Dr. Maria Sukkar, UTS)

Figure 10. HDM exposure induces *in vivo* airway hyperresponsiveness. Airway resistance in control (CTRL) and house dust mite (HDM) mice was assessed via invasive plethysmography in response to increasing concentrations of nebulised MCh. Reponses are expressed as resistance as a change from baseline. Data is expressed as mean ± s.e.m for n=7 per group. $^{**}P<0.01$ vs CTRL.

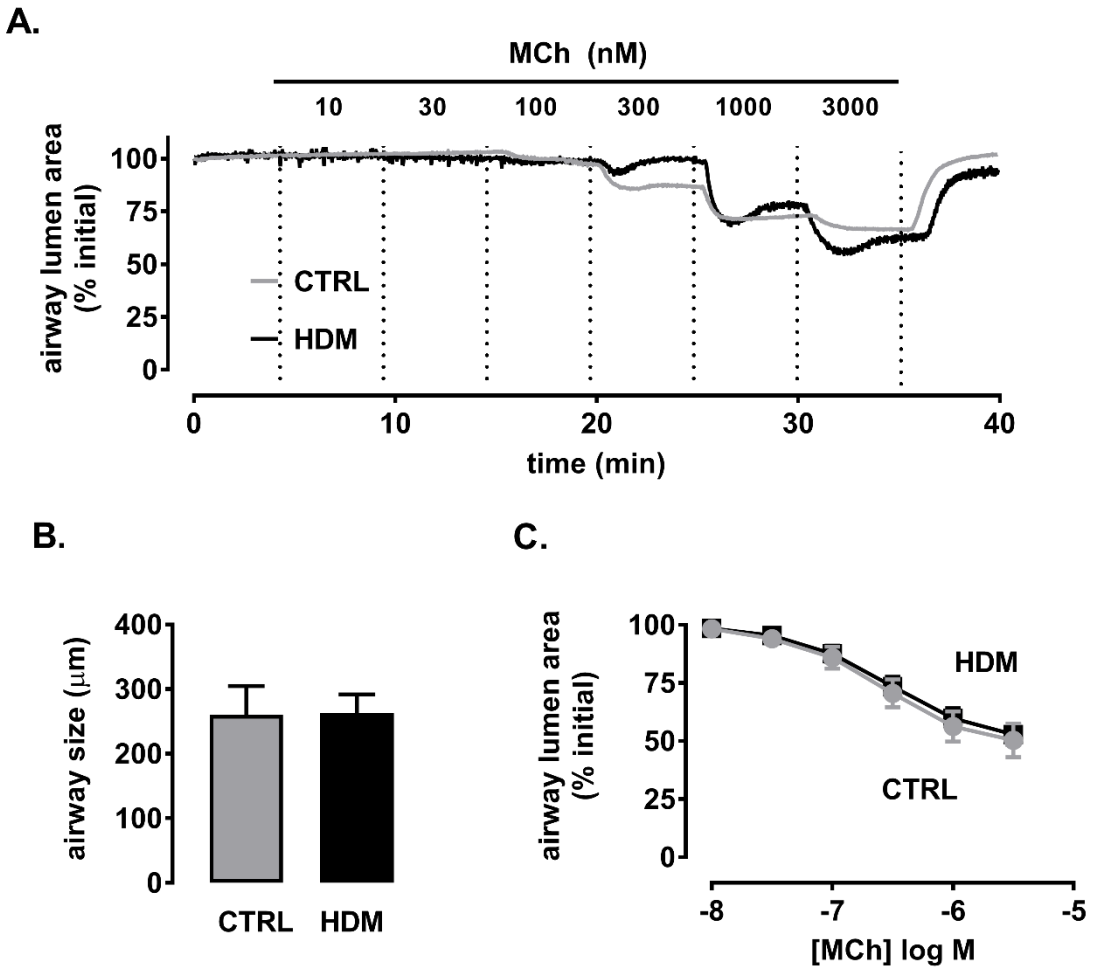


Figure 11. HDM does not alter MCh-induced contraction in small intrapulmonary mouse airways. Mouse lung slices were assessed for changes in intrapulmonary airway area by phase-contrast microscopy. Airways were contracted to increasing concentrations of MCh (0.01-3 μM) at 5 min intervals. (A) Representative trace shows airway area values in pixels determined from gray-scale analysis of images using ImageJ. (B) Airway diameter was noted prior to (C) perfusion with MCh. Data is expressed as % initial airway area (mean ± s.e.m. for n= 12-15 per group).

7.3.6 SALB-mediated relaxation is decreased but potentiation by RLX is maintained after acute house dust mite challenge

The relaxation response elicited by SALB in small intrapulmonary airways was assessed after submaximal pre-contraction to MCh (0.3 µM). Although the potency of SALB was similar in PCLS from CTRL- and HDM-challenged mice, maximum relaxation was significantly attenuated in small airways following allergen challenge (% relaxation to SALB alone: CTRL 49.5±3.5 vs HDM 27.1±9.6, $P<0.05$, Figure 12C).

Small airway relaxation to the combination of RLX and SALB was assessed following HDM-challenge. RLX (0.1 µM) alone did not cause relaxation in either CTRL or HDM airways (not shown). In the presence of RLX, the dilator response elicited by SALB was enhanced by more than 30% in airways from both CTRL and HDM mice, despite impaired SALB responses post-allergen challenge (% relaxation to SALB+RLX: CTRL 82.8 ± 10.2, HDM 68.6 ± 8.2, both $P<0.05$ compared to SALB alone) (Figure 13B, C).

Immunohistochemistry for RXFP1 receptor showed that the intensity of RXFP1 expression in airway epithelium and smooth muscle was maintained following allergen challenge (Figure 14).

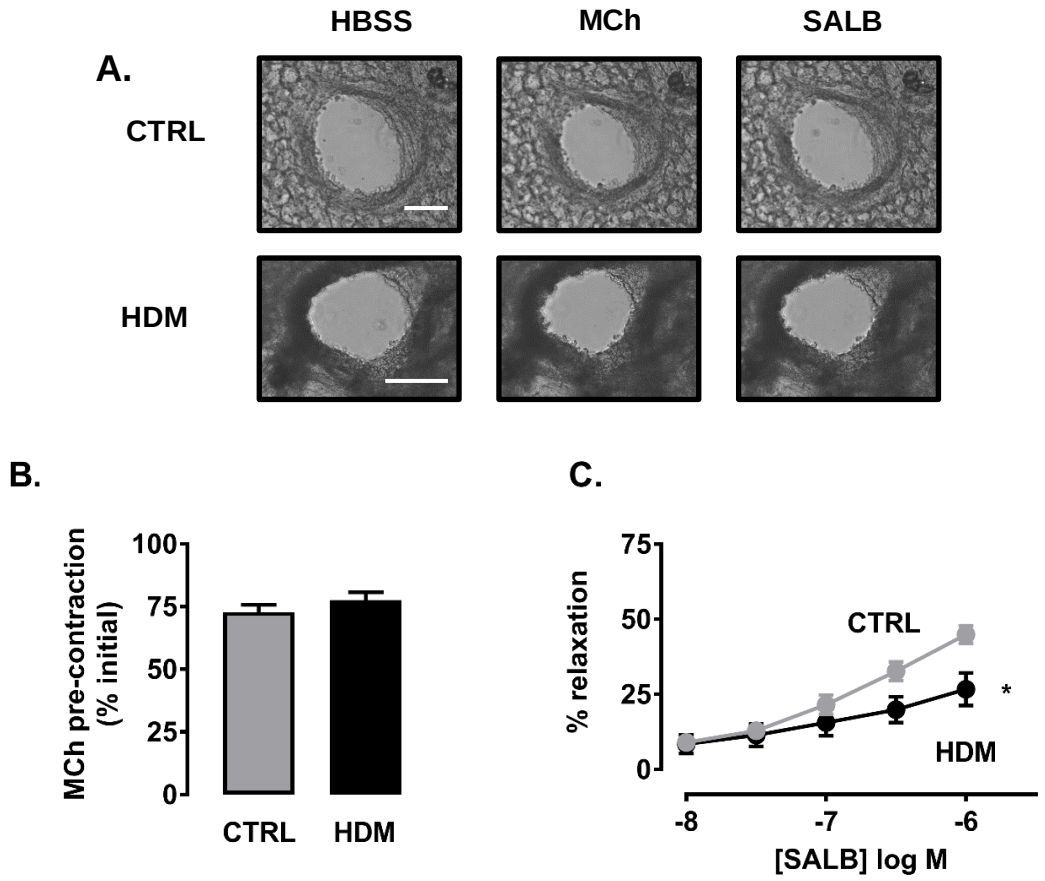


Figure 12. HDM challenge attenuates SALB-mediated relaxation. Mouse lung slices were assessed for changes in intrapulmonary airway area by phase-contrast microscopy. (A) Representative phase-contrast images are derived from the last frame depicting MCh contraction (0.3 µM) and SALB relaxation (1 µM). (B) Airways were pre-contracted to MCh (0.3 µM) prior to (C) relaxation with increasing concentrations of SALB. Data is expressed as % initial airway area (mean ± s.e.m. for n= 9-11 per group). $^{*}P<0.05$ vs control (CTRL). Scale bar= 100 µm.

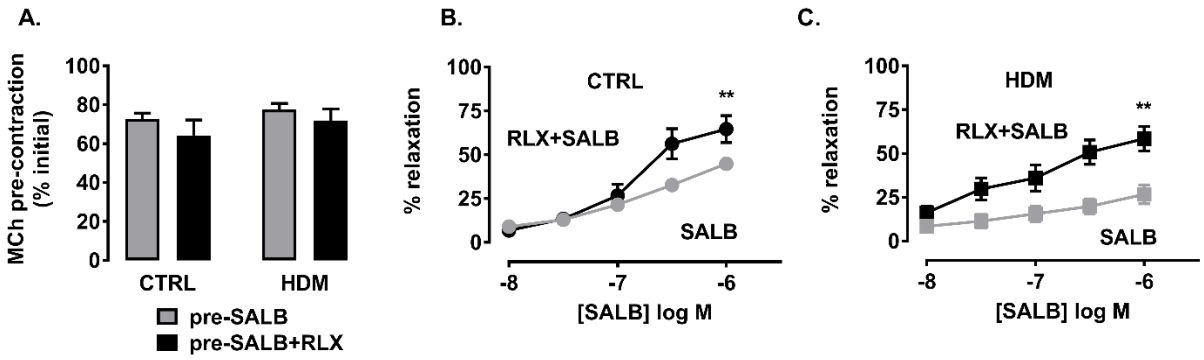


Figure 13. Potentiation of SALB-mediated relaxation by RLX is maintained following HDM exposure. Mouse lung slices were assessed for changes in intrapulmonary airway area by phase-contrast microscopy. (A) Airways were pre-contracted to MCh (0.3 µM) prior to relaxation with SALB in the absence or presence of RLX (0.1 µM) in (B) control (CTRL) or (C) house dust mite (HDM) mice. Data is expressed as % initial airway area (mean ± s.e.m. for n= 7-8 per group). $^{**}P<0.01$ vs SALB alone.

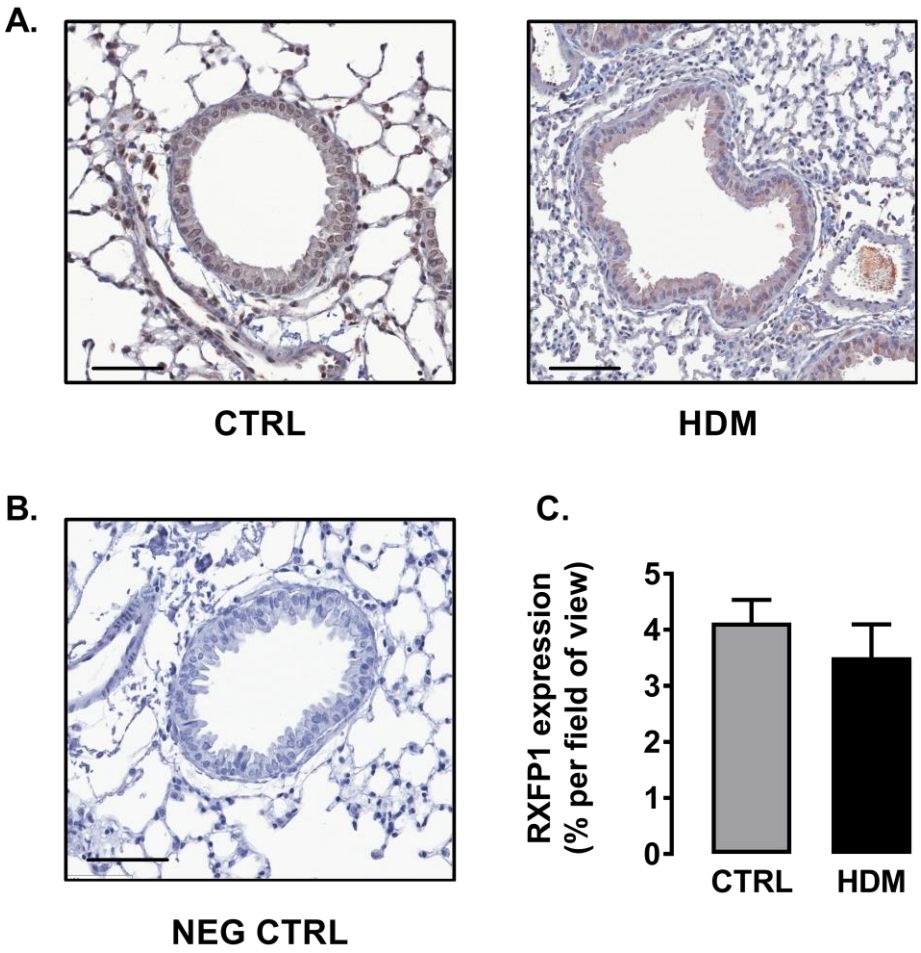


Figure 14. HDM exposure does not alter RXFP1 expression in mouse lung. (A) RXFP1 protein was localised in airways of control (CTRL) and house dust mite (HDM) mice compared to (B) a negative control by immunohistochemistry. (C) Grouped data shows the level of RXFP1 expression as a % per field of view for each group. Data is expressed as mean ± s.e.m. for n= 6 per group averaged from multiple fields of view per section. Scale bar= 100 µm.

7.3.7 Short-term TGFβ1 overexpression induces increased levels in BALF

Two-week doxycycline administration did not induce airway inflammation or cause any apparent increase in airway fibrosis (Figure 15A, B). TGFβ1 levels in BALF were undetectable at baseline but levels were significantly increased at 2 weeks, Although TGFβ1 levels were sustained at 6 h post-withdrawal, this was followed by a time-dependent decrease back to control levels within 48 h (Figure 15C). TGFβ1 levels in serum were unaffected by doxycycline administration (Figure 15D).

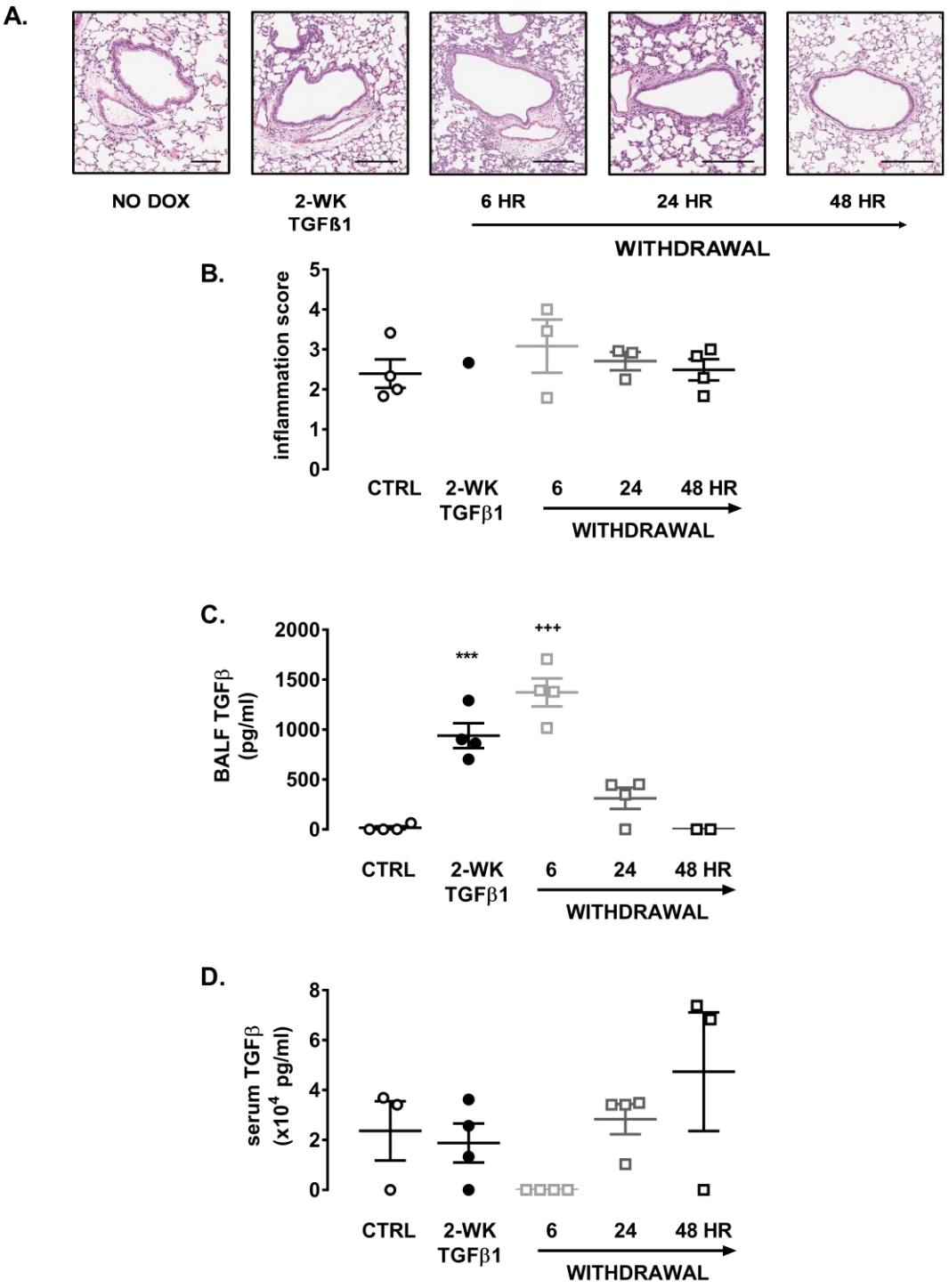


Figure 15. Regression of TGFβ1 levels following dox withdrawal is evident in TGFβ1-overexpressing mice. (A) Lung sections from control (CTRL) and 2-week (2-WK) TGFβ1 mice were stained with H&E to demonstrate the extent of airway inflammation. (B) Sections (5 per mouse) were also scored based on the number and density of inflammatory aggregates on a scale of 0 (no inflammation) - 4 (severe inflammation). TGFβ1 levels were measured from (C) bronchoalveolar lavage fluid (BALF) and (D) blood serum Data is expressed as mean±s.e.m. for n= 1-4 per group. ***P<0.001 vs CTRL; $^{+++}$P<0.001 vs 2-WK TGFβ1. Scale bar= 100 µm.

7.3.8 TGFβ1-overexpression increases airway fibrosis but not inflammation

Inflammation and airway remodelling were assessed via Masson's trichrome staining and Histoindex scanning. Representative H&E lung sections show differences in airway morphology but no obvious inflammation following 1- or 2-month doxycycline administration (Figure 16A). Scoring of blinded sections did not reveal any differences in airway inflammation in TGFβ1 mice compared to time-matched controls (Figure 16B). The number of inflammatory cells was also similar in BALF from CTRL and TGFβ1 mice (Figure 16C).

Although lungs from TGFβ1-overexpressing mice did not demonstrate increased inflammation, thinning of the epithelial layer was evident following both 1- and 2-month doxycycline administration ($P<0.05$, Figure 16D).

Significant airway fibrosis was evident in both Masson's trichrome sections (Figure 16E) and Histoindex scans (Figure 17C). Subepithelial collagen thickness was increased 3-fold with TGFβ overexpression at either 1- or 2-month timepoints compared to control. Examination of individual airway values from TGFβ1-overexpressing mice showed that ~98% of all airways assessed were more fibrotic than the average control (subepithelial thickness: CTRL 4.3±1.4 µm, Figure 16E).

After 8 weeks doxycycline, the area of collagen, expressed as a % of the total lung section area, was slightly increased ($P<0.05$, Figure 17B). When the analysis was limited to areas surrounding multiple airways within each section, the difference between control and TGFβ1 groups was amplified, with airway localised collagen deposition increasing by 25% after 8 weeks doxycycline ($P<0.01$, Figure 17C).

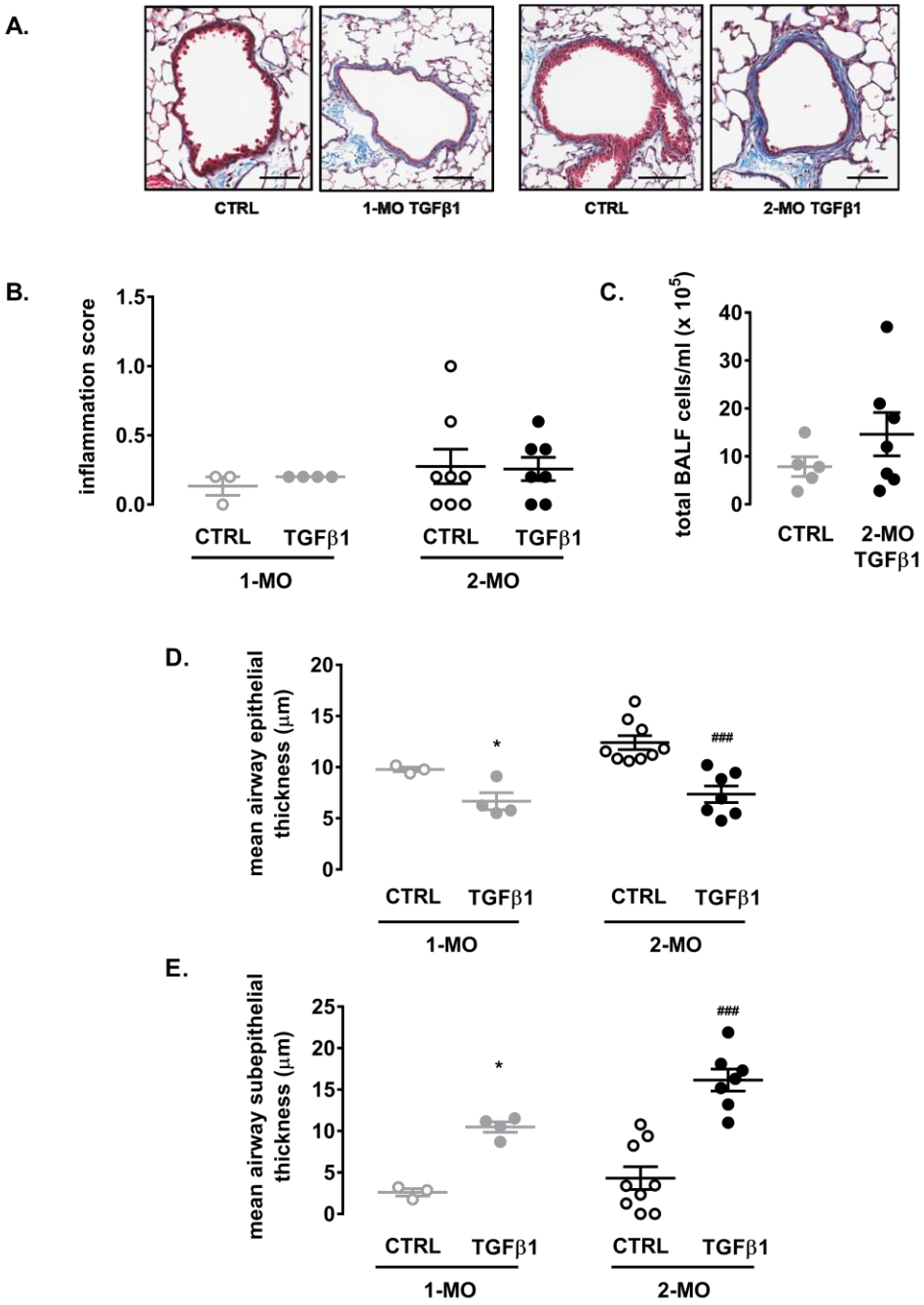


Figure 16. Chronic TGFβ1-overexpression does not cause airway inflammation, but reduces epithelial thickness and increases subepithelial fibrosis. (A) Lung sections from control (CTRL) mice and mice administered doxycycline for 1- or 2-months to induce TGFβ1 overexpression (TGFβ1-1MO, TGFβ1-2MO) were stained with Masson's trichrome to assess inflammation and airway remodelling. (B) Sections (5 per mouse) were scored based on the number and density of inflammatory aggregates on a scale of 0 (no inflammation) - 4 (severe inflammation). (C) Bronchoalveolar lavage fluid (BALF) was also collected for total cell counts. Analysis of sections provided measurements for (D) epithelial and (E) subepithelial thickness (μm). (Data is expressed as mean ± s.e.m. for n= 3-8 per group. $^{*}P<0.05$ vs 1-MO CTRL. $^{###}P<0.001$ vs 2-MO CTRL. Scale bar= 100 μm.

