The Role of Prostaglandin E_2 in Airway Microvascular Leak

Victoria Clare Jones

2014

A thesis submitted for the Degree of Doctor of Philosophy

in the Faculty of Medicine, Imperial College London

Respiratory Pharmacology Group

National Heart and Lung Institute

Faculty of Medicine

Imperial College London

Sir Alexander Fleming Building

Exhibition Road

London

SW7 2AZ

Abstract

Airway microvascular leak (MVL) is the extravasation of plasma and plasma proteins from post-capillary venules into surrounding tissue. MVL is thought of as a cardinal sign of inflammation and is an important aspect in the pathogenesis of airway inflammatory diseases, including allergic asthma. Prostaglandin E_2 (PGE_2) is a product of cyclooxygenase mediated metabolism of arachidonic acid and acts via the activation of 4 different receptor subtypes, termed $EP_{1\text{-}4}$. PGE_2 has demonstrated a wide variety of effects within the airway, such as on inflammation, sensory nerve activation and airway tone. However, its effects on airway MVL have not been extensively studied. This formed the basis of the thesis hypothesis; PGE_2 acts through specific EP receptor(s) to induce microvascular leak within the airways.

Using Evans Blue dye as a marker of leak, PGE_2 significantly induced airway MVL in mice. I have shown, for the first time that this response is mediated via the activation of the EP_2 and the EP_4 receptors. This was confirmed using selective EP receptor agonists and antagonists, as well as EP receptor deficient mice. Importantly, this airway MVL was not just exclusive to the mouse; PGE_2 significantly increased airway MVL in guinea pigs. To investigate MVL in a disease setting, the ovalbumin model of allergic asthma was employed to study the role of EP receptors in mediating OVA induced MVL. OVA-induced Evans Blue leak was almost completely absent in the airways of EP_2 and EP_4 receptor knock-out mice. Finally, preliminary work investigating four other prostanoid ligands (PGD_2, $PGF_{2\alpha}$, U46619 and Iloprost) indicates that they too cause airway MVL to some extent and appear to do so via activation of their own receptor subtype (DP_1, FP, TP and IP, respectively).

Therefore, the findings outlined in this thesis suggest that PGE_2 activates the EP_2 and EP_4 receptors to induce airway microvascular leak and may also be responsible for the MVL observed in the OVA model.

Acknowledgements

First I would like to thank my supervisors Professor Maria Belvisi and Dr Mark Birrell for all their help, expertise, encouragement and support throughout my PhD. I will always be grateful for the time they have given me during this project and for making my experience here truly enjoyable. Special thanks must also go to Dr Sarah Maher who has always been so generous with her time, support and friendship over the last 4 years.

My thanks go to the entire Respiratory Pharmacology Group, past and present, whose expertise and help I would have been lost without. They really have made this a wonderful experience. In no particular order, thanks to Liang Yew-Booth, Sara Bonvini, Katie Baker, Michael Wortley, Matt Baxter, James Buckley, Joe Rastrick, Kristof Raemdonck, Eric Dubuis, Megan Grace, Nicole Dale, Suffwan Eltom, Abdel Dekkak and Bilel Dekkak. Special thanks must also go to John Adcock and Meirion Davies for their help and expertise with the blood pressure measurements. I must also thank ONO Pharmaceuticals for the prostanoid receptor knock-outs and EP agonists.

I would especially like to thank my “Les Mills” crew (Sarah, Sara and Liang) for their support and friendship over the years; I don’t know what I would have done without you. I must also thank Georgie, who started this adventure with me. I am so fortunate to have had her with me along the way, as a course mate, a flat mate and of course a fantastic friend.

Finally I would like to express my thanks to my family, Mum, Dad, Emily and Laura (and Bentley). Their kindness, support and unconditional love mean so much to me and I wouldn’t be where I am without them. I can’t thank my family without mentioning Lucy, whose love and friendship means more to me than she will ever know. She is a true inspiration. Last, but certainly not least, my love and thanks go to Adam for his encouragement, patience and love during my PhD and for never failing to make me smile.

Statement

This thesis is the product of my own work, except where appropriately referenced.

Carotid artery cannulation and blood pressure measurements were performed by Dr John Adcock and Meirion Davies, with my assistance (Chapters 3 and 6).

Copyright Declaration

The copyright of this thesis rests with the author and is made available under a Creative Commons Attribution Non-Commercial No Derivatives licence. Researchers are free to copy, distribute or transmit the thesis on the condition that they attribute it, that they do not use it for commercial purposes and that they do not alter, transform or build upon it. For any reuse or redistribution, researchers must make clear to others the licence terms of this work.

Table of Contents

Abstract **2**
Acknowledgements **3**
Statement **4**
Copyright Declaration **5**
Table of Contents **6**
List of Figures **11**
List of Tables **14**
List of Abbreviations **15**
Chapter One **18**
1. Introduction **19**
1.1. Airway Inflammatory Diseases 19
1.1.1. Asthma 20
1.1.1.1. Allergic Sensitisation and Responses 20
1.1.1.1.1. EAR and LAR 21
1.1.1.1.2. Airway Hyperresponsiveness 22
1.1.1.2. Treatments 22
1.1.1.3. In vivo models of Asthma 23
1.1.1.3.1. Ovalbumin Model 24
1.1.1.3.2. House Dust Mite Model 25
1.1.2. Chronic Obstructive Pulmonary Disease 26
1.1.2.1. Treatments 27
1.1.2.2. In vivo models of COPD 28
1.1.2.2.1. Lipopolysaccharide Model 28
1.1.2.2.2. Cigarette Smoke Model 29
1.2. Prostanoids and Prostanoid Receptors 30
1.2.1. Synthesis 30
1.2.2. Prostanoid Receptors 31
1.2.2.1. Prostaglandin E_2 and EP receptors 33
1.2.2.1.1. EP_1 Receptors 33
1.2.2.1.2. EP_2 Receptors 34
1.2.2.1.3. EP_3 Receptors 35
1.2.2.1.4. EP_4 Receptors 37
1.2.2.2. Prostaglandin D_2 and DP receptors 38
1.2.2.2.1. DP_1 Receptors 38
1.2.2.2.2. DP_2 Receptors 39
1.2.2.3. Prostaglandin $F_{2\alpha}$ and the FP receptor 40

1.2.2.4. Prostacyclin and the IP receptor 41
1.2.2.5. Thromboxane A_2 and the TP receptor 42
1.2.3. Prostanoids in Airway Disease 43
1.2.3.1. Airway Tone 44
1.2.3.2. Immune and Inflammatory Responses 45
1.2.3.3. Sensory Nerve Activation 47
1.3. Microvascular Leak 48
1.3.1. Introduction 48
1.3.2. Regulation of Endothelial Permeability 50
1.3.2.1. Myosin Light Chain Kinase-Dependent Hyperpermeability 50
1.3.2.2. Endothelial Cell-Cell Junctions and Hyperpermeability 51
1.3.3. Intracellular Signalling Pathways Modulating Endothelial Permeability 53
1.3.3.1. Calcium 54
1.3.3.2. RhoA/Rho Kinase 54
1.3.3.3. Cyclic AMP 55
1.3.4. Airway Microvascular Leak 57
1.3.4.1. Asthma 57
1.3.4.2. COPD 59
1.3.5. Methods for Investigating Microvascular Leak 60
1.3.5.1. In vitro 60
1.3.5.1.1. Cell Culture 60
1.3.5.1.2. Whole Vessel Preparations 62
1.3.5.2. In vivo 63
1.3.5.2.1. Intact Microvessels 63
1.3.5.2.2. Miles Assay 63
1.3.5.2.3. Intravital Fluorescence Microscopy 64
1.3.5.2.4. Magnetic Resonance Imaging 65
1.3.6. Prostanoids in airway microvascular leak 65
1.4. Thesis Plan 67
Chapter Two 68
2. Methodology **69**
2.1. Breeding of Knock-Out Mice 69
2.2 Genotyping of KO Mice 70
2.2.1 DNA Extraction and Quantification 70
2.2.2 Polymerase Chain Reaction 70
2.2.3 Visualisation of PCR Products 71
2.3 Microvascular Leak 73
2.3.1 Compound Administration 73

2.3.2 Measurement of Plasma Extravasation 73
2.3.3 Analysis 75
2.4 Blood Pressure Measurements 76
2.4.1 Cannulation of Carotid Artery 76
2.4.2 Measuring Blood Pressure 77
2.5 Ovalbumin Model of Allergic Asthma 78
2.5.1 Assessing airway Microvascular Leak 79
2.6 *In vitro* Organ Bath Experiments 80
2.6.1. Physiological salt solutions 80
2.6.2. Isolated airway preparation 80
2.6.3. Isolated airway organ bath protocols 81
2.6.3.1. General protocol 81
2.6.3.2. Induced tone protocol 81
2.6.4. Data Analysis 82
2.7 Human Cell Based Assay of Microvascular Leak 83
2.7.1 Cell Culture 83
2.7.2 Measuring Endothelial Barrier Permeability 83
2.7.3 MTT Assay 85
2.8 Measurement of Gene Expression 86
2.8.1 RNA Extraction 86
2.8.2 Reverse Transcription 87
2.8.3 Real Time Polymerase Chain Reaction (PCR) 87
2.8.4 Analysis of Real Time PCR Results 88
2.9 Statistical Analysis 89
Chapter Three 90
3. Characterisation of an *in vivo* preparation to measure airway MVL and the effect of Prostaglandin E_2 **91**
3.1. Rationale 91
3.2. Methods 93
3.2.1. Characterisation of airway MVL in mice using 5-HT 93
3.2.2. Dose Response of PGE_2 - induced airway MVL in Mice 93
3.2.3. Time Course of PGE_2 - induced airway MVL in Mice 94
3.2.4. Effect of Diclofenac on PGE_2-induced airway MVL in Mice 94
3.2.5. Effect of intranasal dosing of PGE_2 on airway MVL in Mice 94
3.2.6. PGE_2-induced airway MVL in Guinea Pigs 94
3.2.7. Effect of PGE_2 on Systemic Blood Pressure in Mice 95
3.2.8. Statistical Analysis 95
3.3. Results 96

3.3.1. Characterisation of airway MVL in mice using 5-HT 96
3.3.2. Dose Response of PGE_2 - induced airway MVL in Mice 98
3.3.3. Time Course of PGE_2 - induced airway MVL in Mice 99
3.3.4. Effect of Diclofenac on PGE_2-induced airway MVL in Mice 101
3.3.5. Effect of intranasal dosing of PGE_2 on airway MVL in Mice 102
3.3.6. PGE_2-induced airway MVL in Guinea Pigs 104
3.3.7. Effect of PGE_2 on Systemic Blood Pressure in Mice 105
3.4. Discussion 106
Chapter Four 110
4. Identifying the EP Receptor Mediating PGE_2-induced MVL in mouse airways **111**
4.1. Rationale **111**
4.2. Methods 112
4.2.1. The Effect of PGE_2-induced MVL in EP Receptor Knock-Out Mice 112
4.2.2. Effect of ONO-D1-004, ONO-AE1-259, ONO-AE-248 and ONO-AE1-329 on MVL 112
4.2.3. Investigating the EP_2 receptor in airway MVL 112
4.2.4. Investigating the EP_4 receptor in airway MVL 113
4.2.4.1. Rat Tracheal Organ Bath Preparation 113
4.2.4.2. In Vivo Characterisation 114
4.2.5. Effect of the EP_2 and EP_4 receptor antagonists against PGE_2-induced MVL in mice 114
4.2.7. Genotyping of KO Mice 115
4.2.8. Statistical Analysis 116
4.3. Results 117
4.3.1. The Effect of PGE_2-induced MVL in EP Receptor Knock-Out Mice 117
4.3.2. Effect of ONO-D1-004, ONO-AE1-259, ONO-AE-248 and ONO-AE1-329 on MVL 121
4.3.3. Investigating the EP_2 receptor in airway MVL 122
4.3.4. Investigating the EP_4 receptor in airway MVL 125
4.3.5. Effect of the EP_2 and EP_4 receptor antagonists against PGE_2-induced MVL in mice 130
4.4. Discussion 132
Chapter Five 140
5. Identifying the Role of EP Receptors in Ovalbumin-Induced MVL in Mouse Airways **141**
5.1 Rationale 141
5.2. Methods 143
5.2.1. Characterisation of Ovalbumin-induced Microvascular Leak in Mouse Airways 143
5.2.2. Effect of Diclofenac on Ovalbumin-induced MVL 143
5.2.3. The role of EP receptors in Ovalbumin-induced MVL 144
5.2.4. Statistical Analysis 144
5.3. Results 145
5.3.1. Characterisation of Ovalbumin-induced Microvascular Leak in Mouse Airways 145

5.3.2. Effect of Diclofenac on Ovalbumin-induced MVL 147
5.3.3. The Role of EP_2 and EP_4 Receptor Knock-outs in Ovalbumin-Induced Airway MVL 148
5.3.4. The Role of EP_2 and EP_4 Receptor Antagonists in Ovalbumin-Induced Airway MVL 150
5.4. Discussion 151
Chapter Six 154
6. The role of additional prostanoids in mediating airway MVL in the mouse **155**
6.1. Rationale 155
6.2. Methods 156
6.2.1. Effect of PGD_2, $PGF_{2\alpha}$, U46619 and Iloprost on airway MVL in mice 156
6.2.2. Effect of PGD_2, $PGF_{2\alpha}$, U46619 and Iloprost in individual prostanoid receptor knock-out mice 156
6.2.3. Effect of PGD_2, $PGF_{2\alpha}$, U46619 and Iloprost on systemic blood pressure 156
6.2.4. Genotyping of Prostanoid Receptor Knockouts 157
6.2.5. Statistical Analysis 158
6.3. Results 159
6.3.1. Effect of PGD_2, $PGF_{2\alpha}$, U46619 and Iloprost on airway MVL in mice 159
6.3.2. Effect of PGD_2, $PGF_{2\alpha}$, U46619 and Iloprost in individual prostanoid receptor knock-out mice 160
6.3.3. Effect of PGD_2, $PGF_{2\alpha}$, U46619 and Iloprost on systemic blood pressure 164
6.4. Discussion 166
Chapter Seven 172
7. Summary and Future Studies **173**
7.1. Summary and discussion 173
7.1.1. Limitations of the thesis 176
7.2. Future Studies 178
7.2.1. Effect of EP agonists on systemic blood pressure 178
7.2.2. The role of PGE_2 and the EP receptors in the guinea pig 178
7.2.3. Investigating the prostanoid receptors mediating airway microvascular leak 179
7.2.4. Modelling OVA-induced airway MVL in prostanoid receptor knock-out mice 179
7.2.5 Modelling airway MVL in other airway disease models 179
7.2.6. Modelling MVL in Human Lung Microvascular Endothelial Cells 181
7.2.7. Modelling MVL in Mouse Lung Microvascular Endothelial Cells 182
7.3. Concluding Remarks 183
Reference List 184
Appendix 227

List of Figures

Figure 1.1. Schematic representation of the inflammatory cascade in allergic asthma…… 21
Figure 1.2. Schematic representation of the biosynthesis of prostanoids…….. 31
Figure 1.3. Signalling cascade of prostanoid receptors……. 32
Figure 1.4. Schematic representation of microvascular leakage in the airways…… 49
Figure 1.5. Schematic representation of the mechanisms involved in endothelial barrier disruption……... 53
Figure 2.1. Schematic of injection site used for intra venous administration and anatomy of the mouse heart illustrating where incisions were made for saline perfusion…… 74
Figure 2.2. Diagram of right carotid artery cannulation……. 76
Figure 2.3. Timeline demonstrating the protocol used for the ovalbumin model…….. 78
Figure 2.4. Diagram illustrating method used for measuring endothelial barrier permeability in human lung microvascular endothelial cells (HLMVECs)…….. 84
Figure 3.1. Photograph of mouse airway tissue illustrating the effects of intravenous 5-HT on microvascular leak…….. 96
Figure 3.2. Effect of 5-HT on MVL into the trachea and the bronchi and intra-pulmonary airways of mice…… 97
Figure 3.3. Effect of methysergide on 5-HT-induced MVL into the trachea and the bronchi and intra-pulmonary airways of mice……... 98
Figure 3.4. Effect of PGE_2 or 5-HT on MVL into the trachea and the bronchi and intra-pulmonary airways of mice……. 99
Figure 3.5. Effect of PGE_2 on MVL after 30 minutes or 5 minutes into the trachea and the bronchi and intra-pulmonary airways of mice..... 100
Figure 3.6. Effect of PGE_2 on MVL after 30 minutes into the trachea and the bronchi and intra-pulmonary airways of mice…… 101
Figure 3.7. Effect of diclofenac on MVL in vehicle and PGE_2 treated mice…… 102
Figure 3.8. Effect of an intranasal dose of PGE_2 on MVL after 30 minutes into the trachea and the bronchi and intra-pulmonary airways of mice…… 103
Figure 3.9. Effect of PGE_2 after 30 minutes or 5-HT after 5 minutes (respectively) into the upper trachea, lower trachea and bronchi and intra-pulmonary airways of Dunkin Hartley Guinea Pigs…….. 104
Figure 3.10. Effect of PGE_2, Iloprost and U46619 on the mean arterial blood pressure of C57BL/6 mice……... 105
Figure 4.1. Effect of PGE_2 on MVL after 30 minutes into the trachea and the bronchi and intra-pulmonary airways of wild-type (WT) and $EP_{1\text{-}4}$ receptor deficient mice……... 118
Figure 4.2. Effect of 5-HT on MVL into the trachea and the bronchi and intra-pulmonary airways of wild-type (WT) and EP_2 and EP_4 receptor deficient mice……... 119
Figure 4.3. Example gel of genotyping for the *Ptger3*$^{-/-}$ mice…… 120

Figure 4.4. Effect of PGE_2, ONO-D1-004 (EP_1), ONO-AE1-259 (EP_2), ONO-AE-248 (EP_3) and ONO-AE1-329 (EP_4) on MVL into the trachea and the bronchi and intra-pulmonary airways of mice……. 121
Figure 4.5. Effect of ONO-AE1-259 (EP_2) on MVL into the trachea and the bronchi and intra-pulmonary airways of mice……. 122
Figure 4.6. Effect of PF-04418948 on ONO-AE1-259-induced MVL into the trachea and the bronchi and intra-pulmonary airways of mice…….. 123
Figure 4.7. Effect of PF-04418948 on ONO-AE1-259-induced MVL into the trachea and the bronchi and intra-pulmonary airways of mice…….. 124
Figure 4.8. Relaxation of rat isolated rat tracheal tissue…….. 125
Figure 4.9. Schild plot analysis of the antagonism of ONO-AE1-329 with ER-819762…… 126
Figure 4.10. Effect of ONO-AE1-329 (EP_4) on MVL into the trachea and the bronchi and intra-pulmonary airways of mice…… 127
Figure 4.11. Effect of ER-819762 on ONO-AE1-329-induced MVL into the trachea and the bronchi and intra-pulmonary airways of mice…….. 128
Figure 4.12. Effect of ER-819762 on ONO-AE1-329-induced MVL into the trachea and the bronchi and intra-pulmonary airways of mice…….. 129
Figure 4.13. Effect of PF-04418948 and ER-819762 on PGE_2-induced MVL into the trachea and the bronchi and intra-pulmonary airways of mice…… 130
Figure 4.14. Effect of simultaneous dosing of PF-04418948 and ER-819762 on PGE_2-induced MVL into the trachea and the bronchi and intra-pulmonary airways of mice……. 131
Figure 4.15. Schematic of possible $EP_{2/4}$ heterodimer and how this interaction influences PGE_2-induced airway microvascular leak…… 138
Figure 5.1. Effect of Ovalbumin-challenge on airway MVL in C57BL/6 mice into the trachea and the bronchi and intra-pulmonary airways of mice, 2 and 24 hours after final intra nasal OVA challenge……. 145
Figure 5.2. Effect of Ovalbumin-challenge on wet weight of the bronchi and intra-pulmonary airways at 2 hours and 24 hours after final intranasal OVA challenge…… 146
Figure 5.3. Effect of diclofenac on OVA induced MVL into the trachea and the bronchi and intra-pulmonary airways of mice…… 147
Figure 5.4. Effect of OVA induced MVL into the trachea and the bronchi and intra-pulmonary airways of EP_2 receptor knockout mice…… 148
Figure 5.5. Effect of OVA induced MVL into the trachea and the bronchi and intra-pulmonary airways of EP_4 receptor knockout mice…… 149
Figure 5.6. Effect of PF-04418948 and ER-819762 on OVA induced MVL into the trachea and the bronchi and intra-pulmonary airways of mice…… 150
Figure 6.1. Effect of PGE_2, PGD_2, $PGF_{2\alpha}$, U46619 and Iloprost on MVL into the trachea and the bronchi and intra-pulmonary airways of mice…….. 159
Figure 6.2. Effect of PGD_2 in wild type, DP_1 and DP_2 receptor knockouts……. 160
Figure 6.3. Effect of $PGF_{2\alpha}$ on MVL in the trachea and the bronchi and intra-pulmonary 161

airways of wild type and FP receptor knockout mice…….
Figure 6.4. Effect of U46619 on MVL in the trachea and the bronchi and intra-pulmonary airways of wild type and TP receptor knockout mice……. 162
Figure 6.5. Effect of Iloprost on MVL in the trachea and the bronchi and intra-pulmonary airways of wild type and IP receptor knockout mice…….. 163
Figure 6.6. An example gel showing the PCR products of DNA extracted from wild type, DP_{1-2}, FP, IP and TP KO mice……... 164
Figure 6.7. Effect of PGD_2, $PGF_{2\alpha}$, U46619 and Iloprost on mean arterial blood pressure in C57BL/6 mice…….. 165
Figure 7.1. The effect of LPS and Cigarette smoke on total protein in BAL fluid of C57Bl6 mice…… 180
Figure 7.2. Effect of the TP agonist, U46619, on Human Lung Microvascular Endothelial Cell (HLMVEC) permeability…….. 181
Figure 7.3. mRNA expression of prostanoid receptors (EP_{1-4}, DP_{1-2}, FP, IP, TP) in HLMVEC... 182

List of Tables

Table 2.1. Primer sequences used to genotype prostanoid receptor KO mice…….. 71
Table 4.1. Optimised PCR conditions for genotyping prostanoid receptor KO mice……... 116
Table 4.2. Antagonism of ONO-AE1-329-induced relaxation by ER-819762 in rat trachea…… 126
Table 4.3. Schild plot analysis data for ONO-AE1-329 vs. ER-819762 in rat trachea…….. 127
Table 6.1. Optimised PCR conditions for genotyping prostanoid receptor KO mice……... 158

List of Abbreviations

5-HT	5-Hydroxytryptamine
AC	Adenylyl Cyclase
ACh	Acetylcholine
AHR	Airway hyperresponsiveness
AOD	Assay on Demand
APC	Antigen Presenting Cell
ASM	Airway Smooth Muscle
BAL	Bronchoalveolar Lavage
BRET	Bioluminescence resonance energy transfer
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
COPD	Chronic Obstructive Pulmonary Disease
COX	Cyclooxygenase
CR	Concentration response
$CRTH_2$	Chemoattractant receptor-homologous molecule expressed on T helper Type 2 cells
CS	Cigarette Smoke
DAG	Diacylglycerol
Der f	*Dermatophagoides farina*
Der p	*Dermatophagoides pteronyssinus*
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic acid
DP	D-prostanoid receptor
EAR	Early asthmatic response
EB	Evans Blue
ECIS	Electric cell-substrate impedance sensing
EDTA	Ethylenediaminetetraacetic acid
EP	E-prostanoid receptor

FBS	Foetal bovine serum
FcεR1	Fcε receptors
FITC	Fluorescein isothiocyanate conjugate
Gd-DTPA	Gadolinium diethylenetriamine pentaacetic acid
GOLD	Global Initiative for Chronic Obstructive Lung Disease
GPCR	G-protein coupled receptor
HDM	House dust mite
hEGF	Human endothelial growth factor
h-FGF-B	Human fibroblast growth factor basic
HLMVEC	Human lung microvascular endothelial cells
i.p.	Intra peritoneal
i.v.	Intra venous
ICS	Inhaled corticosteroids
IFN	Interferon
IL-	Interleukin
IP	Prostacyclin receptor
IP_3	Inositol trisphosphate
IPA	Intrapulmonary airways
IVM	Intra vital microscopy
KHS	Krebs-Henseleit solution
KO	Knock-out
LABA	Long acting beta-adrenoceptor agonist
LAMA	Long acting muscarinic antagonist
LAR	Late asthmatic response
LPS	Lipopolysaccharide
MHC-II	Major histocompatibility complex class II molecules
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
mRNA	messenger ribonucleic acid

MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
MVL	Microvascular leak
NF-κB	Nuclear factor kappa-B
OVA	Ovalbumin
p.o.	*per os*
PCR	Polymerase chain reaction
PET	Polyethylene terephthalate
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGG_2	Prostaglandin G_2
PGH_2	Prostaglandin H_2
PGI_2	Prostacyclin
PGJ_2	Prostaglandin J_2
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
PLC	Phospholipase C
R3-IGF-1	Insulin-like growth factor 1
RNA	ribonucleic acid
s.e.m.	Standard error of the mean
TCR	T cell receptor
TEER	Transendothelial electrical resistance
TNF_α	Tumour Necrosis Factor alpha
TP	Thromboxane A_2 receptor
TXA_2	Thromboxane A_2
VE-cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
WT	Wild type

Chapter One

Introduction

1. Introduction

1.1. Airway Inflammatory Diseases

Asthma and Chronic Obstructive Pulmonary Disease (COPD) are two chronic lung diseases which are associated with significant morbidity and mortality worldwide (Edwards *et al*, 2009). Both are characterised by airway obstruction that is variable and reversible in asthma but irreversible and progressive in COPD. Additionally, chronic inflammation of the respiratory tract is apparent in both diseases. Asthma and COPD represent an increasing burden on health services as their incidence increases worldwide. Currently they stand as the fourth leading cause of death in the world, and it has been predicted by the World Health Organisation that the death toll from smoking related diseases, such as COPD, could reach 8 million by 2030 (Minino *et al*, 2007; The World Health Organisation, 2011). Furthermore, it has been estimated that approximately 300 million people globally are affected by asthma and it has been predicted that this will continue to rise with a further 100 million people affected by 2025 (Masoli *et al*. 2004).

Microvascular leak, the exudation of plasma and plasma proteins, is a key feature in airway inflammatory diseases. Increased levels of albumin and other plasma proteins have been measured in the airways of asthma (Meerschaert *et al*, 1999; Svensson *et al*, 1995; Van Rensen *et al*, 2002) and COPD patients (Hill *et al*, 1999; Minikata *et al*, 2005; Bessa *et al*, 2012). Airway microvascular leak is thought to play an important role in the thickening of bronchial mucosa, airway hyperresponsiveness and exacerbations seen in asthmatic patients (Van de Graaf *et al*, 1991; Khor *et al*, 2009; Paredi & Barnes, 2009). Similarly, in COPD this increase in plasma exudation and thickening of airway mucosa is thought to influence the obstructive processes of this disease (Bosken *et al*, 1990; Kuwano *et al*, 1993). Additionally the exuded plasma will be abundant in pro-inflammatory mediators that possess the ability to induce various events, such as inflammatory cell recruitment, compromised epithelial integrity and cilia function, as well as the activation of sensory neural pathways.

1.1.1. Asthma

Bronchial asthma is usually defined as a chronic inflammatory pathology characterised by reversible airflow obstruction resulting from bronchoconstriction, inflammation and airway hyperresponsiveness (AHR). This leads to recurrent episodes of wheezing, dyspnoea, coughing, and chest tightness, associated with a decrease in lung function (James & Wenzel, 2007). A wide range of endogenous and environmental stimuli have been shown to induce an asthma attack. These triggers can include allergic stimuli, such as pollen, animal dander and dust mite faeces, or non-allergic stimuli, such as exercise and cold air (Holgate, 2008a,b; McKenzie & Boulet, 2008; Passalacqua & Ciprandi, 2008). Allergic or 'atopic' asthma represents the largest overall phenotype among patients, while few are defined as non-atopic (or intrinsic) (Wenzel, 2006).

1.1.1.1. Allergic Sensitisation and Responses

The immune response in allergic asthma involves two phases; the sensitisation phase following allergen exposure, and then the challenge or allergic response phase. During the sensitisation phase allergens are detected by antigen presenting cells (APCs), primarily dendritic cells in the airways, where they are internalised and processed onto a major histocompatibility complex class II molecules (MHC-II) which they express on their cell surface. This then enables the allergen to be 'presented' to naïve $CD4^+$ T cells and a complex to be formed between the MHC-II/allergen molecule and T cells that express the specific T cell receptor (TCR). Accordingly, the antigen must be presented to the relevant T cell that expresses the right TCR. However, it has been recognised that each TCR can in fact recognise a number of different antigenic peptides (Wooldridge *et al*, 2012). This complex formation and additional co-stimulatory signals subsequently leads to T cell proliferation and differentiation; predominantly Th1 or Th2 effector cells. In asthma, the Th2 phenotype is a key immunological feature and factor in the pathogenesis of the disease where it dominates the allergic immune response and is most likely to be responsible for on-going chronic inflammation. It is the Th2 cells that then release interleukin (IL)-4 and IL-13 which in turn stimulates B cells to produce and release

allergen specific IgE (Grünig *et al*, 1998; McKenzie *et al*, 1999). These IgE molecules bind to high affinity Fcε receptors (FcεR1) expressed on mast cells and basophils thus priming them for future allergen exposure (Figure 1.1).

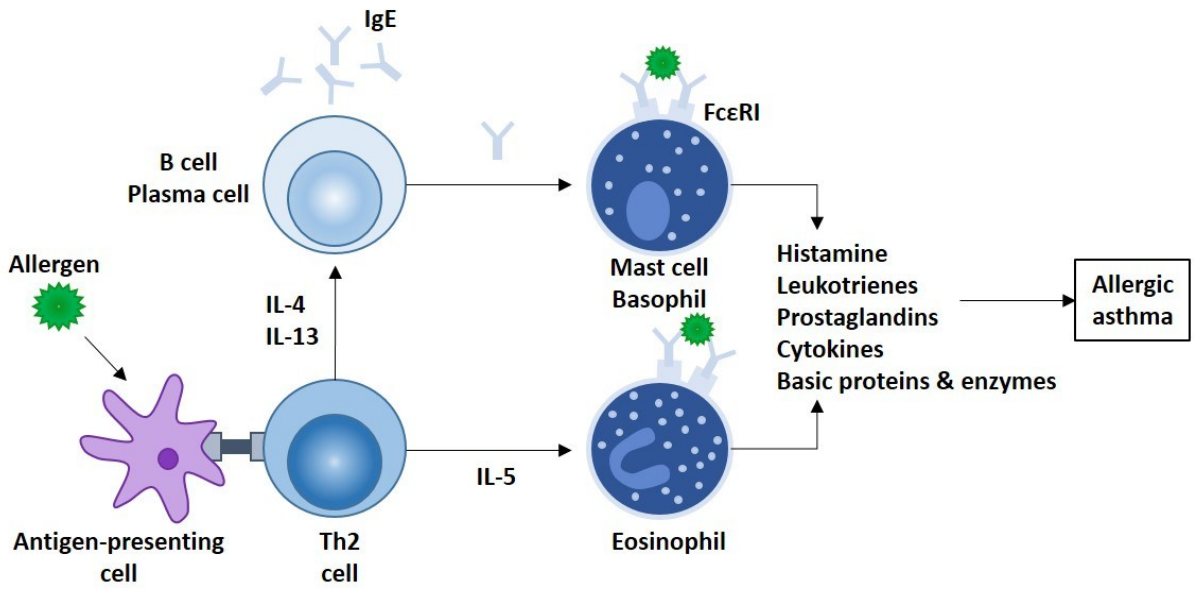


Figure 1.1. Schematic representation of the inflammatory cascade in allergic asthma. (Adapted from Bradding *et al*, 2006).

1.1.1.1.1. EAR and LAR

Subsequent allergen exposure results in an allergic response, activating sensitised mast cells by crosslinking surface bound IgE molecules to release several bronchoconstrictor mediators, including histamine, cysteinyl leukotrienes and prostaglandin D_2 (PGD_2) (Barnes 2008). This reaction is termed the early asthmatic response (EAR) and is characterised by rapid bronchoconstriction, oedema and plasma extravasation, and mucus secretion which all contribute to airflow obstruction. Approximately 4-8 hours after initial allergen challenge the late asthmatic response (LAR) occurs in about 50% of patients (Booij-Noord *et al* 1971, 1972). This is usually characterised by bronchoconstriction and an influx of inflammatory cells, such as eosinophils, mast cells, neutrophils and T cells (Hogan *et al* 1998).

1.1.1.1.2. Airway Hyperresponsiveness

One of the defining physiological characteristics of asthma is AHR. AHR is defined as an increased narrowing of the airways of asthmatic patients in response to stimuli which do not usually cause bronchoconstriction in healthy individuals. Such stimuli include pollution, allergens, cold air and spasmogens such as methacholine and histamine (Spina & Page, 2002). This response leads to excessive bronchoconstriction and airflow limitation, which results in shortness of breath and chest tightness. Several mechanisms are thought to contribute to AHR including hypertrophy and hyperplasia of airway smooth muscle (Ebina *et al*, 1993; Lambert *et al*, 1993) and airway wall oedema (Van de Graaf *et al*, 1991; Kimura *et al*, 1992; Chung *et al*, 1999). It has also been suggested that inflammation and inflammatory mediators are driving AHR, which is supported by the fact that inhaled and oral corticosteroids are able to attenuate AHR in asthmatic patients (Djukanović *et al*, 1992, 1997; Laitinen *et al*, 1992; Chalmers *et al*, 2002; Clearie *et al*, 2012). Increases in cytokines (IL-4, IL-5 and IL-13), eosinophils and IgE can also lead to hyperresponsiveness (Grünig *et al*, 1998; Walter *et al*, 2001; Taube *et al*, 2002), with eosinophilic inflammation correlating with asthma severity and AHR (Bousquet *et al*, 1990).

1.1.1.2. Treatments

Current management of asthma involves the use of short and long acting β_2-adrenergic receptor agonists and inhaled corticosteroids (ICS). β_2-agonists act as bronchodilators for symptomatic relief as they are able to relax airway smooth muscle, reversing airflow obstruction. Short acting β_2-agonists, such as salbutamol, are commonly prescribed to those with mild intermittent asthma symptoms. However, for moderate to severe cases the most common, and highly effective, strategy is regular ICS with long-acting β_2-agonists (LABAs) in combination inhalers. LABAs most frequently used include salmeterol and formoterol. Briefly, β_2-agonists exert their effects through the activation of the β_2 adrenoceptor on airway smooth

muscle, which leads to an increase in cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA) or PKG activity. This induces a relaxation of the airway smooth muscle thus causing bronchodilation. In addition, there is evidence to suggest that anti-spasmogenic effects of these compounds may be mediated via PKA/PKG-independent mechanisms (Torphy *et al*, 1982; Torphy, 1994; Spicuzza *et al*, 2001).

ICS, such as budesonide or fluticasone, have become a mainstay of asthma management and are recommended as a first-line therapy for patients of all ages with persistent asthma (Bateman *et al*, 2008). Furthermore, they are extremely effective at controlling varying degrees of asthma severity. ICS are potent anti-inflammatories that act at glucocorticoid receptors (GR) to activate various anti-inflammatory genes as well as suppressing pro-inflammatory genes (Barnes, 2011). The main action is the switching off of multiple activated inflammatory genes, encoding for cytokines, chemokines, adhesion molecules and inflammatory receptors. Furthermore, there have been concerns over the safety of LABAs when administered alone but there has been no evidence of this risk when administered in combination with ICS (Jaeschke *et al*, 2008; Sears, 2009; Weatherall *et al*, 2010).

Although combination therapy with inhaled ICS and LABAs is highly effective, approximately 5 to 10% of patients are not well controlled even with maximum doses of inhaled therapies (Heaney & Robinson, 2005; Wenzel 2005). Although this percentage appears small, it accounts for a huge amount of health care spending, highlighting an urgent need for new treatments.

1.1.1.3. In vivo models of Asthma

Although there are both allergic and non-allergic variants of asthma, the majority of *in vivo* models are based on the allergic form. This section will therefore outline two of the most commonly used experimental models of allergic asthma; the ovalbumin (OVA) and house dust mite (HDM) models. It is important to note that these *in vivo* models are not exact recreations of human disease but they allow us to investigate the mechanistic, pathophysiological and physiological characteristics of asthma. In

addition, most laboratory animals do not spontaneously develop an allergic response so this must be artificially induced (Slauson & Hahn, 1980; Kurucz & Szelenyi, 2006).

1.1.1.3.1. Ovalbumin Model

Ovalbumin (OVA) is a protein allergen that is found in chicken egg white, which has been used for decades to induce an allergic response in rodents. To produce an allergic response to OVA, the model requires an initial sensitisation phase followed by a challenge phase. Typically, animals are sensitised systemically (by intraperitoneal or subcutaneous administration) with OVA, in combination with an adjuvant. The adjuvant, such as Alum or aluminium hydroxide, acts by priming the immune system towards a Th2 phenotype. Sensitisation to OVA usually requires two systemic doses, separated by at least one week. This phase is then followed by allergen exposure to the airways one to two weeks later. The exact protocols in terms of timings, doses and routes of administration can vary between different research groups. The OVA model has proved effective in replicating some of the major features of allergic asthma, including IgE production, mucus secretion, EAR and LAR, non-specific AHR and oedema (as reviewed in Stevenson & Belvisi, 2008). In addition, these responses to OVA can be modulated by glucocorticoids and β_2-agonists (Birrell *et al*, 2003; Eum *et al*, 2003; Wyss *et al*, 2007). The OVA model has been utilised in guinea pigs, rats and mice, with the brown-Norway rat and BALB/C mouse being the most commonly used strains (Trifilieff *et al*, 2000; Hylkema *et al*, 2002). The C57/BL6 mice are also regularly used, as there are a number of genetically modified mice created using this strain.

However, this model is not without any shortcomings. Firstly, the use of OVA is not a clinically relevant antigen as it is rarely associated with triggering asthma in humans. Secondly, the route of sensitisation and use of an adjuvant does not replicate what would occur in man, where airborne allergens are probably more relevant. Finally, there is evidence to suggest that tolerance occurs in chronic OVA models (Swirski *et al*, 2002; Van Hove *et al*, 2007), with suppression of eosinophilia, AHR and OVA-specific IgE having been demonstrated (Sakai *et al*, 2001). In contrast some groups have demonstrated airway remodelling (e.g. goblet cell hyperplasia and airway

fibrosis) following chronic OVA-exposure (Temelkovski *et al*, 1998; McMillan & Lloyd, 2004; Van Hove *et al*, 2009).

1.1.1.3.2. House Dust Mite Model

The house dust mite (HDM) model of allergic asthma was developed as an alternative to the OVA model, using a more clinically relevant allergen. House dust mite has been shown to induce a positive skin prick test in approximately 80% of asthmatic patients (Smith *et al*, 1969) and is associated with a number of other allergic disorders, including allergic rhinitis and atopic dermatitis (Platt-Mills, 1995). The two commonly used house dust mite allergens are *Dermatophagoides pteronyssinus (Der p)* and *Dermatophagoides farina (Der f)*, which can produce proteins and macromolecules that induce IgE antibodies in allergic individuals (Chapman & Platts-Mills, 1980). Typical protocols using HDM involves multiple topical instillations over several weeks, rather than separate sensitisation and challenge phases. Modelling allergic asthma using HDM has several advantages over the OVA model. Firstly, no adjuvant or systemic sensitisation is required as animals can be sensitised to HDM via the respiratory mucosa. Secondly, eosinophilic inflammation is sustained following chronic exposure in addition to structural remodelling (Johnson *et al*, 2004; Southam *et al*, 2007). However, unlike the OVA model, very little is known about the HDM model and the role played by immune cell populations and inflammatory mediators involved. In addition, it has been shown that a single topical dose of HDM in the rat was able to induce asthma-like airway inflammation, suggesting that initial inflammation in this model is innate in nature (De Alba *et al*, 2010).

1.1.2. Chronic Obstructive Pulmonary Disease

Chronic obstructive pulmonary disease (COPD) is a term that encompasses a group of respiratory diseases including emphysema, chronic bronchitis and small airways disease (Stockley *et al*, 2009). It is characterised by progressive and irreversible airflow obstruction caused by chronic inflammation of the small airways and lung parenchyma. This aberrant inflammatory response is associated with various different stimuli, including cigarette smoke, pollution and viral or bacterial infections. COPD is a major public health problem, which the World Health Organisation (2008) has estimated to become the third leading cause of death worldwide in 2030. Unfortunately there are no effective treatments that halt the progression of this disease, with current therapies only alleviating symptoms and acute exacerbations. Classical symptoms of COPD include cough, sputum production and dyspnoea, eventually leading to a loss in lung function. Additionally, there are several extra-pulmonary comorbidities that affect COPD patients, including skeletal muscle dysfunction, cardiovascular disease, metabolic diseases, depression and osteoporosis (Gan *et al*, 2004; Agustí, 2005; Sin *et al*, 2006; Barnes & Celli, 2009; Barr *et al*, 2010). These comorbidities probably stem from the systemic inflammation observed in COPD (Gan *et al*, 2004; Agustí, 2005; Barnes, 2010).

As described above COPD describes a group of diseases, often divided into chronic bronchitis, small airways disease and emphysema. Chronic bronchitis involves persistent inflammation of the epithelium, characterised by mucus hypersecretion, goblet cell hypertrophy and hyperplasia and epithelial barrier disruption (Hogg, 2004). Small airways disease (or bronchiolitis) describes inflammation and remodelling specific to conducting airways less than 2mm in diameter, which are the major site of airflow obstruction (Hogg, 2004). Finally, emphysema refers to the destruction of structural lung tissue, reducing the maximum expiratory airflow and lung elastic recoil (Mead *et al*, 1967).

Cigarette smoking accounts for the majority of COPD cases in high income countries, while in lower-income countries other risk factors are important, such as indoor air pollution from biomass fuels (Salvi & Barnes, 2009). The harmful effects of cigarette smoke are thought to arise from the toxic constituents that it is composed

of (including acrolein, benzene and formaldehyde) as well as an increase in reactive oxygen species (Owen, 2008; Bodnar *et al*, 2012). α1-antitrypsin deficiency is one of the only known genetic causes of COPD and is involved in approximately 1-3% of cases (Stoller & Aboussouan, 2012). Despite tobacco smoke being a primary risk factor (about 80-90% of COPD patients) (Stevensen *et al*, 2006), only about 25% of smokers develop the disease (Løkke *et al*, 2006). This suggests that there may be genetic, epigenetic or environmental factors that determine susceptibility.

The Global Initiative for Chronic Obstructive Lung Disease (GOLD) has outlined a classification system of airflow limitation which is divided into four grades (GOLD 1, mild; GOLD 2, moderate; GOLD 3, severe; and GOLD 4, very severe). These grades are based upon the fixed ratio, post-bronchodilator FEV_1/FVC (Forced Expiratory Volume in 1 second and Forced Vital Capacity, respectively) less than 0.7, as an indication of airflow limitation (Vestbo *et al*, 2013). Additional COPD assessment is based upon symptom severity, history of exacerbations, spirometric abnormality and comorbidities. A combined COPD assessment then splits patients into four different categories; group A, low risk less symptoms; group B, low risk, more symptoms; group C, high risk, less symptoms; group D, high risk, more symptoms. Assessing COPD using this combined approach reflects the complexity of the disease and provides a more robust guide to treatment and disease management (Vestbo *et al*, 2013).

1.1.2.1. Treatments

One of the main risk factors associated with COPD is smoking; however smoking cessation strategies have not been hugely successful, especially in patients that are more severely affected (Scanlon *et al*, 2000; Willemse *et al*, 2005).

In terms of pharmacological therapies, bronchodilators are commonly used to manage the symptoms of COPD. Bronchodilators used include β_2-agonists, anti-cholinergics and methylxanthines, with long-acting formulations favourable as they are both more effective and convenient compared to short-acting (Dahl *et al*, 2001; Oostenbrink *et al*, 2004; Vestbo *et al*, 2013). These drugs may be effective at

relaxing the airway smooth muscle but they fail to treat or reduce the inflammation associated with the disease.

The use of corticosteroids (e.g. fluticasone or budesonide) is also common among COPD patients. Like asthma, COPD involves chronic inflammation of the airways. However, most patients with COPD respond poorly to inhaled corticosteroids (ICS) showing little improvement in lung function or symptom severity (Suissa & Barnes, 2009; Barnes, 2010; Yang *et al*, 2012). Nevertheless, approximately 80% of COPD patients are using high-dose ICS. The overuse of glucocorticoids can have various long term side effects, including osteoporosis, muscle breakdown and an increased risk of pneumonia (Drummond *et al*, 2008; Barnes, 2010). However, several large clinical trials have shown that combination therapy with ICS and long acting β_2-agonists (LABAs) produces better control of respiratory symptoms, lung function and quality of life (Calverley *et al*, 2003a; Calverley *et al*, 2003b; Szafranski *et al*, 2003; Calverley *et al*, 2007; Nannini *et al*, 2007; Bathoorn *et al*, 2008). Furthermore, combining a LABA and a long-acting muscarinic antagonist (LAMA) have additive effects on bronchodilation with greater clinical benefit than each given alone. Combinations inhalers include indacaterol-glycopyrrolate, olodaterol-tiotropium and vilanterol-umeclidinium (Barnes, 2013).

1.1.2.2. In vivo models of COPD

1.1.2.2.1. Lipopolysaccharide Model

Lipopolysaccharide (LPS) is a bacterial endotoxin that is found in cigarette smoke. It is a widely used model of acute lung injury, where it is characterised by prominent lung neutrophilia, peaking between 6 and 24 hours (Ferretti *et al*, 2003). The neutrophil is a primary source of neutrophil elastase in COPD and thus regarded as one of the most important mediators driving emphysema. In addition, the LPS model has been favourable as the protocol is quick and easy, involving just a single dose of LPS. Furthermore, chronic administration of LPS has demonstrated airway remodelling, emphysema and changes in lung function (Stolk *et al*, 1992; Savov *et*

al, 2003; Brass *et al*, 2008). However, LPS-induced inflammation, unlike human COPD, is sensitive to glucocorticoid treatment (Birrell *et al*, 2005; Håkansson *et al*, 2012), bringing into question the clinical relevance of this model. Although no longer used as a COPD-like model it does provide a model of acute innate immunity-driven inflammation and also serves as a model of bacterial exacerbation (Wright *et al*, 2008).

1.1.2.2.2. Cigarette Smoke Model

The use of cigarette smoke (CS) to model COPD in small animals has become favourable over LPS and elastase models (a single stimulus) as CS smoke contains over 4700 chemical compounds and more than 10^{15} oxidants/free radicals (reviewed in Vlahos & Bozinovski, 2014). In addition, CS is the primary etiological agent in human COPD. Several hallmark features of human COPD have been observed in rodent CS models, including chronic lung inflammation, impaired lung function, emphysema, mucus hypersecretion, airway wall remodelling, and vascular remodelling. In addition, CS-induced inflammation in rodents is insensitive to glucocorticoids, resembling that seen in human COPD (Marwick *et al*, 2004; Marwick *et al*, 2009). However, it can be difficult to compare findings from different research groups as protocols for CS exposure can vary immensely. For example, differences occur with delivery systems (nose-only or whole-body), types of cigarettes, smoke concentration, duration and frequency as well as species and strain of animal. Furthermore, a lot of the changes seen in rodent CS models are very mild in comparison to those seen in humans. Subsequently, for lung function, small airway remodelling and emphysematous changes to develop chronic exposures of 6 months or more are required (Churg *et al*, 2004; Barreiro *et al*, 2012; Yao *et al*, 2012), which is both time consuming and expensive. With that being said, acute exposure models (<1 week) are effective as primary models to explore mechanisms of smoke-induced lung inflammation as well as testing potential treatments.

1.2. Prostanoids and Prostanoid Receptors

Within the airways prostanoids have been shown to contribute to airflow regulation, bronchoconstriction, mucus secretion and microvascular leak, all of which are prominent features in asthma and COPD. In addition, increased levels of prostanoids, in particular PGE_2 and PGD_2, have been measured in the airways of asthma and COPD patients (Murray *et al*, 1986; Montushi *et al*, 2003; Long *et al*, 2004). It has also been suggested that prostanoids initiate protective mechanisms in the airways and inhibit the release of inflammatory mediators from airway cells (Clarke *et al*, 2004; Kay *et al*, 2006; Pavord & Tattersfield, 1995). There appears to be a fine balance between the pro-inflammatory and anti-inflammatory effects of prostanoids but it is clear that they have important functions in the airways.

1.2.1. Synthesis

Prostanoids are described as lipid mediators that belong to a diverse family of eicosanoids synthesised by cyclooxygenase (COX)-mediated metabolism of the unsaturated 20-carbon fatty acid arachidonic acid. Arachidonic acid is liberated from the cell membrane by phospholipase A_2 and oxidised by COX enzymes into PGG_2. This then undergoes a reduction reaction by COX to form an unstable endoperoxidase PGH_2. PGH_2 subsequently acts as a substrate for a number of specific synthase enzymes which produce the five bioactive prostanoids (Flower, 1978; Goulet *et al*, 2004; Hata & Breyer, 2004). These are PGE_2, $PGF_{2\alpha}$, PGD_2, Prostacyclin (PGI_2) and thromboxane A_2 (TXA_2), as shown in Figure 1.2. There are two forms of COX enzyme, simply termed COX-1 and COX-2. In the majority of cell types within the body COX-1 is expressed constitutively, while COX-2 is inducible in response to inflammatory stimuli, such as endotoxin, pro-inflammatory cytokines, Interleukin 1β (IL-1β) and tumour necrosis factor α (TNF-α) and regulated by nuclear factor κ B (NF-κB) (Newton *et al*, 1997).

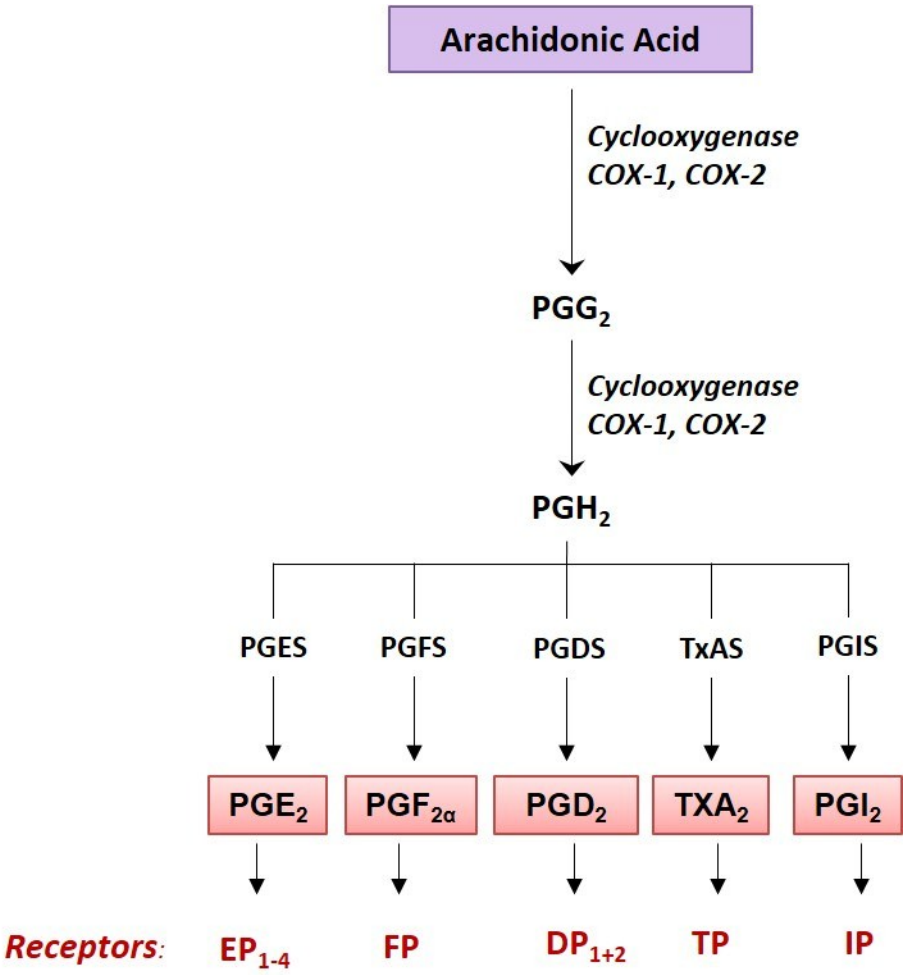


Figure 1.2. Schematic representation of the biosynthesis of prostanoids. *Arachidonic acid is metabolised by cyclooxygenase-1 or -2 (COX-1/2) to PGH_2. The prostanoids PGE_2, $PGF_{2\alpha}$, PGD_2, TxA_2 and PGI_2 are formed by their individual synthase enzymes PGES, PGFS, PGDS, TxAS and PGIS respectively.*

1.2.2. Prostanoid Receptors

In order to exert their biological effects prostanoids bind to specific cell surface receptors. These receptors belong to the Rhodopsin-like family of the seven transmembrane domain G-Protein Coupled Receptors (GPCRs). Currently there are nine known prostanoid receptors, FP, IP, TP, DP_{1+2}, and $EP_{1-4,}$ named according to the prostanoid affinity profiles i.e. of all the prostanoids PGE_2 has the most affinity for the 4 EP receptors (Figure 1.2) (Coleman *et al*, 1994). Although individual prostaglandins have the highest affinity for their cognate receptor, there is

considerable cross reactivity between a given prostanoid and other receptors within the family. For example, PGD_2 has been shown to act via TP receptors to mediate different responses (Coleman & Sheldrick, 1989; Nishimura *et al*, 2001). Furthermore, the observation that PGE_2 can have multiple and often opposing functional effects on a single tissue, is likely accounted for by the existence of multiple receptors (Woodward *et al*, 2011). The G-proteins and downstream signalling pathways activated by each prostanoid receptor are discussed in more detail later. The schematic in figure 1.3 outlines the main signalling pathways for each prostanoid receptor.

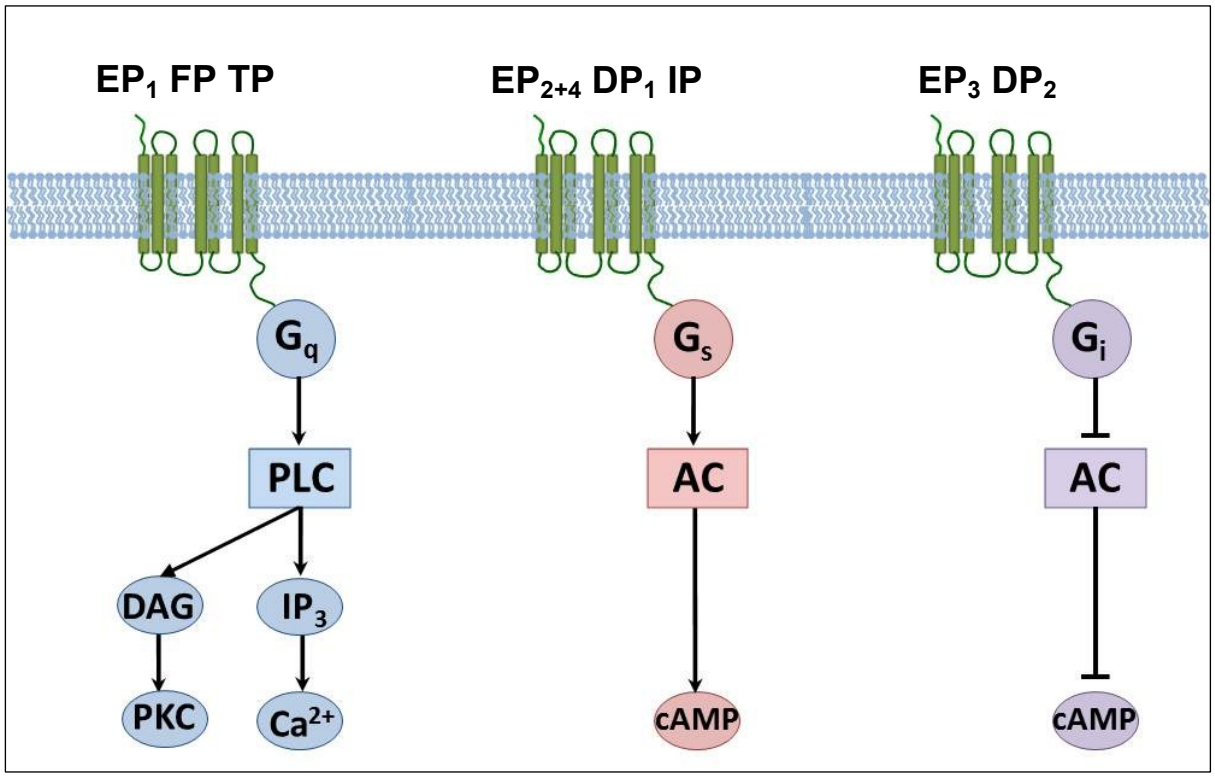


Figure 1.3. Signalling cascade of prostanoid receptors. *The EP_1, FP and TP receptors predominantly couple to G_q, the EP_2, EP_4, DP_1 and IP receptors to G_s and the EP_3 and DP_2 receptors to G_1. Although the existence of EP_3 splice variants may alter the signalling pathway. Activation of G_q causes phospholipase C (PLC) activation and an increase in calcium/protein kinase C (PKC). G_s coupling activates adenylyl cyclase (AC) and subsequent increase in cAMP, while G_i inhibits AC and decreases cAMP.*

1.2.2.1. Prostaglandin E_2 and EP receptors

Prostaglandin E_2 (PGE_2) is generated by PGE synthase that catalyses the isomerization of PGH_2 to PGE_2. As previously mentioned, PGE_2 has the highest affinity for EP receptors, EP_{1-4}, which are encoded for by the genes *Ptger1-4* (Coleman *et al*, 1994). Original discovery of multiple receptor subtypes was confirmed through molecular cloning from human tissue (Adam *et al*, 1994; Bastien *et al*, 1994; Funk *et al*, 1993; Regan *et al*, 1994) as well as mouse tissue (Sugimoto *et al*, 1992; Honda *et al*, 1993; Watabe *et al*, 1993; Katsuyama *et al*, 1995). Additionally, it was demonstrated that the EP_3 receptor has multiple splice variants (Adam *et al*, 1994). In terms of affinity, the EP_3 and EP_4 receptors bind PGE_2 with the highest affinity (K_d < 1nM) while the EP_1 and EP_2 receptors show a slightly lower affinity (K_d > 10nM) (Abramovitz *et al*, 2000).

With four different receptor subtypes it is unsurprising that PGE_2 has the most diverse range of physiological effects. In the gastrointestinal tract, PGE_2 protects the integrity of the gastric mucosa, while the use of non-steroidal anti-inflammatory drugs (NSAIDs) has been associated with significant adverse gastrointestinal effects (Woo *et al*, 1986; Warner *et al*, 1999). In the kidney, PGE_2 is crucial in preserving renal blood flow and glomerular filtration rate (reviewed in Breyer & Breyer, 2001). PGE_2 also plays an important role in the cardiovascular system where it is able to maintain blood pressure following salt overload (Kennedy *et al*, 1999). Opposing effects of PGE_2 has also been noticed; on airway and vascular smooth muscle it can cause both relaxation and contraction and within the immune system it appears to have both pro- and anti-inflammatory actions. The signal transduction mechanisms mediating their effects differ between each receptor subtype, which accounts for the diverse role of PGE_2. More detail on individual EP receptors and their biological functions is discussed below.

1.2.2.1.1. EP_1 Receptors

The human EP_1 receptor cDNA encodes a 402-amino-acid sequence which shares 83% and 85% homology with the rat and mouse EP_1 receptors, respectively (Funk *et al*, 1993). It is predominantly thought to couple to the G_q pathway with the activation

of phospholipase C (PLC) and Ca^{2+} mobilisation (Nicola *et al*, 2005; Tang *et al*, 2005). However, in mouse EP_1 receptors, this increase in Ca^{2+} correlated with negligible IP_3 response, indicating a signalling pathway independent of G_q (Narumiya *et al*, 1999).

EP_1 receptors are not widely distributed in higher species and are considerably more common in guinea pigs and mice (Coleman *et al*, 1994b). However, functional EP_1 receptors have been observed in the myometrium (Senior *et al*, 1991), pulmonary veins (Norel *et al*, 2004), mast cells (Wang & Lau, 2006) and keratinocytes (Konger *et al*, 2009). The physiological functions of EP_1 receptors include allodynia (Stock *et al*, 2001), cardiovascular homeostasis (Audoly *et al*, 1999; Stock *et al*, 2001), inflammatory pain and hyperalgesia (Minami *et al*, 1994; Nakayama *et al*, 2004) and stress-induced impulse behaviour (Matsuoka *et al*, 2005). In the airways, EP_1 receptors mediate airway constriction in mice (Tilley *et al*, 2003) and guinea pigs (Coleman & Kennedy, 1985; Ndukwu *et al*, 1997) and surfactant secretion from rat alveolar type II cells (Morsy *et al*, 2001).

Previously, 17-Phenyl PGE_2 was the selective agonist used to investigate the EP_1 receptor but its selectivity was only modest (Lawrence *et al*, 1992). The ligand ONO-D1-004 produced by ONO pharmaceuticals (Japan) is more favourable due to its superior selectivity (Okada *et al*, 2000; Suzawa *et al*, 2000). The most suitable selective antagonist is considered to be GW848687X (Giblin *et al*, 2007; Jones *et al*, 2009).

1.2.2.1.2. EP_2 Receptors

The human EP_2 receptor cDNA encodes a 358-amino-acid polypeptide which has also been cloned in the rat, mouse and rabbit (Boie *et al*, 1997; Guan *et al*, 2002; Katsuyama *et al*, 1995; Regan *et al*, 1994). EP_2 receptors mediate their effects through G_s coupling and an increase in cAMP (Regan *et al*, 1994). Additionally, it has been suggested that a positive feedback loop exists whereby cAMP signalling enhances the expression of EP_2 receptors (Sagana *et al*, 2009).

Earlier studies on EP_2 receptor mRNA expression indicated relatively low levels and an uncertain distribution pattern (Regan *et al*, 1994; Narumiya *et al*, 1999; Smock *et*

al, 1999). However, in the past decade it has become apparent that it is more widely distributed. In the airways, expression has been reported throughout, including airway smooth muscle, epithelial cells and inflammatory cells (Coleman *et al*, 1994). According to functional studies, EP_2 receptor activation invariably leads to relaxation. Indeed, EP_2-mediated relaxation of vascular smooth muscle and vasodepressor responses has long been known (Armstrong *et al*, 1976; Audoly *et al*, 1999). Kennedy *et al* (1999) demonstrated that mice deficient in the EP_2 receptor developed salt-sensitive hypertension. In addition, EP_2 receptors were thought to mediate PGE_2-induced bronchodilation in isolated airway tissue in a variety of species including humans (Norel *et al*, 1999), mice (Tilley *et al*, 2003), guinea pigs (Nials *et al*, 1993) and cats (Gardiner 1986). However, more recently it has been shown that in rat and human airway smooth muscle EP_4 receptor activation mediates relaxation and bronchodilation (Buckley *et al*, 2011).

Currently, ONO-AE1-259 is the preferred selective EP_2 agonist and has proved valuable in characterising EP_2 receptor systems (Cao *et al*, 2002; Clarke *et al*, 2004). AH6809 was previously used as a mixed EP_1/EP_2 receptor antagonist as there were no other potent or selective compounds in this class (Woodward *et al*, 1995). However, Forselles *et al* (2011) recently reported on the development of a selective EP_2 antagonist, PF-04418948. Results from our group confirmed its selectivity, relative to other prostanoid receptors, using a range of isolated tissue based systems (Birrell *et al*, 2013).

1.2.2.1.3. EP_3 Receptors

Following pharmacological investigations into the EP_3 receptor it was evident that its activation mediated a diverse range of effects. This has been attributed to the existence of multiple splice variants, with at least eight having been identified in human tissue (Bos *et al*, 2004). The major signalling pathway for EP_3 receptors is through G_i coupling and an inhibition of adenylyl cyclase (Narumiya *et al*, 1999). However, the presence of alternative splice variants has demonstrated a wide repertoire of signalling pathways. EP_3 receptor activation has been reported to show activation of the G_s pathway, increases in intracellular Ca^{2+} and Rho kinase stimulation (An *et al*, 1994; Namba *et al*, 1993; Hasegawa *et al*, 1997; Macias-Perez

et al, 2008). Currently, there is no consistent nomenclature for these alternative splice variants.

Through Northern blotting, *in situ* hybridisation and functional studies the EP_3 receptor has demonstrated a wide distribution, including high expression levels in the central nervous system, cardiovascular system, reproduction system, kidney, uterus, adrenal gland and stomach (Yang *et al*, 1994; Abramovitz *et al*, 1995; Sugimoto & Narumiya, 2007; Woodward *et al*, 2011). This wide distribution and existence of multiple receptor isoforms appears to correlate with the diverse range of effects EP_3 activation has. The EP_3 receptor mediates PGE_2-induced hyperalgesia (Minami *et al*, 2001) and plays a major role in hyperpyrexia (Ushikubi *et al*, 1998). Within the cardiovascular system EP_3 receptors can mediate platelet aggregation by potentiation of the effects of pro-aggregatory agents (Matthews & Jones, 1993; Armstrong, 1996; Fabre *et al*, 2001; Ma *et al*, 2001; Gross *et al*, 2007; Singh *et al*, 2009). EP_3 receptors also have a range of effects in the airways including sensory nerve activation (Maher *et al*, 2009), constriction of human pulmonary artery (Qian *et al*, 1994), bronchoconstriction and allergic responses in mice (Tilley *et al*, 2003; Kunikata *et al*, 2005) and anti-proliferative effects on human airway smooth muscle (Burgess *et al*, 2004). Furthermore, opposing effects on oedema formation have been described. In rat and mouse skin, EP_3 receptor activation inhibited oedema formation (Ahluwalia & Perretti, 1994), while PGE_2-induced mouse paw oedema was EP_3 receptor-mediated (Claudino *et al*, 2006).

Sulprostone has been used as an EP_3 agonist in functional studies (Coleman & Sheldrick, 1989; Maher *et al*, 2009) but it has only modest EP_3 versus EP_1 selectivity which has limited its utility (Coleman *et al*, 1987). ONO-AE-248 is a more suitable agonist as it has proved to be highly selective at the EP_3 receptors (Okada *et al*, 2000; Suzawa *et al*, 2000) despite having only modest potency (Jones *et al*, 2008). EP_3 antagonist L-826266 demonstrated high potency and selectivity (Gallant *et al*, 2002; Jones *et al*, 2009) but does have a slow onset of action owing to its lipophilicity (Jones *et al*, 2008).

1.2.2.1.4. EP_4 Receptors

The human EP_4 receptor cDNA encodes a 488-amino-acid sequence (Bastien *et al*, 1994) and this sequence has been cloned in the mouse, rat, rabbit and cow (An *et al*, 1994; Boie *et al*, 1997; Breyer *et al*, 1996; Honda *et al*, 1993). Originally, the pharmacologically defined EP_4 receptor was named EP_2 (Honda *et al*, 1993; Bastien *et al*, 1994) until the authentic EP_2 receptor was cloned (Regan *et al*, 1994b). The EP_4 receptor primarily couples to G_s and the subsequent increase in cAMP (Narumiya *et al*, 1999) but when compared with EP_2 receptor signalling the functional coupling to cAMP appeared less efficient for the EP_4 receptor (Fujino *et al*, 2002, 2005). In addition, several cAMP-independent signalling cascades have been identified following EP_4 receptor activation (Fiebich *et al*, 2001; Pozzi *et al*, 2004; Mendez & LaPointe, 2005; Frias *et al*, 2007; George *et al*, 2007; Rao *et al*, 2007).

EP_4 receptors are widely distributed and expression has been detected in tissues including the small intestine, lung, kidney, spleen, adrenal glands and thymus (Honda *et al*, 1993; Bastien *et al*, 1994; Breyer *et al*, 1996). Functionally, EP_4 receptor activation mediates relaxation of vascular smooth muscle of pulmonary arterial veins (Foudi *et al*, 2008) and is essential in closing the ductus arteriosus in newborns (Nguyen *et al*, 1997). Relaxation of airway smooth muscle can also be mediated via EP_4 receptor activation in both the rat (Lydford & McKechnie, 1994; Buckley *et al*, 2011) and human trachea (Buckley *et al*, 2011). Additionally, the EP_4 receptor has demonstrated anti-inflammatory effects in various cell based assays (Takayama *et al*, 2002; Clarke *et al*, 2004; Okano *et al*, 2006; Ratcliffe *et al*, 2007) as well as Th1 cell differentiation and Th17 expansion (Yao *et al*, 2009).

One of the most extensively used EP_4 agonists is ONO-AE1-329 which has demonstrated high selectivity as a full EP_4 agonist with relatively high potency (Okada *et al*, 2000; Suzawa *et al*, 2000; Cao *et al*, 2002). AH23848 was one of the first EP_4 receptor antagonists (Coleman *et al*, 1994a) but has since been overtaken by more selective and potent antagonists. These more selective antagonists include GW627368X (Wilson *et al*, 2006), ONO-AE3-208 (Kabashima *et al*, 2002) and ER-819762 (Chen *et al*, 2010).

