THE UNIVERSITY OF HULL

Effects of Hypoxia on the Human Lung

Being a Thesis Submitted for the Degree of Doctor of Philosophy in the Department of Cardiovascular and Respiratory Studies, Post Graduate Medical Institute, University of Hull

by

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Abstract

A large number of animal studies have investigated the effects of hypoxia on the pulmonary vasculature and hypoxic pulmonary vasoconstriction (HPV) is now established as an important homeostatic mechanism for perfusion-ventilation matching in the lung. However, there is a conspicuous lack of studies investigating HPV in human pulmonary vascular preparations. In comparison to the pulmonary vasculature only a limited number of studies have previously investigated the effects of acute hypoxia on the airways. In this thesis the effects of hypoxia on the human pulmonary vasculature and airways was investigated in a number of *ex vivo* human lung models.

In isolated human pulmonary arteries and veins and in *ex vivo* perfused and ventilated human lungs it was found that hypoxia caused a vasodilation. This finding is at variance with a large body of published literature and it is suggested that a neuronal mechanism could play an important role in the regulation of pulmonary vascular tone under hypoxic conditions *in vivo* which is not present in the *ex vivo* models used in this thesis.

In exploring the effects of hypoxia on the pulmonary vasculature it was identified that the pulmonary veins could play a more significant role in the regulation of pulmonary vascular resistance than was previously believed. It was also found that exposure of the pulmonary veins to hypoxia precipitated a phasic activity which was suggestive of an automaticity and that the isolated human pulmonary vein model could therefore be used to investigate arrhythmic activity and the efficacy of new antiarrhythmic agents.

Exposure of *ex vivo* perfused and ventilated human lungs to hypoxia caused a reduction in the rate of oedema formation and it is hypothesised that this effect could be mediated by an inhibition of pulmonary vascular endothelial cell contraction and an increase in endothelial barrier function.

In isolated human bronchi and *ex vivo* human lungs hypoxia caused a robust and reversible bronchodilation. The mechanism of hypoxic bronchodilation (HBD) was investigated and it was found that HBD could be mediated by a reversal of the calcium sensitisation mechanism. This mechanism represents a significant therapeutic target for the future development of effective bronchodilator therapies which are not subject to the limitations of GPCR agonists (desensitisation).

Studies investigating the effects of changing oxygen concentrations on bronchial tone identified that oxygen concentrations above ambient levels (21%) caused a robust contraction of human airways which could have significant implications for the clinical use of oxygen therapy in constrictive airway disease.

In investigating the putative role of hydrogen sulphide (H_2S) as an oxygen sensor it was found that H_2S is an effective vasodilator and bronchodilator in the human lung and could have significant therapeutic potential in the treatment of human pulmonary disease.

Further studies have been planned to build on the significant findings in this thesis.

For my parents

Robert Bennett BSc OBE

and

Elizabeth Lucas Bennett

Sorry you didn't get the chance to read this

"An amalgamation of experience and total bewilderment" Ken Bennett (c.2011)

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Effects of Hypoxia on the Human Lung

Contents

Abbreviations

AA	Arachidonic acid
$[Ca^{2+}]_i$	Intracellular calcium concentration
4-AP	4-Aminopyridine
ACh	Acetylcholine chloride
ALI	Acute lung injury
AMPK	Adenosine monophosphate activated kinase
AP	Airway pressure
ARDS	Acute respiratory distress syndrome
ASM	Airway smooth muscle
ATP	Adenosine triphosphate
BK_{ca}	Large- conductance Ca^{2+}-dependent K^+-channel
cADPR	Cyclic adenosine diphosphate ribose
cAMP	Cyclic adenosine monophosphate
CAT	Cysteine aminotransferase
cGMP	Cyclic guanosine monophosphate
CBS	Glutathione β-synthase
CHPH	Chronic hypoxic pulmonary hypertension
CO	Carbon monoxide
COX	Cyclooxygenase
CPB	Cardio pulmonary bypass
CRC	Concentration response curve
CSE	cystathionine γ-lyase
DAG	1,2-diacyl-glycerol
DMSO	Dimethyl sulfoxide
DRC	Dose response curve
EC	Endothelial cell
EC_{50}	Concentration of agonist required to elicit 50% of maximum response
ECMO	Extra corporeal membrane oxygenation
EDCF	Endothelium derived constricting factor
EDHF	Endothelium derived hyperpolarising factor
EDRF	Endothelium derived relaxing factor
E_{max}	Maximum effect of a drug
EpDRF	Epithelium derived relaxing factor
ER	Endoplasmic reticulum
ET-1	Endothelin-1
ETC	Electron transport chain
EVLP	Ex vivo lung perfusion
FCCP	Carbonyl cyanide 4-(triflouromethoxy)phenylhydrazone
FEV1	Forced expiratory volume in 1 second
F_iO_2	Fraction of inspired oxygen
gf	Gram force
GJ	Gap junction
GPCRs	G-protein coupled receptors

GTP	Guanosine triphosphate
H_2S	Hydrogen sulphide
HA	Hydroxylamine
HAPE	High altitude pulmonary oedema
HBD	Hypoxic bronchodilation
HIF	Hypoxia inducible factor
HO	Haem oxygenase
HPV	Hypoxic pulmonary vasoconstriction
HSV	Hypoxic systemic vasodilation
IC_{50}	Concentration of antagonist required to elicit 50% inhibition of maximum response
IP_3	Inositol-1, 4, 5-trisphosphate
IP_3R	IP_3 receptor
IPAH	Idiopathic pulmonary arterial hypertension
IPL	Isolated perfused lung
IU	International unit
K_{ATP}	ATP-dependent K^+ channel
Kca	Ca^{2+}-dependent K^+-channel
KCl	Potassium chloride
K_{fc}	Capillary filtration coefficient
KPa	Kilo pascal
K_v	Voltage-dependent K^+ channel
L-NAME	N-Nitro-L-arginine methyl ester hydrochloride
3MST	3-mercaptopyruvate sulfurtransferase
MCh	Methacholine (Acetyl-β-methylcholine chloride)
MLC	Myosin light chain
MLCK	MLC kinase
MLCP	MLC phosphatase
mmHg	Milimeters of mercury
mV	Mili volts
MYPT1	Myosin phosphatase target subunit 1
NA	Noradrenaline
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-kB	Nuclear Factor -kB
NO	Nitric oxide
NOS	Nitric oxide synthase
$O^{2\cdot-}$	Superoxide radical
PA	Pulmonary artery
PAH	Pulmonary arterial hypertension
PASMC	Pulmonary artery smooth muscle cell
PCO_2	Partial pressure of carbon dioxide
pEC_{50}	Negative logarithm of the EC_{50} molar concentration (formerly pD_2)
PD_2	Negative logarithm of the EC_{50} molar concentration
PDTC	Pyrrolidinedithiocarbamate ammonium
pGC	Particulate guanylate cyclase
PIP_2	Phosphatidyl-1, 4-bisphosphate
PKA	Protein Kinase A

PKC	Protein Kinase C
PKG	Protein Kinase G
PLC	phospholipase C
PP	Perfusion pressure
PPG	Propargyl glycine
PO_2	Partial pressure of oxygen
PVR	Pulmonary vascular resistance
RMM	Relative molecular mass
Rho	Monomeric G protein
ROCC	Receptor operated calcium channel
ROCK	Rho-activated protein kinase
ROS	Reactive oxygen species
Rp-8-Br-cGMPs	Rp-8-Bromoguanosine-3',5'-cyclic monophosphorothioate
RyR	Ryanodine receptor
SERCA	Sarco/endoplasmic reticulum calcium ATPase
SD	Standard deviation
sGC	Soluble guanylate cyclase
Sk_{ca}	Small conductance Ca^{2+} activated K^{+}-channel
SNP	Sodium nitroprusside
Sn-PP	Tin protoporphyrin IX dichloride
SOCE	Store operated calcium entry
SOD	Super oxide dismutase
SR	Sarcoplasmic reticulum
TAS2R	Bitter taste receptor
TPPO	Triphenylphosphine oxide
TTX	Tetrodotoxin
Tv	Tidal volume
VOCC	Voltage operated calcium channel
VSMC	Vascular smooth muscle cell

CHAPTER 1

Introduction

Contents

1.1 Oxygen Sensing and Metabolic Homeostasis

The ability of multicellular organisms to sense and respond to acute changes in oxygen concentrations is an essential homeostatic mechanism for matching oxygen supply with demand.

In the systemic circulation hypoxia causes a relaxation of blood vessels (hypoxic systemic vasodilation (HSV)) which increases the perfusion of oxygen deprived tissue and matches oxygen supply with demand.

In the pulmonary circulation the opposite occurs where hypoxia causes vasoconstriction of pulmonary arteries (hypoxic pulmonary vasoconstriction (HPV)) which decreases blood flow to under ventilated regions of the lung and increases blood flow to better ventilated regions. A large body of research, mostly in animal models, has investigated the effects of hypoxia on the pulmonary vasculature and HPV is now recognised as an important homeostatic mechanism for optimising perfusion-ventilation matching in the lung. However, HPV is also the primary mechanism mediating the pathophysiological effects of hypoxia in the lung. Chronic global hypoxia can occur in the lung as a result of pulmonary disease such as chronic obstructive pulmonary disease, sleep apnoea, emphysema and fibrosing alveolitis. Prolonged HPV causes vascular remodelling, a sustained increase in pulmonary artery pressure and eventually irreversible chronic hypoxic pulmonary hypertension (CHPH). Prolonged exposure to high altitude (with low oxygen concentrations) can cause pulmonary arterial hypertension which increases the hydrostatic pressure in the pulmonary circulation leading to the development of high altitude pulmonary oedema (HAPE). There are also some surgical procedures which result in periods of lung hypoxia and

post-operative pulmonary dysfunction is associated with a significant effect on clinical outcome. During cardiac surgery cardiopulmonary bypass (CPB) is used to divert blood away from the heart and lungs and the lungs are often hypoxic for long periods of time (1-3 hours). Cardiopulmonary bypass is often followed by pulmonary dysfunction- so called “pump lung” [1-4] which is manifest as an increase in PAP and pulmonary oedema which is the most frequent and significant contributor to morbidity and mortality after cardiac surgery. Thoracic surgery of the lung is associated with a high incidence of post-operative atrial fibrillation (AF) [5, 6] and it is suggested that periods of pulmonary hypoxia could be responsible for precipitating AF during thoracic surgery.

Understanding the mechanisms mediating the effects of hypoxia on the pulmonary vasculature is essential for the future development of effective therapeutics in the treatment of hypoxic pulmonary hypertension. There is general agreement that the vasopressor effect of hypoxia in the lung is mediated by a direct effect on the pulmonary artery smooth muscle cells (PASMCs) of the small resistance arteries and is largely independent of external neural and hormonal influences. However, despite intense research effort the exact sensor, transduction and effector mechanisms mediating HPV have not been clearly defined (comprehensively reviewed by Sylvester *et al.* [7]).

In contrast to the pulmonary vasculature relatively few studies have investigated the effects of hypoxia on airway smooth muscle (ASM) tone and the results from these studies were inconsistent; showing either no effect, constriction or dilation of airways on exposure to hypoxia and it is generally believed that acute, local changes in ASM tone in response to changes in oxygen tension serve no physiological function.

There are a number of significant inconsistencies in animal studies investigating the effects of changing oxygen concentrations on pulmonary vascular and airway tone which suggests that significant species differences may exist. There is also a conspicuous lack of data from studies in human tissue preparations which is a significant gap in the current scientific literature. The aim of studies in this thesis was to expand the current scientific understanding by investigating the effects of hypoxia on pulmonary vascular and airway tone in human lung preparations.

1.2 Mechanisms of Smooth Muscle Contraction and Relaxation

Airway tone and vascular tone is regulated by the smooth muscle cell layers and ultimately is determined by the contraction-relaxation status of the constituent smooth muscle cells (SMCs). An understanding of the cellular mechanisms mediating contraction and relaxation of SMCs is central to any study investigating effects on vascular or airway tone.

1.2.1 Contractile Mechanisms

Some of the established mechanisms mediating smooth muscle contraction are shown in **figure 1.1**.

Under normal resting conditions the intracellular calcium concentration $[Ca^{2+}]_i$ is maintained at a low level by a number of homeostatic mechanisms including extrusion of intracellular calcium by the membrane Na^+-Ca^{2+} exchanger which is driven by the Na^+ gradient maintained by the Na^+-K^+-ATPase and by sequestration of $[Ca^{2+}]_i$ into intracellular stores by sarco/endoplasmic reticulum ATPase (SERCA). An increase in $[Ca^{2+}]_i$ (calcium transient) causes contraction of the smooth muscle by the formation of the calcium-calmodulin complex which activates myosin light chain kinase (MLCK).

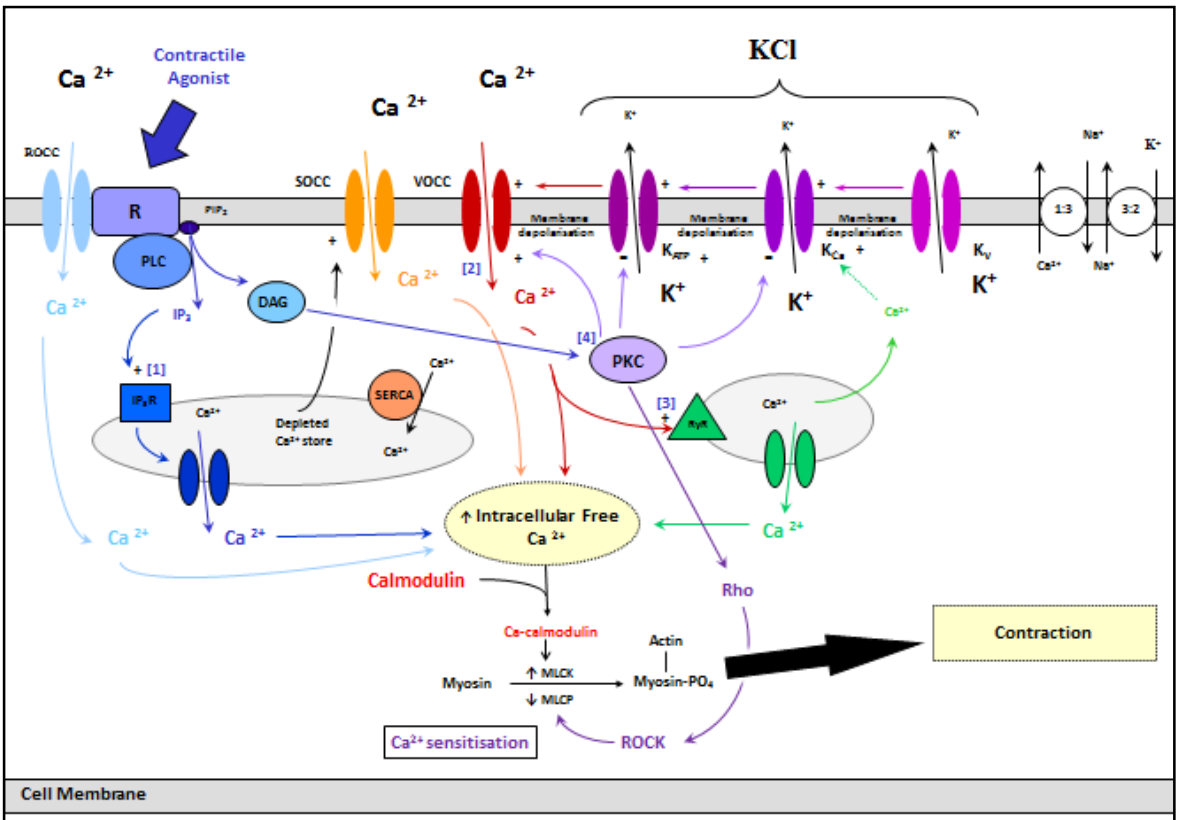


Figure 1.1 Simplified schematic showing established mechanisms of smooth muscle contraction. (Blue numbers in square brackets refer to putative site of action for antagonists used in ***Chapter 7*, 7.2.3** and ***Chapter 8*, 8.2.4**. DAG: diacylglycerol; IP_3: inositol-1, 4, 5-triphosphate; K_{ATP}: ATP- sensitive K^+ channels; K_{Ca}: calcium- activated K^+ channel; K_v: voltage operated calcium channel; MLCK: myosin light chain kinase; MLCP: myosin light chain phosphatase; PIP_2: phosphatidyl inositol-4, 5-biphosphate; PKC: protein kinase C; PLC: phospholipase C; R: g-protein coupled receptor; Rho: monomeric G protein; ROCK: Rho-activated kinase; RyR: ryanodine receptor; SERCA: sarco/endoplasmic reticulum calcium ATPase; SOCC: store operated calcium channel; VOCC: voltage operated calcium channel

MLCK causes phosphorylation of the 20 KDa myosin light chains (MLC_{20}) which activates myosin ATPase causing the formation of actin-myosin cross-bridges and cell contraction. The effects of MLCK are opposed by myosin light chain phosphatase (MLCP) which dephosphorylates myosin light chains.

Agonist induced contraction of smooth muscle is mediated by binding to specific membrane bound G-protein coupled receptors. Activation of the receptor causes

activation of the enzyme phospholipase C (PLC) which cleaves the membrane phospholipid phosphatidyl inositol-4, 5-biphosphate (PIP_2) into inositol-1, 4, 5-triphosphate (IP_3) and diacylglycerol (DAG).

IP_3 causes a rapid increase in $[Ca^{2+}]_i$ by activating IP_3 receptors (IP_3R) on the sarcoplasmic reticulum causing release of calcium from the intracellular stores. Increases in intracellular calcium stimulate further calcium release from the sarcoplasmic reticulum by activation of the ryanodine receptors (RyR). DAG causes an increase in $[Ca^{2+}]_i$ by opening receptor operated calcium channels (ROCC). Further increases in $[Ca^{2+}]_i$ occur due to opening of store operated calcium channels when the intracellular stores become depleted. DAG also activates protein kinase C which in turn inhibits large conductance calcium activated potassium channels (BK_{ca}) to prevent hyperpolarisation and increases calcium sensitivity. Increased calcium sensitivity facilitates sustained contraction despite decreasing $[Ca^{2+}]_i$ (due to extrusion and sequestration of intracellular calcium). There is currently debate about the exact mechanism mediating calcium sensitivity; however, the current opinion is that inhibition of MLCP is the prevailing mechanism. Inhibition of MLCP is mediated via activation of the monomeric G-protein Rho which activates Rho-activated kinase (ROCK) which in turn inhibits MLCP activity.

KCl induced contraction is induced by increasing the extracellular potassium concentration which prevents potassium extrusion through the potassium channels (due to a decreased concentration gradient) causing a membrane depolarisation, opening of VOCC and calcium influx.

1.2.2 Relaxant Mechanisms

Some of the established mechanisms mediating relaxation of smooth muscle cells are shown in **figure 1.2.**

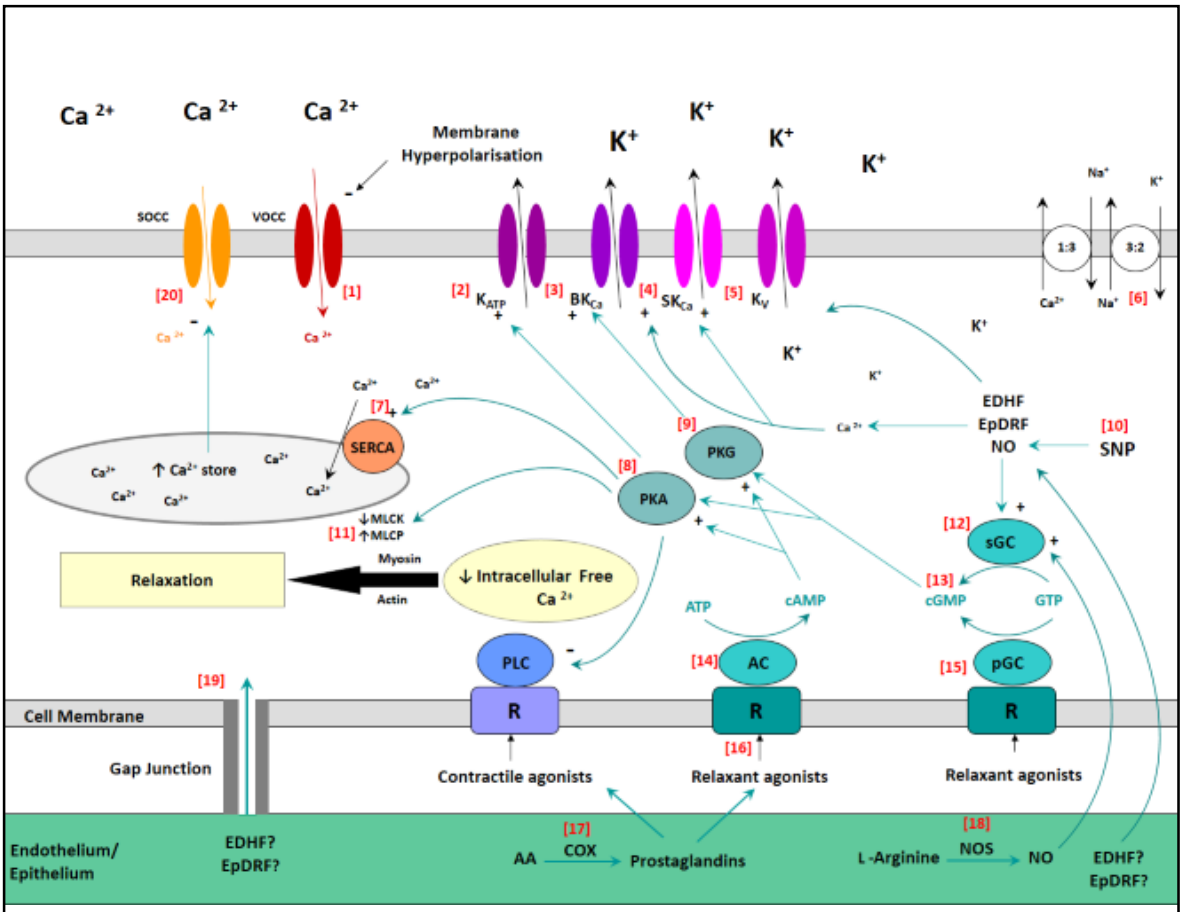


Figure 1.2 Simplified schematic showing established mechanisms of smooth muscle relaxation. (Red numbers in square brackets refer to putative site of action for antagonists used in ***Chapter 7*, 7.2.3** and ***Chapter 8*, 8.2.4**). AA: arachidonic acid; AC adenylate cyclase; ATP: adenosine triphosphate; BK_{Ca}: large conductance calcium-activated K^+ channel; cAMP: cyclic adenosine monophosphate; cGMP: cyclic guanosine monophosphate; COX: cyclooxygenase; EDHF: endothelium derived hyperpolarising factor; EpDRF: epithelium derived relaxing factor; GTP: guanosine triphosphate; K_{ATP}: ATP- sensitive K^+ channel; K_v: voltage operated calcium channel; MLCK: myosin light chain kinase; MLCP: myosin light chain phosphatase; NO: nitric oxide; NOS: nitric oxide synthase; pGC: particulate guanylate cyclase; PKA: protein kinase A; PG: prostaglandin; PKG: protein kinase G; PLC: phospholipase C; SERCA: sarco/endoplasmic reticulum calcium ATPase; sGC: soluble guanylate cyclase; SK_{Ca}: small conductance calcium-activated K^+ channel; SNP: sodium nitroprusside; SOCC: store operated calcium channel; VOCC: voltage operated calcium channel.

Relaxation of smooth muscle is mediated by activation of soluble guanylate cyclase (sGC) by NO or by agonist binding to G protein coupled receptors which activate adenylate cyclase (AC) or particulate guanylate cyclase (pGC). AC and GC cause the catalytic conversion of ATP or GTP respectively to cAMP and cGMP. cAMP and cGMP then bind to and activate cAMP dependent protein kinase A (PKA) or cGMP dependent protein kinase G (PKG). PKA and PKG phosphorylate intracellular proteins to mediate relaxation via a number of mechanisms including; opening of potassium channels (hyperpolarisation), stimulation of SERCA (calcium sequestration), inhibition of PLC, inhibition of MLCK, augmentation of Na^+-Ca^{2+} exchange by stimulation of Na^+-K^+-ATPase and decreasing calcium sensitivity by increasing MLCP activity.

1.2.3 Mechanisms Regulating Airway Smooth Muscle Tone

Previous studies investigating the mechanisms of contraction and relaxation of airway smooth muscle have relied heavily on extrapolations from mechanisms determined in vascular smooth muscle studies (VSM) (described above). However, it is now recognised that there are significant differences in the mechanisms regulating airway smooth muscle (ASM) tone and that extrapolations from vascular smooth muscle studies can lead to misleading results. Furthermore, it has also been recognised that there are significant species differences in the regulation of airway tone.

1.2.3.1 Species Differences and Calcium Oscillations

Airway hyperresponsiveness (AHR), where the ASM cells respond to stimuli with excessive contraction, is a key feature in the pathophysiology of asthma and consequently the mechanisms regulating ASM tone have been extensively investigated. From these studies it has been recognised that significant species

differences exist [8-11] in the regulation of ASM tone, which emphasises the need for studies in human tissue for the future development of effective therapeutics, and also that there are significant differences in the way that ASMCs handle Ca^{2+} compared to VSMCs (extensively investigated and reviewed by Luke Janssen and co-workers [12-18] and other groups [19-21]) . It has also been recognised from *in vitro* studies that there are significant differences in the responses of airway preparations depending on their anatomical position in the airway tract.

An interesting characteristic of ASMCs is that they exhibit rhythmic fluctuations in intracellular calcium-so called 'calcium oscillations'. (Although not unique to ASMCs-they have been observed in some other types of SMC). Calcium oscillations have been shown to occur in ASMCs from most species including humans and a decrease in the frequency (but not the amplitude) of calcium oscillations has been shown to correlate with the degree of relaxation to isoprenaline in murine airways [22] and an increase in the frequency of calcium oscillations has been shown to correlate with the magnitude of contraction in murine [23] and rodent airways [24]. Debate continues in the literature about the intracellular mechanisms mediating calcium oscillations, which is likely confounded by species differences, and a number of intracellular mechanisms have been suggested for example; IP_3 –receptor based mechanisms, Ryanodine-receptor based mechanisms, changes in SERCA activity and modulation of the Na^+/Ca^{2+} exchanger activity (reviewed by Hirota *et al.* [17], Sanderson *et al.*[20] , Wright *et al.* [25]).

1.2.3.2 The Calcium Paradox and the Superficial Buffer Barrier Model

The advent of fluorescence imaging led to an enormous number of studies investigating calcium signalling in smooth muscle cells. However, in numerous early

studies it was observed that exposure of ASMCs (and some other types of smooth muscle cells) to relaxants caused an increase in intracellular calcium which was clearly contrary to the accepted paradigm that contraction of smooth muscle cells is mediated by an increase in intracellular calcium and relaxation is mediated by a decrease in intracellular calcium. One explanation for this apparent paradox was suggested by Chen and van Breemen in the early 1990's [26, 27] who proposed the "superficial buffer barrier hypothesis". This hypothesis holds that there is a physical cytoplasmic 'superficial buffer barrier' between the inner plasmalemmal surface and the peripheral sarcoplasmic reticulum (SR). The superficial buffer barrier facilitates relaxation of smooth muscle cells by reducing the $[Ca^{2+}]_i$ in the centre of the cell where the contractile myofilaments are located. This is achieved by restricting the flow of extracellular calcium into the deep cytosol and by the sequestration of $[Ca^{2+}]_i$ from the centre of the cell into the SR and subsequent extrusion into the subplasmalemmal and extracellular space. Whole cell photometry using conventional optics in earlier studies would not detect regional changes in calcium concentrations and an increase in subplasmalemmal calcium concentrations on exposure of smooth muscle cells to relaxant agents would give the impression of a paradoxical increase in global cellular calcium concentrations. More refined Ca^{2+} imaging techniques were needed to investigate the intricacies of Ca^{2+} handling in smooth muscle cells and in 1995 Yamaguchi published a seminal paper [28] in which they were able to demonstrate temporal and spatial separation of calcium in the superficial buffer barrier. In bovine airway smooth muscle cells Yamaguchi *et al.* used confocal microscopy to observe regional changes in the calcium signal and found that exposure of the cells to

isoprenaline decreased $[Ca^{2+}]_i$ in the inner cytosol but increased $[Ca^{2+}]_i$ in the peripheral cytosol.

The superficial buffer barrier model is now generally accepted as a ubiquitous mechanism for calcium handling in airway smooth muscle cells (although it should be noted that this mechanism is not exclusive to ASM cells and has been observed in other smooth muscle cell types; for example, in the seminal studies by Chen and van Breemen [26, 27] in isolated rabbit vena cava smooth muscle cells). Luke Janssen's group from McMaster University have extensively investigated the physiological and pathophysiological mechanisms of calcium handling by ASM cells and the central role of the superficial buffer barrier in the regulation of ASM tone [14, 17]. Janssen and co-workers have investigated the mechanisms regulating transport of calcium into the superficial buffer barrier and suggest that the ryanodine receptors (RyRs), which are localised to the side of the SR closest to the cell membrane, play a key role in maintaining the transport of calcium into the buffer barrier [17, 29] and that cell membrane chloride channels are essential for maintaining the electrochemical gradient in the superficial buffer barrier [30] (see **figure 1.3**).

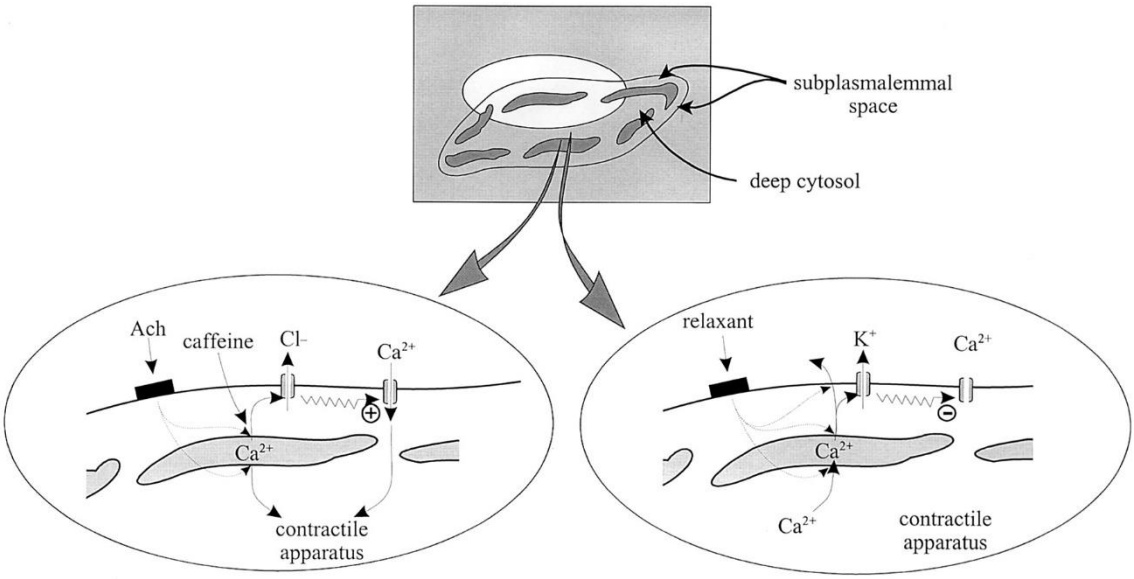


Figure 1.3 Superficial buffer barrier model. The sarcoplasmic reticulum (SR) divides the cytosol into 2 spatially and functionally distinct compartments: the subplasmalemmal space and the deep cytosolic space. *Bottom left*: excitatory agonists release Ca^{2+} into the deep cytosol to trigger contraction, as well as into the subplasmalemmal space to activate Ca^{2+}-dependent Cl^- channels. *Bottom right*: relaxants, on the other hand, promote Ca^{2+}uptake and Ca^{2+} extrusion but also trigger Ca^{2+}release via ryanodine receptors. The latter effect allows for unloading of the SR, increasing its buffering capacity, without triggering contraction. It may also lead to activation of Ca^{2+}-dependent K^+ channels and membrane hyperpolarization. Reproduced from Janssen [14].

1.2.4 Role of the Vascular Endothelium in the Modulation of Vascular Smooth Muscle Tone

For many years the vascular endothelium was considered to be an inert monolayer functioning only to facilitate non-turbulent blood flow in the vasculature by forming a 'non-stick' barrier between the blood and the sub-intimal layers of the vascular wall. However, in 1976 Caldwell, Seegal and Hsu published a seminal paper showing that angiotensin- converting enzyme is largely localised in the vascular endothelium [31] and in 1980 Furchgott and Zawadzki published a ground breaking study showing that acetylcholine induced relaxation of rabbit aorta was mediated via the release of an

endothelium derived relaxing factor (EDRF) [32]. In 1987 Palmer, Ferrige and Moncada identified EDRF as nitric oxide (NO) [33]. Since the 1980's considerable research effort has established the importance of the endothelium in the regulation of vascular tone. Modulation of vascular tone by the vascular endothelium is an intricate interplay between EDRFs (for example; NO, endothelium derived hyperpolarising factor (EDHF) and prostacyclin) and endothelium-derived contracting factors (EDCF) (for example; endothelin-1, 5-hydroxytryptamine, arachidonic acid and adenosine diphosphate) and it has now been established that endothelial dysfunction can play an important role in the pathophysiology of cardiovascular disease such as hypertension, coronary vascular disease and atherosclerosis (see themed edition on Vascular Endothelium in Health and Disease in the British Journal of Pharmacology, edited by Garland and Weston [34], and review by Vanhoutte *et al.* [35]).

1.2.5 Role of the Airway Epithelium in the Modulation of Airway Smooth Muscle Tone

The airway epithelium is a much more robust layer than the simple monolayer of the vascular endothelium and can play a significant role in the modulation of ASM tone via a number of mechanisms; (1) providing a diffusion barrier to prevent penetration of contractile and relaxant agents to the smooth muscle layers and protecting the intra-epithelial nerves from stimulation by bioactive agents (2) metabolising bioactive agents before they reach the underlying ASM layer and (3) releasing so called epithelium derived relaxing factors (EpDRFs) to cause relaxation of airway smooth muscle. There is debate in the literature about the relative importance of these different mechanisms in health and disease (Reviewed by Folkerts and Nijkamp [36],

Spina [37] , Knight and Holgate [38]). There is also a lack of consensus about the molecular identity of EpDRFs. Epithelial cells have been shown to metabolise arachidonic acid (AA) and a number of AA metabolites have been proposed as potential EpDRFs. Similarly nitric oxide synthase (NOS) has been demonstrated to produce nitric oxide (NO) in airway epithelium and may act as an EpDRF. However, debate continues in the literature about the relative importance of arachidonic acid metabolites and NO in contributing to the regulation of airway tone by the epithelium [36-40]. It is thought that a loss of the airway epithelial layer itself or a loss of functional integrity of the airway epithelium plays a significant role in the bronchial hyperactivity seen clinically in asthma and airway infection [38, 40, 41].

1.3 Effects of Hypoxia on the Pulmonary Vasculature

1.3.1 Hypoxic Pulmonary Vasoconstriction (HPV)

Hypoxia causes constriction of the pulmonary arteries and hypoxic pulmonary vasoconstriction (HPV) is now accepted as an important homeostatic mechanism for optimising perfusion-ventilation matching in the lung. Hypoxic pulmonary vasoconstriction optimises perfusion-ventilation matching by diverting blood away from areas of low oxygenation to areas of higher oxygenation.

Von Euler and Liljestrand [42] are generally accredited with being the first to recognise the physiological role of changing oxygen concentrations in the regulation of pulmonary vasculature tone. In anaesthetised cats they found that breathing pure oxygen decreased pulmonary arterial pressure and a lack of oxygen increased pulmonary arterial pressure. They recognised the significance of this observation and concluded that “regulation of the pulmonary blood flow is mainly mediated by a local

action of the blood and alveolar gases leading to an adequate distribution of the blood through the various parts of the lungs according to the efficiency of aeration." Within a year of von Euler and Liljestrand's publication, Motley *et al*. [43] reported that pulmonary hypertension was rapidly induced in healthy volunteers by breathing 10 percent oxygen for short periods of time and subsequent *in vivo* studies [44-57] have confirmed that hypoxic pulmonary vasoconstriction (HPV) occurs in man.

Since the 1940's the effect of hypoxia on the pulmonary vasculature has been extensively investigated in numerous species (reviewed by Sylvester *et al.* [7]) and there is general agreement that the vasopressor effect of hypoxia in the lung is mediated by a direct effect on the pulmonary artery smooth muscle cells (PASMCs) of the small resistance arteries and is largely independent of external neural and hormonal influences and it is generally believed that it is not modulated by the endothelium. However, despite intense research effort the exact sensor, transduction and effector mechanisms mediating HPV have not been clearly defined.

1.3.2 Mechanisms of HPV

Although the exact mechanisms mediating changes in PASMC tone in response to hypoxia have not been clearly defined it is generally agreed that the mitochondria play a central role in oxygen sensing; however, the exact mechanism by which mitochondria signal changes in oxygen concentration and mediate changes in PASMC tone is the subject of considerable debate (reviewed by Ward [58] and Evans *et al.* [59]). There are currently three prevailing theories for oxygen sensing by the mitochondria in the pulmonary vasculature; the redox hypothesis, the reactive oxygen species (ROS) hypothesis and the energy state or AMPK (AMP- activated protein kinase) hypothesis.

1.3.2.1 The Redox Hypothesis

The redox hypothesis suggests that hypoxia suppresses mitochondrial oxidative phosphorylation which causes a shift in the redox state of the cytosol towards a more reduced state and reduces ROS production. Under normoxic conditions it is proposed that the oxidative state of the cytosol and ROS maintain plasmalemma voltage- gated potassium channels (K_v) in the open state. Under hypoxic conditions a shift in the redox state of the cytosol towards a more reduced state and a reduction in ROS therefore causes closing of the K_v channels, PASMC membrane depolarisation, calcium influx via opening of voltage- gated calcium channels and subsequent vasoconstriction (**figure 1.4a**).

1.3.2.2 The Reactive Oxygen Species (ROS) Hypothesis

In contrast to the redox hypothesis the reactive oxygen species hypothesis holds that inhibition of mitochondrial oxidative phosphorylation by hypoxia causes an increased production of ROS (primarily superoxide and its more stable dismutase product hydrogen peroxide) which mediate contraction of PASMCs. Debate continues in the literature about the mechanisms by which ROS can mediate a change in PASM tone and a number of different pathways have been suggested; for example, release of intracellular calcium from ryanodine sensitive stores and opening of store operated calcium channels (SOCCs) (**figure 1.4b**) and studies by a group from Kings College, London [60-62] have provided compelling evidence to suggest that the Rho kinase mediated Ca^{2+} sensitisation mechanism plays a central role in the effector mechanism of ROS induced contraction of PASMCs.

1.3.2.3 The Energy State or AMPK (AMP- Activated Kinase) Hypothesis

The energy state hypothesis suggests that the decreased mitochondrial ATP production, under hypoxic conditions, causes an increase in the AMP to ATP ratio which activates AMP- activated kinase (AMPK). Activation of AMPK causes an increase in the intracellular concentration of cyclic adenosine diphosphate ribose (cADPR) which causes release of Ca^{2+} from the sarcoplasmic reticulum and contraction of PASMCs (**figure 1.4c**).

There is extensive experimental evidence both for and against the three theories for mitochondrial oxygen sensing and there is currently no consensus about which mechanism or combination of mechanisms likely prevails (see reviews by Sylvester *et al.* [7] , Evans *et al.* [59] and Ward [58]). Furthermore, other potential mechanisms for oxygen sensing in PASMCs are now emerging; for example, it has been shown that NADPH oxidases could play an important role in oxygen sensing and signalling [63] and there is currently growing interest in the potential role of the gasotransmitters nitric oxide (NO) [64] and carbon monoxide (CO) [65, 66] in oxygen sensing and recently it has also been suggested that the gasotransmitter hydrogen sulphide (H_2S) has a role in oxygen sensing and signalling [58, 67, 68].

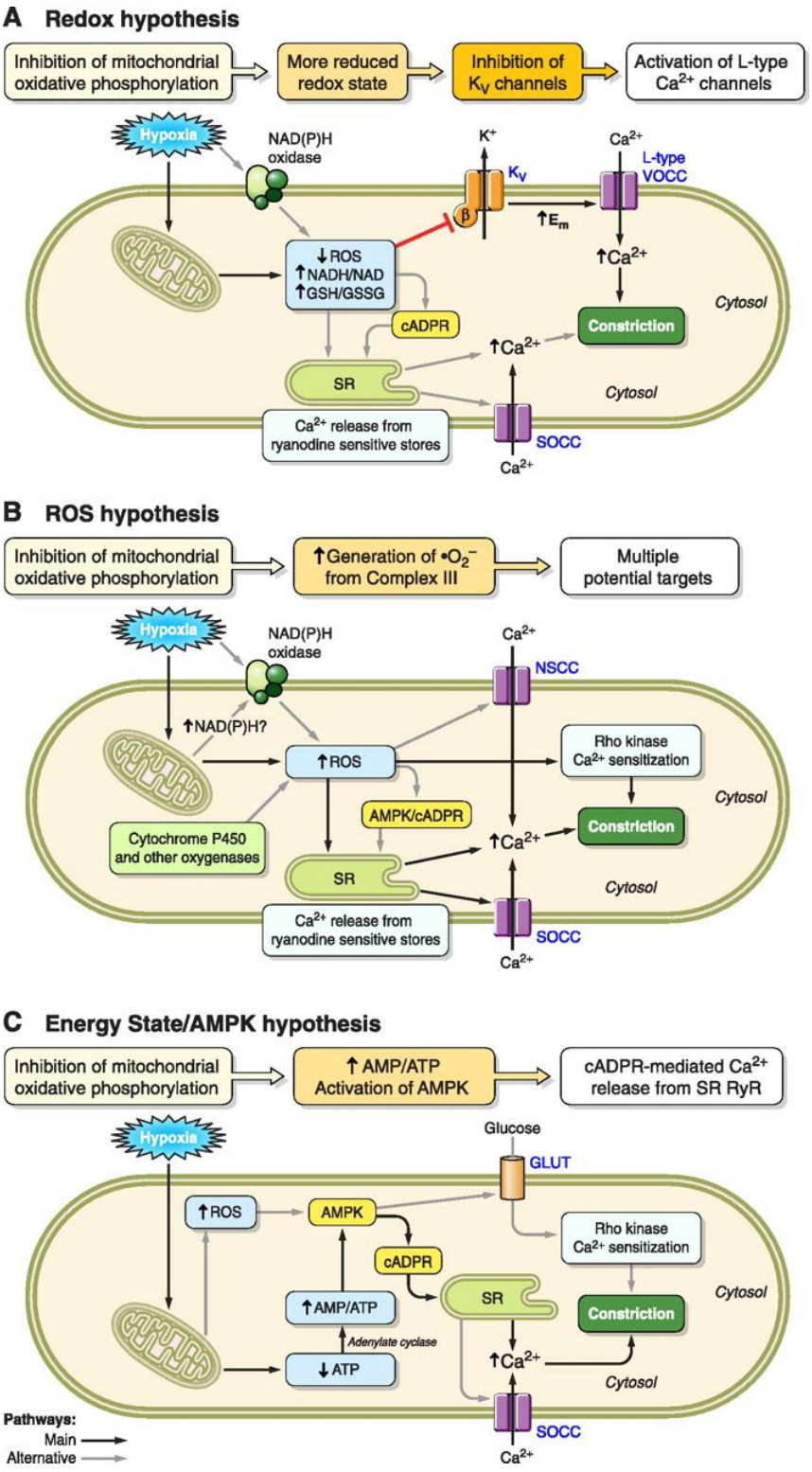


Figure 1.4 Diagrams of possible mechanisms that explain how hypoxia causes constriction in pulmonary arterial smooth muscle, as proposed by the Redox **(A)**, ROS **(B)**, and energy state/AMP kinase **(C)** hypotheses. Main and alternative pathways are indicated by black and gray arrows, respectively. K_V, VOCC, SOCC, and NSCC, voltage-dependent K^+, voltage-operated Ca^{2+}, store-operated Ca^{2+}, and nonselective cation channels, respectively; E_m, membrane potential; cADPR, cyclic ADP ribose; AMPK, AMP kinase; SR, sarcoplasmic reticulum. Reproduced from Sylvester *et al.* [7].

1.3.3 Pathophysiological Effects of Hypoxia on the Pulmonary Vasculature

As well as being an important homeostatic mechanism HPV is also the primary mechanism mediating the pathophysiological effects of hypoxia in the lung. Chronic global hypoxia can occur in the lung as a result of prolonged exposure to high altitude, constrictive airway disease such as chronic obstructive pulmonary disease, sleep apnoea, emphysema and fibrosing alveolitis. Prolonged HPV leads to vascular remodelling, a sustained increase in pulmonary artery pressure and eventually irreversible chronic hypoxic pulmonary hypertension (CHPH) [69].

Prolonged exposure to high altitude can cause pulmonary arterial hypertension and high altitude pulmonary oedema (HAPE).There is currently some debate in the literature regarding the mechanisms of HAPE; some groups argue that HAPE is mediated exclusively by an increase in hydrostatic pressure (due to HPV) [70-72] whereas others suggest that an increase in pulmonary vascular permeability, due to an inflammatory response, is also involved [73, 74].

There are also some clinical situations where the lungs are exposed to hypoxia and post-operative lung problems are significant indicators of morbidity and a protracted recovery. During cardiac surgery cardiopulmonary bypass (CPB) is used to divert blood away from the heart and lungs and the lungs are often hypoxic for long periods of time (1-3h). Cardiopulmonary bypass is often followed by pulmonary dysfunction- so called "pump lung" [1-4] which is manifest as an increase in PAP and pulmonary oedema which is the most frequent and significant contributor to morbidity and mortality after cardiac surgery.

Thoracic surgery of the lung is also associated with periods of cardiac and pulmonary hypoxia and a high incidence of post-operative atrial fibrillation (AF) [5, 6] (as is cardiac surgery [75]) and evidence suggests that hypoxia could be responsible for precipitating AF during cardiac and thoracic surgery.

1.4 Effects of Hypoxia on the Airways

In contrast to the pulmonary vasculature surprisingly few studies have investigated the effect of hypoxia on the airways and the reported results were inconsistent.

1.4.1 Effects of Hypoxia on Airway Tone

Although it is not generally believed that acute changes in oxygen concentrations have a physiological role in modulating airway tone a number of studies have investigated the effects of changing oxygen tension on bronchial tone. However, the results were inconsistent. An early study in normal human subjects found that acute hypoxia caused an increase in airway resistance [76] and the bronchoconstrictor effect of hypoxia in man was supported by subsequent studies which showed that; chronic hypoxic bronchoconstriction in COPD patients was relieved by breathing 30% oxygen [77], in asthmatic patients hypoxia was found to potentiate methacholine induced contraction [78] and changes in lung mechanics induced by isocapnic hypoxia in healthy volunteers were interpreted as bronchoconstriction of the large airways [79]. However, other studies showed a different effect of hypoxia. In healthy human volunteers the cross-sectional area (measured using an acoustic reflection technique) of the extrathoracic trachea, intrathoracic trachea and main bronchi all increased under hypoxic conditions [80] and in another study in healthy human subjects transient hypoxia caused a decrease in upper airway resistance [81]. In healthy volunteers and patients

with chronic pulmonary disease acute hypoxia caused a decrease in airway resistance but did not change dynamic compliance, which was interpreted as indicating a bronchodilator effect [82]. However, in other studies hypoxia did not affect small airway function in healthy volunteers [83] or FEV_1 in asthmatic patients [84].

In vivo animal studies were also inconsistent. In anaesthetised dogs [85-89] , rabbits [90] and cats [91] hypoxia was found to cause constriction of the airways as manifest by an increase in respiratory resistance. In contrast, high resolution computed tomography was used to directly visualise the airways of minipigs and demonstrated a significant dilation of the large and small airways in response to hypoxia [92]. Likewise, exposure of anaesthetised and ventilated guinea-pigs to hypoxic conditions reduced histamine induced increases in total pulmonary resistance [93] and hypoxic ventilation of anaesthetised rats caused a slight reduction in airway resistance [94].

Isolated tissue preparations have been used to directly measure the effect of hypoxia on ASM tone. However, the reported results were variable depending on species, experimental conditions and level in the respiratory tract of the tissue used. Hypoxia consistently causes a robust and reversible relaxation of pre-constricted porcine bronchial rings [95-97] and porcine tracheal smooth muscle strips [98, 99]. In histamine contracted guinea-pig tracheal rings hypoxia caused a significant relaxation [100] and pre exposure to hypoxia inhibited maximal cholinergic and histaminergic contractions of guinea-pig tracheal smooth muscle strips [101]. In bovine tracheal smooth muscle strips hypoxia reversibly reduced resting tension and attenuated contraction to electrical field stimulation [102] whereas in bovine bronchi hypoxia enhanced contraction to methacholine [103]. Exposure to hypoxia caused relaxation of canine isolated bronchi [104]

and canine lung parenchyma strips [105]. However, in canine tracheal muscle strips moderate hypoxia caused a relaxation [106, 107] whereas severe hypoxia caused an increase in resting tension [106]. In reviewing the literature, only one report was found describing the effect of hypoxia on isolated human airways; in isolated human bronchi hypoxia caused only a slight reduction in resting tone, did not significantly alter contraction to methacholine and attenuated relaxation to salbutamol [84].

1.4.2 Pathophysiological Effects of Hypoxia on the Airways

Hypoxia has been shown to cause or exacerbate pulmonary oedema in acute lung injury (ALI) [108], high altitude pulmonary oedema (HAPE) [72, 109] and decompensated heart failure.

In the critical care setting and during anaesthetic procedures high FiO_2 (fraction of inspired oxygen) concentrations are frequently used. It is recognised that high FiO_2 can cause 'hyperoxic acute lung injury' (HALI). HALI is characterised as a progressive inflammatory response which is ultimately fatal (reviewed by Kallet and Matthay [110]).

1.5 Human Lung Models

In this thesis a number of *ex vivo* human tissue models were used to investigate the effects of hypoxia on the human pulmonary vasculature and airways.

1.5.1 *Ex vivo* Isolated Perfused and Ventilated Human Lungs

Isolated perfused lung (IPL) preparations from a number of species (mouse, rat, rabbit, ferret, pig and dog) have been used extensively to investigate physiological and pharmacological effects on the lung as an intact system. In this thesis whole human lungs or lobes were obtained at resection for cancer and used in an e*x vivo* isolated

perfused and ventilated human lung model. The isolated human lung preparation is an established model which has previously been used to investigate the vasoconstrictor [111] and oedemagenic effects [112] of endothelin agonists and the vasodilator effects of testosterone [113] and oestrogen [114] on the human pulmonary circulation. The advantage of the human lung model is that it is possible to measure effects on the pulmonary circulation and airways as intact systems.

Other groups have also described and validated human IPL models using lung specimens resected for cancer [115, 116] and human IPL models have previously been used to investigate the pharmacokinetics of inhaled glucocorticoids [117], the pharmacokinetics and pharmacodynamics of β_2-agonists [118] and to identify potential biochemical markers for pulmonary oedema formation in *ex vivo* human lungs [119]. Furthermore, because human IPL models using lung samples resected for carcinoma contain a tumour they have proved particularly effective for investigating the pharmacokinetics of anticancer agents [120-123] and have previously been used to investigate the effects of laser ablation on human lung tumours [124, 125].

The IPL model used in this thesis was unique in that a method was included to continuously replace the CO_2 removed by the lungs and maintain the buffering capacity of the perfusate solution (based on a method previously described in isolated rat lungs [126]). During the course of studies in this thesis further improvements were made to the human IPL model; in ***Chapter 5*** the perfusion system was modified to facilitate determination of the capillary filtration coefficient $K_{f,c}$ and in ***Chapters 4*** and ***6*** adaptations were made to the system to induce hypoxia in the lung.

1.5.2 Isolated Human Pulmonary Vessels and Airways

Isolated tissue preparations are a simple organ bath technique which can be used to investigate physiological and pharmacological mechanisms at tissue level. Rings of tissue are mounted between stainless steel wires and direct measurements of smooth muscle contractile function are made by measuring changes in isometric tension.

1.5.3 Parallel Organ Baths and Perfusion Circuit

A novel parallel organ bath system, with continuously recirculating buffer, was developed to facilitate accurate control of gas partial pressures in organ bath buffer solutions and was used in ***Chapter 6*** to investigate the effect of hypercapnia on bronchial tone.

1.6 Hypothesis and Aims

A considerable number of animal studies and *in vivo* human studies have established that hypoxic pulmonary vasoconstriction (HPV) is an important homeostatic mechanism for optimising perfusion-ventilation matching in the lung.

In contrast to the vasculature only a limited number of previous studies have investigated the effects of hypoxia on airway tone and the results were inconsistent. It is generally believed that acute changes in airway tone in response to changing oxygen concentrations have no physiological function.

There are a number of inconsistencies in the scientific literature investigating the effects of hypoxia on pulmonary vascular and airway tone which suggests that significant species differences may exist. There is also a conspicuous lack of data from studies in human tissue preparations which is a significant gap in the current scientific

literature. The aim of studies in this thesis was to expand the current scientific understanding by investigating the effects of hypoxia on *in vitro* human pulmonary vascular and airway preparations.

Having considered the balance of evidence in the scientific literature it was hypothesised that exposure of the human pulmonary vasculature to hypoxia would cause hypoxic pulmonary vasoconstriction and exposure of human airways to hypoxia would cause bronchodilation.

CHAPTER 2

Materials and Methods

Contents

In Vitro experiments were performed in the Centre for Cardiology and Cardiothoracic Surgery, Castle Hill Hospital, Cottingham, UK

2.1 Approvals

Local research ethics committee and institutional (Hull & East Yorkshire Hospitals NHS Trust) research and development department approval was obtained for the use of lung specimens and surplus lung tissue from patients undergoing elective lobe or lung resection for cancer.

Patients gave written consent for the use of surplus tissue for research purposes.

In accordance with the recommendations of the human tissue act (2004) [127] and the conditions of the local ethics committee approval the donor patient was anonymous to the researcher.

2.2 *Ex Vivo* Isolated Perfused and Ventilated Human lungs

2.2.1 Lung Samples

2.2.1.1 Sample Procurement

Whole lungs (left or right) or lobes or were obtained from patients undergoing elective thoracotomy for cancer. Following surgical resection lung specimens were taken immediately to the laboratory.

2.2.1.2 Exclusion Criteria

Lung specimens were not collected from patients with; Mesothelioma, Pulmonary Arterial Hypertension (PAH), an active lung infection (TB) or an infectious blood born virus (HIV, Hepatitis etc.).

Lung samples were not used if there was extensive tumour invasion of the airways and/or vasculature or excessive surgical manipulation had caused significant damage to the lung tissue during the operative procedure.

2.2.2 Patient Data

Patient demographics (age, sex, height, and weight), relevant medical history, current medication and details of the anaesthetic and analgesic regimen the patients were exposed to whilst undergoing lung resection were recorded by the consultant anaesthetist.

2.2.3 Sample Preparation

All surgical clips and sutures were removed. To provide the sample with oxygen and remove blood clots the pulmonary circulation was flushed with approximately 300 ml of oxygenated Krebs-Henseleit solution containing 1000 IU heparin. The pulmonary arteries and bronchi were dissected down and freed from surrounding tissue to expose an adequate length of tissue for cannulation. Bifurcating arteries and bronchi were divided to allow separate cannulation of segmental arteries and bronchi. The number of arteries and bronchi requiring cannulation depended on the point of surgical resection and the anatomy of the specimen.

2.2.4 Cannulation

The pulmonary arteries and bronchi were cannulated using a range of different sized (3.3-8 mm external diameter) plastic cannulae; DeBakey coronary perfusion cannulae (L Series 1870 coronary perfusion Cannulae, Terumo Europe Cardiovascular Systems) or arterial perfusion cannulae (Type 1860, Terumo Europe Cardiovascular Systems).

The largest cannulae possible were inserted into each artery and bronchus. Cable ties were wrapped round the artery or bronchus and tightened to secure and seal the cannulation site. If complete cannulation could not be achieved the specimen was not used.The arterial cannulae were connected together using 'Y' connectors to form a single inlet. Air was displaced from the pulmonary circulation and arterial cannulae by using a bladder syringe to retrograde flush Krebs-Henseleit solution through the pulmonary veins. When all the air had been displaced the cannulae were clamped. Flushing the system in this way also identified any leaks and obstructions to flow which could be corrected by repositioning the cannulae.

The bronchial cannulae were connected together using 'Y' connectors to form a single inlet. A bladder syringe was connected to the inlet and the lung inflated with air to confirm that the cannulation sites were patent and airtight.

The lung was then connected to the Perfusion-Ventilation system.

2.2.5 Perfusion-Ventilation System

The Perfusion-ventilation system is illustrated in **figure 2.1**.

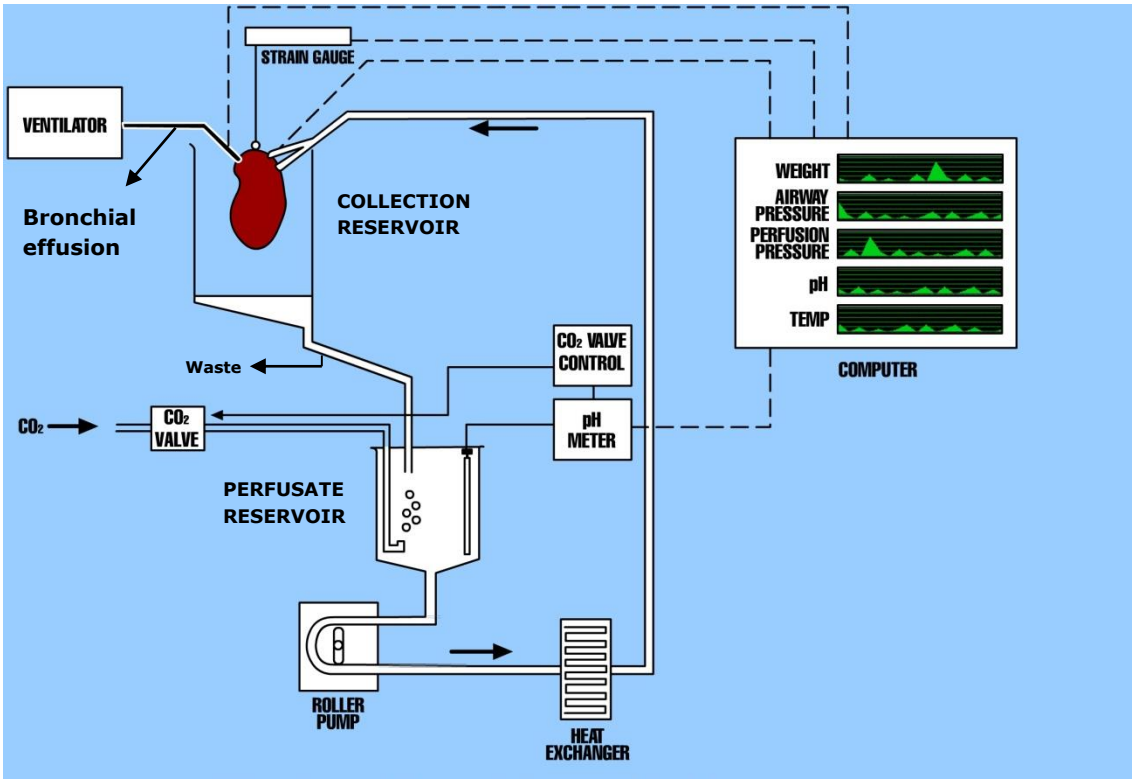


Figure 2.1 Perfusion-ventilation system

2.2.5.1 Ventilation Circuit

The ventilation circuit consisted of ½", ⅜" and ¼" connective PVC tubing (Chalice Medical, UK) connected to a piston ventilator (Harvard Apparatus Ltd, Edenbridge, Kent, UK). Anti-bacterial heat and moisture exchanging filters (Clear-Therm® 3, Intersurgical, Complete Respiratory Systems, UK) were connected to the inlet and outlet of the ventilator. A 'Y' connector was positioned proximal to the bronchial cannulae inlet and connected to a 50 ml syringe to allow collection and quantification of bronchial effusion. Airway pressure was measured via a pressure transducer (Datex-Ohmeda Ltd., Hatfield, Hertfordshire, UK) connected to a pressure line situated proximal to the bronchial cannulae. The total volume of the circuit was 250 ml.

2.2.5.2 Perfusion Circuit

The perfusion circuit consisted of ⅜" and ¼" connective PVC tubing (Chalice Medical, UK), a peristaltic roller pump (Watson-Marlow, Falmouth, Cornwall, UK), a heat exchanger (Sorin Biomedica, Quedgeley, Gloucester, UK), an in-line temperature probe (Terumo UK, Knowsley, Merseyside, UK), a pressure line and transducer (Datex-Ohmeda Ltd., Hatfield, Hertfordshire, UK) and two poly-carbonate reservoirs; the collection reservoir (Sorin Biomedica, Quedgeley, Gloucester, UK) and the Perfusate reservoir (Baxter Healthcare, Compton, Berkshire, UK).

The perfusion circuit was primed with 1 l Krebs-Henseleit solution which was recirculated from the perfusate reservoir to the collection reservoir, using the roller pump, ensuring all air was displaced from the circuit. The pressure monitoring line, which was connected to the circuit proximal to the arterial cannulae inlet, was primed. The perfusate was warmed and maintained at 36-38^{o}C by the heat exchanger which was connected to a heater-cooler unit (HCU- 20-602. Jostra, Sweden).

2.2.5.3 Carbon Dioxide Replacement

Carbon dioxide (CO_2) removed from the perfusate by the lungs was automatically replaced; A pH probe (Thermo Russell, Combination pH electrode) was suspended in the Krebs-Henseleit solution in the perfusate reservoir and connected to a pH meter (Philips Medical Systems, Leeds, West Yorkshire, UK) for continuous measurement of perfusate pH. The pH meter was connected to a pH control box (Medical Physics Department, Hull Royal Hospitals, Hull, UK) which opened a solenoid valve (CO_2 Valve, Medical Physics Department, Hull Royal Hospitals, Hull, UK) to allow the flow of CO_2 into the perfusate when the pH was greater than 7.40. When the CO_2 valve was closed

to the perfusate the exhaust CO_2 was directed through a reservoir containing soda lime (Intersurgical) to scavenge CO_2.

Replacing CO_2 kept the partial pressure of CO_2 (pCO_2) in the perfusate within normal limits and maintained the buffering capacity of the Krebs-Henseleit solution.

2.2.6 Measured Parameters

Perfusate temperature and pH were continuously measured, and maintained within set limits, but not recorded.

