CHARACTERISATION OF PROTECTIVE SECRETORY RESPONSES OF THE AIRWAY EPITHELium ELICITED BY ACUTE ACROLEIN EXPOSURE IN AN EX VIVO MODEL OF MOUSE AIRWAYS

Esther Yiern Cheah
BSc (Hons)

This thesis is presented for the degree of Doctor of Philosophy of The University of Western Australia

School of Medicine and Pharmacology
Faculty of Medicine, Dentistry and Health Sciences
The University of Western Australia
2016
THESIS DECLARATION

The research described in this Thesis was performed between February 2013 and August 2016 in the School of Medicine and Pharmacology at the University of Western Australia. I, Esther Cheah, certify that:

This thesis has been substantially accomplished during enrolment in the degree.

This thesis does not contain material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution.

No part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of The University of Western Australia and where applicable, any partner institution responsible for the joint-award of this degree.

This thesis does not contain any material previously published or written by another person, except where due reference has been made in the text.

The work(s) are not in any way a violation or infringement of any copyright, trademark, patent, or other rights whatsoever of any person.

The research involving animal data reported in this thesis was assessed and approved by The University of Western Australia Animal Ethics Committee (Approval #100/1258).

The research involving animals reported in this thesis followed The University of Western Australia and national standards for the care and use of laboratory animals.

This thesis contains published work and/or work prepared for publication, some of which has been co-authored (included in the Appendix). The bibliographical details of the work are outlined below:


   Participated in study design: E.Y.C, T.S.M, P.J.H
   Performed experiments and conducted data analysis: E.Y.C, T.S.M, P.J.H
   Prepared the manuscript: E.Y.C, P.C.B, T.S.M, P.J.H

*Participated in study design: E.Y.C, T.S.M, P.J.H
Performed experiments and conducted data analysis: E.Y.C, T.S.M, P.J.H
Prepared the manuscript: E.Y.C, P.C.B, T.S.M, P.J.H*


*Participated in study design: E.Y.C, T.S.M, P.J.H
Performed experiments and conducted data analysis: E.Y.C, T.S.M, P.J.H
Prepared the manuscript: E.Y.C, P.C.B, T.S.M, P.J.H*


Prepared the manuscript: E.Y.C, P.J.H, J.J.P, P.B.N*

---

**Candidate**

[Signature]

*Esther Cheah*

**Coordinating Supervisor**

[Signature]

*A/Prof Peter Henry*

**Co-supervisor**

[Signature]

*A/Prof Philip Burcham*
DEDICATED TO MY FAMILY AND THE M-BLOCK PHARMILY
ACKNOWLEDGEMENTS

So here we are, closing out the end of a long Chapter (well, technically it’s the beginning of a very long Thesis, but you get my drift…) of my student life. The last 3 and a half years have been nothing short of challenging (but rewarding!) and this Thesis is a testament of the support of a countless number of people who have helped me through my doctoral studies.

First and foremost, to my primary supervisor A/Prof Peter Henry, who was gracious enough to take me on as a young, wide-eyed, and somewhat naïvely optimistic Honours student 4 and a half years ago, and then again as a slightly older (but slightly more pragmatic) PhD student a year later. The best supervisor and mentor any student could hope for, your scientific acumen, positive mindset, resilience, and meticulous approach to research is something I will continue to aspire towards for the rest of my career. Not only have you played a pivotal role in directing the scientific trajectory of my project, but without your tremendous support and sustained encouragement, none of the work in this Thesis would have been possible and my enthusiasm for the project would surely have petered out. I am absolutely privileged to have spent the last 5 years in your lab and to have picked up the trademark “Henry Lab writing style” (aka an affinity for transition words, so I’ve been told…). Thank you also for introducing me to the wonders of organ bathing and for giving me the opportunity to forcefully bestow pass on my love for organ bathing to the unsuspecting third year pharmacology students during their labs. Your unwavering support for everything that I do (except for my support for the Dockers) has made completing my PhD an extremely enjoyable, rewarding, and memorable experience, for which I will always be grateful.

To Peter’s right-hand woman, Tracy Mann; lab whiz, keeper of all things in the Henry Lab, and co-supplier of the cookie jar. Thanks for letting me be the “Henry Lab minion” for the last 5 years and for keeping everything (and everyone) in line. You’ve taught me most of the lab skills and techniques required to complete my project (including how not to “butcher the mouse trachea with a knife and fork”) with such patience and generosity. Also, without your constant threats encouragements to “Mann Up™” and reminders of that “broomstick in the corner”, I would probably still be staring at blank computer screen. In between all your expert travel tips and supplies of lab snacks, my experience in the Henry lab has been extremely well-rounded (in more ways than one). I am indebted to you for all your help and now probably owe you an endless supply of good-quality champagne and wine.

To my co-supervisor A/Prof Phil Burcham, thank you for always being willing to answer my many questions (and requests) and for your words of encouragement throughout my project. And also for introducing me to acrolein and encouraging me to “fly the acrolein flag” at various conferences.
To the rest of the kids of the M-Block pharmily - (Dr) Sam “Samwise” Taylor (fellow pun enthusiast), (Dr) Amy “Amzworth” Dwyer (fellow Dockers enthusiast), Eloise “Elle Elle” Greenland (fellow squash enthusiast), and Kim “Kimchi” Burton (fellow acrolein enthusiast) - thank you all for sharing your lunchtimes with me and for helping to keep M-Block kid-friendly. I am truly grateful to have journeyed through my PhD with such an awesome and fun bunch of equally nerdy scientists and will always cherish the science and non-science memories we made together. I’m sure that not even a covalent bond can rival the strength of the bond and camaraderie we have formed (I can practically hear the groans, but I couldn’t let this acknowledgments section go pun-less, right?). No matter what part of the globe we each end up, I have no doubt we’ll be seeing more of each other in the future, and not just at conferences, where we can continue to see who can garner the most impressive set of vendor goodies. To the extended M-Block Pharmily - Jo Duvestyn, Emma Dishington, Kellie Mouchemore, Maxine Janka, and Kyla Jamieson - I also thank you all immensely for the fun times we shared at M-Block.

To the adults of the M-Block pharmily - Richard Claudius for always keeping us entertained with his well-embelished stories and for hunting down that compound for my final experiment, Melissa Underwood and Rochelle Horsley for always processing my orders with a smile, Mary Anne O’Hara and Succorin Fernades for keeping the pharmacology admin affairs in order, Prof Wally Langdon for pardoning my intrusions into your lab (sometimes up to 16 times a day!) to use your warm room for my pump experiments, the Animal House staff for taking good care of our mice, and to all the other academics and technical staff (Ke and Suraj in particular) at M-Block - thanks for your continual support. I also thank the extended UWA Pharmily - Mary Lee and Shirley Chang for processing many (many!) of my tissue samples all these years, and A/Prof Peter Noble and Prof Jane Pillow (and other members of their laboratories) for allowing me to get involved (somewhat sheepishly) in their ovine work.

Last but not least, to my actual family, and closest friends who have become my family, whose bemused expressions did not become any less bemused no matter how many times I tried to explain my PhD project to them - Mum, Dad, Aunty Edith, Aunty Tee Mien, and Ruth - your unconditional love, prayers, and support go beyond anything I could ever express in words. This work is also dedicated to all of you.

PhinisheD at last, and onward and upward to the next journey!
ABSTRACT

The airway epithelium is the first point of contact with the inhaled environment and is a central component of the lung’s defense system. Through its secretory function, the airway epithelium serves a crucial protective function against noxious airborne compounds, including smoke. Among the compounds contained in smoke, acrolein has emerged as a significant contributor of the respiratory effects elicited by smoke. However, due to ethical limitations associated with the exposure of animals to toxicants, in vitro systems have typically been favoured over in vivo systems as the experimental platform through which to characterise responses elicited by acrolein. Consequently, few findings have been consolidated in more physiologically relevant systems, such as ex vivo systems. Thus, the overarching aim of this Thesis was to characterise protective secretory responses of the airway epithelium elicited by acute acrolein exposure in an ex vivo mouse model.

In the first Results Chapter of the Thesis, two novel dynamic ex vivo systems that more accurately replicated the dynamic in vivo environment of the lungs were developed and were shown to better preserve the integrity of mouse isolated tracheal compared to a conventional static tissue culture system. In the perfusion-superfusion and ventilation-perfusion systems, sterile physiological media or humidified air was passed through the tracheal lumen, respectively, and media was superfused along the exterior surface of tracheal segments. Although both systems preserved the histological and functional integrity of mouse tracheal segments, the perfusion-superfusion system preserved the integrity for a longer period of culture.

By preserving the integrity of mouse isolated tracheal segments, the perfusion-superfusion system was established as a suitable model through which to conduct subsequent studies of mouse airways. Prior to characterisation of the secretory responses of the epithelium elicited by acrolein, perfusion of mouse tracheal segments with media using this system was demonstrated to produce mucous metaplasia. These observations served as the impetus for further examination of the physical stimuli that drive mucous metaplasia in mouse airways, which were also examined in an in vivo ovine model. In the second Results Chapter of the Thesis, the presence of fluid and perturbations of the mechanical environment were identified as two physical stimuli that influenced epithelial mucin levels. These findings suggested that physical factors are capable of driving changes in the levels of mucin in the epithelium.
As a robust model of mucous metaplasia, the perfusion-superfusion system facilitated the study of epithelial mucin secretory responses elicited by acrolein. In the third Results Chapter of the Thesis, acrolein elicited epithelial mucin secretory responses in mouse isolated tracheal segments, consolidating longstanding observations of the strong mucous secretory responses evoked by acrolein in vivo. Importantly, acrolein-induced mucin secretory responses were further characterised, and these ex vivo studies revealed a U-shaped (biphasic) dose-response curve and a leftward shift of the curve when mucin stores were induced by lipopolysaccharide. Together, these findings revealed a complexity of the protective mucin secretory response elicited by acrolein not previously documented in "whole" animal models or in vitro systems.

In addition to mucins, secretory responses of the airway epithelium were also examined within the context of the production and secretion of bronchoactive mediators. In the fourth Results Chapter of the Thesis, acrolein elicited relaxation of mouse tracheal smooth muscle through a mechanism that involved the activation of airway sensory nerves and subsequent secretion of PGE$_2$ from the epithelium to produce relaxation of the tracheal smooth muscle. Importantly, this relaxation response was postulated to form part of a “sensory nerve inhibitory system” that may help maintain the balance between airway opening and closure. In this system, the epithelium played an important role as the structural link between sensory nerve activation and changes in airway smooth muscle tone.

The fifth and final Results Chapter of the Thesis aimed to determine whether the function of the “sensory nerve inhibitory system” was altered in a model of airway disease. A significant finding was that influenza A infection profoundly attenuated acrolein-induced bronchodilation through disruption of the tracheal epithelium and consequent reduction in PGE$_2$ secretion. Attenuation of the capacity of influenza A-infected tracheal segments to produce bronchoprotective relaxation responses may reflect a dysfunctional “sensory nerve inhibitory system”, thereby providing a novel mechanism by which airway hyperresponsiveness is produced.

In summary, work presented in this Thesis has characterised secretory responses of the airway epithelium elicited by acrolein within the context of two major compounds produced and secreted by the epithelium — mucins and bronchoactive mediators. A
novel dynamic *ex vivo* perfusion model was developed in order to further characterise acrolein-induced mucin secretory responses. In addition, a novel secretory response elicited by acrolein exposure involving epithelial secretion of PGE$_2$ was also characterised and found to be modulated by influenza A infection. Together, these responses serve an important protective function in lung defense against acrolein.
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>AHR</td>
<td>Airway Hyperresponsiveness</td>
</tr>
<tr>
<td>AITC</td>
<td>Allyl Isothiocyanate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis Of Variance</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BDRC</td>
<td>Bolus Dose-Response Curve</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic AMP</td>
</tr>
<tr>
<td>CDRC</td>
<td>Cumulative Dose-Response Curve</td>
</tr>
<tr>
<td>CEMA</td>
<td>2-carboxyethylmercapturic acid</td>
</tr>
<tr>
<td>CGRP</td>
<td>Calcitonin Gene-Related Peptide</td>
</tr>
<tr>
<td>CMV</td>
<td>Conventional Mechanical Ventilation</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic Obstructive Pulmonary Disorder</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>EGR</td>
<td>Early Growth Response</td>
</tr>
<tr>
<td>EIA</td>
<td>Enzyme Immunosorbent Assay</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked Immunosorbent Assay</td>
</tr>
<tr>
<td>ET</td>
<td>Endothelin</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and Eosin</td>
</tr>
<tr>
<td>HFOV</td>
<td>High Frequency Oscillatory Ventilation</td>
</tr>
<tr>
<td>i.m.</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
</tbody>
</table>
### LIST OF ABBREVIATIONS (CONTINUED)

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP</td>
<td>Mitogen-Activated Protein</td>
</tr>
<tr>
<td>MARCKS</td>
<td>Myristoylated Alanine-Rich C Kinase Substrate</td>
</tr>
<tr>
<td>miniTOBs</td>
<td>Mini Tissue Organ Bath System</td>
</tr>
<tr>
<td>NEP</td>
<td>Neutral Endopeptidase</td>
</tr>
<tr>
<td>NK</td>
<td>Neurokinin</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>OCT</td>
<td>Optimal Cutting Temperature</td>
</tr>
<tr>
<td>OPMA</td>
<td>S-(3-oxopropyl)-N-acetylcysteine</td>
</tr>
<tr>
<td>PAS</td>
<td>Periodic Acid-Schiff’s reagent</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>RARs</td>
<td>Rapidly Adapting Receptors</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>S.E.M</td>
<td>Standard Error about the Mean</td>
</tr>
<tr>
<td>SARs</td>
<td>Slowly Adapting Receptors</td>
</tr>
<tr>
<td>SNARE</td>
<td>Soluble N-ethyl-maleimide–sensitive factor Attachment protein Receptor</td>
</tr>
<tr>
<td>SP</td>
<td>Substance P</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TRP</td>
<td>Transient Receptor Potential</td>
</tr>
<tr>
<td>UVC</td>
<td>Unventilated Controls</td>
</tr>
<tr>
<td>% R</td>
<td>Relaxation response expressed as a percentage reversal of the spasmogen-induced pre-contraction</td>
</tr>
<tr>
<td>% SPP</td>
<td>Strong Positive Pixels expressed as a percentage of the total number of pixels forming the epithelium</td>
</tr>
<tr>
<td>% v/v</td>
<td>% volume per volume</td>
</tr>
<tr>
<td>% w/v</td>
<td>% weight per volume</td>
</tr>
</tbody>
</table>
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>THESIS DECLARATION</strong></td>
<td>iii</td>
</tr>
<tr>
<td></td>
<td><strong>ACKNOWLEDGEMENTS</strong></td>
<td>vii</td>
</tr>
<tr>
<td></td>
<td><strong>ABSTRACT</strong></td>
<td>ix</td>
</tr>
<tr>
<td></td>
<td><strong>LIST OF ABBREVIATIONS</strong></td>
<td>xiii</td>
</tr>
<tr>
<td></td>
<td><strong>TABLE OF CONTENTS</strong></td>
<td>xv</td>
</tr>
<tr>
<td></td>
<td><strong>TABLE OF FIGURES</strong></td>
<td>xxvi</td>
</tr>
<tr>
<td></td>
<td><strong>TABLE OF TABLES</strong></td>
<td>xxxi</td>
</tr>
</tbody>
</table>

### Chapter 1: GENERAL INTRODUCTION

#### 1.1 ACROLEIN

1.1.1 A SMALL BUT NOXIOUS IRRITANT .................................................. 1
1.1.1.1 General chemistry and reactivity ............................................. 1
1.1.1.2 Sources .................................................................................. 1
1.1.1.3 Metabolic fate ......................................................................... 2
1.1.2 DIFFERENTIAL RESPIRATORY RESPONSES EVOKED BY ACROLEIN .......... 7

#### 1.2 AIRWAY EPITHELIUM

1.2.1 THE FIRST LINE OF DEFENCE ......................................................... 8
1.2.2 ROLE IN THE PRODUCTION AND SECRETION OF MUCUS .................... 8
1.2.2.1 Mucin structure, subtypes, and synthesis ..................................... 13
1.2.2.2 Mucin exocytosis and secretion ............................................... 17
1.2.2.3 Mucociliary transport ............................................................. 18
1.2.2.4 Epithelium-derived stimulants of mucin secretion ......................... 18
1.2.3 ROLE IN THE PRODUCTION AND SECRETION OF BRONCHOACTIVE MEDIATORS ................................................................. 22
1.2.3.1 Spasmogenic mediators .......................................................... 27
1.2.3.2 Spasmolytic mediators .......................................................... 29

#### 1.3 AIRWAY SENSORY NERVES

1.3.1 POPULATIONS OF AIRWAY SENSORY NERVES ............................... 31
1.3.1.1 Sensory C-fibres .................................................................. 31
4.1.4 CHAPTER AIMS ......................................................................................... 126

4.2 METHODS.................................................................................................127
4.2.1 Ex vivo murine model ........................................................................... 127
4.2.1.1 Time course of perfusion-associated mucous metaplasia ............... 127
4.2.1.2 Fluid as a physical stimulus of mucous metaplasia: perfusion vs ventilation ........................................................................................................... 127
4.2.1.3 The mechanical environment as a physical stimulus of mucous metaplasia: different rates of perfusion ...................................................... 127
4.2.2 In vivo ovine model .............................................................................. 128
4.2.2.1 Fluid as a physical stimulus of epithelial mucin production: fetal lamb vs four-month-old sheep ................................................................. 128
4.2.2.2 The mechanical environment as a physical stimulus of mucous metaplasia: different ventilation strategies of preterm lambs ........................................................................................................... 128
4.2.3 LPS exposure in the perfusion-superfusion and ventilation-superfusion systems ........................................................................................................... 130
4.2.4 Tissue processing and PAS staining ..................................................... 130
4.2.4.1 Quantification of PAS staining levels ................................................ 130
4.2.5 Data and statistical analysis .................................................................. 135

4.3 RESULTS....................................................................................................136
4.3.1 Ex vivo murine model ........................................................................... 136
4.3.1.1 Time course of perfusion-associated mucous metaplasia ............... 136
4.3.1.2 Dependency of perfusion-associated mucous metaplasia on the presence of fluid ........................................................................................................... 136
4.3.1.3 Lack of dependency of perfusion-associated mucous metaplasia on the rate of perfusion ................................................................. 143
4.3.2 In vivo ovine model .............................................................................. 143
4.3.2.1 Comparison between fetal lambs and four-month-old sheep .......... 143
4.3.2.2 Comparison between ventilation strategies of newborn lambs ........ 144
4.3.2.2.1 Trachea ......................................................................................... 144
4.3.2.2.2 Large bronchi ............................................................................... 144
4.3.2.2.3 Small bronchi ............................................................................... 165
4.3.3 Differential effects of LPS exposure in the perfusion-superfusion system and ventilation-superfusion systems ................................................. 165
4.4 DISCUSSION.........................................................................................................................183

4.4.1 FLUID AS A PHYSICAL STIMULUS OF MUCOUS METAPLASIA................................. 184

4.4.2 PERTURBATIONS OF THE MECHANICAL ENVIRONMENT AS A PHYSICAL
STIMULUS OF MUCOUS METAPLASIA....................................................................................... 186

4.4.3 FLUID AS A MODULATOR OF LPS-INDUCED MUCOUS METAPLASIA .................... 188

4.4.4 MAJOR FINDINGS ............................................................................................................. 190

Chapter 5: SECRETION OF EPITHELIAL MUCIN ELICITED BY
ACUTE ACROLEIN EXPOSURE.....................................................................................................191

5.0 PREAMBLE ......................................................................................................................... 191

5.1 INTRODUCTION ................................................................................................................191

5.1.1 EPITHELIAL MUCIN SECRETION .................................................................................. 191

5.1.1.1 In healthy airways ........................................................................................................ 191

5.1.1.2 In airway disease .......................................................................................................... 192

5.1.2 EXPERIMENTAL APPROACHES TO MEASURING MUCIN SECRETION ................. 195

5.1.3 ACROLEIN-INDUCED MUCIN SECRETION .................................................................. 197

5.1.4 CHAPTER AIMS .............................................................................................................. 198

5.2 METHODS ......................................................................................................................... 199

5.2.1 EXPOSURE TO ACROLEIN AND ATP ....................................................................... 199

5.2.1.1 Perfusion-superfusion system ................................................................................... 199

5.2.1.1.1 Time course studies .............................................................................................. 199

5.2.1.1.2 Dose-response studies ......................................................................................... 199

5.2.1.2 LPS-perfusion model ............................................................................................... 199

5.2.1.2.1 Dose-response studies ......................................................................................... 199

5.2.1.3 Standard fixation and tissue processing .................................................................... 200

5.2.2 FIXATION APPROACHES TO VISUALISING MUCIN SECRETION BY
CAPTURING THE MUCUS LAYER IN SITU ............................................................................200

5.2.2.1 Paraformaldehyde/picric acid fixation ..................................................................... 200

5.2.2.1.1 Immersion fixation .............................................................................................. 200

5.2.2.1.2 Aerosol exposure to segretagogues and aerosol fixation .................................. 200

5.2.2.2 Flash freezing with liquid nitrogen ......................................................................... 201

5.2.2.3 Methacarn fixation .................................................................................................... 201

5.2.2.3.1 Standard processing ........................................................................................... 201
5.2.2.3.2 Modified processing .................................................. 202
5.2.2.4 Osmium tetroxide fixation .............................................. 202
5.2.2.4.1 Immersion fixation .................................................. 202
5.2.2.4.2 Aerosol exposure to secretagogues and aerosol fixation .... 202
5.2.3 PAS STAINING ............................................................... 205
5.2.3.1 Quantification of PAS staining levels and thickness of the mucus layer .................................................. 205
5.2.4 DATA AND STATISTICAL ANALYSIS .................................. 206

5.3 RESULTS ................................................................................. 211
5.3.1 REDUCTION IN INTRACELLULAR EPITHELIAL MUCIN STORES AS AN INDIRECT MEASURE OF MUCIN SECRETION .............................................................. 211
5.3.1.1 Time-dependency of acrolein and ATP-induced epithelial mucin secretion .................................................. 211
5.3.1.2 Dose-dependency of acrolein and ATP-induced epithelial mucin secretion .................................................. 212
5.3.1.2.1 Secretion of mucin stores induced by perfusion ............... 212
5.3.1.2.2 Secretion of LPS-induced mucin .................................. 212
5.3.2 INCREASES IN THE IN SITU MUCIN LAYER AS A DIRECT MEASURE OF MUCIN SECRETION .............................................................. 226
5.3.2.1 Paraformaldehyde fixation and flash freezing did not capture the mucus layer in situ .................................................. 226
5.3.2.2 The non-aqueous fixative methacarn partially captured the mucus layer in situ .................................................. 226
5.3.2.3 The non-aqueous fixative OsO₄ in FC-72 successfully captured the mucus layer in situ .................................................. 237

5.4 DISCUSSION ............................................................................. 243
5.4.1 MAJOR FINDINGS ................................................................. 251

Chapter 6: SECRETION OF BRONCHOACTIVE MEDIATORS ELICITED BY ACUTE ACROLEIN EXPOSURE 253

6.0 PREAMBLE ............................................................................. 253
6.1 INTRODUCTION .......................................................................................................................... 253
   6.1.1 EPITHELIUM-DEPENDENT MODULATION OF AIRWAY SMOOTH MUSCLE TONE: ROLE OF PROSTAGLANDINS ......................................................... 253
   6.1.2 MAINTAINING THE BALANCE BETWEEN AIRWAY OPENING AND CLOSURE .............. 257
   6.1.3 KNOWN EFFECTS OF ACROLEIN ON AIRWAY SMOOTH MUSCLE TONE ................. 258
   6.1.4 CHAPTER AIMS ................................................................................................................... 259

6.2 METHODS ................................................................................................................................... 261
   6.2.1 ISOLATION OF MOUSE TRACHEAL SEGMENTS ............................................................. 261
   6.2.2 ISOMETRIC TENSION RECORDING STUDIES .................................................................. 261
       6.2.2.1 Relaxation responses to bolus doses of acrolein and additional TRPA1 channel activators ............................................................... 261
       6.2.2.2 Dose-response curves to acrolein .............................................................................. 261
       6.2.2.3 Antagonist studies to characterise acrolein-induced relaxation responses ........... 262
       6.2.2.4 Antagonist studies to characterise exogenous SP- and CGRP-induced relaxation responses .............................................................. 262
       6.2.2.5 Antagonist studies to characterise exogenous PGE2-induced relaxation responses .............................................................. 263
   6.2.3 QUANTITATION OF PGE2 RELEASE ................................................................................. 263
   6.2.4 DATA AND STATISTICAL ANALYSIS .................................................................................. 264

6.3 RESULTS ................................................................................................................................. 265
   6.3.1 EFFECT OF ACROLEIN AND ADDITIONAL TRPA1 ACTIVATORS ON AIRWAY SMOOTH MUSCLE TONE IN PRE-CONTRACTED MOUSE TRACHEAL SEGMENTS ......................................................................................................................... 265
   6.3.2 CHARACTERISATION OF ACROLEIN-INDUCED RELAXATION RESPONSES .......... 265
       6.3.2.1 Acrolein-induced relaxation responses exhibited a dose-dependent relationship and regional potency differences ..................... 265
   6.3.3 PHARMACOLOGICAL CHARACTERISATION OF THE MECHANISMS UNDERLYING ACROLEIN-INDUCED RELAXATION RESPONSES .................................................... 265
       6.3.3.1 Effect of TRPA1 and TPRV1 channel inhibitors on acrolein-induced relaxation responses ............................................................... 265
       6.3.3.2 Effect of NK1 and CGRP receptor antagonists on acrolein-induced relaxation responses ............................................................... 266
6.3.3.3 Effect of an EP$_2$ receptor antagonist on acrolein-induced relaxation responses ................................................................. 266
6.3.3.4 Effect of TRPA1, NK$_1$ and EP$_2$ receptor antagonists on exogenous Substance P-induced relaxation responses ..................... 266
6.3.3.5 Effect of TRPA1, NK$_1$ and EP$_2$ receptor antagonists on exogenous PGE$_2$-induced relaxation responses ...................................... 281
6.3.3.6 Effect of indomethacin on acrolein-induced relaxation responses ................................................................................................. 281
6.3.4 ACROLEIN-INDUCED CHANGES IN LEVELS OF PGE$_2$ RELEASED BY TRACHEAL SEGMENTS ................................................................. 295

6.4 DISCUSSION ........................................................................................................ 301
6.4.1 MAJOR FINDINGS .......................................................................................... 311

Chapter 7: INFLUENCE OF INFLUENZA A INFECTION ON THE SECRETION OF BRONCHOACTIVE MEDIATORS ELICITED BY ACUTE ACROLEIN EXPOSURE 313

7.0 PREAMBLE .......................................................................................................... 313

7.1 INTRODUCTION .................................................................................................. 313
7.1.1 RESPIRATORY TRACT VIRUSES .................................................................. 313
7.1.1.1 Influenza A virus ....................................................................................... 314
7.1.1.1.1 Biology .................................................................................................. 314
7.1.1.1.2 Animal models of influenza A infection .................................................. 315
7.1.1.2 Effects of influenza A infection on the respiratory epithelium .................... 316
7.1.1.2.1 Loss of epithelial integrity via cytotoxic effects ...................................... 316
7.1.1.2.2 Loss of epithelial integrity via inflammation ........................................... 319
7.1.1.2.3 Loss of epithelial cell mediator generation ............................................ 319
7.1.2 MODULATION OF AIRWAY RESPONSIVENESS BY RESPIRATORY TRACT VIRUSES .................................................................................. 320
7.1.3 CHAPTER AIMS .............................................................................................. 325

7.2 METHODS ........................................................................................................... 326
7.2.1 INFLUENZA A INFECTION ........................................................................... 326
7.2.1.1 Influenza A/PR-8/34 virus .................................................................326
7.2.1.2 Animals and viral inoculation ...........................................................326
7.2.2 PATHOHISTOLOGIC CHARACTERISATION AND IMMUNOHISTOCHEMICAL
DETECTION OF VIRAL INFECTION .................................................................327
7.2.2.1 Tissue processing and histochemical staining ........................................327
7.2.2.2 Immunohistochemical staining ............................................................327
7.2.3 EFFECT OF INFLUENZA A INFECTION ON RESPONSES OF MOUSE
ISOLATED TRACHEAL SMOOTH MUSCLE ...................................................328
7.2.3.1 Isometric tension recordings ...............................................................328
7.2.4 EFFECT OF INFLUENZA A INFECTION ON PGE₂ PRODUCTION IN PRE-
CONTRACTED TRACHEAL SEGMENTS .......................................................328
7.2.5 DATA AND STATISTICAL ANALYSIS ......................................................329