1.2.2.2. Prostaglandin D_2 and DP receptors

Prostaglandin D_2 (PGD_2) is generated by PGD synthase (Urade *et al*, 1995) and is able to bind and activate, with similar affinity, two distinct receptors; DP_1 (Boie *et al*, 1995) and DP_2 (CRTH2) (Nagata *et al*, 1999; Hirai *et al*, 2001). PGD_2 is primarily produced by activated mast cells and/or T cells and is associated with inflammatory conditions (Roberts *et al*, 1980; Lewis *et al*, 1982). In addition, BAL fluid analyses have shown increased levels of PGD_2 in the airways following antigen challenge (Murray *et al*, 1986). The different functions and biological effects of each receptor subtype is described below.

1.2.2.2.1. DP_1 Receptors

The human DP_1 receptor cDNA encodes a 359 amino-acid polypeptide and is expressed in low levels in most tissues (Boie *et al*, 1995). Receptor activation is predominantly coupled to G_s and stimulation of cAMP production (Gorman *et al*, 1977; Halushka *et al*, 1989; Hirata *et al*, 1994; Boie *et al*, 1995) and DP_1 expressing cells have also elicited increases in intracellular Ca^{2+} through cAMP-dependent mechanisms (Boie *et al*, 1995; Zaccolo, 2009).

Compared to the other prostanoid receptors, DP_1 is the least abundant in tissues. Northern blot analyses demonstrated expression in the retina and small intestine (Boie *et al*, 1995) and in mice, expression is moderate in the ileum and weak in the stomach, lung and uterus (Hirata *et al*, 1994). In contrast, functional studies have demonstrated that DP_1 receptor expression is limited to certain areas and/or specific cells, which may explain low levels of detection (Narumiya *et al*, 1999). Activation of the DP_1 receptor mediates many physiological events, including allergic responses and the production of chemokines and cytokines that recruit inflammatory lymphocytes and eosinophils (Matsuoka *et al*, 2000). Further, these cells are known to be involved in the airway inflammation and AHR seen in asthma (Kabashima & Narumiya, 2003). Functional DP_1 receptors also inhibit platelet aggregation (Whittle *et al*, 1983; Seiler *et al*, 1990), induce sleep (Mizoguchi *et al*, 2001), inhibit angiogenesis and plasma leakage (Murata *et al*, 2008) and mediate smooth muscle

relaxation and contraction (Narumiya & Toda, 1985). Differential effects of PGD_2 on airway smooth muscle tone has been reported and attributed to its agonism of contractile TP receptors (Beasley *et al*, 1987; McKenniff *et al*, 1991; Underwood *et al*, 1994).

A commonly used agonist for DP_1 receptors is BW245C as it exhibits minimal DP_2 agonism (Monneret *et al*, 2001; Yoshimura-Uchiyama *et al*, 2004) compared to PGD_2 (Hirai *et al*, 2001). Originally, the DP_1 antagonist AH-6809 was most frequently used (Keery & Lumley, 1988), however, its low affinity and its mixed selectivity with EP_1 and EP_2 receptors has since limited its utility (Abramovitz *et al*, 2000). The use of BWA868C has become favourable and the first choice antagonist as it is a selective and competitive blocker of high affinity (Giles *et al*, 1989; Lydford *et al*, 1996).

1.2.2.2.2. DP_2 Receptors

PGD_2 is also a primary ligand for the DP_2 receptor; however, the DP_2 receptor has no significant homology with the DP_1 receptor or any other prostanoid receptor that was described in the original classification (Abe *et al*, 1999; Hirai *et al*, 2001; Hata *et al*, 2005). In fact, DP_2 receptors are more closely related to chemoattractant receptors, hence its previous name being chemoattractant receptor-homologous molecule expressed on T helper Type 2 cells (CRTH2) (Abe *et al*, 1999; Hirai *et al*, 2001). It is the arginine residue in the seventh domain, which is conserved in all receptors of the original classification that is missing in the DP_2 receptors. In addition, the widespread distribution of the receptor outside of the immune system made its classification as DP_2 more appropriate (Jones *et al*, 2009).

DP_2 receptors primarily signal via a G_i-coupled pathway, resulting in decreases in cAMP levels (Sawyer *et al*, 2002; Gallant *et al*, 2007) and mobilisation of intracellular Ca^{2+} (Hirai *et al*, 2001; Gallant *et al*, 2007). Its distribution appears to be widespread, with high levels of expression found in the human stomach, heart, thymus and small intestine; moderate expression in the colon, blood and spinal cord and lower levels in the brain, skeletal muscle and spleen (Sawyer *et al*, 2002). Subsequent functional analyses found that DP_2 receptors were also present in

smooth muscle, the cardiovascular system and the eye (Jones *et al*, 2009). The expression of DP_2 receptors has also been reported on a number of human leukocytes, including eosinophils, Th2 cells and basophils (Hirai *et al*, 2001; Nagata *et al*, 1999).

DP_2 receptors have been implicated in immune regulation, more specifically in the development of allergic responses such as asthma and atopic dermatitis. Its expression in $CD4^+$ T cells is enhanced in allergic subjects and correlates with disease severity (Kostenis & Ulven, 2006). In addition, activation of the DP_2 receptor leads to increased production of cytokines such as IL-4, IL-5 and IL-13 (Honda *et al*, 2003; Gallant *et al*, 2005; Xue *et al*, 2005; Kostenis & Ulven, 2006; Herlong & Scott, 2006), chemotaxis of eosinophils (Gervais *et al*, 2001; Hirai *et al*, 2001; Monneret *et al*, 2001; Sugimoto *et al*, 2003; Böhm *et al*, 2004) and the activation of basophils (Hirai *et al*, 2001; Cossette *et al*, 2007).

DP_2 receptors have the highest affinity for PGD_2 but have also shown affinity for the PGD_2 metabolites DK-PGD_2 and PGJ_2 (Hirai *et al*, 2001). Selective agonists have also been described, including 15*R*-methyl PGD_2 (Monneret *et al*, 2003) and L-888,607 (Gervais *et al*, 2005). In addition, indomethacin has also been shown to be a DP_2 agonist (Hirai *et al*, 2002). The development of selective antagonists has stemmed from the use of ramatroban, which is a TP antagonist that has demonstrated antagonism of DP_2 receptors, although with lower affinity (Mathiesen *et al*, 2005; Sugimoto *et al*, 2003; Mathiesen *et al*, 2006). Modification of ramatroban led to the development of CAY-10471, which has a higher affinity for DP_2 over TP receptors (Ulven & Kostenis, 2005).

1.2.2.3. Prostaglandin $F_{2\alpha}$ and the FP receptor

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is formed through three pathways from PGE_2, PGD_2 or PGH_2 by PGE 9-ketoreductase, PGD 11-ketoreductase, or PGH 9-,11-endoperoxide reductase, respectively (Watanabe, 2002). $PGF_{2\alpha}$ is known to activate a single prostanoid receptor, FP, that has two splice variants; FP_A, FP_B (Abramovitz *et al*, 1994; Pierce *et al*, 1997). The human FP receptor cDNA encodes an amino acid sequence of 359 residues (Abramovitz *et al*, 1994). The FP receptor is primarily

coupled to G_q and subsequent activation of PLC (Ito *et al*, 1994) but has also demonstrated $G_{12/13}$ coupling and the activation of Rho (Pierce *et al*, 1999).

FP receptors are widely distributed and is expressed in tissues including the kidneys, uterus, placenta, spinal cord and eye (Abramovitz *et al*, 1994; Sugimoto *et al*, 1994a; Schlötzer-Schrehardt *et al*, 2002; Suzuki-Yamamoto *et al*, 2009). Its most prominent function has been in reproduction. Sugimoto *et al* (1997) observed that in mice lacking the FP receptor parturition was abolished. $PGF_{2\alpha}$ has also demonstrated pathophysiological effects in the cardiovascular system where it increases blood pressure and promotes atherosclerosis (Yu *et al*, 2009b). Although no FP receptor expression has been observed in immune cells (Tilley *et al*, 2001; Hata & Breyer, 2004) it has been shown to induce bronchoconstriction *in vitro* (Coleman & Sheldrick, 1989) and in asthmatic patients (Fish *et al*, 1984). In addition, bleomycin-induced pulmonary fibrosis was suppressed in FP-deficient mice while pulmonary inflammation was comparable with wild-type mice (Oga *et al*, 2009).

$PGF_{2\alpha}$ itself is most potent at FP receptors but this receptor is also able to bind both PGD_2 and PGE_2 at nanomolar concentrations (Abramovitz *et al*, 2000). In addition, $PGF_{2\alpha}$ does not have a high degree of selectivity for FP receptors as it is also able to bind to the EP_1 and EP_3 receptors with high affinity (Breyer *et al*, 2001). The most frequently used antagonist for FP receptors is AL8810 (Griffin *et al*, 1999) but has only modest affinity for FP receptors (Sharif *et al*, 2006) and also blocks TP receptors (Hutchinson *et al*, 2003; Vysniauskiene *et al*, 2006). Furthermore, its inhibitory effect on FP receptors has been shown to be through partial agonism and no true competitive antagonism (Woodward *et al*, 2007).

1.2.2.4. Prostacyclin and the IP receptor

Prostaglandin I_2 (PGI_2) or prostacyclin is formed by PGI synthase and binds the prostanoid IP receptor. The human IP receptor cDNA encodes a sequence of 386 amino acids and couples to the stimulatory G_s protein which mediates an increase in cAMP production via stimulation of adenylyl cyclase (Boie *et al*, 1994). The activation of the cAMP-PKA pathway appears to be responsible for several prostacyclin-mediated events (Nasrallah *et al*, 2001; Ritchie *et al*, 2004; Kamio *et al*,

2007; Muja *et al*, 2007). In addition, IP receptors have also demonstrated coupling to other pathways, including G_i and G_q (Hebert *et al*, 1998; Katsuyama *et al*, 1994; Namba *et al*, 1994; Schwaner *et al*, 1995).

IP receptors have long been recognised for their key role in vascular homeostasis, mediating vasodilation and inhibiting thrombosis (Vane & Botting, 1995). PGI_2 production from endothelial cells may confer protection and counteract the vasoconstriction and platelet aggregation induced by thromboxane (TxA_2) during vascular dysfunction (Cheng *et* al, 2002; Tan *et al*, 2007). Additionally, IP receptors play a central role in oedema formation, hyperalgesia and pain (Bley *et al*, 1998; Hata & Breyer, 2004). Anti-inflammatory properties of IP receptors have also been observed including suppression of Th2-mediated responses (Hata & Breyer, 2004), inhibition of Th2 cytokines (IL-4, IL-10, IL-13) and Th1 cytokine (IFNγ) production (Zhou *et al*, 2007) and attenuation of allergic inflammatory responses (Jaffar *et al*, 2002; Takahashi *et al*, 2002).

The endogenous IP receptor ligand, PGI_2, is highly unstable and so has little experimental use. Therefore, more stable IP agonists were developed such as cicaprost, iloprost and beraprost to study this receptor. However, Iloprost has shown potent EP_1 agonism and some EP_3 agonism, while cicaprost is more selective (Dong *et al*, 1986; Lawrence *et al*, 1992; Abramovitz *et al*, 2000; McCormick *et al*, 2010). Chemical screening highlighted two IP antagonists, RO-1138452 and RO-3244794 (Bley *et al*, 2006).

1.2.2.5. Thromboxane A_2 and the TP receptor

Thromboxane A_2 (TxA_2) is generated via thromboxane synthase and signals through the activation of the TP receptor (Nusing *et al*, 1990; Tanabe & Ullrich, 1995). The human TP receptor was the first eicosanoid receptor cloned (Hirata *et al*, 1991) and encodes a 343 amino acid protein. Two isoforms have been described; TPα (placental/platelet) and TPβ (endothelial). These two isoforms are generated through alternative splicing in the carboxy-terminal region after the seventh transmembrane domain (Raychowdhury *et al*, 1994). TP receptors are known to be G_q-coupled, activating PLC and inositol trisphosphate (IP_3)/diacylglycerol (DAG) with

an increase in Ca^{2+}/PKC (Hirata *et al*, 1991; Dorn & Becker, 1993; Kinsella *et al*, 1997; Offermanns, 2006; Nakahata, 2008). However, the range of G protein coupling for TP receptors is extensive and may also couple to G_s (Hirata *et al*, 1996; Walsh *et al*, 1998; Mir & Le Breton, 2008) and G_i (Ushikubi *et al*, 1994; Song *et al*, 2009).

The TP receptor was first identified in human platelets (Ushikubi *et al*, 1989) and has been known to induce shape change and platelet aggregation (Offermanns, 2006; Nakahata, 2008). In addition, TP receptor-deficient mice have prolonged bleeding times and cannot form stable thrombi (Thomas *et al*, 1998). Furthermore, TP receptors are widely expressed throughout the cardiovascular system, including endothelial cells, vascular smooth muscle and cardiomyocytes, and are often associated with cardiovascular disease. Increased production of TxA_2 is thought to contribute to endothelial dysfunction and subsequent vasoconstriction (Gendron & Thorin, 2007; Francois *et al*, 2008; Denniss & Rush, 2009; Félétou *et al*, 2009; Graham & Rush, 2009) and TxA_2 mimetic U46619 can cause arrhythmia (Wacker *et al*, 2009). Outside of the cardiovascular system, TP receptors are prominently expressed in bronchial smooth muscle (Capra *et al*, 2003). TP activation has shown potent bronchoconstriction in isolated tissue from a number of different species (Coleman & Sheldrick, 1989; Armour *et al*, 1989; McKenniff *et al*, 1991; Underwood *et al*, 1994) as well as in clinical and *in vivo* studies (Saroea *et al*, 1995; Lewis & Broadley, 1995; Cyphert *et al*, 2012).

Although TxA_2 can potently activate TP receptors, it is very chemically unstable and so is of limited use in experimental protocols. Stable analogs were therefore developed and the most widely used is U46619. SQ29548 is a highly selective and potent TP antagonist and its use is favourable over older, less selective antagonists such as AH23848 (Abramovitz *et al*, 2000; Jones *et al*, 2009).

1.2.3. Prostanoids in Airway Disease

The role that prostanoids play in airway inflammation has been subject to extensive investigation, in particular their role in asthma and COPD. The role of prostanoids in mediating inflammatory responses in airway diseases, such as asthma and COPD,

is often varied, where pro- and anti-inflammatory actions of prostanoids can present in a single disease process (Narumiya, 2009). Notably, non-steroidal anti-inflammatory drugs (NSAIDs) inhibit many processes mediated by prostanoids, through an inhibition of the cyclooxygenase enzymes (Vane, 1971; Flower & Vane, 1974; Coleman *et al*, 1994; Narumiya *et al* 1999). For example, prostanoids are able to mediate fever (Ushikubi *et al*, 1998), oedema and pain (Murata *et al*, 1997; Ueno *et al*, 2000), hyperalgesia (Moriyama *et al*, 2005; Ueno *et al*, 2001) and arthritic inflammation (Honda *et al*, 2006). This has led to the assumption that they act as a pro-inflammatory mediators in several inflammatory diseases (McIlroy *et al*, 2006). However, it is well documented that exacerbations in asthma can be associated with aspirin. Additionally, this aspirin-sensitivity is associated with the inhibition of COX enzymes (Szczeklik & Stevenson 2003). Their effects in the airways is summarised below and includes their role in mediating airway tone and airway inflammatory processes.

1.2.3.1. Airway Tone

Bronchodilation following inhaled PGE_2 has been demonstrated in both normal subjects and in patients with asthma and chronic bronchitis (Gauvreau *et al*, 1999; Kawakami *et al*, 1973; Seth *et al*, 1981). This effect has also been observed in isolated airway smooth muscle in mice, where several reports have suggested that it is mediated via the activation of the EP_2 receptors (Fortner *et al*, 2001; Sheller *et al*, 2000). Similar findings have also been shown in constricted tracheal tissue from guinea pigs and monkeys (Buckley *et al*, 2011). However, Buckley *et al* (2011) recently demonstrated that it is actually the EP_4 receptor that is key in mediating this effect in humans. Experiments using selective EP ligands on human airway tissue found that an EP_4 agonist was effective in relaxing smooth muscle, while $EP_{1\text{-}3}$ were unable to induce this effect. These findings were also mimicked in isolated rat trachea (Buckley *et al*, 2011). IP receptor activation has also been shown to mediate bronchodilation; in canine (Tamaoki *et al*, 1993) and human (Norel *et al*, 1999) isolated smooth muscle IP receptor agonists induced substantial relaxation, but in mouse (Richter & Sirois, 2000) and rat (Lydford & McKechnie, 1994) IP activation only produced small relaxation responses.

PGD_2-induced bronchoconstriction in normal and asthmatic subjects has been demonstrated (Hardy *et al*, 1984) and expression of DP_1 receptors has been reported in airway smooth muscle (Coleman *et al*, 1994). Additionally, tracheal tissue under basal tone has shown DP-induced increases in airway tone (Beasley *et al*, 1987; McKenniff *et al*, 1991; Underwood *et al*, 1994). However, DP_1 activation in isolated human airway smooth muscle also causes relaxation (Norel *et al*, 1999). An explanation of these discrepancies may be due to PGD_2 agonism at TP receptors. TxA_2 is a potent constrictor of bronchial smooth muscle and is considered an important mediator in asthma and COPD. Increased levels of TxA_2 have been observed in the airways of asthma patients following allergen challenge (Wenzel *et al*, 1991) and its bronchoconstrictor effects in clinical and *in vivo* studies have also been reported (Saroea *et al*, 1995; Lewis & Broadley, 1995; Cyphert *et al*, 2012). In addition, increased levels of TxA_2 metabolites were seen in COPD patients compared to healthy subjects (Davi *et al*, 1997). Its potent effects on platelet aggregation may be important in the pathogenesis of COPD as thromboembolic events and pulmonary hypertension are common in COPD (Zheng *et al*, 1991).

Although FP receptor expression in the lung is low (Coleman *et al*, 1994), $PGF_{2\alpha}$ can induce bronchoconstriction *in vitro* (Coleman & Sheldrick, 1989) and in asthmatic patients (Fish *et al*, 1984).

Another important characteristic of chronic airway disease, such as asthma, is airway smooth muscle (ASM) proliferation which may contribute significantly to airway hyperresponsiveness and decline in lung function (Roscioni *et al*, 2011). Several studies have indicated a role for PGE_2, via activation of EP_2 and EP_4 receptors, in inhibiting this ASM proliferation through an elevation of cAMP (Clarke *et al*, 2005; Roscioni *et al*, 2011; Yan *et al*, 2011).

1.2.3.2. Immune and Inflammatory Responses

PGD_2 is a prominent mediator of airway inflammation and is the major prostanoid produced by mast cells where it is released in large amounts following antigen challenge. Matsuoka *et al* (2000) found DP_1 knock-out mice demonstrated a substantial reduction in asthmatic responses in the OVA model; the levels of Th2

cytokines and eosinophil infiltration were greatly reduced in the knock-outs. Activation of the DP_1 receptor has also been shown to inhibit airway inflammation in mice by modulating regulatory T cells and dendritic cells (Hammad *et al*, 2007). Additionally, the discovery of DP_2 receptor as a Th2 cell chemoattractant further implicated PGD_2 in mediating inflammatory responses (Hirai *et al*, 2001). Enhanced expression of DP_2 receptors on CD^+ T cells has been observed in atopic subjects (Kostenis & Ulven, 2006) and activation of DP_2 causes chemotaxis and degranulation of eosinophils (Gervais *et al*, 2001; Hirai *et al*, 2001; Monneret *et al*, 2001; Sugimoto *et al*, 2003; Böhm *et al*, 2004). In contrast, Chevalier *et al* (2005) examined the effects of OVA-induced allergic asthma in DP_2 knock-outs and saw an increase in eosinophil recruitment into the BAL fluid.

Long *et al* (2004) reported that increased PGE_2 levels in bronchoalveolar lavage (BAL) fluid and in the plasma of patients with asthma may correlate with the severity of airway obstruction. Additionally, in murine B cells, PGE_2 is able to prolong eosinophil survival and Sturm *et al* (2008) demonstrated that the chemotactic responsiveness of isolated human eosinophils is restrained by low concentrations of PGE_2 *in vitro.* In addition they showed that this attenuation in eosinophil locomotion is attenuated via EP_2 receptors (Sturm *et al*, 2008). Furthermore, *in vitro* data from human alveolar macrophages indicates that both EP_2 and EP_4 receptors mediate the anti-inflammatory actions of PGE_2 (Ratcliffe *et al*, 2007). *In vivo,* an agonist selective for the EP_3 receptor was effective in suppressing allergic inflammatory reactions in murine airways, while EP_3 receptor deficient mice demonstrated enhanced allergic reactions in the ovalbumin-challenge model, suggesting that suppression of allergic inflammation is mediated via PGE_2-EP_3 signalling (Kunikata *et al*, 2005). PGE_2 has also been implicated in COPD, where Montuschi *et al* (2003) observed 2.2 fold higher exhaled PGE_2 levels in COPD patients compared to healthy subjects. There is also recent evidence for a possible role for COX-2, along with PGE_2, on the infiltration of neutrophils (Profita *et al*, 2010). Furthermore, cigarette smoke, a major risk factor, may play a significant role by increasing the expression of COX enzymes as well as specific EP receptors in alveolar macrophages and neutrophils (Profita *et al*, 2010).

IP receptor mRNA has been shown to be expressed by Th2 cells but not Th1 cells and Jaffar *et al* (2002) showed elevated production of anti-inflammatory cytokine, IL-

10, following PGI_2-Th2 activation. *In vivo* studies using allergen-induced asthma also demonstrated an inhibitory role for IP receptors (Takahashi *et al*, 2002). Yuhki *et al* (2004), however, demonstrated that IP receptors mediated exudate formation in a carrageenan-induced mouse pleurisy model, which suggests that the PGI_2 can elicit inflammatory responses in a context dependent manner. Additionally, PGI_2 and IP receptor activation causes prominent vasodilation and increased blood flow which is a classic sign of inflammation.

1.2.3.3. Sensory Nerve Activation

The beneficial effects of inhaled PGE_2 have been hindered by its irritancy of the upper airway and associated cough. This cough reflex is initiated by the activation of sensory afferent nerve endings in the airways, which PGE_2 has been shown to mediate (Coleridge *et al*, 1976; Mohammed *et al*, 1993). Furthermore, it appears that this effect is mediated via the EP_3 receptor. Maher *et al* (2009) used prostanoid receptor-deficient mice and pharmacology to provide substantial evidence that this is the receptor that mediates PGE_2 sensory nerve activation. Additionally, depolarization of guinea pig vagus has been observed with $PGF_{2\alpha}$, PGD_2, Iloprost and U46619 (Birrell *et al*, 2009; Maher *et al*, 2009).

1.3. Microvascular Leak

1.3.1. Introduction

Microvascular leakage (MVL) and plasma exudation represent classical features in the pathogenesis of various inflammatory diseases and is thought of as a cardinal sign of inflammation leading to tissue swelling ("tumor") (Paredi & Barnes, 2009).

In a healthy endothelium the semipermeable barrier carefully regulates the movement of water, solutes and proteins between the blood and tissue. In this state, water and low molecular weight solutes are able to pass via the paracellular route. Larger macromolecules such as albumin are only able to move across via the transcellular route. However, during an inflammatory response such as in sepsis and acute lung injury, this barrier is disrupted allowing fluid and large macromolecules to move across through interendothelial gaps (Figure 1.4). Briefly, during an inflammatory challenge there is the release of several mediators, such as histamine, 5-hydroxytryptamine (5-HT), leukotrienes and cytokines, which act directly upon post capillary venules to cause the opening of these intercellular gaps and thus increasing vascular permeability. This increase in permeability causes plasma to 'leak' into extravascular sites due to hydrostatic pressure gradients (Olivenstein *et al*, 1997; Reynolds *et al*, 2002). These hydrostatic pressure gradients form between the microvasculature and the lamina propria, resulting in a rapid and widespread distribution of plasma in the lamina propria and epithelium (Greiff *et al*, 2003).

This phenomena is a very distinctive feature of acute inflammation but is also observed in more chronic diseases such as asthma (Laitinen *et al*, 1987; Li & Wilson, 1997, Innes *et al*, 2009; Khor, 2009) and COPD (Hill *et al*, 1999; Minikata *et al*, 2005; Bessa *et al*, 2012). Microvascular leak can often present as an adverse side effect of disease, exacerbating a patient's condition, or it can be the main underlying cause of a disease. For example, in pathological states where there is a significant increase in vascular permeability, excessive leak can aggravate acute life-threatening disorders, such as airway obstruction in asthma, acute lung injury and related pulmonary diseases. Vascular leakage is also a prominent feature in sepsis where it causes circulatory collapse (Amerongen & Hinsburgh, 2002).

In the early sixties, Majno and Palade were able to study the effects of histamine and 5-HT on vascular leakage using an electron microscope. They showed that this plasma exudation was occurring only in postcapillary venules and not in capillaries of the systemic circulation (Majno & Palade, 1961). The use of particulate tracers, such as Monastral blue and colloidal gold, has allowed the visualisation of where plasma leak is occurring. Erjefalt *et al* (1998) administered Monastral blue to OVA-challenged mice and identified the extravasating vessels of the trachea as postcapillary venules. These venules had a mean diameter of 30 ± 2.3μm (Erjefalt *et al*, 1998). McDonald (1994) also observed a similar response in rat airways where Monastral blue particles were trapped in interendothelial gaps of postcapillary venules.

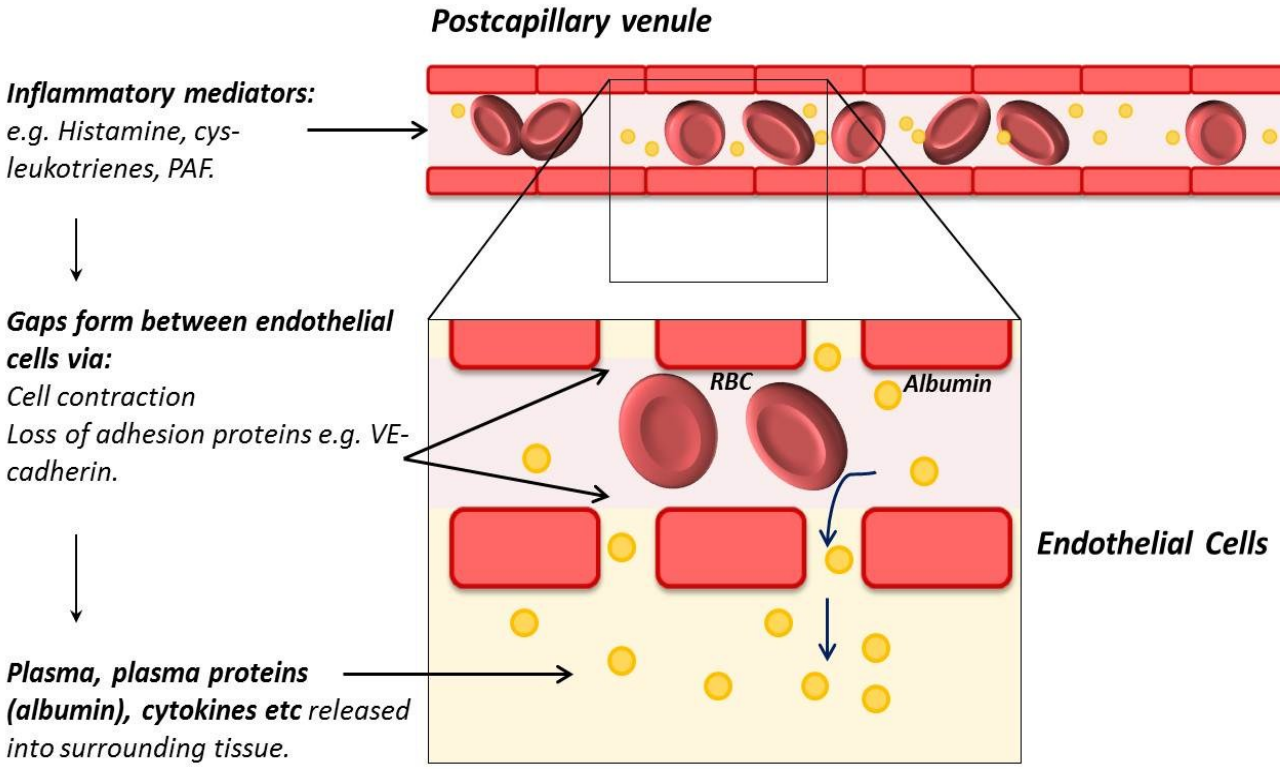


Figure 1.4. Schematic representation of microvascular leakage in the airways. *During an inflammatory insult various mediators at upon endothelial cells in postcapillary venules causing them to contract producing intercellular gaps. This gap formation allows the movement of plasma (and plasma proteins) out into extravascular sites, such as the airway lumen. This can contribute to the formation and exacerbation of oedema.*

1.3.2. Regulation of Endothelial Permeability

Current hypotheses regarding the mechanisms that cause an increase in microvascular permeability suggest a disruption in the endothelial barrier and the opening of interendothelial gaps allowing fluid and macromolecules to leak out. Specific signalling pathways may lead to an increase in the contractility of the acto-myosin cytoskeleton, or a decrease in junction integrity by weakening intercellular junctions, primarily tight and adherens junctions (Schweitzer *et al*, 2011).

1.3.2.1. Myosin Light Chain Kinase-Dependent Hyperpermeability

It was Majno in the 1960s that first suggested that the formation of these gaps was due to endothelial cells actively contracting in response to inflammatory stimuli (Majno & Palade, 1961; Majno *et al*, 1961). From microscopy studies he noticed the nuclei of these cells formed deep membrane indentations suggesting cell shortening and contraction. The thought is that the contraction of adjacent endothelial cells will modulate cell shape causing a 'rounding' of the cell and the formation of these intercellular gaps (Mehta & Malik, 2006). Most *in vitro* studies working on isolated and cultured endothelial cells have indicated that a balance of competing contractile and adhesive forces lead to the modulation of cell shape and gap formation (Garcia *et al*, 1995). Additionally, there is evidence revealing an association of myosin with actin, thus enhancing the cell contraction-cell separation theory (Drenckhahn & Wagner, 1986; Schnittler *et al*, 1990). More specifically, it has been shown that increasing the activity of myosin light chain kinase (MLCK) induces barrier dysfunction while a normal basal level of MLCK activity is required to maintain physiological microvascular permeability. MLCK is a serine/threonine-specific protein kinase whose primary biological function is to phosphorylate the regulatory myosin light chain II (MLC-II) (Takashima, 2009; Shen *et al*, 2010). Phosphorylation occurs at Ser-19 and then Thr-18 which subsequently causes an ATP-dependent contraction of the actomyosin complex (Verin *et al*, 1998; Lazar & Garcia, 1999; Shen *et al*, 2010). This MLCK-dependent contractility is associated with arteriolar vasoconstriction and capillary and venule permeability. In order to control basal cytoskeletal tension a balance is needed between phosphorylation by MLCK and dephosphorylation by MLC phosphatase (MLCP). Dephosphorylation by MLCP

decreases actomyosin contraction causing the actin cytoskeleton to relax and permeability to decrease. (Schnittler *et al*, 1990; Verin *et al*, 1995; Chrzanowska-Wodnicka & Burridge, 1996; Cai *et al*, 1998). Furthermore, inhibition of MLCP can increase the phosphorylation of MLC and subsequent cytoskeletal contraction without an increase in MLCK activity.

1.3.2.2. Endothelial Cell-Cell Junctions and Hyperpermeability

As well as an increase in endothelial cell contraction, cell-cell interactions play an enormous role in modulating barrier function and the development of interendothelial gaps. Indeed, Moy *et al* (1996) demonstrated that in human umbilical vein endothelial cells histamine caused a decrease in monolayer resistance without endothelial cell contraction. Simultaneously, they also showed that thrombin produced a similar effect and at the same time caused endothelial contraction (Moy *et al*, 1996). This data suggests that neither process acting alone is sufficient to impair endothelial barrier integrity. Subsequently, others have also confirmed that gap formation occurred with a loss of cell-cell adhesion but not always with cell contraction (Corada *et al*, 1999; Winter *et al*, 1999; Moy *et al*, 2000). What does appear to be the case is that endothelial contraction can increase the magnitude of the gap size and the duration that the gap remains open but only in the presence of junctional reorganisation (Ochoa & Stevens, 2012).

Endothelial cell-cell junctions are made up of membrane proteins that are expressed on the lateral surface of endothelial cells and consist of both extracellular and intracellular domains. It is the interaction of the extracellular domains which forms the barrier between adjacent endothelial cells and the passage of large macromolecules across the endothelium. The intracellular domain of these junctional proteins is linked to adaptor proteins that form connections with the actin cytoskeleton (Rigor *et al*, 2013). The two main types of junctions involved in mediating this response include adherens and tight junctions. Adherens junctions play the more prominent role as they are present in the vascular endothelium of most organs and tissues (Mehta & Malik, 2006; Vandenbroucke *et al*, 2008; Komarova *et al*, 2010). Tight junctions, however, are primarily found in the microvasculature of specialised tissues, for example, the blood-brain barrier or blood-retinal barrier

(Hawkins & Davis, 2005; Engelhardt & Sorokin, 2009; Cosine & Engelhardt, 2011). The principal structural element of adherens junctions between endothelial cells is vascular endothelial cadherin (VE-cadherin), which is able to connect neighbouring cells in a calcium dependent manner (Goldenberg *et al*, 2011). For VE-cadherin, intracellular connections to the actin cytoskeleton occur via catenins (α, β, γ and p120), while tight junctions have specialised proteins: occludin and claudins (see Figure 1.5) (Dudek & Garcia, 2001; Vandenbroucke *et al*, 2008; Prasain & Stevens, 2009; Shen *et al*, 2009). It is the disassembly of these junctional proteins during an inflammatory response that weakens the endothelial barrier and causes vascular leakage. The cadherin-catenin complex is phosphorylated and then internalised via endocytosis. For example, inflammatory mediators such as thrombin and VEGF are able to induce MVL though the phosphorylation and internalisation of VE-cadherin (Rabiet *et al*, 1996; Cohen *et al*, 1999; Ukropec *et al*, 2000; Gavard & Gutkind, 2006; Gavard *et al*, 2009).

However, modulation of these responses involves complex signalling pathways which are not fully understood and various signalling cascades have been proposed. Furthermore, the majority of research into these pathways is carried out on cultured endothelial cells which do not develop the permeability characteristics of intact endothelium and so have therefore not been validated *in vivo* (Jiang *et al*, 2008).

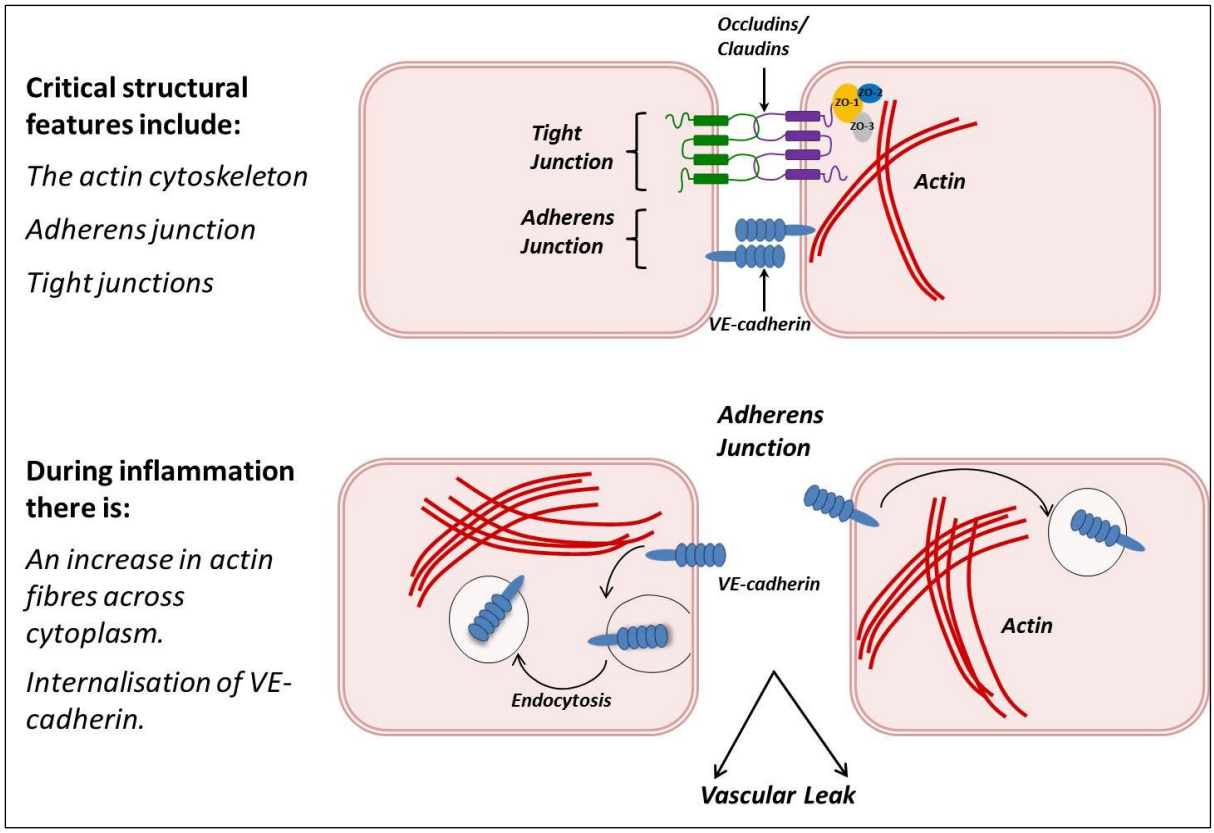


Figure 1.5. Schematic representation of the mechanisms involved in endothelial barrier disruption. *The critical features include myosin-actin interactions leading to cell contraction and a loss of junctional integrity.*

1.3.3. Intracellular Signalling Pathways Modulating Endothelial Permeability

Most of the literature describing the signalling pathways involved in the disruption or enhancement of the endothelial barrier focus on research conducted in cultured endothelial cells and intact microvessels, including a variety of species and subtypes. As described the main endothelial subtype that is important in regulating permeability are the microvascular endothelial cells from postcapillary venules. While a lot of information has been obtained from such research, it cannot be a true representation of what occurs *in vivo*.

Endothelial barrier dysfunction occurs due to a misregulation of intracellular signalling that is associated with maintaining barrier integrity, or via the activation of signalling pathways that lead to a hyperpermeable endothelium (Yuan, 2000; Yuan, 2002; Shen *et al*, 2010). In either case, the subsequent increase in myosin light

chain kinase (MLCK) activity is the primary cause of barrier dysfunction. In a 'healthy' endothelium a basal level of MLCK activity is needed to maintain and regulate the permeability of the microvasculature. However, when MLCK activity is elevated in disease an increase in microvascular leak is observed (Shen *et al*, 2010). Additionally, cell to cell interactions via adherens and tight junctions play a vital role in regulation barrier integrity. How these factors induce hyperpermeability was described in section 1.3.2. Outlined below is the role that key cell signalling pathways play in mediating microvascular permeability, and how they influence MLCK and cell-cell interactions.

1.3.3.1. Calcium

Increasing intracellular calcium (Ca^{2+}) is pivotal to the control of MLCK activity and subsequent increases in endothelial permeability. Ca^{2+}/calmodulin binding to MLCK is known to release the MLCK autoinhibition and thus increase its activity (Ito *et* al, 1991; Blue *et al*, 2002; Hong *et al*, 2011). This pathway is thought to be responsible for the rapid and transient increase in permeability to vasoactive agents such as histamine and substance P. Histamine has been shown to increase cytoplasmic Ca^{2+} concentration in endothelial cells (Shasby *et al*, 1997) as well as increasing endothelial permeability. This increase in endothelial permeability has been inhibited by a Ca^{2+} chelator as well as a calmodulin inhibitor (van Niew Amerongen *et al*, 1998).

In smooth muscle the binding of Ca^{2+} to calmodulin activates smooth muscle (sm)MLCK and induces actin-myosin contraction. However, in the endothelium this alone does not appear to be sufficient to cause cytoskeletal contraction, suggesting other signalling pathways are involved (Garcia *et al*, 1997).

1.3.3.2. RhoA/Rho Kinase

Rho GTPases are a family of small signalling G proteins that are involved in the regulation of endothelial cell contractility. This regulation is dominated by three of the most prominent members; RhoA, Rac and cdc42 (Steinberg *et al*, 2012; van Nieuw

Amerongen & van Hinsbergh, 2002). Stimulation by inflammatory mediators, such as thrombin, destabilises the endothelial junction and increases vascular permeability by signalling through RhoA. Thrombin and VEGF have both been shown to initiate endothelial barrier disruption through RhoA/Rho Kinase signalling (Lorenzo *et al*, 2013; Sun *et al*, 2006; Qiao *et al*, 2003; van Nieuw Amerongen *et al*, 2000). RhoA controls endothelial cell contractility by stabilising the phosphorylated state of MLC, which is thought to occur via the activation of Rho kinase. Rho kinase inactivates myosin light chain phosphatase thus increasing MLC phosphorylation and the subsequent cytoskeletal contraction (Amano et al, 1996; Kimura *et al*, 1996; Kawano *et al*, 1999, Katoh *et al*, 2001). Additionally, Rho kinase activity leads to a reduction in VE-cadherin stability and so disassembly of junctional proteins (Essler *et al*, 1998).

1.3.3.3. Cyclic AMP

In contrast to Ca^{2+} and RhoA signalling, increasing concentrations of cyclic AMP (cAMP) have been associated with a reduction in endothelial permeability. In addition, increasing endothelial cAMP does not only reduce the increase in permeability caused by inflammatory mediators but also reduces baseline levels (reviewed by Michel & Curry, 1999). It was earlier studies that demonstrated β-adrenergic stimuli inhibited histamine-induced leakage in animals and this effect appeared to be related to enhanced cAMP concentrations in the endothelial cells (Rippe & Grega, 1978). Later studies have also established the effectiveness of elevated endothelial cAMP in reducing vascular permeability *in vitro* (Stelzner *et al*, 1989; Langeler & van Hinsbergh, 1991; Moy *et al*, 1993) as well as reducing histamine-induced leak *in vivo* (Kobayashi *et al*, 2013). This elevation of cAMP stabilises endothelial junctions, inhibits MLCK and actomyosin contraction and the formation of stress fibres (He & Curry, 1993; Moy *et al*, 1993; Moy *et al*, 1998; Siflinger-Birnboim *et al*, 1993; Adamson *et al*, 1998). Furthermore, the effects of cAMP have been linked to protein kinase A (PKA) activation where studies have demonstrated the effectiveness of cAMP and PKA in reducing endothelial permeability (Garcia *et al*, 1995; Garcia *et al*, 1997; Qiao *et al*, 2003). Liu *et al* (2001) pharmacologically reduced PKA activity in endothelial cells which induced

actin stress fibre formation and subsequent cell contraction. It has also been suggested that activation of the cAMP/PKA pathway inhibited endothelial contraction by inhibiting the activation of the RhoA/Rho kinase pathway (Qiao *et al*, 2003; Birukova *et al*, 2004; Baumer *et al*, 2008; Baumer *et al*, 2009; Birukova *et al*, 2010). Qiao *et al* (2003) found that cAMP-elevating agents inhibited thrombin-induced RhoA activation in human microvascular endothelial cells and protected against endothelial barrier dysfunction. Birukova *et al* (2004) also demonstrated PKA-mediated inhibition of RhoA and Rho kinase resulted in decreased MLC phosphorylation, actomyosin contraction and endothelial permeability.

1.3.4. Airway Microvascular Leak

Within healthy airways, the bronchial microvasculature provides a multitude of important functions that are essential for maintaining pulmonary homeostasis. For example, it is able to provide oxygen and appropriate nutrients, aids in the regulation of temperature and in humidifying air, as well as acting as the initial site for immune responses (Zanini *et al*, 2010). During chronic inflammation, in airway diseases such as asthma and COPD, vascular remodelling can be extensive. One of the main features of bronchial vascular remodelling is angiogenesis, the formation of new vessels within the airway wall. What is often observed is that newly generated vessels tend to be hyperpermeable and increase oedema (Bousquet *et al*, 2000). Furthermore, an increase in bronchial subepithelial blood flow has also been observed as well as increased vascular permeability and plasma exudation. These factors all contribute to the narrowing of the airway lumen seen in inflamed airways (Zanini *et al*, 2010).

1.3.4.1. Asthma

Since the sixties post-mortem observations have highlighted oedematous bronchial mucosa with dilated and congested blood vessels within the airways of those with fatal asthma (Dunnill *et al*, 1969). Additionally, bronchial biopsies from mild asthmatics appear more vascular and endothelial gaps are prominent in the bronchial mucosal microvasculature than control subjects (Laitinen *et al*, 1987; Li & Wilson, 1997). More recent biopsy studies have also demonstrated an increase in the number of microvessels within the airways of chronic asthma patients with additional endothelial proliferation (Vrugt *et al*, 2000; Wilson & Robertson, 2002; Barbato *et al*, 2006). Despite the importance of microvascular leak in the pathogenesis of asthma, it is not often measured when monitoring airway inflammation in asthmatic patients (Van Rensen *et al*, 2002). Several studies have investigated microvascular permeability in asthma by measuring albumin levels in induced sputum and/or BAL fluid, as well as other proteins such as fibronectin and alpha 2-macroglobulin (Meerschaert *et al*, 1999; Svensson *et al*, 1995; Van Rensen *et al*, 2002). Albumin is one of the most abundant plasma proteins, at least 60%, and is found in higher concentrations in airways secretions from acute asthmatic patients

(List *et al*, 1978; Travis, 2003). Innes *et al* (2009) postulated that the leakage of these proteins into the airway lumen can reduce mucus clearance in acute severe asthma and may be an underlying cause of fatal asphyxiation. Following *ex vivo* analyses of airway mucus from patients in acute asthma exacerbations (and control subjects), they found that albumin within the mucus inhibits normal mucus degradation. Additionally, Khor (2009) demonstrated that reduction in inhaled corticosteroid dosage was associated with an increase in albumin exudation and they hypothesise that this is an early feature of asthma deterioration. Unlike other human studies, they also assessed biopsies from these patients by staining sections for albumin and found this was also elevated in asthmatic subjects (Khor 2009). Animal models of allergic asthma have also illustrated a measurable increase in airway microvascular permeability. Using the ovalbumin model of allergic asthma several groups have demonstrated significant increases in airway microvascular leak following antigen challenge in guinea pigs (Erjefält *et al*, 1995; Liaw & Chao, 2001), rats (Olivenstein *et al*, 1997; Hele *et al*, 2001; Tillie-Leblond *et al*, 2007) and mice (Erjefält *et al*, 1998; Lee *et al*, 2004; Kim *et al*, 2012).

Airway MVL is thought to have a critical role in thickening the bronchial mucosa and the resulting airway hyperresponsiveness (Van de Graaf *et al*, 1991; Paredi & Barnes, 2009). In addition, leakage of plasma proteins is often associated with exacerbations of asthma due to mucosal oedema and so worsening airflow obstruction and reducing airway calibre (Khor *et al*, 2009). Particularly, extravasation into the airway lumen can cause this increase in airway resistance. During bronchospasm the constricted airways compress the mucosa forming epithelial folds and so exuded plasma can then fill the interstices between these folds, thus amplifying bronchoconstriction (Olivenstein *et al*, 1997). Furthermore, during chronic inflammation blood vessels in the airways are in a hypervascularised and destabilised state and so increasing microvascular permeability (Kanazawa *et al*, 2004).

1.3.4.2. COPD

Vascular remodelling and changes in endothelial permeability are also prominent in COPD, where vascular intimal thickening in small pulmonary arteries has been attributed to chronic inflammatory processes (Peinado *et al*, 1998; de Boer *et al*, 2007). COPD is characterised by this chronic inflammation that occurs along the entire bronchial tree, with airways becoming oedematous, having excess mucus production and impaired epithelial ciliary function (Bailey *et al*, 2009). Cigarette smoking is thought to lead to endothelial dysfunction as a result of exogenous oxidative stress and/or the endogenous production of oxygen-free radicals (Mendes *et al*, 2006). This dysfunction precipitates attenuated vascular relaxation responses in the systemic circulation. The importance of such vascular changes in the development of bronchitis is subject to speculation but these changes would certainly exacerbate inflammatory cell recruitment, leukocyte migration and the formation of endothelial gaps and vascular leak (Baluk *et al*, 1998). Nevertheless, endothelial function has been shown to be impaired in smokers and ex-smokers with COPD (Mendes *et al*, 2006) and increased levels of albumin have been measured in the sputum of COPD patients (Hill *et al*, 1999; Minikata *et al*, 2005; Bessa *et al*, 2012). Minikata *et al* (2005) collected airway epithelial lining fluid from patients with differing severities of COPD and noticed a strong correlation with airway albumin levels and the forced expiratory volume in 1 second values. This suggests that an increase in airway microvascular leak may contribute to the subsequent obstructive process of COPD. Studies have also reported that the airways of patients with COPD were thicker compared to healthy control subjects (Bosken *et al*, 1990; Kuwano *et al*, 1993) and several groups have shown alterations in the vascularity of COPD airways (Hashimoto *et al*, 2005 ; Calabrese *et al*, 2006; Zanini *et al*, 2009). Calabrese *et al* (2006) looked at bronchial biopsies from the central airways of smokers and observed an increase in vascular area as well as an increase in the number of vessels in smokers with COPD and symptomatic smokers with normal lung function. In addition, Zanini *et al* (2009) saw a higher vascular area but no change in vessel number in clinically stable COPD patients compared to controls.

Similar characteristics have been observed in animal models of COPD. Cigarette smoke (CS) is the most common etiological factor of COPD and so CS exposure *in*

vivo provides an excellent model for examining disease processes. Several groups have demonstrated increases in protein extravasation in the airways of various species in response to CS exposure (Lundberg & Saria, 1983; Geppetti *et al*, 1993; Andrè *et al*, 2008; Nassini *et al*, 2012). Schweitzer *et al* (2011) examined the effects of CS on vascular permeability both *in vitro* and *in vivo*. Using primary rat and human lung microvascular endothelial cells they demonstrated a dose-dependent decrease in barrier function following CS exposure. Furthermore, they noted significant increases in ceramide production following CS exposure (*in vitro* and *in vivo*) which itself caused a dose-dependent increase in FITC-albumin extravasation from the alveolar microcirculation of conscious rats (Schweitzer *et al*, 2011).

1.3.5. Methods for Investigating Microvascular Leak

Current methods used to investigate microvascular leak use varying degrees of complexity, ranging from cultured endothelial cells to conscious animal preparations. All models come with distinct advantages over others as well as a number of limitations. The most frequently used methods to study vascular permeability will be outlined below, with discussion of the advantages and disadvantages of each.

1.3.5.1. In vitro

1.3.5.1.1. Cell Culture

One of the most widely employed *in vitro* models of microvascular leak uses cultured microvascular endothelial cells. Either human or animal microvascular endothelial cell monolayers are grown on semi-permeable tissue culture well inserts, which allows the movement of large substrates across the endothelial barrier to be measured. The most commonly used substrates are albumin or dextran conjugated to a fluorophore such as fluorescein isothiocyanate (FITC) and come in a variety of polymer sizes. The principle behind this method is measuring the movement of FITC-albumin/dextran across the endothelial monolayer from the upper chamber of the well insert into the bottom chamber under various conditions. This allows simple and accurate quantification of barrier integrity (Imai-Sasaki *et al*, 1995; Gavard & Gutkind, 2006; Tharakan *et al*, 2009; Li *et al*, 2010; Diebel & Liberati, 2011;

Tharakan *et al*, 2012; Yu *et al*, 2013). The use of cell culture also enables easier dissection of the complex signalling pathways activated during hyperpermeability. Additionally, *in vitro* methods using microvascular endothelial cells allows translation between animal and human which is not possible with most *in vivo* methods examining microvascular permeability.

An additional *in vitro* assay that is often employed uses an electrical current to assess the integrity of the endothelial monolayer (Qiao *et al*, 2003; Michail & Abernathy, 2004; Liu *et al*, 2008; Bogatcheva *et al*, 2009; Konya *et al*, 2011; Schweitzer *et al*, 2011; Booth & Kim, 2012; Griep *et al*, 2012). Transendothelial electrical resistance (TEER) assays involve an electrical current being passed across the cell monolayer and uses impedance as a measure of barrier integrity. The voltage across the monolayer then allows the TEER to be calculated. A higher TEER value would indicate a stable barrier while lower TEER values would suggest disruption of the barrier and an increase in permeability. TEER is often measured in combination with FITC-albumin flux, thus providing a more accurate representation of barrier function (Armstrong *et al*, 2012; Birukova *et al*, 2012). However, more recently electric cell-substrate impedance sensing (ECIS) has replaced TEER measurements (Lo *et al*, 1995; Birukova *et al*, 2004; Solodushko *et al*, 2008; Lorenzo *et al*, 2013). The difference with this assay is that the endothelial monolayer is grown in specialised plates which have electrodes embedded into it. Briefly, as cells grow and form a monolayer, the current that is applied is impeded. When cells are then stimulated with a permeability increasing mediator the changes in cell morphology and function alter the impedance. A significant advantage of this technique over TEER is the ability to continuously measure barrier integrity and permeability in real time (Fordjour & Harrington, 2009; Wang *et al*, 2010).

One of the major limitations of cultured endothelial cells is that they do not accurately reflect the permeability characteristics of intact microvascular endothelium *in vivo*. For example, they lack the shear stress that the endothelium is exposed to *in vivo*, including changes in blood flow (Dai *et al*, 2004; Davies, 2009). Additionally, the interaction and effects of other cell types, such as smooth muscle cells is also eliminated in these preparations. Structural differences have also been observed between cultured endothelial monolayers and *in situ* microvessels; inter-endothelial gaps in cultured monolayers are upwards of 5-10 μm while in isolated microvessels

these gaps are usually less than 1 μm (Baluk *et al*, 1997; Curry & Adamson, 2010; McDonald *et al*, 1999). Furthermore, using primary cells over immortalised cell lines does not necessarily eliminate certain problems; specifically, the de-differentiation that occurs when cultured without their specific microenvironment (Cleaver & Melton, 2003).

1.3.5.1.2. Whole Vessel Preparations

The use of intact microvessels in specialised perfusion systems aims to eliminate some of the limitations outlined for cultured monolayers and acts as an intermediate between cell culture and whole animal studies. They allow investigation of whole vessels in a more physiological environment where sheer stress and alterations in flow dynamics can be applied (Gunther *et al*, 2010). Vessels are either dissected from animals or humans and are mounted in a microfluidic perfusion rig where intraluminal perfusion pressure and flow can be controlled (Yuan *et al*, 1993). Vascular permeability can be measured by transvascular flux of a labelled tracer, such as FITC-albumin, or by confocal microscopy using fluorescent microspheres to mark endothelial gaps (Yuan *et al*, 1993; Breslin *et al*, 2006; Sun *et al*, 2006; Jiang *et al*, 2008). These methods represent a more physiologically accurate model of endothelial dynamics and more closely mimic an *in vivo* environment. However, there are still a number of limitations with this method. Firstly, they involve the dissection and direct interference with the vessel and secondly, they are often restricted to convenient vessel types. The microcirculation may differ between different vascular beds. Moss *et al* (2010) described a similar preparation, developed by Biopta, whereby vascular function and vascular permeability could be measured simultaneously. Vascular permeability was measured by the extravasation of Evans Blue dye into the wall of isolated vessels. However, this was achieved through image analysis rather than direct quantification. Additionally, they looked at small subcutaneous arteries and not post capillary venules.

1.3.5.2. In vivo

1.3.5.2.1. Intact Microvessels

Similar to whole mounted vessel preparations described above, the permeability of individual vessels can be studied *in situ* in unconscious animals (Michel *et al*, 1974; He *et al*, 2006; Jiang *et al*, 2008; Ereso *et al*, 2009). Briefly, animals are anaesthetised and the vascular bed of interest is removed and placed on the pillar of an inverted microscope. Here, an individual venule is cannulated and perfused with a physiological solution. Measurements of vascular permeability are based upon hydraulic conductivity, assessing the volume flux of water across the microvessel wall (Clough *et al*, 1988). These preparations allow quantitative measurements of vascular permeability under well-controlled experimental conditions. One of the main drawbacks of this technique is the vascular bed examined needs to be easily accessible. The mesentery is most commonly used as it can be excised with ease while the animal remains unconscious. This would not be possible for deeper tissues, such as post capillary venules within the lower airways.

1.3.5.2.2. Miles Assay

One of the most widely used techniques to detect microvascular permeability *in vivo* is the Miles assay, which was first developed to assess inflammatory plasma leak in guinea pig skin using Evans blue (EB) dye (Miles & Miles, 1952). The principle behind this method is that intravenous EB dye binds non-covalently to circulating albumin, the most abundant plasma protein, and then leaks out of the microcirculation in response to various mediators. The target tissue is dissected out at a given time point and the dye extracted and quantified by spectrophotometry. Rapid perfusion of the circulation to remove intravascular dye prior to tissue dissection improves the sensitivity of this assay (Renkin & Tucker, 1998; Nedrebo *et al*, 2003; Curry *et al*, 2010). Additionally, this technique is easily applicable to most species and the extravasation can be simultaneously determined in several tissue types. Subsequently, it has been used for decades to quantitatively assess vascular permeability in various different tissues across a variety of species (Miles & Miles, 1952; Evans *et al*, 1987; Rogers *et al*, 1988; Baluk *et al*, 1999; Reynolds *et al*, 2002; Xle *et al*, 2003; Zhuang *et al*, 2011). Studies using this assay have yielded important

information regarding the nature of inflammatory mediators and their impact on the microvasculature. Furthermore, the use of EB dye as a marker of plasma leak has extended into disease models of inflammation (Rogers *et al*, 1988; Geppetti *et al*, 1993; Olivenstein *et al*, 1997; Mirza *et al*, 1998; Hele *et al*, 2000; Hele *et al*, 2001; Irie *et al*, 2001; Lee *et al*, 2004; Kim *et al*, 2012).

The major disadvantage of this method is that it is a terminal procedure. This means that each animal is limited to a single intervention or time point so large cohorts are required for adequate comparisons between groups. In addition, the data only provides a reference to the net accumulation and doesn't pin point any fluctuations that may be occurring in vascular permeability. The use of radiolabelled albumin and a double tracer method has been used in an attempt to eliminate some of these problems. However, the use of radiolabelled tracers as an alternative to Evans Blue dye does not provide a more accurate measure and comes with the obvious safety hazards associated with radiation (Udaka *et al*, 1970; Rogers *et al,* 1989).

1.3.5.2.3. Intravital Fluorescence Microscopy

More recently, the use of intravital microscopy (IVM) has become popular for examining microvascular permeability as well as leukocyte endothelial interactions. This technique uses epifluorescence, confocal or two-photon microscopy to visualise the microvasculature in living animals (Chappell *et al*, 2006). Similar to the intact microvessels, the tissue being studied is exteriorised for assessment but here a fluorescent plasma marker, such as FITC-albumin, is administered intravenously to visualise plasma leak in real time (Svensjo *et al*, 1979). One of the main advantages of this technique over the Miles assay is that is allows both temporal and spatial characteristics of plasma exudation to be determined. This means that changes in leakage over a given time can be measured. Additionally, it has contributed immensely to our knowledge of the localisation of endothelial gaps to the postcapillary venules. However, since tissue needs to be exteriorised this preparation is limited to thin tissues or easily accessible organs. For example, IVM is commonly used for the skin, mesentery, cremaster muscle and superficial veins of the brain (Boric *et al*, 1987; Wu & Baldwin, 1992; Raud & Lindbom, 1994; Kubes & Gaboury, 1996; Jin *et al*, 2010). Attempting to view microvasculature of deeper

tissues would result in blurry images and fluorescent quenching. Studying the bronchial circulation and airway microvascular leak would be extremely difficult using IVM. Furthermore, this technique is time-consuming and requires delicate surgery making it very low throughput with only one animal being examined at a time. Maintenance of physiological conditions of the excised tissue is pivotal to ensure limited inflammation occurs following surgery (Raud & Lindbom, 1994).

1.3.5.2.4. Magnetic Resonance Imaging

Magnetic Resonance Imaging (MRI) has been used to visualise permeability changes in the brain (Jiang *et al*, 2005; Knotkova & Pappagallo, 2007; Nagaraja *et al*, 2008) and the lung (Tournebize *et al*, 2006; Tigani *et al*, 2007; Serkova *et al*, 2008; Ble *et al*, 2009) in clinical settings as well as in experimental animals. High molecular weight contrast agents are often used to carry out longitudinal studies of plasma leakage in selected tissues and organs (Fu *et al*, 2004; Huxley *et al*, 2007). A commonly used marker is gadolinium diethylenetriamine pentaacetic acid (Gd-DTPA) which has a molecular weight of 550 Da (Pautler, 2004; Nagaraja *et al*, 2008). An advantage of using MRI is that it enables visualisation of the whole organ without any invasive techniques. This allows measurements to be carried out continually over days, weeks or months. However, the images obtained are usually low resolution, making quantification of plasma leakage difficult. In addition, MRI scanners are expensive and complex.

1.3.6. Prostanoids in airway microvascular leak

As previously discussed, prostanoids are involved in various inflammatory processes, including those seen in asthma and COPD. With high concentrations of certain prostaglandins found at inflammatory sites, it is possible that they may in part mediate the microvascular leakage observed. PGE_2-induced airway MVL has not been widely examined, especially *in vivo*, but has been investigated for its effects in cutaneous plasma leak. These studies have demonstrated that PGE_2 induces significant MVL and oedema in mouse (Goulet *et al*, 2004) and rat (Flower & Kingston, 1975; Takagi *et al*, 1987) skin. Additionally, the EP_2 and EP_4 receptors

have been implicated in acute skin inflammation in mice following ultraviolet B exposure (Kabashima *et al*, 2007). *In vitro* studies, however, have illustrated opposing effects where PGE_2 appears to enhance microvascular endothelial barrier function (Birukova *et al*, 2007; Konya *et al*, 2013b). PGI_2 and the IP receptor have also been implicated in plasma leak and oedema formation (Murata *et al*, 1997; Yuhki *et al*, 2004) but, similar to PGE_2, have also demonstrated inhibitory effects on induced MVL (Moller & Grande, 1997; Cureton *et al*, 2010). The effects of PGD_2 in airway disease are well documented but its role in MVL has not been highly investigated. It has been shown to induce increases in vascular permeability in rat skin (Flower & Kingston, 1975; Flower *et al*, 1976) and long-lasting erythema in human skin (Flower *et al*, 1976). Nishimura *et al* (2001) observed a dose-dependent increase in Evans Blue dye in guinea pig airways with PGD_2 administration, but this response appeared to be mediated via TP receptor activation. Others have also demonstrated TP-induced airway MVL in rats and in guinea pigs (Lötvall *et al*, 1992; Bertolino *et al*, 1995).

As outlined in this introduction airway microvascular leak (MVL) represents a cardinal sign of inflammation and is a prominent feature in the pathogenesis of airway inflammatory diseases, including asthma and COPD. Understanding the mechanism and the role of possible mediators in producing airway MVL is an important step in the development of new and effective therapies. PGE_2 has been subject to extensive investigation as a potential therapy due to its beneficial effects in the airways. PGE_2 has been shown to act as a bronchodilator and an anti-inflammatory in cell based systems, *in vivo* models and clinically in healthy and asthmatic patients. However, inhaled PGE_2 caused airway irritancy and cough. Recent work in our lab has identified that EP_3 receptor activation mediates this tussive effect, while EP_2 and EP_4 receptors mediate bronchodilation. The aim of this thesis is to further investigate the role of PGE_2 and the EP receptors in airway inflammation, specifically their role in airway microvascular leak. It is prudent to identify the receptor(s) responsible for mediating any adverse effects especially if an agonist could be developed as a bronchodilator and/or anti-inflammatory.

1.4. Thesis Plan

1.4.1. Hypothesis

The hypothesis that formed the basis of this project was;

"PGE_2 acts through specific EP receptor(s) to induce microvascular leak within the airways."

1.4.2. Aims

A multidisciplinary approach was used to investigate PGE_2-induced airway microvascular leak and to identify the receptor(s) involved. Therefore, the aims of this thesis are to:

- Characterise an *in vivo* model to measure airway microvascular leak using a known mediator of MVL (5-HT) and establish the effect of exogenous PGE_2 administration in this system (Chapter 3).

- Investigate the EP receptor mediating PGE_2-induced MVL using genetically modified mice and a range of pharmacological tools. This will involve characterisation of a novel EP_4 receptor antagonist that has not been previously used in our lab (Chapter 4).

- Characterise the measurement of airway MVL in an allergic model of asthma (OVA model) and use EP receptor-deficient mice and selective antagonists to investigate the role of PGE_2 (Chapter 5).

- Begin preliminary work exploring the role of the other prostanoids in mediating airway MVL (Chapter 6).

Chapter Two

Methodology

2. Methodology

2.1. Breeding of Knock-Out Mice

Homozygous breeding pairs of mice genetically modified to disrupt one of the following genes; *Ptger1* (EP_1), *Ptger2* (EP_2), *Ptger3* (EP_3) (Ushikubi *et al*. 1998), *Ptger4* (EP_4), *Ptgdr* (DP) (Matsuoka *et al*. 2000), *Gpr44* (DP_2), *Ptgfr* (FP) (Sugimoto *et al*. 1997), *Ptgir* (IP) (Murata *et al*. 1997), *Tbxa2r* (TP) (Kabashima *et al*. 2003) were kindly provided by Dr S Narumiya, Kyoto University, *Gpr44* (DP_2) (Satoh *et al*, 2006) knock-out mice were kindly supplied by Dr. Hiroyuki Hirai and Professor Masataka Nakamura, Tokyo Medical and Dental University, and breeding colonies maintained at Imperial College London. All strains had been backcrossed at least 8 times onto the C57BL/6 background, except the *Ptger4*$^{-/-}$ (EP_4) mice as they do not survive on this background due to patent ductus arteriosus (Segi *et al*. 1998) and so were backcrossed on a mixed background of 129/Ola X C57bl/6. These wild type mice were also provided by Kyoto University while C57BL/6 wild type mice were bred internally. The DP_2 knockout mice were originally bred on a balb/c background and so were backcrossed onto a C57BL/6 strain before used in experiments.

2.1.1. Animal Husbandry

Male Dunkin Hartley guinea pigs and male Brown Norway rats were purchased from Harlan (Bichester, UK). Mice were bred in house as described above. All animals were housed in individually ventilated cages (IVC) and provided with food and water *ad libitum* in a controlled environment. A 12 hour light-dark cycle was maintained for all animals. All studies and procedures were performed in accordance with Home Office guidelines under the Animals (Scientific Procedures) Act of 1986.

2.2 Genotyping of KO Mice

2.2.1 DNA Extraction and Quantification

DNA was extracted from mouse tail tips using Quanta Biosciences Extracta DNA prep for PCR kit. Tail tips were transferred into autoclaved 0.5 ml eppendorfs and 75µl of extraction reagent was added. These were then heated for 30 minutes at 95ºC. Once samples had cooled to room temperature, 75µl of stabilisation reagent was added.

The purity and integrity of each DNA sample was then assessed by A_{260}/A_{280} spectrophotometry on the GeneQuant RNA/DNA quantifier (Amersham Pharmacia Biotech, U.K.). Following quantification samples were diluted to 10 ng μl^{-1} and stored at -20 ºC.

2.2.2 Polymerase Chain Reaction

PCR reactions were run using mouse genomic DNA primers that had been previously optimised and validated. Primer sequences are listed in table 2.1. The PCR reaction contained 1x Green GoTaq Flexi buffer, 0.2mM dNTPs, 2mM $MgCl_2$, 1.25µl enzyme (GoTaq polymerase), 10pmol forward and 10pmol reverse primers, and a volume of DNA (50ng) or Nuclease-free water for control, made up to 25µl with Nuclease-free water (Promega U.K. Ltd). The reaction was used to exponentially amplify the sections of DNA in between the primer pair.

Using the ABI Prism 7000 (Applied Biosystems, Warrington, Cheshire, UK) the samples were heated to 94ºC for 2 minutes followed by 40 cycles of denaturing, annealing and extension steps. All samples were denatured at 94ºC for 30 seconds as this is dependent on the enzyme. The annealing temperature is specific to the primer pair and temperatures are between 58-65ºC for 30-45 seconds. The temperature of the extension step is also primer dependent and the length of time that the reaction is held for depends on the product size. This was followed by a final extension step for 10 minutes at 68-75ºC. The reaction was then stopped by cooling to 4ºC.

2.2.3 Visualisation of PCR Products

The PCR products and DNA ladder (Hyperladder II or IV, Bioline Ltd, London) were run on a 2% agarose gel in Tris Borate EDTA (TBE) buffer containing 0.05 µl ml^{-1} of Safeview (NBS Biologicals Ltd, Huntingdon, U.K.) at 80 V for at least one hour. The gel was then visualised under ultra-violet light and photographed using the Molecular Imager® ChemiDoc™ XRS System with Image Lab™ software (Bio-Rad, UK).

Table 2.1. Primer sequences used to genotype prostanoid receptor KO mice.