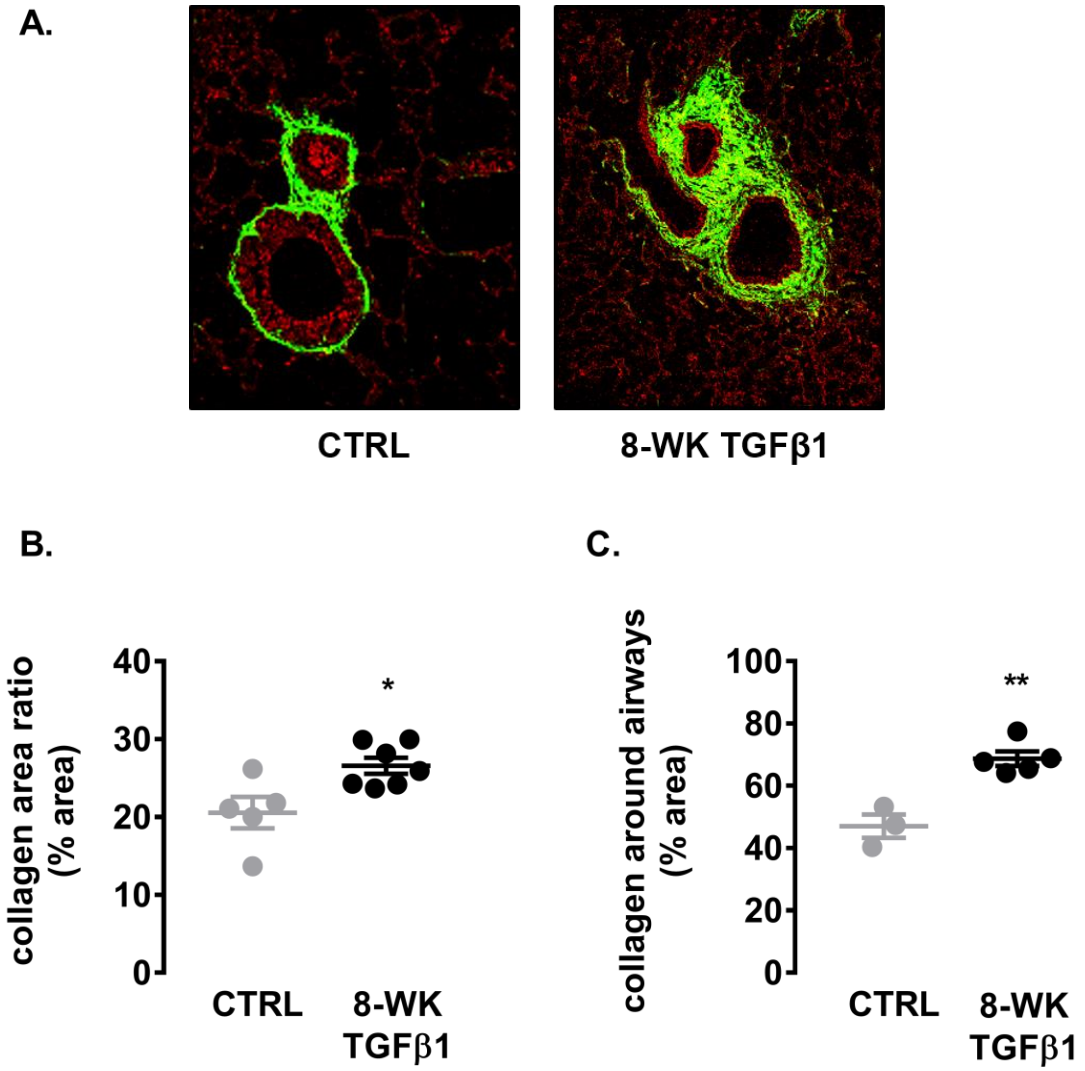
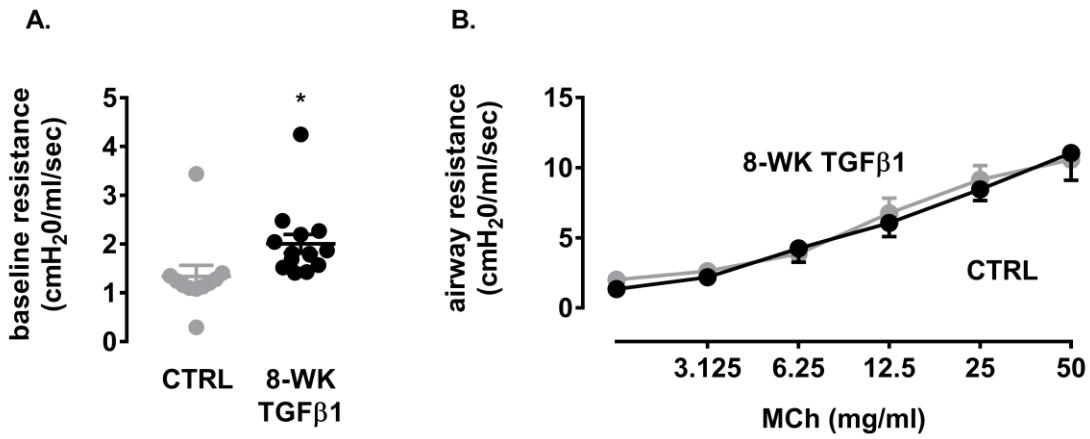


Figure 17. Chronic TGFβ1-overexpression increases airway collagen deposition. (A) Representative sections of airways from control (CTRL) and TGFβ1-overexpressing mice after 8 weeks doxycycline (8-WK TGFβ1) were scanned with the Genesis 200, using second-harmonic generation and two-photon excited fluorescence to detect collagen accumulation. Collagen area ratio in the (B) whole tissue and (C) around the airways (averaged from 5-9 airways/section) was measured and expressed as % area (mean ± s.e.m, n= 3-6 per group). $^{*}P<0.05$, $^{**}P<0.01$ vs CTRL.

7.3.9 TGFβ1-overexpression increases airway responsiveness to MCh *ex vivo* but not *in vivo*

Following 8 weeks doxycycline administration, mice underwent invasive whole-body plethysmography to assessing the effect of TGFβ1 overexpression on *in vivo* lung function. TGFβ1-overexpressing mice demonstrated 1.5-fold higher baseline airway resistance compared to CTRL mice (P<0.05, Figure 18A). However, there was no evidence of AHR to MCh *in vivo* (Figure 18B).

To measure changes in small airway reactivity, PCLS were prepared. Although there was no difference in MCh potency, the maximum reduction in initial airway area was increased by more than 20% in size-matched airways from TGFβ1-overexpressing mice compared to CTRL mice (P<0.05, Figure 19).



(data provided by Vivian Mao and Dr. Simon Royce, Monash University)

Figure 18. Chronic TGFβ1 overexpression does not lead to *in vivo* AHR. Airway resistance in response to nebulised MCh was assessed via invasive plethysmography in control (CTRL) and TGFβ1 mice after 8 week doxycycline (8-WK TGFβ1). Reponses are expressed at (A) baseline and (B) in response to increasing concentrations of MCh. Data is expressed as mean ± s.e.m for n= 11-14 per group. *P<0.05 vs CTRL.

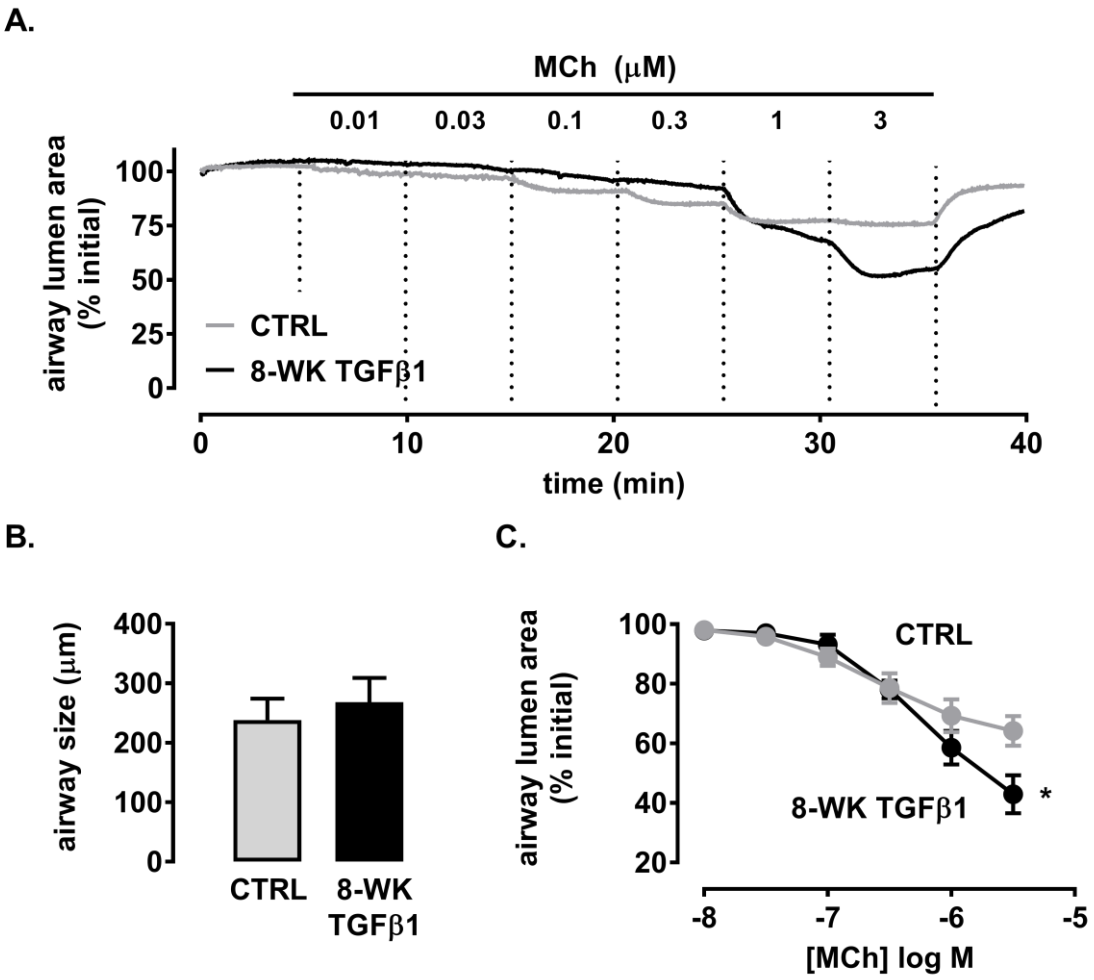


Figure 19. Chronic TGFβ1-overexpression increases MCh-induced contraction in small intrapulmonary mouse airways. Mouse lung slices were prepared from control (CTRL) and TGFβ1 overexpressing mice after 8 week doxycycline (8-WK TGFβ1) and assessed for changes in intrapulmonary airway area by phase-contrast microscopy. Airways were perfused with increasing concentrations of MCh (0.01-3 μM). (A) Representative trace shows airway area values in pixels determined from gray-scale analysis of images using ImageJ. (B) Airway diameter was noted prior to (C) perfusion with MCh. Data is expressed as % initial airway area (mean±s.e.m, n=6 per group). *P<0.05 vs CTRL.

7.3.10 SALB-mediated relaxation and potentiation by RLX are maintained after chronic TGFβ1 overexpression

SALB-mediated relaxation in small intrapulmonary airways was assessed after submaximal pre-contraction to MCh (0.3 μM). The potency and maximum dilator response of SALB was comparable in both control and TGFβ1 groups (Figure 20C).

The combined relaxation effect of RLX and SALB was assessed in small airways from TGFβ1-overexpressing mice. The trend towards increased relaxation to SALB, in the presence of RLX, did not reach significance in CTRL lungs (Figure 21B). The enhanced relaxation of SALB-mediated relaxation by RLX was however demonstrated in TGFβ1-overexpressing mice where there was a 44.1% increase in maximum relaxation ($P<0.05$, Figure 21C).

To determine RXFP1 distribution and density, receptor expression was determined via immunohistochemistry. Examination of histology sections showed that there was no difference in RXFP1 expression in the airway smooth muscle and epithelial layer in CTRL and TGFβ1-overexpressing mice (Figure 22).

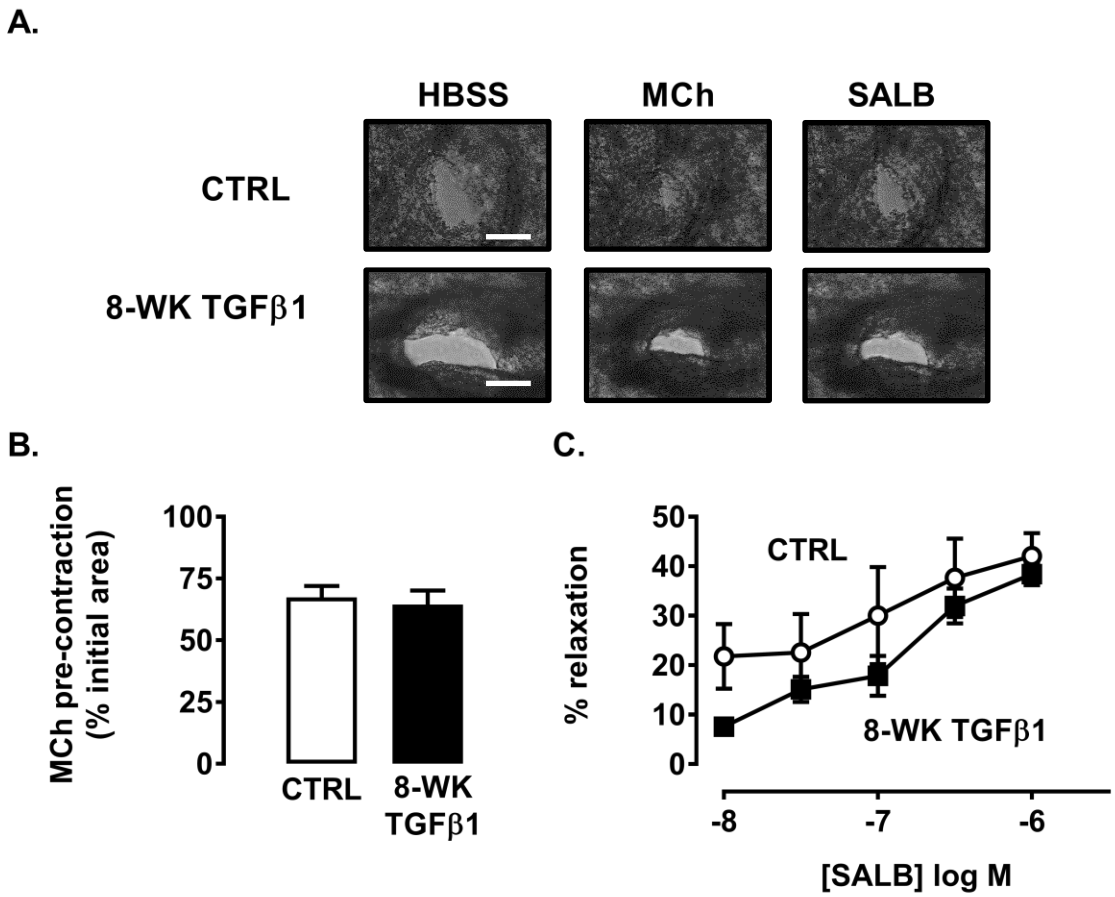


Figure 20. SALB-mediated relaxation is maintained in small intrapulmonary airways after chronic TGFβ1 overexpression. Mouse lung slices were prepared from control (CTRL) and TGFβ1 overexpressing mice after 8 week doxycycline (8-WK TGFβ1) and assessed for changes in intrapulmonary airway area by phase-contrast microscopy. (A) Representative phase-contrast images are derived from the last frame depicting MCh contraction (0.3 µM) and SALB relaxation (1 µM). (B) Airways were pre-contracted to MCh (0.3 µM) prior to (C) relaxation with increasing concentrations of SALB. Data is expressed as % initial airway area (mean ± s.e.m, for n= 4-5 per group). $^{*}P<0.05$ vs control (CTRL). Scale bar= 150 µm.

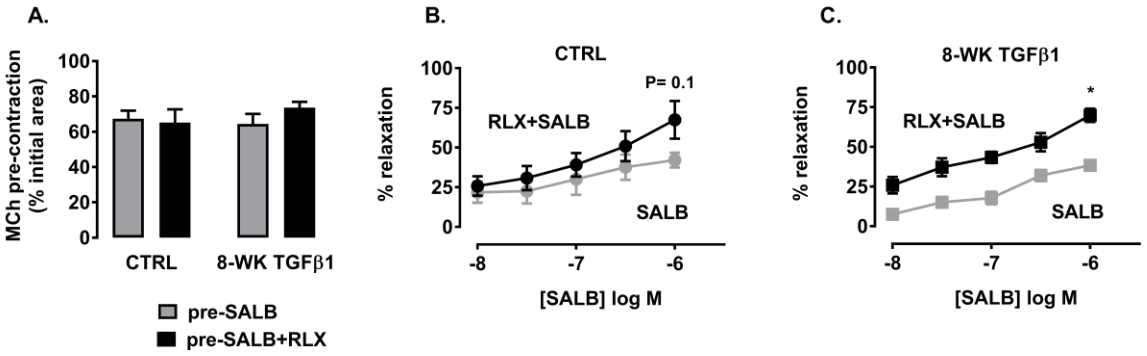


Figure 21. Potentiation of SALB-mediated relaxation by RLX is maintained after chronic TGFβ1-overexpression. Mouse lung slices were prepared from control (CTRL) and TGFβ1-overexpressing mice after 8-weeks doxycycline (8-WK TGFβ1) and assessed for changes in intrapulmonary airway area by phase-contrast microscopy. (A) Airways were pre-contracted to MCh (0.3 µM) prior to relaxation with SALB in the absence or presence of RLX (0.1 µM) in (B) CTRL or (C) 8-WK TGFβ1 mice. Data is expressed as % initial airway area (mean ± s.e.m for n= 5 per group). *P<0.05 vs SALB alone.

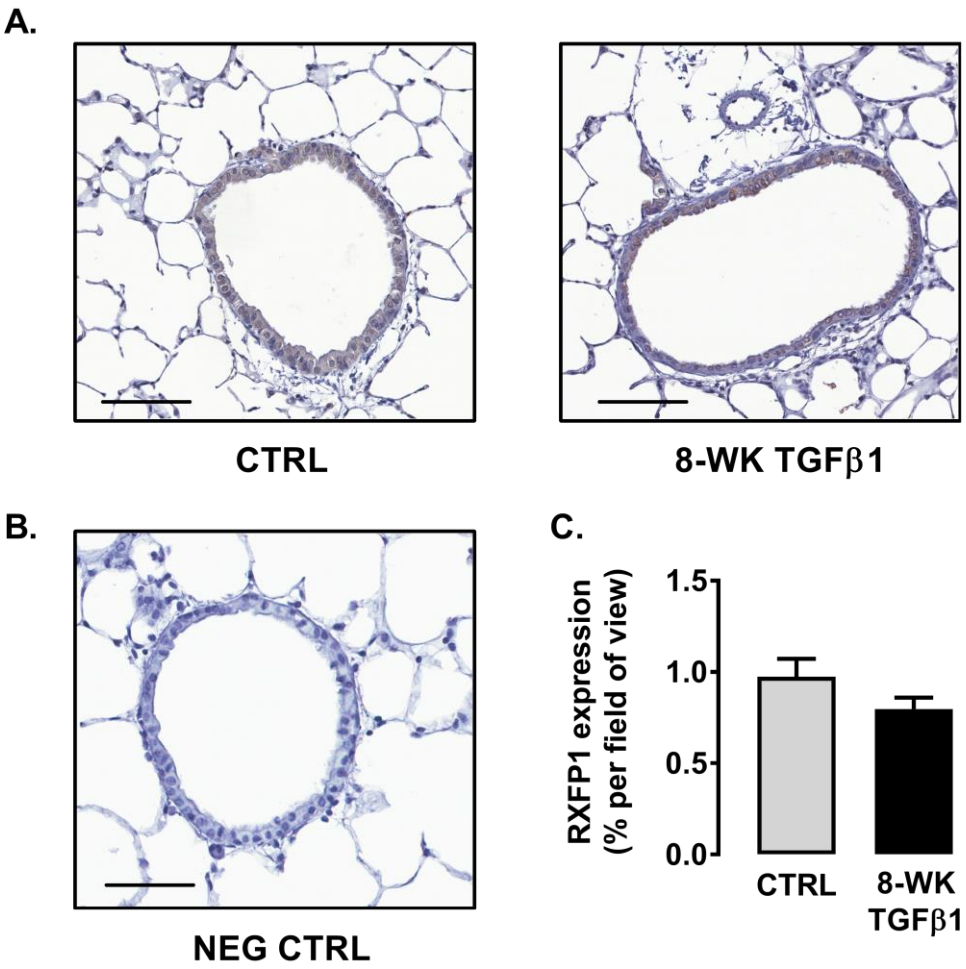


Figure 22. RXFP1 expression is maintained after chronic TGFβ1-overexpression. (A) RXFP1 protein was localised in airways from control mice (CTRL) and TGFβ1-overexpressing mice after 8-week doxycycline (8-WK TGFβ1) by immunohistochemistry and compared to (B) a negative control. (C) Grouped data shows the level of RXFP1 expression as a % per field of view for each group. Data is expressed as mean ± s.e.m. for n= 5-6 per group averaged from multiple fields of view per section. Scale bar= 100 µm.

7.4 Discussion

In this study, bleomycin (BLM) or acute house dust mite (HDM) models were used to reproduce some of the key features of pulmonary fibrosis and acute asthma respectively. The TGFβ1-overexpression model in transgenic mice was used to establish airway fibrosis. The contribution of inflammation and fibrosis to altered reactivity had not been previously well defined in these models. Under these disease conditions, contraction to MCh and relaxation to SALB in the absence and presence of RLX were assessed.

Summary of results

Overall, increased inflammation was evident in lungs exposed to BLM and HDM but not in TGFβ1-overexpressing mice. As predicted, the extent of fibrosis was variable between the disease models where BLM caused wide-spread lung fibrosis, TGFβ1-overexpression induced localised airway fibrosis, while acute HDM challenge did not affect collagen deposition. *In vivo* functional tests showed that only acute HDM challenge induced AHR. These findings were not consistent with in *ex vivo* reactivity experiments in which small airway contraction to MCh was unaltered following BLM and HDM treatment, but was increased in PCLS from TGFβ1 overexpressing mice. In addition, the dilator response of SALB was only diminished following HDM exposure but not with BLM or TGFβ1-overexpression. In the presence of RLX, relaxation to SALB was increased in HDM and TGFβ1-overexpression models but not following BLM administration, where RXFP1 expression appeared to be downregulated. Our novel findings show that relaxation to SALB is potentiated by RLX in inflamed or fibrotic airways but may be limited when parenchymal fibrosis is also present.