7.3 RESULTS ...........................................................................................................330
7.3.1 CLINICAL, HISTOCHEMICAL, AND IMMUNOHISTOCHEMICAL DETECTION
OF INFLUENZA A INFECTION .....................................................................330
7.3.2 EFFECT OF INFLUENZA A INFECTION ON RELAXATION RESPONSES
EVOKE BY ACROLEIN .................................................................................330
7.3.3 EFFECT OF INFLUENZA A INFECTION ON RELAXATION RESPONSES
EVOKE BY SUBSTANCE P ............................................................................330
7.3.4 EFFECT OF INFLUENZA A INFECTION ON RELAXATION RESPONSES
EVOKE BY EXOGENOUS PGE₂ AND CONTRACTILE RESPONSES
EVOKE BY CARBACHOL ..................................................................................337
7.3.5 EFFECT OF INFLUENZA A INFECTION ON EPITHELIAL SECRETION OF
PGE₂ .............................................................................................................337

7.4 DISCUSSION .....................................................................................................347
7.4.1 MAJOR FINDINGS .......................................................................................354

Chapter 8: GENERAL DISCUSSION ........................................................................355

8.1 USE OF NOVEL EXPERIMENTAL APPROACHES TO FURTHER
CHARACTERISE ESTABLISHED AIRWAY RESPONSES TO
ACROLEIN .......................................................................................................356
8.1.1 DEVELOPMENT OF DYNAMIC EX VIVO SYSTEMS ..................................356
8.1.2 Using the dynamic ex vivo systems to investigate mucous metaplasia ............................................................... 359
8.1.3 Using the dynamic ex vivo system to investigate acrolein-induced secretion of mucin from the surface epithelium ...................... 360

8.2 Use of established experimental approaches to characterise novel airway responses to acrolein ............... 361
8.2.1 Acrolein-induced secretion of bronchoactive mediators from the surface epithelium in health and disease ......................... 361

8.3 Concluding remarks .......................................................................................................................... 364

Chapter 9: References .............................................................................................................. 365

Chapter 10: Appendix ................................................................................................................. 401

10.1 Research Publications .............................................................................................................. 401
10.1.1 Original research publications .......................................................................................... 401
10.1.2 Published F1000 prime reviews ...................................................................................... 401

10.2 Research communications ........................................................................................................ 402
### TABLE OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>The chemical structure of acrolein.</td>
<td>3</td>
</tr>
<tr>
<td>1.2</td>
<td>The sources and metabolic fate of acrolein.</td>
<td>5</td>
</tr>
<tr>
<td>1.3</td>
<td>Major cell types of the airway epithelium vary with airway region.</td>
<td>9</td>
</tr>
<tr>
<td>1.4</td>
<td>Mucin structure, synthesis, and assembly.</td>
<td>15</td>
</tr>
<tr>
<td>1.5</td>
<td>Primary events of mucin granule exocytosis.</td>
<td>19</td>
</tr>
<tr>
<td>1.6</td>
<td>Innervation of key airway structures.</td>
<td>33</td>
</tr>
<tr>
<td>1.7</td>
<td>Innervation of the airway epithelium.</td>
<td>35</td>
</tr>
<tr>
<td>1.8</td>
<td>Structures of the TRPA1 and TRPV1 channels showing ligand interaction and regulatory sites.</td>
<td>39</td>
</tr>
<tr>
<td>1.9</td>
<td>Diverse mechanisms of TRPA1 channel activation.</td>
<td>41</td>
</tr>
<tr>
<td>1.10</td>
<td>Local release of neuropeptides by peripheral projections of C-fibres.</td>
<td>47</td>
</tr>
<tr>
<td>1.11</td>
<td>Immunohistochemical evidence of the innervation of the airway epithelium by sensory nerves.</td>
<td>53</td>
</tr>
<tr>
<td>1.12</td>
<td>Schematic outlining aims of individual Results Chapters.</td>
<td>69</td>
</tr>
<tr>
<td>2.1</td>
<td>Quantification of PAS staining using a Positive Pixel algorithm.</td>
<td>79</td>
</tr>
<tr>
<td>3.1</td>
<td>The ex vivo perfusion-superfusion system.</td>
<td>89</td>
</tr>
<tr>
<td>3.2</td>
<td>The ex vivo ventilation-superfusion system.</td>
<td>91</td>
</tr>
<tr>
<td>3.3</td>
<td>Preservation of tissue histology was dependent on the flow rate of media perfusion.</td>
<td>97</td>
</tr>
<tr>
<td>3.4</td>
<td>Preservation of epithelial histology for up to 48 h by perfusion with media at a flow rate of 0.1 ml min^-1.</td>
<td>99</td>
</tr>
<tr>
<td>3.5</td>
<td>Preservation of epithelial histology for up to 18 h by ventilation with humidified air.</td>
<td>101</td>
</tr>
<tr>
<td>3.6</td>
<td>Preservation of functional responses in perfused tracheal segments.</td>
<td>103</td>
</tr>
<tr>
<td>3.7</td>
<td>Preservation of functional responses in ventilated tracheal segments.</td>
<td>105</td>
</tr>
</tbody>
</table>
Figure 4.1. Molecular pathways activated by allergic inflammation and LPS that initiate mucous metaplasia................................................................. 123

Figure 4.2. Schematic illustrating the differences between the CMV and HFOV modalities................................................................. 131

Figure 4.3. Time course of mucous metaplasia development in mouse tracheal epithelium over 48 h of perfusion.................................................. 137

Figure 4.4. Quantification of the time course of mucous metaplasia development in mouse tracheal epithelium over 48 h of perfusion................................................................. 139

Figure 4.5. Development of mucous metaplasia associated with perfusion of mouse tracheal segments with media supplemented with 0% or 2% fetal bovine serum (FBS) over 48 h of perfusion.................................................. 141

Figure 4.6. Lack of mucous metaplasia development in ventilated tracheal segments.................................................................................. 145

Figure 4.7. Quantification of levels of PAS-positive epithelial mucin in tracheal segments isolated from control mice (0 h) or those ventilated or perfused for 18 h...................................................... 147

Figure 4.8. Effect of the flow rate of perfusion on the mucous metaplastic response observed in mouse tracheal epithelium associated with the perfusion-superfusion system........................................................................ 149

Figure 4.9. Quantification of effect of the rate of perfusion on the mucous metaplastic response observed in mouse tracheal epithelium after 6 h of perfusion.................................................................................. 151

Figure 4.10. Tracheal epithelial mucin levels in fluid-filled vs air-filled ovine airways.................................................................................. 153

Figure 4.11. Quantification of tracheal epithelial mucin levels in fluid-filled vs air-filled ovine airways.................................................................................. 155

Figure 4.12. Tracheal epithelial mucin levels of lambs subjected to CMV and HFOV compared to control lambs.................................................. 157

Figure 4.13. Quantification of tracheal epithelial mucin levels of lambs subjected to CMV and HFOV compared to control lambs.................................................. 159

Figure 4.14. Large bronchial epithelial mucin levels of lambs subjected to CMV and HFOV compared to control lambs.................................................. 161

Figure 4.15. Quantification of large bronchial epithelial mucin levels of lambs subjected to CMV and HFOV compared to control lambs.................................................. 163

Figure 4.16. Small bronchial epithelial mucin levels of lambs subjected to CMV and HFOV compared to control sheep.................................................. 167

Figure 4.17. Quantification of small bronchial epithelial mucin levels of lambs subjected to CMV and HFOV compared to control sheep.................................................. 169
Figure 5.11. PAS-stained sections of mouse tracheal segments fixed with PFA by immersion or aerosolisation. ................................................................. 231

Figure 5.12. PAS-stained sections of mouse tracheal segments flash-frozen with liquid nitrogen prior to paraformaldehyde fixation post-staining. .................................................................................. 233

Figure 5.13. PAS-stained sections of mouse tracheal segments fixed with methacarn and processed under standard or modified conditions. ........................................................................................................ 235

Figure 5.14. PAS-stained sections of mouse tracheal segments fixed with 0.1% OsO4 in FC-72 by immersion and aerosolisation. ................................................................. 239

Figure 5.15. Measurements of the thickness of the in situ PAS-positive mucus layer. ................................................................................................................................. 241

Figure 6.1. Prostaglandin production by epithelial cells and target receptors that mediate contractile or relaxation responses.............................................................. 255

Figure 6.2. Relaxation responses elicited by TRPA1 channel activators. ................................................................................................................................. 267

Figure 6.3. Bolus dose-response curves to acrolein in upper and lower mouse tracheal segments. ........................................................................................................ 269

Figure 6.4. Effects of AP-18 (30 µM), RP-67580 (20 nM), HC-030031 (20 µM) and PF-04418948 (100 nM) on acrolein-induced relaxation responses in upper (A, B) and lower (C, D) mouse tracheal segments............................................................ 271

Figure 6.5. Effect of capsazepine (3µM) on acrolein-induced relaxation responses (A) and capsaicin-induced relaxation response (B) in upper and lower mouse tracheal segments. .................................................. 273

Figure 6.6. Effect of CGRP 8-37 (1 µM) on acrolein-induced relaxation responses in upper and lower mouse tracheal segments. ........................................ 275

Figure 6.7. Effects of AP-18 (30 µM), RP-67580 (20 nM), HC-030031 (20 µM), and PF-04418948 (100 nM) on substance P (SP)-induced relaxation responses in upper (A, B) and lower (C, D) mouse tracheal segments .................................................................................. 283

Figure 6.8. Cumulative dose-response curves to PGE2 in upper and lower mouse tracheal segments. ........................................................................................................ 285

Figure 6.9. Effects of AP-18 (30 µM), capsazepine (10 µM), RP-67580 (20 nM), HC-030031 (20 µM), and PF-04418948 (100 nM) on PGE2-induced relaxation responses in upper (A, B) and lower (C, D) mouse tracheal segments. .................................................................................. 287

Figure 6.10. Effect of indomethacin (5 µM) on acrolein-induced relaxation responses of carbachol pre-contracted upper and lower mouse tracheal segments. .................................................................................. 289
Figure 6.11. Quantitation of PGE$_2$ released by mouse tracheal segments. .......... 297
Figure 6.12. Relationship between acrolein-induced PGE$_2$ secretion and airway smooth muscle relaxation. ................................................................. 299
Figure 6.13. Schematic outlining the proposed paradigm of acrolein-induced relaxation of mouse tracheal smooth muscle. ......................... 303
Figure 6.14. The balance between airway opening and closure. ....................... 309

Figure 7.1. Effect of influenza A infection on the tracheal epithelium.............. 317
Figure 7.2. An imbalance between airway closure and opening may drive airway obstruction. ........................................................... 323
Figure 7.3. Clinical, histopathologic, and immunohistochemical evaluation of influenza A infection in mice................................................ 331
Figure 7.4. Relaxation responses evoked by acrolein (30 µM) in tracheal segments isolated from SHAM and VIRUS mice. ...................... 333
Figure 7.5. Bolus dose-response curves to substance P in tracheal segments isolated from SHAM and VIRUS mice. ...................... 335
Figure 7.6. Cumulative dose-response curves to PGE$_2$ in tracheal segments isolated from SHAM and VIRUS mice. ...................... 339
Figure 7.7. Cumulative dose-response curve to isoprenaline (A) and carbachol (B) in tracheal segments isolated from SHAM and VIRUS mice. ............................................................... 341
Figure 7.8. Quantitation of PGE$_2$ released by SHAM and VIRUS tracheal segments and corresponding relaxation responses. .............. 343
Figure 7.9. Schematic representation of the postulated mechanism through which influenza A virus (V) attenuates acrolein-induced relaxation responses. ............................................................... 349

Figure 8.1. Schematic outlining major findings of individual Results Chapters........................................................................................................... 357
TABLE OF TABLES

Table 1.1. Major human airway epithelial cell types and their primary features and functions ................................................................. 11
Table 1.2. Ligands known to induce mucin secretion from airway epithelial cells. .................................................................................. 23
Table 1.3. Classes of mediators secreted by the airway epithelium and their major features and functions ........................................... 25
Table 1.4. Airborne activators of the TRPA1 channel ................................................. 43
Table 1.5. Sensory neuropeptide receptors in the airways and associated responses mediated by their activation ............................. 49

Table 2.1. General drugs and reagents ........................................................................ 73

Table 3.1. Summary of functional responses of perfused and ventilated tracheal segments ...................................................................... 109

Table 4.1. Input parameters for the Positive Pixel Algorithm used to quantify the level of PAS staining in the epithelial layer of mouse tracheal sections ......................................................... 133

Table 5.1. Classes of mucin secretagogues. ................................................................. 193
Table 5.2. Input parameters for the Positive Pixel Algorithm used to quantify levels of PAS staining in the epithelial layer of mouse tracheal sections ............................................................. 209

Table 6.1. Relaxation responses induced by TRPA1 channel activators in upper and lower mouse tracheal segments .............................. 277
Table 6.2. Effects of selected inhibitors and antagonists on relaxation responses induced by acrolein, capsaicin and substance P in mouse tracheal segments ....................................................... 279
Table 6.3. Relaxation responses evoked by neuropeptides substance P and CGRP in upper and lower mouse tracheal segments .............. 291
Table 6.4. Effects of selected antagonists on relaxation responses evoked by PGE₂ in mouse tracheal segments ....................................... 293
Table 7.1. Differences in the responses of tracheal segments isolated from SHAM and VIRUS mice to spasmogenic and spasmolytic agents.
CHAPTER 1: GENERAL INTRODUCTION

1.1 ACROLEIN

1.1.1 A small but noxious irritant

The respiratory tract serves as a major port of entry for a host of airborne compounds and irritants that are ubiquitously distributed in the environment, such as smoke. Although smoke is a complex cocktail of noxious compounds, acrolein has emerged as a prevalent and highly toxic constituent of smoke produced by the combustion of tobacco and other organic materials (Bein and Leikauf, 2011; Faroon et al., 2008; Moghe et al., 2015; Stevens and Maier, 2008; Yeager et al., 2016). By virtue of its toxicity and prevalence in smoke, acrolein has been proposed as a key mediator contained in smoke. Adding further credence to this postulate are studies that have demonstrated that many smoke-related responses and pathophysiological changes are reproducible with acrolein exposure alone (Bein and Leikauf, 2011; Burcham et al., 2010c; Kehrer and Biswal, 2000). Collectively, these findings have led to recognition of acrolein as a significant contributor of the respiratory effects elicited by smoke (Cunningham et al., 2011; Fowles and Dybing, 2003; Haussmann, 2012; Yeager et al., 2016).

1.1.1.1 General chemistry and reactivity

Acrolein is a small, 3-carbon α, β-unsaturated aldehyde and its reactivity is conferred by the presence of two reactive moieties: a highly electrophilic, α,β-bond, which reacts readily with nucleophilic sites on macromolecules including DNA (Chung et al., 1984) and proteins (Aldini et al., 2011; Uchida et al., 1998) via Michael addition at the β-carbon, and a carbonyl group, which forms Schiff bases with primary amines (LoPachin and Gavin, 2016; Spiess et al., 2011) (Figure 1.1). Of these two reactive moieties, reactions at the β-carbon with nucleophilic targets typically predominate over formation of Schiff bases (Burcham and Fontaine, 2001).

1.1.1.2 Sources

Although generated endogenously as a by-product of lipid peroxidation and introduced to the body through dietary sources, the major source of acrolein for human exposure comes exogenously by way of inhalation of smoke generated by the combustion of a
Due to continual introduction into the ambient environment, acrolein is recognised as a major common health hazard (Bein and Leikauf, 2011; Logue et al., 2011). By far, the most common source of acrolein is cigarette smoke, where up to 450 µg of acrolein can be released by just a single cigarette (Sarkar and Hayes, 2007; Stevens and Maier, 2008). However, additional sources of acrolein may also contribute to human exposure. For example, ambient (outdoor) levels have been reported to range from 8.7 to 410 µg per m$^3$, while higher levels of acrolein exposure have been recorded in enclosed (indoor) areas where smoke is generated by cooking of vegetable and animal fats (Moghe et al., 2015), or in the context of acute smoke inhalation resulting from fires (Bein and Leikauf, 2011; Faroon et al., 2008).

Although measurements of airborne acrolein are readily made in the ambient air and in experimental systems, these estimations are not easily translated into concentrations reached in respiratory tissue due to technical challenges associated with making accurate measurements of the contents of lung fluid (Burcham et al., 2010c; Moghe et al., 2015). Collection of lung samples typically involves bronchoalveolar lavage, which often results in the dilution of lung fluids and consequent underestimation of acrolein concentrations. Moreover, as a highly reactive molecule, any airborne acrolein that reaches the lung likely reacts with proteins in the lung fluid, rendering measurements of “free” (unbound) acrolein an unreliable estimation of levels reached in the lung tissue. Notwithstanding these challenges, concentrations of acrolein reached in the lung during smoking have been reported to reach micromolar levels, and these concentrations are conceivably exceeded during acute intoxication with larger volumes of smoke (Eiserich et al., 1995).

1.1.1.3 Metabolic fate

Due to its small molecular size and high lipophilicity, airborne acrolein that enters the lungs is able to travel directly across cell membranes by passive diffusion into tissues and the bloodstream. Upon entry into tissues, acrolein reacts readily with nucleophilic cellular targets, including protein and DNA, or is metabolised. The main pathway for acrolein metabolism occurs via conjugation with cellular glutathione (GSH), which then undergoes proteolytic processing and N-acetylation to S-(3-oxopropyl)-N-
Figure 1.1. The chemical structure of acrolein.

Acrolein is a small, 3-carbon unsaturated aldehyde (A) and its chemical reactivity is conferred by two reactive moieties: a highly electrophilic $\alpha,\beta$- bond (circled in blue) and a carbonyl group (circled in red) (A). A colour-coded electrostatic potential map illustrates how charge is distributed across the acrolein molecule, where red signifies the most negative electrostatic potential, and orange-yellow-green indicates intermediate electrostatic potential (from negative to positive, respectively) (B). The black arrow indicates C$\beta$, the site at which Michael addition takes place (adapted from LoPachin and Gavin, 2016).
Although acrolein is endogenously generated and introduced to the body via dietary sources (dotted black line), the major source of exposure for humans comes via inhalation of a range of exogenous airborne substances (solid black line). Acrolein can then form adducts with protein and DNA (solid grey line), or is conjugated with glutathione (GSH) to form metabolites including S-(3-oxopropyl)-N-acetylcysteine (OPMA), 2-carboxyethylmercapturic acid (CEMA), and S-(3-hydroxypropyl)-N-acetylcysteine (3-HPMA) (solid grey line). Minor metabolites can also be formed (dotted grey line) (adapted from Mogue et al., 2015).
acetylcysteine (OPMA) in the liver and kidneys (Stevens and Maier, 2008). Oxidation of OPMA yields 2-carboxyethylmercapturic acid (CEMA) while reduction of OPMA yields S-(3-hydroxypropyl)-N-acetylcysteine (3-HPMA), which is the major metabolite excreted in urine. Minor metabolites of acrolein may also be formed, including acrylic acid, glutaraldehyde (formed upon oxidation and epoxidation of acrolein prior to GSH conjugation), and malonic acid (formed upon metabolism with aldehyde dehydrogenase) (Figure 1.2) (Moghe et al., 2015).

1.1.2 Differential respiratory responses evoked by acrolein

In characterising the responses elicited by acrolein, it has become clear that the effects of acrolein are intimately related to both the magnitude and duration of exposure. Moreover, there has emerged a clear distinction between the effects elicited acutely and those that develop in the hours and days following the initial acrolein insult. Upon acute exposure to high doses of acrolein, a series of powerful and co-ordinated respiratory reflexes are rapidly evoked that function to limit further inhalation of acrolein and promote its removal from the airways (Faroon et al., 2008). Many of these immediate protective reflexes are mediated by key structures within the airways, including the airway epithelium and the complex network of sensory nerves that innervate it. However, if the defensive capacity of the airways is overwhelmed, acute lung injury may ensue (Bein and Leikauf, 2011; Moretto et al., 2012). In the hours following the initial acrolein insult, susceptible cellular components, such as proteins and DNA, are subsequently targeted by acrolein; this leads to a host of responses including transcriptional changes, alterations in cell-cycle control, and sustained activation of pro-inflammatory signals in a number of different cell types such as airway epithelial cells (Burcham et al., 2010c). In addition, chronic or repeated exposure to low doses of acrolein may also activate inflammatory signals, thus promoting development of a number of chronic pulmonary diseases (Burcham et al., 2010c; Moghe et al., 2015) (see Section 1.5.2.2.2).

The work presented in this Thesis has focused on the acute respiratory responses elicited by acrolein. As many of these protective responses involve the airway epithelium and the sensory nerves, and also the co-operative actions between these two airway structures, they will first be discussed in the following sections.
1.2 AIRWAY EPITHELium

1.2.1 The first line of defence

The human lung is the largest surface in the body in continuous contact with the environment; approximately 23 generations of bifurcating airways deliver air to over 300 million alveoli with a total estimated surface area of 80 m² (Wiebe and Laursen, 1995). On average, 10 000 litres of air containing a wide array of physical, chemical, and biological agents passes through the lungs per day (Widdicombe and Wine, 2015). Consequently, there is immense potential for exposure to insults, such as acrolein, and the lung is equipped with a complex defence system to combat potential injury. A key component of this defence system is the epithelial layer that lines the entire respiratory tract, which serves as the first point of contact with the inhaled environment (Holgate, 2011). Once viewed as an inert physical barrier between the host and external milieu, the airway epithelium is now recognised as a complex, multi-cellular modulator of the airways capable of eliciting dynamic homeostatic responses to maintain airway integrity in the face of exposure to inhaled pathogens, toxins, and pollutants (Proud and Leigh, 2011).

Distinct cell populations within the airway epithelium provide diverse protective functions that work not only to prevent particulate matter from detrimental penetration into the underlying submucosal regions of the airways but that also promote clearance of those particles (Figure 1.3). Importantly, these functions are achieved via the secretion of a host of bioactive and biophysical agents, which regulate aspects of airway function, including the mucociliary system and bronchomotor tone (Table 1.1).

1.2.2 Role in the production and secretion of mucus

Present on the lumenal surface of the airway epithelium is a thin, mobile layer of mucus that functions to trap inhaled particles and toxicants. The mucus layer serves as a physical barrier between the extracellular milieu and the intracellular regions and consists of two distinct layers: an inner, low-viscosity serous phase through which the cilia beat, and an outer gel phase that lies on top of the cilia and is moved towards the mouth by beating cilia (Ali and Pearson, 2007). In addition to the entrapment and removal of particles, the mucus layer also serves a number of other important functions, including the humidification of air, insulation, lubrication, and is also a source
Figure 1.3. Major cell types of the airway epithelium vary with airway region.

In the large airways ($2^0-2^5$ generations; diameter $>2$mm), the major cell types are ciliated cells, undifferentiated columnar cells, secretory (mucous) cells, and basal cells. In the small airways ($2^6-2^{23}$ generations; diameter $<2$mm), the cell types are similar, although there is shift from secretory (mucous) cells to club cells. After $2^{23}$ generations, the airway epithelium merges with the alveolar epithelium, which consist of type I and II cells (modified from Crystal et al., 2008).
Table 1.1. Major human airway epithelial cell types and their primary features and functions.

<table>
<thead>
<tr>
<th>Epithelial cell type</th>
<th>Features and functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Columnar cell</td>
<td>• Abundantly expressed in airways (~50% of the cell population)</td>
</tr>
<tr>
<td></td>
<td>• Ciliated</td>
</tr>
<tr>
<td></td>
<td>• Terminally differentiated</td>
</tr>
<tr>
<td></td>
<td>• Required for mucociliary clearance</td>
</tr>
<tr>
<td>Secretary (mucous) cell</td>
<td>• Mucus-producing</td>
</tr>
<tr>
<td></td>
<td>• Mucus glycoproteins (mucins) form an extensive network throughout the mucus layer</td>
</tr>
<tr>
<td>Basal cell</td>
<td>• Anchor superficial cells into basement membrane</td>
</tr>
<tr>
<td></td>
<td>• Primary stem cell</td>
</tr>
<tr>
<td>Club cell</td>
<td>• Found in large and small human airways</td>
</tr>
<tr>
<td></td>
<td>• Produce bronchial surfactants</td>
</tr>
<tr>
<td></td>
<td>• Contain electron-dense granules</td>
</tr>
<tr>
<td></td>
<td>• Possess stem cell-like properties</td>
</tr>
<tr>
<td>Type I cell</td>
<td>• Very thin cytoplasmic extensions</td>
</tr>
<tr>
<td></td>
<td>• Possible biosynthetic function</td>
</tr>
<tr>
<td>Type II cell</td>
<td>• Small, cuboidal cells</td>
</tr>
<tr>
<td></td>
<td>• Characterised by distinct secretory granules</td>
</tr>
<tr>
<td></td>
<td>• Synthesise and secrete pulmonary surfactants</td>
</tr>
</tbody>
</table>
of antibacterial enzymes (Proud and Leigh, 2011; Rogers, 2007; Thornton et al., 2008). Through the secretion of mucus, the epithelium is uniquely primed to mount a rapid and critical protective response to noxious airborne agents. Together, the processes of mucus production and secretion serve a crucial protective role against inhaled insults and have been recognised as the cornerstones of airway host defence (Ballard and Spadafora, 2007).

Within the airways, the secretory (mucous) cells and submucosal glands represent the two primary sources of mucus. Although they constitute 5-15% of the cell population in the human large airways, the presence of secretory cells decreases dramatically with airway branching such that they are rarely observed below the sixteenth generation (Widdicombe, 2012) (Figure 1.3). In the human distal airways, the basal rates of production and secretion are balanced, resulting in little to no accumulation of mucous-producing cells under normal conditions (Adler et al., 2013).

Mucus secretions themselves are comprised of 97% water, with the remaining 3% a combination of solid constituents including salts, lipids, antimicrobial and immunomodulatory molecules, as well as highly glycosylated proteins known as mucins (Rogers, 2003). The capacity of mucus to trap and eliminate particulates and pathogens is largely attributable to mucins, which confer the gel-like properties and viscoelasticity required for effective particulate entrapment and mucociliary transport (Adler et al., 2013; Ali and Pearson, 2007; Proud and Leigh, 2011).

1.2.2.1 Mucin structure, subtypes, and synthesis

Mucins are high-weight proteins that constitute up to 2% of the total weight in normal airway mucus. Individual mucin molecules are comprised of a flexible amino acid backbone formed from alternating subunits of glycosylated and non-glycosylated regions (Ali and Pearson, 2007; Rogers, 2007). Within the highly glycosylated regions are tandemly repeated amino acid sequences rich in serine and threonine residues that serve as O−glycosylation sites (Thornton et al., 2008). Two distinct classes of mucins have been defined: membrane-associated mucins, which possess a hydrophobic tail anchoring the mucin to the plasma membrane, and secreted mucins, which are stored in intracellular secretory granules in preparation for release. Distinct mucin genes encode the amino acid backbone of each specific mucin, with nine of these genes
identified in the human respiratory tract. The major secreted mucins in mammalian airways are MUC5AC, MUC5B, MUC2 and MUC19, and a subset of these are secreted in normal respiratory tract secretions and also in respiratory disease (Evans and Koo, 2009; Hovenberg et al., 1996; Wickstrom et al., 1998). In human airways, MUC5AC is predominantly localised to secretory cells of the surface epithelium and, though sparse under basal conditions, its expression is profoundly elevated in the airway epithelium of patients with asthma and chronic obstructive pulmonary disease (COPD) (Kirkham et al., 2002) as well in the sputum produced by cystic fibrotic patients (Groneberg et al., 2002). The expression profile of these mucins is fundamentally mirrored in mouse airways, where an elevation in Muc5ac has been reported in numerous mouse models of asthma (Young et al., 2007; Zuhdi et al., 2000) and in response to viruses, such as parainfluenza type I (Walter et al., 2002). MUC5B is the second most abundant mucin subtype expressed and is predominantly produced by mucous cells in submucosal glands of the tracheobronchial airways (Buisine et al., 1999; Groneberg et al., 2002; Thornton et al., 2008). In contrast to Muc5ac, levels of Muc5b are typically unchanged in allergic murine models of asthma (Young et al., 2007). Other minor mucin subtypes, including Muc2 and Muc19, are produced by surface epithelial cells and tracheal submucosal glands, respectively, but at levels far lower than Muc5ac and Muc5b (Young et al., 2007).