Primer Name		Sequence
EP_1	forward	5'-TGA GCC TAG CGG ATG AGG CAG C-3'
	reverse	5'-GCG GAG AGT CCG GCT AGA GAA G-3'
	neomycin	5'-CCT GGG TGG AGA GGC TTT TT-3'
EP_2	forward	5'-CTG GCC ATT ATG ACC ATC ACC TTC GCC A-3'
	reverse	5'-CTG AGC AAC ACC CAT GTT TCT ATC CTG G-3'
	neomycin	5'-TGA TAT TGC TGA AGA GCT TGG CGG CGA A-3'
EP_3	forward	5'-AAA GTG ACT AGC ACC CAG AAT TCC TGC C-3'
	reverse	5'-GAG TCC TCC ACT TTG GTG TAC ACA GTA C-3'
	neomycin	5'-TGC CGA ATA TCA TGG TGG AAA ATG GCC G-3'
EP_4	forward	5'-TCT ACT TGC TCC CAG TGG ACA TAG A-3'
	reverse	5'-GAA CAG ACT CCT GAA CTG GGT ATG G-3'
	neomycin	5'-TGC CGA ATA TCA TGG TGG AAA ATG G-3'
FP	forward	5'-GCC CAT CCT TGG ACA CCG AGA TTA TCA A-3'
	reverse	5'-AGA GTT GGC AAG CTG TGA CTT CGT CTT T-3'
	neomycin	5'-TGA TAT TGC TGA AGA GCT TGG CGG CGA A-3'
IP	forward	5'-GAG CAG AAA AAT TCC CAG AGG CTT-3'
	reverse	5'-GTA TCT TTC AGT ACC TGA GGA CTG-3'
	neomycin	5'-TGA CCG CTT CCT CGT GCT TTA C-3'
TP	forward	5'-ACT TCA TGG GGT CAG AAG CA-3'
	reverse	5'-ACA TGG CTC CTA AGC CTG AC-3'

	neomycin	5'-CTC GTC CTG CAG TTC ATT CA-3'
DP_1	forward	5'-TCG GTC TTT TAT GTG CTC GTG-3'
	reverse	5'-GGA TCA TCT GGA TGA AAC ACC-3'
	neomycin	5'-CCC GTG ATA TTG CTG AAG AGC-3'
DP_2 (CRTH2)	wt forward	5'-CGT AGC CGT GAG CCT GCG ACT G-3'
	wt reverse	5'-GGC GAT TGC GGA GCC CAC CAC T-3'
	KO forward	5'-GAG CAC GTA CTC GGA TGG AA-3'
	KO reverse	5'-TCC TCG AGG CTA CTT TTG C-3'

2.3 Microvascular Leak

Airway wall microvascular leak was measured using Evans Blue dye as a marker for serum albumin extravasation. As discussed in the introduction, Evans Blue binds avidly to albumin so allows us to track the movement of this protein out of the circulation and into airway tissue. An increase in the concentration of Evans Blue in the tissue is indicative of an inflammatory response.

2.3.1 Compound Administration

Animals were anaesthetised with intra-peritoneal (i.p) Urethane (2 g kg^{-1}; 200 µl of a 25% solution given at two sites, followed by a further 50-100 µl when supine if necessary). Mice breathe spontaneously under urethane and so were therefore not artificially ventilated. Depth of anaesthesia was assessed using the pedal and corneal reflex. Once surgical anaesthesia was reached the jugular veins were then exposed following a midline incision over the thorax. The left jugular vein was most often used, except when a third injection was needed or it was unsuitable for anatomical reasons. Intravenous administration was achieved by passing the injection needle through the pectoralis major as this helped to prevent bleeding on withdrawal.

Animals would receive Evans Blue dye (5 mg ml^{-1} at 4 ml kg^{-1}) followed by the specific mediator or its vehicle one minute later (4 ml kg^{-1}). The doses and timings used were those shown to be effective in previous studies within the group and from the literature (Hele *et al*, 2001).

2.3.2 Measurement of Plasma Extravasation

Five or 30 minutes after vehicle or compound administration, the thoracic cavity was opened and a small incision was made in the left ventricle and also the left atria of the heart (Figure 2.1). A cannula was then inserted into the left ventricle and the

systemic circulation perfused with sterile saline (0.9%) at a pressure of approximately 100mmHg. This was continued until the perfusate ran clear (2-5 minutes). The purpose of this was to remove any intravascular dye.

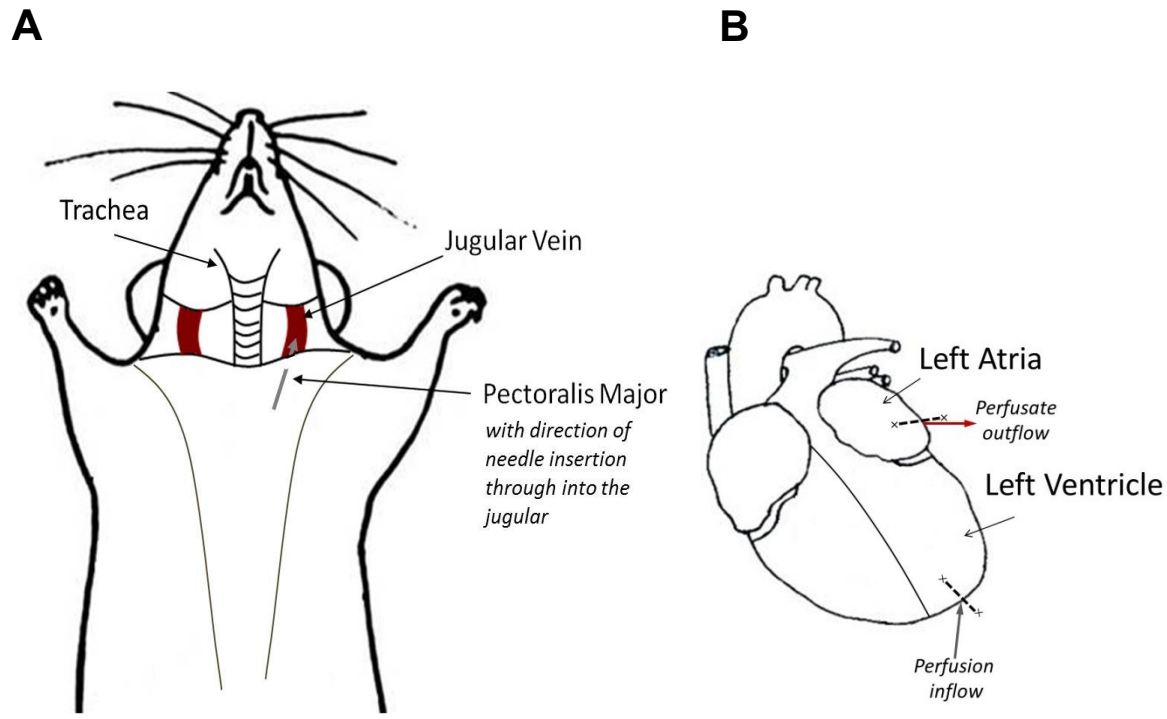


Figure 2.1. Schematic of (A) injection site used for intra venous administration in mice and (B) anatomy of the mouse heart illustrating where incisions were made for saline perfusion.

The heart, lungs and oesophagus were removed *en bloc* and then the trachea and lungs separated from the heart and oesophagus. The oesophagus and bladder were taken from all animals and used as non-airway tissue controls (data not shown). The trachea was isolated by cutting just above the bifurcation of the bronchi and the larynx removed. The parenchyma was then carefully scraped off using a scalpel to reveal the intra pulmonary airways (IPA). The trachea, the bronchi and IPA, the oesophagus and the bladder were then all weighed and the wet tissue weight recorded. Each tissue was then placed into an eppendorf with 120µl of formamide and incubated at 37.5˚C for at least 18 hours to facilitate the extraction of Evans Blue dye.

2.3.3 Analysis

The concentration of Evans Blue extracted from each tissue was determined by light absorbance at 620nm using a spectrophotomer (Biotek Powerwave XS Plate Reader, Biotek, UK). 100µl of formamide was removed from each eppendorf and pipetted into a 96 well plate alongside a standard curve of Evans Blue in formamide (0, 0.3125, 0.625, 1.25, 2.5, 5, 10 and 20 µg ml^{-1}). The concentration was then calculated by interpolation from the standard curve and expressed as ng mg^{-1} of tissue.

2.4 Blood Pressure Measurements

2.4.1 Cannulation of Carotid Artery

Mice were anaesthetised with Urethane (2 g kg^{-1}; 200-300 µl of a 25% solution given at two sites i.p). Mice breathe spontaneously under urethane and so were therefore not artificially ventilated. Depth of anaesthesia was assessed using the pedal reflex. Body temperature was continuously monitored with a rectal thermometer and maintained at 37ºC with a heated blanket and control unit (Harvard). Mice were placed under a dissecting microscope.

Once surgical anaesthesia was reached, a midline incision in the neck region was made, the trachea exposed and the salivary glands carefully separated. The right carotid artery was then isolated and three ligatures applied; one fixed tightly at the cranial end, one loose at the caudal end pulled taught and a third loose loop in the middle. A small incision was made using vannas scissors and a polyethylene catheter was then inserted and secured with the two caudal ligatures (Figure 2.2).

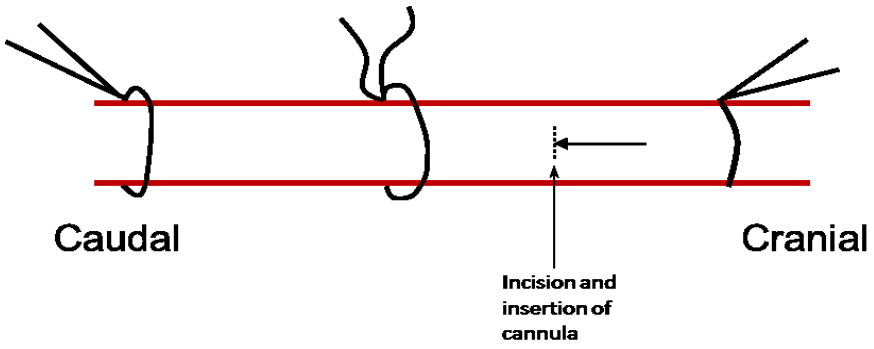


Figure 2.2. Diagram of right carotid artery cannulation Illustrating ligature placement on the vessel as well as the incision site for inserting the cannula. *The ligature at the cranial end is fixed, occluding the vessel, while the two ligatures caudal to this are only loosely applied until the cannula is inserted. The arrow indicates the direction the cannula is inserted in. Once the cannula is in place these ligatures are pulled tightly to secure the cannula.*

2.4.2 Measuring Blood Pressure

Once the carotid was cannulated the systemic arterial blood pressure was measured using a pressure transducer. Heart rate was also obtained from the arterial pulse trigger using Spike 2 software (CED). Systemic arterial blood pressure and heart rate were continuously recorded on the Spike 2 software data acquisition system via a CED Micro1401 interface.

Following this the left jugular vein was exposed to allow for intra venous administration. After surgery the animals were allowed to stabilize and after a control baseline recording of 5 minutes, vehicle or compound (all at 4 ml kg^{-1}) were administered via the jugular vein. Blood pressure was monitored and recorded for 30 minutes following which animals were culled via cervical dislocation.

2.5 Ovalbumin Model of Allergic Asthma

A standard Ovalbumin (OVA) model of allergic asthma that has been previously developed and characterised 'in house' (Birrell *et al*, 2003) was used to assess airway microvascular leak in a disease setting. Four different treatment groups were initially used. These were vehicle sensitised/saline challenged, vehicle sensitised/OVA challenged, OVA sensitised/saline challenged and OVA sensitised/OVA challenged. Following a small time course study it was deemed practical to include just two groups for all other experiments; OVA sensitised/saline challenged and OVA sensitised/OVA challenged. Alum™ was used as an adjuvant for the sensitisation of all mice and was diluted 1:1 with saline. Alum™ is made up of 20 mg ml^{-1} of aluminium hydroxide and 20 mg ml^{-1} of magnesium.

Male C57BL/6 mice (20-25 g) were sensitised with OVA in Alum™ (10µg per mouse, 100 µl i.p.) or vehicle (Alum™ diluted 1:1 with saline, 100µl i.p) on days 0 and 14 (Figure 2.3). Subsequently on days 24, 25 and 26 mice were given a single intranasal (i.n) challenge of either OVA (50 µg in 50µl) or saline. For the intranasal dosing, mice were placed into a Perspex™ chamber connected to an anaesthetic machine (Bowring Medical Engineering Ltd, Witney, UK) and exposed to 4% isoflurane in 0.5% oxygen. Once mice were lightly anaesthetised 50 µl was slowly dropped with a pipette onto both nostrils until passively inhaled. Mice were monitored closely during recovery.

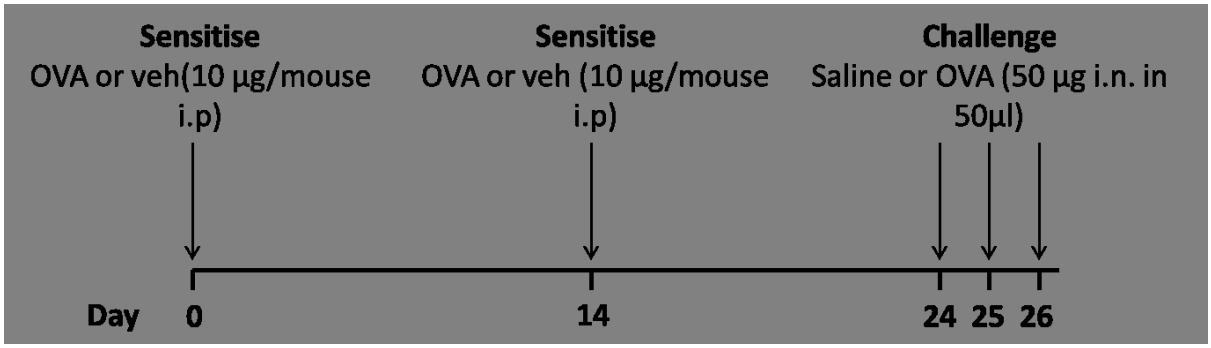


Figure 2.3. Timeline demonstrating the protocol used for the ovalbumin model.

2.5.1 Assessing airway Microvascular Leak

Evans Blue dye was used as a marker of airway microvascular leak induced by OVA. 2 and 24 hours following the final intra nasal challenge mice were anaesthetised (Urethane 2 g kg^{-1} 200-300 µl i.p) and the jugular veins exposed to allow for a single i.v dose of Evans Blue dye (5 mg ml^{-1} in 4 ml kg^{-1}). Thirty minutes later, the circulation was perfused and airways dissected out as described before. In the first study only wet tissue weight was recorded and then in subsequent experiments the tissue was dried for one hour at 60°C and wet and dry weights were recorded. As before, the tissue was incubated in formamide and concentration of Evans Blue dye assessed using a spectrophotometer.

2.6 *In vitro* Organ Bath Experiments

To assess the selectivity of the EP_4 antagonist (ER-819762) in a functional bioassay system, isolated rat trachea was used. PGE_2-induced relaxation of induced tone in rat tracheal tissue has previously been shown to be mediated by the EP_4 receptor (Lydford & McKechnie, 1994; Buckley *et al*, 2011; Benyahia *et al*, 2012) and so this was used as an assay system to assess the functionality at this receptor subtype.

2.6.1. Physiological salt solutions

For all experiments, tissues were cleaned, dissected and bathed in modified Krebs-Henseleit solution (KHS). This solution was composed of (mM); NaCl (118), KCl (5.9), $MgSO_4$ (1.2), NaH_2PO_4 (1.2), $CaCl_2$ (2.5), Glucose (6.6), $NaHCO_3$ (25.5), dissolved in distilled water. The KHS was maintained at 37°C and a 95% O_2/5% CO_2 gas mixture bubbled through. Additionally, 10μM indomethacin (a non-selective COX inhibitor) was added to the solution to prevent the formation and release of any endogenous prostanoids (Patel *et al*. 1995; Takahashi *et al*. 1994).

2.6.2. Isolated airway preparation

Male Sprague Dawley rats (225-300g) were euthanized with an overdose of pentobarbitone (200 mg kg^{-1} i.p) and the trachea carefully dissected out and quickly placed in KHS. All extraneous connective tissue was dissected clear and the trachea opened via a longitudinal incision along the cartilage opposite the smooth muscle. The trachea was then divided into 4 equal transverse segments and sutured appropriately for attachment to the transducer. Once the tracheal strips were set up in the organ bath they were left to equilibrate for 1 hour under a resting tension of 1000mg.

2.6.3. Isolated airway organ bath protocols

2.6.3.1. General protocol

Each prepared tracheal section was attached to force-displacement transducers (Model FT-03; Grass Instruments Co., Quincy, MA, USA) connected to a data acquisition system (MP100) operating on a Windows PC using AcqKnowledge software (BIOPAC systems, USA). The tissue was left to equilibrate in 10 ml organ baths in KHS for one hour and washed at 30 minutes with fresh KHS. Following this, a supramaximal response to 1mM acetylcholine (ACh) was obtained three times, with three washes and re-equilibration between each ACh administration.

2.6.3.2. Induced tone protocol

Once the tissues had returned and stabilised to resting tension levels, an increase in muscular tone was induced by administering 1μM carbachol to the organ bath. The tissues were then left to equilibrate at this increased resting tension until stable, typically about 20 minutes. The EP_4 antagonist (ER-819762) or vehicle was then administered to the baths and left to incubate for 20 minutes, during which time the baseline tension was monitored to ensure that the antagonist was not affecting the tension itself. Following this, a cumulative concentration response curve to the EP_4 agonist (ONO-AE1-329) was performed. Finally, once a plateau was reached to the highest agonist concentration, 100μM of papaverine was added to produce standard maximal relaxation responses.

2.6.4. Data Analysis

Data was expressed as mean ± s.e.m. of n individual observations. Concentration response data was analysed using PRISM 5 (GraphPad Software, CA, USA) and curves were fitted by non-linear regression of data using a four parameter logistic equation (below) from which EC_{50} values were then calculated:

$$Y = \text{Bottom} + \frac{(\text{Top - Bottom})}{1 + 10^{(LogEC_{50} - X)\cdot \text{HillSlope}}}$$

Where: Bottom = Y value at the bottom plateau, Top = Y value at the top plateau, $LogEC_{50}$ = X value when the response is halfway between Bottom and Top, HillSlope = Description of gradient of curve.

Estimates of antagonist affinity were calculated using the Gaddum-Schild equation as described previously (Gaddum 1957):

$$pKb = \log (CR - 1) - \log [B]$$

Where: CR = Concentration ratio between individual EC_{50} values.

2.7 Human Cell Based Assay of Microvascular Leak

To further explore the mechanisms by which prostanoids induce microvascular leak, human lung microvascular endothelial cells were used. Eliminating physiological parameters such as blood pressure and blood flow using a cell based system allowed investigation into the actions of prostanoids directly on endothelial cells. Endothelial barrier permeability to albumin was used as an indicator of microvascular leak. Data is presented in chapter 7 as future work (section 7.2.6.).

2.7.1 Cell Culture

Human Lung Microvascular Endothelial cells (HLMVECs) were purchased from Lonza (Lonza Walkersville, Inc., Walkersville, MD, USA) and arrived at passage 5. These cells were isolated from small vessels within normal human lung but not specified as post capillary venules. Cells were grown and used at passage 8. They were cultured in Endothelial Growth Medium (EGM^{TM}-2 MV BulletKit^{TM} Medium, Lonza.) containing the following growth factors; FBS, hydrocortisone, hFGF-B, VEGF, R3-IGF-1, ascorbic acid, hEGF, Gentamicin and Amphotericin B. Cells were maintained at 37˚C in a humidified atmosphere of 5% CO_2-95% air. Cells were passaged using a Trypsin/EDTA solution when they reached 90% confluency and seeded at 800,000 cells per flask.

2.7.2 Measuring Endothelial Barrier Permeability

Cell culture inserts with a 3.0 micron, transparent polyethylene terephthalate (PET) membrane (BD Falcon^{TM}) were used in all experiments. Before seeding, complete medium was added to both insert and well and the membrane equilibrated for 3 hours at 37˚C in 5% CO_2-air. Cells were seeded onto culture inserts at 4 x 10^4 cells per insert. Seeded inserts were incubated for 48 hours at 37˚C in 5% CO_2-air. At the end of incubation cultures were examined under light-microscopy and those with damaged monolayers were discarded.

Vehicle (DMSO, final concentration 0.1%, V/V) or compound was added to the upper chamber and incubated for one hour in a humidified atmosphere (95% air, 5% (v/v) CO_2) at 37˚C. After the 60 minute incubation cultures were once again examined and any with a damaged monolayer were discarded. To detect changes in monolayer permeability fluorescein isothiocyanate conjugate (FITC)-albumin (100 µg/ml) was then added to the upper chamber of each insert in a volume of 250µl and 750µl of assay medium was also added to each lower well to ensure similar hydrostatic pressure on both sides of the monolayer. Cells were incubated with FITC-albumin for a further 60 minutes (Figure 2.4).

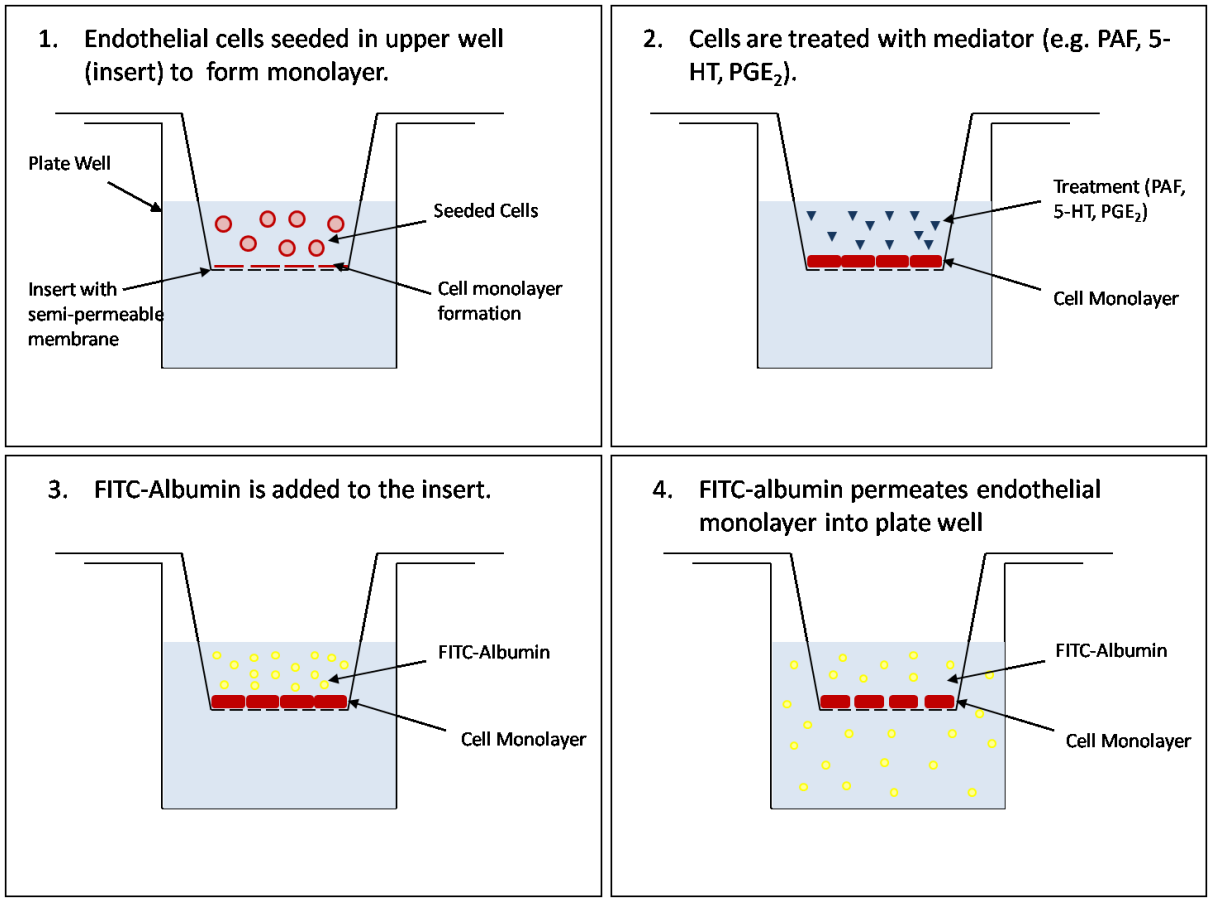


Figure 2.4. Diagram illustrating method used for measuring endothelial barrier permeability in human lung microvascular endothelial cells (HLMVECs).

At the end of the incubation, inserts were carefully removed and medium in each lower well thoroughly mixed. 100 µl samples were then collected from the lower chambers were analysed for FITC fluorescent intensity using a fluorometric plate reader (Ettan Dige Imager) at excitation 492nm and emission 520nm.

After assay of culture permeability, cultures were examined under phase-contrast microscopy for intactness of monolayers. Data from cultures with damaged monolayers was excluded. Additionally cell viability will be assessed by MTT assay.

2.7.3 MTT Assay

Mitochondrial enzymes produced from viable cells cleave the yellow tetrazolium salt MTT to blue formazan. Hence, formazan is directly proportional to the number of living cells present during MTT colorimetric analysis and can be used to quantitatively determine cell viability (Sylvester 2011). 500µL of MTT in RPMI (1mg/mL) was added to each well of the 24 well plates post J774 cell treatment and transfer. After incubating for 20 minutes, the plates were emptied and dried overnight before absorbance was recorded in a microplate reader at 570nm.

2.8 Measurement of Gene Expression

2.8.1 RNA Extraction

RNA was extracted from the human lung microvascular endothelial cells (3 patients) to detect and quantify the presence of prostanoid receptors in this cell type. The cells were first isolated by centrifugation of a cell suspension at 12,000 x g for 5 minutes and discarding the supernatant. 1 ml of Tri Reagent (Sigma-Aldrich Co., Poole, UK) was then added to each pellet of cells. Tri Reagent is a mixture of guanidine thiocyanate and phenol in a non-phase solution which is able to effectively dissolve DNA, RNA and protein on lysis with the sample. Samples were then left to stand at room temperature for 5 minutes to ensure the complete dissociation of nucleoprotein complexes. After this, 0.2 ml of chloroform was added to each tube, mixed and left to stand for approximately 15 minutes. Samples were then centrifuged at 15,000 x g for 15 minutes at 4ºC in a microcentrifuge. This step causes the mixture to separate into three phases: a colourless upper aqueous phase (containing RNA), an interphase (containing DNA) and a red organic phase at the bottom (containing protein). Only the RNA was required so the aqueous phase was removed and placed into an autoclaved 1.5ml centrifuge tube. To this, 1/10 volume of isopropanol was added, mixed and left at room temperature for 5 minutes. Samples were then centrifuged at 12,000 x g for 10 minutes at 4ºC and the supernatant transferred to an autoclaved 1.5ml tube. The remaining volume of isopropanol was then added (a total of 0.5ml of isopropanol is needed per 1ml of Tri Reagent used), mixed and left to stand at room temperature for 10 minutes. This will lead to the formation of an RNA precipitate in the sample. Samples were then centrifuged at 12,000 x g for 10 minutes at 4ºC and this time the supernatant was carefully discarded leaving an RNA pellet. This pellet was then washed with 1ml of 75% ethanol, vortexed and centrifuged at 12,000 x g for 5 minutes at 4ºC. Following this, the ethanol was removed, being careful not to disturb the RNA pellet, and left to air dry for 5-10 minutes. The RNA pellet was subsequently resuspended in 50µl of nuclease-free water. The purity and integrity of the RNA was assessed via A_{260}/A_{280} spectrophotometric measurements on the GeneQuant RNA/DNA quantifier

(Amersham Pharmacia Biotech, U.K). Samples were then diluted to a concentration of 0.05 ng ml^{-1}.

2.8.2 Reverse Transcription

RNA samples (1µg) were reverse transcribed to complementary deoxyribonucleic acid (cDNA), using a master mix (TaqMan reverse transcription reagents, Applied Biosystems, Warrington, Cheshire, U.K.) containing 1X TaqMan RT buffer, 5.5mM $MgCl_2$, deoxyNTPs mixture (500µM per NTP), 2.5µM random hexamers, 0.4U $µl^{-1}$ RNase inhibitor and 1.25U $µl^{-1}$ Multiscribe reverse transcriptase, in a final reaction volume of 50µl (10µl RNA sample). The samples were incubated in a Perkin Elmer 480 thermal cycler (Boston, MA, U.S.A.) at 25ºC for 10 minutes, then reverse transcribed at 48ºC for 30 minutes and then the enzyme was denatured at 95ºC for 2 minutes. Samples were stored at -80ºC until required for gene expression analysis.

2.8.3 Real Time Polymerase Chain Reaction (PCR)

Real time polymerase chain reaction (PCR) was carried out to detect transcriptional expression of target mRNA transcripts in RNA samples by using fluorescently labelled Taqman probes (Applied Biosystems, Warrington, Cheshire, UK) and analysed with the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Warrington, Cheshire, UK). The oligonucleotide probes are constructed so that they contain a reporter fluorescent dye on the 5' end and a quencher dye on the 3' end. While intact, the close proximity of the quencher dye to the reporter dye greatly reduces the level of fluorescence emitted. If the target sequence is present, the probe anneals downstream from one of the primer sites and is cleaved by the 5' nuclease activity of Taq DNA polymerase as this primer is extended. This cleavage separates the reporter dye from the quencher dye and so increases the signal of the reporter dye. The TaqMan machine is able to measure the fluorescence emitted cycle by cycle.

Pre-developed Assays on Demand for the target genes of interest were purchased from Applied Biosystems and contained the primers and probe in one reagent. The TaqMan probe has a fluorescent reporter dye, FAM, covalently linked to its 5' end and a downstream quencher dye, TAMRA, linked to the 3' end. The probe is designed to anneal to a DNA sequence in between primers on the gene of interest. Reactions were internally controlled with 18s rRNA internal control (Applied Biosystems, Warrington, Cheshire, UK). 18s is designed in the same way as the assay probe but uses VIC as its quencher dye so that both reporter signals in the reaction can be measured.

The PCR reactions were performed using 3µl of cDNA (2.5ng ul^{-1}), 12.5µl of TaqMan Universal Master Mix, 1.25µl 18s internal control, 1.25µl of the assay on demands (AOD) and 7µl of Nuclease-free water, to make a total reaction volume of 25µl. The amplification and detection was carried out using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Warrington, Cheshire, UK) using the following protocol; 1 cycle at 50ºC for 2 minutes, 1 cycle at 95ºC for 10 minutes, 40 cycles at 95ºC for 15 seconds, and 60ºC for 1 minute.

2.8.4 Analysis of Real Time PCR Results

Real Time PCR results were analysed using the Sequence Detection Software where the relative amount of target gene transcript was normalised to the amount of 18s internal control transcript in the same cDNA sample. The critical threshold (ct), which is defined as the cycle at which the fluorescence from the TaqMan probe becomes detectable above background, is inversely proportional to the logarithm of the initial number of template molecules. Therefore the higher the target concentration, the lower the number of amplification cycles needed to measure the rise above baseline. The ct was set during the exponential phase of cDNA replication and the relative difference between 18s and AOD ct (Δct) was then calculated. Data was expressed using the following equation; $2^{-\text{delta ct}}$ i.e. $2^{-(\text{target ct-18s ct})}$, written as $2^{-\Delta ct}$.

2.9 Statistical Analysis

All data was analysed using GraphPad Prism 5. Parametric data was expressed as mean ± s.e.m and non-parametric data presented as the median with the interquartile range. Multiple measurements were analysed by one-way ANOVA, non-parametric Kruskal-Wallis test and post-hoc comparisons were performed by Dunn's multiple comparison test, comparing selected columns to a control. Additionally, an unpaired t test and/or non-parametric Mann Whitney test, with two-tailed p values, were carried out to determine statistical significance between two groups. Differences were considered statistically significant if $p<0.05$. The figures have been graphically presented on a range-specific axis

Chapter Three

Characterisation of an *in vivo* preparation to measure airway MVL and the effect of Prostaglandin E_2

3. Characterisation of an *in vivo* preparation to measure airway MVL and the effect of Prostaglandin E_2

3.1. Rationale

Airway microvascular leakage and plasma exudation are prominent features in both asthma and COPD representing signs of airway inflammation (Paredi & Barnes, 2009). Increased levels of PGE_2 have been reported in BAL fluid and plasma of asthma patients, and enhanced levels in the condensate of COPD patients (Montushi *et al*, 2003; Long *et al*, 2004). However, its role in airway inflammation remains varied and inconclusive and there appears to be a fine balance between the pro and anti-inflammatory effects of this prostanoid in human airways. Microvascular leak has been extensively studied in larger species, such as the guinea pig, using various different methods, as described in Chapter 2. However the aim of this chapter was to establish whether the mouse was an appropriate species to use to investigate PGE_2's effects on airway MVL. There are several reasons why using the mouse would be advantageous; it is the species of choice to model asthma and COPD, the availability of prostanoid receptor knock-out mice, cost and lesser quantities of compound are required.

3.1.1. Chapter Hypothesis

"Exogenous PGE_2 is able to cause microvascular leak in rodent airways."

3.1.2. Aims

- Characterise the Evans Blue model in the mouse using 5-HT, which is a known and potent inducer of airway MVL (Germonpre *et al*, 1997; Olivenstein *et al*, 1997; Hele *et al* 2001).
- To investigate a dose dependent relationship to PGE_2 and determine the ideal length of time over which to measure this effect.
- Compare the response to PGE_2 in a larger species (guinea pig).
- Investigate whether the MVL measured coincided with a change in systemic blood pressure, as PGE_2 has been implicated in the vasodilation observed in inflammatory processes and additionally bronchial vasodilators have

demonstrated increases in plasma extravasation (Narumiya *et al*, 1999; Paredi & Barnes, 2009).

3.2. Methods

The following experiments were carried out as described in Chapter 2. Details of individual experiments are described below.

3.2.1. Characterisation of airway MVL in mice using 5-HT

A dose response to 5-HT was first established to help characterise the microvascular leakage model in mice. Animals received Evans Blue (EB; 5 mg kg^{-1} at 4 ml kg^{-1}) and one minute later received 5-HT (0.1, 1 or 10 mg kg^{-1} at 4 ml kg^{-1}) or vehicle (1% ethanol in saline). Five minutes after vehicle/5-HT administration, animals were culled for the measurement of EB extravasation as described in section 2.3. The doses and timings used were those shown to be effective in previous studies within the group and from the literature (Hele *et al*, 2001).

To confirm that 5-HT induced MVL was through activation of 5-HT receptors the non-selective 5-HT receptor antagonist methysergide was used. Methysergide (10 mg kg^{-1}; Hele *et al*., 2001) or vehicle (0.9% saline; 4 ml kg^{-1}) was given i.v. 1 minute after EB. Five minutes after this a sub maximal dose of 5-HT (1 mg kg^{-1}) or vehicle was administered (1% ethanol in saline; 4 ml kg^{-1}). After a further 5 minutes EB extravasation was measured.

3.2.2. Dose Response of PGE_2 - induced airway MVL in Mice

A dose response to PGE_2 was established where male C57BL/6 mice were given PGE_2 (0.1, 0.3, 1, 3, or 10 mg kg^{-1} at 4 ml kg^{-1}), 5-HT (10 mg kg^{-1} at 4 ml kg^{-1}) as a positive control or vehicle (1% ethanol in saline). 5 minutes following administration EB extravasation was measured.

3.2.3. Time Course of PGE_2 - induced airway MVL in Mice

As only a small increase in MVL was observed in response to PGE_2, the PGE_2 exposure time was increased to 30 minutes and compared to 5 minutes. In this study a single dose of PGE_2 (10 mg kg^{-1}) was used, as well as a vehicle control. As an enhanced increase in MVL was demonstrated at 30 minutes, the dose response to PGE_2 was repeated.

3.2.4. Effect of Diclofenac on PGE_2-induced airway MVL in Mice

A non-selective COX inhibitor, diclofenac (30 mg kg^{-1} in 10 ml kg^{-1}), or vehicle (0.5% methylcellulose, 0.2% Tween 80 in water) was administered orally 60 minutes prior to PGE_2 administration. This was to block any endogenous prostanoids that may be influencing the baseline or PGE_2-induced response.

3.2.5. Effect of intranasal dosing of PGE_2 on airway MVL in Mice

Having assessed the effect of intravenous administration of PGE_2, I wanted to examine whether I could reproduce the same effect via topical delivery straight to the airways to more closely mimic what might happen in airway disease. Here mice were anaesthetised and then dosed intra-nasally (i.n) with either vehicle (1% ethanol in saline) or PGE_2 (3 mg kg^{-1}) in 50µl. Once surgical anaesthesia was reached, mice were then given Evans Blue and culled 30 minutes later.

3.2.6. PGE_2-induced airway MVL in Guinea Pigs

Guinea Pigs were chosen as a second (larger) species to assess PGE_2-induced airway MVL as they more closely resemble humans in terms of their respiratory anatomy (lobing and branching) and physiology (mediator release). In this study it was more suitable to look at only one dose of PGE_2 for reasons pertaining to cost and time. Male Dunkin Hartley Guinea Pigs (300-600g) were anaesthetised with urethane (2 g kg^{-1} i.p of a 25% solution). Once surgical anaesthesia was reached

animals received EB (5 mg ml^{-1} at 1 ml kg^{-1} i.v) and then one minute later vehicle (1% ethanol in saline), PGE_2 (3 mg kg^{-1}) or 5-HT (1 mg kg^{-1}), all intravenously at 1 ml kg^{-1}. 5 minutes following 5-HT or 30 minutes after PGE_2 EB extravasation was measured.

3.2.7. Effect of PGE_2 on Systemic Blood Pressure in Mice

Male C57BL/6 mice were anaesthetised as previously described and their carotid artery cannulated (as outlined in 2.4). Once a baseline blood pressure recording was achieved (approximately 5 minutes), the jugular veins were exposed and an intravenous dose of PGE_2 (3 mg kg^{-1}) or vehicle (1% ethanol in saline) administered. Mean arterial blood pressure (MAP) was measured over 30 minutes after which animals were culled. Along with PGE_2, the IP agonist Iloprost and TP agonist U46619 were given as positive controls (Walch *et al*, 1999; 2001).

3.2.8. Statistical Analysis

All data was analysed using GraphPad Prism 5. Parametric data was expressed as mean $\pm$ s.e.m and non-parametric data presented as the median with the interquartile range. Multiple measurements were analysed by one-way ANOVA, non-parametric Kruskal-Wallis test and post-hoc comparisons were performed by Dunn's multiple comparison test, comparing selected columns to a control. Additionally, an unpaired t test and/or non-parametric Mann Whitney test, with two-tailed p values, were carried out to determine statistical significance between two groups. Differences were considered statistically significant if $p<0.05$. The figures have been graphically presented on a range-specific axis.

3.3. Results

3.3.1. Characterisation of airway MVL in mice using 5-HT

In order to validate this model in mice, a known inducer of MVL, 5-HT, was used (Hele *et al*, 2001). An increase in Evans Blue dye concentration correlates with an increase in microvascular leak and is expressed in ng mg^{-1} of tissue. Intravenous administration of 5-HT (0.1, 1 and 10 mg kg^{-1}) caused substantial increases in Evans blue extravasation into the trachea and bronchi and IPA (Figure 3.1 and Figure 3.2). However, from the photo in figure 3.1 it appears that EB leak in the lower airways (IPA) is substantially less than in the bronchi. This could possibly 'dilute' the final value of EB leak. From this data a first effective dose of 1 mg kg^{-1} 5-HT was chosen. As a dose ranging study an n number of 3 was appropriate and complied with the 3R's (Replacement, Reduction and Refinement). Additionally statistical analysis with an n of 3 was not possible.

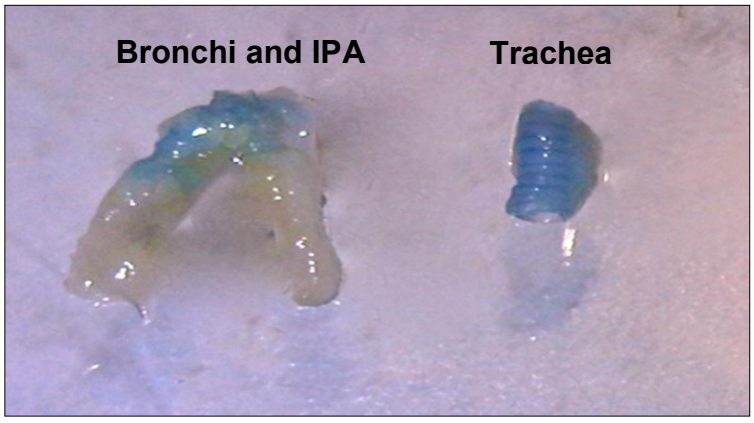


Figure 3.1. Photograph of mouse airway tissue illustrating the effects of intravenous 5-HT on microvascular leak.

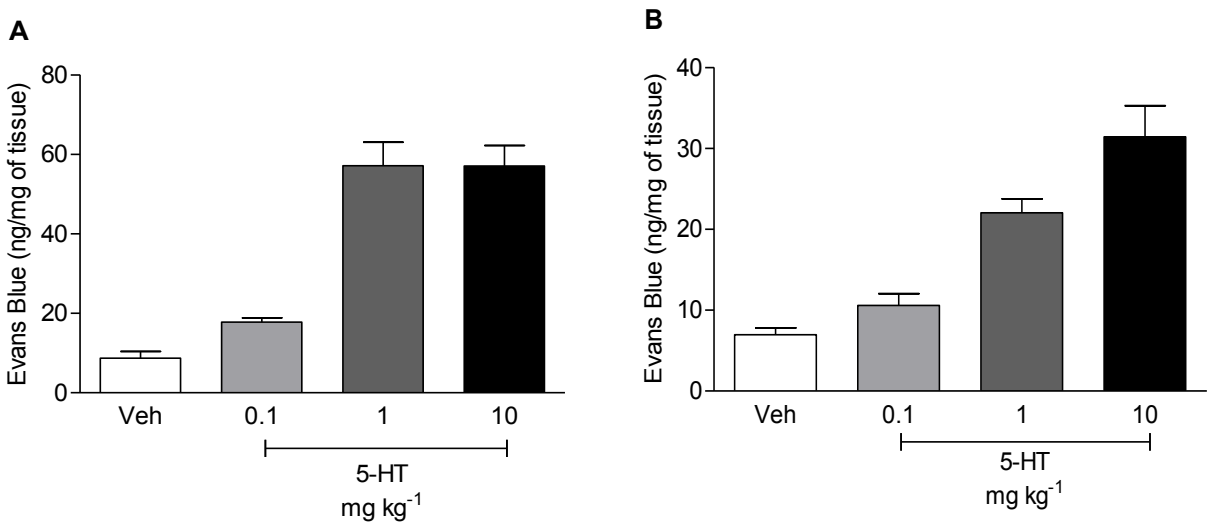


Figure 3.2. Effect of 5-HT (0.1, 1 and 10 mg kg^{-1} i.v) after 5 minutes on MVL into (A) the trachea or (B) the bronchi and intra-pulmonary airways (IPA) of mice. Data expressed as mean ± s.e.m of the concentration of Evans blue dye (ng mg^{-1} of tissue). *n=3.*

A non-selective 5-HT receptor antagonist, methysergide, was then used to determine whether 5-HT was inducing MVL via activation of 5-HT receptors. Figure 3.3 clearly shows that methysergide is able to significantly inhibit MVL induced by the sub-maximal dose of 5-HT (1 mg kg^{-1}).

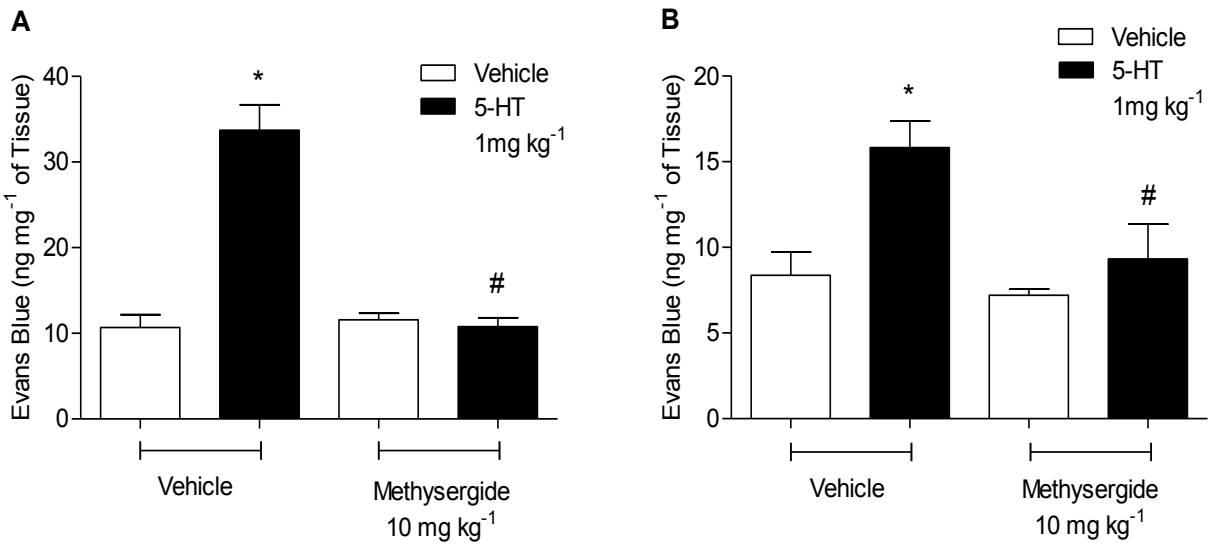


Figure 3.3. Effect of methysergide (10 mg kg^{-1} i.v; 5 minutes) on 5-HT (1 mg kg^{-1} i.v; 5 minutes) induced MVL into (A) the trachea or (B) the bronchi and intra-pulmonary airways of mice. Data expressed as mean ± s.e.m of the concentration of Evans blue dye (ng mg^{-1} of tissue). *n=6. Unpaired t test for parametric data. * p<0.05 representing a significant increase from vehicle control and # p<0.05 a significant decrease from treatment control.*

3.3.2. Dose Response of PGE_2 - induced airway MVL in Mice

Intravenous administration of PGE_2 for 5 minutes (0.1, 0.3, 1, 3 and 10mg kg^{-1}) caused only minor increases in MVL in the trachea and bronchi and IPA of mice (Figure 3.4). Although not significant, PGE_2 induced MVL in a dose dependent manner in the trachea. The positive control, 5-HT produced a significant increase in MVL in both the trachea and bronchi and IPA further validating this model.

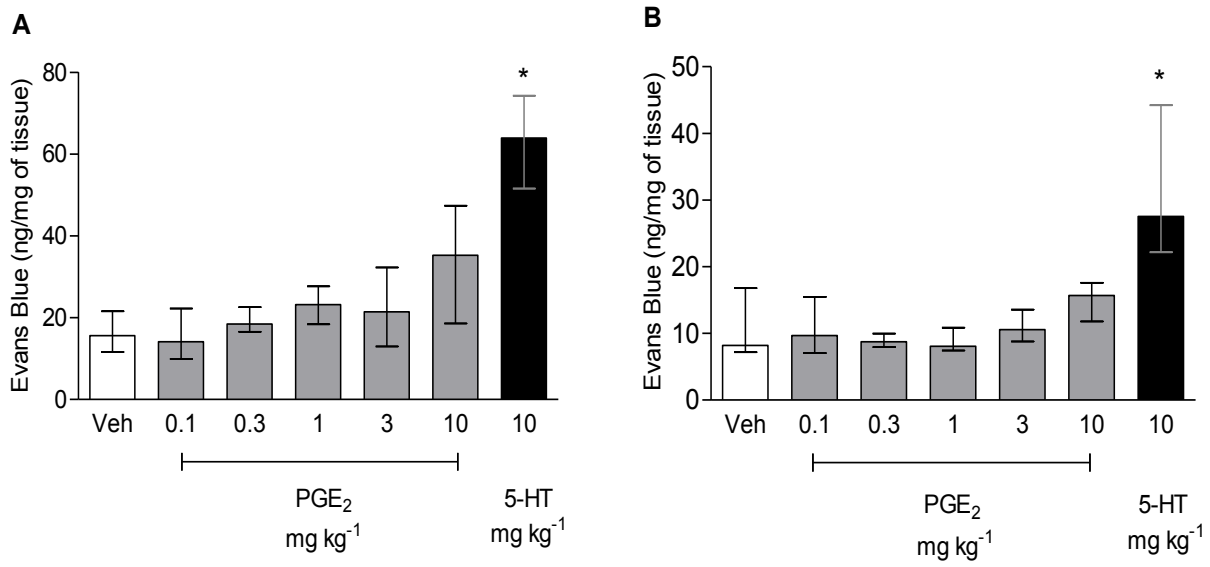


Figure 3.4. Effect of PGE_2 (0.1, 0.3, 1, 3 and 10 mg kg^{-1} i.v; 5 minutes) or 5-HT (10 mg kg^{-1} i.v; 5 minutes) on MVL into (A) trachea or (B) bronchi and intra-pulmonary airways of mice. Data expressed as median, with interquartile range, of the concentration of Evans blue dye (ng mg^{-1} of tissue). *n=6. One-way ANOVA non-parametric Kruskal Wallis test. * p<0.05.*

3.3.3. Time Course of PGE_2 - induced airway MVL in Mice

To determine whether PGE_2 could induce greater MVL after a longer period of time, the exposure time was increased from 5 minutes to 30 minutes. After this length of time PGE_2 significantly increased extravasation of Evans blue dye into the trachea as well as the bronchi and IPA, which is comparable to the increase seen with the 5 minute time point (Figure 3.5). Additionally, there was no difference in MVL in the vehicle treated group over time.

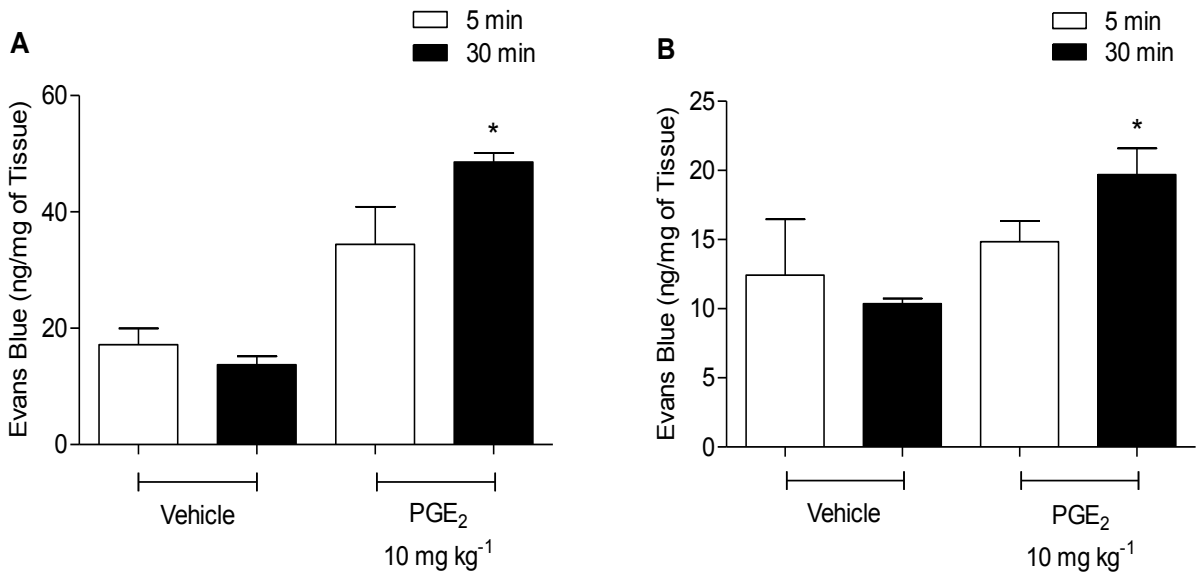


Figure 3.5. Effect of PGE_2 (10 mg kg^{-1} i.v) on MVL after 30 minutes (n=5) compared to 5 minutes (n=6), into (A) trachea or (B) bronchi and intra-pulmonary airways of mice. Data expressed as mean ± s.e.m of the concentration of Evans blue dye (ng mg^{-1} of tissue). *n=5-6. Unpaired t test for parametric data. *p<0.05.*

A dose response to intravenous PGE_2 (0.1, 0.3, 1, 3, 10 mg kg^{-1}) was repeated using the 30 minute time point and this induced an enhanced dose dependent increase in MVL in both the trachea and the bronchi and IPA (Figure 3.6). From this a sub maximal dose of 3 mg kg^{-1} was chosen. Again, as this was a dose ranging study an n number of 3-4 was appropriate and complied with the 3R's. Additionally statistical analysis with an n of 3-4 was not possible. What is also interesting to note is that in general, the responses to PGE_2 are more pronounced and higher in the tracheal segment in comparison to the lower airways. However, as seen with 5-HT this could be due to variation between the bronchi and the IPA.

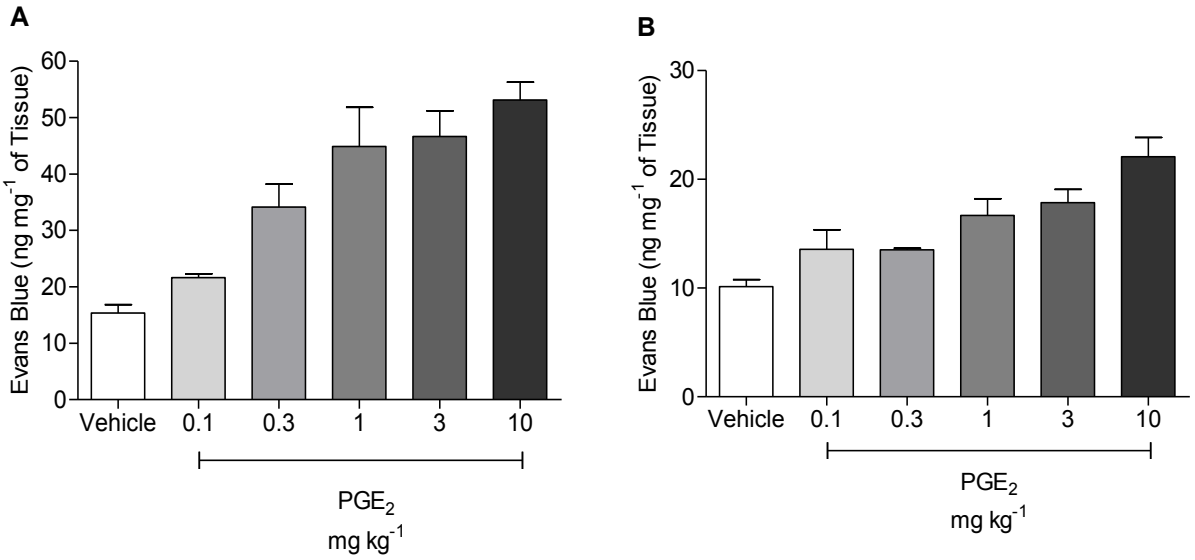


Figure 3.6. Effect of PGE_2 (0.1 – 10 mg kg^{-1} i.v) on MVL after 30 minutes into (A) trachea or (B) bronchi and intra-pulmonary airways of mice. Data expressed as mean ± s.e.m of the concentration of Evans blue dye (ng mg^{-1} of tissue). *n=3-4.*

3.3.4. Effect of Diclofenac on PGE_2-induced airway MVL in Mice

Diclofenac, a non-selective COX inhibitor, was administered prior to PGE_2 administration to block the production and release of endogenous prostanoids to ensure that they were not influencing baseline or PGE_2-induced airway leak. The data clearly shows that diclofenac had no effect on baseline or PGE_2 induced leak in the trachea or bronchi and IPA (Figure 3.7). In both the vehicle and diclofenac treated groups, PGE_2 significantly increased airway MVL.

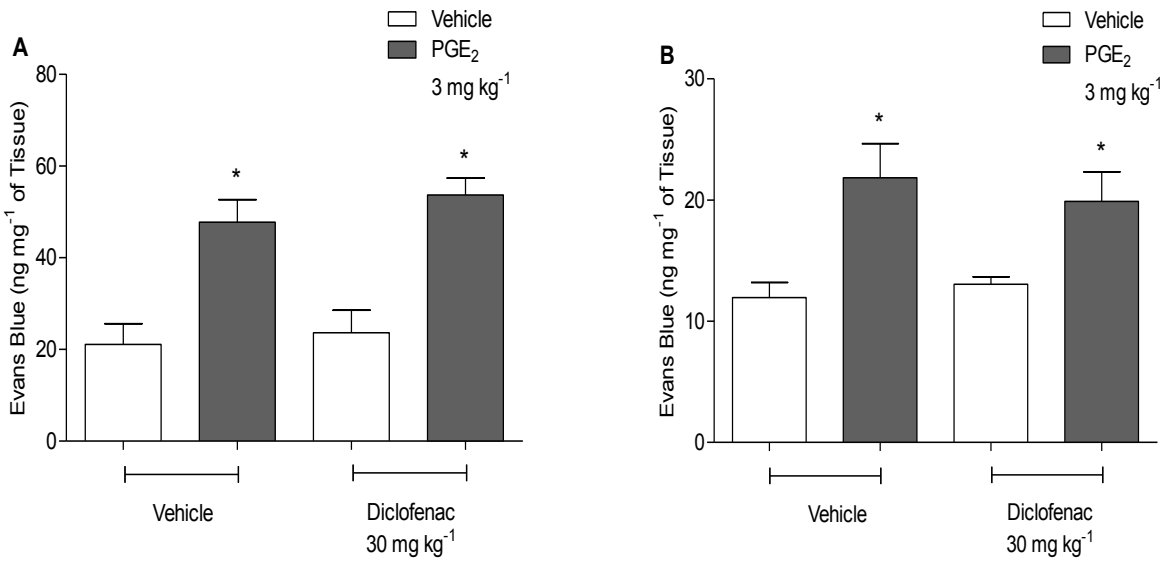


Figure 3.7. Effect of diclofenac (30 mg kg^{-1} in 10 ml kg^{-1} p.o; 1 hour) on MVL in vehicle and PGE_2 (3 mg kg^{-1} i.v; 30 minutes) treated mice. **(A) Trachea and (B) Bronchi and intra-pulmonary airways.** Data expressed as mean ± s.e.m of the concentration of Evans blue dye (ng mg^{-1} of tissue). *n=4. Unpaired t test for parametric data. *p<0.05.*

3.3.5. Effect of intranasal dosing of PGE_2 on airway MVL in Mice

Having established that when given intravenously PGE_2 is able to induce significant MVL in murine airways I wanted to see whether such a response could be replicated via topical administration direct to the airways. This would mimic what may occur in disease when there is an increase of PGE_2 in the airways. Therefore I administered a single intranasal dose of PGE_2 (3 mg kg^{-1}) and measured EB leak after 30 minutes. Interestingly, via this route of administration PGE_2 was able to induce significant MVL and the extent to which was almost identical to that seen with i.v administration (Figure 3.8).

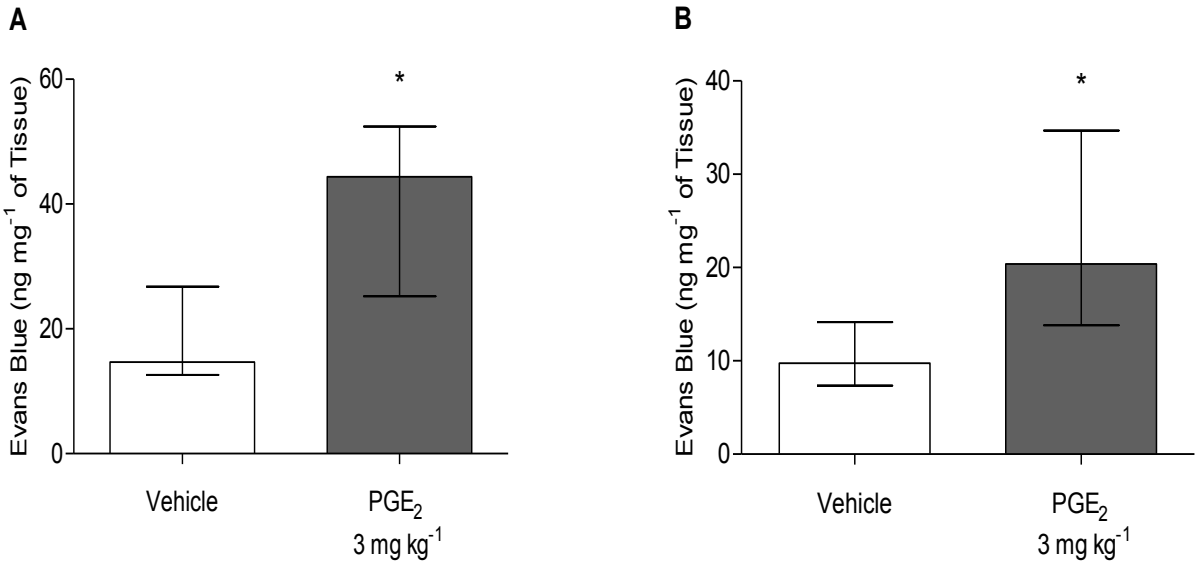


Figure 3.8. Effect of an intranasal dose of PGE_2 (3 mg kg^{-1} i.n) on MVL after 30 minutes into (A) trachea or (B) bronchi and intra-pulmonary airways (IPA) of mice. Data expressed as median, with interquartile range, of the concentration of Evans blue dye (ng mg^{-1} of tissue). *n=6. Non-parametric Mann Whitney test. * p<0.05.significant increase from veh control.*

3.3.6. PGE_2-induced airway MVL in Guinea Pigs

To rule out whether the effect seen with PGE_2 was exclusive to the mouse, I gave the sub maximal dose of PGE_2 to guinea pigs alongside 5-HT, acting as a positive control. When PGE_2 (3 mg kg^{-1}) was given intravenously there was a remarkable increase in airway microvascular leak in the trachea and in the bronchi and intrapulmonary airways (Figure 3.9). This response was comparable to 5-HT.

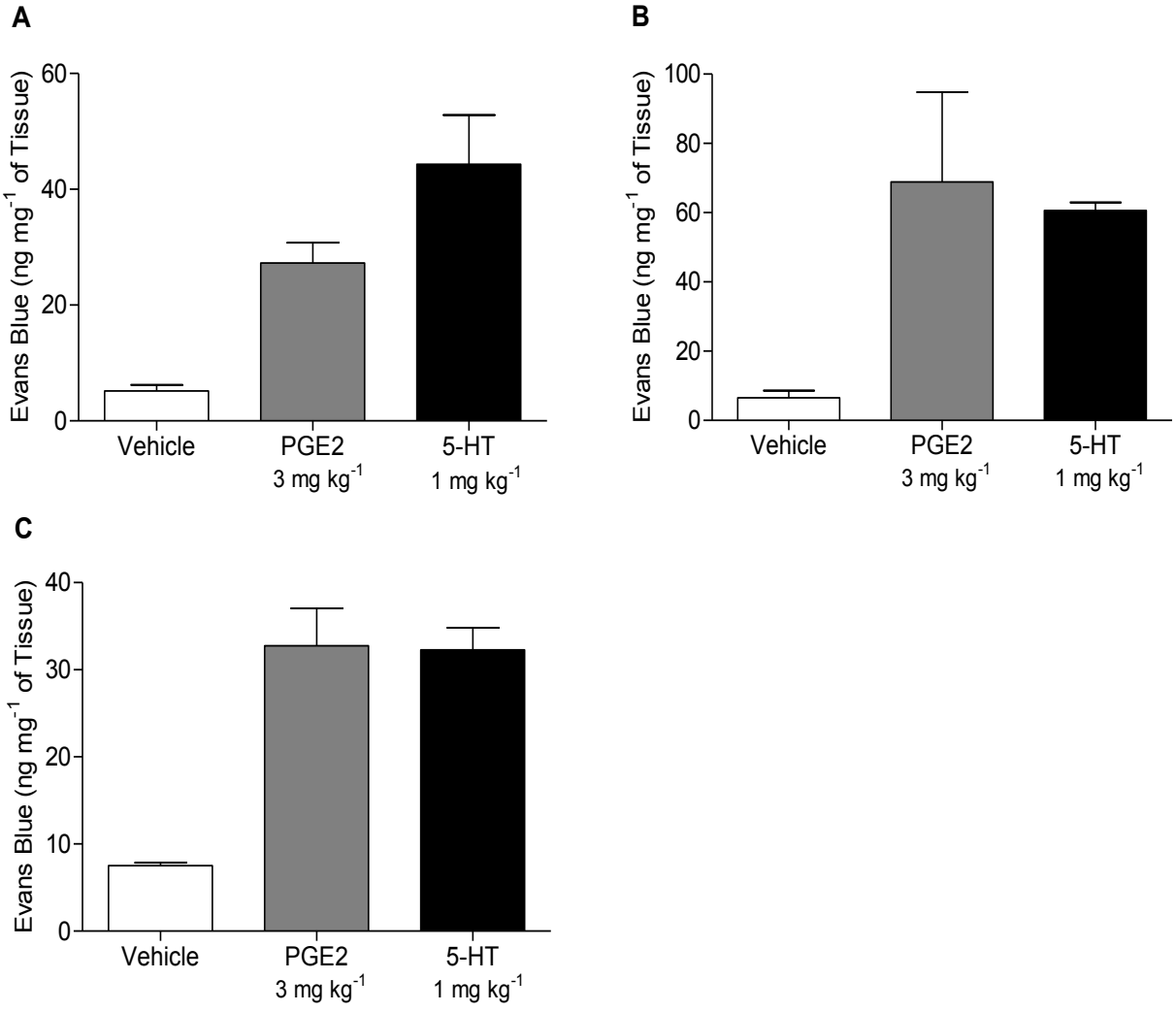


Figure 3.9. Effect of PGE_2 (3 mg kg^{-1} i.v) after 30 minutes or 5-HT (1 mg kg^{-1}) after 5 minutes (respectively) into (A) upper trachea, (B) lower trachea or (C) bronchi and intra-pulmonary airways (IPA) of Dunkin Hartley Guinea Pigs. Data expressed as mean ± s.e.m of the concentration of Evans blue dye (ng mg^{-1} of tissue). *n= 3*

3.3.7. Effect of PGE_2 on Systemic Blood Pressure in Mice

Following PGE_2 administration there was little change in the systemic blood pressure, with only a small decrease apparent (Figure 3.10). This is different to the effects seen with the TP and IP agonists. As expected the TP agonist U46619 caused a rapid and substantial increase in blood pressure compared to the vehicle response, while the IP agonist Iloprost had a completely opposite effect, demonstrating an almost instant drop in the mean arterial pressure. While the effect of U46619 seemed to be relatively transient, the decrease seen with Iloprost appeared to be sustained (Figure 3.10).

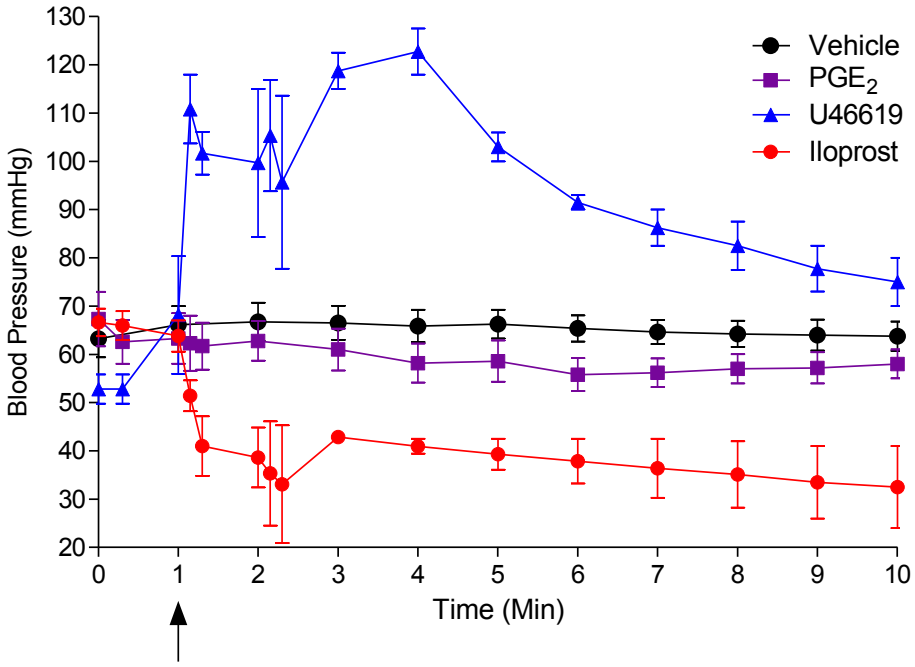


Figure 3.10. Effect of PGE_2, Iloprost and U46619 (3 mg kg^{-1} i.v) on the mean arterial blood pressure of C57BL/6 mice. Following carotid artery cannulation mice were given an i.v dose of either PGE_2, Iloprost, U46619 or vehicle and systemic blood pressure measured and recorded over a 20 minute period. Arrow indicates time of administration. *n=3-4*.

3.4. Discussion

The aim of this chapter was to characterise a method for measuring airway microvascular leak *in vivo* using Evans Blue dye and to establish the effect of prostaglandin E_2 in both mouse and guinea pig airways.

Airway microvascular leakage and plasma exudation are prominent features in both asthma and COPD representing signs of airway inflammation (Paredi & Barnes, 2009). Many mediators (e.g. cysteinyl leukotrienes) are capable of inducing this effect when released in response to an inflammatory insult in the airway. These inflammatory mediators act upon the endothelium of post capillary venules to open intercellular gaps. This effect causes plasma to 'leak' out into extravascular sites (Reynolds *et al*, 2002; Greiff *et al*, 2003). Measuring this event *in vivo* provides a specific index representing to what degree the airway mucosa has been affected by the inflammatory process of a mediator (Greiff *et al*, 2003). Events such as these have been associated with the development and exacerbations of airway inflammation, thus modulation of this response represents a key pathway in developing new treatments. What I wanted to investigate was whether prostaglandin E_2 (PGE_2) was involved in mediating this response.