A force transducer (Strain gauge) (Thames Side Maywood Instruments Ltd, Tilehurst, Berkshire, UK) was used to measure the weight of the lung. The force transducer and pressure transducers in the perfusion and ventilation circuits were connected to a quad bridge amplifier (Power lab/400 system, ADInstruments) for continuous recording of sample weight, airway pressure and perfusion pressure on a personal computer using data acquisition software (Chart, version 3.4, ADInstruments).

2.2.7 Calibration

All transducers and probes were calibrated before each experiment.

The pH probe was calibrated in pH 7 and pH 10 standard reference buffer solutions (Sigma-Aldrich).

The transducers were calibrated using the software calibration procedure. The force transducer was calibrated against a 500 g weight. The pressure transducers were calibrated against a known pressure applied to the monitoring line using a transducer calibration system (XCaliber, Spectramed, Oxnard, California).

2.2.8 Connection to the Perfusion-Ventilation Circuit and Initiation of an Experiment

A suture was tied round the bronchial cannulae and the lung sample suspended from the force transducer. The sample was suspended over the collection reservoir and covered with plastic to prevent desiccation of the surface. The bronchial cannulae were connected to the ventilation circuit and the lung ventilated with room air. The pulmonary arterial cannulae were connected to the perfusion circuit taking care to displace any air from the connection site. The roller pump was then turned on and the pulmonary circulation perfused with Krebs-Henseleit solution from the perfusate reservoir. Pulmonary venous drainage flowed freely into the collection reservoir before being returned to the perfusate reservoir for re-circulation. The temperature was maintained at 36-38^{o}C by the heat exchanger and the pH and PCO_2 maintained within normal physiological parameters by automatically replacing exhaled CO_2 ***(2.2.5.3)***. When the pulmonary vasculature had been filled and circulation established a note was made of the volume of fluid in the perfusate reservoir. The circulating perfusate volume was kept constant at 1 litre by replenishing the volume in the perfusate reservoir with Krebs-Henseleit solution.

2.2.9 Baseline Parameters

2.2.9.1 Ventilation

The respiration rate was set at 10 breaths per minute (BPM) for the duration of the experiment. The tidal volume was set, depending on the size and airway resistance of the lung sample, to achieve a peak airway pressure of less than 18 mmHg, and ranged between 100 and 300 ml.

2.2.9.2 Perfusion

The perfusion flow rate was gradually increased to achieve a mean perfusion pressure between 15 and 20 mmHg and ranged between 100 and 500 ml min^{-1}. Because the flow rate was constant, changes in perfusion pressure were proportional to pulmonary vascular resistance.

2.2.9.3 Weight

The weight of the lung sample was continuously recorded by the force transducer. Increase in the weight of the lung was taken to be a measure of oedema formation and was quantified as % of initial weight min^{-1}.

2.2.9.4 Equilibration

Lungs were allowed to equilibrate for at least 30 minutes before starting experimental procedures.

2.2.10 Criteria for Early Termination of an Experiment

Experiments were abandoned if:

i) There was excessive weight gain in the lung (> 1.0 % of initial weight min^{-1}) during the equilibration period, indicating excessive oedema formation.

ii) The flow rate was limited (< 100 ml min^{-1}) by a high perfusion pressure, indicating obstruction of the vasculature.

iii) Ventilation was limited (Tv < 100ml) by a high airway pressure, indicating obstruction of the airways.

2.2.11 Collection and Analysis of Perfusate Samples

Regular 'arterial' and 'venous' perfusate samples were collected for analysis of pH, pCO_2, pO_2, Na^+, K^+, iCa^{2+}, HCO_3, HCO_3std, TCO_2 and base excess with a blood gas analyser (Mallinckrodt, Bicester, Oxfordshire, UK). 'Arterial' samples were obtained from the perfusion line just proximal to the arterial cannulae. 'Venous' samples were obtained by collecting a small volume of the effluent from the sample in the collection reservoir. Analysis of perfusate samples confirmed that the system efficiently replaced exhaled CO_2 and returned the pH, pCO_2, HCO_3 and TCO_2 of the perfusate to within normal physiologic parameters before it was re-circulated to the lung sample.

2.2.12 Experimental Protocols

2.2.12.1 Dose Response Curves and Antagonist studies

When stable baseline airway and perfusion pressures were obtained dose response curves to agonists were obtained by cumulative addition to the perfusate reservoir. After a maximum dose response had been obtained the perfusate was completely exchanged by flushing the system with three litres of fresh Krebs-Henseleit solution over a 45 minute period. This was achieved by clamping the line from the collection reservoir to the perfusate reservoir (**figure 2.1**) and directing the effluent from the lung to a waste reservoir. When the perfusate reservoir was empty it was refilled with fresh Krebs-Henseleit solution. When at least three litres of fresh Krebs-Henseleit solution had been flushed through the lung the recirculation of perfusate was restarted by opening the clamp between the collection and perfusate reservoir and closing the line to the waste reservoir. Antagonists were injected directly into the

perfusate reservoir and recirculated. Agonist dose response curves were repeated after a minimum of 45 minutes and when the baseline pressures were stable.

2.2.12.2 Collection of Bronchial Effusion

In some studies samples of bronchial effusion were collected for analysis in further *in vitro* studies (collaboration with Y Hayman (PhD Thesis, University of Hull (2012)). Samples were obtained by aspirating from the ventilation circuit with a 50 ml syringe connected to a 'Y' connector situated proximal to the bronchial cannulae in the ventilation circuit.

2.2.12.3 Modified Perfusion-Ventilation System to Create Hypoxic Conditions in Isolated Lungs

When baseline airway and perfusion pressures were stable the lungs were exposed to hypoxia by modifying the perfusion-ventilation system as follows (**figure 2.2**); Ventilation was switched from room air to 95% N_2: 5% CO_2 by connecting a 2 litre flexible reservoir bag (Intersurgical Ltd.), continuously filled with 95% N_2: 5% CO_2, to the ventilator inlet. The collection reservoir was sealed by means of a thin plastic membrane and filled with 95% N_2: 5% CO_2 (3-4 litres min^{-1}) to prevent ambient uptake of oxygen through the lung surface. An oxygen sensor (Viamed, Keighley,UK) (Calibrated in 100% O_2 and air) was suspended in the collection reservoir to confirm displacement of oxygen by continuously measuring the percentage oxygen concentration (Teledyne Analytical Instruments, California, USA) in the reservoir. A membrane lung (Dideco D905®, paediatric hollow fibre membrane oxygenator (Sorin Group Italia), Quadrox® oxygenator (Maquet Getinge Group) or Inspire® oxygenator (Sorin Group Italia)) was incorporated in the circuit to remove oxygen from the

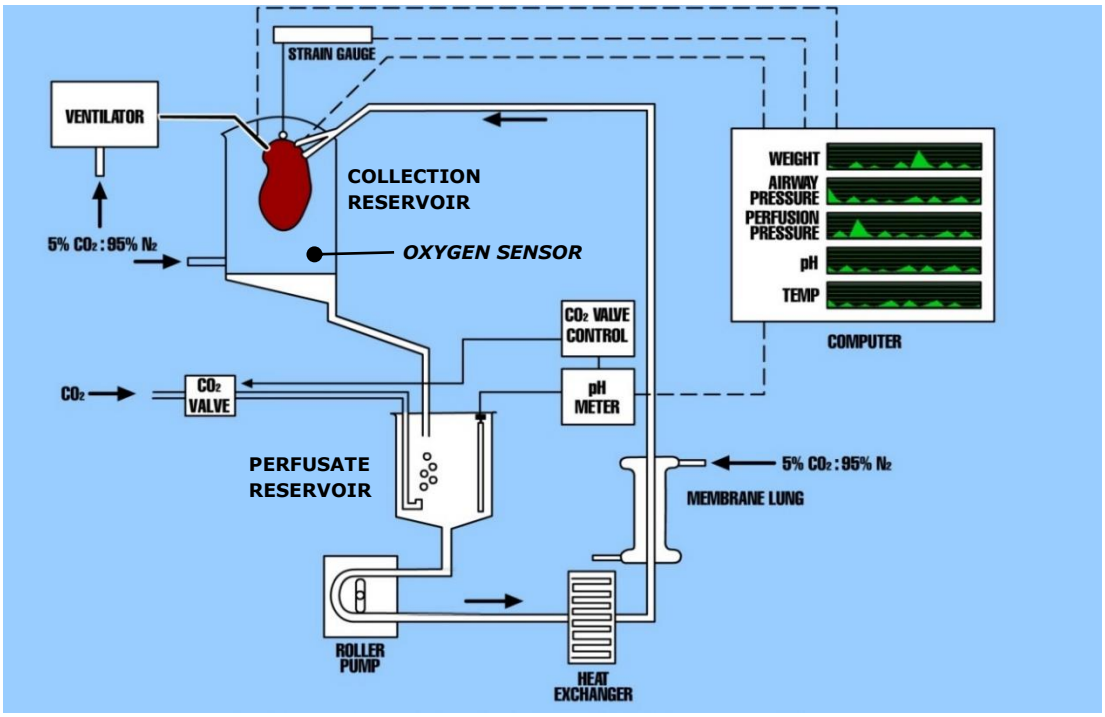
perfusate by using 95% N_2: 5% CO_2 (1-2 litres min^{-1}) as the sweep gas across the hollow fibre membrane.

Perfusate samples were taken from the circuit proximal to the arterial cannula for analysis of perfusate pH, pO_2, pCO_2 and HCO_3 with a blood gas analyser (Gem Premier 3000, Instrumentation Laboratory Company, Lexington, MA, USA).

2.2.13 Processing of lung Specimens After Experimentation

On completion of isolated lung experiments all cannulae were removed. The specimen was immersed in 10% formaldehyde solution and transferred to the pathology department for histopathological examination as per normal institutional practice.

a



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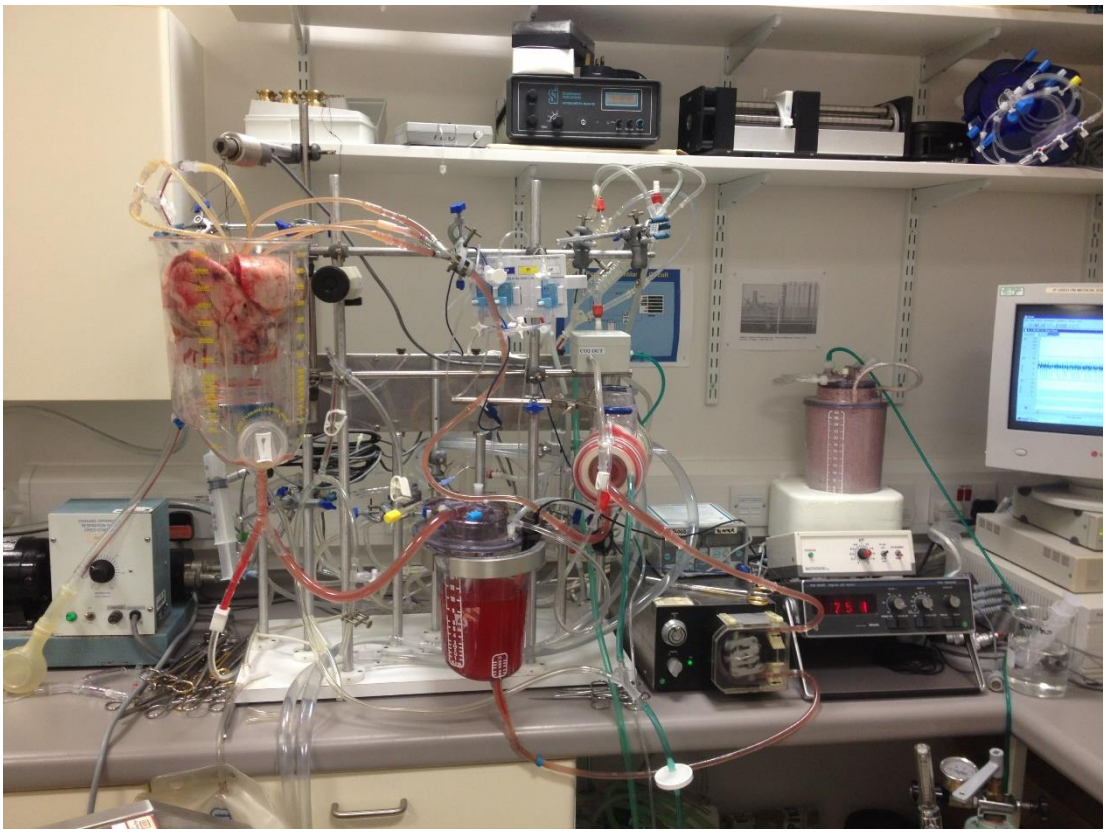

Figure 2.2 Modified perfusion-ventilation system for creating hypoxic conditions in isolated human lungs **(a)** Schematic of modified perfusion-ventilation system **(b)** Photograph of modified perfusion-ventilation system in use.

2.3 *In Vitro* Studies: Isolated Human Pulmonary Arteries, Veins and Bronchi

2.3.1 Sample Procurement

Sections of human bronchi (primary, second order or tertiary), pulmonary arteries or pulmonary veins were obtained from human lungs or lobes immediately following resection for cancer. Lengths of healthy bronchial or vascular tissue were exposed and resected by the operating surgeon, taking care to avoid the tumour margins, and placed in oxygenated Krebs-Henseleit solution before being transferred to the laboratory.

2.3.2 Sample Preparation

Lengths of normal bronchial or vascular tissue were dissected free from surrounding tissue and cut into 3-5 mm sections with an internal diameter of 2-16 mm. Care was taken during dissection to avoid excessive manipulation of the bronchial epithelium or vascular endothelium. Rings of tissue were then mounted in organ baths (***2.3.4***) or stored in oxygenated Krebs-Henseleit solution at 5^{o}C for future use.

2.3.3 Sample Storage

Preliminary experiments showed that bronchi remain viable for at least 72 hours and pulmonary vessels remain viable for at least 24 hours when stored in oxygenated Krebs-Henseleit solution at 5^{o}C.

2.3.4 Organ Baths

Rings of tissue were mounted between stainless steel wires and immersed in 25 ml water jacketed organ baths (Radnoti) containing Krebs-Henseleit solution aerated with 95% O_2: 5% CO_2 (**figure 2.3**). The lower wire was fixed and the upper wire was

connected to a force transducer (Harvard UF1) to measure isometric tension. A heater-cooler unit (HCU- 20-602. Jostra, Sweden) was connected to the water jacket system of the organ baths to maintain the Krebs-Henseleit temperature in the organ baths at 37^{o}C and to heating coils which were used to pre-heat the Krebs-Henseleit solution when refilling the organ baths.

The force transducers were connected to a quad bridge amplifier (power lab/400 system, ADInstruments) for continuous recording of isometric tension as gram force (gf) using data acquisition software (Chart, version 3.4, ADInstruments) on a personal computer.

2.3.5 Isometric Tension

Contraction of the vascular or airway smooth muscle caused an increase in isometric tension which was quantified as active tension in gf or % increase in baseline resting tension where appropriate. Active tension was calculated as: isometric tension- resting tension.

Relaxation of vascular or airway smooth muscle caused a decrease in isometric tension. Relaxation from resting tension was quantified as absolute decrease in resting tension (gf) or % decrease in resting tension where appropriate. Relaxation from active tension to contractile agonists was quantified as decrease in active tension (gf) or % reversal of maximum active tension where appropriate.

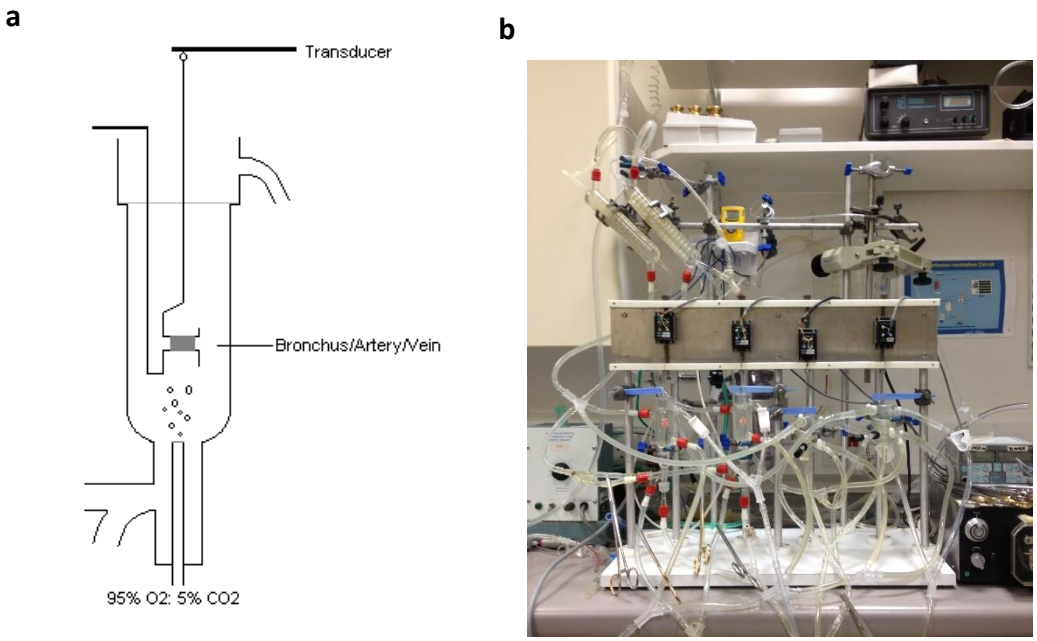

Figure 2.3 Organ baths **(a)** Schematic of organ bath preparation for *in vitro* human bronchi and *in vitro* human pulmonary arteries and veins **(b)** Photograph of four organ bath system; in this example human bronchi are mounted in 2 of 4 organ baths.

2.3.6 Regulation of Organ Bath pO_2

The partial pressure of oxygen (pO_2) in the organ baths was altered by covering the top of the organ bath with a thin plastic film and changing the percentage oxygen concentration (95%, 40%, 21% or 0%) of the aerating gas whilst maintaining a 5% CO_2 concentration to preserve the buffering capacity of the Krebs-Henseleit solution. A Clarke electrode (Micro oxygen electrode, Lazar Industries) was used to continuously measure the pO_2 of the Krebs-Henseleit solution in one of the organ baths. Changing the percentage oxygen concentration of the aerating gas caused a rapid (< 4 minutes) and sustained change in the oxygen concentration of the Krebs-Henseleit solution in the organ bath (**figure 2.4**). Output from the oxygen electrode was in mV. The pO_2 in samples of Krebs-Henseleit solution was measured at different oxygen concentrations using a blood gas analyser (Gem Premier 3000, Instrumentation Laboratory Company,

Lexington, MA, USA) to determine the pO_2 corresponding to a given mV output: 10 mV ~ 1 KPa ~ 7.5 mmHg. The resultant oxygen electrode output (mV) and corresponding pO_2 (KPa and mmHg) in the organ bath Krebs-Henseleit solution at the standard oxygen concentrations used in experiments (95%, 40%, 21% or 0% oxygen in the aerating gas) is shown in **table 2.1.**

2.3.7 Calibration

The transducers were calibrated against a 20 g weight before each experiment using the software calibration procedure. The oxygen electrode was calibrated in 95%, 21% and 0% oxygen prior to each experiment.

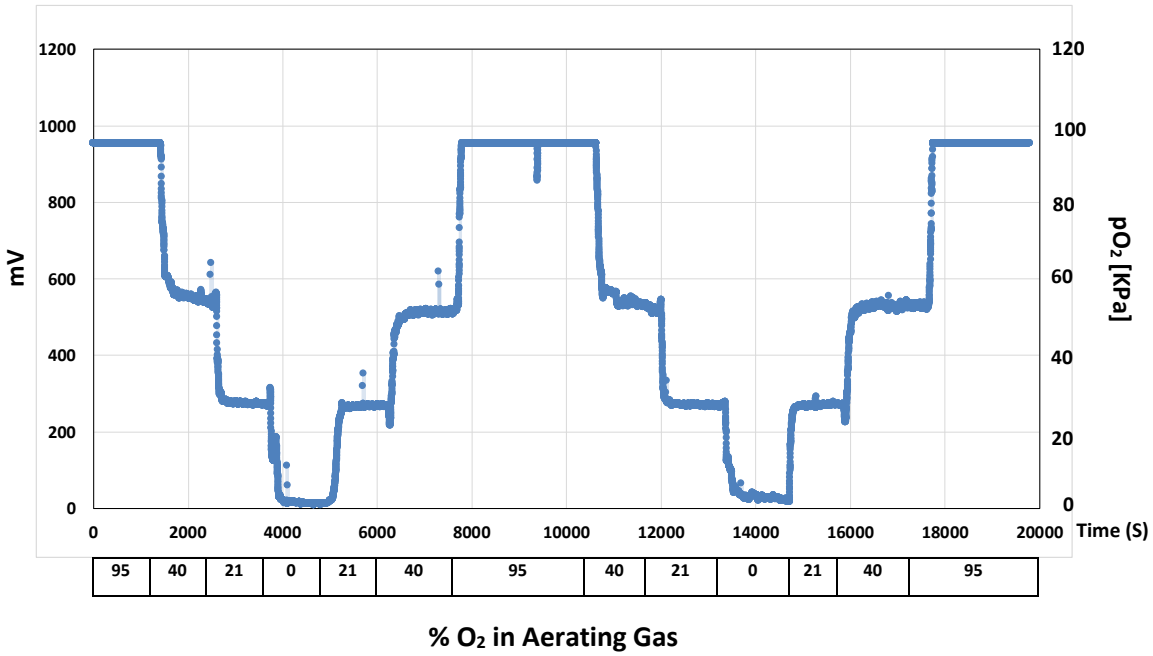

Figure 2.4 Experimental trace showing change in the partial pressure of oxygen (pO_2) in the Krebs-Henseleit solution of an organ bath with changes in the % oxygen concentration of the aerating gas. The trace was obtained using a Clarke electrode and the pO_2 at a given output determined by measuring the pO_2 of the Krebs-Henseleit solution using a blood gas analyser (10 mV ~ 1 KPa ~ 7.5 mmHg).

Table 2.1 Oxygen electrode output (mV) and corresponding pO_2 (KPa and mmHg) in the organ bath Krebs-Henseleit solution at standard oxygen concentrations used (% O_2 in the aerating gas)

% O_2 in Aerating Gas	Oxygen Probe Output	Partial Pressure of Oxygen (pO_2) in Krebs-Henseleit Solution	
	mV	mmHg	KPa
95	950	713	95
40	520	390	52
21	270	203	27
0	20	15	2

2.3.8 Experimental Protocols

2.3.8.1 Equilibration – Resting Tension

2.3.8.1.1 Bronchi

A resting tension of 2 gf was applied and the bronchi left to equilibrate for 30-90 minutes before starting experimental protocols. During equilibration the bronchi were frequently washed and the resting tension adjusted to 2 gf.

2.3.8.1.2 Pulmonary Vessels

A resting tension of 1-2 gf (arteries) or 0.5-2.0 gf (veins) was applied and the vessels allowed to equilibrate for 30-90 minutes. During equilibration the vessels were frequently washed and the resting tension adjusted to 1-2 gf (arteries) or 0.5-2.0 gf (veins). Before commencing experimental studies the functional integrity of the endothelium was determined by exposing the tissue to 1 µM acetylcholine (ACh).

(Preliminary experiments determined that 1 μM ACh caused maximum relaxation in human pulmonary arteries). The tissue was then washed for 30 minutes.

2.3.8.2 Contractile Agonist Responses

Contractile agonists were added directly to the organ baths and the effects of contractile agonists expressed as active tension (gf) or % increase in resting tension. The maximum response (gf) to a given concentration of agonist was recorded when the response reached a plateau and active tension (gf) was calculated as: maximum response (gf) – resting tension (gf). Contractile responses were quantified by determining the maximum response (E_{max}) to a single dose of agonist or by constructing cumulative concentration response curves by stepwise increases in agonist concentration when a plateau response had been obtained to the preceding dose.

2.3.8.3 Relaxant Responses

Relaxant responses from resting tension were expressed as absolute change in tension (gf) or % change from resting tension and relaxation from active tension was expressed as change in active tension (gf) or % reversal of maximum active tension. Relaxant responses from resting and active tension were quantified by determining the maximum relaxant response (E_{max}) to a single concentration of agonist or by constructing cumulative concentration response curves by stepwise increases in agonist concentration when a plateau response had been obtained to the preceding dose.

2.3.8.4 Agonist Potency

Agonist EC_{50} concentrations (concentration required to elicit 50% of the maximum agonist response) were estimated manually from individual concentration response curves and agonist potency expressed as EC_{50} (as a molar unit concentration) and pEC_{50} (the negative logarithm to base 10 of the molar EC_{50} concentration).

2.3.8.5 Antagonist Studies

When a stable baseline tension was achieved control responses to agonists and/or changes in oxygen tension from resting tension and/or active tension were obtained. When control responses were completed the vessels were washed by rapidly replacing the Krebs-Henseleit solution three times every 5-10 minutes over 30-90 minutes. Antagonists were then added directly to the organ bath. Repeat responses in the presence of the antagonist were then obtained after a minimum of 30 minutes and when the baseline tension was stable.

The effects of antagonists on agonist responses were quantified either by determining the effect on the maximum agonist effect (E_{max}) obtained to a single concentration of agonist and expressed as % change in E_{max}, or by determining the effect on an agonist cumulative concentration response curve (CRC) (expressed as change in EC_{50} and % change in E_{max}).

2.3.8.6 Vehicle Controls

2.3.8.6.1 Bronchi

Preliminary experiments identified that DMSO and ethanol, which were used to dissolve some of the drugs, caused a dose dependent dilation of the bronchi at

volumes greater than 25 µl (0.1%). Therefore, in experiments using drugs dissolved in ethanol or DMSO, minimum volumes of drug solution were used. When a volume of drug solution greater than 25 µl was used a control response was obtained to determine the proportionate effect of the excipient by repeating the experimental protocol in the same preparation or a parallel preparation using the same volume of solvent without the active agent.

2.3.8.6.2 Pulmonary Vessels

For experiments using drugs dissolved in DMSO or ethanol control responses to the vehicle were obtained in parallel preparations by the addition of corresponding volumes of solvent without the active agent.

2.3.9 Disposal of tissue

Surplus tissue and tissue used in organ bath studies were immersed in disinfectant solution (3% Vircon solution, Antec International) before disposal by incineration.

2.4 Materials

2.4.1 Physiological Salt Solutions

Krebs-Henseleit solution contained: (mM) NaCl 118, KCl 4.7, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, $CaCl_2$ 2.4 and glucose 11.

A Krebs stock solution was prepared by dissolving 53.9 g KCl, 24.6 g KH_2PO_4 and 44.5 g $MgSO_4.7H_2O$ in 1 litre of deionised water (Purite Select Deioniser) and stored at $4^{O}C$.

10 litres of fresh Krebs-Henseleit solution was made and used on the same day as follows; 69 g NaCl, 21 g $NaHCO_3$ and 20 g of glucose were dissolved in 2 litres of

deionised water and added to 7.9 litres of deionised water. 65 ml of Krebs stock solution was then added and the solution mixed. Finally 26 ml of 1 Molar $CaCl_2$ was added and the solution immediately bubbled with 95% O_2: 5% CO_2 to prevent precipitation of calcium carbonate. Solutions were bubbled for at least 15 minutes before use.

Calcium free Krebs-Henseleit solution was prepared as above with the omission of $CaCl_2$.

2.4.2 Reagents

All reagents were obtained from Fisher Scientific unless otherwise stated.

2.4.3 Gases

All gases (**table 2.2**) were supplied by the British Oxygen Company Ltd, Guildford, Surrey, UK.

Table 2.2 Gases used.

95% O_2: 5% CO_2
95% Air: 5% CO_2
95% N_2: 5% CO_2
40% O_2: 5% CO_2
100% O_2
100% CO_2

2.4.4 Drugs

Stock solutions of drugs were prepared using the solvent recommended by the supplier (**Appendix I**). Solutions were stored at the temperature recommended by the supplier and protected from light if necessary. Fresh serial dilutions were made, using the appropriate solvent, for each experiment.

2.5 Statistical Analysis

Values are expressed as mean ± standard deviation (SD) of the mean. One-sample Kolmogorov-Smirnov test was used to test for normality of distribution. Parametric data was compared using a one or two-tailed paired or unpaired t-test. For non-parametric data analysis was made via Wilcoxon signed-rank test for paired variables or by Mann Whitney U test for unpaired variables using IBM SPSS statistics software (version 19). Significance was assumed for values of $p < .05$. Box-whisker plots display median, the interquartile range (box) and maximum and minimum values (whiskers). Outliers (1.5 – 3 x the interquartile range) and extreme outliers (> 3 x the interquartile range) were automatically removed from the data analysis by the statistics software. Outliers and extreme outliers are displayed in box-whisker plots by the statistical package and denoted as ○ and * respectively. Extreme outlier symbols were circled on box-whisker plots to differentiate from markers of statistical significance.

CHAPTER 3

Effects of Hypoxia on the Human Pulmonary Vasculature: *In Vitro* Human Pulmonary Arteries and Veins

Contents

3.1 Introduction

In 1946 Von Euler and Liljestrand published a paper [42] describing the effects of breathing different concentrations of oxygen on the pulmonary artery pressure of cats. They found that breathing high concentrations of oxygen caused a decrease in pulmonary artery pressure and breathing low concentrations of oxygen caused an increase in pulmonary artery pressure. They recognised the significance of this observation and concluded that "regulation of the pulmonary blood flow is mainly mediated by a local action of the blood and alveolar gases leading to an adequate distribution of the blood through the various parts of the lungs according to the efficiency of aeration." The occurrence of hypoxic pulmonary vasoconstriction (HPV) in man was described by Motley *et al*. [43] within a year of von Euler and Liljestrand's publication and has been confirmed by a number of subsequent *in vivo* studies [44-57] in man.

Since the 1940's the effect of hypoxia on the pulmonary vasculature has been extensively investigated in numerous species using a variety of *in vivo* and *in vitro* models (reviewed by Sylvester *et al*. [7]) and HPV is now accepted as an important homeostatic mechanism for optimising perfusion-ventilation matching in the lung. There is also general agreement that the vasopressor effect of hypoxia in the lung is mediated by a direct effect on the pulmonary artery smooth muscle cells of the small resistance arteries and is largely independent of external neural and hormonal influences. However, although HPV has been shown to occur in most species it is not a uniform and ubiquitous effect. There are considerable differences in the time course

and magnitude of HPV amongst species and inconsistencies between *in vitro* and *in vivo* results [7].

In vitro pulmonary artery preparations from numerous species have been used to investigate the effects of hypoxia on pulmonary vascular tone. However, significant differences exist in the magnitude and temporal effects of hypoxia in pulmonary artery preparations from different species. For example in rat isolated pulmonary arteries the response to hypoxia consists of four distinct phases; brief relaxation (phase I), rapid contraction (phase II), slow relaxation (phase III) and sustained contraction (phase IV) [128]. In ferret pulmonary arteries hypoxia causes an initial transient contraction followed by a sustained relaxation [129] and in pre-contracted sea lion pulmonary arteries acute hypoxia causes a relaxation [130]. Given the assumed significance of HPV as an important homeostatic mechanism there is a conspicuous lack of data on HPV from *in vitro* human pulmonary artery studies. A small number of studies have shown a constriction to hypoxia in isolated human pulmonary arteries; however, the results were variable. In some studies HPV could be induced from resting tension [131, 132] in isolated human pulmonary arteries (PAs) whereas in other studies [133-135] an element of precontraction was necessary to reveal a pressor effect of hypoxia. The role of the endothelium in human HPV is also uncertain. In one study [136] removal of the endothelium was found to either attenuate the pressor effect of hypoxia or convert the effect of hypoxia to a relaxation from resting or active tension in human PAs but in another study [137] removal of the endothelium augmented HPV in human PAs.

The majority of *in vitro* studies have focused on the effects of hypoxia on the pulmonary arterial system. It is becoming increasingly recognised that the pulmonary

veins make a significant contribution to the total pulmonary vascular resistance (PVR) [138]. Although there are species differences in the relative contribution of the veins to PVR, and a tendency for the contribution of the venous system to change with age, it would seem likely that the pulmonary veins will play a significant role in the overall effect of hypoxia on pulmonary vascular pressure. It is therefore surprising that only a small number of studies have investigated the effects of hypoxia on *in vitro* pulmonary veins and the results were variable; in guinea-pig [139], rat [140, 141] and pig [142] isolated pulmonary veins acute hypoxia caused an increase in isometric tension whereas in sheep pulmonary veins pre exposure to prolonged hypoxia was needed to expose a pressor effect of hypoxia [143]. Only one *in vitro* study was found describing the effect of hypoxia on human pulmonary veins. Savineau *et al*. [132] found that hypoxia had no effect on resting tension in strips of human pulmonary vein.

Acute regional or global hypoxia can occur in the lung as a result of pathological episodes such as acute exacerbations of asthma, airway obstruction and sleep apnoea and during surgical procedures such as cardiopulmonary bypass and thoracic surgery. A considerable body of literature has described the effect of acute hypoxia on the pulmonary vasculature in numerous species. However, there are significant species differences in the response to hypoxia and there is a conspicuous lack of *in vitro* data describing the effects of hypoxia on the human pulmonary vasculature which is a significant gap in the current scientific understanding. Having considered the evidence from animal studies, *in vivo* human studies and a small number of previous *in vitro* human studies it was hypothesised that exposure to hypoxia would cause a constriction of *in vitro* human pulmonary arteries and veins.

The aim of the studies in this chapter was to:

1. Determine the effect of hypoxia on vascular tone in isolated human pulmonary arteries

2. Determine the effect of hypoxia on vascular tone in isolated human pulmonary veins

3.2 Methods

3.2.1 Tissue Preparation

Human lobar or inter lobar pulmonary arteries and human pulmonary veins were obtained at resection for lung cancer. Samples were prepared and mounted between stainless steel wires in organ baths to measure changes in isometric tension **(*Chapter 2, 2.3*).**

Resting tension was set at 1-2 gf and the vessels allowed to equilibrate. During equilibration the vessels were frequently washed and the resting tension adjusted to 1-2 gf.

3.2.2 Contractile Agonist Dose Responses

Preliminary experiments were performed to determine the dose of contractile agonists, noradrenaline (NA) or endothelin-1 (ET-1), required to produce the maximum increase in active tension in the arteries and veins.

A dose response to contractile agonists was obtained by stepwise addition of increasing concentrations of agonist to the organ bath. The effect of each drug concentration was measured after the response had reached plateau and was the mean tension (gf) for the 2 minute period preceding the next increase in drug concentration (obtained using the data pad function of the Chart software). Active tension was calculated as maximum response (at plateau) – resting (baseline) tension. Changes in active tension were quantified as absolute change in active tension (gf) or % change from baseline.

In pulmonary arteries (n=12 from 4 patients, mean internal diameter 4.0 ± 2.0 mm, mean width 4.3 ± 2.5 mm) and pulmonary veins (n= 6 from two patients, mean internal diameter 2.0 ± 0 mm, 3.0 ± 0 mm wide) noradrenaline caused a dose dependent (1-10 μM) increase in active tension with a maximum effect at 10 μM. However, in pulmonary veins contractions to NA were not sustained for more than 10 minutes and therefore the effects of hypoxia on active tension to NA in pulmonary veins could not be determined.

ET-1 dose dependently (10 pM - 30 nM) contracted human pulmonary arteries (n = 9 from 3 patients, mean internal diameter 4.6 ± 2.0 mm, 3.7 ± 0.9 mm wide) and human pulmonary veins (n = 9 from 3 patients, mean internal diameter 6.0 ± 3.7 mm, 3.9 ± 1.5 mm wide). (***Chapter 5, 5.3.2***).

3.2.3 Effect of Hypoxia on Resting Tension

After equilibration for 60-90 minutes and when a stable resting tension was achieved the vessels were exposed to 1 μM acetylcholine (ACh) to determine the functional integrity of the endothelium. When a maximum response to ACh was obtained or after a minimum of 15 minutes the vessels were washed. After a minimum washout of 30 minutes and when a stable resting tension was achieved the vessels were exposed to hypoxia by covering the top of the organ bath with a plastic film and changing the aerating gas to 95% N_2: 5% CO_2.

In preliminary experiments a Clarke electrode (Micro oxygen electrode, Lazar Industries) was used to measure the pO_2 of the Krebs-Henseleit solution in one of the organ baths and confirmed that aerating the buffer solution with 95% N_2: 5% CO_2 caused a rapid (< 4 minutes) displacement of the oxygen from the buffer solution. The

final concentration of oxygen measured in the buffer solution was approximately 2 KPa which was confirmed by analysis of a sample from the buffer solution using a blood gas analyser (Gem Premier 3000, Instrumentation Laboratory Company, Lexington, MA, USA) (***Chapter 2, 2.3.6, Table 2.1***).

When a maximum response to hypoxia was obtained or after a minimum of 30 minutes the aerating gas was changed back to 95% O_2: 5% CO_2 which resulted in an increase in the buffer pO_2 to approximately 95 KPa (***Chapter 2, 2.3.6, Table 2.1***).

The effects of hypoxia on resting tension were determined by calculating the mean resting tension (gf) for three 5-minute periods (using the data pad function in Chart); before exposure to hypoxia, 5 minutes before reoxygenation and on reaching plateau after reoxygenation.

The effect of hypoxia was quantified as absolute change in resting tension (gf) and % change in resting tension.

3.2.4 Effect of Hypoxia on Active Tension

The vessels were contracted to noradrenaline or endothelin-1. When a maximum increase in active tension was obtained and sustained for more than 10 minutes the effect of hypoxia on active tension was determined by covering the top of the organ bath with a plastic film and changing the aerating gas to 95% N_2: 5% CO_2. When a maximum response to hypoxia was obtained or after a minimum of 30 minutes the aerating gas was changed back to 95% O_2: 5% CO_2.

The effect of hypoxia on active tension was quantified as change in active tension (gf) or % change in maximum active tension.

3.2.5 Oscillatory Phasic Activity in Pulmonary Veins: Effect of Antiarrhythmic Agents

Preliminary experiments revealed that the pulmonary veins displayed an oscillatory phasic contraction- relaxation activity, which was initiated spontaneously or precipitated by exposure of the veins to hypoxia and reoxygenation. Given the established role of the pulmonary veins in the initiation and maintenance of atrial fibrillation further studies were performed to investigate the possible effect of antiarrhythmic agents on the oscillatory activity of the veins. When oscillatory phasic activity was established the veins were exposed to increasing concentrations of amiodarone or flecainide by direct addition to the organ baths.

3.3 Results

3.3.1 *In Vitro* Human Pulmonary Arteries

3.3.1.1 Effect of Hypoxia on Resting Tension

21 human lobar or segmental pulmonary arteries (mean internal diameter 4.3 ± 2.0 mm, mean width 4.0 ± 1.9 mm) were obtained from 7 patients.

Control responses to 1 μM acetylcholine (ACh) were obtained in all arteries before starting experimental protocols. The effect of ACh on resting tension was variable. In 7 pulmonary artery preparations ACh caused a relaxation (mean relaxation -16.67 ± 7.58 % of baseline resting tension, raw data Appendix II). In 5 preparations ACh caused an increase in resting baseline tension (mean increase 15.00 ± 7.46 % of baseline resting tension, raw data Appendix II). In the remainder of the preparations (n=9) ACh had no effect on resting tension. . These results suggest that either the endothelium was not functionally intact in some of the vessels or that there is an inherent variability in the response of human pulmonary arteries to ACh.

The effect of hypoxia on resting tension was also variable. None of the arteries studied contracted to hypoxia. In 5 arteries hypoxia caused a significant relaxation from resting tension (mean relaxation -34 ± 14 % of baseline resting tension, $p < .05$ vs. pre- hypoxia via Wilcoxon signed-rank test, raw data Appendix II) which was not reversed on reoxygenation (**table 3.1, figure 3.1**). In the remaining arteries (n=16) hypoxia had no effect on resting tension

There was no obvious association between response of the arteries to ACh and response to hypoxia. Not all arteries that relaxed to ACh relaxed to hypoxia and one artery relaxed to hypoxia but not to ACh.

Table 3.1 Effect of hypoxia and reoxygenation on resting tension in isolated human pulmonary Arteries. n=5. *$p < .05$ vs. pre-hypoxia via Wilcoxon signed-rank test

	Resting Tension (gf)		
	Pre-Hypoxia	**Hypoxia**	**Reoxygenation**
Mean	1.45	0.97*	0.92*
SD	0.44	0.39	0.40

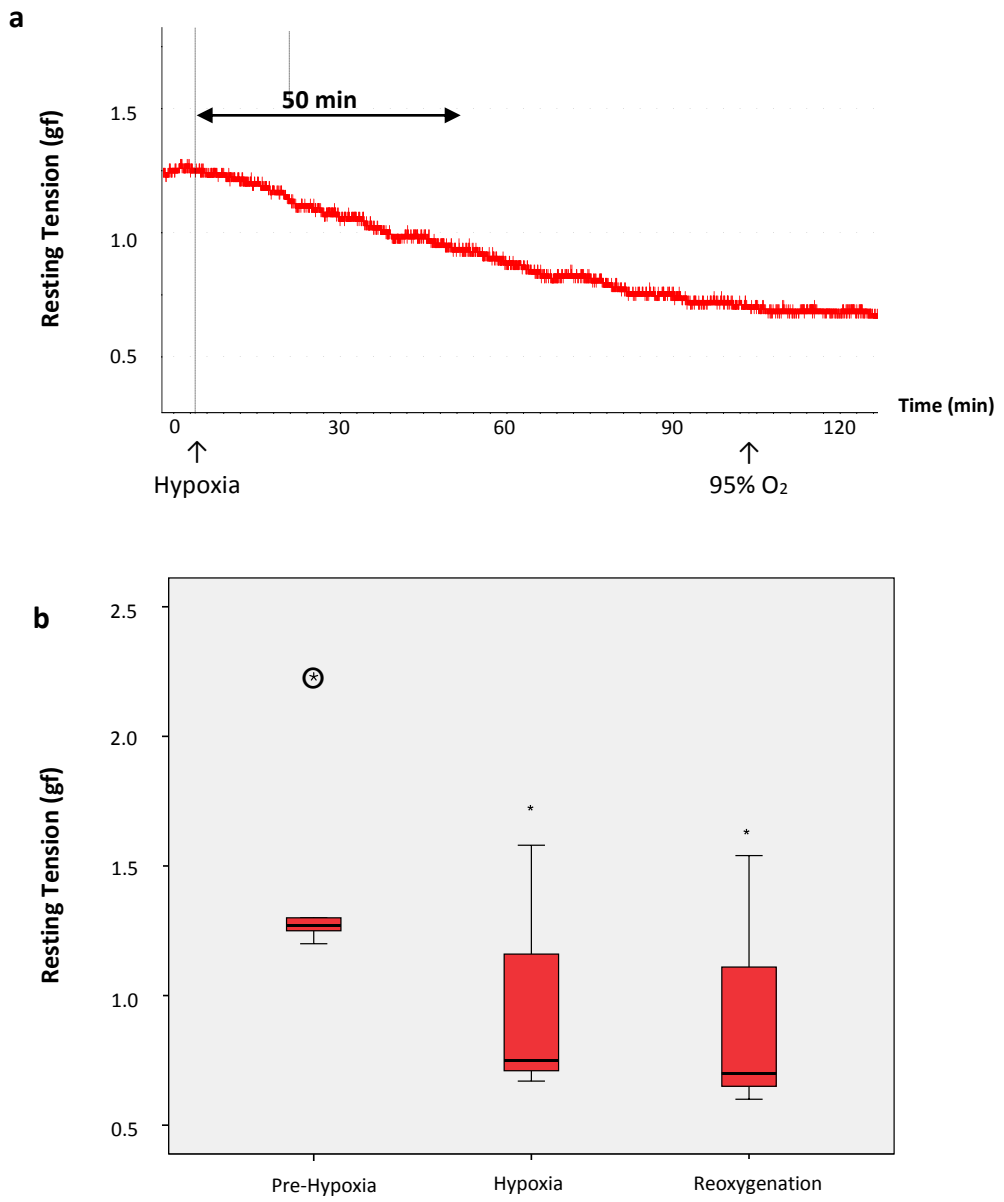

Figure 3.1 Effect of hypoxia on resting tension in isolated human pulmonary arteries. (**a**) Experimental trace showing effect of hypoxia and reoxygenation on resting tension. (**b**) Box whisker plot of resting tension at baseline, on exposure to hypoxia and on reoxygenation in five pulmonary arteries that relaxed on exposure to hypoxia. The remaining arteries in this study (n=16) did not relax to hypoxia from resting tension. Circled asterisk indicates extreme outlier. **p* < .05 vs. pre- hypoxia via Wilcoxon signed-rank test.

3.3.1.2 Effect of Hypoxia on Active Tension

Active Tension to Noradrenaline

The effect of hypoxia on active tension in human pulmonary arteries (n=12 from 4 patients, mean internal diameter 4.0 ± 2.0 mm, mean width 4.3 ± 2.5 mm) contracted to 10 µM noradrenaline (NA) was investigated. In three arteries the contraction to NA was not sustained and the effects of hypoxia on active tension in these preparations could not be determined. In the remaining 9 preparations hypoxia caused a significant relaxation from active tension (-52 ± 26 % of active tension, $p < .01$ via paired, 2-tailed, t-test, n=9, raw data Appendix II) which was not reversed on reoxygenation (**table 3.2, figure 3.2**).

Table 3.2 Effect of hypoxia and reoxygenation on active tension in human pulmonary arteries contracted to 10 µM noradrenaline .n=9, $^{**}p < .01$ vs. pre-hypoxia via paired samples, 2-tailed t-test.

	Δ Active Tension (gf)		**Δ Active Tension (%)**	
	Hypoxia	**Reoxygenation**	**Hypoxia**	**Reoxygenation**
Mean	-0.26**	-0.40**	-52**	-75**
SD	0.16	0.32	26	38

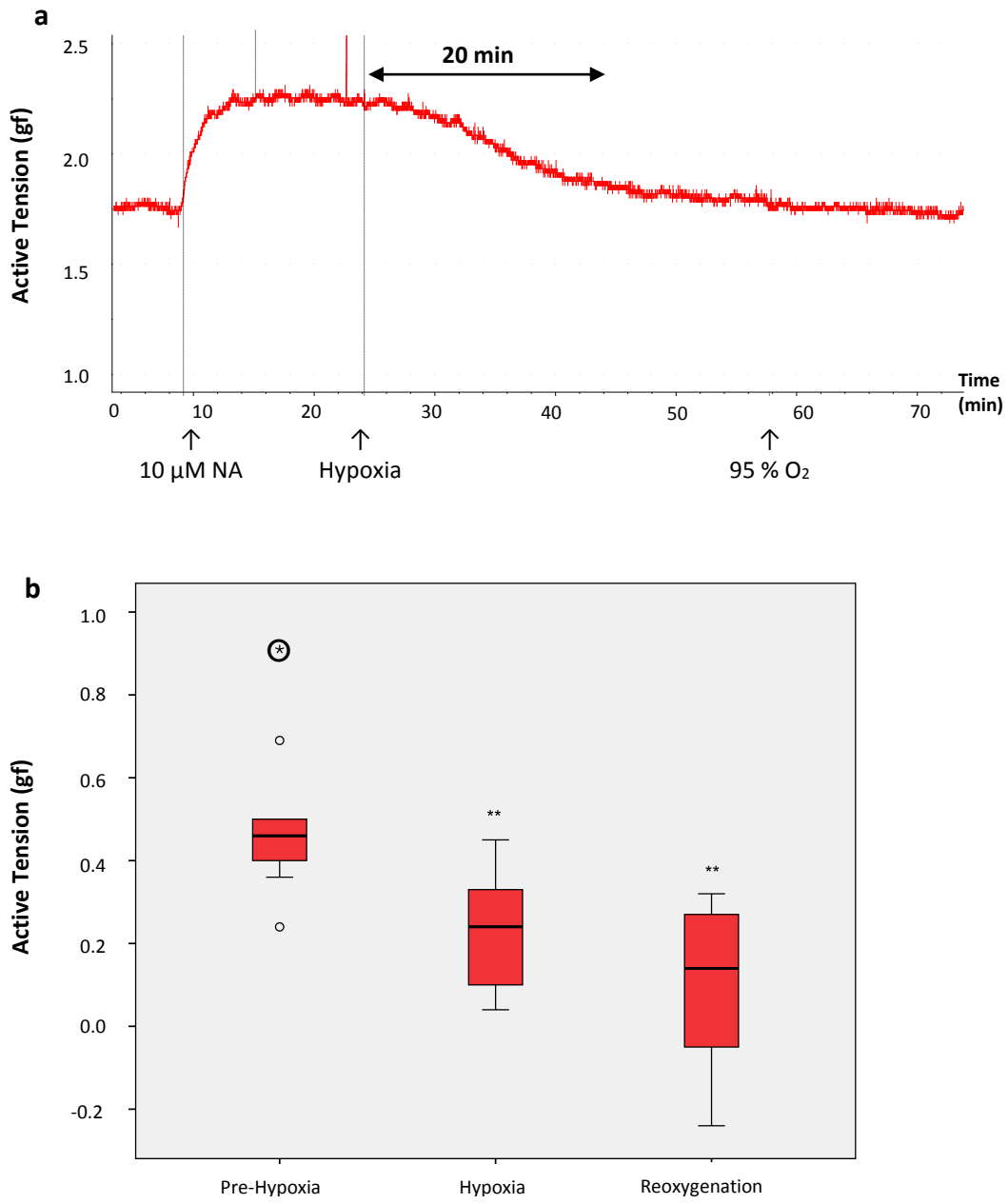

Figure 3.2 Effect of hypoxia on active tension in isolated human pulmonary arteries contracted to 10 µM noradrenaline. (**a**) Experimental trace showing effect of hypoxia and reoxygenation on active tension. (**b**) Box whisker plot of active tension pre-hypoxia, on exposure to hypoxia and on reoxygenation. The pre-hypoxia data contained two outliers (circles) and one extreme outlier (circled asterisk). n=9, $^{**}p < .01$ vs. pre- hypoxia via paired samples, 2- tail, t- test.

Active Tension to Endothelin-1

Hypoxia caused a significant relaxation from active tension (-155 ± 213 % of active tension, $p < .01$ via paired, 2-tailed, t-test, raw data Appendix II) in human pulmonary arteries (n=9 from 3 patients, mean internal diameter 4.6 ± 2.0 mm, 3.7 ± 0.9 mm wide) maximally contracted to ET-1 (10 or 30 nM) which was not reversed on reoxygenation (**table 3.3, figure 3.3**).

Table 3.3 Effect of hypoxia and reoxygenation on active tension in human pulmonary arteries contracted to ET-1. n=9, $^{**}p < .01$ vs. pre-hypoxia via Wilcoxon signed-rank test

.

	Δ Active Tension (gf)		**Δ Active Tension (%)**	
	Hypoxia	**Reoxygenation**	**Hypoxia**	**Reoxygenation**
Mean	-0.36**	-0.05**	-155**	-30**
SD	0.32	0.05	213	39

Exposure of pre-contracted vessels to hypoxia did not induce a further increase in active tension in any of the preparations studied.

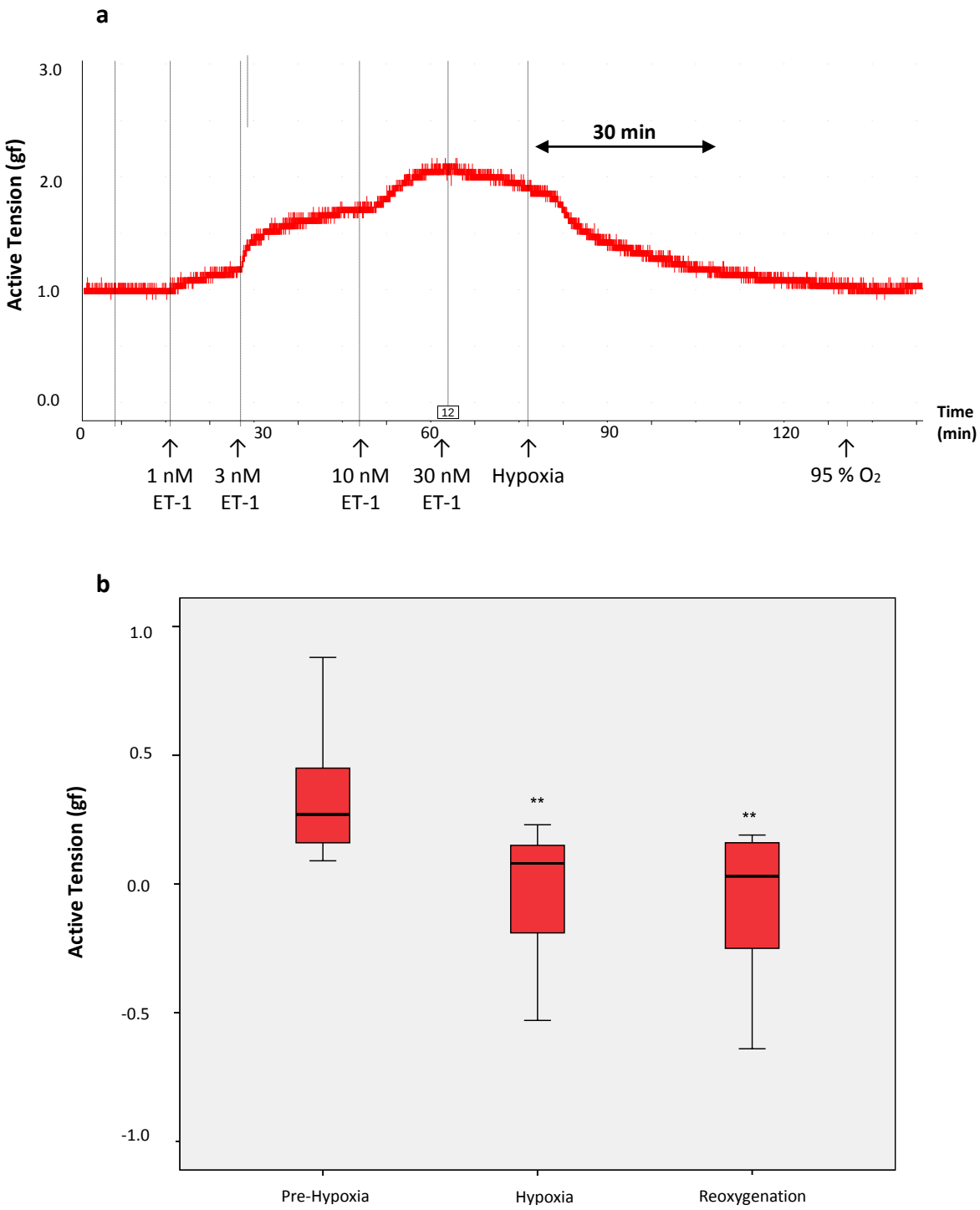

Figure 3.3 Effect of hypoxia on active tension in isolated human pulmonary arteries contracted to ET-1 (10 or 30 nM) (**a**) Experimental trace showing effect of hypoxia and reoxygenation on active tension to ET-1. (**b**) Box whisker plot of active tension pre-hypoxia, on exposure to hypoxia and on reoxygenation. n=9, $^{**}p < .01$ vs. pre- hypoxia via Wilcoxon signed-rank test.

3.3.2 *In Vitro* Human Pulmonary Veins

3.3.2.1 Effect of Hypoxia on Resting Tension

Sixteen human pulmonary veins were obtained from 7 patients (mean internal diameter 4.8 ± 3.2 mm, 3.8 ± 1.1 mm wide).

Control responses to 1 μM ACh on resting tension were obtained in 12 veins (mean internal diameter 5.6 ± 3.3 mm, 3.9 ± 1.2 mm wide) before starting experimental protocols. ACh had no effect on resting tension in 10 veins and caused a relaxation in two veins.

In thirteen of the sixteen veins studied hypoxia caused a significant relaxation from resting tension (-12 ± 15 % of resting tension, n=16, $p < .01$ vs. pre-hypoxia via Wilcoxon signed-rank test, raw data Appendix II) which was reversed on reoxygenation (**table 3.4, figure 3.4**). In five of the veins hypoxic relaxation was preceded by a transient contraction. In three veins hypoxia had no effect on resting tension.

Table 3.4 Effect of hypoxia and reoxygenation on resting tension in isolated human pulmonary veins. n=16. $^{**}p < .01$ vs. pre-hypoxia via Wilcoxon signed-rank test

	Resting Tension (gf)		
	Pre-Hypoxia	**Hypoxia**	**Reoxygenation**
Mean	1.41	1.21**	1.45
SD	0.67	0.55	0.76

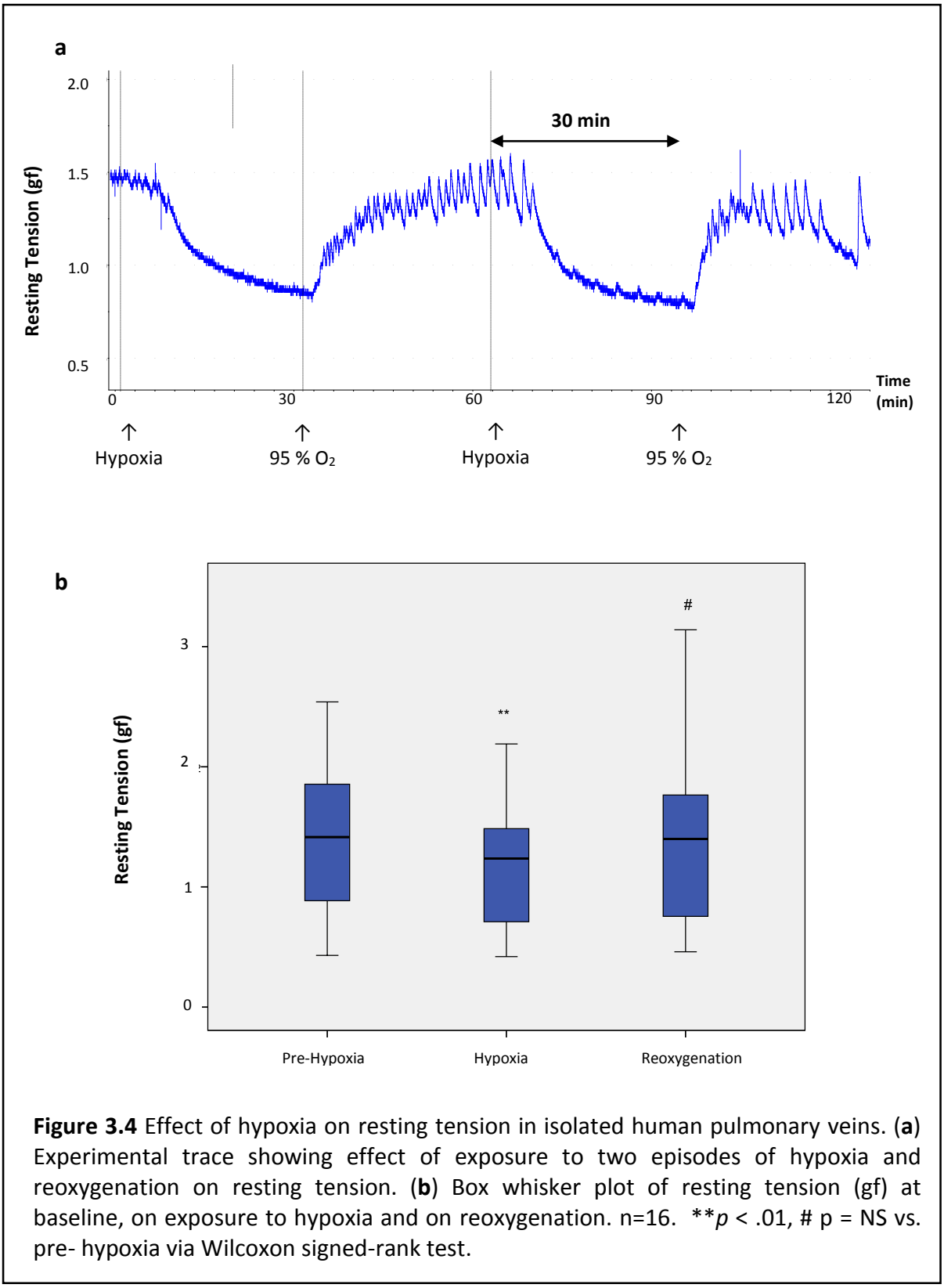

Figure 3.4 Effect of hypoxia on resting tension in isolated human pulmonary veins. (**a**) Experimental trace showing effect of exposure to two episodes of hypoxia and reoxygenation on resting tension. (**b**) Box whisker plot of resting tension (gf) at baseline, on exposure to hypoxia and on reoxygenation. n=16. ***p* < .01, # p = NS vs. pre- hypoxia via Wilcoxon signed-rank test.

3.3.2.2 Effect of Hypoxia on Active Tension

Hypoxia caused a significant relaxation from active tension (- 55 ± 16 %, $p < .01$ via Wilcoxon signed-rank test) in veins (n=8, from 4 patients, mean internal diameter 5.8 ± 3.9 mm, 4.0 ± 1.5 mm wide) maximally contracted to ET-1 (10 or 30 nM) which was not significantly reversed by reoxygenation (**table 3.5, figure 3.5).** (Raw data Appendix II).

Table 3.5 Effect of hypoxia and reoxygenation on active tension in human pulmonary veins contracted to ET-1 .n=8, $^{**}p < .01$ vs. pre-hypoxia via Wilcoxon signed-rank test.