Comparison of inflammation in disease models

Distinct inflammation patterns were demonstrated in the mouse models tested. Although differential cell counts were not conducted for BALF collected from BLM mice, previous studies have shown total cell, neutrophil and lymphocyte numbers to be increased following intratracheal administration of BLM (Izbicki *et al.*, 2002; Lv *et al.*, 2017). The increase in cell types appears to be time-dependent whereby early induction of inflammation is due to elevated neutrophil levels whilst latter inflammatory events may be regulated by lymphocytes (Izbicki *et al.*, 2002). Neutrophil levels have been shown to peak by day 6 and subside by day 21 whilst lymphocytes progressively increase (Izbicki *et al.*, 2002). Since the current model involved the re-administration of BLM every 7 days, it is likely that the inflammation that occurred was mediated by both neutrophils and lymphocytes.

Following HDM challenge, both eosinophil and neutrophils levels were increased which is consistent with the development of severe asthma where both cell types are elevated (Fahy *et al.*, 1995; Nath *et al.*, 2014). This is consistent with 4-week and 8-week HDM models where BAL consisted primarily of eosinophils and neutrophils (Salehi *et al.*, 2017; Woo *et al.*, 2018). Despite the increased levels of these leukocytes, studies have shown that acute 2 week HDM challenge does not increase HDM-specific IgE which can indicate an allergic response (Johnson *et al.*, 2003; Woo *et al.*, 2018). This lack of allergic response may explain the failure to show increased mast cells in the current model. Subsequent studies demonstrated that consecutive HDM exposure for 5 days/week for 2 weeks was required to induce increases in IgE levels. Therefore, the current model can be improved by more frequently exposing mice to HDM in order to better mimic the inflammatory changes that occur in chronic asthma.

In comparison to both the BLM and HDM model, there was no evidence of inflammation in lungs from TGFβ1-overexpressing mice following 8-week doxycycline administration. This contrasts with previous studies which show a significant increase in total cell, macrophage and eosinophil numbers following 2-days of doxycycline (Lee *et al.*, 2004). Due to the self-resolving nature of this model, we cannot exclude inflammation to TGFβ1 at earlier timepoints.

Comparison of fibrosis in disease models

The site and severity of fibrosis across the models were also determined. Structural changes including epithelial and subepithelial thickness can be differentiated by the conventional Masson's Trichrome staining (which cannot distinguish between interstitial vs basement membrane collagen) whilst Genesis 200 analysis not only detects interstitial collagen localised to the airways but also quantifies interstitial collagen deposition in the lung (collagen area ratio). The Genesis 200 objectively analyses fibrosis via second-harmonic generation and two-photon excited fluorescence with greater sensitivity compared to subjective analysis with Masson's Trichrome where sections were manually traced.

Using Masson's Trichrome staining, airways of BLM mice demonstrated increased subepithelial and interstitial fibrosis whilst airway epithelial thickness was unaltered. Although not assessed here, studies in mice and sheep have also showed hyperplasia of Clara and cuboidal epithelial cells as well as enlarged alveoli (Lee *et al.*, 2004; Lee, 2006; Organ *et al.*, 2015). Despite emulating key features of pulmonary fibrosis, a major limitation of the BLM model is that fibrosis has been shown to spontaneously resolve (Degryse *et al.*, 2010) which contrasts with the progressive nature of fibrosis in chronic lung diseases. Repeated BLM administration, as performed in this study, may overcome this limitation. However, some studies have shown that although fibrosis can persist for up to 3-6 months, inflammation

becomes less prominent (Lawson *et al.*, 2005; Moore and Hogaboam, 2008). This complicated relationship between inflammation and remodelling in IPF is difficult to target and as a consequence, there are no current effective treatments available.

In contrast to the BLM model, short term HDM challenge did not promote fibrosis due to the relatively acute duration of the model. While HDM exposure did not induce fibrosis in the current study, it has previously been shown to increase PAS-positive cells, a marker of goblet cells following 4 week HDM challenge (Woo *et al.*, 2018). Goblet cell metaplasia and hyperplasia are prominent structural alterations seen in asthmatic airways which contribute to airway obstruction. Future studies should quantify changes in goblet cell number to determine whether this characteristic is present in the acute model used in the current study. Aside from the potential effects of HDM on goblet cells, there was no evidence of other features of airway remodelling. There is a clear need to establish a more chronic model using HDM to better replicate the multiple components of asthma.

While fibrosis induced by BLM was diffuse throughout the parenchyma, only localised airway fibrosis was evident with TGFβ1-overexpression. This localised airway fibrosis appeared to be maximal within 1 month and stabilised with continuous doxycycline induction for up to 2 months. The specific effects of doxycycline on Clara cells located in the epithelium only is consistent with release of TGFβ1 from the epithelium and local pro-fibrotic effects on the airway wall. This finding differed from a single study in these transgenic mice, where TGFβ1-overexpression induced both airway and parenchymal fibrosis (Lee *et al.*, 2004). Other studies in AdV-TGFβ models also demonstrate increased α-SMA expression and collagen deposition in interstitial cells (Kolb *et al.*, 2002, 2011). Whether the current findings represent a failure to establish parenchymal fibrosis or resolution despite continuous induction of TGFβ1-overexpression is unclear. Differences in mouse strain may also contribute to these variable findings. Although mice with a C57/Bl6 background are more susceptible to developing lung fibrosis, transgenic mice of the same background may have different fibrotic phenotypes. Future studies should compare the differences in fibrotic markers such as α-SMA to determine whether altered expression is consistent with the broader differences in lung and airway fibrosis.

TGFβ1 overexpression also caused thinning of the epithelial layer. In these same transgenic mice, it had been reported that TGFβ1 induced an early transient increase in epithelial apoptosis that was not sustained with prolonged TGFβ1-overexpression (Lee *et al.*, 2004). However, according to our findings, the epithelial layer did not recover from any early induction

of apoptosis by 2-months of doxycycline administration. In addition to the potential loss of its protective barrier function, a reduced epithelial layer has implications for altered airway reactivity due to the potential impairment in the production of epithelial-derived relaxing factors that oppose contraction, and may be a target to protect dilator function in severe airway disease where there is evidence of amplified TGFβ1 activity.

Comparison of airway contraction to MCh in disease models

Increased inflammation and fibrosis can lead to the development of airway hyperresponsiveness (AHR), whereby airways become more sensitive to contractile mediators. The impact of BLM treatment, HDM challenge and TGFβ1 overexpression on airway contraction to MCh was assessed to examine the potential differential effects of inflammation and fibrosis both *in vivo* and *in vitro.*

BLM administration increased baseline airway resistance and reduced compliance *in vivo* compared to controls. This finding differed from a single study in BLM mice, airway resistance was unaltered while total lung compliance was reduced (Phillips *et al.*, 2012), but was consistent with structural evidence of both airway and parenchymal fibrosis in the current study.

Post-BLM, *in vivo* reactivity to MCh was not determined. However, PCLS from this model were assessed, since lung injury in IPF is often localised to the distal airways. In this first study to assess contraction of airways from a BLM model, there was a trend toward reduced MCh contraction in airways from PCLS from BLM mice. This finding may reflect the combined influence of fixed airway obstruction and parenchymal fibrosis. In contrast, trachea from BLM-treated rat demonstrated increased contraction to carbachol which suggests that differences in species or airway size may influence contraction since tracheal responses are not impacted by parenchymal changes (Barrio *et al.*, 2006).

Following HDM challenge, increased airway sensitivity to MCh was evident *in vivo* . This was associated with increased inflammatory cell infiltration, even in the absence of airway remodelling. However, contraction to MCh was unaltered in PCLS from the same model, which suggests the requirement for the continued presence of the *in vivo* inflammatory milieu. Previously, acute OVA challenge did not affect MCh potency in small airways compared to controls (Kim *et al.*, 2015) whilst chronic OVA exposure was shown to reduce MCh potency by 15-fold associated with fibrosis and potentially fixed airway obstruction (Donovan *et al.*,

2013). Thus, further studies should extend the length of HDM exposure to better mimic the hypersensitivity of small airways in asthma.

Following TGFβ1 overexpression, airway responsiveness to MCh was unaltered *in vivo* but increased *in vitro*. As such, it appears that airway fibrosis in the absence of persistent inflammation was not sufficient to induce *in vivo* AHR, or that TGFβ1-induced changes in reactivity are restricted to the small airways. The increased airway contraction to MCh in airways from PCLS from TGFβ1-overexpressing mice is consistent with a study in guinea pig PCLS where prolonged contraction to MCh mediated the release of TGFβ1 which in turn increased the expression of contractile proteins (Oenema *et al.*, 2013). Future studies should measure levels of contractile proteins including α-SMA in PCLS from TGFβ1-overexpressing mice to better understand the mechanisms regulating contraction.

model		**pEC_{50}**	**fitted maximum (% reduction in airway area)**
BLM *Balb/C*	CTRL	6.7 ± 0.4	76.4 ± 5.7
	BLM	6.5 ± 0.2	53.7 ± 9.5
HDM *C57Bl/6*	CTRL	6.6 ± 0.2	58.0 ± 8.1
	HDM	6.5 ± 0.1	55.5 ± 5.0
TGFβ1 *C57Bl/6*	CTRL	6.5 ± 0.2	44.0 ± 5.5
	TGFβ1	6.2 ± 0.1	71.5 ± 8.3*

Table 1. Comparison of fitted maximum airway contraction in PCLS across the BLM, HDM and TGFβ1-overexpression models. *P<0.05 vs CTRL.

Comparison of relaxation to SALB in disease models

The transient nature of MCh-induced responses *in vivo* means that assessment of dilator responses as reversal of contraction cannot be easily achieved. In the current study, airway and/or lung fibrosis did not impair SALB-mediated relaxation. Because SALB was shown to still be effective at eliciting dilation in the presence of excessive collagen deposition in both the BLM and TGFβ1-overexpression model, bronchodilator treatment for fibrotic diseases may be beneficial for IPF patients who experience reversible airflow limitation (Assayag *et al.*, 2015). Although TGFβ1 is upregulated following BLM (Cutroneo *et al.*, 2007), its effects do not appear to be limiting airway relaxation to SALB in these model. A recent clinical study in IPF patients showed that nebulisation of SALB via monodispersed particles (1.5 µm) allowed for better lung penetration where delivery of SALB to peripheral airways was possible (Usmani *et al.*, 2018). While SALB may be able to elicit relaxation under conditions of increased fibrosis, its clinical effects will still only provide relief of symptoms without inhibiting the progression of disease.

In the short-term HDM model, increased airway inflammation in the absence of fibrosis was shown to impair SALB-mediated relaxation in mouse airways in PCLS. In previous literature, HDM-sensitised mice demonstrated elevated levels of IL-4 and IFN-γ (Doras *et al.*, 2018), while a single report showed that SALB-mediated relaxation in isolated airways pre-treated with a combination of IL-1β and TNFα was reduced (Horiba *et al.*, 2011). Whether these endogenous cytokines directly or indirectly impair relaxation to SALB via downregulation of β-AR expression or signalling remains to be determined, but targeting these cytokines in asthma may provide additional relief for patients with difficult-to-control asthma. Although bronchial provocation by HDM in asthmatic patients is significantly relieved by SALB inhalation (Giulekas *et al.*, 1984), there is still the potential to improve the benefits of β-AR agonists in asthma.

		SALB		SALB+RLX	
models		**pEC50**	**% relaxation**	**pEC50**	**% relaxation**
BLM *Balb/C*	CTRL	6.7 ± 0.4	43.5 ± 7.2	6.9± 0.3	109.7 ± 12.5
	BLM	6.5 ± 0.2	66.4 ± 15.5	7.1 ± 0.2	66.4 ± 9.2
HDM *C57Bl/6*	CTRL	6.5 ± 0.2	49.5 ± 3.5	6.8 ± 0.1	82.8 ± 10.2
	HDM	6.9 ± 0.3	27.1 ± 9.6	6.8 ± 0.2	68.6 ± 8.2
TGFβ1 *C57Bl/6*	CTRL	6.5 ± 0.3	50.5 ± 3.9	6.5 ± 0.2	86.9 ± 17.4
	TGFβ1	6.7 ± 0.4	55.5 ± 9.4	6.5 ± 0.3	92.6 ± 9.5

Table 2. Comparison of fitted maximum airway relaxation in PCLS across the BLM, HDM and TGFβ1-overexpression model.

Comparison of relaxation to SALB in the presence of RLX in disease models

Having assessed the influence of the models on SALB responses in the small airways, we were then finally able to validate the potentiation of SALB by RLX in disease, using the information gained from these models to understand specific pathologies that may or may not limit its application.

SALB only elicits partial relaxation in mice airways compared to complete relaxation of guinea pig and human airways, and RLX causes an increase in SALB efficacy in mice airways rather than the increase in potency shown in guinea pig and human airways (Chapters 5,6). In the mouse disease models examined here, potentiation by RLX was maintained in the presence of acute inflammation, despite impaired SALB-induced relaxation (HDM model) or stable airway fibrosis alone (TGFβ1-overexpression model), suggesting that RLX could contribute to greater symptom relief even in inflamed or fibrotic airways. There is still a need to assess the acute *in vitro* response to SALB in the presence of RLX from a chronic HDM model where both pathologies are present.

In the BLM model, the potentiation of relaxation by RLX was reduced, associated with parenchymal fibrosis and apparent downregulation of the RXFP1 receptor. Chronic RLX administration may be required to overcome this limitation, since this has been shown to upregulate RXFP1 receptor expression in a chronic AAD model, where OVA challenge reduced levels of endogenous RLX and RXFP1 (Royce *et al.*, 2013).

In conclusion, each model provided unique insights into the effect of RLX and SALB on small airway reactivity. The study showed that while inflammation reduced relaxation to SALB, increased fibrosis augmented contraction to MCh. Despite these differences, RLX was still able to potentiate relaxation to SALB, and could therefore provide additional relief for patients with increased airway narrowing or impaired dilator responsiveness to β_2-AR agonists.

The effects of chronic treatment with RLX remain to be fully elucidated, particularly under settings of increased inflammation and parenchymal fibrosis, where potentiation of SALB by RLX was shown to be reduced. Future studies may show that chronic RLX is required to oppose fibrosis and preserve or restore RXFP1 expression in order to maintain its actions on airway contraction in specific disease settings. Nevertheless, the accumulating evidence that RLX elicits direct relaxation (as established in non-inflamed, non-fibrotic airways in Chapter 5) and potentiates relaxation to SALB (as shown here in HDM and TGFβ1-overexpression models), provides further support for RLX as a novel agent that may provide benefit when current treatment options are limited by their loss of potency or efficacy.

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Chapter 8: General Discussion

8.1 Introduction

The major goals of this thesis were to assess relaxin as a novel therapeutic for chronic lung diseases, especially for asthma, by providing further evidence of its antifibrotic properties and establishing its bronchodilator actions. Airway fibrosis is not targeted by chronic anti-inflammatory steroids, and existing treatments for pulmonary fibrosis are not used in asthma and only slow the progression of other lung diseases such as IPF. While β_2-adrenoceptor agonists are the mainstay treatment for acute asthma, repeated use of high doses with increasing disease severity can result in loss of bronchodilator efficacy.

The anti-fibrotic effects of the peptide hormone, relaxin (RLX), have been clearly established in many organ systems (Lam *et al.*, 2018). It has been proposed that RLX exerts its effects through its cognate RXFP1 receptors by increasing nitric oxide bioavailability as well as upregulating collagen-degrading enzymes, while inhibiting the TGFβ/Smad signalling pathway that promotes fibrosis and associated tissue remodelling. The role of RLX in regulating collagen turnover was initially established in lung myofibroblasts *in vitro* (Unemori *et al.*, 1996), but had yet to be confirmed in the context of asthma by using myofibroblasts from patients with asthma.

Consistent findings have demonstrated the ability of RLX to inhibit collagen deposition in models of lung disease (Lam *et al.*, 2019). Of relevance to asthma, chronic RLX treatment not only inhibited but also reversed OVA-induced remodelling, and also reduced AHR, suggesting that additional actions that opposed airway contraction (Royce *et al.*, 2009, 2015). Evidence for direct vasodilator actions of RLX had previously been shown in mesenteric, renal and uterine arteries (McGuane *et al.*, 2011; Vodstrcil *et al.*, 2012; Leo *et al.*, 2014). However, assessment of potential bronchodilator effects of RLX had previously been limited to an isolated study which showed that a single concentration of 1000 nM, caused relaxation of isolated mouse lung strips precontracted with carbachol (Samuel *et al.*, 2003).

Intrapulmonary airways are the site of increased inflammation and remodelling in asthmatic patients (Mauad *et al.*, 2007), and are more sensitive to contractile mediators *ex vivo* (Wohlsen *et al.*, 2003). Application of the precision cut lung slice (PCLS) technique would provide a novel approach to assess the bronchodilator efficacy of RLX in these small intrapulmonary airways. Furthermore, the clinical relevance of positive findings for RLX could be supported by additional studies using tracheal preparations and validation in human tissues and mouse disease models.

8.1.1 Hypothesis and Aims

It was hypothesised that relaxin would oppose fibrosis in airway fibroblasts, elicit airway relaxation of both large (trachea) and small airways (PCLS) and maintain its antifibrotic and dilator efficacy in disease context. The studies in this thesis aimed to:

6. optimise the preparation of viable precision cut lung slices (PCLS) in order to extend their applicability (Chapter 3)
7. compare the antifibrotic actions of RLX to existing therapies in healthy and asthmatic human airway fibroblasts from non-asthmatic and asthmatic subjects (Chapter 4)
8. assess the *in vitro* dilator efficacy of relaxin in large and small airways and define its underlying mechanisms of action (Chapter 5)
9. establish combined dilator effects of RLX and salbutamol (SALB) across multiple species and under conditions of reduced β-adrenoceptor-responsiveness, and to define the mechanism of potentiation (Chapter 6)
10. determine whether the effects of the combination of RLX and SALB are maintained in a chronic bleomycin model (BLM, lung fibrosis), an acute house dust mite model (HDM, airway inflammation) and a chronic TGFβ-overexpression model (airway fibrosis) (Chapter 7)

8.2 Discussion

8.2.1 RLX inhibits collagen remodelling by human asthmatic fibroblasts

Chapter 4 / Aim 2

Airway fibrosis and remodelling are key contributors to disease progression in asthma. Current treatments that target fibrosis in IPF include pirfenidone, which only slows the progressive decline in lung function. Inhaled glucocorticoids are used to reduce inflammation in asthma but do not affect airway remodelling. This is the first study to compare the anti-fibrotic actions of RLX with these currently used therapies using a gel contraction assay, in which collagen gels are seeded with airway fibroblasts with reductions in gel area ("gel contraction") occurring over hours to days used as a measure of cell-mediated remodelling of their surrounding matrix.

We showed that gels seeded with asthmatic fibroblasts (which have differentiated into myofibroblasts) contract more than gels seeded with non-asthmatic fibroblasts, and that contraction was increased by the pro-fibrotic mediator TGFβ1. RLX and pirfenidone inhibited TGFβ1-mediated contraction of collagen gels seeded with non-asthmatic fibroblasts, but RLX was markedly more potent and only RLX was effective in reducing collagen gel contraction from asthmatic fibroblasts. Dexamethasone was unable to inhibit collagen gel contraction, consistent with its primary use in asthma to reduce inflammation rather than fibrosis. It could be hypothesised that the potent anti-fibrotic actions of RLX combined with the anti-inflammatory effects of glucocorticoids could further improve asthma outcomes than glucocorticoid alone. Indeed, two studies previously showed that the addition of chronic RLX treatment to glucocorticoids caused greater inhibition of the development of subepithelial fibrosis than either alone (Royce *et al.*, 2013; Patel *et al.*, 2016). Dual therapy to target both persistent inflammation and established fibrosis would clearly be beneficial for the treatment of asthma.

A major limitation of the collagen gel assay is that it was not possible to assess potential reversal of fibroblast-mediated remodelling of collagen gels by RLX, or whether changes in collagen remodelling would affect functional responses of intact airways. However, in future studies, the PCLS preparation could be used to evaluate the influence of chronic RLX treatment on established fibrosis in intact airways, integrated with assessment of potential acute effects airway reactivity. *In vitro* studies have recently shown that treatment of PCLS with a cocktail of pro-fibrotic growth factors and signalling molecules induces fibrosis, shown as the upregulation of profibrotic proteins and increased deposition of ECM (Alsafadi *et al.*,

2017). This would provide a setting for integrated assessment of *in vitro* treatment with RLX to demonstrate its ability to reverse these fibrotic changes, as well as whether it can directly mediate airway relaxation and improve responsiveness to dilator treatment in this fibrotic setting.

8.2.2 RLX is a novel bronchodilator which potentiates relaxation to current treatment

Chapter 3 / Aim 1

Small airways are the site of increased inflammation and remodelling, but their functional responses are relatively understudied compared to those of larger airways. The PCLS technique, in which airway contraction and relaxation can be visualised *in situ* as in the intact lung, provides an innovative approach to provide insights into intrapulmonary airway function. Although most commonly approved to study mouse airways, the findings presented in this thesis confirmed that viable lung slices can also be generated from different species including the marmoset and human (Ch 5, 6), providing a potential bridge to support clinical translation of positive findings with novel therapeutics.

Importantly, multiple conditions for the preparation of functional PCLS from mouse lungs were established, as demonstrated by consistent airway responses to MCh between PCLS prepared from different lobes, after delayed filling or slicing, or assessed after cryopreservation. These validated techniques were successfully applied in multiple chapters, summarised in Table 8.1.

The reproducibility of airway responses in PCLS prepared using these various approaches was established, showing similar reactivity between lung lobes and irrespective of the timing of preparation of lung slices. This enabled key comparisons to be made in subsequent experiments, including relaxation to SALB in different PCLS (Ch 6) in the absence or presence of RLX, or the effect of disease models on reactivity to MCh and dilators (Ch 7).

We also validated responsiveness of airways following cryopreservation of PCLS. Whilst past studies have demonstrated airway contraction to MCh and relaxation to formoterol was maintained after cryopreservation (Bai *et al.*, 2016), this was the first study to confirm that SALB-mediated relaxation was similarly unaffected. This validation was a strategic approach to maximise experimental output from later studies when multiple PCLS were available from human lungs (Ch 5, 6) or disease models (Ch 7).

Table 8.1. The application of validated methods for the preparation of PCLS.

PCLS methods	Chapter 3	Chapter 5	Chapter 6	Chapter 7
different species	✓	✓	✓	
different lobes	✓			
delayed filling and slicing	✓		✓	✓
cryopreservation	✓	✓	✓	✓
cytokine pre-incubation	✓			

It is important to assess the influence of inflammation and fibrosis on airway responsiveness. Evidence of altered reactivity *in vitro* in response to treatment with specific cytokines has implicated their contribution to changes in disease. The marked increase in MCh-induced contraction in PCLS treated with TNFα is consistent with findings in larger airways (Makwana *et al.*, 2012). Studies also have shown that incubation of PCLS with TGFβ1 induces fibrosis but whether fibrosis affects reactivity is unknown (Huang *et al.*, 2011; Oenema *et al.*, 2013; Lehmann *et al.*, 2018). Our findings suggest that future studies could utilise PCLS as a novel experimental tool to assess the direct influence of specific fibrotic mediators as well as inflammatory cytokines on reactivity. This approach could inform interpretation of findings from *in vivo* models where multiple direct and indirect influences are likely to affect airway reactivity.

Chapter 5 / Aim 4

There is an unmet medical need for alternative treatments for obstructive lung diseases as current dilator treatments can become ineffective with frequent use or more severe disease. Our findings demonstrated that RLX is both a novel bronchoprotective agent and an effective bronchodilator, based on our published (Lam *et al.*, 2016) and additional outcomes summarised in Table 8.2.

RLX effectively inhibited the development of airway contraction to both MCh and ET-1 in mouse, rat and guinea pig airways (Table 8.2). Notably, RLX was equally as effective as a 100-fold higher concentration of the β-AR agonist, isoprenaline. These findings suggest that RLX could prevent the development of bronchoconstriction without the requirement for the high doses of β-AR agonist known to cause homologous desensitisation of its receptors. Future studies should establish the extent of bronchoprotection by RLX in human airways in comparison to the selective β_2-AR agonist, SALB, since the latter is often used in this way prior to exercise by asthmatic athletes to prevent exercise-induced asthma.