The process of mucin synthesis begins with a common regulated process of assembly. Prior to granule formation, individual mucin subunits are linked end-to-end by disulphide bonds to form long, linear, thread-like molecules (Figure 1.4 A). Current evidence indicates that mucin monomers are disulphide-linked via their C-terminals in the rough endoplasmic reticulum and become linked via their N-terminals upon moving to the Golgi. Within secretory epithelial cells, the process of assembly begins in the rough endoplasmic reticulum, where it undergoes oligomerisation before being transported to the Golgi for glycosylation and packaging into highly condensed mature mucin secretory granules (Rogers, 2007). However, the precise mechanisms involved in the assembly of mucin monomers into polymers and formation of mucin granules remain incompletely understood (Verdugo, 2012). Early studies proposed that mucin granules are bound together by high concentrations of Ca$^{2+}$ ions, which serve as a shielding cation that promotes close packaging of the polyanionic granules, thereby aiding mucins to achieve a condensed state (Verdugo, 1990) (Figure 1.4 B). However, more recent studies have proposed a more complex model in which the concomitant fall in pH and increase in Ca$^{2+}$ concentration in the late Golgi facilitates specific
In preliminary stages of synthesis, mucin subunits are joined end-to-end by disulphide bonds (S-S) to form long, thread-like molecules (A). A classic model of granule packaging proposes that polyanionic mucin granules are highly condensed and kept together by $\text{Ca}^{2+}$ ions (from Rogers, 2007) (B). A more complex model of packaging proposes that linear mucin polymers undergo specific conformational changes in the dimeric N termini (C). Subsequent formation of noncovalent, calcium-mediated interactions forms focal links or “nodes” that organise polymers into granules (from Ridley et al., 2014).

**Figure 1.4. Mucin structure, synthesis, and assembly.**
conformational changes that lead to formation of covalent, Ca²⁺-mediated interactions between dimeric N-termini on adjacent mucin subunits (Ridley et al., 2014). As a consequence, mucin monomers centre around “nodes” formed by dimeric N-termini interactions to form highly organised mucin polymers (Figure. 1.4 C). The entire process of synthesis and assembly is thought to take up to 2 hours to complete and is highly demanding of cellular metabolism. Thus, mucins are pre-synthesised and stored in the cytoplasm in preparation for rapid release in response to noxious stimuli without a delay from responsive transcriptional upregulation or a need for de novo synthesis (Thornton et al., 2008).

1.2.2.2 Mucin exocytosis and secretion

When triggered by a stimulus, the secretion of pre-assembled mucin granules by airway epithelial cells is extremely rapid, with exocytosis thought to take place in just tens of milliseconds. The secretion process, which occurs exclusively via the exocytotic process, is initiated upon activation of a range of cell-surface receptors that results in the activation of protein kinases C and G (Davis and Dickey, 2008) (Figure. 1.5). Myristoylated alanine-rich C kinase substrate (MARCKS) protein is a key molecule purported to regulate movement of mucin granules to the cell surface (Green et al., 2011; Li et al., 2001; Singer et al., 2004). This was revealed by peptide blocking studies in which the administration of MANS, a peptide analogous to the MARCKS N-terminal sequence, strongly attenuated mucin secretion by airway epithelial cells, both in vitro (Li et al., 2001; Park et al., 2008) and in animal models (Agrawal et al., 2007; Foster et al., 2010; Singer et al., 2004). Subsequent phosphorylation of MARCKS by activated protein kinase C is thought to lead to translocation of MARCKS from the apical membrane into the cytoplasm, where it attaches to the membrane of the mucin granule. The attachment becomes stabilised by protein phosphatase-2A-dependant dephosphorylation of MARCKS, which is activated by protein kinase G via the nitric oxide-cGMP pathway (Li et al., 2001; Rogers, 2003). In addition, dephosphorylated MARCKS may also interact with actin and myosin to facilitate granule movement towards the cell membrane. Thus, MARCKS is postulated to play a role in co-ordinating the actions of protein kinases C and G and in linking secretagogue-induced activation of cell surface receptors to mucin granule movement. However, a recent study has challenged this paradigm by bringing into question the specificity of the MANS inhibitory peptide by work demonstrating that it inhibits the in vitro mucin binding assay rather than the secretory process (Haddock et al., 2014). Therefore, further studies are required to fully uncover the involvement of MARCKS in the mucin exocytotic process.
Mucin granules that translocate to the cell surface dock are subsequently tethered to the plasma membrane. A number of key proteins are involved in this process, including soluble n-ethyl-maleimide–sensitive factor attachment protein receptor (SNARE), which is regulated by members of the Rab GTPase family that are anchored on the cell surface (Zerial and McBride, 2001). Activation of an additional regulatory protein, Munc13, is required to prime the mucin granule for release by exocytosis. In this step, Munc13 interacts with RAB3 effector or Rab27 and displaces Munc18, thereby enabling formation of the SNARE complex (Burgoyne and Morgan, 2007; Rizo and Südhof, 2002). Following an appropriate Ca$^{2+}$ signal, the mucin granule fuses with the plasma membranes to complete exocytosis and granule contents are expelled into the airway lumen. The auto-repellent, polyanionic mucin granules then spread out across the epithelial surface and expand up to 400-fold times their original size to form a tangled mucin network (Rogers, 2003; Rogers, 2007).

1.2.2.3 Mucociliary transport

The mucins secreted onto the epithelial surface by secretory cells are crucial to the entrapment of particulates and pathogens subsequently removed from the airways by mucociliary transport. This transport process engages the co-ordinated actions of an additional population of epithelial cells that express cilia that move in a co-ordinated beat cycle. The active stroke pushes the mucins and the entire mucus blanket towards the mouth and continues until the cilia lie flat on the airway surface in the “rest phase”. The process is reset during the “recovery phase”, wherein cilia move backwards to return to the start point of the active stroke (Widdicombe, 2012). In this way, ciliated cells beat synchronously to waft the gel-forming, pathogen-containing mucus layer up and out of the airways, where it is either expelled or swallowed.

1.2.2.4 Epithelium-derived stimulants of mucin secretion

Upstream of mucin granule secretion, cellular mediators, termed secretagogues, initiate the mucin secretory process via activation of cell-surface receptors. A number of these secretagogues act directly on the epithelium, while others are thought to target
Figure 1.5. Primary events of mucin granule exocytosis.

The major steps involve receptor activation, translocation to the cell surface, tethering/docking, priming, and exocytosis of the mucin secretory granule (SG) (from Davis and Dickey, 2008).
cells that bring about the indirect activation of epithelial cells (Table 1.2). Of these ligands, the most extensively studied and well-defined secretagogue is ATP, which, along with UTP, is capable of eliciting maximal mucin secretory responses from surface airway epithelial cells in all mammalian species examined thus far, including humans (Chen et al., 2001; Conway et al., 2003; Davis and Abdullah, 1997; Ehre et al., 2007; Kemp et al., 2004; Kim and Lee, 1991). Studies strongly indicate that ATP activates P2Y2 receptors to activate Gq and phospholipase C (PLC)-β1, leading to the generation of the second messengers diacylglycerol (DAG) and IP3. DAG activates its downstream target Munc13 via the SNARE complex to promote mucin granule translocation and fusion with the cell membrane (Fahy and Dickey, 2010). An intermediary role of the P2Y2 receptor has been unequivocally established by studies demonstrating that P2Y2 receptor-deficient mice exhibit severely compromised secretory responses to ATP (Ehre et al., 2007). Moreover, mucin secretion was successfully suppressed by selective pharmacological inhibition of the P2Y2 receptor, as well as in animal models involving targeted genetic knockout of molecules downstream of P2Y2 receptor activation, such as PLC and Munc13 (Kemp et al., 2004; Shin et al., 2001; Zhu et al., 2008a). Thus, current literature indicates that ATP and P2Y2 is a major regulatory system for mucin secretion.

The airway epithelium represents a primary source of ATP within the respiratory tract. Cellular release of ATP by the ciliated cells and the secretory (mucous) cells provide feedback on the epithelium through P2Y2 receptor activation in a paracrine and autocrine manner, respectively (Lazarowski and Boucher, 2009; Tarran et al., 2006). Under normal conditions, low levels of ATP are released constitutively from epithelial cells, with in vitro studies reporting that resting airway epithelial cells release ATP at a rate of 500 fmol min⁻¹ m⁻¹ (Lazarowski et al., 2000). This constitutive release may be important in regulating basal levels of mucin release and additional cellular processes (Corriden and Insel, 2010). In addition to basal ATP release, in vitro studies have demonstrated that an array of airway stresses, including mechanical irritation (Ransford et al., 2009; Tarran et al., 2006), allergens (Kouzaki et al., 2011), and cigarette smoke (Baxter et al., 2014), can greatly enhance ATP release (Schwiebert and Zsembery, 2003; Seminario-Vidal et al., 2011). Intriguingly, there is also evidence that P2Y2 receptor activation by ATP not only initiates the release of mucin but activates additional cellular signalling pathways promoting further secretion of ATP. Therefore, P2Y2 receptor activation may be part of an important positive feedback mechanism through which secretory cells produce paracrine signals to regulate key processes.
such as mucin release, hydration status, and cilia beat frequency (Burnstock et al., 2012; Kreda et al., 2007; Kreda et al., 2010; Okada et al., 2011). Furthermore, there is evidence that mucin secretory granules themselves contain nucleotides, which sets up a feed-forward loop whereby the secretion of mucins and concomitant release of nucleotides leads to further mucin release via purinoceptor activation (Kreda et al., 2010). Therefore, ATP and associated purinergic signalling pathways may serve as vital “danger signals” that promote crucial protective responses such as epithelial mucin secretion to combat potential injury (Burnstock et al., 2012; Kouzaki et al., 2011).

1.2.3 Role in the production and secretion of bronchoactive mediators

While the airway epithelium is an important source of mucins that are secreted to entrap exogenous agents, it is an equally important source of mediators that target the function of endogenous cellular targets. These mediators include cytokines, chemokines, growth factors, reactive oxygen and nitrogen species, peptides, and lipids, and together they serve a diverse range of biological functions (Table 1.3).

A subset of the bioactive mediators synthesised and secreted by the epithelium possess important bronchoactive properties. Similar to the capacity of the endothelium to control vascular smooth muscle tone, evidence has emerged that the epithelium is capable of modulating airway smooth muscle tone (Morrison et al., 1990; Vanhoutte et al., 1986). These findings were initially revealed by studies that examined functional responses of airway smooth muscle in epithelium-denuded isolated airway preparations. In these studies, removal of the airway epithelium augmented responses of the airway smooth muscle to contractile agents including histamine, but not carbachol or potassium chloride (Goldie et al., 1986; Preuss et al., 1992). Conversely, removal of the epithelium attenuated relaxation responses induced by bronchodilators including isoprenaline and capsaicin (Goldie et al., 1986), while completely abolishing relaxation responses to substance P (SP) (Szarek et al., 1998). Thus, it appears that the presence of a healthy epithelium can modulate responses of airway smooth muscle to both contractile and relaxant agents. A wide array of bronchoactive mediators have since been identified to be synthesised and secreted by the epithelium, including endothelins (Black et al., 1989; Goldie and Henry, 1999; Hay et al., 1993b), acetylcholine (Kummer and Krasteva-Christ, 2014; Kummer et al., 2008), prostaglandins (PGs) (Folkerts and Nijkamp, 1998), and nitric oxide (Di Maria et al., 2000), which may also be produced by subepithelial structures (Vanhoutte, 2013).
Table 1.2. Ligands known to induce mucin secretion from airway epithelial cells.

The major receptors mediating the secretory response and primary site of action of these ligands are also listed, where known.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Receptor</th>
<th>Primary site of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP/UTP</td>
<td>P2Y₂</td>
<td>Epithelium</td>
</tr>
<tr>
<td>Adenosine</td>
<td>A3AR</td>
<td>Epithelium</td>
</tr>
<tr>
<td>Proteases</td>
<td>PAR₁, PAR₂</td>
<td>Epithelium</td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>Unknown</td>
<td>Indirect</td>
</tr>
<tr>
<td>Histamine</td>
<td>Unknown</td>
<td>Indirect</td>
</tr>
<tr>
<td>Serotonin</td>
<td>Unknown</td>
<td>Indirect</td>
</tr>
<tr>
<td>Capsaicin (via substance P)</td>
<td>NK₁</td>
<td>May be indirect</td>
</tr>
</tbody>
</table>
Table 1.3. Classes of mediators secreted by the airway epithelium and their major features and functions.

<table>
<thead>
<tr>
<th>Class of mediator</th>
<th>Examples</th>
<th>Features and functions</th>
</tr>
</thead>
</table>
| Cytokines                             | IL-6, IL-11, thymic stromal lymphopoietin (TSLP) | • Released in response to allergen challenge  
• IL-6 enhances production of IgA  
• IL-11 activates B-cells  
• TSLP primes dendritic cells which promote naïve T-cell differentiation |
| Chemokines                            | Eotaxin-2, Eotaxin-3                          | • Recruits immune cells including eosinophils and basophils to the airways               |
| Growth factors                        | Epidermal growth factor                      | • Regulate proliferation and differentiation in response to epithelial injury  
• Crucial for epithelial repair                                               |
| Reactive oxygen and nitrogen species  | Nitric oxide                                  | • Activates transcription factors that increase cytokines and chemokine expression  
• Spasmolytic effect                                                              |
| Lipids                                | Prostaglandins F₂α, E₂                        | • PGE₂ is spasmolytic  
• Anti-inflammatory effects                                                          |
| Peptides                              | Endothelin-1                                  | • Spasmogenic effect                                                                  |
| Other                                 | Acetylcholine                                 | • May be released by epithelial cells, which may be a non-neuronal source of ACh, spasmogenic |
Upon secretion, these mediators activate target receptors on the neighbouring smooth muscle cells to engage downstream signalling pathways that elicit changes in airway smooth muscle tone.

1.2.3.1 Spasmogenic mediators

Airway epithelial cells are an important source of mediators that exhibit spasmogenic activity. Of these spasmogenic mediators, endothelins (ET) including ET-1 and ET-3 are among the most potent spasmogens in mammalian airways including humans. ET-1 induced a slow but long-lasting contraction in isolated preparations of tracheal and bronchial airway smooth muscle (Goldie et al., 1994; Goldie et al., 1995; Hay et al., 1993a; Henry et al., 1990), findings that are consistent with its potent in vivo bronchoconstrictor effects (Chalmers et al., 1997; Lagente et al., 1989). Functional studies indicated that ET-1-induced contractile responses are mediated by ET$_A$ and ET$_B$ receptors, which are differentially expressed in different mammalian species. For example, the ET$_B$ receptor is 10-fold more abundant than the ET$_A$ receptor in human bronchi, a ratio that is reversed in sheep airways (Ergul et al., 1995; Goldie et al., 1994; Goldie et al., 1995), whereas in rodent airways, ET$_A$ and ET$_B$ receptors are present in equal proportions (Henry, 1993). In addition to their direct spasmogenic effects, ETs have more recently been shown to promote airway hyperresponsiveness (Gregory et al., 2013). ETs have received attention due to their putative role in asthma, as levels are clearly elevated in asthmatic patients and compelling data has revealed a strong correlation between ET levels and the degree of asthma severity (Ackerman et al., 1995; Ninomiya et al., 1992; Zietkowski et al., 2008). Targeting ETs in a therapeutic context has yielded mixed results; early studies demonstrated that ET$_A$-selective antagonists successfully inhibited the early and late phase of asthma in a rodent model (Uchida et al., 1992), but in more recent clinical trials, a non-selective ET-1 receptor antagonist, Bosentan, did not improve clinical outcomes of patients with poorly controlled asthma (Coyle and Metersky, 2013). Thus, the therapeutic potential of targeting the ET system within a clinical context remains the subject of ongoing investigation (Coyle and Metersky, 2013; Goldie, 1999; Goldie and Henry, 1999; Gosens and Grainge, 2015; Kedzierski and Yanagisawa, 2001).

More recently, evidence has emerged that the epithelium may be a source of another important spasmogen, acetylcholine (ACh). ACh has traditionally been studied as a neurotransmitter of the neuronal cholinergic system that regulates bronchomotor tone.
Within the airways, the release of ACh from parasympathetic nerve endings elicits powerful contractile responses of the airway smooth muscle as well as secretory responses from the submucosal glands (Racké and Matthiesen, 2004). While these neuronal-driven signalling pathways play an undisputed role in the control of bronchomotor tone and glandular secretion, non-neuronal pathways of cholinergic signalling have recently been elucidated (Kummer and Krasteva-Christ, 2014; Kummer et al., 2008). Non-neuronal cells, such as those of the immune system and airway epithelium, possess the capacity to synthesise ACh, as proteins responsible for ACh synthesis in parasympathetic nerve fibres have been variably detected in these cells by immunohistochemical and Western blot approaches (Kummer et al., 2008). Although the production processes are comparable, the mechanisms of ACh secretion from airway epithelial cells appear to be distinct from those that exist in parasympathetic nerves. Upon stimulation, nerves typically liberate highly concentrated ACh vesicles by exocytosis, whereas epithelial ACh release is achieved via membrane transporters OCT1 and OCT2 that facilitate the active bidirectional movement of ACh (Lips et al., 2005).

Studies that examine epithelium-derived ACh are driven by a need to uncover its potential contribution to bronchoconstriction in asthma and COPD (Grainge et al., 2011). While neuronal ACh unequivocally contributes to these contractile responses, a potential role for epithelium-derived ACh remains to be evaluated. However, separating the relative contributions of each is not straightforward, and to determine whether bronchoconstrictor responses observed in vivo are mediated by one or the other, or a combination of the two, has proven to be experimentally challenging. Nonetheless, studies performed on mouse isolated tracheal preparations have demonstrated that stimuli such as 5-hydroxytryptamine (5-HT) evoke a contractile response sensitive to both epithelium removal and atropine but unaffected by the neurotoxin tetrodotoxin and an inhibitor of vesicle exocytosis BTXA (Moffatt et al., 2004). Taken together, these findings are suggestive of a non-neuronal source of ACh and implicate epithelial-derived ACh in the contraction of mouse tracheal smooth muscle. Interestingly, similar experiments performed in mouse bronchi did not yield similar conclusions, as 5-HT was capable of eliciting bronchoconstriction in bronchi isolated from M2/M3 knockout mice, suggesting a mechanism independent of ACh release (Kummer et al., 2006). It is possible that regional differences in the expression of cholinergic epithelial cells exist between the trachea and bronchi, which might account for the apparent disparity (Krasteva et al., 2011). Nonetheless, the epithelium may represent an important non-
neuronal source of ACh in selected regions of the airways. Further studies are required to fully characterise involvement of epithelial-derived ACh in bronchoconstriction, as well as a host of other responses including proliferation, mucociliary clearance, and immune functions (Wessler and Kirkpatrick, 2001).

1.2.3.2 Spasmolytic mediators

Airway epithelial cells are an important source of a range of bioactive mediators that also exhibit spasmolytic activity. Lipid mediators are synthesised in epithelial cells via three pathways: cyclooxygenase (COX), lipooxygenase, and cytochrome P450 monoxygenase. Of these, mediators produced via the COX pathway have been most extensively characterised for their spasmogenic and spasmolytic properties. Although COX-1 and COX-2 enzymes are both constitutively expressed by epithelial cells, only the latter enzyme is upregulated in response to stimuli including cytokines and pollutants (Zhao et al., 2009), as well as in asthmatic airways (Redington et al., 2001). Consistent with COX upregulation, bronchoalveolar lavage fluids collected from asthmatic subjects contained elevated levels of the COX-derived prostaglandins PGE$_2$ and PGF$_{2\alpha}$ (Liu et al., 1991). While PGF$_{2\alpha}$ stimulates its receptor to elicit bronchoconstriction, the effects of PGE$_2$ are more complex. PGE$_2$ interacts with four receptor subtypes expressed on airway smooth muscle cells to differentially elicit bronchoconstriction (via EP$_1$ or EP$_3$) and bronchodilation (via EP$_2$ or EP$_4$) (Tilley et al., 2003a). In particular, potent bronchodilatory responses elicited by PGE$_2$ have been observed in both small and large airways (Birrell et al., 2013; Buckley et al., 2011; Fortner et al., 2001). Since those initial observations, concerted efforts have been made to evaluate the therapeutic potential of exploiting PGE$_2$-mediated bronchodilatory responses for the treatment of asthma, particularly as an adjunct or alternative therapy to β-adrenoceptor agonists. Although studies remain ongoing, early findings suggest that exogenous PGE$_2$ and selective EP$_2$ receptor agonists possess limited bronchodilatory efficacy and are associated with undesirable side effects such as airway irritation and cough, whereas selective EP$_4$ receptor agonists show more promise as a potential treatment option (Benyahia et al., 2012; Buckley et al., 2011; Dowell et al., 2014; Gerthoffer et al., 2013).
Another mediator generated and secreted by the epithelium with well-documented spasmolytic properties is nitric oxide (NO). Although mRNA for all three isoforms of NO synthase have been detected in airway epithelial cells, the inducible, type 2 isoform (iNOS) is most abundantly expressed (Proud and Leigh, 2011; Robbins et al., 1994). NO secretion appears to confer a bronchoprotective function, as revealed by studies that have demonstrated the capacity of inhaled NO to counteract bronchoconstriction induced by spasmogens including methacholine (Dupuy et al., 1992), bradykinin (Nijkamp et al., 1993), and citric acid (Ricciardolo et al., 1999) in a range of animal models. Unexpectedly, these findings were not replicated in human subjects, as NO did not influence resting bronchomotor tone in healthy airways. However, small relaxation responses were evoked by NO in asthmatic airways (Hogman et al., 1993; Kacmarek et al., 1996). Thus, the in vivo effects of NO are likely more complex in human airways than in other mammalian species but its putative bronchoprotective properties warrant further investigation (Di Maria et al., 2000).

1.3 AIRWAY SENSORY NERVES

Although the airway epithelium is the first line of defence in the lungs, the respiratory tract contains additional structures that are equally crucial to conferring bronchoprotection. Intimate cross-talk between different structural components of the airways is often required to evoke more complex reflex responses. A number of these reflex responses involve activation of the network of nerves that innervate key structures within the entire respiratory tract. This neural network is comprised of three divisions: the parasympathetic, sympathetic, and afferent (sensory) systems (Jordan, 2001; Rogers, 2001; van der Velden and Hulsmann, 1999). The complex nature of the neural control of the airways is attributed to the capacity of neurotransmitters to activate a wide range of receptor types, each engaging differential second messenger systems in order to produce a spectrum of downstream effects. Many crucial aspects of airway function are under neural control, including a number of epithelial cell functions, submucosal gland secretion, and airway smooth muscle and bronchial vascular tone (Barnes, 1986b; van der Velden and Hulsmann, 1999). Whilst the parasympathetic and sympathetic divisions regulate other structural components in the respiratory tract, the airway epithelium is predominantly under direct neural control of the afferent (sensory nerve) system, and will be discussed in the following sections.
1.3.1 Populations of airway sensory nerves

The afferent (sensory nerve) system innervates a range of key structures within the respiratory tract including the airway epithelium, submucosal glands, the smooth muscle layer, and bronchial vessels (Figure 1.6). Innervation of the afferent system is primarily carried by branches of the vagus nerve, with neuronal bodies located in the nodose and jugular ganglia (Hunter and Undem, 1999). The vagus nerve then divides into the superior and recurrent laryngeal nerves that supply fibres to the trachea and main bronchi, while minor vagal branches innervate the remainder of the small airways (Belvisi, 2003b). Neuropeptides are preferentially released by high frequency firing of sensory nerves and subsequently produce a spectrum of downstream responses (Barnes and Thomson, 2009). The importance of sensory nerves in the production of reflex responses was first revealed by early studies demonstrating that blockade of sensory nerves alone was capable of abolishing reflex responses typically evoked by many mechanical and chemical stimuli (Coleridge and Coleridge, 1984; Widdicombe, 1981). Through the initiation of powerful and co-ordinated reflex responses such as cough, sneezing, mucus secretion, and bronchoconstriction, the sensory nerve system plays a crucial role in pulmonary defense (Bautista et al., 2013).

1.3.1.1 Sensory C-fibres

By far, the most abundant group of sensory fibres in the airways are those of the unmyelinated C-fibre family, comprising over 80% of all afferent sensory fibres in mammals (Jammes et al., 1982). The superficial positioning of the sensory nerve endings within the epithelium is crucial for the detection of airborne irritants. In contrast to nociceptor C-fibres in the somatosensory system, which transmit pain messages when activated, bronchopulmonary C-fibres evoke a number of defensive reflexes in response to noxious stimuli. Although activated by a range of thermal and mechanical stimuli, C-fibres are characterised by their distinct sensitivity to chemical irritants (Bessac and Jordt, 2008). Upon stimulation by these chemical irritants, chemosensitive fibres transmit information to the central nervous system (Lee and Pisarri, 2001). Alternatively, neuropeptides may be released peripherally from sensory efferents into the tissue microenvironment via a local axonal reflex (Figure 1.7). Together, the central and local axon reflex pathways combine to initiate reflex responses including bronchoconstriction, mucus secretion, and cough, which are critical for protecting the lungs against inhaled insults (Lee, 2009). Desensitisation of C-fibres was associated with an exacerbation of damage inflicted by a number of airborne irritants including
acrolein (Turner et al., 1993), endotoxin (Long et al., 1993) and ozone (Vesely et al., 1999), which supports the notion that C-fibres actively contribute to respiratory tract defences.

### 1.3.1.2 SARs and RARs

A smaller subpopulation of sensory nerves is highly myelinated and is characterised primarily by its sensitivity to mechanical stimuli (Figure 1.6). The two major myelinated fibre types, namely the slowly adapting receptors (SARs) and rapidly adapting receptors (RARs), innervate the airway smooth muscle and the epithelium, respectively. A principal function of these mechanosensitive fibre types is to monitor basal resting airway tension and to participate in feedback control (Bessac and Jordt, 2008). Additionally, SARs are thought to be involved with initiation of the cough reflex and bronchodilatory responses via activation of vagal efferent fibres (Barnes and Thomson, 2009). RARs, which belong to the Aδ fibre type, also demonstrate responsivity to chemical stimuli such as histamine and acids (Belvisi, 2003b).

### 1.3.2 Pathways of sensory C-fibre activation

#### 1.3.2.1 Transient receptor potential channels

The process of sensory C-fibre activation by chemical stimuli involves an important superfamily of ion channels that are highly expressed on sensory nerve terminals known as the Transient Receptor Potential (TRP) channels. This diverse family consists of 28 cation channels that are classified into 6 subfamilies: TRPV (vanilloid), TRPA (ankyrin), TRPC (canonical), TRPM (melastatin), TRPP (polycystin), and TRPML (mucolipin) (Banner et al., 2011; Moran et al., 2011). Although these subfamilies each contain distinct molecular motifs, they activate related molecular signalling systems. Evidence of a role for these ion channels in the activation of sensory C-fibres has emerged from work detailing the capacity of a range of exogenous irritants to activate specific members of the TRP superfamily. Capsaicin, a pungent constituent of hot chilli peppers, is by far the most extensively characterised of these irritants and readily activates the TRPV type 1 (TRPV1) channel. Consequently, capsaicin has become a common tool for the identification and characterisation of sensory C-fibre processes (Bessac and Jordt, 2008; Nagy et al., 2004). As the majority of C-fibres (80-90%) have demonstrated responsiveness to capsaicin, TRPV1 expression is considered one of the most prominent features of afferent C-fibres (Bessac and Jordt, 2010).
Figure 1.6. Innervation of key airway structures.

Slowly adapting receptors (SARs) innervate the airway smooth muscles (blue), while myelinated rapidly adapting receptors (RARs) (purple) and unmyelinated C-fibres (green) innervate the airway mucosa (modified from Barnes and Thompson, 2009).
Figure 1.7. Innervation of the airway epithelium.

Cholinergic (parasympathetic) nerves are the dominant pathway, where acetylcholine (ACh) interacts with muscarinic receptors (red). Adrenergic (sympathetic) innervation is species-specific and has not been demonstrated in human airways (black). Sensory nerve endings detect inhaled irritants and relay impulses via sensory (afferent) pathways to the central nervous system (CNS) to initiate reflex responses via the vagus nerve. Axonal neurotransmission via collateral sensory-efferent pathways leads to the release of sensory neuropeptides (blue) (adapted from Rogers, 2002).
1.3.2.1.1 TRPV1 channels

Structure-function studies have revealed the TRPV1 channel to be a ~95kDa, tetrameric, non-selective cation channel that possesses a vanilloid binding domain localised to transmembrane domains 3-4, which serves as the site of interaction for endogenous agonists of TRPV1 (Figure 1.8) (Caterina et al., 1997). In addition to capsaicin, TRPV1 has now been demonstrated to be activated by a variety of other stimuli including protons (Tominaga et al., 1998), products of lipoxygenase (Hwang et al., 2000), and thermal stimuli above 42°C (Caterina et al., 1997). Activation of TRPV1 leads to channel opening thereby producing an influx of calcium and other cations and subsequent depolarisation (Banner et al., 2011).

Despite its polymodality, there are stimuli and irritants that remain undetected by the TRPV1 channel. This was revealed by studies in which TRPV1-deficient mice were found to display normal respiratory sensitivity to agents such as acrolein, the major toxic component of smoke, and styrene, a lipophilic solvent (Symanowicz et al., 2004). Furthermore, whole-cell voltage clamp studies revealed that many electrophilic irritants, including acrolein, do not evoke any detectable currents in TRPV1-expressing CHO cells (Dinis et al., 2004). Collectively, these findings implicated involvement of additional ion channels in mediating responses to these irritants.