	Δ Active Tension (gf)		**Δ Active Tension (%)**	
	Hypoxia	**Reoxygenation**	**Hypoxia**	**Reoxygenation**
Mean	-0.73**	-0.66**	-55**	-49**
SD	0.63	0.61	16	15

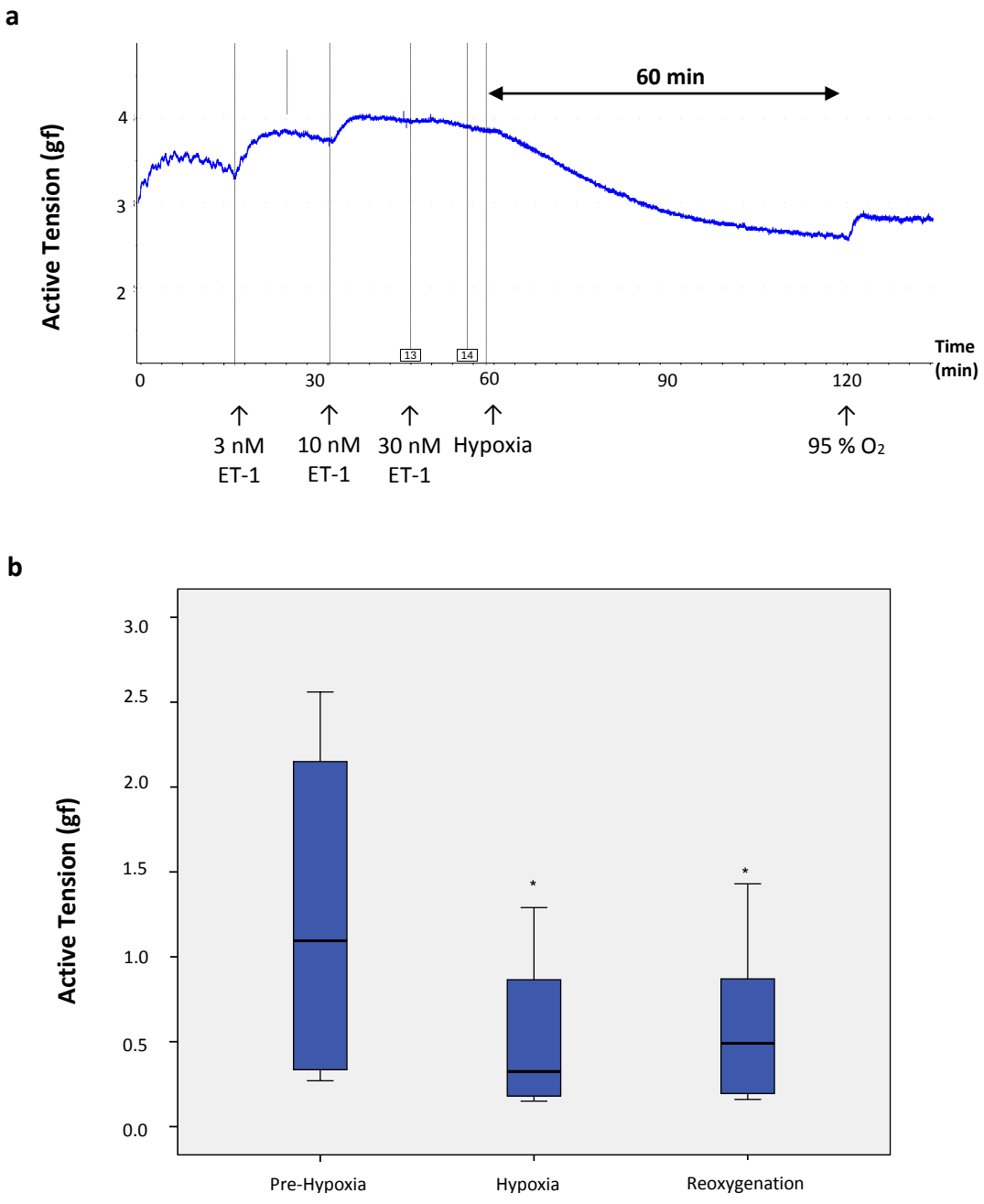

Figure 3.5 Effect of hypoxia on active tension in isolated human pulmonary veins contracted to ET-1 (10 or 30 nM) (**a**) Experimental trace showing effect of hypoxia and reoxygenation on active tension to ET-1. (**b**) Box whisker plot of active tension pre-hypoxia, on exposure to hypoxia and on reoxygenation. n=8, **p* < .05 vs. pre- hypoxia via Wilcoxon signed-rank test.

3.3.2.3 Oscillatory Phasic Activity

In the studies described in this chapter a total of 17 pulmonary vein rings were used from 7 patients (mean internal diameter 4.9 ± 3.2 mm, 3.7 ± 1.1 mm wide). The pulmonary veins developed oscillatory phasic contraction- relaxation activity. Oscillatory phasic activity occurred spontaneously, before exposure to hypoxia, in 11 veins after 49 ± 21 min (**figure 3.6a)** or was precipitated by exposure to hypoxia/ reoxygenation in 5 veins (**figure 3.6b**).

One preparation was not exposed to hypoxia and did not develop oscillatory phasic activity and one vein, which was exposed to hypoxia/reoxygenation, did not develop phasic activity.

3.3.2.4 Effect of Antiarrhythmic Agents on Oscillatory Phasic Activity

Amiodarone caused a dose dependent (10-300 μM) relaxation from resting tension and abolished oscillatory phasic activity in one isolated pulmonary vein (**figure 3.7a**). Flecainide 10-30 μM had no effect on resting tension or oscillatory phasic activity in one isolated pulmonary vein (**figure 3.7b**).

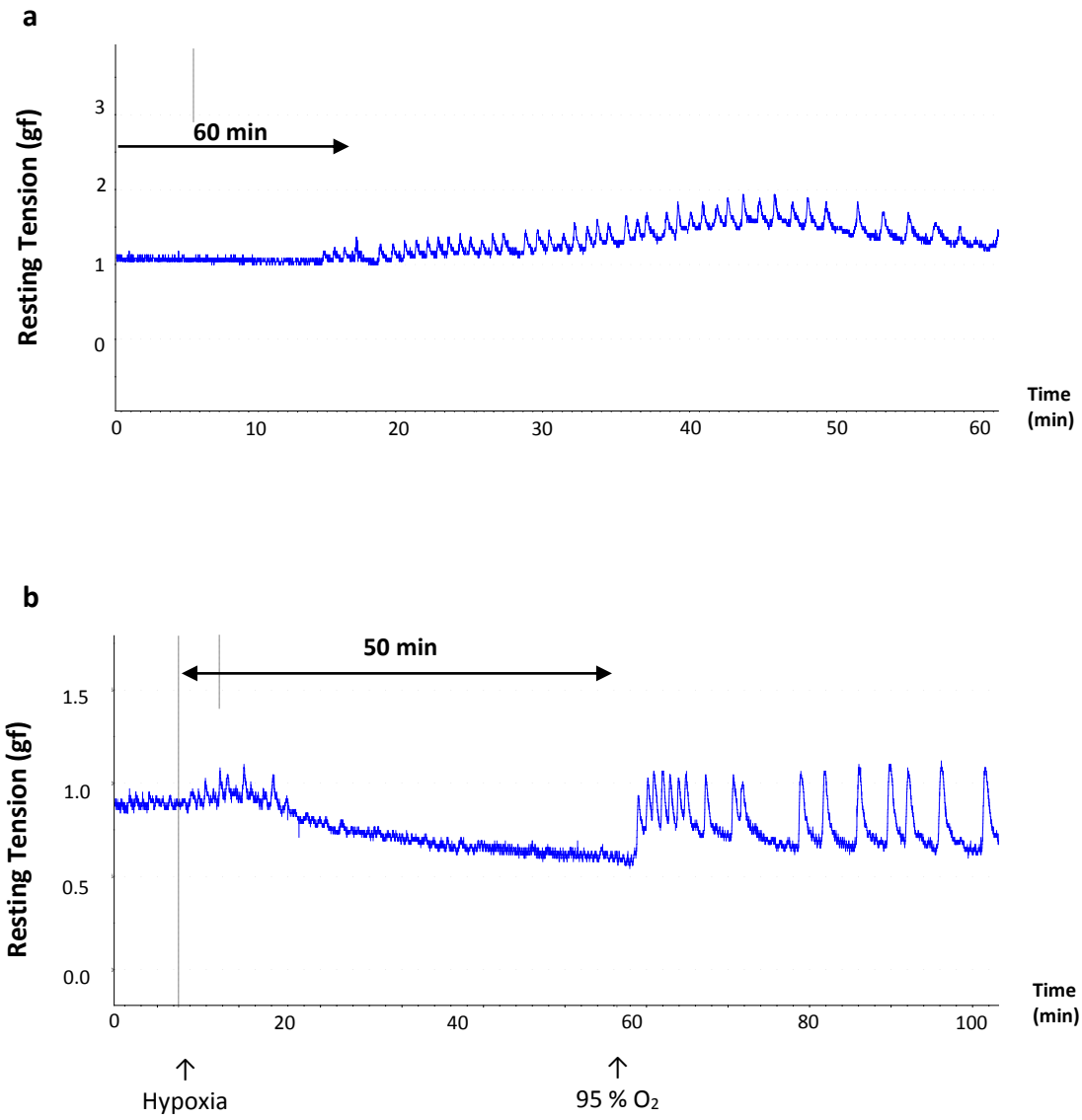

Figure 3.6 Oscillatory phasic activity in human pulmonary veins (**a**) Experimental trace showing spontaneous initiation of oscillatory phasic activity in an isolated human pulmonary vein 60 minutes from initiation of the experiment (**b**) Experimental trace showing precipitation of oscillatory phasic activity by hypoxia/reoxygenation in an isolated human pulmonary vein.

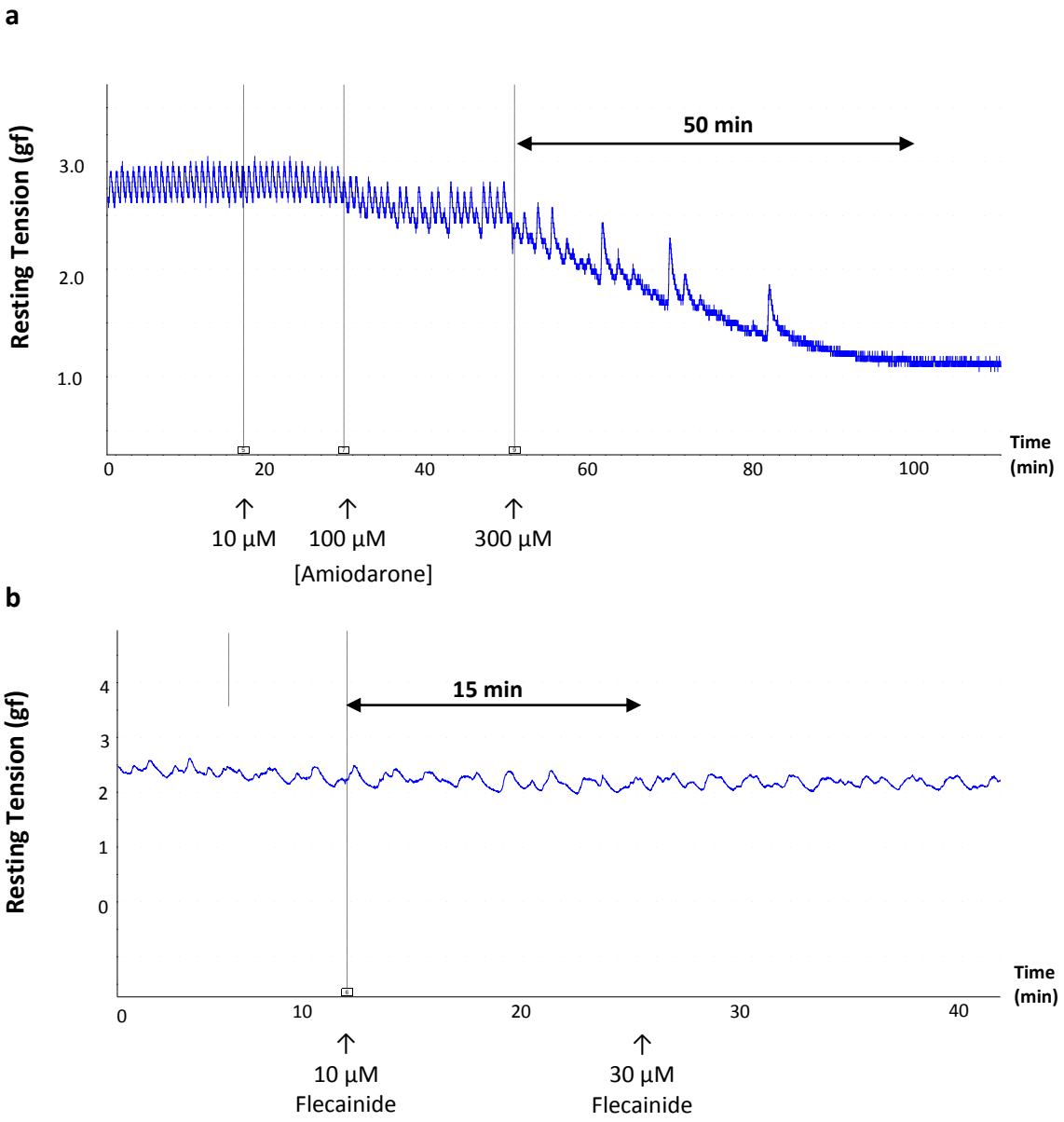

Figure 3.7 Effect of antiarrhythmic agents on oscillatory phasic activity in isolated human pulmonary veins. (**a**) Effect of amiodarone (10-300 µM) on resting tension and oscillatory phasic activity (**b**) Effect of flecainide (10-30 µM) on resting tension and oscillatory phasic activity.

3.4 Discussion

In this chapter the effects of hypoxia on resting and active tension in isolated human pulmonary arteries and isolated human pulmonary veins was investigated. It was hypothesised that hypoxia would cause a contraction of in vitro human pulmonary vessels. However, the results were unexpected and at variance with the published literature.

The effect of hypoxia on resting tension in isolated human pulmonary arteries was found to be variable. In most vessels hypoxia had no effect on resting tension but in some vessels hypoxia caused a significant relaxation which was not reversed by reoxygenation. None of the vessels contracted to hypoxia from resting tension. The effect of hypoxia on active tension was more consistent and hypoxia was found to cause a significant relaxation from active tension in vessels contracted to noradrenaline and endothelin-1, which was not reversed on reoxygenation. None of the arteries constricted to hypoxia.

The finding that hypoxia caused a relaxation in isolated human pulmonary arteries was surprising. A small number of previous studies have shown a constriction to hypoxia in isolated human pulmonary artery preparations which could be induced from resting tension [131, 132, 136] or required an element of precontraction [133-135]. It is difficult to explain the opposing effects of hypoxia on the human pulmonary arteries in this study and those reported in the literature. Some differences in experimental design could offer an explanation. In studies by Peinado *et al*. [133, 134] indomethacin was added to the buffer solution to inhibit cyclooxygenase activity. It would seem inappropriate in a physiological study of this nature to inhibit a potential vasodilator pathway in a system

that is inherently dependent on the balance between vasoconstrictor and vasodilator mechanisms and could have altered the results in the studies by Peinado *et al*. Of the remaining four studies reported in the literature the key experimental conditions were very similar to those of the current study; specimens were large lobar or segmental arteries (2-5mm external diameter) obtained at resection for carcinoma, the buffer solutions used were Krebs-Henseleit or Krebs-Ringers, resting tension was set at 1.6-3 gf and hypoxia was induced by aerating the buffer solution with 95% N_2: 5% CO_2. In the current study the effects of hypoxia on resting tension were variable and hypoxic vasodilation was only observed in five of twenty one arteries. In previous studies using human pulmonary arteries the effect of hypoxia on resting tension was also inconsistent; in some studies HPV could be induced from resting tension [131, 132, 136] whereas in another study it could not [135]. These results suggest an inherent variability in the response of large human pulmonary arteries to hypoxia. It is also possible that in the current study there was variability in the functional integrity of the endothelium that could account for the differences in the results. The role of the endothelium in the contractile response of human pulmonary arteries to hypoxia has previously been investigated. In one study [136] removal of the endothelium was found to either attenuate the pressor effect of hypoxia or convert the effect of hypoxia to a relaxation from resting or active tension in human PAs but in another study [137] removal of the endothelium augmented HPV in human PAs. In the current study control responses to acetylcholine were obtained in all preparations to determine the functional integrity of the endothelium. However, there was no obvious association between the response of the arteries to acetylcholine and the response to hypoxia. Not all arteries that relaxed to ACh relaxed to hypoxia and one artery relaxed to hypoxia but not to ACh. These

results suggest that the presence or absence of a functional endothelium was not the reason why HPV could not be induced in the current study and furthermore that the endothelium was not involved in the relaxant effect of hypoxia. However, it should be noted that a significant limitation of the current study is that the presence of an intact endothelium was determined by exposure of the vessels to 1 μM ACh from resting tension whereas in the majority of animal experiments the presence of a functional endothelium in isolated vessels is confirmed by exposure of precontracted vessels to 10 μM ACh and therefore the experimental protocol used in this study might not have been sensitive enough to accurately determine the presence or absence of a functional endothelium. It is therefore possible that the endothelium was not functionally intact in the vessels studied which could account for a lack of hypoxic vasoconstriction. These experiments should be repeated using a more appropriate method to determine the functional integrity of the endothelium in order to fully understand the role of the endothelium in the response of human pulmonary arteries to hypoxia. Another difference between this study and those described in the literature is that in the current study vessels were maximally precontracted to noradrenaline or ET-1, in contrast to previous studies where sub-maximal contractions were used, which may have precluded any further contraction to hypoxia. Experiments need to be repeated to investigate the effect of hypoxia on sub-maximally contracted human pulmonary arteries. It might be the case that large human pulmonary arteries do, overall, dilate to hypoxia and that previous groups have observed this effect but not reported it. The accepted paradigm, from animal studies, is that HPV occurs mostly at the level of the small resistance arterioles and that the larger pulmonary arteries are not involved [7].

Further experiments are needed using a myograph to measure the effects of hypoxia on vascular tone in small human pulmonary resistance arteries.

In human pulmonary veins hypoxia caused a significant relaxation from resting tension which was reversed on reoxygenation. Hypoxia also caused a significant relaxation in veins contracted to ET-1 which was not reversed on reoxygenation. This finding is at variance with a small number of animal studies which showed contraction of isolated pulmonary veins on exposure to hypoxia and suggests that there may be species differences in the response of the pulmonary veins to hypoxia. Only one previous study has described the effects of hypoxia on human pulmonary veins and found, in five isolated human pulmonary vein strips, that hypoxia had no effect on isometric tension [132]. In the current study the effect of hypoxia on the pulmonary veins was more robust and reproducible than in the pulmonary arteries. This finding suggests that the pulmonary veins might play a more significant role than the pulmonary arteries in the response of the human pulmonary circulation to hypoxia. However, the veins and arteries used in the studies described in this chapter were from different patients and it is therefore not possible to directly compare the magnitude of effects between the arteries and veins. The experimental design could have been improved by using vein and arterial samples from the same patient which would allow a direct comparison in the magnitude of the responses.

In this chapter an interesting and serendipitous discovery was made in the isolated human pulmonary vein preparation. It was found that human pulmonary veins exhibit pulsatile activity (as manifest by oscillatory phasic changes in isometric tension) which occurred spontaneously or was precipitated by exposure to hypoxia and

reoxygenation. Pulsatile activity in pulmonary veins, following exposure to hypoxia, has been recognised for some time. In 1757 Haller [144] observed pulsation of the pulmonary veins in open chest animals which lasted for two hours after the heart had stopped beating. In 1876 Brunton and Lauder [145] observed pulsations in the pulmonary veins of rabbits and cats after stopping artificial ventilation. The pulsations observed in the pulmonary veins were independent of pulsations originating from the heart and suggested that the pulmonary veins exhibit an independent electrical automaticity. In 1998, Haissaguerre *et al*. [146] published a landmark study showing that the pulmonary veins are a source of ectopic beats in the heart and can cause paroxysmal atrial fibrillation (PAF) in man. Subsequent anatomical studies [147, 148] have shown that the left atrial myocardium extends over the pulmonary veins, forming myocardial sleeves, and electrophysiological studies [149, 150] have confirmed that ectopic electrical activity in the pulmonary vein myocardium is involved in the pathogenesis of AF. Radiofrequency catheter or surgical ablation of the pulmonary veins is now routinely used to treat refractory AF. The vein specimens used in this study were from the distal portions of the pulmonary veins, near to the point of resection, which in most cases was near to or involving the myocardial sleeves. It would seem likely that the pulsatile activity observed in the human pulmonary veins is the result of an electrical automaticity and would therefore be arrhythmogenic in nature. Preliminary experiments were performed to investigate the effect of anti-arrhythmic agents on the pulsatile activity in the veins. In one vein preparation the anti-arrhythmic amiodarone caused a dose dependent relaxation and abolished pulsatile activity; however, in another vein preparation the antiarrhythmic flecainide had no effect on pulsatile activity. It is not possible to draw any conclusions about the nature of the pulsatile activity from these

experiments and electrophysiological studies will be needed to confirm if the pulsations in the veins represent true action potentials. If the pulsations in the pulmonary veins are arrhythmogenic in origin then *in vitro* human pulmonary veins will be a simple and effective model to investigate arrhythmogenic activity in the pulmonary veins. Furthermore, there is currently a lack of consensus on the optimum pharmacotherapy for AF [149] and isolated human pulmonary veins could be an excellent model in which to investigate the actions of antiarrhythmic drugs and to screen for potential new therapeutic agents.

Further evidence to suggest that the pulsatile activity in the veins is arrhythmogenic in nature is that in some of the pulmonary veins studied pulsations were precipitated by exposure to hypoxia and reoxygenation. A recent electrophysiological study [151] measured transmembrane action potentials in isolated rabbit pulmonary veins and found that arrhythmogenic activity was increased by exposure of the vessels to hypoxia and reoxygenation. Furthermore, there are a number of surgical procedures and clinical conditions which result in exposure of the lungs to hypoxia and reoxygenation and are associated with an increased incidence of AF. Atrial fibrillation is a frequent postoperative complication of cardiac surgery [75]. During cardiac surgery with cardiopulmonary bypass (CPB) the lungs are often hypoxic for long periods of time (1-3h) and on termination of CPB are exposed to high concentrations of oxygen. Thoracic surgery of the lung is also associated with periods of cardiac and pulmonary hypoxia and a high incidence of post-operative AF [5,6]. Sleep apnoea can cause intermittent periods of hypoxaemia and reoxygenation and the magnitude of nocturnal oxygen desaturation has been shown to be an independent risk factor for the development of AF in sleep apnoea subjects [152]. Future studies in isolated human

pulmonary veins could investigate the mechanisms of hypoxia/reoxygenation induced AF and provide a basis for the development of therapeutics to prevent or treat hypoxia/reoxygenation induced AF.

It should be mentioned that two of the veins in this study did not display pulsatile activity; one vein which was not exposed to hypoxia and one which was. One possible explanation for the lack of pulsatile activity might be that these vessels did not contain myocytes, being obtained from sections of vein proximal to the lung. It is known that the myocardial sleeves extend to different extents over the pulmonary veins, being longer in the superior veins and shortest in the right inferior vein [147, 148], and that the longer myocardial sleeves are associated with more abnormal electrical activity [146]. In future studies it will be important to record the anatomical origin of the specimens used and the proximity of the specimen to the myocardial sleeve.

In this chapter it was found, unexpectedly, that hypoxia caused a relaxation from resting and active tension in isolated human pulmonary arteries and isolated human pulmonary veins. None of the vessels studied exhibited a contraction to hypoxia. The effects of hypoxia were more robust and reproducible in the pulmonary veins than in the pulmonary arteries suggesting that the pulmonary veins play a more important role than the pulmonary arteries in the response of the human pulmonary circulation to hypoxia. These findings are at variance with the published literature and inconsistent with *in vivo* studies in man and further studies will be needed to clarify some of the inconsistencies between the results described in this chapter and the currently accepted paradigms.

A serendipitous discovery in this chapter was the pulsatile activity observed in the pulmonary veins. It is thought that this activity represents an intrinsic automaticity in the veins and is arrhythmogenic in nature. It is hoped that the *in vitro* human pulmonary vein preparation will prove to be a simple and effective model for investigating arrhythmic activity in the pulmonary veins and facilitate the development of new and effective treatments for atrial fibrillation.

CHAPTER 4

Effects of Hypoxia on the Human Pulmonary Vasculature: *Ex Vivo* Human Lungs

Contents

4.1 Introduction

The effects of hypoxia on pulmonary vascular tone have been investigated in *ex vivo* isolated perfused lung (IPL) preparations from numerous species [7]. The vast majority of studies found that exposure of lungs to hypoxia caused an increase in pulmonary vascular resistance (PVR). However, some studies have shown inconsistency. Sylvester *et al*. [153] and Peake *et al*. [154] compared the effects of hypoxia in isolated lungs from pigs, dogs, cats, ferrets and rabbits. They were unable to induce HPV in isolated dog lungs and in the remaining species observed marked differences in the magnitude of HPV. Furthermore, at low partial pressures of oxygen they observed a steady fall in pulmonary artery pressure in all species studied. To date, no previous studies have investigated the effect of hypoxia in an *ex vivo* human lung preparation.

In addition to the effect of hypoxia on pulmonary vascular tone, hypoxia has also been shown to have an oedemagenic effect in the lung. Hypoxia has been shown to either cause or exacerbate pulmonary oedema in acute lung injury (ALI) [108], high altitude pulmonary oedema (HAPE) [72, 109] and decompensated heart failure. The oedemagenic effects of hypoxia in the lung can result from different pathophysiological effects including increased hydrostatic pressure, due to hypoxic vasoconstriction, an increase in pulmonary vascular permeability [155] and disruption of alveolar epithelial cell function [156] . Although the majority of published studies have shown that hypoxia augments pulmonary oedema a significant number of studies in animals [157-159] and man [160, 161] have reported that hypoxia attenuates or even prevents oedema formation in the lung. To date the effects of hypoxia on pulmonary vascular permeability have not been investigated in *ex vivo* human lungs.

Ex vivo isolated perfused lung (IPL) preparations from numerous species have been used to investigate the effect of hypoxia on pulmonary vascular tone and pulmonary vascular permeability. However, significant species differences have been identified in the response to hypoxia and to date no studies have investigated the effects of hypoxia in an *ex vivo* human lung model. In considering the weight of evidence from previous animal studies in IPL preparations it was hypothesised that exposure of human IPLs to hypoxia would cause an increase in pulmonary vascular tone and an increase in oedema formation. The specific aims of studies in this chapter were, for the first time, to:

1. Determine the effect of hypoxia on pulmonary vascular tone in *ex vivo* human lungs

2. Determine the effect of hypoxia on oedema formation in *ex vivo* human lungs

4.2 Methods

4.2.1 Sample Preparation and Exposure to Hypoxia

The studies described in this section, to investigate the effects of hypoxia on the pulmonary circulation, are from the same lung preparations described in ***6.2.1.1***.

Lung samples were obtained from patients undergoing resection for cancer. Samples were prepared and connected to the modified perfusion-ventilation system for creating hypoxic conditions (***Chapter 2, 2.2.12.3***)**.**

Lung samples were ventilated with room air. The respiration rate was set at 10 breaths per minute (BPM) for the duration of the experiment. The tidal volume was set, depending on the size and airway resistance of the lung sample, to achieve a peak airway pressure of less than 18 mmHg, and ranged between 100 and 200 ml. The perfusion flow rate was gradually increased to achieve a mean perfusion pressure between 15 and 20 mmHg and ranged between 100 and 200 ml min^{-1}.

Weight (g), perfusion pressure (PP) (mmHg) and airway pressure (AP) (mmHg) was continuously recorded.

Lungs were allowed to equilibrate for at least 60 minutes before starting experimental procedures. When baseline airway and perfusion pressures were stable the lungs were exposed to hypoxia **(*Chapter 2, 2.2.12.3*).**

4.2.2 Effect of Hypoxia on Perfusion Pressure

Perfusion flow rate was constant for each experiment and changes in perfusion pressure were taken to represent changes in pulmonary vascular resistance.

Perfusate samples were taken from the circuit proximal to the arterial cannulae for analysis of perfusate pH, pO_2, pCO_2 and HCO_3 with a blood gas analyser (Gem Premier 3000, Instrumentation Laboratory Company, Lexington, MA, USA) at four time points; Pre hypoxia, 20 minutes post hypoxia, 50 minutes post hypoxia and after return to normoxic ventilation and perfusion.

Mean perfusion pressure was obtained for 10 minute periods at five time points; 10 minutes pre hypoxia, 10, 20 and 50 minutes post hypoxia and 10 minutes after resuming ventilation with room air.

4.2.3 Effect of Hypoxia and Reoxygenation on Pulmonary Vascular Permeability

An increase in the rate of weight gain of the lungs was taken as an indication of oedema formation and was quantified as % of the initial lung weight per minute (% min^{-1}). The initial weight of the lungs was taken 5 minutes after initiation of perfusion to allow for filling of the circulation.

The rate of weight gain of the lungs was calculated for 3 time periods; (1) 30 minutes before exposure to hypoxia (baseline), (2) during exposure to hypoxia and (3) after reoxygenation.

4.3 Results

4.3.1 Effect of Hypoxia on Perfusion Pressure

Lung samples (10 lobes and 2 whole lungs) were obtained from twelve male patients (age 68 ± 11 years) undergoing resection for cancer. Patient and specimen details with individual experimental parameters are shown in **table 4.1.**

Table 4.1 *Ex Vivo* Human lungs: Patient and specimen data with experimental parameters.

	Patient		**Specimen**		**Experimental Parameters**		
Prep	**Sex**	**age (years)**	**Lobe**	**Weight (g)**	**Perfusate Flow (ml/min)**	**Tv (ml)**	**BPM**
Hull 15	m	79	Left Upper	190	100	100	10
Hull 15 (h)	m	65	Right Upper	398	100	100	10
Hull 17 (h)	m	75	Right Lower	310	100	100	10
Hull 21	m	61	Right Upper	348	100	100	10
Hull 25	m	53	Left Upper	177	100	100	10
Hull 28	m	73	Right Upper	244	100	100	10
Hull 30	m	76	Left Lower	265	100	100	10
Hull 31	m	55	Left Lung	480	200	200	10
Hull 32	m	75	Right Upper	330	100	100	10
Hull 33	m	51	Right Upper	240	100	100	10
Hull 34	m	79	Right Upper	421	150	100	10
Hull 41	m	79	Left Upper	261	100	100	10
Mean		**68**		**305**			
SD		**11**		**94**			

Four of the lungs in this series of experiments demonstrated excessive oedema formation (> 0.5 % min^{-1}); however, the oedema formation did not affect perfusion pressure and the results were included in the analysis. Two lungs (hull 28 and hull 41) were excluded from the analysis (of effects on perfusion pressure) because pressure measurements were unreliable. Exposure to hypoxia caused a significant decrease in

perfusate pO_2 after 20 and 50 minutes which returned to pre-exposure concentrations 10 minutes after return to ventilation with room air (**table 6.2, figure 4.1**). The lungs were exposed to hypoxia for 67 ± 45 minutes which resulted in a reduction in mean perfusion pressure which was significant after 20 and 50 minutes and was reversed on return to ventilation with room air. Mean perfusion pressure at baseline, after 10, 20 and 50 minutes exposure to hypoxia and on return to ventilation with room air are shown in **table 4.2** and change in perfusion pressure with corresponding perfusate pO_2 at 20 and 50 minutes post hypoxia shown in **figure 4.1** (Raw data Appendix II). The effects of hypoxia on perfusion pressure were not obtained at every time point in some experiments which precluded the use of a repeated measures ANOVA (analysis of variance) to analyse the effects of hypoxia on perfusion pressure over time. The effects of hypoxia on perfusion pressure at different time points were therefore compared to the baseline perfusion pressure at each time point via related samples Wilcoxon signed-rank test and the number of paired values for each time point shown in **table 4.2** and **figure 4.1**. The pH, pCO_2 and electrolyte concentrations of the perfusate remained within normal physiological parameters during the experiments.

Table 4.2 Change in mean perfusion pressure (mmHg) on exposure to hypoxia in *ex vivo* human lungs. **p* < .05 vs. baseline via related samples Wilcoxon signed-rank test. n = number of paired values obtained at each time point

	Change in Mean Perfusion Pressure (mmHg)				
	Base Line (Room Air)	**10 min Post Hypoxia**	**20 min Post Hypoxia**	**50 min Post Hypoxia**	**Room Air**
Mean	0.00	-0.70	-1.24*	-1.88*	-0.12
SD	0.00	1.17	1.44	2.23	1.63
n	10	10	9	6	7

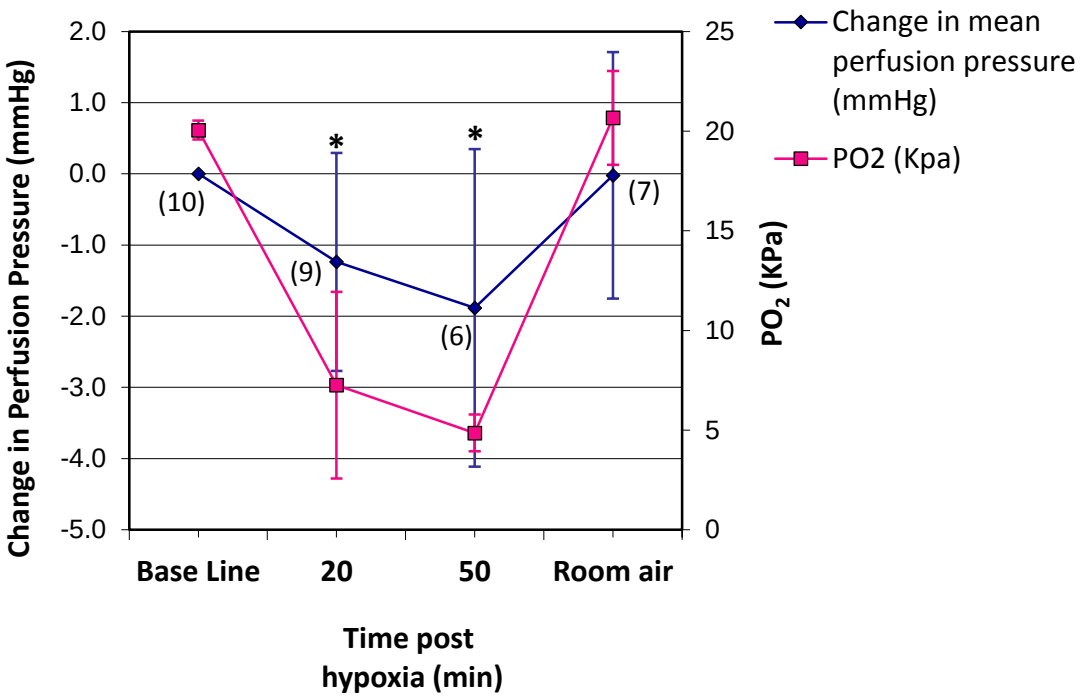

Figure 4.1 Change in mean perfusion pressure with change in perfusate pO_2 in *ex vivo* perfused and ventilated human lungs. Numbers in parentheses denote number of values obtained at each time point and compared to baseline. *$p < .05$ via related samples Wilcoxon signed-rank test vs. baseline.

4.3.2 Effect of Hypoxia on Pulmonary Vascular Permeability

There was considerable variability in the rate of weight gain data for the lung samples studied. Rate of weight gain was significantly less under hypoxia compared to pre hypoxia. Rate of weight gain after reoxygenation was not significantly different to the rate of weight gain pre hypoxia or during hypoxia (**table 4.3, figure 4.2)** (Raw data Appendix II).

Table 4.3 Rate of weight gain (% of initial Wt. min^{-1}) pre hypoxia, during hypoxia and on reoxygenation in *ex vivo* isolated perfused human lungs. n=12, **p* < .05 vs. base line via related samples Wilcoxon signed-rank test

	Rate of weight gain (% min^{-1})		
	Pre Hypoxia	**Hypoxia**	**Reoxygenation**
Mean	0.77	0.30*	0.27
SD	0.83	0.35	0.32

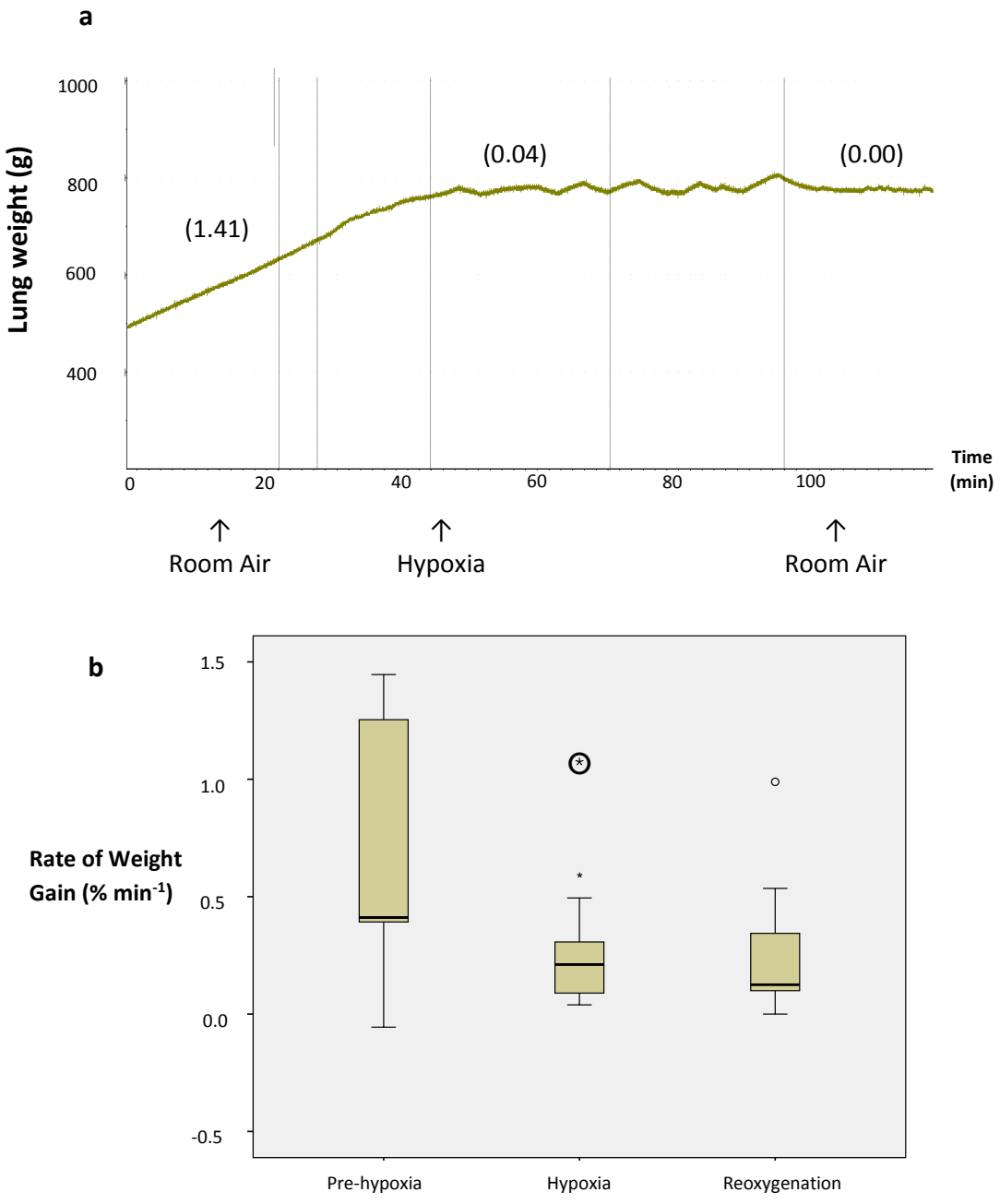

Figure 4.2 Effect of hypoxia and reoxygenation on rate of weight gain (% of initial weight min^{-1}) in *ex vivo* human lungs. **(a)** Experimental trace showing effect of hypoxia and reoxygenation on rate of weight gain in an *ex vivo* perfused and ventilated human lung preparation. Rate of weight gain (% of min^{-1}) shown in parentheses. Dotted lines are comment markers. **(b**) Box whisker plot showing rate of weight gain in isolated lungs under different conditions: pre- hypoxia, during hypoxia and on reoxygenation (n=12). The hypoxia data contained one extreme outlier (circled asterisk) and the reoxygenation data had three missing data points and one outlier (circle). **p* < .05 vs. pre- hypoxia via Wilcoxon signed-rank test.

4.4 Discussion

The original hypothesis to this chapter was that hypoxia would cause an increase in pulmonary vascular pressure and an increase in oedema formation in isolated perfused human lungs. However, all of the results in this investigation were unexpected and at variance with a large body of published literature. The predominant effect of hypoxia on pulmonary vascular tone was found to be a relaxation and not a constriction as expected and hypoxia inhibited oedemagenesis where it was expected to increase pulmonary vascular permeability.

In this chapter the effects of hypoxia on pulmonary vascular resistance (PVR) was investigated for the first time in *ex vivo* human lungs. Hypoxia in the *ex vivo* human lung was found to cause a marginal but significant fall in PVR which was reversed on returning to ventilation with room air. This finding was unexpected and inconsistent with a large body of published literature. It is difficult to explain why hypoxia caused a vasorelaxation in the human lung whereas in isolated perfused lung (IPL) preparations from numerous other species [7] hypoxia has been shown to cause a vasoconstriction. Some limitations in the experimental design could explain why hypoxia induced vasodilation rather than vasoconstriction in the human lung model:

1. The buffer solution used in this model was asanguineous. Some studies in *ex vivo* rat lungs have shown that blood is required in the perfusate in order to induce HPV and that HPV did not occur in buffer perfused rat lungs [162] . One explanation for this is that buffer solutions are not capable of retaining oxygen (compared to blood) and that oxygen partial pressures in the buffer and alveoli will quickly equilibrate to a pO_2 in the alveoli and perfusate which is too high to trigger a HPV

response. This could not have occurred in the present study because hypoxia was induced in both the perfusate and alveoli. Another explanation which has been suggested is that the erythrocytes play an instrumental role in mediating HPV. However, this also seems unlikely because HPV has been demonstrated in numerous buffer perfused lung preparations [163, 164]. It would seem that the need for blood in the perfusate is peculiar to rats; however, further studies using a blood perfusate are warranted in the human lung model to resolve this issue.

2. The isolated lungs used in this study are flushed with 1000 iu heparin to prevent clot formation and pulmonary emboli. In addition to its anticoagulant effect heparin also inhibits intracellular IP_3 receptor- mediated Ca^{2+} release in human airway smooth muscle [165] and it is possible that heparin may have prevented vasoconstriction via this mechanism. However, this seems unlikely for two reasons; firstly, the perfusate is regularly replaced during the normalisation period which would wash out the majority of heparin and secondly the half-life for heparin is 1.5 hours and it is largely metabolised in macrophage and endothelial cells and would therefore have been mostly removed before the lungs were exposed to hypoxia.

3. The lungs release vasodilators which are recirculated and counteract vasoconstriction to hypoxia. Although this explanation is possible the most likely vasodilators to be released by the lungs would be prostanoids or nitric oxide and addition of the prostanoid antagonist indomethacin and the nitric oxide synthase inhibitor L-NAME (n=1) (results not shown) to the perfusate failed to unmask a constriction to hypoxia.

4. An element of precontraction is necessary to induce HPV. Some studies in isolated lungs have shown that an element of precontraction is needed to reveal a pressor response to hypoxia [162, 166]. However, in this study precontraction to KCl (n=3) failed to unmask a pressor response to hypoxia.

5. Age could be a contributing factor. Animal studies generally use lungs from young animals whereas the lung specimens used in this study were obtained from comparatively old patients (68 ± 11 years). In isolated rat lungs HPV has been shown to diminish with advancing age [167]. It is possible that the vasopressor response to hypoxia is lost with advancing age in humans to reveal the vasodilator effects of hypoxia described in this chapter. Further studies are needed to determine if the pulmonary vascular response to hypoxia alters with age in humans.

6. It is possible that the tumour which is present in the *ex vivo* lung preparations could release pharmacologically active agents which alter the vascular response. A significant limitation of the human lung model is the presence of a tumour which is of unknown size and type. Further studies are needed to determine if different types of tumour do release pharmacologically active agents which could alter vascular responses.

Although the majority of animal studies have shown a pressor response to hypoxia in isolated lungs there are some inconsistencies in the literature. Sylvester *et al*. [153] and Peake *et al*. [154] compared the effects of hypoxia in isolated lungs from pigs, dogs, cats, ferrets and rabbits. They were unable to induce HPV in isolated dog lungs. In the remaining species they observed marked differences in the magnitude of HPV and the

pO_2 required to elicit a hypoxic response. Furthermore, at low partial pressures of oxygen (below that needed to induce HPV) they observed a steady fall in pulmonary artery pressure in all species studied. The vasodepressor effect observed by Peak *et al*. at low partial pressures of oxygen is consistent with the findings of the current study where the hypoxic perfusate pO_2 was 5-7 mmHg. However, in the current study decreasing pO_2 did not induce an initial hypoxic contraction.

In isolated human pulmonary arteries and isolated human pulmonary veins hypoxia caused a relaxation from resting and active tension (***Chapter 3***) which was consistent with the findings in the *ex vivo* human lung model. This finding is contrary to the accepted paradigm and at variance with *in vivo* studies which have convincingly shown that hypoxia causes pulmonary vasoconstriction in man [43-50]. The currently accepted paradigm is that the effects of hypoxia in the lung are mediated by a local effect on the vascular smooth muscle of the pulmonary arteries and are not subject to major hormonal or neuronal control. However, one possible explanation for the discrepancy between the *in vitro* results in this thesis (***Chapter 3 and this chapter***) and *in vivo* results is that there is a significant neurogenic component to the hypoxic response in human lungs which is missing *in vitro.* Further support for this suggestion comes from two recent studies by Chen *et al.* [168, 169] investigating the neural modulation of pulmonary vascular tone in IPAH (Idiopathic pulmonary arterial hypertension). Like HPV, the pathogenesis of IPAH is thought to be a local effect originating in the pulmonary vascular smooth muscles (being an imbalance in the production of vasodilators and vasoconstrictors). However, some studies have shown the existence of baroreceptors at the bifurcation of the main pulmonary artery which exert a pulmo-pulmonary reflex regulation of pulmonary vascular tone [170, 171]. It was shown that

distension of the main PA in the region of the bifurcation causes an increase in pulmonary artery pressure and that this reflex is abolished by surgical denervation of the bifurcation but not by vagotomy. Chen *et al*. investigated the therapeutic potential of pulmonary artery denervation in the treatment of pulmonary arterial hypertension (PAH). In dogs they showed that radiofrequency ablation of the pulmonary artery bifurcation completely abolished PAH induced by balloon occlusion of the left pulmonary inter lobar artery [168]. In a recent clinical study Chen *et al*. showed that pulmonary artery denervation caused a significant reduction in mean PAP and had significant clinical benefits in IPAH patients [169]. Furthermore, these findings could also offer another explanation as to why it was not possible to induce HPV in *ex vivo* human lungs; the lungs used for these studies were resected at the level of the inter lobar or segmental arteries and did not include the large pulmonary branches where the pulmonary baroreceptors are thought to be located. In contrast, the lung specimens used in *ex vivo* animal experiments are resected at the origin of the main pulmonary artery and perfusion through the main pulmonary artery will clearly include perfusion of the large pulmonary artery branches and the putative pulmonary baroreceptors. Future *ex vivo* animal lung experiments could easily determine if denervation of the putative baroreceptor region in the bifurcation of the main pulmonary artery alters the HPV response in isolated lungs. These recent results suggest a significant neural involvement in the regulation of pulmonary vascular tone and further studies are indicated to investigate the neurogenic mechanisms in HPV.

In this chapter the effects of hypoxia on pulmonary oedema formation has been investigated for the first time in *ex vivo* human lungs. Exposure of the lungs to hypoxia caused a significant reduction in the rate of weight gain and the reduction in oedema

formation continued after reoxygenation. This result was surprising because numerous previous studies in *ex vivo* lungs from different species including rat [172], dog [173] and pig [174] found that hypoxia caused an increase in oedema formation. Furthermore, reoxygenation after a period of hypoxia has previously been shown to cause acute lung injury in isolated rat lungs [175] which manifests as an increase in filtration coefficient and weight gain.

Although most studies have shown an oedemagenic effect of hypoxia in the lung a significant number of studies have also shown a different effect of hypoxia in the lung. *In vivo* studies in sheep [157] and dogs [158] showed no effect of hypoxia on oedema formation in the lung and other studies in rats [159] and healthy human subjects [160, 161] showed that hypoxia increased fluid clearance in the lung.

It was not possible in the current study to determine if the reduction in oedema formation was caused by the reduction in hydrostatic pressure (due to pulmonary vasodilation) or a reduction in vascular permeability. It would seem unlikely that the marginal reduction in perfusion pressure observed in the lungs could account for the reduced oedema formation and furthermore the rate of weight gain did not increase again on reoxygenation when the perfusion pressure returned to baseline. However, in ***Chapter 3*** it was found that the vasodilator effect of hypoxia was more consistent, robust and reproducible in the pulmonary veins than in the pulmonary arteries. A proportionately larger fall in pulmonary venous pressure may have accounted for the decrease in oedemagenesis. Again, this seems unlikely because oedema formation did not increase on reoxygenation. It would therefore seem likely that the observed reduction in oedemagenesis with exposure to hypoxia was due, at least in part, to a

reduction in vascular permeability. Vascular permeability is determined by the barrier function of the vascular endothelial cells (ECs). Vascular endothelial cells contain an abundance of contractile elements, for example actin, myosin and an actin based cytoskeleton and the balance between contraction and relaxation of vascular ECs is thought to play a central role in the regulation of vascular permeability. An increase in the contractile status of vascular endothelial cells causes opening of intercellular gaps (adherens junctions and tight junctions) and a resulting increase in vascular permeability (for a full description see Dudek and Garcia [176]). Given that hypoxia was found to cause a relaxation of human pulmonary vascular smooth muscle, as manifest by a relaxation of human pulmonary arteries and veins (***Chapter 3***) and a decrease in perfusion pressure in isolated human lungs in this chapter, it is tempting to suggest that hypoxia could have caused a reduction in the rate of oedemagenesis in the isolated lungs by inhibiting EC contraction and increasing endothelial barrier function. However, it has previously been identified that there are significant differences in the regulation of the MLCK isoform in endothelial cells compared to vascular smooth muscle cells [176] (and presumably ASM cells) and that there are distinct differences in the signalling pathways regulating barrier function in the pulmonary and systemic vasculature [177]. Further functional and molecular studies are warranted, using human pulmonary vascular EC models, to investigate the effect of hypoxia on human pulmonary EC contractility and barrier function.

One method which can be used to distinguish between the hydrostatic and permeability components of oedema formation is to measure the pulmonary capillary filtration coefficient using the hydrostatic challenge method described in ***Chapter 5, 5.2.1***. Further studies are needed to investigate the effects of hypoxia on vascular

permeability in the *ex vivo* human lung model and to determine the mechanism by which hypoxia causes a reduction in pulmonary vascular permeability which could lead to the development of novel therapeutics for the treatment of pulmonary oedema in lung diseases such as acute lung injury (ALI) and acute respiratory distress syndrome (ARDS).

If hypoxia also reduces pulmonary vascular permeability *in vivo* then there is a therapeutic potential for hypoxic ventilation. For instance, during cardiac surgery with cardiopulmonary bypass (CPB) patients sometimes develop pulmonary oedema – so called "pump lung" [1]. Since patients are systemically oxygenated during CPB it may be possible to prevent oedemagenesis during CPB by ventilating the lungs with a hypoxic gas mixture. Similarly for patients on ECMO (extra corporeal membrane oxygenation) for respiratory failure periods of hypoxic ventilation could potentially attenuate pulmonary oedema. Further studies are needed to investigate the therapeutic potential of lung hypoxia in reducing pulmonary oedema during cardiac surgery with CPB and in the critical care setting for patients undergoing ECMO therapy. Another potential application for the oedema reducing effects of hypoxia is in *ex vivo* lung perfusion (EVLP) for clinical lung transplantation. EVLP is increasingly being used for the preservation, optimisation and assessment of donor lungs before transplantation [178, 179] and clinical trials have shown that EVLP can be used to successfully recondition donor lungs, to within acceptable physiological parameters, which would otherwise have been deemed unsuitable for transplantation [178, 180]. However, a significant limitation of EVLP is pulmonary oedema [181] and it is possible that periods of hypoxic perfusion and ventilation could help to reduce oedemagenesis and improve lung function during EVLP of donor lungs. Further studies are warranted to investigate the

potential benefits of hypoxic ventilation and/or perfusion in reducing oedema and preserving lung function during EVLP of donor lungs in clinical lung transplantation.

In this chapter it has been shown for the first time in *ex vivo* human lungs that hypoxia causes a decrease in pulmonary vascular resistance which is consistent with the *in vitro* results described in ***Chapter 3***. The prevailing *in vitro* effect of hypoxia on the human pulmonary vasculature has therefore been shown to be a relaxation. This finding is at variance with a large body of published literature and is inconsistent with *in vivo* studies in man which have convincingly shown that hypoxia causes pulmonary vasoconstriction. The discrepancy between the *in vitro* studies described in this thesis and *in vivo* studies suggests that, contrary to the accepted paradigm, there could be a significant neural component to the hypoxic pulmonary vascular response in man. This suggestion is supported by recent clinical evidence [169] and is in need of further investigation.

Another significant finding in this chapter was that hypoxia reduces oedema formation in the human lung which is in agreement with previous studies which have shown that hypoxia reduces pulmonary oedema by reducing pulmonary vascular permeability. Further studies are warranted to determine the mechanism by which hypoxia reduces pulmonary oedema which could identify potential therapeutic targets for the treatment of pulmonary oedema in conditions such as ARDS and ALI. Further studies are warranted to investigate the therapeutic potential of hypoxic lung ventilation in reducing pulmonary oedema during surgical procedures and therapies using cardiopulmonary bypass and the potential for lung hypoxia in reducing oedemagenesis during EVLP of donor lungs in clinical lung transplantation.

CHAPTER 5

Effects of Endothelin-1 on the Human Pulmonary Vasculature

Contents

5.1 Introduction

A large body of literature has investigated the effects of hypoxia in the lung. However, despite extensive research efforts the exact sensor, mediator and effector mechanisms of hypoxia in the lung have not been clearly defined. The potential of the potent vasoconstrictor endothelin-1 (ET-1) in mediating at least some of the effects of hypoxia in the lung has received considerable interest in the literature.

ET-1 was first identified by Yanagisawa in 1988 [182] and remains the most potent vasoconstrictor so far identified in man. The highest levels of ET-1 in the body are found in the lung and ET-1 has been implicated in the pathogenesis of numerous pulmonary diseases [183, 184]. Extensive research has realised the clinical benefits of endothelin antagonists in the treatment of pulmonary arterial hypertension of various aetiologies [185-190]. ET-1 has also been shown to play a role in the effects of hypoxia in the lung. *In vivo* studies in rats [191], pigs [192] and humans [50] found that endothelin receptor antagonists attenuate hypoxic pulmonary vasoconstriction. In isolated perfused rat lungs hypoxia caused an increase in perfusate ET-1 concentrations, perfusion pressure and lung weight and changes in perfusion pressure and lung weight were attenuated by endothelin antagonists or endothelin converting enzyme inhibitors [193]. Another study in isolated blood and buffer perfused rat lungs also showed that endothelin antagonists attenuate HPV [191]. In contrast, other studies in isolated rat lungs found that perfusate ET-1 concentrations were increased on exposure to hypoxia but concluded that ET-1 did not mediate the pressor effect of hypoxia [194, 195]. In primary cultures of rat pulmonary artery smooth muscle cells hypoxia induced synthesis of HIF-1 α (hypoxia- inducible factor-1 α) was inhibited by the selective ET_A antagonist BQ-123

[196]. ET-1 has also been implicated in the pathogenesis of high altitude pulmonary oedema (HAPE). At high altitude plasma ET-1 concentrations in healthy volunteers were shown to correlate with decreased arterial oxygen saturations and increased pulmonary arterial pressure (PAP) [197, 198]. In another study the endothelin antagonist bosentan significantly attenuated the increase in PAP caused by high altitude in mountaineers compared to control subjects [199]. There is currently some debate in the literature regarding the mechanisms of HAPE; some groups argue that HAPE is mediated exclusively by an increase in hydrostatic pressure (due to HPV) [70-72] whereas others suggest that an increase in pulmonary vascular permeability, due to an inflammatory response, is also involved [73, 74]. Whether the effects of ET-1 in HAPE are mediated solely by an increase in PAP or also involves an inflammatory effect, causing an increase in pulmonary vascular permeability, has not been investigated.

Previous studies by this group have shown that ET-1 is a potent vasoconstrictor [111] and causes a fulminant pulmonary oedema [112] in *ex vivo* human lungs. These effects are similar to the effects of hypoxia reported in the literature and suggested that ET-1 might mediate the effects of hypoxia in the lung. In these previous studies it was noted that the magnitude of contraction to ET-1 did not correlate with the magnitude of oedema formation which suggests that, in addition to causing oedema by increasing hydrostatic pressure, ET-1 was also causing an increase in vascular permeability. However, in these previous studies it was not possible to differentiate between the hydrostatic and vascular permeability components of the oedema formation. In order to differentiate between hydrostatic effects and permeability effects on oedema formation it is necessary to determine the capillary filtration coefficient ($K_{f,c}$) which is a measure of membrane permeability. $K_{f,c}$ is determined by increasing the pulmonary

venous pressure and measuring the gravimetric rate of weight gain at a specified time point during the hydrostatic challenge.. The effect of drugs or changing experimental conditions on membrane permeability can be investigated by comparing baseline and post exposure $K_{f,c.}$ values. *Ex* v*ivo* animal lungs have previously been used to measure capillary filtration coefficient [200-202] (reviewed by Parker and Townsley and Bhattacharya [203-205]). In this chapter the *ex vivo* human lung model was modified to allow determination of $K_{f,c}$. Furthermore, a novel method has previously been described to differentiate between the hydrostatic and permeability effects of ET-1 on oedema formation in the lung. Kuklin *et al*. [206] used papaverine in *ex vivo* rat lungs to induce a vasoplegia and prevent vasoconstriction to ET-1 before measuring the effects of ET-1 on vascular permeability. In the current chapter this method was employed to determine the vascular permeability component of ET-1 induced pulmonary oedema in *ex vivo* human lungs.

Previous *in vitro* animal [207] and human studies [208] have shown that there is a differential response of pulmonary arteries and veins to contractile and relaxant agonists. A number of *in vitro* animal studies have compared the effects of ET-1 on pulmonary arterial and venous tone and shown that ET-1 is a more potent and more efficacious constrictor in the pulmonary veins than the pulmonary arteries [194, 207, 209-212] and in isolated rat lungs the preferential venoconstriction by ET-1 was responsible for oedema formation [194]. These findings suggest that the contractile effects of ET-1 on the pulmonary veins is of more pathophysiological significance than the effect of ET-1 on the pulmonary arteries; however, to date no previous studies have investigated the differential contractile effects of ET-1 in human pulmonary arteries and veins, which is a significant gap in the literature.

Reports in the literature suggest that ET-1 could have a role in mediating the effects of hypoxia in the lung. We have previously shown that ET-1 is a potent vasopressor and causes a significant increase in oedema formation in *ex vivo* human lungs. These effects of ET-1 are similar to the effects of hypoxia previously described in *ex vivo* animal lung studies and it was hypothesised that if ET-1 does play a role in mediating the effects of hypoxia in the lung then it would have a similar effect to hypoxia on pulmonary vascular permeability and vascular tone in human lung preparations. The purpose of the studies in this chapter was to further examine the effects of ET-1 on pulmonary vascular permeability and vascular tone with a view to comparing the effects of ET-1 to the effects of hypoxia on the human pulmonary vasculature. The specific aims of this chapter were:

1. To determine the effect of ET-1 on vascular permeability in *ex vivo* human lungs
2. To compare the effect of ET-1 on vascular tone in isolated human pulmonary arteries and isolated human pulmonary veins

5.2 Methods

5.2.1 *Ex Vivo* Human lungs: Effect of Endothelin-1 on Pulmonary Vascular Permeability

5.2.1.1 Capillary Filtration Coefficient ($K_{f,c}$)

In order to differentiate between the effects of changes in hydrostatic pressure and changes in membrane permeability on oedema formation it is necessary to determine the capillary filtration coefficient ($K_{f,c}$). The $K_{f,c}$ is a measure of membrane permeability and is given by the equation:

$$K_{f,c} = \frac{(\Delta W/\Delta t)}{\Delta P_{pc}}$$

Where $(\Delta W/\Delta t)$ = rate of weight gain and P_{pc} = capillary filtration pressure and is given by the equation:

$$P_{pc} = (P_{pa} - P_{pv})/2$$

Where P_{pa} = pulmonary arterial pressure and P_{pv} = pulmonary venous pressure.

$K_{f,c}$ is determined by increasing the pulmonary venous pressure and measuring the gravimetric rate of weight gain at a specified time point during the hydrostatic challenge. Where the baseline weight gain is not isogravimetric then the baseline $\Delta W/\Delta t$ is subtracted from the $\Delta W/\Delta t$ value obtained after increasing the $P_{pv.}$ The effect of drugs or changing experimental conditions on membrane permeability can be investigated by comparing baseline and post exposure $K_{f,c.}$ values.

5.2.1.2 Modified Perfusion Circuit for Determination of $K_{f,c}$

The perfusion system was modified to allow determination of $K_{f,c}$; after the airways and arteries had been cannulated the pulmonary veins were cannulated with the largest cannulae possible. The cannulae were connected together to form a single outlet and the air displaced by perfusing through the arterial system. The lung was then connected to the perfusion-ventilation system as described in ***Chapter 2, 2.2.8*** and the venous outlet connected to the perfusion circuit as illustrated in **figure 5.1**. A pressure line was connected to the venous outlet line for continuous measurement of venous pressure.

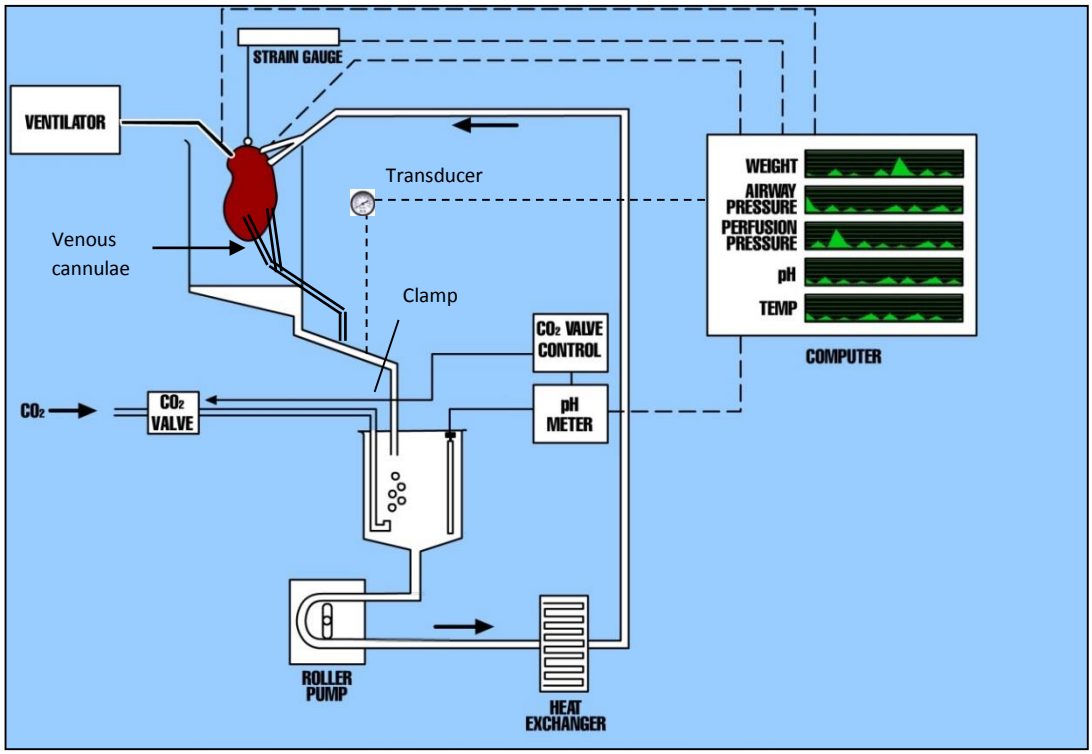

Figure 5.1 Modified perfusion-ventilation circuit for determination of $K_{f,c}$

Venous pressure was increased by partially occluding the venous outlet with a gate clamp. $K_{f,c}$ was determined by increasing the pulmonary venous pressure and measuring the rate of weight gain (over 5 minutes) after 5 minutes. The increase in P_{pv}

was at least 5 mmHg to ensure a measurable change in filtration rate. If the rate of weight gain before the hydrostatic challenge was not isogravimetric then the pre exposure ΔW/Δt was subtracted from the ΔW/Δt after increasing the $P_{pv.}$

5.2.1.3 Preliminary Experiments to Determine Dose of Papaverine to Prevent Contraction to ET-1

ET-1 causes pulmonary oedema by increasing hydrostatic pressure and increasing vascular permeability. In order to differentiate between the effects of increasing hydrostatic pressure and changes in vascular permeability it was necessary to prevent the vasopressor effect of ET-1. Papaverine was used to prevent vasoconstriction to ET-1.