This is the first study to characterise the direct bronchodilator effects of RLX (Table 8.2). RLX was not an effective dilator in mouse PCLS or in guinea pig trachea, but whether this is related to species or airway size remains to be determined since comparisons between trachea and PCLS were not made within these species. Of note, relaxation to 100 nM RLX was evident in rat airways irrespective of size, where it was more potent than isoprenaline. RLX also elicited rapid relaxation in intrapulmonary airways in human PCLS. There is still a need to construct full-concentration response curves to RLX in order to more accurately determine its potency and maximum effects, particularly in comparison to the clinically used β_2-AR agonist salbutamol (SALB) in human tissue. However, the requirement for only low doses of RLX to reverse airway contraction could potentially overcome the loss of efficacy associated with the overuse of β-AR agonists as reliever treatments for asthma.

The mechanisms underlying the dilator action of RLX remain to be fully established. Relaxation of rat, but not human, airways was shown to require conditions where epithelial relaxing factors accumulated. This suggests that the role of epithelium may be less significant in mediating RLX-induced relaxation in human airways, and that RLX may still be an effective dilator even if the epithelial layer is damaged in disease.

Findings in human airways were limited by the availability of tissue, relying on provision of donor lungs unsuitable for transplant for the preparation of PCLS. Since functional responses have been validated in mouse cryopreserved PCLS (Ch 3), this approach should also be applied to human PCLS to overcome the shortage of samples, whereby multiple PCLS from each precious sample could be cryopreserved and stored for future experiments to maximise outcomes. Another limitation is the scarcity of human lung samples from disease settings, particularly from asthmatic sufferers. Treatment of human PCLS with cytokines and fibrotic mediators have previously been used to mimic disease conditions (Temann *et al.*, 2017) and would permit novel treatments to be tested. Although the findings with RLX in human airway are preliminary, its promising dilator effects support further preclinical investigation and future clinical translation.

Table 8.2. The effects of relaxin on large airways (trachea) and small airways (PCLS) of multiple species.

	mouse		**rat**		**guinea pig**	**marmoset**	**human**
	large	small	large	small	large	small	small
bronchoprotection	✓	n.d	✓	n.d	✓	n.d.	n.d.
bronchodilation	x	x	✓	✓	n.d.	n.d.	✓
potentiation of SALB	n.d	✓	✓	✓	✓	x	✓

Chapter 6 / Aim 5

Additional dilator treatments are required for patients with severe asthma when SALB alone does not relieve symptoms. Currently used combination treatments targeting excess airway contraction include short-acting muscarinic antagonists, leukotriene antagonists and theophylline but these are not always effective. This is the first study to demonstrate the ability of RLX to potentiate relaxation to SALB across multiple species and to reveal the potential mechanisms involved in this potentiation. Having established the differential effects of RLX to directly oppose contraction across species, it was shown that SALB mediated complete relaxation in airways from rats and guinea pigs, similar to human airways, but only partial relaxation of mouse and marmoset airways in PCLS. There was clear evidence of that RLX could potentiate SALB-mediated relaxation in rat and guinea pig trachea as well as in mouse, rat and human intrapulmonary airways in PCLS, but not marmoset PCLS (Table 8.2). Findings in marmoset suggest that these other species may provide better models of human airways.

Several mechanisms involving both the airway epithelium and smooth muscle layer have been implicated in the multiple actions of RLX. The beneficial effects of RLX are mediated by inhibiting the downstream signalling pathways of fibrosis, and by opposing multiple mechanisms driving inflammation or contraction (Lam *et al.*, 2018). Previous studies have demonstrated that RLX exerts its anti-fibrotic effects through the NO/sGC/cGMP pathway (Chow *et al.*, 2012; Wang *et al.*, 2016). RLX-mediated inhibition of collagen synthesis in lung myofibroblasts was dependent on increases in NO synthesis and cGMP levels (Huang et al., 2011). From our findings, the effect of RLX on smooth muscle tone was mediated via epithelial-derived mediators which opposed contraction. In rat trachea, the inhibition of ET-1-induced contraction by RLX was partially prevented by either L-NAME or the COX inhibitor, indomethacin highlighting the importance of NO and prostaglandin synthesis in mediating its actions. However, epithelial denudation of rat airways only partially suppressed the

bronchoprotective effects of RLX. Furthermore, since ODQ and the adenylate cyclase inhibitor, SQ2236, caused further inhibition of RLX-induced relaxation in these epithelial-denuded trachea, RLX may also be having direct actions on airway smooth muscle via cGMP- and cAMP-dependent pathways to contribute to the relaxation response.

RLX potentiated relaxation to SALB by increasing maximum relaxation when the efficacy of SALB was limited (mouse) or increasing SALB potency when full relaxation occurred (rat, guinea pig, human). Therefore, it is likely that RLX and SALB mediate their effects via different mechanisms. Potentiation of SALB-mediated relaxation by RLX was also maintained in the presence of increased contraction to MCh in guinea pig trachea despite reduced SALB potency, suggesting that the mechanism of potentiation is not limited by functional antagonism. Although the addition of high dose exogenous MCh was used to mimic the contribution of increased contraction as a component of severe asthma, it clearly does not entirely replicate disease conditions such as inflammation and fibrosis. This was further investigated using mouse models of disease in Chapter 7. The mechanism of RLX-mediated potentiation of SALB still needs to be fully defined, but our preliminary evidence suggests that RLX may be more effective in opposing contraction mediated via PKC-dependent pathways than contraction due to calcium sensitivity. This may have implications in disease settings where contractile signalling is dysregulated.

Chapter 7/ Aim 6

In chronic lung diseases, combination therapy targets different aspects of disease. For example, inhaled GCS/LABA preventer medication opposes both inflammation and airway contraction, while inhaled SABA/SAMA oppose airway contraction by different mechanisms. This chapter is the first study to assess the potentiation of relaxation to SALB by RLX in different mouse models of lung disease conditions (summarised in Table 8.3). Although RLX alone had no direct dilator action in mouse airways (Ch 5), it was able to increase the effects of SALB in PCLS from naïve mice (Ch 6). It was therefore possible to assess its ability to potentiate relaxation to SALB in diseased settings, using bleomycin (BLM), house dust mite (HDM) and TGFβ-overexpression to increased fibrosis and/or inflammation

The BLM model used was characterised by established parenchymal fibrosis and diffuse inflammation. Chronic RLX treatment has been previously been shown to inhibit the development of BLM-induced fibrosis (Unemori *et al.*, 1996). In the current study, SALB-mediated relaxation was maintained in PCLS from this model, but acute treatment with RLX failed to potentiate this response. This was attributed to the marked downregulation of RXFP1

expression in airways from BLM-treated mice, as has been previously reported in fibrotic lungs of patients with IPF (Tan *et al.*, 2016). It is possible that chronic RLX administration could restore receptor expression, as shown in an allergic airway disease (AAD) model, where RLX also reversed established fibrosis (Royce *et al.*, 2013). If potentiation by RLX could be re-established by chronic treatment, acute RLX may also offer benefit for the treatment of IPF in the subgroup of patients who have a degree of reversible airflow limitation and rely on SALB to relieve breathlessness (Assayag *et al.*, 2015).

Given the lack of significant benefit with anti-inflammatory glucocorticoids on disease progression in IPF, or the failure of anti-fibrotic agents such as pirfenidone to reverse established fibrosis, it is critical that chronic RLX as a treatment should be explored further. Although the aetiology of IPF is unknown, it has consistently been shown to involve repeated epithelial cell injury and activation. It would also be of interest to determine if RLX could target the airway epithelium, where there is an abundance of RXFP1, to limit its contribution to lung scarring and potential loss of dilator sensitivity.

In a short-term HDM model of AAD, increased airway inflammation was associated with reduced dilator sensitivity to SALB in mouse PCLS, but maximum relaxation to SALB was still increased by RLX. The chronic TGFβ-overexpression model demonstrated increased airway fibrosis at 8 weeks and increased *ex vivo* airway contraction to MCh. Unlike the BLM model of diffuse fibrosis, RXFP1 expression was maintained and RLX was also able to potentiate SALB-mediated relaxation. Thus, potentiation of relaxation by RLX persisted under settings of impaired dilator responsiveness or increased airway contraction associated with acute inflammation and established airway fibrosis respectively. There is a clear need for further assessment in chronic AAD disease models, and to measure dilator responses to and potentiation by RLX *in vivo* in order to translate these potentially beneficial effects.

Whether these effects are evident in diseased human airways also remains unknown. However, our preliminary findings in non-diseased human airways where RLX increased the potency of SALB would suggest that the use of RLX as an adjunct dilator would be of benefit. Reducing the dose of SALB required to relieve symptoms could preserve β-AR sensitivity in severe asthma by limiting the receptor desensitisation and loss of dilator efficacy due to frequent use of high doses of SALB.

Table 3. Summary of outcomes from the bleomycin (BLM), house dust model (HDM) and TGFβ-overexpression (TGFβ) models.

Outcomes	**Models**		
	BLM (2 week)	HDM (2 week)	TGFβ (8 week)
fibrosis	✓ (parenchyma and airway)	x	✓ (airway)
airway inflammation	✓	✓	x
↑ MCh contraction *in vivo*	n.d.	✓	x
↑ MCh contraction *in vitro*	x	x	✓
↓ SALB relaxation *in vitro*	x	✓	x
potentiation of SALB relaxation by RLX *in vitro*	↓	maintained	maintained

8.3 Future directions and conclusion

Our overall findings with RLX have provided further support of its anti-fibrotic efficacy and the first evidence of its bronchoprotective and bronchodilator effects of RLX in small and large airways. The ability of RLX to inhibit collagen remodelling by human asthmatic fibroblasts coupled with airway relaxation and potentiation of SALB-mediated relaxation could oppose both persistent remodelling as well as bronchoconstriction in chronic disease. Our accumulating evidence suggests that potentiation of relaxation to SALB by RLX is maintained with increased airway contraction, and in inflamed or fibrotic airways. The host of beneficial effects of RLX were variable between species but were evident in human airways.

We have identified RLX as the only known chronic anti-fibrotic treatment that also opposes airway smooth muscle contraction. Our novel findings need to be extended to confirm the bronchodilator effects of RLX *in vivo*, particularly in models of allergic airways disease. Although we have demonstrated the bronchodilator effects of RLX *in vitro*, its ability to oppose AHR *in vivo* has only been demonstrated with chronic, rather than acute treatment, so could be mediated by its effects via reduced remodelling alone rather than direct actions on airway tone (Royce *et al.*, 2009, 2013). Our preliminary findings showing bronchodilator actions of RLX in human airways are promising but also require further assessment to confirm efficacy, particularly under conditions of established fibrosis or in diseased airways. This is critical since the dilator effects of RLX only after continuous administration in clinical trials for heart failure (Teerlink *et al.*, 2017) was less effective than in animal models of disease (Royce *et al.*, 2009; Samuel *et al.*, 2011). Furthermore, in these clinical trials, RLX was administered via intravenous infusion. It is imperative that further studies assess the acute and chronic effects of inhaled RLX in lung diseases in which early and late-stage fibrosis are present.

A potential limitation the clinical translation of RLX for asthma treatment maybe its short half-life (10 minutes *in vivo*) (Chen *et al.*, 1993a, 1993b). However, an allosteric agonist of the RXFP1 receptor, ML290, has recently been developed and shown to have a plasma half-life of 8.5 h in mice (Kocan *et al.*, 2017), comparable to the half-life of inhaled SALB (Hindle and Chrystyn, 1992). As such, ML290 may be a more reliable treatment for asthma than native RLX. To date, ML290 has been shown to inhibit fibrosis (Kocan *et al.*, 2017) but has yet to be assessed for bronchodilator efficacy. It also remains to be determined whether like RLX, ML290 is beneficial when used in combination to increase the effectiveness of current treatments.

Current guidelines for the treatment of asthma recommend combination treatment with a long acting β-agonist (LABA) and an inhaled corticosteroid (Global Initiative for Asthma, 2019). Although this combination primarily reduces inflammation and induces ongoing relaxation to reduce symptoms in mild asthma, both drugs may become less effective in severe disease where there is increased airway contraction and fibrosis. We envisage RLX as a long-term triple therapy, where its combination with a glucocorticoid and a long-acting β-adrenoceptor agonist may overcome the limited anti-remodelling actions of current preventer medication (Bergeron et al., 2010). The acute use of RLX combined with a short-acting β- adrenoceptor agonist as a reliever may also reduce the dilator dose required to reverse symptoms associated with airway hyperresponsiveness, and limit the loss of bronchoprotection evident with frequent high-dose use of SALB (Yim and Koumbourlis, 2013). In the absence of off-target effects when administered systemically in the clinical RELAX-AHF trials, RLX itself appears to have a positive safety profile (Teerlink *et al.*, 2013) which would be further limited when administered by inhalation. Given its safe clinical profile (Dschietzig *et al.*, 2009; Díez, 2014), there is a strong impetus for the assessment of RLX as a novel multifunctional therapeutic for the treatment of asthma and other lung diseases.

Conclusion

This thesis presents compelling evidence demonstrating dual beneficial actions of RLX in the airways by inhibiting collagen remodelling by airway fibroblasts, and inducing / potentiating airway relaxation. While the clinical relevance of our findings remains to be tested, its effects in myofibroblasts from patients with asthma and in human airways support further characterisation of its effects when used both alone and in combination with currently available therapies under conditions where their efficacy is limited.

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Appendix

Appendix

Publications

First author

Lam M, Lamanna E, Bourke J (2019). Regulation of airway smooth muscle contraction in health and disease. Smooth Muscle Spontaneous Activity, H Hashitani, R Lang, eds. (Springer Singapore).

Lam M, Royce SG, Samuel CS, Bourke JE (2018). Serelaxin as a novel therapeutic opposing fibrosis and contraction in lung diseases. *Pharmacology & Therapeutics* **187**: 61-70.

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Royce S, Nold M, Bui C, Donovan C, **Lam M**, Lamanna, E, Rudloff I, Bourke JE, Nold, C (2016). Airway remodelling and hyperreactivity in a model of bronchopulmonary dysplasia and their modulation by IL- 1Ra. *Am J Respir Cell Mol Biol* **55**: 858-868.

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Abstracts titles (for conference attendance):

Lam M, Royce S, Donovan C, Jelinic M, Parry L, Samuel C, Bourke J. Serelaxin is a novel bronchodilator which enhances β-adrenoceptor-mediated relaxation in multiple species (2017). *Respirology* **23**: 152. **(oral, Ann Woolcock Young Investigator finalist)**

Lam M, FitzPatrick M, Harper R, Reynolds PN, Bourke JE (2017). Intrapulmonary artery and airway reactivity is altered in precision cut lung slices from a rat model of pulmonary arterial hypertension. *Am J Respir Crit Care Med* **195**: A4876. **(poster, awarded international trainee scholarship and respiratory structure and function (RSF) award)**

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Lam M, Royce S, Samuel C, Bourke J. Relaxin potentiates salbutamol-mediated airway relaxation in multiple species**.** *TSANZ Victorian Branch Meeting, 2015* ***(oral, best presentation award)***

Lam M, Royce SG, Samuel CS, Bourke JE, Serelaxin is an epithelial-dependent bronchodilator in rat precision cut lung slices and trachea. *Australasian Society for Experimental Pharmacologist and Toxicologists (ASCEPT), Hobart 2015 (poster and oral*)

Lam M, Samuel CS, Royce SG, Bourke JE. Relaxation to β-adrenoceptor agonists is enhanced by the novel potent bronchodilator serelaxin. *TSANZ Victorian Branch Meeting, 2015* ***(oral, best presentation award)***

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frontiers in Pharmacology

ORIGINAL RESEARCH
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Serelaxin Elicits Bronchodilation and Enhances β-Adrenoceptor-Mediated Airway Relaxation

*Maggie Lam[1], Simon G. Royce[1], Chantal Donovan[1,2], Maria Jelinic[3], Laura J. Parry[3], Chrishan S. Samuel[1] and Jane E. Bourke[1,2]**

[1] Biomedicine Discovery Institute and Department of Pharmacology, Monash University, Clayton, VIC, Australia, [2] Department of Pharmacology, Lung Health Research Centre, The University of Melbourne, Parkville, VIC, Australia, [3] School of BioSciences, The University of Melbourne, Parkville, VIC, Australia

Treatment with β-adrenoceptor agonists does not fully overcome the symptoms associated with severe asthma. Serelaxin elicits potent uterine and vascular relaxation via its cognate receptor, RXFP1, and nitric oxide (NO) signaling, and is being clinically evaluated for the treatment of acute heart failure. However, its direct bronchodilator efficacy has yet to be explored. Tracheal rings were prepared from male Sprague-Dawley rats (250–350 g) and tricolor guinea pigs, and precision cut lung slices (PCLSs) containing intrapulmonary airways were prepared from rats only. Recombinant human serelaxin (rhRLX) alone and in combination with rosiglitazone (PPARγ agonist; recently described as a novel dilator) or β-adrenoceptor agonists (isoprenaline, salbutamol) were added either to pre-contracted airways, or before contraction with methacholine or endothelin-1. Regulation of rhRLX responses by epithelial removal, indomethacin (cyclooxygenase inhibitor), L-NAME (nitric oxide synthase inhibitor), SQ22536 (adenylate cyclase inhibitor) and ODQ (guanylate cyclase inhibitor) were also evaluated. Immunohistochemistry was used to localize RXFP1 to airway epithelium and smooth muscle. rhRLX elicited relaxation in rat trachea and PCLS, more slowly than rosiglitazone or isoprenaline, but potentiated relaxation to both these dilators. It markedly increased β-adrenoceptor agonist potency in guinea pig trachea. rhRLX, rosiglitazone, and isoprenaline pretreatment also inhibited the development of rat tracheal contraction. Bronchoprotection by rhRLX increased with longer pre-incubation time, and was partially reduced by epithelial removal, indomethacin and/or L-NAME. SQ22536 and ODQ also partially inhibited rhRLX-mediated relaxation in both intact and epithelial-denuded trachea. RXFP1 expression in the airways was at higher levels in epithelium than smooth muscle. In summary, rhRLX elicits large and small airway relaxation via epithelial-dependent and -independent mechanisms, likely via RXFP1 activation and generation of NO, prostaglandins and cAMP/cGMP. rhRLX also enhanced responsiveness to other dilators, suggesting its potential as an alternative or add-on therapy for severe asthma.

Keywords: airway, β-adrenoceptor agonist, bronchodilation, epithelium, precision-cut lung slice, relaxin, rosiglitazone, trachea

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Rajasekaran Subbiah, Anna University, India

****Correspondence:***
Jane E. Bourke
jane.bourke@monash.edu

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INTRODUCTION

Asthma is a chronic inflammatory disease of the airways, affecting 300 million world-wide (Masoli et al., 2004). Recruitment of inflammatory cells and stimulation of resident structural cells in asthma promotes epithelial damage, goblet cell metaplasia, fibrosis and the accumulation of airway smooth muscle (ASM) (Mauad et al., 2007). These inflammatory and structural changes contribute to the development of airway hyperresponsiveness (AHR), characterized by excessive bronchoconstriction to allergic and non-allergic stimuli (Yeganeh et al., 2013).

While treatment with the β_2-adrenoceptor agonist salbutamol (SAL) generally reverses asthma symptoms, dilator responses in many patients may be limited by factors such as frequent use and/or viral infection leading to tolerance and receptor desensitization (Duechs et al., 2014; Spina, 2014). β_2-adrenoceptor agonists have also been shown to have relatively lower efficacy in small airways in the distal lung, where increased inflammation and AHR contributes to asthma severity (Donovan et al., 2013; Bourke et al., 2014). Thus, it is important to identify novel agents to reverse or inhibit the development of contraction to act as bronchodilators or bronchoprotective agents in both large and small airways.

Serelaxin (rhRLX or RLX) is the recombinant drug-based version of the major stored and circulating form of the human gene-2 relaxin hormone, which mediates its physiological actions via activation of Relaxin Family Peptide Receptor 1 (RXFP1) (Samuel et al., 2007). Although rhRLX is generally associated with vascular changes in pregnancy and childbirth, its recent clinical assessment for acute heart failure was associated with beneficial effects on hemodynamics and reduced mortality (Teerlink et al., 2013). These findings were consistent with rhRLX-enhanced vasorelaxation in isolated rodent small renal and mesenteric arteries (McGuane et al., 2011; Leo et al., 2014) and human gluteal and subcutaneous resistance arteries (Fisher et al., 2002; McGuane et al., 2011). rhRLX has also been shown to influence gastrointestinal motility in mice via the generation of nitric oxide (NO) from an intact epithelial layer (Baccari et al., 2013).

Of relevance to asthma, chronic *in vivo* administration of rhRLX inhibited fibrosis and the development of AHR in a mouse model of ovalbumin-induced allergic airways disease (AAD) mimicking key features of asthma (Royce et al., 2009, 2013a). Although rhRLX is able to exert protective effects in the lung, and has also been identified as a safe and efficacious relaxant of vascular and uterine smooth muscle (Bani et al., 1998; Tan et al., 1998), its acute effects on airway contraction have yet to be characterized.

Another potential novel bronchodilator of interest is rosiglitazone (RGZ), originally identified as a potent agonist of peroxisome proliferator activated receptor γ (PPARγ). RGZ has recently been shown to elicit acute airway relaxation independently of PPARγ activation in mouse precision cut lung slices (PCLS) (Donovan et al., 2014) and mouse trachea (Donovan et al., 2015). Furthermore, RGZ was more efficacious than β_2-adrenoceptor agonists in mouse airways, albeit at lower potency (Donovan et al., 2014). Like rhRLX, RGZ has been shown to inhibit fibrosis and the development of AHR in mouse models of allergic AAD (Honda et al., 2004; Ward et al., 2004; Donovan et al., 2012).

Given this background, the aims of this study were to assess the potential bronchodilator and bronchoprotective effects of rhRLX in comparison to RGZ and the β-adrenoceptor agonists salbutamol and isoprenaline (ISO). We hypothesized that rhRLX would both reverse established airway contraction and inhibit the development of contraction, with potential for additivity with these other dilators.

MATERIALS AND METHODS

Materials

Acetylcholine (ACh), methacholine (MCh), substance P, isoprenaline (ISO), salbutamol (SAL), and *N*ω-nitro-L-arginine methyl ester (L-NAME) (all from Sigma-Aldrich, St. Louis, MO, USA); endothelin-1 (ET-1, GenScript, Piscatawa, NJ, USA); indomethacin and rosiglitazone (RGZ) (both from Cayman Chemical, Ann Arbor, MI, USA); recombinant human gene-2 relaxin (serelaxin/ rhRLX, kindly provided by Novartis AG, Basel, Switzerland).

Animals and Tissue Collection

All procedures were approved by a Monash University Animal Ethics Committee (AEC number: MARP2/2014/066), which adheres to the Australia Code of Practice for the Care and Use of Animals for Scientific Purposes. Male Sprague-Dawley rats (250–350 g) (Monash University Animal Services, Melbourne, VIC, Australia) and Tricolor guinea pigs (8–10 weeks) (Pipers, Cowra, NSW, Australia) were housed in the Animal Facility, Monash University and maintained on a fixed 12 h light, 12 h dark lighting schedule, with free access to food and water at all times.

Rats were humanely euthanized by sodium pentobarbitone overdose (60 mg/mL administered i.p. at a volume of 1 mL per 100 g body weight) for preparation of tracheal tissue and PCLS. Guinea pigs were gently restrained and stunned by a single blow to the head. Loss of consciousness was confirmed by the lack of withdrawal reflex, followed by immediate exsanguination of the carotid artery.

Myograph Experiments

Trachea were quickly dissected and mounted in Krebs-Henseleit (59 mM NaCl, 2.3 mM KCl, 0.69 mM $MgSO_4 \cdot 7H_2O$, 2.5 mM $CaCl_2 \cdot 6H_2O$, 0.6 mM KH_2PO, 10 mM EDTA, 25 mM $NaHCO_3$, and 6 mM glucose) buffer solution for experiments using myograph (Danish MyoTechnology, Arthuus Denmark, 5 ml bath for rat trachea) or standard organ baths (10 ml bath for guinea pig trachea). Tissues were set to optimum resting tension for maximum development of contraction to potassium physiological salt solution (KPSS; 123.7 mM K^+). A maximal contraction to ACh (30 μM) was also obtained in all tissues. To assess airway relaxation, cumulative additions of MCh were made to establish a submaximal contraction (50–70% of ACh maximum). Concentration-response curves were then constructed to rhRLX, RGZ, ISO, or SAL or selected

concentrations of rhRLX (0.1 μM), RGZ (10, 100 μM) or ISO (1, 10 μM) were added alone or in combination.