1.3.2.1.2 TRPA1 channels

The TRPA1 channel was demonstrated by a series of seminal studies to be the major chemosensory receptor for many irritants, in particular electrophilic irritants and oxidants produced by both exogenous (Bautista et al., 2006; Bautista et al., 2005; Bessac et al., 2008) and endogenous (Andersson et al., 2008) sources (Table 1.4). TRPA1 is the sole member of the TRPA subfamily expressed in mammals and is characterised by distinct ankyrin repeats located in the intracellular N-terminus established to be crucial to the activation process (Figure 1.8, Figure 1.9) (Escalera et al., 2008). More widespread than initially characterised, TRPA1 is now known to be co-expressed in 30-40% of TRPV1-expressing neurons, which amounts to approximately 20-35% of all sensory neurons (Bessac and Jordt, 2008; Jordt et al., 2004; Shapiro et al., 2013). Studies have revealed that TRPA1-expressing C-fibres innervate the upper
and conducting airways down to regions of gas exchange, including the respiratory bronchioles, alveolar ducts, and alveoli (Bautista et al., 2013).

In addition to electrophilic irritants, a number of other airborne irritants have also been found to activate TRPA1, including natural pungent compounds such as cinnamaldehyde (found in cinnamon) (Bandell et al., 2004), allicin (found in garlic extract) (Bautista et al., 2005) and polygodial (found in water pepper and Tasmanian pepper) (Escalera et al., 2008), alongside oxidising agents, isocyanates, heavy metals, and general anaesthetics (Table 1.4). It has now become clear that activation of TRPA1 is essential to initiating many of the physiological and protective responses associated with exposure to these agents (Bessac and Jordt, 2008).

The capacity of TRPA1 to detect such a diverse and structurally disparate range of irritants has been attributed to the distinctive ankyrin repeats present in the cytoplasmic N-terminus, which have been identified as critical sites for channel activation (Bautista et al., 2013) (Figure 1.9). Noting that many TRPA1-activating compounds were electrophilic and possess the capacity to react with nucleophilic cysteine residues in these ankyrin domains via Michael Addition reactions, Hinman and co-workers sought to determine whether these sites were relevant to channel activation (Hinman et al., 2006). The process by which the electrophilic compound allyl isothiocyanate activates TRPA1 was shown to involve reversible covalent modification of a crucial cluster of cysteine residues within the ankyrin domain (Hinman et al., 2006). In a subsequent study that performed systematic site-directed mutation of each of the 31 cysteine residues in the mouse TRPA1 channel, at least three specific cysteine residues (C415S, C422, C622S) were identified to be critical in the activation process (Macpherson et al., 2007). In addition to covalent modification of cysteine residues, TRPA1 activation is also thought to be modified by G-protein coupled receptors (Figure 1.9), however the mechanisms underlying activation are not as well-understood (Bautista et al., 2013). For example, bradykinin, a peptide produced in response to tissue injury, activates the BK$_2$ receptor, which is coupled to G$_s$ and phospholipase C, and may sensitise TRPA1 to activation by other stimuli (Bautista et al., 2006).
Figure 1.8. Structures of the TRPA1 and TRPV1 channels showing ligand interaction and regulatory sites.

Structures of TRPA1 (left) and TRPV1 (right) monomers, modelled after the X-ray crystal structure of the potassium channel, Kv1.2, a channel of the same superfamily of P-loop. TRPV1 is gated by vanilloids and endogenous inflammatory fatty acid metabolites binding to sites close to the intracellular face of the membrane. The intracellular NH$_2$-terminal segment of TRPA1 has ~15 ankyrin repeats that contain several cysteine and lysine residues (highlighted in red circle) that are crucial for activation by reactive agonists (from Bessac and Jordt, 2008).
Figure 1.9. Diverse mechanisms of TRPA1 channel activation.

Endogenous and exogenous agonists covalently modify cysteines in the TRPA1 N terminus to promote channel activity. Signalling molecules downstream of G protein-coupled receptors (GPCRs) also regulate TRPA1 channel activity (from Bautista et al., 2013).
Table 1.4. Airborne activators of the TRPA1 channel.

<table>
<thead>
<tr>
<th>Irritant Class</th>
<th>Chemical name</th>
<th>Sources</th>
<th>TRPA1-mediated effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>α,β-unsaturated aldehydes</td>
<td>Acrolein</td>
<td>Smoke</td>
<td>Vagus nerve activation, bronchial contraction, cough</td>
</tr>
<tr>
<td></td>
<td>Crotonaldehyde</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxidising agents</td>
<td>Hypochlorite</td>
<td>Industrial manufacturing</td>
<td>Reduced respiratory rate</td>
</tr>
<tr>
<td></td>
<td>H₂O₂</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isocyanates</td>
<td>Toluene diisocyanate</td>
<td>Industrial manufacturing</td>
<td>Reduced respiratory rate</td>
</tr>
<tr>
<td></td>
<td>Allyl isothiocyanate</td>
<td>Mustard oil</td>
<td>Reduced respiratory rate, cough</td>
</tr>
<tr>
<td>Heavy metals</td>
<td>Zinc</td>
<td>Industrial manufacturing</td>
<td>Reduced respiratory rate</td>
</tr>
<tr>
<td>General anaesthetics</td>
<td>Desflurane</td>
<td>Surgery</td>
<td>Bronchial contraction</td>
</tr>
</tbody>
</table>
1.3.3 Pathways of sensory C-fibre impulse propagation

Downstream of activation of sensory C-fibres by aforementioned stimuli, action potential impulses are conducted down one of two pathways. Firstly, impulses may propagate up the vagus nerve to the central nervous system, resulting in initiation of reflex responses mediated via the vagal efferent pathway (Lee and Pisarri, 2001; Lee and Undem, 2005). Alternatively, impulses may propagate down collateral sensory efferent nerve endings, resulting in the peripheral release of sensory neuropeptides that mediate reflex responses via a local axonal pathway (Rogers, 2001) (Figure 1.7).

1.3.3.1 Central reflex pathway

Action potentials propagated in an orthodromic manner travel up the vagus nerve to the commissural subnucleus of the nucleus tractus solitaries and elicit chemoreflexes via the efferent pathway (Figure 1.6) (Lee and Undem, 2005). Information relayed to the central nervous system is processed accordingly, resulting in the initiation of respiratory reflex responses such as apnoea, airway constriction, mucus secretion, and cough, in addition to a range of cardiovascular responses including bradycardia and bronchial vasodilatation (Belvisi, 2003b; Jordan, 2001; Lee, 2009; Lee and Pisarri, 2001). Together, these responses function to limit further inhalation of the irritant and to promote its removal from the airways.

1.3.3.2 Local axonal reflex pathway

Action potentials propagated in an antidromic manner travel down collateral sensory efferents to elicit reflexes via peripheral release of neuropeptides contained within sensory nerve terminals in a local axonal reflex (Barnes, 1986a; Belvisi, 2003b). Release of these neuropeptides initiates reflexes including bronchoconstriction, mucus secretion, vasodilatation, and plasma extravasation (Barnes, 1992; Belvisi, 2003b; van der Velden and Hulsmann, 1999) (Figure 1.10). Among the neuropeptides released by the local axonal reflex, the most well-characterised neuropeptides include the 37-amino acid peptide calcitonin gene-related peptide (CGRP) and the tachykinins SP, neurokinin A (NKA), and neurokinin B (NKB), which share the common C-terminus sequence Phe-X-Gly-Ley-Met-NH₂ (van der Velden and Hulsmann, 1999). Expression of the target receptor of CGRP, the CGRP type 1 receptor, has been predominantly confined to arterial vessels rather than airway smooth muscle or epithelial cells, which is consistent with its potent vasodilatory actions but limited bronchoactive properties
(Carstairs, 1987; Solway and Leff, 1991). In addition to its direct effects, CGRP works synergistically to amplify the effects of tachykinins (Brain et al., 1985). The presence of tachykinin-immunoreactive fibres has been demonstrated throughout the human airways, innervating key structures such as the airway epithelium, submucosal glands, bronchial smooth muscle, and blood vessels (Lundberg et al., 1984; Martling, 1987; Ollerenshaw et al., 1991). Activation of these nerves fibres results in the release of tachykinins that differentially activate members of the neurokinin receptor family 1-3 (NK₁-₃) to elicit specific responses (Table 1.5).
Figure 1.10. Local release of neuropeptides by peripheral projections of C-fibres.

In the peripheral tissues, neuropeptides can be released locally as a consequence of axon reflexes that produce the responses described in the figure (from Belvisi, 2003).
Table 1.5. Sensory neuropeptide receptors in the airways and associated responses mediated by their activation.

CGRP, calcitonin gene-related peptide; NK, neurokinin; SP, substance P.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Activation preferences</th>
<th>Expression</th>
<th>Responses evoked</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGRP</td>
<td>CGRP</td>
<td>Arterial vessels</td>
<td>Vasodilatation</td>
</tr>
<tr>
<td>NK₁</td>
<td>SP (&gt;NKₐ&gt;NK₈)</td>
<td>Airway smooth muscle, pulmonary vessels, epithelial cells, submucosal gland</td>
<td>Mucus secretion, microvasular leak</td>
</tr>
<tr>
<td>NK₂</td>
<td>NKₐ (&gt;SP&gt;NK₈)</td>
<td>Airway smooth muscle (not definitively characterised in humans)</td>
<td>Bronchoconstriction, greater contractile effect in smaller bronchi than proximal airways</td>
</tr>
<tr>
<td>NK₃</td>
<td>NK₈ (&gt;NKₐ&gt;SP)</td>
<td>Not expressed in humans</td>
<td>-</td>
</tr>
</tbody>
</table>
1.4 Interaction between the Airway Sensory Nerves and Airway Epithelium

The close spatial association between the sensory nerves and the epithelium facilitates an intimate degree of interaction between these two airway structures. On one level, the network of sensory nerves innervates the epithelium, exerting neuronal control over several aspects of epithelial function. A reciprocal level of control is also exerted, with changes in the epithelium having profound functional consequences on the sensory nerves and associated neuropeptides released, which may have implications in airway disease. Thus, the bi-directional interaction that exists between these two structures adds another degree of complexity to the airways.

1.4.1 Influence of Airway Sensory Nerves on the Epithelium

1.4.1.1 Innervation of the Epithelium

In mammalian airways, the airway epithelium is richly innervated by sensory nerves originating from the sub-epithelial plexus. Staining with the neuronal marker protein gene product 9.5 or SP-immunoreactive nerves in mammalian airways has revealed a dense plexus of sub-epithelial nerves that lie beneath the basement membrane lumen (Figure 1.11). Extending from this plexus are fibres that form focal points that interdigitate beneath and penetrate the basement membrane before spreading in between individual epithelial cells and for some fibres, terminating in the airway lumen (Adriaensen and Scheuermann, 1993; Laitinen, 1985). Using retrograde tracing and immunohistochemical techniques, early studies established that approximately 30% of all afferent fibres innervating the guinea pig trachea are sensory nerves that originate from cell bodies in the jugular ganglia and contain both CGRP and the tachykinins SP and NKA (Kummer et al., 1992; Ricco et al., 1996). Although SP-immunoreactive nerves are more abundant in rodent airways (Howarth et al., 1995; Hunter and Undem, 1999; Lamb and Sparrow, 2002), immunohistochemical studies have confirmed the presence of SP-containing nerves innervating the epithelium in human airways (Bowden and Gibbins, 1992; Ollerenshaw et al., 1991). These nerves exhibited a similar pattern of distribution in human airways and there is evidence that expression increases further in asthmatic airways (Ollerenshaw et al., 1991). The lack of strong levels of staining for these nerves in healthy human airways is thought to be partly attributable to peptide degradation in denervated post-mortem tissue (Belvisi, 2003a; Knight and Holgate, 2003). Thus, while the distribution of sensory C-fibres has been
well-mapped in guinea pig airways, the expression of these nerves has not been as well-characterised in human airways, but is thought to be comparatively sparse.

Neuropeptides released by sensory C-fibres participate in the control of airway epithelial function through interaction with cognate receptors expressed by surface epithelial cells and epithelial cells that line submucosal glands. In particular, NK₁ receptor immunoreactivity has been strongly demonstrated in the human airway epithelium (Chu et al., 2000). More specifically, through a combination of in vitro and in vivo studies, activation of NK₁ by SP and additional tachykinins are known to activate signalling pathways that elicit a number of downstream effects in the airway epithelium, including surface epithelial secretion of inflammatory cytokines, secretion of mediators that modulate bronchomotor tone (Devillier et al., 1992; Manzini, 1992; Veronesi et al., 1999), glandular secretion of mucus (Trout et al., 2001), and epithelial cell proliferation (Kim et al., 1995).

1.4.2 Influence of the airway epithelium on sensory nerves

1.4.2.1 Epithelial production of neuropeptide degradative enzymes

In addition to expressing cognate neuropeptide receptors, longstanding work has demonstrated that epithelial cells regulate the actions of sensory nerve-derived neuropeptides through production of neutral endopeptidase (NEP), a membrane bound metallopeptidase and an important degradative enzyme responsible for the breakdown of tachykinins, amongst other peptides (Borson, 1991; Di Maria et al., 1998; Nadel, 1991). Although it is also produced by airway smooth muscle cells, submucosal glands, and fibroblasts, NEP is located in high concentrations at the surface of airway epithelial cells, where it preferentially cleaves peptides or hydrolyses peptide bonds (Gln-Phe, Phe-Phe and Gly-Leu in SP, Ser-Phe and Gly-Leu in NKA) to yield peptide fragments that lack the carboxy terminal required for tachykinin receptor binding (Di Maria et al., 1998). A role for NEP in the regulation of airway responses evoked by tachykinins was further supported by early studies that demonstrated that epithelium removal or pharmacologic inhibition of NEP by agents such as thiorphan markedly potentiated NK₁-dependent responses including mucous secretion (Geppetti et al., 1993) and bronchoconstriction (Cheung et al., 1992) in rodent and human airways, respectively. Enhanced inflammatory responses observed in asthmatic airways have been partly attributed to epithelial damage and consequent potentiation of tachykinin activity as a
Figure 1.11. Immunohistochemical evidence of the innervation of the airway epithelium by sensory nerves.

Confocal projections of the apical bronchial epithelium of a pig stained with phalloidin reveals the margins of epithelial cells (scan depth 1 \( \mu \text{m} \), bar = 10 \( \mu \text{m} \)) (A). Tufts of cilia on the epithelial cells are seen as white speckles (Ai). SP-immunoreactive fibres in the apical bronchial epithelium of a pig (B). The same field as concurrently stained for PGP immunoreactive nerves (C, D). Varicose fibres with nerve endings that pass around the apices of goblet cells (white arrowheads). The apical epithelium of human bronchial tissue showing PGP immunoreactive stained varicose fibres (white arrow heads, scan depth 14 \( \mu \text{m} \), bar = 50 \( \mu \text{m} \)) (E, F). Autofluorescence arising from 488 nm excitation has been overlaid to show the goblet cells (G) (adapted from Lamb et al., 2002).
result of reduced NEP levels (Di Maria et al., 1998; Joos et al., 2000). Therefore, the effects of tachykinins released by sensory C-fibres appear to tightly regulated by the epithelium through production of NEP, a process that may become dysregulated in certain airway pathologies.

1.4.2.2 Exposure of sensory nerves caused by epithelial shedding

Although a proportion of sensory nerve endings terminate in the airway lumen, the epithelium remains the primary interface between the airways and the inhaled environment. For this reason, a principal function of the epithelium is to protect the underlying structures, including the network of sensory nerves, from inappropriate levels of exposure to noxious airborne agents. Thus, disruption of the epithelial layer may heighten exposure of efferent nerve endings to these agents resulting in inappropriate stimulation of sensory nerves and exaggerated production of neurogenic inflammatory responses (Belvisi, 2003a; b).

The pathological consequences of inappropriate levels of sensory nerve stimulation have been demonstrated in rodent models of asthma and viral infection, whereby depletion of sensory neuropeptides by capsaicin desensitisation, inhibition of neuropeptide release, and pharmacological inhibition of neurokinin receptors have all strongly attenuated neurogenic inflammatory responses (Barnes, 2001; Jacoby and Fryer, 1990; Undem and Carr, 2002; Widdicombe, 2003). However, findings from these animal models have not yet been fully extrapolated to the human airways. For this reason, the role of exaggerated sensory nerve activation and associated neurogenic inflammatory responses in airway disease requires further clarification and is the subject of ongoing debate (Belvisi, 2003a; Veres et al., 2009). Discrepancies between animal models and human airways may be partly attributed to the scant expression of sensory nerves in human airways compared to rodent airways, although increases in the number of SP-immunoreactive nerves in airways of asthma patients coupled to observations of elevated concentrations of SP in bronchoalveolar lavage fluids and enhanced expression of both the NK₁ and NK₂ receptors seem to favour a role of neurogenic inflammation in these diseases (Adcock et al., 1993; Bai and Bramley, 1993; Ollerenshaw et al., 1991). However, conflicting reports of the clinical efficacy of NK receptor antagonists in combating responses such as bronchoconstriction and cough in asthmatic patients have brought into question the relative contribution of neurogenic inflammation in human airway disease, and more work is needed before
definitive conclusions can be made (Belvisi, 2003a; Groneberg et al., 2004). A more effective approach might be to inhibit both local reflexes and the central reflex through a generalised action that targets both the afferent and efferent fibres. Nonetheless, neuropeptides released locally from peripheral nerve endings may still play an important role and thus merit consideration as a potential target in combination with the afferent pathway.

1.5 ACROLEIN AND THE AIRWAYS

As discussed in Sections 1.2 and 1.3, both the airway epithelium and the network of sensory nerves form part of the lung defence system. As such, these structures play a key role in protecting the airways against a host of noxious airborne agents, including acrolein. While protective reflexes form part of the acute physiological responses to acrolein, pathophysiological responses to acrolein are incurred through various mechanisms of toxicity, and both will be discussed in the following sections.

1.5.1 Physiological responses to acrolein — protective respiratory reflexes

1.5.1.1 Acrolein-induced effects on the airway epithelium

As the first point of contact with airborne acrolein, responses of the airway epithelium participate in the initial defence against this noxious molecule. As discussed in Section 1.2.2, the stimulation of mucin secretion from the airway epithelium facilitates entrapment of airborne particles and is therefore an important protective response.

1.5.1.1.1 Mucus secretion

Previous studies conducted in a variety of mammalian species have demonstrated that acute exposure to high doses of acrolein elicits a strong mucous secretory response (Dahlgren et al., 1972; Gusev et al., 1966; Harkema et al., 1987; Lamb and Reid, 1968; Le Bouffant et al., 1980; Lyon et al., 1970). In rat and guinea pigs, inhalational exposure to acrolein markedly increased the levels of mucus secretions deposited onto the bronchial surface (Dahlgren et al., 1972; Gusev et al., 1966). However, beyond these longstanding observations, the precise cellular mechanisms controlling acrolein-induced mucous secretion remain incompletely understood. For example, the relative
contribution of mucus released from goblet cells and from glandular epithelium to the overall mucus secretions observed with acrolein exposure has not been yet defined. These mucin secretory responses are further explored in Chapter 5.

1.5.1.2 Acrolein-induced activation of sensory nerves

As a highly potent irritant, inhalational exposure to acrolein produces immediate irritation to the upper respiratory tract. Previous work has demonstrated that acute exposure to acrolein initiates a host of reflexes typically associated with sensory nerve activation, including lacrimation, depressed breathing rate, cough, bronchoconstriction, vasodilation, and plasma protein extravasation (Alarie and Stokinger, 1973; Feron et al., 1978; Lyon et al., 1970; Morris et al., 1999; Morris et al., 2003). Desensitisation of sensory C-fibres by capsaicin pre-treatment exacerbated acrolein-induced pulmonary damage and was associated with elevated mortality rates in guinea pigs (Turner et al., 1993). Thus, sensory nerve-mediated responses serve a critical function in protecting the airways against acrolein.

1.5.1.2.1 TRPA1 channel activation

While earlier studies focused on the acute respiratory effects of acrolein, more recent studies have utilised a combination of in vitro and ex vivo approaches to examine the cellular mechanisms through which sensory nerves are activated by acrolein, upstream of observed respiratory effects. Through a series of seminal experiments conducted in human and rodent airways, acrolein was demonstrated to be a potent activator of the TRPA1 channel (Andrè et al., 2008; Bautista et al., 2006; Birrell et al., 2009; Hinman et al., 2006). In these studies, acrolein activated a substantial influx of calcium into TRPA1-transfected HEK293 cells, as well as in TRPA1-expressing vagal sensory neurons cultured from mice, guinea pig, and human airways. Compellingly, selective inhibition of the TRPA1 channel significantly abated this influx of calcium, a response that was completely absent in normal (non-TRPA1-transfected) HEK293 cells (Andrè et al., 2008; Bautista et al., 2006; Birrell et al., 2009). Furthermore, sensory neurons cultured from TRPA1 gene-deleted mice lacked any responsiveness to acrolein but retained their sensitivity to TRPV1-activating agents such as capsaicin. Taken together, these observations supported a role for the TRPA1 channel in not only the activation of sensory neurons by acrolein but for basic detection of acrolein by sensory nerves.
Consequently, TRPA1 has been recognised as an essential chemosensory receptor for acrolein (Bautista et al., 2006; Birrell et al., 2009).

Acrolein is thought to activate the TRPA1 channel through a distinct mechanism involving covalent modification of a cluster of key cysteine residues within the intracellular ankyrin repeat domains (Hinman et al., 2006; Macpherson et al., 2007). The strong electrophilic nature of acrolein permits it to react readily with these nucleophilic cysteine residues via Michael addition, which, in turn, initiates channel activation. This is also thought to be a common mechanism through which additional noxious stimuli activate the TRPA1 channel to signal potential tissue damage.

1.5.1.2.2 TRPA1-mediated responses

Despite functioning as a chemosensory receptor for acrolein, a clear mechanistic link between TRPA1 channel activation and production of downstream responses remains to be established for several sensory nerve-mediated responses. For the few sensory nerve-mediated responses that have been examined, evidence for TRPA1 involvement has been demonstrated through direct modulation of responses by genetic manipulation and pharmacologic inhibition of the TRPA1 channel. While this has been established for both acrolein-induced cough and neurogenic inflammation (Andrè et al., 2008;andrè et al., 2009), whether TRPA1 channel is involved in the regulation of additional responses, such as those associated with the secretory function of the epithelium, are unknown; this is further explored in Chapter 6.

1.5.1.2.2.1 Cough reflex

The cough reflex represents one of the most powerful mechanisms for lung defence and is crucial for the clearance of environmental irritants, particles, and airway mucus from the respiratory tract. Activation of afferent vagal fibres that densely innervate the upper and lower respiratory tracts play a critical functional role in mediating the cough reflex (Bautista et al., 2013). Although tussive stimuli activate a range of plasma membrane receptors and ion channels localised to vagal bronchopulmonary C-fibres, there are several lines of evidence implicating involvement of the TRPA1 channel in the cough reflex. Firstly, TRPA1 agonists are capable of depolarising TRPA1-expressing vagal C-fibres (Birrell et al., 2009; Nassenstein et al., 2008). Secondly, vagus nerve
activation was strongly attenuated by pharmacologic blockade of the TRPA1 channel and genetic deletion of TRPA1 (Birrell et al., 2009; Taylor-Clark et al., 2008). Thirdly, selective TRPA1 activators are potent triggers of the cough reflex in rodents and humans (Andrè et al., 2009; Birrell et al., 2009).

As a potent tussive agent, interest surrounds the mechanisms underpinning acrolein-induced cough. Currently, there exists compelling evidence for a role of the TRPA1 channel in mediating the cough response to acrolein (Geppetti et al., 2010). Demonstrated through a series of elegant in vivo experiments, aerosolised acrolein, as well as additional TRPA1 channel activators including cinnamaldehyde and crotonaldehyde, was shown to evoke powerful, dose-dependent cough reflexes in guinea pigs. Importantly, these responses were inhibited by pre-treatment with the selective TRPA1 channel inhibitor HC-030031 (but not by the selective TRPV1 channel inhibitor capsazepine) and by desensitisation from prolonged capsaicin exposure (Andrè et al., 2009; Birrell et al., 2009).

The majority of in vivo studies have modelled cough responses in guinea pigs rather than in mice, which lack a cough reflex (Bautista et al., 2013). Nonetheless, mice do exhibit altered breathing patterns such as a reduction in breathing frequency and lengthening of respiratory pause in response to irritants, and these indications of airway irritation are routinely used as experimental endpoints akin to cough. Importantly, these indications of irritation were completely ablated in TRPA1−/− mice, firmly implicating a role for TRPA1 in these irritant responses (Gu and Lin, 2010; Macpherson et al., 2007; Taylor-Clark et al., 2009). Whilst we still await results of studies examining tussive responses elicited by acrolein in human subjects, existing studies have strongly indicated that cough responses evoked in human volunteers by other TRPA1 channel activators including cinnamaldehyde closely mirror that of the (conscious) guinea pig model (Birrell et al., 2009).

1.5.1.2.2 Neurogenic inflammation
The inflammatory response associated with cigarette smoke exposure is a well-documented effect and was purported by early studies to be partly neurogenic in origin (Lundberg and Saria, 1983). Since those initial observations, α, β- unsaturated aldehydes, including crotonaldehyde and acrolein, have been identified as major
causative agents of neurogenic inflammation in cigarette smoke via TRPA1 activation (Andrè et al., 2008; Bautista et al., 2006; Trevisani et al., 2007). Upon TRPA1 activation, release of neuropeptides from sensory nerve endings results in the activation of target cells that elicit a complex array of downstream inflammatory responses such as increased vascular permeability, elevated blood flow, plasma protein extravasation, local recruitment of inflammatory cells, as well as the pain sensation (Andrè et al., 2008; Morris et al., 1999).

While the general consensus is that protracted and unmodulated neurogenic inflammation contributes to inflammatory diseases such as asthma and COPD, acute neurogenic inflammation also may form part of a protective mechanism termed the “nocifensor system” that exists in various tissue systems including the airways (Jimba et al., 1995; Light, 2004; Peskar et al., 1990). By limiting insult exposure through early and acute inflammatory responses, this system may be crucial for protection against the detrimental actions of airborne insults, including cigarette smoke (Turner et al., 1993).

1.5.2 Pathophysiologi cal responses to acrolein — mechanisms of toxicity

Although protective respiratory reflexes are elicited in response to acute acrolein exposure, defensive capacities of the lung may be overwhelmed, particularly in the face of exposure to high or extended doses of acrolein (Bein and Leikauf, 2011). When lung defences are breached, acrolein has a tremendous capacity to react with a wide array of cellular targets, often with deleterious functional outcomes. For example, acrolein doses in excess of 50 µM typically produce massive cell disruption and necrosis (Kehrer and Biswal, 2000; Tanel and Averill-Bates, 2007) whereas doses under 50 µM produce apoptosis (Nardini et al., 2002). To delineate the mechanisms of acrolein-induced toxicity, most work has focused on identifying cellular components targeted by acrolein; less is known about the functional consequences of acrolein’s effects on these targets. Furthermore, the potential interdependence and cross-talk between distinct mechanisms of acrolein toxicity and their relative contribution to the pathogenesis of human disease is not well-defined. Thus, the broader focus of toxicological studies is to rationalise the pathological influence of acrolein with its profound chemical reactivity and molecular effects. Although this was not the focus of the work presented in this Thesis, the pathological influence of acrolein will be
discussed in the following sections to provide a comprehensive overview of the known effects of acrolein.

### 1.5.2.1 Cellular targets of acrolein

#### 1.5.2.1.1 Protein adduction

As a “soft” electrophile (small atomic radius, low effective nuclear charge, high polarisability), acrolein readily targets soft cellular nucleophiles such as the sulfhydryl group of cysteine residues found in proteins. To a lesser degree, acrolein also displays reactivity with “hard” nucleophiles (large atomic radius, high effective nuclear charge, low polarisability) such as the nitrogen atom in the imidazole group of histidine and amino group of lysine to form Michael addition adducts or Schiff base cross-links (Pizzimenti et al., 2013; Zarkovic et al., 2013). Although GSH is an important acrolein-scavenging molecule, free (unbound) acrolein reacts readily with these proteins, which may be subjected to further structural and functional modification (Nardini et al., 2002; Spiess et al., 2011). Susceptible protein targets have major physiological roles in a range of cellular functions, including enzyme catalysis, redox signalling, and maintaining the structural integrity of the cells. Thus, interest in protein adduction is driven by the premise that identification of protein targets may provide insight into cellular pathways dysregulated by acrolein and the functional deficits that ensue (Aldini et al., 2011; Burcham et al., 2010c).