Preliminary experiments were performed to determine the dose of papaverine needed to prevent a vasopressor response to 30 μM ET-1. Human lungs or lobes were obtained at resection for carcinoma and connected to the standard perfusion-ventilation system as described in ***Chapter 2, 2.2.8***. Lungs were ventilated with room air. After normalisation for 30-120 minutes, and when baseline parameters were stable, the lungs were exposed to 100 mM KCl to confirm vascular viability. Lungs that did not vasoconstrict to KCl were discarded. Washout of KCl was then performed over 10-20 minutes by regularly replacing the perfusate with fresh buffer solution until the perfusion pressure returned to baseline. Papaverine (0.1 μg – 120 mg) was then added to the perfusate and recirculated. When a stable baseline perfusion pressure was achieved the lungs were exposed to 30 μM ET-1.

5.2.1.4 Effect of ET-1 on capillary filtration coefficient ($K_{f,c}$)

Human lungs or lobes were obtained at resection for carcinoma, cannulated and connected to the perfusion-ventilation system as described in ***Chapter 2, 2.2.8***. Lungs were ventilated with room air. After equilibration for 60-90 minutes and when baseline parameters were stable 120 mg of papaverine was added to the perfusate (to prevent vasoconstriction to ET-1) and a baseline $K_{f,c}$ value obtained. Lungs were then exposed to 30 μM ET-1 for 5 minutes before determining the $K_{f,c}$ in the presence of ET-1.

5.2.2 *In Vitro* Human Pulmonary Arteries and *In Vitro* Human Pulmonary Veins

5.2.2.1 Tissue Preparation

Human lobar or inter lobar pulmonary arteries and human pulmonary veins were obtained at resection for lung cancer. Samples were prepared and mounted between stainless steel wires in organ baths to measure changes in isometric tension (***Chapter 2, 2.3***).

Resting tension was set at 1-2 gf and the vessels allowed to equilibrate. During equilibration the vessels were frequently washed and the resting tension adjusted to 1-2 gf.

5.2.2.2 Dose Response to ET-1

Dose responses to ET-1 were obtained in the arteries and veins by stepwise addition of increasing concentrations of ET-1 to the organ bath. The effect of each drug concentration was measured after the response had reached plateau and was the mean tension (gf) for the 2 minute period preceding the next increase in drug

concentration (obtained using the data pad function of the Chart software). Active tension was calculated as maximum response (at plateau) – resting (baseline) tension. Changes in active tension were quantified as absolute change in active tension (gf) or % change from baseline.

5.3 Results

5.3.1 Effect of Endothelin-1 (ET-1) on Pulmonary Vascular Permeability in *Ex Vivo* Human Lungs

5.3.1.1 Preliminary Experiments to Determine Dose of Papaverine to Prevent Contraction to ET-1

Lung samples were obtained from 7 patients. Patient demographics, sample data and experimental parameters are shown in **table 5.1.** In this series of experiments 6/7 lungs vasoconstricted to 100 mM KCl. Vasoconstriction to KCl was associated with an increase in the rate of weight gain which was reversed on washout. Previous studies in the *ex vivo* human lung model found that ET-1 caused a dose dependent increase in perfusion pressure with an EC_{50} of 10.83 ± 8.47 nM. In these experiments the lungs were exposed to 30 µM ET-1. Papaverine was added to the perfusate either cumulatively to reverse contraction to 30 µM ET-1 (n=2) or before exposure to 30 µM ET-1 (n=4). Results from these preliminary studies determined that 120mg of papaverine (0.3 mM) was needed to prevent pulmonary vasoconstriction to 30 µM ET-1 in the human lung model. This concentration of papaverine (≈ 240 mg kg^{-1}) was considerably higher than the concentration of papaverine previously reported to induce vasoplegia in isolated rat lungs (0.1 µg Kg^{-1}) [206].

Table 5.1 Patient and specimen data with experimental parameters for *ex vivo* lung experiments to determine required dose of papaverine. Tv; tidal volume, BPM; breaths per minute.

	Patient		Specimen		Experimental Parameters		
Prep	**Sex**	**age (years)**	**Lobe**	**Weight (g)**	**Perfusate Flow (ml/min)**	**Tv (ml)**	**BPM**
ET1	F	74	Left Upper Lobe	264	250	200	10
ET2	M	70	Left Upper Lobe	383	200	200	10
ET3	F	64	Left Lower Lobe	242	200	200	10
ET4	M	50	Left Upper Lobe	271	100	200	10
ET5	F	57	Left Lower Lobe	260	200	200	10
ET6	F	81	Left Upper Lobe	256	200	100	10
ET7	M	67	Right Lower and Middle Lobe	494	200	300	10
Mean		**66**		**310**	**193**	**200**	**10**
SD		**10**		**94**	**45**	**58**	**0**

5.3.1.2 Effect of ET-1 on vascular permeability

Lung samples were obtained from 11 patients (9 lobes and 2 lungs) (Raw data Appendix II). Two lungs in this series of experiments showed marginally high baseline weight gain (1.87 and 1.51 % min^{-1}). Given that the aim of these experiments was to investigate changes in vascular permeability under different conditions these lungs were included. In one lung preparation the perfusion pressure was greater than 50 mmHg, indicating tumour obstruction of the vasculature, and this specimen was excluded. Two lungs were excluded due to excessive baseline weight gain (> 2 % min^{-1}) and two experiments failed for technical reasons. Patient, lung data and experimental parameters for the remaining lung samples are shown in **table 5.2**.

Table 5.2 Patient and specimen data with experimental parameters for ex vivo lung experiments to determine the effect of ET-1 on capillary filtration coefficient. Tv; tidal volume, BPM; breaths per minute.

	Patient		Specimen		Experimental Parameters		
Prep	**Sex**	**age (years)**	**Lobe**	**Weight (g)**	**Perfusate Flow (ml/min)**	**Tv (ml)**	**BPM**
ET9	M	81	Right Upper Lobe	275	200	200	10
ET12	M	62	Left Upper Lobe	315	100	200	10
ET13	M	75	Right Upper Lobe	411	100	100	10
ET16	M	71	Right Lower Lobe	296	200	100	10
ET18	F	57	Right Lower Lobe	266	100	100	10
ET20	M	62	Left Lung	1060	350	200	10
Mean		**68**		**437**			
SD		**9**		**310**			

5.3.1.2.1 Effect of ET-1 on rate of weight gain

Addition of 120 mg papaverine to the perfusate significantly attenuated vasoconstriction to 30 µM ET-1 in all lung preparations. In five of six lung preparations ET-1 caused an observable increase in the rate of weight gain (**table 5.3, figure 5.2**). However, the increase in the rate of weight gain did not reach statistical significance (p = .10 via paired samples, 2-tail, t-test). Addition of ET-1 also caused a rapid and observable bronchial effusion in three of six lungs.

Table 5.3 Rate of weight gain in isolated human lungs (% of initial weight min^{-1}) pre and post exposure to 30 µM ET-1. n = 6. *p* = .10 via paired, 2-tail, t-test.

Prep	Rate of Wt. gain pre ET-1 (%min^{-1})	Rate of Wt. gain post ET-1 (%min^{-1})
ET9	0.95	1.60
ET12	0.63	1.84
ET13	0.34	-0.29
ET16	3.24	5.61
ET18	1.43	3.38
ET20	-0.08	0.04
Mean	**1.09**	**2.03**
SD	**1.17**	**2.20**

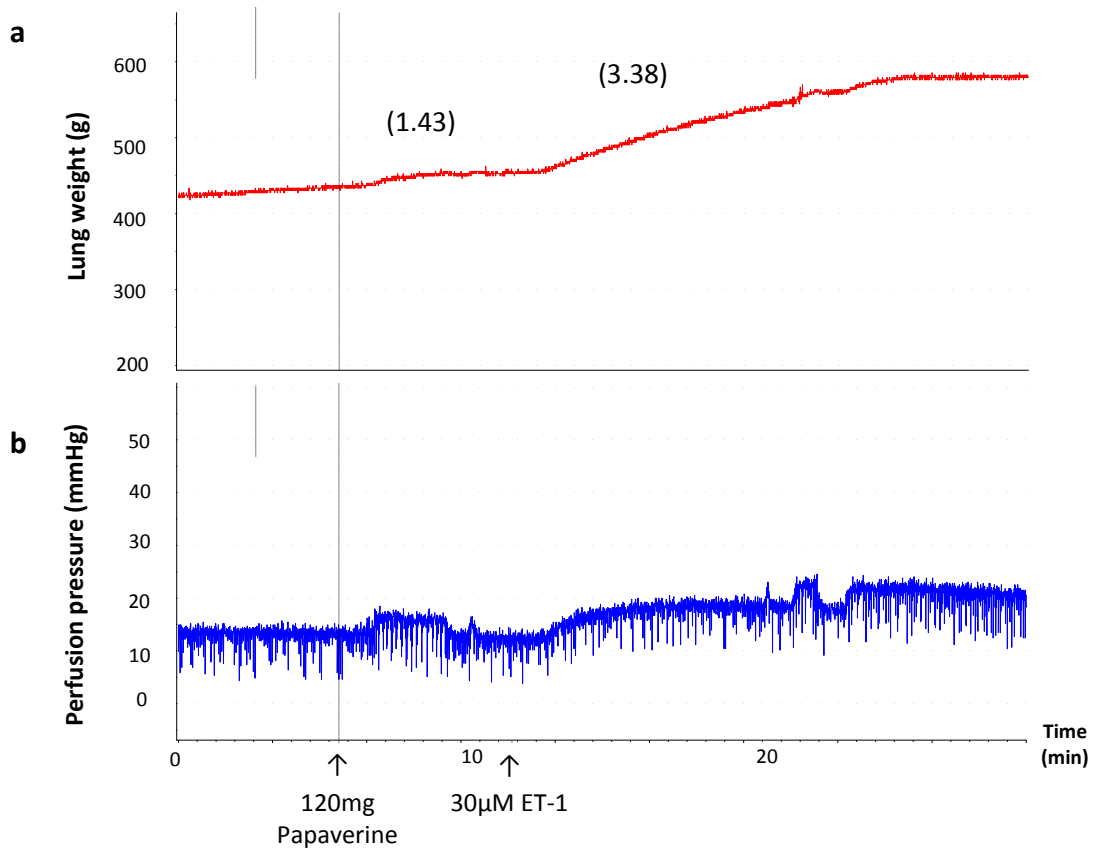

Figure 5.2 Experimental trace showing effect of ET-1 on rate of weight gain and perfusion pressure in an isolated human lung preparation. (**a**) Lung weight (g). Rate of weight gain (% min^{-1}) shown in parentheses. (**b**) Perfusion pressure (mmHg). Fluctuations in the pressure waveform are due to the pressure generated by the pulsatile pump.

5.3.1.2.2 Effect of ET-1 on capillary filtration coefficient ($K_{f,c}$)

On calculating the $K_{f,c}$ two anomalous results were generated: one false positive result when the rate of weight gain after exposure to ET-1 was negative and the change in P_{pc} was negative and one false negative result when the rate of weight gain after ET-1 was positive and the change in P_{pc} was negative (Raw data Appendix II). These results were excluded from the analysis. Of the remaining four preparations the $K_{f,c}$ was not significantly greater following exposure to ET-1 (**table 5.4**).

Table 5.4 Capillary filtration coefficient ($K_{f,c}$) (%/min/mmHg) in isolated human lungs pre and post exposure to 30 µM ET-1. n = 4. *p* = .18 via paired, 2-tail, t-test.

Prep	$K_{f,c}$ Pre ET-1	$K_{f,c}$ Post ET-1
ET9	0.11	0.05
ET12	0.09	0.26
ET18	0.00	0.39
ET20	-0.10	0.04
Mean	**0.03**	**0.18**
SD	**0.10**	**0.17**

5.3.2 Dose Response Effect of ET-1 in Isolated Human Pulmonary Arteries and Isolated Human Pulmonary Veins

ET-1 dose dependently (10 pM - 30 nM) contracted human pulmonary arteries (n = 9 from 3 patients, mean internal diameter 4.6 ± 2.0 mm, 3.7 ± 0.9 mm wide) and human pulmonary veins (n = 9 from 3 patients, mean internal diameter 6.0 ± 3.7 mm, 3.9 ± 1.5 mm wide).

ET-1 was a significantly more potent vasoconstrictor in the pulmonary veins than in the pulmonary arteries (EC_{50} 0.50 ± 0.22 nM vs. 2.75 ± 2.58 nM, pEC_{50} 9.36 ± 0.26 vs. 8.71

± 0.38 for veins and arteries respectively. p = .03 and < .01 for EC_{50} and pEC_{50} respectively via 2-tail, unpaired t-test for parametric data with unequal variance, EC_{50} concentrations were estimated manually from individual concentration response curves). The efficacy of ET-1 was also greater in the pulmonary veins compared to the pulmonary arteries, which was statistically significant at most concentrations tested (**table 5.5, figure 5.3,** raw data Appendix II).

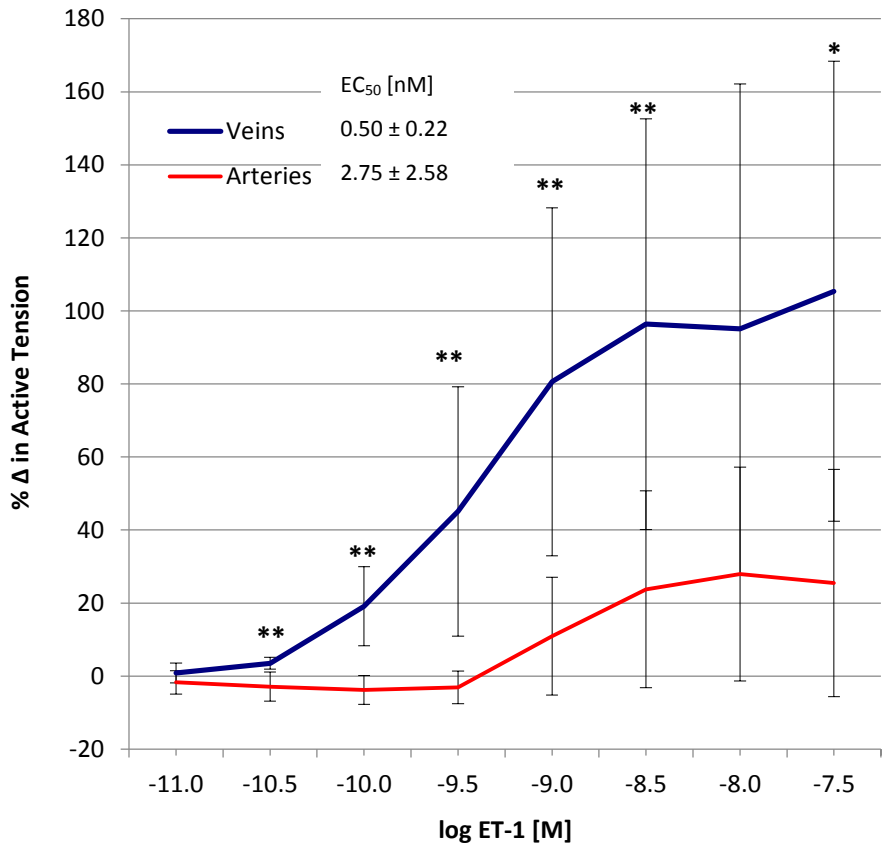

Figure 5.3 Log dose response curve to ET-1 in isolated human pulmonary arteries (n = 9) and veins (n = 9). Effect of ET-1 is shown as % change in baseline tension. ** p < .01, * p < .05 arteries vs. veins via 2-tail, unpaired t-test for parametric data with unequal variance.

Table 5.5 Dose response effects and potency of ET-1 in isolated human pulmonary arteries (n = 9) and veins (n = 9). Dose dependent effect of ET-1 is shown as % change in baseline tension and potency as EC_{50} (nM) and pEC_{50} (-log EC_{50} [M]). *p* value for arteries vs. veins via 2-tail, unpaired t-test for parametric data with unequal variance. Values shown as mean ± (SD)

	Resting Tension (gf)	**% Change in tension from baseline**								**EC_{50} [nM]**	**pEC_{50}**
		ET-1 [nM]									
		0.01	**0.03**	**0.1**	**0.3**	**1**	**3**	**10**	**30**		
Arteries	1.52 (0.52)	-1.69 (3.2)	-2.89 (4.00)	-3.77 (3.97)	-3.11 (4.48)	10.97 (16.12)	23.78 (26.95)	27.94 (29.30)	25.52 (31.12)	2.25 (2.58)	8.71 (0.38)
Veins	1.17 (0.45)	0.88 (2.71)	3.52 (1.64)	19.13 (10.84)	45.10 (34.15)	80.59 (47.64)	96.37 (56.24)	95.08 (67.06)	105.36 (62.97)	0.50 (0.22)	9.36 (0.26)
***p* value: arteries vs. veins**	**0.14**	**0.12**	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	**0.06**	**0.05**	**0.03**	**< 0.01**

5.4 Discussion

The original aim of this study was to compare the effects of ET-1 with the effects of hypoxia in the human pulmonary vasculature and it was hypothesised that ET-1 would have the same effects as hypoxia. However, it was found, surprisingly, that the effects of ET-1 were the opposite to the effects of hypoxia in *ex vivo* human lung preparations. In isolated pulmonary arteries and veins ET-1 caused a dose dependent constriction whereas hypoxia dilated the vessels and reversed the effect of ET-1. In isolated lungs hypoxia attenuated oedema formation whereas ET-1 caused an increase in pulmonary vascular permeability. Therefore, it would seem unlikely that the effects of hypoxia are mediated by ET-1. However, there were a number of novel and significant findings to arise from the studies in this chapter.

In this chapter it has been shown for the first time in *ex vivo* human lungs that ET-1 causes an increase in pulmonary vascular permeability. The increase in oedema formation in the lungs was not due to an increase in hydrostatic pressure because the vasoconstrictor effect of ET-1 was prevented by papaverine. These results confirmed previous studies by this group, which suggested that the fulminant oedemagenic effect of ET-1 in the lung was a combination of increased hydrostatic pressure and increased pulmonary vascular permeability. ET-1 has been implicated in the increased pulmonary vascular permeability associated with the pathogenesis of pulmonary oedema in a number of lung conditions including high altitude pulmonary oedema (HAPE) [109, 198] acute lung injury (ALI)/ acute respiratory distress syndrome (ARDS) [213-216], flash pulmonary oedema [217] and post cardiotomy lung [218]. Further studies are needed to investigate the mechanisms of ET-1 induced changes in vascular permeability and to

characterise the endothelin receptor subtypes responsible, which could identify the therapeutic potential for endothelin antagonists in treating permeability pulmonary oedema in a number of clinical settings. It is hoped that the isolated human lung model will facilitate the development of new and improved therapeutics, such as selective endothelin antagonists, for treating pulmonary oedema. It should be noted that a limitation of this study was that control experiments were not performed to determine the possible oedemagenic effects of papaverine on the lungs. Although there is currently no evidence in the literature to suggest that papaverine does cause oedema it is possible that papaverine (especially at the high concentrations used in this study) could have modulated the effects of ET-1 on vascular permeability. Further experiments are needed to determine if papaverine does affect ET-1 induced increases in vascular permeability.

Cannulation of the pulmonary veins in the *ex vivo* human lung model made it possible to measure the capillary filtration coefficient. Furthermore, measuring pulmonary venous pressure also allows quantification of total pulmonary vascular resistance (which is given by the equation; RT= (Ppa-Ppv)/Q. Where Ppa is pulmonary arterial pressure, Ppv is pulmonary venous pressure and Q is flow). This represents a significant improvement of the *ex vivo* human lung model and will facilitate future studies to investigate effects on pulmonary vascular tone and oedemagenesis in the human lung.

In this chapter the vasoconstrictor effect of ET-1 on human pulmonary arteries and veins has been compared for the first time. It was found that ET-1 is a more potent constrictor in human pulmonary veins than human pulmonary arteries (pEC_{50} 9.36 ±

0.26 vs. 8.71 ± 0.38 for veins and arteries respectively. $p < .01$) and also that the magnitude of constriction was significantly greater in the veins than the arteries at most concentrations tested. This finding is consistent with previous animal studies which found that ET-1 preferentially constricts the pulmonary veins with greater potency and efficacy than the pulmonary arteries [194, 207, 209-212]. In some of the previous animal studies it was also found that the selective ET_B receptor agonist sarafotoxin 6 c was more potent and induced a significantly greater magnitude of contraction in the pulmonary veins than the pulmonary arteries [212, 219] and that contraction to sarafotoxin 6 c was augmented in the pulmonary veins but not in the pulmonary arteries by exposure to endotoxin [219] or chronic hypoxia [212]. Taken together these findings imply that the vasopressor effect of ET-1 in the lungs is mediated predominantly by activation of the ET_B receptors on the pulmonary veins. These results highlight the importance of considering the pulmonary venous response in therapeutic drug development and clinically when treating pulmonary hypertension. In treating pulmonary hypertension it is not uncommon for physicians to use the pulmonary artery pressure to monitor the effectiveness of treatment. However, dilating the pulmonary arterial system without a concomitant dilation of the pulmonary veins is likely to cause pulmonary oedema which has been shown to be the case in pulmonary veno-occlusive disease where the use of selective arterial dilators causes severe pulmonary oedema [220, 221]. Selective endothelin A (ET_A) or dual endothelin A/B (ET_A/ET_B) receptor antagonists are one of the pharmacotherapeutic options for the treatment of pulmonary hypertension. If it is the case that the vasopressor effect of ET-1 in the lung is predominantly due to a venoconstriction, mediated by activation of the ET_B receptors, then selective ET_A receptor antagonists could increase the risk of

oedema formation by causing a preferential dilation of the pulmonary arterial system without reducing pulmonary venous resistance. The use of dual ET_A/ET_B or even selective ET_B receptor antagonists would therefore be indicated. Further studies are needed in isolated human pulmonary arteries and veins to characterise the receptors responsible for the vasoconstrictor effects of ET-1 and in *ex vivo* human lungs to determine the relative contribution of the arterial and venous systems in the vasopressor effect of ET-1. A better understanding of the pathologic effects of ET-1 in the human lung would provide a more informed basis for the choice of endothelin antagonists used to treat pulmonary hypertension and could lead to the development of more effective therapeutics in the future.

In this chapter it has been shown for the first time that ET-1 causes an increase in vascular permeability in the human lung. Further studies are needed to investigate the mechanisms of ET-1 induced increases in pulmonary vascular permeability which could lead to the development of effective treatments for permeability oedema. It has also been shown for the first time that ET-1 is a more potent vasoconstrictor in human pulmonary veins than in human pulmonary arteries which suggests that the pulmonary veins might play a more significant role than the pulmonary arteries in mediating the vasopressor effects of ET-1 in the human lung. Further studies are needed to investigate the differential role of the arterial and venous systems in the vasopressor effect of ET-1 in the human lung. Previous animal studies have shown that different ET receptors are responsible for contraction of the pulmonary arteries and veins. Further studies are needed to characterise the endothelin receptor subtype mediating constriction to ET-1 in the human pulmonary arteries and veins which could facilitate

the development of more effective treatments for pulmonary vascular disease in the future.

CHAPTER 6

Hypoxic Bronchodilation (HBD) in *Ex Vivo* Human Lungs and *In Vitro* Human Bronchi

Contents

6.1 Introduction

Obstructive and inflammatory respiratory diseases can cause global or regional hypoxia in the lung. The effect of hypoxia on the pulmonary vasculature has been extensively investigated and hypoxic vasoconstriction is now generally accepted as an important homeostatic mechanism for optimising perfusion-ventilation matching in the lung. In contrast, surprisingly few studies have investigated the effect of hypoxia on airway tone and the reported results are inconsistent.

Isolated tissue preparations have been used to directly measure the effect of hypoxia on airway smooth muscle (ASM) tone. However, the reported results were variable depending on species, experimental conditions and level in the respiratory tract of the tissue used. Hypoxia consistently caused a robust and reversible relaxation of pre-constricted porcine bronchial rings [95-97] and porcine tracheal smooth muscle strips [98, 99]. In histamine contracted guinea-pig tracheal rings hypoxia caused a significant relaxation [100] and pre exposure to hypoxia inhibited maximal cholinergic and histaminergic contractions of guinea-pig tracheal smooth muscle strips [101]. In bovine tracheal smooth muscle strips hypoxia reversibly reduced resting tension and attenuated contraction to electrical field stimulation [102] whereas in bovine bronchi hypoxia enhanced contraction to methacholine [103]. Exposure to hypoxia caused relaxation of canine isolated bronchi [104] and canine lung parenchyma strips [105]. However, in canine tracheal muscle strips moderate hypoxia caused a relaxation [106, 107] whereas severe hypoxia caused an increase in resting tension [106]. In reviewing the literature, only one report was found describing the effect of hypoxia on isolated human airways; in isolated human bronchi hypoxia caused only a slight reduction in

resting tone, did not significantly alter contraction to methacholine and attenuated relaxation to salbutamol [84].

In vivo animal studies investigating the effect of hypoxia were similarly inconsistent. In anaesthetised and ventilated dogs hypoxia was shown to cause constriction of the airways (as determined from derived values of lung resistance) [85-89] which was diminished [87] or abolished [85, 86] by vagotomy [85, 87] or inhibition of vagal tone [86]. Exposure of anaesthetised and ventilated rabbits to hypoxia caused an increase in respiratory resistance [90] which in a subsequent study was shown to be caused by a direct effect on the lung parenchyma [222]. In decerebrate cats, hypoxia also caused an increase in airway resistance [91]. In contrast, high resolution computed tomography was used to directly visualise the airways of minipigs and demonstrated a significant dilation of the large and small airways in response to hypoxia [92]. Likewise, exposure of anaesthetised and ventilated guinea-pigs to hypoxic conditions reduced histamine induced increases in total pulmonary resistance [93] and hypoxic ventilation of anaesthetised rats caused a slight reduction in airway resistance [94].

Results from *in vivo* studies in man were also inconsistent. An early study in normal subjects found that acute hypoxia caused an increase in airway resistance and it was concluded that hypoxia acts directly on bronchial smooth muscle to cause bronchoconstriction [76]. The bronchoconstrictor effect of hypoxia in man was supported by subsequent studies; chronic hypoxic bronchoconstriction in COPD patients was relieved by breathing 30% oxygen [77] , in asthmatic patients hypoxia was found to potentiate methacholine induced contraction [78] and changes in lung mechanics induced by isocapnic hypoxia in healthy volunteers were interpreted as

bronchoconstriction of the large airways [79]. However, other studies showed a different effect of hypoxia. In healthy human volunteers the cross-sectional area (measured using an acoustic reflection technique) of the extrathoracic trachea, intrathoracic trachea and main bronchi all increased under hypoxic conditions [80] and in another study in healthy human subjects transient hypoxia caused a decrease in upper airway resistance [81]. In healthy volunteers and patients with chronic pulmonary disease acute hypoxia caused a decrease in airway resistance but did not change dynamic compliance, which was interpreted as indicating a bronchodilator effect [82]. However, in other studies hypoxia did not affect small airway function in healthy volunteers [83] or FEV_1 in asthmatic patients [84].

It should be noted that *in vivo* studies measuring effects on airway tone are often controversial, due to disagreement about the true meaning of derived values and the number of confounding experimental factors which can affect airway tone.

Some studies have compared the effects of hypercapnia to the effects of hypoxia on the airways. In intact animals the effect of hypercapnia was found to be very similar to that of hypoxia. In anaesthetised and ventilated dogs hypoxia and hypercapnia caused a similar increase in airway resistance [85-87] and both effects were dependent on an intact vagal tone. Similarly in ventilated cats [91] and rabbits [90] hypoxia and hypercapnia both increased airway resistance. In healthy subjects and patients with pulmonary disease hypercapnia and hypoxia both caused decreased airway resistance [82] and in healthy anaesthetised and ventilated volunteers hypercapnia caused bronchodilation [223] and in another study hypocapnia increased resistance to flow in healthy volunteers [224]. The similarity and consistency (relaxation or contraction) of the response to

hypoxia and hypercapnia suggests that there may be a causal relationship between the effects of hypoxia and hypercapnia on the airways and in an early canine study [85] the authors even assumed 'hypercapnia to be the principal factor responsible for tracheal constriction during re-breathing, hypoventilation and asphyxia'.

In contrast, *in vitro* studies found that hypercapnia had only a marginal effect on airway tone compared to hypoxia. For instance; in field stimulated bovine tracheal muscle strips hypoxia caused a robust decrease in the magnitude of contractions whereas hypercapnia caused a much smaller (but still significant) decrease in contractions [102] and in carbachol contracted porcine tracheal muscle strips, hypoxia caused a robust (60%) relaxation whereas hypercapnia caused a much smaller (7%) relaxation [98].

Clearly there are considerable inconsistencies in the reported effects of hypoxia on the airways depending on species and experimental technique used, and to date the effect of hypoxia on human airways has not been clearly defined. There is also inconsistency in the literature regarding the role of carbon dioxide in modulating, or even causing, the response of the airways to hypoxia. However, in considering the balance of evidence from animal studies and *in vivo* studies in man it was hypothesised that hypoxia would cause a relaxation of human airways and that hypercapnia would cause a relaxation of human airways and augment the effects of hypoxia.

Given the potential physiological and pathophysiological importance of the hypoxic response in perfusion-ventilation matching the aims of this study were;

1. To determine the effect of hypoxia on human airways.

2. To determine the effect of hypercapnia on human airway tone and the modulatory effect of CO_2 on the response of airways to hypoxia.

Three human tissue preparations were used in these studies: (1) *Ex vivo* isolated perfused human lungs (2) isolated human bronchi and (3) a novel parallel organ bath system with continuously recirculating buffer, which was developed to facilitate accurate control of gas partial pressures in the organ bath buffer solutions, and was used in this chapter to investigate the effect of hypercapnia on bronchial tone.

6.2 Methods

6.2.1 *Ex Vivo* Human Lungs

6.2.1.1 Effect of Hypoxia on Airway Pressure

Lung samples were obtained from patients undergoing resection for cancer. Samples were prepared and connected to the perfusion-ventilation circuit (***Chapter 2, 2.2***).

Lung samples were ventilated with room air. The respiration rate was set at 10 breaths per minute (BPM) for the duration of the experiment. The tidal volume was set, depending on the size and airway resistance of the lung sample, to achieve a peak airway pressure of less than 18 mmHg, and ranged between 100 and 200ml.

The perfusion flow rate was gradually increased to achieve a mean perfusion pressure between 15 and 20 mmHg and ranged between 100 and 200 ml min^{-1}.

Lungs were allowed to equilibrate for at least 60 minutes before starting experimental procedures.

When baseline airway and perfusion pressures were stable the lungs were exposed to hypoxia (***Chapter 2, 2.2.12.3***) for 71 ± 45 minutes followed by return to normoxic ventilation and perfusion.

Perfusate samples were taken from the circuit proximal to the arterial cannula for analysis of perfusate pH, pO_2, pCO_2 and HCO_3 with a blood gas analyser (Gem Premier 3000, Instrumentation Laboratory Company, Lexington, MA, USA) at four time points; Pre hypoxia, 20 minutes post hypoxia, 50 minutes post hypoxia and after return to normoxic ventilation and perfusion.

Mean airway pressure and mean peak airway pressure were calculated for 10 minute periods at five time points; 10 minutes pre hypoxia, 20, 30 and 50 minutes post hypoxia and 10 minutes after resuming ventilation with room air.

6.2.1.2 Effect of Normoxic, Hypoxic and Hyperoxic ventilation with 5% CO_2 on Airway Pressure

In the experiments described above *(**6.2.1.1***) the gas used to ventilate the lungs during hypoxic conditions contained 5% CO_2 which may have had an effect on airway tone. Furthermore, because both airways and the perfusate were made hypoxic simultaneously it was not possible to determine if alveolar or perfusate oxygen concentrations were responsible for changes in airway pressure. The experiments described in this section were therefore performed to investigate if:

(1) 5% CO_2 per se in the ventilation gas had an effect on airway pressure

(2) 5% CO_2 affected the pCO_2 and/or pH of the perfusate

(3) Changing the pO_2 of the ventilating gas had a direct effect on airway pressure

Lung samples were obtained from patients undergoing resection for cancer and connected to the standard perfusion-ventilation system (***Chapter 2, 2.2***).

Lung samples were ventilated with room air. The respiration rate was set at 10 breaths per minute (BPM) and the tidal volume at 200 ml for the duration of the experiment.

The perfusion flow rate was gradually increased to achieve a mean perfusion pressure between 15 and 20 mmHg and was 100 - 300 ml min^{-1}.

After 60 minutes ventilation with room air a baseline perfusate sample was taken for analysis. Ventilation was then switched to 95% air: 5% CO_2 (30min), 95% N_2: 5% CO_2 (30min) and 95% O_2: 5% CO_2 (30min) by connecting a 2 litre flexible reservoir bag (Intersurgical Ltd.), continuously filled with the appropriate gas, to the ventilator inlet. Perfusate samples were taken 30 minutes after changing the ventilation gas for analysis with a blood gas analyser.

Mean peak airway pressure was calculated over 10 minute periods; 10 minutes before and 20 minutes after changing the ventilator gas composition.

6.2.1.3 Relative influence of perfusate and airway pO_2 on the hypoxic response

Preliminary experiments were designed to investigate the effect of changing the perfusate pO_2 independently of the alveolar pO_2 in order to determine the relative contribution of perfusate and/or airway pO_2 to the hypoxic response.

Lungs were connected to the modified perfusion circuit for creating hypoxic conditions (***Chapter 2, 2.2.12.3***) and ventilated with 95% air: 5% CO_2. Ventilation rate was set at 10 BPM and the tidal volume at 200 ml for the duration of the experiment. The perfusion flow rate was gradually increased to achieve a mean perfusion pressure between 15 and 20 mmHg and was 100- 300 ml min^{-1}.

After a minimum equilibration time of 60 minutes and when airway and perfusion pressure baselines were stable the lungs were exposed to different combinations of perfusate and airway pO_2 concentrations; Airway ventilation was either hyperoxic (95% O_2: 5% CO_2), normoxic (21% O_2: 5% CO_2), or hypoxic (95% N_2: 5% CO_2). Likewise, the perfusate oxygen concentration was altered, independent of ventilation, to

hyperoxia or hypoxia by changing the sweep gas across the membrane oxygenator to 95% or 0% O_2 in 5% CO_2 (1-2 l min^{-1}). After changing a ventilator or perfusate oxygenation parameter the lungs were allowed to equilibrate for at least 15 minutes. A perfusate sample was then taken, post membrane oxygenator, to confirm the pO_2 in the perfusate entering the lung.

Mean peak airway pressure was calculated over 5 minute periods- 5 minutes before and 10 minutes after changing ventilator or perfusate oxygen concentration.

6.2.2 *In Vitro* Human Bronchi

Human bronchi (primary, second order or tertiary) with an internal diameter of 4-10 mm were obtained at resection for lung cancer. Samples were prepared and mounted between stainless steel wires in organ baths to measure changes in isometric tension (***Chapter 2, 2.3***).

A resting tension of 1-3 gf was applied and the bronchi allowed to equilibrate for 60-90 minutes.

6.2.2.1 Effect of Hypoxia on Resting Tension

In a number of experiments described in this thesis control responses to hypoxia from resting tension were obtained before starting experimental protocols. When a stable baseline tension was achieved hypoxia was induced by covering the top of the organ bath with a thin plastic film and aerating with 95% N_2: 5% CO_2. When a plateau effect on resting tension was obtained the aerating gas was switched back to 95% O_2: 5% CO_2.

6.2.2.2 Effect of Hypoxia on Resting Tension; Comparison to β-adrenergic Relaxation

When a stable baseline tension was achieved hypoxia was induced by covering the top of the organ bath with a thin plastic film and aerating with 95% N_2: 5% CO_2. When a plateau effect on resting tension was obtained the aerating gas was switched back to 95% O_2: 5% CO_2. When the resting tension returned to pre hypoxic baseline, or a stable plateau, the bronchi were exposed to 10 µM isoprenaline. Effect on resting tension was expressed as % change in resting tension.

6.2.2.3 Effect of Hypoxia on Contraction to Methacholine; Comparison to β-adrenergic Relaxation

When a stable baseline tension was achieved bronchi were contracted to 1 mM methacholine (MCh). Active tension to MCh was calculated as maximum response (at plateau) – resting (baseline) tension. When contractions reached plateau the bronchi were exposed to hypoxia. When a maximum effect of hypoxia was obtained oxygenation was restarted. When the bronchi returned to active baseline a control relaxation to 10 µM isoprenaline was performed. Maximum relaxation was expressed as % change in active tension.

6.2.2.4 Concentration Dependent Effect of Changes in Oxygen Partial Pressure (pO_2) on Bronchial Tone

The concentration dependent effects of oxygen on resting tension and after pre contraction to 1mM MCh were obtained by stepwise changes in the aerating gas from 95% to 40%, 21% and 0% O_2 in nitrogen with 5% CO_2. Oxygen concentration in one of the organ baths was continuously recorded with an oxygen electrode (Micro oxygen electrode, Lazar Industries). Oxygen concentration was expressed as mV reading (10

mV ~ 1 KPa ~ 7.5 mmHg) and the recorded oxygen concentration in the organ bath Krebs-Henseleit solution validated by comparison with pO_2 values obtained using a blood gas analyser (Gem Premier 4000, Instrumentation Laboratory).

6.2.2.5 Effect of Hypercapnia on Resting Tension, Cholinergic Contraction and Hypoxic Bronchodilation (Parallel Organ Baths and Perfusion Circuit)

In order to change the partial pressure of carbon dioxide (pCO_2) independently of the partial pressure of oxygen (pO_2) in the organ bath a continuous perfusion system with a membrane oxygenator was designed to facilitate changes in the partial pressure of gases in the perfusate. The parallel organ bath and perfusion circuit is shown in **figure 6.1**; the outlets from each organ bath were connected to a peristaltic roller pump (Watson-Marlow, Falmouth, Cornwall, UK) using UPVC tubing to allow continuous recirculation (100 ml min^{-1}) of the Krebs-Henseleit solution through a membrane oxygenator (Dideco D905, paediatric hollow fibre membrane oxygenator, Sorin Group Italia). A gas blender (Sechrist Inc.) was used to regulate the proportion of gases in the 'sweep' gas across the membrane oxygenator which facilitated accurate regulation of the partial pressure of oxygen (pO_2) and carbon dioxide (pCO_2) in the recirculating perfusate. The temperature of the perfusate was maintained at 37^{o}C using the integral heat exchanger of the membrane oxygenator which was connected in parallel with the organ bath water jackets to a heater-cooler unit (HCU- 20-602. Jostra, Sweden). The circulating volume of the circuit was 210 ml.

When the perfusion circuit had been primed, and all air removed, bronchi were mounted in organ baths as previously described (*Chapter 2*) and left to equilibrate for 60-90 minutes at a sweep gas of 95% O_2: 5% CO_2 (1 l min^{-1}). Washout was performed

every 10 minutes by opening the waste line and replacing the perfusate with fresh buffer from the perfusate reservoir. Baseline resting and active tensions (contraction to MCh) were obtained with a sweep gas of 95% O_2: 5% CO_2 (1l min^{-1}). Hypoxia was induced by changing the sweep gas across the membrane oxygenator to 95% N_2: 5% CO_2.

The effect of hypercapnia was investigated by the addition of CO_2 (0.3 l min^{-1}) to the sweep gas across the membrane oxygenator. The partial pressure of CO_2 in the perfusate (pCO_2) was confirmed by analysis of perfusate samples with a blood gas analyser. The effect of hypercapnia on resting tension, hypoxia from resting tension, cholinergic contraction and hypoxia post cholinergic contraction was determined.

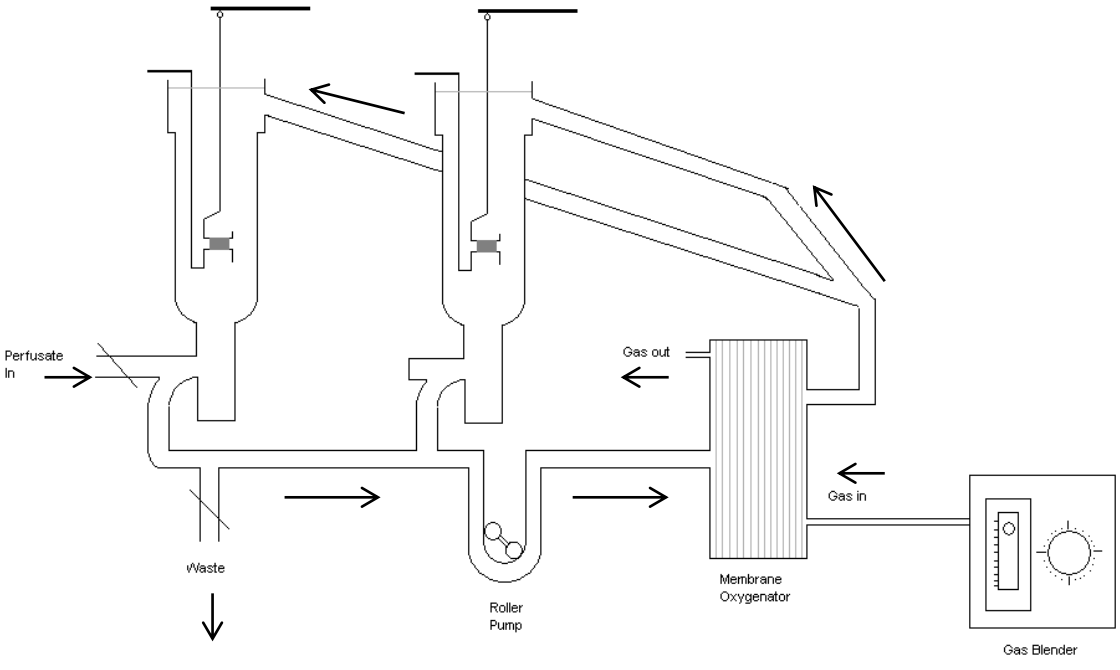

Figure 6.1 Parallel organ baths with perfusion circuit for regulation of gas partial pressures

6.3 Results

6.3.1 *Ex Vivo* Human Lungs

6.3.1.1 Effect of Hypoxia on Airway Pressure

The results reported in this section are from the same experiments described in ***4.3.1***. Lung samples (10 lobes and 2 whole lungs) were obtained from twelve male patients (age 68 ± 11 years) undergoing resection for cancer. Patient and specimen details with individual experimental parameters are shown in **table 4.1**.

Four of the lungs in this series of experiments demonstrated excessive oedema formation (> 1.0 % min^{-1}); however, the oedema formation did not affect airway pressure and the results were included in the analysis. Exposure to hypoxia caused a significant reduction in perfusate pO_2 after 20 and 50 minutes which returned to pre-exposure concentrations 10 minutes after return to ventilation with room air (**table 6.1 and figure 6.2b**). The lungs were exposed to hypoxia for 68 ± 41 minutes which resulted in a significant and sustained reduction in peak airway pressure over 10-50 minutes (18.28 ± 5.74 vs. 14.36 ± 5.42 mmHg baseline peak airway pressure pre hypoxia and lowest peak airway pressure post hypoxia respectively, or -22 ± 14%, p < .01 via 2-tail paired t-test, n = 12) in 10 of 12 isolated human lung preparations which was reversed on return to ventilation with room air **(figure 6.2a)** (Raw data Appendix II). The time taken to reach maximum hypoxic bronchodilation was 11 ± 9 minutes (range 3-30 minutes). The effects of hypoxia on peak airway pressure after 10, 20 and 50 minutes and on returning to ventilation with room air are shown in **table 6.2, and figure 6.2c** (Raw data Appendix II). The effects of hypoxia on peak airway pressure were not obtained at every time point in some experiments which precluded the use

of a repeated measures ANOVA (analysis of variance) to analyse the effects of hypoxia on peak airway pressure over time. The effects of hypoxia on peak airway pressure at different time points were therefore compared to the baseline peak airway pressure at each time point via paired samples (2-tailed) t-test and the number of paired values for each time point shown in **table 6.2** and in parentheses in **figure 6.2c**. Hypoxia caused a significant reduction in peak airway pressure at all time points which was rapidly reversed 10 minutes after returning to ventilation with room air although the peak airway pressure remained statistically significantly lower than the pre-exposure baseline. The pH, pCO_2 and electrolyte concentrations of the perfusate remained within normal physiological parameters during the experiments.

Table 6.1 Change in perfusate pO_2 with hypoxia. *p < .05 vs. baseline via related samples Wilcoxon signed-rank test. n = number of values obtained at each time point

	Perfusate pO_2 (KPa)			
	Baseline (Room Air)	**20min post hypoxia**	**50min post hypoxia**	**Post hypoxia (Room Air)**
Mean	20.1	7.3*	4.9*	20.7
SD	0.5	4.7	0.9	2.4
n	8	9	5	3

Table 6.2 Changes in peak airway pressure with hypoxia. **p < .01, *p < .05 vs. baseline via paired samples (2-tailed) t-test. n = number of values obtained at each time point

	Peak Airway Pressure (mmHg)				
	Baseline (Room Air)	**10 min Post Hypoxia**	**20 min Post Hypoxia**	**50 min Post Hypoxia**	**Room Air**
Mean	20.14	16.65**	15.20**	14.93**	19.42*
SD	6.33	5.93	5.99	6.36	7.82
n	12	12	11	8	8

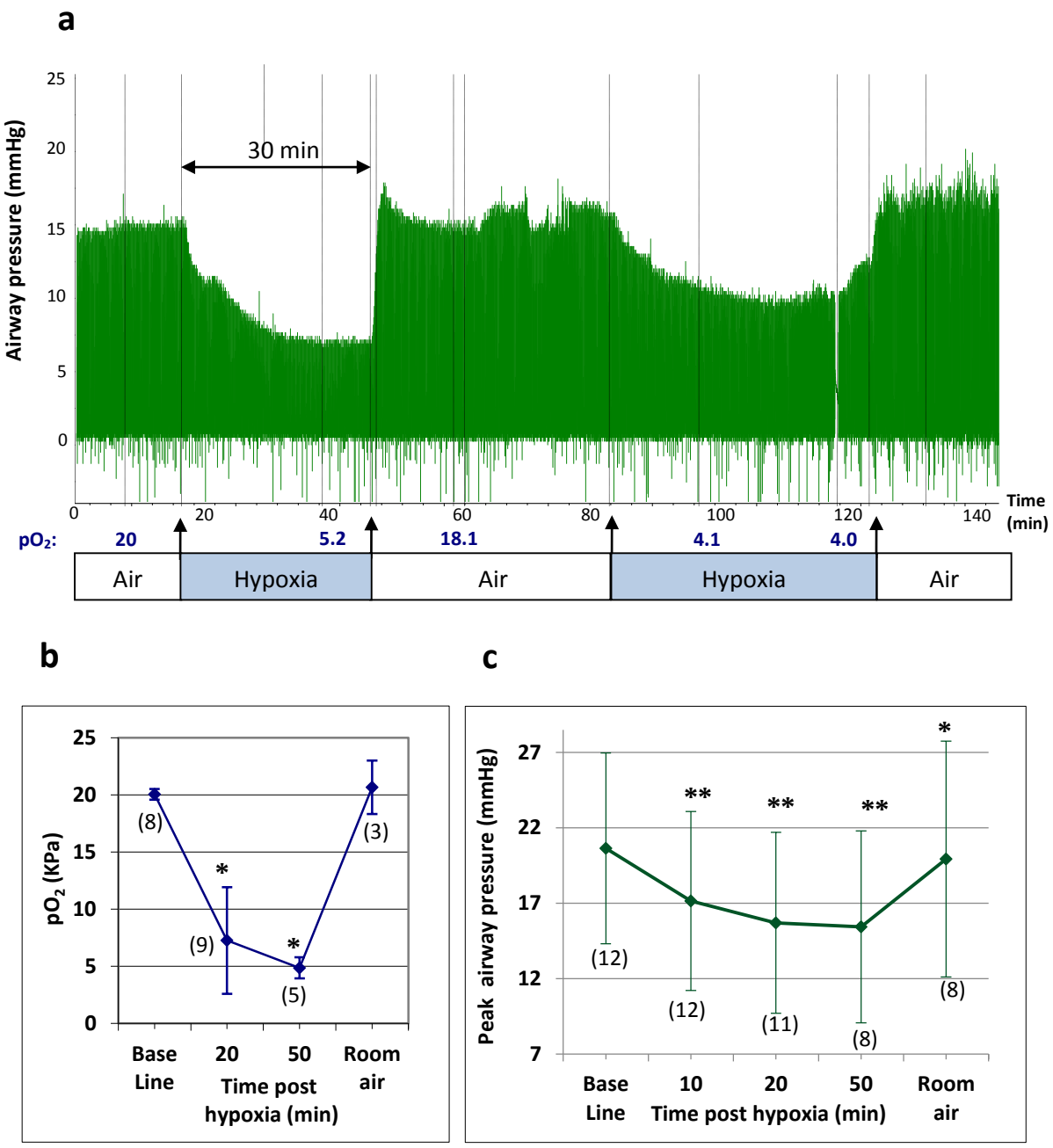

Figure 6.2 Effect of hypoxia on airway pressure in ex vivo perfused and ventilated human lungs. **(a)** Experimental trace showing effect of hypoxia on airway pressure in an isolated perfused and ventilated human lung preparation.pO_2; partial pressure of oxygen in the perfusate (KPa). **(b)** Mean perfusate pO_2 before exposure to hypoxia (baseline), after 20 and 50 minutes exposure to hypoxia and on return to ventilation with room air. **(c)** Mean peak airway pressure (mmHg) before exposure to hypoxia (baseline), after 10, 20 and 50 minutes exposure to hypoxia and on return to ventilation with room air. Numbers in parentheses denote number of values obtained at each time point and compared to baseline. *$p < .05$, **$p < .01$ via related-samples Wilcoxon signed-rank test vs. baseline (perfusate pO_2) or via paired samples (2-tailed) t-test (peak airway pressure).

6.3.1.2 Effect of Normoxic, Hypoxic and Hyperoxic ventilation with 5% CO_2 on Airway Pressure

Lung samples were obtained from one female (66years, right upper lobe) and one male (58 years, whole left lung) patient.

Ventilation with gases containing 5% CO_2 did not affect the pCO_2 or pH of the perfusate which remained within normal physiological parameters (pCO_2 3.3-5.2 KPa, pH 7.30-7.51). Switching ventilation from room air to ventilation with Air: 5% CO_2 did not significantly affect airway pressure. 30 minutes of hypoxic ventilation (95% N_2: 5% CO_2) reduced perfusate pO_2 (from 21.65 ± 1.48 to 18.70 ± 2.26 KPa) but did not affect airway pressure. Hyperoxic ventilation (95% O_2: 5% CO_2) increased perfusate pO_2 (from 18.70 ± 2.26 to 35.35 ± 17.04 KPa) but did not affect airway pressure (raw data Appendix II).

6.3.1.3 Relative influence of perfusate and airway pO_2 on the hypoxic response

Using a sweep gas of 95% N_2: 5% CO_2 across the gas phase of the membrane oxygenator caused a rapid and sustained hypoxia in perfusate entering the lung (pO_2 5.7 -16.0 KPa) and using a sweep gas of 95% O_2: 5% CO_2 caused a rapid and sustained perfusate hyperoxia (pO_2 50.0 KPa).

These results confirm that the isolated human lung model can be used effectively to investigate the relative contribution of changes in perfusate and/or airways oxygen concentration on the response of the airways and pulmonary vasculature to changes in oxygen tension.

The effects of independently changing the perfusate pO_2 and the concentration of oxygen in the ventilation gas on peak airway pressure were investigated in one whole left lung (male, 58 years).

The ventilation/perfusion combinations investigated and perfusate pO_2 are shown in **table 6.3.**

Table 6.3 Perfusion and ventilation combinations and perfusate pO_2 in *ex vivo* human lung.

Ventilation	**Perfusate**	**Perfusate pO_2 (KPa)**
Normoxia	Hypoxia	5.7
Hypoxia	Hypoxia	16.0
Normoxia	Hyperoxia	50.0
Hyperoxia	Hyperoxia	-

None of the experimental combinations performed in this experiment caused a significant change in airway pressure (raw data Appendix II). Measurement of perfusate pO_2 did however confirm that this method is effective for investigating the effect of changing the pO_2 of the perfusate independently of the pO_2 in the airways.

6.3.2 *In Vitro* Human Bronchi

6.3.2.1 Effect of Hypoxia on Resting Tension

Without exception, exposure to hypoxia caused a significant relaxation of all bronchi (n=50 from 25 patients, mean internal diameter 4.6 ± 2.2 mm) from resting tension in 95% O_2: 5% CO_2 (**figure 6.3**, **table 6.4**) (Raw data Appendix II).

Table 6.4 Magnitude and rate of relaxation of human bronchi to hypoxia from resting tension in 95% O_2. (n=50). Values are mean ± (SD) $^{**}p < .01$ vs. baseline tension via paired samples, 2-tailed t-test.

	Baseline pre hypoxia (gf)	Baseline post hypoxia (gf)	Hypoxic Dilation from baseline (gf)	Hypoxic Dilation from baseline (% Change)	Time to HBD plateau (min)
Mean (SD)	2.21(0.42)	1.07 (0.44)**	-1.14 (0.60)	-50.10 (21.27)	16.04 (7.02)

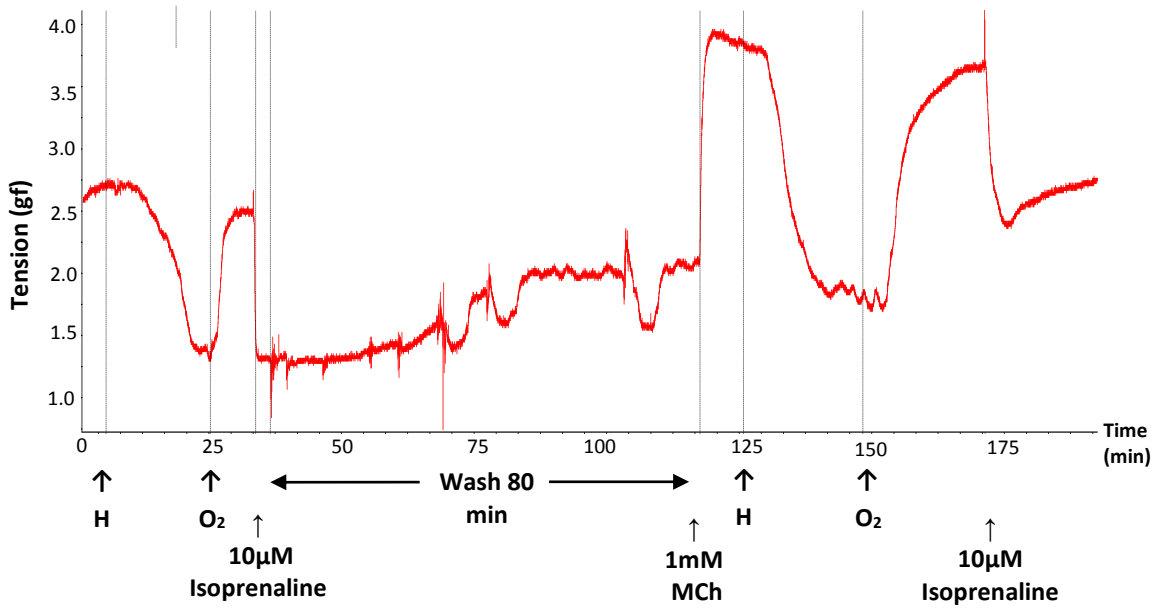

Figure 6.3 Experimental trace showing relaxation of a human bronchus to hypoxia and isoprenaline (10 µM) from resting tension and maximum active tension to 1 mM methacholine (MCh). H: hypoxia; O_2: 95% Oxygen

6.3.2.2 Effect of Hypoxia on Resting Tension; Comparison to β-adrenergic Relaxation

Hypoxia caused a robust and reversible relaxation of human bronchi (n=8 from 3 patients, mean internal diameter 3.9 ± 1.5 mm) from resting tension in 95% oxygen (**figure 6.3**). In individual bronchi there was no significant difference in the maximum magnitude of relaxation from resting tension to hypoxia or 10 μM isoprenaline (minimum baseline 0.9 ± 0.3 vs. 0.9 ± 0.4 gf or -58 ± 17 vs. 63 ± 24 % change from baseline for hypoxia and isoprenaline respectively) (**table 6.5, figure 6.4a**). The time taken to reach maximum relaxation from resting tension was significantly longer for hypoxia than relaxation to 10 μM isoprenaline (10.63 ± 3.38 vs. 2.56 ± 1.67 min for hypoxia and isoprenaline respectively, $p < .001$, via paired samples, 2-tailed t-test) (**table 6.5, figure 6.4b**) (Raw data Appendix II).

Table 6.5 Rate and magnitude of relaxation to hypoxia and 10 μM isoprenaline of bronchi (n=8) from resting tension in 95% O_2. Values are mean ± (SD). $^{**}p < .01$ for HBD vs. Isoprenaline via paired samples, 2-tailed t-test.

	Baseline pre exposure (gf)	**Baseline post exposure (gf)**	**Relaxation from baseline (gf)**	**% Change**	**Time to maximum relaxation (min)**
Hypoxia	2.17 (0.50)	0.87 (0.33)	-1.30 (0.51)	-58 (17)	11 (3)**
Isoprenaline	2.59 (0.47)	0.86 (0.37)	-1.73 (0.78)	-63 (24)	3 (2)

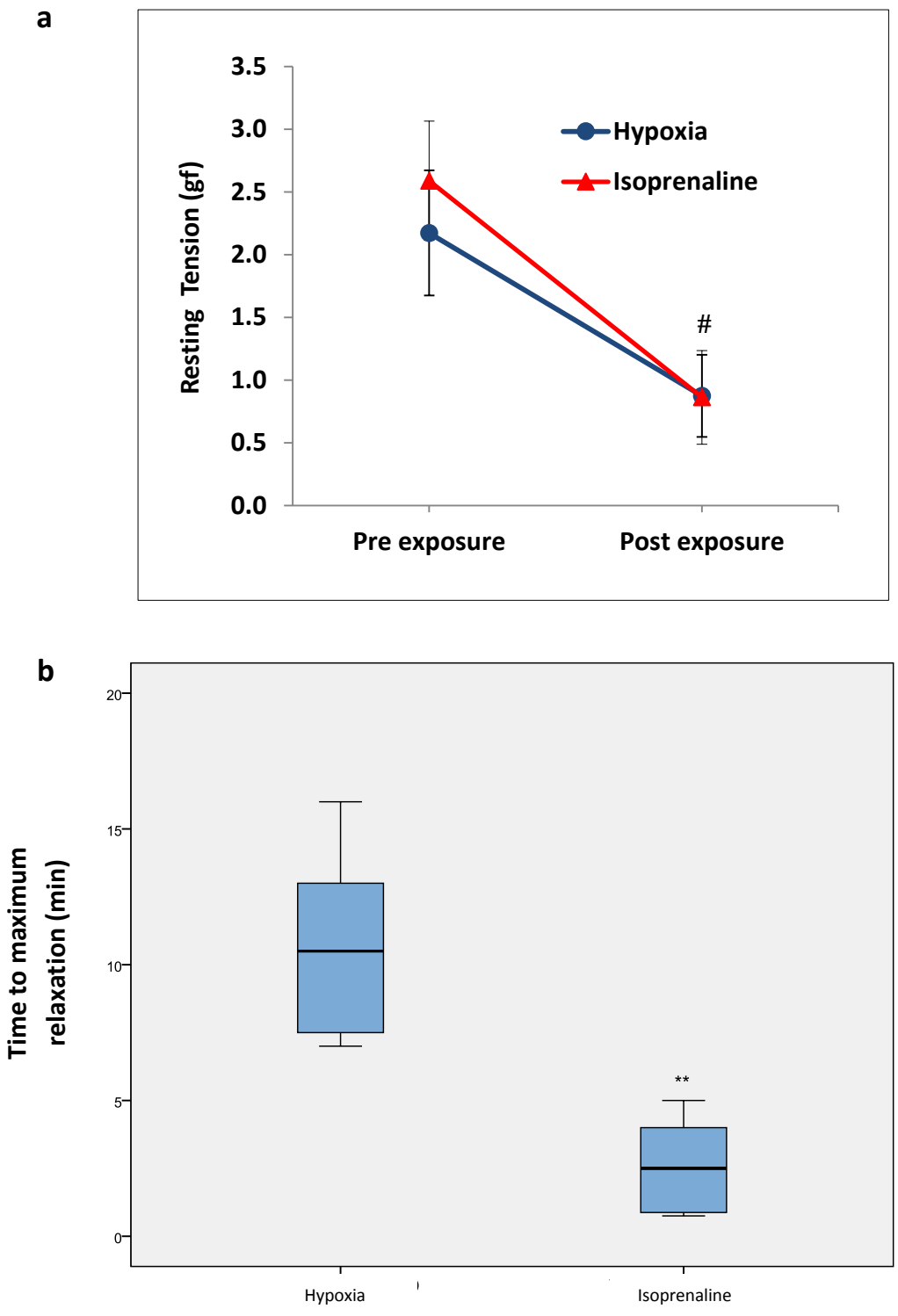

Figure 6.4 Magnitude and rate of relaxation to hypoxia and isoprenaline in isolated human bronchi **(a)** Change in resting tension (gf) from baseline on exposure to hypoxia and 10 µM isoprenaline in the same bronchi (n=8) **(b)** Box-whisker plot of time to maximum relaxation from baseline (min) on exposure to hypoxia and 10 µM isoprenaline (n=8) $^{**}p < .001$, $^{\#}p$ = NS, via 2-tail, paired t-test.