To assess potential inhibition of airway contraction, trachea were pre-incubated with selected concentrations of rhRLX (0.1 μM), RGZ (100 μM), or ISO (10 μM) for 30 min prior to the construction of cumulative MCh or ET-1 concentration-response curves. The contribution of epithelial-derived factors to inhibition of airway contraction was assessed in trachea pre-incubated in the absence or presence of rhRLX (0.1 μM) for 2 h. The bath solution was then either left or replaced before concentration-response curves were generated to ET-1. Alternatively, the epithelium was removed by gentle agitation of the lumen using a wooden toothpick and confirmed by loss of relaxation to the epithelial-dependent dilator substance P (1 μM) and H&E staining of tracheal sections after experiments. The contribution of NO and cyclooxygenase (COX)-derived mediators was assessed using the NO synthase (NOS) inhibitor, L-NAME (100 μM) and/or the COX inhibitor, indomethacin (3 μM) added during the rhRLX pre-incubation period before ET-1 curves.

Finally, SQ22536 (10 μM) and/or ODQ (1 μM), inhibitors of adenylate cyclase and guanylate cyclase respectively, were added to both intact and denuded trachea before a submaximal contraction to MCh was established for assessment of relaxation to rhRLX (0.1 μM).

Precision Cut Lung Slice (PCLS) Experiments

Precision cut lung slice were prepared with minor modifications from previously published methods (Bourke et al., 2014; Donovan et al., 2014). Rats were euthanized as described previously and dissected to expose the heart and trachea. Heparin sodium (500IU) was injected into the left ventricle to empty the lungs of blood before cannulation. Trachea were cannulated with a catheter containing two ports (20 G Intima, Becton Dickinson, VIC, Australia) and lungs were inflated with ∼10 mL agarose gel (2% in 1x HBSS at 37°C), followed by a bolus of ∼3 mL air. Lungs were cooled by bathing in cold HBSS/HEPES and the rats were then kept at 4°C to allow the agarose to solidify, before the lungs were removed. The upper right lobe was isolated and adhered with cyanocrylate to a mounting plate in a vibratome (Compresstome, Precisionary Instruments, Greenville, NC, USA). PCLS of 200 μm thickness were prepared and transferred into cell culture plates containing DMEM, supplemented with 1% penicillin-streptomycin and incubated for 24 h (37°C, 5% CO_2) prior to experiments.

Precision cut lung slice were transferred to HBSS/HEPES and mounted in custom-made perfusion chambers (∼100 μL volume). A viable airway (∼200 μm diameter) was selected from each slice based on the presence of an intact layer of epithelial cells with ciliary activity. PCLS were perfused at a constant rate (∼0.5 mL/min) through an eight-channel gravity-fed perfusion system under vacuum. Slices were initially perfused with MCh (0.3 μM) to establish a submaximal pre-contraction, then perfused with MCh in combination with rhRLX (0.1 μM) or ISO (10 μM). Perfusion was then stopped to permit assessment of airway responses to MCh in the presence of dilator under static conditions.

Phase contrast microscopy was conducted using an inverted microscope (Nikon Ti-U, Melville, NY, USA) to observe drug-induced airway changes, employing 10X objective lens, zoom adapter, reducing lens and camera (CCD camera model TM-62EX; Pulnix, Takex, Japan). Changes in airway lumen area were captured as digital images (744 × 572 pixels) in time lapse (0.5 Hz) using image acquisition software (Video Savant; IO Industries, Inc., London, ON, Canada). Obtained files were converted to TIFF files and analyzed using NIH/Scion software (Scion Corporation; download www.scioncorpcom).

Immunohistochemistry

Lungs were dissected whole and fixed in neutral buffered formalin before being embedded in paraffin wax. Sections (5 μm) were cut and mounted on SuperFrost PLUS slides (Menzel-Gläser, Braunschweig, Germany). Procedures for bright field immunohistochemistry are detailed in (Jelinic et al., 2014). Lung sections were incubated overnight at 4°C with 3 μg/ml rat RXFP1 antiserum (#107, raised against amino acid residues 107–119 of the rat RXFP1 protein) or preimmune serum (rabbit IgG). Immunoreactivity was detected using the MACH 2^{TM} system (Biocare Medical, Concord, CA, USA) and 3, 3′ diaminobenzidine (DAB) as the chromagen substrate (Vector Laboratories, Burlingame, CA, USA). The rat RXFP1 antibody used has previously been shown to be selective, with no cross-reactivity to another relaxin-family peptide receptor (RXFP2) (Jelinic et al., 2014).

Statistical Analysis

Contraction was normalized to the maximum ACh contraction for MCh or the KPSS response for ET-1 in trachea, or expressed as % initial airway area in PCLS. Relaxation was expressed as % of the MCh submaximal pre-contraction. All data are expressed as the mean ± SEM. Non-linear regression of concentration-response curves to obtain fitted maxima and pEC50 values where possible. Results were analyzed via *t*-test or one-way ANOVA for multiple comparisons between groups as appropriate; where statistical significance was accepted at $P < 0.05$. All data analysis was performed using GraphPad Prism v6 (GraphPad Software, San Diego, CA, USA).

RESULTS

rhRLX, RGZ, and ISO Elicit Relaxation of Rat Trachea

Dilator effects of rhRLX, RGZ, and ISO in rat trachea were compared after pre-contraction to similar submaximal levels with MCh (300 nM), measuring changes in isometric force in a static organ bath (5 ml volume; **Figure 1A**). When added at 5–10 min intervals, rhRLX did not cause any relaxation up to 0.1 μM, the highest concentration available (**Figure 1B**). In contrast, RGZ and ISO induced concentration-dependent relaxation (fitted maximum % relaxation: ISO 45.6 ± 5.3; RGZ

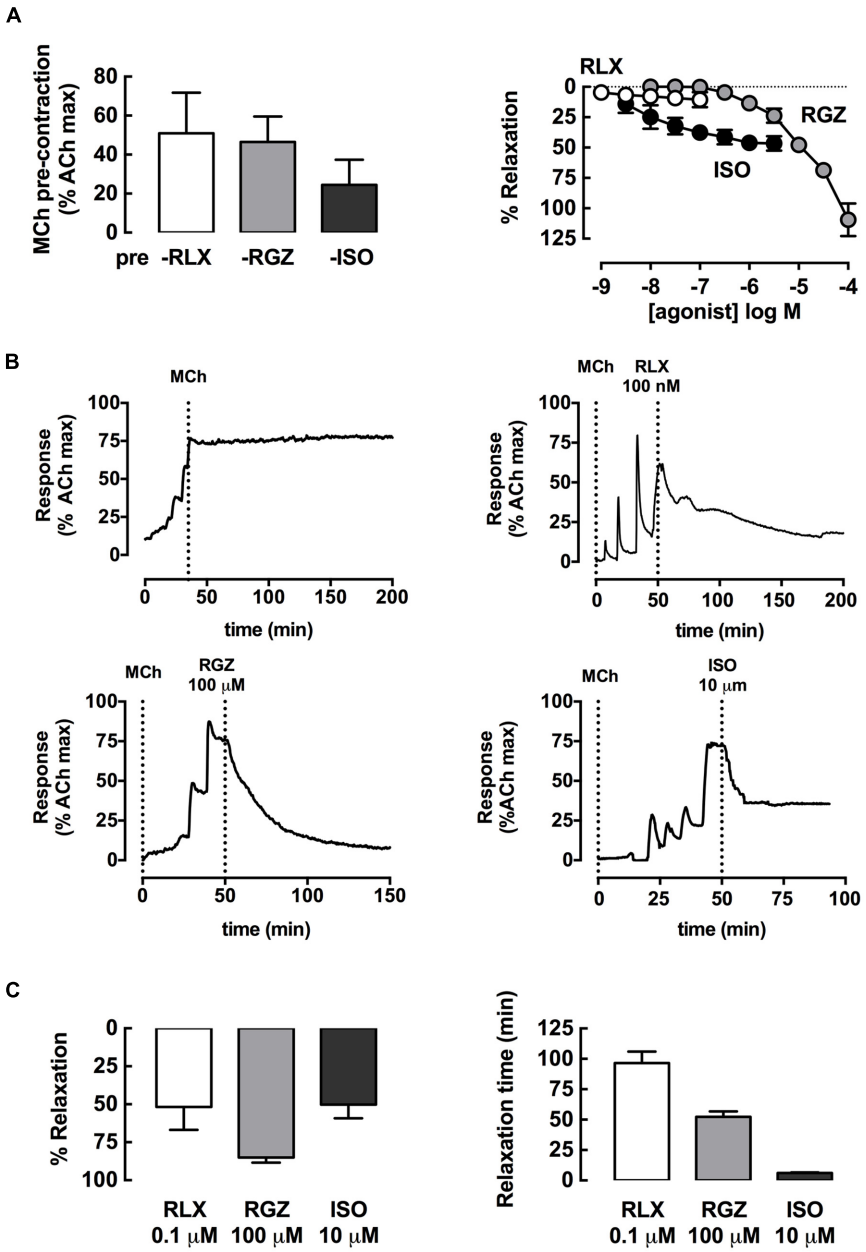

FIGURE 1 | rhRLX, RGZ, and ISO elicit relaxation of rat trachea. Rat trachea were mounted under isometric conditions for optimum development of contraction. Trachea were **(A)** precontracted with MCh, prior to preparation of concentration-response curves to rhRLX (RLX), RGZ, or ISO. **(B)** Representative traces show relaxation to RLX, RGZ, and ISO after MCh, as well as a time control for MCh alone. **(C)** Relaxation responses and time to maximum relaxation to single additions of RLX (0.1 μM), RGZ (100 μM), or ISO (1 μM) were also assessed. Extent of relaxation is expressed as % of submaximal MCh pre-contraction, with relaxation time expressed in min (mean ± SEM for $n = 4$ per group).

138.1 ± 30.0; $P < 0.05$). When compared to RGZ, ISO was more potent (pEC_{50}: ISO 8.0 ± 0.3; RGZ 4.7 ± 0.2; $P < 0.001$), but only RGZ elicited complete relaxation (**Figure 1A**).

When the highest concentrations of each drug tested were allowed to remain in contact with the pre-contracted trachea, marked relaxation to rhRLX, as well as to RGZ and ISO, became evident. Representative traces show the effects of addition of rhRLX (0.1 μM), RGZ (100 μM), and ISO (1 μM) relative to a time control showing maintained contraction to MCh alone over 200 min (**Figure 1B**). Partial relaxation to rhRLX occurred at slower rate than either RGZ or ISO, which caused near complete and partial tracheal relaxation within approximately 50 and 5 min respectively (**Figures 1B,C**). Notably, the extent of relaxation to 0.1 μM rhRLX-mediated (% relaxation: 52 ± 15%) was comparable to the maximal ISO-induced airway relaxation (50 ± 9%).

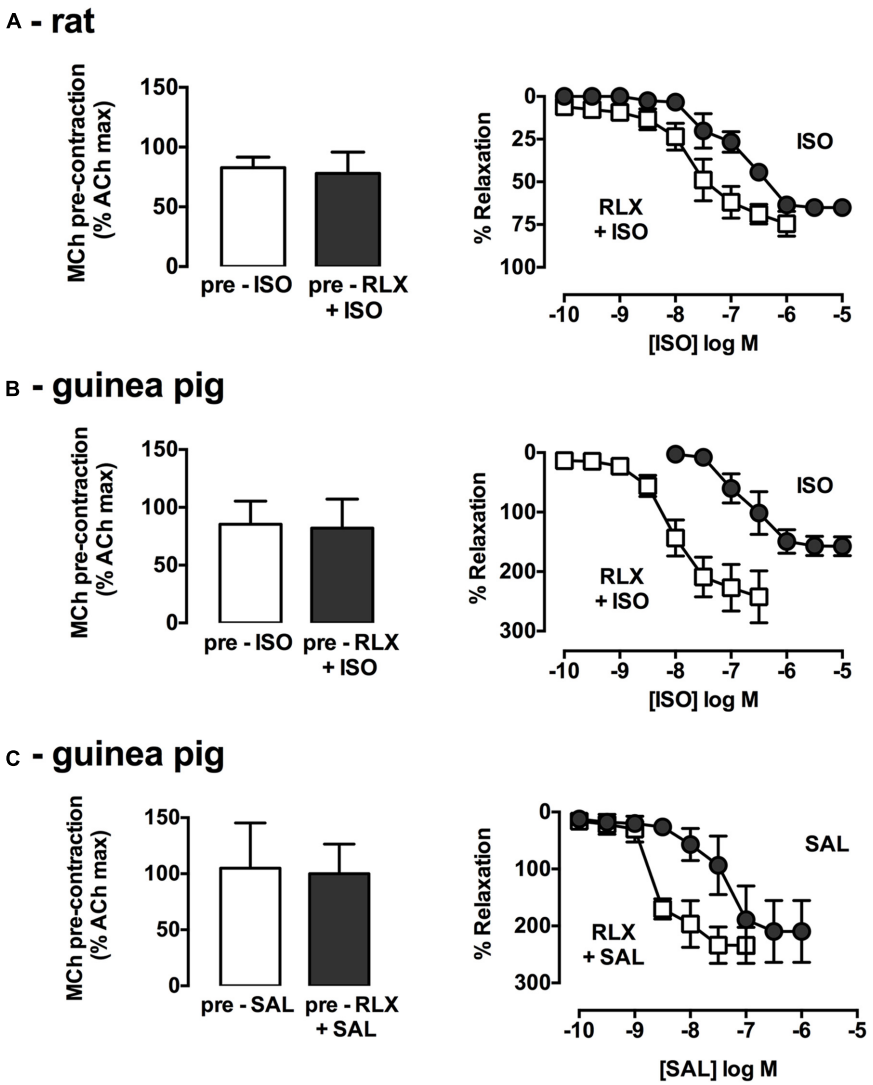

FIGURE 2 | rhRLX potentiates relaxation to β-adrenoceptor agonists. Trachea were precontracted with MCh, prior to measurement of relaxation responses in the absence and presence of rhRLX (100 nM) to ISO (0.001–10 μM) in **(A)** rat and **(B)** guinea pig trachea or SAL (0.001–10 μM) in **(C)** guinea pig trachea. Extent of relaxation is expressed as % of submaximal MCh pre-contraction (mean ± SEM for $n = 4$–5 per group).

rhRLX Potentiates Tracheal Relaxation to β-Adrenoceptor Agonists and RGZ

The combined effects of rhRLX and β-adrenoceptor agonists were explored. In rat trachea, where activation of both β_1 and β_2 adrenoceptors contributes to relaxation, ISO alone did not elicit complete relaxation, and the apparent leftward shift in its concentration-response in the presence of rhRLX did not reach significance (potency $P = 0.11$; efficacy $P = 0.08$, $n = 4$–6, **Figure 2A**).

As in rat trachea, rhRLX alone did not cause rapid relaxation of guinea pig trachea (data not shown). Consistent with the predominant role of β_2-adrenoceptor in guinea pig airways, the selective β_2-adrenoceptor agonist SAL was more potent than the β_1/β_2-adrenoceptor agonist ISO (pEC_{50}: SAL 7.4 ± 0.3, ISO 6.6 ± 0.2, **Figures 2B,C**), with both dilators causing relaxation below the baseline prior to MCh. Pre-incubation with rhRLX resulted in a 27.1-fold increase in ISO potency ($P < 0.001$, $n = 4$, **Figure 2B**) with a similar leftward shift seen for SAL in the presence of rhRLX ($P < 0.05$, $n = 3$, **Figure 2C**).

Effective single dilator concentrations of rhRLX, RGZ, and ISO were also tested in combination in rat trachea (**Figure 3**). Combining rhRLX (0.1 μM) with a submaximal concentration of RGZ (10 μM) did not induce greater relaxation compared to RGZ alone, while near-maximal relaxation to RGZ (100 μM) was maintained in the presence of rhRLX (**Figure 3A**). However, the addition of rhRLX markedly increased the rate of relaxation to both concentrations of RGZ tested ($P < 0.001$, $P < 0.01$ vs. RGZ alone, **Figure 3B**).

In contrast, the addition of rhRLX resulted in greater relaxation to ISO (0.1, 10 μM) ($P < 0.05$ vs. ISO alone),

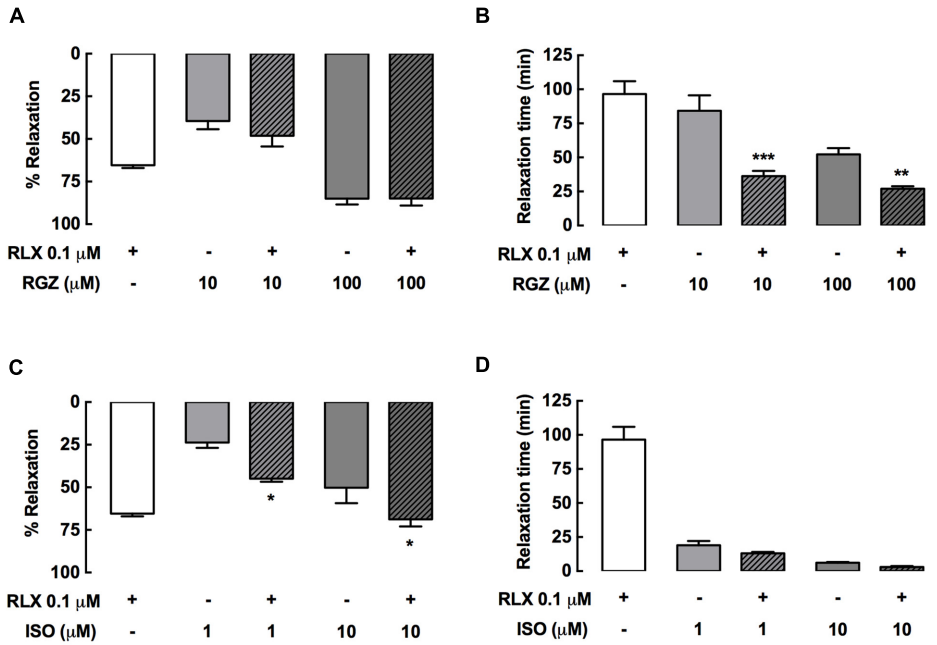

FIGURE 3 | rhRLX potentiates tracheal relaxation to RGZ and ISO. Rat trachea were precontracted with MCh, prior to measurement of **(A,C)** relaxation responses and **(B,D)** time to relaxation for rhRLX (RLX, 0.1 μM), RGZ (10, 100 μM) and ISO (0.1, 1 μM) alone and in combination. Extent of relaxation is expressed as % of submaximal MCh pre-contraction, with relaxation time expressed in min (mean ± SEM for $n = 4$–5 per group). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. the matched concentration of RGZ or ISO alone.

increasing the efficacy of both the submaximal and maximally effective concentrations of ISO (**Figure 3C**). The rapid rate of ISO-mediated relaxation was not further increased by rhRLX (**Figure 3D**).

rhRLX Elicits Relaxation of Rat Intrapulmonary Airways in Lung Slices

Using rat PCLS, the effect of rhRLX (0.1 μM) on intrapulmonary airway contraction was also assessed, measuring changes in airway area under perfused and static conditions in a small volume customized chamber (∼100 μl volume). A representative trace and sequential images of the same airway in PCLS show that the stable contraction to MCh during continuous perfusion over the lung slice was maintained under static conditions (**Figure 4A**). rhRLX rapidly and partially reversed the MCh-induced reduction in airway area (**Figure 4B**), with greater relaxation achieved under static than perfused conditions (% relaxation: perfused 7 ± 3%, static 64 ± 8%, $P < 0.001$; **Figure 4C**). In contrast, relaxation to both RGZ and ISO was similar under perfused and static conditions, causing near-complete and partial relaxation respectively, similar to that seen in trachea (**Figure 4C**).

rhRLX and RGZ, but not ISO, Inhibit the Development of Tracheal Contraction

The bronchoprotective effects of rhRLX, RGZ, or ISO in inhibiting the development of rat tracheal contraction were then compared. Concentration-dependent contraction was established, with full and partial curves obtained for MCh and ET-1 respectively over the concentration ranges tested (**Figure 5**).

Pre-incubation with an effective dilator concentration of RGZ (100 μM) for 30 min inhibited the development of contractile responses to MCh by 90% (**Figure 5B**). Although, the maximum contraction to MCh was reduced by 25% with either rhRLX (0.1 μM) (**Figure 5A**) or ISO (10 μM) (**Figure 5C**), this did not reach significance (**Figure 5D**).

rhRLX, RGZ, and ISO all significantly inhibited contraction to ET-1 (**Figures 5E–H**), but only rhRLX was effective when these drugs were compared at the same concentration (0.1 μM), where rhRLX reduced contraction to 10^{-7} M ET-1 by 50% (**Figures 5E,H**).

rhRLX-Mediated Inhibition of Tracheal Contraction is Increased with Time and Dependent on Released Factors

The effect of increasing rhRLX preincubation time on the inhibition of development of ET-1 contraction by rhRLX was assessed (**Figure 6A**). Pre-incubation with rhRLX for 120 min completely inhibited the development of ET-1 contraction ($P < 0.01$ vs. control), while 30 min preincubation only partially inhibited contraction.

Trachea were also preincubated in the absence or presence of rhRLX, and contraction to ET-1 assessed after the bathing solution was replaced with buffer without rhRLX (washout).

In the absence of rhRLX preincubation, ET-1 mediated contraction after washout was lower than without washout

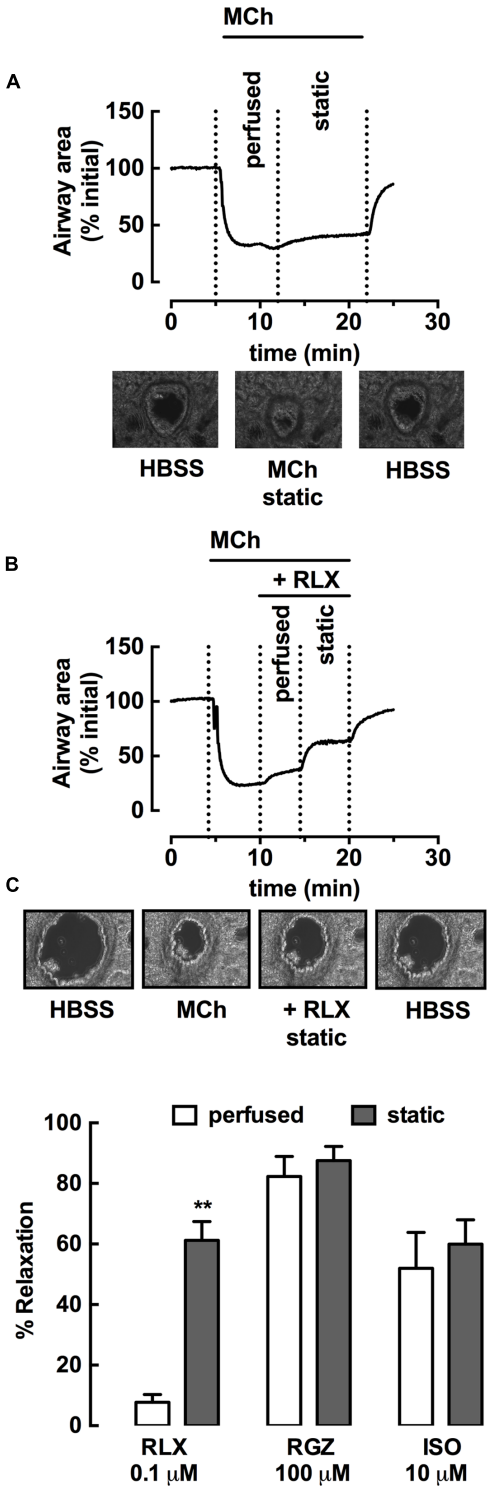

FIGURE 4 | rhRLX elicits relaxation of rat intrapulmonary airways in lung slices. Rat lung slices were mounted in a customized chamber for assessment of changes in intrapulmonary airway area by phase-contrast microscopy. Airways were pre-contracted with MCh (0.3 μM) under perfused and static conditions, in the absence and presence of rhRLX (RLX, 0.1 μM). Representative time-course and phase-contrast images of the last frame of *(Continued)*

FIGURE 4 | Continued
each condition for **(A)** MCh alone and **(B)** MCh followed by RLX (0.1 μM). Traces show area values in pixels determined from gray-scale analysis of images using VideoSavant. **(C)** Grouped data shows relaxation by RLX, RGZ (100 μM) and ISO (10 μM) under perfused and static conditions. Data is expressed as % relaxation of MCh pre-contraction (mean ± SEM, $n = 4$ per group). $^{**}P < 0.01$ vs. relaxation to RLX under perfused conditions.