Several in vitro studies have revealed the identity of major proteins targeted by acrolein for adduction. Particular interest has been paid to carbonylation, a process by which acrolein reacts with soft nucleophiles via Michael addition to the unsaturated β-carbon resulting in the formation of carbonyl-retaining adducts (Suzuki et al., 2010). By Western blot analysis, a range of cytosolic proteins including vimentin, keratin-18, and other intermediate filaments were identified as targets for acrolein carbonylation in A549 lung cells, even at low concentrations of exposure (25-75 μM). Higher concentrations of acrolein (100-150 μM) resulted in carbonylation of membrane and nuclear proteins (Burcham et al., 2010a). Importantly, use of the carbonyl scavenger bisulfite strongly attenuated adduction of proteins and suppressing the cytotoxicity of acrolein, as assessed through measurements of cell impedance (Burcham et al., 2010b). Additional targets for adduction by acrolein have also emerged, including the transcription factor NF-κB (Horton et al., 1999; Lambert et al., 2007; Valacchi et al.,
2005; Wu et al., 2010) and caspases that regulate apoptotic cell death (Finkelstein et al., 2005; Hristova et al., 2007; Kern and Kehrer, 2002; Myers and Myers, 2009). Using comprehensive global proteomic profiling approaches, a wide array of proteins were revealed to be alkylated by acute acrolein exposure in rat lung epithelial cells and human bronchial epithelial cells (Sarkar and Hayes, 2009; Spiess et al., 2011). More specifically, proteins with an enhanced susceptibility to modification by acrolein were revealed to be the stress proteins, cytoskeletal proteins, and proteins involved in redox signalling.

Structural modification of proteins likely has deleterious functional consequences for the proteins targeted. For example, adduction of intermediate filaments within Calu-3 lung cells was shown to be associated with a loss of cellular integrity and transepithelial resistance with concomitant increases in cellular permeability (Burcham et al., 2014). Although studies have identified a range of proteins targeted by acrolein and have begun to examine the functional consequences of adduction in vitro, much more work is required to appraise the significance of these observations in vivo. Thus, the current state of knowledge concerning the relationship between adduction of specific protein targets and functional outcomes elicited remains rudimentary, particularly within an in vivo setting.

1.5.2.1.2 DNA adduction

Although less thermodynamically favoured than protein adduction, acrolein may also react with harder nucleophilic sites such as those present within DNA, and is capable of forming adducts and cross-linkages with all four DNA bases (Pawłowicz et al., 2007; Pawłowicz and Kronberg, 2008; Stevens and Maier, 2008) and inhibiting DNA repair proteins (Feng et al., 2006; Wang et al., 2012). For example, acrolein has been shown to target guanine, the most nucleophilic DNA base, to form two pairs of stereoisomers of cyclic 1,N\(^2\)-propanodeoxyguanosine (Acr-dGuo) adducts: \(\alpha\)-OH-Acr-dGuo, and \(\gamma\)-OH-AcrdGuo isomer, and the latter is particularly mutagenic in vitro (Zhang et al., 2007). If left unrepaired, acrolein-DNA adducts may lead to mutations in a number of critical genes, a process that is often the first step in the mechanism through which many carcinogens exert their effect. Indeed, a seminal study revealed through a sequencing approach that the pattern of DNA damage inflicted by acrolein to the \(p53\) tumour suppressor gene in human bronchial epithelial cells correlated closely with
those observed in lung cancer (Feng et al., 2006). However, subsequent studies have not yielded consistent results and do not provide a unanimous view on the mutagenicity of acrolein. For example, several in vitro studies provided further evidence in support of an aetiological role for acrolein in cigarette smoke-related lung cancer by demonstrating acrolein-induced G-to-T and G-to-A mutations in CpG sites in the p53 gene (Tang et al., 2011; Wang et al., 2012; Wang et al., 2013; Wang et al., 2009). In contrast, other studies have strongly refuted a mutagenic effect of acrolein. While they recognise the capacity of acrolein to form DNA adducts, these studies have brought into question the mutagenic potential of such adducts by arguing that existence of highly accurate replication bypass of acrolein-DNA lesions and rapid repair systems preclude acrolein-DNA adducts from exerting a mutagenic effect in vivo (Besaratinia, 2009; Kim et al., 2007; Liu et al., 2010). Thus, while acrolein readily forms DNA adducts, these studies concluded that there is insufficient evidence that acrolein produces mutagenesis in vivo.

1.5.2.1.3 Transcriptional changes

1.5.2.1.3.1 Acute changes

Acrolein is also capable of eliciting profound changes in gene expression. Early studies identified members of the classic antioxidant response element locus as important targets for acrolein, with hallmark genes including heme-oxygenase 1, NAD(P)H:quinone oxidoreductase-1, and GSH all strongly upregulated with acute exposure (Tirumalai et al., 2002) and may blunt toxicological consequences of acrolein exposure. A more comprehensive appraisal of the short-term transcriptional changes elicited by high-dose acrolein exposure was performed in human lung A549 cells. In keeping with its pronounced chemical reactivity, acrolein exposure altered transcript levels of hundreds of genes involved in apoptosis, cell cycle control, cytoskeletal maintenance, and transcription factors (Thompson and Burcham, 2008). Initial downregulation of a large cohort of genes preceded rapid recovery of transcript levels within 2 h of exposure and by 4 h, more than half of the genes affected were elevated over control levels. Notably, acrolein strongly upregulated expression of members of the early growth response (EGR) family in a time-dependent manner, with more than a 400-fold increase in EGR-1 transcript levels reported and confirmed by real time-PCR and Western blot (Thompson and Burcham, 2008). Transcript levels of the heat shock protein Hsp70 and the antioxidant response gene HMOX-1 also increased strongly in response to acrolein. Although incompletely characterised, the toxicological
significance of upregulation of EGR-1 appears to lie in its pro-inflammatory and pro-apoptotic functions (Ngiam et al., 2007). Additionally, EGR-1 may control expression of repressors that control tight junction protein expression, thus offering a plausible mechanism by which acrolein disrupts cell-cell interactions (Grotegut et al., 2006).

1.5.2.1.3.2 Chronic changes
The transcriptional changes elicited by acute acrolein exposure stand in contrast to those observed within a chronic setting. Of the transcriptional changes associated with chronic exposure to acrolein, the most pronounced and well-characterised response involves upregulation of the mucin gene MUC5AC (Borchers et al., 1999a; Borchers et al., 1998; Chen et al., 2013; Deshmukh et al., 2005; Deshmukh et al., 2009; Deshmukh et al., 2008; Leikauf et al., 2002; Liu et al., 2009a; Liu et al., 2009b). In these studies, increases in the levels of intracellular mucin associated with sub-chronic to chronic acrolein exposure were driven exclusively by upregulation of MUC5AC gene expression. For example, exposure of mice and rat airways to acrolein (3 ppm) for 6 hours a day, 5 days a week, for 2 weeks was clearly associated with increases in MUC5AC mRNA levels in both the trachea and lung (Borchers et al., 1998; Borchers et al., 1999b). In these animals, changes in mucin expression were preceded by marked inflammatory changes, consistent with an inflammatory-driven mucous metaplastic response. In airway epithelial cells, acrolein, and associated inflammatory mediators such as PGE2 and TNF-α, produced comparable increases in MUC5AC mRNA expression (Haswell et al., 2010; Leikauf et al., 2002).

Although more work is required to fully delineate the mechanisms underlying transcriptional upregulation of MUC5AC elicited by chronic acrolein exposure, several candidate molecules and pathways have been identified. At concentrations directly comparable to those measured in sputum from COPD patients (100-300 nM), acrolein was strongly associated with increases in transcript levels of matrix metalloproteinases (MMP) in human airway cells (Deshmukh et al., 2008). MMP-9, 12, and 14 have been identified as key initiators of the epidermal growth factor receptor (EGFR) pathway, which is crucial to regulating mucin production. More specifically, MMPs are involved in the cleavage and release of cell surface EGFR ligands that activate EGFR to trigger downstream signalling pathways that lead to upregulation of MUC5AC gene expression. Marked suppression of acrolein-induced increases in transcriptional and protein levels of MUC5AC in MMP9−/− mice and in mice treated with specific EGFR
kinase inhibitors or neutralising antibodies provided evidence in support of a role for these molecules in the mucous metaplastic response (Deshmukh et al., 2005; Deshmukh et al., 2008). Additional signalling pathways may also be involved, with acrolein-induced MUC5AC expression also suppressed by inhibitors of mitogen-activated protein kinase (MAP) 3/2 and 8 (Deshmukh et al., 2009), MAPK14 (Liu et al., 2009b), simvastatin (Chen et al., 2010), and rosiglitazone (Liu et al., 2009a). Successful attenuation of MUC5AC overexpression by these drugs suggests that targeting specific molecules in these pathways may have potential therapeutic applications in the treatment of airway diseases characterised by mucous metaplasia.

1.5.2.2 Involvement in respiratory disease

In keeping with its overt toxicity and widespread effects, acrolein has been implicated in the pathogenesis of a number of respiratory diseases. While acute exposure to high doses of smoke is relevant in the context of environmental or industrial fire exposure, chronic exposure to lower doses of smoke is relevant in the context of passive or habitual smoking. Under both of these conditions, there is accumulating evidence that acrolein is a key mediator of associated respiratory diseases.

1.5.2.2.1 Acute exposure

Acute exposure to acrolein has been reported to elicit a number of respiratory effects. The severity of the acute respiratory effects elicited by large volumes of smoke is dependent on aspects of exposure, including dose and depth of the respiratory tract reached by airborne acrolein. Mild irritation to the nasal cavity and eyes can be caused within seconds of exposure to lower doses of acrolein (<0.3 ppm), whereas higher airborne concentrations (2-5 ppm) can bring about strong irritation over the whole respiratory tract (Faroon et al., 2008). Excessive mucous secretion, hypopnea, dyspnea, cyanosis, and even lung haemorrhage have all been observed in response to high-dose acrolein exposure in a variety of animal species, including rodents, dogs, sheep and humans (Borchers et al., 1998; Catilina et al., 1966; Faroon et al., 2008; Hales et al., 1988; Hales et al., 1992), and may be directly relevant to doses of acrolein inhaled by fire victims.
The respiratory condition most strongly associated with acute acrolein exposure is smoke inhalation injury (SII). This severe respiratory condition develops following inhalation of high doses of smoke generated from house, industrial or bush fires, and is associated with poor clinical prognosis and high mortality rates. Respiratory failure is observed in SII patients alongside severe bronchospasm, breathing abnormalities, bronchorrhea, pulmonary oedema, and retrograde alveolar flooding (Demling, 2008; Enkhbaatar and Traber, 2004). A role of acrolein in SII was implicated by studies that identified acrolein as the primary oedematogenic component of cigarette smoke. In a series of compelling studies conducted in sheep, a clear association between levels of acrolein present in synthetic smoke and the degree of pulmonary oedema observed firmly implicated acrolein as a key mediator of oedema (Efimova et al., 2000; Hales et al., 1988; Hales et al., 1992). Furthermore, acrolein has been reported to disrupt tight junctions that might also contribute to oedema and associated airway swelling, reduction of luminal diameter, and resulting hypoxia (Romet-Haddad et al., 1993). In spite of the unambiguous role of acrolein in pulmonary oedema, current treatments of SII serve only as symptomatic interventions rather than measures directed specifically against underlying toxicological events (Burcham et al., 2010c). Thus, shedding more light on these responses on a cellular or molecular level may reveal potential targets for therapeutic intervention.

1.5.2.2.2 Chronic exposure

Chronic cigarette smoking remains amongst the most important risk factors for COPD, a debilitating respiratory disease characterised by progressive air flow limitation associated with abnormal inflammatory responses (Mannino and Buist). Based on results of large-scale clinical trials and additional studies, inflammation alone remains insufficient in accounting for the complexity of this disease. There is accumulating evidence that mucous dysfunction is a crucial yet untargeted pathology in COPD (Cerveri and Brusasco, 2010; Rogers, 2000). Acrolein has attracted attention as a potential mediator of COPD through observations that levels of acrolein are significantly elevated in the lung fluid of COPD patients (Moretto et al., 2012). More direct evidence of causality comes through findings that have identified acrolein as the component of smoke that most strongly influences mucous production. In addition to its effects on mucous production, acrolein-induced ciliotoxicity impairs clearance of excess mucus, which may further exacerbate airway obstruction (Romet et al., 1990). Acrolein may also have direct effects on the inflammatory component of COPD through
upregulation of the pro-inflammatory transcription factor EGR-1, which is a predictive marker of COPD (Ning et al., 2004). Targeting acrolein in a therapeutic context has been evaluated through use of the acrolein-scavenging molecule N-acetylcysteine, and beneficial effects have been reported in COPD sufferers in small-scale clinical trials (Black et al., 2004; Schermer et al., 2009; Stav and Raz, 2009). However, these findings were not supported by results of a larger multi-centre study (BRONCUS), which demonstrated that low doses of N-acetylcysteine were ineffective in arresting deterioration of lung function or reducing exacerbations over a three year period, and it was concluded that higher doses are likely needed for any therapeutic effect (Decramer et al., 2005).

In addition to COPD, cigarette smoking is a leading cause of lung cancer. Indeed, smoking is postulated to account for approximately 80% and 50% of the global lung cancer burden in male and female sufferers, respectively (Jemal et al., 2011). Clinical evidence of elevated levels of acrolein biomarkers in a subset of chronic smokers led to tentative speculation about the possible role of acrolein in the development of lung cancer (Park et al., 2015b). However, despite observations that acrolein readily forms adducts with DNA in vitro, experimental evidence of a carcinogenic role for acrolein in humans is still lacking and its mutagenic potential remains ill-defined in vivo (Moghe et al., 2015). Thus, whilst acrolein has an established role in the development of COPD, controversy continues to surround its role in the aetiology of smoking-related lung cancers.
1.6 THESIS AIMS

As discussed in the preceding sections, responses to acrolein have been broadly divided into several experimental contexts: acute physiological responses to acrolein elicited in a protective capacity (Section 1.5.1), pathophysiological consequences of acute acrolein exposure incurred through mechanisms of toxicity (Section 1.5.2.2.1), and pathophysiological consequences of chronic acrolein exposure that develop over a sustained period (Section 1.5.2.2.2). Although all three contexts remain incompletely characterised, in examining the acute effects of high-dose acrolein exposure, the work in this Thesis will focus largely on the first experimental context.

The overarching aim of the work described in this Thesis is to characterise acute responses of the airways to acrolein that involve the secretory function of the airway epithelium, which are currently poorly characterised within an ex vivo setting. While Chapters 3 to 5 will examine the production and secretion of mucins, Chapters 6 and 7 will examine the bronchoactive mediators secreted by the airway epithelium in response to acute acrolein exposure. As responses of the airway epithelium to acrolein are also dependent on integrated actions of surrounding airway structures, the interaction between the airway epithelium, sensory nerves, and smooth muscle will also be explored. Specific aims of individual Results Chapters and the relationship between these five Chapters are summarised in Figure 1.12.
Figure 1.12. Schematic outlining aims of individual Results Chapters.
CHAPTER 2: GENERAL METHODS

2.1 GENERAL DEFINITIONS

The metric system and appropriate metric system abbreviations were used for units of measurement. Room temperature (RT) referred to temperatures between 22°C-25°C.

2.2 GENERAL DRUGS AND REAGENTS

The general drugs and reagents used in the experiments described in this Thesis are summarised in Table 2.1. Unless otherwise specified, drugs were stored and reconstituted according to guidelines provided by the manufacturer, as indicated.

2.3 MICE

Specific pathogen-free male BALB/c mice aged 7-8 weeks were purchased from the Animal Resources Centre (Murdoch, WA) and housed at the M-block Animal House (QEII medical centre, University of Western Australia, Crawley, WA). All mice were specific pathogen-free and kept on a 12 h light/dark cycle and given access to autoclaved food and water ad libitum. All studies were conducted with the approval of the University of Western Australia Animal Ethics Committee and adhered to the Australian National Health and Medical Research Council’s Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (8th Edition, 2013).

2.3.1 Isolation of murine airway tissue

Mice were sacrificed with an overdose of sodium pentobarbitone (160 mg kg⁻¹, i.p.) and exsanguinated by severing the right renal artery. With the aid of a dissecting microscope, the thoracic region was opened and viscera exposed, following which the trachea (from the larynx down to the bifurcation) and individual lung lobes were surgically excised from the mouse. Tracheae were freed from surrounding connective tissue before flushed with sterile physiological media (RPMI 1640 supplemented with HEPES (25 mM), GlutaMAX™ (2 mM), fetal bovine serum (2%), amphotericin B (2.5 mg ml⁻¹), gentamicin (100 μg ml⁻¹) prior to examination.
2.4 TISSUE HISTOLOGY

2.4.1 Tissue fixation and processing

Unless otherwise indicated, tracheal segments were fixed by immersion in 2% (w/v) paraformaldehyde/1.5% picric acid for 24 h at 4°C. Fixed tissues were then rinsed in 50% ethanol (EtOH) before being dehydrated and processed in a standard 15 h cycle (Leica ASP200 automated tissue processor, Leica Microsystems Pty Ltd, North Ryde, NSW) and then embedded in paraffin wax in a cross-sectional (upright) orientation. Transverse sections were cut using a standard wax microtome (Leica RM2255 rotary microtome, Leica Microsystems Pty Ltd, North Ryde, NSW) at 5 μm onto Superfrost Plus glass slides (Lomb Scientific, Scoresby, VIC).

2.4.2 Periodic acid-Schiff (PAS) staining

Slide-mounted tissue sections were de-waxed in xylene and rehydrated to water through descending ethanol solutions before staining with periodic acid-Schiff’s reagent (PAS) by sequential exposure to periodic acid and Schiff’s reagent (each for 10 min) to examine the morphology of tracheal segments and to demonstrate the presence of (neutral) mucins, the principal glycoprotein constituent of mucus, in the surface epithelium. Following sequential exposure to PAS, sections were counterstained with Mayer’s haematoxylin and blued with Scott’s tap water substitute before being dehydrated by passing through ascending EtOH solutions, mounted with DePex (BDH Chemical Company, Kilsyth, VIC), and coverslipped.

2.4.3 Quantification of PAS staining

All stained sections were examined by brightfield microscopy and digital images were generated using ScanScope XT (automated digital microscope slide scanner, Aperio Technologies, Vista, CA) at 10X or 40X lens magnification and analysed using ImageScope v12.3.0 and the Positive Pixel Count v9 algorithm (Aperio Technologies). To quantify levels of PAS staining, a highly specific approach was developed. Firstly, the epithelial layer was digitally traced to isolate for analysis. Input parameters (hue, saturation threshold, and intensity ranges for weak positive, positive and strong positive pixels) were then defined for each separate experiment to generate algorithms capable of identifying PAS-positive stores of mucin in the epithelial layer stained pink. “Mark-up”
Table 2.1. General drugs and reagents.

<table>
<thead>
<tr>
<th>Drug/reagent</th>
<th>Notes</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-calcitonin gene related peptide</td>
<td>Reconstituted in sterile MilliQ water and frozen in aliquots at -20°C until thawed for use</td>
<td>Tocris Bioscience</td>
</tr>
<tr>
<td>Acrolein</td>
<td>Diluted with sterile saline and used immediately</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Adenosine 5′Triphosphosphate</td>
<td>Reconstituted in sterile saline and used immediately</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Allyl isothiocyanate</td>
<td>—</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>AP-18</td>
<td>Reconstituted in EtOH and frozen in aliquots at -20°C until thawed for use</td>
<td>Tocris Bioscience</td>
</tr>
<tr>
<td>Atropine</td>
<td>Reconstituted in sterile saline and stored in aliquots at -20°C until thawed for use</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Calcitonin gene related peptide fragment 8-37</td>
<td>Reconstituted in sterile MilliQ H2O and frozen in aliquots at -20°C until thawed for use</td>
<td>Auspep Pty Ltd</td>
</tr>
<tr>
<td>(human)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Capsaicin</td>
<td>Reconstituted in EtOH and frozen in aliquots at -20°C until thawed for use. Serially diluted in sterile saline</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Capsazepine</td>
<td>Reconstituted in EtOH and frozen in aliquots at -20°C until thawed for use. Diluted further in sterile saline</td>
<td>Tocris Bioscience</td>
</tr>
<tr>
<td>Carbachol Carbamylcholine chloride</td>
<td>Reconstituted in sterile saline and frozen in aliquots at -20°C until thawed for use. Serially diluted in sterile saline</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Cinnamaldehyde Trans-cinnamaldehyde</td>
<td>Diluted with EtOH</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Crotonaldehyde</td>
<td>—</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Drug/reagent</td>
<td>Notes</td>
<td>Source</td>
</tr>
<tr>
<td>------------------------------</td>
<td>--------------------------------------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>DePex mounting medium</td>
<td>Xylene/dibutyl phthalate</td>
<td>BDH Laboratory Supplies</td>
</tr>
<tr>
<td></td>
<td>Reconstituted in TBS at 4 mg ml⁻¹ and frozen at -20°C until thawed for use. Diluted 1:10 and contained H₂O₂ (0.1% v/v)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Diaminobenzidine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endothelin-1</td>
<td>Reconstituted in sterile saline</td>
<td>Auspep Pty Ltd</td>
</tr>
<tr>
<td>FC-72 (Fluorinert™)</td>
<td></td>
<td>3M</td>
</tr>
<tr>
<td>Formalin</td>
<td></td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Haematoxylin (Mayer's)</td>
<td>Filtered through filter paper (Grade 4 (25 µm), cellulose fibre) immediately before use</td>
<td>Amber Scientific</td>
</tr>
<tr>
<td>HC-030031</td>
<td>Reconstituted in DMSO and frozen in aliquots at -20°C until thawed for use</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>Reconstituted in 100 mM Na₂CO₃ in aliquots at -20°C until thawed for use</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Immun-Mount®</td>
<td></td>
<td>Shandon</td>
</tr>
<tr>
<td>Isoprenaline</td>
<td>Reconstituted in 0.1mM ascorbic acid in aliquots at -20°C until thawed for use</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Isoproterenol hydrochloride</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Krebs solution</td>
<td>Composition: NaCl 117mM, KCl 5.36 mM, NaHCO₃ 25mM, KH₂PO₄ 1.03mM, MgSO₄·7H₂O 0.57mM, CaCl₂ 2.5mM, D-glucose 11.1mM. Salts dissolved in deionised H₂O (CaCl₂ added following carbogen bubbling) and used immediately</td>
<td>(Various)</td>
</tr>
<tr>
<td>Lethabarb</td>
<td>Diluted 1/5 with sterile saline and stored at RT</td>
<td>Virbac Australia</td>
</tr>
</tbody>
</table>

Krebs solution Composition: NaCl 117mM, KCl 5.36 mM, NaHCO₃ 25mM, KH₂PO₄ 1.03mM, MgSO₄·7H₂O 0.57mM, CaCl₂ 2.5mM, D-glucose 11.1mM. Salts dissolved in deionised H₂O (CaCl₂ added following carbogen bubbling) and used immediately.
<table>
<thead>
<tr>
<th>Drug/reagent</th>
<th>Notes</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipopolysaccharides from Escherichia coli (serotype 0111:B4)</td>
<td>Reconstituted in sterile saline and frozen in aliquots at -20°C until thawed for use</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Methacarn</td>
<td>Mixture of methanol, chloroform, glacial acetic acid solution (60:30:10)</td>
<td>(Various)</td>
</tr>
<tr>
<td>Methoxyflurane</td>
<td>—</td>
<td>Medical Developments International Ltd</td>
</tr>
<tr>
<td>Optimal Cutting Temperature (OCT) embedding medium</td>
<td>—</td>
<td>Sakura Finetek USA Inc</td>
</tr>
<tr>
<td>Osmium tetroxide</td>
<td>Reconstituted in FC-72 (Fluorinert™)</td>
<td>Sigma-Aldrich, Fluorinert™ obtained from 3M</td>
</tr>
<tr>
<td>Paraformaldehyde</td>
<td>Reconstituted in mouse PBS</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Periodic acid</td>
<td>Salt dissolved in distilled deionised H₂O</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>PF-04418948</td>
<td>Reconstituted in DMSO and frozen in aliquots at -20°C until thawed for use</td>
<td>Tocris Bioscience</td>
</tr>
<tr>
<td>Phosphate-buffered saline (mouse)</td>
<td>Composition: NaCl 9.06 g l⁻¹, KCl 0.2 g l⁻¹, Na₂HPO₄ 0.91 g l⁻¹, KH₂PO₄ 0.12 g l⁻¹</td>
<td>(Various)</td>
</tr>
<tr>
<td>Picric acid</td>
<td>Solution mixed with mouse PBS and 2% paraformaldehyde</td>
<td>Amber Scientific</td>
</tr>
<tr>
<td>Propranolol</td>
<td>Reconstituted with sterile saline and frozen in aliquots at -20°C until thawed for use</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Drug/reagent</td>
<td>Notes</td>
<td>Source</td>
</tr>
<tr>
<td>--------------------------------------------------</td>
<td>-----------------------------------------------------------------------</td>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>Prostaglandin E₂</td>
<td>Reconstituted in sterile saline and frozen in aliquots at -20°C until thawed for use</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>PGE₂ enzyme immunoassay kit (cat. No. 514010)</td>
<td>Kit was used in accordance with manufacturer’s instructions</td>
<td>Cayman Chemicals</td>
</tr>
<tr>
<td>RPMI 1640 medium Hepes modification (25 mM)</td>
<td>Supplemented with GlutaMAX™, felt bovine serum (%) 2 mM, amphotericin B 2.5 mg ml⁻¹, gentamicin 100 μg ml⁻¹</td>
<td>GIBCO (ThermoFisher Scientific) and (Various)</td>
</tr>
<tr>
<td>RP-67580</td>
<td>Reconstituted in EIOH and frozen in aliquots at -20°C until thawed for use</td>
<td>Tocris Bioscience</td>
</tr>
<tr>
<td>Schiff’s reagent</td>
<td>—</td>
<td>Australian Biostain</td>
</tr>
<tr>
<td>Scotts tap water substitute</td>
<td>NaHCO₃ (3.5 g) and MgSO₄ (20 g) dissolved in 1 l of distilled water</td>
<td>Amber Scientific</td>
</tr>
<tr>
<td>Substance P</td>
<td>Reconstituted in sterile saline and frozen in aliquots at -20°C until thawed for use</td>
<td>Tocris Bioscience</td>
</tr>
<tr>
<td>Tris buffered saline (TBS)</td>
<td>—</td>
<td>(Various)</td>
</tr>
</tbody>
</table>

Alphabetical listing of general drugs and reagents. Reagents were purchased from: Amber Scientific, Midvale, WA, AUS; Auspep Pty Ltd, Tullamarine, VIC, AUS; Australian Biostain, Traralgon, VIC, AUS; BDH Laboratory Supplies, Poole, England, UK; Cayman Chemicals, Ann Arbor, MI, USA; GIBCO (ThermoFisher Scientific), Waltham, MA, USA; Medical Developments International Ltd, Scoresby, VIC, AUS; Sakura Finetek USA Inc, Torrance, CA, USA; Shandon Pittsburgh, PA, USA; Sigma-Aldrich, St. Louis, MI, USA; Tocris Bioscience, Ellisville, MO, USA; Virbac Australia, Peakhurst, NSW, AU; 3M, Maplewood, MI, USA.
images were pseudocoloured with negative (blue), weak positive (yellow), positive (orange), and strong positive (red) pixels were then generated, whereby strong positive pixels (%SPP) corresponded to the PAS-positive mucins of interest (Figure 2.1). Using the algorithm, SPP in the epithelial layer were then enumerated and expressed as a percentage of the total pixels within the epithelial layer (%SPP). %SPP was calculated to provide a means of quantifying changes in levels of mucin staining. In addition, the total intensity of SPP (arbitrary values) and total number of pixels in the epithelial layer were also measured and enumerated, respectively. Multiple sections were assessed for quantitative analysis of PAS staining, where a minimum of 2 sections from tracheae isolated from at least 3 mice in each treatment group was quantified and analysed.