PGE_2 is a ubiquitous eicosanoid that has been implicated in the pathogenesis of both asthma and COPD. It exerts its biological effects via activation of four cell-surface GPCRs, termed $EP_{1\text{-}4}$, encoded for by the genes *Ptger1-4* (Coleman *et al*, 1994). Increased levels of PGE_2 have been reported in BAL fluid and plasma of asthma patients (Brightling *et al*, 2000; Birring *et al*, 2003; Long *et al*, 2004; Sastre *et al*, 2008), and enhanced levels in the condensate of COPD patients (Montushi *et al*, 2003; Chen *et al*, 2008; Antczak *et al*, 2012). Additionally, increased levels of PGE_2 have been observed in the BAL fluid of allergen-sensitised mice (Herrerias *et al*. 2009). However, its role in airway inflammation remains varied and inconclusive and there appears to be a fine balance between the pro and anti-inflammatory effects of this prostanoid in human airways.

Before investigating the effect of PGE_2, however, I needed to determine whether the mouse was a suitable species to model MVL in. Therefore I used 5-hydroxytryptamine (5-HT) as a tool for validating this model, as previous reports

have shown potent effects in rats (Hele *et al*, 2001). Here, 5-HT induced a dose related increase in MVL in both the trachea and the bronchi and IPA of mice. However, from the photograph illustrating a typical EB response following 5-HT, it appears that the leak in the IPA is much less than what occurred in the bronchi. This effect may mask a larger increase following 5-HT. Furthermore, I determined that 5-HT was acting via 5-HT receptors by using a non-selective 5-HT receptor antagonist methysergide (Hele *et al*, 2001).

Once the model of MVL was successfully validated in the mouse, the effects of PGE_2 were then investigated. In an initial study PGE_2 produced only a moderate increase in MVL, using timings taken from the 5-HT study. This was evident in both the trachea and the bronchi and IPA, where only a marginal dose response relationship was obvious. The effect of PGE_2 on airway microvascular leak has not been shown previously, and so timings of administration were based upon previous methods within the group and the literature. However, increasing the time post PGE_2 administration from 5 minutes to 30 minutes produced a greater increase in MVL in the airways of mice. Additionally, a dose response relationship was more evident using this time point in both the trachea and bronchi and IPA. Five minutes was based on the data from 5-HT and it is interesting that it was after 30 minutes that PGE_2 was able to cause significant microvascular leak as PGE_2 has been shown to have a short biological half-life, around 30 seconds (Kozak *et al*, 2001; Brochhausen *et al*, 2009). To fully elucidate why this may be the case would take a considerable amount of time. One explanation may be that PGE_2 is causing the release of other mediators that are involved in inducing microvascular leak. Aso *et al* (2012) demonstrated that *in vitro* activation of human pulmonary microvascular endothelial cells by PGE_2 enhanced IL-8 production. IL-8 itself has subsequently been shown to promote endothelial permeability, as well as other cytokines such as TNF-α and IL-1β (Yu *et al*, 2013). In addition, LPS effects on the endothelium may be occurring secondary to the release of TNF-α, IL-1β and IL-8 (Horgan *et al*, 1993; Ishii *et al*, 1993). Furthermore, PGE_2 has been demonstrated in several studies to stimulate the production and release of the pro-angiogenic mediator VEGF (Pai *et al*, 2001; Bradbury *et al*, 2005). The potent permeability enhancing effects of VEGF are well documented both *in vivo* and *in vitro* (Esser *et al*, 1998; Feng *et al*, 1999; Paul *et al*, 2001; Gavard & Gutkind 2006). One other possible explanation may be a positive

feedback loop amplifying the initial signal and sustaining the inflammatory response, whereby activation of EP receptors by PGE_2 leads to production and release of PGE_2. Such positive feedback loops have been shown in animal models of chronic inflammatory diseases (Aoki *et al*, 2011). However, I demonstrated that blocking the production of endogenous prostanoids with the COX inhibitor diclofenac did not impact on PGE_2-induced MVL. It is therefore unlikely that a positive feedback loop is occurring in this situation.

Having demonstrated that i.v. dosing of PGE_2 caused dose dependent increases in airway MVL, an alternative route of administration was then investigated. The data showed that an intranasal dose of PGE_2 was just as effective at inducing airway MVL in the mice as with i.v. dosing which shows that the effects we are seeing are not specific to a certain route of administration.

As previously mentioned, the majority of research into airway MVL has been achieved using larger species, including the rat and the guinea pig. I wanted to rule out that what was being observed with PGE_2 was not exclusive to the mouse. In the guinea pig PGE_2 produced a large, substantial increase in airway MVL that was comparable to that seen with 5-HT. Both the mouse and the guinea pig produced similar profiles in the magnitude of their responses demonstrating that the effect of PGE_2 is translated across species.

The effect of PGE_2 on airway microvascular leak has not been extensively investigated, especially *in vivo*. A lot of research appears to be focused on cutaneous inflammation in relation to prostanoids, particularly PGE_2 (Takagi *et al*, 1987; Goulet *et al* 2004; Kabashima *et al*, 2007). Goulet *et al* (2004) examined the effect of arachidonic acid on mouse ear tissue and established a primary role for PGE_2 in mediating the oedema formation in this model. Prior to this, Takagi *et al* (1987) observed, both pharmacologically and histologically, that in a rat air pouch model PGE_2 increases vascular permeability via the degranulation of mast cells. Data from *in vitro* investigations is often contradictory, with some reporting barrier enhancing effects and others describing an increase in permeability following PGE_2 (Moreno 2009; Konya *et al*, 2013). These opposing effects are most likely due to the fact that PGE_2 activates four different receptor subtypes ($EP_{1\text{-}4}$). Investigation into

the roles of each EP receptor in mediating PGE_2-induced leak is described in the following chapter (Chapter 4).

As described above small changes in blood flow may impact on MVL, especially in the mouse where the bronchial circulation is small, so it could be possible that the PGE_2-induced MVL may be caused by vasodilation and an increase in blood flow to the airways. Rogers *et al* (1990) suggested that the vasodilator prostaglandins PGE_2 and PGI_2 are able to potentiate MVL in the skin by increasing blood flow. In contrast to the rapid and substantial drop in blood pressure (vasodilation) seen with the IP agonist Iloprost, PGE_2 appeared to have very little effect on the systemic blood pressure in mice. Although this does not represent local changes in blood pressure and flow (i.e. in the bronchial circulation) it does suggest that there is not a clear and defined association between vasodilation and increased airway microvascular leak in this study. Zhang *et al* (2000), however, did observe that i.v. administration of PGE_2 caused a significant decrease in mean arterial pressure (MAP) in wild type (C57BL/6) mice at doses lower than what was used in this present study. Audoly *et al* (1999) reported almost identical findings, where PGE_2 caused a marked and immediate fall in blood pressure, where maximal blood pressure reduction occurred within the initial 20 seconds after PGE_2 infusion (using 129/SvEv mice). However, as already mentioned, deciphering which EP receptor is mediating this effect will further elucidate the role PGE_2 is playing.

To summarise, this chapter has demonstrated that the mouse is a suitable species to investigate the effects of PGE_2 on airway MVL and most importantly that PGE_2 is able to induce substantial and significant MVL in the trachea and the bronchi and IPA of mice and guinea pigs. Additionally this effect appears to be distinct from any changes in systemic blood pressure as using the same dose produced no significant increase or decrease in blood pressure. The data presented in this chapter therefore allows me to accept the hypothesis stated in section 3.1.1. The next step is to decipher which of the four EP receptors ($EP_{1\text{-}4}$) is mediating this response (Chapter 4).

Chapter Four

Identifying the EP Receptor Mediating PGE_2-induced MVL in mouse airways

4. Identifying the EP Receptor Mediating PGE_2-induced MVL in mouse airways

4.1. Rationale

In chapter 3 I demonstrated that the mouse was a suitable model to investigate airway microvascular leak using Evans Blue dye and that PGE_2 induced significant extravasation in both the trachea and bronchi and IPA. PGE_2 acts via the activation of four different receptor subtypes, termed $EP_{1\text{-}4}$, and has already demonstrated a wide range of effects in the airways. Therefore it seemed logical to determine which EP receptor may be mediating this airway microvascular leak in the mouse.

4.1.1. Chapter Hypothesis

"PGE_2 activates a specific EP receptor to mediate microvascular leak in mouse airways"

4.1.2. Aims

- Investigate the effect of PGE_2 in individual EP receptor knock out mice ($EP_{1\text{-}4}$).
- Investigate the effect of selective EP receptor agonists from ONO Pharmaceuticals.
- Charactise selective EP_2 (PF-04418948) and EP_4 (ER-819762) receptor antagonists against relevant selective EP agonists.
- Use selective EP antagonists against PGE_2 to confirm KO data.

4.2. Methods

The following experiments were carried out as described in chapter 2. Details of individual experiments are described below.

4.2.1. The Effect of PGE_2-induced MVL in EP Receptor Knock-Out Mice

The same microvascular leakage protocol was used as before with a single sub maximal dose of PGE_2 (3 mg kg^{-1}) or vehicle (1% ethanol in saline) used as well as the 30 minute time point. Tail tips were also taken to determine genetic status by genotyping.

Additionally, the response to 5-HT in EP receptor knock-outs (*ptger2*$^{-/-}$ *and ptger4*$^{-/-}$) and their wild types was investigated to ensure their lack of response to PGE_2 was not due to an overall disruption in MVL. A sub maximal dose of 5-HT (1 mg kg^{-1}) was used as a known-inducer of MVL.

4.2.2. Effect of ONO-D1-004, ONO-AE1-259, ONO-AE-248 and ONO-AE1-329 on MVL

To parallel the effects seen in the EP receptor deficient mice, the response to selective EP agonists was studied. Following EB administration, animals were given selective EP receptor agonists (Okada *et al*, 2000; Suzawa *et al*, 2000; Cao *et al*, 2002); ONO-D1-004 (EP_1), ONO-AE1-259 (EP_2), ONO-AE-248 (EP_3), ONO-AE1-329 (EP_4) or PGE_2 (3 mg kg^{-1}) i.v at 4 ml kg^{-1} or Vehicle (1% ethanol in saline). The 30 minute time point was used for all agonists.

4.2.3. Investigating the EP_2 receptor in airway MVL

Having shown using EP receptor knockout mice that the EP_2 receptor appears to have a role in mediating airway MVL I wanted to validate an EP_2 receptor antagonist

for use *in vivo* to confirm this data. First of all I carried out a dose response to ONO-AE1-259 (EP_2 agonist) to establish whether 3 mg kg^{-1} was a suitable dose to use. The doses used were 0.1, 0.3, 1 and 3 mg kg^{-1} (i.v at 4 ml kg^{-1}) and unfortunately I was not able to go higher due to compound availability.

The EP_2 antagonist PF-04418948 was used in subsequent studies and first dosed orally at 100 mg kg^{-1} or vehicle control (0.5% methylcellulose, 0.2% tween 80 in water) one hour prior to ONO-AE1-259 administration (3 mg kg^{-1} in 4 ml kg^{-1} i.v). The dosing was based on data shown by Forselles *et al* (2011), however, I decided to go higher as their top dose (30 mg kg^{-1}) was unable to block a PGE_2-induced response and the EP_2 agonist they used (Butaprost) is less potent than ONO-AE1-259.

An alternative route of antagonist administration (intraperitoneal; i.p.) was then investigated. Here mice were dosed with PF-04418948 at 3, 10, 30 or 100 mg kg^{-1} (10 ml kg^{-1} i.p.) or vehicle (0.5% methylcellulose, 0.2% tween 80 in saline) one hour before ONO-AE1-259 administration (3 mg kg^{-1} in 4 ml kg^{-1} i.v.)

It must be noted that PF-04418948 did not go into solution in the vehicles being used and was therefore delivered as a suspension.

4.2.4. Investigating the EP_4 receptor in airway MVL

4.2.4.1. Rat Tracheal Organ Bath Preparation

Before beginning *in vivo* work, I wanted to validate a reported EP_4 antagonist, ER-819762 (Chen *et al*, 2010) using a functional bioassay. This antagonist was chosen as it proved effective in their *in vivo* model. To do this I used rat trachea organ bath set up as outlined in chapter 2.6. PGE_2-induced relaxation of induced tone in rat tracheal tissue has previously been shown to be mediated via the activation of EP_4 receptors (Lydford and McKechnie, 1994; Buckley *et al*, 2011; Benyahia *et al*, 2012) and so was used as an assay system to assess functionality at this receptor subtype.

In these experiments, carbachol (CCh; 1 mM) was used to induce a stable contraction of the tissues. The tissues were then incubated with vehicle or ER-

819762 (100 nM to 10 μM) for 30 minutes before cumulative concentration–response curves were performed to ONO-AE1-329 (10^{-9} to 10^{-5} M). At the end of the experiment, the non-specific PDE inhibitor, papaverine (100 μM), was added to assess the maximum capacity for relaxation of each tissue.

4.2.4.2. In Vivo Characterisation

Next a dose response to ONO-AE1-329 (EP_4 agonist) was carried out in C57BL/6 mice. The doses used were 0.1, 0.3, 1 and 3 mg kg^{-1} (i.v at 4 ml kg^{-1}) and unfortunately I was not able to go higher due to compound availability.

The EP_4 antagonist ER-819762 was used in subsequent studies and was first dosed orally at 100 mg kg^{-1} (Chen *et al*, 2010) or vehicle control (0.5% methylcellulose, 0.2% tween 80 in water) one hour prior to ONO-AE1-329 administration (3 mg kg^{-1} in 4 ml kg^{-1} i.v). Dosing was based on data from Chen *et al* (2010).

An alternative route of antagonist administration was then investigated (intraperitoneal; i.p.). Here mice were dosed with ER-819762 at 3, 10, 30 or 100 mg kg^{-1} (10 ml kg^{-1} i.p.) or vehicle (0.5% methylcellulose, 0.2% tween 80 in saline) one hour before ONO-AE1-329 administration (3 mg kg^{-1} in 4 ml kg^{-1} i.v.)

Doses were initially based on those used by Chen *et al* (2010). Additionally, it must be noted that ER-819762 did not go into solution in the vehicles being used and was therefore delivered as a suspension.

4.2.5. Effect of the EP_2 and EP_4 receptor antagonists against PGE_2-induced MVL in mice

Having determined suitable doses and route of administration for the EP_2 and EP_4 receptor antagonists, the next step was to try and replicate the findings from the EP receptor knockout mice. Here mice were dosed with PF-04418948 (10 mg kg^{-1} at 10 ml kg^{-1} i.p), ER-819762 (30 mg kg^{-1} at 10 ml kg^{-1} i.p) or vehicle (0.5%

methylcellulose, 0.2% tween 80 in saline) one hour prior to PGE_2 administration (3 mg kg^{-1} at 4 ml kg^{-1} i.v.).

Additionally, in a separate study, mice were dosed with both PF-04418948 and ER-819762 simultaneously one hour before PGE_2.

4.2.7. Genotyping of KO Mice

DNA was extracted from tail tips (0.5-1cm) of wild type and prostanoid receptor knockout mice using Quanta Biosciences Extracta DNA prep for PCR kit. The purity and integrity of the DNA samples was assessed using a PowerWave XS Microplate Spectrophotometer (Biotek, Bedfordshire, UK). PCR reactions were run using mouse genomic DNA primers that had been previously optimised and validated. The PCR reaction contained 1x Green GoTaq Flexi buffer, 0.2mM dNTPs, 2mM $MgCl_2$, 1.25µl enzyme (GoTaq polymerase), 10pmol forward and 10pmol reverse primers, and a volume of DNA (50ng) or Nuclease-free water for control, made up to 25µl with Nuclease-free water. Samples were then run on a Taqman machine under the conditions detailed in table 4.1. EP_1 and EP_2 primers were run in singleplex reactions, so wild type and knock-out primers had to be run as separate reactions, while EP_3 and EP_4 primers were run as multiplex reactions, so wild type and knock-out primers could be run in the same PCR reaction.

The PCR products and DNA ladder (Hyperladder II or IV) were run on a 2% agarose gel in Tris Borate EDTA (TBE) buffer containing 5µl per 100ml Safeview at 80V for about 1 hour. The gel was then visualised under ultra-violet light and photographed using the Molecular Imager® ChemiDoc™ XRS System with Image Lab™ software (Bio-Rad, UK).

Table 4.1. Optimised PCR conditions for genotyping prostanoid receptor KO mice. *EP_1 and EP_2 were run as singleplex (indicated by a "-" where that primer isn't included so no band expected) while EP_3 and EP_4 were run as multiplex reactions.*

Receptor	PCR Conditions			Expected Primer Bands (bp)	
	Denaturing	Annealing	Extension	Wild Type	Knockout
EP_1 (w.t) for/rev	94ºC / 30 s	59ºC / 30 s	68ºC / 120 s	1000	2100
EP_1 (KO) neo/rev	95ºC / 30 s	60ºC / 30 s	68ºC / 90 s	-	1000
EP_2 (w.t) for/rev	94ºC / 30 s	58ºC / 30 s	68ºC / 60 s	650	-
EP_2 (KO) neo/rev	94ºC / 30 s	65ºC / 30 s	75ºC / 60 s	-	470
EP_3 for/rev/neo	95ºC / 30 s	65ºC / 30 s	75ºC / 60 s	350	550
EP_4 for/rev/neo	95ºC / 30 s	65ºC / 30 s	75ºC / 60 s	450	700

4.2.8. Statistical Analysis

All data was analysed using GraphPad Prism 5. Parametric data was expressed as mean $\pm$ s.e.m and non-parametric data presented as the median with the interquartile range. Multiple measurements were analysed by one-way ANOVA, non-parametric Kruskal-Wallis test and post-hoc comparisons were performed by Dunn's multiple comparison test, comparing selected columns to a control. Additionally, an unpaired t test and/or non-parametric Mann Whitney test, with two-tailed p values, were carried out to determine statistical significance between two groups. Differences were considered statistically significant if $p<0.05$. The figures have been graphically presented on a range-specific axis. Analysis of the organ bath work is detailed in chapter 2 (section 2.6.3.3.).

4.3. Results

4.3.1. The Effect of PGE_2-induced MVL in EP Receptor Knock-Out Mice

The effect of PGE_2 (3 mg kg^{-1}) on MVL was compared in WT and mice deficient in individual EP receptors (*Ptger1-4*$^{-/-}$). The data has demonstrated a significant increase in MVL in wild-type, *Ptger1*$^{-/-}$ and *Ptger3*$^{-/-}$ mice in response to PGE_2. However, a substantial and significant reduction in PGE_2-induced MVL was shown in *Ptger2*$^{-/-}$ and *Ptger4*$^{-/-}$ mice (Figure 4.1). This was apparent in the trachea and bronchi and IPA.

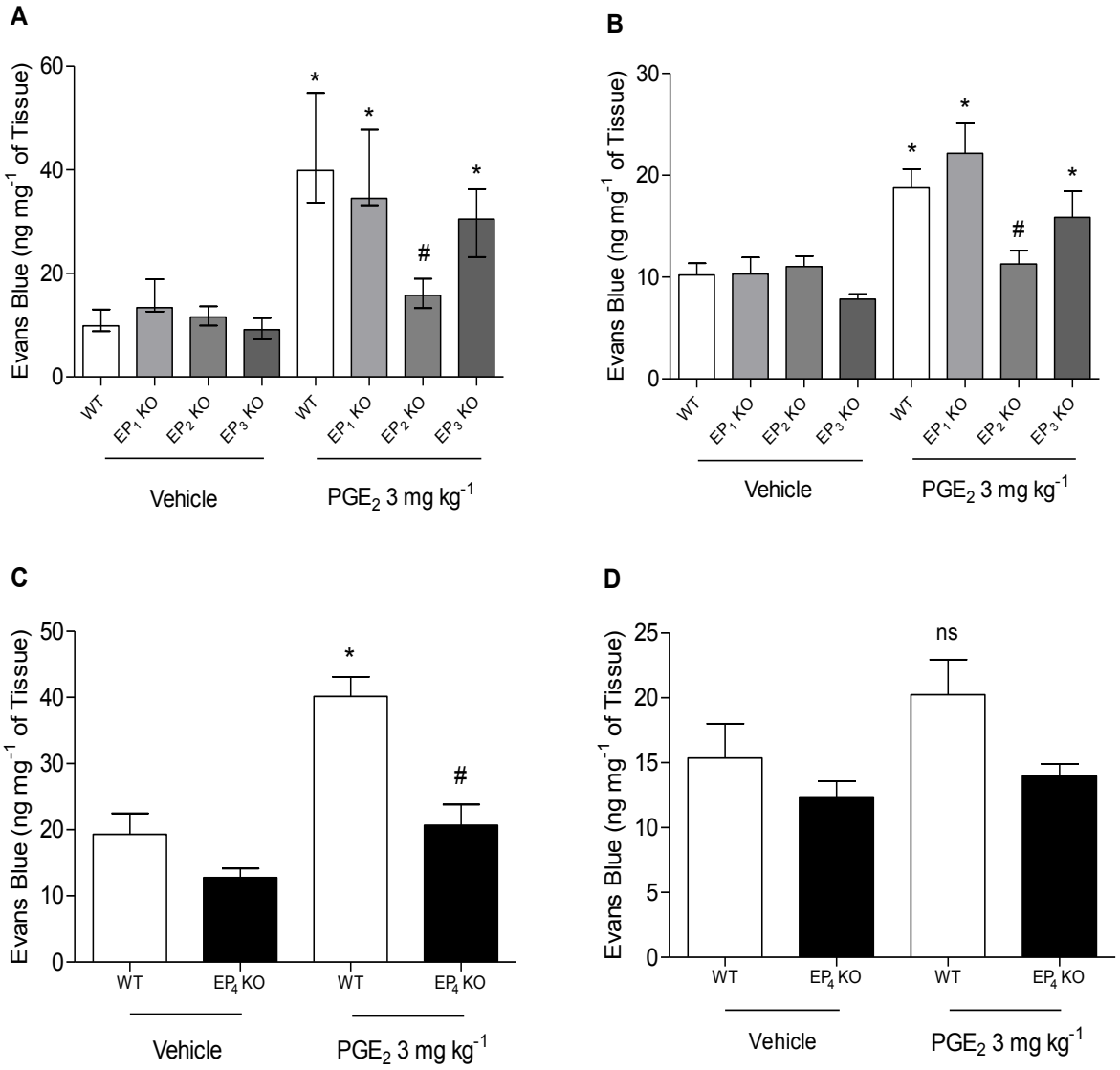


Figure 4.1. Effect of PGE_2 (3 mg kg^{-1} i.v) on MVL after 30 minutes into (A) trachea or (B) bronchi and intra-pulmonary airways (IPA) of wild-type (WT) and EP_{1-3} receptor deficient mice and (C) trachea or (D) bronchi and IPA of WT and EP_4 receptor deficient mice. Non-parametric data expressed as median, with interquartile range, and parametric data expressed as mean ± s.e.m. of the concentration of Evans blue dye (ng mg^{-1} of tissue). *n=6. Non-parametric Mann Whitney test or unpaired t test for parametric data. * p<0.05.significant increase from veh control. # p<0.05 significant decrease from WT. ns = non significant*

To determine whether their lack of response to PGE_2 was not due to an overall disruption in MVL, 5-HT was administered to *Ptger2*$^{-/-}$ and *Ptger4*$^{-/-}$ mice. Here, 5-HT produced a significant increase in MVL in the trachea and in the bronchi and IPA, with no difference seen between the EP-receptor deficient mice and their wild types (Figure 4.2.)

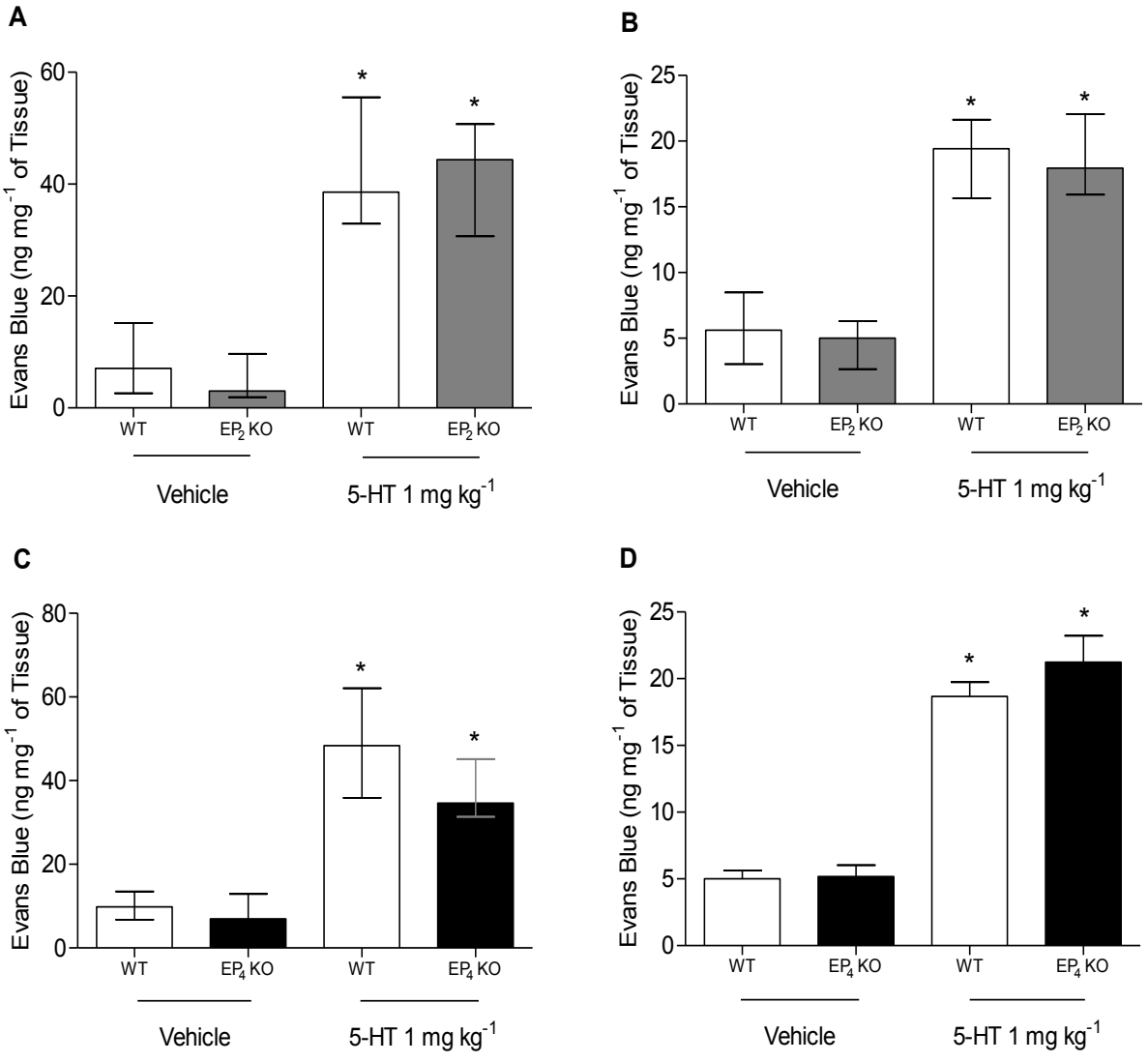


Figure 4.2. **Effect of 5-HT (1 mg kg^{-1} i.v.; 5 minutes) on MVL into (A) trachea or (B) bronchi and intra-pulmonary airways (IPA) of wild-type (WT) and EP_2 receptor deficient mice and (C) trachea or (D) bronchi and IPA of WT and EP_4 receptor deficient mice.** Non-parametric data expressed as median, with interquartile range, and parametric data expressed as mean ± s.e.m. of the concentration of Evans blue dye (ng mg^{-1} of tissue). *n=6. Unpaired t test for parametric data or Non-parametric Mann Whitney test. * p<0.05.significant increase from vehicle control.*

Figure 4.3 shows an example gel from genotyping for the *Ptger3* gene in wild-type and *Ptger3*$^{-/-}$ mouse tail tips. Primers for the wild-type gene produced a band at 400bp while *Ptger3*$^{-/-}$ at 550bp. This confirms that the *Ptger3* gene was present in WT, *Ptger1*$^{-/-}$ and *Ptger2*$^{-/-}$ as primers specific for the WT gene produce a band corresponding to 400bp. In addition, the band at 550bp is specific for the primer sequence of the knock out *Ptger3* gene, thus confirming the *Ptger3*$^{-/-}$ mice were deficient of the correct gene.

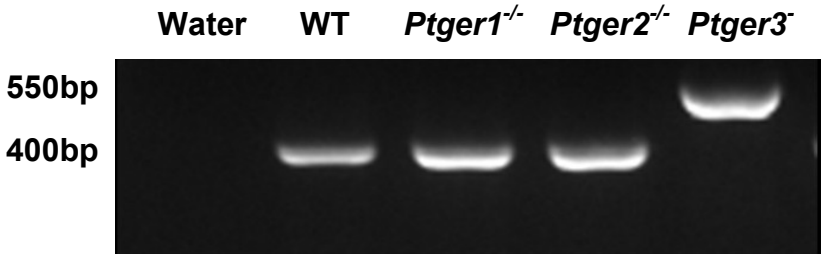


Figure 4.3. Example gel of genotyping for the *Ptger3*$^{-/-}$ mice. Primers for the wild-type gene produced a band at 400bp while *Ptger3*$^{-/-}$ at 550bp.

4.3.2. Effect of ONO-D1-004, ONO-AE1-259, ONO-AE-248 and ONO-AE1-329 on MVL

Having shown an inhibition in PGE_2-induced MVL in EP_2 and EP_4 receptor deficient mice, selective EP receptor agonists were investigated in order to parallel these results. ONO-AE1-259 (EP_2) and ONO-AE1-329 (EP_4) both significantly increased MVL in both the trachea and bronchi and IPA of mice, while ONO-D1-004 (EP_1) and ONO-AE-248 (EP_3) had little or no effect on MVL in either trachea or bronchi and IPA (Figure 4.4).

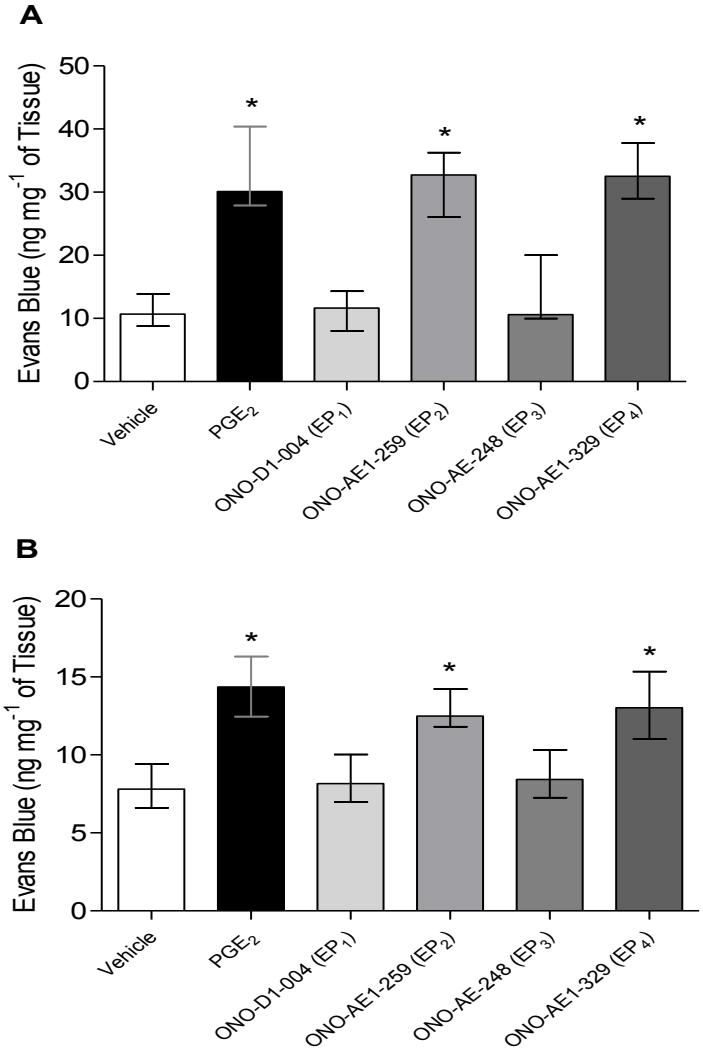


Figure 4.4. Effect of PGE_2, ONO-D1-004 (EP_1), ONO-AE1-259 (EP_2), ONO-AE-248 (EP_3) and ONO-AE1-329 (EP_4) (3 mg kg^{-1} i.v.; 30 minutes) on MVL into (A) trachea or (B) bronchi and intra-pulmonary airways of mice. Data presented as the median, with interquartile range, of the concentration of Evans blue dye (ng mg^{-1} of tissue). *n=6 One-way ANOVA, non-parametric Kruskall-Wallis test with post-hoc comparisons using Dunn's multiple comparison test. * p<0.05.significant increase from vehicle control.*

4.3.3. Investigating the EP_2 receptor in airway MVL

The data shown from the knock-out mice and the EP receptor agonists clearly demonstrates a role for the EP_2 and the EP_4 receptors in mediating airway MVL in mice. Although knock-out mice are excellent tools for investigating the role these receptors play in disease they aren't without their limitations. Therefore, I wanted to confirm this data using selective EP_2 and EP_4 antagonists.

First of all I wanted to establish a dose response with the EP_2 agonist (ONO-AE1-259) to see if 3 mg kg^{-1} was the most suitable dose to use. Due to the quantity of compound available this was the highest dose possible and from figure 4.5 it is evident that 3 mg kg^{-1} induced a significant increase in MVL in the trachea. Therefore I carried on using this dose in the antagonist studies.

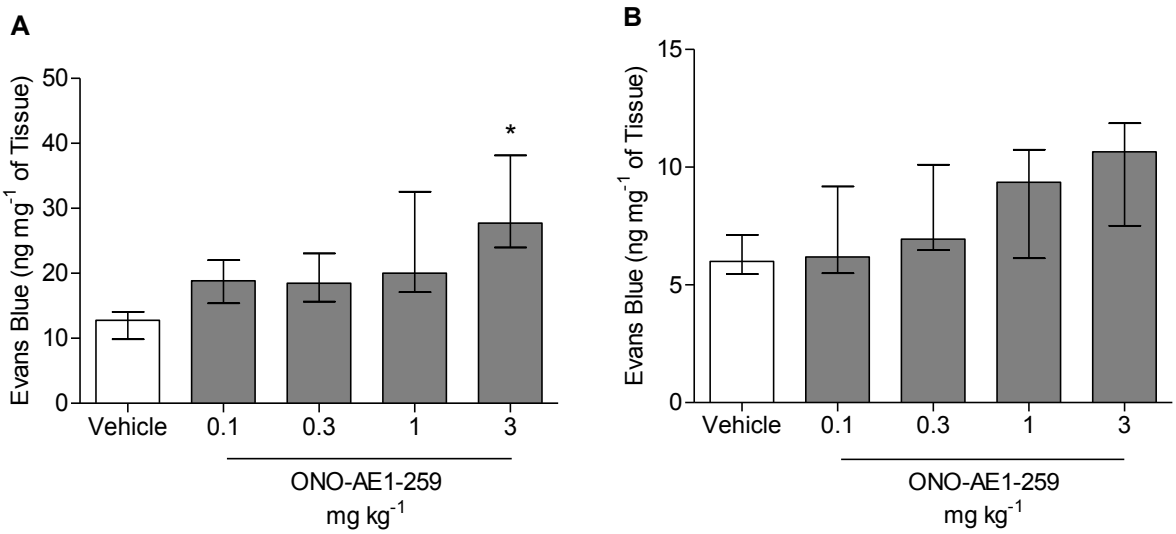


Figure 4.5. Effect of ONO-AE1-259 (EP_2) (0.1, 0.3, 1 and 3 mg kg^{-1} i.v.; 30 minutes) on MVL into (A) trachea or (B) bronchi and intra-pulmonary airways of mice. Non-parametric data presented as median, with interquartile range, of the concentration of Evans blue dye (ng mg^{-1} of tissue). *n=6. One-Way ANOVA non-parametric Kruskall-Wallis test with post-hoc comparisons using Dunn's multiple comparison test. * p<0.05.significant increase from vehicle control.*

Next I wanted to examine what would be the best route of administration for the EP_2 receptor antagonist (PF-04418948). A single oral dose of 100 mg kg^{-1} had no significant effect on airway MVL induced by ONO-AE1-259 (Figure 4.6).

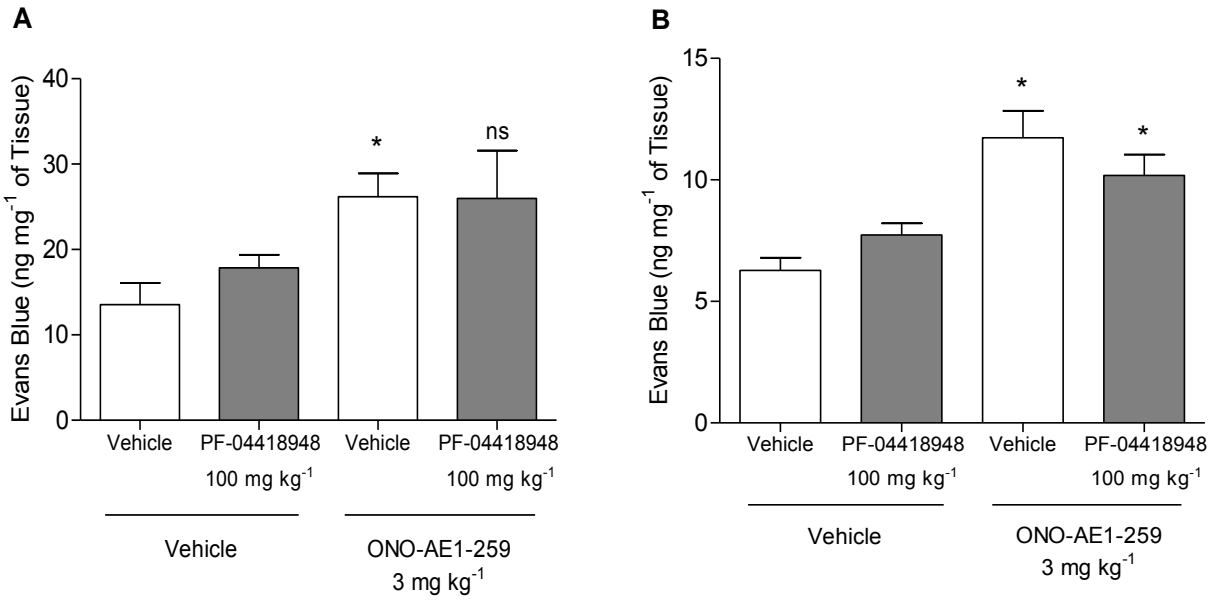


Figure 4.6. Effect of PF-04418948 (100 mg kg^{-1} p.o; 1 hour) on ONO-AE1-259-induced MVL (3 mg kg^{-1} i.v.; 30 minutes) into (A) trachea or (B) bronchi and intra-pulmonary airways of mice. Data expressed as mean ± s.e.m of the concentration of Evans blue dye (ng mg^{-1} of tissue). *n=6. Unpaired t test for parametric data. * p<0.05.significant increase from vehicle control. ns = non significant.*

I then wanted to investigate whether giving this same antagonist by an intraperitoneal injection would yield a better result and inhibit the MVL-induced by ONO-AE1-259. Indeed, as demonstrated in figure 4.7, there appears to be a dose dependent trend in inhibiting MVL with a dose of 30 mg kg^{-1} showing a significant inhibition of EP_2-induced MVL. Therefore, I decided to continue using 30 mg kg^{-1} i.p.in future studies.

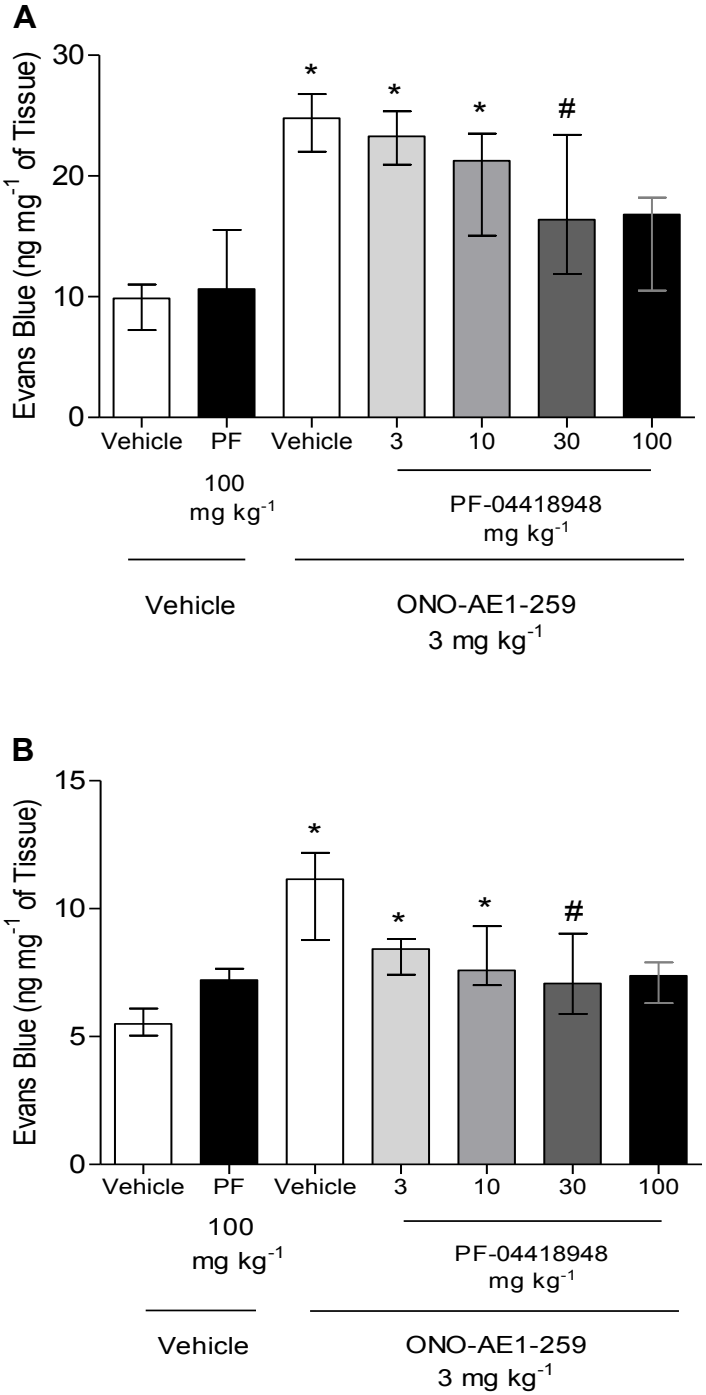


Figure 4.7. Effect of PF-04418948 (3, 10, 30 and 100 mg kg^{-1} i.p.; 1 hour) on ONO-AE1-259-induced MVL (3 mg kg^{-1} i.v.; 30 minutes) into (A) trachea or (B) bronchi and intra-pulmonary airways of mice. Data presented as median, with interquartile range, of the concentration of Evans blue dye (ng mg^{-1} of tissue). *n=6 One-Way ANOVA, non-parametric Kruskall-Wallis test with post-hoc comparisons using Dunn's multiple comparison test. * p<0.05.significant increase from vehicle/vehicle control. # p<0.05 significant decrease from agonist/vehicle control.*

4.3.4. Investigating the EP_4 receptor in airway MVL

Prior to utilising the EP_4 antagonist (ER-819762) I wanted to characterise it *in vitro* first, so I looked at a concentration response against ONO-AE1-329-induced relaxation in an EP_4 receptor mediated system. ONO-AE1-329 caused a concentration-dependent relaxation of pre-contracted rat trachea (38.8% ± 3.4) that was inhibited by the EP_4 receptor antagonist (ER-819762). ER-819762, compared to its vehicle control (0.1% DMSO), produced a graded, parallel rightward shift of the ONO-AE1-329 concentration response curves (Figure 4.8 and Table 4.1).

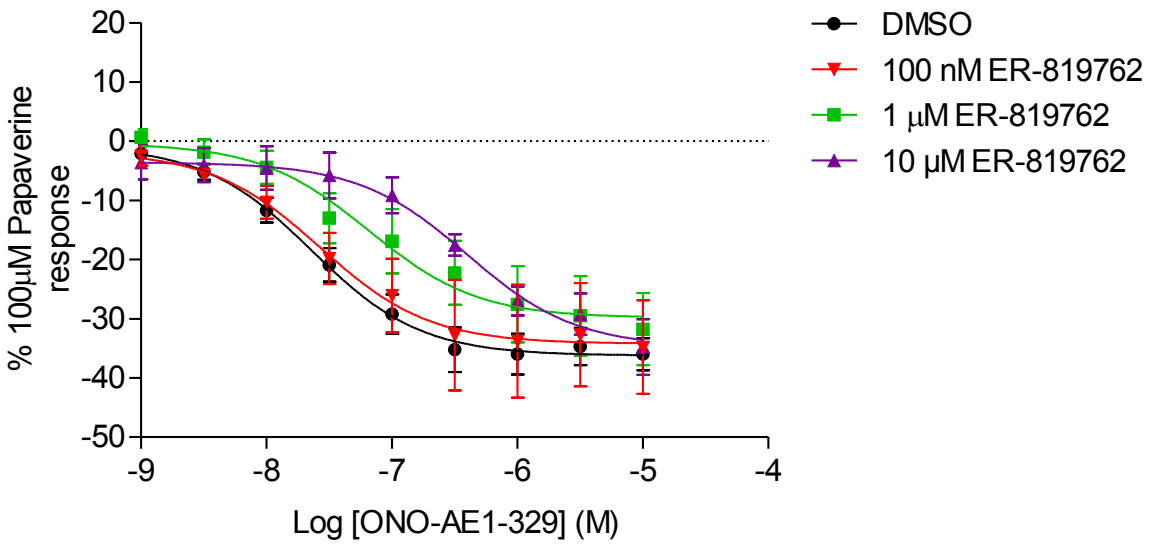


Figure 4.8. Relaxation of rat isolated rat tracheal tissue. Effect of vehicle (DMSO, 0.1%) or ER-819762 (10^{-8} M- 10^{-5} M) on ONO-AE1-329-induced relaxation of carbachol pre-contracted rat tracheal tissue. Data represent mean ± s.e.m. *n=6-13*

Table 4.2. Antagonism of ONO-AE1-329-induced relaxation by ER-819762 in rat trachea.

Antagonist	n	Maximum Relaxation		EC_{50} (M) of papaverine response	Mean CCh baseline tension (mg)
		(mg)	% papaverine		
DMSO	13	394 ± 47	38.8 ± 3.4	2.3×10^{-8}	2121 ± 108
100 nM ER-819762	7	305 ± 51	36.0 ± 9.2	2.7×10^{-8}	1940 ± 147
1 μM ER-819762	7	353 ± 98	32.4 ± 6.2	6.5×10^{-8}	2171 ± 149
10 μM ER-819762	6	332 ± 62	34.8 ± 4.7	4.0×10^{-7}	2548 ± 214

Schild plot analysis indicated a linear relationship between the concentration of ER-819762 and degree of antagonism produced, indicating surmountable competitive antagonism at the EP_4 receptor. Accordingly, the Schild slope factor value obtained was 1.01, from which a pK_B value of 6.236 was derived (Figure 4.9 and Table 4.2).

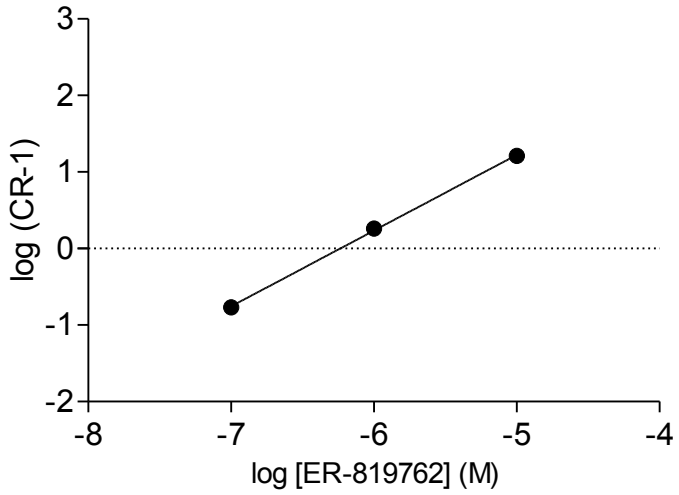


Figure 4.9. Schild plot analysis of the antagonism of ONO-AE1-329 with ER-819762.

Table 4.3. Schild plot analysis data for ONO-AE1-329 vs. ER-819762 in rat trachea.

[ER-819762] (M)	Log (CR-1)
10^{-7}	-0.77
10^{-6}	0.26
10^{-5}	1.21

Just as I characterised the EP_2 receptor antagonist, I also wanted to investigate the novel EP_4 receptor antagonist ER-819762 *in vivo* using ONO-AE1-329 (EP_4 agonist) before examining its effect against PGE_2. First, a dose response to ONO-AE1-329 was established which is clearly demonstrated in figure 4.10 below. As with ONO-AE1-259, the highest dose I was able to use was 3 mg kg^{-1} and as shown this was the only dose to induce a significant increase in airway MVL.

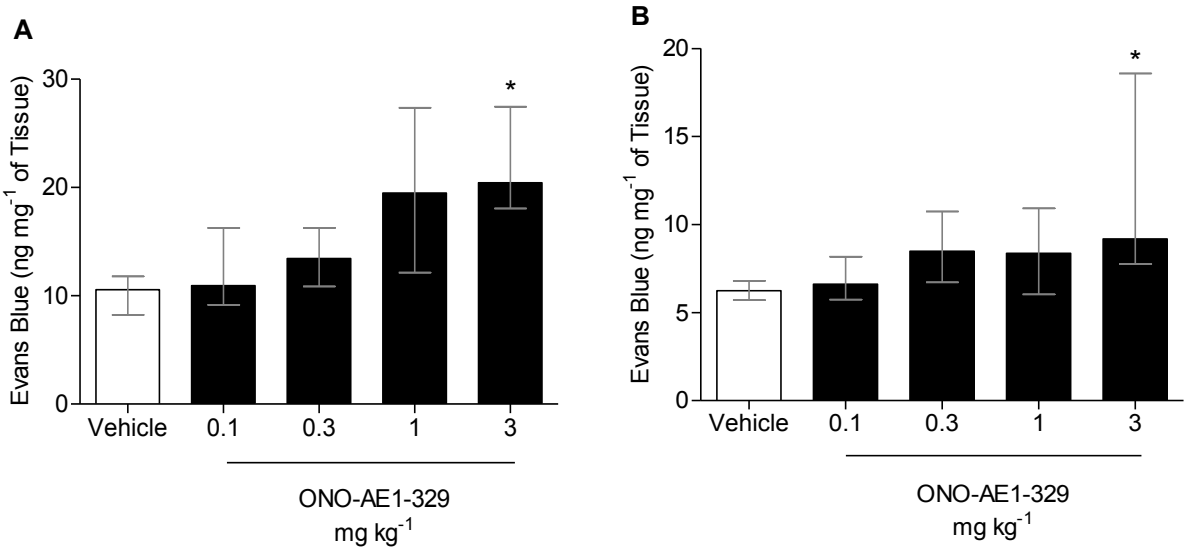


Figure 4.10. Effect of ONO-AE1-329 (EP_4) (0.1, 0.3, 1 and 3 mg kg^{-1} i.v.; 30 minutes) on MVL into (A) trachea or (B) bronchi and intra-pulmonary airways of mice. Data presented as median, with interquartile range, of the concentration of Evans blue dye (ng mg^{-1} of tissue). *n=6. One-Way ANOVA, non-parametric Kruskall-Wallis test with post-hoc comparisons using Dunn's multiple comparison test. * p<0.05 significant increase from vehicle control.*

Next I looked at a single oral dose of ER-819762 (100 mg kg^{-1}) against ONO-AE1-329 induced MVL. At this dose and route of administration, the antagonist did not inhibit MVL induced by the EP_4 agonist (Figure 4.11).

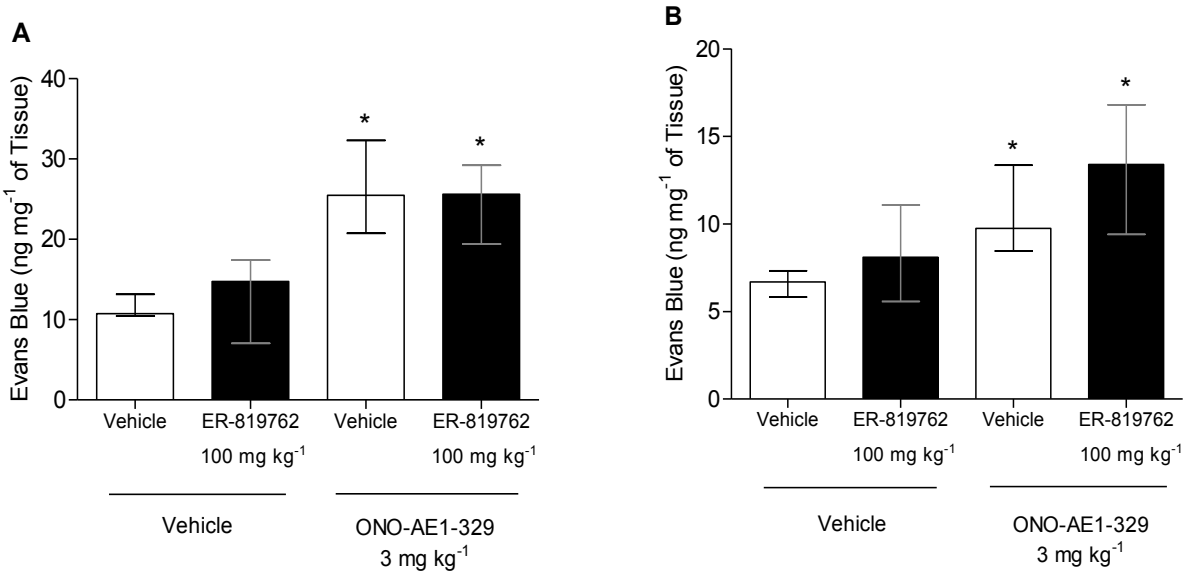


Figure 4.11. Effect of ER-819762 (100 mg kg^{-1} p.o.; 1 hour) on ONO-AE1-329-induced MVL (3 mg kg^{-1} i.v.; 30 minutes) into (A) trachea or (B) bronchi and intra-pulmonary airways of mice. Data presented as the median, with interquartile range, of the concentration of Evans blue dye (ng mg^{-1} of tissue). *n=6. Non-parametric Mann Whitney test. * p<0.05.significant increase from vehicle control.*

As with the EP_2 antagonist I then decided to explore whether an alternative route of administration would be better and so carried out a dose response via i.p dosing. The doses used were 3, 10, 30 and 100 mg kg^{-1}. Figure 4.12 shows that at 10 and 100 mg kg^{-1} ER-819762 was able to significantly inhibit MVL in the trachea induced by ONO-AE1-329. Although not a dose dependent relationship there was a trend towards reducing airway MVL induced by the EP_4 agonist. From this data I decided to use this compound at 10 mg kg^{-1} (i.p) in the following PGE_2 experiments.

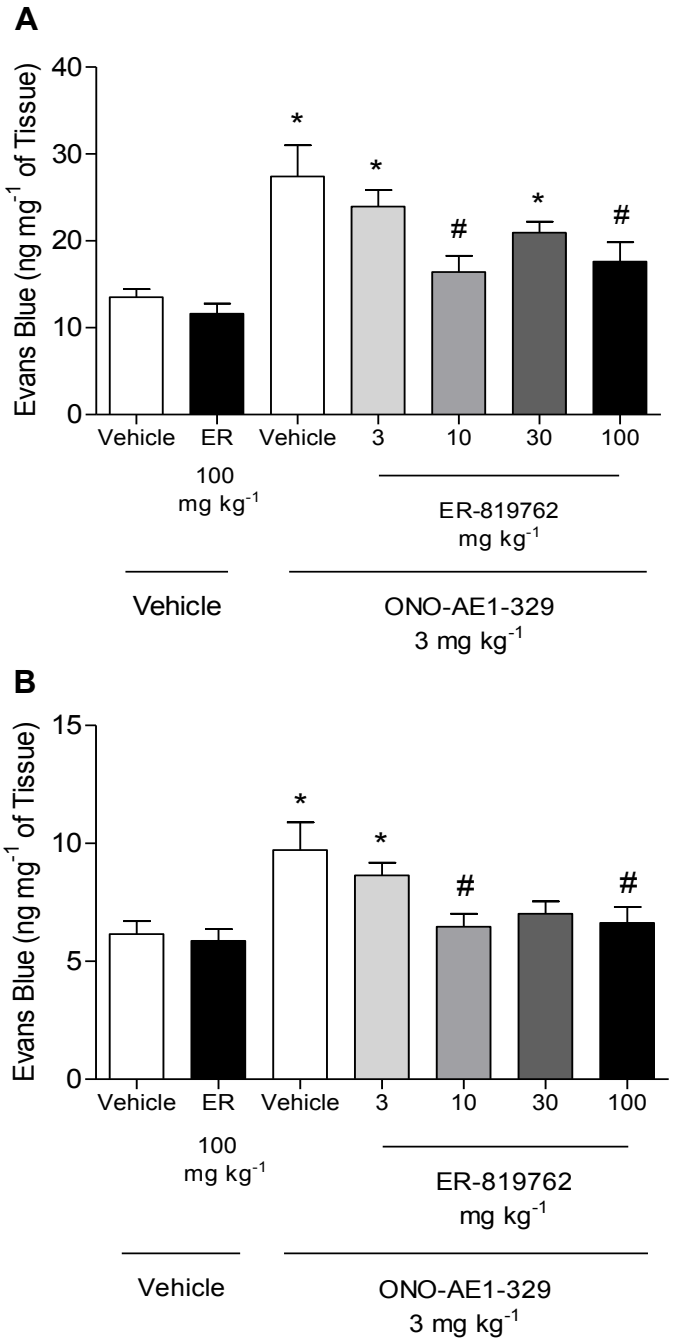


Figure 4.12. Effect of ER-819762 (3, 10, 30 and 100 mg kg^{-1} i.p.; 1 hour) on ONO-AE1-329-induced MVL (3 mg kg^{-1} i.v.; 30 minutes) into (A) trachea or (B) bronchi and intra-pulmonary airways of mice. Data expressed as mean ± s.e.m of the concentration of Evans blue dye (ng mg^{-1} of tissue). *n=6 One-Way ANOVA with post-hoc comparisons using Dunnett's multiple comparison test. *p<0.05.significant increase from vehicle control. # p<0.05 significant decrease from treatment control.*

4.3.5. Effect of the EP_2 and EP_4 receptor antagonists against PGE_2-induced MVL in mice

Having shown that the EP_2 antagonist (PF-04418948) and the EP_4 antagonist (ER-819762) are able to block airway MVL induced by their respective receptor agonists, I then wanted to replicate the data from the knockout mice. Initially each antagonist was dosed separately, however as demonstrated in figure 4.13 neither antagonist was able to inhibit PGE_2-induced airway MVL. When both antagonists were given simultaneously there was a significant reduction in PGE_2-induced MVL in the trachea and in the bronchi and IPA (Figure 4.14).

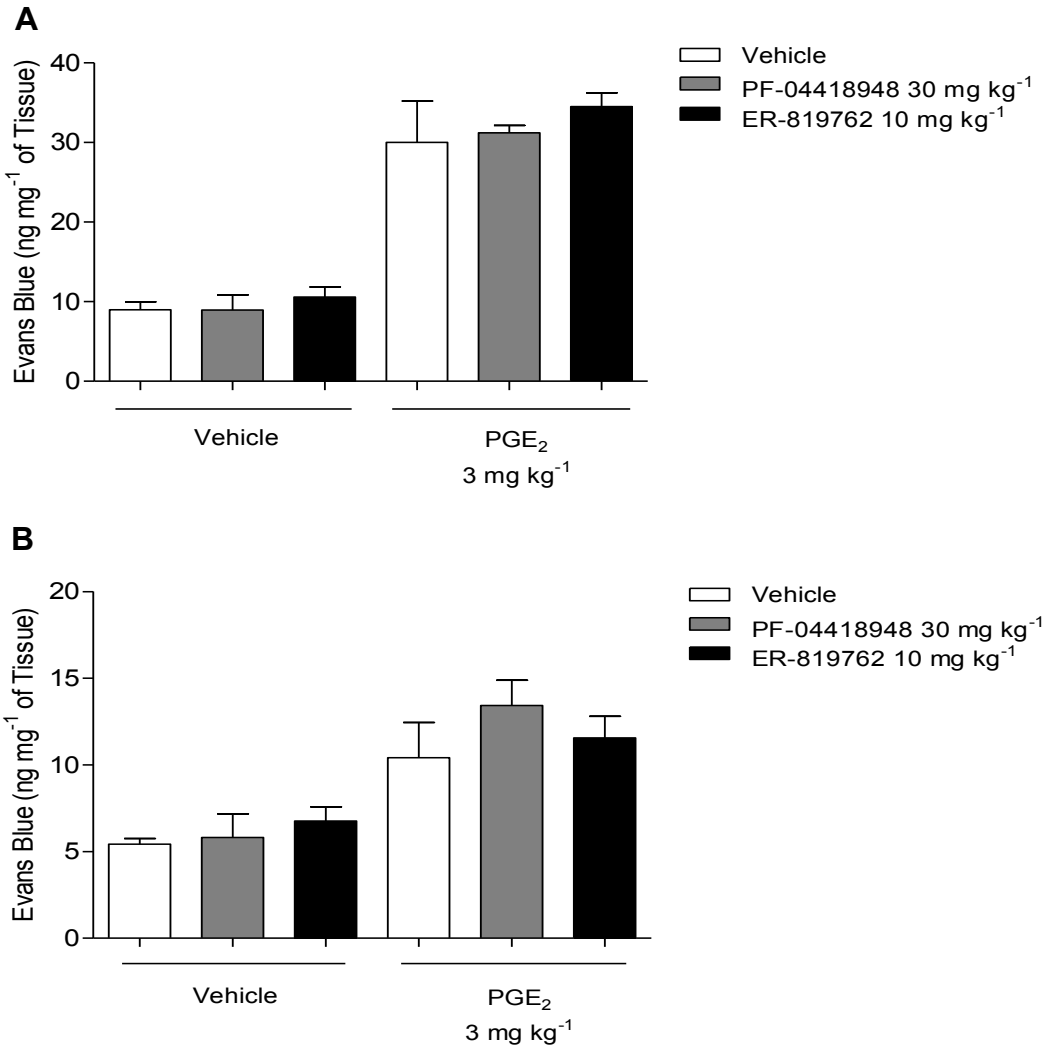


Figure 4.13. Effect of PF-04418948 (30 mg kg^{-1} i.p.; 1 hour) and ER-819762 (10 mg kg^{-1} i.p.; 1 hour) on PGE_2-induced MVL (3 mg kg^{-1} i.v.; 30 minutes) into (A) trachea or (B) bronchi and intra-pulmonary airways of mice. Data expressed as mean ± s.e.m of the concentration of Evans blue dye (ng mg^{-1} of tissue). *n=3*

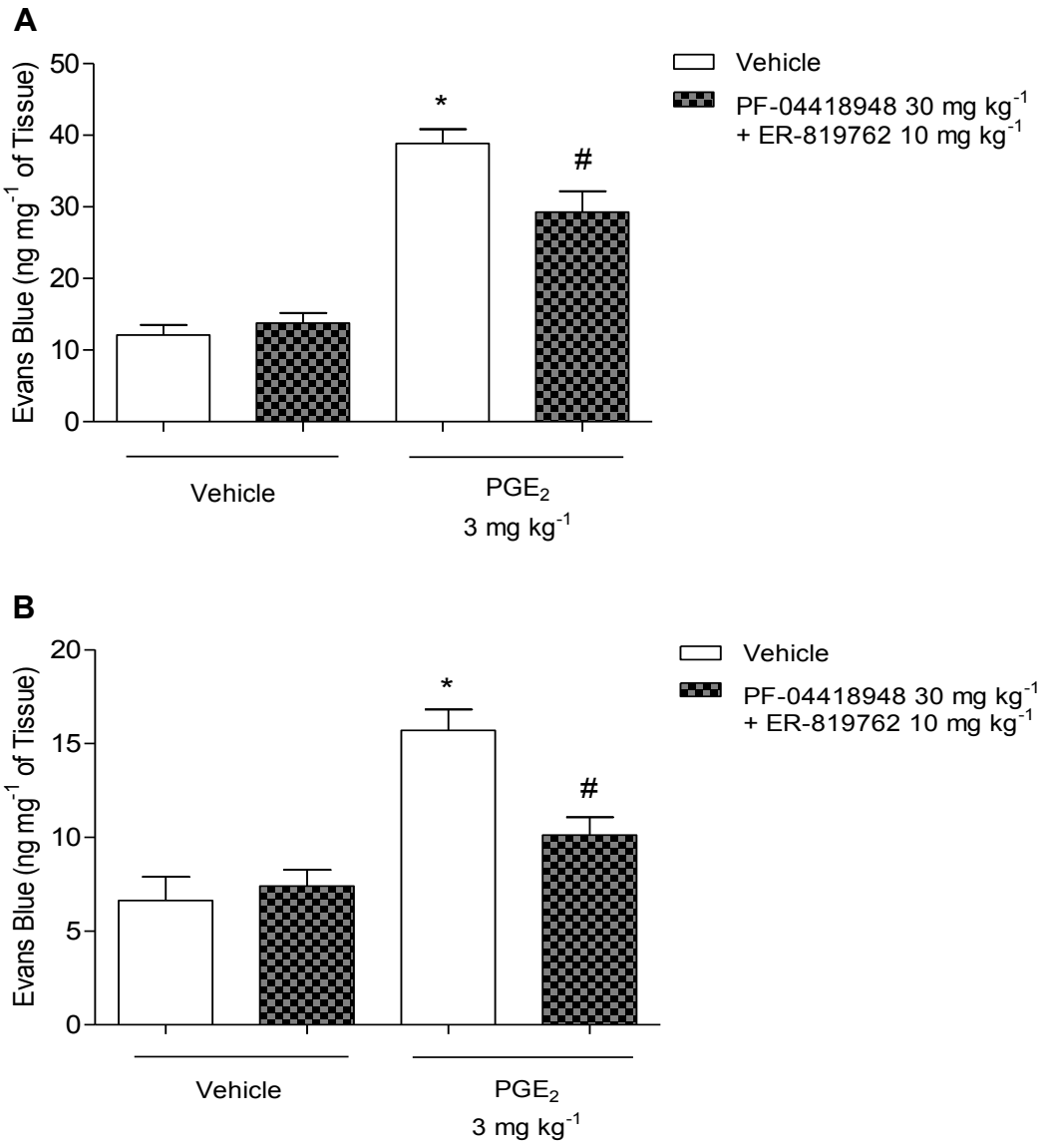


Figure 4.14. Effect of simultaneous dosing of PF-04418948 (30 mg kg^{-1} i.p.; 1 hour) and ER-819762 (10 mg kg^{-1} i.p.; 1 hour) on PGE_2-induced MVL (3 mg kg^{-1} i.v.; 30 minutes) into (A) trachea or (B) bronchi and intra-pulmonary airways of mice. Data expressed as mean ± s.e.m of the concentration of Evans blue dye (ng mg^{-1} of tissue). *n=5-6 Unpaired t test for parametric data. * p<0.05 significant increase from vehicle control. # p<0.05 significant decrease from treatment control.*

4.4. Discussion

Prostaglandin E_2 (PGE_2) is an endogenous lipid eicosanoid synthesised by cyclooxygenase (COX)-mediated metabolism of free arachidonic acid. It is produced in a variety of cells, including airway smooth muscle, epithelial cells, alveolar macrophages and pulmonary endothelial cells (Meyrick *et al*, 1989; Widdicombe *et al*, 1989). In addition, PGE_2 has been shown to have immunomodulatory effects, effects on airway smooth muscle and nerves, allowing it to modulate airway calibre (Buckley *et al*, 2011; Cocks *et al*, 1999; Maher *et al*, 2009; Portanova *et al*, 1996; Walters *et al*, 1984). It mediates its actions via 4 distinct G-protein coupled receptors, termed $EP_{1\text{-}4}$, encoded for by the genes *Ptger1-4* (Coleman *et al*, 1994).

In Chapter 3 I demonstrated a clear role for PGE_2 mediating airway microvascular leak in mice and guinea pigs. Furthermore, using Evans Blue dye as a marker of plasma protein exudation proved effective. The aim of this chapter was to investigate the role of each EP receptor in mediating this PGE_2-induced leak. To do this I used mice deficient of each individual EP receptor as well as a range of pharmacological tools, including agonists and antagonists targeting each receptor subtype. As discussed previously the effect of PGE_2 on airway microvascular leak has not been extensively investigated, especially *in vivo*. As microvascular leak and plasma exudation are both prominent features in airway inflammatory diseases, such as asthma, elucidating the role of endogenous mediators is an important step in developing novel therapies.

Having shown that PGE_2 induces MVL in the mouse I wanted to determine which of the EP receptors were central to this response. Differing effects of PGE_2 have been demonstrated *in vivo* and these, often opposing, effects can be attributed to the activation of different receptor subtypes ($EP_{1\text{-}4}$) with different signalling characteristics (Breyer *et al*, 2001). Through gene targeting and the development of mice deficient in specific prostanoid receptors we are able to identify the receptor/s mediating the inflammatory actions of PGE_2, a step that has been difficult with current selective EP agonists and antagonists (Goulet *et al*, 2004). What was evident in both the trachea and the bronchi and IPA was that the response to PGE_2 in the EP_2 and EP_4 knock-out mice was completely abolished. This data suggests

that both the EP_2 and EP_4 receptors may be essential in this process. For these knock-out mice to be a suitable tool for investigating airway microvascular permeability, it was important to determine if there were any major differences in their ability to produce this inflammatory response. 5-HT was administered to wild type, EP_2 and EP_4 knock-out mice and the leakage of Evans Blue measured. A significant increase in airway leak was observed in EP_2 and EP_4 knock-out mice in response to 5-HT, identical to that seen in the wild-type mice. So lacking either the EP_2 or the EP_4 receptor did not impact the effectiveness of other inflammatory mediators, supporting these knock-outs as suitable tools for future studies.