6.3.2.3 Effect of Hypoxia on Contraction to Methacholine; Comparison to β-adrenergic Relaxation

Hypoxia caused a complete and reversible relaxation to resting tension or below in human bronchi (n=34 from 18 patients, mean internal diameter 5.2 ± 2.6 mm) pre-contracted to 1 mM MCh in 95% oxygen (**figure 6.3**). In individual bronchi the magnitude of relaxation to hypoxia was significantly greater than the magnitude of relaxation to 10 μM isoprenaline (minimum baseline 1.6 ± 0.5 vs. 2.5 ± 1.4 gf or -117 ± 31 vs. -93 ± 39 % of MCh contraction for hypoxia and isoprenaline respectively, n=34, $p < .001$ via paired samples, 2-tailed t-test) (**table 6.6, figure 6.5a**). The time taken to reach maximum dilation was significantly longer for hypoxia than dilation to 10 μM isoprenaline (32.24 ± 14.46 vs. 4.56 ± 2.92 min for hypoxia and isoprenaline respectively, n=34, $p < .001$, via paired samples, 2-tailed t-test) (**table 6.6, figure 6.5b**) (Raw data Appendix II).

Table 6.6 Rate and magnitude of relaxation to hypoxia and 10 μM isoprenaline (Iso) in bronchi contracted to 1 mM MCh in 95% O_2 (n=34). Values are mean ± (SD). $^{**}p < .01$ for HBD vs. Isoprenaline via paired samples, 2-tailed t-test.

	Baseline Pre MCh (gf)	Active Tension to MCh (gf)	Baseline post exposure (gf)	Relaxation (gf)	% Reversal of MCh contraction	Time to maximum relaxation (min)
Hypoxia	1.89 (0.62)	4.23 (2.37)	1.56 (0.54)	-4.56 (2.13)**	-117 (31)**	32 (14)**
Iso	1.89 (0.62)	4.23 (2.37)	2.53 (1.36)	-3.59 (2.02)	-93 (39)	4.6 (2.9)

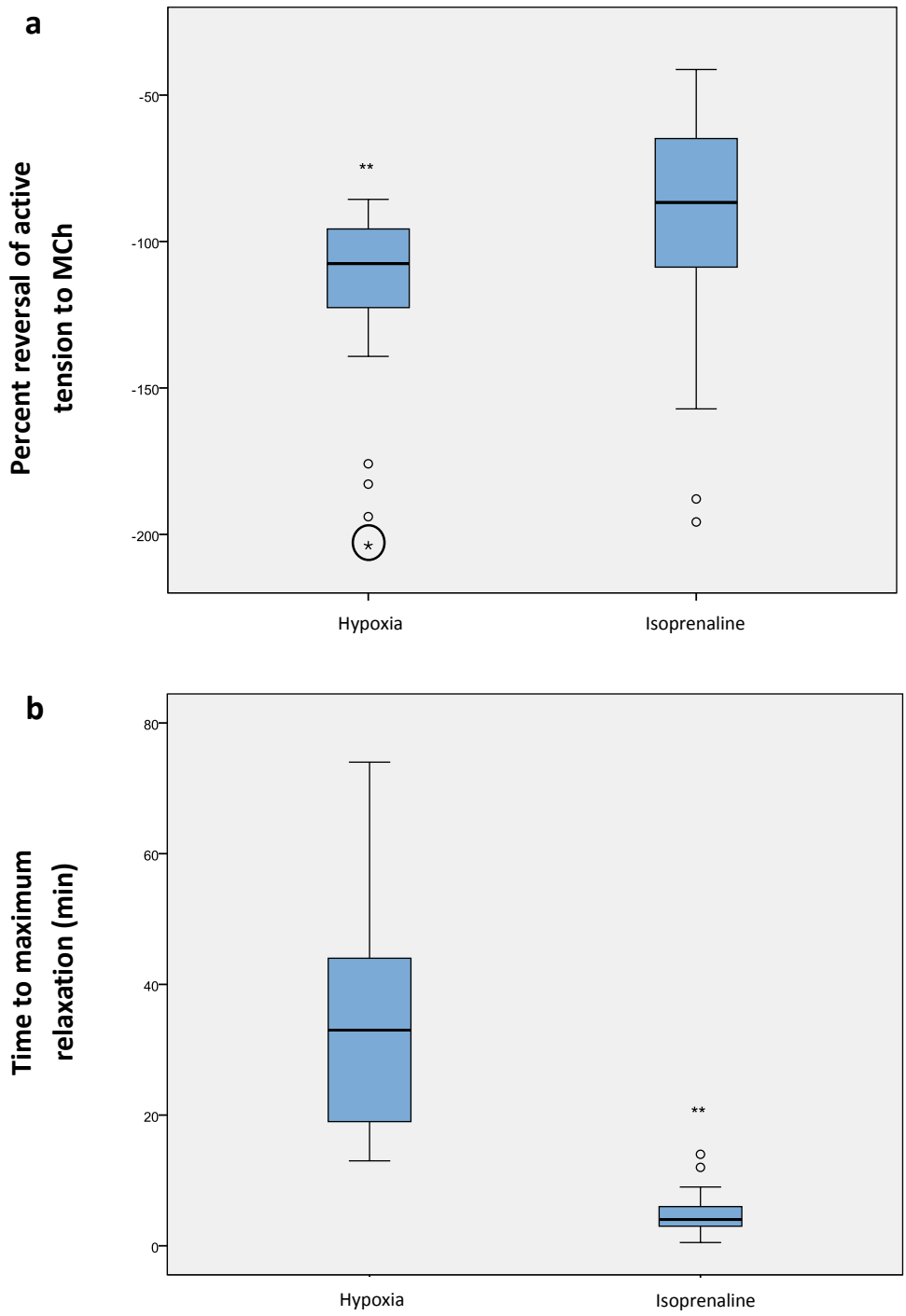

Figure 6.5 Magnitude and rate of relaxation to hypoxia and isoprenaline from maximum active tension to 1 mM MCh in isolated human bronchi **(a)** Box-whisker plot showing percentage reversal of active tension to 1 mM MCh on exposure to hypoxia and 10 µM isoprenaline (n=34) **(b)** Box-whisker plot of time to maximum relaxation to hypoxia and 10 µM isoprenaline from active tension to MCh (n=34). $^{**}p < .001$, via 2-tail, paired t-test. Circles denote outliers and circled asterisk denotes extreme outlier.

6.3.2.4 Concentration Dependent Effect of Changes in Oxygen Partial Pressure (pO_2) on bronchial tone

Reducing the oxygen concentration of the aerating gas in the organ baths from 95% to 40%, 21% and 0% caused a concentration dependent reduction in bronchial tone from resting tension (n=8 from 2 patients, mean internal diameter 4.0 ± 1.1 mm) (**table 6.7, figure 6.6a**) and in bronchi contracted to 1mM MCh (n=11 from 4 patients, mean internal diameter 4.7 ± 2.5 mm) (**table 6.8, figure 6.6b**) which was concentration dependently reversed on reoxygenation (**figure 6.6**) (Raw data Appendix II). (The resultant pO_2 concentration (KPa and mmHg) in the Krebs-Henseleit solution at different % oxygen concentrations in the aerating gas are shown in ***Chapter 2, 2.3.6, table 2.1***).

Table 6.7 Concentration dependent effect of O_2 on resting tension. % O_2; % O_2 concentration of aerating gas. Values are mean ± (SD). n=8

	% O_2			
	95	**40**	**21**	**0**
Resting Tension (gf)	1.81 (0.78)	1.37 (0.58)	1.04 (0.47)	0.75 (0.32)
Change in Resting Tension (gf)	-	-0.44 (0.33)	-0.77 (0.51)	-1.06 (0.66)
% Change	-	-22 (13)	-39 (20)	-53 (23)

Table 6.8 Concentration dependent effect of O_2 on active tension in bronchi contracted to MCh. %O_2; % O_2 concentration of aerating gas. % Change; % reversal of MCh contraction. Values are mean ± (SD). n=11

	% O_2			
	95	**40**	**21**	**0**
Active Tension (gf)	4.54 (1.01)	3.35 (0.78)	2.31 (0.66)	1.17 (0.53)
Change in Active Tension (gf)	-	-1.19 (0.57)	-2.23 (0.76)	-3.37 (0.95)
% Change	-	-43 (23)	-80 (28)	-120 (25)

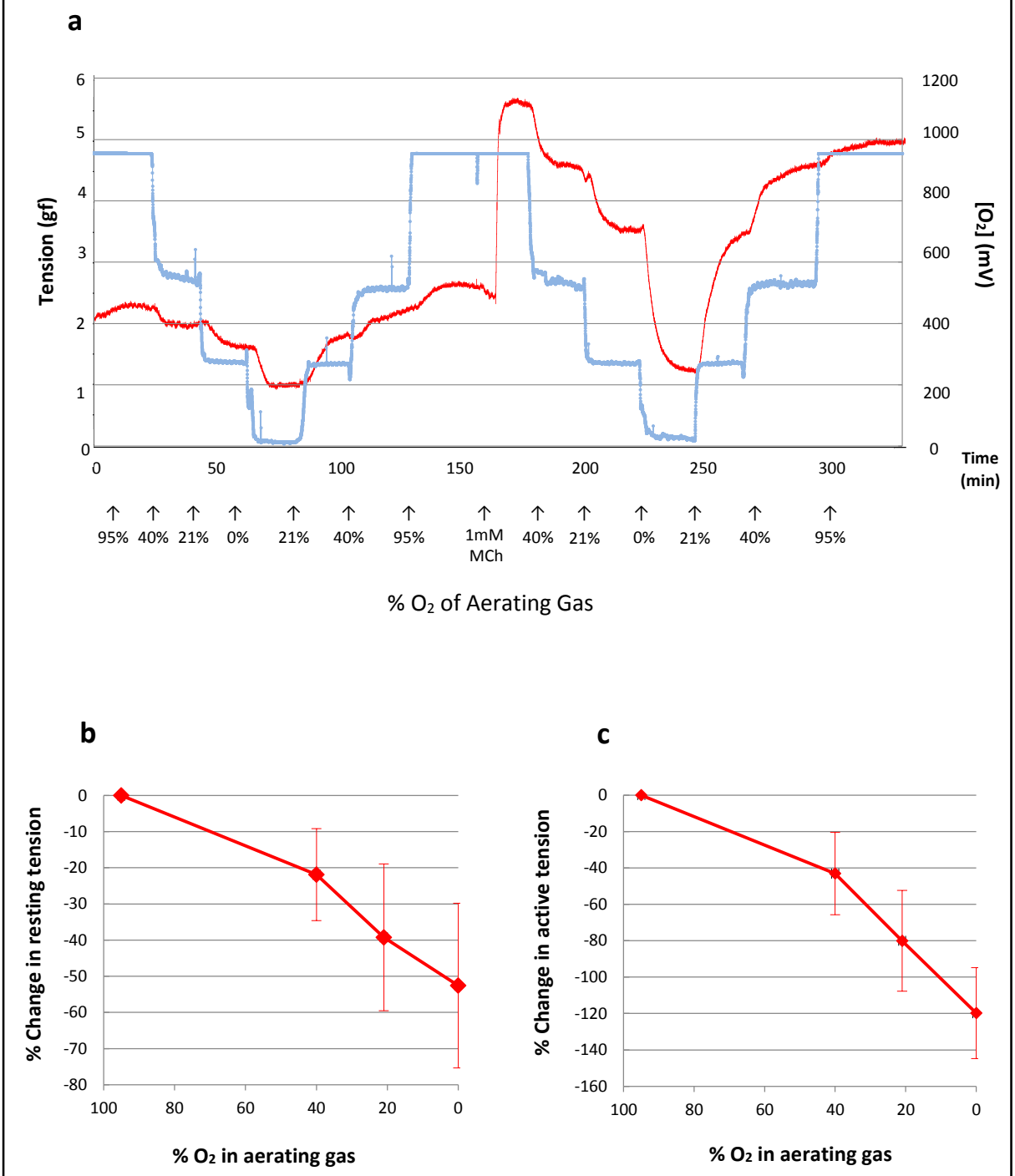

Figure 6.6 Concentration dependent effects of oxygen on resting tension and active tension to 1 mM MCh in isolated human bronchi. **(a)** Experimental trace (red) shows change in isometric tension (gf) with change in oxygen concentration of the organ bath buffer solution (blue) (mV) (10 mV ~ 1 KPa ~ 7.5 mmHg). **(b)** Oxygen concentration response curve from resting tension in isolated human bronchi (% inhibition of resting tension, n = 8). **(c)** Oxygen concentration response curve in isolated human bronchi contracted to 1 mM MCh (% inhibition of active tension, n = 11).

6.3.2.5 Effect of Hypercapnia on Resting Tension, Cholinergic Contraction and HBD

Hypercapnia (pCO_2 10-15 KPa) caused a small and significant relaxation of the bronchi from resting tension (-0.22 ± 0.6 gf or -12.80 ± 9.00 % of baseline, p = .01, n=6 responses) and a small but non-significant relaxation after cholinergic precontraction (-0.42 ± 0.18 gf or -19.65 ± 4.10 % of maximum MCh contraction, p = .10). Hypercapnia did not significantly affect HBD from baseline (-26.49 ± 1.68 vs -16.88 ± 7.55 % of baseline for normocapnic and hypercapnic conditions respectively. p = .22) or HBD following cholinergic contraction (-103.11 ± 13.66 vs -106.98 ± 15.71 % of maximum MCh contraction for normocapnic and hypercapnic conditions respectively. p = .23) (n=2 from one patient, mean internal diameter 2.0 ± 0.0 mm) (Raw data Appendix II).

6.4 Discussion

In this chapter it has been shown, for the first time, that hypoxia causes a robust and reversible relaxation of human airways. In a unique *ex vivo* perfused and ventilated human lung model exposure to hypoxia caused a rapid, robust and sustained reduction in airway pressure which was completely reversed on returning to ventilation with room air. In isolated human bronchi hypoxia cause a significant relaxation, in every preparation studied, from resting tension or from active tension induced by MCh.

In isolated human bronchi the magnitude of hypoxic bronchodilation (HBD) from resting tension was equal to that of the full β-adrenoceptor agonist isoprenaline. From active tension (induced by cholinergic contraction) the magnitude of HBD was significantly greater than the magnitude of relaxation to isoprenaline. However, the rate of relaxation to isoprenaline was significantly faster than the rate of HBD. Taken together these results suggest that the mechanism of HBD is different to that of β-adrenergic relaxation and may therefore represent a novel mode of bronchodilation. It could be argued that the dose of MCh used in these studies was supramaximal which could account for the disparity between the maximal responses to hypoxia and isoprenaline. However, this seems unlikely because the dose of isoprenaline used was also supramaximal. Given the magnitude of relaxation to hypoxia, a better understanding of the mechanisms involved could lead to the development of novel bronchodilator therapies with greater efficacy than those currently available.

The rate and magnitude of hypoxic bronchodilation in the pre-contracted human bronchi, described in this chapter, is very similar to that previously reported in isolated porcine [95-97] and canine [104] bronchi. These animal tissue preparations may therefore

represent good alternatives to human tissue preparations for investigating the effects of hypoxia on the airways. However, caution should be applied when interpreting results from animal tissue experiments, especially airway preparations, where significant species differences are thought to exist.

Only one previous study could be found in the literature describing the effect of hypoxia on *in vitro* human bronchi. Dagg *et al.* [84] used a standard organ bath preparation, with very similar experimental conditions to those used in this study, to investigate the effect of changing oxygen concentrations on resting tension and contractile responses of human bronchial rings. They reported that lowering the oxygen tension from hyperoxia to normoxia or hypoxia caused only a marginal and non-significant reduction in resting tension. They also found that reducing the oxygen tension did not significantly attenuate cholinergic contractions. However, in the results reported by Dagg *et al*., the maximum contractions evoked by methacholine were 761.3 ± 178.9 mg under hyperoxic conditions compared to 613.0 ± 156.2 mg and 483.5 ± 149.9 mg under normoxic and hypoxic conditions respectively. These results suggest a trend towards a reduction in cholinergic contraction with reduced oxygen concentration; however, the results were not statistically different and were not considered significant. In contrast, in the current study, hypoxia was found to cause a significant and reversible reduction in resting tension and a complete and reversible relaxation of cholinergic contraction. There are some differences between the experimental protocols used by Dagg *et al.* and those used in the present study which might explain these discrepancies. Dagg *et al*. used 4% O_2 as their hypoxic gas mixture whereas in this study 0% O_2 was used which might have had a greater effect. However, this explanation would seem unlikely because in the present study hypoxia was shown

to have a marked concentration dependent effect, both on resting and active tension, which was near to maximum at 4% O_2. Another possible explanation is that Dagg *et al.* reapplied the resting tension, following relaxation, in the normoxic and hypoxic bronchi. Increasing the resting tension to compensate for a relaxation in an isometric preparation can increase the length of the smooth muscle fibres which could increase subsequent isometric contraction. Reapplying the resting tension in the normoxic and hypoxic bronchi but not the hyperoxic bronchi would have increased the length of the smooth muscle fibres in the hypoxic and normoxic bronchi and could have caused a misleading increase in the subsequent cholinergic contractions which would account for the marginal results reported by Dagg *et al.* Another difference between this study and that of Dagg *et al* is that in this study bronchi were contracted to 1 mM of methacholine whereas Dagg *et al.* used 3 µM methacholine. It might be that larger contractions, induced by higher concentrations of methacholine, are needed to reveal the full effect of hypoxia. An alternative explanation is that in Dagg *et al*.'s study contractions to methacholine were performed under hyperoxic, normoxic or hypoxic conditions whereas in this study all contractions were initiated under hyperoxic conditions and the effects of hypoxia on the maintenance phase of cholinergic contraction was investigated. It has previously been shown in isolated porcine bronchi [95] that initiation of cholinergic contraction is not affected by hypoxia but that the maintenance phase of contraction is reversibly inhibited by hypoxia. This finding is consistent with Dagg *et al*.'s results and the results from the current study and suggests that different mechanisms are involved in the initiation and maintenance phases of cholinergic contractions which are differentially affected by hypoxia. Further

studies are needed to investigate the effects of hypoxia on the initiation phase of cholinergic contractions in human airways.

In this chapter the effect of hypercapnia on airway tone was investigated in *ex vivo* human lungs and *in vitro* human bronchi for the first time. In *ex vivo* lungs switching ventilation from room air to ventilation with Air: 5% CO_2 did not significantly affect airway pressure. However, in these experiments, switching to air with 5% CO_2 did not increase the pCO_2 of the perfusate above physiological parameters, possibly due to the buffering capacity of the perfusate, and in future experiments it may be necessary to increase the CO_2 concentration of the ventilating gas to induce a hypercapnic response. However, these experiments did act as controls for the experiments investigating the effects of hypoxia on the isolated lungs, in that it proved that 5% CO_2 in the ventilation gas was not the cause of bronchodilation in lungs ventilated with 95% N_2: 5% CO_2. In isolated human bronchi hypercapnia (pCO_2 10-15 KPa) caused a small and reversible relaxation from resting (-13 ± 9 %) and active tension (-20 ± 4 %) which was consistent with findings in porcine [98] and bovine tracheal muscle strips [102]. However, hypercapnia had no effect on the rate or magnitude of hypoxic bronchodilation from resting or active tension. These results suggest that, at a local level, hypercapnia does not mediate or modulate the effect of hypoxia on bronchial tone. However, one of the limitations of the models used in the current study is that they are exempt from neural and hormonal control mechanisms. Previous *in vivo* studies [85-87, 90, 91] have shown similar and consistent responses of the airways to hypoxia and hypercapnia and in some canine studies [85-87] both responses were dependent on an intact vagal tone which suggests that the effects of CO_2 are mediated

systemically via modulation of vagal activity. Further studies are warranted to determine the central/autonomic effects of carbon dioxide in regulating airway tone.

The parallel perfused organ bath system developed for this study is a novel method for accurately regulating the partial pressure of any gas in the buffer solution of isolated tissue preparations. In future studies this system will facilitate studies to investigate the effects of different gases on *in vitro* tissue preparations.

Debate continues in the literature regarding the relative importance of alveolar and pulmonary vascular hypoxia in triggering the hypoxic response in the lung [7, 89, 163, 225, 226]. In this study a novel system was developed to investigate the effect of independently changing the perfusate and alveolar oxygen concentrations on airway pressure. In a single experiment the effect of various combinations of perfusate and alveolar oxygen concentrations on airway pressure was investigated. However, no significant effects were observed. It was surprising that a combination of alveolar and perfusate hypoxia did not cause a reduction in airway pressure. However, in this particular experiment the perfusate reservoir was not flooded with nitrogen to prevent the uptake of ambient oxygen and the perfusate pO_2 was quite high (16 KPa). It is likely that the oxygen in the perfusate would have equilibrated with the alveolar gas and may have raised the airway pO_2 above the concentration necessary to trigger HBD. An improvement of this model would be the ability to directly measure or calculate the alveolar pO_2. Clearly it is not possible to draw any conclusions from a single experiment; however, this experiment did confirm that this is an effective model in which to investigate the differential effects of alveolar and perfusate pO_2 on the hypoxic response.

The finding in this chapter that oxygen caused a concentration dependent constriction of the bronchi from resting and active tension has significant implications both scientifically and clinically. The vast majority of *in vitro* tissue experiments are performed in solutions bubbled with 95% O_2. Historically it was believed that it was necessary to use high oxygen concentrations in isolated tissue experiments, to compensate for the lack of haemoglobin in the perfusate solutions used, and ensure adequate bioavailability of oxygen. It was also assumed that oxygen was relatively inert and did not affect responses in the tissue under investigation. Findings from this study have shown that this is not true- that the oxygen concentration of the aerating gas can have a significant effect on the contractile state of the airway smooth muscle. Furthermore, when measuring the oxygen concentration in the buffer solutions it was found that the pO_2 did not fall for an extended period even when the aerating gas was turned off- indicating that the tissue was using very little of the available oxygen. Using hyperoxia in isolated tissue preparations could therefore lead to misleading results which are not relevant in physiological conditions. This suggestion is supported by studies in isolated airway preparations; in isolated bovine bronchi changes in oxygen tension (hyperoxia, normoxia and hypoxia) had a marked effect on the contractile response to methacholine and the efficacy of bronchodilators [103, 227] and in isolated human bronchi relaxation to salbutamol was found to be significantly greater in hyperoxia and normoxia compared to hypoxia [84].

These findings could explain why some drugs show promising efficacy *in vitro* but do not demonstrate an equivalent clinical efficacy. Considering that all tissue is sensitive to oxygen the validity of millions of *in vitro* experiments could be questioned. It should also be mentioned that the glucose concentrations used in most physiological buffer

solutions are also high (11 mM in Krebs-Henseleit solution); based on the same reasoning for using a high oxygen concentration. This concentration of glucose represents a hyperglycaemic state and can also affect responses in the tissue under investigation. For future *in vitro* experiments the physiological buffer used should be 'tailored' to more closely reflect the normal physiological conditions of the tissue being studied.

In the critical care setting and during anaesthetic procedures high FiO_2 (fraction of inspired oxygen) concentrations are frequently used. It is recognised that high FiO_2 can cause 'hyperoxic acute lung injury' (HALI). HALI is characterised as a progressive inflammatory response which is ultimately fatal (reviewed by Kallet and Matthay [110]). The findings of the current study suggest that hyperoxia may also cause a significant bronchoconstriction which could contribute to the pathophysiological effects of oxygen in the lung (possibly by increasing barotrauma) and this is in need of further investigation.

In this chapter it has been shown, for the first time, that hypoxia causes a robust and reversible relaxation of human airways. Obstructive airway disease can result in acute regional hypoxia in the lung. It is likely that hypoxic bronchodilation will play an important physiological role in increasing airflow to under ventilated portions of the lung and improve perfusion-ventilation matching. Further chapters of this thesis will examine the mechanism of HBD. It is hoped that a better understanding of the mechanisms behind this robust effect will lead to a better understanding of lung physiology and the development of more effective bronchodilator therapies for the treatment of obstructive airway diseases such as asthma and COPD.

CHAPTER 7

Mechanism of Hypoxic Bronchodilation: Role of the Epithelium, Adrenergic Receptors and Intracellular Signalling Pathways

Contents

7.1 Introduction

In ***Chapter 6*** it was shown that hypoxia causes a robust and reversible relaxation of human airways. Characterisation of the mechanisms responsible for hypoxic bronchodilation (HBD) in human airways could lead to the development of improved therapeutics for the treatment of constrictive airway diseases such as asthma and COPD. However, only a limited number of studies have previously described HBD and consequently even fewer studies have attempted to characterise the mechanisms of HBD and the results from those studies were inconsistent. In the 1990's a group from The John Hopkins University, Baltimore attempted to characterise the mechanism of HBD in porcine isolated bronchi. In an initial study [95] they found that HBD was independent of an intact epithelium and was not attenuated by the soluble guanylate cyclase inhibitor methylene blue, the cyclooxygenase inhibitor indomethacin or the β-adrenoceptor antagonist propranolol. In this study they also found that pre exposure of porcine bronchi to hypoxia inhibited subsequent contraction to KCl but not to carbachol [95] which suggested that hypoxia modulates airway tone in porcine bronchi by altering extracellular calcium entry into the smooth muscle. In subsequent studies they investigated the role of the K_{ATP} channel [96] and the role of dihydropyridine-sensitive calcium entry [97] in HBD and concluded that opening of K_{ATP} channels is involved in HBD during moderate hypoxia and that hypoxia causes relaxation by inhibiting Ca^{2+} entry through dihydropyridine-sensitive channels and another unknown mechanism. However, other mechanistic studies have shown different effects to the results found in porcine bronchi. In porcine tracheal smooth muscle strips hypoxia was found to inhibit cholinergic contraction independently of changes in intracellular calcium [99]. Hypoxic relaxation of guinea-pig tracheal rings was shown to be dependent

on an intact epithelium and independent of extra and intra-cellular Ca^{2+} mobilisation [100] and pre exposure to hypoxia inhibited maximal cholinergic and histaminergic contractions of guinea-pig tracheal strips [101, 228] whereas in bovine bronchi hypoxia augmented contraction to methacholine [103]. A study in canine isolated bronchi found that hypoxia caused an epithelium dependent relaxation which was not blocked by methylene blue, indomethacin, propranolol or tetrodotoxin and it was concluded that in canine bronchi hypoxia was causing release of a relaxing factor from the airway epithelium [104].

The inconsistencies in previous animal studies could be explained by species differences. In contrast to vascular smooth muscle it is recognised that there are considerable species differences in the contractile and relaxant mechanisms of airway smooth muscle [8, 9, 11, 229, 230] which emphasises the need for studies in human tissue to identify novel targets in the development of effective therapeutics for the treatment of obstructive airway diseases in man.

To date no studies have attempted to characterise the mechanism of HBD in human airways. Given that significant species differences are known to exist in the regulation of airway tone and that previous animal studies investigating the mechanisms of hypoxic airway relaxation were inconsistent, in attempting to characterise the mechanism of HBD in human bronchi it was necessary to start from the basic mechanisms which are known to modulate airway tone. Relaxation of the airways can result from an inhibition of contractile mechanisms and/or activation of dilator mechanisms. The G-protein coupled receptor systems and classical signalling pathways which mediate contraction and relaxation of the airway smooth muscle are described

in ***Chapter 1, Figure 1.1 and Figure 1.2*** and were used as a starting point to identify potential membrane receptors and cellular pathways which could mediate HBD and provide rationale for the functional studies undertaken in this chapter. A number of specific agonists, antagonists and enzyme inhibitors were used to investigate the potential role of selected receptor systems and intracellular signalling pathways in the mechanism of hypoxic bronchodilation.

In addition to the regulation of airway smooth muscle tone by the G-protein coupled receptor systems previous studies have shown that the airway epithelium can play an important role in modulating airway bronchomotor tone and it is suggested that a loss of functional integrity of the airway epithelium is responsible, at least in part, for the bronchial hyperactivity seen in asthma and airway infection [38, 40, 41]. The epithelium can affect airway smooth muscle contractility via a number of mechanisms; (1) providing a diffusion barrier to prevent penetration of contractile and relaxant agents to the smooth muscle layers and protecting the intra-epithelial nerves from stimulation by bioactive agents (2) metabolising bioactive agents and (3) releasing so called epithelium derived relaxing factors (EpDRFs) to cause relaxation of airway smooth muscle. There is debate in the literature about the relative importance of these different mechanisms in health and disease (reviewed by Folkerts and Nijkamp [36], Spina [37] , Knight and Holgate [38]). There is also a lack of consensus about the molecular identity of EpDRFs. Epithelial cells have been shown to metabolise arachidonic acid (AA) and a number of AA metabolites have been proposed as potential EpDRFs. Similarly nitric oxide synthase (NOS) has been demonstrated to produce nitric oxide (NO) in airway epithelium and may act as an EpDRF. However, debate continues in the literature about the relative significance of arachidonic acid metabolites and NO in

contributing to the regulation of airway tone by the epithelium [36-40]. Given that the epithelium has been shown to play a significant role in the regulation of airway tone and that previous studies found that hypoxic relaxation of guinea-pig trachea [100] and canine bronchi [104] was dependent on an intact epithelium the role of the epithelium and its constituent enzyme systems in the hypoxic relaxation of human bronchi was investigated in this chapter.

Another interesting potential signalling pathway in HBD is carbon monoxide (CO). CO is released endogenously within cells by the catalytic degradation of haem to biliverdin and CO by the enzyme haem oxygenase (HO). Haem oxygenase has two isoforms; HO-1 and HO-2. HO-2 is constitutively expressed whereas HO-1 is inducible. There is growing interest in the physiological role of CO and the therapeutic potential of CO and carbon monoxide releasing molecules (CO-RMs) in cardiovascular and respiratory disease [231-235]. In the 1990's Cardell and co-workers investigated the *in vivo* effects of CO and hypoxia on airway tone in guinea-pigs. In an initial study [93] it was shown that inhalation of CO caused a dose dependent inhibition of histamine induced increases in total pulmonary resistance. In this study pre-treatment of the animals with the competitive cGMP analogue Rp-8Br-cyclic GMPs caused a 60% inhibition of the CO induced bronchodilation whereas pre-treatment with a nitric oxide synthase inhibitor (L-NAME) had no effect and it was concluded that CO was causing bronchodilation *in vivo* via cyclic GMP. In a subsequent study [236] Cardell *et al*. investigated the role of endogenously produced CO in hypoxic bronchodilation. It was shown that HO-2 was constitutively expressed in airway smooth muscle and epithelium and that HO-1 expression was up-regulated by exposure to hypoxia. Furthermore, it was found that HBD (following histaminergic contraction of the airways) was more than 75% inhibited

by pre-treatment with Rp-8Br-cyclic GMPs and that this inhibitory effect was mimicked by pre-treatment with the haem oxygenase inhibitor zinc protoporphyrin-IX (ZnPP). Taken together these findings suggested a role for CO in the hypoxic regulation of airway tone which is mediated via a cGMP dependent pathway. A more recent study [237] demonstrated the expression of HO-2 and the induction of HO-1 expression in human airway epithelial cells which suggests that CO could be acting as an EpDRF in mediating HBD. In this chapter the haem oxygenase inhibitor tin protoporphyrin-IX (Sn-PP) and selective cGMP pathway inhibitors, including Rp-8Br-cyclic GMPs, were used to investigate the putative signalling role of CO in the hypoxic relaxation of human bronchi.

An interesting study by Kizub *et al*. [238] has recently identified a role for gap junctions (GJs) in the maintenance phase of hypoxic pulmonary vasoconstriction. Gap junctions are channels, composed of six connexin proteins, which connect cells and provide a direct pathway for electrical and signalling molecule communication between cells. Connexins are extensively expressed in the lung including the respiratory epithelium, trachea, vascular endothelium, vascular smooth muscle and fibroblasts and gap junctions facilitate communication between the different functional compartments of the lung [239]. Kizub *et al*. [238] investigated the effects of GJ inhibitors on the transient Phase 1 and the sustained Phase 2 of HPV in rat isolated intrapulmonary arteries and in anaesthetised rats. They found that GJ inhibitors completely abolished Phase 2 but had little effect on Phase 1 of HPV and provided evidence that the GJs are critically involved in signalling pathways between the vascular endothelium and vascular smooth muscle, leading to calcium sensitization of the smooth muscle which is essential for the sustained Phase 2 of HPV. These findings also provided an explanation

for why an intact endothelium is necessary for Phase 2 of HPV. In the current study it was postulated that an analogous GJ signalling pathway could exist between the epithelium and airway smooth muscle and play a role in the mechanism of HBD. In the previous study by Kizub *et al*. [238] it was shown that 2-aminoethoxyldiphenyl borate (2-APB), which is an IP_3 receptor antagonist and store-operated Ca^{2+} entry (SOCE) inhibitor, is also an effective inhibitor of GJs. In the current study the effect of 2-APB on HBD was investigated with a view to investigating the role of the IP_3 receptor pathway, SOCE and the putative role of GJ signalling in HBD.

In this chapter it was hypothesised that a number of intracellular or epithelial cell pathways could play a role in the mechanism of HBD.

The specific aims of the studies in this chapter were;

1. To determine the role of the epithelium in the hypoxic relaxation of human bronchi
2. To investigate the role of adrenergic receptors and intracellular signalling pathways in the mechanism of hypoxic bronchodilation
3. To investigate the role of carbon monoxide in HBD

7.2 Methods

7.2.1 Tissue Preparation

Human bronchi (primary, second order or tertiary) were obtained at resection for lung cancer, prepared and mounted in 25 ml organ baths as described in ***Chapter 2, 2.3*****.** A resting tension of 1-3g was applied and the bronchi allowed to equilibrate for 60-90 minutes.

7.2.2 Effect of Removing the Epithelium

Two bronchial rings from the same donor were mounted in separate organ baths.

After equilibration for 70 minutes and when a stable resting tension was achieved control responses to hypoxia from resting tension and to hypoxia and isoprenaline after contraction to methacholine [1mM] were obtained as described in ***6.2.2.2*** and ***6.2.2.3***.

After a 30 minute wash out the bronchi were removed from the organ baths and the epithelium removed from one bronchus by gently rubbing the internal lumen with a 1.5mm diameter brush (Endoscope cleaning brush, Pentax UK Ltd.) for 3-5 minutes. The bronchi were then re-mounted in the organ baths and set at the same resting tension which was recorded before removal from the organ bath. After equilibration for 90 minutes and when a stable resting tension was achieved repeat responses were obtained to hypoxia and isoprenaline.

7.2.3 Relaxation of Bronchi to Hypoxia and Sodium Nitroprusside (SNP)

Preliminary experiments found that the maximum relaxation of bronchi to SNP was achieved at a concentration of 100 μM. This concentration of SNP was therefore used

to compare the magnitude of relaxation to SNP with the magnitude HBD in the same bronchi from active tension to 1 mM MCh.

7.2.4 Antagonist studies

After obtaining control responses to hypoxia from resting tension and to hypoxia and isoprenaline [10 μM] from active tension to methacholine [1 mM] the bronchi were washed for 30 minutes before adding antagonists directly to the organ bath. After a minimum incubation of 30 minutes repeat responses to hypoxia and isoprenaline were obtained from resting and active tension. The effect of antagonists on resting baseline tension, cholinergic contraction, HBD from resting and active tension and β-adrenergic relaxation from active tension was determined by comparison with pre-exposure controls and quantified as % change from control. In some experiments the effects of antagonists were only investigated on relaxant responses from active tension.

7.2.4.1 Adrenergic Receptors and Extracellular Enzymes

The effect of adrenoceptor antagonism and haem oxygenase, cyclooxygenase and nitric oxide synthase inhibitors were investigated. Concentration of drugs used, pharmacological action and putative site of action referred to in ***figure 1.2*** (***Chapter 1***) are shown in **table 7.1**.

Table 7.1 Receptor antagonist and enzyme inhibitors used. Pharmacological action, concentration used and reference to site of action in **figure 1.2** (red)

Drug	**Pharmacological Action**	**Concentration [µM]**	**Site of action**
Labetalol hydrochloride	Non- selective adrenergic antagonist	100	[16]
Indomethacin	Non selective Cyclooxygenase (COX) 1 and 2 inhibitor	10	[17]
L-NAME	Nitric oxide (NO) synthase inhibitor	1000	[18]
Tin protoporphyrin IX dichloride (Sn-PP)	Haem oxygenase inhibitor	100	

7.2.4.2 Intracellular Signalling Mechanisms

A number of intracellular enzyme inhibitors and intracellular receptor antagonists were used to investigate the intracellular signalling and putative Gap junction pathway in the mechanism of HBD. The concentration of drug used, pharmacological action and putative site of action referred to in ***figure 1.1*** and ***figure 1.2*** (***Chapter 1***) are shown in **table 7.2**. For experiments investigating the effects of the selective protein kinase A inhibitor KT 5720 positive controls were obtained to isoprenaline and for experiments investigating the effects of the selective protein kinase G inhibitor KT5823 or the competitive cGMP analogue Rp-8-Br-cGMPs positive controls were obtained to sodium nitroprusside (100 µM).

Table 7.2 Intracellular inhibitors and agonists used to investigate the intracellular signalling mechanisms of HBD. Pharmacological action, concentration used and reference to site of action in ***figure 1.1*** (blue) and ***figure 1.2*** (red)

Drug	**Pharmacological Action**	**Concentration [µM]**	**Site of action**
2-APB	IP_3 receptor antagonist and gap junction inhibitor	50	[1] [19] [20]
KT 5720	Selective Protein Kinase A (PKA) inhibitor	0.2	[8]
KT 5823	Selective Protein Kinase G (PKG) inhibitor	0.4	[9]
Methylene Blue	Soluble guanylate cyclase inhibitor	50	[12]
Rp-8-Br-cGMPs	Competitive cGMP analogue	10/30	[13]
Ryanodine	Ryanodine receptor (RyR) antagonist at µM and partial agonist at nM concentrations	0.01/10	[3]

7.3 Results

7.3.1 Effect of Removing the Epithelium

Eight paired bronchi were obtained from three patients (mean internal diameter 8.1 ± 3.3 mm). The individual internal diameters, mucosal layer depth and width of the four paired control and experimental preparations did not differ significantly (**table 7.3**).

Table 7.3 Individual dimensions of paired control and experimental preparations used to investigate the effect of epithelium removal on HBD

	Control			Experimental		
Pair	**Internal diameter (mm)**	**Mucosa (mm)**	**Width (mm)**	**Internal diameter (mm)**	**Mucosa (mm)**	**Width (mm)**
1	8	3	4	9	2	7
2	13	2	4	10	3	6
3	8	3	4	10	2	5
4	4	2	5	3	1	4
Mean	**8.3**	**2.5**	**4.3**	**8.0**	**2.0**	**5.5**
SD	**3.7**	**0.6**	**0.5**	**3.4**	**0.8**	**1.3**

7.3.1.1 Effect on Cholinergic Contraction

The magnitude of cholinergic contraction was not changed significantly by denudation of the epithelium or in paired time- matched control preparations (**figure 7.1**, **table 7.4**). (Raw data Appendix II).

Table 7.4 Effect of denuding the epithelium on maximum cholinergic contraction (gf) in experimental and paired time-matched control preparations (n=4 in both groups). Mean ± (SD)

	Maximum Cholinergic Contraction (gf)	
	Pre Denudation	**Post Denudation**
Experimental	1.76 (0.32)	1.79 (0.80)
Paired controls	1.55 (1.33)	1.29 (0.58)

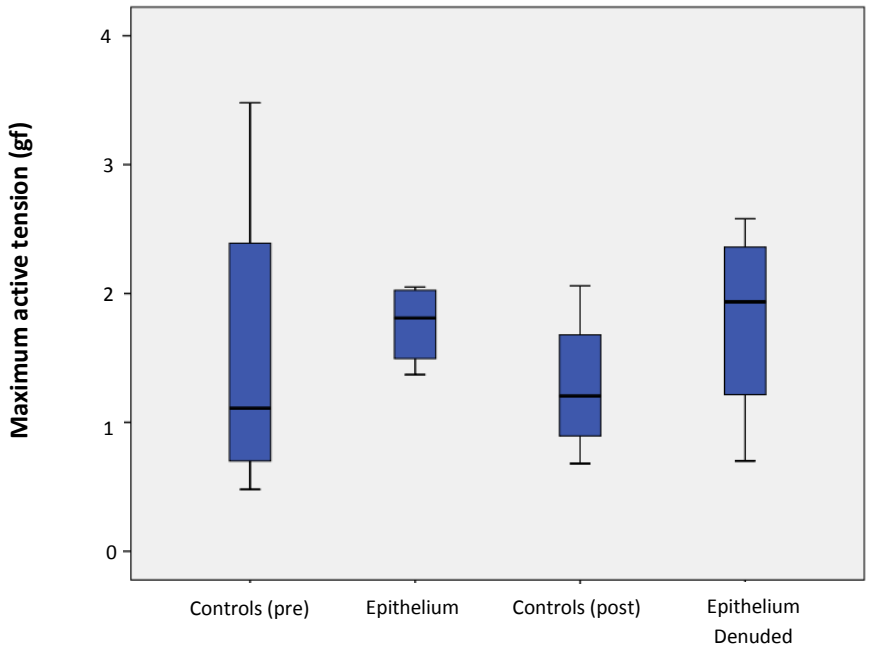

Figure 7.1 Effect of removing the epithelium on maximum cholinergic contraction. Box-whisker plots of maximum active tension to 1 mM MCh in experimental preparations (n=4) before (epithelium) and after (epithelium denuded) denudation of the epithelium and in paired time-matched control groups pre and post (n=4). There were no significant statistical differences between the groups (via paired t-test, 2-tail)

7.3.1.2 Effect on Hypoxic Bronchodilation

7.3.1.2.1 Effect on HBD from Resting Tension

There was considerable variability in the magnitude of HBD from resting tension. Removing the epithelium reduced the magnitude of HBD from resting tension in experimental preparations whereas in paired time-matched control preparations the magnitude of HBD was unchanged (**table 7.5, figure 7.2a**, raw data Appendix II). The attenuation of HBD from resting tension after denudation of the epithelium was not statistically significant and in two of the four experimental preparations the magnitude of HBD was augmented by removal of the epithelium.

The time taken to reach maximum relaxation from resting tension was not significantly different after removal of the epithelium and did not change significantly in paired time-matched control preparations (**table 7.6**, raw data Appendix II).

7.3.1.2.2 Effect on HBD from Active Tension

The magnitude of HBD from active tension to 1mM MCh was significantly reduced after denudation in experimental preparations. In paired time-matched controls the magnitude of HBD was also reduced over time; however, the magnitude of HBD in denuded preparations was significantly less than in paired time-matched preparations and the % attenuation of the maximum HBD from active tension was significantly greater in denuded experimental preparations than in paired time-matched control preparations. (**Table 7.5, figure 7.2b**, raw data Appendix II).

Table 7.5 Effect of denuding the epithelium on the magnitude of HBD from resting and active tension in experimental and paired time-matched control preparations (n=4 in both groups). Mean ± (SD) *$p < .05$, **$p < .01$ pre vs. post denudation via paired t-test (2-tail), $^{\#}p = .05$, $^{\#\#}p = .01$ denuded vs. control via unpaired t-test (2 tail)

	% Relaxation from resting tension		**% Relaxation from active tension**		**% Attenuation of max HBD from active tension**
	Pre Denudation	**Post Denudation**	**Pre Denudation**	**Post Denudation**	
Experimental	-20 (17)	-14 (3)	-108 (11)	-72 (7)* ##	-33 (10)#
Paired controls	-25 (25)	-23 (17)	-106 (5)	-85 (1)**	-20 (3)

The time taken to reach maximum relaxation to hypoxia from maximum active tension to 1mM MCh was not significantly different after removal of the epithelium or in time-matched controls and there was no significant difference between experimental and control preparations (**table 7.6**, raw data Appendix II).

Table 7.6 Effect of denuding the epithelium on the rate of HBD from resting and active tension in experimental and paired time-matched control preparations (n=4 in both groups). Mean ± (SD)

	Time to maximum HBD from resting tension (min)		**Time to maximum HBD from active tension (min)**	
	Pre Denudation	**Post Denudation**	**Pre Denudation**	**Post Denudation**
Experimental	12 (4)	18 (8)	14 (2)	13 (2)
Paired controls	17 (9)	20 (8)	24 (9)	16 (3)

7.3.1.3 Effect on β-adrenergic Relaxation

Removing the epithelium did not significantly affect the rate or magnitude of β-adrenergic relaxation to 10 µM isoprenaline from resting or active tension (**table 7.7, table 7.8, figure 7.3**, raw data Appendix II).

Table 7.7 Effect of denuding the epithelium on the magnitude of β-adrenergic relaxation from resting and active tension in experimental and paired time-matched control preparations (n=4 in both groups). Mean ± (SD)

	% Relaxation from resting tension		% Relaxation from active tension	
	Pre Denudation	**Post Denudation**	**Pre Denudation**	**Post Denudation**
Experimental	-22 (10)	-25 (10)	-96 (30)	-84 (21)
Paired controls	-25 (24)	-36 (24)	-78 (16)	-78 (27)

Table 7.8 Effect of denuding the epithelium on the rate of β-adrenergic relaxation from resting and active tension in experimental and paired time-matched control preparations (n=4 in both groups). Mean ± (SD)

	Time to maximum relaxation from resting tension (min)		Time to maximum relaxation from active tension (min)	
	Pre Denudation	**Post Denudation**	**Pre Denudation**	**Post Denudation**
Experimental	2 (1)	1 (1)	3 (1)	2 (1)
Paired controls	2 (1)	1 (1)	3 (1)	3 (2)

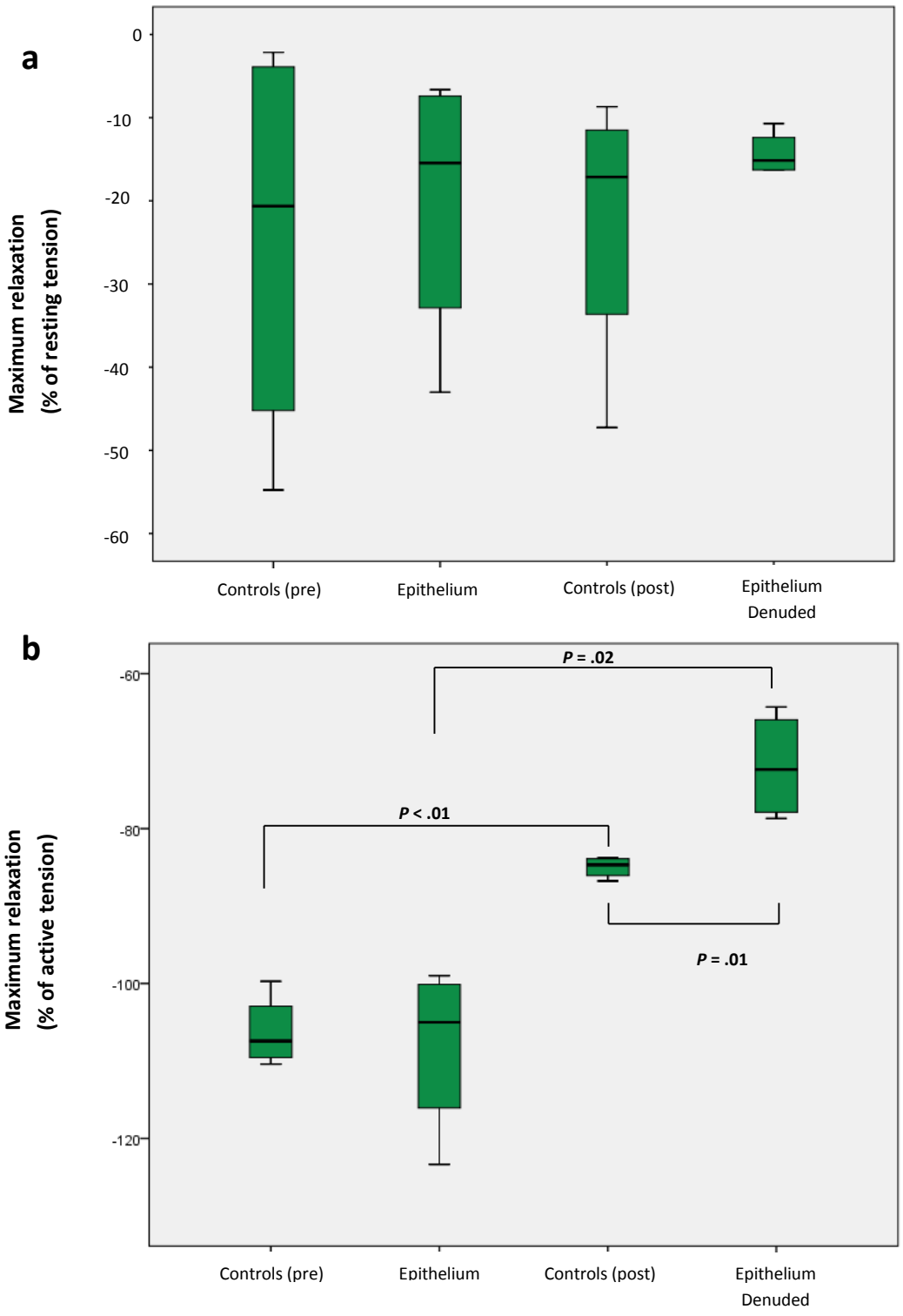

Figure 7.2 Effect of removing the epithelium on HBD from **(a)** resting tension (% reduction in resting tension) and **(b)** active tension to 1mM MCh (% reduction in active tension) in experimental preparations (n=4) before (epithelium) and after (epithelium denuded) removal of the epithelium and in paired time-matched control preparations pre and post (n=4). *P* values via paired t-test (2-tail) for pre and post samples and via unpaired t-test (2-tail) for denuded vs. controls

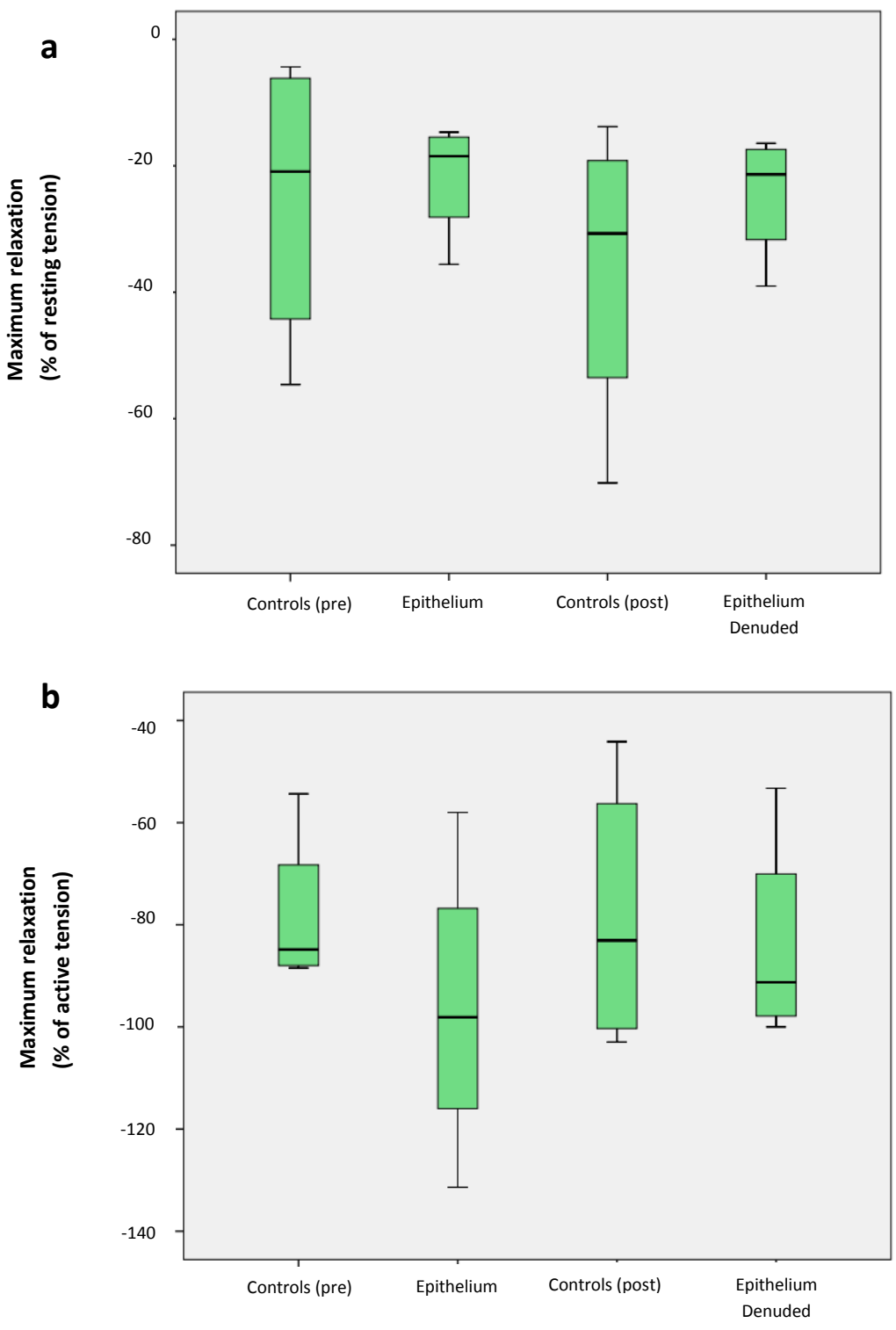

Figure 7.3 Effect of removing the epithelium on β-adrenergic relaxation to 10 µM isoprenaline from **(a)** resting tension (% reduction in resting tension) and **(b)** active tension to 1mM MCh (% reduction in active tension) in experimental preparations (n=4) before (epithelium) and after (epithelium denuded) removal of the epithelium and in paired time-matched control preparations pre and post (n=4). There were no statistically significant differences between the groups (via paired t-test, 2-tail)

7.3.2 Relaxation of Bronchi to Hypoxia and Sodium Nitroprusside (SNP)

Five bronchi (mean internal diameter 5.4 ± 1.7 mm) were obtained from three patients. The maximum magnitude of relaxation to SNP (100 µM) (-37 ± 30% reversal of maximum MCh active tension) was 31 ± 25 % less than the magnitude of HBD (-128 ± 28 % reversal of maximum active tension to MCh) (**figure 7.4**, raw data Appendix II, *p* = .01 via paired t-test, 2-tail).

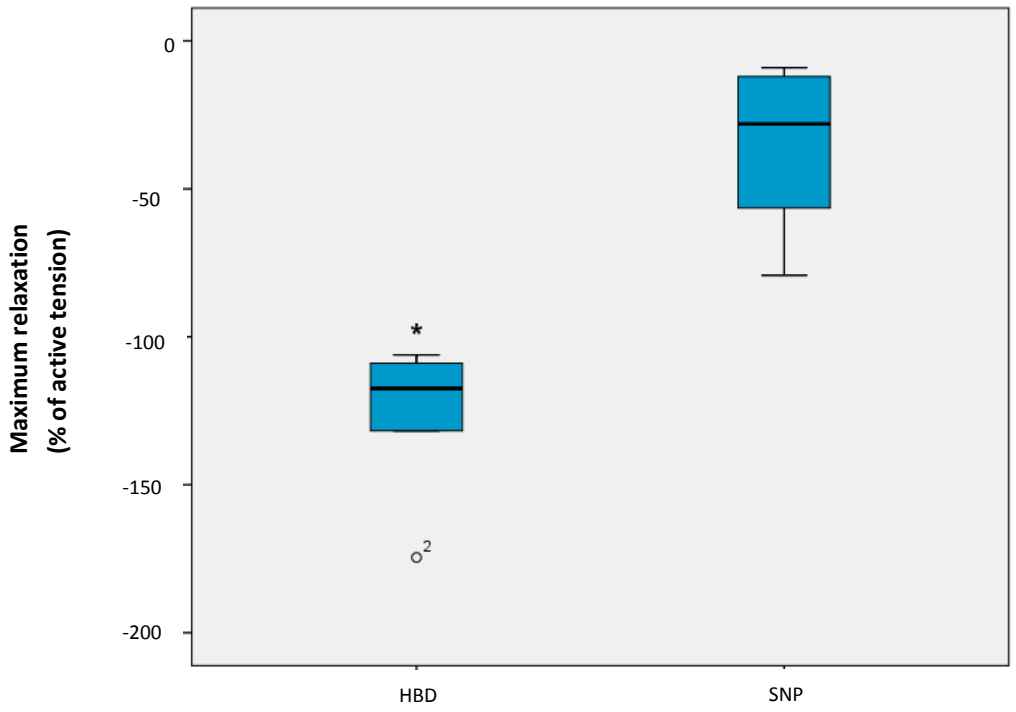

Figure 7.4 Box-whisker plot of maximum relaxation of bronchi to hypoxia (HBD) and SNP from active tension to MCh (1mM). n=5. **p* = .01 for maximum HBD vs. maximum SNP relaxation via paired t-test (2-tail). Data for HBD contained two outliers (circle).

7.3.3 Antagonist Studies

7.3.3.1 Adrenergic Receptors and Extracellular Enzymes

Seven bronchi (mean internal diameter 4.9 ± 2.9 mm) were obtained from five patients. The effect of labetalol and enzyme inhibitors on resting baseline tension, maximum cholinergic contraction and magnitude of HBD and β-adrenergic relaxation from active tension are summarised in **table 7.9** as % change from pre antagonist exposure. (Raw data Appendix II).

Table 7.9 Effects of receptor antagonists and enzyme inhibitors on resting baseline tension, maximum cholinergic contraction and HBD and β-adrenergic relaxation from active tension. Effects are expressed as % change from pre antagonist exposure. Values are shown as mean ± (SD). n: number of preparations; p: number of patients.

Antagonist	% Change vs. Pre Antagonist Exposure				n/p
	Baseline Resting Tension	Maximum Contraction to MCh	Max HBD from Active Tension	Max Relaxation to Isoprenaline from active tension	
Labetalol hydrochloride	-4	-18	0	-96	1/1
Indomethacin	-	2 (3)	4 (4)	-	2/1
L-NAME	15 (29)	-1 (43)	-19 (1)	-	2/2
Tin protoporphyrin IX dichloride (Sn-PP)	-25 (9)	-23 (32)	-19 (39)	1 (17)	2/1

None of the enzyme inhibitors investigated or labetalol had a significant effect on resting baseline tension, cholinergic contraction or maximum HBD from active tension. The adrenergic antagonist, labetalol, abolished relaxation to isoprenaline.

7.3.3.2 Intracellular Signalling Mechanisms

Eleven bronchi (mean internal diameter 8.2 ± 4.5 mm) were obtained from seven patients. The effects of intracellular signalling pathway inhibitors on resting baseline tension, maximum cholinergic contraction, magnitude of HBD from resting and active tension and β-adrenergic relaxation from active tension are summarised in **table 7.10** as % change from pre antagonist exposure. (Raw data Appendix II).

None of the antagonists investigated in this study had a statistically significant effect on any of the measured parameters; however, the number of individual experiments was small and is in need of repeating. Some of results in this study were inconsistent with the mechanism of action of the compounds used; the soluble guanylate cyclase inhibitor, methylene blue, and the competitive cGMP analogue, Rp-8-Br-cGMPs, caused an attenuation of maximum cholinergic contraction. These anomalous results could be explained by changes in the sensitivity of the preparations to methacholine over time and are in need of repeating with paired, time-matched control preparations.

Control responses to isoprenaline for the selective PKA inhibitor KT 5720 were negative and control responses to SNP for Rp-8-Br-cGMPs and the selective PKG inhibitor KT 5823 were negative. In fact Rp-8-Br-cGMPs and KT 5823 augmented relaxation to sodium nitroprusside and KT 5720 augmented relaxation to isoprenaline. These negative control responses suggest that the compounds used were not active.

Table 7.10 Effects of intracellular signalling pathway inhibitors on resting baseline tension, maximum cholinergic contraction, HBD from resting and active tension and β-adrenergic relaxation or relaxation to sodium nitroprusside (SNP) ‡ from active tension. Effects are expressed as % change from pre antagonist exposure. Values are shown as mean ± (SD). n: number of preparations; p: number of patients.

Antagonist	% Change vs. Pre Antagonist Exposure					n/p
	Baseline Resting Tension	**Maximum Contraction to MCh**	**Max HBD from Resting Tension**	**Max HBD from Active Tension**	**Max Relaxation to Isoprenaline or SNP‡ from active tension**	
2-APB	-31 (20)	-30 (8)	-24 (55)	-7 (24)	-	2/2
KT 5720	19	6	-31	-6	41	1/1
KT 5823	11	-3	-4	6	90‡	1/1
Methylene Blue	0 (7)	-69 (19)	-8 (4)	17 (74)	-	2/1
Rp-8-Br-cGMPs	-3 (6)	-34 (48)	-	16 (34)	78 (58)‡	3/2
Ryanodine [10 nM]	32	0.9	-54	-39	-25	1/1
Ryanodine [10 µM]	-10	-32	-42	-30	-2	1/1

7.4 Discussion

In this chapter the role of the epithelium in modulating HBD was investigated for the first time in isolated human bronchi. In one series of experiments the effects of removing the epithelium on HBD from resting and active tension was investigated. It was found that denudation of the epithelium caused a significant attenuation of the maximum relaxation to hypoxia from active tension, compared to paired time-matched control preparations, and a small but variable and statistically non-significant attenuation in the magnitude of HBD from resting tension. In the same bronchi removing the epithelium did not significantly affect the magnitude of β-adrenergic relaxation to isoprenaline from resting or active tension or the magnitude of cholinergic contraction. The finding that removal of the epithelium attenuated the magnitude of HBD from active tension but not from resting tension suggests that the epithelium is acting to inhibit active contraction and has an additive effect in HBD rather than a mediator role. This suggestion is consistent with previous studies in human [240-243], canine [244], rabbit [245], bovine [242] and guinea-pig [246] *in vitro* airway preparations which found that removal of the epithelium caused a leftward shift in contractile agonist dose responses but did not alter maximum contractions suggesting that the epithelium has a role in inhibiting airway constriction. One of the shortfalls of the current study is that active tension in the bronchi was induced by a supramaximal dose of methacholine and the magnitude of this maximum contraction, consistent with the previous studies reported in the literature, was not affected by removal of the epithelium. In this study the effect of removing the epithelium on the dose dependent contraction of the bronchi to methacholine should have been investigated. It has also previously been shown that removal of the epithelium attenuates the dose dependent

relaxation of porcine bronchioles [247] to isoprenaline and the effect of removing the epithelium on the dose dependent relaxation of human bronchi to isoprenaline should also have been investigated in the current study. It should be mentioned that there was an anomalous finding in this study in that the magnitude of HBD from active tension in paired time-matched control preparations was also significantly attenuated over time which was inconsistent with numerous other experiments reported in this thesis where it was found that the magnitude of HBD was reproducible and did not decrease with time. The experimental method used in this study could offer one explanation for this discrepancy; in this study all bronchi were removed from the organ baths and then remounted with the epithelium being removed in the experimental preparations but left intact in the control preparations. It is possible that this procedure *per se* attenuated the subsequent relaxant effect of hypoxia. However, this suggestion does not explain why the magnitude of relaxation to isoprenaline was not affected by this experimental procedure. The finding that HBD in human bronchi is not dependent on an intact epithelium is in agreement with studies in porcine bronchi [95] but at variance with studies in canine bronchi [104] and guinea-pig trachea [100] in which HBD was dependent on an intact epithelium. This suggests that significant species differences exist in the role that the epithelium plays in modulating or even mediating the effects of hypoxia on airway tone and emphasises the need for studies in human tissue to investigate the therapeutic potential of HBD.