2.5 ISOMETRIC TENSION RECORDING STUDIES OF TRACHEAL SMOOTH MUSCLE TONE

Isolated tracheal segments (either whole segments or segments bisected into upper and lower, as indicated in Chapters) were mounted onto two L-shaped stainless steel hooks and suspended in a 20 ml mini tissue organ bath system (miniTOBs) (Danish, Myotechnology, Denmark) or a 2 ml glass organ bath filled with Krebs solution (117 mM NaCl, 5.36 mM KCl, 25 mM NaHCO$_3$, 1.03 mM KH$_2$PO$_4$, 0.57 mM MgSO$_4$.7•H$_2$O, 2.5 mM CaCl$_2$, 11.1 mM D-glucose). The organ baths were maintained at 37°C and bubbled continuously with 5% CO$_2$ in O$_2$. Changes in tension were recorded via an isometric force transducer connected to a PowerLab® data acquisition program (ADInstruments Pty Ltd, Castle Hill, NSW). Following a 30 min equilibration period in which tissues were washed with fresh Krebs solution every 10 min and the resting tension periodically readjusted to ~0.23 g, tracheal segments were sequentially exposed to submaximal (0.2 μM) and supramaximal (10 μM) doses of carbachol in a viability test (Henry et al., 1991). After a 15 min washout and recovery period, the viability test was repeated, and preparations that responded weakly (<0.50 g tension increase) were considered unsuitable for subsequent testing. For viable preparations, the response to the supramaximal dose of carbachol (10 μM) in the second viability test was deemed $C_{\text{max}}$. Viable preparations were then exposed to agents that induced contraction, or pre-contracted with carbachol to 50-60% $C_{\text{max}}$ prior to exposure to agents that induced relaxation. Unless otherwise stated, maximum contractile or relaxation responses ($E_{\text{max}}$) were obtained by fitting individual dose-response curves to obtain maxima. All relaxation responses were expressed as a percentage reversal of the level of contracture induced by carbachol (% R), where 100% R represented a complete reversal of the pre-contraction. Estimations of –logEC$_{50}$ were obtained by
fitting individual dose-response curves and measuring the concentration required to elicit 50% R.

2.6 DATA AND STATISTICAL ANALYSIS

Unless otherwise stated, all data are expressed as mean ± S.E.M. Multiple group data were compared using one- and two-way ANOVA with post-hoc comparisons (Holm-Sidak method), where appropriate, to determine if there were significant differences between groups. To compare two groups of data only, Student’s t-test (unpaired, two-tailed) was performed, and for data that was not normally distributed, the non-parametric Mann-Whitney rank sum test was performed, as indicated. Differences were considered significant if p<0.05. All statistical analyses were performed using SigmaPlot 12.5 (Systat Software, San Jose, CA) and data was graphed using GraphPad Prism 6.04 (GraphPad Software, Inc., San Diego, CA).
Figure 2.1. Quantification of PAS staining using a Positive Pixel algorithm.

The epithelial layer of tracheal sections was digitally traced (red outline) to isolate from the surrounding tissue for analysis (A, B). The epithelial layer was then analysed using the Positive Pixel algorithm which pseudo-coloured the epithelium with blue (negative pixels), yellow (weak positive pixels), orange (positive pixels), and red (strong positive pixels) hues that corresponded to the levels of PAS-positive staining (C, D). Pixels were enumerated by the algorithm, which also computed additional attributes including total intensity of pixels and the total number of pixels (E).
CHAPTER 3: DEVELOPMENT OF A DYNAMIC EX VIVO PERFUSION-SUPERFUSION MODEL AND A VENTILATION-SUPERFUSION MODEL FOR THE STUDY OF MOUSE TRACHEA

3.1 INTRODUCTION

3.1.1 Experimental models for the study of acrolein and the airways

Studies of acrolein are driven by a need to rationalise the physiological and pathophysiological influences of acrolein with its cellular and molecular targets. On one hand, establishing a relationship between the cellular targets of acrolein and the physiological responses observed provides critical insights into the body’s defence system. On the other hand, uncovering the mechanistic link between the cellular targets of acrolein and the pathophysiological influence of acrolein may help to clarify its role in respiratory disease. To perform these studies, numerous experimental models have been developed and they fall under two broad categories, each with distinct advantages and drawbacks.

3.1.1.1 “Whole” animal models

To provide direct insights into the responses elicited by acrolein, studies would ideally be conducted using human subjects. However, the obvious ethical limitations of conducting toxicological studies in human subjects coupled with an intermittent supply of viable ex vivo human tissue have necessitated development of animal models. Thus, early investigations of airborne toxicants, including acrolein, were conducted in animals exposed directly to toxicants in inhalation chambers or that were delivered by an endotracheal tube (Cotgreave et al., 1987; Lane et al., 1976; Leach et al., 1987; Lyon et al., 1970). Closely mimicking conditions of human exposure, these rudimentary studies provided important and highly relevant insights into the overall effects of toxicants and the respiratory responses elicited on a whole-body level. However, limitations of these in vivo approaches quickly became apparent. Administering toxicants at higher doses placed animals at undue risk by compromising their respiratory function, as well as other body systems, thereby restricting experimental design. For example, although head/nose/mouth-only exposure facilitated localised delivery of higher doses of toxicants to animals, it introduced additional stress to the animals, which were cut off from a supply of food and water throughout the period of...
exposure. This approach may not be feasible for chronic, repeated administration of toxicants (Rothen-Rutishauser et al., 2008). Furthermore, the large numbers of animals required to produce meaningful and scientifically valid results rendered in vivo testing of toxicants an undesirable approach, both in terms of cost and from the perspective of animal welfare (Hartung, 2009). These inherent limitations have precluded the use of "whole" (live) animal models as a primary experimental system through which to examine responses of the airways to highly noxious agents, resulting in a progressive shift away from in vivo testing towards alternative models (Hartung and Leist, 2008; Hartung et al., 2012).

3.1.1.2 Isolated models

3.1.1.2.1 In vitro models

Serving as a suitable alternative to in vivo systems, in vitro models have long been considered to be a useful tool through which to investigate the toxic influence of chemicals (Davidson et al., 2000). The principal advantage of in vitro models is that it affords an opportunity to perform comprehensive toxicological studies under tightly controlled conditions, independent of the ethical considerations associated with exposure of live animals to toxicants. For this reason, a large body of our current knowledge of acrolein has been derived from work conducted using isolated cell culture systems that range from simple single-cell to more complex co-culture systems (Burcham et al., 2010c; LoPachin and Gavin, 2014; Moghe et al., 2015). Early in vitro studies were conducted using either primary airway epithelial cells, which were harvested from a range of animal species and subsequently cultured as a monolayer of undifferentiated cells (Blanquart et al., 1991; Grafström et al., 1988; Romet-Haddad et al., 1993), or immortalised cell lines (Borchers et al., 1999a; Horton et al., 1999; Nardini et al., 2002; Tirumalai et al., 2002). However, both of these systems were severely limited by their inability to reproduce the inherent biological complexity of the in vivo epithelium. Although favoured due to their ability to be cultured over a prolonged period, immortalised cell lines do not reflect normal airway physiology. Primary undifferentiated cell cultures, which are formed from identical cells grown on foreign substances without normal extracellular matrix, lack many characteristics of naïve tissue. Thus, the inability of these models to culture isolated cells into the full population of epithelial cells that form the naïve epithelium has brought their biological relevance into question. Consequently, these models have been largely superseded by air-liquid interface (ALI) models that more closely reproduce in vivo conditions.
compared to conventional submerged cultures and in doing so, develop donor epithelial cells into “organotypic” pseudostratified epithelium (Blank et al., 2006; Gaschen et al., 2010; Lam et al., 2011). In the ALI model, epithelial cells are grown on a porous membrane support and warmed culture medium and a gaseous atmosphere is maintained below and above the cells, respectively. Under these conditions, epithelial cells are cultured into a population of differentiated, polarised cells that exhibit tight junctions and characteristics of ciliated and non-ciliated cells, goblet cells, and basal cells (Karp et al., 2002). In addition to these histological features, more recent work has showed that ALI-cultured epithelial cells also closely recapitulate the transcriptional profile of in vivo human airway epithelium (Pezzulo et al., 2011). ALI models also allow cells to be directly exposed to solid, liquid, and gas suspensions, which more closely mimics inhalational exposure in vivo (Chortarea et al., 2015; Mathis et al., 2013). Together, these findings support the use of the ALI system over traditional cultured monolayer models to examine responses of the epithelium to noxious agents, including acrolein.

As with in vivo models, in vitro models are not without their drawbacks. In spite of efforts to develop cell cultures that better reproduce features of naïve epithelium using the ALI system, isolated cells are unable to interact with tissues normally encountered in their native environment and may not always fully reflect behaviour exhibited in vivo (Davidson et al., 2000; Rothen-Rutishauser et al., 2008). Thus, while it is difficult but not impossible to develop a culture of a mixed population of cells using an ALI system, reproducing potential interactions between epithelial cells and associated structures in an isolated cell culture model is not currently possible. More complex 3D co-cultures improve upon this but are only able to reproduce interactions between the two or three cell types selected for co-culture (Blank et al., 2007; Rothen-Rutishauser et al., 2005). This inherent limitation may bring into question the biological relevance of isolated systems, particularly as a model of responses to agents such as acrolein, which may depend on the integrated actions of the various effector cells. Consequently, the degree to which conclusions drawn from these studies can be extrapolated to responses of naïve epithelium are limited and remain speculative at best (Sanderson, 2011).
3.1.1.2 Ex vivo models

Ex vivo models serve an important function by bridging the gap between isolated cell culture systems and “whole” animal models. These models maintain tissue explants or whole organ preparations harvested directly from animals and closely retain characteristics of the in vivo epithelium and underlying mesenchymal tissues in their normal anatomic arrangement. In closely reproducing the complexity of the intact organ, ex vivo models represent a powerful platform through which to examine coordinated responses between the epithelium and surrounding structures, including the airway smooth muscle or the network of nerves. Thus, ex vivo models facilitate functional and physiological measurements currently not possible with in vitro models, such as integrated assessments of smooth muscle dynamics and vascular and bronchial reactivity with the airway epithelium (Wright et al., 2013). Importantly, as they are not constrained by the ethical concerns surrounding exposure of animals to toxicants, ex vivo models also facilitate intrusive procedures or toxicological studies not permissible in live animals.

In traditional tissue explant models, tissue preparations are isolated and maintained in culture medium under static conditions. In addition to these traditional models, several novel ex vivo systems have emerged in recent years, including a precision-cut tissue slice model whereby lungs are gently inflated with agarose before being sliced between 500-700 µm thick and maintained in culture medium (Liberati et al., 2010; Sanderson, 2011). The ability to obtain multiple lung slices from a single animal drastically reduces the number of animals required for toxicological studies and can also aid in reducing experimental error through statistical blocking. As lung slices are able to be maintained in small volumes of medium (typically 0.5-1 ml), they facilitate the use of rare or expensive drugs. Furthermore, lung slices that are exposed to an air-liquid interface may be exposed to compounds in an aerosolised form, which more closely mimics the physiochemical interactions that occur in vivo compared with exposure to compounds in solution (Morin et al., 2013). For these reasons, ex vivo lung slice models have been increasingly utilised in a number of experimental contexts, including examination of the toxic influence of noxious agents on the airways, for modelling lung fibrosis (Westra et al., 2013), and for the study of peripheral airway pharmacology (Donovan et al., 2013; FitzPatrick et al., 2014; Ressmeyer et al., 2006).
To maintain isolated airway preparations, *ex vivo* models have traditionally relied on static medium conditions, which fail to reproduce the dynamic conditions generated by the flow of air through the lungs *in vivo*. As such, existing *ex vivo* models are largely limited to short-term studies. Isolated tissues have reportedly been maintained anywhere between several hours and 7 days of culture, after which deterioration of the morphology and general viability of tissue preparations have proven to be problematic for longer-term toxicological studies (Biesalski *et al.*, 1996; Dai *et al.*, 2003; Wang *et al.*, 2003). Nonetheless, improvements in the capacity of *ex vivo* systems to maintain isolated airway preparations for longer culture periods have been achieved through the development of dynamic organ culture systems. In these systems, dynamic conditions are generated through a variety of techniques, including floating lung slices onto scintillation vials placed on rollers within incubators (Harrigan *et al.*, 2004; Hays *et al.*, 2003; Morin *et al.*, 2013; Morin *et al.*, 2008), or incubating lung slices in multi-well plates subjected to a gentle shaking or rocking motion (Pushparajah *et al.*, 2008; Pushparajah *et al.*, 2007; Sturton *et al.*, 2008; Umachandran *et al.*, 2004). In moving lung slices through an air-liquid interface under dynamic conditions, these models exhibited a greater capacity to preserve the viability of tissues, with some reporting up to 21 days of successful culture (Liberati *et al.*, 2010). Although dynamic models have been established for lung slices, models that possess the capacity to maintain whole airway preparations have not yet been fully developed.
3.1.2 Chapter aims

In vitro systems have been used extensively to characterise responses of the airways to acrolein and few studies have extended these in vitro findings beyond isolated cell culture systems. In bridging the gap between in vivo and in vitro systems, ex vivo models represent an important platform through which to consolidate in vitro findings in a more physiologically relevant model, as well as to conduct further mechanistic studies of the observed responses. However, to be a useful experimental tool, ex vivo models must firstly exhibit a capacity to preserve the morphology of isolated tissue preparations. Equally important is the capacity of ex vivo models to preserve the viability of tissue preparations on a functional level. Thus, the principal aim of the work described in this Chapter was to develop and characterise two dynamic ex vivo models - a perfusion-superfusion system and a ventilation-superfusion system - that would successfully preserve the viability of mouse isolated tracheal segments for subsequent studies. Specifically, experiments aimed:

1. To optimise the experimental setup and the flow rate of media perfusion in the perfusion-superfusion and ventilation-superfusion systems by evaluating the capacities of these systems to preserve the histological integrity of the epithelium of mouse isolated tracheal segments.

2. To evaluate the capacities of the perfusion-superfusion and ventilation-superfusion systems to preserve the functional integrity of the airway epithelium, as well as the smooth muscle and sensory nerves, of mouse isolated tracheal segments using functional isometric tension recording studies.
3.2 METHODS

3.2.1 Experimental models

3.2.1.1 Conventional static tissue culture system

Tracheae were excised from mouse airways and cleared from surrounding connective tissue, as previously described (see Chapter 2, Section 2.3.1), prior to incubation in 24-well plates with 1 ml sterile physiological media (RPMI 1640 supplemented with HEPES (25 mM), GlutaMAX™ (2 mM), fetal bovine serum (2%), amphotericin B (2.5 mg ml\(^{-1}\)), gentamicin (100 μg ml\(^{-1}\)) at 37°C with 5% CO\(_2\) for 24 h under conventional static tissue culture conditions.

3.2.1.2 Novel ex vivo perfusion-superfusion system

Tracheae were excised from mouse airways and cleared from surrounding connective tissue before being secured using 3.0 surgical thread onto a blunted 18 G needle inserted into the filter cap of a 225 cm\(^2\) tissue culture flask (Figure 3.1). Sterile physiological media (RPMI 1640 supplemented with HEPES (25 mM), GlutaMAX™ (2 mM), fetal bovine serum (2%), amphotericin B (2.5 mg ml\(^{-1}\)), gentamicin (100 μg ml\(^{-1}\)) was perfused through the lumen of the tracheal segments using a peristaltic pump (Model P720, Instech Laboratories, Plymouth Meeting, PA) before collected in the waste collection flask (Figure 3.1). Media was also pumped through a second, shorter needle over the exterior surface of the tracheal segment in a process termed “superfusion” (Gaddum, 1997). Flow rates of perfusion and superfusion were equal in any single experiment. Initially, peristaltic pumps were calibrated to deliver media at flow rates of 0.05, 0.1, or 0.2 ml min\(^{-1}\) at 37°C for a total period of 24 h. Based on the results of these preliminary experiments, a standard (optimum) flow rate of 0.1 ml min\(^{-1}\) was chosen for subsequent experiments. Henceforth, tracheal segments were perfused-superfused at 0.1 ml min\(^{-1}\) for up to 48 h. The perfusion-superfusion system was housed in a warm room maintained at 37°C for the entire period of culture.

3.2.1.3 Novel ex vivo ventilation-superfusion system

Tracheae were excised from the mouse respiratory tract and cleared from surrounding connective tissue before being secured onto a blunted 18G needle, as described above. Humidified air was generated using a sealed 225 cm\(^2\) tissue culture flask
containing 400 ml of distilled water at 37°C, which was mechanically ventilated through the lumen of isolated tracheal segments using a SAR-830 ventilator (CWE Inc, Ardmore, PA) for 18 h or 24 h (Figure 3.2). Ventilator settings were adjusted to ventilate the lumen of tracheal segments at a rate of 60 breaths per minute, with an inspiration time of 0.8 s, and at an approximate flow rate of 100 cc min⁻¹. Liken to the perfusion-superfusion system, sterile physiological media (RPMI 1640 supplemented with HEPES (25 mM), GlutaMAX™ (2 mM), fetal bovine serum (2%), amphotericin B (2.5 mg ml⁻¹), gentamicin (100 μg ml⁻¹)) was pumped using a peristaltic pump through a second, shorter needle and superfused over the exterior surface of the tracheal segment at a standard flow rate of 0.1 ml min⁻¹. The ventilation-perfusion system was housed in a warm room maintained at 37°C for the entire period of culture.

3.2.2 Tissue processing and histochemical staining

For evaluation of tissue histology, tracheal segments were removed from the conventional organ culture system, the perfusion-superfusion system, or the ventilation-perfusion system, and immersion-fixed in 2% (w/v) paraformaldehyde/1.5% picric acid for 24 h at 4°C before being processed to paraffin wax and sectioned at 5 μm onto Superfrost slides, as previously described (see Chapter 2, Section 2.4.1). Slide-mounted tracheal sections were then stained with periodic acid-Schiff's reagent (PAS) and counterstained with haematoxylin before being dehydrated through ascending EtOH solutions, mounted with DePex, and coverslipped, as previously described (see Chapter 2, Section 2.4.2).

3.2.3 Isometric tension recording studies

For evaluation of the functional integrity of the airway epithelium, smooth muscle, and sensory nerves of perfused and ventilated-perfused tracheal segments, tracheal segments were removed from the perfusion-superfusion system or the ventilation-perfusion system after 24 and 18 h, respectively, before being mounted in an organ bath system to conduct isolated tension recording studies, as previously described (Chapter 2, Section 2.5). These periods of perfusion and ventilation were chosen based on the results of aforementioned studies that evaluated tissue histology.
Figure 3.1. The ex vivo perfusion-superfusion system.

Components of this system include: a media reservoir (RPMI 1640) (1), a peristaltic pump (2), and a tissue culture flask containing the tracheal segment (black arrow) and waste media (3) (A). Mouse tracheal segments were secured onto the end of a blunted 18G needle through which media perfused (white arrow, magnified in inset) while a second, shorter needle superfused media over the exterior surface of the tracheal segment (grey arrow) (B). The system was housed in a 37°C warm room.
Figure 3.2. The ex vivo ventilation-superfusion system.

Components of this system include: a humidified chamber containing distilled water at 37°C (1), a media reservoir (2), a tissue culture flask containing the tracheal segment (magnified in inset) and waste media (3), a peristaltic pump (4), and a SAR-830 ventilator (5), where the respiration rate (white arrow), inspiration time (blue arrow), and inspiratory flow rate (red arrow) were manually adjusted. The lumen of the mouse tracheal segment was ventilated with humidified air supplied from the humidified chamber while the exterior surface of the tracheal segment was superfused with media. The system was housed in a 37°C warm room.
3.2.3.1 Perfused-superfused tracheal segments

Following initial carbachol viability tests, cumulative dose-response curves (CDRCs) were constructed to carbachol on perfused tracheal segments (0.01-10 µM, half-log increments). After a 15 min washout and recovery period, preparations were pre-contraction with carbachol (0.3 µM) before exposed to a single, bolus dose of substance P (SP; 0.1 nM), a neuropeptide that elicits relaxation responses in rodent airways via an epithelium-dependent pathway (Cheah et al., 2014; Taylor et al., 2012). This process of a 20 min washout and rest period, pre-contraction with carbachol (0.3 µM), and exposure to a single, bolus dose of SP was repeated three more times with successively higher concentrations of SP (1, 10, 100 nM) and extensive washing in between doses. After an additional 15 min washout period, preparations were then pre-contraction with carbachol (0.3 µM) before exposed to a single dose of exogenous prostaglandin E₂ (PGE₂; 1 µM), a prostanoid generated endogenously by epithelial cells that produces direct relaxation responses of mouse tracheal smooth muscle (Ruan et al., 2011). Preparations did not lose their sensitivities to repeated carbachol pre-contraction doses. All relaxation responses were expressed as a percentage reversal of the level of contraction induced by 0.3 µM carbachol (% R), where 100% R represented a complete reversal of the pre-contraction.

To test the viability of airway sensory nerves, separate tracheal segments were mounted in the organ bath system prior to pre-contraction with endothelin-1 (80 nM) in the presence of atropine (10 µM) and propranolol (10 µM) to ensure complete blockade of muscarinic cholinergic and β-adrenergic responses, respectively. Upon reaching a plateau level of contracture, preparations were subjected to electrical field stimulation (EFS; 20 V, 2 ms, 20 Hz for 15 s) to activate sensory nerves, and relaxation responses were measured.

3.2.3.2 Ventilated-superfused tracheal segments

Following initial carbachol viability tests, CDRCs were constructed to carbachol on ventilated tracheal segments (0.01-30 µM, half-log increments). After a 15 min washout and recovery period, preparations were pre-contraction with carbachol (0.3-1 µM) to produce a level of contracture approximately 50-60% of C_max before being exposed to a single, bolus dose of the prototypical TRPV1 channel and sensory C-fibre activator capsaicin (10 µM). After an additional 15 min washout and recovery period,
preparations were pre-contracted with carbachol to 50-60% $C_{\text{max}}$ before exposed to a single, bolus dose of SP (0.01 nM). This process of a 20 min washout and rest period, pre-contraction with carbachol (50-60% $C_{\text{max}}$), and exposure to a single, bolus dose of SP was repeated four more times with successively higher concentrations of SP (0.1, 1, 10, 100 nM) and extensive washing in between doses. Preparations were then pre-contracted with carbachol in the same manner before a CDRC to PGE$_2$ (0.003-3 µM, half-log increments) was completed. This process was then repeated to generate a CDRC to isoprenaline, a β-adrenoceptor agonist and a direct spasmolytic agent (0.001-1 µM, half-log increments). For some preparations, the entire protocol was conducted in the presence of the non-selective cyclooxygenase (COX) inhibitor indomethacin (5 µM), which was administered 20 min prior to the addition of bronchoactive agents. All relaxation responses were expressed as a percentage reversal of the level of contracture induced by carbachol (% R), where 100% R represented a complete reversal of the pre-contraction.

### 3.2.4 Data and Statistical analysis

All data are presented as mean ± S.E.M. For the perfusion-superfusion system, maximum relaxation/contraction responses ($E_{\text{max}}$) and $-\log\text{EC}_{50}$ data was analysed by Student’s t-test (two-tailed, unpaired) and Mann-Whitney rank sum test, respectively. For tracheal segments maintained by the ventilation-perfusion system, maximum relaxation/contraction responses ($E_{\text{max}}$) and $-\log\text{EC}_{50}$ data were analysed using one-way ANOVA with post-hoc comparisons (Holm-Sidak method) or Student’s t-test (two-tailed, unpaired), where appropriate. All photomicrographs were representative of at least 3 mice.
3.3 RESULTS

3.3.1 Preservation of histological integrity

3.3.1.1 Conventional static tissue culture

Examination of PAS-stained sections revealed that the epithelium of control tracheal segments fixed immediately following excision from the mouse airways (control, non-perfused naïve airways) was characterised by a mixed population of ciliated columnar cells, non-ciliated club cells, and basal cells, which were present over an intact basement membrane for the entire circumference of the tracheal cross-sectional area examined (Figure 3.3 A-B). When cultured under static conditions in a conventional tissue culture system for 24 h, tracheal segments exhibited severe epithelial damage characterised by extensive luminal shedding (Figure 3.3 C-D). All photomicrographs shown were representative of at least 3 mice.

3.3.1.2 Perfused-superfused tracheal segments

Examination of PAS-stained sections revealed that perfusion of the lumen and superfusion of the exterior surface of tracheal segments with media preserved the morphology of the tracheal epithelium in a flow rate-dependent manner. Using the perfusion-superfusion system, perfusion of tracheal segments at a flow rate of 0.05 ml min\(^{-1}\) markedly improved epithelial integrity, although some luminal shedding was evident (Figure 3.3 E-F). Increasing the flow rate further to 0.1 ml min\(^{-1}\) (Figure 3.3 G-H) and 0.2 ml min\(^{-1}\) (Figure 3.3 I-J) was associated with further improvements in epithelial morphology. At these higher flow rates, the major histological features of the epithelium were preserved without any evidence of epithelial shedding or additional damage, and were histologically comparable to control, non-perfused segments. Extending the time course of perfusion to 48 h at flow rate of 0.1 ml min\(^{-1}\) revealed similar preservation of the epithelium (Figure 3.4). Thus, the epithelium of tracheal segments retained its primary histological features when segments cultured under dynamic conditions of media perfusion through the lumen and media superfusion along its exterior surface. In addition to the tracheal epithelium, other key airway structures including the smooth muscle band and underlying connective tissue were also successfully preserved by the perfusion-superfusion system (Figure 3.3, Figure 3.4).
3.3.1.3 Ventilated-superfused tracheal segments

Examination of PAS-stained sections revealed that ventilation of tracheal lumens with humidified air at an approximate flow rate of 100 cc min\(^{-1}\) for 18 h and concomitant superfusion of the exterior surface of tracheal segments with media at a flow rate of 0.1 ml min\(^{-1}\) preserved morphology of the tracheal epithelium (Figure 3.5 A-D). Tracheal segments ventilated for 18 h were morphologically comparable to those isolated from control, non-ventilated mice, whereby a pseudostratified epithelium containing a population of ciliated, non-ciliated and basal cells was observed (Figure 3.5 C-D). However, by 24 h of ventilation, there was evidence of a partial loss of epithelial integrity characterised by thinning of the epithelial layer and epithelial shedding in some regions of the epithelium (Figure 3.5 E-F).

3.3.2 Preservation of functional integrity

3.3.2.1 Perfused-superfused tracheal segments

Isometric tension recording studies revealed that tracheal segments perfused for 24 h with media exhibited normal relaxation responses to exogenous SP, which produces relaxation responses via an epithelium-dependent mechanism. Maximal relaxation responses elicited by SP and the potency of SP in perfused segments were not significantly different from control (non-perfused) segments (Figure 3.6 A, Table 3.1). In addition, maximal contractions and sensitivity to carbachol in perfused segments were comparable to control (non-perfused) segments (Figure 3.6 B, Table 3.1). Similarly, relaxation responses evoked by PGE\(_2\) (1 µM) were also unchanged in perfused preparations (Figure 3.6 C). In addition, EFS evoked comparable relaxation responses in perfused and control segments (Figure 3.6 D).

3.3.2.2 Ventilated-superfused tracheal segments

Isometric tension recording studies revealed that tracheal segments ventilated for 18 h with humidified air retained their ability to produce epithelium-dependent relaxation responses to exogenous SP (Figure 3.7 A). Both maximal relaxation and potency of SP were similar in ventilated and control (non-ventilated) tracheal segments. As expected, indomethacin pre-treatment abolished all SP-induced relaxation responses, thereby confirming involvement of a COX-derived product (Figure 3.7 A).
Figure 3.3. Preservation of tissue histology was dependent on the flow rate of media perfusion.

PAS-stained sections of control (non-perfused) tracheal segments (A, B), or tracheal segments perfused at a flow rate of 0 ml min\(^{-1}\) (static incubation) (C, D), 0.05 ml min\(^{-1}\) (E, F), 0.1 ml min\(^{-1}\) (G, H), 0.2 ml min\(^{-1}\) (I, J) for 24 h. 10X (A, C, E, G, I) and 40X (B, D, F, H, J) lens magnification. Black arrowheads indicate the epithelial layer and white arrowheads indicate the smooth muscle band. Black bar = 100 µm.
Figure 3.4. Preservation of epithelial histology for up to 48 h by perfusion with media at a flow rate of 0.1 ml min⁻¹.

PAS-stained sections of control (non-perfused) tracheal segments (A, B), or tracheal segments perfused for 24 h (B, C) or 48 h (E, F). 10X (A, C, E) and 40X (B, D, F) lens magnification. Black arrowheads indicate the epithelial layer and white arrowheads indicate the smooth muscle band. Black bar = 100 µm.
Figure 3.5. Preservation of epithelial histology for up to 18 h by ventilation with humidified air.

PAS-stained sections of tracheal segments isolated from control (non-ventilated) mice (A, B) or tracheal segments subjected to 18 h (C, D) or 24 h (E, F) of ventilation. Ventilation for 18 h preserved epithelial morphology but by 24 h of ventilation, there was evidence of damage characterised by thinning and shedding of the epithelium. 10X (A, C, E) and 40X (B, D, F) lens magnification. Black arrowheads indicate the epithelial layer and white arrowheads indicate the smooth muscle band. Black bar = 100 µm.
Figure 3.6. Preservation of functional responses in perfused tracheal segments.

Relaxation or contractile responses of segments to substance P (SP) (A), carbachol (B), PGE$_2$ (1 μM) (C), and electrical field stimulation (20 V, 20 Hz, 2 ms for 15 s) (D) in tracheal segments perfused with media for 24 h compared to control (non-perfused) tracheal segments. Mean ± S.E.M are shown (n=4 for panel A; n=12-28 for panel B; n=3-8 for panel C; n= 6-8 for panel D, where n denotes the number of segments).
Figure 3.7. Preservation of functional responses in ventilated tracheal segments.