As well as the knock-outs I was also able to use selective EP receptor agonists that were kindly provided by ONO Pharmaceuticals. These allowed direct comparisons to be made with the knock-out data. The four agonists, ONO-D1-004 (EP_1), ONO-AE1-259 (EP_2), ONO-AE-248 (EP_3) and ONO-AE1-329 (EP_4) were administered in the same way as PGE_2. The data clearly demonstrated that, similar to PGE_2, the EP_2 and EP_4 agonists significantly increased the airway plasma leakage. This confirmed that activation of these EP receptors mediates microvascular leak in mouse airways, while activation of EP_1 and EP_3 produced no measurable increase.

EP_2 and EP_4 receptors both couple to G_s and signal through an increase in cAMP (Sugimoto & Narumiya, 2007). Various intracellular signalling pathways are thought to lead to an increase in microvascular fluid leak. In particular increases in intracellular calcium concentration have been closely linked. It has been suggested that an increase in calcium ions is an essential signal that is responsible for the phosphorylation of myosin light-chain kinase (MLCK) in endothelial cells. This causes actin-myosin stress fibres to develop force and a contraction of the cytoskeleton leading to the formation of intercellular gaps between endothelial cells (Ereso *et al*, 2009; Goeckeler & Wysolmerski, 1995). EP_1 receptors have been shown to mediate their effects through this intracellular pathway; however the data presented clearly demonstrated a role for EP_2 and EP_4 receptors in mediating PGE_2-induced MVL and both these receptors couple to G_s and signal through an increase in cAMP (Sugimoto & Narumiya, 2007). Furthermore, an increase in cAMP has been associated with restoration and strengthening of endothelial barrier function thus preventing an increase MVL in response to certain mediators (Mehta & Malik,

2006). There is evidence to suggest that activation of Protein Kinase A (PKA) through this increase in cAMP inhibits the activation of MLCK and so preventing the contraction of endothelial cells and opening of gap junctions (Garcia *et al*, 1995). It is interesting that the EP_3 receptor does not appear to mediate any PGE_2-induced MVL as this receptor is coupled to G_i and a decrease in cAMP. It must be emphasised, however, that the majority of research into the signalling pathways involved in vascular permeability has been studied *in vitro*, and so is not a true representation of the permeability characteristics of intact microvascular endothelium (Jiang *et al*, 2008).

In chapter 3 I was also unable to detect any major change in systemic blood pressure following PGE_2 administration. It would have been reasonable to assume that the response being observed was a result of an increase in bronchial blood flow as it has been suggested that vasodilator prostaglandins PGE_2 and PGI_2 are able to potentiate MVL (Rogers *et al*, 1990). However, the lack of response to PGE_2 may be explained by opposing effects of the four EP receptors. There is considerable evidence indicating that EP_2 and EP_4 receptors are pivotal in mediating the vasodepressor actions in mouse and human vascular preparations (Davis *et al*, 2004; Imig *et al*, 2002; Kennedy *et al*, 1999; Zhang *et al*, 2000). Additionally, the EP_1 and EP_3 receptors have previously been shown to mediate contraction of vascular smooth muscle in wild type mice (Baxter *et al*, 1995; Qian *et al*, 1994; Stock *et al*, 2001; van Rodijnen *et al*, 2007; Walch *et al*, 2001). Measuring changes in blood pressure in response to individual EP agonists is a key study for future investigations and is outlined in more detail in Chapter 7.

It has only been in recent years that novel EP_2 and EP_4 receptor antagonists that are potent and selective for their respective receptor have emerged. This has enabled me to further examine the data that I have generated, and explore the role of these two receptors further. Although knock-out mice for each prostanoid receptor subtype has helped to investigate the physiological and pathological roles of this receptor family, they are only limited to the mouse. Having demonstrated a clear role for EP_2 and EP_4 receptors in mediating airway microvascular leak, I wanted to examine these further using pharmacological tools.

Previously, the EP_2 antagonist relied on by most investigators was AH6809 but this has been shown to act at EP_1 and DP_1 receptors (Jones *et al*, 2009; Woodward *et al*, 2011). However, Forselles *et al* (2011) recently described a novel and selective EP_2 receptor antagonist, PF-04418948. This antagonist was then further characterised by our lab where it was clear that PF-04418948 was only selective for the EP_2 receptor (Birrell *et al*, 2013). Before using PF-04418948 a dose response to ONO-AE1-259 was carried out to establish an appropriate dose to use against the antagonist. A dose-dependent increase in MVL was evident with 3 mg kg^{-1} showing a significant increase. It would have been ideal to investigate all agonists at higher doses, however, this would have required large amounts of compound while increasing the cost in terms of reagents and number of animals used. As such, 3 mg kg^{-1} was then used to test the antagonist. To investigate its effects on EP_2-induced airway microvascular leak, PF-04418948 was given orally one hour prior to EP_2 agonist, ONO-AE1-259. However, no inhibition in Evans Blue leak was seen. Interestingly, when delivered via the intra peritoneal (i.p) route PF-04418948 demonstrated a dose-dependent inhibition in EP_2-induced MVL, with a significant block at 30 mg kg^{-1}. However, Forselles *et al* (2011) showed that orally administered PF-04418948 attenuated EP_2-induced responses in their rat model of cutaneous blood flow. They also report that the oral absorption was high with an oral bioavailability of 78%, however, the plasma protein binding was very high (unbound fraction of 0.003) (Forselles *et al*, 2011). Unfortunately, I was not able to measure plasma levels of this compound and asses its pharmacokinetic properties in mice. One problem that was encountered was the solubility of the compound. It did not go into solution in the vehicle used (0.5% methylcellulose, 0.2% tween 80 in saline) and so was delivered as a suspension.

In 2010, Chen *et al* described a novel EP_4 receptor antagonist, ER-819762, that was highly selective for EP_4 receptors in a number of different *in vitro* assays as well as being effective in both mouse and rat models of arthritis and chronic pain (Chen *et al*, 2010). So this antagonist was chosen as a suitable tool to investigate the EP_4 receptor in MVL in mouse airways. Before studying the effects of this antagonist *in vivo*, I wanted to confirm its functionality and potency in a suitable EP_4 receptor mediated system. PGE_2-induced relaxation of induced tone in rat and human trachea has been shown to be mediated by the activation of the EP_4 receptor

(Lydford and McKechnie, 1994; Buckley *et al*, 2011; Benyahia *et al*, 2012). Here, ER-819762 behaved as a surmountable competitive antagonist, producing rightward parallel shifts of the ONO-AE1-329 (EP_4 agonist) concentration response curves with a pK_B value of 6.24. For the *in vivo* work I first demonstrated that ONO-AE1-329 induced a dose-dependent increase in Evans Blue leak in murine airways, with 3 mg kg^{-1} producing a significant rise. Similarly, the effect of ER-819762 was initially examined against EP_4-mediated responses. A single oral dose of ER-819762 (100 mg kg^{-1}) did not show any inhibition of EP_4-induced airway MVL. However, when given i.p ER-819762 produced a dose-dependent inhibition of EP_4-induced MVL, with a significant drop seen at 10 and 100 mg kg^{-1}. The doses used in this study were based on those used by Chen *et al* (2010) and so it is interesting that when given orally it was unable to block EP_4-induced leak. In their models ER-819762 successfully suppressed clinical signs of arthritis in two different mouse models, as well as inhibiting chronic pain in a rat model of inflammatory pain (Chen *et al*, 2010). Unfortunately I cannot comment on the pharmacokinetic properties of the antagonist, but it may simply be due to differences in dosing regimen and the animal models used. Animals were dosed on a daily basis and disease progression over several weeks was monitored and scored. With the MVL model, only a single dose was given and the response measured was an acute inflammatory reaction.

Having established that both the EP_2 receptor (PF-04418948) and EP_4 receptor antagonists (ER-819762) were able to inhibit airway MVL induced via their respective agonists, the next step was to examine their effects on PGE_2-induced MVL. In initial experiments, neither antagonist was able to inhibit PGE_2-mediated airway MVL. Looking back at the previous data it is clear that both the EP_2 and EP_4 receptor are able to mediate leak to the same extent, so it seemed possible that blocking either was not enough to inhibit PGE_2. Indeed, when both antagonists were dosed simultaneously, at the same doses and time points, a decrease in PGE_2-induced MVL was evident, with a significant inhibition seen the trachea and in the bronchi and IPA. This emphasised the possibility for a dual role for EP_2 and EP_4 receptors in mediating PGE_2 induced responses and it is obvious here that both receptors are important in airway MVL. Little research has been published on their role in airway MVL so the data presented demonstrates an EP receptor activity profile which will aid in further understanding its role in airway inflammation. Yuhki *et*

al (2004) presented evidence that suggested EP_2 and EP_3 participate in inflammatory exudation in their Carrageenin-induced mouse pleurisy model. Furthermore, EP_2 and EP_4 receptor signalling has been implicated in acute skin inflammation by enhancing blood flow in the microenvironment (Kabashima *et al*, 2007). In contrast, Goulet *et al* (2004) found that PGE_2 promotes acute cutaneous inflammation by activating EP_3 receptors. In addition, data from *in vitro* studies has illustrated that PGE_2 actually reduces endothelial permeability to macromolecules, such as dextran or albumin, and achieves this via activation of the EP_4 receptor (Birukova *et al*, 2007; Konya *et al*, 2013b). Konya *et al* (2013) used human pulmonary endothelial cells and observed that only the EP_4 agonist, ONO-AE1-329, mimicked the barrier enhancing effects of PGE_2 (Konya *et al*, 2013b). It is also important to note that expression of EP_4 receptors has been detected in pulmonary endothelial cells in human, rat and mouse (Aso *et al*, 2012; Martin *et al*, 2002; Rao *et al*, 2007).

An interesting observation from the data here was that both EP_2 and EP_4 agonists caused almost identical responses in airway MVL and in the knock-out mice response to PGE_2 were almost completely abolished in both EP_2 and EP_4 receptor knockouts. Moreover, the selective receptor antagonists required simultaneous dosing to block the stimulatory effects of PGE_2. This suggests that EP_2 and EP_4 may be functionally interdependent. Subbaramaiah *et al* (2008) saw a similar effect when studying the activity of PGE_2 in human adipocytes and breast cancer cells. They observed that both receptors played a role in mediating the induction of aromatase and that inhibiting either receptor abrogated the effects of PGE_2 (Subbaramaiah *et al*, 2008). One hypothesis is that the EP_2 and EP_4 receptors form heterodimers. The data from the knock-outs indicates that without functional EP_2 or EP_4 receptors PGE_2 is unable to induce MVL. Furthermore, when EP_2 and EP_4 antagonists were administered alone, with both functional receptors present, PGE_2-induced significant MVL. Only when both receptors were antagonised simultaneously was PGE_2-induced leak attenuated (see figure 4.15).

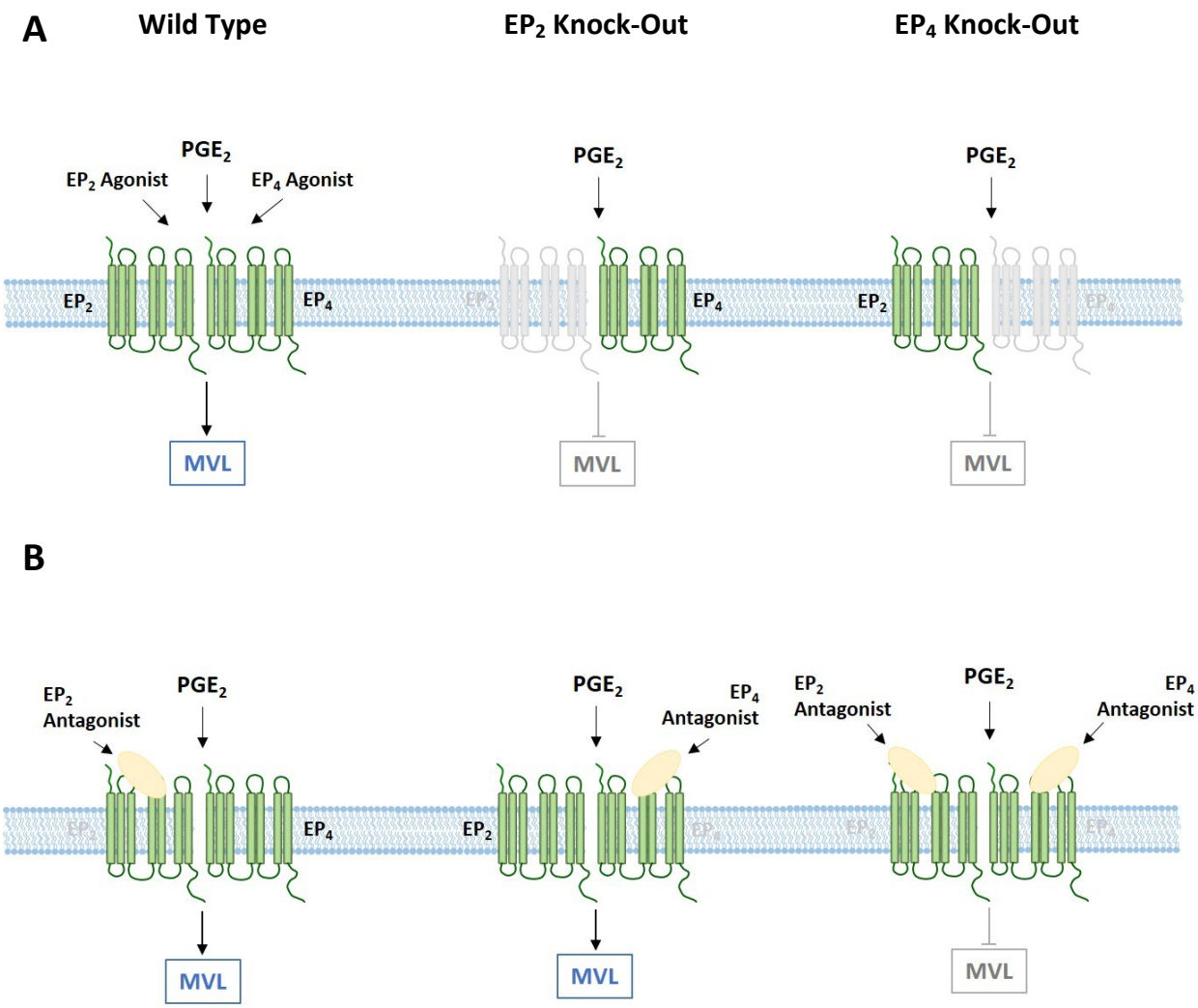


Figure 4.15. Schematic of possible $EP_{2/4}$ heterodimer and how this interaction influences PGE_2-induced airway microvascular leak. *(A) PGE_2 and EP_2 and EP_4 agonists are all able to induce a significant increase in airway MVL. However, in EP_2 and EP_4 receptor knock outs this response to PGE_2 is absent. With either functional receptor missing the $EP_{2/4}$ heterodimer cannot form and so stimulation with PGE_2 cannot elicit a response. (B) Pharmacological inhibition of either receptor with an antagonist does not inhibit airway MVL. When both EP_2 and EP_4 antagonists are administered simultaneously both receptors are blocked and PGE_2-induced MVL is attenuated.*

There is increasing evidence that GPCRs are able to heterodimerise and that this interaction can affect the function of either receptor (Wilson *et al*, 2007; McGraw *et al*, 2006). Wilson *et al* (2007) demonstrated a functionally important

heterodimerisation of TP and IP receptors in human and mouse aortic smooth muscle cells. Additionally, McGraw *et al* (2006) used fluorescence microscopy in airway smooth muscle cells and bioluminescence resonance energy transfer (BRET) and co-immunoprecipitation in a cell line to show heterodimerisation between EP_1 receptors and β_2 adrenoceptors. However, more investigation is obviously needed to establish whether this is occurring between the EP_2 and EP_4 receptors.

Airway microvascular leak represents a prominent feature in airway inflammation and the data presented here highlights a key role for PGE_2 and the EP_2 and EP_4 receptors. These findings suggest that my hypothesis is correct and that activation of specific EP receptors mediates PGE_2-induced microvascular leak in rodent airways. Only agonists selective for the EP_2 and EP_4 receptors were able to induce airway MVL and PGE_2-induced MVL was absent in EP_2 and EP_4 receptor deficient mice. Furthermore, by antagonising both the EP_2 and the EP_4 receptors simultaneously, PGE_2-induced MVL was attenuated. However, as discussed not all findings were significant, so further investigation may be warranted. As I have stated previously, little research has been published on the effects of PGE_2 and the EP receptors in mediating airway plasma exudation and so these findings are novel and make an exciting addition to the expanding repertoire of the effects of EP receptors in airway disease.

Chapter Five

Identifying the Role of EP Receptors in Ovalbumin-induced MVL in Mouse Airways

5. Identifying the Role of EP Receptors in Ovalbumin-Induced MVL in Mouse Airways

5.1 Rationale

Having established in Chapters 3 and 4 that PGE_2 is able to mediate airway microvascular leak when given exogenously, I wanted to investigate its role in a disease model of airway inflammation. To do this I decided to study airway leak in our Ovalbumin mouse model of allergic asthma. It has been widely reported that vascular permeability is common among asthmatics, where it is thought to have an essential role in thickening the bronchial mucosa and the resulting airway hyperresponsiveness. Additionally, albumin has been found in high concentrations in airway secretions from acute asthmatic patients (Svensson *et* al. 1995, Van Rensen *et al*. 2002). Therefore, the next step was to examine PGE_2 and the EP_2 and EP_4 receptors in a disease model of asthma.

The ovalbumin (OVA) model of allergic asthma has been well characterised in our lab and our standard protocol was used, as described in section 2.5. Furthermore, our group and others have demonstrated that OVA-induced MVL is measureable in in a variety of species (Hele *et al*, 2001; Lee *et al*, 2004; Kim *et al*, 2012). However the role of EP receptors in mediating these effects has not been extensively investigated.

5.1.1. Chapter Hypothesis

"Activation of EP_2 and EP_4 receptors by endogenous PGE_2 causes airway microvascular leak during allergic inflammation in mice"

5.1.2. Aims

- Establish a time point post OVA challenge to measure Evans Blue MVL in mouse airways and whether Evans Blue was still an appropriate marker in this model.
- Examine the role of prostanoids in mediating OVA-induced MVL using a non-selective COX inhibitor, diclofenac.

- Study the effects of OVA-induced airway MVL in EP_2 and EP_4 receptor deficient mice.
- Confirm data from the KOs using the EP_2/EP_4 antagonist protocol outlined in the previous chapter.

5.2. Methods

5.2.1. Characterisation of Ovalbumin-induced Microvascular Leak in Mouse Airways

C57BL/6 mice were sensitised and challenged as described in section 2.5. As previously mentioned, I first wanted to establish at which time point was it suitable to measure MVL. 2 hours and 24 hours after final challenge were chosen to measure for Evans blue extravasation. These time points were loosely based on previous model development in our lab where total protein in BAL samples was found to be highest at these two time points. Additionally, 4 groups were included in the characterisation; vehicle sensitised/saline challenged, vehicle sensitised/OVA challenged, OVA sensitised/saline challenged and OVA sensitised/OVA challenged.

5.2.2. Effect of Diclofenac on Ovalbumin-induced MVL

Following on from the characterisation it was appropriate to continue using two groups; OVA sensitised/saline challenged and OVA sensitised and OVA challenged. Additionally, the two hour time point was preferable over 24 hours and was used for all other studies. Finally, the dissected tissue was dried out at 60ºC for one hour prior to extraction of dye in formamide. Wet and dry tissue weight was recorded and the data from this study onwards are presented as the concentration of Evans blue in ng mg^{-1} of dry tissue weight. To examine whether prostanoids are responsible for the MVL seen in this ovalbumin model, a non-selective cyclooxygenase inhibitor, diclofenac, was used (Mitchell *et al*. 1993). Diclofenac or vehicle (0.5% methylcellulose, 0.2% tween 80 in water) was dosed orally (30 mg kg^{-1} at 10 ml kg^{-1}) one hour prior to Evans blue administration.

5.2.3. The role of EP receptors in Ovalbumin-induced MVL

Wild type (C57Bl/6 and 129/Ola X C57Bl/6), *Ptger2*$^{-/-}$ and *Ptger4*$^{-/-}$ mice were used to investigate whether PGE_2, acting via the EP_2 and/or EP_4 receptors, was inducing the MVL demonstrated. These two receptors were chosen based on data presented in Chapter 4 which indicated that it was the EP_2 and EP_4 receptors that were mediating PGE_2-induced MVL seen in mouse airways. For reasons pertaining to cost and time it was not possible to investigate ovalbumin-induced MVL in all prostanoid receptor deficient mice.

In Chapter 4, EP_2 and EP4 receptor antagonists were characterised *in vivo* and it appears that both need to be administered simultaneously to significantly block PGE_2-induced MVL. Therefore, this next experiment was designed to establish whether the knock out data could be replicated by co-administering the two antagonists to block OVA-induced MVL. One hour after final intranasal challenge and one hour before MVL assessment, mice were dosed with vehicle (0.5 % methylcellulose, 0.2% tween 80 in saline) or PF-0441898 (30 mg kg^{-1}) and ER-819762 (10 mg kg^{-1}) at 10 ml kg^{-1} i.p.

5.2.4. Statistical Analysis

All data was analysed using GraphPad Prism 5. Parametric data was expressed as mean $\pm$ s.e.m and non-parametric data presented as the median with the interquartile range. Multiple measurements were analysed by one-way ANOVA, non-parametric Kruskal-Wallis test and post-hoc comparisons were performed by Dunn's multiple comparison test, comparing selected columns to a control. Additionally, an unpaired t test and/or non-parametric Mann Whitney test, with two-tailed p values, were carried out to determine statistical significance between two groups. Differences were considered statistically significant if $p<0.05$. The figures have been graphically presented on a range-specific axis.

5.3. Results

5.3.1. Characterisation of Ovalbumin-induced Microvascular Leak in Mouse Airways

Although the ovalbumin model of allergic asthma has been well characterised in our group, its effect on airway microvascular leak had not been previously established. Firstly, I needed to determine the appropriate time point to use to investigate this response using the same Evans Blue method employed in Chapters 3 and 4. It was clear that 2 hours following a final intra nasal challenge of OVA in OVA-sensitised mice there was a measurable and significant increase in EB leak into the trachea and in the bronchi and IPA, whereas 24 hours after this response is absent (Figure 5.1)

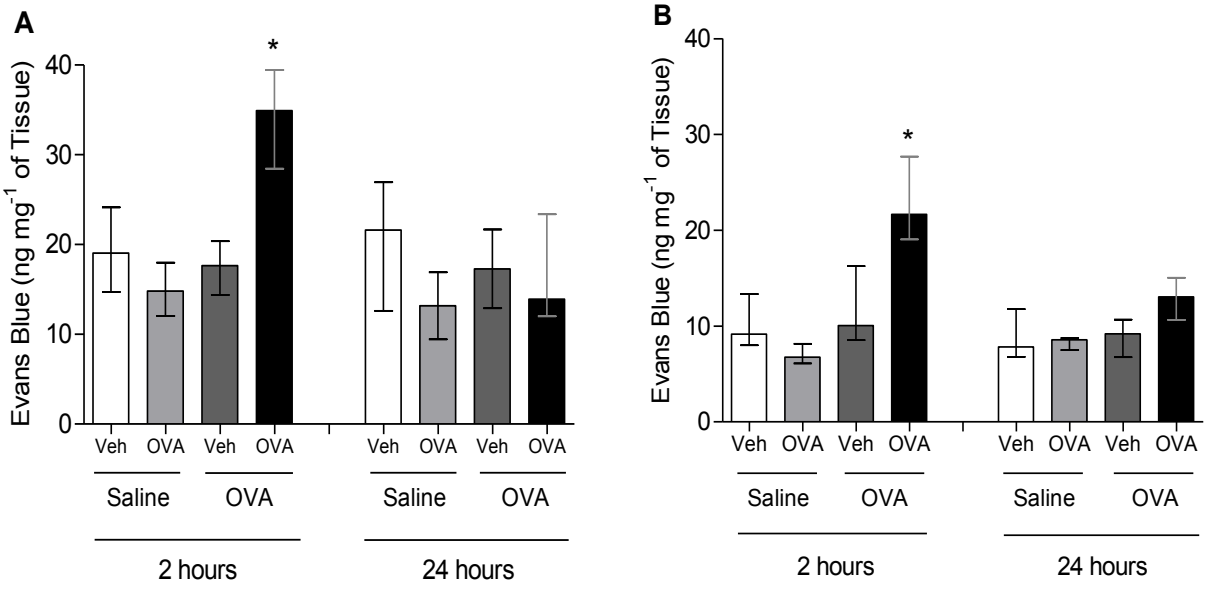


Figure 5.1. Effect of Ovalbumin-challenge on airway MVL in C57BL/6 mice into (A) the trachea or (B) the bronchi and intra-pulmonary airways, 2 and 24 hours after final intra nasal OVA challenge. Four groups were included in this study, vehicle-sensitised/saline challenged, OVA-sensitised/saline challenged, vehicle-sensitised/OVA challenged and OVA-sensitised/OVA challenged. Data presented as the median, with the interquartile range, of the concentration of Evans blue dye (ng mg^{-1} of tissue). *n=4. Mann Whitney t-test for non-parametric data. * p<0.05 representing a significant increase from OVA/Saline control.*

One important observation in this study was that the wet weight of the airway tissue was considerably heavier in the OVA-sensitised/OVA-challenged group (Figure 5.2). As the concentration of EB in the tissue is calculated based on the weight of the tissue, this increase in weight underestimated the EB leak in the airways. To overcome this I decided in future studies to dry out the tissue prior to incubating in formamide.

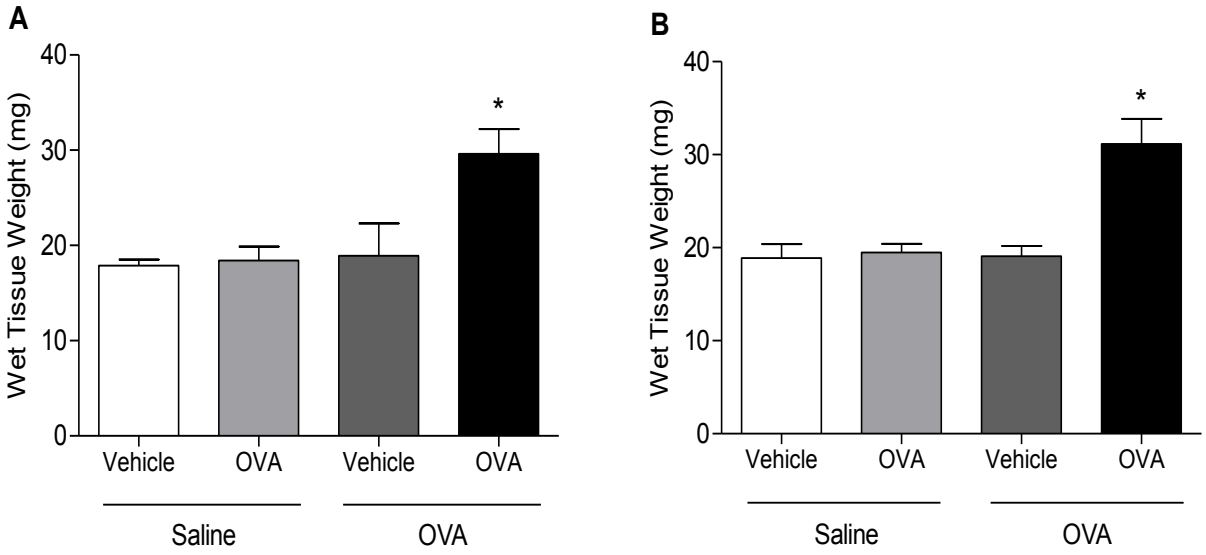


Figure 5.2. Effect of Ovalbumin-challenge on wet weight of the bronchi and intra-pulmonary airways at (A) 2 hours or (B) 24 hours after final intranasal OVA challenge. Four groups were included in this study, vehicle-sensitised/saline challenged, OVA-sensitised/saline challenged, vehicle-sensitised/OVA challenged and OVA-sensitised/OVA challenged. Data expressed as mean ± s.e.m of the wet tissue weight (mg of tissue). *n=4. Unpaired t test for parametric data. * p<0.05 representing a significant increase from OVA/Saline control*

5.3.2. Effect of Diclofenac on Ovalbumin-induced MVL

As outlined in the rationale, I wanted to investigate whether prostanoids were involved in mediating the MVL observed in this ovalbumin model. So to begin with I used a non-selective COX inhibitor, diclofenac. Interestingly, different effects were seen between the trachea and the bronchi and IPA. In the trachea diclofenac (30 mg kg^{-1}) partially reduced the OVA-induced microvascular leak, whereas in the bronchi and IPA it had no effect (Figure 5.3). As before, 2 hours following OVA-challenged there was a large and statistically significant increase in microvascular leak in mouse airways.

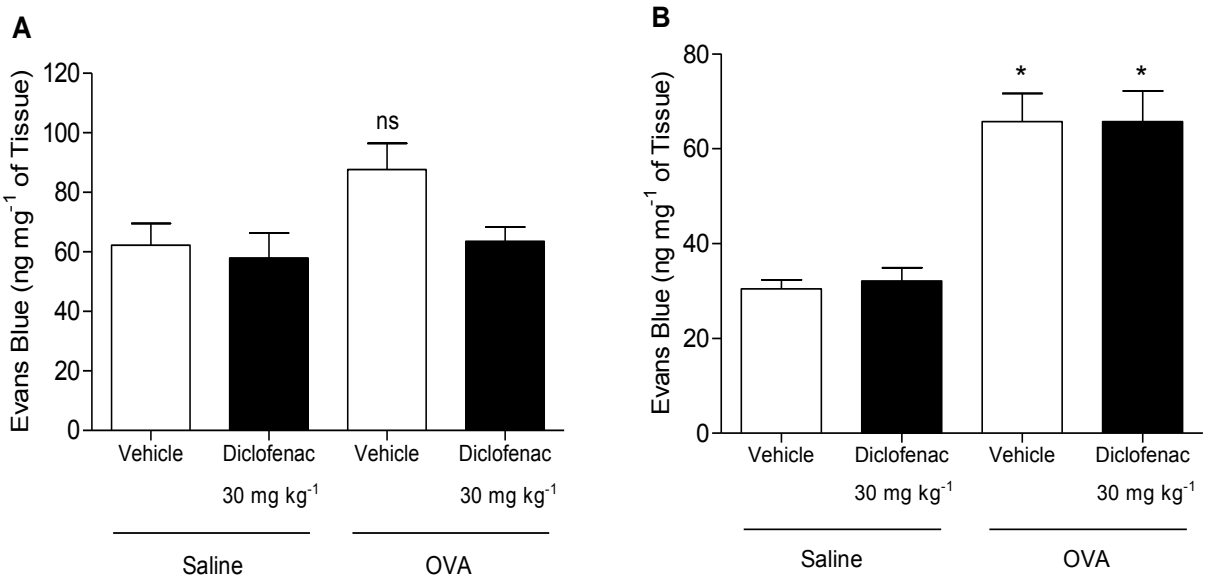


Figure 5.3. Effect of diclofenac (30 mg kg^{-1} p.o) on OVA induced MVL into (A) the trachea or (B) the bronchi and intra-pulmonary airways of mice, 2 hours following final intra nasal challenge. Two groups were included in this study, OVA-sensitised/saline challenged and OVA-sensitised/OVA challenged. Additionally, airway tissue was dried prior to extraction of EB in formamide. Data expressed as mean ± s.e.m of the concentration of Evans blue dye (ng mg^{-1} of dry tissue). *n=7-8 Unpaired t test for parametric data. * p<0.05 representing a significant increase from vehicle control. ns = non significant increase*

5.3.3. The Role of EP_2 and EP_4 Receptor Knock-outs in Ovalbumin-Induced Airway MVL

Following inconclusive data using diclofenac to inhibit OVA-induced leak, I wanted to investigate the role of the EP receptors more specifically. Having already demonstrated that the EP_2 and the EP_4 receptors are the key receptors in PGE_2-induced MVL, I used the EP_2 and EP_4 receptor knockout mice in the OVA model and measured the level of EB leak into the airways.

As both receptor knock outs are bred on different backgrounds, so different wild type controls, they are presented on separate graphs. Additionally, due to difficulties with breeding, the n numbers for the EP_4 knock outs are varied. C57BL/6 wild types demonstrated a measurable and significant increase in EB leak in the trachea and the bronchi and IPA. However, in the EP_2 receptor knock-outs there was a significant reduction in MVL (Figure 5.4).

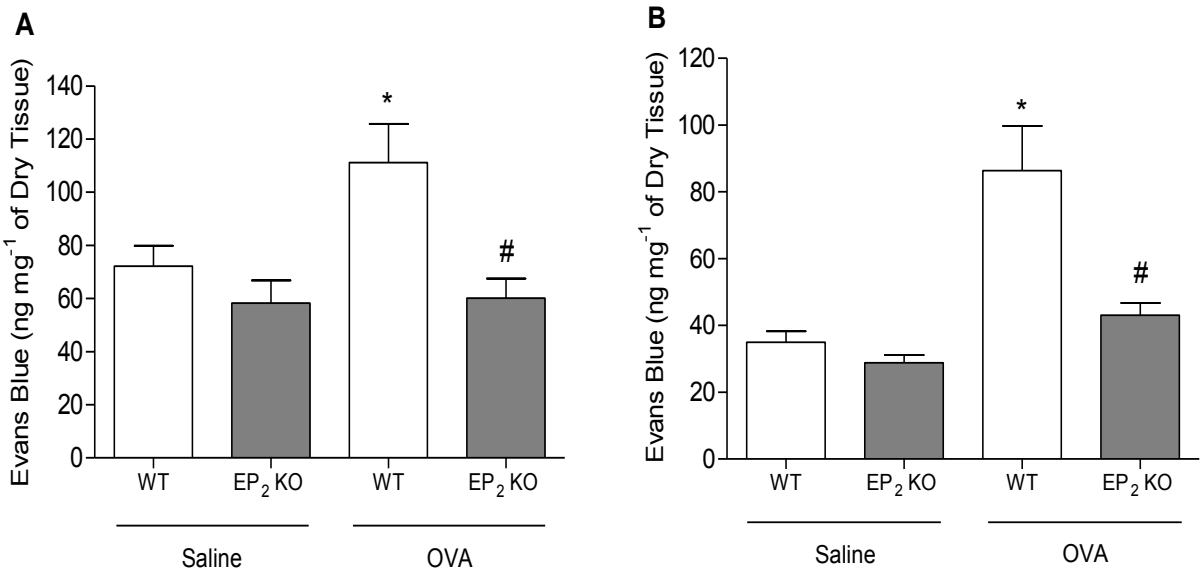


Figure 5.4. Effect of OVA induced MVL into (A) the trachea or (B) the bronchi and intra-pulmonary airways of EP_2 receptor knockout mice, 2 hours following final intra nasal challenge. Two groups were included in this study, OVA-sensitised/saline challenged and OVA-sensitised/OVA challenged. Additionally, airway tissue was dried prior to extraction of EB in formamide. Data expressed as mean ± s.e.m of the concentration of Evans blue dye (ng mg^{-1} of dry tissue). *n=8 Unpaired t test for parametric data. * p<0.05 representing a significant increase from vehicle control and # p<0.05 a significant decrease from wild type control.*

Similarly, the EP_4 wild type controls showed a significant increase in EB leak, while in the knock outs this response was abolished in both the trachea and the bronchi and IPA (Figure 5.5).

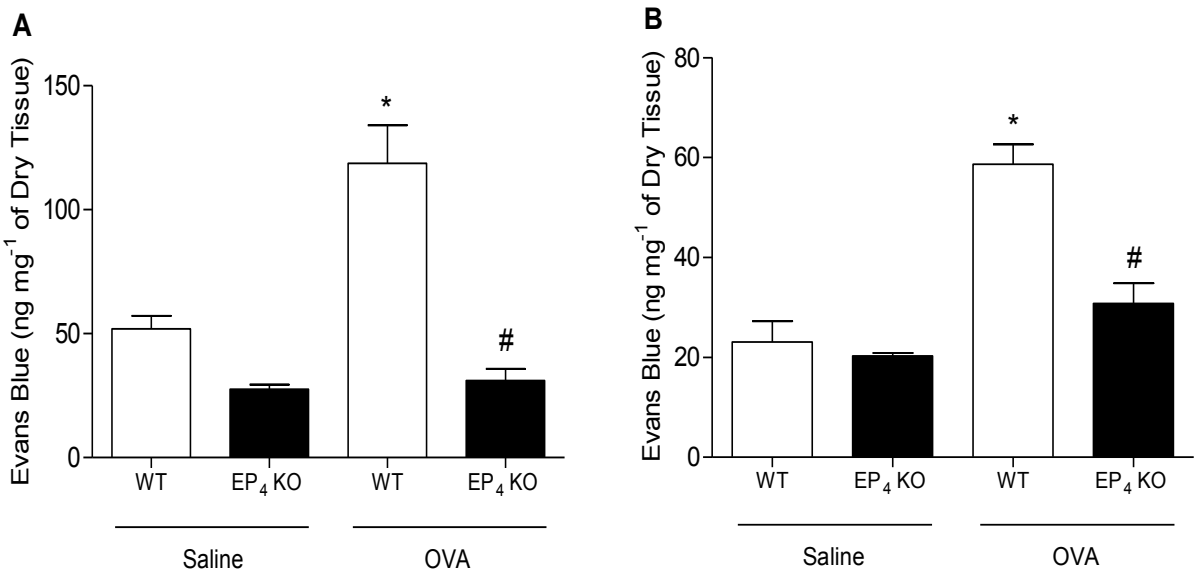


Figure 5.5. Effect of OVA induced MVL into (A) the trachea or (B) the bronchi and intra-pulmonary airways of EP_4 receptor knockout mice, 2 hours following final intra nasal challenge. Two groups were included in this study, OVA-sensitised/saline challenged and OVA-sensitised/OVA challenged. Additionally, airway tissue was dried prior to extraction of EB in formamide. Data expressed as mean $\pm$ s.e.m of the concentration of Evans blue dye (ng mg^{-1} of dry tissue). *n=4-8 Unpaired t test for parametric data. * $p<0.05$ representing a significant increase from vehicle control and # $p<0.05$ a significant decrease from wild type control.*

5.3.4. The Role of EP_2 and EP_4 Receptor Antagonists in Ovalbumin-Induced Airway MVL

Having shown that mice with no functional EP_2 or EP_4 receptors have significantly less MVL in the airways after OVA-challenge, I then wanted to determine whether this data could be replicated using selective EP_2 and EP_4 receptor antagonists. Previously, both antagonists were characterised *in vivo* and it was apparent that both needed to be administered simultaneously to significantly block PGE_2-induced MVL (Chapter 4) and so the same dosing regimen was used in this study.

In both the trachea and the bronchi and IPA, OVA-induced MVL was reduced following PF-04418948 (30 mg kg^{-1}) and ER-819762 (10 mg kg^{-1}), but this did not reach statistical significance (Figure 5.6).

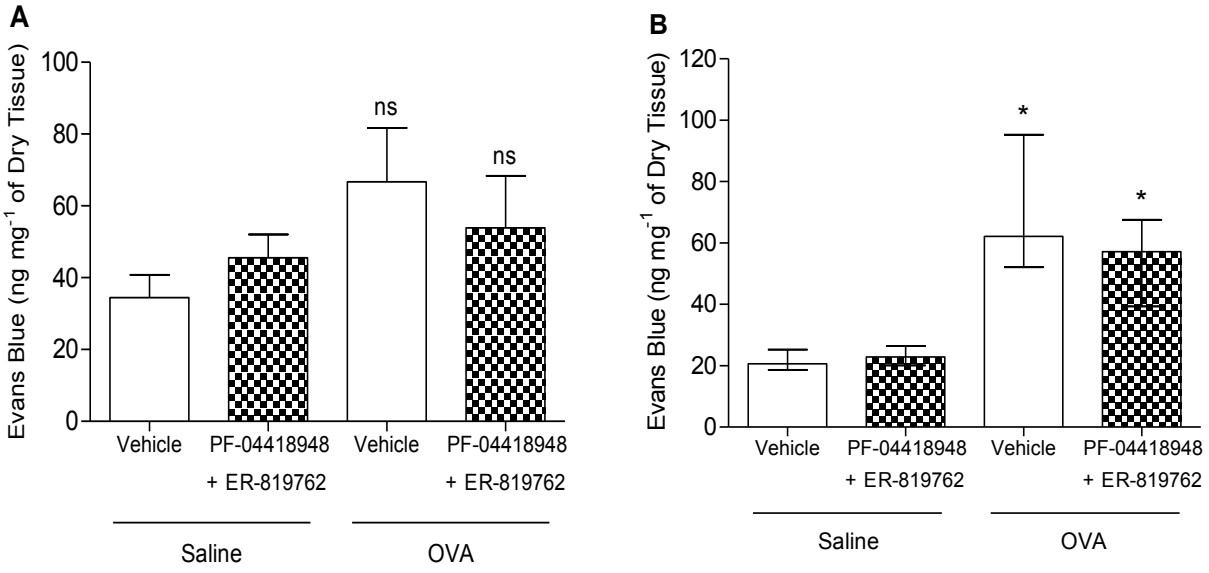


Figure 5.6. Effect of PF-04418948 (30 mg kg^{-1} i.p) and ER-819762 (10 mg kg^{-1} i.p) or vehicle (0.5% methylcellulose, 0.2% tween 80 in saline, i.p) on OVA induced MVL into (A) the trachea or (B) the bronchi and intra-pulmonary airways of mice, 2 hours following final intra nasal challenge. Two groups were included in this study, OVA-sensitised/saline challenged and OVA-sensitised/OVA challenged. Additionally, airway tissue was dried prior to extraction of EB in formamide. Non-parametric data presented as the median, with the interquartile range, and parametric data expressed as mean $\pm$ s.e.m of the concentration of Evans blue dye (ng mg^{-1} of dry tissue). *n=6 Unpaired t test for parametric data or Mann-Whitney for non-parametric data. * $p<0.05$ representing a significant increase from saline control. ns = non significant increase*

5.4. Discussion

It has been well documented that microvascular leak and oedema are prominent features in allergic airway diseases such as asthma, which is associated with thickening of the airway wall and narrowing of the airway (Kanazawa *et al,* 2000, 2002; Khor *et al,* 2009). Additionally, high concentrations of plasma proteins, such as albumin, have been measured in airway secretions from acute asthmatic patients (Meerschaert *et al*, 1999; Tseliou *et al*, 2012). Similar effects have also been observed in animal models of allergic airway disease, including the Ovalbumin model of allergic asthma (Lee *et al*, 2004, 2006; Kim *et al*, 2012).

Having already demonstrated that prostaglandin E_2 was able to induce airway microvascular leak in mice via the EP_2 and EP_4 receptors, I wanted to investigate this response in a disease model. I chose the Ovalbumin model of allergic asthma as it has been well characterised within our group. However, I had to first validate the measurement of Evans blue extravasation into the airways as this marker had not previously been used. Prior time course studies measured total protein in the bronchoalveolar lavage fluid at various time points and so from this data I chose to measure MVL at 2 and 24 hours following a final intra nasal challenge.

The data demonstrated a measurable increase in airway MVL 2 hours after a final intra nasal challenge of OVA, while after 24 hours this response was absent. It must also be emphasised that what is being measured here is a snapshot of airway leak that is occurring for 30 minutes following OVA challenge. What was also apparent was that in the OVA-sensitised/OVA-challenged there was an increase in the bronchi and IPA weights which led to an underestimation in the EB concentration. So in all other studies the airway tissue was dried prior to EB extraction in formamide. Other groups have also used Evans Blue dye to successfully measure OVA-induced MVL in mouse (Lee *et al*. 2004, 2006, Kim *et al*. 2012), rat (Olivenstein *et al*. 1997, Hele *et al*. 2001) and guinea pig airways (Mirza *et al*. 1998). They all showed an increase in Evans Blue dye in the airways and/or lung tissue following OVA challenge providing a valuable measure for investigating MVL in allergic asthma.

As mentioned before, my primary aim was to determine whether PGE_2 and the EP receptors are involved in this response. So firstly I used diclofenac, a non-selective

COX inhibitor, to block endogenous prostanoid production (Mitchell *et al*. 1993). Diclofenac was dosed orally 1 hour prior to EB administration and so one hour following OVA challenge. The data demonstrated that diclofenac was able to significantly inhibit EB MVL in the trachea but not within the lower airways (bronchi and IPA) suggesting that prostanoids may have a tissue specific role in OVA-induced MVL. Although this doesn't indicate which prostanoid may be mediating this effect, it does indicate that they may be of importance here. Of course, diclofenac is only being used as a tool as it is well known that the use of NSAIDs is contraindicated in asthma patients. Following on from the previous two chapters, I wanted to continue and look more specifically at PGE_2 and the EP_2 and EP_4 receptors.

In all wild type controls there was a significant increase in MVL within the trachea and in the bronchi and IPA following OVA challenge, but in the EP_2 and the EP_4 knock-out mice this response was absent. This data indicates a role for PGE_2 in mediating airway MVL in this model of allergic airway inflammation via the activation of the EP_2 and the EP_4 receptors. Of course knock outs do not represent the perfect model, as it is not clear at which point this gene knock out is important, i.e. is it at the point of sensitisation or challenge. It may be different for each different knock out. Unfortunately, a significant attenuation of OVA-induced MVL was not observed when the EP_2 antagonist (PF-04418948) and the EP_4 antagonist (ER-819762) were administered to OVA challenged wild-type mice.

There has been little research on the EP receptors and their effects on MVL in asthma models but their effects on other aspects of asthma have been investigated. Interestingly, Herrerias *et al* (2009) observed an increase in EP_2 mRNA expression in the lungs of mice sensitised and challenged with house dust mite (HDM), as well as in increase in PGE_2 in their BAL fluid. Additionally, a similar finding has been observed in airway smooth muscle cells from asthmatic patients where a selective upregulation of EP_2 receptors was seen (Burgess *et al*. 2004). Swedin *et al* (2009) observed increased levels of PGE_2 in the BAL fluid of OVA challenged mice. Moreover, they demonstrated significant reductions in eosinophils and IL-4 levels following diclofenac administration (Swedin *et al*, 2009). Kunikata *et al* (2005) investigated all four EP receptor knock-outs in their OVA model and indicated a primary role for the EP_3 receptors. They demonstrated that mice lacking EP_3 receptors developed more pronounced allergic inflammation while a selective EP_3

agonist suppressed airway inflammation. Furthermore, their immunohistochemical studies showed that microsomal prostaglandin E synthase 1 is highly expressed in the airway epithelial cells of antigen-challenged mice (Kunikata *et al*. 2005). Unfortunately I was unable to look into all four EP knock-outs in this part of the thesis, but from the data obtained in chapter 2 there was a clear rationale for only looking into the EP_2 and EP_4 receptor knock-outs.

In summary, this chapter has demonstrated that Evans Blue dye is a suitable marker for measuring airway MVL seen in the murine OVA model of allergic asthma and that there may be a role for prostanoids in mediating this effect. Furthermore investigations using EP receptor knock-out mice has elucidated a role for EP_2 and EP_4 receptors in mediating this airway MVL. However, I cannot fully accept the chapter hypothesis as the data using the EP_2 and EP_4 antagonists proved inconclusive and does not indicate a definitive role for these receptors. This is a novel finding that will hopefully further understanding into the role that PGE_2 plays in airway inflammation.

Chapter Six

The Role of Additional Prostanoids in Mediating Airway MVL in the Mouse

6. The role of additional prostanoids in mediating airway MVL in the mouse

6.1. Rationale

Having extensively investigated PGE_2 and the EP receptors in mediating airway MVL throughout this thesis, the next step was to begin studying the role that other prostanoids play. Like PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2 and TXA_2 are products of cyclooxygenase mediated metabolism of arachidonic acid and respective synthases. They exert there biological effects via the activation of specific G-protein coupled receptors; $DP_{1\text{-}2}$, FP, IP and TP, respectively (Coleman *et al*, 1994). Within the airways prostanoids have been known to powerfully contribute to airflow regulation, constricting bronchial smooth muscles, increasing mucous secretion, microvascular leakage and functioning as a chemoattractant for inflammatory cells (Barnes *et al*, 1998)

As mentioned previously we had all prostanoid receptor knock-out mice available to use (DP_1, DP_2, FP, IP and TP) as well as agonists for each receptor subtype. This enabled me to begin preliminary work on prostanoid-induced airway MVL. As both PGI_2 and TXA_2 are chemically unstable and very short acting, stable analogues of each were used. Iloprost was used as an IP agonist and U46619 as a TP agonist.

6.1.1. Chapter Hypothesis

“Activation of additional prostanoid receptors can result in an increase in microvascular leak in mouse airways”.

6.1.2. Aims

- Investigate the effect of a single dose of each prostanoid ligand (PGD_2, $PGF_{2\alpha}$, Iloprost, U46619).
- Investigate whether each agonist is acting via their own receptor subtype to mediate this effect.
- Examine the effect of each agonist on systemic blood pressure.

6.2. Methods

The following experiments were carried out as described in Chapter 2. Details of individual experiments are described below.

6.2.1. Effect of PGD_2, $PGF_{2\alpha}$, U46619 and Iloprost on airway MVL in mice

Having established that PGE_2 induces MVL in the airways of mice, the role of the other prostanoids was then investigated. Following EB administration, animals received PGD_2, $PGF_{2\alpha}$, U46619 (TP receptor agonist), Iloprost (IP receptor agonist) or PGE_2 (3 mg kg^{-1}) i.v. at 4 ml kg^{-1} or Vehicle (1% ethanol in saline). The 30 minute time point was used for all agonists.

6.2.2. Effect of PGD_2, $PGF_{2\alpha}$, U46619 and Iloprost in individual prostanoid receptor knock-out mice

To establish whether the prostanoid receptor agonists were mediating MVL via their own receptor, DP_1, DP_2, TP, IP and FP receptor deficient mice were used. One minute after Evans Blue WT animals received PGD_2, $PGF_{2\alpha}$, U46619, or Iloprost or vehicle, DP_{1+2} KOs vehicle or PGD_2, FP KOs vehicle or $PGF_{2\alpha}$, TP KOs vehicle or U46619 and IP KOs vehicle or Iloprost. All wild types were C57BL/6 littermate controls. The 30 minute time point still applied here.

6.2.3. Effect of PGD_2, $PGF_{2\alpha}$, U46619 and Iloprost on systemic blood pressure

Male C57BL/6 mice were anaesthetised as previously described and their carotid artery cannulated (as outlined in 2.4). Once a baseline blood pressure recording was achieved (approximately 5 minutes), the jugular veins were exposed and an intravenous dose of PGD_2, $PGF_{2\alpha}$, U46619, Iloprost (3 mg kg^{-1}) or vehicle (1% ethanol in saline) administered. Mean arterial blood pressure (MAP) was measured over 30 minutes after which animals were culled.

6.2.4. Genotyping of Prostanoid Receptor Knockouts

DNA was extracted from tail tips (0.5-1cm) of wild type and prostanoid receptor knockout mice using Quanta Biosciences Extracta DNA prep for PCR kit. The purity and integrity of the DNA samples was assessed using a PowerWave XS Microplate Spectrophotometer (Biotek, Bedfordshire, UK). PCR reactions were run using mouse genomic DNA primers that had been previously optimised and validated. The PCR reaction contained 1x Green GoTaq Flexi buffer, 0.2mM dNTPs, 2mM $MgCl_2$, 1.25µl enzyme (GoTaq polymerase), 10pmol forward and 10pmol reverse primers, and a volume of DNA (50ng) or Nuclease-free water for control, made up to 25µl with Nuclease-free water. Samples were then run on a Taqman machine under the conditions detailed in table 2. DP_1 and DP_2 primers were run in singleplex reactions, so wild type and knock-out primers had to be run as separate reactions, while FP, IP and TP primers were run as multiplex reactions, so wild type and knock-out primers could be run in the same PCR reaction.

The PCR products and DNA ladder (Hyperladder II or IV) were run on a 2% agarose gel in Tris Borate EDTA (TBE) buffer containing 5µl per 100ml Safeview at 100V for about 1 hour. The gel was then visualised under ultra-violet light and photographed using the Molecular Imager® ChemiDoc™ XRS System with Image Lab™ software (Bio-Rad, UK).

Table 6.1. Optimised PCR conditions for genotyping prostanoid receptor KO mice. *DP_1 and DP_2 were run as singleplex (indicated by a "-" where that primer isn't included so no band expected) while FP, IP and TP were run as multiplex reactions.*

Receptor	PCR Conditions			Expected Primer Bands (bp)	
	Denaturing	Annealing	Extension	Wild Type	Knockout
FP for/rev/neo	95ºC / 30 s	65ºC / 30 s	75ºC / 45 s	750	500
IP for/rev/neo	94ºC / 30 s	58ºC / 30 s	68ºC / 60 s	1350	900
TP for/rev/neo	95ºC / 30 s	65ºC / 30 s	75ºC / 45 s	250	650
DP_1 (w.t) for/rev	95ºC / 30 s	60ºC / 30 s	75ºC / 90 s	400	1500
DP_1 (KO) neo/rev	95ºC / 30 s	62ºC / 30 s	72ºC / 45 s	-	340
DP_2 (w.t) for/rev	94ºC / 30 s	63ºC / 45 s	72ºC / 60 s	340	-
DP_2 (KO) neo/rev	94ºC / 30 s	55ºC / 45 s	72ºC / 60 s	-	1000

6.2.5. Statistical Analysis

All data was analysed using GraphPad Prism 5. Parametric data was expressed as mean $\pm$ s.e.m and non-parametric data presented as the median with the interquartile range. Multiple measurements were analysed by one-way ANOVA, non-parametric Kruskal-Wallis test and post-hoc comparisons were performed by Dunn's multiple comparison test, comparing selected columns to a control. Additionally, an unpaired t test and/or non-parametric Mann Whitney test, with two-tailed p values, were carried out to determine statistical significance between two groups. Differences were considered statistically significant if $p<0.05$. The figures have been graphically presented on a range-specific axis.

6.3. Results

6.3.1. Effect of PGD_2, $PGF_{2\alpha}$, U46619 and Iloprost on airway MVL in mice

Following the positive data from PGE_2-induced MVL, preliminary work looking into the role of other prostanoids was carried out. PGD_2 significantly increased MVL in both the trachea and bronchi and IPA of mice, whilst $PGF_{2\alpha}$ caused a significant increase in the trachea. Additionally, the TP agonist U46619 induced a significant increase in MVL in the bronchi and IPA. In the trachea U46619 increased MVL but failed to reach statistical significance. Similarly with the IP agonist Iloprost, it induced a small increase in airway MVL but this did not reach statistical significance (Figure 6.1).

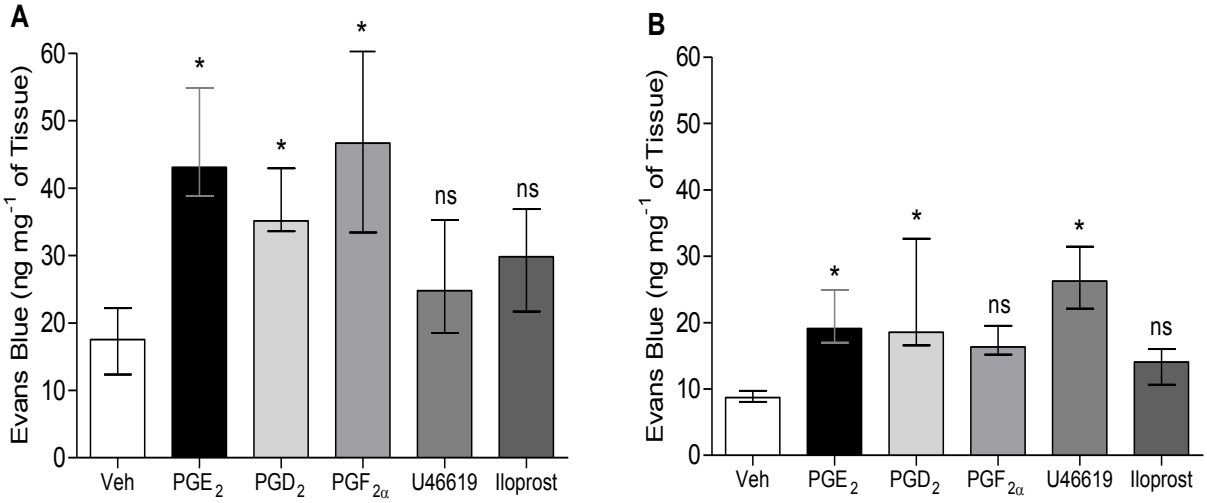


Figure 6.1. **Effect of PGE_2, PGD_2, $PGF_{2\alpha}$, U46619 and Iloprost (3 mg kg^{-1} i.v.; 30 minutes) on MVL into (A) trachea or (B) bronchi and intra-pulmonary airways of mice.** Data presented as the median, with the interquartile range, of the concentration of Evans blue dye (ng mg^{-1} of tissue). *n=6. One-way ANOVA with non-parametric Kruskal-Wallis test and post-hoc comparisons using Dunn's multiple comparisons test. * p<0.05 significant increase from vehicle control. ns = non significant increase*

6.3.2. Effect of PGD_2, $PGF_{2\alpha}$, U46619 and Iloprost in individual prostanoid receptor knock-out mice

Having demonstrated that the other prostanoids were able to induce airway MVL, their effect in individual receptor knock-out mice was then investigated. Unfortunately, I only had the opportunity to look at each prostanoid agonist with their respective receptor knockout. Firstly, PGD_2 demonstrated a measureable (and significant) increase in airway MVL in the wild type and DP_2 knock outs. This response, however, was absent in the trachea and bronchi and IPA of DP_1 knock outs (Figure 6.2).

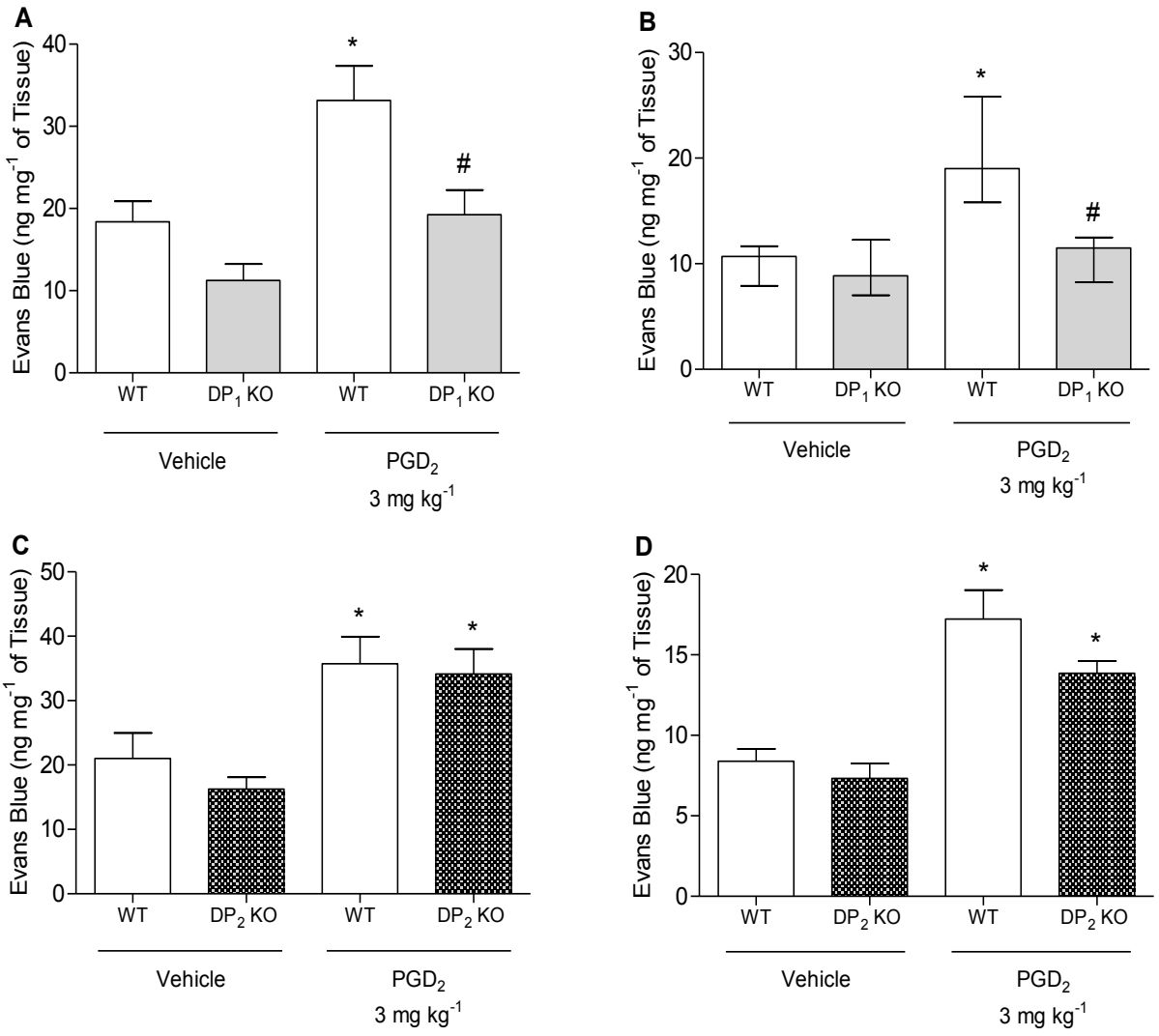


Figure 6.2. Effect of PGD_2 (3 mg kg^{-1} i.v.; 30 minutes) in wild type, (A+B) DP_1 and (C+D) DP_2 receptor knockouts. A and C represent the trachea and B and D the bronchi and IPA. Non-parametric data presented as the median, with the interquartile range, and parametric data expressed as mean ± s.e.m of the concentration of Evans blue dye (ng mg^{-1} of tissue). *n=5-7. Non-parametric*

*Mann Whitney test or unpaired t test for parametric data. * p<0.05 significant increase from vehicle control. # p<0.05 significant decrease from treated wild type control.*

In this study, $PGF_{2\alpha}$ induced a substantial and significant increase in MVL in the trachea and bronchi and IPA of wild type mice compared to vehicle treated controls. However, when administered to FP receptor knockouts this response was significantly reduced (Figure 6.3).

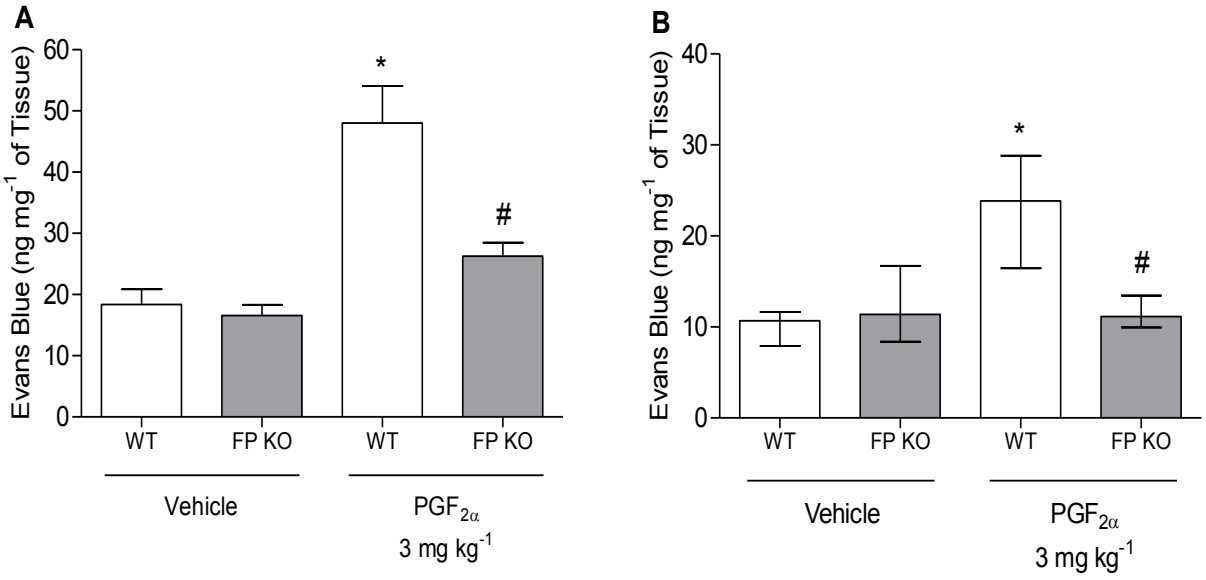


Figure 6.3. Effect of $PGF_{2\alpha}$ (3 mg kg^{-1} i.v.; 30 minutes) on MVL in the (A) trachea and (B) bronchi and IPA of wild type and FP receptor knockout mice. Non-parametric data presented as the median, with the interquartile range, and parametric data expressed as mean ± s.e.m of the concentration of Evans blue dye (ng mg^{-1} of tissue). *n=6. Non-parametric Mann Whitney test or unpaired t test for parametric data. * p<0.05 significant increase from vehicle control. # p<0.05 significant decrease from treated wild type control.*

In wild type mice the TP agonist U46619 significantly increased MVL in both the trachea and bronchi and IPA. In the TP knockout mice U46619 was unable to induce any Evans blue leak (Figure 6.4).

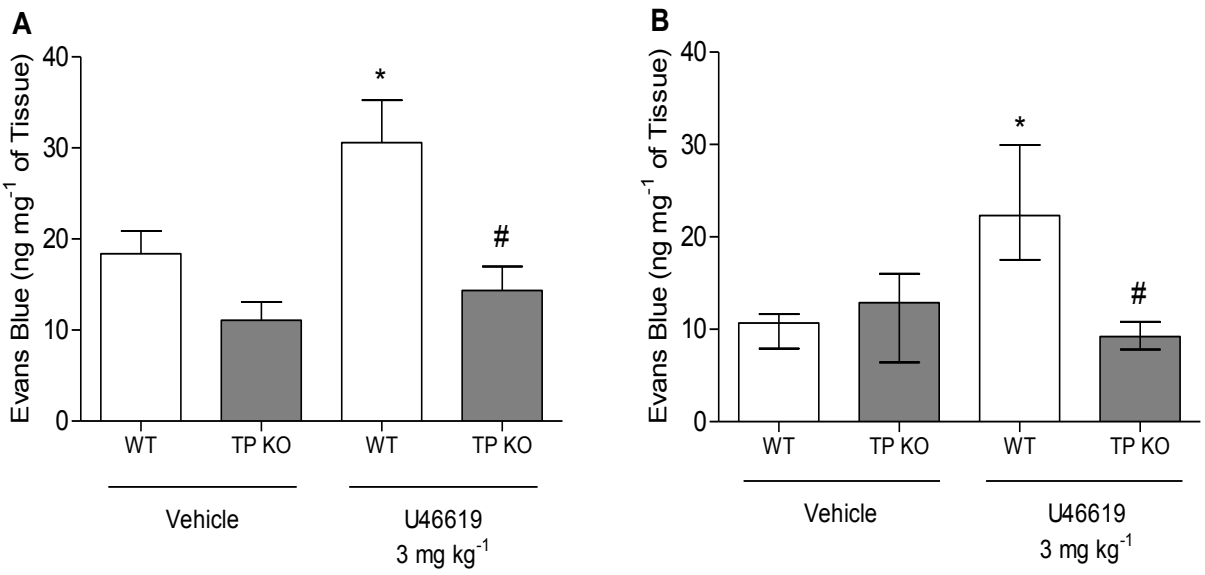


Figure 6.4. Effect of U46619 (3 mg kg^{-1} i.v.; 30 minutes) on MVL in the (A) trachea and (B) bronchi and lPA of wild type and TP receptor knockout mice. Non-parametric data presented as the median, with the interquartile range, and parametric data expressed as mean ± s.e.m of the concentration of Evans blue dye (ng mg^{-1} of tissue). *n=6. Non-parametric Mann Whitney test or unpaired t test for parametric data. * p<0.05 significant increase from vehicle control. # p<0.05 significant decrease from treated wild type control.*

In the previous study the IP agonist Iloprost did not demonstrate a significant increase in MVL in either airway tissues, however on this occasion Iloprost produced a significant increase in MVL in the trachea. This response was then completely absent in IP receptor knockout mice (Figure 6.5).