(**Figure 6B** washout compared to **Figure 6A** control). When pre-treatment with rhRLX was followed by washout, there was no inhibition of contraction to ET-1 (**Figure 6B**).

rhRLX Acts via Epithelial-Dependent and -Independent Mechanisms

To explore potential sites of action of rhRLX, expression of its cognate receptor RXFP1 was determined in rat lungs by immunohistochemistry. RXFP1 was localized in rat airways, with a greater intensity of staining detected in airway epithelium than smooth muscle (**Figure 7A**).

To explore potential mediators of inhibition of contraction by rhRLX, responses to ET-1 were determined in intact trachea after 120 min preincubation with rhRLX in the absence or presence of indomethacin and/or L-NAME. Contraction to 100 nM ET-1 (125 ± 24% KPSS) was almost completely abolished in the presence of rhRLX (17 ± 6% KPSS). Neither inhibitor alone significantly reduced this inhibitory effect of rhRLX (data not shown). However, the marked inhibition of contraction to ET-1 by rhRLX was reduced in the presence of the combination of indomethacin and L-NAME (56 ± 7% KPSS, $P < 0.01$ cf. ET-1+rhRLX alone) (**Figure 7B**). This implicates both COX- and NOS-dependent pathways in the inhibitory effect of rhRLX on contraction.

The tracheal epithelium was then removed in order to assess epithelial-dependence. Denudation was confirmed by conventional hematoxylin staining of formalin-fixed sections (data not shown) and reduced responsiveness to substance P (% relaxation: intact 92.4 ± 2.7%; denuded 10.1 ± 4.0, $P < 0.01$). In contrast to results obtained in intact trachea (control, **Figure 7A**), preincubation with rhRLX for 120 min only partially inhibited contraction to ET-1 following epithelial removal (**Figure 7C**).

The effects of epithelial removal on relaxation to rhRLX following pre-contraction with MCh was also assessed (**Figure 7D**). The level of MCh pre-contraction was similar in epithelial -intact and -denuded trachea (ΔmN: intact 2.4 ± 0.1, denuded 2.2 ± 0.7, NS). Although relaxation to rhRLX (0.1 μM) was reduced by 40% with epithelial removal, a small but significant reversal of the contraction to MCh was still evident (**Figure 7D**). Inhibition of adenylate cyclase and guanylate cyclase, with SQ22536 and ODQ respectively, reduced relaxation to rhRLX in both intact and epithelial-denuded trachea.

DISCUSSION

This study provides the first evidence that rhRLX is an effective bronchodilator and bronchoprotective agent in rat and guinea

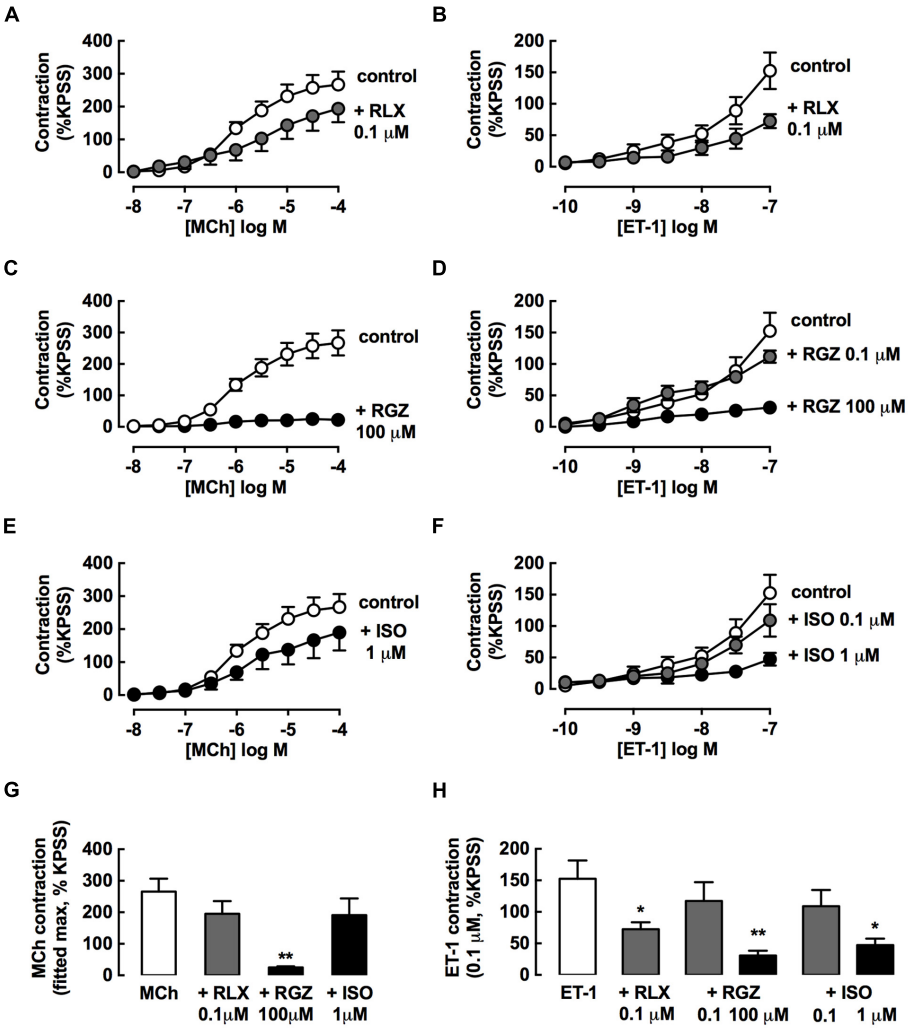

FIGURE 5 | rhRLX and RGZ, but not ISO, inhibit the development of tracheal contraction. The effects of pretreatment with rhRLX (RLX), RGZ and ISO (30 min) on the development of contraction to MCh (left panels) and ET-1 (right panels) were assessed, testing **(A,E)** RLX (0.1 μM), **(B,F)** RGZ (0.1, 100 μM), and **(C,G)** ISO (0.1, 1 μM). For clarity, the same control data is shown on panels **(A–C)** for MCh and panels E-G for ET-1. **(D)** Maximum contraction to MCh determined from fitted concentration-responses curves using GraphPad Prism. **(H)** Contraction to the highest tested concentration of ET-1 (0.1 μM). All responses are expressed as % KPSS standard contraction (mean ± SEM, $n = 4$–5 per group). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. MCh or ET-1 alone.

pig airways. Compared to the β-adrenoceptor agonist ISO and the novel dilator RGZ, rhRLX induced slower relaxation in precontracted rat trachea, with more rapid relaxation elicited in intrapulmonary airways in rat PCLS. The actions of rhRLX appeared to be mediated by the release of endogenous factors [NO, prostaglandin E_2 (PGE_2)], but were only partially epithelial-dependent. rhRLX also markedly increased the potency of β-adrenoceptor agonists ISO and SAL in guinea pig trachea, and potentiated the extent and rate of ISO- and RGZ-mediated relaxation respectively in rat trachea. These data suggest that rhRLX may target alternative dilator mechanisms and offer potential benefit in combination treatments to overcome excessive bronchoconstriction in asthma.

Bronchodilators are the mainstay of pharmacologic therapy for relief of asthma symptoms. However, there is an unmet medical need for more effective treatment under conditions of reduced responsiveness to β-adrenoceptor agonists. Identifying novel bronchodilators that target alternative mechanisms may offer additional benefit during a severe asthma attack or following β-adrenoceptor desensitization (Cazzola et al., 2012).

To date, the acute effects of rhRLX in the regulation of airway contraction have not been examined. Relaxin is a pregnancy-related hormone, but has also been shown to elicit relaxation of rat renal and mesenteric arteries (McGuane et al., 2011; Leo et al., 2014). In the context of the lung, chronic treatment with rhRLX protected against the development of airway remodeling, reversed established fibrosis and reduced AHR in chronic AAD models (Royce et al., 2009, 2013a).

In this study, we assessed potential dilator effects of rhRLX in comparison with the non-selective β-adrenoceptor agonist

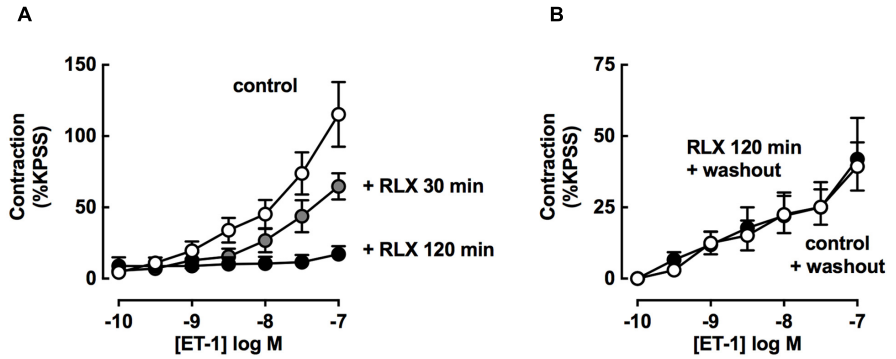

FIGURE 6 | rhRLX-mediated inhibition of tracheal contraction is increased with time and dependent on released factors. ET-1-induced contractions were assessed **(A)** in the absence or presence of rhRLX (RLX, 0.1 μM) following pre-treatment with for 30 and 120 min and **(B)** in the absence of RLX following 120 min in the absence or presence of RLX (0.1 μM) followed by replacement of the bathing solution. All responses are expressed as % KPSS standard contraction (mean ± SEM, $n = 4$–7 per group).

ISO and the selective β_2-adrenoceptor agonist SAL. We also compared rhRLX with the PPARγ agonist RGZ, which was recently shown to exert acute relaxation in mouse trachea and small airways (Bourke et al., 2014; Donovan et al., 2014). We utilized MCh, the gold standard to induce airway contraction both *in vitro* and *in vivo* (Donovan et al., 2013), and ET-1, which is upregulated in asthma (Trakada et al., 2000) and contributes to both airway contraction and fibrosis (Ahmedat et al., 2013).

rhRLX was shown to be an effective but slowly acting dilator, reversing an established MCh-induced contraction in both trachea and intrapulmonary airways in PCLS. Of note, the efficacy of the highest concentration of rhRLX tested was similar to the maximal response to ISO, but this partial relaxation to rhRLX in both large and small airways was achieved at a 10-fold lower concentration than the β-adrenoceptor agonist. RGZ was confirmed as a maximally effective dilator of rat airways, with potency in the high μM range consistent with previous data obtained in mice (Donovan et al., 2015). Future studies will apply higher concentrations of rhRLX than those available for this study to see if complete relaxation can be achieved with greater potency and efficacy than β-adrenoceptor agonists.

Pretreatment with rhRLX (0.1 μM) also inhibited the development of tracheal contraction to the potent bronchoconstrictor ET-1. Similar inhibition was seen with effective dilator concentrations of ISO and RGZ (1 and 100 μM respectively), but only rhRLX was effective when comparisons were made at the same concentration and preincubation time (0.1 μM, 30 min). These findings support a greater bronchoprotective action of rhRLX, in addition to its dilator actions, both evident at lower concentrations than ISO and RGZ.

To identify its site of action, we showed that RXFP1, the cognate receptor for rhRLX, was expressed in both airway epithelium and smooth muscle. In addition, inhibition of airway contraction and relaxation to rhRLX was reduced, but not abolished, by epithelial removal. This is in contrast to acute vascular relaxation to rhRLX that was abolished by endothelial removal alone (McGuane et al., 2011) despite localization of RXFP1 in both endothelial and vascular smooth muscle cells (Novak et al., 2006; Jelinic et al., 2014). In the absence of a commercially available RXFP1 antagonist to confirm receptor-dependence, rhRLX appears to be regulating airway contraction via actions on both epithelium and ASM. Further assessment when RXFP1 expression is reduced or abolished, either by using siRNA strategies *ex vivo* or using airway preparations from a knockout model, should be considered to confirm this mechanism of action.

In exploring the mechanisms underlying the effects of rhRLX, it was notable that rhRLX-mediated relaxation was more rapid in small airways than trachea, occurring within minutes under static conditions. In addition, rhRLX-mediated inhibition of tracheal contraction was almost completely abolished with longer pretreatment, and prevented when the bathing solution was removed prior to assessing contraction. Potentially higher levels of endogenous mediators of relaxation released in response to rhRLX would be present in the relatively smaller chamber used for the lung slice studies or with longer time of exposure. While it remains to be confirmed whether the increased rate and extent of relaxation to RGZ and ISO respectively seen in trachea when in combination with rhRLX is also evident when tested in PCLS, these combined findings are consistent with the requirement for the release and accumulation of endogenous factors for relaxation in response to rhRLX.

The potential identity and origin of these endogenous mediators were then explored. In intact trachea, inhibition of contraction by rhRLX was reduced in the presence of the combination of the NOS inhibitor L-NAME or the COX inhibitor indomethacin, and also by epithelial removal. This implicates both PGE_2 and NO, released from the epithelium, as endogenous mediators contributing to the effects of rhRLX. However, PGE_2 release from ASM has been shown to exert autocrine effects on proliferation and cytokine release (Holgate et al., 2003). Since inhibitory effects of rhRLX on contraction were still evident in epithelial-denuded tissues, rhRLX may also act via RXFP1 on ASM to increase levels of ASM-derived mediators such as PGE_2

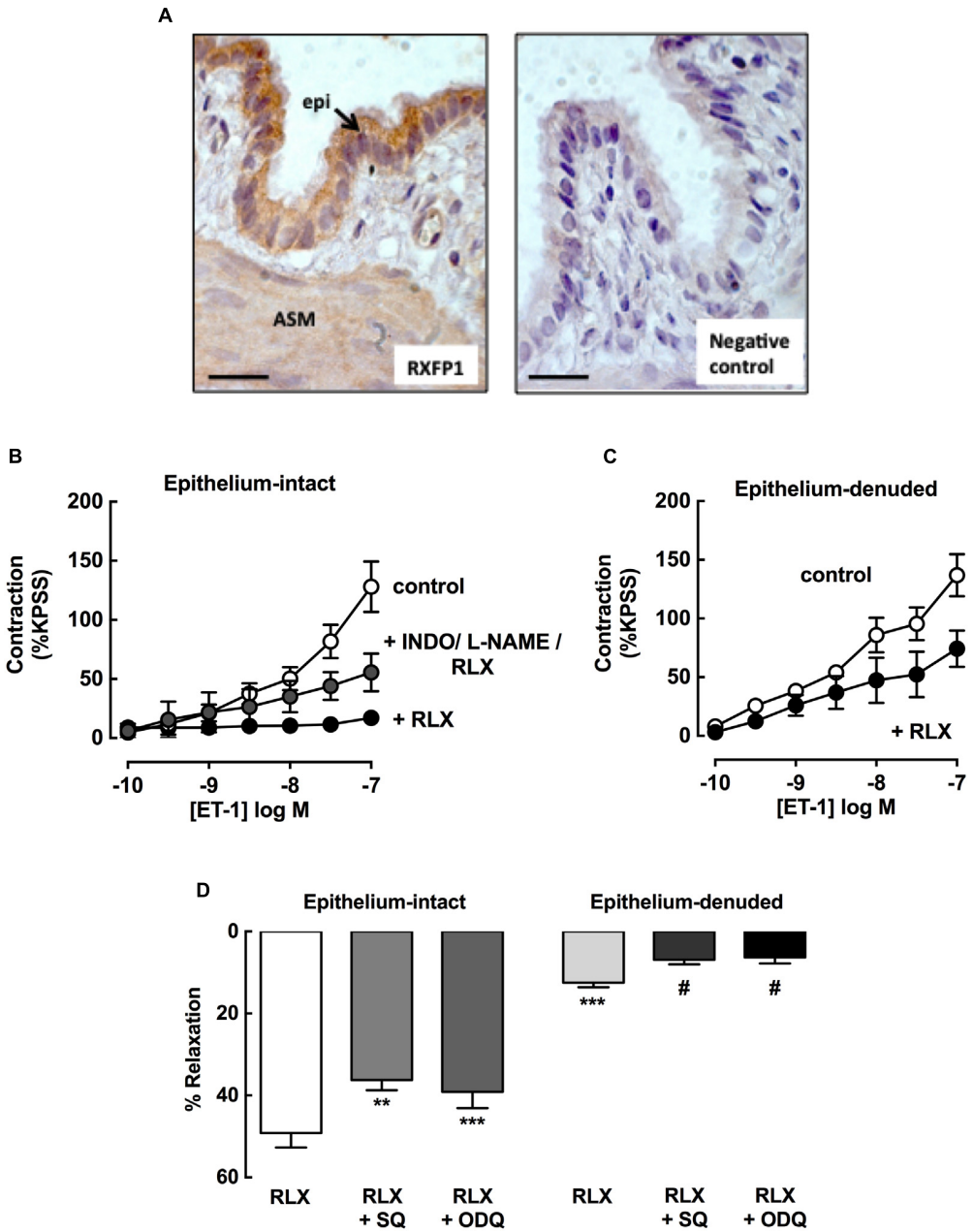

FIGURE 7 | rhRLX acts via epithelial-dependent and -independent mechanisms. RXFP1 protein was localized in rat lungs by immunohistochemistry, and responses to rhRLX (RLX) in epithelial-intact and -denuded trachea were compared in the absence and presence of specific inhibitors. **(A)** Immunoreactive RXFP1 is predominantly localized in airway epithelium (epi) and smooth muscle (ASM). Contraction to ET-1 in **(B)** intact trachea under control conditions, and with RLX (0.1 μM) in the absence and presence of INDO (3 μM) + L-NAME (100 μM) and **(C)** in denuded trachea in the absence and presence of RLX (0.1 μM). Responses to ET-1 are expressed as a % of KPSS contraction. **(D)** Responses to RLX (0.1 μM) in intact and denuded trachea in the absence or presence of SQ22536 (10 μM) or ODQ (1 μM), expressed as % relaxation of MCh pre-contraction. Data is expressed as mean ± SEM (n = 4–8 per group). $^{**}P < 0.01$, $^{***}P < 0.001$, vs. RLX in intact trachea, $\#P < 0.05$ vs. RLX in denuded trachea.

and/or exert direct effects on ASM to oppose contraction. In recent studies, rhRLX was shown to increase prostacyclin release to exert relaxation in vascular tissue (Jelinic et al., 2014; Leo et al., 2014; Sarwar et al., 2016). This further suggests that prostaglandins are key contributors to rhRLX-mediated relaxation.

The guanylate cyclase inhibitor, ODQ and the adenylate cyclase inhibitor SQ22536, in both intact and epithelial-denuded trachea, reduced relaxation to rhRLX to reverse MCh-induced contraction. This finding is also consistent with contributions of NO and PGE_2 released from epithelium and/or ASM in response to rhRLX, as these mediators activate GC and AC respectively in

intact tissue to oppose ASM contraction. However, rhRLX itself has been shown to directly increase cAMP and activate PKA in both transfected HEK cells expressing RXFP1 (Halls et al., 2007) and bronchial epithelial cells to stimulate migration and ciliary beat frequency (Wyatt et al., 2002). Overall, the mechanisms driving relaxation appear to involve indirect effects of rhRLX mediated by NO, and PGE_2 and direct effects of rhRLX on both epithelial cells and ASM.

Characterization of the mechanisms and relative efficacy of novel dilators is necessary to support studies of their potential benefit in combination with existing therapy with β-adrenoceptor agonists. We have previously shown that RGZ reversed MCh-induced contractions in mouse trachea and lung slices through PPARγ- and epithelial-independent mechanisms involving the attenuation of Ca^{2+} oscillations (Bourke et al., 2014; Donovan et al., 2015). We assessed the effects of rhRLX in combination with RGZ, ISO and SAL, to determine whether the mechanism of action of rhRLX, newly identified here, would increase the extent or rate of relaxation to RGZ or the β-adrenoceptor agonists.

Although, the combination of RGZ and rhRLX was not more effective at eliciting relaxation in rat trachea, the response was markedly more rapid than either treatment alone. In addition to its epithelial-independent mechanisms, RGZ has also been shown to inhibit PGE_2 breakdown (Henry et al., 2005), so the PGE_2-dependent pathways contributing to rhRLX-mediated relaxation may have been further enhanced in this particular combination.

The effects of rhRLX in combination with ISO were tested in rat trachea, where relaxation is mediated via both β_1- and β_2-adrenoceptors and ISO elicits rapid but incomplete relaxation. Under these conditions, rhRLX increased ISO-mediated relaxation at a similar rate compared to ISO alone, despite the slow relaxation seen with rhRLX alone. In guinea pig trachea, complete relaxation was mediated via β_2-adrenoceptor agonism by SAL alone, overcoming the contraction due to MCh and any endogenous contractile mediators that may have accumulated during the experiment. SAL was more potent than ISO, and rhRLX markedly increased the potency of both bronchodilators. Thus, although the rapid accumulation of cAMP in response to ISO or SAL may be maximal, the additional ability of rhRLX to generate cGMP (via NO) may contribute to greater relaxation to β-adrenoceptor agonists when their efficacy is limited as in rat trachea, or increase their potency to further enhance dilator responsiveness as in guinea pig trachea.

rhRLX therefore offers intriguing possibilities as an alternative or additional therapy for asthma. Phase 3 clinical trials are already underway for the use of rhRLX to treat acute heart failure, while its safety and efficacy have been established (RELAX-AHF trial, Teerlink et al., 2013). rhRLX has previously been demonstrated to oppose the development of airway remodeling and AHR, and shown to have greater anti-fibrotic effects when used in combination with prednisolone in an experimental model of AAD (Royce et al., 2013b). Here we demonstrate further potential benefits of rhRLX, mediating bronchodilator actions by mechanisms that differ from and potentially enhance responses to β-adrenoceptor agonists. It would therefore be of interest to assess both its bronchodilator efficacy and potential anti-inflammatory actions in this disease context to support its further preclinical evaluation. Future studies should define the therapeutic potential of Serelaxin as an add-on reliever medication for asthma, particularly when β-adrenoceptor responsiveness is limited.

AUTHOR CONTRIBUTIONS

ML designed and performed experiments and assays, and contributed to the writing of the manuscript. CD and MJ performed experiments and contributed to the interpretation of the results and the preparation of the manuscript. SR, LP, and CS contributed to the design of experiments, interpretation of the results and the preparation of the manuscript. JB conceived and organized the study and was the PI, contributed to the design of experiments, interpretation of the results, and wrote the first draft of the manuscript.

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Serelaxin as a novel therapeutic opposing fibrosis and contraction in lung diseases☆

Maggie Lam [a,b], Simon G. Royce [c], Chrishan S. Samuel [a,b], Jane E. Bourke [a,b,*]

[a] Biomedicine Discovery Institute, Monash University, Melbourne, Australia
[b] Department of Pharmacology, School of Biomedical Sciences, Monash University, Melbourne, Australia
[c] Department of Medicine, Central Clinical School, Monash University, Melbourne, Australia

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ABSTRACT

The most common therapies for asthma and other chronic lung diseases are anti-inflammatory agents and bronchodilators. While these drugs oppose disease symptoms, they do not reverse established structural changes in the airways and their therapeutic efficacy is reduced with increasing disease severity. The peptide hormone, relaxin, is a Relaxin Family Peptide Receptor 1 (RXFP1) receptor agonist with unique combined effects in the lung that differentiates it from these existing therapies. Relaxin has previously been reported to have cardioprotective effects in acute heart failure as well anti-fibrotic actions in several organs. This review focuses on recent experimental evidence of the beneficial effects of chronic relaxin treatment in animal models of airways disease demonstrating inhibition of airway hyperresponsiveness and reversal of established fibrosis, consistent with potential therapeutic benefit. Of particular interest, accumulating evidence demonstrates that relaxin can also acutely oppose contraction by reducing the release of mast cell-derived bronchoconstrictors and by directly eliciting bronchodilation. When used in combination, chronic and acute treatment with relaxin has been shown to enhance responsiveness to both glucocorticoids and β_2-adrenoceptor agonists respectively. While the mechanisms underlying these beneficial actions remain to be fully elucidated, translation of these promising combined preclinical findings is critical in the development of relaxin as a novel alternative or adjunct therapeutic opposing multiple aspects of airway pathology in lung diseases.