Relaxation or contractile responses of tracheal segments to substance P (SP) (A), carbachol (B), PGE$_2$ (C), isoprenaline (D), and capsaicin (10 µM; E) in the absence or presence of indomethacin (Indo, 5 µM) in tracheal segments ventilated with humidified air for 18 h compared to control (non-ventilated) tracheal segments. Mean ± S.E.M are shown (n=4-10 for panel A; n=10-12 for panel B; n=12 for panel C; n=9-12 for panel D; n=3-9 for panel E, where n denotes the number of segments). *p<0.05, ***p<0.001 compared to EC$_{50}$ or relaxation response of control, †††p<0.001 compared to EC$_{50}$ of ventilated (18 h) + indo.
Tracheal segments ventilated for 18 h were also responsive to carbachol. However, although maximal responses were comparable to control segments, ventilated segments were significantly less sensitive to carbachol, as indicated by the rightward shift of the dose-response curve (logEC\(_{50}\) 6.38 ± 0.09 vs 6.82 ± 0.08, n=10-11, p<0.001; ~3-fold less sensitive, Figure 3.7 B). Intriguingly, pre-treatment of ventilated segments with indomethacin restored the sensitivity of preparations to carbachol (logEC\(_{50}\) 7.08 ± 0.04 in the presence of indomethacin vs 6.38 ± 0.09 in the absence of indomethacin, n=10-11, p<0.001).

Similar to carbachol, ventilated tracheal segments were also responsive to exogenous PGE\(_2\). However, although maximal responses were comparable to control segments, ventilated segments were significantly less sensitive to PGE\(_2\), as reflected by the rightward shift of the dose-response curve (logEC\(_{50}\) 6.68 ± 0.11 vs 7.39 ± 0.05, n=7-12, p<0.001; Figure 3.7 C). As expected, pre-treatment with indomethacin did not affect PGE\(_2\) responses in either ventilated or control tracheal preparations (Figure 3.7 C).

Similar to both carbachol and PGE\(_2\), ventilated tracheal segments were responsive to isoprenaline. However, although ventilated and control segments were equally sensitive to isoprenaline, maximal relaxation responses to isoprenaline were significantly smaller in ventilated segments compared to those produced by control segments (81 ± 4% vs 99 ± 1%, n=9-12, p<0.05; Figure 3.7 D). As expected, pre-treatment with indomethacin did not affect isoprenaline responses in either ventilated or control tracheal preparations (Figure 3.7 D).

In contrast to carbachol, PGE\(_2\), and isoprenaline, ventilated tracheal preparations were significantly less responsive to the relaxant effects of capsaicin (Figure 3.7 E). The relaxation response elicited by a bolus dose of capsaicin (10 µM) was significantly smaller in ventilated segments compared to those elicited in control segments (9 ± 2% vs 68 ± 4%, n=3-9, p<0.001).

Comparisons between the relaxation and contractile responses of perfused tracheal segments and ventilated tracheal segments and those of control segments (non-perfused or non-ventilated) are summarised in Table 3.1.
Table 3.1. Summary of functional responses of perfused and ventilated tracheal segments.

Responses of control (non-perfused, non-ventilated) tracheal segments, and tracheal segments perfused with media for 24 h using the perfusion-superfusion system or ventilated with humidified air for 18 h using the ventilation-superfusion system to selected agonists/stimuli. BDRC, bolus dose-response curve; CDRC, cumulative dose-response curve; EFS, electrical field stimulation; E_{max}, maximum relaxation or contractile response; indo, indomethacin.

<table>
<thead>
<tr>
<th>Agonist or stimuli</th>
<th>-log EC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>E_{max} (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CONTROL</td>
<td>PERFUSED</td>
</tr>
<tr>
<td>Substance P</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1.0 log BDRC; 0.1-100 nM for perfused, 0.01-100 nM for ventilated)</td>
<td>9.00 ± 0.47</td>
<td>9.04 ± 0.20</td>
</tr>
<tr>
<td>Carbachol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ indo (5 µM)</td>
<td>6.83 ± 0.02</td>
<td>-</td>
</tr>
<tr>
<td>PGE&lt;sub&gt;2&lt;/sub&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bolus (1 µM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isoprenaline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(0.5 log CDRC; 0.003-3 µM)</td>
<td>7.39 ± 0.05</td>
<td>-</td>
</tr>
<tr>
<td>EFS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(20 V, 2 ms, 20 Hz for 15 s)</td>
<td>6.99 ± 0.10</td>
<td>-</td>
</tr>
<tr>
<td>Capsaicin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(10 µM)</td>
<td>68 ± 4</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> p<0.001 compared to -log EC<sub>50</sub> control
<sup>b</sup> p<0.05 compared to E_{max} control
<sup>c</sup> p<0.001 compared to E_{max} control
3.4 DISCUSSION

In this Chapter, two novel dynamic ex vivo models were developed and characterised. As established by histological and functional studies, the perfusion-superfusion and ventilation-perfusion systems successfully preserved the viability of the epithelium of isolated mouse tracheal segments. The perfusion-superfusion system preserved the morphology and the functional integrity of the airway epithelium, smooth muscle, and sensory nerves of mouse isolated tracheal segments for up to 24 h of culture, whereas the ventilation-perfusion system preserved the histological and functional integrity of the epithelium for up to 18 h of culture. Together, these findings indicated that these systems are useful for conducting ex vivo studies of the mouse airways.

Histological examination of perfused tracheal segments revealed that the perfusion-superfusion system successfully preserved epithelial morphology. Conceptually, the system was developed based on a model first presented by Pittet and co-workers as a means for investigating influenza A infection and bacterial adherence in mouse isolated tracheal and lung preparations (Pittet et al., 2010). Results from this Chapter indicate that the system may be have additional applications for further ex vivo studies. Closer histological examination of tracheal segments revealed a direct association between the rate of media perfusion and the degree of epithelial morphology preservation. Under static conditions of conventional tissue culture, mouse isolated tracheal segments exhibited a clear loss of integrity by 24 h of culture that was characterised by epithelial shedding into the airway lumen. Increasing the rate of media flow to 0.05, 0.1, and 0.2 ml min\(^{-1}\) using the perfusion system was associated with progressive improvements in epithelial integrity, indicating that the dynamic nature of the perfusion system was crucial to preservation of epithelial morphology and the general tissue architecture. However, as histological comparisons were made on a qualitative level, more precise observations including differences in the epithelial cell number or thickness of the epithelial layer were not made, and is a limitation of the present study. Nonetheless, qualitative improvements of tissue histology with increasing flow rates are consistent with findings that dynamic rocking systems that move lung slices through an air-liquid interface using shaking or rocking platforms markedly improved the viability of cultured cells and tissues compared to conventional culture systems (Liberati et al., 2010; Pushparajah et al., 2007; Umachandran et al., 2004). In addition to better reproducing dynamic conditions generated by the flow of air in vivo, perfusion of tracheal segments with media likely prevented development of a chemical gradient.
within the media and aided in removal of waste metabolites released by cultured tissue segments, which would not have been removed by conventional static culture. By providing a continuous supply of fresh media at a physiological temperature to the tracheal segments, the full population of airway epithelial cells was preserved on a histological level. Although dynamic systems have been developed for lung slices, work performed in this Chapter has demonstrated that it is possible to preserve whole tissue preparations using a dynamic ex vivo perfusion-superfusion system.

Histological examination of ventilated tracheal segments revealed that the ventilation-superfusion system also successfully preserved epithelial morphology. Conditions of this system were directly comparable to the experimental setup of the established ALI system; the media that superfused over the exterior surface of the tracheal segment is analogous to the warmed culture medium maintained underneath cells grown on a porous membrane support in the ALI system, while the humidified air ventilated through the tracheal lumen is analogous to the gaseous environment to which the luminal side of ALI-cultured cells are exposed (Lam et al., 2011; Mathis et al., 2013). Under these conditions, the morphology of the epithelium of tracheal segments ventilated for 18 h was superior to those segments cultured under static tissue culture conditions. However, ventilation was unable to preserve epithelial morphology beyond 18 h, with epithelial damage characterised by thinning of the epithelial layer and epithelial shedding evident by 24 h of ventilation. Despite recreating dynamic conditions generated by the flow of air in vivo, it is conceivable that ventilation failed to promote adequate removal of waste metabolites released during the culture period, resulting in epithelial damage. The ventilation-superfusion system may also have additional applications by enabling aerosol solutions to be passed directly through the tracheal lumen, and was utilised in subsequent Chapters (see Chapter 5, Sections 5.2.2.1.2 and 5.2.2.4.2). As the perfusion-superfusion system exhibited a greater capacity to preserve the histological integrity of the epithelium over a sustained period of time (up to 48 h) compared to ventilation with humidified air (up to 18 h), the flow of media through the tracheal lumen appears to be crucial for histological preservation of mouse isolated tracheal segments over a sustained period of culture.

In addition to preserving to epithelial morphology, the capacities of the two dynamic ex vivo systems to preserve the functional viability of mouse isolated tracheal segments...
were also evaluated. Preservation of epithelial morphology by the perfusion-
superfusion system (for up to 24 h) and by the ventilation-perfusion system (for up to
18 h) corresponded to a preservation of functional integrity when examined at their
respective time points. The functional integrity of the epithelium was assessed by
examining its response to SP, a neuropeptide that elicits relaxation responses in rodent
airways via an epithelium-dependent pathway involving activation of NK1 receptors and
subsequent generation and release of the spasmolytic agent PGE2 from airway
epithelial cells (Devillier et al., 1992; Fortner et al., 2001; Manzini, 1992) (discussed
further in Chapter 6, Section 6.1.1). Epithelial damage incurred deliberately by
mechanical disruption was strongly associated with an attenuation of SP-elicited
relaxation responses of the airway smooth muscle (Kao et al., 1999; Liu et al., 2006;
Szarek et al., 1998; Szarek et al., 1995). Thus, normal relaxation responses to SP may
serve as an important functional readout of epithelial integrity, indicative of the capacity
of the epithelium to generate cellular mediators, including PGE2, that elicit important
downstream responses such as bronchodilation. Tracheal segments perfused for 24 h
or ventilated for 18 h exhibited normal responses to SP, which elicited maximal
relaxation responses and exhibited potencies comparable to non-perfused, non-
ventilated control tracheal segments. Abolition of SP-induced relaxation responses by
the non-selective COX inhibitor indomethacin confirmed involvement of a COX-derived
mediator in the relaxation response, consistent with PGE2. Thus, preservation of the
functional integrity of the epithelium of perfused and ventilated tracheal segments was
observed alongside preservation of histological integrity.

In addition to the airway epithelium, the capacities of the perfusion-superfusion and
ventilation-perfusion systems to preserve the functional integrity of additional structures
within the airways, namely the smooth muscle and the sensory nerves, were also
examined. To isolate responses of the smooth muscle from surrounding structures,
tracheal segments were exposed to agents that elicited their effects via direct activation
of receptors expressed on smooth muscle cells. Tracheal segments perfused with
media for 24 h exhibited normal responses to carbachol and exogenous PGE2, which
produce their spasmogenic and spasmolytic effects via activation of muscarinic
receptors and EP2 receptors, respectively (Fortner et al., 2001; Struckmann et al.,
2003; Tilley et al., 2003b). Normal responses to these agents confirmed full
preservation of normal smooth muscle function via these cellular pathways.
Tracheal segments ventilated with humidified air for 18 h exhibited distinct, but altered, responses to carbachol and exogenous PGE$_{2}$. Although maximum contractile responses elicited by carbachol remained unchanged, ventilated tracheal segments were significantly less sensitive to carbachol when compared to unventilated control preparations. Intriguingly, carbachol sensitivity was restored with indomethacin administration. Taken together, these observations are consistent with elevated production of basal PGE$_{2}$ in ventilated tracheal preparations, which may have functionally opposed contractile responses to produce a rightward shift of the carbachol dose-response curve. This was consistent with restoration of normal contractile responses to carbachol by indomethacin administration, which inhibited PGE$_{2}$ production by non-selective inhibition of COX enzymes. In further support of elevated basal PGE$_{2}$ production, ventilated tracheal segments exhibited a reduced sensitivity to exogenous PGE$_{2}$ that may have developed as a compensatory mechanism in response to elevated levels of basal PGE$_{2}$ production. As ventilated tracheal segments exhibited a normal sensitivity to isoprenaline, an agonist that elicits bronchodilation via an epithelium-independent pathway involving β-adrenoceptor activation, the possibility of a general defect in the relaxation capacity of the tracheal smooth muscle could be excluded. The reasons for elevated production of PGE$_{2}$ in ventilated tracheal segments remain unclear, however it may be reflective of a stress response that has previously been described in airway and intestinal epithelial cells following exposure to infectious agents or other stresses, including cigarette smoking (Kagnoff and Eckmann, 1997; Ruba et al., 2013). Interestingly, while levels of basal PGE$_{2}$ were elevated, the generation of PGE$_{2}$ in response to SP was normal, indicating that the capacity of the epithelium of tracheal segments to generate PGE$_{2}$ in response to exogenous stimuli was fully preserved by the ventilation-perfusion system. Future studies could make direct measurements of PGE$_{2}$ levels using biochemical assays (ELISA etc.), which would provide more direct evidence of changes in basal PGE$_{2}$ production. Furthermore, examining the components of the PGE$_{2}$ biosynthetic pathway (e.g. COX, tissue-specific prostaglandin synthases (Cha et al., 2006; Zaslona and Peters-Golden, 2015)) or downstream signalling pathways (e.g. EP$_{2}$ receptor activation, cyclic AMP generation (Billington et al., 2013)) that are altered by the ventilation-perfusion system might reveal the mechanistic basis for the putative elevations of basal PGE$_{2}$ production.
Examination of the functional integrity of sensory nerves revealed that the perfusion-superfusion system was capable of preserving sensory nerve function for up to 24 h of culture. Pharmacologic blockade of adrenergic and cholinergic nerves ensured that relaxation responses elicited by EFS were produced exclusively by non-adrenergic, non-cholinergic, sensory nerve activation. Relaxation responses elicited by EFS in perfused tracheal segments were comparable to those produced by control segments, reflecting full preservation of sensory nerve function. To test the functionality of a more specific subset of sensory nerves, relaxation responses elicited by the prototypical TRPV1 channel activator, capsaicin (Bessac and Jordt, 2008; Nagy et al., 2004), were evaluated in ventilated tracheal segments. Although small relaxation responses indicated partial preservation of sensory nerve function, ventilated tracheal segments were significantly less responsive to capsaicin than control segments, indicating a deterioration in the function of capsaicin-sensitive, TRPV1-expressing sensory nerves. Therefore, in comparing the two dynamic models, it is clear that perfusion of the tracheal lumen with media better preserved sensory nerve function compared to ventilation with humidified air. Observations from this Chapter pertaining to the deterioration of sensory nerve function with culture are consistent with findings that the viability of sensory nerves deteriorates at a faster rate in comparison to other airway structures, including the epithelium and smooth muscle, and may possibly reflect a reduction in the density of PGP 9.5-immunoreactive nerve fibres in tracheal segments shown to occur over time with tissue culture (Bachar et al., 2004).
3.4.1 Major Findings

In summary, the data obtained in Chapter 3 demonstrated that:

1. A novel dynamic ex vivo perfusion-superfusion system successfully preserved the histology of the epithelium of mouse isolated tracheal segments for up to 48 h of culture.

2. A novel dynamic ex vivo ventilation-superfusion system also successfully preserved the histology of the epithelium of mouse isolated tracheal segments, but only for up to 18 h of culture.

3. The tracheal epithelium retained its primary histological features when maintained under dynamic conditions of perfusion-superfusion and ventilation-superfusion but was severely damaged when cultured for 24 h under static conditions of conventional tissue culture.

4. Both the ex vivo perfusion-superfusion and ventilation-superfusion systems successfully preserved the functional integrity of the epithelium of mouse isolated tracheal segments for up to 24 h and 18 h of culture, respectively. The functional integrity of the airway smooth muscle was preserved by both systems, whereas sensory nerve function was preserved by the perfusion-superfusion system but not the ventilation-superfusion system.

5. Overall, the perfusion-superfusion system exhibited a greater capacity to preserve the histological and functional viability of mouse tracheal segments for a longer period of culture than the ventilation-superfusion system.

6. In successfully preserving both the histological and functional integrity of mouse isolated tracheal epithelium, the ex vivo perfusion-superfusion system was established as a superior experimental model through which to conduct further ex vivo studies of mouse airways compared to the ventilation-superfusion and conventional static tissue culture systems.
CHAPTER 4: CHARACTERISATION OF MODELS OF MUCOUS METAPLASIA

4.0 PREAMBLE

In Chapter 3, a novel ex vivo perfusion-superfusion system better preserved the morphological and functional integrity of mouse isolated tracheal preparations compared to a novel perfusion-ventilation system and a conventional static system. Consequently, this system was established as a suitable model through which to conduct subsequent studies of mouse airways. Although the original intent of developing the system was for the study of acrolein, an additional application became evident upon closer histological examination of tracheal segments. Perfusion of tracheal segments with media in the system was associated with the development of a mucous metaplastic response, and these observations served as the impetus to further examine the physical factors that drive mucous metaplasia in mouse airways. Additionally, physical factors that influence the expression of mucin in the airway epithelium were also examined in an in vivo ovine model.

4.1 INTRODUCTION

4.1.1 Mucous metaplasia and the respiratory tract

4.1.1.1 Expression of mucous cells

Mucus-producing epithelial cells are widely distributed throughout the upper respiratory tract and are characterised by the presence of large stores of secretory granules containing mucin glycoproteins. Through the expression of these mucous cells, the airway epithelium is uniquely primed to mount a rapid response to airborne environmental cues without a requirement for slower, de novo synthesis (Davis and Dickey, 2008; Thornton et al., 2008).

The expression and distribution of mucous cells in the respiratory tract exhibit distinct species-specific differences. In human airways, mucous cells are present throughout the upper airways and are differentially expressed in the surface and glandular epithelium, which producing MUC5AC and MUC5B, respectively (Groneberg et al., 2002; Thornton et al., 2008; Zhu et al., 2008b). Mucous cells constitute approximately one-sixth of columnar cells in the tracheobronchial epithelium, but their expression becomes increasingly sparse with airway branching such that they are rarely present in
the distal airways (Davis and Dickey, 2008). More recent work established that there is a basal level of MUC5B production in the surface epithelial cells of distal airways. However, the rates of mucin production in these regions are carefully balanced by the rates of mucin secretion, thereby preventing the accumulation of mucins under normal conditions (Adler et al., 2013; Davis and Dickey, 2008; Zhu et al., 2015). Similar to epithelial mucous cells, the presence of submucosal glands is largely restricted to the large (cartilaginous) airways. This expression profile is closely reflected in large mammals (e.g. sheep, primates (Cox et al., 2003; Jackson, 2001)) but not in smaller mammals (e.g. mice), where glands are confined to the laryngeal region of the trachea rather than extending throughout the entire cartilaginous airways, and surface mucous cells are rarely present. In these smaller species, club cells are the predominant secretory cells throughout the tracheobronchial tree. As mouse airways more closely resemble the human non-cartilaginous airways than human large airways, murine models are widely used as model of the human small airways (Davis and Dickey, 2008; Evans and Koo, 2009; Fahy and Dickey, 2010).

4.1.1.2 Changes in the expression of mucous cells

While increases in the abundance of epithelial mucous cells in the human large airways occur predominantly by selective cellular proliferation and hyperplasia of existing mucous cells (Boucherat et al., 2013; Lambrecht and Hammad, 2012), increases in the presence of mucous cells in the human small airways occur by metaplasia (Davis and Dickey, 2008; Fahy and Dickey, 2010). In rodent models, which closely resemble the human small airways, increases in the abundance of mucous cells observed in response to allergen exposure are produced in the absence of epithelial proliferation, indicating that cell division is not involved in this process. Rather, longstanding work has provided several lines of evidence that transition of the epithelium to a mucin-producing phenotype arises exclusively as a result of the transformation of club cells to mucous cells (Curran and Cohn, 2010; Evans et al., 2004; Hayashi et al., 2004; Lumsden et al., 1984; Reader et al., 2003). Firstly, in the airways of allergen-challenged mice, accumulation of mucin-containing secretory granules were detected in the apical region of club cells using electron microscopy (Hayashi et al., 2004; Reader et al., 2003). Secondly, fluorescent periodic acid Schiff-labelled mucins strongly co-localised with CC10, a club cell-specific marker (Boucherat et al., 2012; Evans et al., 2004). Finally, through in vivo cell lineage tracing experiments, mucous cells were shown to express β-galactosidase, a marker expressed by an inducible transgene under the control of the C10 promoter. Collectively, these experiments identified club
cells as the main progenitors of mucous cells induced following allergen exposure in mice (Chen et al., 2009a).

Within club cells, the transition to a mucin-expressing phenotype reflects a change in the balance of mucin production and secretion. As neither expression of the molecular components nor activity of the mucin secretory machinery were found to be significantly diminished in mucous metaplasia, changes in the secretory capacity of club cells do not appear to contribute substantially to the mucous metaplastic process (Davis and Dickey, 2008; Evans et al., 2009). Rather, the accumulation of mucin stores appears to be driven by increases in mucin production. As both MUC5AC and MUC5B production are tightly regulated at the transcriptional level, changes in mucin expression that result in the acquisition of a mucous cell phenotype are predominantly controlled by transcriptional changes that occur within club cells (Fahy and Dickey, 2010; Ordonez et al., 2001). The pathways regulating these transcriptional changes are dependent on the stimuli inducing the mucous metaplastic process (see Section 4.1.3).

4.1.2 Mucous metaplasia and respiratory disease

Mucous metaplasia has been examined within the context of its pathogenic role in a range of respiratory diseases, including asthma, chronic obstructive pulmonary disorders (COPD), cystic fibrosis, and bronchiectasis. In these diseases, a global mucous dysfunction is observed in which the accumulation of pathologic mucus of an abnormal viscosity and elasticity contributes to airway lumenal obstruction and a deterioration in lung function (Fahy and Dickey, 2010; Voynow, 2002). With the exception of cystic fibrosis and severe cases of COPD, changes in submucosal glands do not contribute significantly to the overproduction of mucus in comparison to changes in the expression of mucus-producing epithelial cells (Bergeron et al., 2009; Davis, 2006; Hogg, 2004a; Hogg et al., 2004b). Within mucous cells, changes in the expression of MUC5AC and MUC5B are differentially observed in airway disease. For example, during allergic inflammation, MUC5AC production increases profoundly (40 to 200-fold) whilst increases in MUC5B are comparatively modest (3 to 10-fold) (Evans et al., 2004; Kirkham et al., 2002; Reader et al., 2003; Young et al., 2007; Zhen et al., 2007; Zhu et al., 2008b). Importantly, in many chronic respiratory diseases, mucous cell expression spreads to the peripheral airways, where they are not normally present (Holgate, 2011).
While mucous metaplasia alone does not appear to produce any airway pathology, elevated mucin production is thought to be an important step in priming the airways for subsequent mucus hypersecretion. In retrospective cases of severe and fatal asthma, the proportion of airway epithelial cells that express a goblet cell phenotype in distal airways was reported to increase from 5% to greater than 25%. Importantly, the volume of lumenal mucus was markedly elevated in these airways and correlated strongly with the number of goblet cells in the airway epithelium (Aikawa et al., 1992; Shimura et al., 1996). Mucin overproduction may also be prevalent in mild to moderate cases of asthma (Ordonez et al., 2001). Together, the excessive production and secretion of mucus play a well-recognised role in fatal asthma in which patients die from severe airway obstruction, presumably due to the combined effects of widespread mucous plugging and uncontrolled bronchoconstriction (Kuyper et al., 2003). Thus, mucous metaplasia contributes to airway pathology by conferring a predisposition to mucus dysfunction and is a significant contributor to airway obstruction (Adler et al., 2013).

4.1.3 Stimuli of mucous metaplasia

4.1.3.1 Biochemical stimuli

Among the biochemical stimuli identified as strong stimulants of mucous metaplasia, inflammatory mediators have by far attracted the most attention. Indeed, the most well-characterised mucous metaplasia phenotype is the response produced by allergic airway inflammation induced experimentally in rodents through antigen sensitisation and challenge, typically with ovalbumin. Within 2-6 h of an allergen challenge, an acute neutrophilic response is observed, followed by a Th2 lymphocytic and eosinophilic response 1-7 days later (Evans et al., 2009). While mucous metaplasia is absent in the acute phase, mucin production is profoundly enhanced in the late phase at both the mRNA and protein levels. Modest increases in the expression of Muc5ac mRNA have been reported within 1-2 days post-challenge (<10-fold increase), and by 3-7 days post-challenge, levels are profoundly elevated over controls (>30-fold increase) and may remain elevated for up to 28 days post-challenge (Agrawal et al., 2007; Evans et al., 2004; Reader et al., 2003; Young et al., 2007; Zuhdi et al., 2000). Importantly, these changes in mRNA expression correlated closely with changes in histochemically-detectable mucin.
Although allergic airway inflammation is the most well-characterised model of mucous metaplasia, a number of additional inflammatory stimuli also elicit increases in epithelial mucin expression, including environmental irritants (Takeyama et al., 2001b; Wagner et al., 2003), viruses (Buchweitz et al., 2007; Grayson et al., 2007; Tyner et al., 2006), and bacteria (Kohri et al., 2002) or specific bacterial products such as lipopolysaccharide (LPS), a major pro-inflammatory glycolipid product secreted by gram-negative bacteria and an important component of the bacterial wall (Chen et al., 2009b; Hauber et al., 2007; Kim et al., 2004; Ou et al., 2008a; Ou et al., 2008b; Shao et al., 2003; Silva and Bercik, 2012; Yanagihara et al., 2001). The most common approach to examining LPS-induced metaplasia in vivo has involved a single, intratracheal installation of LPS (from Pseudomonas aeruginosa or Escherichia coli) into rodent airways. Through these studies, temporal associations between inflammatory responses and mucous metaplastic responses have been established, whereby massive recruitment of neutrophils into the lungs of mice were shown to precede increases in MUC5AC mRNA expression (Yanagihara et al., 2001).

A number of pathways regulate the transcriptional changes that drive mucous metaplasia in response to the aforementioned stimuli. Of these, intracellular pathways activated by the epidermal growth factor receptor (EGFR) have been identified to control mucin expression (Boucherat et al., 2013; Curran and Cohn, 2010). Involvement of the EGFR pathway was first revealed by a cohort of studies whereby pro-inflammatory EGFR ligands such as EGF, TNF-α, and TGF-α triggered profound mucous metaplastic responses in airway epithelial cells and these findings were later validated in animal models (Perrais et al., 2002; Takeyama et al., 1999). Conversely, pre-treatment with selective EGFR tyrosine kinase inhibitors prevented goblet cell formation in an allergic ovalbumin model (Takeyama et al., 1999). Since those initial studies, a role for EGFR has been established for mucous metaplasia driven by a diverse range of biochemical stimuli, including allergens and viruses (Burgel et al., 2001; Hewson et al., 2010; Shim et al., 2001; Tyner et al., 2006; Zhu et al., 2009), bacteria (Kohri et al., 2002; Shao et al., 2003), oxidants (Casalino-Matsuda et al., 2006), and cigarette smoke (Takeyama et al., 2001b), as revealed by a combination of in vitro and in vivo models. Therefore, EGFR stimulation and subsequent activation of downstream signalling pathways such as MAP kinase may represent a point of convergence for both ligand and ligand-independent induced mucous metaplasia (Burgel and Nadel, 2004; Matsuoka et al., 2005; Young et al., 2007) (Figure 4.1).
4.1.3.2 Physical stimuli

In addition to biochemical stimuli, there is accumulating evidence that physical stimuli may also shift the airway epithelium towards a mucin secretory phenotype. Of the physical factors identified, perturbations of the mechanical environment have been identified as a key driver of a number of changes within the airways. More recently, the mechanical environment has attracted attention as a stimulus of airway remodelling, a postulate that challenges the current dogma of inflammation-driven remodelling (Gosens and Grainge, 2015; Kistemaker et al., 2014; Manuyakorn, 2014; Park et al., 2015a). Increases in mucin expression are a characteristic feature of airway remodelling, alongside airway smooth muscle thickening, sub-epithelial fibrosis, and airway neovascularisation (Grainge et al., 2011). There is emerging evidence that these structural changes may be driven by pathophysiologic mechanical forces generated by symptomatic bronchoconstriction responses in asthma, independent of inflammation (Gosens and Grainge, 2015; Manuyakorn, 2014). With respect to epithelial mucin, supporting in vitro evidence for a role of the mechanical environment in driving epithelial mucin expression has arisen from studies demonstrating that short periods of apical compressive or lateral stress mimicking pathophysiologic mechanical forces associated with symptomatic bronchoconstriction can induce the plasminogen activator system (Chu et al., 2006). These same mechanical stresses can also increase the levels of growth factors such as endothelin-1 and TGF-β, which have purported roles in the modulation of bronchomotor tone and mucin expression, respectively (Tschumperlin et al., 2003). Furthermore, intermittent apical stress applied to cultured epithelial cells was capable of directly inducing a mucous metaplasia response (Chu et al., 2005; Park and Tschumperlin, 2009). Collectively, these observations provide evidence that airway epithelial cells exhibit receptivity to mechanical stress and that perturbations of the mechanical environment can drive changes in mucin expression. Whether additional physical stimuli also influence mucin expression is largely unknown.