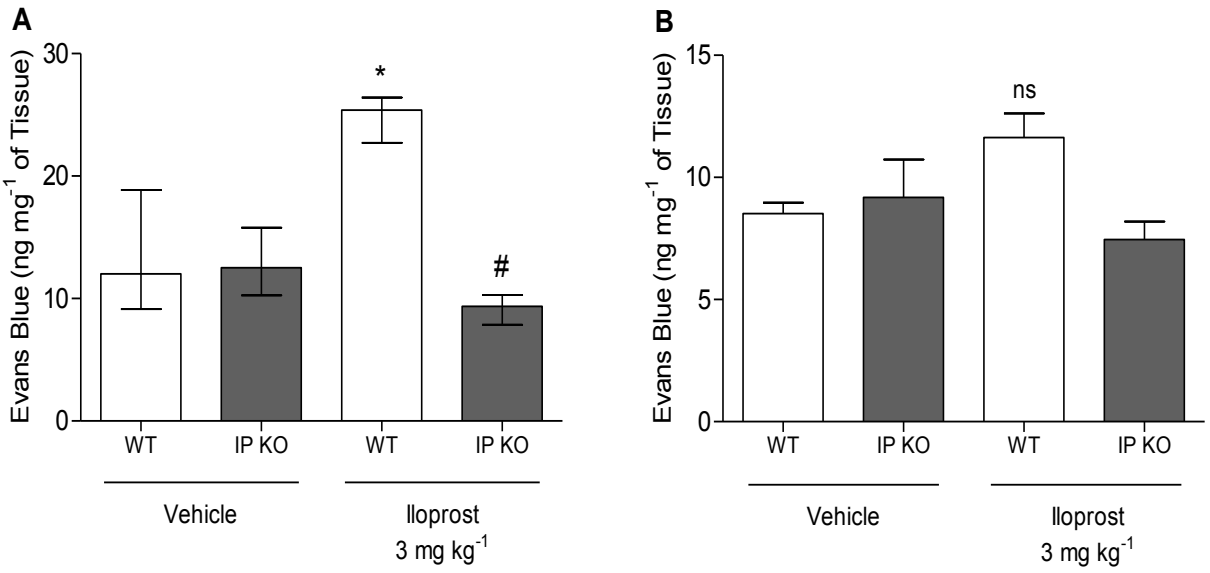


Figure 6.5. Effect of Iloprost (3 mg kg^{-1} i.v.; 30 minutes) on MVL in the (A) trachea and (B) bronchi and IPA of wild type and IP receptor knockout mice. Non-parametric data presented as the median, with the interquartile range, and parametric data expressed as mean ± s.e.m of the concentration of Evans blue dye (ng mg^{-1} of tissue). *n=6. Non-parametric Mann Whitney test or unpaired t test for parametric data. ns = non significant increase. * p<0.05 significant increase from vehicle control. # p<0.05 significant decrease from treated wild type control.*

All of the mice used for these experiments were genotyped using the methods outlined in Chapter 2. As an example, a gel is shown to confirm the disruption of the gene encoding the FP receptor in FP receptor knockout mice. Using the FP primers, the PCR products were run on a gel which allowed confirmation of wild type and FP knockouts, as well as the presence of the wild type allele and the absence of the knock out allele in the DP_1, DP_2, IP, and TP knockouts (Figure 6.6).

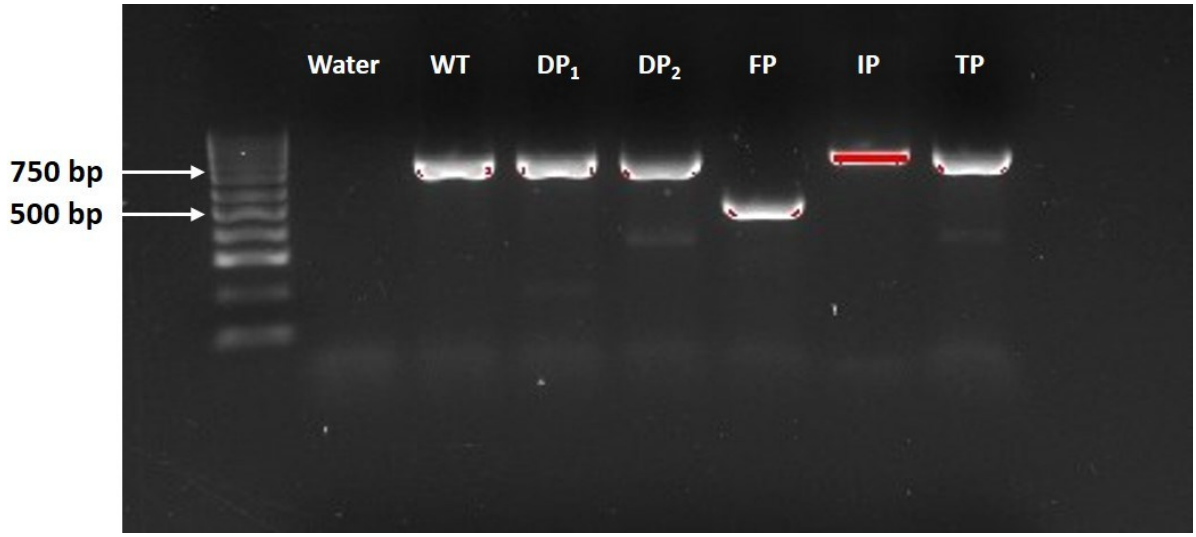


Figure 6.6. An example gel showing the PCR products of DNA extracted from wild type, DP_{1-2}, FP, IP and TP KO mice. This representation was using the FP primers. Product size in wild type was 750 bp and FP KO was 500 bp.

6.3.3. Effect of PGD_2, $PGF_{2\alpha}$, U46619 and Iloprost on systemic blood pressure

Having shown each prostanoid had the ability to induce airway microvascular leak, the effect on systemic blood pressure was then determined. All prostanoid agonists were given intravenously at a dose of 3 mg kg^{-1}. Firstly, PGD_2 and $PGF_{2\alpha}$ only caused a small rise in blood pressure compared to vehicle treated animals. The TP receptor agonist, U46619, caused a substantial and rapid rise in blood pressure that appeared transient while the IP agonist, Iloprost, induced a large drop in blood pressure that was sustained over the 10 minutes.

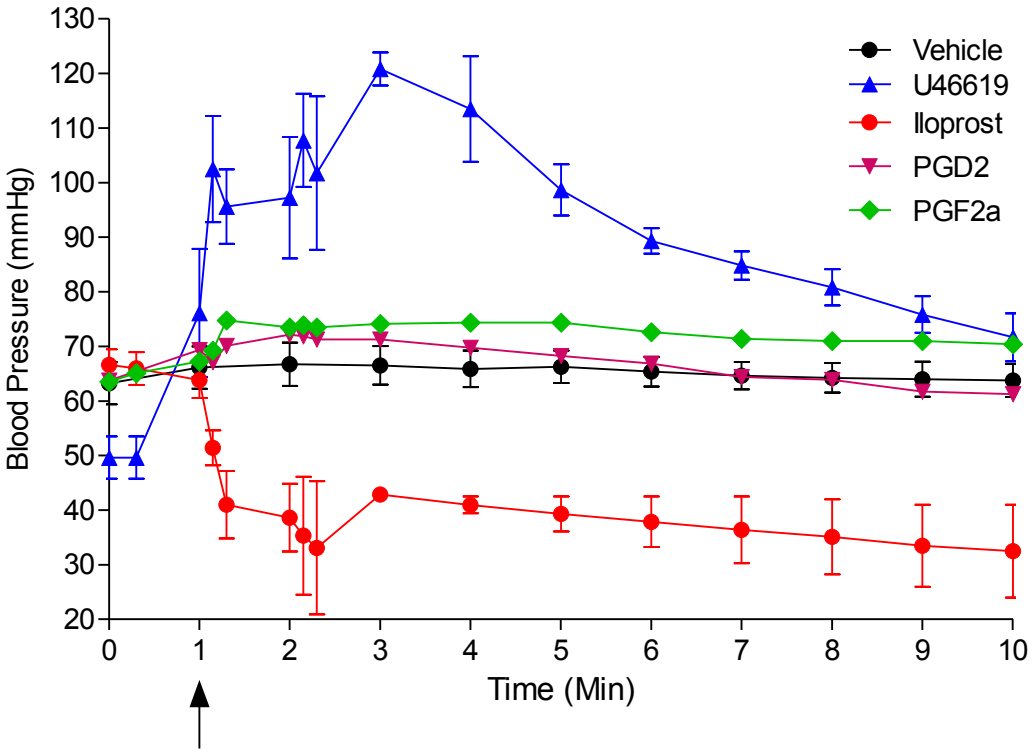


Figure 6.7. Effect of PGD_2, $PGF_{2\alpha}$, U46619 and Iloprost (3 mg kg^{-1} at 4 ml kg^{-1} i.v.) on mean arterial blood pressure in C57BL/6 mice. Data expressed as mean ± s.e.m of mean arterial pressure. Arrow indicates approximate time of prostanoid administration. Blood pressure was recorded for 10 minutes. n=3-4

6.4. Discussion

Prostanoids, including PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2 and TXA_2 are products of cyclooxygenase mediated metabolism of arachidonic acid and respective synthases. They are formed and released following various, sometimes noxious, stimuli and act within the vicinity of their production where they maintain local homeostasis. In order to exert their biological functions they bind to specific cell surface receptors, all of which are G-protein-coupled receptors. Currently there are nine known prostanoid receptors, $EP_{1\text{-}4}$, $DP_{1\text{-}2}$, FP, IP and TP (Coleman *et al*, 1994). Bronchial asthma is a complex disease that is characterised by recurrent episodes of breathlessness, wheezing, chest tightness and airway obstruction (Bousquet *et al*, 2000). Both inflammatory cells and structural cells play a central role and release many different mediators that have been implicated in allergic disease (Barnes, 2011). Of these mediators, prostanoids have been known to powerfully contribute to airflow regulation, constricting bronchial smooth muscles, increasing mucous secretion, microvascular leakage and functioning as a chemoattractant for inflammatory cells (Coleman & Sheldrick, 1989; Hardy *et al*, 1984; Jones *et al*, 1992; Lötvall *et al*, 1992; Tokuyama *et al*, 1991; Barnes *et al*, 1998).

In Chapters three, four and five I have extensively investigated the role of PGE_2 and the EP_2 and EP_4 receptors in mediating airway microvascular leak. I demonstrated that these two receptors are central to the airway microvascular leak observed following PGE_2 administration. Furthermore, using the OVA model of asthma I saw a reduction in OVA-induced MVL in the airways of EP_2 and EP_4 receptor knockout mice. In this chapter I present some preliminary data investigating additional prostanoids and their role in airway microvascular leak. PGD_2, $PGF_{2\alpha}$, PGI_2 and TXA_2 have all, at some point, been implicated in airway inflammation and disease. This preliminary work has illustrated a possible role for all prostanoids in inducing plasma leakage in either the trachea or bronchi and IPA or both.

Following intravenous administration of PGD_2 a substantial and significant increase in airway MVL was observed, a response comparable to that seen with PGE_2. Additionally, when PGD_2 was given to DP_1 and DP_2 receptor knockout mice a

reduction in airway MVL was seen only in the DP_1 knockout mice while an increase in MVL was still apparent in the DP_2 knockouts. This suggests that the airway microvascular leak induced by PGD_2 is via the activation of the DP_1 receptor and subsequent downstream signalling. PGD_2 is the major prostanoid that is produced by activated mast cells and is highly associated with inflammatory diseases (Roberts *et al*, 1980; Lewis *et al*, 1982). Furthermore, analysis of BAL fluid has indicated that PGD_2 is released in the airways following antigen challenge (Murray *et al*, 1986) and can lead to bronchoconstriction (Hardy *et al*, 1984) and eosinophil infiltration (Emery *et al*, 1989). More recently, Nishimura *et al* (2001) studied the effects of PGD_2 in guinea pigs and found that it induced a dose-dependent increase in Evans Blue dye extravasation at all airway levels. However, they observed large increases in systemic blood pressure following PGD_2 administration while in our mouse studies no significant change in pressure was seen. Conversely, PGD_2 has consistently been shown to cause vasodilation and a decrease in blood pressure (Spada *et al*, 2002; Walch *et al*, 1999; Walch *et al*, 2001).

Like the EP_2 and EP_4 receptors DP_1 receptors couple to G_s and stimulate cAMP formation which is the prominent signalling pathway that leads to relaxation responses. As I outlined in Chapter four, increases in intracellular cAMP concentrations are usually associated with an increase in barrier function and so inhibition of microvascular leak while decreasing cAMP is associated with an increase in vascular permeability (Garcia *et al*, 1995; Patterson *et al*, 1995; Chetham *et al*, 1997; Moore *et al*, 1998). It is interesting that DP_2 does not appear to mediate any PGD_2-induced MVL as this receptor is coupled to G_i and a decrease in cAMP (Sawyer *et al*, 2002; Gallant *et al*, 2007). Although no changes in systemic blood pressure were seen, local changes in pressure and blood flow to the lung and airways may have occurred. It could be suggested that PGD_2 is causing vasodilation within the airways and increasing blood flow, an effect which has been known to potentiate microvascular leak (Williams, 1982).

The main implications for $PGF_{2\alpha}$ have been in mammalian reproduction (Sugimoto *et al*, 1997) and it has been long known that functional expression of the FP receptor in the myometrium mediates contraction (Senior *et al*, 1992; Carrasco *et al*, 1996). However, $PGF_{2\alpha}$ has been shown to cause bronchoconstriction of human airways *in*

vitro (Coleman and Sheldrick, 1989) as well as in asthmatic patients (Fish *et al*, 1984). Here I have demonstrated that $PGF_{2\alpha}$ is able to induce significant airway microvascular leak, and from using FP receptor knock outs this effect appears to be via the activation of the FP receptor. Arakawa *et al* (1993) studied the effects of $PGF_{2\alpha}$ in guinea pig airways, and in concordance with my data they demonstrated an increase in Evans Blue extravasation following $PGF_{2\alpha}$ administration. Furthermore, I saw no real change in systemic blood pressure following FP administration. However, Yu *et al* (2009) saw that $PGF_{2\alpha}$ elevates blood pressure and that systolic blood pressure in FP receptor knockout mice was significantly lower than that of wild type mice.

FP receptors are predominantly G_q-coupled and activate the classic pathway. So, following PLCβ activation, there is PI turnover and a transient Ca^{2+} signal in response to inositol trisphosphate formation (Nakao *et al*, 1993; Sugimoto *et al*, 1994; Woodward and Lawrence, 1994). Downstream of this pathway is the activation of myosin light-chain kinase (Ansari *et al*, 2003). According to the dogma on endothelial gap formation and vascular leak, activation of this protein kinase is essential. Briefly, activation of this kinase leads to an increase in myosin light-chain phosphorylation, an increase in actin stress fibres, leading to cell contraction and gap formation (Curry *et al*, 1983; Lu & Wolf, 1993). With no apparent change in blood pressure it could be assumed that the effect of $PGF_2\alpha$ in these experiments is exclusively through endothelial gap formation.

To investigate the role of TXA_2 and the TP receptor U46619, a stable TXA_2 mimetic, was used. TXA_2 has been well documented as a potent constrictor of both vascular and airway smooth muscle (Svensson *et al*, 1977; Coleman *et al*, 1981). Following allergen challenge the stable metabolite TXB_2 has been shown to be elevated in the airways of sensitised guinea pigs and asthma patients (Burka *et al*, 1981; Lupinetti *et al*, 1987) while allergen-induced bronchoconstriction has been inhibited using a TXA_2 antagonist. In initial experiments U46619 only produced a significant increase in MVL in the bronchi and IPA, a response which actually exceeded that seen with any other prostanoid. In subsequent experiments, increases in MVL in the trachea did reach significance. Furthermore, when responses to U46619 were investigated in TP receptor knockout mice the MVL was significantly attenuated, again suggesting

that this prostanoid ligand was acting via its own receptor to induce airway MVL. Others have also shown an increase in Evans Blue extravasation in the airways of rats and guinea pigs following U46619 administration (Lötvall *et al*, 1992; Bertolino *et al*, 1995). In addition, Lötvall *et al* (1992) also found that Evans Blue dye leak was higher in the intrapulmonary airways compared with the trachea. More recently, Nishimura *et al* (2001) examined the role of TP receptors in airway MVL in guinea pigs but looked at the interaction of PGD_2 with this receptor. Interestingly, they found that PGD_2-induced increases in Evans Blue dye extravasation were completely abolished following pre-treatments with TP receptor antagonists. They also used a TXA_2 synthase inhibitor, OKY-046, but found no inhibition of PGD_2 responses indicating that it was the activation of the TP receptor only causing these effects (Nishimura *et al*, 2001).

As well as investigating its effect on airway MVL, systemic blood pressure was also measured using the same dose. U46619 induced a massive and immediate increase in systemic blood pressure in mice. This increase in blood pressure and vasoconstriction induced by TP receptor activation is well documented across various species both *in vitro* (Norel *et al*, 2002; Svensson *et al*, 1977; Coleman *et al*, 1981; Yoshimura *et al*, 1989; Félétou *et al*, 2009) and *in vivo* (Nossaman *et al*, 1992; Bertolino *et al*, 1993; Francois *et al*, 2008; Denniss & Rush, 2009; Graham & Rush, 2009). It has been suggested that mediators that cause plasma extravasation are those that usually produce vasodilation and a drop in systemic blood pressure (Rogers *et al*, 1990; Persson 1987), however having shown that U46619 causes MVL and an increase in systemic blood pressure this may not always be the case. It could be feasible that such an increase in blood pressure may have eventually caused an increase in bronchial blood flow and therefore potentiating Evans Blue exudation. Alternatively the effect seen may be a direct action on the endothelial cells themselves, which have been shown to express TP receptors (Raychowdhury *et al*, 1994; Miggin & Kinsella, 1998). TP receptors have shown coupling to G_q, thus activating PLCβ, generation of IP_3/DAG and mobilisation of intracellular calcium (Hirata *et al*, 1991; Kinsella *et al*, 1997; Offermanns, 2006; Nakahata, 2008). Additional studies have also implicated $G_{12/13}$-Rho pathway (Huang *et al*, 2004; Song *et al*, 2009; Zhang *et al*, 2009). As I described for $PGF_{2\alpha}$ G_q coupling has been associated with endothelial barrier disruption and in addition Rho signalling is also an

important pathway regulating endothelial cell contraction. Briefly, signalling through RhoA destabilises the endothelial junction via Rho Kinase which inactivates myosin light chain phosphatase, causing myosin contraction and the destabilisation of the actin cytoskeleton (Essler *et al*, 1998; Qiao *et al*, 2003; Sun *et al*, 2006). The data I have presented, along with others, outlines clear evidence for the involvement of the TP receptors in mediating airway microvascular leak and contributing to airway inflammation seen in asthma.

Finally, I examined the role of IP receptors in mediating MVL in mouse airways. It was not suitable to use PGI_2 itself in these experiments as it is too chemically unstable with a short duration of action (Coleman *et al*, 1994) so the stable analogue Iloprost was used. PGI_2 plays a central role in oedema formation, hyperalgesia and pain (Bley *et al*, 1998; Hata & Breyer, 2004; Murata *et al*, 1997), while also suppressing Th2-mediated allergic inflammatory responses (Jaffar *et al*, 2002). When first tested, Iloprost caused only modest increases in MVL, failing to reach significance in either the trachea or the bronchi and IPA. In subsequent experiments Iloprost caused a significant increase in MVL in the trachea, which coincided with an attenuated response in IP receptor knockouts. So although only a small response was observed it does appear to occur via the activation of the IP receptor. The IP receptor has been investigated in several models of inflammatory swelling where it appears to play a key role. For example, Murata *et al* (1997) examined carrageenan-induced paw swelling in IP receptor knockout mice and saw a 50% reduction in paw oedema. Yuhki *et al* have also demonstrated that IP receptors participate in exudate formation in a carrageenin-induced mouse pleurisy model, suggesting a role for PGI_2 in plasma extravasation (Yuhki *et al*, 2004). However, others have demonstrated that PGI_2 is able to inhibit and reduce vascular permeability (Moller & Grande, 1997; Cureton *et al*, 2010).

As with the other prostanoid ligands, I also examined the effect of Iloprost on systemic blood pressure in mice. Most studies on vascular tone have described relaxations induced by PGI_2 analogues and Iloprost has been used clinically as a vasodilator to treat pulmonary hypertension and Raynaud phenomenon (Moncada *et al*, 1982; Haye-Legrand *et al*, 1987; Coleman *et al*, 1994; Walch *et al*, 1999; Benedict *et al*, 2007). Indeed, when a dose of 3 mg kg^{-1} was administered, Iloprost

caused a rapid drop in systemic blood pressure that was sustained for at least 10 minutes. It could be suggested that this vasodilatory response may cause in increase in blood flow to airways and thus potentiate MVL. However, as I have described the MVL induced by Iloprost in this model was unremarkable so this assumption may be incorrect. As for DP_1 receptors, IP signals through the G_s pathway, which is the classic pathway responsible for relaxant responses in smooth muscle beds. Conversely, G_s mediated increases in adenylyl cyclase and cAMP levels are associated with barrier protective responses. It is possible that in this model Iloprost is acting to maintain barrier integrity but the substantial increase in blood flow may be counteracting this through hydrostatic changes.

In summary, the data presented in this chapter indicates a role for DP_1, FP, IP and TP receptors in mediating microvascular leak in mouse airways and I am therefore able to accept the chapter hypothesis. However, the significance for some of the prostanoids (noteably Iloprost) varied between studies and between airway tissue types. It must be emphasised that the data presented here is preliminary work and more extensive investigation is required to fully elucidate the role that each prostanoid is playing in mediating airway microvascular leak *in vivo*. Currently, it is only possible to speculate what may be occurring in this model and it is difficult to come to any firm conclusions. The future work that is required is outlined in detail in the following chapter. Nevertheless, prostanoid-induced MVL in murine airways has not been highly reported and is an exciting new finding that will further our understanding into the role that they play in airway inflammation.

Chapter Seven

Summary and Future Studies

7. Summary and Future Studies

7.1. Summary and discussion

Airway microvascular leak is a cardinal sign of inflammation and is a prominent feature in both asthma and COPD. Various inflammatory mediators are capable of inducing this effect following an inflammatory insult to the airways. These inflammatory mediators act upon the endothelium of post capillary venules to open intercellular gap junctions. This effect causes plasma to 'leak' out into extravascular sites (Reynolds *et al*, 2002; Greiff *et al*, 2003). Measuring this event *in vivo* provides a specific index representing to what degree the airway mucosa has been affected by the inflammatory process of a mediator. Events such as these have been associated with the development and exacerbations of airway inflammation, thus modulation of this response represents a key pathway in developing new treatments. What I wanted to investigate was whether prostaglandin E_2 (PGE_2) was involved in mediating this response.

PGE_2 is a ubiquitous eicosanoid that has been implicated in the pathogenesis of both asthma and COPD. It exerts its biological effects via activation of four cell-surface GPCRs, termed $EP_{1\text{-}4}$, encoded for by the genes *Ptger1-4* (Coleman *et al*, 1994). Increased levels of PGE_2 have been reported in BAL fluid and plasma of asthma patients, and enhanced levels in the condensate of COPD patients (Montushi *et al*, 2003; Long *et al*, 2004). Additionally, increased levels of PGE_2 have been observed in the BAL fluid of allergen-sensitised mice (Herrerias *et al*. 2009). However, its role in airway inflammation remains varied and inconclusive and there appears to be a fine balance between the pro and anti-inflammatory effects of this prostanoid in human airways. The aim of this thesis was to determine whether PGE_2 could induce airway microvascular leak *in vivo* and if so which EP receptor was mediating this effect.

Before investigating the effect of PGE_2, however, I needed to determine whether the mouse was a suitable species to model MVL in. Therefore I used 5-hydroxytryptamine (5-HT) as a tool for validating this model, as previous reports have shown potent effects in rats (Hele *et al*, 2001). Here, 5-HT induced a dose

related increase in MVL in both the trachea and the bronchi and IPA of mice. Once the model of MVL was successfully validated in the mouse, the effects of PGE_2 were then investigated. In an initial study PGE_2 produced only a moderate increase in MVL, using timings taken from the 5-HT study. This was evident in both the trachea and the bronchi and IPA, where only a marginal dose response relationship was obvious. The effect of PGE_2 on airway microvascular leak has not been shown previously, and so timings of administration were based upon previous methods within the group and the literature. However, increasing the time post PGE_2 administration from 5 minutes to 30 minutes produced a greater increase in MVL in the airways of mice. Additionally, a dose response relationship was more evident using this time point in both the trachea and bronchi and IPA.

As previously mentioned, the majority of research into airway MVL has been achieved using larger species, including the rat and the guinea pig. I wanted to rule out that what was being observed with PGE_2 was not exclusive to the mouse. In the guinea pig PGE_2 produced a large, significant increase in airway MVL that was comparable to that seen with 5-HT. Both the mouse and the guinea pig produced similar profiles in the magnitude of their responses demonstrating that the effect of PGE_2 is translated across species.

As described previously small changes in blood flow may impact on MVL, so it could be possible that the PGE_2-induced MVL may be caused by vasodilation and an increase in blood flow to the airways. However, in contrast to the rapid and substantial drop in blood pressure (vasodilation) seen with the IP agonist Iloprost, PGE_2 appeared to have very little effect on the systemic blood pressure in mice. Although this does not represent local changes in blood pressure and flow (i.e. in the bronchial circulation) it does suggest that there is not a clear and defined association between vasodilation and increased airway microvascular leak in this study.

Having shown that PGE_2 induces MVL in the mouse I wanted to determine which of the EP receptors were central to this response. To do this I used mice deficient of each individual EP receptor as well as a range of pharmacological tools, including agonists and antagonists targeting each receptor subtype. What was evident in both the trachea and the IPA was that the response to PGE_2 in the EP_2 and EP_4 knock-out mice was completely abolished. This data suggests that both the EP_2 and EP_4

receptors may be essential in this process. As well as the knock-outs I was fortunate enough to use selective EP receptor agonists that were kindly provided by ONO pharmaceuticals. These allowed direct comparisons to be made with the knock-out data. The data clearly demonstrated that, similar to PGE_2, the EP_2 and EP_4 agonists significantly increased the airway plasma leakage. This confirmed that activation of these EP receptors mediates microvascular leak in mouse airways, while activation of EP_1 and EP_3 produced no measurable increase.

Although knock-out mice for each prostanoid receptor subtype has helped to investigate the physiological and pathological roles of this receptor family, they are only limited to the mouse. Having demonstrated a clear role for EP_2 and EP_4 receptors in mediating airway microvascular leak, I wanted to examine these further using pharmacological tools. An EP_2 antagonist (PF-04418948) was ineffective at inhibiting EP_2-induced MVL when dosed orally, but when delivered via the i.p route it produced a dose-dependent decrease in MVL. Similarly, an EP_4 antagonist (ER-819762) was unable to block EP_4-mediated MVL following oral administration, while after i.p administration it attenuated the response to the EP_4 agonist. The next step was to investigate their effects against PGE_2-induced MVL. In initial experiments neither antagonist was effective at inhibiting PGE_2-induced airway MVL. However, when both antagonists were dosed simultaneously, a prominent inhibition in PGE_2 leak was seen. This data further suggests a dual role for the EP_2 and EP_4 receptors in mediating PGE_2 induced responses.

The next aim was to establish whether PGE_2 and the EP receptors were important in mediating airway MVL in a disease model of asthma. Firstly, I established that Evans Blue was a suitable marker to measure MVL in the Ovalbumin model of allergic asthma. The data showed a significant increase in airway MVL two hours after a final intranasal challenge of OVA. As mentioned before, my primary aim was to determine whether PGE_2 and the EP receptors are involved in this response. So firstly I used diclofenac, a non-selective COX inhibitor, to block endogenous prostanoid production (Mitchell *et al*. 1993). Diclofenac was able to partially inhibit EB MVL in the trachea but not within the lower airways (bronchi and IPA) suggesting that prostanoids may have a tissue specific role in OVA-induced MVL. Following on from this, EP_2 and EP_4 receptor knock-out mice were used. In all wild type controls

there was a significant increase in airway MVL following OVA challenge, but in the EP_2 and the EP_4 knock-out mice this response was absent. This effect was obvious in both the trachea and in the bronchi and IPA, unlike that seen following diclofenac administration. Finally, this data was mimicked when the EP_2 antagonist (PF-04418948) and the EP_4 antagonist (ER-819762) were administered to OVA challenged wild-type mice. Simultaneous dosing of the antagonists attenuated the OVA-induced airway MVL. This data indicates a role for PGE_2 in mediating airway MVL in this model of allergic airway inflammation via the activation of the EP_2 and the EP_4 receptors.

Finally, having extensively investigated PGE_2 and the EP receptors in mediating airway MVL, the next step was to begin preliminary work studying the role that other prostanoids play. PGD_2, $PGF_{2\alpha}$, Iloprost (IP) and U46619 (TP) all induced airway MVL in mice and it appeared that they did so via the activation of their own receptor subtype. For PGD_2, it was evident that it was the activation of the DP_1 and not the DP_2 receptor that mediated airway MVL. Additionally, their effect on systemic blood pressure was also measured. The TP agonist, U46619, produced a substantial increase in blood pressure while the IP agonist, Iloprost, showed an opposing effect, causing a sharp drop in mean arterial blood pressure. PGD_2 and $PGF_{2\alpha}$, however, did not cause any remarkable change in blood pressure at the dose tested. It must be emphasised that this data is preliminary work and more extensive investigation, outlined in section 7.2 below, is required to fully elucidate the role that each prostanoid is playing in mediating airway microvascular leak *in vivo*.

7.1.1. Limitations of the thesis

The experiments that have been used throughout this thesis have provided data indicating that PGE_2 activates both the EP_2 and EP_4 receptors to mediate microvascular leak in murine airways. However, there are several limitations associated with some of the experimental techniques used. Evans Blue dye has been used for decades as a marker of albumin leak in a variety of species (Miles & Miles, 1952; Rogers *et al*, 1988; Baluk *et al*, 1999; Reynolds *et al*, 2002; Xle *et al*, 2003; Zhuang *et al*, 2011). It has been extremely useful in helping to understand the

mechanisms and mediators that are involved in microvascular leak *in vivo* as it is a relatively straightforward and reliable method. One of the main problems that is associated with this technique is that it is a terminal procedure, so each animal is limited to a single intervention. This means that fairly large cohorts are required, especially for studies that examine the effects over time. Furthermore, the data obtained only provides a reference to the net accumulation of dye in the tissues and so it is difficult to measure any fluctuations that may be occurring in vascular permeability. As I have outlined in previous chapters, microvascular leak has been shown to be influenced by other physiological factors, namely changes in blood pressure and blood flow to the microvasculature. Additionally, it could be possible that the perfusion of the circulation is not enough to remove all of the intravascular dye. However in comparison to some of the alternatives it is safer, cheaper and easier to carry out while still providing an accurate measure of microvascular permeability (reviewed by Kenne and Lindbom, 2011). For example, with intravital microscopy there are often major difficulties in quantifying the plasma leakage from the images obtained and is very difficult for deep tissues such as the lung. Furthermore, the use of radiolabelled tracers as an alternative to Evans Blue dye does not provide a more accurate measure and comes with the obvious safety hazards associated with radiation (Rogers *et al,* 1989).

As I mentioned above changes in blood pressure have been shown to potentiate microvascular leak which is why I measured mean arterial blood pressure in mice following administration of prostanoids. However, this was done independently of the MVL studies so direct correlations cannot be made. More importantly, the blood pressure was measured via the cannulation of the carotid artery, which measured systemic pressure changes that does not indicate any changes occurring within the airway circulation.

7.2. Future Studies

7.2.1. Effect of EP agonists on systemic blood pressure

Unfortunately, due to time and compound availability, the effect of the selective EP agonists on systemic blood pressure was never tested. When PGE_2 was given very little effect was seen but this may be down to the opposing effects of the EP receptors. As I described in previous chapters, EP_2 and EP_4 receptors have been shown to mediate relaxation of vascular smooth muscle and a fall in blood pressure (Davis *et al*, 2004; Imig *et al*, 2002; Kennedy *et al*, 1999; Zhang *et al*, 2000), while the EP_1 and EP_3 receptors are associated with vasoconstriction (Baxter *et al*, 1995; Qian *et al*, 1994; Stock *et al*, 2001; van Rodijen *et al*, 2007; Walch *et al*, 2001). Data from this would fit nicely with what I have shown so far and may aid in understanding how PGE_2-$EP_{2/4}$ signalling causes airway MVL.

7.2.2. The role of PGE_2 and the EP receptors in the guinea pig

Having demonstrated that PGE_2 was able to induce substantial MVL in the guinea pig at 3 mg kg^{-1} it seems appropriate to continue this work and look at a dose response to establish a more suitable and perhaps lower dose to use. Additionally the EP_2 and EP_4 receptor agonists and antagonists would be used to explore whether these were responsible for PGE_2 induced MVL in guinea pig airways like in mice. As demonstrated in chapter 4 I would dose the guinea pigs i.p with the antagonists at various doses.

Furthermore I would investigate the effects of PGE_2 and the EP receptor agonists on systemic blood pressure in guinea pigs. Having shown that in the mouse that little effect was seen on blood pressure, which may have in turn had an effect on the level of airway microvascular leak it seems appropriate to decipher whether there are any differences between species.

7.2.3. Investigating the prostanoid receptors mediating airway microvascular leak

Preliminary data illustrated in chapter 6 showed that all the other prostanoids (PGD_2, $PGF_{2\alpha}$, Iloprost and U46619) are able to induce airway MVL in mice. I also demonstrated that when each agonist was given to the respective receptor knock out it appeared their responses were mediated via their own receptor. However it has been reported that PGD_2 can induce its effects via the activation of the TP receptor (Nishimura *et al*, 2001). Therefore, I would give each agonist to each knock out mouse, i.e., PGD_2 would be administered to the FP, IP and TP receptor knock outs. Additionally, to follow up the data from the DP_2 knock outs I would use specific DP_2 agonists or antagonists.

7.2.4. Modelling OVA-induced airway MVL in prostanoid receptor knock-out mice

I have previously shown that diclofenac (a non-selective COX inhibitor) is capable of inhibiting OVA-induced MVL which suggests that prostanoids may be mediating this effect. However this alone does not pin point a specific prostanoid or prostanoid receptor. I did then show that the EP_2 and EP_4 receptors may play a key role in this response and in chapter 6 I observed that PGD_2, $PGF_{2\alpha}$, Iloprost and U46619 are all able to induce airway MVL in mice. Therefore the next step would be to investigate the other prostanoid receptor knock outs (DP_{1+2}, FP, IP and TP) in this OVA-induced MVL model.

7.2.5 Modelling airway MVL in other airway disease models

Microvascular leak is a prominent feature in both acute and chronic airway diseases and increased levels of plasma proteins has been measured in the sputum of COPD patients (Hill *et al*, 1999; Minikata *et al*, 2005; Bessa *et al*, 2012). Studies would include acute Lipopolysaccharide (LPS)-exposure, which provides a model of acute

innate immunity-derived inflammation and is a contaminant of tobacco smoke that has been implicated in the development of COPD. Additionally, both *in vitro* and *in vivo* LPS models have yielded significant protein extravasation (Hele *et al*, 2000; Irie *et al*, 2001; Bogatcheva *et al*, 2009; Schlegal *et al*, 2012; Jian *et al*, 2013; Sun *et al*, 2013). Acute cigarette smoke exposure acts as a model of airway inflammation using the same causative agent for the vast majority of COPD cases (Rabe *et al*, 2007) and has shown considerable MVL in a number of different models (Lundberg & Saria, 1983; Geppetti *et al*, 1993; Andrè *et al*, 2008; Sharma *et al*, 2008; Schweitzer *et al*, 2011). Additionally, data generated in our lab (unpublished, personal commincation by Birrell *et al*) using both of these models have demonstrated significant increases in protein levels in the BAL fluid (see figure 7.1).

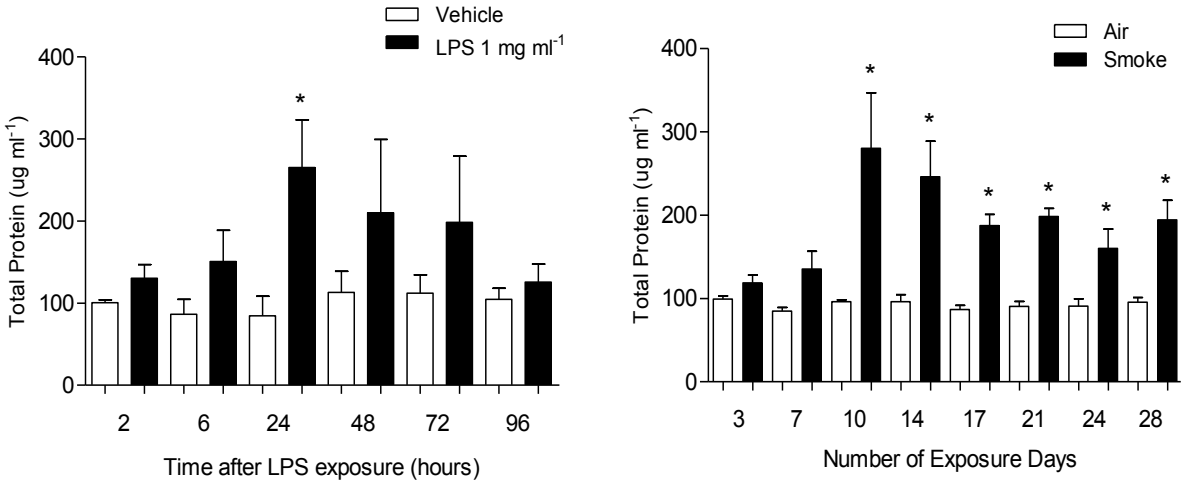


Figure 7.1. The effect of (A) LPS and (B) Cigarette smoke on total protein in BAL fluid of C57Bl6 mice. *(A) Mice were exposed to aerosolised saline or LPS (1 mg ml^{-1} for 30 minutes) and BAL fluid samples were taken 2, 6, 24, 48, 72 and 96 hours after challenge and analysed using a Bradford Assay to quantify protein levels. (B) Mice were exposed (for 50 minutes) twice a day (four hours apart) to cigarette smoke or air for 3, 7, 10, 14, 17, 21, 24 and 28 days. 24 hours after last exposure BAL fluid samples were taken and analysed using a Bradford Assay to quantify protein levels. n=5. * p<0.05; Unpaired t-test for parametric data or Mann-Whitney t-test for non-parametric data.*

Having demonstrated measurable increases in airway MVL in our OVA it would be interesting to replicate this in other airway disease models. Furthermore, the role that EP receptors place in mediating these effects has not been extensively investigated.

7.2.6. Modelling MVL in Human Lung Microvascular Endothelial Cells

All the data that I have presented on airway MVL has been observed *in vivo* in rodents. It would therefore be important to investigate the role of PGE_2 in a human cell based system. The method for measuring endothelial cell permeability to albumin was outlined in chapter 2. Preliminary work using Human Lung Microvascular Endothelial Cells (HLMVECs) highlighted a possible role for the TP receptor in mediating endothelial monolayer permeability (Figure 7.2) and gene expression studies have demonstrated that HLMVECs express EP_1, EP_4, FP, IP, TP and DP_2 receptors (Figure 7.3).

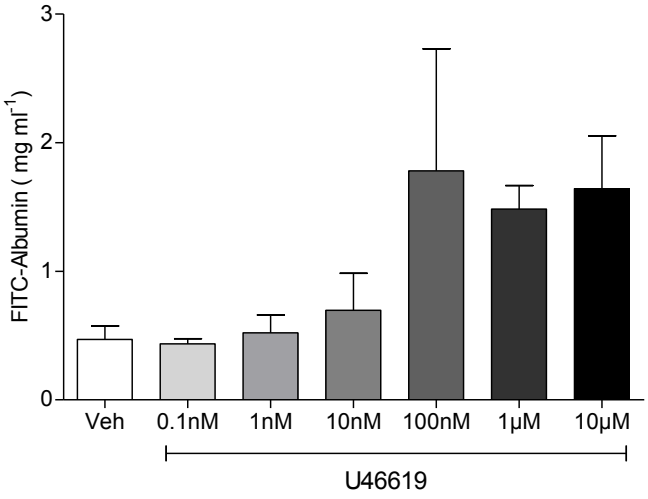


Figure 7.2. Effect of the TP agonist, U46619, on Human Lung Microvascular Endothelial Cell (HLMVEC) permeability. *The movement of FITC-albumin was used as a measure of permeability across the endothelial cell monolayer. N=1, n=6. The MTT Assay indicated that none of prostanoid agonists had an effect on cell viability.*

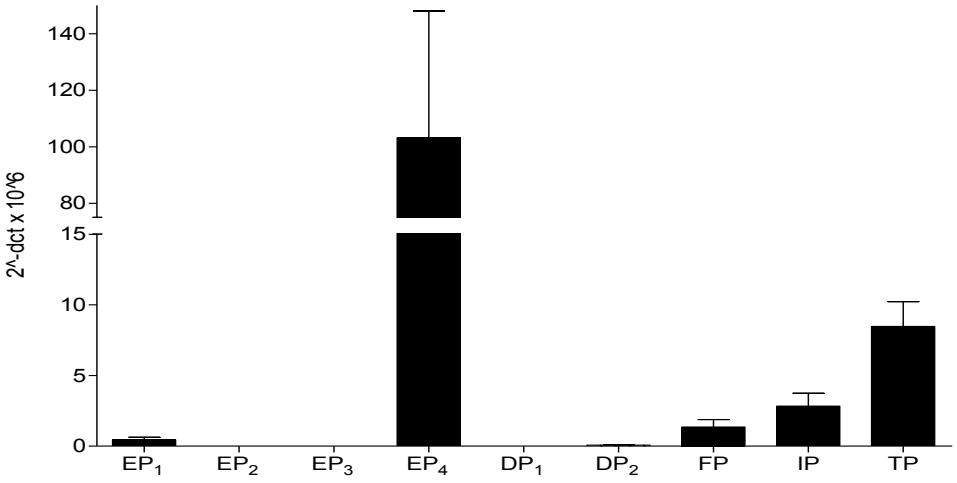


Figure 7.3. mRNA expression of prostanoid receptors (EP_{1-4}, DP_{1-2}, FP, IP, TP) in HLMVEC. *n=2.*

This work with the human lung microvascular endothelial cells is preliminary and only performed in one patient. Therefore it would be important to repeat this set of experiments with more patients if and when they become available. Additionally I would also investigate the various prostanoid receptor antagonists in this system. As I outlined in chapter 6, this may not be the best way of modelling MVL *in vitro*, certainly not by itself. However, in terms of equipment, costs and expertise this was all that was available for this thesis. If I could repeat this I would run this alongside a system that used electrical resistance or impedance (TEER or ECIS) as a measure of barrier integrity as these provide a more accurate representation of the stability of the endothelial barrier.

7.2.7. Modelling MVL in Mouse Lung Microvascular Endothelial Cells

The preliminary work described above was done using human endothelial cells. However, the majority of the work presented in this thesis was from mice, therefore to make the data more translatable I would repeat the cell work in mouse lung microvascular endothelial cells (MLMVEC). The extraction of these cells has been described by several groups (Licht *et al*, 2006; Rao *et al*, 2007; Farley *et al*, 2008; Schniedermann *et al*, 2010) and would be a great addition to the data that I have

obtained through my *in vivo* studies. In addition, Rao *et al* (2007) also examined EP receptor mRNA expression on primary mouse microvascular endothelial cells and detected expression of EP_1, EP_2 and EP_4. Finally, I would examine the effects of prostanoids in microvascular endothelial cells from the knock-out mice.

7.3. Concluding Remarks

In summary, the data presented in this thesis provides evidence that PGE_2 is able to increase airway microvascular leak via the activation of EP_2 and EP_4 receptors and so I am therefore able to accept the hypothesis for this thesis. In addition, both receptors also appear to play a key role in OVA-induced airway MVL. However, this data is only illustrated using EP receptor knock-out mice and so warrants further investigation. The exact mechanism behind PGE_2-induced MVL is not clear and it is possible that haemodynamic changes may be responsible. Furthermore, preliminary data indicates a role for the other prostanoids in mediating plasma extravasation. The future work outlined in section 7.2 will hopefully provide further insight into prostanoid-induced airway MVL.

Reference List

Abe, H., Takeshita, T., Nagata, K., Arita, T., Endo, Y., Fujita, T., … Sugamura, K. (1999). Molecular cloning, chromosome mapping and characterization of the mouse CRTH2 gene, a putative member of the leukocyte chemoattractant receptor family. *Gene*, *227*(1), 71–7.

Abramovitz, M., Adam, M., Boie, Y., Carrière, M., Denis, D., Godbout, C., … Metters, K. M. (2000). The utilization of recombinant prostanoid receptors to determine the affinities and selectivities of prostaglandins and related analogs. *Biochimica et Biophysica Acta*, *1483*(2), 285–93.

Abramovitz, M., Adam, M., Boie, Y., Grygorczyk, R., Rushmore, T. H., Nguyen, T., Funk, C. D., Bastien, L., Sawyer, N., Rochette, C., Slipetz, D. M. & Metters, K. M. (1995). Human prostanoid receptors: cloning and characterisation. *Advances in Prostaglandin, Thromboxane and Leukotriene Research*, 23, 499-504.

Adam, M., Boie, Y., Rushmore, T. H., Müller, G., Bastien, L., McKee, K. T., … Abramovitz, M. (1994). Cloning and expression of three isoforms of the human EP3 prostanoid receptor. *FEBS Letters*, *338*(2), 170–4.

Adamson, R. H., Liu, B., Fry, G. N., Rubin, L. L., & Curry, F. E. (1998). Microvascular permeability and number of tight junctions are modulated by cAMP. *The American Journal of Physiology*, *274*(6 Pt 2), H1885–94.

Agustí, A. G. N. (2005). Systemic effects of chronic obstructive pulmonary disease. *Proceedings of the American Thoracic Society*, *2*(4), 367–70; discussion 371–2.

Ahluwalia, A., & Perretti, M. (1994). Effect of Prostanoids Evidence for a Role of EP3-Receptors ' in Mouse and Rat Skin : *The Journal of Pharmacology and Experimental Therapeutics*, *268*(3), 1526–1531.

Amano, M., Ito, M., Fukata, Y., Chihara, K., Nakano, T., Matsuura, Y., & Kaibuchi, K. (1996). Phosphorylation and Activation of Myosin by Rho-associated. *The Journal of Biological Chemistry*, *271*, 20246–20249.

Amerongen, G. P. V. N., Draijer, R., Vermeer, M. a., & van Hinsbergh, V. W. M. (1998). Transient and Prolonged Increase in Endothelial Permeability Induced by Histamine and Thrombin : Role of Protein Kinases, Calcium, and RhoA. *Circulation Research*, *83*(11), 1115–1123.

Amerongen, G. P. V. N., Vermeer, M. a., & van Hinsbergh, V. W. M. (2000). Role of RhoA and Rho Kinase in Lysophosphatidic Acid-Induced Endothelial Barrier Dysfunction. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *20*(12), e127–e133.

Armstrong, M. J., Boura, A. L. A., Hamberg, M., & Samuelsson, B. (1976). A Comparison of the Vasodepressor Effects of the Cyclic Endoperoxides PGG_2 and PGH_2 with those of PGD_2 and PGE_2 in Hypertensive and Normotensive rats. *European Journal of Pharmacology*, *39*, 251–258.

An, S., Yang, J., So, S. W., Zeng, L., & Goetzl, E. J. (1994). Isoforms of the EP3 subtype of human prostaglandin E2 receptor transduce both intracellular calcium and cAMP signals. *Biochemistry*, *33*(48), 14496–502.

Ansari, H. R., Davis, A. M., Kaddour-Djebbar, I., & Abdel-Latif, A. a. (2003). Effects of prostaglandin F2alpha and latanoprost on phosphoinositide turnover, myosin light chain phosphorylation and contraction in cat iris sphincter. *Journal of Ocular Pharmacology and Therapeutics : The Official Journal of the Association for Ocular Pharmacology and Therapeutics*, *19*(3), 217–31.

Antczak, A., Ciebiada, M., Pietras, T., Piotrowski, W. J., Kurmanowska, Z., & Górski, P. (2012). Exhaled eicosanoids and biomarkers of oxidative stress in exacerbation of chronic obstructive pulmonary disease. *Archives of Medical Science : AMS*, *8*(2), 277–85.

Aoki, T., Nishimura, M., Matsuoka, T., Yamamoto, K., Furuyashiki, T., Kataoka, H., … Narumiya, S. (2011). PGE(2) -EP(2) signalling in endothelium is activated by haemodynamic stress and induces cerebral aneurysm through an amplifying loop via NF-κB. *British Journal of Pharmacology*, *163*(6), 1237–49.

Arakawa, H., Lötvall, J., Kawikova, I., Löfdahl, C. G., & Skoogh, B. E. (1993). Leukotriene D4- and prostaglandin F2 alpha-induced airflow obstruction and airway plasma exudation in guinea-pig: role of thromboxane and its receptor. *British Journal of Pharmacology*, *110*(1), 127–32.

Armour, C. L., Johnson, P. R., Alfredson, M. L., & Black, J. L. (1989). Characterization of contractile prostanoid receptors on human airway smooth muscle. *European Journal of Pharmacology*, *165*(2-3), 215–22.

Armstrong, R. a. (1996). Platelet prostanoid receptors. *Pharmacology & Therapeutics*, *72*(3), 171–191.

Aso, H., Ito, S., Mori, A., Morioka, M., Suganuma, N., Kondo, M., … Hasegawa, Y. (2012). Prostaglandin E2 enhances interleukin-8 production via EP4 receptor in human pulmonary microvascular endothelial cells. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *302*(2), L266–73.

Audoly, L. P., Tilley, S. L., Goulet, J., Key, M., Nguyen, M., Stock, J. L., … Coffman, T. M. (1999). Identification of specific EP receptors responsible for the hemodynamic effects of PGE2. *The American Journal of Physiology*, *277*(3 Pt 2), H924–30. Retrieved from

Bailey, S. R., Boustany, S., Burgess, J. K., Hirst, S. J., Sharma, H. S., Simcock, D. E., … Weckmann, M. (2009). Airway vascular reactivity and vascularisation in human chronic airway disease. *Pulmonary Pharmacology & Therapeutics*, *22*(5), 417–25.

Baluk, P., Bolton, P., Hirata, a, Thurston, G., & McDonald, D. M. (1998). Endothelial gaps and adherent leukocytes in allergen-induced early- and late-phase plasma leakage in rat airways. *The American Journal of Pathology*, *152*(6), 1463–76. Retrieved from

Baluk, P., Hirata, a, Thurston, G., Fujiwara, T., Neal, C. R., Michel, C. C., & McDonald, D. M. (1997). Endothelial gaps: time course of formation and closure in inflamed venules of rats. *The American Journal of Physiology*, *272*(1 Pt 1), L155–70.

Baluk, P., Thurston, G., Murphy, T. J., Bunnett, N. W., & McDonald, D. M. (1999). Neurogenic plasma leakage in mouse airways. *British Journal of Pharmacology*, *126*(2), 522–8.

Barbato, A., Turato, G., Baraldo, S., Bazzan, E., Calabrese, F., Panizzolo, C., … Saetta, M. (2006). Epithelial damage and angiogenesis in the airways of children with asthma. *American Journal of Respiratory and Critical Care Medicine*, *174*(9), 975–81.

Barnes, P. J. (1993). Effect of nedocromil sodium on microvascular leakage. *The Journal of Allergy and Clinical Immunology*, *92*(1 Pt 2), 197–9.

Barnes, P. J. (2000). Pro / Con Editorials Inhaled Corticosteroids Are Beneficial in Chronic Inhaled Corticosteroids Are Not Beneficial in Chronic. *American Journal of Respiratory and Critical Care Medicine*, *161*, 341–344.

Barnes, P. J. (2006). New therapies for asthma. *Trends in Molecular Medicine*, *12*(11), 515–20.

Barnes, P. J. (2008). Immunology of asthma and chronic obstructive pulmonary disease. *Nature Reviews. Immunology*, *8*(3), 183–92.

Barnes, P. J. (2010a). Chronic obstructive pulmonary disease: effects beyond the lungs. *PLoS Medicine*, *7*(3), e1000220.

Barnes, P. J. (2010b). Inhaled corticosteroids in COPD: a controversy. *Respiration; International Review of Thoracic Diseases*, *80*(2), 89–95.

Barnes, P. J. (2011). Glucocorticosteroids: current and future directions. *British Journal of Pharmacology*, *163*(1), 29–43.

Barnes, P. J. (2012). Severe asthma: advances in current management and future therapy. *The Journal of Allergy and Clinical Immunology*, *129*(1), 48–59.

Barnes, P. J. (2013). New anti-inflammatory targets for chronic obstructive pulmonary disease. *Nature Reviews Drug Discovery*, *12*(7), 543–559.

Barnes, P. J., & Celli, B. R. (2009). Systemic manifestations and comorbidities of COPD. *The European Respiratory Journal*, *33*(5), 1165–85.

Barnes, P. J., Chung, K. F., & Page, C. P. (1998). Inflammatory mediators of asthma: an update. *Pharmacological Reviews*, *50*(4), 515–96. Retrieved from

Barr, R. G., Bluemke, D. a, Ahmed, F. S., Carr, J. J., Enright, P. L., Hoffman, E. a, … Watson, K. E. (2010). Percent emphysema, airflow obstruction, and impaired left ventricular filling. *The New England Journal of Medicine*, *362*(3), 217–27.

Barreiro, E., del Puerto-Nevado, L., Puig-Vilanova, E., Pérez-Rial, S., Sánchez, F., Martínez-Galán, L., … Peces-Barba, G. (2012). Cigarette smoke-induced oxidative stress in skeletal muscles of mice. *Respiratory Physiology & Neurobiology*, *182*(1), 9–17.

Bastien, L., Sawyer, N., Grygorczyks, R., Metters, K. M., & Adam, M. (1994). Cloning , Functional Expression , and Characterization of the Human Prostaglandin E2 Receptor EP2 Subtype. *The Journal of Biological Chemistry*, *269*(16), 11873–11877.

Bateman, E. D., Hurd, S. S., Barnes, P. J., Bousquet, J., Drazen, J. M., FitzGerald, M., … Zar, H. J. (2008). Global strategy for asthma management and prevention: GINA executive summary. *The European Respiratory Journal*, *31*(1), 143–78.

Bathoorn, E., Liesker, J. J. W., Postma, D. S., Boorsma, M., Bondesson, E., Koëter, G. H., … Kerstjens, H. a M. (2008). Anti-inflammatory effects of combined budesonide/formoterol in COPD exacerbations. *Copd*, *5*(5), 282–90.

Baumer, Y., Drenckhahn, D., & Waschke, J. (2008). cAMP induced Rac 1-mediated cytoskeletal reorganization in microvascular endothelium. *Histochemistry and Cell Biology*, *129*(6), 765–78.

Baumer, Y., Spindler, V., Werthmann, R. C., Bünemann, M., & Waschke, J. (2009). Role of Rac 1 and cAMP in endothelial barrier stabilization and thrombin-induced barrier breakdown. *Journal of Cellular Physiology*, *220*(3), 716–26.

Baxter, G. S., Clayton, J. K., Coleman, R. a, Marshall, K., Sangha, R., & Senior, J. (1995). Characterization of the prostanoid receptors mediating constriction and relaxation of human isolated uterine artery. *British Journal of Pharmacology*, *116*(1), 1692–6.

Beasley, C. R. W., Robinson, C., Featherstone, R. L., Varley, J. G., Hardy, C. C., Church, M. K., & Holgate, S. T. (1987). 9α,11β-Prostaglandin F2, a Novel Metabolite of Prostaglandin D2, Is a

Potent Contractile Agonist of Human and Guinea Pig Airways. *Journal of Clinical Investigation*, *79*, 978–983.

Belvisi, M. G., Patel, H. J., Takahashi, T., Barnes, P. J., & Giembycz, M. a. (1996). Paradoxical facilitation of acetylcholine release from parasympathetic nerves innervating guinea-pig trachea by isoprenaline. *British Journal of Pharmacology*, *117*(7), 1413–20.

Benedict, N., Seybert, A., & Mathier, M. a. (2007). Evidence-based pharmacologic management of pulmonary arterial hypertension. *Clinical Therapeutics*, *29*(10), 2134–53.

Benyahia, C., Gomez, I., Kanyinda, L., Boukais, K., Danel, C., Leséche, G., … Norel, X. (2012). PGE(2) receptor (EP(4)) agonists: potent dilators of human bronchi and future asthma therapy? *Pulmonary Pharmacology & Therapeutics*, *25*(1), 115–8. doi:10.1016/j.pupt.2011.12.012

Bertolino, F., Valentin, J. P., Maffre, M., Bessac, a M., & John, G. W. (1995). TxA2 receptor activation elicits organ-specific increases in microvascular permeability in the rat. *The American Journal of Physiology*, *268*(2 Pt 2), R366–74.

Bertolino, F., Valentin, J. P., Maffre, M., Jover, B., Bessac, a M., & John, G. W. (1994). Prevention of thromboxane A2 receptor-mediated pulmonary hypertension by a nonpeptide angiotensin II type 1 receptor antagonist. *The Journal of Pharmacology and Experimental Therapeutics*, *268*(2), 747–52.

Birrell, M. a, Battram, C. H., Woodman, P., McCluskie, K., & Belvisi, M. G. (2003). Dissociation by steroids of eosinophilic inflammation from airway hyperresponsiveness in murine airways. *Respiratory Research*, *4*, 3.

Birrell, M. a, Maher, S. a, Buckley, J., Dale, N., Bonvini, S., Raemdonck, K., … Belvisi, M. G. (2013). Selectivity profiling of the novel EP2 receptor antagonist, PF-04418948, in functional bioassay systems: atypical affinity at the guinea pig EP2 receptor. *British Journal of Pharmacology*, *168*(1), 129–38.

Birrell, M. A., Mccluskie, K., Wong, S., Donnelly, L. E., Barnes, P. J., & Belvisi, M. G. (2005). Resveratrol, an extract of red wine, inhibits lipopolysaccharide induced airway neutrophilia and inflammatory mediators through an NF-κB-independent mechanism. *The FASEB Journal*, *22*(2), 1–22.

Birring, S. S., Parker, D., Brightling, C. E., Bradding, P., Wardlaw, A. J., & Pavord, I. D. (2004). Induced sputum inflammatory mediator concentrations in chronic cough. *American Journal of Respiratory and Critical Care Medicine*, *169*(1), 15–9.

Birukova, A. a, Burdette, D., Moldobaeva, N., Xing, J., Fu, P., & Birukov, K. G. (2010). Rac GTPase is a hub for protein kinase A and Epac signaling in endothelial barrier protection by cAMP. *Microvascular Research*, *79*(2), 128–38.

Birukova, A. a, Liu, F., Garcia, J. G. N., & Verin, A. D. (2004). Protein kinase A attenuates endothelial cell barrier dysfunction induced by microtubule disassembly. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *287*(1), L86–93.

Birukova, A. a, Zagranichnaya, T., Fu, P., Alekseeva, E., Chen, W., Jacobson, J. R., & Birukov, K. G. (2007). Prostaglandins PGE(2) and PGI(2) promote endothelial barrier enhancement via PKA- and Epac1/Rap1-dependent Rac activation. *Experimental Cell Research*, *313*(11), 2504–20.

Blé, F.-X., Cannet, C., Zurbruegg, S., Gérard, C., Frossard, N., Beckmann, N., & Trifilieff, a. (2009). Activation of the lung S1P(1) receptor reduces allergen-induced plasma leakage in mice. *British Journal of Pharmacology*, *158*(5), 1295–301.

Bley, K. R., Bhattacharya, A., Daniels, D. V, Gever, J., Jahangir, A., O'Yang, C., … Jett, M.-F. (2006). RO1138452 and RO3244794: characterization of structurally distinct, potent and selective IP (prostacyclin) receptor antagonists. *British Journal of Pharmacology*, *147*(3), 335–45.

Bley, K. R., Hunter, J. C., Eglen, R. M., & Smith, J. a. (1998). The role of IP prostanoid receptors in inflammatory pain. *Trends in Pharmacological Sciences*, *19*(4), 141–7.

Blue, E. K., Goeckeler, Z. M., Jin, Y., Hou, L., Dixon, S. a, Herring, B. P., … Gallagher, P. J. (2002). 220- and 130-kDa MLCKs have distinct tissue distributions and intracellular localization patterns. *American Journal of Physiology. Cell Physiology*, *282*(3), C451–60.

Bodnar, J. a, Morgan, W. T., Murphy, P. a, & Ogden, M. W. (2012). Mainstream smoke chemistry analysis of samples from the 2009 US cigarette market. *Regulatory Toxicology and Pharmacology : RTP*, *64*(1), 35–42.

Boer, W. I., Alagappan, V. K. T., & Sharma, H. S. (2007). Molecular mechanisms in chronic obstructive pulmonary disease. *Cell Biochemistry and Biophysics*, *47*(1), 131–147.

Böhm, E., Sturm, G. J., Weiglhofer, I., Sandig, H., Shichijo, M., McNamee, A., … Heinemann, A. (2004). 11-Dehydro-thromboxane B2, a stable thromboxane metabolite, is a full agonist of chemoattractant receptor-homologous molecule expressed on TH2 cells (CRTH2) in human eosinophils and basophils. *The Journal of Biological Chemistry*, *279*(9), 7663–70.

Boie, Y., Rushmore, T. H., Darmon-Goodwin, A., Grygorczyk, R., Slipetz, D. M., Metters, K. M., & Abramovitz, M. (1994). Cloning and Expression of a cDNA for the Human Prostanoid IP receptor. *The Journal of Biological Chemistry*, *269*(16), 12173–12178.

Boie, Y., Sawyer, N., Slipetz, D. M., Metters, K. M., & Abramovitz, M. (1995). Molecular Cloning and Characterization of the Human Prostanoid DP Receptor. *The Journal of Biological Chemistry*, *270*(32), 18910–18916.

Boie, Y., Stocco, R., Sawyer, N., Slipetz, D. M., Ungrin, M. D., Neuschäfer-Rube, F., … Abramovitz, M. (1997). Molecular cloning and characterization of the four rat prostaglandin E2 prostanoid receptor subtypes. *European Journal of Pharmacology*, *340*(2-3), 227–41.

Booij-Noord, H., Orie, N. G., & De Vries, K. (1971). Immediate and late bronchial obstructive reactions to inhalation of house dust and protective effects of disodium cromoglycate and prednisolone. *The Journal of Allergy and Clinical Immunology*, *48*(6), 344–54.

Booij-Noord, H., De Vries, K., Sluiter, H. J. & Orie, N. G. (1972). Late bronchial obstructive reaction to experimental inhalation of house dust extract. *Clinical Allergy*, 2, 43-61.

Booth, R., & Kim, H. (2012). Characterization of a microfluidic in vitro model of the blood-brain barrier (μBBB). *Lab on a Chip*, *12*(10), 1784–92.

Borić, M. P., Roblero, J. S., & Durán, W. N. (1987). Quantitation of bradykinin-induced microvascular leakage of FITC-dextran in rat cremaster muscle. *Microvascular Research*, *33*(3), 397–412.

Bos, C. L., Richel, D. J., Ritsema, T., Peppelenbosch, M. P., & Versteeg, H. H. (2004). Prostanoids and prostanoid receptors in signal transduction. *The International Journal of Biochemistry & Cell Biology*, *36*(7), 1187–205.

Bousquet, J., Chanez, P., Lacoste, J. Y., Barneon, G., Ghavanian, N., Enander, I., … Michel, F.-B. (1990). Eosinophilic Inflammation in Asthma. *The New England Journal of Medicine*, *323*(15), 1033–1039.

Bousquet, J., Jeffery, P. K., Busse, W. W., Johnson, M., & Vignola, A. M. (2000). Asthma. From Bronchoconstriction to Airways Inflammation and Remodeling. *American Journal of Respiratory and Critical Care Medicine*, *161*, 1720–1745.

Bradbury, D., Clarke, D., Seedhouse, C., Corbett, L., Stocks, J., & Knox, A. (2005). Vascular endothelial growth factor induction by prostaglandin E2 in human airway smooth muscle cells is mediated by E prostanoid EP2/EP4 receptors and SP-1 transcription factor binding sites. *The Journal of Biological Chemistry*, *280*(34), 29993–30000.

Bradding, P., Walls, A. F., & Holgate, S. T. (2006). The role of the mast cell in the pathophysiology of asthma. *The Journal of Allergy and Clinical Immunology*, *117*(6), 1277–84.

Brass, D. M., Hollingsworth, J. W., Cinque, M., Li, Z., Potts, E., Toloza, E., … Schwartz, D. a. (2008). Chronic LPS inhalation causes emphysema-like changes in mouse lung that are associated with apoptosis. *American Journal of Respiratory Cell and Molecular Biology*, *39*(5), 584–90.

Breyer, M. D., & Breyer, R. M. (2001). G Protein-Coupled Prostanoid Receptors and the Kidney. *Annual Review of Physiology*, *63*, 579–605.

Breyer, R. M., Bagdassarian, C. K., Scott, A., & Breyer, M. D. (2001). Prostanoid Receptors: Subtypes and Signaling. *Annual Review of Pharmacology and Toxicology*, *41*, 661–690.

Breyer, R. M., Davis, L. S., Nian, C., Redha, R., Stillman, B., Jacobson, H. R., & Breyer, M. D. (1996). Cloning and expression of the rabbit prostaglandin EP4 receptor. *The American Journal of Physiology*, *270*(3 Pt 2), F485–93.

Brightling, C. E., Ward, R., Woltmann, G., Bradding, P., Sheller, J. R., Dworski, R., & Pavord, I. D. (2000). Induced Sputum Inflammatory Mediator Concentrations in Eosinophilic Bronchitis and Asthma. *American Journal of Respiratory and Critical Care Medicine*, *162*, 878–882.

Brochhausen, C., Zehbe, R., Watzer, B., Halstenberg, S., Gabler, F., Schubert, H., & Kirkpatrick, C. J. (2009). Immobilization and controlled release of prostaglandin E2 from poly-L-lactide-co-glycolide microspheres. *Journal of Biomedical Materials Research. Part A*, *91*(2), 454–62.

Buckley, J., Birrell, M. a, Maher, S. a, Nials, A. T., Clarke, D. L., & Belvisi, M. G. (2011). EP4 receptor as a new target for bronchodilator therapy. *Thorax*, *66*(12), 1029–35.

Burgess, J. K., Ge, Q., Boustany, S., Black, J. L., & Johnson, P. R. a. (2004). Increased sensitivity of asthmatic airway smooth muscle cells to prostaglandin E2 might be mediated by increased numbers of E-prostanoid receptors. *The Journal of Allergy and Clinical Immunology*, *113*(5), 876–81.

Burka, J. F., Ali, M., Mcdonald, J. W. D., & Paterson, N. A. M. (1981). Immunological and Non-Immunological Synthesis and Release of Prostaglandins and Thromboxanes from Isolated Guinea Pig Trachea. *Prostaglandins*, *22*(5), 683–691.

Cai, S., Pestic-Dragovich, L., O'Donnell, M. E., Wang, N., Ingber, D., Elson, E., & De Lanerolle, P. (1998). Regulation of cytoskeletal mechanics and cell growth by myosin light chain phosphorylation. *The American Journal of Physiology*, *275*(5 Pt 1), C1349–56. Retrieved from

Calverley, P. M. A., Anderson, J. A., Celli, B., Ferguson, G. T., Jenkins, C., Jones, P. W., … Vestbo, J. (2007). Salmeterol and Fluticasone Propionate and Survival in Chronic Obstructive Pulmonary Disease. *The New England Journal of Medicine*, *356*(8), 775–789.

Calverley, P. M., Boonsawat, W., Cseke, Z., Zhong, N., Peterson, S., & Olsson, H. (2003). Maintenance therapy with budesonide and formoterol in chronicobstructive pulmonary disease. *European Respiratory Journal*, *22*(6), 912–919.

Calverley, P., Pauwels, R., Vestbo, J., Jones, P., Pride, N., Gulsvik, A., … Maden, C. (2003). Combined salmeterol and fluticasone in the treatment of chronic obstructive pulmonary disease: a randomised controlled trial. *Lancet*, *361*(9356), 449–56.

Cao, J., Shayibuzhati, M., Tajima, T., Kitazawa, T., & Taneike, T. (2002). In vitro pharmacological characterization of the prostanoid receptor population in the non-pregnant porcine myometrium. *European Journal of Pharmacology*, *442*(1-2), 115–23.

Capra, V. (2003). Thromboxane prostanoid receptor in human airway smooth muscle cells: a relevant role in proliferation. *European Journal of Pharmacology*, *474*(2-3), 149–159.

Carrasco, M. P., Phaneuf, S., Asboth, G. & Bernal A. L. (1996). Fluprostenol activates phospholipase C and Ca2+ mobilization in human myometrial cells. *Journal of Clinical Endocrinology & Metabolism*, 81, 2104-2110.

Chalmers, G. W., Macleod, K. J., Little, S. a, Thomson, L. J., McSharry, C. P., & Thomson, N. C. (2002). Influence of cigarette smoking on inhaled corticosteroid treatment in mild asthma. *Thorax*, *57*(3), 226–30.

Chapman, M. D., & Platts-mills, T. A. E. (1980). Purification and Characterization of the Major Allergen from Dermatophagoides Pteronyssinus-Antigen P1. *The Journal of Immunology*, *125*(2), 587–592.

Chappell, D., Beiras-Fernandez, A., Hammer, C., & Thein, E. (2006). In vivo visualization of the effect of polyclonal antithymocyte globulins on the microcirculation after ischemia/reperfusion in a primate model. *Transplantation*, *81*(4), 552–8.

Chen, Q., Muramoto, K., Masaaki, N., Ding, Y., Yang, H., Mackey, M., … Lamphier, M. (2010). A novel antagonist of the prostaglandin E(2) EP(4) receptor inhibits Th1 differentiation and Th17 expansion and is orally active in arthritis models. *British Journal of Pharmacology*, *160*(2), 292–310.

Chen, Y., Chen, P., Hanaoka, M., Droma, Y., & Kubo, K. (2008). Enhanced levels of prostaglandin E2 and matrix metalloproteinase-2 correlate with the severity of airflow limitation in stable COPD. *Respirology (Carlton, Vic.)*, *13*(7), 1014–21.

Cheng, Y., Austin, S. C., Rocca, B., Koller, B. H., Coffman, T. M., Grosser, T., … FitzGerald, G. a. (2002). Role of prostacyclin in the cardiovascular response to thromboxane A2. *Science (New York, N.Y.)*, *296*(5567), 539–41.