Contents

Abbreviations: AAD, allergic airways disease; AECs, amnion epithelial cells; AHF, acute heat failure; AHR, airway hyperresponsiveness; Ang II, angiotensin II; AT2, angiotensin type 2 receptor; COPD, chronic obstructive pulmonary disease; CS, cigarette smoke; ERK, extracellular signal-regulated kinases; ET-1, endothelin-1; HEK, human embryonic kidney cells; IPF, idiopathic pulmonary fibrosis; I/R, ischemia-reperfusion; L-NAME, *N*-nitroarginine methyl ester; L-NMMA, L-N^G-monomethyl arginine citrate; MAPK, mitogen-activated protein kinases; MMP, matrix metalloproteinases; MLC20, myosin light chain; MCh, methacholine; MSCs, mesenchymal stem cells; NOS, nitric oxide synthase; OVA, ovalbumin; PKA, protein kinase A; PPARγ, peroxisome proliferator-activated receptor gamma; PKG, protein kinase G; RXFP1, relaxin/insulin-like family peptide receptor 1; sGC, soluble guanylate cyclase; TGFβ, transforming growth factor beta.

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* Corresponding author at: 9 Ancora Imparo Way, Room B112, Clayton Campus, VIC 3800, Australia.
E-mail address: jane.bourke@monash.edu (J.E. Bourke).

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1. Introduction

Chronic respiratory diseases are the third leading cause of death worldwide, accounting for over 3 million deaths globally each year (Lozano et al., 2012). These diseases, including asthma, chronic obstructive pulmonary disease (COPD) and idiopathic pulmonary fibrosis (IPF), are commonly characterized by inflammation and structural changes, leading to a loss of function. In the case of asthma, this persistent airway inflammation and remodelling, which includes fibrosis and increased airway smooth muscle thickness, leads to the development of airway hyperresponsiveness (AHR). Current anti-inflammatory glucocorticoids are unable to reverse remodelling or fibrosis of the airways and/or lung parenchyma, and in more severe disease, both glucocorticoids and bronchodilators become less efficacious. Multifunctional drugs that reverse these established structural changes, reduce AHR and elicit dilator responses would provide novel alternative or additional treatments for severe asthma and other chronic lung diseases characterized by fibrosis and excessive airway contraction.

Relaxin is a peptide originally identified as a hormone of pregnancy and primarily produced in the ovaries and placenta. It is also produced to a lesser extent in the prostate and testes (Bathgate et al., 2013) as well as several non-reproductive organs including the heart (Dschietzig et al., 2001) and lung (Royce, Samuel, and Tang, 2013). It is now known that the actions of relaxin are not restricted to reproductive organs but extend to other organs including the heart, kidneys, skin, vasculature and lungs in both genders (Bathgate et al., 2013; Royce, Moodley, and Samuel, 2014; Samuel, Summers, and Hewitson, 2016).

Relaxin has been shown to promote collagen turnover and abrogate fibrosis. Secretion of endogenous relaxin in pregnancy was thought to facilitate parturition by softening the extracellular matrix of the cervix and relaxing pelvic ligaments (Burger and Sherwood, 1995; Hisaw and Zarrow, 1950; Timmons, Akins, and Mahendroo, 2011). Indeed, treatment of pregnant rats with a neutralizing antibody to relaxin led to more dense collagen bundles in the cervix (Lee, Hwang, Haab, Fields, and Sherwood, 1992), while relaxin-deficient mice demonstrated incomplete cervical ripening and complications during labour that could be prevented by relaxin treatment (Parry et al., 2009). However, a recent Phase II clinical trial showed that intravenous treatment with relaxin to induce labour did not advance the softening of the cervix (Weiss et al., 2016). It has now been suggested that relaxin regulates collagen turnover in pregnancy to maintain vascular compliance and uterine blood flow. Vodstrcil *et al.* (Vodstrcil et al., 2012) showed that rats treated with a monoclonal antibody against relaxin during late pregnancy developed increased uterine artery wall stiffness. The expression of RXFP1 receptors in the smooth muscle layer of the uterine artery (Vodstrcil et al., 2012) also implicated relaxin as a potential vasodilator.

In humans, relaxin belongs to a family of seven peptide hormones involved in the activation of G-protein coupled receptors (Bathgate, 2006). This family comprises of three relaxin peptides, termed H1 relaxin, H2 relaxin and H3 relaxin, and four insulin-like peptides (Wilkinson et al., 2005). The recombinant human form of H2 relaxin (the major stored and circulating form of relaxin), serelaxin, has been shown to possess unique combined abilities to reduce fibrosis, inhibit inflammation, and induce smooth muscle relaxation in multiple organ systems. Its therapeutic potential was assessed in the 2013 RELAX-AHF (phase III) study, where early worsening of acute heart failure in hospital was reduced in serelaxin-treated patients (30 μg/kg/day continuous i.v. infusion for 48 h). Following 6 h serelaxin treatment, these patients demonstrated reduced dyspnoea, which persisted throughout the next 5 days. Results at 180 days also demonstrated lower cardiovascular-related mortality and fewer adverse effects (Teerlink et al., 2013). However, these findings were not confirmed in a larger follow-up RELAX-AHF2 (phase III) study (Novartis press release; March 22nd, 2017). While it remains to be determined whether further assessment of serelaxin for the treatment of acute heart failure is warranted, its safe clinical profile has been established (Díez, 2014; Dschietzig et al., 2009) and accumulating evidence suggests benefit in other disease settings.

This review describes recent experimental findings in the respiratory context supporting the potential therapeutic application of relaxin to oppose airway inflammation, fibrosis and AHR, as well as to directly elicit airway relaxation. Given its capacity to oppose aspects of disease that are not adequately targeted by existing therapy, there is a strong impetus for the clinical assessment of relaxin as a novel multifunctional therapeutic for the treatment of asthma and other lung diseases.

2. Relaxin and RXFP1 expression

Relaxin, a member of the relaxin peptide family, is encoded by the relaxin genes *RLN1*, *RLN2* and *RLN3* and other insulin-like genes in humans. *RLN2* codes for the main circulating form of relaxin, human gene-2 relaxin (relaxin). Relaxin consists of two chains (A and B) linked by disulfide bonds. The B chain contains two arginine residues at positions 13 and 17 required for the binding of relaxin to its cognate G-protein coupled receptor, RXFP1. The expression and distribution of RXFP1 in human and rodent cardiac, vascular and pulmonary tissues have been thoroughly evaluated (Dschietzig et al., 2011; Jelinic et al., 2014; Lam et al., 2016; Royce et al., 2013).

Potential roles for relaxin/RXFP1 in the regulation of contractility, remodelling and fibrosis were suggested by the localization of RXFP1 in neonatal rat cardiac myocytes and endothelial cells combined with relatively high expression of RXFP1 in fibroblasts (The Human Protein Atlas, 2017; Samuel et al., 2004). In addition, RXFP1 was co-localised with α-smooth muscle actin (α-SMA) in smooth muscle in rat abdominal aorta, vena cava and pulmonary, renal and mesenteric arteries (Jelinic et al., 2014). Furthermore, higher expression of RXFP1 in endothelial cells relative to vascular smooth muscle cells suggested that relaxin may regulate contractile function through endothelial-dependent and/or endothelial-independent mechanisms (Jelinic et al., 2014). In the setting of cardiovascular disease, RXFP1 gene expression was similar in mesenteric arteries of spontaneously hypertensive and normal rats (Drongelen et al., 2013), but RXFP1 protein expression was reported to be lower in the atrial myocardium of patients with heart failure compared to non-diseased patients (Dschietzig et al., 2011). To date, the effects of serelaxin on cardiovascular expression of RXFP1 in health and disease have not been assessed. Nevertheless, a potential explanation for the lack of positive findings for serelaxin in the follow-up phase III study (Novartis press release; March 22nd, 2017) compared to the smaller phase III trial (Teerlink et al., 2013) could be due to reduced RXFP1 expression associated with heart failure and/or treatment with serelaxin itself.

In the context of the lung, both relaxin and RXFP1 protein are expressed in human lung biopsies (Royce et al., 2013) and mouse lung tissue (Royce et al., 2013). Evidence of co-expression of relaxin and its receptor have been detected in epithelial cells and to a lesser extent, fibroblasts and airway smooth muscle, suggestive of an autocrine/paracrine mode of hormone action. Decreased expression of relaxin itself was also observed in asthmatic biopsies, and associated with increased thickness of both the epithelial layer and reticular basement membrane (Royce et al., 2013). RXFP1 was shown to be relatively lower in biopsies from subjects with asthma and in fibroblasts from IPF patients compared to healthy controls (Royce et al., 2013; Tan et al., 2016). Somewhat surprisingly, treatment with relaxin increased RXFP1 expression in a mouse model of allergic airways disease (AAD), by a yet to be determined mechanism (Royce, Sedjahtera, Samuel, and Tang, 2013). This suggests that, unlike heart failure, the potential reduction in RXFP1 expression associated with asthma may not limit the therapeutic potential of relaxin, and that the efficacy of relaxin may be maintained or even increased in human lung disease context. These combined findings provide further impetus for assessing the therapeutic benefit of supplementing a potential lack of endogenous

relaxin (Royce, Bathgate, and Samuel, 2016). As detailed below, broad beneficial actions of relaxin in validated animal models of acute and chronic lung diseases have now been clearly established.

3. Anti-inflammatory effects of relaxin

Chronic inflammatory cascades can potentiate fibrosis and contribute to increased airway contraction and disease progression with worsening symptoms in lung diseases (Auger et al., 2016). Although glucocorticoids are commonly used to treat inflammation, they become less effective with increasing disease severity and more prominent fibrosis. This relative insensitivity has been attributed to a variety of intrinsic mechanisms such as increased expression of the inactive glucocorticoid receptor isoform and changes in transcription factor abundance and/or localization (Keenan, Radojicic, Li, Radwan, and Stewart, 2015). However, extrinsic factors, notably changes in extracellular matrix composition including increased collagen, have also been implicated in loss of steroid responsiveness ((Keenan et al., 2015).

4. Animal models of inflammation

There is increasing evidence that serelaxin may elicit acute anti-inflammatory effects in the lung as well as in other organs. In perfused rat lungs *ex vivo*, ischemia-reperfusion (I/R) injury increased neutrophil accumulation, neutrophil elastase and ET-1 levels (Alexiou, Matschke, Westphal, Stangl, and Dschietzig, 2010; Alexiou, Wilbring, Matschke, and Dschietzig, 2013). In the presence of serelaxin, these pro-inflammatory markers were significantly reduced compared to vehicle-treated I/R rat lungs. Although a direct comparison between serelaxin and glucocorticoids has yet to be made in this setting of acute inflammation, *in vivo* pre-treatment with dexamethasone was shown to markedly attenuate I/R-induced oedema, tissue inflammation and inflammatory cytokines (Sun et al., 2009).

Further anti-inflammatory actions of serelaxin have been demonstrated in chronic lung disease models. In a cigarette-smoke (CS) model of COPD, guinea pigs were exposed to 8 week CS treatment, followed by daily serelaxin (1 or 10 μg/day s.c., or 10 μg/mL/day via aerosol) (Pini et al., 2016). Via either route, serelaxin markedly inhibited CS-induced inflammation, reducing pro-inflammatory cytokines, TNFα and IL-1β, in lung tissue whilst increasing levels of the anti-inflammatory cytokine, IL-10. Although the effect of serelaxin on the neutrophilia seen in this chronic model was not assessed, *Ban*i and colleagues have shown that porcine relaxin can inhibit the activation of human neutrophils (Masini et al., 2004) as well as the lipopolysaccharide-induced adhesion of neutrophils to coronary endothelial cells in rats (Nistri, Chiappini, Sassoli, and Bani, 2003) (via a nitric oxide (NO)-dependent mechanism; suggesting that serelaxin may inhibit neutrophil infiltration and/or activation as part of its anti-inflammatory actions.

In the context of airway inflammation associated with asthma and allergy, serelaxin shows promise but its effects have yet to be fully defined. In ovalbumin (OVA)-sensitized guinea pigs, pretreatment with serelaxin (twice daily for 4 days before each antigen challenge) inhibited the migration of pro-inflammatory cells into the lung and reduced mast cell degranulation and release of histamine after allergen challenge (Bani, Ballati, Masini, Bigazzi, and Sacchi, 1997). However, in an OVA-induced mouse model of chronic AAD, the increase in eosinophils and inflammatory cell infiltration was not affected by relaxin treatment administered after the onset of established inflammation (Royce et al., 2009; Royce et al., 2013). Dexamethasone has consistently been shown to reduced levels of both eosinophils and Th2-cytokines including IL-5, -6, -13 and 17A in chronic asthma models (Herbert et al., 2008). These effects of dexamethasone (Patel, Giraud, Samuel, and Royce, 2016) or other corticosteroids (Royce et al., 2013) were not enhanced, but only maintained by serelaxin co-administration. Serelaxin was more recently found to promote macrophage polarization in the kidney towards a potentially tissue-repairing M2 macrophage phenotype (Chen et al., 2017); and may thus promote similar effects in the lung (Jiang and Zhu, 2016).

5. Anti-fibrotic effects of relaxin

Fibrosis is the major contributor to the pathology of chronic respiratory diseases and accumulation of fibrotic tissue is associated with more severe disease and potential loss of sensitivity to therapy (Bergeron, Tulic, and Hamid, 2010). While extracellular matrix deposition is critical for normal wound repair and restoration of organ function, dysregulation of the balance of synthesis and degradation of collagen can lead to excessive scarring, reducing compliance and dilator responsiveness in fibrotic airways. There is an unmet need for effective therapeutics that abrogate the increasing contribution of fibrosis to airway remodelling seen in more severe asthma, as this is unresponsive to steroid treatment. Accumulating evidence demonstrates the significant potential of serelaxin as a novel anti-fibrotic therapy for chronic lung diseases.

5.1. Fibroblasts in vitro

Serelaxin has been shown to limit excessive fibrosis by promoting the degradation of collagen via the increased activation of matrix metalloproteinases (MMPs) such as collagenases and gelatinases. Several studies have characterised the *in vitro* effects of serelaxin on dermal, renal and cardiac fibroblasts, showing that relaxin consistently reduces transforming growth factor beta-1 (TGFβ1)-stimulated increases in proliferation, procollagen-α1 mRNA, collagen synthesis and secretion (Masterson et al., 2004; Samuel et al., 2004; Unemori and Amento, 1990). In TGF-β1-stimulated human dermal fibroblasts and primary rat renal myofibroblasts, MMP-1, -2, -9 and -13 respectively were increased with serelaxin treatment (Chow et al., 2012). Similar findings have been obtained in human lung fibroblasts, where enhanced collagen and fibronectin expression in response to TGF-β1 was inhibited by via the increased expression of pro-collagenase (MMP-1) (Unemori et al., 1996). These actions of serelaxin would be predicted to effectively inhibit and potentially reverse excessive fibrosis and remodelling in the context of lung diseases.

5.2. Animal models of fibrotic disease

In animal models, relaxin deficiency has been shown to be related to more severe age-related lung fibrosis (Samuel et al., 2003). Parenchymal fibrosis, bronchial epithelial thickening and enhanced collagen deposition around pulmonary arterioles were higher in relaxin-deficient mice compared to wild-type mice by 9 months of age (Samuel et al., 2003). This lung phenotype, reminiscent of increased pulmonary fibrosis, resulted in lower peak expiratory flow and lung recoil. In addition, these mice demonstrated airway wall thickening and a focal loss of alveolar structure. With 14 day serelaxin treatment, total collagen content in the lungs of 9- and 12-month old relaxin-deficient mice was reduced to levels exhibited by age-matched wild-type mice (Samuel et al., 2003).

Serelaxin has also been demonstrated to have beneficial effects on key histological markers of bronchial remodelling in *in vivo* models of COPD, IPF and asthma. In a chronic model of COPD, daily systemic or aerosol administration of serelaxin to guinea pigs abrogated increases in airway smooth muscle thickness and collagen accumulation induced over 8 week exposure to CS (Pini et al., 2016). In contrast, studies in numerous preclinical animal models have shown that the inflammatory and structural changes associated with CS exposure are steroid-insensitive (Stevenson and Belvisi, 2008).

In mouse models utilising bleomycin to induce key features of IPF, pirfenidone was shown to have potential of as an anti-fibrotic agent (Mouratis and Aidinis, 2011), while glucocorticoids reduced oedema

but failed to suppress pulmonary fibrosis (Langenbach et al., 2007; Oku et al., 2008). Relaxin-deficient mice had exaggerated fibrosis in response to bleomycin (Huang et al., 2011), while treatment with serelaxin itself or continuous subcutaneous infusion of a relaxin-like peptide, CGEN25009, reduced bleomycin-induced lung fibrosis (Huang et al., 2011; Pini et al., 2010; Unemori et al., 1996). In these studies, serelaxin reduced parenchymal collagen deposition, as measured by histological scoring of alveolar fibrosis and hydroxyproline assays of total lung collagen (Huang et al., 2011; Pini et al., 2010; Unemori et al., 1996). A direct comparison of the relative efficacy of serelaxin and pirfenidone on reversal on established fibrosis would therefore be of interest in determining their relative therapeutic potential in the treatment of IPF.

In models of AAD, airway remodelling including fibrosis is implicated in the development of AHR (Russell and Brightling, 2017). Increases in subepithelial collagen were amplified in relaxin-deficient mice following allergen challenge, with these mice having lower levels of MMP-9 and failing to respond to OVA with the compensatory increase in MMP-2 seen in wild-type mice. Despite this, AHR was not greater in OVA-challenged relaxin-deficient mice compared to their wild-type counterparts. However, when infused over the second half of a 4-week exposure to OVA, serelaxin (1 mg/mL, s.c.; 1 μL/h for 2 weeks) reduced airway collagen (Kenyon, Ward, and Last, 2003). Treatment was also able to reverse established OVA-induced remodelling and fibrosis (Royce et al., 2009; Royce et al., 2013; Royce et al., 2014). These effects were mediated via the upregulation of MMP-2 and -9 to increase collagen turnover and inhibition of TGF-β1 signal transduction at the level of Smad2 phosphorylation to decrease collagen synthesis (Royce et al., 2009; Royce et al., 2013; Royce et al., 2014). When serelaxin was administered either during or post-allergen challenge, AHR to methacholine (MCh) was reduced (Royce et al., 2009; Royce et al., 2013; Royce et al., 2014). This reduction in AHR was more likely due to the effects of serelaxin on inflammation and fibrosis rather than directly mediated through inhibition of ASM contraction as it was still evident when MCh-induced contraction was assessed approximately 24 h after the last drug treatment.

Further studies in chronic allergen challenge models have compared the effects of treatment with serelaxin, human bone marrow-derived mesenchymal stem cells (MSCs) and human amnion epithelial cells (AECs), alone and in combination (Royce et al., 2015; Royce et al., 2016; Royce, Rele, Broughton, Kelly, and Samuel, 2017). In the absence of serelaxin, induced pluripotent stem cell-derived MSCs normalized allergen-induced airway and lung fibrosis and AHR, but only partially reduced peribronchial inflammation, goblet cell metaplasia and epithelial thickening (Royce et al., 2017). Although AECs alone demonstrated greater protection against the airway wall remodeling compared with MSCs alone, combining serelaxin with MSCs or AECs reversed airway fibrosis to an even greater extent as well as reversing established AHR (Royce et al., 2015; Royce et al., 2016). Thus, while many of the beneficial effects of stem cells alone were similar to relaxin, it is likely that combined treatment with relaxin may aid those stem cells that offer limited therapeutic efficacy on their own when administered to chronic disease settings.

The effects of combination treatment with serelaxin and methylprednisolone has also been assessed with both drugs administered during chronic allergen challenge (Royce et al., 2013). In this particular model, glucocorticoid treatment alone did not reduce fibrosis or AHR, but the combined effects of methylprednisolone and serelaxin reduced subepithelial collagen thickness to a greater extent than corticosteroid treatment alone. Additionally, the inhibition of AHR induced by serelaxin alone was maintained with the combination treatment (Royce et al., 2013).

These combined findings extend the positive *in vitro* findings in human lung fibroblasts to provide strong evidence of the antifibrotic efficacy of serelaxin *in vivo* when administered either systemically or to the lung in multiple mouse models relevant to asthma, COPD and IPF.

6. Inhibition of contractility by relaxin

6.1. Fibroblast contractility

Fibrosis is associated with differentiation of fibroblasts to myofibroblasts, characterized by increased expression of contractile proteins as well as greater synthesis of collagen (Westergren-Thorsson et al., 2010). In addition to its actions in promoting collagen degradation, serelaxin has been shown to abrogate TGF-β1-stimulated increases in alpha smooth muscle actin (α-SMA) expression and contraction of collagen I gel matrices by cardiac and renal fibroblasts (Masterson et al., 2004; Samuel et al., 2004). Both fibroblast foci in lung biopsies from IPF patients and the lungs of mice following bleomycin treatment show increased levels of MLC20, a biomarker of *in vivo* cellular contractility (Huang et al., 2011). *In vitro* treatment with exogenous serelaxin prevented both collagen deposition and the increase in MLC20 in fibroblasts from IPF patients, while MLC20 overexpression prevented the protective effects of serelaxin. The functional consequences of serelaxin-mediated inhibition of MLC20 were confirmed by the reduction in the number of wrinkle-forming fibroblasts. In the same study, an even greater increase in bleomycin-induced MLC20 expression was evident in relaxin-null mice and prevented by *in vivo* treatment with serelaxin (14 days after bleomycin administration) (Huang et al., 2011).

6.2. Airway contractility in vitro

Chronic lung diseases are often associated with increased airway contraction that may not be adequately treated with current bronchodilators (Cazzola, Page, Calzetta, and Matera, 2012). Of note, saline-treated relaxin-deficient mice exhibit AHR even in the absence of allergen challenge (Samuel et al., 2007) and chronic treatment with serelaxin has consistently been shown to reverse established AHR in mouse models of AAD (Royce et al., 2009; Royce et al., 2014), identifying a potential role for serelaxin in the regulation of airway contractility as well as fibrosis. Indeed, the expression of RXFP1 in uterine, vascular as well as airway smooth muscle and their associated endothelium and epithelium suggests that serelaxin may directly regulate muscle tone.

The vasodilator actions of serelaxin have been well-established *in vitro*, and include partial relaxation of rat uterine arterial rings precontracted with phenylephrine (Longo et al., 2003). Ang-II-induced maximum constriction was greater in small mesenteric arteries from pregnant relaxin-deficient mice compared to matched wild-type mice, further implicating the protective effects of endogenous levels of this hormone (Marshall et al., 2016).

Until recently, only one study had assessed the effects of serelaxin on airway contraction. In mouse lung strips precontracted with carbachol, a single addition of 1000 nM serelaxin elicited approximately 75% of the maximal relaxation to the non-selective β-adrenoceptor agonist isoprenaline (Samuel et al., 2003). More recently, the potential bronchodilator and bronchoprotective actions of serelaxin relative to β-adrenoceptor agonists have been characterized in more detail, using both large and small airway preparations from rat and guinea pig (Lam et al., 2016). In rat trachea precontracted with MCh, serelaxin (100 nM) elicited partial relaxation with greater potency than isoprenaline (10 μM), albeit at a slower rate. Bronchoprotection by serelaxin was also evident, with MCh and endothelin (ET)-1-induced contraction almost completely abolished with increasing pre-incubation time of serelaxin, suggesting that some of the *in vivo* effects of chronic treatment with serelaxin in reducing AHR may be due to direct inhibition of contraction.