One mechanism by which the epithelium is thought to modulate airway smooth muscle tone is via the release of epithelium derived relaxing factors (EpDRFs). Debate continues in the literature about the exact molecular nature of EpDRFs; however, numerous studies have shown that cyclooxygenase metabolites of arachidonic acid

and the nitric oxide synthase product nitric oxide can act as EpDRFs (Reviewed in [36-38, 40]). In this study the cyclooxygenase inhibitor, indomethacin, the nitric oxide synthase inhibitor, L-NAME and the soluble guanylate cyclase inhibitor, methylene blue, did not affect the rate or magnitude of HBD in human bronchi. Soluble guanylate cyclase is the primary intracellular receptor for NO and these results, which are consistent with previous findings in canine [104] and porcine bronchi [95], provide evidence that NO and arachidonic acid metabolites (which are considered to be primarily epithelium derived) are not involved in the mechanism of HBD.

Previous *in vivo* animal studies provided evidence that CO, acting via a cGMP dependent pathway, is involved in mediating the effects of hypoxia on airway tone [93, 236]. In the current study inhibition of haem oxygenase (the enzyme responsible for CO production in the cell) with Tin protoporphyrin (Sn-PP) or inhibition of the cGMP dependent pathway had no effect on the rate or magnitude of HBD in human bronchi. These results suggest that CO is not involved in the mechanism of HBD in human airways and suggests that species differences may exist in the effects of CO on airway tone. However, these results should be treated with caution. In this study the efficacy of Sn-PP in inhibiting haem oxygenase activity was not confirmed and furthermore controversy exists in the literature about the non-specific effects of protoporphyrins and the validity of using protoporphyrins as selective haem oxygenase inhibitors [248]. It should also be noted that in the studies described here positive controls were not obtained to confirm inhibition of the cGMP dependent pathway. Another possible explanation for the variance between the results obtained in the current *in vitro* studies and previous *in vivo* studies is that *in vivo* hypoxia causes release of CO which modulates airway tone via a neuronal mechanism, either centrally or at the level of the

autonomic nervous system, which is not present or is not active in the isolated human bronchi preparation used here. Indeed CO has previously been shown to have a transmitter role in the non-adrenergic, non-cholinergic (NANC) electrically induced relaxations of guinea-pig whole tracheal segments [249]. It should be noted that one of the limitations of the current study is that the possible role of a neuronal mechanism in mediating the effects of hypoxia on airway tone was not investigated and further studies are needed to investigate the effect of neurotoxins (for example, tetrodotoxin (TTX)) on HBD in order to establish if a neuronal component is involved in the mechanism of HBD. Further *in vitro* and *in vivo* studies are needed to investigate the role of CO in HBD and the therapeutic potential of CO in the treatment of constrictive airway diseases. Future studies are planned to investigate the *in vitro* effects of carbon monoxide releasing molecules (CO-RMs) on airway tone in isolated human bronchi.

In this study the effect of the IP_3 receptor antagonist and SOCE inhibitor 2-APB was investigated. 2-APB did not significantly affect resting tension, cholinergic contraction or HBD from resting or active tension. These results suggest that inhibition of intracellular IP_3 receptors or SOCE is not involved in the mechanism of HBD. The fact that 2-APB did not significantly attenuate the maximum cholinergic contraction to methacholine (which is reliant at least in part on IP_3 and SOCE mechanisms to mediate increases in $[Ca^{2+}]_i$) resides in the fact that in this study the dose of MCh used was supramaximal which could have surmounted the effects of 2-APB. It has recently been shown that 2-APB is a GJ inhibitor [238] and the negative result from the current study suggests that the GJs are not involved in signalling pathways of HBD. However, it should be noted that in the previous study by Kizub *et al*. [238] the concentration of 2-APB used to inhibit GJs in vascular preparations was 75 µM compared to 50 µM used in

the present study and it is possible that a higher concentration was needed to inhibit GJ function in the airways. Further studies are needed using selective gap junction inhibitors to investigate the potential role of the gap junctions in facilitating communication between the different cellular compartments of the airways and their potential role in HBD.

In this study labetalol, a non-selective adrenoceptor antagonist, had no effect on the rate or magnitude of HBD but completely abolished relaxation to isoprenaline. This finding confirmed the conclusion in chapter 6 that hypoxia does not cause relaxation of the bronchi via the β-adrenergic receptor system and is consistent with previous studies in porcine [95] and canine bronchi [104] in which the selective β-adrenoceptor antagonist propranolol did not block HBD.

It has previously been postulated that the ryanodine receptors (RyRs) play a role in the "superficial buffer barrier" model of bronchodilation [14]. This model suggests that relaxation of ASM can be achieved by the diversion of intracellular calcium away from the contractile apparatus in the centre of the cell to a superficial buffer barrier between the sarcoplasmic reticulum (SR) and the cell membrane. It is suggested that the RyRs, which are localised to the side of the SR closest to the cell membrane, are responsible for maintaining the transport of calcium into the buffer barrier [17, 29] (see ***Chapter 1, 1.2.3.2***). Ryanodine has been shown to be a RyR antagonist at μM concentrations and a partial agonist at nM concentrations. In the current study it was hypothesised that an antagonist concentration of ryanodine would attenuate HBD and a partial agonist concentration of ryanodine would augment HBD. At 10 μM ryanodine did attenuated (-30%) the magnitude of HBD. However, at 10 nM ryanodine also

inhibited maximum HBD (-39%). Clearly it is not possible to draw any conclusions from these two experiments and further studies are warranted to investigate the role of this calcium transfer mechanism in HBD; preferably with the use of calcium imaging and confocal microscopy in human ASMs.

Agonist induced relaxation of airway smooth muscle is mediated by increasing intracellular concentrations of the second messengers cAMP or cGMP. In turn cAMP causes relaxation via activation of protein kinase A (PKA) and cGMP relaxation is mediated via activation of protein kinase G (PKG) (***Chapter 1, 1.2.2***). In this study a number of selective antagonists were used to investigate the role of the cAMP and cGMP signalling pathways in the mechanism of HBD. Rp-8-Br-cGMPs (a competitive cGMP analogue), KT 5823 (selective PKG inhibitor) and methylene blue (soluble guanylate cyclase inhibitor) were used to investigate the role of the cGMP pathway and KT 5720 (selective PKA inhibitor) was used to investigate the role of the cAMP pathway. None of the antagonists investigated in this study had a significant effect on the rate or magnitude of HBD which suggests that hypoxia is not causing relaxation of the airway smooth muscle by activation of the cAMP or cGMP pathways. However, control responses to isoprenaline (cAMP pathway) and sodium nitroprusside (SNP) (cGMP pathway) were negative. In fact Rp-8-Br-cGMPs and KT 5823 augmented relaxation to SNP and KT 5720 augmented relaxation to isoprenaline which suggests that the antagonists used in this study were not active and therefore no conclusions can be made about the role of the cAMP or cGMP pathways in the mechanism of HBD. The finding that the antagonists used in this study didn't work is in need of further investigation in itself. It is believed that different isoforms of PKA and PKG exist between species and in different tissues [19, 250]. It could be that the antagonists used in

this study, which have proven effective in most animal studies, are not effective in human airway tissue because of differences in enzyme isoforms and further studies are needed to establish the validity of using these antagonists in human airway studies. Another possible explanation for why positive control responses to isoprenaline and SNP were not obtained in this study is that relaxation to these agents occurs via both pathways. Convention has it that relaxation to isoprenaline is mediated almost exclusively via the cAMP pathway and relaxation to SNP is mediated via the cGMP pathway. However, there is good evidence for cross-activation of PKG by cAMP and PKA by cGMP [19, 250, 251] and the high concentration of SNP and isoprenaline used in this study could have surmounted inhibition of the individual signalling pathways causing relaxation via the alternative pathway. Furthermore, it is becoming increasingly recognised that β_2-adrenoceptor mediated relaxation of smooth muscle is not mediated exclusively via activation of PKA by cAMP and involves multiple mechanisms [19, 251] which would not have been antagonised in this study. The methodology in this study could have been improved by antagonising PKA and PKG simultaneously and using submaximal agonist concentrations to obtain control responses. Further studies are needed to establish effective antagonism of the cAMP and cGMP pathways in human airway smooth muscle in order to investigate the role of these pathways in the mechanism of HBD.

The role of nitric oxide in mediating relaxation of human bronchi was investigated in this chapter. The finding that the nitric oxide donor SNP had a limited relaxant effect, compared to HBD, and that L-NAME and methylene blue had no effect on HBD suggests that NO is not involved in the intracellular mechanism of HBD.

In this chapter the role of the epithelium, the adrenergic receptor system, classical intracellular signalling pathways and some putative pathways in the mechanism of HBD were investigated. Some of the key findings were:

- HBD in human bronchi is not dependent on an intact epithelium but the epithelium may have a role in modulating airway tone by inhibiting cholinergic contraction via a mechanism which does not involve nitric oxide or arachidonic acid metabolites.
- HBD is not mediated via activation of the β-adrenergic system.
- Nitric oxide is not an effective dilator in human bronchi and is not involved in the mechanism of HBD.
- Carbon monoxide is unlikely to act as a signalling molecule in HBD at the tissue level but further studies are needed to investigate the role of CO in HBD.
- HBD is not mediated via inhibition of intracellular IP_3 receptors or SOCE and Gap Junction signalling pathways do not modulate HBD.
- With the limited number of antagonists used in this study it was not possible to determine the role of the cAMP or cGMP signalling pathways in the mechanism of HBD and further studies are needed.

CHAPTER 8

Mechanism of Hypoxic Bronchodilation: Role of the Potassium Channels and Intra and Extra-Cellular Calcium

Contents

8.1 Introduction

An increase in intracellular calcium concentration $[Ca^{2+}]_i$ causes contraction of airway smooth muscle (ASM). Regulation of the intracellular calcium concentration and modulation of the sensitivity of the contractile apparatus to calcium by airway smooth muscle cells plays a pivotal role in the regulation of airway tone. A large body of research has investigated the mechanisms of calcium handling in ASM. These studies have identified that there are significant differences in the mechanism of calcium handling in ASM between species and at different levels of the respiratory tract. Furthermore, it has also been recognised that there are important differences between the regulation of $[Ca^{2+}]_i$ in vascular smooth muscle (VSM) and ASM and that extrapolation from mechanistic studies in VSM to ASM has in the past led to misleading results. Ongoing research continues to provide a better understanding of the unique intracellular mechanisms responsible for the regulation of ASM contraction and relaxation which are ultimately responsible for the regulation of airway tone *in vivo*.

The role of extracellular and intracellular calcium in the mechanism of HBD has previously been investigated in a small number of animal studies with conflicting results. In the 1990's a group from The John Hopkins University, Baltimore investigated the role of the voltage operated calcium channels (VOCCs) in the effects of hypoxia on isolated porcine bronchi. In a preliminary study [95] they found that pre-exposure of bronchi to hypoxia inhibited the subsequent initiation phase of contraction to KCl and the maintenance phase of contraction to both KCl and carbachol but not the initiation phase of carbachol contraction. These results suggested that hypoxia was modulating

airway tone by inhibiting extracellular calcium influx. In a follow on study [97] they further investigated the role of dihydropyridine-sensitive calcium entry in HBD. They found that BAY K 8644 (an L-type calcium channel opener) completely inhibited HBD in KCL contracted but not carbachol contracted bronchi and that nifedipine completely relaxed contraction to KCl but did not affect HBD in carbachol contracted bronchi. From these results they concluded that hypoxia was causing relaxation of the airways by inhibiting entry of calcium through the dihydropyridine pathway and another unknown mechanism.

However, subsequent studies provided evidence that HBD was independent of changes in intracellular calcium. Studies in porcine tracheal smooth muscle [99] and guinea-pig trachea [100] simultaneously measured the effects of hypoxia on active tension and intracellular calcium concentrations (using fura-2 and spectrometry). These studies found that hypoxia caused relaxation of the smooth muscle preparations but had little effect on intracellular calcium concentrations and it was concluded that, unlike the studies in porcine bronchi, hypoxic relaxation of the airways is independent of changes in intracellular calcium. These anomalous findings could be explained by the buffer barrier model [14, 17] which suggests that relaxation of ASM is achieved by the translocation of intracellular calcium away from the contractile apparatus in the centre of the cell into the "buffer barrier" between the sarcoplasmic reticulum and cell membrane (***Chapter 1, 1.2.3.2***). The fluorescence techniques used in these studies would only have measured the overall intracellular calcium concentration in the cells and would not have been able to detect spatial changes in calcium distribution which is misleading and lead to the conclusion that the intracellular calcium concentration did not change in the tissues on exposure to hypoxia.

Clearly the results from these earlier animal studies were inconclusive with regard to the role of calcium in the mechanism of HBD. However, the current understanding of the mechanisms of calcium handling by ASM cells can now explain some of the discrepancies in the conclusions made in these studies. In this chapter the role of the voltage operated calcium channels (VOCC) and intracellular calcium in the mechanism of HBD in human bronchi was investigated.

One mechanism by which a reduction in intracellular calcium concentration and relaxation of ASM can be achieved is via opening of membrane potassium channels. Opening of cell membrane potassium channels reduces influx of extracellular calcium by hyperpolarising the cell membrane which causes closing of the voltage gated calcium channels. The role of the potassium channels in the regulation of $[Ca^{2+}]_i$ by ASM cells has been extensively investigated and the potassium channels have previously been shown to play a significant functional role in the mechanism of adrenergic relaxation in isolated human [252,253] and guinea-pig [253,254] airway preparations. However, other studies have suggested that the potassium channels do not play an important role in the relaxation mechanism in human bronchi [254] and there is now some debate in the literature about the relative importance of these channels in the regulation of airway tone [14, 18].

Only one previous study has investigated the role of a potassium channel mechanism in HBD. Linderman *et al*. (from the Baltimore group) investigated the role of the ATP dependent potassium channel in the mechanism of HBD in porcine isolated bronchi [96]. They first established the presence of functional K_{ATP} channels in porcine bronchi by demonstrating a dose dependent relaxation to levcromakalim (K_{ATP} selective channel

opener) which was inhibited by the K_{ATP} antagonist glibenclamide. They then went on to investigate the effect of glibenclamide on the dose dependent relaxation of the bronchi to decreasing oxygen concentrations. It was found that glibenclamide attenuated the rate and magnitude of hypoxic relaxation of porcine bronchial rings exposed to 50% O_2 or 28% O_2 (% oxygen in the aerating gas) but did not significantly alter HBD to 0% O_2.

In light of the findings in porcine bronchi for a role of the K_{ATP} channels in mediating bronchodilation, and having a role in the mechanism of HBD, studies were undertaken in this chapter to investigate the bronchodilator effects levcromakalim and the effects of glibenclamide on HBD in human bronchi. Furthermore, because the potassium channels have previously been shown to play a significant part in other bronchodilator mechanisms a number of selective potassium channel blockers were used to investigate the role of all of the known cell membrane potassium channels in the mechanism of HBD. In this chapter it was hypothesised that the membrane potassium or calcium channels could play a role in the mechanism of HBD.

The aim of the studies in this chapter were;

1. To determine the role of the cell membrane potassium channels in the mechanism of HBD
2. To determine the effect of levcromakalim on bronchial tone and further investigate the role of K_{ATP} channels in the mechanism of HBD in human bronchi
3. To determine the role of the VOCCs and intracellular calcium in the mechanism of HBD

8.2 Methods

8.2.1 Tissue Preparation

Human bronchi (primary, second order or tertiary) were obtained at resection for lung cancer, prepared and mounted in 25 ml organ baths as described in ***Chapter 2, 2.3.*** A resting tension of 1-3g was applied and the bronchi allowed to equilibrate for 60-90 minutes.

8.2.2 Effect of Contraction to Methacholine or Potassium Chloride on HBD

After equilibration for 90 minutes and when a stable resting tension was achieved bronchi were contracted to 1 mM methacholine or 100 mM KCl. When maximum contraction reached plateau the bronchi were exposed to hypoxia. When maximum hypoxic bronchodilation was achieved the bronchi were again aerated with 95 % O_2: 5 % CO_2. When re-contraction reached plateau a control relaxation to isoprenaline [10 µM] was obtained. The bronchi were then washed for a minimum of sixty minutes before being contracted to the alternative agent (methacholine or KCl) and responses to hypoxia and isoprenaline repeated.

8.2.3 Effect of Extracellular and Intracellular Calcium Concentration on HBD

8.2.3.1 Extracellular Calcium

After equilibration for 90 minutes and when a stable resting tension was achieved control responses to hypoxia from resting tension and to hypoxia and isoprenaline after contraction to methacholine [1mM] were obtained as described in ***6.2.2.2*** and ***6.2.2.3***. After a 30 minute washout the effect of low extracellular calcium was determined by changing the buffer solution to a calcium free Krebs-Henseleit solution

(***Chapter 2***, ***2.4.1***) and repeating responses to MCh, hypoxia and isoprenaline after 30 minutes. The effect of high extracellular calcium was investigated by contracting the bronchi to 80 mM $CaCl_2$ before exposure to hypoxia.

8.2.3.2 Intracellular Calcium

Two pharmacological agents, thapsigargin and ouabain, which are known to cause an increase in intracellular calcium and bronchoconstriction via distinct cellular mechanisms were used to investigate the role of increasing intracellular calcium concentration on HBD. When a stable resting tension was achieved thapsigargin (10 μM) or ouabain (5 μM) were added directly to the organ bath. When a stable contraction plateau was achieved the effect of hypoxia and reoxygenation on the contraction to thapsigargin or ouabain was determined before obtaining a control response to isoprenaline.

8.2.4 Effect of Potassium and Calcium Channel Antagonists on HBD

After obtaining control responses to hypoxia from resting tension and to hypoxia and isoprenaline after contraction to methacholine [1mM] the bronchi were washed for 30 minutes before adding antagonists directly to the organ bath. After a minimum incubation of 30 minutes repeat responses to hypoxia from resting tension and to hypoxia and isoprenaline after contraction to methacholine [1mM] were obtained. The effect of antagonists on resting tension, cholinergic contraction, HBD and β-adrenergic relaxation was determined by comparison with pre-exposure controls.

The effects of a number of potassium channel blockers and the L-type calcium channel blocker nifedipine on HBD and β-adrenergic relaxation were investigated. The

concentration of each antagonist used, specific cation channels blocked and putative site of action referred to in ***figure 1.1*** and ***figure 1.2*** **(*Chapter 1*)** are shown in **table 8.1**.

Table 8.1 Potassium and calcium channel antagonists investigated. Pharmacological action, concentration used and reference to site of action in **figure 1.1** (blue) and **figure 1.2** (red)

Drug (s)	**Channels Blocked***	**Concentration [µM]**	**Site of action**
4-aminopyridine	K_v 1.4, 3.1, 3.2	100	[5]
Apamin	K_{Ca} 2.1,2.2,2.3 (SK_{Ca})	0.3	[4]
Glibenclamide	K_{ir}6.1, K_{ir}6.2 (K_{ATP})	100	[2]
Iberiotoxin	K_{Ca} 1.1,4.1,4.2,5.1 (BK_{Ca})	0.1	[3]
Nifedipine	Ca_V 1.1,1.2	20	[2] [1]
Tetraethylammonium (TEA)	K_{Ca} 1.1, 4.1, 4.2, 5.1 K_v 1.1, 1.3, 1.6, 2.1, 2.2, 3.1, 3.2, 3.3, 3.4, 7.2, 7.4	1000	[3] [5]
TRAM-34	K_{Ca} 3.1	0.2	[4]
Apamin **Iberiotoxin** **Glibenclamide**	K_{Ca} 2.1,2.2,2.3 K_{Ca} 1.1,4.1,4.2,5.1 K_{ir}6.1, K_{ir}6.2	0.3 0.1 100	[2] [3] [4]

*According to the International Union of Basic and Clinical Pharmacology IUPHAR database (IUPHAR-DB) [255] Kv: Voltage- gated potassium channel; BK_{Ca} and SK_{ca}: Large and small conductance calcium activated potassium channels; K_{ir}: Inwardly rectifying potassium channel; Ca_V 1.1, 1.2: high-voltage activated dihydropyridine-sensitive (L-type) channels.

8.2.5 Effect of Glibenclamide on Oxygen Concentration Responses

Previous animal studies [96] reported that the K_{ATP} antagonist glibenclamide attenuated the rate and magnitude of hypoxic relaxation of porcine bronchial rings exposed to 50% O_2 or 28% O_2 (% O_2 in the aerating gas) but did not significantly alter the magnitude of HBD to 0% O_2. Studies were therefore designed to investigate the effects of glibenclamide on the concentration dependent effects of oxygen on bronchial tone in human bronchi.

Bronchi were contracted to 1 mM MCh. Oxygen concentration dependent relaxation responses from maximum active tension in 95% O_2 were obtained at 40%, 21% and 0% O_2 (% O_2 in aerating gas) and to re-contraction from 0% O_2 at 21%, 40% and 95% O_2 (expressed as % of maximum active tension). When a stable re-contraction plateau was achieved the bronchi were exposed to 50 µM glibenclamide or 25µl DMSO (controls). Concentration responses to oxygen were repeated after incubation for 30 minutes.

8.2.6 Effects of Levcromakalim on Bronchial Tone and HBD

To further investigate the role of the K_{ATP} channel in HBD the effects of the K_{ATP} channel opener levcromakalim were investigated.

Bronchi were contracted to 1 mM MCh and control responses to hypoxia and reoxygenation obtained. When a stable re-contraction baseline was achieved dose response curves were constructed to levcromakalim (0.1-30 µM) by cumulative addition to the organ baths. When a maximum response to levcromakalim was obtained the bronchi were again exposed to hypoxia and reoxygenation.

8.3 Results

8.3.1 Effect of Contraction to Methacholine or Potassium Chloride on HBD

Eight bronchi were obtained from five patients (mean internal diameter 6.0 ± 2.2 mm). Active tension to KCl (100 mM) and MCh (1 mM) was determined in all preparations. Reversal of active tension to KCl and MCh by hypoxia was determined in all bronchi and control responses to isoprenaline (10 μM) after contraction to KCl and MCh obtained in six bronchi. Results are summarised in **table 8.2** (Raw data Appendix II). Maximum active tension to KCl was greater than maximum active tension to MCh but the difference was not statistically significant. The magnitude of relaxation to isoprenaline (percent reversal of active tension) was less after contraction to KCl compared to MCh but did not reach statistical significance. The rate of relaxation to isoprenaline post KCl and post MCh was not significantly different. The magnitude of HBD (% reversal of active tension) after KCl contraction was less than the magnitude of HBD after MCh contraction but this did not reach statistical significance. The time taken to reach maximum relaxation to hypoxia was significantly longer in bronchi contracted to KCl than bronchi contracted to MCh (**figure 8.1**).

Table 8.2 Summary of contractile and relaxant responses in bronchi after contraction to KCl (100mM) and MCh (1mM). n=8. %; percent reversal of agonist active tension. Values are mean ± (SD). *P* via paired t-test (2-tail)

Parameter	KCl	MCh	*p* value
Maximum active tension (gf)	4.56 (3.13)	3.86 (2.41)	**0.30**
Magnitude of relaxation to isoprenaline (%) (n=6)	-43 (17)	-112 (73)	**0.08**
Time to maximum isoprenaline relaxation (S) (n=6)	345 (62)	420 (283)	**0.51**
Magnitude of HBD (%)	- 95 (28)	-129 (47)	**0.19**
Time to maximum HBD (min)	89 (44)	30 (11)	**0.01**

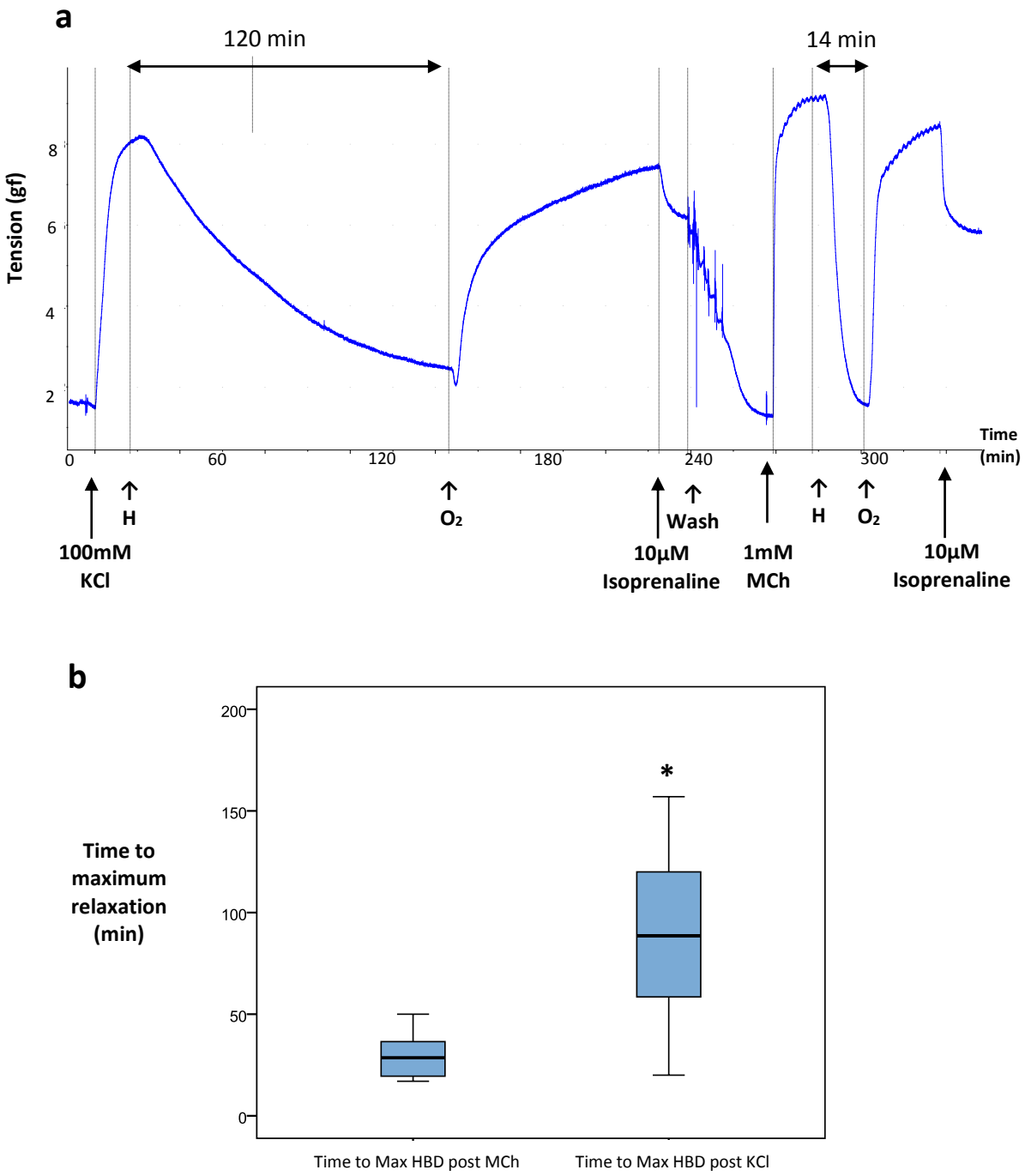

Figure 8.1 Rate of HBD post contraction to KCl and MCh **(a)** Experimental trace showing relaxation to hypoxia after contraction to KCl and MCh **(b)** Box-whisker plots of time taken (min) to reach maximum hypoxic relaxation in the same bronchi contracted to KCl (100 mM) and MCh (1 mM). n=8. H; hypoxia, O_2; 95% O_2 *p < .01 via paired t-test (2-tail) for MCh vs. KCl

8.3.2 Effect of Extracellular and Intracellular Calcium Concentration on HBD

8.3.2.1 Extracellular Calcium

Two bronchi were obtained from one patient (internal diameters 13 and 4 mm). The effects of reducing extracellular calcium were inconsistent. In one preparation (large bronchus, internal diameter 13 mm) changing the buffer solution to calcium free Krebs-Henseleit solution almost completely abolished HBD from resting tension and contraction to MCh (- 94% of control) but did not affect the magnitude of HBD from active tension (% reversal of active tension) or the magnitude of relaxation to isoprenaline compared to control responses in normal Krebs. In the other, smaller, bronchus (Internal diameter 4mm) changing to a calcium free buffer had no effect on HBD from resting tension, contraction to MCh, HBD from active tension or relaxation to isoprenaline from active tension.

Increasing the extracellular calcium concentration to 80 mM by the addition of calcium chloride to the organ baths caused a robust contraction (66 ± 62 % of cholinergic contraction) which was reversed by hypoxia (**figure 8.2)** (Raw data Appendix II).

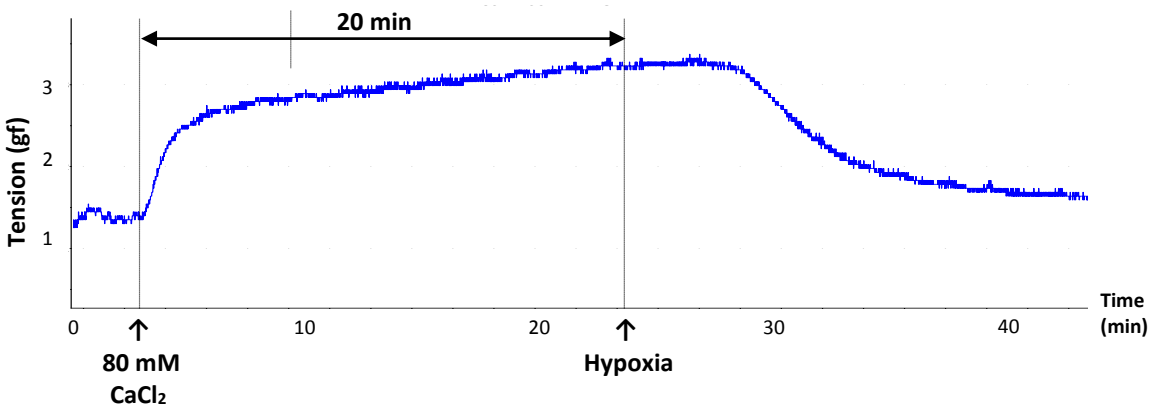

Figure 8.2 Experimental trace showing the effect of hypoxia on active tension to 80 mM $CaCl_2$ in an isolated bronchus

8.3.2.2 Intracellular Calcium

Thapsigargin [10 µM] caused a robust and sustained contraction of bronchi (85 ± 14% of maximum cholinergic contraction) (n= 3 from 2 patients, mean internal diameter 5 ± 2 mm) which was completely reversed by hypoxia and restored on reoxygenation. The magnitude of HBD was significantly greater after contraction to thapsigargin than after contraction to MCh (-111 ± 47 vs. -121 ± 51% of active tension for MCh and thapsigargin respectively. n=3, p = .05 via 2-tail paired t-test). Isoprenaline (10 µM) also caused a complete reversal of contraction to thapsigargin (**figure 8.3**, raw data Appendix II).

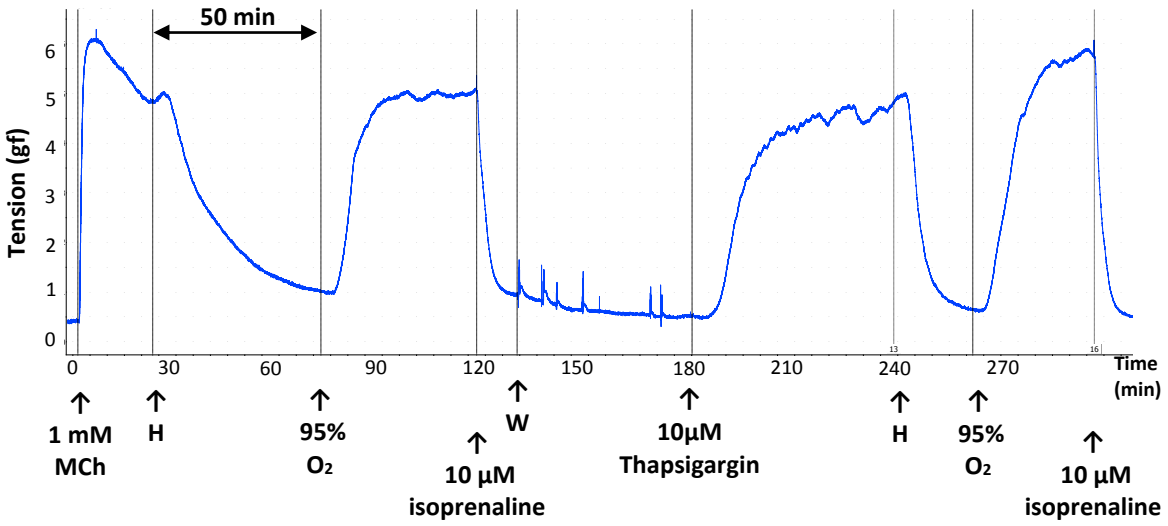

Figure 8.3 Experimental trace showing effect of hypoxia, reoxygenation and 10 µM isoprenaline on active tension to 1 mM MCh and active tension to 10 µM thapsigargin. H: hypoxia; W: wash

Ouabain [5 µM] caused a slow (90 ± 0 minutes) but robust (65 ± 21% of maximum cholinergic contraction) and sustained contraction of bronchi (n= 2 from 2 patients, mean internal diameter 4.5 ± 0.7 mm) which was completely reversed by hypoxia and restored on reoxygenation. The magnitude and rate of HBD was not significantly

different after contraction to ouabain than after contraction to MCh (-99 ± 13 vs. -93 ± 34% of active tension and 50 ± 25 vs. 34 ± 2 minutes for MCh and ouabain respectively. n=3). Isoprenaline (10 µM) also caused a complete reversal of contraction to ouabain (**figure 8.4**, raw data Appendix II).

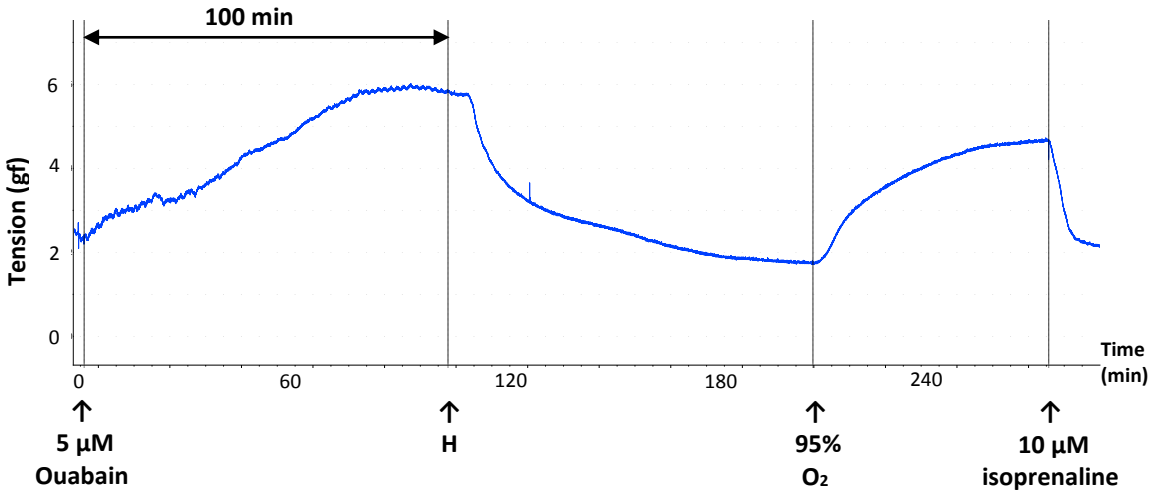

Figure 8.4 Experimental trace showing effect of hypoxia, reoxygenation and 10 µM isoprenaline on active tension to ouabain (5 µM). H: hypoxia.

8.3.3 Effect of Potassium and Calcium Channel Antagonists on HBD

Seventeen bronchi (mean internal diameter 4.6 ± 1.9 mm) were obtained from eight patients. The effects of potassium channel antagonists and the calcium channel antagonist nifedipine on resting tension, maximum cholinergic contraction, magnitude of HBD and β-adrenergic relaxation are summarised in **table 8.3** as % change from pre antagonist exposure. (Raw data Appendix II).

The selective K_{Ca} 3.1 channel antagonist TRAM-34 caused a slow (98 ± 31 min to plateau) but sustained and significant increase in resting tension (142 ± 54% increase, n=3, p = .02 via 1-tail paired t-test) which was 44 ± 23% of maximum active tension to MCh (n=2) and 55% of maximum active tension to KCl (n=1). The Kv channel antagonist 4-AP caused a slow (91 ± 8 minutes), large (47 ± 26% of maximum active tension to MCh) and sustained contraction from resting tension which was not statistically significant. A combination of apamin, iberiotoxin and glibenclamide caused a small (20 ± 0% increase in resting tension) but significant (p < .05, n=2, via paired 1-tail t-test) increase in resting tension. TEA, glibenclamide and the calcium antagonist nifedipine caused a relaxation from resting tension.

Hypoxia caused a complete and reversible relaxation from active tension to TRAM-34 (**figure 8.5**).

All of the antagonists investigated attenuated the magnitude of cholinergic contraction with the exception of combined apamin, iberiotoxin and glibenclamide which potentiated cholinergic contraction. However, none of these results were statistically significant.

Iberiotoxin and 4-AP augmented the magnitude of HBD and β-adrenergic relaxation; however, this did not reach statistical significance. The remainder of the antagonists investigated caused a small and non- significant attenuation of maximum HBD and did not significantly alter β-adrenergic relaxation. None of the antagonists used in this study had a significant effect on the rate (not shown) of HBD or β-adrenergic relaxation.

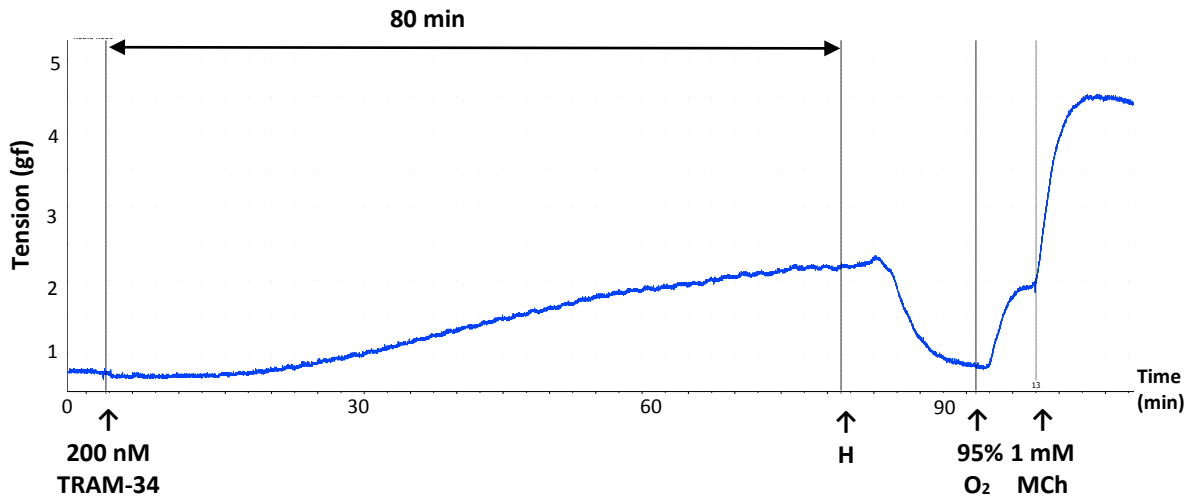

Figure 8.5 Experimental trace showing the effect of hypoxia and reoxygenation on active tension to TRAM-34 [200 nM]. H: hypoxia.

Table 8.3 Effects of potassium channel antagonists and nifedipine on resting baseline tension, maximum cholinergic contraction, HBD from resting and active tension and β-adrenergic relaxation from active tension. Effects are expressed as % change from pre antagonist exposure. Values are shown as mean ± (SD). n: number of preparations; p: number of patients; *$p < .05$ via paired t-test (2-tail)

	% Change vs. Pre Antagonist					
Antagonist	**Baseline Resting Tension**	**Max Contraction to MCh**	**Max HBD from Resting Tension**	**Max HBD from Active Tension**	**Max Relaxation to Isoprenaline from active tension**	**n/p**
4-aminopyridine	139 (23)	-39 (13)	-	44 (36)	68 (49)	2/1
Apamin	5 (25)	-24 (16)	-	-10 (7)	-	2/1
Glibenclamide	-22 (14)	-46 (1)	-	-31 (2)	4 (4)	2/1
Iberiotoxin	34 (3)	-32 (19)	-	43 (48)	87 (56)	2/1
Nifedipine	-36 (17)	-14 (2)	-	-8 (39)	-	2/1
Tetraethylamonium (TEA)	-36 (12)*	-16 (4)	8 (8)	-12 (18)	-17 (31)	2/1
TRAM-34	142 (54)*	-7 (19)	-7 (31)	-30 (7)	-9 (1)	3/2
Apamin Iberiotoxin Glibenclamide	20 (0)*	56 (43)	-	-9 (14)	7 (12)	2/1

8.3.4 Effect of Glibenclamide on Oxygen Concentration Responses

Glibenclamide [50 µM] or solvent (DMSO) did not cause a significant shift in oxygen concentration dependent relaxation from maximum active tension in 95% O_2 (**figure. 8.6a**) or oxygen concentration dependent re-contraction from maximum relaxation to 0% O_2 (**Figure 8.6b**). (n=2 in control and experimental groups from one patient, mean internal diameter 3 ± 0 mm) (Raw data Appendix II). Corresponding pO_2 concentrations (KPa and mmHg) at different % oxygen concentrations in the aerating gas are shown in ***Chapter 2, 2.3.6, table 2.1.***

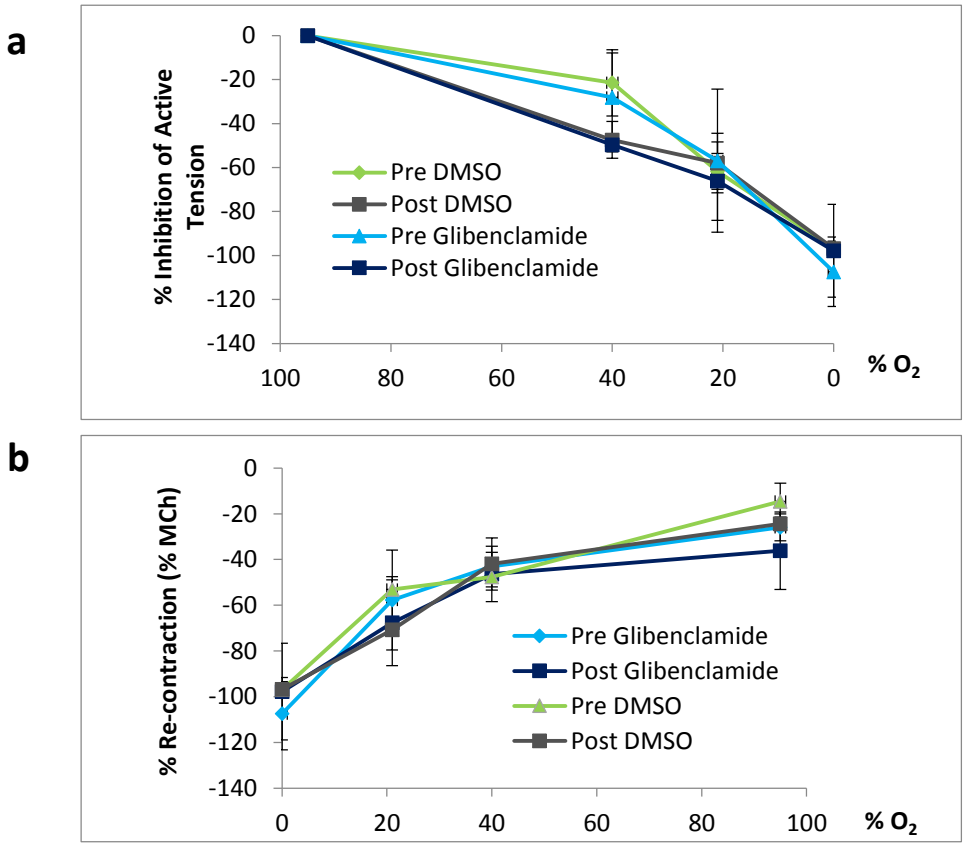

Figure 8.6 Effect of glibenclamide on oxygen concentration dependent responses in isolated human bronchi **(a)** Oxygen concentration dependent relaxation curves (% change from maximum active tension in 95% O_2) in bronchi pre and post glibenclamide [50 µM] (n=2) and control preparations pre and post DMSO (n=2) **(b)** Oxygen concentration dependent re-contraction curves (% of maximum active tension) from 0% O_2 in bronchi pre and post glibenclamide [50 µM] (n=2) and control preparations pre and post DMSO (n=2). % O_2: % O_2 in aerating gas.

8.3.5 Effects of Levcromakalim on Bronchial Tone and HBD

Levcromakalim caused a dose dependent (0.3-30 μM) relaxation of human bronchi (EC_{50} 2.75 ± 2.62 μM, pEC_{50} 5.69 ± 0.5) (n=2 from two patients, mean internal diameter 6.0 ± 1.4 mm) (**figure 8.7**, raw data Appendix II). After maximum relaxation to levcromakalim hypoxia caused a further relaxation. The maximum relaxation to levcromakalim was 41 ± 20% of maximum HBD (**figure 8.7**). Relaxation to levcromakalim did not affect the rate of HBD (21 ± 1 vs. 20 ± 3 min pre and post levcromakalim respectively). The magnitude of HBD after maximum relaxation to levcromakalim was marginally but significantly greater than the magnitude of HBD pre levcromakalim (-170 ± 41 vs. -183 ± 39 % of maximum MCh contraction pre and post levcromakalim respectively. *P* = .05 via paired t-test, 2-tail).

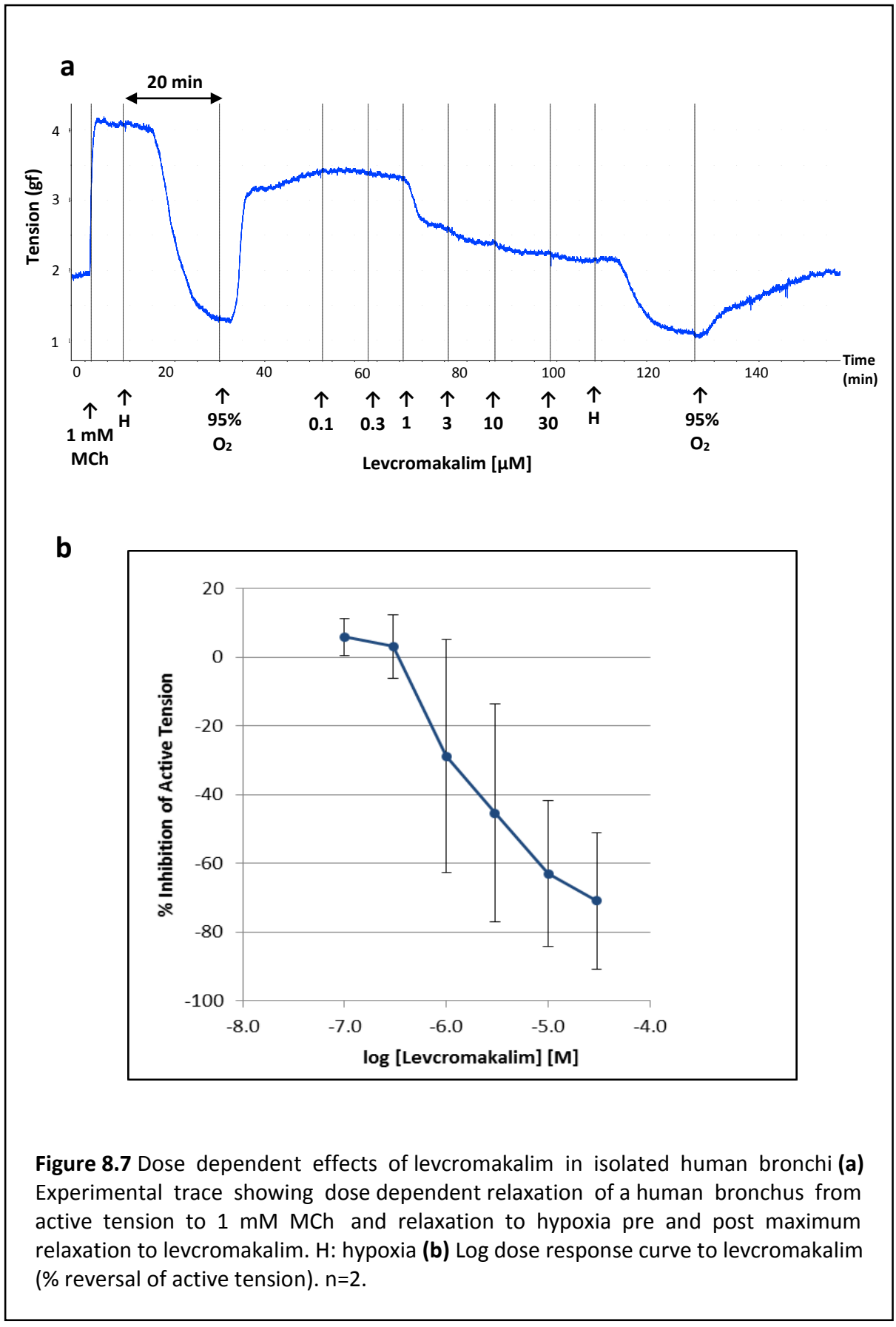

Figure 8.7 Dose dependent effects of levcromakalim in isolated human bronchi **(a)** Experimental trace showing dose dependent relaxation of a human bronchus from active tension to 1 mM MCh and relaxation to hypoxia pre and post maximum relaxation to levcromakalim. H: hypoxia **(b)** Log dose response curve to levcromakalim (% reversal of active tension). n=2.

8.4 Discussion

Studies in this chapter have investigated the role of the potassium channels, L-type voltage gated calcium channel and intra and extracellular calcium in the mechanism of HBD. Results from these studies provide evidence for the mechanism of HBD.

A number of potassium channel antagonists were used to determine the role of the known cell membrane potassium channels in the mechanism of HBD. None of the antagonists used in this study had a significant effect on the rate or magnitude of HBD which suggests that the potassium channels are not involved in the mechanism of HBD in human bronchi.

In this study it was also found that the L-type voltage gated calcium channel blocker nifedipine did not inhibit HBD which suggests that HBD is not mediated by inhibiting calcium influx via the voltage operated calcium channels (VOCC). Furthermore, this finding is evidence in itself that the potassium channels are not involved in the mechanism of HBD. Opening of the potassium channels mediates relaxation by hyperpolarising the cell membrane, causing closure of the voltage gated calcium channels thereby reducing calcium influx into the cell and ultimately reducing intracellular calcium concentration which causes relaxation. The fact that the voltage gated calcium channels were shown not to be involved in the mechanism of HBD therefore negates a role for the potassium channels in the first place. However, this finding does not rule out a role for other voltage gated calcium channels such as the N-type gated channels and further studies would be needed using selective N-type voltage gated calcium channel blockers to investigate the role of these channels in HBD.

The finding that nifedipine did not attenuate contraction to MCh is consistent with the current evidence that extracellular calcium influx through the voltage gated calcium channels is not an important mechanism in agonist induced contraction of ASM (in contrast to VSM).

An interesting and potentially significant finding in the potassium antagonist studies described here is that, at the concentrations used in this study (200 nM), the selective $K_{Ca\ 3.1}$ channel antagonist TRAM-34 caused a slow but robust and sustained contraction of the bronchi from resting tension. The presence of functional $K_{Ca\ 3.1}$ channels in human airway smooth muscle has not been previously demonstrated and it was previously thought that the $K_{Ca\ 3.1}$ channel did not play a role in the maintenance of resting tone in human airways. However, the finding in this study that $K_{Ca3.1}$ antagonism caused a robust and sustained contraction of the bronchi from resting tension implies the presence of functional $K_{Ca\ 3.1}$ channels in human bronchi and the magnitude of contraction following $K_{Ca\ 3.1}$ antagonism suggests a significant role for these channels in the regulation of basal airway tone. Selective $K_{Ca\ 3.1}$ channel openers could therefore prove to be an effective and novel class of therapeutic agents in the treatment of constrictive airway disease and further studies are warranted to investigate the role of the $K_{Ca\ 3.1}$ channel in the regulation of basal tone in human airways. A recent study [256] has shown the potential for TRAM-34 in the treatment of asthma. In a mouse model of chronic asthma TRAM-34 was found to abolish bronchial smooth muscle remodelling, attenuate eosinophilia and reduce bronchial hyperresponsiveness to methacholine. In the current study TRAM-34 caused a robust contraction of human bronchi and further studies in human tissue are needed to

define the effect of TRAM-34 before considering the use of TRAM-34 to treat pulmonary disease.

A previous study in porcine isolated bronchi [96] found that opening of the K_{ATP} channels caused a dose dependent bronchodilation and that antagonism of the K_{ATP} channels attenuated the relaxant effect of decreasing oxygen concentrations but did not alter the maximum relaxation to 0% oxygen. In the current study these experiments were repeated to investigate these potentially significant effects in human bronchi. It was found that levcromakalim caused a dose dependent (0.3-30 μM) relaxation of human bronchi with an EC_{50} of 2.75 ± 2.62 μM which was consistent with the study in porcine bronchi and previous studies in isolated human bronchi [257-259]. The maximum relaxation to levcromakalim was 41 ± 20% of the maximum magnitude of HBD which confirms previous findings that levcromakalim has limited bronchodilator efficacy (previously compared to β-agonists) in human bronchi. After maximum relaxation to levcromakalim hypoxia caused a further relaxation which suggests that opening of the K_{ATP} channel is not a constituent mechanism in HBD.

A significant limitation of the current studies is that the effects of antagonists were only determined on the rate and maximum magnitude of HBD between the two extremes of 95% O_2 and 0% O_2 which could have obscured the effects of antagonists. Given the previous finding in porcine bronchi, that glibenclamide attenuated the rate and magnitude of HBD at higher O_2 but did not affect the maximum magnitude of HBD to 0% O_2, the effect of glibenclamide on the concentration dependent effects of oxygen on bronchial tone were investigated in this study. It was found that glibenclamide had no effect on oxygen concentration dependent relaxation from

maximum active tension in 95% O_2 or oxygen concentration dependent re-contraction from maximum relaxation to 0% O_2. This finding suggests that the K_{ATP} channel is not involved in the mechanism of HBD in human bronchi.

The most interesting and significant finding in the current studies was that the rate of HBD was significantly prolonged after contraction to KCl compared to MCh which suggests a possible mechanism for HBD. On first interpretation this finding suggested a role for the VOCCs or potassium channels in the mechanism of HBD. Contraction of the bronchi to KCl is mediated by depolarisation of the cell membrane and opening of the VOCCs, causing an influx of extracellular calcium whereas contraction to MCh is mediated by the release of calcium from intracellular stores. If HBD was mediated by an inhibition of calcium influx by closing the VOCCs then sustained opening of the VOCCs by depolarisation of the membrane by KCl could have slowed the rate of hypoxic relaxation. Similarly, if hypoxia was causing a relaxation by closing the VOCCs via hyperpolarisation of the cell membrane, by opening the potassium channels, then increasing the extracellular potassium concentration would have inhibited the efflux of potassium and slowed the rate of HBD. However, antagonist studies have shown that the calcium and potassium channels are not involved in the mechanism of HBD. Another explanation for the difference in the rate of relaxation could be functional antagonism; if the magnitude of contraction to KCl was greater than the magnitude of cholinergic contraction then reversal of KCl contraction by hypoxia would take longer. However, there was no significant difference in the magnitude of contraction to KCl or MCh in this study. Likewise it could be that contraction to KCl caused a greater increase in intracellular calcium compared to MCh and that relaxation to hypoxia is mediated by extrusion of intracellular calcium, or translocation of calcium from the

centre of the cell to the buffer barrier (see buffer barrier model, ***Chapter 1***, ***1.2.3.2***), and the rate of relaxation was proportionate to the intracellular calcium concentration. However, in this study bronchi contracted to a high extracellular calcium concentration were rapidly and reversibly relaxed by hypoxia which suggests that extrusion of intracellular calcium is not one of the mechanisms of HBD because if it was then a high extracellular calcium concentration would have increased the calcium concentration gradient across the cell membrane and prevented or at least inhibited the rate of HBD by slowing calcium extrusion (due to the increased calcium concentration gradient across the cell membrane) . An alternative explanation resides in the different mechanisms mediating the maintenance phases of KCl and MCh induced bronchoconstriction. The maintenance phase of contraction to KCl is thought to be mediated primarily by a sustained increase in intracellular calcium concentration, via influx through the open VOCCs, whereas maintenance of cholinergic contraction is mediated by an increase in the sensitivity of the contractile apparatus to calcium which sustains contraction despite falling intracellular calcium concentrations. The finding that the rate of relaxation to hypoxia was faster after contraction to MCh than after KCl contraction could therefore suggest that one of the mechanisms mediating hypoxic relaxation is a reversal of calcium sensitisation which would lead to a more rapid relaxation in bronchi contracted to MCh. However, it should be mentioned that while it has generally been accepted that contraction to KCl does not evoke an increase in calcium sensitivity Luke Janssen's group from McMaster University, Canada have provided evidence for an increase in calcium sensitivity with KCl evoked contraction in isolated airway preparations from a number of species including humans [260, 261].

Although the exact mechanism of calcium sensitisation is the subject of debate [16, 19, 20, 261] it is generally agreed that the primary mechanism is an inhibition of myosin light chain phosphatase (MLCP). Reversal of calcium sensitisation could therefore be mediated be an increase in MLCP activity, which has previously been shown to be one of the mechanisms mediating β-adrenergic relaxations in bovine and porcine (but not human) bronchi [9]. Further studies have been planned to use molecular methods (western blots) to directly measure the effects of changing oxygen concentrations on the calcium sensitisation mechanism in human airway smooth muscle by measuring changes in the intracellular activity of MLCK and MLCP.

It is also possible that hypoxia is mediating relaxation of the bronchi by directly inhibiting the contractile mechanism at the level of Ca^{2+} -calmodulin formation or the fundamental level of actin myosin cross-bridge formation by myosin ATPase. In this study the effects of hypoxia on bronchoconstriction induced by directly increasing intracellular calcium via a number of different mechanisms was investigated. In bronchi contracted to thapsigargin (sarco/endoplasmic reticulum Ca^{2+} ATPase inhibitor), ouabain (cell membrane Na+/K+-ATPase inhibitor), high extracellular calcium concentration or TRAM-34 (K^+ channel blocker) (incidental finding) hypoxia caused a complete and reversible relaxation. It is assumed that these agents all caused bronchoconstriction purely by increasing intracellular calcium which would suggest that hypoxia is acting at a fundamental level in the contractile mechanism. Given that hypoxia likely causes a decrease in available intracellular ATP it is tempting to suggest that hypoxia could mediate relaxation simply by depriving the contractile apparatus of ATP which is essential for actin myosin cross-bridge formation by myosin ATPase; however, this suggestion will need further investigation.

Further studies are needed to define the effects of hypoxia on the contractile apparatus of ASM cells in order to explore the therapeutic potential of this robust relaxant mechanism in the treatment of constrictive airway disease.

Chronic use of GPCR agonists, such as β_2- adrenoceptor agonists, in the treatment of constrictive airway disease such as asthma and COPD can cause a reduction in receptor function or desensitisation [251, 262, 263]. Desensitisation is a significant limitation in the therapeutic application of GPCRs. The studies in ***Chapter 7*** suggested that HBD is not mediated by a GPCR pathway and is therefore unlikely to be subject to desensitisation.

Studies in this chapter have shown that the mechanism of HBD does not involve the cell membrane calcium or potassium channels. The findings in this study suggest that hypoxic relaxation of human bronchi is mediated either by a direct effect at the level of the ASM contractile apparatus or by an inhibition of the calcium sensitisation mechanism. This mechanism offers a unique target for the development of novel bronchodilator therapies with potentially greater efficacy than those currently available and which are not subject to the limitations of desensitisation.

CHAPTER 9

Oxygen Sensing in Human Bronchi: Role of the Mitochondria, Reactive Oxygen Species and ATP

Contents

9.1 Introduction

The ability of different cells in the body to sense and respond to changes in oxygen concentrations is an essential homeostatic mechanism to match oxygen supply with demand. Vascular smooth muscle cells can sense and respond to changes in oxygen concentrations. In the pulmonary vasculature of most species falling oxygen concentrations cause a vasoconstriction which optimises perfusion- ventilation matching by diverting blood away from poorly ventilated areas of the lung. In systemic vessels reducing O_2 concentrations causes a relaxation which increases perfusion to oxygen deprived tissue thereby matching supply with demand. It is thought that the mechanism by which vascular smooth muscle senses and responds to changing oxygen concentrations could be a significant target in the development of therapeutics to treat systemic and pulmonary hypertension. However, there is currently debate in the literature, and a lack of consensus, about the mechanisms mediating oxygen sensing and signalling in the vasculature (Reviewed by Ward [58] and Evans *et al*. [59]).