Chetham, P. M., Alexandra, H., Brough, G. H., Bridges, J. P., Joseph, W., Stevens, T., … Brough, H. (1997). Ca2+-inhibitable adenylyl cyclase and pulmonary microvascular permeability. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *273*, L22–L30.

Chevalier, E., Stock, J., Fisher, T., Dupont, M., Fric, M., Fargeau, H., … Li, B. (2005). Cutting Edge: Chemoattractant Receptor-Homologous Molecule Expressed on TH2 Cells Plays a Restricting Role on IL-5 Production and Eosinophil Recruitment. *The Journal of Immunology*, *175*(4), 2056–2060.

Chrzanowska-Wodnicka, M., & Burridge, K. (1996). Rho-stimulated contractility drives the formation of stress fibers and focal adhesions. *The Journal of Cell Biology*, *133*(6), 1403–15.

Chung, K. F., Godard, P., Adelroth, E., Ayres, J., Barnes, N., Barnes, P., … Sterk, P. (1999). Difficult / therapy-resistant asthma. The need for an integrated approach to define clinical phenotypes, evaluate risk factors, understand pathophysiology and find novel therapies. *European Respiratory Journal*, *13*, 1198–1208.

Churg, A., Wang, R. D., Tai, H., Wang, X., Xie, C., & Wright, J. L. (2004). Tumor necrosis factor-alpha drives 70% of cigarette smoke-induced emphysema in the mouse. *American Journal of Respiratory and Critical Care Medicine*, *170*(5), 492–8.

Clarke, D. L., Belvisi, M. G., Catley, M. C., Yacoub, M. H., Newton, R., & Giembycz, M. a. (2004). Identification in human airways smooth muscle cells of the prostanoid receptor and signalling pathway through which PGE2 inhibits the release of GM-CSF. *British Journal of Pharmacology*, *141*(7), 1141–50.

Clarke, D. L., Belvisi, M. G., Hardaker, E., Newton, R., & Giembycz, M. A. (2005). E-Ring 8-Isoprostanes Are Agonists at EP 2 - and EP 4 - Prostanoid Receptors on Human Airway Smooth Muscle Cells and Regulate the Release of Colony-Stimulating Factors by Activating cAMP-Dependent Protein Kinase. *Molecular Pharmacology*, *67*(2), 383–393.

Claudino, R. F., Kassuya, C. A. L., Ferreira, J., & Calixto, B. (2006). Pharmacological and Molecular Characterization of the Mechanisms Involved in Prostaglandin E 2 -Induced Mouse Paw Edema. *The Journal of Pharmacology and Experimental Therapeutics*, *318*(2), 611–618.

Clearie, K. L., McKinlay, L., Williamson, P. a, & Lipworth, B. J. (2012). Fluticasone/Salmeterol combination confers benefits in people with asthma who smoke. *Chest*, *141*(2), 330–8.

Cleaver, O., & Melton, D. a. (2003). Endothelial signaling during development. *Nature Medicine*, *9*(6), 661–8.

Clough, B. Y. G., & Michel, C. C. (1988). Quantitative comparisons of Hydraulic Permeability and Endothelial Intercellular Cleft Dimensions in Single Frog Capillaries. *Journal of Physiology*, *405*, 563–576.

Cocks, T. M., Fong, B., Chow, J. M., Anderson, G. P., Frauman, A. G., Goldie, R. G., … Moffatt, J. D. (1999). A protective role for protease-activated receptors in the airways. *Letters to Nature*, *398*, 156–160.

Cohen, a W., Carbajal, J. M., & Schaeffer, R. C. (1999). VEGF stimulates tyrosine phosphorylation of beta-catenin and small-pore endothelial barrier dysfunction. *The American Journal of Physiology*, *277*(5 Pt 2), H2038–49. Retrieved from

Coisne, C., & Engelhardt, B. (2011). Tight junctions in brain barriers during central nervous system inflammation. *Antioxidants & Redox Signaling*, *15*(5), 1285–303.

Coleman, R. A., Kennedy, I., Sheldrick, E. L. (1987). New evidence with selective agonists and antagonists for the subclassification of PGE2-sensitive (EP) receptors. *Advances in prostaglandin, thromboxane, and leukotriene research*, 17A, 467-70.

Coleman, R. a, Grix, S. P., Head, S. a, Louttit, J. B., Mallett, a, & Sheldrick, R. L. (1994). A novel inhibitory prostanoid receptor in piglet saphenous vein. *Prostaglandins*, *47*(2), 151–68.

Coleman, R. a, Humphrey, P. P., Kennedy, I., Levy, G. P., & Lumley, P. (1981). Comparison of the actions of U-46619, a prostaglandin H2-analogue, with those of prostaglandin H2 and thromboxane A2 on some isolated smooth muscle preparations. *British Journal of Pharmacology*, *73*(3), 773–8.

Coleman, R. a, & Sheldrick, R. L. (1989). Prostanoid-induced contraction of human bronchial smooth muscle is mediated by TP-receptors. *British Journal of Pharmacology*, *96*(3), 688–92.

Coleman, R. A., & Kennedy, I. (1985). Characterisation of the Prostanoid Receptors Mediating Contraction fo Guinea-Pig Isolated Trachea. *Prostaglandins*, *29*(3), 363–375.

Coleridge, H. M., Coleridge, J. C. G., Ginzel, K. H., Baker, D. G., Banzett, R. B., & Morrison, M. A. (1976). Stimulation of “irritant” receptors and afferent C-fibred in the lungs by prostaglandins. *Nature*, *264*, 451–453.

Connor, T. I. M. O., Neville, C., Burns, K. D., He, R. L., Laneuville, O., Peterson, L. N., … Peter-, L. N. (1998). Prostanoid signaling, localiazation, and expression of IP receptors in rat thick ascending limb cells. *American Journal of Physiology. Renal Physiology*, *275*, 904–914.

Corada, M., Mariotti, M., Thurston, G., Smith, K., Kunkel, R., Brockhaus, M., … Dejana, E. (1999). Vascular endothelial-cadherin is an important determinant of microvascular integrity in vivo. *Proceedings of the National Academy of Sciences of the United States of America*, *96*(17), 9815–20.

Cossette, C., Walsh, S. E., Kim, S., Lee, G., Lawson, J. A., Bellone, S., … Powell, W. S. (2007). Agonist and Antagonist Effects of 15 R -Prostaglandin (PG) D2 and 11-Methylene-PGD2 on Human Eosinophils and Basophils. *The Journal of Pharmacology and Experimental Therapeutics*, *320*(1), 173–179.

Cureton, E. L., Chong, T. J., Kwan, R. O., Dozier, K. C., Sadjadi, J., Curran, B., & Victorino, G. P. (2010). Endothelin 1 and prostacyclin attenuate increases in hydraulic permeability caused by platelet-activating factor in rats. *Shock*, *33*(6), 620–5.

Curry, F. E. (2002). Microvascular Injury: Mechanisms and Modulation. *International Journal of Angiology*, *6*, 1–6.

Curry, F.-R. E., & Adamson, R. H. (2010). Vascular permeability modulation at the cell, microvessel, or whole organ level: towards closing gaps in our knowledge. *Cardiovascular Research*, *87*(2), 218–29.

Curry, F., Huxley, V. & Sarelius, I. (1983). *Cardiovascular Physiology. Techniques in the Life Sciences*. New York, Elsevier, 1-34.

Curry, F.-R. E., Rygh, C. B., Karlsen, T., Wiig, H., Adamson, R. H., Clark, J. F., … Reed, R. K. (2010). Atrial natriuretic peptide modulation of albumin clearance and contrast agent permeability in mouse skeletal muscle and skin: role in regulation of plasma volume. *The Journal of Physiology*, *588*(Pt 2), 325–39.

Cyphert, J. M., Allen, I. C., Church, R. J., Latour, A. M., Snouwaert, J. N., Coffman, T. M., & Koller, B. H. (2012). Allergic inflammation induces a persistent mechanistic switch in thromboxane-mediated airway constriction in the mouse. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *302*(1), L140–51.

Dahl, R., Greefhorst, L. A. P. M., Nowak, D., Nonikov, V., Byrne, A. M., Thomson, M. H., … Cioppa, G. Della. (2001). Inhaled Formoterol Dry Powder Versus Ipratropium Bromide in Chronic Obstructive Pulmonary Disease. *American Journal of Respiratory and Critical Care Medicine*, *164*, 778–784.

Dai, G., Kaazempur-Mofrad, M. R., Natarajan, S., Zhang, Y., Vaughn, S., Blackman, B. R., … Gimbrone, M. a. (2004). Distinct endothelial phenotypes evoked by arterial waveforms derived from atherosclerosis-susceptible and -resistant regions of human vasculature. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(41), 14871–6.

Davì, G., Basili, S., Vieri, M., Cipollone, F., Santarone, S., Alessandri, C., … Enhanced, G. (1997). Enhanced Thromboxane Biosynthesis in Patients with Chronic Obstructive Pulmonary Disease. *American Journal of Respiratory and Critical Care Medicine*, *156*, 1794–1799.

Davies, P. F. (2009). Hemodynamic Shear Stress and the endothelium in cardiovascular pathophysiology. *Nature Clinical Practice Cardiovascular Medicine*, *6*(1), 16–26.

Davis, R. J., Murdoch, C. E., Ali, M., Purbrick, S., Ravid, R., Baxter, G. S., … Coleman, R. a. (2004). EP4 prostanoid receptor-mediated vasodilatation of human middle cerebral arteries. *British Journal of Pharmacology*, *141*(4), 580–5.

De Alba, J., Raemdonck, K., Dekkak, a, Collins, M., Wong, S., Nials, a T., … Birrell, M. a. (2010). House dust mite induces direct airway inflammation in vivo: implications for future disease therapy? *The European Respiratory Journal*, *35*(6), 1377–87.

Denniss, S. G., & Rush, J. W. E. (2009). Impaired hemodynamics and endothelial vasomotor function via endoperoxide-mediated vasoconstriction in the carotid artery of spontaneously hypertensive rats. *American Journal of Physiology. Heart and Circulatory Physiology*, *296*, 1038–1047.

Di Lorenzo, A., Lin, M. I., Murata, T., Landskroner-Eiger, S., Schleicher, M., Kothiya, M., … Sessa, W. C. (2013). eNOS-derived nitric oxide regulates endothelial barrier function through VE-cadherin and Rho GTPases. *Journal of Cell Science*, *126*, 5541–52.

Djukanović, R., Homeyard, S., Gratziou, C., Madden, J., Walls, a, Montefort, S., … Howarth, P. (1997). The effect of treatment with oral corticosteroids on asthma symptoms and airway inflammation. *American Journal of Respiratory and Critical Care Medicine*, *155*(3), 826–32.

Djukanović, R., Wilson, J. W., Britten, K. M., Wilson, S. J., Walls, A. F., Roche, W. R., Howarth, P. H. & Holgate, S. T. (1992). Effect of an inhaled corticosteroid on airway inflammation and symptoms in asthma. *The American review of respiratory disease*, 145, 669-674.

Dong, Y. J., Jones, R. L., & Wilson, N. H. (1986). Prostaglandin E receptor subtypes in smooth muscle: agonist activities of stable prostacyclin analogues. *British Journal of Pharmacology*, *87*(1), 97–107.

Dorn, G. W., & Becker, M. W. (1993). Thromboxane A2 Stimulated Signal Transduction in Vascular Smooth Muscle. *The Journal of Pharmacology and Experimental Therapeutics*, *265*(1), 447–456.

Drenckhahn, D., & Wagner, J. (1986). Stress fibers in the splenic sinus endothelium in situ: molecular structure, relationship to the extracellular matrix, and contractility. *The Journal of Cell Biology*, *102*(5), 1738–47.

Drummond, M. B., Dasenbrook, E. C., Pitz, M. W., Murphy, D. J., & Fan, E. (2008). Inhaled Corticosteroids in Patients with Stable Chronic Obstructive Pulmonary Disease. A Systematic Review and Meta Analysis. *JAMA*, *300*(20), 2407–2416.

Dudek, S. M., & Garcia, J. G. N. (2001). Cytoskeletal regulation of pulmonary vascular permeability. *Journal of Applied Physiology*, *91*, 1487–1500.

Dunnill, M. S., Massarella, G. R., & Anderson, J. a. (1969). A comparison of the quantitative anatomy of the bronchi in normal subjects, in status asthmaticus, in chronic bronchitis, and in emphysema. *Thorax*, *24*(2), 176–9.

Ebina, M., Takahashi, T., Chiba, T., & Motomiya, M. (1993). Cellular Hypertrophy and Hyperplasia of Airway Smooth Muscles Underlying Bronchial Asthma. A 3-D Morphometric Study. *American Reviews of Respiratory Disease*, *148*, 720–726.

Edwards, M. R., Bartlett, N. W., Clarke, D., Birrell, M., Belvisi, M., & Johnston, S. L. (2009). Targeting the NF-kappaB pathway in asthma and chronic obstructive pulmonary disease. *Pharmacology & Therapeutics*, *121*(1), 1–13.

Emery, D. L., Djokic, T. D., Graf, P. D., & Nadel, J. A. (1989). Prostaglandin D2 causes accumulation of eosinophils in the lumen of the dog trachea. *Journal of Applied Physiology*, *67*(3), 959–962.

Engelhardt, B., & Sorokin, L. (2009). The blood-brain and the blood-cerebrospinal fluid barriers: function and dysfunction. *Seminars in Immunopathology*, *31*(4), 497–511.

Ereso, A. Q., Cureton, E. L., Cripps, M. W., Sadjadi, J., Dua, M. M., Curran, B., & Victorino, G. P. (2009). Lipoxin a(4) attenuates microvascular fluid leak during inflammation. *The Journal of Surgical Research*, *156*(2), 183–8.

Erjefält, J. S., Andersson, P., Gustafsson, B., Korsgren, M., Sonmark, B., & Persson, C. G. (1998). Allergen challenge-induced extravasation of plasma in mouse airways. *Clinical and Experimental Allergy : Journal of the British Society for Allergy and Clinical Immunology*, *28*(8), 1013–20.

Esser, S., Wolburg, K., Wolburg, H., Breier, G., Kurzchalia, T., & Risau, W. (1998). Vascular endothelial growth factor induces endothelial fenestrations in vitro. *The Journal of Cell Biology*, *140*(4), 947–59.

Essler, M. (1998). Thrombin Inactivates Myosin Light Chain Phosphatase via Rho and Its Target Rho Kinase in Human Endothelial Cells. *Journal of Biological Chemistry*, *273*(34), 21867–21874.

Eum, S.-Y., Maghni, K., Hamid, Q., Eidelman, D. H., Campbell, H., Isogai, S., & Martin, J. G. (2003). Inhibition of allergic airways inflammation and airway hyperresponsiveness in mice by dexamethasone: Role of eosinophils, IL-5, eotaxin, and IL-13. *Journal of Allergy and Clinical Immunology*, *111*(5), 1049–1061.

Fabre, J. E., Nguyen, M., Athirakul, K., Coggins, K., McNeish, J. D., Austin, S., … Koller, B. H. (2001). Activation of the murine EP3 receptor for PGE2 inhibits cAMP production and promotes platelet aggregation. *The Journal of Clinical Investigation*, *107*(5), 603–10.

Félétou, M., Verbeuren, T. J., & Vanhoutte, P. M. (2009). Endothelium-dependent contractions in SHR: a tale of prostanoid TP and IP receptors. *British Journal of Pharmacology*, *156*(4), 563–74.

Feng, D., Nagy, J. a, Hipp, J., Dvorak, H. F., & Dvorak, a M. (1996). Vesiculo-vacuolar organelles and the regulation of venule permeability to macromolecules by vascular permeability factor, histamine, and serotonin. *The Journal of Experimental Medicine*, *183*(5), 1981–6.

Ferretti, S., Bonneau, O., Dubois, G. R., Jones, C. E., & Trifilieff, a. (2003). IL-17, Produced by Lymphocytes and Neutrophils, Is Necessary for Lipopolysaccharide-Induced Airway Neutrophilia: IL-15 as a Possible Trigger. *The Journal of Immunology*, *170*(4), 2106–2112.

Fiebich, B. L., Schleicher, S., Spleiss, O., Czygan, M., & Hüll, M. (2001). Mechanisms of prostaglandin E2-induced interleukin-6 release in astrocytes: possible involvement of EP4-like receptors, p38 mitogen-activated protein kinase and protein kinase C. *Journal of Neurochemistry*, *79*(5), 950–8.

Fish, J. E., Jameson, L. S., Albright, A., Norman, P. S. (1984). Modulation of the bronchomotor effects of chemical mediators by prostaglandin F2 alpha in asthmatic subjects. *American Review of Respiratory Disease*, 130 **(4)**, 571-574.

Flower, R. J. (1973). Aspirin-like drugs and prostaglandins. *American Heart Journal*, *86*(6), 844–846.

Flower, R. J. (1978). Biosynthesis of prostaglandins. *CIBA Foundation Symposium*, 65, 123-142.

Flower, R. J., Harvey, E. A., & Kingston, W. P. (1976). Inflammatory Effects of Prostaglandin D2 in Rat and Human Skin. *British Journal of Pharmacology*, *56*, 229–233.

Flower, R. J., & Kingston, W. P. (1975). Prostglandin D1 inhibits the increase in vascular permeability in rat skin produced by prostaglandin E1, E2, and D2. *Proceedings of the British Pharmacological Society*.

Flower, R. J., & Vane, J. R. (1974). Inhibition of prostaglandin biosynthesis. *Biochemical Pharmacology*, *23*, 1439–1450.

Fordjour, A. K., & Harrington, E. O. (2009). PKCdelta influences p190 phosphorylation and activity: events independent of PKCdelta-mediated regulation of endothelial cell stress fiber and focal adhesion formation and barrier function. *Biochimica et Biophysica Acta*, *1790*(10), 1179–90.

Fortner, C. N., Breyer, R. M., & Paul, R. J. (2001). EP2 receptors mediate airway relaxation to substance P, ATP, and PGE2. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *281*(2), L469–74.

Forselles, K. J., Root, J., Clarke, T., Davey, D., Aughton, K., Dack, K., & Pullen, N. (2011). In vitro and in vivo characterization of PF-04418948, a novel, potent and selective prostaglandin EP_2 receptor antagonist. *British Journal of Pharmacology*, *164*(7), 1847–56.

Foudi, N., Kotelevets, L., Louedec, L., Leséche, G., Henin, D., Chastre, E., & Norel, X. (2008). Vasorelaxation induced by prostaglandin E2 in human pulmonary vein: role of the EP4 receptor subtype. *British Journal of Pharmacology*, *154*(8), 1631–9.

Francois, H., Makhanova, N., Ruiz, P., Ellison, J., Mao, L., Rockman, H. A., & Coffman, T. M. (2008). A role for the thromboxane receptor in L -NAME hypertension. *American Journal of Physiology. Renal Physiology*, *295*, 1096–1102.

Frias, M. a, Rebsamen, M. C., Gerber-Wicht, C., & Lang, U. (2007). Prostaglandin E2 activates Stat3 in neonatal rat ventricular cardiomyocytes: A role in cardiac hypertrophy. *Cardiovascular Research*, *73*(1), 57–65.

Fu, B. M., & Shen, S. (2004). Acute VEGF effect on solute permeability of mammalian microvessels in vivo. *Microvascular Research*, *68*(1), 51–62.

Fujino, H., Salvi, S., & Regan, J. W. (2005). Differential Regulation of Phosphorylation of the cAMP Response Element-Binding Protein after Activation of EP 2 and EP 4 Prostanoid Receptors by Prostaglandin E 2. *Molecular Pharmacology*, *68*(1), 251–259.

Fujino, H., West, K. a, & Regan, J. W. (2002). Phosphorylation of glycogen synthase kinase-3 and stimulation of T-cell factor signaling following activation of EP2 and EP4 prostanoid receptors by prostaglandin E2. *The Journal of Biological Chemistry*, *277*(4), 2614–9.

Funk, C. D., Furci, L., FitzGerald, G. a, Grygorczyk, R., Rochette, C., Bayne, M. a, … Metters, K. M. (1993). Cloning and expression of a cDNA for the human prostaglandin E receptor EP1 subtype. *The Journal of Biological Chemistry*, *268*(35), 26767–72.

Gallant, M. a, Samadfam, R., Hackett, J. a, Antoniou, J., Parent, J.-L., & de Brum-Fernandes, A. J. (2005). Production of prostaglandin D(2) by human osteoblasts and modulation of osteoprotegerin, RANKL, and cellular migration by DP and CRTH2 receptors. *Journal of Bone and Mineral Research : The Official Journal of the American Society for Bone and Mineral Research*, *20*(4), 672–81.

Gallant, M. a, Slipetz, D., Hamelin, E., Rochdi, M. D., Talbot, S., de Brum-Fernandes, A. J., & Parent, J.-L. (2007). Differential regulation of the signaling and trafficking of the two prostaglandin D2 receptors, prostanoid DP receptor and CRTH2. *European Journal of Pharmacology*, *557*(2-3), 115–23.

Gallant, M., Carrière, M. C., Chateauneuf, a, Denis, D., Gareau, Y., Godbout, C., … Labelle, M. (2002). Structure-activity relationship of biaryl acylsulfonamide analogues on the human EP(3) prostanoid receptor. *Bioorganic & Medicinal Chemistry Letters*, *12*(18), 2583–6.

Gan, W. Q. (2004). Association between chronic obstructive pulmonary disease and systemic inflammation: a systematic review and a meta-analysis. *Thorax*, *59*(7), 574–580.

Garcia, J. G., Davis, H. W. & Patterson, C. E. (1995). Regulation of endothelial cell gap formation and barrier dysfunction: role of myosin light chain phosphorylation. *Journal of Cellular Physiology*, 163, 510-522.

Garcia, J. G. N., Lazar, V., Gilbert-mcclain, L. I., Gallagher, P. J., & Verin, A. D. (1997). Myosin Light Chain Kinase in Endothelium: Molecular Cloning and Regulation. *American Journal of Respiratory Cell and Molecular Biology*, *16*, 489–494.

Gardiner, P. J. (1986). Characterization of prostanoid relaxant/inhibitory receptors (psi) using a highly selective agonist, TR4979. *British Journal of Pharmacology*, *87*(1), 45–56.

Gauvreau, G. M., Watson, R. M., & O'Byrne, P. M. (1999). Protective effects of inhaled PGE2 on allergen-induced airway responses and airway inflammation. *American Journal of Respiratory and Critical Care Medicine*, *159*(1), 31–6.

Gavard, J., & Gutkind, J. S. (2006). VEGF controls endothelial-cell permeability by promoting the beta-arrestin-dependent endocytosis of VE-cadherin. *Nature Cell Biology*, *8*(11), 1223–34.

Gendron, M.-E., & Thorin, E. (2007). A change in the redox environment and thromboxane A 2 production precede endothelial dysfunction in mice. *American Journal of Physiology. Heart and Circulatory Physiology*, *293*, 2508–2515.

George, R. J., Sturmoski, M. a, Anant, S., & Houchen, C. W. (2007). EP4 mediates PGE2 dependent cell survival through the PI3 kinase/AKT pathway. *Prostaglandins & Other Lipid Mediators*, *83*(1-2), 112–20.

Germonpré, P. R., Joos, G. F., Mekeirele, K., & Pauwels, R. a. (1997). Role of the 5-HT receptor in neurogenic inflammation in Fisher 344 rat airways. *European Journal of Pharmacology*, *324*(2-3), 249–55.

Gervais, F. G., Cruz, R. P., Chateauneuf, a, Gale, S., Sawyer, N., Nantel, F., … O'neill, G. P. (2001). Selective modulation of chemokinesis, degranulation, and apoptosis in eosinophils through the PGD2 receptors CRTH2 and DP. *The Journal of Allergy and Clinical Immunology*, *108*(6), 982–8.

Gervais, F. G., Morello, J., Beaulieu, C., Sawyer, N., Denis, D., Greig, G., … Neill, G. P. O. (2005). Identification of a Potent and Selective Synthetic Agonist at the CRTH2 Receptor. *Molecular Pharmacology*, *67*(6), 1834–1839.

Giblin, G. M. P., Bit, R. a, Brown, S. H., Chaignot, H. M., Chowdhury, A., Chessell, I. P., … Wilson, R. (2007). The discovery of 6-[2-(5-chloro-2-{[(2,4-difluorophenyl)methyl]oxy}phenyl)-1-cyclopenten-1-yl]-2-pyridinecarboxylic acid, GW848687X, a potent and selective prostaglandin EP1 receptor antagonist for the treatment of inflammatory pain. *Bioorganic & Medicinal Chemistry Letters*, *17*(2), 385–9.

Giles, H., Leff, P., Bolofo, M. L., Kelly, M. G., & Robertson, a D. (1989). The classification of prostaglandin DP-receptors in platelets and vasculature using BW A868C, a novel, selective and potent competitive antagonist. *British Journal of Pharmacology*, *96*(2), 291–300.

Goeckeler, Z. M., & Wysolmerski, R. B. (1995). Myosin light chain kinase-regulated endothelial cell contraction: the relationship between isometric tension, actin polymerization, and myosin phosphorylation. *The Journal of Cell Biology*, *130*(3), 613–27.

Goldenberg, N. M., Steinberg, B. E., Slutsky, A. S., & Lee, W. L. (2011). Broken barriers: a new take on sepsis pathogenesis. *Science Translational Medicine*, *3*(88), 88ps25.

Gorman, R. R., Bunting, S., & Miller, O. V. (1977). Modulation of Human Platelet Adenylate Cyclase by Prostacyclin. *Prostaglandins*, *13*(3), 377–388.

Goulet, J. L., Pace, A. J., Key, M. L., Byrum, R. S., Nguyen, M., Tilley, S. L., … Koller, B. H. (2004). E-prostanoid-3 receptors mediate the proinflammatory actions of prostaglandin E2 in acute cutaneous inflammation. *Journal of Immunology (Baltimore, Md. : 1950)*, *173*(2), 1321–6.

Graham, D. A., & Rush, J. W. E. (2009). Cyclooxygenase and thromboxane / prostaglandin receptor contribute to aortic endothelium-dependent dysfunction in aging female spontaneously hypertensive rats. *Journal of Applied Physiology*, *107*, 1059–1067.

Greiff, L., Andersson, M., Erjefält, J. S., Persson, C. G. a, & Wollmer, P. (2003). Airway microvascular extravasation and luminal entry of plasma. *Clinical Physiology and Functional Imaging*, *23*(6), 301–6.

Griep, L. M., Wolbers, F., de Wagenaar, B., ter Braak, P. M., Weksler, B. B., Romero, I. a, … van den Berg, a. (2013). BBB on chip: microfluidic platform to mechanically and biochemically modulate blood-brain barrier function. *Biomedical Microdevices*, *15*(1), 145–50.

Griffin, B. W., Klimko, P., Crider, J. Y., & Sharif, N. a. (1999). AL-8810: a novel prostaglandin F2 alpha analog with selective antagonist effects at the prostaglandin F2 alpha (FP) receptor. *The Journal of Pharmacology and Experimental Therapeutics*, *290*(3), 1278–84.

Gross, S., Tilly, P., Hentsch, D., Vonesch, J.-L., & Fabre, J.-E. (2007). Vascular wall-produced prostaglandin E2 exacerbates arterial thrombosis and atherothrombosis through platelet EP3 receptors. *The Journal of Experimental Medicine*, *204*(2), 311–20.

Grünig, G. (1998). Requirement for IL-13 Independently of IL-4 in Experimental Asthma. *Science*, *282*(5397), 2261–2263.

Guan, Y., Stillman, B. a, Zhang, Y., Schneider, A., Saito, O., Davis, L. S., … Breyer, M. D. (2002). Cloning and expression of the rabbit prostaglandin EP2 receptor. *BMC Pharmacology*, *2*, 14.

Günther, A., Yasotharan, S., Vagaon, A., Lochovsky, C., Pinto, S., Yang, J., … Bolz, S.-S. (2010). A microfluidic platform for probing small artery structure and function. *Lab on a Chip*, *10*(18),

Håkansson, H. F., Smailagic, A., Brunmark, C., Miller-Larsson, A., & Lal, H. (2012). Altered lung function relates to inflammation in an acute LPS mouse model. *Pulmonary Pharmacology & Therapeutics*, *25*(5), 399–406.

Halushka, P. V, Mais, D. E., Mayeux, P. R., & Morinelli, T. a. (1989). Thromboxane, prostaglandin and leukotriene receptors. *Annual Review of Pharmacology and Toxicology*, *29*, 213–39.

Hammad, H., Kool, M., Soullié, T., Narumiya, S., Trottein, F., Hoogsteden, H. C., & Lambrecht, B. N. (2007). Activation of the D prostanoid 1 receptor suppresses asthma by modulation of lung dendritic cell function and induction of regulatory T cells. *The Journal of Experimental Medicine*, *204*(2), 357–67.

Haque, R., Hakim, A., Moodley, T., Torrego, A., Essilfie-Quaye, S., Jazrawi, E., … Usmani, O. S. (2013). Inhaled long-acting β2 agonists enhance glucocorticoid receptor nuclear translocation

and efficacy in sputum macrophages in COPD. *The Journal of Allergy and Clinical Immunology*, *132*(5), 1166–73.

Hardy, C. C., Robinson, C., Tattersfield, A. E. & Holgate, S. T. (1984). The Bronchoconstrictor Effect of Inhaled Prostaglandin-D2 in Normal and Asthmatic Men. *New England Journal of Medicine*, 311 **(4)**, 209-213.

Hasegawa, H., Negishi, M., Katoh, H., & Ichikawa, a. (1997). Two isoforms of prostaglandin EP3 receptor exhibiting constitutive activity and agonist-dependent activity in Rho-mediated stress fiber formation. *Biochemical and Biophysical Research Communications*, *234*(3), 631–6.

Hata, A. N., & Breyer, R. M. (2004). Pharmacology and signaling of prostaglandin receptors: multiple roles in inflammation and immune modulation. *Pharmacology & Therapeutics*, *103*(2), 147–66.

Hata, A. N., Lybrand, T. P., & Breyer, R. M. (2005). Identification of determinants of ligand binding affinity and selectivity in the prostaglandin D2 receptor CRTH2. *The Journal of Biological Chemistry*, *280*(37), 32442–51.

Hawkins, B. T., & Davis, T. P. (2005). The Blood-Brain Barrier / Neurovascular Unit in Health and Disease. *Pharmaco*, *57*(2), 173–185.

Haye-Legrand, I., Bourdillat, B., Labat, C., Cerrina, J., Norel, X., Benveniste, J., & Brink, C. (1987). Relaxation of Isolated Human Pulmonary Muscle Preparations with Prostacyclin (PGI2) and Its Analogs. *Prostaglandins*, *33*(6), 845–854.

He, P., & Curry, F. E. (1993). Differential actions of cAMP on endothelial [Ca2+]i and permeability in microvessels exposed to ATP. *The American Journal of Physiology*, *265*(3 Pt 2), H1019–23.

Heaney, L. G., & Robinson, D. S. (2005). Severe asthma treatment: need for characterising patients. *Lancet*, *365*(9463), 974–6.

Hele, D. J., Birrell, M. a, Webber, S. E., Foster, M. L., & Belvisi, M. G. (2001). Mediator involvement in antigen-induced bronchospasm and microvascular leakage in the airways of ovalbumin sensitized Brown Norway rats. *British Journal of Pharmacology*, *132*(2), 481–8.

Herlong, J. L., & Scott, T. R. (2006). Positioning prostanoids of the D and J series in the immunopathogenic scheme. *Immunology Letters*, *102*(2), 121–31.

Herrerias, A., Torres, R., Serra, M., Marco, A., Pujols, L., Picado, C., & de Mora, F. (2009). Activity of the cyclooxygenase 2-prostaglandin-E prostanoid receptor pathway in mice exposed to house dust mite aeroallergens, and impact of exogenous prostaglandin E2. *Journal of Inflammation (London, England)*, *6*, 30.

Hill, a T., Bayley, D., & Stockley, R. a. (1999). The interrelationship of sputum inflammatory markers in patients with chronic bronchitis. *American Journal of Respiratory and Critical Care Medicine*, *160*(3), 893–8.

Hirai, H., Tanaka, K., Takano, S., Ichimasa, M., Nakamura, M., & Nagata, K. (2002). Cutting Edge: Agonistic Effect of Indomethacin on a Prostaglandin D2 Receptor, CRTH2. *The Journal of Immunology*, *168*(3), 981–985.

Hirai, H., Tanaka, K., Yoshie, O., Ogawa, K., Kenmotsu, K., Takamori, Y., … Nagata, K. (2001). Prostaglandin D2 selectively induces chemotaxis in T helper type 2 cells, eosinophils, and basophils via seven-transmembrane receptor CRTH2. *The Journal of Experimental Medicine*, *193*(2), 255–61.

Hirata, M., Hayashi, Y., Ushikubi, F., Yokota, Y., Kageyama, R., Nakanishi, S., & Narumiya, S. (1991). Cloning and expression of cDNA for a human thromboxane A2 receptor. *Nature*, *349*, 617–620.

Hirata, M., Kakizuka, a, Aizawa, M., Ushikubi, F., & Narumiya, S. (1994). Molecular characterization of a mouse prostaglandin D receptor and functional expression of the cloned gene. *Proceedings of the National Academy of Sciences of the United States of America*, *91*(23), 11192–6.

Hirata, T., Ushikubi, F., Kakizuka, A., Okuma, M., & Narumiya, S. (1996). Two Thromboxane A2 Receptor Isoforms in Human Platelets. *Journal of Clinical Investigation*, *97*(4), 949–956.

Hogan, S. P., Koskinen, A., Matthaei, K. I., Young, I. G. & Foster, P. S. (1998). Interleukin-5-producing CD4+ T cells play a pivotal role in aeroallergen-induced eosinophilia, bronchial hyperreactivity, and lung damage in mice. *American Journal of Respiratory and Critical Care Medicine*, 157, 210-218.

Hogg, J. C. (2004). Pathophysiology of airflow limitation in chronic obstructive pulmonary disease. *Lancet*, *364*(9435), 709–21.

Holgate, S. T. (2008a). Pathogenesis of asthma. *Clinical and Experimental Allergy : Journal of the British Society for Allergy and Clinical Immunology*, *38*(6), 872–97.

Holgate, S. T. (2008b). The airway epithelium is central to the pathogenesis of asthma. *Allergology International : Official Journal of the Japanese Society of Allergology*, *57*(1), 1–10.

Honda, a, Sugimoto, Y., Namba, T., Watabe, a, Irie, a, Negishi, M., … Ichikawa, a. (1993). Cloning and expression of a cDNA for mouse prostaglandin E receptor EP2 subtype. *The Journal of Biological Chemistry*, *268*(11), 7759–62. Retrieved from

Honda, K., Arima, M., Cheng, G., Taki, S., Hirata, H., Eda, F., … Fukuda, T. (2003). Prostaglandin D2 reinforces Th2 type inflammatory responses of airways to low-dose antigen through bronchial expression of macrophage-derived chemokine. *The Journal of Experimental Medicine*, *198*(4), 533–43.

Honda, T., Segi-Nishida, E., Miyachi, Y., & Narumiya, S. (2006). Prostacyclin-IP signaling and prostaglandin E2-EP2/EP4 signaling both mediate joint inflammation in mouse collagen-induced arthritis. *The Journal of Experimental Medicine*, *203*(2), 325–35.

Hong, F., Haldeman, B. D., Jackson, D., Carter, M., Baker, J. E., & Cremo, C. R. (2011). Biochemistry of smooth muscle myosin light chain kinase. *Archives of Biochemistry and Biophysics*, *510*(2), 135–46.

Horgan, M. J., Palace, G. P., Everitt, J. E., & Malik, a B. (1993). TNF-alpha release in endotoxemia contributes to neutrophil-dependent pulmonary edema. *The American Journal of Physiology*, *264*(4 Pt 2), H1161–5.

Huang, J. (2004). Cell signalling through thromboxane A2 receptors. *Cellular Signalling*, *16*(5), 521–533.

Hutchinson, J., Marshall, K. M. & Senior, J. (2003). Preliminary studies using a putative FP-receptor antagonist, AL-8810, on isolated mouse uterus. *Proceedings of the British Pharmacological Society*. Available at: http://www.pa2online.org/Vol1Issue3abst038P.html.

Huxley, V. H., Wang, J. J., & Sarelius, I. H. (2007). Adaptation of coronary microvascular exchange in arterioles and venules to exercise training and a role for sex in determining permeability responses. *American Journal of Physiology. Heart and Circulatory Physiology*, *293*, 1196–1205.

Hylkema, M. N., Hoekstra, M. O., Luinge, M., & Timens, W. (2002). The strength of the OVA-induced airway inflammation in rats is strain dependent. *Clinical and Experimental Immunology*, *129*(3), 390–6.

Ichinose, M., Sugiura, H., Yamagata, S., Koarai, a., Tomaki, M., Ogawa, H., … Hattori, T. (2003). Xanthine oxidase inhibition reduces reactive nitrogen species production in COPD airways. *European Respiratory Journal*, *22*(3), 457–461.

Ichinose, M., Sugiura, H., Yamagata, S., Koarai, A., & Shirato, K. (2000). Increase in Reactive Nitrogen Species Production in Chronic Obstructive Pulmonary Disease Airways. *American Journal of Respiratory and Critical Care Medicine*, *162*, 701–706.

Imig, J. D., Breyer, M. D., & Breyer, R. M. (2002). Contribution of prostaglandin EP(2) receptors to renal microvascular reactivity in mice. *American Journal of Physiology. Renal Physiology*, *283*(3), F415–22.

Innes, A. L., Carrington, S. D., Thornton, D. J., Kirkham, S., Rousseau, K., Dougherty, R. H., … Fahy, J. V. (2009). Ex vivo sputum analysis reveals impairment of protease-dependent mucus degradation by plasma proteins in acute asthma. *American Journal of Respiratory and Critical Care Medicine*, *180*(3), 203–10.

Ishii, Y., Wang, Y., Haziot, a., del Vecchio, P. J., Goyert, S. M., & Malik, a. B. (1993). Lipopolysaccharide binding protein and CD14 interaction induces tumor necrosis factor-alpha generation and neutrophil sequestration in lungs after intratracheal endotoxin. *Circulation Research*, *73*(1), 15–23.

Ito, M., Guerriero, V. Jr. & Hartshorne, D. J. (1991). Structure-function relationships in smooth muscle light chain kinase. *Advances in Experimental Medicine and Biology*, 304, 3-10.

Ito, S., Sakamoto, K., Mochizuki-Oda, N., Ezashi, T., Miwa, K., Okuda-Ashitaka, E., Shevchenko, V. I., Kiso, Y. & Hayaishi, O. (1994). Prostaglandin $F_{2\alpha}$ receptor is coupled to G_q in cDNA-transfected Chinese hamster ovary cells. *Biochemical and biophysical research communications*, 200, 756-762.

Jaeschke, R., O'Byrne, P. M., Mejza, F., Nair, P., Lesniak, W., Brozek, J., … Guyatt, G. (2008). The safety of long-acting beta-agonists among patients with asthma using inhaled corticosteroids: systematic review and metaanalysis. *American Journal of Respiratory and Critical Care Medicine*, *178*(10), 1009–16.

Jaffar, Z., Wan, K.-S., & Roberts, K. (2002). A key role for prostaglandin I2 in limiting lung mucosal Th2, but not Th1, responses to inhaled allergen. *Journal of Immunology (Baltimore, Md. : 1950)*, *169*(10), 5997–6004.

James, a L., Paré, P. D., & Hogg, J. C. (1989). The mechanics of airway narrowing in asthma. *The American Review of Respiratory Disease*, *139*(1), 242–6.

James, a L., & Wenzel, S. (2007). Clinical relevance of airway remodelling in airway diseases. *The European Respiratory Journal*, *30*(1), 134–55.

Jiang, Q., Ewing, J. R., Ding, G. L., Zhang, L., Zhang, Z. G., Li, L., … Chopp, M. (2005). Quantitative evaluation of BBB permeability after embolic stroke in rat using MRI. *Journal of Cerebral Blood Flow and Metabolism : Official Journal of the International Society of Cerebral Blood Flow and Metabolism*, *25*(5), 583–92.

Jiang, Y., Wen, K., Zhou, X., Schwegler-Berry, D., Castranova, V., & He, P. (2008). Three-dimensional localization and quantification of PAF-induced gap formation in intact venular microvessels. *American Journal of Physiology. Heart and Circulatory Physiology*, *295*(2), H898–906.

Jin, A. Y., Tuor, U. I., Rushforth, D., Kaur, J., Muller, R. N., Petterson, J. L., … Barber, P. a. (2010). Reduced blood brain barrier breakdown in P-selectin deficient mice following transient ischemic stroke: a future therapeutic target for treatment of stroke. *BMC Neuroscience*, *11*, 12.

Johnson, J. R., Wiley, R. E., Fattouh, R., Swirski, F. K., Gajewska, B. U., Coyle, A. J., … Jordana, M. (2004). Continuous exposure to house dust mite elicits chronic airway inflammation and structural remodeling. *American Journal of Respiratory and Critical Care Medicine*, *169*(3), 378–85.

Jones, G. L., Saroea, H. G., Watson, R. M., & O'Byrne, P. M. (1992). Effect of an inhaled thromboxane mimetic (U46619) on airway function in human subjects. *The American Review of Respiratory Disease*, *145*(6), 1270–4.

Jones, R. L., Giembycz, M. a, & Woodward, D. F. (2009a). Prostanoid receptor antagonists: development strategies and therapeutic applications. *British Journal of Pharmacology*, *158*(1), 104–45.

Jones, R. L., Giembycz, M. A., & Woodward, D. F. (2009b). Prostanoid receptor antagonists : development strategies and therapeutic applications. *British Journal of Pharmacology*, *158*, 104–145.

Jones, R., Woodward, D., & Wang, J. (2008). Investigation of the slow kinetics of the prostanoid EP3 receptor antaonists L-798106 and L-826266 on guinea pig aorta. *Fundamental & Clinical Pharmacology*, *22 (Suppl),* 69–79.

Kabashima, K., Murata, T., Tanaka, H., Matsuoka, T., Sakata, D., Yoshida, N., … Narumiya, S. (2003b). Thromboxane A2 modulates interaction of dendritic cells and T cells and regulates acquired immunity. *Nature Immunology*, *4*(7), 694–701

Kabashima, K., Nagamachi, M., Honda, T., Nishigori, C., Miyachi, Y., Tokura, Y., & Narumiya, S. (2007). Prostaglandin E2 is required for ultraviolet B-induced skin inflammation via EP2 and EP4 receptors. *Laboratory Investigation; a Journal of Technical Methods and Pathology*, *87*(1), 49–55.

Kabashima, K., & Narumiya, S. (2003a). The DP receptor, allergic inflammation and asthma. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, *69*(2-3), 187–194

Kabashima, K., Saji, T., Murata, T., Nagamachi, M., Matsuoka, T., Segi, E., … Narumiya, S. (2002). The prostaglandin receptor EP4 suppresses colitis, mucosal damage and CD4 cell activation in the gut. *Journal of Clinical Investigation*, *109*, 883–893.

Kamio, K., Liu, X., Sugiura, H., Togo, S., Kobayashi, T., Kawasaki, S., … Rennard, S. I. (2007). Prostacyclin analogs inhibit fibroblast contraction of collagen gels through the cAMP-PKA pathway. *American Journal of Respiratory Cell and Molecular Biology*, *37*(1), 113–20.

Kanazawa, H., Asai, K., Hirata, K., & Yoshikawa, J. (2002). Vascular involvement in exercise-induced airway narrowing in patients with bronchial asthma. *Chest*, *122*(1), 166–70.

Kanazawa, H., Hirata, K., & Yoshikawa, J. (2000). Role of endogenous nitric oxide in exercise-induced airway narrowing in patients with bronchial asthma. *The Journal of Allergy and Clinical Immunology*, *106*(6), 1081–7.

Kanazawa, H., Nomura, S., & Yoshikawa, J. (2004). Role of microvascular permeability on physiologic differences in asthma and eosinophilic bronchitis. *American Journal of Respiratory and Critical Care Medicine*, *169*(10), 1125–30.

Katoh, K., Kano, Y., Amano, M., Onishi, H., Kaibuchi, K., & Fujiwara, K. (2001). Rho-kinase--mediated contraction of isolated stress fibers. *The Journal of Cell Biology*, *153*(3), 569–84.

Katsuyama, M., Nishigaki, N., Sugimoto, Y., Morimoto, K., Negishi, M., Narumiya, S., & Ichikawa, a. (1995). The mouse prostaglandin E receptor EP2 subtype: cloning, expression, and northern blot analysis. *FEBS Letters*, *372*(2-3), 151–6.

Katsuyama, M., Sugimoto, Y., Namba, T., Irie, a, Negishi, M., Narumiya, S., & Ichikawa, a. (1994). Cloning and expression of a cDNA for the human prostacyclin receptor. *FEBS Letters*, *344*(1), 74–8.

Kawakami, Y., Uchiyama, K., Irie, T., & Murao, M. (1973). Evaluation of aerosols of prostaglandins E1 and E2 as bronchodilators. *European Journal of Clinical Pharmacology*, *6*(2), 127–32.

Kawano, Y., Fukata, Y., Oshiro, N., Amano, M., Nakamura, T., Ito, M., … Kaibuchi, K. (1999). Phosphorylation of Mysoin-binding Subunit (MBS) of Myosin Phosphatase by Rho-Kinase In Vivo. *The Journal of Cell Biology*, *147*(5), 1023–1037.

Kay, L. J., Yeo, W. W., & Peachell, P. T. (2006). Prostaglandin E2 activates EP2 receptors to inhibit human lung mast cell degranulation. *British Journal of Pharmacology*, *147*(7), 707–13.

Keery, R. J., & Lumley, P. (1988). AH6809, a prostaglandin DP-receptor blocking drug on human platelets. *British Journal of Pharmacology*, *94*(3), 745–54.

Kellend, K. (2011, May 31). Smoking could kill 8 million a year by 2030:WHO. *Reuters.* http://uk.reuters.com/article/2011/05/31/health-us-smoking-deaths-idUKTRE74U29O20110531

Kendall, S., & Michel, C. C. (1995). The measurement of permeability in single rat venules using the red cell microperfusion technique. *Experimental Physiology*, *80*(3), 359–72.

Kenne, E., & Lindbom, L. (2011). Imaging inflammatory plasma leakage in vivo. *Thrombosis and Haemostasis*, *105*(5), 783–9.

Kennedy, C. R., Zhang, Y., Brandon, S., Guan, Y., Coffee, K., Funk, C. D., … Breyer, R. M. (1999). Salt-sensitive hypertension and reduced fertility in mice lacking the prostaglandin EP2 receptor. *Nature Medicine*, *5*(2), 217–20.

Khor, Y. H., Teoh, a K. Y., Lam, S. M., Mo, D. C. Q., Weston, S., Reid, D. W., & Walters, E. H. (2009). Increased vascular permeability precedes cellular inflammation as asthma control deteriorates. *Clinical and Experimental Allergy : Journal of the British Society for Allergy and Clinical Immunology*, *39*(11), 1659–67.

Kim, S. R., Lee, K. S., Lee, K. B., & Lee, Y. C. (2012). Recombinant IGFBP-3 inhibits allergic lung inflammation, VEGF production, and vascular leak in a mouse model of asthma. *Allergy*, *67*(7), 869–77.

Kimura, K., Inoue, H., Ichinose, M., Miura, M., Katsumata, U., Takahashi, T., & Takishima, T. (1992). Bradykinin Causes Airway Hyperresponsiveness and Enhances Maximal Airway Narrowing. *American Reviews of Respiratory Disease*, *146*, 1301–1305.

Kimura, K., Ito, M., Amano, M., Chihara, K., Fukata, Y., Nakafuku, M., … Kaibuchi, K. (1996). Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). *Science (New York, N.Y.)*, *273*(5272), 245–8.

Kinsella, B. T., O'Mahony, D. J., & Fitzgerald, G. a. (1997). The human thromboxane A2 receptor alpha isoform (TP alpha) functionally couples to the G proteins Gq and G11 in vivo and is

activated by the isoprostane 8-epi prostaglandin F2 alpha. *The Journal of Pharmacology and Experimental Therapeutics*, *281*(2), 957–64.

Knotkova, H., & Pappagallo, M. (2007). Imaging intracranial plasma extravasation in a migraine patient: a case report. *Pain Medicine (Malden, Mass.)*, *8*(4), 383–7.

Komarova, Y., & Malik, A. B. (2010). *Regulation of endothelial permeability via paracellular and transcellular transport pathways. Annual review of physiology*,72, 463–93.

Konger, R. L., Billings, S. D., Prall, N. C., Katona, T. M., Dasilva, S. C., Kennedy, C. R. J., … Lacelle, P. T. (2009). The EP1 subtype of prostaglandin E2 receptor: role in keratinocyte differentiation and expression in non-melanoma skin cancer. *Prostaglandins, Leukotrienes, and Essential Fatty Acids*, *81*(4), 279–90.

Konya, V., Marsche, G., Schuligoi, R., & Heinemann, A. (2013a). E-type prostanoid receptor 4 (EP4) in disease and therapy. *Pharmacology & Therapeutics*, *138*(3), 485–502.

Konya, V., Üllen, A., Kampitsch, N., Theiler, A., Philipose, S., Parzmair, G. P., … Heinemann, A. (2013b). Endothelial E-type prostanoid 4 receptors promote barrier function and inhibit neutrophil trafficking. *The Journal of Allergy and Clinical Immunology*, *131*(2), 532–40.e1–2.

Kostenis, E., & Ulven, T. (2006). Emerging roles of DP and CRTH2 in allergic inflammation. *Trends in Molecular Medicine*, *12*(4), 148–58.

Kozak, K. R., Crews, B. C., Ray, J. L., Tai, H. H., Morrow, J. D., & Marnett, L. J. (2001). Metabolism of prostaglandin glycerol esters and prostaglandin ethanolamides in vitro and in vivo. *The Journal of Biological Chemistry*, *276*(40), 36993–8.

Kubes, P., & Gaboury, J. P. (1996). Rapid mast cell activation causes leukocyte-dependent and -independent permeability alterations. *The American Journal of Physiology*, *271*(6 Pt 2), H2438–46.

Kunikata, T., Yamane, H., Segi, E., Matsuoka, T., Sugimoto, Y., Tanaka, S., … Narumiya, S. (2005). Suppression of allergic inflammation by the prostaglandin E receptor subtype EP3. *Nature Immunology*, *6*(5), 524–31.

Kurucz, I. & Szelenyi, I. (2006). Current animal models of bronchial asthma. *Current Pharmaceutical Design*, 12, 3175-3194.

Laitinen, L. A., Laitinen, A., & Haahtela, T. (1992). A comparative study of the effects of an inhaled corticosteroid , budesonide, and a β2-agonist, terbutaline, on airway inflammation in newly diagnosed asthma: A randomized, double-blind, parallel-group controlled trial. *Journal of Allergy and Clinical Immunology*, *90*, 32–42.

Laitinen, L. A., Laitinen, A. & Widdicombe, J. (1987). Effects of inflammatory and other mediators on airway vascular beds. *American Review of Respiratory Disease*, 135, S67-S70.

Lambert, R. K., Wiggs, B. R., Kuwano, K., Hogg, J. C., & Paré, P. D. (1993). Functional significance of increased airway smooth muscle in asthma and COPD. *Journal of Applied Physiology (Bethesda, Md. : 1985)*, *74*(6), 2771–81.

Langeler, E. G., & van Hinsbergh, V. W. (1991). Norepinephrine and iloprost improve barrier function of human endothelial cell monolayers: role of cAMP. *The American Journal of Physiology*, *260*(5 Pt 1), C1052–9.

Lawrence, R. a, Jones, R. L., & Wilson, N. H. (1992). Characterization of receptors involved in the direct and indirect actions of prostaglandins E and I on the guinea-pig ileum. *British Journal of Pharmacology*, *105*(2), 271–8.

Lee, H., Kim, S., Chung, M., Kim, J. H., & Jeon, N. L. (2014). A bioengineered array of 3D microvessels for vascular permeability assay. *Microvascular Research*, *91*, 90–8.

Lee, K. S., Kim, S. R., Park, H. S., Jin, G. Y., & Lee, Y. C. (2004). Cysteinyl leukotriene receptor antagonist regulates vascular permeability by reducing vascular endothelial growth factor expression. *The Journal of Allergy and Clinical Immunology*, *114*(5), 1093–9.

Lee, K. S., Kim, S. R., Park, S. J., Park, H. S., Min, K. H., Jin, S. M., … Lee, Y. C. (2006). Peroxisome proliferator activated receptor-gamma modulates reactive oxygen species generation and activation of nuclear factor-kappaB and hypoxia-inducible factor 1alpha in allergic airway disease of mice. *The Journal of Allergy and Clinical Immunology*, *118*(1), 120–7.

Lee, K. S., Park, S. J., Kim, S. R., Min, K. H., Jin, S. M., Puri, K. D., & Lee, Y. C. (2006). Phosphoinositide 3-kinase-delta inhibitor reduces vascular permeability in a murine model of asthma. *The Journal of Allergy and Clinical Immunology*, *118*(2), 403–9.

Lewis, C. a, & Broadley, K. J. (1995). Airway hyper- or hyporeactivity to inhaled spasmogens 24 h after ovalbumin challenge of sensitized guinea-pigs. *British Journal of Pharmacology*, *116*(5), 2351–8.

Lewis, R. a, Soter, N. a, Diamond, P. T., Austen, K. F., Oates, J. a, & Roberts, L. J. (1982). Prostaglandin D2 generation after activation of rat and human mast cells with anti-IgE. *Journal of Immunology (Baltimore, Md. : 1950)*, *129*(4), 1627–31.

Li, X., & Wilson, J. W. (1997). Increased vascularity of the bronchial mucosa in mild asthma. *American Journal of Respiratory and Critical Care Medicine*, *156*(1), 229–33.

Licht, A. H., Pein, O. T., Florin, L., Hartenstein, B., Reuter, H., Arnold, B., … Schorpp-Kistner, M. (2006). JunB is required for endothelial cell morphogenesis by regulating core-binding factor beta. *The Journal of Cell Biology*, *175*(6), 981–91.

Lima, C., Souza, V. M. O., Soares, a L., Macedo, M. S., Tavares-de-Lima, W., & Vargaftig, B. B. (2007). Interference of methysergide, a specific 5-hydroxytryptamine receptor antagonist, with airway chronic allergic inflammation and remodelling in a murine model of asthma. *Clinical and Experimental Allergy : Journal of the British Society for Allergy and Clinical Immunology*, *37*(5), 723–34.

List, S. J., Findlay, B. P., Forstner, G. G., & Forstner, J. F. (1978). Enhancement of the viscosity of mucin by serum albumin. *The Biochemical Journal*, *175*(2), 565–71.

Liu, F., Verin, a D., Borbiev, T., & Garcia, J. G. (2001). Role of cAMP-dependent protein kinase A activity in endothelial cell cytoskeleton rearrangement. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *280*(6), L1309–17.

Lo, C. M., Keese, C. R., & Giaever, I. (1995). Impedance analysis of MDCK cells measured by electric cell-substrate impedance sensing. *Biophysical Journal*, *69*(6), 2800–7.

Løkke, a, Lange, P., Scharling, H., Fabricius, P., & Vestbo, J. (2006). Developing COPD: a 25 year follow up study of the general population. *Thorax*, *61*(11), 935–9.

Long, J. a, Fogel-Petrovic, M., Knight, D. a, Thompson, P. J., & Upham, J. W. (2004). Higher prostaglandin e2 production by dendritic cells from subjects with asthma compared with normal subjects. *American Journal of Respiratory and Critical Care Medicine*, *170*(5), 485–91.

Lötvall, J., Elwood, W., Tokuyama, K., Sakamoto, T., Barnes, P. J., & Chung, K. F. (1992). A thromboxane mimetic, U-46619, produces plasma exudation in airways of the guinea pig. *Journal of Applied Physiology, 72*(6), 2415–9.

Lu, Z. & Wolf, M. B. (1993). Platelet activating factor-induced microvascular permeability increases in the cat hindlimb. *Circulatory Shock*, 41, 8-18.

Lupinetti, M. D., Sheller, J. R., Catella, F. & Fitzgerald, G. A. (1989). Thromboxane biosynthesis in allergen-induced bronchospasm, evidence for platelet activation. *American Review of Respiratory Disease*, 140 **(4)**, 932-935.

Lydford, S. J., & McKechnie, K. (1994). Characterization of the prostaglandin E2 sensitive (EP)-receptor in the rat isolated trachea. *British Journal of Pharmacology*, *112*(1), 133–6.

Lydford, S. J., McKechnie, K. C., & Dougall, I. G. (1996). Pharmacological studies on prostanoid receptors in the rabbit isolated saphenous vein: a comparison with the rabbit isolated ear artery. *British Journal of Pharmacology*, *117*(1), 13–20.

Ma, H., Hara, a., Xiao, C. Y., Okada, Y., Takahata, O., Nakaya, K., … Ushikubi, F. (2001). Increased Bleeding Tendency and Decreased Susceptibility to Thromboembolism in Mice Lacking the Prostaglandin E Receptor Subtype EP3. *Circulation*, *104*(10), 1176–1180.

Macias-Perez, I. M., Zent, R., Carmosino, M., Breyer, M. D., Breyer, R. M., & Pozzi, A. (2008). Mouse EP3 alpha, beta, and gamma receptor variants reduce tumor cell proliferation and tumorigenesis in vivo. *The Journal of Biological Chemistry*, *283*(18), 12538–45.

Maher, S. a, Birrell, M. a, & Belvisi, M. G. (2009). Prostaglandin E2 mediates cough via the EP3 receptor: implications for future disease therapy. *American Journal of Respiratory and Critical Care Medicine*, *180*(10), 923–8.

Majno, G., Gilmore, V., & Leventhal, M. (1967). On the Mechanism of Vascular Leakage Caused by Histamine-Type Mediators: A Microscopic Study In Vivo. *Circulation Research*, *21*(6), 833–848.

Majno, G., & Palade, G. E. (1961). Studies on inflammation. 1. The effect of histamine and serotonin on vascular permeability: an electron microscopic study. *The Journal of Biophysical and Biochemical Cytology*, *11*(2), 571–605.

Majno, G., Palade, G. E., & Schoefl, G. I. (1961). Studies on inflammation. II. The site of action of histamine and serotonin along the vascular tree: a topographic study. *The Journal of Biophysical and Biochemical Cytology*, *11*, 607–26.

Majno, G., Shea, S. M., & Leventhal, M. (1969). Endothelial Contraction Induced by Histamine-Type Mediators. An Electron Microscopic Study. *The Journal of Cell Biology*, *42*(1), 647–672.

Martin, J. G., Suzuki, M., Maghni, K., Pantano, R., Ramos-barbón, D., Ihaku, D., … Powell, W. S. (2002). The Immunomodulatory Actions of Prostaglandin E2 on Allergic Airway Responses in the Rat. *Journal of Immunology*, *169*, 3963–3969.

Marwick, J. a, Caramori, G., Stevenson, C. S., Casolari, P., Jazrawi, E., Barnes, P. J., … Papi, A. (2009). Inhibition of PI3Kdelta restores glucocorticoid function in smoking-induced airway inflammation in mice. *American Journal of Respiratory and Critical Care Medicine*, *179*(7), 542–8.

Marwick, J. a, Kirkham, P. a, Stevenson, C. S., Danahay, H., Giddings, J., Butler, K., … Rahman, I. (2004). Cigarette smoke alters chromatin remodeling and induces proinflammatory genes in rat lungs. *American Journal of Respiratory Cell and Molecular Biology*, *31*(6), 633–42.

Masoli, M., Fabian, D., Holt, S., & Beasley, R. (2004). The global burden of asthma : executive summary of the GINA Dissemination Committee Report. *Allergy*, *59*, 469–478.

Mathiesen, J. M., Christopoulos, A., Ulven, T., Royer, J. F., Campillo, M., Heinemann, A., … Kostenis, E. (2006). On the Mechanism of Interaction of Potent Surmountable and Insurmountable Antagonists with the Prostaglandin D2 Receptor CRTH2. *Molecular Pharmacology*, *69*, 1441–1453.

Mathiesen, J. M., Ulven, T., Martini, L., Gerlach, L. O., Heinemann, A., & Kostenis, E. (2005). Identification of Indole Derivatives Exclusively Interfering with a G Protein-Independent Signaling Pathway of the Prostaglandin D2 Receptor CRTH2. *Molecular Pharmacology*, *68*(2), 393–402.

Matsuoka, T. (2000). Prostaglandin D2 as a Mediator of Allergic Asthma. *Science*, *287*(5460), 2013–2017.

Matsuoka, Y., Furuyashiki, T., Yamada, K., Nagai, T., Bito, H., Tanaka, Y., … Narumiya, S. (2005). Prostaglandin E receptor EP1 controls impulsive behavior under stress. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(44), 16066–71.

Matthews, J. S., & Jones, R. L. (1993). Potentiation of aggregation and inhibition of adenylate cyclase in human platelets by prostaglandin E analogues. *British Journal of Pharmacology*, *108*(2), 363–9.

McCormick, C., Jones, R. L., Kennedy, S., & Wadsworth, R. M. (2010). Activation of prostanoid EP receptors by prostacyclin analogues in rabbit iliac artery: implications for anti-restenotic potential. *European Journal of Pharmacology*, *641*(2-3), 160–7.

Mcdonald, D. M. (1994). Endothelial gaps and permeability of venules in rat tracheas exposed to inflammatory stimuli. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *266*, 61–83.

Mcdonald, D. M., Thurston, G., & Baluk, P. (1999). Endothelial Gaps as Sites for Plasma Leakage in Inflammation. *Microcirculation*, *6*, 7–22.

Mcgraw, D. W., Mihlbachler, K. A., Schwarb, M. R., Rahman, F. F., Small, K. M., Almoosa, K. F., & Liggett, S. B. (2006). Airway smooth muscle prostaglandin-EP1 receptors directly modulate β2 – adrenergic receptors within a unique heterodimeric complex. *The Journal of Clinical Investigation*, *116*, 1400–1409.

McIlroy, A., Caron, G., Blanchard, S., Frémaux, I., Duluc, D., Delneste, Y., … Jeannin, P. (2006). Histamine and prostaglandin E up-regulate the production of Th2-attracting chemokines (CCL17 and CCL22) and down-regulate IFN-gamma-induced CXCL10 production by immature human dendritic cells. *Immunology*, *117*(4), 507–16.

McKenniff, M. G., Norman, P., Cuthbert, N. J., & Gardiner, P. J. (1991). BAY u3405, a potent and selective thromboxane A2 receptor antagonist on airway smooth muscle in vitro. *British Journal of Pharmacology*, *104*(3), 585–90.

Mckenzie, B. G. J., Fallon, P. G., Emson, C. L., Grencis, R. K., & Mckenzie, A. N. J. (1999). Simultaneous Disruption of Interleukin (IL)-4 and IL-13 Defines Individual Roles in T Helper Cell Type 2-mediated Responses. *Journal of Experimental Medicine*, *189*(10), 1565–1572.

Mckenzie, D. C., & Boulet, L. (2008). Asthma, outdoor air quality and the Olympic Games. *Canadian Medical Association Journal*, *179*(6), 543–548.

McMillan, S. J., & Lloyd, C. M. (2004). Prolonged allergen challenge in mice leads to persistent airway remodelling. *Clinical and Experimental Allergy : Journal of the British Society for Allergy and Clinical Immunology*, *34*(3), 497–507.

Mead, J., Turner, J. M., Macklem, P. T., & Little, J. B. (1967). Significance of the relationship between lung recoil and maximum expiratory flow. *Journal of Applied Physiology*, *22*(1), 95–108.

Meerschaert, J., Kelly, E. a, Mosher, D. F., Busse, W. W., & Jarjour, N. N. (1999). Segmental antigen challenge increases fibronectin in bronchoalveolar lavage fluid. *American Journal of Respiratory and Critical Care Medicine*, *159*(2), 619–25.

Mehta, D., & Malik, A. B. (2006). Signaling Mechanisms Regulating Endothelial Permeability. *Phy*, *86*, 279–367..

Mendes, E. S., Campos, M. a, & Wanner, A. (2006). Airway blood flow reactivity in healthy smokers and in ex-smokers with or without COPD. *Chest*, *129*(4), 893–8.

Mendez, M., & Lapointe, M. C. (2005). PGE 2 -induced hypertrophy of cardiac myocytes involves EP 4 receptor-dependent activation of p42/44 MAPK and EGFR transactivation. *American Journal of Physiology. Heart and Circulatory Physiology*, *288*, 2111–2117.

Meyrick, B., Hoover, R., Jones, M. R., Berry, L. C. & Brigham, K. L. (1989). In Vitro Effects of Endotoxin on Bovine and Sheep Lung Microvascular and Pulmonary-Artery Endothelial Cells. *Journal of Cellular Physiology*, 138, 165-175.

Michel, C. C., & Curry, F. E. (1999). Microvascular permeability. *Physiological Reviews*, *79*(3), 703–61.

Michel, C. C., Mason, J. C., Curry, F. E., Tooke, J. E., & Hunter, P. J. (1974). A development of the Landis technique for measuring the filtration coefficient of individual capillaries in the frog mesentery. *Quarterly Journal of Experimental Physiology and Cognate Medical Sciences*, *59*(4), 283–309.

Miggin, S. M., & Kinsella, B. T. (1998). Expression and tissue distribution of the mRNAs encoding the human thromboxane A2 receptor (TP) alpha and beta isoforms. *Biochimica et Biophysica Acta*, *1425*(3), 543–59.

Miles, A. A., & Miles, E. M. (1952). Vascular Reactions to Histamine, Histamine-Liberator and Leukotaxine in the Skin of Guinea Pigs. *Journal of Physiology*, *118*, 228–257.

Minakata, Y., Nakanishi, M., Hirano, T., Matsunaga, K., Yamagata, T., & Ichinose, M. (2005). Microvascular hyperpermeability in COPD airways. *Thorax*, *60*(10), 882.

Minami, T., Nakano, H., Kobayashi, T., Sugimoto, Y., Ushikubi, F., Ichikawa, a, … Ito, S. (2001). Characterization of EP receptor subtypes responsible for prostaglandin E2-induced pain responses by use of EP1 and EP3 receptor knockout mice. *British Journal of Pharmacology*, *133*(3), 438–44.

Minami, T., Nishihara, I., Uda, R., Ito, S., Hyodo, M., & Hayaishi, O. (1994). Characterization of EP-receptor subtypes involved in allodynia and hyperalgesia induced by intrathecal administration of prostaglandin E2 to mice. *British Journal of Pharmacology*, *112*(3), 735–40.

Miniño, A. M., Heron, M. P., Murphy, S. L., & Kochanek, K. D. (2007). Deaths: final data for 2004. *National Vital Statistics Reports : From the Centers for Disease Control and Prevention, National Center for Health Statistics, National Vital Statistics System*, *55*(19), 1–119.

Mir, F., & Le Breton, G. C. (2008). A novel nuclear signaling pathway for thromboxane A2 receptors in oligodendrocytes: evidence for signaling compartmentalization during differentiation. *Molecular and Cellular Biology*, *28*(20), 6329–41.

Mirza, Z. N., Tokuyama, K., Arakawa, H., Kato, M., Mochizuki, H., & Morikawa, a. (1998). Inhaled procaterol inhibits histamine-induced airflow obstruction and microvascular leakage in guinea-pig airways with allergic inflammation. *Clinical and Experimental Allergy : Journal of the British Society for Allergy and Clinical Immunology*, *28*(5), 644–52.

Mitchell, J. a, Akarasereenont, P., Thiemermann, C., Flower, R. J., & Vane, J. R. (1993). Selectivity of nonsteroidal antiinflammatory drugs as inhibitors of constitutive and inducible cyclooxygenase. *Proceedings of the National Academy of Sciences of the United States of America*, *90*(24), 11693–7.

Mizoguchi, a, Eguchi, N., Kimura, K., Kiyohara, Y., Qu, W. M., Huang, Z. L., … Hayaishi, O. (2001). Dominant localization of prostaglandin D receptors on arachnoid trabecular cells in mouse basal forebrain and their involvement in the regulation of non-rapid eye movement sleep. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(20), 11674–9.

Mohammed, S. P., Higenbottam, T. W., & Adcock, J. J. (1993). Effects of Aerosol-Applied Capsaicin, Histamine and Prostaglandin E2 on Airway Sensory Receptors of Anaesthetized Cats. *Journal of Physiology*, *469*, 51–66.

Moller, A. D., & Grande, P.-O. (1997). Low-dose prostacyclin has potent capillary permeability-reducing effect in cat skeletal muscle in vivo. *American Journal of Physiology. Heart and Circulatory Physiology*, *273*, 200–207.

Moncada, S. (1982). Biological Importance of Prostacyclin. *British Journal of Pharmacology*, *76*, 3–31.

Monneret, G. (2001). Prostaglandin D2 is a potent chemoattractant for human eosinophils that acts via a novel DP receptor. *Blood*, *98*(6), 1942–1948.

Monneret, G., Cossette, C., Gravel, S., Rokach, J., & Powell, W. S. (2003). 15R-Methyl-Prostaglandin D2 Is a Potent and Selective CRTH2/DP 2 Receptor Agonist in Human Eosinophils. *The Journal of Pharmacology and Experimental Therapeutics*, *304*(1), 349–355.

Montuschi, P. (2003). Exhaled leukotrienes and prostaglandins in COPD. *Thorax*, *58*(7), 585–588.

Moore, M. L., & Peebles, R. S. (2006). Update on the role of prostaglandins in allergic lung inflammation: separating friends from foes, harder than you might think. *The Journal of Allergy and Clinical Immunology*, *117*(5), 1036–9.