Most *in vitro* studies of novel dilators have been limited to assessment of their effects in trachea and readily accessible bronchi. However, small intrapulmonary airways are important sites of structural injury and inflammation in disease (Cottini, Lombardi, and Micheletto, 2015). The precision cut lung slice (PCLS) technique allows analysis of reactivity of intrapulmonary airways *in situ* that cannot be achieved in

organ bath/myograph set-ups (Sanderson, 2011). Using both of these techniques, rat airways were precontracted with MCh and shown to relax to 100 nM serelaxin with similar efficacy in PCLS as in trachea (Lam et al., 2016). In contrast, β-adrenoceptor agonists have been shown to be less effective in small airways compared to larger airways (Finney, Karlsson, and Persson, 1985). The maintained efficacy of serelaxin in the distal airways would thus appear to be of potential relative benefit in asthma. However, the slower rate of serelaxin-mediated relaxation compared to β-adrenoceptor agonists suggests that it may not be suitable as a stand-alone therapy for rapid relief of symptoms.

Of note, serelaxin has been demonstrated to increase dilator responsiveness of guinea pig airways to β-adrenoceptor agonists. The potency of both isoprenaline and the clinically used selective β_2-adrenoceptor agonist, salbutamol were increased almost 30-fold in the presence of a submaximal concentration of serelaxin, suggesting the potential of serelaxin as an effective add-on therapy for asthma (Lam et al., 2016). Defining the mechanism of this marked serelaxin-induced potentiation will be important in its further preclinical development. As outlined later in this review, the signaling pathways whereby serelaxin elicits relaxation and increases salbutamol potency could be independent of cAMP-dependent pathways downstream of β_2-adrenoceptor signaling in airway smooth muscle, but may at least in part rely on generation of epithelial-derived relaxing factors. Confirmation that these direct effects of serelaxin to oppose airway contraction and enhance relaxation to current bronchodilators are maintained in animal disease models both in isolated airways and *in vivo* and in human airways is required to provide further support for clinical assessment of serelaxin as a novel bronchodilator.

7. Signalling mechanisms of relaxin

7.1. Receptor-dependence

Although serelaxin is thought to mediate its actions via activation of RXFP1, selective RXFP1 antagonists have yet to be used to confirm the receptor dependence underlying its beneficial effects in the lung. However, RXFP1-deficient mice have provided some insights into the mechanism underlying its protective roles.

RXFP1-dependence has been implicated in the potential effects of endogenous relaxin on age-related fibrosis *in vivo*, since both peribronchial and perivascular fibrosis occurred more rapidly in the lungs of RXFP1-null mice than their age-matched wild-type counterparts (Feng, Bogatcheva, Kamat, and Agoulnik, 2005; Kamat et al., 2004; Samuel et al., 2009).

The relaxin analogue B7–33, a single B-chain mimetic of serelaxin, has been shown to activate RXFP1, and showed similar anti-fibrotic efficacy to serelaxin in a chronic AAD model (Hossain et al., 2016), supporting a key role for RXFP1 in mediating the actions of serelaxin. However, gene deletion of RXFP1 did not exacerbate OVA-induced inflammation, with similar increases in serum IgE, and circulating or bronchoalveolar lavage (BAL) cells seen in RXFP1-deficient mice and wild-type mice (Samuel et al., 2009). OVA-treated RXFP1-knockout mice also had comparable levels of myofibroblast differentiation markers, collagen deposition and gelatinase expression compared to OVA-treated wild-type mice. These combined findings suggest that while serelaxin may act via RXFP1 in regulating/protecting against collagen deposition and fibrosis to maintain homeostasis, it may act through both RXFP1-dependent and -independent pathways to oppose

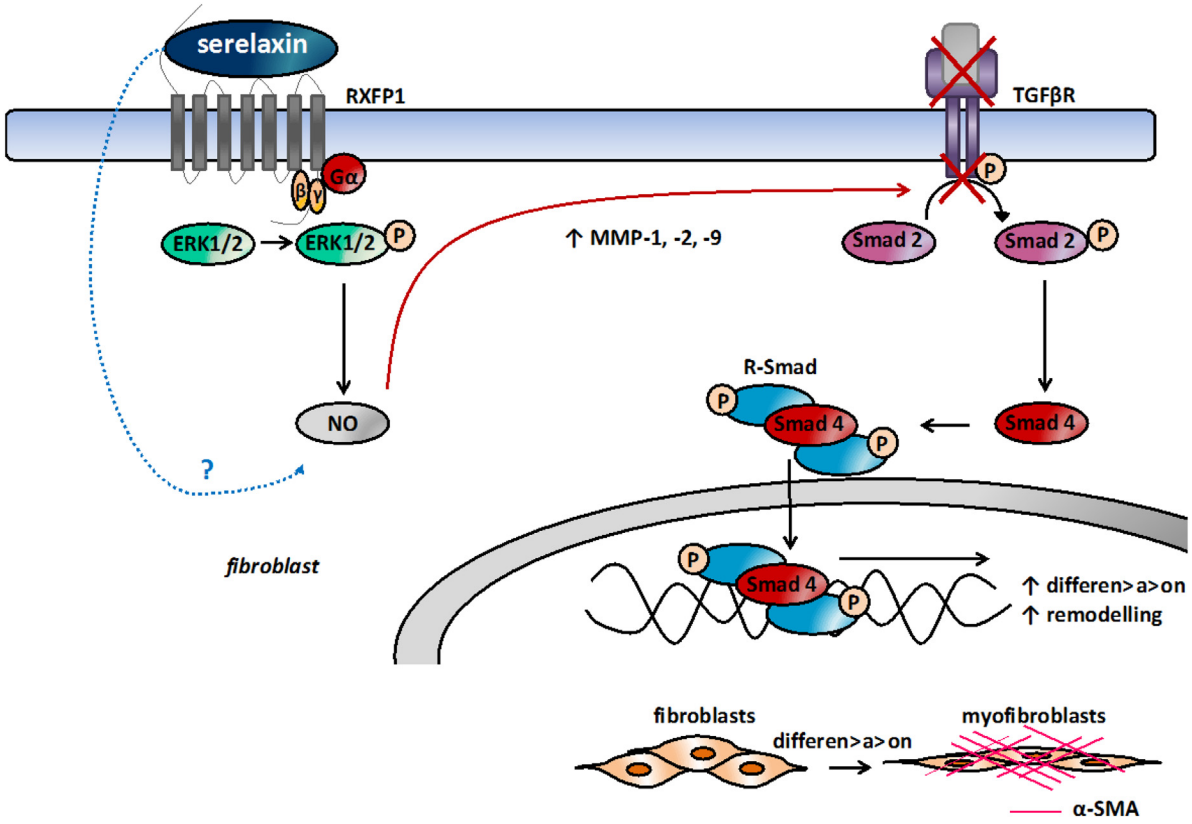

Fig. 1. Serelaxin opposes TGF-β1-induced fibrosis. (revised from (Chow et al., 2012)). TGFβ-1 activates its receptor on fibroblasts to cause Smad2 (and Smad3) phosphorylation (the complexing of phosphorylated Smad2 and phosphorylated Smad3 with Smad4; and the translocation of Smad2/3/4 complexes from the cytoplasm to the nucleus) leading to fibroblast differentiation into myofibroblasts. This is evidenced by increased α-smooth muscle actin (α-SMA), excessive collagen production and fibrosis. Serelaxin acts on its cognate RXFP1 receptor to activate two subclasses of G-proteins, $G_{\alpha s}$ and $G_{\alpha oB}$ (not shown), but may also act via RXFP1-independent pathways. Stimulation of the RXFP1 receptor by serelaxin causes ERK1/2 phosphorylation and stimulation of neuronal nitric oxide (NO) synthase (nNOS)-induced NO/sGC/cGMP signalling. NO/sGC/cGMP inhibits Smad2 phosphorylation and increases the expression of matrix metalloproteinases-1, -2 and -9 (MMP-1, -2, -9). These gelatinases help to break down extracellular matrix proteins to ultimately reduce the severity of fibrosis.

the fibrosis associated with inflammation in AAD. The role of RXFP1 in the regulation of airway smooth muscle contraction by serelaxin remains to be confirmed.

Serelaxin may also mediate effects via glucocorticoid receptor (GR) activation. Inhibition of endotoxin-stimulated secretion of cytokines from human macrophages by serelaxin was prevented by the GR antagonist RU-486 (Dschietzig, Bartsch, Baumann, and Stangl, 2009), and associated with GR binding and activation, nuclear translocation and DNA binding (Dschietzig, Bartsch, Stangl, Baumann, and Stangl, 2004). However, the protective effects of serelaxin in reducing I/R-induced inflammatory changes in rat lungs were not affected by RU-486 (Alexiou et al., 2013). Thus, it appears that serelaxin may inhibit inflammation via actions as an agonist at both GR and RXFP1, and therefore has the potential to offer additional benefit over commonly used glucocorticoids acting via GR alone. Despite these findings, the direct activation of GR by relaxin is highly controversial and has yet to be replicated by other studies.

In addition, serelaxin has been implicated as an indirect mediator of the angiotensin type 2 (AT2) receptor pathway to oppose renal fibrosis. Through bioluminescence resonance energy transfer, serelaxin was shown to act through heterodimers that could form between RXFP1 and the AT2 receptor to increase pERK1/2 and nNOS expression (Chow et al., 2014). While angiotensin II signalling has not been associated with fibrosis in the airways, further investigation into this pathway may provide insight into the potential role of serelaxin in vascular remodelling in pulmonary arterial hypertension via its actions through AT2 receptors.

Another alternative signalling pathway mediating the effects of serelaxin has been demonstrated in cells overexpressing (HEK-RXFP1) or naturally expressing (THP-1) RXFP1, whereby antifibrotic effects were mediated through activation of PPARγ via the cAMP/PKA pathway (Singh and Bennett, 2009; Singh, Simpson, and Bennett, 2015). Although this mechanism for serelaxin has yet to be tested in the lung, PPARγ receptor agonists such as rosiglitazone are well-established anti-fibrotic agents in chronic lung diseases (Honda, Marquillies, Capron, and Dombrowicz, 2004; Tan, Dagher, Hutton, and Bourke, 2010) and like serelaxin itself, have been shown inhibit the development of AHR and cause direct relaxation of both large and small airways (Bourke et al., 2014; Honda et al., 2004; Ward, Gould, Harris, Bonacci, and Stewart, 2004).

7.2. Downstream signalling

Multiple mechanisms have been implicated in the actions of serelaxin in opposing inflammation, reducing fibrosis and eliciting relaxation. Serelaxin has been shown to exert its beneficial effects by inhibiting the downstream signalling pathways of specific drivers of fibrosis, and by opposing multiple mechanisms driving inflammation or contraction.

The coupling of RXFP1 activation to ERK1/2/NO/sGC/cGMP signalling has been well characterized, leading to anti-inflammatory, anti-fibrotic and dilator effects. In a model of I/R injury of rat lungs, the inhibition of neutrophilic inflammation by serelaxin was associated with protection against I/R induced-downregulation of eNOS expression and NOS activity. This anti-inflammatory effect was blunted by PD-98059 and wortmannin implicating PI3K and ERK1/2 as downstream mediators of serelaxin-induced NO production (Alexiou et al., 2013). Additionally, *in vitro* activation of human neutrophils, induced in response by different proinflammatory agents (N-formylmethionine-leucyl-phenylalanine and phorbol-12-myristate-13-acetate), was inhibited by serelaxin. This inhibition was prevented by co-treatment with L-NMMA, an inhibitor of NO production (Masini et al., 2004). Thus, serelaxin and associated increases in NO signalling could confer protection following acute lung injury, and may also be of relevance in limiting neutrophil activation in lung diseases such as severe asthma and COPD.

Serelaxin may also exert its anti-fibrotic effects through the NO/sGC/cGMP pathway to negate the pro-fibrotic influence on TGF-β1 signalling (Fig. 1). In rat renal myofibroblasts (activated fibroblasts), inhibition of TGF-β1-induced collagen synthesis and gelatinase activity by serelaxin was prevented by the ERK1/2 inhibitor, PD98059, the NOS inhibitor, L-NAME, and the guanylate cyclase inhibitor, ODQ (Chow et al., 2012; Wang et al., 2016). Similar findings were seen in lung myofibroblasts where serelaxin-mediated inhibition of collagen synthesis was cAMP-independent, but associated with increases in NO synthesis and cGMP levels, and prevented by inhibition of NOS (Huang et al., 2011).

In terms of specifically regulating TGF-β1 signalling, serelaxin was shown to activate RXFP1 to inhibit TGF-β1-induced phosphorylation and translocation of Smad2 to the nucleus in human renal fibroblasts, in the absence of any direct effects on Smad3, Smad4, Smad7 or ERK/MAPK (Heeg et al., 2005). An additional potential mechanism has been described in cardiac fibroblasts, whereby serelaxin enhances Notch signaling to inhibit TGF-β-induced differentiation of fibroblast to myofibroblast (Sassoli et al., 2013). Consistent with these findings, *in vivo* treatment with serelaxin was able to disrupt TGF-β1 signal transduction in a chronic model of AAD (Royce et al., 2014). Allergen challenge increased TGF-β1, airway collagen deposition and AHR, with subsequent serelaxin treatment reducing Smad2 phosphorylation without affecting TGF-β1 expression, leading to partial reversal of the pro-fibrotic actions of TGF-β1 in the lung as well as reduced AHR (Royce et al., 2014).

The effects of serelaxin on smooth muscle tone may be mediated either by direct effects or via increased synthesis of endothelial/epithelial-derived factors to oppose contraction (Fig. 2). Evidence from vascular studies implicates endothelial-derived NO (Bani et al., 1998;

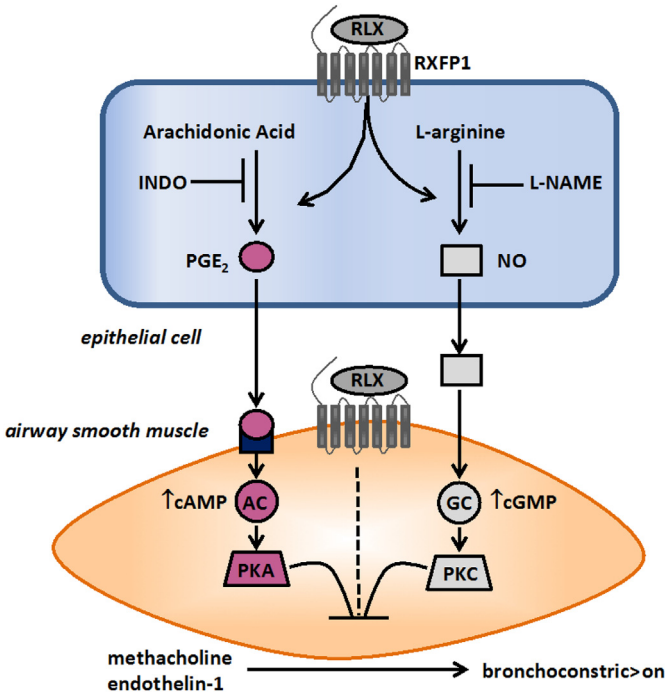

Fig. 2. Serelaxin opposes airway contraction. Serelaxin inhibits the development of contraction, reverses established precontraction and potentiates relaxation to β-adrenoceptor agonists in trachea and small intrapulmonary airways (Lam et al., 2016). Airway relaxation to serelaxin may be mediated via its cognate receptor, although RXFP1-independent pathways (not shown) may also be involved. Relaxation is reduced but not abolished by epithelial removal, indomethacin (INDO) and/or L-NAME. Dilator effects of serelaxin in epithelial-denuded airways are further reduced by adenylate cyclase (AC) and guanylate cyclase (GC) inhibition. Thus, the mechanism of action of serelaxin appears to involve COX-derived prostaglandin E_2 (PGE_2), NOS-derived nitric oxide (NO) signalling as well as direct effects on airway smooth muscle cAMP and cGMP. cAMP-independent mechanisms may contribute to increased potency of β-adrenoceptor agonists in the presence of serelaxin.

Failli et al., 2002) and prostaglandins, and subsequent increases in cGMP and cAMP since serelaxin-mediated relaxation of rat uterine artery was decreased by L-NAME, ODQ, and the adenylate cyclase inhibitor, SQ22–22 (Longo et al., 2003), while endothelial removal abolished acute serelaxin-induced vasodilation of small rat renal arteries (McGuane et al., 2011). Similar downstream mechanisms to those established in the vascular context have been implicated in the regulation of airway smooth muscle contraction by serelaxin. In rat trachea, the inhibition of ET-1-induced contraction by serelaxin was partially prevented by either L-NAME or the COX inhibitor, indomethacin (Lam et al., 2016), highlighting the importance of NO and prostaglandin synthesis in mediating its actions. However, epithelial denudation of airways only partially suppressed the bronchoprotective effects of serelaxin (Lam et al., 2016). Furthermore, since ODQ and the adenylate cyclase inhibitor, SQ2236, caused further inhibition of serelaxin-induced relaxation in these epithelial-denuded trachea, serelaxin may also be having direct actions on airway smooth muscle via cGMP- and cAMP-dependent pathways to contribute to the relaxation response. The capacity of serelaxin to elicit relaxation via these multiple mechanisms may allow its efficacy to be maintained even if epithelial-mediated signalling is impaired as part of the disease process.

7.3. Serelaxin as a novel therapeutic in asthma

Given the prevalence and associated morbidity of chronic lung diseases, improved therapies are clearly required. Serelaxin possesses a unique suite of complementary actions (summarized in Table 1) that may overcome the limitations of current treatments by ameliorating the multiple pathologies contributing to these diseases.

As previously described, serelaxin inhibits the inflammatory response to acute lung injury and to exposure to allergens and cigarette smoke, by limiting the influx of inflammatory cells and reducing cytokine levels in the lung (Alexiou et al., 2013; Pini et al., 2016). Notably, unlike glucocorticoids, serelaxin inhibited cigarette-smoke-induced neutrophilia, suggesting potential benefit in a setting of steroid resistance.

The anti-fibrotic effects of serelaxin have been established in lung disease context, including *in vitro* models using fibroblasts from IPF patients and *in vivo*, inhibiting the development of parenchymal fibrosis in a bleomycin-injured mouse model. Furthermore, a significant body of evidence supports the efficacy of serelaxin in reversing established steroid-resistant airway fibrosis in chronic AAD, with potential additive effects when combined with glucocorticoids or stem cell-based therapies to further reduce fibrosis and AHR. In trials for scleroderma, serelaxin had either beneficial or limited effects (Khanna et al., 2013; Seibold et al., 2000). Inconsistencies in its ability to reduce skin thickening may relate to differences in the severity of disease in treated patients in different trials and/or insufficient duration of serelaxin treatment for benefit against skin fibrosis, which is known to be particularly severe in established scleroderma. The consistently positive in vitro and in vivo findings observed for serelaxin in the lung may therefore be due to the relatively lower levels of collagen seen in these models of asthma, COPD and IPF. This could lead to more effective inhibition or reversal of fibrosis in the lung compared to skin when administered for an appropriate length of time. As such, clinical evaluation of serelaxin is warranted, particularly given the lack of effective treatments targeting this key aspect of disease pathology.

Although yet to be confirmed in disease context, serelaxin has recently been shown to exert direct effects on airway contraction, exerting both bronchoprotective and bronchodilator actions and the ability to increase the potency of β-adrenoceptor agonists (Lam et al., 2016). These findings suggest that while relaxin may be a useful monotherapy, it is likely to be more beneficial when used in combination, by

Table 1
Acute and chronic effects of relaxin.

		Effect of serelaxin	[Serelaxin]	Species
Cells	Neutrophils	↓ activation of neutrophils ↑ nitric oxide synthase expression ↑ nitric oxide generation	1–100 nM	Human (Kamat et al., 2004)
	Lung fibroblasts	↓ interstitial collagen type I and III ↓ collagen deposition ↑ MMP-2 ↓ contractile protein (MLC20)	0.02–16.8 nM (Masterson et al., 2004) 100 nM (Patel et al., 2016)	Human Normal (Masterson et al., 2004) IPF (Patel et al., 2016)
Isolated tissues	Airways (pre-treatment)	↓ development of MCh, ET-1 contraction in trachea ↑ dilator potency of β-adrenoceptor agonists	100 nM	Rat (Dschietzig et al., 2009) Guinea pig (Dschietzig et al., 2009)
	Airways (post-treatment)	↑ relaxation after MCh, ET-1 pre-contraction of trachea/small airways ↑ relaxation after carbachol pre-contraction of lung strips	100 nM (Dschietzig et al., 2009) 1000 nM (McGuane et al., 2011)	Mouse (Dschietzig et al., 2009; McGuane et al., 2011) Rat (Dschietzig et al., 2009) Guinea pig (Dschietzig et al., 2009)
	Lung ischemia-reperfusion	↓ neutrophil accumulation ↓ neutrophil eastase ↓ ET-1 levels	5 nM	Rat (Hossain et al., 2016; Huang et al., 2011)
Models	Cigarette smoke/COPD (co-treatment)	↓ TNF-α, IL-1β ↑ IL-10 ↓ airway smooth muscle thickness ↓ collagen accumulation	1, 10 μg/day s.c. or 10 μg/mL/day i.n. for 8 weeks	Guinea pig (Jiang and Zhu, 2016)
	Bleomycin/IPF (post-bleomycin)	↓ lung injury (TBARS) ↓ leukocyte infiltration (MPO) ↓ bronchial smooth muscle layer thickness ↓ parenchymal collagen deposition ↓ contractile protein (MLC20)	0.6 mg/kg s.c.; 0.25 μL/h for 14–17 days (Heeg et al., 2005) 100 μg i.p for 7 days (Pini et al., 2016)	Mouse (Heeg et al., 2005; Pini et al., 2016)
	Ovalbumin/AAD/asthma (co-treatment)	↓ mast cell degranulation ↓ leukocyte adhesion ↓ subepithelial thickness ↓ collagen deposition ↓ AHR ↓↓ AHR with methylprednisolone	30 μg/kg, twice daily for 4 days (Kenyon et al., 2003) 0.8 mg/mL; 0.5 μL/h for 14 days (Khanna et al., 2013)	Guinea pig (Kenyon et al., 2003) Mouse (Khanna et al., 2013)
	Ovalbumin/AAD/asthma (post-allergen)	↔ BAL cell count ↑ MMP-2 and MMP-9 ↓ collagen deposition ↓ AHR	0.5 mg/kg for 14 days	Mouse (Khanna et al., 2013; Royce et al., 2014; Royce et al., 2016; Samuel et al., 2004)

increasing the effectiveness of current treatments. It remains to be determined whether this is applicable to related drugs, such as the relaxin-like peptide CGEN25009, which has been shown to inhibit fibrosis and inflammation (Tan et al., 2016) but has yet to be assessed for bronchodilator efficacy.

As a long-term triple therapy, combining relaxin with a glucocorticoid and a long-acting β-adrenoceptor agonist may overcome the limited anti-remodelling actions of current preventer medication (Bergeron et al., 2010). The acute use of relaxin combined with a short-acting β-adrenoceptor agonist as a reliever may reduce the dilator dose required to reverse symptoms associated with airway hyperresponsiveness, and limit the loss of bronchoprotection evident with frequent high-dose use of salbutamol (Yim and Koumbourlis, 2013). In the absence of off-target effects in the clinical RELAX-AHF trials, serelaxin itself appears to have a positive safety profile when administered systemically which would be further limited when administered by inhalation.

Future studies assessing serelaxin are required to better define the mechanisms underlying these promising findings, including its receptor dependence and downstream signalling pathways. In particular, it is important to establish whether serelaxin elicits its chronic actions in regulating inflammation and fibrosis through the same mechanisms as its acute bronchodilator responses. It is critical to elucidate the beneficial effects of serelaxin when used alone under conditions where current treatments are ineffective, and to assess its potential for additivity or synergy in combination with suboptimal therapies. Further confirmation of efficacy in validated models of chronic lung diseases relative to current therapy will support clinical translation of serelaxin as a novel therapeutic opposing fibrosis and contraction in lung diseases.

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