Numerous studies have shown that inhibition of mitochondrial oxidative phosphorylation can inhibit hypoxic pulmonary vasoconstriction which suggests a central role for the mitochondria in oxygen sensing in the vasculature. The exact mechanism by which the mitochondria can sense and signal changes in oxygen concentration has been the subject of much investigation and debate and a number of different mechanisms have been proposed. The energy state hypothesis suggests that oxygen sensing is mediated by a fall in intracellular ATP concentrations (due to a lack of oxygen causing a reduction in mitochondrial ATP production) and a decrease in the ratio of ATP/ADP. Many intracellular enzymes and ion transporters are ATP dependent

and it is suggested that falling ATP concentrations affect these systems and mediate oxygen sensing. The ATP dependent potassium channel has also received considerable interest as a possible mediator of oxygen sensing by ATP being open when the ATP/ADP ratio is high and closed when the ATP/ADP ratio is low which can mediate diverse effects in different tissues [264, 265]. However, there are a number of inconsistencies in the results from studies investigating the effects of mitochondrial inhibitors which are not consistent with the energy state hypothesis in that inhibitors of oxidative phosphorylation do not always mimic the effects of hypoxia and that intracellular ATP concentrations remain stable under hypoxic conditions (see Ward [58] and Evans *et al*. [59]). A recent study has offered an explanation for these inconsistencies. Evans *et al.* [266] showed that under hypoxic conditions a rise in the ratio of ADP/ATP is converted (by adenylate kinase) into an increase in the ratio of AMP/ATP and that AMP binds to and activates AMP-activated protein kinase (AMPK) which mediates the effects of hypoxia.

It has also been suggested that reactive oxygen species (ROS) (primarily superoxide which is rapidly dismuted to the more stable peroxide by super oxide dismutase) play a pivotal role in hypoxic sensing/signalling. Under hypoxic conditions ROS are produced by the mitochondria and/or membrane bound NADPH oxidase. A large body of research has investigated the role of ROS derived from the mitochondria and the prevailing evidence supports a role for these oxygen species in the mechanism of hypoxic sensing/signalling although it should be mentioned that some studies have suggested otherwise (reviewed by Ward [58], Evans *et al.* [59] , Sylvester *et al*. [7], Chandel [267], Hamanaka and Chandel [268] and discussed in the literature by Ward [269] and Weir and Archer [270]). Previous studies have also shown that inhibition of NADPH oxidase by

Diphenyleneiodonium (DPI) inhibits the hypoxic response in isolated rat pulmonary arteries [128], rat myocytes [271] and isolated rabbit lungs [272] which suggested an oxygen sensing role for NADPH in the vasculature.

To date the oxygen sensing/signalling role of the mitochondria and ROS has not been previously investigated in human airway smooth muscle which is a significant gap in the current scientific understanding. Evidence in the literature suggests a central role for the mitochondria and ROS in oxygen sensing/signalling in the vasculature and it was hypothesised that the mitochondria and ROS would also play an important role in oxygen sensing/signalling in the airways. The aims of this study were;

1. To investigate the role of ATP as an oxygen sensor in human airways
2. To Investigate the role of mitochondrial pathways in oxygen sensing and the signalling role of ROS in the effects of hypoxia on human airway tone
3. To investigate the role of NADPH oxidase as an oxygen sensor in human airways

9.2 Methods

9.2.1 *In Vitro* Human Bronchi

Human bronchi were obtained at resection for lung cancer and mounted in organ baths to measure changes in isometric tension **(*Chapter 2, 2.3*).**

A resting tension of 2 gf was applied and the bronchi left to equilibrate for 30-90 minutes. During equilibration the bronchi were frequently washed and the resting tension adjusted to 2 gf.

9.2.2 Effect of ATP on HBD from Resting and Active Tension

When a stable resting tension was achieved the bronchi were exposed to hypoxia. When a maximum relaxation to hypoxia from resting tension was obtained the effect of ATP on HBD from resting tension was determined by cumulative addition of ATP (1-100 μM) to the organ bath. A minimum of 5 minutes was allowed between cumulative additions of ATP to measure effects on resting tension. Five minutes after addition of the final concentration of ATP the bronchi were reoxygenated with 95% O_2: 5% CO_2. When a stable resting tension was achieved the bronchi were washed for 10 minutes. When the resting tension was stable the bronchi were contracted to 1 mM MCh. When a plateau contraction to MCh was obtained the bronchi were maximally relaxed to hypoxia. The effect of ATP on HBD from active tension was then determined by addition of a bolus dose (100 μM) to the organ baths.

9.2.3 Effect of Electron Transport Chain Inhibitors on HBD

Mitochondrial electron transport chain (ETC) inhibitors were used to investigate the role of the mitochondrial pathways in oxygen sensing (**table 9.1**). Control responses to

hypoxia from resting tension and active tension to MCh were obtained. After a 30 minute washout the bronchi were incubated with ETC inhibitors for a minimum of 30 minutes before obtaining repeat responses to hypoxia from resting and active tension to MCh. The effect of ETC inhibitors on resting tension, cholinergic contraction and HBD from resting and active tension was determined and the data expressed as % change from control responses. In some experiments control responses were obtained to the vehicle (DMSO) by addition of an equal volume of vehicle without active agent to paired, time-matched preparations.

Table 9.1 Electron transport chain inhibitors used. Pharmacological action and concentration used.

Drug	Pharmacological Action	Concentration [µM]
Carbonyl cyanide 4-(triflouromethoxy) phenylhydrazone (FCCP)	Cytochrome oxidase inhibitor	3
Rotenone	Mitochondrial electron transport chain (complex I) inhibitor	1/3

9.2.4 Effect of Reactive Oxygen Species (ROS) Donors and Scavengers on HBD

Some known reactive oxygen species (ROS) donors and scavengers were used to investigate the signalling role of ROS in HBD (**table 9.2**). The effect of ROS scavengers was determined as follows; control responses to hypoxia from resting tension and active tension to MCh were obtained. After a 30 minute washout the bronchi were incubated with ROS scavengers for a minimum of 30 minutes before obtaining repeat responses to hypoxia from resting and active tension to MCh. The effect of ROS scavengers (tempol and PDTC) on resting tension, cholinergic contraction and HBD from resting and active tension was determined and the data expressed as % change

from control responses. The effect of hydrogen peroxide (H_2O_2) and the superoxide generator LY 83583 on resting and active tension was investigated by cumulative addition to the organ baths.

In one experiment the effect of tempol on the maximum relaxation to LY 83583 was investigated in two paired bronchi. One bronchus was used as the experimental preparation and the other bronchus was used to control for the effects of the corresponding vehicles for LY 83583 (ethanol) and tempol (DMSO). A maximum relaxation response to LY 83583 (30 µM) or 75 µl ethanol (control prep) from active tension to MCh was obtained. After washout the bronchi were again maximally contracted to MCh (1 mM) before the addition of tempol (3 mM) or DMSO (0.75 ml) (control). When a plateau response was obtained the bronchi were again relaxed to LY 83583 (30 µM). When a maximum response was obtained the bronchi were washed for 60 minutes before again contracting to MCh and relaxing to LY 83583. When a maximum plateau response to LY 83583 was obtained the bronchi were exposed to hypoxia.

Table 9.2 Reactive oxygen species donors and scavengers used. Pharmacological action and concentration used.

Drug	**Pharmacological Action**	**Concentration [µM]**
Pyrrolidinedithiocarbamate ammonium (PDTC)	Augments catalytic degradation of H_2O_2	5/10
Tempol	Superoxide scavenger	3000
6-Anilino-5,8-quinolinedione (LY 83583)	Superoxide generator	1-30
Hydrogen peroxide (H_2O_2)	ROS	10-1000

9.2.5 Effect of the NADPH Oxidase Inhibitor Diphenyleneiodonium (DPI) on HBD

Control responses to hypoxia from resting tension and active tension to MCh were obtained. After a 30 minute washout the bronchi were incubated with DPI (30-300 μM for a minimum of 30 minutes before obtaining repeat responses to hypoxia from resting and active tension to MCh. The effect of DPI on resting tension, cholinergic contraction and HBD from resting and active tension was determined and the data expressed as % change from control responses. In some experiments control responses were obtained to the vehicle (DMSO) by addition of an equal volume of vehicle without active agent to paired, time-matched preparations. The effect of DPI on contraction to KCl (60mM) and HBD was investigated in some experiments.

9.3 Results

9.3.1 Effect of ATP on HBD from Resting and Active Tension

Exposure of bronchi (n=4 from one patient, mean internal diameter 3.8 ± 1.5 mm) to cumulative concentrations of exogenous ATP (1-100 µM) had no effect on maximum HBD from resting tension and a bolus dose of ATP (100 µM) had no effect on maximum HBD from active tension to MCh (**figure 9.1**).

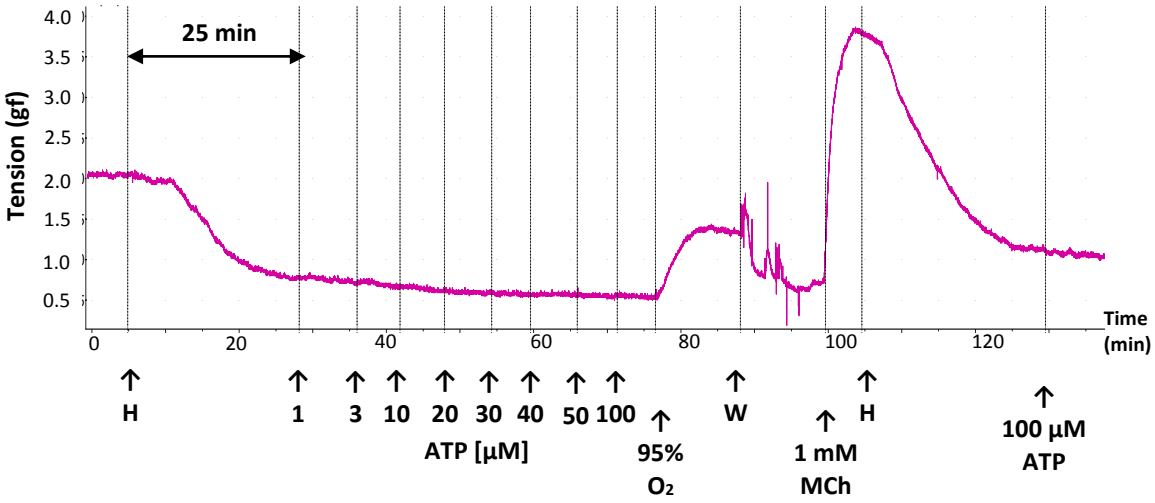

Figure 9.1 Experimental trace showing effect of ATP on HBD. ATP (1 -100 µM) had no effect on HBD from resting tension and a 100 µM bolus of ATP had no effect on HBD from active tension (n=4). H: hypoxia; W: wash

9.3.2 Effect of Electron Transport Chain Inhibitors on HBD

Six bronchi were obtained from three patients (mean internal diameter 7.2 ± 3.0 mm). The effects of the cytochrome oxidase inhibitor (complex IV) FCCP and the mitochondrial electron transport chain (complex I) inhibitor rotenone on resting tension, cholinergic contraction and HBD from resting and active tension are shown in **table 9.3** as % change from control responses. FCCP and rotenone caused attenuation in the majority of responses compared to control responses; however, none of the

effects were statistically significant and could have been due to changes over time or an effect of the vehicle (DMSO). A limitation of these studies is that vehicle and time matched control preparations were not used in all experiments and in future studies should be included in the experimental design.

Table 9.3 Effect of FCCP and Rotenone on resting tension, cholinergic contraction and HBD from resting and active tension. Data expressed as % change from control responses. Mean ± (SD); n/p: number of bronchi/number of patients

	% change from control				
Drug [µM]	**Resting tension**	**Cholinergic Contraction**	**HBD from Resting Tension**	**HBD from Active Tension**	**n/p**
FCCP [3]	-35 (0)	-79 (11)	-75 (5)	-23 (14)	2/2
Rotenone [1]	-12 (2)	-43 (27)	22 (66)	-32 (5)	2/1
Rotenone [3]	-31 (9)	-49 (17)	-	-16 (27)	2/1

9.3.3 Effect of ROS Donors and Scavengers on HBD

The effects of PDTC (augments catalytic degradation of H_2O_2) and tempol (superoxide scavenger) were investigated in four bronchi from two patients (mean internal diameter 6.0 ± 1.8 mm) and are shown in **table 9.4** as % change from control response.

Table 9.4 Effect of PDTC and tempol on resting tension, cholinergic contraction and HBD from resting and active tension. Data expressed as % change from control responses. Mean ± (SD); n/p: number of bronchi/number of patients; *$p < .05$ via 2 tail paired t-test

	% change from control				
Drug [µM]	**Resting tension**	**Cholinergic Contraction**	**HBD from Resting Tension**	**HBD from Active Tension**	**n/p**
PDTC [5]	9	-44	-	-10	1/1
PDTC [10]	-4	27	-	-29	1/1
Tempol [3000]	-26 (15)	-71 (10)	-27 (5)*	39 (1)*	2/1

9.3.3.1 Role of Hydrogen Peroxide in HBD

In one bronchus (2 mm internal diameter) hydrogen peroxide (H_2O_2) caused a dose dependent (0.01-3 mM) and reversible increase in resting tension but had little or no effect on active tension to 1 mM MCh (**figure 9.2**). This finding is not consistent with a role for H_2O_2 in the relaxant effects of hypoxia on the bronchi. However, at the extracellular concentrations used in this experiment it is possible that H_2O_2 was causing a non-specific effect on resting tension. At 5 µM and 10 µM PDTC, which augments the catalytic degradation of H_2O_2, did not substantially affect resting tension, cholinergic contraction or the magnitude of HBD from resting or active tension (**table 9.4**). Taken together these results suggest that an increase in intracellular H_2O_2 is not involved in O_2 sensing during hypoxia in the airways. However, the number of individual experiments in this study was very small and further studies are needed to confirm whether H_2O_2 has a role, or not, in the mechanism of HBD. In one experiment the effect of cumulative concentrations on the inhibition of HBD mediated by DPI was investigated and is described in **9.3.4**.

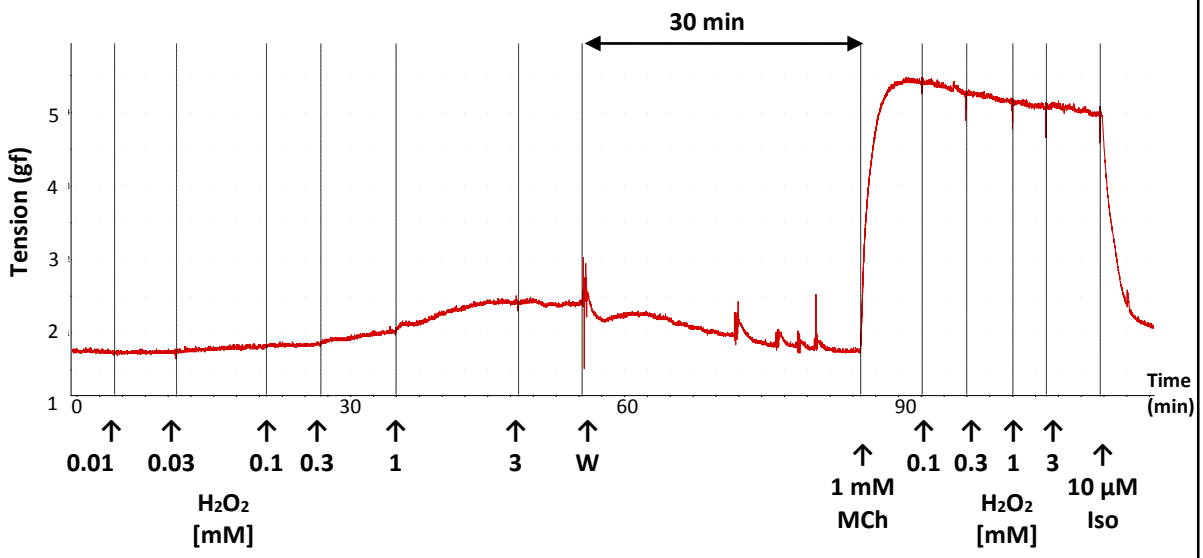

Figure 9.2 Experimental trace showing dose dependent effect of H_2O_2 on resting and active tension to 1 mM MCh in an isolated human bronchus. W: wash; Iso: isoprenaline

9.3.3.2 Role of Superoxide in HBD

The super oxide donor LY 83583 caused a robust, reversible and dose dependent relaxation (1-30 µM) in two bronchi (mean internal diameters 6 and 9 mm from one patient) from resting and active tension to 1 mM MCh (**figure 9.3a**). The magnitude of relaxation to LY 83583 was very similar to the magnitude of HBD previously observed and suggested that superoxide could have a signalling role in HBD. However, the effects of the superoxide scavenger tempol on HBD were not consistent with this suggestion. Tempol was found to cause a significant attenuation in the magnitude of HBD from resting tension but significantly augmented the magnitude of HBD from active tension compared to control responses (**table 9.4**). The apparent attenuation of HBD from resting tension could be explained by the relaxation to tempol from resting tension (-26 ± 15% from control, not statistically significant) which could have reduced the magnitude of subsequent HBD and implied an inhibitory effect.

In another experiment the effect of tempol on the maximum relaxation to LY 83583 was investigated (**figure 9.3b**) in two bronchi (both 2 mm internal diameter) from one patient. One bronchus was used as the experimental preparation and the other bronchus was used to control for the effects of the corresponding vehicles for LY 83583 (ethanol) and tempol (DMSO). In this experiment tempol was found to cause a robust relaxation from active tension, which was in part due to DMSO (Control responses in this experiment found that ethanol, the vehicle for LY 83583, did not contribute to the effects of LY 83583), but bid not inhibit the subsequent magnitude of relaxation to LY 83583. This finding shows either that tempol is not an effective super oxide scavenger or that, at the concentrations used in this experiment, tempol is not capable of scavenging the superoxide generated by LY 83583. In this experiment it was also found that hypoxia caused a further and complete relaxation of bronchi after maximum relaxation to LY 83583 which suggests that superoxide is not a primary effector in HBD. These findings were inconclusive and further studies are needed, for example to determine the effect of super oxide dismutase on HBD, to investigate the signalling role of superoxide in HBD.

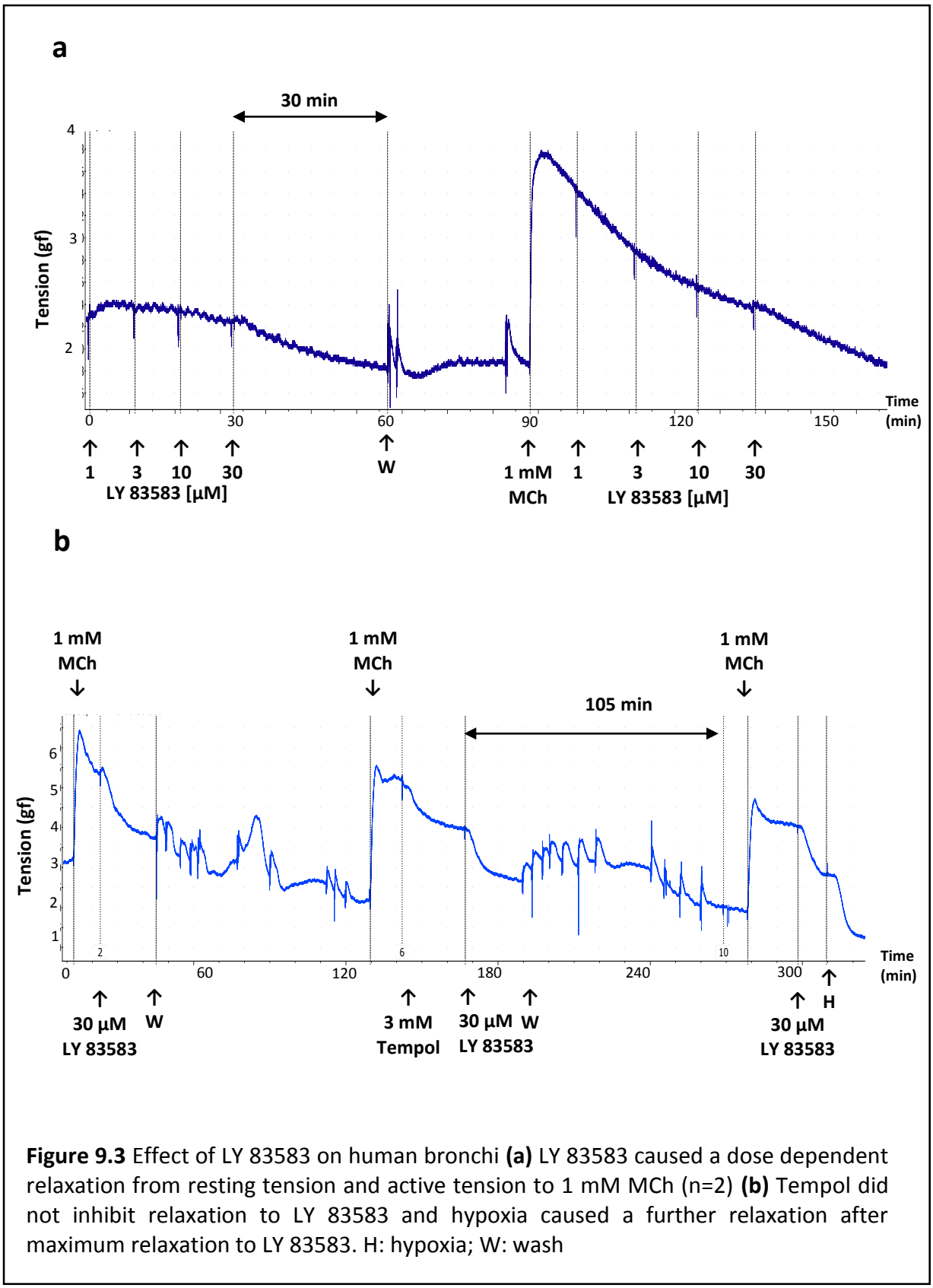

Figure 9.3 Effect of LY 83583 on human bronchi **(a)** LY 83583 caused a dose dependent relaxation from resting tension and active tension to 1 mM MCh (n=2) **(b)** Tempol did not inhibit relaxation to LY 83583 and hypoxia caused a further relaxation after maximum relaxation to LY 83583. H: hypoxia; W: wash

9.3.4 Effect of the NADPH Oxidase Inhibitor Diphenyleneiodonium (DPI) on HBD

A total of 13 bronchi were obtained from six patients (mean internal diameter 3.5 ± 1.3 mm).

9.3.4.1 Effect on Resting Tension and HBD from Resting Tension

The effects of DPI on resting tension and HBD from resting tension at concentrations of 30-300 μM did not appear to be dose related and were therefore collated (effects of different concentrations shown in **table 9.5**).

At 300 μM (n=1), 200 μM (n=1), 100 μM (n=4) and 50 μM (n=3) DPI caused a significant relaxation from resting tension (-34 ± 25% of resting tension, p = .01 via paired, 2 tail, t-test, raw data Appendix II). In paired time-matched control preparations (n=4) exposure to the vehicle (DMSO) did not cause a significant relaxation from resting tension (-16 ± 22% of resting tension, p = .29 via paired, 2 tail, t-test, raw data Appendix II).

At 300 μM (n=1), 200 μM (n=1), 100 μM (n=3) and 50 μM (n=1) DPI completely abolished HBD from resting tension in five of the six bronchi studied (-94 ± 33% of pre DPI controls, p = .01 via paired, 2 tail, t-test, raw data Appendix II, **figure 9.4a**). In paired time-matched control preparations (n=4) exposure to the vehicle (DMSO) did not cause a significant inhibition in the magnitude of HBD from resting tension (-49 ± 48% of control, p = .22 via paired, 2 tail, t-test, raw data Appendix II).

Table 9.5 Effect of different concentrations of DPI on resting tension and HBD from resting tension. Data expressed as % change from control response. Mean ± (SD); n/p: number of bronchi/number of patients; *p < .05 via 2 tail paired t-test vs. pre DPI

DPI [μM]	% Change from Control			
	Change in Resting Tension	n/p	Change in HBD from Resting Tension	n/p
300	-39	1/1	-98	1/1
200	-41	1/1	-131	1/1
100	-10 (8)	4/4	-78 (41)	3/3
50	-59 (6)	2/1	-	-
30	-66	1/1	-100	1/1
Mean	**-34**	**9/8**	**-94**	**6/6**
SD	**25**		**33**	

9.3.4.2 Effect on Active Tension and HBD from Active Tension

At 300 μM (n=1), 200 μM (n=1), 100 μM (n=2) and 50 μM (n=2) DPI completely abolished contraction to MCh (-96 ± 6% of pre DPI controls, p = .01 via paired, 2 tail, t-test, raw data Appendix II). At 30 μM in one bronchus DPI attenuated contraction to MCh by 64% and completely abolished HBD from active tension (**figure 9.4b**). In paired time-matched control preparations (n=3) exposure to the vehicle (DMSO) did not significantly attenuate the magnitude of contraction to MCh (-45 ± 12% of control, p = .23, via paired, 2 tail, t-test) or the magnitude of HBD (0 ± 15% of control, p = .98, via paired, 2 tail, t-test).

In two bronchi 100 μM DPI attenuated contraction to KCl (-80 ± 12% of control) and completely abolished HBD from active tension (raw data Appendix II) and in one of these bronchi the inhibitory effect of DPI was not reversed by cumulative addition of H_2O_2 (**figure 9.5a,b**) indicating that the effects of DPI are not mediated by an inhibition of H_2O_2 release by NADPH oxidase. In one bronchus in which contraction to MCh was

abolished by DPI a diminished contraction to KCl was obtained (**figure 9.5c**). In this bronchus HBD from active tension to KCl was abolished.

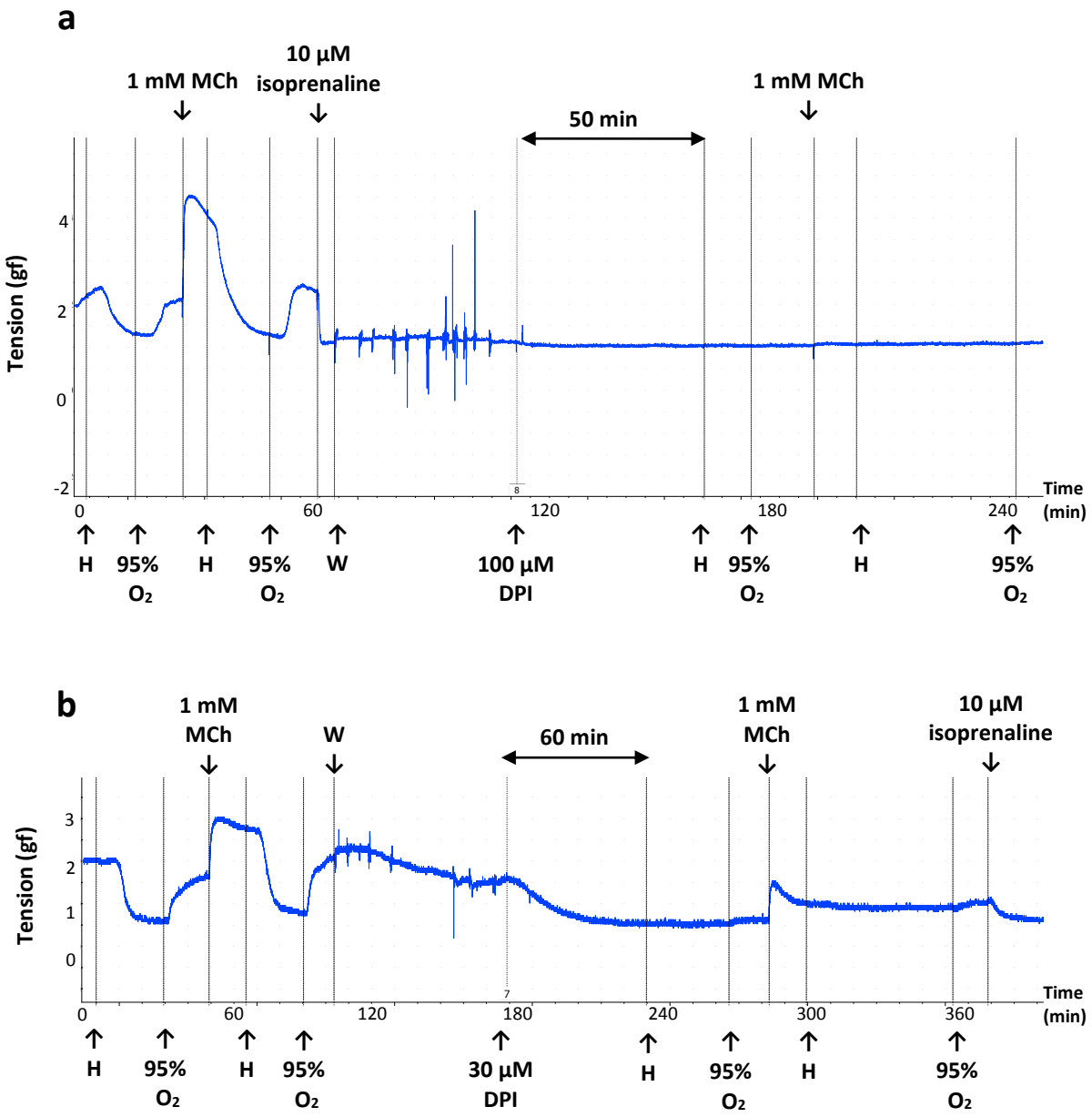

Figure 9.4 Effect of DPI on contraction to MCh and HBD **(a)** Experimental trace showing effect of DPI on HBD from resting tension and contraction to MCh (1 mM). At 50-300 µM DPI completely abolished HBD from resting tension and abolished contraction to MCh (n=6) **(b)** At 30 µM DPI completely abolished HBD from resting tension, attenuated contraction to MCh and abolished HBD from active tension. H: hypoxia; W: wash

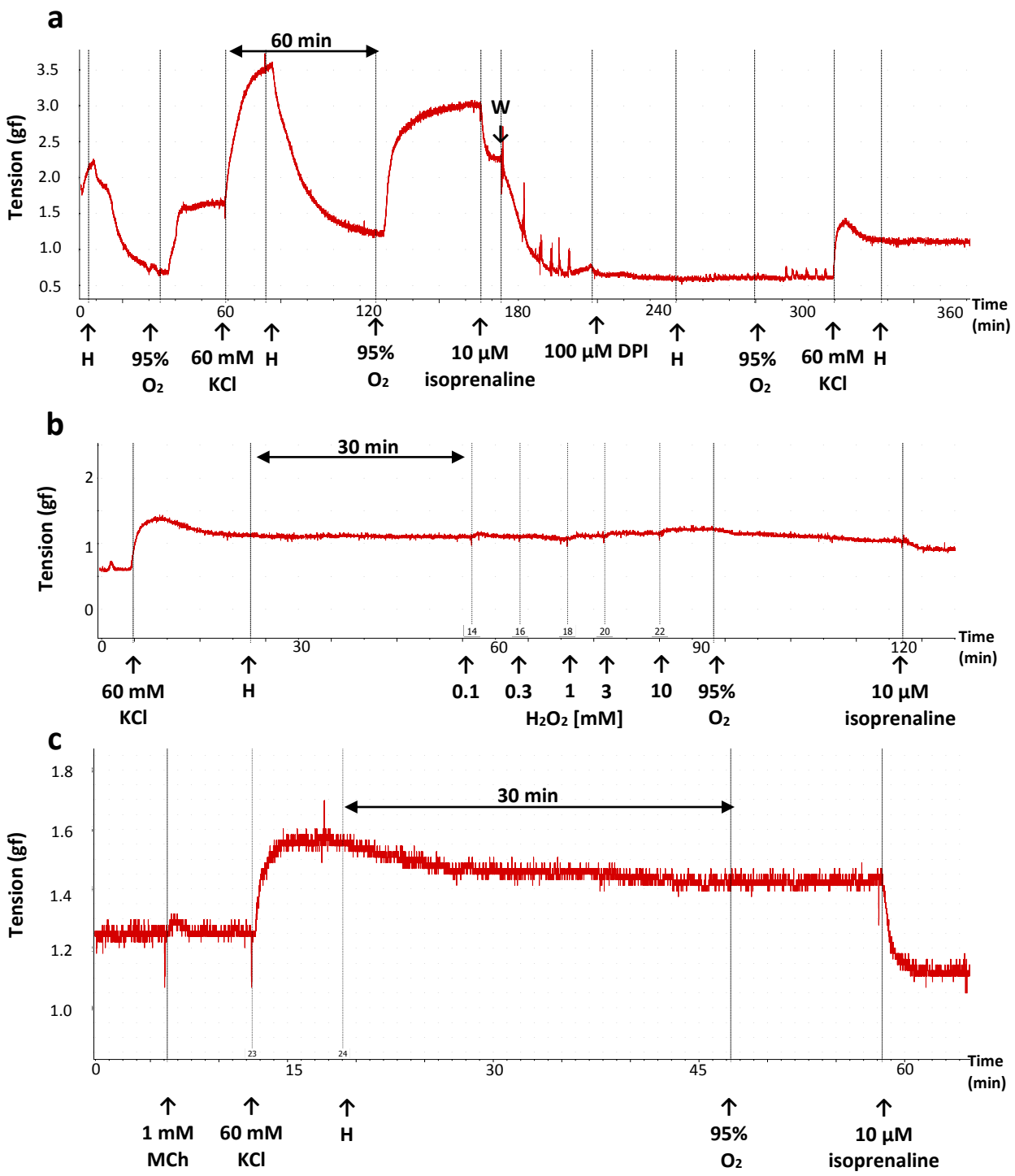

Figure 9.5 Effect of DPI on contraction to KCl and HBD **(a)** Experimental trace showing effect of DPI (100 µM) on contraction to 60 mM KCl. In two bronchi DPI significantly attenuated contraction to KCl and abolished HBD from active tension to KCl **(b)** Inhibition of HBD by DPI was not reversed by H_2O_2 **(c)** In one bronchus a diminished contraction to KCl was obtained after contraction to MCh had been abolished by DPI (100 µM) and HBD from contraction to KCL was abolished. H: hypoxia; W: wash

The finding in this study that DPI completely abolished contraction to MCh and significantly attenuated contraction to KCl and that HBD after contraction to KCl was completely abolished provides further evidence to support the suggestion made in ***Chapter 8*** that HBD could be mediated by an effect on the calcium sensitisation mechanism and will be discussed further in **9.4.**

9.4 Discussion

In this study the role of the mitochondrial ETC, reactive oxygen species and ATP in the mechanism of HBD have been investigated for the first time in human airways.

In this study cumulative addition of ATP (1-100 μM) did not reverse HBD from resting or active tension which suggests that ATP does not act as an O_2 sensor in the human bronchi. This finding is at variance with a previous study in guinea-pig tracheal rings [100] where it was found that in tissue with an intact epithelium (but not denuded trachea) pre exposure of the tissue to 100 μM ATP before contraction to histamine and hypoxia attenuated the subsequent hypoxic relaxation from active tension but that addition of ATP after relaxation to hypoxia caused a further relaxation. In this previous study it was also found that hypoxia caused a reduction in ATP content of the tracheal tissue which was partially, but significantly, regained on reoxygenation. The finding in this previous study that hypoxia decreased ATP concentrations and pre exposure to ATP attenuated subsequent relaxation could be taken to suggest a role for ATP in the mechanism of hypoxic airway relaxation; however, the finding that ATP caused a further relaxation after maximum relaxation to hypoxia is not consistent. The results in the current study should be treated with some caution. In an isolated tissue preparation such as the isolated bronchus exogenous ATP may not reach the cellular level to mediate an effect. Further studies are needed to investigate the O_2 sensor role of ATP at a cellular level for example using caged ATP in cell culture models.

The putative role of the mitochondria in oxygen sensing in HBD was investigated in this study. It was found that the ETC complex IV inhibitor FCCP and the ETC complex I inhibitor rotenone did not significantly affect HBD at concentrations which have

previously been shown to inhibit the effects of hypoxia in vascular preparations and suggests that the mitochondria do not play a role in the oxygen sensing mechanism of HBD.

The role of the ROS, hydrogen peroxide and superoxide, in the mechanism of HBD was also investigated in this study. It was found that hydrogen peroxide when added directly to the organ baths caused a dose dependent contraction (0.01-3 mM) of bronchi from resting tension but had no effect on active tension and that PDTC, which augments the catalytic degradation of H_2O_2, at concentrations which have previously been shown to attenuate the effects of hypoxia in the vasculature (5 and 10 μM) did not significantly affect the magnitude of HBD from resting or active tension. These results are not consistent with a role for hydrogen peroxide mediating the effects of hypoxia in human bronchi. In contrast to hydrogen peroxide, the superoxide donor, LY 83583 was found to cause a robust, reversible and dose dependent relaxation (1-30 μM) of the bronchi from resting and active tension and the magnitude of relaxation to LY 83583 was very similar to the magnitude of HBD previously observed which suggested that superoxide could have a signalling role in HBD. However, the superoxide scavenger tempol was found to significantly attenuate the magnitude of HBD from resting tension (which was probably due to a relaxation to tempol) and significantly augment the magnitude of HBD from active tension. Furthermore, after maximum relaxation to LY 83583 hypoxia was found to cause a further relaxation. Taken together these results suggest that superoxide is not involved in the signalling mechanism of HBD in human airways.

An important finding in this study was that diphenyleneiodonium (DPI) completely abolished HBD from resting and active tension. DPI is the only agent so far identified which can prevent HBD and the effects of DPI in this study further support the hypothesis that HBD is mediated by a calcium desensitisation mechanism. In earlier studies DPI has been used pharmacologically as an inhibitor of NADPH oxidase (as it was originally in this study) and in previous studies DPI was found to inhibit hypoxic pulmonary vasoconstriction in rat pulmonary arteries [128], rat myocytes [271] and perfused rabbit lungs [272] which was taken to indicate an O_2 sensing role for NADPH oxidase in the effects of hypoxia. However, other studies have shown that DPI has multiple pharmacological actions such as inhibition of potassium channels [273], inhibition of nitric oxide synthase [272, 274, 275], inhibition of ETC complex I [271] and recently it has been shown that DPI inhibits cholinesterase activity and sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) activity in bovine tracheal strips [276] . In the current study it was found that the ROS are probably not involved in the mechanism of HBD and that the inhibitory effect of DPI was not reversed by the addition of hydrogen peroxide and therefore it would seem unlikely that the inhibitory effect of DPI on HBD is mediated by an inhibition of NADPH oxidase activity. It was also found in this study that the selective complex I inhibitor Rotenone had no effect on HBD which suggests that the inhibitory effect of DPI on HBD is not mediated by an inhibition of complex I in the bronchi. Furthermore, in ***Chapter 8*** of this thesis it was shown that the potassium channels and SERCA are not involved in the mechanism of HBD and in ***Chapter 7*** it was found that inhibition of the nitric oxide pathway did not affect HBD which suggests that none of these pathways are involved in the inhibitory effect of DPI on HBD. The most significant finding in the current study was that DPI completely abolished

contraction to MCh and attenuated contraction to KCl and that KCl could elicit a contraction in a bronchus in which contraction to MCh had been abolished by DPI. Sustained contraction of the bronchi to cholinergic agonists is thought to be mediated predominantly by an increase in calcium sensitivity of the airway smooth muscle, whereas contraction to KCl is mediated by a combination of an increase in intracellular calcium (due to cell membrane depolarisation and opening of voltage gated calcium channels) and an increase in calcium sensitisation. The finding in this study that DPI completely abolished contraction to MCh and attenuated contraction to KCl could be interpreted to mean that DPI is inhibiting the calcium sensitisation mechanism and that the residual contraction elicited by KCl is mediated purely by an increase in intracellular calcium. The finding that hypoxia did not cause a relaxation in bronchi contracted only to calcium therefore suggests that hypoxic relaxation could be mediated by an inhibition or reversal of the calcium sensitisation mechanism. Further studies have been planned to determine the effects of changing oxygen concentrations on the calcium sensitisation mechanism in human airway smooth muscle cells at a molecular level using western blots to measure changes in the intracellular activity of MLCK and MLCP.

The finding that DPI caused a significant relaxation from resting tension and abolished HBD from resting tension is also consistent with the suggestion that hypoxia inhibits the calcium sensitisation mechanism . Maintenance of basal tone has been shown to involve a significant calcium sensitisation element and an inhibition of calcium sensitisation by DPI would account for the relaxation to DPI from resting tension and an inhibition of the calcium sensitisation mechanism by DPI would preclude any further relaxation to hypoxia being mediated by a desensitisation effect. Furthermore,

the suggestion that hypoxia could be acting on the calcium sensitisation mechanism is also consistent with studies reported by a group from King's College, London (led by Professor Jeremy Ward) which have shown that the Rho kinase inhibitor Y27632 inhibits hypoxic pulmonary vasoconstriction and suggests a pivotal role for calcium sensitisation in the mechanism of HPV [60, 61].

In ***Chapter 8*** it was proposed that hypoxia could also be acting at a fundamental level in the contractile mechanism; mediating relaxation simply by depriving the contractile apparatus of ATP. However, the finding in this study that ATP did not reverse the effects of hypoxia suggests that this might not be the case.

The hypothesis that hypoxia could be causing relaxation of the airway smooth muscle by directly inhibiting the cellular contractile apparatus and/or the calcium sensitisation mechanism adds further support for the suggestion in ***Chapter 4*** that hypoxia could be attenuating oedemagenesis by inhibiting the contractile response in pulmonary vascular endothelial cells. Phosphorylation of regulatory myosin light chain (MLC) by myosin light chain kinase (MLCK) causes actin- myosin interaction and cell contraction and it is known that the level of MLC phosphorylation has a central role in determining the contractile status of endothelial cells and the endothelial barrier function [176, 277]. Furthermore, in bovine pulmonary artery monolayers it has been shown that inhibition of RhoA (mediator of calcium sensitisation) enhances endothelial barrier function [278] and in porcine pulmonary artery endothelial cells [279] it was found that hypoxia caused an increase in intracellular RhoA and an increase in endothelial permeability (contrary to the current study) which suggests a possible role for the calcium sensitisation mechanism in the regulation of endothelial cell contractility. If hypoxia does indeed

inhibit the fundamental contractile mechanism and/or the calcium sensitisation mechanism then it would seem reasonable to suggest that hypoxia could also cause relaxation of pulmonary vascular ECs which augments endothelial barrier function and attenuates oedemagenesis. However, it is recognised that there are significant differences in the regulation of the MLCK isoform in endothelial cells compared to vascular smooth muscle cells [176] (and presumably ASM cells). Further studies have therefore been planned using cultured human pulmonary vascular endothelial cells, to test the hypothesis that hypoxia causes relaxation of pulmonary vascular ECs. Studies will measure the functional effects of hypoxia on the contractile status of endothelial cells (microscopy and calcium imaging), the effects of hypoxia on the contractile apparatus at a molecular level (western blots to quantify changes in MLCK and MLCP activity) and the effects of hypoxia on the barrier function of endothelial cell monolayers (permeability assay).

In this study it has been shown for the first time that the mitochondrial pathways, ATP and reactive oxygen species are probably not involved in the mechanism of hypoxic bronchodilation in human airways. The inhibitory effects of DPI on HBD have provided further evidence to support the hypothesis that HBD is mediated by a desensitisation of the contractile apparatus and future studies to investigate this mechanism could lead to the development of novel and effective therapeutics for the treatment of constrictive airway diseases such as COPD and asthma.

CHAPTER 10

Role of Hydrogen Sulphide as an Oxygen Sensor in the Human Pulmonary Vasculature and Airways

Contents

10.1 Introduction

Hydrogen sulphide (H_2S) is now considered to be the third endogenous gaseous signalling molecule, alongside nitric oxide (NO) and carbon monoxide (CO). Abe and Kimura [280] were the first to describe a role for H_2S as an endogenous neuromodulator in 1996 and since then a considerable body of research has investigated the physiological and pathophysiological role of H_2S. Hydrogen sulphide has been shown to have a physiological function in the cardiovascular, respiratory, endocrine, nervous, immune and reproductive systems and abnormal H_2S metabolism has been implicated in the pathophysiology of numerous diseases such as asthma, chronic respiratory diseases, atherosclerosis, cancer, essential hypertension, pulmonary hypertension, diabetes and neurodegenerative diseases (reviewed by Wang and Chen [281] and Wang [282]). There is currently growing interest in the therapeutic potential of H_2S and H_2S releasing compounds in numerous pathologies including cardiovascular and respiratory disease [283-286].

One important physiological function of H_2S is thought to be oxygen sensing. Although there is some debate in the literature about the role of H_2S in oxygen sensing studies by a group from Indiana, led by Ken Olson, have provided compelling evidence for a role of H_2S as an oxygen sensor in the effects of hypoxia on vascular tissue. In an initial study [287] they compared the effects of hypoxia and H_2S on the electrical and mechanical responses of vascular tissue preparations from a number of different species. It was found that the response of the vessels to hypoxia and H_2S was almost identical, temporally and quantitatively, regardless of whether the response was a relaxation (rat aorta), constriction (lamprey dorsal aorta) or a multiphasic response (rat

and bovine pulmonary arteries). In a subsequent study [130] Olson *et al.* compared the effect of H_2S and hypoxia in pulmonary arteries from two mammalian species. In bovine large conductance pulmonary arteries and small resistance pulmonary arteries hypoxia and H_2S caused a vasoconstriction. To their surprise, in sea lion conductance pulmonary arteries H_2S and hypoxia were found to cause a constriction or relaxation and vessels that relaxed to hypoxia relaxed to H_2S and those that contracted to hypoxia also contracted to H_2S. In smaller resistance pulmonary arteries from the sea lion hypoxia or H_2S caused only a relaxation. Clearly the similarities in the response of the blood vessels to hypoxia and H_2S suggested a role for H_2S in mediating the effects of hypoxia in the vessels and in these studies Olson and co-workers provided further evidence to support this suggestion; (1) H_2S was shown to be synthesised in lamprey and bovine vessels, (2) inhibition of the enzymes responsible for H_2S synthesis inhibited the hypoxic response, (3) addition of cysteine, the precursor for H_2S synthesis, significantly augmented the hypoxic response, (4) the effects of hypoxia and H_2S were competitive and (5) in bovine pulmonary arteries H_2S and hypoxia caused the same degree of membrane depolarisation and contraction. Furthermore, in these studies cell homogenates were used to simultaneously measure oxygen and H_2S concentrations. It was found that H_2S was constitutively synthesised in the cytoplasm but in the presence of oxygen was rapidly oxidised in the mitochondria. Under normoxic conditions H_2S concentrations remained low but when the oxygen concentration fell below 20 mmHg the H_2S concentration increased. These findings led Olson *et al*. to propose a simple yet plausible oxygen sensing/signalling mechanism for H_2S in the response of tissue to hypoxia [288]; when the tissue oxygen concentration falls the rate of H_2S oxidation decreases and the cellular concentration of H_2S increases

proportionately which then mediates an effect on the particular tissue affected. To date, the effect of H_2S on vascular tone and the putative role of H_2S in hypoxic signalling has not been investigated in the human pulmonary vasculature which is a significant gap in the current scientific understanding.

Only a small number of studies have previously described the effect of H_2S on airway tone. Kubo *et al*. [289] investigated the effect of the H_2S donor sodium hydrogen sulphide (NaHS) on resting and active tension in mouse and guinea-pig bronchial rings. They found that NaHS had no effect on resting tone in bronchial rings from either species but caused a robust and dose dependent relaxation of mouse bronchial rings after contraction to carbachol but only a small relaxation in pre-contracted guinea-pig rings. These results suggest significant species differences in the response of the airways to H_2S. A recent study [290] in porcine bronchi investigated the effect of H_2S on airway tone. It was shown that the enzymes responsible for H_2S synthesis were expressed in the airways and could be induced to produce H_2S and that L-cysteine the precursor for H_2S caused a relaxation of the bronchi, proving that H_2S could be produced endogenously and cause a relaxation of the bronchi. It was also found that NaHS caused a large but transient relaxation of the airways, which was shown to be mediated in part by a K^+ channel mechanism. In this previous paper an experimental trace was presented showing the transient effect of NaHS on active tension to carbachol in porcine bronchi. It was observed that this trace showed remarkable similarity, both temporally and quantitatively, to the effects of hypoxia and reoxygenation seen in pre-contracted human bronchi shown in this thesis (***Chapter 6***) and suggested that H_2S could play a role in the mechanism of HBD.

To date, the effect of H_2S on human airway tone and its role in hypoxic signalling has not been investigated which is a significant gap in the current scientific literature.

Previous animal studies have shown that H_2S is an effective vasodilator in the pulmonary vasculature and some animal studies have shown that H_2S is an effective bronchodilator. To date, the effect of H_2S on human pulmonary vascular tone and human airway tone has not been previously investigated. Compelling evidence has also been provided which suggests a role for H_2S in oxygen sensing/signalling. It was hypothesised that H_2S would be an effective vasodilator and bronchodilator in the human lung and could have a central role in sensing/signalling changes in oxygen concentrations in the human lung. The aim of the studies in this chapter were;

1. To compare the effect of H_2S and hypoxia on human pulmonary vascular tone in isolated human pulmonary arteries and veins
2. To compare the effect of H_2S and hypoxia on bronchial tone in isolated human bronchi
3. To investigate the putative oxygen sensing role of H_2S in hypoxic bronchodilation

10.2 Methods

10.2.1 *In Vitro* Human Pulmonary Arteries and Veins

10.2.1.1 Tissue Preparation

Human lobar and inter lobar pulmonary arteries or veins were obtained at resection for lung cancer. Four vessels were prepared and mounted between stainless steel wires in separate organ baths to measure changes in isometric tension **(*Chapter 2, 2.3*).**

Resting tension was set at 1 gf and the vessels allowed to equilibrate. During equilibration the vessels were frequently washed and the resting tension adjusted to 1 gf.

After equilibration for 60-90 minutes and when a stable resting tension was achieved the vessels were exposed to 1 μM acetylcholine (ACh) to determine the functional integrity of the endothelium. When a maximum response to ACh was obtained or after a minimum of 15 minutes the vessels were washed for a minimum of 30 minutes before starting experimental procedures.

10.2.1.2 Effect of Hypoxia and NaHS on Resting Tension

When a stable resting tension was achieved the vessels were exposed to either hypoxia (by covering the top of the organ bath with a plastic film and changing the aerating gas to 95% N_2: 5% CO_2) (two vessels) or cumulative concentrations of NaHS (two vessels). The effect of each concentration of NaHS was measured after the response had reached plateau and was the mean tension (gf) for the 2 minute period preceding the next increase in drug concentration (obtained using the data pad function of the Chart software). When a maximum response to hypoxia or NaHS was

obtained the aerating gas was changed back to 95% O_2: 5% CO_2 (hypoxic vessels) or the vessels washed (vessels exposed to NaHS). After a minimum of 30 minutes reoxygenation or washout and when the resting tension was stable the vessels were exposed to the alternative procedure (hypoxia or NaHS) in order to compare the effects of hypoxia and NaHS in the same vessels. When a maximum response to hypoxia or NaHS was obtained all vessels were washed and aerated with 95% O_2: 5% CO_2 before determining the effect of hypoxia and NaHS on active tension.

The effect of hypoxia and NaHS on resting tension was quantified as absolute change in resting tension (gf) or % change in resting tension where appropriate. Because experiments were performed in the same vessels absolute baseline values were used to compare the maximum relaxation to hypoxia and NaHS.

10.2.1.3 Effect of Hypoxia and NaHS on Active Tension

Studies in ***Chapter 3*** (***3.3.1.2***) found that contraction of the pulmonary arteries to noradrenaline and adrenaline was not sustained. Therefore in this study active tension was induced to endothelin-1 (ET-1). Studies in ***Chapter 5*** (***5.3.2***) found that ET-1 was less potent in the pulmonary arteries than the pulmonary veins and therefore, in order to achieve a maximum increase in active tension, in this study arteries were contracted to 3 nM ET-1 and veins to 1 nM ET-1.

When a stable resting tension was achieved the vessels were maximally contracted to ET-1. When a stable plateau in active tension was achieved the vessels were exposed to either hypoxia (two vessels) or cumulative concentrations of NaHS (two vessels). The effect of each concentration of NaHS was measured after the response had reached plateau and was the mean tension (gf) for the 2 minute period preceding the

next increase in drug concentration (obtained using the data pad function of the Chart software). When a maximum response to hypoxia or NaHS was obtained the aerating gas was changed back to 95% O_2: 5% CO_2 (hypoxic vessels) or the vessels washed (vessels exposed to NaHS). After a minimum of 30 minutes reoxygenation or washout and when the resting tension was stable the vessels were again contracted to ET-1 and exposed to the alternative procedure (hypoxia or NaHS) in order to compare the effects of hypoxia and NaHS on active tension in the same vessels.

The effect of hypoxia and NaHS on active tension was quantified as change in active tension (gf) or % change in maximum active tension. Because experiments were performed in the same vessels absolute baseline values were used to compare the maximum relaxation to hypoxia and NaHS.

10.2.2 *In Vitro* Human Bronchi

Human bronchi were obtained at resection for lung cancer and mounted in organ baths to measure changes in isometric tension **(*Chapter 2, 2.3*).**

A resting tension of 2 gf was applied and the bronchi left to equilibrate for 30-90 minutes. During equilibration the bronchi were frequently washed and the resting tension adjusted to 2 gf.

10.2.2.1 Effect of Hypoxia and NaHS on Resting Tension

When a stable resting tension was achieved the bronchi were exposed to hypoxia by covering the top of the organ bath with a plastic film and changing the aerating gas to 95% N_2: 5% CO_2. When a maximum response to hypoxia was obtained the aerating gas was changed back to 95% O_2: 5% CO_2. When the reoxygenation resting tension was

stable the effect of NaHS on resting tension was determined either by cumulative addition of NaHS to the organ bath, to determine the dose response effect, or by the addition of a single dose (1 mM) to determine the maximum effect.

The effect of hypoxia and NaHS on resting tension was quantified as absolute change in resting tension (gf) or % change in resting tension where appropriate. Because experiments were performed in the same bronchi absolute baseline values were also used to compare the maximum relaxation to hypoxia and NaHS.

10.2.2.2 Effect of Hypoxia and NaHS on Active Tension

Bronchi were contracted to 1 mM MCh and control responses to hypoxia and reoxygenation obtained. When a stable re-contraction baseline was achieved the effect of NaHS on active tension was determined either by cumulative addition of NaHS to the organ bath, to determine the dose response effect, or by the addition of a single dose (1 mM) to determine the maximum effect.

The effect of hypoxia and NaHS on active tension was quantified as change in active tension (gf) or % change in maximum active tension. Because experiments were performed in the same bronchi absolute baseline values were used to compare the maximum relaxation to hypoxia and NaHS.

10.2.2.3 Role of The Potassium Channels in the Mechanism of NaHS relaxation

A previous study in porcine bronchi [290] found that the relaxation to NaHS was significantly reduced in bronchi contracted to KCl compared to bronchi pre-contracted to carbachol which suggests a possible role for the potassium channels in the mechanism of relaxation to NaHS. In this study the effect of NaHS on contraction to KCl

was compared to the effect of NaHS in a paired bronchus contracted to MCh. One bronchus was contracted to 1 mM MCh and the other bronchus contracted to 60 mM KCl. When a stable contraction plateau was achieved control responses to hypoxia were obtained. When a maximum relaxation to hypoxia was achieved the bronchi were re-contracted to oxygen. When a maximum re-contraction plateau was achieved the effect of NaHS on active tension was determined by cumulative addition to the organ bath.

In two bronchi the effect of the non-selective K^+ channel blocker Tetraethylammonium (TEA) was investigated on the relaxation to NaHS. These experiments were originally designed to determine the effect of TEA on HBD. After exposure of the bronchi to TEA for 30 minutes repeat responses to hypoxia from resting and active tension were obtained. After re-contraction of the bronchi to reoxygenation from active tension a control relaxation to isoprenaline was obtained. In this experiment the relaxation to isoprenaline was small in both bronchi and a further response to hypoxia and reoxygenation was obtained in the presence of isoprenaline. After the final re-contraction of the bronchi to reoxygenation the bronchi were exposed to 1 mM NaHS.

10.2.2.4 Competitive Effects of Hypoxia and NaHS

In a previous study Olson *et al*. [287] showed that the effects of H_2S and hypoxia were mutually competitive; in a number of different vessel preparations they found that exposure to one agent (hypoxia or H_2S) inhibited the subsequent effect of the other agent. This suggested a common mechanism and was taken as further evidence for the role of H_2S as an oxygen sensor. In this study the effect of hypoxia after maximum

relaxation to NaHS from active and resting tension was determined in a number of bronchi and the observations reported.

10.2.2.5 Effect of H_2S Enzyme Synthesis Inhibitors on HBD

H_2S can be produced by cells from cysteine via a number of biochemical pathways (See Olson [284]). However, two enzymatic pathways are thought to predominate in the production of H_2S; cystathionine β-synthase (CBS) and cystathionine γ–lyase (CSE). In this study two recognised H_2S synthesis inhibitors, Propargylglycine (PPG) a selective CSE inhibitor and hydroxylamine (HA) a non-selective CSE and CBS inhibitor, were used to investigate the role of H_2S as an oxygen sensor in human bronchi.

After obtaining control responses to hypoxia from resting tension and from active tension to methacholine [1 mM] the bronchi were washed for 30 minutes before adding H_2S synthesis inhibitors (PPG) (10 or 30 mM) or Hydroxylamine (HA) (0.3 or 1 mM) directly to the organ bath. After a minimum incubation of 30 minutes repeat responses to hypoxia from resting and active tension were obtained. The effect of the enzyme inhibitors on resting baseline tension, cholinergic contraction and HBD from resting and active tension was determined by comparison with pre-exposure controls and quantified as % change from control.

10.2.2.6 Effect of DPI on relaxation to NaHS

In ***Chapter 8*** it was found that DPI abolished HBD. In this study the effect of DPI (100 μM) on relaxation to NaHS from active tension to KCl was investigated in a single preparation. After obtaining control responses to hypoxia and reoxygenation from resting tension and active tension to KCl a maximum response to NaHS from active

tension to KCl was obtained. The bronchus was then washed for 75 minutes before exposure to 100 µM DPI. After a 30 minute incubation repeat responses were obtained for hypoxia/reoxygenation from resting tension, contraction to KCl and for hypoxia/reoxygenation and NaHS (1 mM) from active tension to KCl.

10.3 Results

10.3.1 *In Vitro* Human Pulmonary Arteries

Eight human pulmonary arteries (mean internal diameter 5.3 ± 1.7 mm, 3.1 ± 0.6 mm wide) were obtained from two patients. Five of the eight pulmonary arteries relaxed to ACh before commencing experimental procedures.

10.3.1.1 Effect of Hypoxia and NaHS on Resting Tension

NaHS caused a dose dependent (0.1-3 mM) relaxation in 6 of 8 human pulmonary arteries from resting tension with an EC_{50} of 0.44 ± 0.49 mM, pEC_{50} 3.51 ± 0.36 which was statistically significant at 0.3, 1 and 3 mM NaHS ($p < .05$ resting tension vs. baseline resting tension via paired 2 tail t-test, n=8) (**table 10.1, figure 10.1,** raw data Appendix II, EC_{50} concentrations were estimated manually from individual concentration response curves). In paired preparations hypoxia also caused a significant relaxation ($p = .01$ via paired 2 tail t-test, n=8) of human pulmonary arteries from resting tension which was not reversed on reoxygenation (**table 10.1,** raw data Appendix II). The maximum relaxation to hypoxia was not significantly different to the maximum magnitude of relaxation to NaHS (-0.10 ± 0.09 gf and -0.19 ± 0.17 gf or -8 ± 7% and -10 ± 8% of resting tension for hypoxia and NaHS respectively, $p = .19$ and 0.43 via paired 2 tail t-test, n=8) and the absolute baseline was not significantly different after relaxation to NaHS and hypoxia (**table 10.1, figure 10.1c**, raw data Appendix II). However, the effects of NaHS and hypoxia on resting tension were not consistent; in one preparation hypoxia caused a relaxation but NaHS had no effect on resting tension and in another artery NaHS caused a robust relaxation whereas hypoxia had no effect. There was no obvious association between the presence of a functional endothelium

(as determined by a positive control relaxation to ACh) and effect, or lack of effect, of NaHS and hypoxia in the vessels.

Table 10.1 Dose dependent effect of NaHS and maximum effect of hypoxia on resting tension in human pulmonary arteries. Mean change in resting tension (gf) ± (SD). n=8. EC_{50} n=6. *p < .05 vs. baseline resting tension and $^{\#}p$ = NS vs. maximum relaxation to NaHS both via paired 2-tail t-test

	NaHS [mM]				**EC_{50} (mM)**	**pEC_{50}**	**Maximum Relaxation to hypoxia (gf)**
	0.1	**0.3**	**1**	**3**			
Change in Resting Tension (gf)	-0.03 (0.06)	-0.12 (0.13)*	-0.15 (0.15)*	-0.19 (0.17)*	0.44 (0.49)	3.51 (0.36)	-0.10 (0.09)#

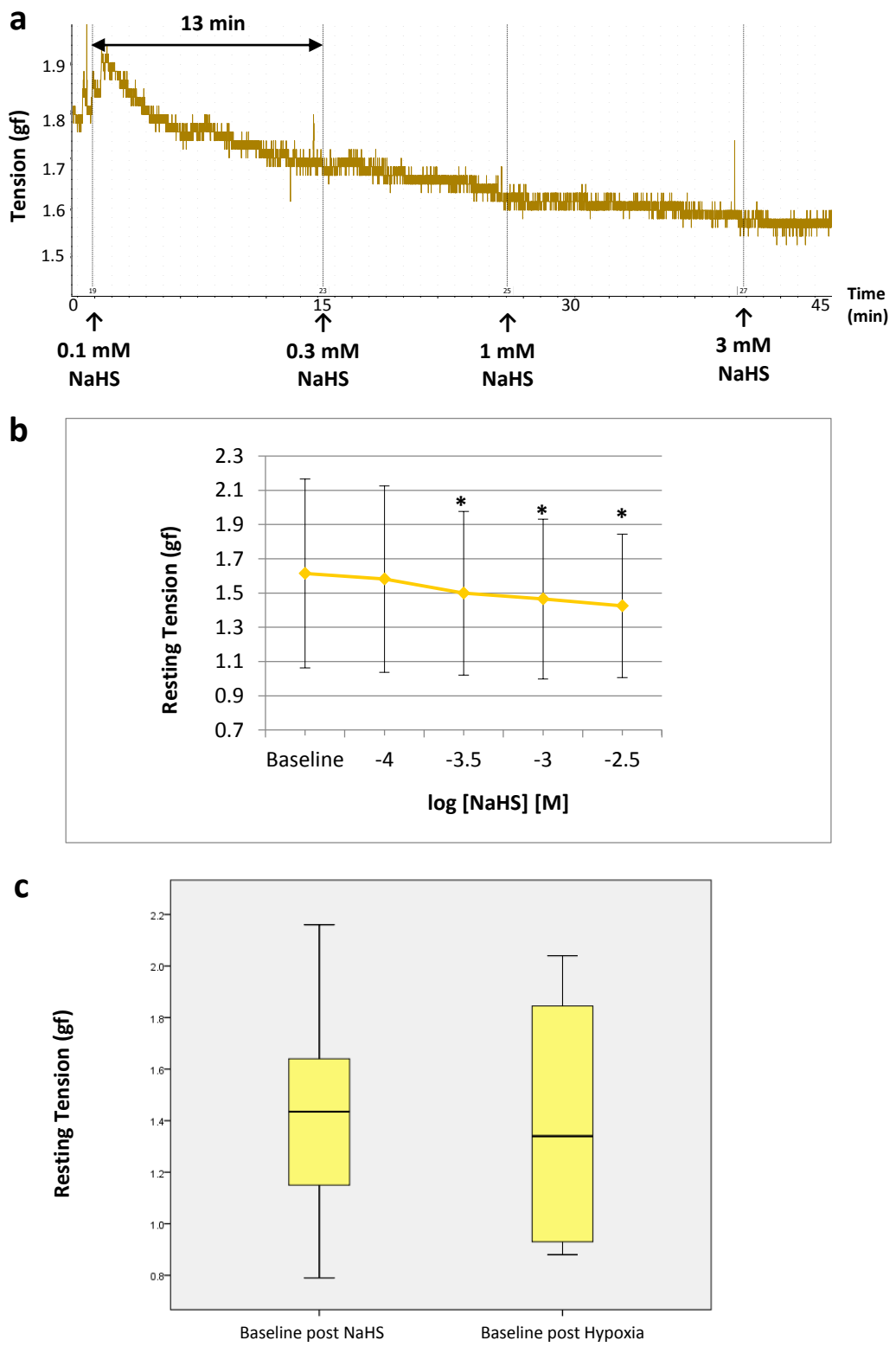

Figure 10.1 Effect of NaHS and hypoxia on resting tension in isolated human pulmonary arteries (**a**) Experimental trace showing dose dependent relaxation of a human pulmonary artery to NaHS from resting tension (**b**) log dose response curve to NaHS from resting tension. Absolute tension (gf) shown. n=8, *p < .05 vs. baseline resting tension via paired 2 tail t-test (**c**) Box-whisker plot of absolute baseline tension post maximum relaxation to NaHS and hypoxia in the same vessels. n=8.