Moore, T. M., Chetham, P. M., Kelly, J. J., Stevens, T., & Timothy, M. (1998). Signal transduction and regulation of lung endothelial cell permeability. Interaction between calcium and cAMP. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *275*, 203–222.

Moreno, J. J. (2009). Differential Effects of Arachidonic and Eicosapentaenoic Acid- Derived Eicosanoids on Polymorphonuclear Transmigration Across Endothelial Cell Cultures. *The Journal of Pharmacology and Experimental Therapeutics*, *331*(3), 1111–1117.

Moriyama, T., Higashi, T., Togashi, K., Iida, T., Segi, E., Sugimoto, Y., … Tominaga, M. (2005). Sensitization of TRPV1 by EP1 and IP reveals peripheral nociceptive mechanism of prostaglandins. *Molecular Pain*, *1*, 3.

Morsy, M. a, Isohama, Y., & Miyata, T. (2001). Prostaglandin E(2) increases surfactant secretion via the EP(1) receptor in rat alveolar type II cells. *European Journal of Pharmacology*, *426*(1-2), 21–4.

Moy, a B., Bodmer, J. E., Blackwell, K., Shasby, S., & Shasby, D. M. (1998). cAMP protects endothelial barrier function independent of inhibiting MLC20-dependent tension development. *The American Journal of Physiology*, *274*(6 Pt 1), L1024–9.

Moy, a B., Shasby, S. S., Scott, B. D., & Shasby, D. M. (1993). The effect of histamine and cyclic adenosine monophosphate on myosin light chain phosphorylation in human umbilical vein endothelial cells. *The Journal of Clinical Investigation*, *92*(3), 1198–206.

Moy, a B., Van Engelenhoven, J., Bodmer, J., Kamath, J., Keese, C., Giaever, I., … Shasby, D. M. (1996). Histamine and thrombin modulate endothelial focal adhesion through centripetal and centrifugal forces. *The Journal of Clinical Investigation*, *97*(4), 1020–7.

Moy, a B., Winter, M., Kamath, a, Blackwell, K., Reyes, G., Giaever, I., … Shasby, D. M. (2000). Histamine alters endothelial barrier function at cell-cell and cell-matrix sites. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *278*(5), L888–98.

Muja, N., Nelson, J. K., & Devries, G. H. (2007). Schwann Cells Express IP Prostanoid Receptors Coupled to an Elevation in Intracellular Cyclic AMP. *Journal of Neuroscience Research*, *85*, 1159–1169.

Murata, T., Lin, M. I., Aritake, K., Matsumoto, S., Narumiya, S., Ozaki, H., … Sessa, W. C. (2008). Role of prostaglandin D2 receptor DP as a suppressor of tumor hyperpermeability and angiogenesis in vivo. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(50), 20009–14.

Murata, T., Ushikubi, F., Matsuoka, T., Hirata, M., Yamasaki, A., Sugimoto, Y., … Narumiya, S. (1997). Altered pain perception and inflammatory response in mice lacking prostacyclin receptor. *Nature*, *388*, 678–682.

Murray, C. J., & Lopez, a D. (1997). Alternative projections of mortality and disability by cause 1990-2020: Global Burden of Disease Study. *Lancet*, *349*(9064), 1498–504.

Murray, J. J., Tonnel, A. B., Brash, A. R., Roberts, L. J., Gosset, P., Workman, R., Capron, A. & Oates, J. A. (1986). Release of Prostaglandin-D2 into Human Airways During Acute Antigen Challenge. *New England Journal of Medicine*, 315, 800-804.

Nagaraja, T. N., Karki, K., Ewing, J. R., Croxen, R. L., & Knight, R. a. (2008). Identification of variations in blood-brain barrier opening after cerebral ischemia by dual contrast-enhanced magnetic resonance imaging and T 1sat measurements. *Stroke; a Journal of Cerebral Circulation*, *39*(2), 427–32.

Nagata, K., Hirai, H., Tanaka, K., Ogawa, K., Aso, T., Sugamura, K., … Takano, S. (1999). CRTH2, an orphan receptor of T-helper-2-cells, is expressed on basophils and eosinophils and responds to mast cell-derived factor(s). *FEBS Letters*, *459*(2), 195–9.

Nagata, K., Tanaka, K., Ogawa, K., Kemmotsu, K., Imai, T., Yoshie, O., … Takano, S. (1999). Selective expression of a novel surface molecule by human Th2 cells in vivo. *Journal of Immunology*, *162*(3), 1278–86.

Nakahata, N. (2008). Thromboxane A2: physiology/pathophysiology, cellular signal transduction and pharmacology. *Pharmacology & Therapeutics*, *118*(1), 18–35.

Nakao, A., Watanabe, T., Taniguchi, S., Nakamura, M., Honda, Z. I., Shimizu, T. & Kurokawa, K. (1993). Characterization of Prostaglandin-F2-alpha Receptor of Mouse 3T3 Fibroblasts and its Functional Expression in Xenopus-laevis Oocytes. *Journal of Cellular Physiology*, 155, 257-264.

Nakayama, Y., Omote, K., Kawamata, T., & Namiki, A. (2004). Role of prostaglandin receptor subtype EP1 in prostaglandin E2-induced nociceptive transmission in the rat spinal dorsal horn. *Brain Research*, *1010*(1-2), 62–8.

Namba, T., Oida, H., Sugimoto, Y., Kakizuka, A., Negishi, M., Ichikawa, A., & Narumiya, S. (1994). cDNA Cloning of a Mouse Prostacyclin Receptor. *The Journal of Biological Chemistry*, *269*(13), 9986–9992.

Namba, T., Sugimoto, Y., Negishi, M., Irie, A., Ushikubi, F., Kakizuka, A., … Narumiya, S. (1993). Alternative splicing of C-terminal tail of prostaglandin E receptor subtype EP3 determines G-protein specificity. *Nature*, *365*, 166–170.

Nannini, L. J., Poole, P., Milan, S. J., & Kesterton, A. (2013). Combined corticosteroid and long-acting beta2-agonist in one inhaler versus inhaled corticosteroid alone for chronic obstructive pulmonary disease. *The Cochrane Collaboration*, (8), 1–136.

Narumiya, S. (2009). Prostanoids and inflammation: a new concept arising from receptor knockout mice. *Journal of Molecular Medicine (Berlin, Germany)*, *87*(10), 1015–22.

Narumiya, S., Sugimoto, Y., & Ushikubi, F. (1999). Prostanoid receptors: structures, properties, and functions. *Physiological Reviews*, *79*(4), 1193–226.

Narumiya, S., & Toda, N. (1985). Different responsiveness of prostaglandin D2-sensitive systems to prostaglandin D2 and its analogues. *British Journal of Pharmacology*, *85*(2), 367–75.

Nasrallah, R., Zimpelmann, J., Singh, S., & Hébert, R. L. (2001). Molecular and biochemical characterization of prostacyclin receptors in rat kidney. *American Journal of Physiology. Renal Physiology*, *280*(2), F266–77.

Nassini, R., Pedretti, P., Moretto, N., Fusi, C., Carnini, C., Facchinetti, F., … Materazzi, S. (2012). Transient receptor potential ankyrin 1 channel localized to non-neuronal airway cells promotes non-neurogenic inflammation. *PloS One*, *7*(8), e42454.

Ndukwu, I. M., White, S. R., Leff, a R., & Mitchell, R. W. (1997). EP1 receptor blockade attenuates both spontaneous tone and PGE2-elicited contraction in guinea pig trachealis. *The American Journal of Physiology*, *273*(3 Pt 1), L626–33.

Nedrebø, T., Reed, R. K., & Berg, A. (2003). Effect of alpha-trinositol on interstitial fluid pressure, edema generation, and albumin extravasation after ischemia-reperfusion injury in rat hind limb. *Shock (Augusta, Ga.)*, *20*(2), 149–53.

Negishi, M., Sugimoto, Y., & Ichikawa, A. (1995). Molecular mechanisms of diverse actions of prostanoid receptors. *Biochimica et Biophysica Acta*, *1259*, 109–120.

Nelson, H. S., Weiss, S. T., Bleecker, E. R., Yancey, S. W., & Dorinsky, P. M. (2006). The Salmeterol Multicenter Asthma Research Trial: a comparison of usual pharmacotherapy for asthma or usual pharmacotherapy plus salmeterol. *Chest*, *129*(1), 15–26.

Newton, R., Kuitert, L. M., Bergmann, M., Adcock, I. M., & Barnes, P. J. (1997). Evidence for involvement of NF-kappaB in the transcriptional control of COX-2 gene expression by IL-1beta. *Biochemical and Biophysical Research Communications*, *237*(1), 28–32.

Nguyen, M., Camenisch, T., Snouwaert, J. N., Hicks, E., Coffman, T. M., Anderson, P. A. W., … Koller, B. H. (1997). The prostaglandin receptor EP4 triggers remodelling of the cardiovascular system at birth. *Nature*, *390*(6), 78–81.

Nials, A. T., Vardey, C. J., Denyer, L. H., Thomas, M., Sparrow, S. J., Shepherd, G. D. & Coleman, R. A. (1993). AH 13205, a selective prostanoid EP_2-receptor agonist. *Cardiovascular Drug Reviews*, 11, 165-179.

Nicola, C., Timoshenko, A. V, Dixon, S. J., Lala, P. K., & Chakraborty, C. (2005). EP1 receptor-mediated migration of the first trimester human extravillous trophoblast: the role of intracellular calcium and calpain. *The Journal of Clinical Endocrinology and Metabolism*, *90*(8), 4736–46.

Nishimura, H., Tokuyama, K., Inoue, Y., Arakawa, H., Kato, M., Mochizuki, H., & Morikawa, a. (2001). Acute effects of prostaglandin D2 to induce airflow obstruction and airway microvascular leakage in guinea pigs: role of thromboxane A2 receptors. *Prostaglandins & Other Lipid Mediators*, *66*(1), 1–15.

Norel, X. (2007). Prostanoid receptors in the human vascular wall. *TheScientificWorldJournal*, *7*, 1359–74.

Norel, X., Montpreville, V. De, & Brink, C. (2004). Vasoconstriction induced by activation of EP1 and EP3 receptors in human lung: effects of ONO-AE-248, ONO-DI-004, ONO-8711 or ONO-8713. *Prostaglandins & Other Lipid Mediators*, *74*(1-4), 101–112.

Norel, X., Walch, L. & Brink, C. (2002). U46619 induced contractions: Role of prostanoids and nitric oxide in human pulmonary vessels. *British Journal of Pharmacology*, 135, Proceedings Supplement, 282P.

Norel, X., Walch, L., Labat, C., Gascard, J. P., Dulmet, E., & Brink, C. (1999). Prostanoid receptors involved in the relaxation of human bronchial preparations. *British Journal of Pharmacology*, *126*(4), 867–72.

Nossaman, B. D., McMahon, T. J., Ragheb, M. S., Ibrahim, I. N., Babycos, C. R., Hood, J. S., & Kadowitz, P. J. (1992). Blockade of thromboxane/endoperoxide receptor-mediated responses in the pulmonary vascular bed of the cat by sulotroban. *European Journal of Pharmacology*, *213*(1), 1–7.

Nusing, R., Lesch, R., Ullrich, V. (1990). Immunohistochemical localization of thromboxane synthase in human tissues. *Eicosanoids*, 3, 53-58.

Ochoa, C. D., & Stevens, T. (2012). Studies on the cell biology of interendothelial cell gaps. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *302*(3), L275–86.

Offermanns, S. (2006). Activation of Platelet Function Through G Protein-Coupled Receptors. *Circulation Research*, *99*(12), 1293–1304.

Oga, T., Matsuoka, T., Yao, C., Nonomura, K., Kitaoka, S., Sakata, D., … Narumiya, S. (2009). Prostaglandin F(2alpha) receptor signaling facilitates bleomycin-induced pulmonary fibrosis independently of transforming growth factor-beta. *Nature Medicine*, *15*(12), 1426–30.

Okada, Y., Hara, a, Ma, H., Xiao, C. Y., Takahata, O., Kohgo, Y., … Ushikubi, F. (2000). Characterization of prostanoid receptors mediating contraction of the gastric fundus and ileum: studies using mice deficient in prostanoid receptors. *British Journal of Pharmacology*, *131*(4), 745–55.

Okano, M., Sugata, Y., Fujiwara, T., Matsumoto, R., Nishibori, M., Shimizu, K., … Nishizaki, K. (2006). E prostanoid 2 (EP2)/EP4-mediated suppression of antigen-specific human T-cell responses by prostaglandin E2. *Immunology*, *118*(3), 343–52.

Olivenstein, R., Du, T., Xu, L. J., & Martin, J. G. (1997). Microvascular leakage in the airway wall and lumen during allergen induced early and late responses in rats. *Pulmonary Pharmacology & Therapeutics*, *10*(4), 223–30.

Oostenbrink, J. B., Rutten-van Molken, M. P. M. H., Al, M. J., Van Noord, J. a., & Vincken, W. (2004). One-year cost-effectiveness of tiotropium versus ipratropium to treat chronic obstructive pulmonary disease. *European Respiratory Journal*, *23*(2), 241–249.

Owen, C. a. (2008). Roles for proteinases in the pathogenesis of chronic obstructive pulmonary disease. *International Journal of Chronic Obstructive Pulmonary Disease*, *3*(2), 253–68.

Pai, R., Szabo, I. L., Soreghan, B. a, Atay, S., Kawanaka, H., & Tarnawski, a S. (2001). PGE(2) stimulates VEGF expression in endothelial cells via ERK2/JNK1 signaling pathways. *Biochemical and Biophysical Research Communications*, *286*(5), 923–8.

Paredi, P., & Barnes, P. J. (2009). The airway vasculature: recent advances and clinical implications. *Thorax*, *64*(5), 444–50.

Passalacqua, G., & Ciprandi, G. (2008). Allergy and the lung. *Clinical and Experimental Immunology*, *153 Suppl* , 12–6.

Patel, H. J., Barnes, P. J., Takahashi, T., Tadjkarimi, S., Yacoub, M. H., & Belvisi, M. G. (1995). Evidence for prejunctional muscarinic autoreceptors in human and guinea pig trachea. *American Journal of Respiratory and Critical Care Medicine*, *152*(3), 872–8.

Patterson, C. E., Stasek, J. E., Schaphorst, K. L., Davis, H. W., & Garcia, J. G. N. (1995). Mechanisms of pertussis toxin-induced barrier dysfunction in bovine pulmonary artery endothelial cell monolayers. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *268*, 926–934.

Paul, R., Zhang, Z. G., Eliceiri, B. P., Jiang, Q., Boccia, a D., Zhang, R. L., … Cheresh, D. a. (2001). Src deficiency or blockade of Src activity in mice provides cerebral protection following stroke. *Nature Medicine*, *7*(2), 222–7.

Pautler, R. G. (2004). Mouse MRI: concepts and applications in physiology. *Physiology (Bethesda, Md.)*, *19*, 168–75.

Pavord, I. D., & Tattersfield, A. E. (1994). Bronchoprotective role for endogenous prostaglandin E2. *The Lancet*, *344*, 436–438.

Peinado, V. I., Barbera, J. a, Ramirez, J., Gomez, F. P., Roca, J., Jover, L., … Rodriguez-Roisin, R. (1998). Endothelial dysfunction in pulmonary arteries of patients with mild COPD. *The American Journal of Physiology*, *274*(6), L908–13.

Persson, C. G. A. (1987). Leakage of Macromolecules From the Tracheobronchial Microcirculation. *American Review of Respiratory Disease*, 135 **(6)**, S71-S75.

Pierce, K. L., Bailey, T. J., Hoyer, P. B., Gil, D. W., Woodward, D. F., & Regan, J. W. (1997). Cloning of a Carboxyl-terminal Isoform of the Prostanoid FP Receptor. *Journal of Biological Chemistry*, *272*, 883–887.

Pierce, K. L., Fujino, H., Srinivasan, D., & Regan, J. W. (1999). Activation of FP prostanoid receptor isoforms leads to Rho-mediated changes in cell morphology and in the cell cytoskeleton. *The Journal of Biological Chemistry*, *274*(50), 35944–9.

Platt-Mills, T. a, Sporik, R. B., Chapman, M. D., & Heymann, P. W. (1995). The role of indoor allergens in asthma. *Allergy*, *50*(22 Suppl), 5–12.

Portanova, J. P., Zhang, Y., Anderson, G. D., Hauser, S. D., Masferrer, J. L., Seibert, K., … Isakson, P. C. (1996). Selective neutralization of prostaglandin E2 blocks inflammation, hyperalgesia, and interleukin 6 production in vivo. *The Journal of Experimental Medicine*, *184*(3), 883–91.

Pozzi, A., Yan, X., Macias-Perez, I., Wei, S., Hata, A. N., Breyer, R. M., … Capdevila, J. H. (2004). Colon carcinoma cell growth is associated with prostaglandin E2/EP4 receptor-evoked ERK activation. *The Journal of Biological Chemistry*, *279*(28), 29797–804.

Prasain, N., & Stevens, T. (2009). The actin cytoskeleton in endothelial cell phenotypes. *Microvascular Research*, *77*(1), 53–63.

Profita, M., Sala, A., Bonanno, A., Riccobono, L., Ferraro, M., La Grutta, S., … Gjomarkaj, M. (2010). Chronic obstructive pulmonary disease and neutrophil infiltration: role of cigarette smoke and cyclooxygenase products. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *298*(2), L261–9.

Profita, M., Sala, A., Bonanno, A., Riccobono, L., Siena, L., Melis, M. R., … Vignola, A. M. (2003). Increased prostaglandin E2 concentrations and cyclooxygenase-2 expression in asthmatic subjects with sputum eosinophilia. *Journal of Allergy and Clinical Immunology*, *112*, 709–716.

Qian, Y. M., Jones, R. L., Chan, K. M., Stock, a I., & Ho, J. K. (1994). Potent contractile actions of prostanoid EP3-receptor agonists on human isolated pulmonary artery. *British Journal of Pharmacology*, *113*(2), 369–74.

Qiao, J., Huang, F., & Lum, H. (2003). PKA inhibits RhoA activation: a protection mechanism against endothelial barrier dysfunction. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *284*(6), L972–80.

Rabe, K. F., Hurd, S., Anzueto, A., Barnes, P. J., Buist, S. a, Calverley, P., … Zielinski, J. (2007). Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. *American Journal of Respiratory and Critical Care Medicine*, *176*(6), 532–55.

Rabiet, M. J., Plantier, J. L., Rival, Y. Genoux, Y. Lampugnani, M. G. & Dejana E. (1996). Thrombin-induced increase in endothelial permeability is associated with changes in cell-to-cell junction organization. *Arteriosclerosis, Thrombosis and Vascular Biology*, 16, 488-496.

Rao, R., Redha, R., Macias-Perez, I., Su, Y., Hao, C., Zent, R., … Pozzi, A. (2007). Prostaglandin E2-EP4 receptor promotes endothelial cell migration via ERK activation and angiogenesis in vivo. *The Journal of Biological Chemistry*, *282*(23), 16959–68.

Ratcliffe, M. J., Walding, a, Shelton, P. a, Flaherty, a, & Dougall, I. G. (2007). Activation of E-prostanoid4 and E-prostanoid2 receptors inhibits TNF-alpha release from human alveolar macrophages. *The European Respiratory Journal*, *29*(5), 986–94.

Raud, J. & Lindbom, L. (1994). Studies by intravital microscopy of basic inflammatory reactions and acute allergic inflammation. In: The Handbook of Immunopharmacology. Academic Press, London, 1994, 127-170.

Raychowdhury, M. K., Yukawa, M., Collins, L. J., Mcgrail, S. H., Kent, K. C., & Ware, J. A. (1994). Alternative Splicing Produces a Divergent Cytoplasmic Tail in the Human Endothelial Thromboxane A2 Receptor. *The Journal of Biological Chemistry*, *269*(30), 19256–19261.

Regan, J. W., Bailey, T. J., Pepperl, D. J., Pierce, K. L., Bogardus, A. M., Donello, J. E., … Gil, D. W. (1994). Cloning of a Novel Human Prostaglandin Receptor with Characteristics of the Pharmacologically Defined EP2 Subtype. *Molecular Pharmacology*, *46*, 213–220.

Renkin, E. M., & Tucker, V. L. (1998). Measurement of Microvascular Transport Parameters of Macromolecules in Tissues and Organs of Intact Animals. *Microcirculation*, *5*, 139–152.

Reynolds, A. M., Holmes, M. D., & Scicchitano, R. (2002). Interleukin-1beta and tumour necrosis factor-a increase microvascular leakage in the guinea pig trachea. *Respirology*, *7*, 23–28.

Richter, M., & Sirois, P. (2000). Effects of eicosanoids, neuromediators and bioactive peptides on murine airways. *European Journal of Pharmacology*, *389*(2-3), 225–34.

Rippe, B. & Grega, G. J. (1978) Effects of isoprenaline and cooling on histamine induced changes of capillary permeability in the rat hindquarter vascular bed. *Acta Physiologica Scandinavica*, 103, 252-262

Ritchie, R. H., Rosenkranz, a C., Huynh, L. P., Stephenson, T., Kaye, D. M., & Dusting, G. J. (2004). Activation of IP prostanoid receptors prevents cardiomyocyte hypertrophy via cAMP-dependent signaling. *American Journal of Physiology. Heart and Circulatory Physiology*, *287*(3), H1179–85.

Roberts, L. J. II., Sweetman, B. J., Lewis, R. A., Austen, K. F. & Oates, J. A. (1980). Increased Production of Prostaglandin D-2 in Patients with Systemic Mastocytosis. *New England Journal of Medicine*, 303, 1400-1404.

Rodijnen, W. F. Van, Korstjens, I. J., Legerstee, N., Wee, P. M., & Tangelder, G. (2007). Direct vasoconstrictor effect of prostaglandin E 2 on renal interlobular arteries : role of the EP3 receptor. *American Journal of Physiology. Renal Physiology*, *292*, 1094–1101..

Rogers, D. F., Belvisi, M. G., Aursudkij, B., Evans, T. W., & Barnes, P. J. (1988). Effects and interactions of sensory neuropeptides on airway microvascular leakage in guinea-pigs. *British Journal of Pharmacology*, *95*, 1109–1116.

Rogers, D. F., Boschetto, P., & Barnes, P. J. (1989). Plasma exudation. Correlation between Evans blue dye and radiolabeled albumin in guinea pig airways in vivo. *Journal of Pharmacological Methods*, *21*(4), 309–15.

Rogers, D. F., Dijk, S., & Barnes, P. J. (1990). Bradykinin-induced plasma exudation in guinea-pig airways: involvement of platelet activating factor. *British Journal of Pharmacology*, *101*(3), 739–45.

Rolin, S., Masereel, B., & Dogné, J.-M. (2006). Prostanoids as pharmacological targets in COPD and asthma. *European Journal of Pharmacology*, *533*(1-3), 89–100.

Roscioni, S. S., Dekkers, B. G. J., Prins, A. G., Menzen, M. H., Meurs, H., Schmidt, M., & Maarsingh, H. (2011). cAMP inhibits modulation of airway smooth muscle phenotype via the exchange protein activated by cAMP (Epac) and protein kinase A. *British Journal of Pharmacology*, *162*(1), 193–209.

Royce, S. G., Cheng, V., Samuel, C. S., & Tang, M. L. K. (2012). The regulation of fibrosis in airway remodeling in asthma. *Molecular and Cellular Endocrinology*, *351*(2), 167–75.

Sagana, R. L., Yan, M., Cornett, A. M., Tsui, J. L., Stephenson, D. a, Huang, S. K., … White, E. S. (2009). Phosphatase and tensin homologue on chromosome 10 (PTEN) directs prostaglandin E2-mediated fibroblast responses via regulation of E prostanoid 2 receptor expression. *The Journal of Biological Chemistry*, *284*(47), 32264–71.

Sakai, K., Yokoyama, A., Kohno, N., Hamada, H., & Hiwada, K. (2001). Prolonged Antigen Exposure Ameliorates Airway Inflammation But Not Remodeling in a Mouse Model of Bronchial Asthma. *International Archives of Allergy and Immunology*, *126*, 126–134.

Salvi, S. S., & Barnes, P. J. (2009). Chronic obstructive pulmonary disease in non-smokers. *Lancet*, *374*(9691), 733–43.

Saroea, H. G., Inman, M. D., & O'Byrne, P. M. (1995). U46619-induced bronchoconstriction in asthmatic subjects is mediated by acetylcholine release. *American Journal of Respiratory and Critical Care Medicine*, *151*(2 Pt 1), 321–4.

Sastre, B., & del Pozo, V. (2012). Role of PGE2 in asthma and nonasthmatic eosinophilic bronchitis. *Mediators of Inflammation*, *2012*, 645383.

Sastre, B., Fernández-Nieto, M., Mollá, R., López, E., Lahoz, C., Sastre, J., … Quirce, S. (2008). Increased prostaglandin E2 levels in the airway of patients with eosinophilic bronchitis. *Allergy*, *63*(1), 58–66.

Satoh, T., Moroi, R., Aritake, K., Urade, Y., Kanai, Y., Sumi, K., … Nakamura, M. (2006). Prostaglandin D2 Plays an Essential Role in Chronic Allergic Inflammation of the Skin via CRTH2 Receptor. *The Journal of Immunology*, *177*(4), 2621–2629.

Savov, J. D., Brass, D. M., Berman, K. G., McElvania, E., & Schwartz, D. a. (2003). Fibrinolysis in LPS-induced chronic airway disease. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *285*(4), L940–8.

Sawyer, N., Cauchon, E., Chateauneuf, A., Cruz, R. P. G., Nicholson, D. W., Metters, K. M., … Gervais, F. G. (2002). Molecular pharmacology of the human prostaglandin D2 receptor, CRTH2. *British Journal of Pharmacology*, *137*(8), 1163–72.

Scanlon, P. D., Connett, J. E., Waller, L. A., Altose, M. D., Bailey, W. C., Buist, A. S., & Tashkin, D. P. (2000). Smoking Cessation and Lung Function in Mild-to- Moderate Chronic Obstructive Pulmonary Disease. The Lung Health Study. *American Journal of Respiratory and Critical Care Medicine*, *161*, 381–390.

Schlötzer-Schrehardt, U., Zenkel, M., & Nüsing, R. M. (2002). Expression and localization of FP and EP prostanoid receptor subtypes in human ocular tissues. *Investigative Ophthalmology & Visual Science*, *43*(5), 1475–87.

Schniedermann, J., Rennecke, M., Buttler, K., Richter, G., Städtler, A.-M., Norgall, S., … Weich, H. a. (2010). Mouse lung contains endothelial progenitors with high capacity to form blood and lymphatic vessels. *BMC Cell Biology*, *11*, 50.

Schnittler, H., Wilke, A., Gress, T., Suttorp, N., & Drenckhahn, D. (1990a). Role of Actin and Myosin in the Control of Paracellular Permeability in Pig, Rat and Human Vascular Endothelium. *Journal of Physiology*, *431*, 379–401.

Schnittler, H., Wilke, A., Gress, T., Suttorp, N., & Drenckhahn, D. (1990b). Role of Actin and Myson in the Control of Paracellular Permeability in Pig, Rat and Human Vascular Endothelium. *The Journal of Physiology*, *431*, 379–401.

Schwaner, I., Offermanns, S., Spicher, K., Seifert, R., & Schultz, G. (1995). Differential activation of Gi and Gs proteins by E- and I-type prostaglandins in membranes from the human erythroleukaemia cell line, HEL. *Biochimica et Biophysica Acta*, *1265*, 8–14.

Schweitzer, K. S., Hatoum, H., Brown, M. B., Gupta, M., Justice, M. J., Beteck, B., … Petrache, I. (2011). Mechanisms of lung endothelial barrier disruption induced by cigarette smoke: role of oxidative stress and ceramides. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *301*(6), L836–46.

Sears, M. R. (2009). Safety of long-acting beta-agonists: are new data really required? *Chest*, *136*(2), 604–7.

Segi, E., Sugimoto, Y., Yamasaki, a, Aze, Y., Oida, H., Nishimura, T., … Ichikawa, a. (1998). Patent ductus arteriosus and neonatal death in prostaglandin receptor EP4-deficient mice. *Biochemical and Biophysical Research Communications*, *246*(1), 7–12.

Seiler, S., Brassard, C. L., & Federici, M. E. (1990). SQ-27986 inhibition of platelet aggregation is mediated through activation of platelet prostaglandin D2 receptors. *Prostaglandins*, *40*(2), 119–130.

Senior, J., Marshall, K., Sangha, R., Baxter, G. S., & Clayton, J. K. (1991). In vitro characterization of prostanoid EP-receptors in the non-pregnant human myometrium. *British Journal of Pharmacology*, *102*(3), 747–53.

Senoir, J., Sangha, R., Baxter, G. S., Marshall, K., & Clayton, K. (1992). In vitro characterisation of prostanoid FP-, DP-, IP- and TP-receptors on non-pregnant human myometrium. *British Journal of Pharmacology*, *107*, 215–221.

Serkova, N. J., Van Rheen, Z., Tobias, M., Pitzer, J. E., Wilkinson, J. E., & Stringer, K. A. (2008). Utility of magnetic resonance imaging and nuclear magnetic resonance-based metabolomics for quantification of inflammatory lung injury. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *295*, 152–161.

Seth, R. V, Clarke, V. S., Lewis, R. a, & Tattersfield, a E. (1981). Effect of propranolol on the airway response to prostaglandin E2 in normal man. *British Journal of Clinical Pharmacology*, *12*(5), 731–5.

Sharif, N. a, McLaughlin, M. a, Kelly, C. R., Xu, S., Crider, J. Y., Williams, G. W., & Parker, J. L. (2006). Preclinical pharmacology of AL-12182, a new ocular hypotensive 11-oxa prostaglandin analog. *Journal of Ocular Pharmacology and Therapeutics : The Official Journal of the Association for Ocular Pharmacology and Therapeutics*, *22*(5), 291–309.

Shasby, D. M., Shasby, S. S., Sullivan, J. M., & Peach, M. J. (1982). Role of endothelial cell cytoskeleton in control of endothelial permeability. *Circulation Research*, *51*(5), 657–661.

Shasby, D. M., Stevens, T., Ries, D., Moy, A. B., Kamath, J. M., Kamath, A. M., & Shasby, S. S. (1997). Thrombin inhibits myosin light chain dephosphorylation in endothelial cells. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *272*, 311–319.

Sheller, J. R., Mitchell, D., Meyrick, B., Oates, J., & Breyer, R. (2000). EP2 receptor mediates bronchodilation by PGE2 in mice. *Journal of Applied Physiology*, *88*, 2214–2218.

Shen, Q., Rigor, R. R., Pivetti, C. D., Wu, M. H., & Yuan, S. Y. (2010). Myosin light chain kinase in microvascular endothelial barrier function. *Cardiovascular Research*, *87*(2), 272–80.

Shen, Q., Wu, M. H., & Yuan, S. Y. (2009). Endothelial contractile cytoskeleton and microvascular permeability. *Journal of Cell Health and Cytoskeleton*, *1*, 43–50.

Siflinger-Birnboim, A., Bode, D. C., & Malik, A. B. (1993). Adenosine 3',5'-cyclic monophosphate attenuates neutrophil-mediated increase in endothelial permeability. *American Journal of Physiology. Heart and Circulatory Physiology*, *264*, 370–375.

Sin, D. D., Anthonisen, N. R., Soriano, J. B., & Agusti, a G. (2006). Mortality in COPD: Role of comorbidities. *The European Respiratory Journal*, *28*(6), 1245–57.

Singh, D., Brooks, J., Hagan, G., Cahn, a, & O'Connor, B. J. (2008). Superiority of "triple" therapy with salmeterol/fluticasone propionate and tiotropium bromide versus individual components in moderate to severe COPD. *Thorax*, *63*(7), 592–8.

Singh, J., Zeller, W., Zhou, N., Hategen, G., Mishra, R., Polozov, A., … Gurney, M. E. (2009). Antagonists of the EP3 Receptor for Prostaglandin E2 Are Novel Antiplatelet Agents That Do Not Prolong Bleeding. *ACS Chemical Biology*, *4*(2), 115–126.

Slauson, D. O., & Hahn, F. F. (1980). Criteria for Development of Animal Models of Diseases of the Respiratory System. *American Journal of Pathology*, *101*(3S), 103–122.

Smith, J. M., Disney, M. E., Williams, J. D., & Goels, Z. a. (1969). Clinical significance of skin reactions to mite extracts in children with asthma. *British Medical Journal*, *2*(5659), 723–6.

Smock, S. L., Pan, L. C., Castleberry, T. a, Lu, B., Mather, R. J., & Owen, T. a. (1999). Cloning, structural characterization, and chromosomal localization of the gene encoding the human prostaglandin E(2) receptor EP2 subtype. *Gene*, *237*(2), 393–402.

Song, P., Zhang, M., Wang, S., Xu, J., Choi, H. C., & Zou, M.-H. (2009). Thromboxane A2 receptor activates a Rho-associated kinase/LKB1/PTEN pathway to attenuate endothelium insulin signaling. *The Journal of Biological Chemistry*, *284*(25), 17120–8.

Southam, D. S., Ellis, R., Wattie, J., & Inman, M. D. (2007). Components of airway hyperresponsiveness and their associations with inflammation and remodeling in mice. *The Journal of Allergy and Clinical Immunology*, *119*(4), 848–54.

Spada, C. S., Nieves, A. L., & Woodward, D. F. (2002). Vascular Activities of Prostaglandins and Selective Prostanoid Receptor Agonists in Human Retinal Microvessels. *Experimental Eye Research*, *75*(2), 155–163.

Spicuzza, L., Belvisi, M. G., Birrell, M. a, Barnes, P. J., Hele, D. J., & Giembycz, M. a. (2001). Evidence that the anti-spasmogenic effect of the beta-adrenoceptor agonist, isoprenaline, on guinea-pig trachealis is not mediated by cyclic AMP-dependent protein kinase. *British Journal of Pharmacology*, *133*(8), 1201–12.

Spina, D., & Page, C. P. (2002). Pharmacology of airway irritability. *Current Opinion in Pharmacology*, *2*(3), 264–72.

Spindler, V., Schlegel, N., & Waschke, J. (2010). Role of GTPases in control of microvascular permeability. *Cardiovascular Research*, *87*(2), 243–53.

Steinberg, B. E., Goldenberg, N. M., & Lee, W. L. (2012). Do viral infections mimic bacterial sepsis? The role of microvascular permeability: A review of mechanisms and methods. *Antiviral Research*, *93*(1), 2–15.

Stelzner, T. J., Weil, J. V. & O'Brien, R. F. (1989). Role of cyclic adenosine monophosphate in the induction of endothelial barrier properties. *Journal of Cellular Physiology*, 139, 157-166.

Stevenson, C. S., & Belvisi, M. G. (2008). Preclinical animal models of asthma and chronic obstructive pulmonary disease. *Expert Review of Respiratory Medicine*, *2*(5), 631–43.

Stevenson, C. S., & Birrell, M. a. (2011). Moving towards a new generation of animal models for asthma and COPD with improved clinical relevance. *Pharmacology & Therapeutics*, *130*(2), 93–105.

Stevenson, C. S., Koch, L. G., & Britton, S. L. (2006). Aerobic capacity, oxidant stress, and chronic obstructive pulmonary disease--a new take on an old hypothesis. *Pharmacology & Therapeutics*, *110*(1), 71–82.

Stock, J. L., Shinjo, K., Burkhardt, J., Roach, M., Taniguchi, K., Ishikawa, T., … Audoly, L. P. (2001). The prostaglandin E2 EP1 receptor mediates pain perception and regulates blood pressure. *The Journal of Clinical Investigation*, *107*(3), 325–31.

Stockley, R. a, Mannino, D., & Barnes, P. J. (2009). Burden and pathogenesis of chronic obstructive pulmonary disease. *Proceedings of the American Thoracic Society*, *6*(6), 524–6.

Stolk, J., Rudolphus, a, Davies, P., Osinga, D., Dijkman, J. H., Agarwal, L., … Kramps, J. a. (1992). Induction of emphysema and bronchial mucus cell hyperplasia by intratracheal instillation of lipopolysaccharide in the hamster. *The Journal of Pathology*, *167*(3), 349–56.

Stoller, J. K., & Aboussouan, L. S. (2012). A review of α1-antitrypsin deficiency. *American Journal of Respiratory and Critical Care Medicine*, *185*(3), 246–59.

Sturm, E. M., Schratl, P., Schuligoi, R., Konya, V., Sturm, G. J., Lippe, I. T., … Heinemann, A. (2008). Prostaglandin E2 inhibits eosinophil trafficking through E-prostanoid 2 receptors. *Journal of Immunology (Baltimore, Md. : 1950)*, *181*(10), 7273–83.

Subbaramaiah, K., Hudis, C., Chang, S.-H., Hla, T., & Dannenberg, A. J. (2008). EP2 and EP4 receptors regulate aromatase expression in human adipocytes and breast cancer cells. Evidence of a BRCA1 and p300 exchange. *The Journal of Biological Chemistry*, *283*(6), 3433–44.

Sugimoto, H., Shichijo, M., Iino, T., Manabe, Y., Watanabe, A., Shimazaki, M., … Bacon, K. B. (2003). An Orally Bioavailable Small Molecule Antagonist of CRTH2 , Ramatroban (BAY u3405), Inhibits Prostaglandin D 2 -Induced Eosinophil Migration in Vitro. *The Journal of Pharmacology and Experimental Therapeutics*, *305*(1), 347–352.

Sugimoto, Y., Hasumoto, K., Namba, T., Irie, a, Katsuyama, M., Negishi, M., … Ichikawa, a. (1994). Cloning and expression of a cDNA for mouse prostaglandin F receptor. *The Journal of Biological Chemistry*, *269*(2), 1356–60.

Sugimoto, Y., Hasumoto, K., Namba, T., Irie, A., Katsuyama, M., Negishi, M., … Ichikawa, A. (1994). Cloning and Expression of a cDNA for Mouse Prostaglandin F Receptor. *The Journal of Biological Chemistry*, *269*(2), 1356–1360.

Sugimoto, Y., & Narumiya, S. (2007). Prostaglandin E receptors. *The Journal of Biological Chemistry*, *282*(16), 11613–7.

Sugimoto, Y., Yamasaki, A., Segi, E., Tsuboi, K., Aze, Y., Nishimura, T., … Narumiya, S. (1997). Failure of Parturition in Mice Lacking the Prostaglandin F Receptor. *Science*, *277*, 681–683.

Suissa, S., & Barnes, P. J. (2009). Inhaled corticosteroids in COPD: the case against. *European Respiratory Journal*, *34*, 13–16.

Sun, H., Breslin, J. W., Zhu, J., Yuan, S. Y., & Wu, M. H. (2006). Rho and ROCK Signaling in VEGF-Induced Microvascular Endothelial Hyperpermeability. *Microcirculation*, *13*, 237–247.

Suzawa, T., Miyaura, C., Inada, M., Maruyama, T., Sugimoto, Y., Ushikubi, F., … Suda, T. (200AD). The Role of Prostaglandin E Receptor Subtypes (EP1, EP2, EP3, and EP4) in Bone Resorption : An Analysis Using Specific Agonists for the Respective EPs. *Endocinology*, *141*(4), 1554–1559.

Suzuki-Yamamoto, T., Toida, K., Sugimoto, Y., & Ishimura, K. (2009). Colocalization of prostaglandin F2a receptor FP and prostaglandin F synthase-I in the spinal cord. *Journal of Lipid Research*, *50*, 1996–2003.

Svensjo, E., Arfors, K.-E., Raymond, R. M., & Grega, G. J. (1979). Morphological and physiological correlation of bradykinin-induced macromolecular efflux of. *American Journal of Physiology. Heart and Circulatory Physiology*, *5*(4), 600–606.

Svensson, C., Grinneberg, R., Andersson, M., Alkner, U., Andersson, O., Billing, B., … Persson, C. G. A. (1995). Allergen challenge-induced entry of alpha2-macroglobulin and tryptase into human nasal and bronchial airways. *Journal of Allergy and Clinical Immunology*, *96*, 239–246.

Svensson, J., Strandberg, K., Tuvemo, T., & Hamberg, M. (1977). THROMHOXANE A2 : EFFECTS ON AIRWAY AND VASCULAR SMOOTH MUSCLE. *Prostaglandins*, *14*(3), 425–436.

Swirski, F. K., Sajic, D., Robbins, C. S., Gajewska, B. U., Jordana, M., & Stämpfli, M. R. (2002). Chronic Exposure to Innocuous Antigen in Sensitized Mice Leads to Suppressed Airway Eosinophilia That Is Reversed by Granulocyte Macrophage Colony-Stimulating Factor. *The Journal of Immunology*, *169*, 3499–3506.

Sylvester, P. W. (2011). Optimization of the Tetrazolium Dye (MTT) Colorimetric Assay for Cellular Growth and Viability. *Methods in Molecular Biology*, *716*, 157–168.

Szafranski, W., Cukier, A., Ramirez, A., Menga, G., Sansores, R., Nahabedian, S., … Olsson, H. (2003). Efficacy and safety of budesonide/formoterol in the management of chronic obstructive pulmonary disease. *European Respiratory Journal*, *21*, 74–81.

Szczeklik, A., & Stevenson, D. D. (2003). Aspirin-induced asthma : Advances in pathogenesis, diagnosis, and management. *Journal of Allergy and Clinical Immunology*, *111*, 913–921.

Takagi, T., Forrest, M. J., & Brooks, P. M. (1987). A Pharmacological and Histological Examination of the Microcirculation of the Rat Subcutaneous Air-Pouch: Microcirculation of the Rat Air-Pouch. *Pathology*, *19*, 294–298.

Takahashi, Y., Tokuoka, S., Masuda, T., Hirano, Y., Nagao, M., Tanaka, H., … Nagai, H. (2002). Augmentation of allergic inflammation in prostanoid IP receptor deficient mice. *British Journal of Pharmacology*, *137*, 315–322.

Takayama, K., Garcia-Cardena, G., Sukhova, G. K., Comander, J., Gimbrone Jr, M. A., & Libby, P. (2002). Prostaglandin E2 Suppresses Chemokine Production in Human Macrophages through the EP4 Receptor. *The Journal of Biological Chemistry*, *277*(46), 44147–44154.

Tamaoki, J., Chiyotani, A., Takeyama, K., Yamauchi, F., Tagaya, E., & Konno, K. (1993). Relaxation and Inhibition of Contractile Response to Electrical Field Stimulation by Beraprost Sodium in Canine Airway Smooth Muscle. *Prostaglandins*, *45*, 363–373.

Tan, X., Essengue, S., Talreja, J., Reese, J., Stechschulte, D. J., & Dileepan, K. N. (2007). Histamine Directly and Synergistically with Lipopolysaccharide Stimulates Cyclooxygenase-2 Expression and Prostaglandin I2 and E2 Production in Human Coronary Artery Endothelial Cells. *The Journal of Immunology*, *179*(11), 7899–7906.

Tanabe, T., & Ullrich, V. (1995). Prostacyclin and thromboxane synthases. *Journal of Lipid Mediators and Cell Signalling*, *12*(2-3), 243–55.

Tanaka, H., Kanako, S., & Abe, S. (2005). Prostaglandin E2 receptor selective agonists E-prostanoid 2 and E-prostanoid 4 may have therapeutic effects on ovalbumin-induced bronchoconstriction. *Chest*, *128*(5), 3717–23.

Tang, C.-H., Yang, R.-S., & Fu, W.-M. (2005). Prostaglandin E2 stimulates fibronectin expression through EP1 receptor, phospholipase C, protein kinase Calpha, and c-Src pathway in primary cultured rat osteoblasts. *The Journal of Biological Chemistry*, *280*(24), 22907–16.

Taube, C., Duez, C., Cui, Z.-H., Takeda, K., Rha, Y.-H., Park, J.-W., … Gelfand, E. W. (2002). The Role of IL-13 in Established Allergic Airway Disease. *The Journal of Immunology*, *169*(11), 6482–6489.

Temelkovski, J., Hogan, S. P., Shepherd, D. P., Foster, P. S., & Kumar, R. K. (1998). An improved murine model of asthma: selective airway inflammation, epithelial lesions and increased methacholine responsiveness following chronic exposure to aerosolised allergen. *Thorax*, *53*(10), 849–56.

Thomas, D. W., Mannon, R. B., Mannon, P. J., Latour, a, Oliver, J. a, Hoffman, M., … Coffman, T. M. (1998). Coagulation defects and altered hemodynamic responses in mice lacking receptors for thromboxane A2. *The Journal of Clinical Investigation*, *102*(11), 1994–2001.

Tigani, B., Cannet, C., Karmouty-quintana, H., Ble, F.-X., Zurbruegg, S., Schaeublin, E., … Beckmann, N. (2007). Lung inflammation and vascular remodeling after repeated allergen challenge detected noninvasively by MRI. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *292*, 644–653.

Tilley, S. L., Coffman, T. M., & Koller, B. H. (2001). Mixed messages : modulation of inflammation and immune responses by prostaglandins and thromboxanes. *Journal of Clinical Investigation*, *108*, 15–23.

Tilley, S. L., Hartney, J. M., Erikson, C. J., Jania, C., Nguyen, M., Stock, J., … Panet-, R. A. (2003). Receptors and pathways mediating the effects of prostaglandin E2 on airway tone. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *284*(4), L599–606.

Tokuyama, K., Lötvall, J. O., Morikawa, a, Barnes, P. J., & Chung, K. F. (1991). Role of thromboxane A2 in airway microvascular leakage induced by inhaled platelet-activating factor. *Journal of Applied Physiology (Bethesda, Md. : 1985)*, *71*(5), 1729–34.

Torphy, T. J. (1994). Beta-adrenoceptors, cAMP and airway smooth muscle relaxation: challenges to the dogma. *Trends in Pharmacological Sciences*, *15*(10), 370–4.

Torphy, T. J., Freese, W. B., Rinard, G. a, Brunton, L. L., & Mayer, S. E. (1982). Cyclic nucleotide-dependent protein kinases in airway smooth muscle. *The Journal of Biological Chemistry*, *257*(19), 11609–16.

Tournebize, R., Doan, B.-T., Dillies, M.-A., Maurin, S., Beloeil, J.-C., & Sansonetti, P. J. (2006). Magnetic resonance imaging of Klebsiella pneumoniae-induced pneumonia in mice. *Cellular Microbiology*, *8*(1), 33–43.

Travis, J. (2003). Scientists seek to identify all the proteins in plasma. *Science News Online*, 163, 171.

Trifilieff, A., El-hashim, A., & Bertrand, C. (2000). Time course of inflammatory and remodeling events in a murine model of asthma: effect of steroid treatment. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *279*(6), L1120–8.

Tseliou, E., Bakakos, P., Kostikas, K., Hillas, G., Mantzouranis, K., Emmanouil, P., … Loukides, S. (2012). Increased levels of angiopoietins 1 and 2 in sputum supernatant in severe refractory asthma. *Allergy*, *67*(3), 396–402.

Udaka, K., Takeuchi, Y. & Movat, H. Z. (1970). Simple Method for Quantitation of Enhanced Vascular Permeability. *Proceedings of the Society for Experimental Biology and Medicine*, 133, 1384-1387.

Ueno, a, Matsumoto, H., Naraba, H., Ikeda, Y., Ushikubi, F., Matsuoka, T., … Oh-ishi, S. (2001). Major roles of prostanoid receptors IP and EP(3) in endotoxin-induced enhancement of pain perception. *Biochemical Pharmacology*, *62*(2), 157–60.

Ueno, A., Naraba, H., Ikeda, Y., Ushikubi, F., Murata, T., Narumiya, S., & Oh-ishi, S. (2000). Intrinsic prostacyclic contributes to exudation induced by bradykini or carregeenin: A study on the paw edema induced in IP-receptor-defcient mice. *Life Sciences*, *66*(12), 155–160.

Ukropec, J. A., Hollinger, M. K., Salva, S. M., & Woolkalis, M. J. (2000). SHP2 Association with VE-Cadherin Complexes in Human Endothelial Cells Is Regulated by Thrombin. *Journal of Biological Chemistry*, *275*(8), 5983–5986.

Ulven, T., & Kostenis, E. (2005). Minor structural modifications convert the dual TP/CRTH2 antagonist ramatroban into a highly selective and potent CRTH2 antagonist. *Journal of Medicinal Chemistry*, *48*(4), 897–900.

Underwood, D. C., Muccitelli, R. M., Luttmann, M. a, Hay, D. W., Torphy, T. J., & Wasserman, M. a. (1994). Differential antagonism of airway contractile responses to prostaglandin (PG)D2 and 9 alpha, 11 beta-PGF2 by atropine, SK&F 88046 and SQ 29,548 in the guinea pig. *The Journal of Pharmacology and Experimental Therapeutics*, *268*(1), 304–10.

Urade, Y., Watanabe, K., & Hayaishi, O. (1995). Prostaglandin D, E, and F synthases. *Journal of Lipid Mediators and Cell Signalling*, *12*(2-3), 257–73.

Ushikubi, F., Nakajima, M., Hirata, M., Okuma, M., Fujiwara, M., & Narumiya, S. (1989). Purification of the thromboxane A2/prostaglandin H2 receptor from human blood platelets. *The Journal of Biological Chemistry*, *264*(28), 16496–501.

Ushikubi, F., Nakamura, K., & Narumiya, S. (1994). Functional Reconstitution of Platelet Thromboxane A2 Receptors with Gq and G12 in Phospholipid Vesicles. *Molecular Pharmacology*, *46*, 808–816.

Ushikubi, F., Segi, E., Sugimoto, Y., Murata, T., Matsuoka, T., Kobayashi, T., … Narumiya, S. (1998). Impaired febrile response in mice lacking the prostaglandin E receptor subtype EP3. *Nature*, *395*(6699), 281–4.

Van de Graaf, E. A., Out, T. A., Roos, C. M., & Jansen, H. M. (1991). Respiratory Membrane Permeability and Bronchial Hyperreactivity in Patients with Stable Athma. *American Reviews of Respiratory Disease*, *143*, 362–368.

Van Hinsbergh, V. W. M., & van Nieuw Amerongen, G. P. (2002). Intracellular signalling involved in modulating human endothelial barrier function. *Journal of Anatomy*, *200*(6), 549–60.

Van Hove, C. L., Maes, T., Cataldo, D. D., Guéders, M. M., Palmans, E., Joos, G. F., & Tournoy, K. G. (2009). Comparison of acute inflammatory and chronic structural asthma-like responses between C57BL/6 and BALB/c mice. *International Archives of Allergy and Immunology*, *149*(3), 195–207.

Van Hove, C. L., Maes, T., Joos, G. F., & Tournoy, K. G. (2007). Prolonged inhaled allergen exposure can induce persistent tolerance. *American Journal of Respiratory Cell and Molecular Biology*, *36*(5), 573–84.

Van Nieuw Amerongen, G. P., & van Hinsbergh, V. W. M. (2002). Targets for pharmacological intervention of endothelial hyperpermeability and barrier function. *Vascular Pharmacology*, *39*(4-5), 257–272.

Van Rensen, E. L. J., Hiemstra, P. S., Rabe, K. F., & Sterk, P. J. (2002). Assessment of microvascular leakage via sputum induction: the role of substance P and neurokinin A in patients with asthma. *American Journal of Respiratory and Critical Care Medicine*, *165*(9), 1275–9.

Vandenbroucke, E., Mehta, D., Minshall, R., & Malik, A. B. (2008). Regulation of endothelial junctional permeability. *Annals of the New York Academy of Sciences*, *1123*, 134–45.

Vane, J. R., & Botting, R. M. (1995). Pharmacodynamic Profile of Prostacyclin. *American Journal Cardiology*, *75*, 3–10.

Verin, A. D., Patterson, C. E., Day, M. A., & Garcia, J. G. N. (1995). Regulation of endothelial cell gap formation and barrier function by myosin-associated phosphatase activities. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *269*, 99–108.

Vestbo, J., Hurd, S. S., Agustí, A. G., Jones, P. W., Vogelmeier, C., Anzueto, A., … Rodriguez-Roisin, R. (2013). Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. *American Journal of Respiratory and Critical Care Medicine*, *187*(4), 347–65.

Vrugt, B., Wilson, S., Bron, a, Holgate, S. T., Djukanovic, R., & Aalbers, R. (2000). Bronchial angiogenesis in severe glucocorticoid-dependent asthma. *The European Respiratory Journal*, *15*(6), 1014–21.

Vysniauskiene, I., Allemann, R., Flammer, J., & Haefliger, I. O. (2006). Vasoactive responses of U46619, PGF2alpha, latanoprost, and travoprost in isolated porcine ciliary arteries. *Investigative Ophthalmology & Visual Science*, *47*(1), 295–8.

Wacker, M. J., Kosloski, L. M., Gilbert, W. J. R., Touchberry, C. D., Moore, D. S., Kelly, J. K., … Orr, J. A. (2009). Inhibition of Thromboxane A 2 -Induced Arrhythmias and Intracellular Calcium Changes in Cardiac Myocytes by Blockade of the Inositol Trisphosphate Pathway. *The Journal of Pharmacology and Experimental Therapeutics*, *331*(3), 917–924.

Walch, L., de Montpreville, V., Brink, C., & Norel, X. (2001). Prostanoid EP(1)- and TP-receptors involved in the contraction of human pulmonary veins. *British Journal of Pharmacology*, *134*(8), 1671–8.

Walch, L., Labat, C., Gascard, J. P., de Montpreville, V., Brink, C., & Norel, X. (1999). Prostanoid receptors involved in the relaxation of human pulmonary vessels. *British Journal of Pharmacology*, *126*(4), 859–66.

Walsh, M. T., Foley, J. F., & Kinsella, B. T. (1998). Characterization of the role of N-linked glycosylation on the cell signaling and expression of the human thromboxane A2 receptor alpha and beta isoforms. *The Journal of Pharmacology and Experimental Therapeutics*, *286*(2), 1026–36.

Walter, D. M., Mcintire, J. J., Berry, G., Mckenzie, N. J., Donaldson, D. D., Rosemarie, H., … Dekruyff, R. H. (2001). Critical Role for IL-13 in the Development of Allergen-Induced Airway Hyperreactivity. *The Journal of Immunology*, *167*(8), 4668–4675.

Walters, E. H., O'Byrne, P. M., Fabbri, L. M., Graf, P. D., Holtzman, M. J., & Nadel, J. a. (1984). Control of neurotransmission by prostaglandins in canine trachealis smooth muscle. *Journal of Applied Physiology: Respiratory, Environmental and Exercise Physiology*, *57*(1), 129–34.

Wang, X. S., & Lau, H. Y. a. (2006). Prostaglandin E potentiates the immunologically stimulated histamine release from human peripheral blood-derived mast cells through EP1/EP3 receptors. *Allergy*, *61*(4), 503–6.

Wang, Z., Ginnan, R., Iskandar, F., Trebak, M., Peter, A., Singer, H. A., … Vincent, P. A. (2010). Calcium/Calmodulin-dependent protein kinase II delta 6 (CaMKIIdelta6) and RhoA involvement in thrombin-induced endothelial barrier dysfunction. *The Journal of Biological Chemistry*, *285*(28), 21303–12.

Warner, T. D., Giuliano, F., Vojnovic, I., Bukasa, a, Mitchell, J. a, & Vane, J. R. (1999). Nonsteroid drug selectivities for cyclo-oxygenase-1 rather than cyclo-oxygenase-2 are associated with human gastrointestinal toxicity: a full in vitro analysis. *Proceedings of the National Academy of Sciences of the United States of America*, *96*(13), 7563–8.

Watabe, A., Sugimoto, Y., Honda, A., Irie, A., Namba, T., Negishi, M., … Ichikawa, A. (1993). Cloning and Expression of cDNA for a Mouse EP1 Subtype of Prostaglandin E Receptor. *The Journal of Biological Chemistry*, *268*(27), 20175–20178.

Watanabe, K. (2002). Prostaglandin F synthase. *Prostaglandins & Other Lipid Mediators*, *68-69*, 401–407.

Weatherall, M., Wijesinghe, M., Perrin, K., Harwood, M., & Beasley, R. (2010). Meta-analysis of the risk of mortality with salmeterol and the effect of concomitant inhaled corticosteroid therapy. *Thorax*, *65*(1), 39–43.

Wenzel, S. (2005). Severe asthma in adults. *American Journal of Respiratory and Critical Care Medicine*, *172*(2), 149–60.

Wenzel, S. E. (2006). Asthma : defining of the persistent adult phenotypes. *Lancet*, *368*, 804–813.

Wenzel, S. E., Westcott, J. Y., & Larsen, G. L. (1991). Bronchoalveolar lavage fluid mediator levels 5 minutes after allergen challenge in atopic subjects with asthma: Relationship to the development of late asthmatic responses. *Journal of Allergy and Clinical Immunology*, *87*, 540–548.

Whittle, B. J. R., Moncada, S., Mullane, K., & Vane, J. R. (1983). Platelet and Cardiovascular Activity of the Hydantoin BW245C, A Potent Prostaglandin Analogue. *Prostaglandins*, *25*(2), 205–223.

Widdicombe, J. H., Ueki, I. F., Emery, D., Margolskee, D., Yergey, J., & Nadel, J. a. (1989). Release of cyclooxygenase products from primary cultures of tracheal epithelia of dog and human. *The American Journal of Physiology*, *257*(6 Pt 1), L361–5.

Willemse, B. W. M., ten Hacken, N. H. T., Rutgers, B., Lesman-Leegte, I. G. a T., Postma, D. S., & Timens, W. (2005). Effect of 1-year smoking cessation on airway inflammation in COPD and asymptomatic smokers. *The European Respiratory Journal*, *26*(5), 835–45.

Williams, T. J. (1982). Vasoactive intestinal polypeptide is more potent than prostaglandin E2 as a vasodilator and oedema potentiator in rabbit skin. *British Journal of Pharmacology*, *77*(3), 505–9.

Wilson, J. W., & Robertson, C. F. (2002). Angiogenesis in paediatric airway disease. *Paediatric Respiratory Reviews*, *3*, 219–229.

Wilson, R. J., Giblin, G. M. P., Roomans, S., Rhodes, S. a, Cartwright, K.-A., Shield, V. J., … Giles, H. (2006). GW627368X ((N-{2-[4-(4,9-diethoxy-1-oxo-1,3-dihydro-2H-benzo[f]isoindol-2-yl)phenyl]acetyl} benzene sulphonamide): a novel, potent and selective prostanoid EP4 receptor antagonist. *British Journal of Pharmacology*, *148*(3), 326–39.

Winter, M. C., Kamath, A. M., Ries, D. R., Shasby, S. S., Chen, Y., Shasby, D. M., & Michael, C. (1999). Histamine alters cadherin-mediated sites of endothelial adhesion. *The American Journal of Physiology*, *277*(5 Pt 1), L988–95.

Woo, S. K., Roszkowski, P., Waterbury, L. D., & Garay, G. L. (1986). Gastric Mucosal Binding Studies with Enprostil: A Potent Anti-Ulcer Prostaglandin. *Prostaglandins*, *32*(2), 243–257.

Woodward, D. F., Jones, R. L., & Narumiya, S. (2011). International Union of Basic and Clinical Pharmacology . LXXXIII : Classification of Prostanoid Receptors, Updating 15 Years of Progress. *Pharmacological Reviews*, *63*(3), 471–538.

Woodward, D. F., Krauss, a H., Wang, J. W., Protzman, C. E., Nieves, a L., Liang, Y., … Struble, C. (2007). Identification of an antagonist that selectively blocks the activity of prostamides (prostaglandin-ethanolamides) in the feline iris. *British Journal of Pharmacology*, *150*(3), 342–52.

Woodward, D. F., & Lawrence, R. a. (1994). Identification of a single (FP) receptor associated with prostanoid-induced Ca2+ signals in Swiss 3T3 cells. *Biochemical Pharmacology*, *47*(9), 1567–74.

Woodward, D. F., Pepperl, D. J., Burkey, T. H., & Regan, J. W. (1995). 6-Isopropoxy-9-oxoxanthene-2carboxylic Acid (AH 6809) A Human EP2 Receptor Antagonist. *Biochemical Pharmacology*, *50*(10), 1731–1733.

Wooldridge, L., Ekeruche-makinde, J., Berg, H. A. Van Den, Skowera, A., John, J., Tan, M. P., … Miles, J. J. (2012). A single autoimmune T cell receptor recognizes more than a million different peptides. *The Journal of Biological Chemistry*, *287*(2), 1168–77.

World Health Organisation (2008). *World Health Statistics*. [Online] Available from: http://www.who.int/whosis/whostat/EN_WHS08_Full.pdf

Wright, J. L., Cosio, M., & Churg, A. (2008). Animal models of chronic obstructive pulmonary disease. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *295*, 1–15.

Wu, N. Z., & Baldwin, a L. (1992). Transient venular permeability increase and endothelial gap formation induced by histamine. *The American Journal of Physiology*, *262*(4 Pt 2), H1238–47.

Wyss, D., Bonneau, O., & Trifilieff, a. (2007). Synergistic effect of formoterol and mometasone in a mouse model of allergic lung inflammation. *British Journal of Pharmacology*, *152*(1), 83–90.

Xie, G., Wang, Y., Sharma, M., Gabriel, a, Mitchell, J., Xing, Y., … Palmer, P. P. (2003). 5-Hydroxytryptamine-induced plasma extravasation in the rat knee joint is mediated by multiple prostaglandins. *Inflammation Research : Official Journal of the European Histamine Research Society ... [et Al.]*, *52*(1), 32–8.

Xue, L., Gyles, S. L., Wettey, F. R., Gazi, L., Townsend, E., Hunter, M. G., … Gyles, L. (2005). Prostaglandin D2 Causes Preferential Induction of Proinflammatory Th2 Cytokine Production through an Action on Chemoattractant Receptor-Like Molecule Expressed on Th2 Cells. *The Journal of Immunology*, *175*(10), 6531–6536.

Yan, H., Deshpande, D. a, Misior, A. M., Miles, M. C., Saxena, H., Riemer, E. C., … Penn, R. B. (2011). Anti-mitogenic effects of β-agonists and PGE2 on airway smooth muscle are PKA dependent. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *25*(1), 389–97.

Yang, I. A., Fong, K., Eha, S., Black, P., & Lasserson, T. (2009). Inhaled corticosteroids for stable chronic obstructive pulmonary disease. *The Cochrane Library*, (2), 1–151.

Yang, J. H., Xia, M. H., Goetzl, E. J. & An, S. Z. (1994). Cloning and expression of EP3-subtype of human receptors for prostaglandin E2. *Biochemical and Biophysical Research Communications*, 198, 999-1006.

Yao, C., Sakata, D., Esaki, Y., Li, Y., Matsuoka, T., Kuroiwa, K., … Narumiya, S. (2009). Prostaglandin E2-EP4 signaling promotes immune inflammation through Th1 cell differentiation and Th17 cell expansion. *Nature Medicine*, *15*(6), 633–40.

Yao, H., Chung, S., Hwang, J., Rajendrasozhan, S., Sundar, I. K., Dean, D. A., … Rahman, I. (2012). SIRT1 protects against emphysema via FOXO3-mediated reduction of premature senescence in mice. *The Journal of Clinical Investigation*, *122*(6), 2032–2045.

Yoshimura, K., Tod, M. L., Pier, K. G., & Rubin, L. J. (1989). Role of venoconstriction in thromboxane-induced pulmonary hypertension and edema in lambs. *Journal of Applied Physiology*, *66*(2), 929–935.

Yoshimura-Uchiyama, C., Iikura, M., Yamaguchi, M., Nagase, H., Ishii, a, Matsushima, K., … Hirai, K. (2004). Differential modulation of human basophil functions through prostaglandin D2 receptors DP and chemoattractant receptor-homologous molecule expressed on Th2 cells/DP2. *Clinical and Experimental Allergy : Journal of the British Society for Allergy and Clinical Immunology*, *34*(8), 1283–90.

Yu, H., Huang, X., Ma, Y., Gao, M., Wang, O., Gao, T., … Liu, X. (2013). Interleukin-8 regulates endothelial permeability by down-regulation of tight junction but not dependent on integrins induced focal adhesions. *International Journal of Biological Sciences*, *9*(9), 966–79.

Yu, Y., Lucitt, M. B., Stubbe, J., Cheng, Y., Friis, U. G., Hansen, P. B., … FitzGerald, G. a. (2009). Prostaglandin F2alpha elevates blood pressure and promotes atherosclerosis. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(19), 7985–90.

Yuan, S. Y. (2000). Signal transduction pathways in enhanced microvascular permeability. *Microcirculation*, *7*(6), 395–403.

Yuan, S. Y. (2002). Protein kinase signaling in the modulation of microvascular permeability. *Vascular Pharmacology*, *39*(4-5), 213–223.

Yuan, Y., Huang, Q., & Wu, H. M. (1997). Myosin light chain phosphorylation: modulation of basal and agonist-stimulated venular permeability. *The American Journal of Physiology*, *272*(3 Pt 2), H1437–43.

Yuhki, K., Ueno, A., Naraba, H., Kojima, F., Ushikubi, F., Narumiya, S., & Oh-ishi, S. (2004). Prostaglandin Receptors EP2 , EP3 , and IP Mediate Exudate Formation in Carrageenin-Induced Mouse Pleurisy. *The Journal of Pharmacology and Experimental Therapeutics*, *311*(3), 1218–1224.

Zaccolo, M. (2009). cAMP signal transduction in the heart: understanding spatial control for the development of novel therapeutic strategies. *British Journal of Pharmacology*, *158*(1), 50–60.

Zanini, A., Chetta, A., Imperatori, A. S., Spanevello, A., & Olivieri, D. (2010). The role of the bronchial microvasculature in the airway remodelling in asthma and COPD. *Respiratory Research*, *11*, 132.

Zhang, L., Brass, L. F., & Manning, D. R. (2009). The Gq and G12 Families of Heterotrimeric G Proteins Report Functional Selectivity. *Molecular Pharmacology*, *75*(1), 235–241.

Zhang, Y., Guan, Y., Schneider, A., Brandon, S., Breyer, R. M., & Breyer, M. D. (2000). Characterization of Murine Vasopressor and Vasodepressor Prostaglandin E2 Receptors. *Hypertension*, *35*, 1129–1134.

Zheng, L., Duan, S. F., Zhang, Z. X. (1991). Changes of thromboxane A2 (TXA2) and prostacyclin (PGI2) in COPD patients with pulmonary hypertension. *Zhonghua Nei Ke Za Zhi*, 30, 91-93.

Zhou, W., Blackwell, T. S., Goleniewska, K., O'Neal, J. F., Fitzgerald, G. a, Lucitt, M., … Peebles, R. S. (2007). Prostaglandin I2 analogs inhibit Th1 and Th2 effector cytokine production by CD4 T cells. *Journal of Leukocyte Biology*, *81*(3), 809–17.

Zhuang, J., Xu, J., Zhang, C., & Xu, F. (2011). IL-1β acutely increases pulmonary SP and permeability without associated changes in airway resistance and ventilation in anesthetized rats. *Respiratory Physiology & Neurobiology*, *175*(1), 12–9.

Appendix

Chemicals and Reagents

The following table lists all the chemical reagents and compounds that were used in this thesis. The vehicles have also been described where necessary.

Drug / Reagent	Source	Vehicle
5-Hydroxytryptamine	Sigma-Aldrich	1% ethanol in saline
Albumin from Chicken Egg White (OVA)	Sigma-Aldrich	Saline (challenging); Alum:saline (sensitising)
Agarose	Sigma-Aldrich	2% in 1xTBE
Boric Acid	Sigma-Aldrich	-
$CaCl_2$	VWR	-
Chloroform	Sigma-Aldrich	-
Extracta DNA PCR kit	Quanta Biosciences	-
EDTA	Promega	-
EGM™-2 MV Bulletkit	Lonza	-
ER-819762	Eisai	0.5% methylcellulose, 0.2% tween 80 in water (p.o.) or saline (i.p.)
Ethanol	VWR	-
Evans Blue	Sigma-Aldrich	Saline
FITC-Albumin	Sigma-Aldrich	Cell Media
Formamide	Sigma-Aldrich	-
Glucose	VWR	-
Hyperladder II and IV	Bioline Ltd	-
Iloprost	Cayman Europe	0.1% DMSO in Media (*in vitro*); 1% Ethanol in Saline (*in vivo*)
Isopropanol	Sigma-Aldrich	-
KCl	VWR	-
KH_2PO_4	VWR	-

Methylcellulose	Sigma-Aldrich	-
$MgSO_4$	VWR	-
NaCl	VWR	-
NaH_2PO_4	VWR	-
$NaH_2PO_4.2H_2O$	VWR	-
$NaHCO_3$	VWR	-
Nuclease-free water	Promega	-
PAF	Sigma-Aldrich	0.1% DMSO in Media
PGD_2	Cayman Europe	0.1% DMSO in Media (*in vitro*); 1% Ethanol in Saline (*in vivo*)
PGE_2	Cayman Europe	0.1% DMSO in Media (*in vitro*); 1% Ethanol in Saline (*in vivo*)
$PGF_{2\alpha}$	Cayman Europe	0.1% DMSO in Media (*in vitro*); 1% Ethanol in Saline (*in vivo*)
PF-04418948	Pfizer	0.5% methylcellulose, 0.2% tween 80 in water (p.o.) or saline (i.p.)
Safeview	NBS Biologicals	-
Saline (0.9% sterile)	Fresenius Kabi	-
Sodium pentobarbitone	Merial Animal Health	-
TRI Reagent	Sigma-Aldrich	-
Trizma Base	Sigma-Aldrich	-
Tween 20	Sigma-Aldrich	-
Tween 80	Sigma-Aldrich	-
U46619	Cayman Europe	0.1% DMSO in Media (*in vitro*); 1% Ethanol in Saline (*in vivo*)
Urethane	Sigma-Aldrich	Saline