On a mechanistic level, several studies have substantiated an intermediary role for the EGFR pathway in the mucous metaplasia response associated with mechanical stimulation. Expression of the EGFR is typically sparse in healthy human and rodent airways (Burgel and Nadel, 2004; Takeyama et al., 2001a). Mechanical stress appears capable of inducing EGFR expression and also enhancing expression of EGFR ligands required for EGFR activation (Lee et al., 2000). Resultant activation of the EGFR receptor and initiation of downstream signalling pathways may lead to elevated mucin
Figure 4.1. Molecular pathways activated by allergic inflammation and LPS that initiate mucous metaplasia.

A raft of direct and indirect mechanisms converge to activate transcription factors including AP-1 and NF-κB that upregulate MUC5AC gene expression, resulting in enhanced MUC5AC protein production. EGFR, epidermal growth factor receptor; IRAK, IL-1 receptor-associated kinase; TACE, tumour necrosis factor α-converting enzyme; TAK1, transforming growth factor-activated protein kinase 1; TGF-α, transforming growth factor-α; TLR4, toll-like receptor 4.
expression (Chu et al., 2005; Tschumperlin et al., 2004; Tschumperlin et al., 2002). As selective inhibition of the EGFR and downstream signalling molecules such as ERK and p-Akt profoundly attenuated the mucous metaplasia response associated with mechanical stimulation, the EGFR pathway is likely to be an important regulator of this response (Lee et al., 2000; Liu et al., 2013; Park and Tschumperlin, 2009; Takeyama et al., 1999).
4.1.4 Chapter aims

Work described in this Chapter aimed to use the ex vivo perfusion system developed in Chapter 3 to investigate the physical stimuli that can drive the process of mucous metaplasia in mouse airways. Additionally, the physical factors that influence the levels of epithelial mucin were also explored in an in vivo ovine model. Specifically, experiments aimed:

1. To examine the physical factors that influence the levels of epithelial mucin in an ex vivo murine model by:
   
   (i) Characterising the time-course of the development of mucous metaplasia in perfused mouse isolated tracheal segments
   
   (ii) Evaluating the dependency of perfusion-associated mucous metaplasia on the presence of lumenal fluid by comparing responses to the ventilation-superfusion system, whereby the lumen of tracheal segments was ventilated with humidified air rather than perfused with media
   
   (iii) Evaluating the dependency of perfusion-associated mucous metaplasia on perturbations of the mechanical environment by altering the rate of media flow through the tracheal lumen

2. To examine the physical factors that influence the levels of epithelial mucin in an in vivo ovine model by:

   (i) Evaluating the influence of the presence of lumenal fluid on epithelial mucin levels by comparing mucin levels in sheep airways exposed to a fluid environment (fetal lambs) to those exposed to an air environment (four-month-old sheep, ventilated newborn lambs) in vivo
   
   (ii) Evaluating the influence of perturbations of the mechanical environment on epithelial mucin levels by comparing the effects of ventilation strategies thought to generate different levels of shear stress in the airways of ventilated preterm lambs in vivo

3. To evaluate the effect of the physical environment (fluid vs air) on the development of mucous metaplasia induced by a biochemical stimulus, namely LPS, in mouse isolated tracheal segments.
4.2 METHODS

4.2.1 Ex vivo murine model

4.2.1.1 Time course of perfusion-associated mucous metaplasia

Mouse isolated tracheal segments were set up in the perfusion-superfusion system, as previously described (see Chapter 3, Section 3.2.1.2). To examine the time-course of mucous metaplasia development, tracheal preparations were perfused at a flow rate of 0.1 ml min\(^{-1}\) for 2, 6, 18, 24, or 48 h with sterile physiological media (RPMI 1640 with HEPES (25 mM), GlutaMAX™ (2 mM), amphotericin B (2.5 mg ml\(^{-1}\)), gentamicin (100 μg ml\(^{-1}\)) supplemented with either 0% or 2% fetal bovine serum (FBS).

4.2.1.2 Fluid as a physical stimulus of mucous metaplasia: perfusion vs ventilation

To examine the dependency of the perfusion-associated mucous metaplasia on the presence of lumenal fluid, mouse isolated tracheal segments were set up in the ventilation-superfusion system, as previously described (see Chapter 3, Section 3.2.1.3). In this system, the media that perfused through the tracheal lumen in the perfusion-superfusion system was replaced with humidified air ventilated directly through the tracheal lumen using a SAR-830 ventilator. Media was superfused over the exterior surface of tracheal segments at a flow rate of 0.1 ml min\(^{-1}\) for 18 h at 37°C. The ventilation period of 18 h was selected based on the results of histological studies that examined the time-course of epithelial morphology preservation by the ventilation-superfusion system (see Chapter 3, Section 3.3.1.3).

4.2.1.3 The mechanical environment as a physical stimulus of mucous metaplasia: different rates of perfusion

To examine the dependency of the perfusion-associated mucous metaplastic response on perturbations of the mechanical environment, mouse isolated tracheal segments were set up in the perfusion-superfusion system and peristaltic pumps were calibrated to perfuse media through the tracheal lumen and superfusion along the exterior surface at increasing flow rates of 0.05, 0.1, or 0.2 ml min\(^{-1}\) for a total period of 24 h. These studies evaluated the influence of the rate of media flow on the development of the perfusion-associated mucous metaplasia.
4.2.2 *In vivo* ovine model

4.2.2.1 Fluid as a physical stimulus of epithelial mucin production: fetal lamb vs four-month-old sheep

Fluid conditions of the perfusion-superfusion system were compared to a physiological system in which the entire respiratory tract is exposed to a fluid environment *in vivo*. Fetal Airways serve as a physiological model of fluid-filled airways and an ovine model was selected since it is an established and widely-used model of pre-term and fetal animals (Elovitz and Mrinalini, 2004; Olver *et al.*, 2004). Therefore, epithelial mucin levels in the airways of fetal lambs (128 d gestation; fluid-filled airways) were compared to adult sheep (four-month-old sheep; air-filled airways).

In these studies, date-mated ewes (Merino) were sedated (buprenorphine, 0.1 mg kg\(^{-1}\); acepromazine, 0.5 mg kg\(^{-1}\), i.m.) and anaesthetised (ketamine, 5 mg kg\(^{-1}\); midazolam, 0.25 mg kg\(^{-1}\), i.v.) prior to exteriorisation of the fetal head and chest via hysterotomy. The lamb fetus was then immediately euthanized with pentobarbitone (100 mg kg\(^{-1}\)) and the trachea fixed via an airway infusion of 10% neutral-buffered formalin. For four-month-old sheep, the tracheae were excised and inflation-fixed at 20 cmH\(_2\)O with 10% neutral buffered formalin. These studies were conducted in collaboration with A/Prof Peter Noble and Prof Jane Pillow (School of Anatomy, Physiology and Human Biology, The University of Western Australia). Surgical procedures and ventilation of lambs (experiments described in Sections 4.2.2.1 and 4.2.2.2) were conducted by researchers within the laboratories of A/Prof Peter Noble and Prof Jane Pillow. Subsequent sectioning and staining of tissue (Section 4.2.4), quantification of PAS staining (Section 4.2.4.1), and data analysis and interpretation (Section 4.2.5) were conducted by the candidate.

4.2.2.2 The mechanical environment as a physical stimulus of mucous metaplasia: different ventilation strategies of preterm lambs

To evaluate the influence of perturbations of the mechanical environment on the levels of epithelial mucin within an *in vivo* context, a separate series of studies was conducted on preterm lambs (128 d gestation) that were subjected to two distinct ventilation strategies thought to generate different levels of shear stress in the airways — conventional mechanical ventilation (CMV) or high frequency oscillatory ventilation (HFOV).
In these studies, date-mated ewes (Merino) were sedated and anesthetised as previously described, and cord arterial blood gases were obtained prior to euthanasia of the lamb fetus (unventilated controls; UVC) or delivery of the newborn lamb. Newborn lambs were dried, weighed, and intubated with a cuffed 4.5 mm tracheal tube through which an intratracheal bolus of surfactant (100 mg kg\(^{-1}\)) was administered. Lambs were positioned prone and subjected to their assigned ventilation strategy (CMV or HFOV) with heated, humidified gas. The breath volume was adjusted to achieve permissive hypercapnoea (\(\text{PaCO}_2\) 40-50 mmHg) in accordance with routine clinical practice, and the peripheral oxyhaemoglobin saturation and pulse rate were monitored continuously. Additionally, arterial blood pressure was monitored via an umbilical arterial catheter positioned prior to clamping of the umbilical cord, and the rectal body temperature was monitored and maintained at 38.5°C. Anaesthesia and analgesia were delivered by continuous infusion of propofol (0.1 mg kg\(^{-1}\) min\(^{-1}\)) and remifentanil (0.05 µg kg\(^{-1}\) min\(^{-1}\)) into the umbilical vein.

Under CMV conditions, lungs of lambs were inflated from 5 to 15 cmH\(_2\)O at a rate of 40 breaths min\(^{-1}\) to replicate normal tidal breathing. Using this approach, lungs were ventilated with moderate tidal volumes (20 ± 1 ml) about a moderate distending pressure (Figure 4.2 A). Under HFOV conditions, lungs of lambs were inflated at a constant distending pressure of 15 cmH\(_2\)O before ventilation at a high frequency (15 Hz = 900 breaths min\(^{-1}\); Sensormedics 3100A, Viasys, CA). Using this approach, lungs were ventilated with pressures that oscillated at a high frequency to generate small tidal volumes (9.1 ± 0.3 ml) about a high distending pressure (Figure 4.2 B) and is a clinical rescue ventilation modality used to manage neonates with respiratory failure (Pillow, 2012). While HFOV may reduce shear stress at the alveolar level due to low tidal volumes and damped pressure oscillations, the high velocity of airflow, coupled to the turbulent or transitional airflow caused by the high frequency of ventilation, is thought to generate higher levels of shear stress on the epithelium in the proximal airways (Alzahrany and Banerjee, 2015; Chen et al., 2014). Thus, use of HFOV likely generated higher levels of shear stress on the airway epithelium than CMV in large airways. In addition, HFOV likely generated differential levels of shear stress on different regions within the proximal airways, with levels in the trachea higher than those in the small bronchi. Lambs were ventilated using CMV or HFOV strategies for a total period of 3 h prior to euthanasia with pentobarbitone (100 mg kg\(^{-1}\), i.p.) and exsanguination. The airways (trachea, bronchi, upper lobe of right lung) were then fixed via an airway infusion of 10% neutral buffered formalin.
4.2.3 LPS exposure in the perfusion-superfusion and ventilation-superfusion systems

To evaluate the influence of the physical environment on the development of mucous metaplasia induced by a biochemical stimulus, mouse isolated tracheal segments were set up in the perfusion-superfusion or the ventilation-superfusion systems, as previously detailed (see Chapter 3, Section 3.2.1.2, Section 3.2.1.3). Tracheal segments were then gently instilled with LPS (E. coli serotype 0111: B4, 1 µg ml⁻¹) for 30 min or 1 h before undergoing perfusion-superfusion or ventilation-superfusion for 24 h and 18 h, respectively. During the instillation period, LPS was gently flushed through the lumen of tracheal segments at 15 min intervals.

4.2.4 Tissue processing and PAS staining

For evaluation of epithelial mucin content, mouse tracheal segments were removed from the perfusion-superfusion or the ventilation-superfusion systems and immersion-fixed in 2% (w/v) paraformaldehyde/1.5% picric acid for 24 h at 4°C before being processed to paraffin wax under standard processing conditions and sectioned at 5 µm onto Superfrost Plus slides, as previously described (see Chapter 2, Section 2.4.1). For sheep airways, formalin-fixed tissues were processed to paraffin wax under standard processing conditions and cut at 5 µm onto Superfrost Plus slides. Slide-mounted airway sections were then stained with periodic acid-Schiff's reagent (PAS) and counterstained with haematoxylin before being dehydrated through ascending EtOH solutions, mounted with DePex, and coverslipped, as previously described (see Chapter 2, Section 2.4.2).

4.2.4.1 Quantification of PAS staining levels

All PAS-stained sections were examined by brightfield microscopy and digital images were generated using ScanScope (Aperio Technologies, Vista, CA). PAS-stained sections were quantified, as earlier described (see Chapter 2, Section 2.4.3). Briefly, individual input parameters of the Positive Pixel Count algorithm v9 (within the ImageScope 12.3.0 program, Aperio Technologies) were adjusted and optimised for each study to enable quantification of levels of PAS staining (Table 4.1). The epithelial layer of PAS-stained tracheal sections was then digitally traced to isolate it from the surrounding tissue for analysis. Having isolated the epithelial region of interest, mark-
Figure 4.2. Schematic illustrating the differences between the CMV and HFOV modalities.

In conventional mechanical ventilation (CMV), lamb lungs were ventilated with a moderate tidal volume ($V_T$) and inflated about a moderate distending pressure ($P_{aw}$) at a rate of 40 breaths min$^{-1}$ (A). In high frequency oscillatory ventilation (HFOV), lamb lungs were inflated to a large volume at a constant distending pressure before ventilated with a small $V_T$ at a high frequency about a high $P_{aw}$ at a rate of 900 breaths min$^{-1}$ (B).
Table 4.1. Input parameters for the Positive Pixel Algorithm used to quantify the level of PAS staining in the epithelial layer of mouse tracheal sections.

<table>
<thead>
<tr>
<th>Experiment (Chapter section)</th>
<th>Hue Value (width)</th>
<th>Saturation threshold</th>
<th>Intensity ranges</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Weak positive</td>
</tr>
<tr>
<td><strong>Ex vivo murine model (4.2.1)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time-course (4.2.1.1)</td>
<td>0.96 (0.42)</td>
<td>0.09</td>
<td>225-166</td>
</tr>
<tr>
<td>Perfusion-superfusion vs ventilation-superfusion (4.2.1.2)</td>
<td>0.96 (0.40)</td>
<td>0.09</td>
<td>225-183</td>
</tr>
<tr>
<td>Different rates of perfusion (4.2.1.3)</td>
<td>0.96 (0.42)</td>
<td>0.09</td>
<td>225-180</td>
</tr>
<tr>
<td><strong>In vivo ovine model (4.2.2)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fetal lamb vs four-month-old sheep (4.2.2.1)</td>
<td>0.95 (0.40)</td>
<td>0.09</td>
<td>225-183</td>
</tr>
<tr>
<td>Different ventilation strategies of preterm lambs: trachea (4.2.2.2)</td>
<td>0.95 (0.40)</td>
<td>0.09</td>
<td>225-183</td>
</tr>
<tr>
<td>Different ventilation strategies of preterm lambs: large and small bronchi (4.2.2.2)</td>
<td>0.95 (0.40)</td>
<td>0.09</td>
<td>225-183</td>
</tr>
<tr>
<td><strong>LPS-induced mucous metaplasia (4.2.3)</strong></td>
<td>0.96 (0.40)</td>
<td>0.09</td>
<td>225-183</td>
</tr>
</tbody>
</table>
up images were generated and the strong positive pixels enumerated by the algorithm and expressed as a percentage of the pixels forming the epithelial layer (%SPP). For each study, all tracheal sections were stained and quantified in the same run for consistency. The total intensity of SPP (arbitrary values) and total number of pixels in the epithelial layer were also measured and enumerated, respectively.

4.2.5 Data and Statistical analysis

Unless otherwise stated, data are presented as mean ± S.E.M. and analysed using a one-way ANOVA with post-hoc comparisons (Holm-Sidak). Data pertaining to the time course of perfusion-associated metaplasia was analysed using a one-way ANOVA with a Dunnett's post-hoc test, as all comparisons were made to a single time point (t=0). Data comparing the time course of perfusion-associated metaplasia in the presence of 0% or 2% FBS was analysed using a two-way ANOVA with a Dunnett's post-hoc test. All photomicrographs were representative of at least 3 mice.
4.3 RESULTS

4.3.1 Ex vivo murine model

4.3.1.1 Time course of perfusion-associated mucous metaplasia

Examination of PAS-stained sections of control, non-perfused tracheal segments fixed immediately following excision from mouse airways revealed low levels of staining for mucin in the epithelial layer (1.1 ± 0.2 %SPP, n=4; Figure 4.3 A Figure 4.4). Using the perfusion-superfusion system, perfusion of tracheal segments with media supplemented with 2% FBS at a flow rate of 0.1 ml min\(^{-1}\) produced time-dependent increases in the levels of mucin in the epithelium. Within 2 h of perfusion, faint but distinct areas of PAS-positive, fuchsia-coloured staining were observed in areas of the epithelial layer indicative of immature, developing mucous cells (6.9 ± 2.4 %SPP vs 1.1 ± 0.2 %SPP, n=4-5; Figure 4.3 B). Similar levels of staining were observed at 6 h (6.6 ± 0.8% SPP vs 1.1 ± 0.2 %SPP, n=3-4; Figure 4.3 C) but by 24 h of perfusion, cells replete with PAS-positive mucin were widespread throughout the epithelium (12.4 ± 0.8% SPP vs 1.1 ± 0.2 %SPP n=4-7, p<0.001; Figure 4.3 D). The increase in levels of epithelial mucin was sustained after 48 h of perfusion (8.8 ± 3.4 %SPP vs 1.1 ± 0.2 %SPP, n=3-4, p<0.05; Figure 4.3 E). At all of the time points examined, formation of mucin stores appeared to be localised primarily to club cells identified morphologically by their apical, dome-shaped protrusions.

Perfusion of tracheal segments with media not supplemented with any FBS (0%) resulted in a similar profile of mucous metaplasia development over a 48 h period. In examining the full time course of mucous metaplasia development, levels of PAS-positive staining observed at any of the time points between 0 h and 48 h of perfusion with media supplemented with 0% FBS were indistinguishable from 2% FBS (n=3-7, two-way ANOVA; Figure 4.5).

4.3.1.2 Dependency of perfusion-associated mucous metaplasia on the presence of fluid

In the ventilation-superfusion system, the lumen of tracheal segments was ventilated with humidified air rather than perfused with media. Crucially, ventilation of tracheal segments with air for 18 h was not associated with significant increases in the levels of PAS staining in the surface epithelium (2.4 ± 0.4 %SPP vs 1.1 ± 0.3 %SPP, n=6-7;
Figure 4.3. Time course of mucous metaplasia development in mouse tracheal epithelium over 48 h of perfusion.

PAS-stained sections of tracheal segments perfused-superfused for 0 h (A), 2 h (B), 6 h (C), 24 h (D) and 48 h (E). Black bar = 100 µm.
Figure 4.4. Quantification of the time course of mucous metaplasia development in mouse tracheal epithelium over 48 h of perfusion.

Mean ± S.E.M strong positive pixels (expressed as a percentage of total pixels in the epithelial layer) are shown (n=3–7). *p<0.05, ***p<0.001 compared to t=0 h.
Figure 4.5. Development of mucous metaplasia associated with perfusion of mouse tracheal segments with media supplemented with 0% or 2% fetal bovine serum (FBS) over 48 h of perfusion.

Mean ± S.E.M strong positive pixels (expressed as a percentage of total pixels in the epithelial layer) are shown (n=3–7). *p<0.05, **p<0.01, ***p<0.001 compared to t=0 h.
Figure 4.6, Figure 4.7). In contrast to ventilation, perfusion of tracheal segments with media for 18 h significantly elevated levels of PAS staining compared to controls (6.3 ± 1.2 %SPP vs 1.1 ± 0.3 %SPP, n=4-7, p<0.01; Figure 4.6, Figure 4.7).

4.3.1.3 Lack of dependency of perfusion-associated mucous metaplasia on the rate of perfusion

Perfusion-superfusion of tracheal segments with media at different flow rates influenced the capacity of the system to preserve mouse isolated tracheal segments. Reducing the flow rate had a profound influence on epithelial integrity, where halving the flow rate to 0.05 ml min\(^{-1}\) from the standard rate of 0.1 ml min\(^{-1}\) was associated with marked epithelial damage characterised by shedding of epithelial cells into the trachea lumen, thus confounding the effects of flow rate on mucous metaplasia development. Doubling the flow rate from 0.1 ml min\(^{-1}\) to 0.2 ml min\(^{-1}\) did not significantly increase levels of PAS-positive staining (8.5 ± 1.9 %SPP vs 3.3 ± 1.1 %SPP, n=3-4; Figure 4.8, Figure 4.9). Therefore, a definitive relationship between the rate of media perfusion and mucous metaplasia development response was unable to be established.

4.3.2 In vivo ovine model

4.3.2.1 Comparison between fetal lambs and four-month-old sheep

Examination of PAS-stained sections of naïve tracheal segments excised from the fluid-filled airways of fetal lambs revealed a high level of PAS-positive mucin in the epithelial layer (42.2 ± 6.0 % SPP; Figure 4.10 A, Figure 4.11). PAS staining was specific and highly localised to distinct mucous cells throughout the epithelium, which was characterised by large, distinctive folds not observed in mouse trachea (Figure 4.10 A). Importantly, levels of mucin in the epithelium of trachea isolated from air-filled airways of four-month-old sheep were significantly lower than those in the fetal lambs (18.7 ± 1.9 %SPP vs 42.2 ± 6.0 %SPP, n=4-6, p<0.001; Figure 4.10 B, Figure 4.11). Similarly, subjecting age-matched fetal lambs (128 d gestation) to CMV also reduced the levels of epithelial mucin to a similar degree (17 ± 2 %SPP vs 42.2 ± 6.0 %SPP, n=5-8, p<0.001; Figure 4.10 C, Figure 4.11).
4.3.2.2 Comparison between ventilation strategies of newborn lambs

To examine the effects of the different ventilation strategies on epithelial mucin levels, levels of PAS-positive mucin in the epithelial layer were measured in newborn lambs subjected to CMV and HFOV and were compared to UVC lambs. For each group, the trachea, the large bronchi, and the small bronchi of newborn lamb airways were examined. Large (ratio of 0.4-0.7) and small (ratio of 0.1-0.4) bronchi were defined by calculating the ratio of the internal perimeter of basement membrane (PBM) of the bronchi to the PBM of the trachea (PBM_{bronchi} : PBM_{trachea}).

4.3.2.2.1 Trachea

Examination of PAS-stained sections of trachea excised from UVC fetal lambs revealed high levels of staining in the epithelial layer (42 ± 6 %SPP, n=4; Figure 4.12 A-B, Figure 4.13 A). Ventilation of newborn lambs at a frequency matched to normal tidal breathing (CMV) was associated with a significant reduction in the expression of mucin (17 ± 2 %SPP vs 42 ± 6 %SPP, n=4-7, p<0.001; Figure 4.12 C-D, Figure 4.13 A). Importantly, subjecting newborn lambs to HFOV increased epithelial mucin levels compared to CMV (27 ± 2 %SPP vs 17 ± 2 %SPP, n=7-13, p<0.01; Figure 4.12 E-F, Figure 4.13 A). Changes in the intensity of SPP largely mirrored those observed for %SPP (Figure 4.13 B). As expected, the total number of pixels forming the epithelial layer did not differ significantly between the three groups (16x10^6 ± 4.5x10^6, 9.7x10^6 ± 0.83x10^6, 14x10^6 ± 1.1x10^6 for UVC, CMV, and HFOV respectively; Figure 4.13 C).

4.3.2.2.2 Large bronchi

Examination of PAS-stained sections of large bronchi excised from UVC fetal lambs revealed a moderate level of staining in the epithelial layer (24 ± 3 %SPP, n=3; Figure 4.14 A-B, Figure 4.15 A). Ventilation of newborn lambs at a frequency matched to normal tidal breathing (CMV) did not affect levels of mucin staining in the epithelium of large bronchi (20 ± 4 %SPP vs 24 ± 3 %SPP, n=3-8; Figure 4.14 C-D, Figure 4.15 A). Subjecting newborn lambs to HFOV significantly increased levels of epithelial mucin compared to UVC lambs (33 ± 3 %SPP vs 20 ± 4 %SPP, n=8-9, p<0.05; Figure E-F, Figure 4.15 A). Changes in the intensity of SPP largely mirrored those observed for %SPP, although the differences between HFOV and CMV did not reach statistical significance (Figure 4.15 B). As expected, total number of pixels forming the epithelial
Figure 4.6. Lack of mucous metaplasia development in ventilated tracheal segments.

Levels of PAS-positive epithelial mucin in tracheal segments isolated from control mice (0 h; A) or those ventilated-superfused (B) or perfused-superfused for 18 h (C). Black bar = 100 µm.
Figure 4.7. Quantification of levels of PAS-positive epithelial mucin in tracheal segments isolated from control mice (0 h) or those ventilated or perfused for 18 h.

Mean ± S.E.M % strong positive pixels are shown (n=4–7). **p<0.01, ***p<0.001, as indicated.
Figure 4.8. Effect of the flow rate of perfusion on the mucous metaplastic response observed in mouse tracheal epithelium associated with the perfusion-superfusion system.

PAS-stained sections of non-perfused tracheal segments (A), or those perfused with media at a flow rate of 0.05 ml min\(^{-1}\) (B), 0.1 ml min\(^{-1}\) (C), or 0.2 ml min\(^{-1}\) (D) for 6 h in the perfusion-superfusion system. Black bar = 100 µm.
Figure 4.9. Quantification of effect of the rate of perfusion on the mucous metaplastic response observed in mouse tracheal epithelium after 6 h of perfusion.

Mean ± S.E.M % strong positive pixels are shown (n=3–4). **p<0.01 compared to non-perfused.
Figure 4.10. Tracheal epithelial mucin levels in fluid-filled vs air-filled ovine airways.

Differences between the levels of PAS-positive epithelial mucin tracheal segments isolated from fetal lambs (128 d gestation, fluid-filled airways) (A), four-month old sheep (air-filled airways) (B), and newborn lambs subjected to conventional mechanical ventilation (air-filled airways) (C). Black bar = 500 µm.
Figure 4.11. Quantification of tracheal epithelial mucin levels in fluid-filled vs air-filled ovine airways.

Quantification of the differences between the levels of PAS-positive epithelial mucin in the trachea of fetal lambs (fluid-filled airways), four-month old sheep (air-filled airways), and newborn lambs (air-filled airways) subjected to conventional mechanical ventilation (CMV). Mean ± S.E.M % strong positive pixels are shown (n=4–7) (C). ***p<0.01 compared to fetal lamb.
Figure 4.12. Tracheal epithelial mucin levels of lambs subjected to CMV and HFOV compared to control lambs.

PAS-positive epithelial mucin in tracheal segments isolated from unventilated control sheep (A, B), or lambs subjected to conventional mechanical ventilation (CMV) (C, D) or high frequency oscillatory ventilation (HFOV) (E, F). 1x magnification (A, C, E) and 10x magnification (B, D, F). Black bar = 500 µm.
Figure 4.13. Quantification of tracheal epithelial mucin levels of lambs subjected to CMV and HFOV compared to control lambs.

Levels of PAS-positive epithelial mucin in unventilated control sheep (UVC), or lambs subjected to conventional mechanical ventilation (CMV) or high frequency oscillatory ventilation (HFOV) were quantified by enumerating the strong positive pixels (SPP) within the epithelial layer (A) and by assessment of the total intensity of SPP (B). The total number of pixels forming the epithelial layer was also enumerated for all groups (C). *p<0.05, **p<0.01, ***p<0.001, as indicated (n=4-13).
Figure 4.14. Large bronchial epithelial mucin levels of lambs subjected to CMV and HFOV compared to control lambs.

PAS-positive epithelial mucin in large bronchial segments isolated from unventilated control sheep (A, B), or lambs subjected to conventional mechanical ventilation (CMV) (C, D) or high frequency oscillatory ventilation (HFOV) (E, F). 1x magnification (A, C, E) and 10x magnification (B, D, F). Black bar = 500 µm.
Figure 4.15. Quantification of large bronchial epithelial mucin levels of lambs subjected to CMV and HFOV compared to control lambs

Levels of PAS-positive epithelial mucin in unventilated control sheep (UVC), or lambs subjected to conventional mechanical ventilation (CMV), and high frequency oscillatory ventilation (HFOV) were quantified by enumerating the strong positive pixels (SPP) within the epithelial layer (A) and by assessment of the total intensity of SPP (B). The total number of pixels forming the epithelial layer was also enumerated for all groups (C). *p<0.05 compared to CMV (n=3-13).