10.3.1.2 Effect of Hypoxia and NaHS on Active Tension

NaHS caused a dose dependent (0.1-3 mM) relaxation in 7 of 8 human pulmonary arteries from active tension to 3 nM ET-1 with an EC_{50} of 0.66 ± 0.55 mM, pEC_{50} 3.31 ± 0.36, (n=7) which was statistically significant at 1 and 3 mM NaHS ($p < .05$ active tension vs. maximum active tension to 3 mM ET-1 via paired 2 tail t-test, n=8)) (**table 10.2, figure 10.3,** raw data Appendix II, EC_{50} concentrations were estimated manually from individual concentration response curves). In paired preparations hypoxia also caused a significant ($p = .02$ via paired 2 tail t-test, n=8) relaxation of all human pulmonary arteries which was not reversed on reoxygenation (**table 10.2,** raw data Appendix II). The maximum relaxation to hypoxia was not significantly different to the maximum magnitude of relaxation to NaHS (-0.30 ± 0.14 gf and -0.40 ± 0.40 gf or -73 ± 375 and -52 ± 40% reversal of maximum active tension for hypoxia and NaHS respectively $p = .54$ and .43 via 2 tail t-test, n=8) and the absolute baseline was not significantly different after relaxation to hypoxia and NaHS from active tension which suggests a possible causal relationship between relaxation to hypoxia and NaHS (**table 10.2, figure 10.3c**, raw data Appendix II). However, in one artery NaHS caused a dose dependent contraction from maximum active tension to 3 nM ET-1. In this artery hypoxia caused a robust relaxation (-121% of active tension) (**figure 10.2**). There was no obvious association between the presence of a functional endothelium and an effect, or lack of effect, of NaHS and hypoxia in the vessels.

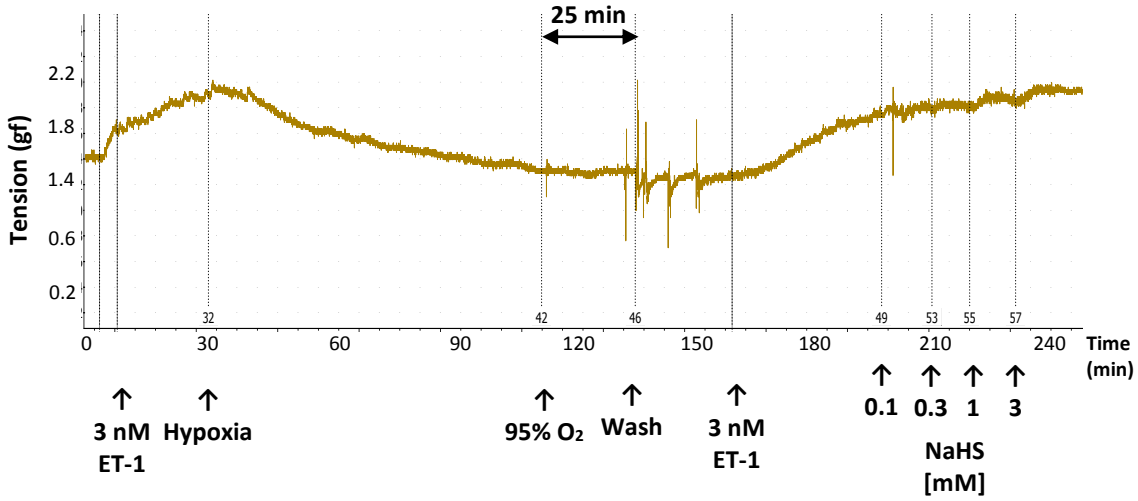

Figure 10.2 Experimental trace showing relaxation to hypoxia from active tension to ET-1 (3 nM) and dose dependent contraction to NaHS from active tension in an isolated human pulmonary artery.

Table 10.2 Dose dependent effect of NaHS and maximum effect of hypoxia on active tension to 3 nM ET-1 in human pulmonary arteries. Change in active tension (gf) and % change in active tension. Mean ± (SD). n=8. EC_{50} n=7. $^{*}p < .05$ vs. maximum active tension and $^{\#}p$ = NS vs. maximum relaxation to NaHS both via paired 2-tail t-test

	NaHS [mM]				Maximum Relaxation to hypoxia	EC_{50} (mM)	pEC_{50}
	0.1	**0.3**	**1**	**3**			
Change in Active Tension (gf)	-0.05 (0.10)	-0.21 (0.27)	-0.34 (0.40)*	-0.40 (0.40)*	-0.30 (0.14)$^{\#}$	0.66 (0.55)	3.31 (0.36)
% Change in Active Tension	-4 (13)	-24 (25)	-43 (38)	-52 (40)	-73 (37)		

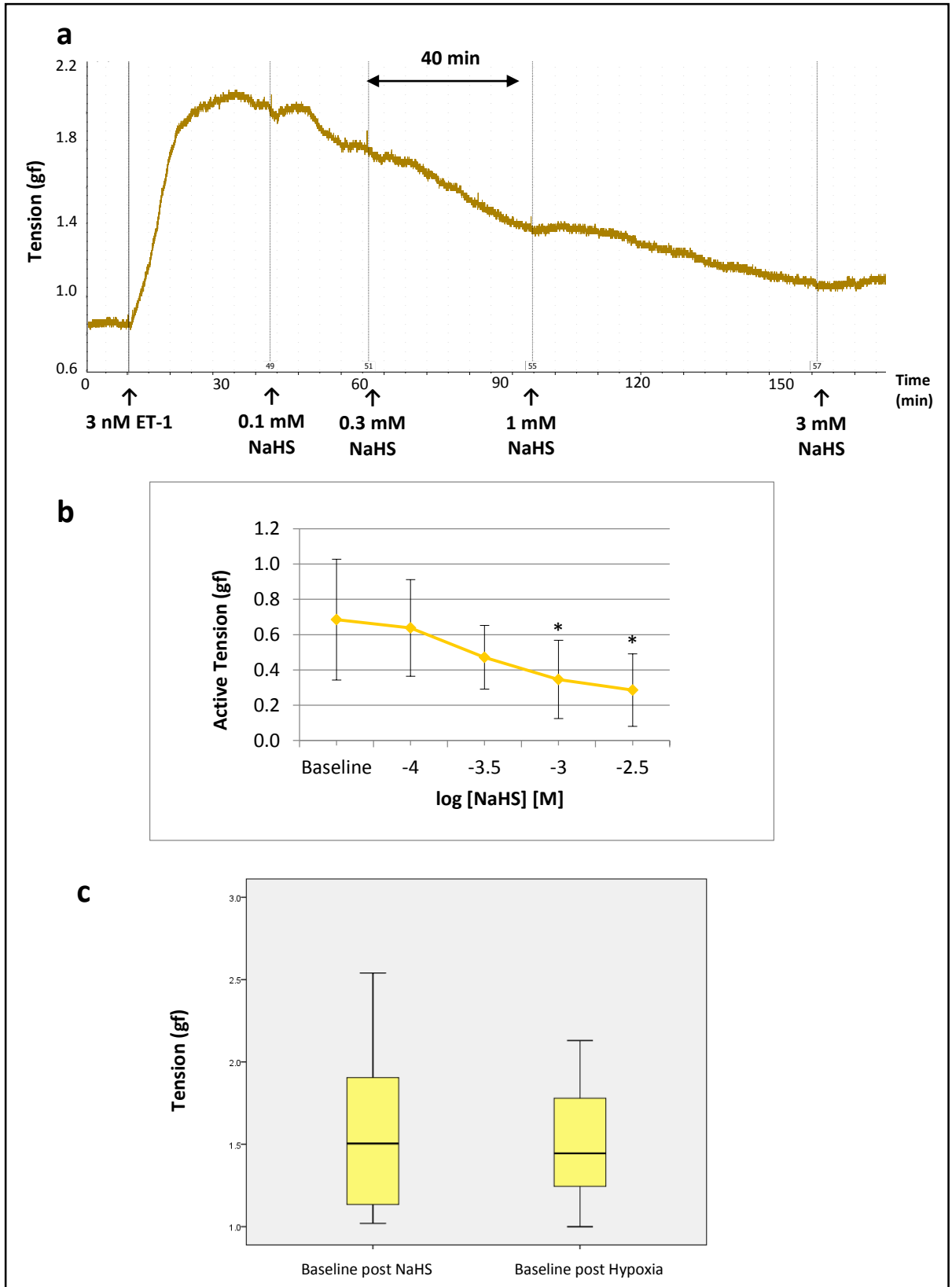

Figure 10.3 Effect of NaHS and hypoxia on active tension in isolated human pulmonary arteries (**a**) Experimental trace showing dose dependent relaxation of a human pulmonary artery to NaHS from active tension to 3 nM ET-1 (**b**) log dose response curve to NaHS from active tension. Absolute tension (gf) shown. *$p < .05$ vs. maximum active tension n=8. (**c**) Box-whisker plot of absolute baseline tension post maximum relaxation from active tension to NaHS and hypoxia in the same vessels. n=8.

10.3.2 *In Vitro* Human Pulmonary Veins

Eight human pulmonary veins (mean internal diameter 5.3 ± 1.6 mm, 3.3 ± 0.9 mm wide) were obtained from two patients. Six of the eight pulmonary veins relaxed to ACh.

10.3.2.1 Effect of Hypoxia and NaHS on Resting Tension

NaHS caused a dose dependent (0.1-1 mM) relaxation from resting tension in 5 of 8 human pulmonary veins studied, EC_{50} 0.68 ± 0.58 mM, pEC_{50} 3.30 ± 0.38 (estimated manually from individual concentration response curves) which was not statistically significant at any of the concentrations of NaHS tested (resting tension vs. baseline resting tension via paired 2 tail t-test, n = 8) (**figure 10.4**, **table 10.3**, raw data Appendix II). In paired preparations hypoxia also caused a relaxation of human pulmonary arteries from resting tension which was not statistically significant (p = .06 via paired 2 tail t-test, n=8) (**table 10.3,** raw data Appendix II). The effects of NaHS and hypoxia were inconsistent; two of the vessels which did not relax to NaHS did not relax to hypoxia and one vessel did not relax to NaHS but did relax to hypoxia and one vessel relaxed to NaHS but not to hypoxia. The magnitude of relaxation to hypoxia and NaHS was not significantly different in paired vessels (-0.13 ± 0.17 gf and -0.17 ± 0.21 gf or -12 ± 15% and -19 ± 25% of baseline for hypoxia and NaHS respectively p = .60 and .42 via 2 tail t-test, n=8) and the absolute baseline was not significantly different after relaxation to hypoxia and NaHS from resting tension (**figure 10.4c**). There was no obvious association between the presence of a functional endothelium and an effect, or lack of effect, of NaHS and hypoxia in the vessels.

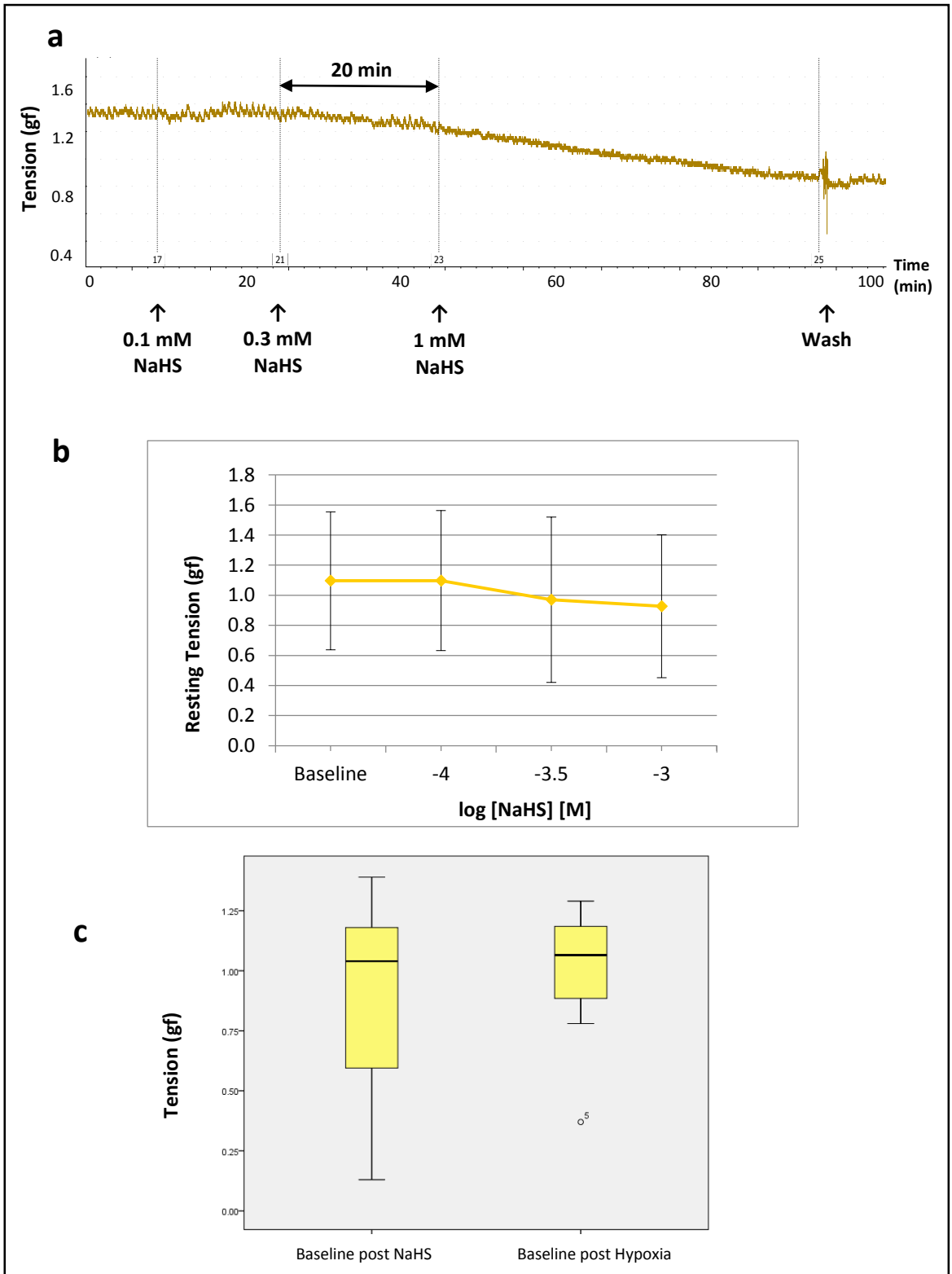

Figure 10.4 Effect of NaHS and hypoxia on resting tension in isolated human pulmonary veins (**a**) Experimental trace showing dose dependent relaxation of a human pulmonary vein to NaHS from resting tension (**b**) log dose response curve to NaHS from resting tension. Absolute tension (gf) shown. n=8. (**c**) Box-whisker plot of absolute baseline tension post maximum relaxation to NaHS and hypoxia in the same vessels. n=8.

Table 10.3 Dose dependent effect of NaHS on resting tension and maximum relaxation to hypoxia in human pulmonary veins. Mean change in resting tension (gf) ± (SD). n=8. EC_{50} n=5, $^{\#}p$ = NS vs. maximum relaxation to NaHS via paired 2-tail t-test

	NaHS [mM]			EC_{50} (mM)	pEC_{50}	Maximum Relaxation to hypoxia (gf)
	0.1	0.3	1			
Change in Resting Tension (gf)	0.0 (0.1)	-0.12 (0.12)	-0.17 (0.21)	0.68 (0.58)	3.30 (0.38)	-0.13 (0.17)$^{\#}$

10.3.2.2 Effect of Hypoxia and NaHS on Active Tension

The effect of NaHS on active tension could not be obtained in one vein for technical reasons. In six of the remaining seven veins NaHS caused a dose dependent (0.1-3 mM) relaxation from active tension to 1 nM ET-1 with an EC_{50} of 0.15 ± 0.10 mM and pEC_{50} of 3.93 ± 0.35 (estimated manually from individual concentration response curves, n=6) which was not statistically significant at any of the concentrations tested (**table 10.4, figure 10.5,** raw data Appendix II). The effect of hypoxia on active tension was not obtained in two preparations. In the remaining six veins hypoxia caused a significant relaxation ($p < .01$ via paired 2 tail t-test, n=6) of all veins which was not reversed on reoxygenation. In paired preparations (n=5) the maximum magnitude of relaxation to hypoxia was not significantly different to the maximum relaxation to NaHS (-0.14 ± 0.07 gf and -0.64 ± 1.08 gf or -73 ± 30 and -55 ± 60% of active tension for hypoxia and NaHS respectively p = .56 and .38 via 2 tail paired t-test, n=5, raw data Appendix II). The absolute baseline tension after maximum relaxation to hypoxia and NaHS was not significantly different (**figure 10.5c**). Taken together these results suggest a possible causal relationship between relaxation to hypoxia and NaHS. There

was no obvious association between the presence of a functional endothelium and an effect, or lack of effect, of NaHS and hypoxia in the vessels.

Table 10.4 Dose dependent effect of NaHS and maximum effect of hypoxia on active tension in human pulmonary veins. Change in active tension (gf) and % change in active tension. Mean ± (SD). n=7, EC_{50} n=6, $^{\#}p$ = NS vs. maximum relaxation to NaHS via paired 2-tail t-test

	NaHS [mM]			**Maximum Relaxation to hypoxia**	**EC_{50} (mM)**	**pEC_{50}**
	0.1	**0.3**	**1**			
Δ Active Tension (gf)	-0.31 (0.53)	-0.59 (1.04)	-0.60 (1.10)	-0.14 (0.07)$^{\#}$	0.15 (0.10)	3.93 (0.35)
% Δ Active Tension	-23 (19)	-55 (55)	-47 (42)	-76 (28)$^{\#}$		

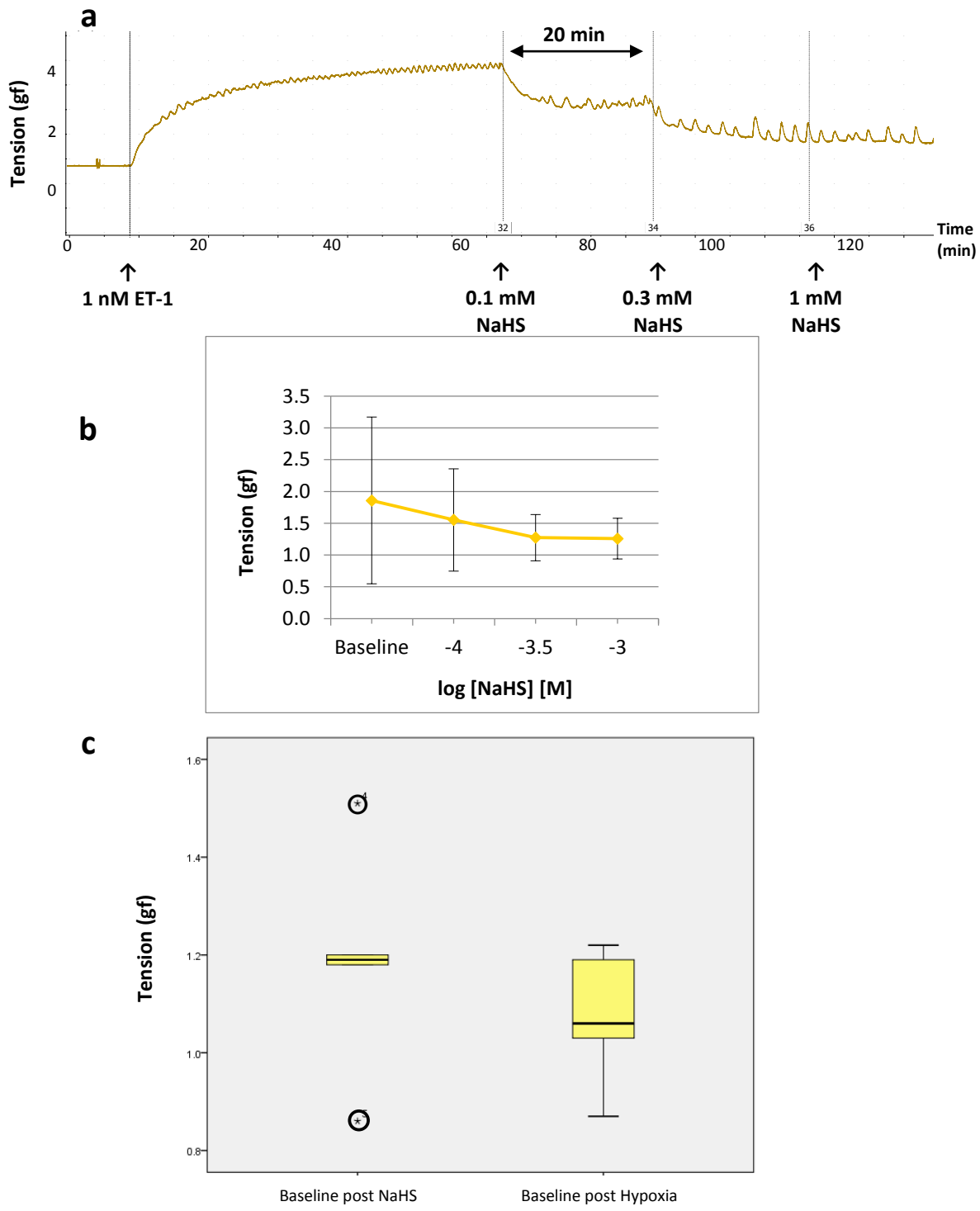

Figure 10.5 Effect of NaHS and hypoxia on active tension to 1 nM ET-1 in isolated human pulmonary veins (**a**) Experimental trace showing dose dependent relaxation of a human pulmonary vein to NaHS from active tension (**b**) log dose response curve to NaHS from active tension. Absolute tension (gf) shown. n=7. (**c**) Box-whisker plot of absolute baseline tension post maximum relaxation to NaHS (two extreme outliers, circled asterisk) and hypoxia in paired vessels. n=5.

10.3.3 *In Vitro* Human Bronchi

10.3.3.1 Effect of Hypoxia and NaHS on Resting Tension

NaHS caused a dose dependent (0.01-1 mM) relaxation from resting tension in all bronchi investigated (n=6 from 2 patients, mean internal diameter 4.5 ± 1.5 mm). EC_{50} 0.40 ± 0.23 mM, pEC_{50} 3.61 ± 0.69 (estimated manually from individual concentration response curves) which was not statistically significant at any of the concentrations tested (**figure 10.6a, 10.6b, table 10.5**, raw data Appendix II).

Table 10.5 Dose dependent effect of NaHS on resting tension in human bronchi. Mean change in resting tension (gf) ± (SD). n=6

	NaHS [mM]				**EC_{50} (mM)**	**pEC_{50}**
	0.01	**0.1**	**0.3**	**1**		
Change in Resting Tension (gf)	-0.07 (0.14)	-0.09 (0.18)	-0.06 (0.04)	-0.42 (0.27)	0.40 (0.23)	3.61 (0.69)

Hypoxia also caused a significant relaxation of all bronchi from resting tension ($p < .01$ via paired 2 tail t-test, n=6). The maximum relaxation to hypoxia was compared to the maximum relaxation to 1 mM NaHS in a further four paired bronchi (mean internal diameter 4.5 ± 1.3 mm from 3 patients). The magnitude of relaxation to hypoxia in all of the bronchi (n=10) was found to be significantly greater than the magnitude of relaxation to NaHS (**table 10.6**).

Table 10.6 Magnitude of relaxation to hypoxia and NaHS from resting tension as change in resting tension (gf) or % change and absolute baseline tension after maximum relaxation to hypoxia and NaHS. Mean ± (SD). n=10. $^{\#}p$ = NS, $*p < .05$ hypoxia vs. NaHS via paired 2 tail t-test

	Change in Tension (gf)	**% Change in Tension**	**Baseline (gf) post maximum relaxation**
Hypoxia	-0.88 (0.65)*	-40 (20)*	1.18 (0.38)$^{\#}$
NaHS	-0.44 (0.36)	-26 (14)	1.17 (0.37)

However, the absolute baseline tension after maximum relaxation to hypoxia and NaHS was not significantly different (**figure 10.6c, table 10.6**) which suggests a possible causal relationship between relaxation to hypoxia and NaHS. The difference in magnitude could be explained because the baseline resting tension was significantly greater before relaxation to hypoxia than NaHS (2.06 ± 0.58 gf and 1.60 ± 0.51 gf pre hypoxia and NaHS respectively. $P = .04$ via 2 tail t-test. n=10) and would have resulted in a larger magnitude of relaxation to reach the same maximum relaxation.

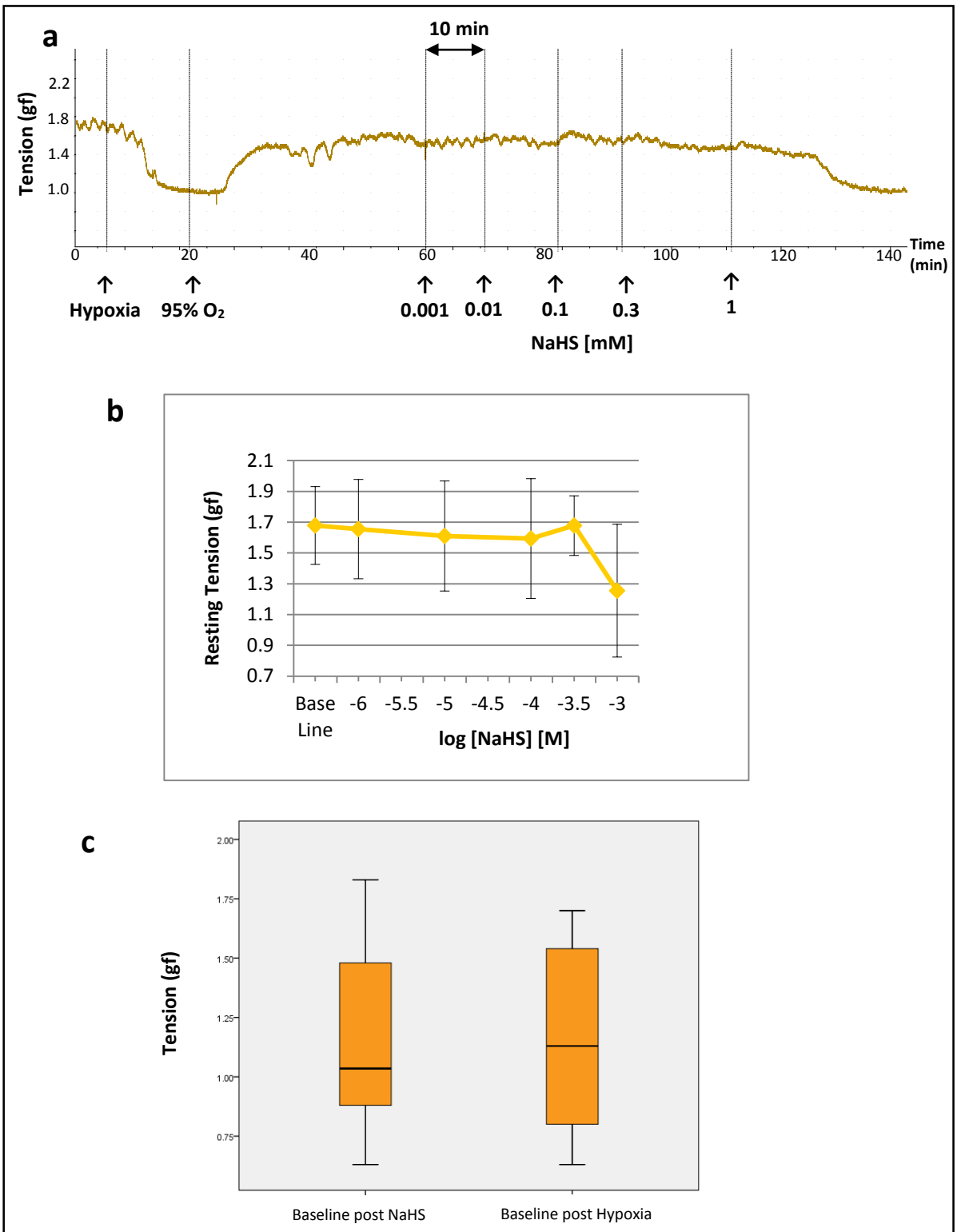

Figure 10.6 Effect of NaHS and hypoxia on resting tension in isolated human bronchi (**a**) Experimental trace showing dose dependent relaxation of a human bronchus to NaHS and hypoxia from resting tension. Note similarity in final baseline (**b**) log dose response curve to NaHS from resting tension. Absolute baseline tension (gf) shown. n=6. (**c**) Box-whisker plot of absolute baseline tension post maximum relaxation to NaHS and hypoxia in the same bronchi. n=10.

10.3.3.2 Effect of Hypoxia and NaHS on Active Tension

NaHS caused a dose dependent (0.1-1 mM) relaxation of all bronchi (n=7 from 3 patients, mean internal diameter 3.6 ± 1.0 mm) from active tension to 1 mM MCh, EC_{50} 0.57 ± 0.11 mM, pEC_{50} 3.25 ± 0.09 (estimated manually from individual concentration response curves) which was statistically significant at 1 mM NaHS (**table 10.7, figure 10.7 a,b**, raw data Appendix II).

Table 10.7 Dose dependent effect of NaHS on active tension in human bronchi. Change in resting tension (gf) and % change in active tension. Mean ± (SD). n=7, *p < .01 via paired 2 tail t-test, n=7

	NaHS [mM]				EC_{50} (mM)	pEC_{50}
	0.01	**0.1**	**0.3**	**1**		
Change in Active Tension (gf)	0.07 (0.08)	0.01 (0.20)	-0.66 (0.82)	-3.41 (0.94)*	0.57 (0.11)	3.25 (0.09)
% Change in Active Tension	2 (3)	1 (6)	-16 (18)	-114 (21)		

Hypoxia also caused a relaxation of all bronchi from active tension to MCh. In a further seven paired bronchi (mean internal diameter 4.4 ± 1.6 mm from 4 patients) the maximum relaxation to hypoxia was compared to the maximum relaxation to 1 mM NaHS. The magnitude of relaxation to hypoxia and NaHS (% reversal of active tension) and the absolute baseline post relaxation to NaHS and hypoxia were not significantly different (n=14) (**table 10.8, figure 10.7c**) which suggests a possible causal relationship between relaxation to hypoxia and NaHS.

Table 10.8 Magnitude of relaxation to hypoxia and NaHS from active tension as change in active tension (gf) or % change in active tension and absolute baseline tension after maximum relaxation to hypoxia and NaHS. Mean ± (SD). n=14. $^{\#}p$ = NS, *p < .05 hypoxia vs. NaHS via paired 2 tail t-test

	Change in Tension (gf)	**% Change in Tension**	**Baseline (gf) post maximum relaxation**
Hypoxia	-4.07 (1.68)*	-102 (18)	1.56 (0.39)$^{\#}$
NaHS	-2.92 (1.30)	-120 (43)	1.45 (0.37)

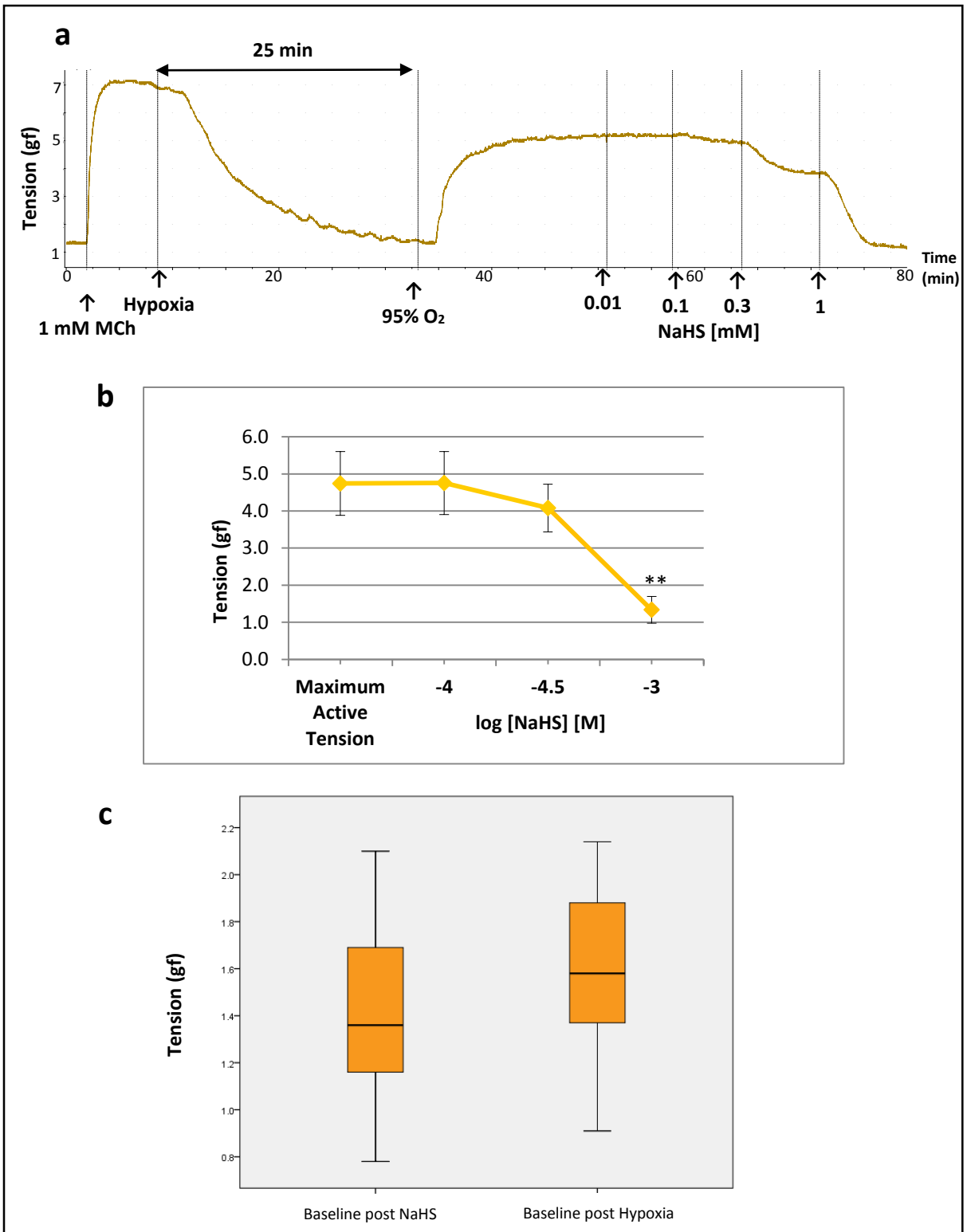

Figure 10.7 Effect of NaHS and hypoxia on active tension in isolated human bronchi (**a**) Experimental trace showing dose dependent relaxation of a human bronchus to NaHS and hypoxia from active tension. Note similarity in final baseline (**b**) log dose response curve to NaHS from active tension to MCh. Absolute baseline tension (gf) shown. $^{**}p < .01$ vs. maximum active tension, n=7. (**c**) Box-whisker plot of absolute baseline tension post maximum relaxation to NaHS and hypoxia in the same bronchi. n=14.

10.3.3.3 Role of The Potassium Channels in the Mechanism of NaHS relaxation

Two bronchi were obtained from one patient (control preparation internal diameter 5 mm, experimental preparation internal diameter 3 mm). In this experiment the dose dependent relaxation of the bronchi after pre-contraction to MCh and KCL was compared. It was found that pre-contraction to KCl did not attenuate the relaxant effect of NaHS compared to relaxation from cholinergic contraction to the same extent as previously reported in porcine bronchi [290] (**figure 10.8**). However, the maximum relaxation to NaHS in the KCl contracted bronchus did not achieve the same maximum relaxation as the control response to hypoxia compared to the control preparation contracted to MCh where the maximum relaxation to NaHS was similar in magnitude to the control relaxation to hypoxia (which is consistent with previous results in this study). Clearly it is not possible to draw a conclusion from a single experiment but this finding does suggest that hypoxia and NaHS could be acting on distinct pathways and further studies are needed to investigate the mechanism of relaxation to NaHS.

The effect of the non-selective K^+ channel blocker Tetraethylammonium (TEA) was investigated on the relaxation to NaHS in two bronchi from one patient (both bronchi 3 mm internal diameter). At the end of an experiment in which the bronchi were exposed to TEA (to determine the effect of TEA on HBD) NaHS (1 mM) caused a robust relaxation in both bronchi (which were partially relaxed to isoprenaline) and the magnitude of relaxation was similar to the magnitude of HBD in both bronchi (**figure 10.9**). Although pharmacologically not good practice this result does suggest that the potassium channels are not involved in the mechanism of relaxation to H_2S which is consistent with the previous finding in porcine bronchi [290].

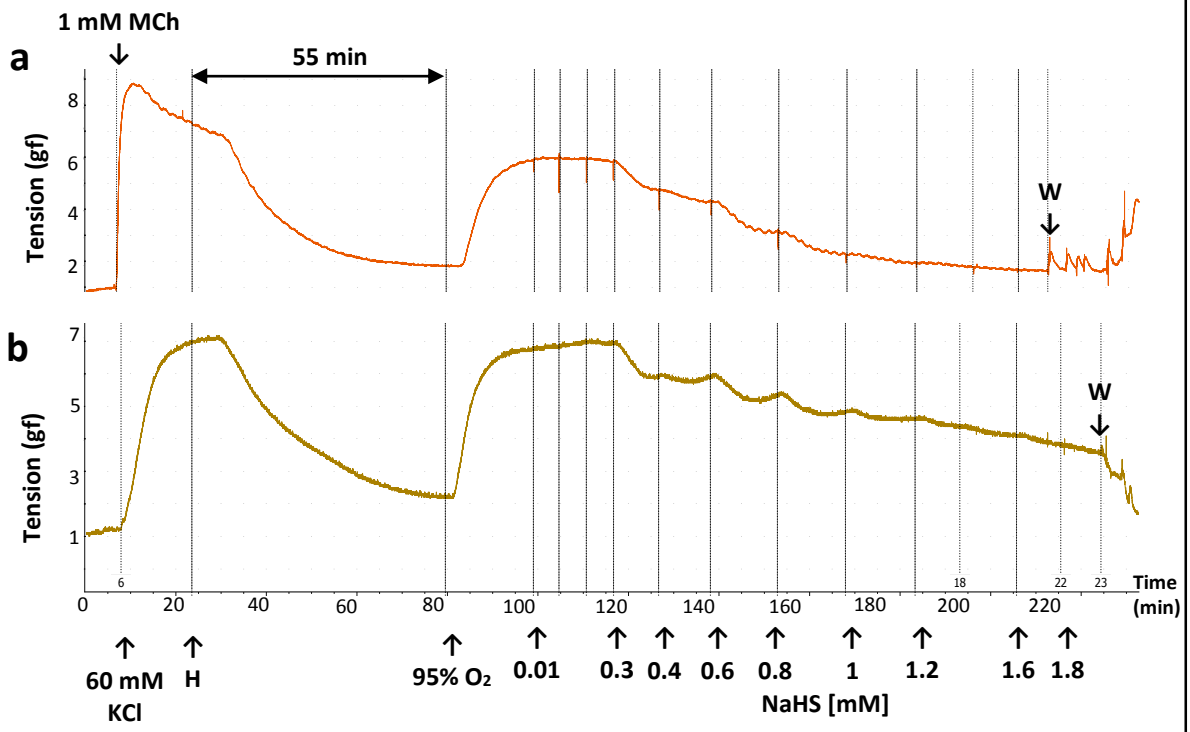

Figure 10.8 Relaxation to hypoxia and dose dependent relaxation to NaHS post (**a**) contraction to MCh and (**b**) after contraction to KCl. H: hypoxia; W: wash.

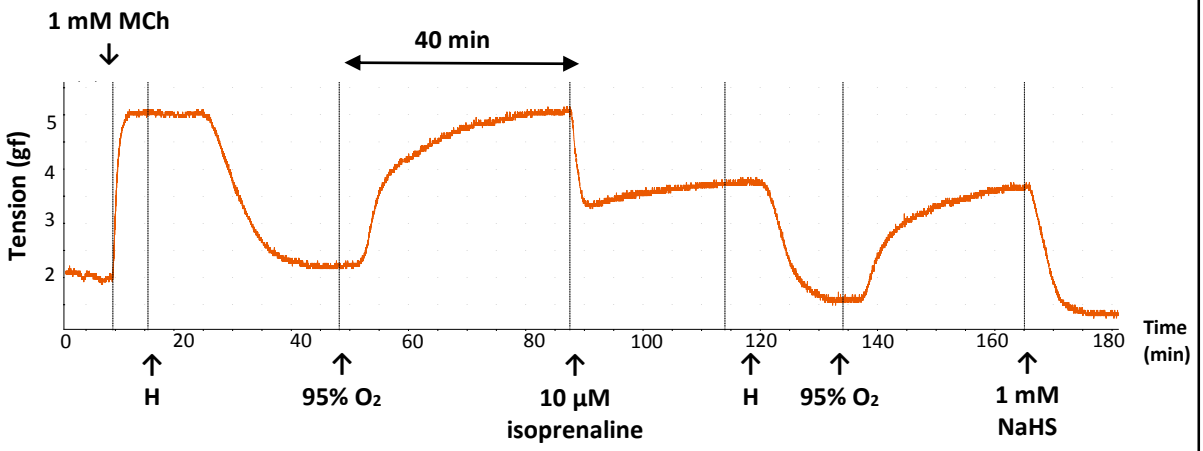

Figure 10.9 Experimental trace showing the effect of NaHS on active tension in a bronchus exposed to TEA (1 mM) and partially relaxed to isoprenaline. Note similarity in the magnitude of relaxation to NaHS and hypoxia. H: hypoxia

10.3.3.4 Competitive Effects of Hypoxia and NaHS

The effect of hypoxia on resting tone after maximum relaxation to NaHS from resting tension and active tension was investigated in this study. In two bronchi maximally relaxed to NaHS from resting tension and in three bronchi maximally relaxed to NaHS from active tension exposure to hypoxia did not cause a further relaxation (**figure 10.10a**). These findings are consistent with previous results observed in vascular preparations [287] which showed that the effects of hypoxia and H_2S are competitive and suggests a common mechanism in the response to hypoxia and H_2S. However, in this study an anomalous effect of hypoxia was observed; in one bronchus after maximum relaxation to NaHS from active tension hypoxia caused a further relaxation and reoxygenation caused a reversal of the relaxation to NaHS (**figure 10.10b**). This finding is inconsistent with the effects of H_2S and hypoxia being competitive and is difficult to interpret. It could be that the effects of NaHS were diminishing over time (due to diffusion from the buffer solution) which allowed a HBD to occur and a re-contraction to active tension on reoxygenation. Further studies are needed to determine the effect of NaHS over time.

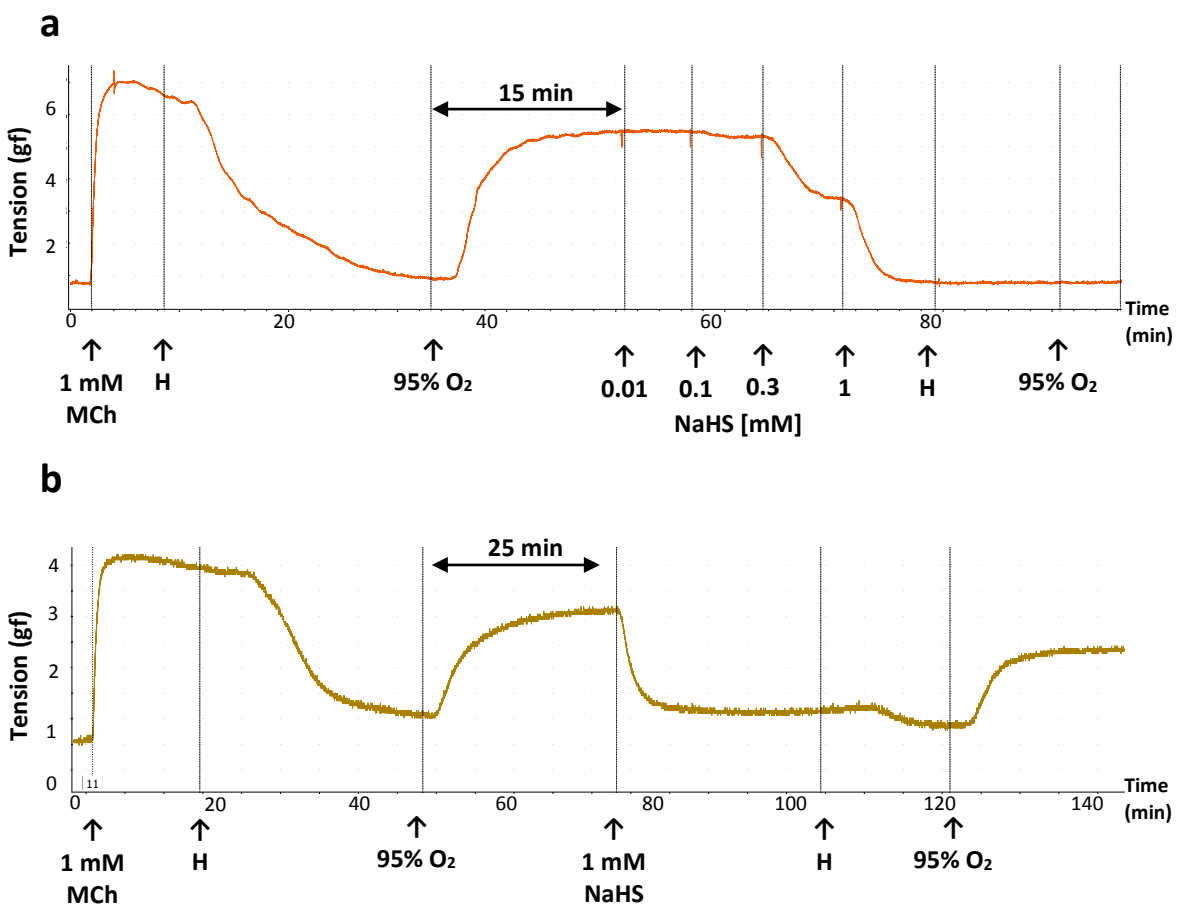

Figure 10.10 Competitive effects of hypoxia and H_2S (**a**) Experimental trace showing effect of hypoxia on resting tension after maximum relaxation to NaHS from active tension. In most bronchi hypoxia did not cause a further relaxation (**b**) In one bronchus hypoxia did cause a further relaxation after maximum relaxation to NaHS from active tension and reoxygenation caused a return to active tension. H: hypoxia

10.3.3.5 Effect of H_2S Enzyme Synthesis Inhibitors on HBD

At 30 mM PPG (n=2, 5 and 9 mm internal diameter, from two patients) caused a sustained contraction from resting tension (35 ± 10% of resting tension, p = .07 via 2 tail paired t-test) which suggests that H_2S could have a relaxant role in the maintenance of basal tone in human bronchi and furthermore suggests that CSE is functionally active in human bronchi. In contrast and at variance with this finding was that HA (0.3 mM) (n=4 from 2 patients, mean internal diameter 9 ± 3.4 mm) caused a

robust relaxation from resting tension (-11 ± 9% of resting tension, p = .05 via 2 tail paired t-test).

The effects of PPG and HA on the rate and magnitude of HBD from resting and active tension are shown in **table 10.9** as % change from control response obtained before exposure to the enzyme inhibitors (raw data Appendix II). PPG had no effect on the rate or magnitude of HBD from resting or active tension. In contrast, HA was found to significantly (p < .05 via 2 tail paired t-test) attenuate the rate and magnitude of HBD from resting tension. However, this finding was likely to be a false result because the magnitude of relaxation to HA from resting tone would have resulted in a maximum relaxation of the bronchi rendering further relaxation to hypoxia unlikely. This suggestion is further supported by the finding that HA had no effect on the rate or magnitude of HBD from active tension. These results suggest that an increase in H_2S synthesis does not mediate the effects of hypoxia on the human bronchi.

Table 10.9 Effect of H_2S synthesis inhibitors on rate and magnitude of HBD from resting and active tension. Data are expressed as % change from control response. Mean ± (SD). *p < .05 via 2 tail paired t-test

	% of Control Response			
	Resting Tension		**Active Tension**	
Enzyme inhibitor [mM]	**Rate**	**Magnitude**	**Rate**	**Magnitude**
PPG [30]	-10 (42)	-6 (22)	13 (19)	18 (1)
HA [0.3]	-66 (14)*	-50 (30)*	6 (24)	13 (19)

10.3.3.6 Effect of DPI on relaxation to NaHS

In single bronchial ring (internal diameter 3 mm, 5 mm wide) the effect of DPI (100 µM) on relaxation to hypoxia and NaHS from active tension to KCl (60 mM) was investigated. It was found that a 30 minute incubation with DPI (100 µM) significantly attenuated contraction to KCl (-85%), abolished relaxation to hypoxia from resting and active tension and abolished relaxation to NaHS but not to isoprenaline (**figure 10.11**). This result suggests that there might be a common mechanism in the relaxation of human bronchi to hypoxia and H_2S and further studies are warranted to investigate the effects of DPI on NaHS mediated relaxation of the bronchi.

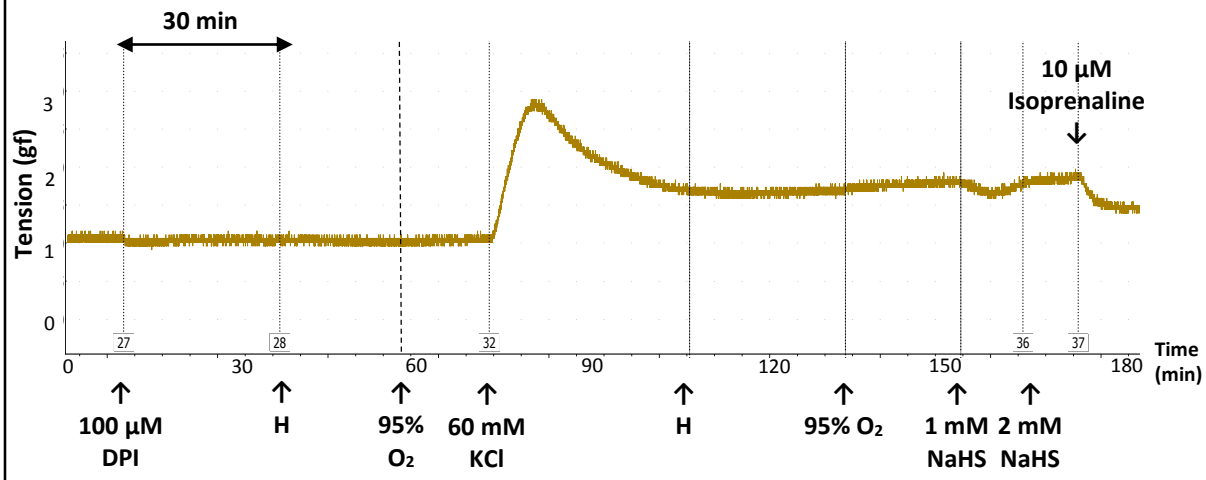

Figure 10.11 Experimental trace showing the effect of DPI (100 µM) on HBD and relaxation to NaHS. H: hypoxia

10.4 Discussion

In this study it has been shown for the first time that H_2S causes a robust, dose dependent and reversible relaxation of the human pulmonary vasculature and airways. Previous studies have suggested a role for H_2S as an oxygen sensor in the lung [130, 287, 291]. In the current study the effects of H_2S were compared to the effects of hypoxia in human pulmonary vascular and airway preparations with a view to investigating the putative oxygen sensing role of H_2S in hypoxia. The previous studies by Olson *et al.* [130, 287, 291] which have proposed a role for H_2S as an oxygen sensor were based on a number of findings in animal vascular studies; (1) H_2S and hypoxia uniquely evoke the same response in blood vessels (contraction or relaxation) (2) H_2S is enzymatically generated in vessels (3) Inhibition of H_2S synthesis inhibits the effects of hypoxia (4) tissue H_2S concentration is inversely related to O_2 concentration (5) precursors of H_2S synthesis (L-cysteine) augment the effects of hypoxia (6) the effects of H_2S and hypoxia are competitive.

In the current study it was found that the absolute baseline tension in pulmonary vessels and bronchi after maximum relaxation to NaHS and hypoxia was very similar in the majority of experiments which is consistent with Olson and co-workers findings and suggested a causal relationship in the effects of hypoxia and H_2S and therefore that H_2S could act as an oxygen sensor in the human lung. In this study it was also found that DPI completely abolished relaxation to hypoxia and H_2S but not relaxation to isoprenaline in isolated human bronchi which further supports the view that the effects of H_2S and hypoxia are one and the same. However, there were a number of inconsistencies in the results from this study which do not support this view. In the current study there was significant variability in the responses of the human

pulmonary arteries and veins to NaHS and hypoxia; in some arteries and veins hypoxia caused a relaxation whereas NaHS did not and in some vessels NaHS caused a relaxation and hypoxia had no effect. In one pulmonary artery NaHS was found to cause a dose dependent contraction from maximum active tension; however, this vessel showed a robust relaxation to hypoxia from active tension. This finding is not consistent with Olson's study in large pulmonary arteries from sea lions [130] where it was found that vessels which were relaxed by hypoxia were also relaxed by H_2S and vessels that were contracted by hypoxia also contracted to H_2S and suggest that H_2S is not acting as an O_2 sensor in the human pulmonary vasculature. In the current study the effects of hypoxia and H_2S on the airways was more consistent than in the vasculature. In all isolated human bronchi hypoxia and H_2S caused a robust and reversible relaxation and the magnitude of relaxation to H_2S and hypoxia was very similar which suggested an oxygen sensing role for H_2S in the airways. Previous studies have shown that H_2S synthesis inhibitors attenuate the effects of hypoxia on blood vessels [287] and isolated lungs [291] which was taken as evidence that H_2S acts as an O_2 sensor in the vasculature. However, in this study it was found that the H_2S enzyme synthesis inhibitors PPG and HA had no effect on the rate or magnitude of HBD in human bronchi and suggests that an increase in H_2S synthesis does not mediate the effects of hypoxia in the human bronchi. Furthermore, previous studies in vascular preparations [287] found that the effects of hypoxia and H_2S were competitive which suggested a common mechanism in the response to hypoxia and H_2S. However, in the current study hypoxia was found to cause a further relaxation in one bronchus after maximum relaxation to NaHS from active tension suggesting that the effects of hypoxia and H_2S are not mutually inhibitive in the human airways. Taken together the

results from these studies suggest that H_2S does not act as an O_2 sensor in the human lung. The similarities in the magnitude of relaxation to hypoxia and H_2S in both the pulmonary vessels and bronchi suggest that both effects could be mediated by the same pathway. A recent study in rat pulmonary arteries [292] showed that H_2S mimics the effects of hypoxia in the pulmonary vasculature by inhibiting the mitochondrial electron transport chain (ETC) (to the same extent as hypoxia). However, results from studies in ***Chapter 9*** suggested that HBD is not mediated via a mitochondrial/ROS mechanism and it would therefore seem unlikely that H_2S mimics the effects of hypoxia (in human bronchi at least) via an inhibition of the mitochondrial ETC. In ***Chapter 9*** it was suggested that DPI could inhibit HBD by inhibiting calcium desensitisation and the finding in this study that DPI abolished HBD and relaxation to H_2S could therefore be taken to indicate that relaxation of the bronchi to H_2S is also mediated by a desensitisation mechanism and further studies will be needed to investigate this mechanism.

In this study it was thought that if H_2S did mimic the effects of hypoxia then exposure of the pulmonary veins to NaHS would precipitate phasic activity as described in ***Chapter 3*** **(*3.3.2.3*)** which would confirm a role for H_2S in hypoxic signalling. However, in this study phasic activity did not occur in any of the vessels from one patient, possibly because the samples did not include myocytes (being distal to the pulmonary sleeves) and only transiently in the veins from the other patient and therefore this hypothesis could not be tested in the current study.

There were also a number of differences in the effect of H_2S on human bronchi described in this study compared to previous studies in isolated animal airway

preparations. In mouse and guinea-pig bronchial rings NaHS was found to have no effect on resting tone but caused a robust and dose dependent relaxation of mouse bronchial rings after contraction to carbachol but only a small relaxation in pre-contracted guinea-pig bronchial rings [289]. In the human bronchi (this study) NaHS was found to cause a robust relaxation from both resting and active tension. A recent study in porcine bronchi [290] found that NaHS had no effect on basal tone and caused a robust but transient bronchorelaxation from active tension to carbachol. In the porcine bronchi relaxation to NaHS was found to be significantly reduced in preparations precontracted to KCl and inhibited by the non-selective potassium channel blocker TEA. In contrast this study has shown that NaHS causes a sustained relaxation of human bronchi from resting and active tension which is not inhibited by TEA and is only marginally attenuated in bronchi pre-contracted to KCl. The differences in these studies suggest that significant species differences exist in the response of the airways to H_2S and emphasises the need to use human tissue in future studies to investigate the therapeutic potential of H_2S.

In this study the effects of H_2S were compared to the effects of hypoxia and the role of H_2S as an oxygen sensor investigated. However, a causal association between the effects of hypoxia and H_2S was not established.

Irrespective of the putative role of H_2S as an O_2 sensor, in this study it has been shown for the first time that H_2S is an effective pulmonary vasodilator and bronchodilator in the human lung with equal efficacy to the robust effects of hypoxia. The therapeutic potential of H_2S in treating pulmonary vascular disease, such as pulmonary

hypertension and constrictive airway diseases such as COPD and asthma is in need of further investigation.

CHAPTER 11

General Discussion

The lungs are exquisitely adapted to respond to changes in oxygen concentration and hypoxic pulmonary vasoconstriction (HPV), which diverts blood away from areas of low oxygenation to areas of higher oxygenation, is now established as an important homeostatic mechanism in optimising perfusion-ventilation matching.

In this thesis the effects of hypoxia on pulmonary vascular tone were investigated in isolated human pulmonary arteries and veins and for the first time in isolated perfused and ventilated human lungs. It was found, surprisingly and at variance with a large number of previous animal studies (reviewed by Sylvester *et al.* [7]), that hypoxia caused a relaxation of the human pulmonary vasculature. It is difficult to account for this tendentious finding. It is possible that species differences exist and that hypoxia causes a relaxation of the human pulmonary vasculature. However, this would seem unlikely given the compelling evidence from previous *in vivo* studies which have convincingly shown that hypoxia causes pulmonary vasoconstriction in man [43-57]. One possible explanation for the discrepancy between the *in vitro* results in this thesis and *in vivo* results is that there is a significant neurogenic component to the hypoxic response in human lungs which is missing *in vitro.* Previous studies [170, 171] have identified baroreceptors at the bifurcation of the main pulmonary artery which exert a pulmo-pulmonary reflex regulation of pulmonary vascular tone and Chen *et al.* have recently shown in dogs [168] that radiofrequency ablation of the pulmonary artery bifurcation completely abolished PAH induced by balloon occlusion of the left pulmonary inter lobar artery and clinically [169] that pulmonary artery denervation caused a significant

reduction in mean PAP and had significant clinical benefits in IPAH patients. Further studies are warranted to investigate the possible neural component in the hypoxic pulmonary vascular response in man which could prove to be a significant therapeutic target for the treatment of hypoxia induced pulmonary hypertension.

In investigating the effects of hypoxia on the pulmonary vasculature and airways in the *ex vivo* human lung it was found that hypoxia attenuated the rate of oedema formation. It was suggested that hypoxia might cause a relaxation of pulmonary vascular endothelial cells which in turn improves endothelial barrier function (by closing intercellular adherens and tight junctions) and reduces oedemagenesis. Further studies have been planned, using cultured human pulmonary endothelial cells, to investigate the effects of hypoxia on pulmonary endothelial cell contractility and barrier function. The finding that hypoxia reduced oedema formation was consistent with previous studies in rats [159] and humans [160, 161] which showed that hypoxia increased fluid clearance in the lung and suggests a therapeutic potential for hypoxic ventilation during cardiac surgery and for patients on extracorporeal membrane oxygenation therapy (ECMO). Patients undergoing cardiac surgery with cardiopulmonary bypass (CPB) sometimes develop pulmonary oedema – so called "pump lung" [1]. Since patients are systemically oxygenated during CPB it may be possible to prevent oedemagenesis during CPB by ventilating the lungs with a hypoxic gas mixture. Similarly for patients on ECMO for respiratory failure periods of hypoxic ventilation could potentially attenuate pulmonary oedema. Further studies are warranted to investigate the therapeutic potential of hypoxic ventilation in the critical care setting (ECMO) and for patients undergoing cardiac surgery with cardio pulmonary bypass. Another potential application for the oedema reducing effects of

hypoxia is in *ex vivo* lung perfusion (EVLP) for clinical lung transplantation. EVLP is increasingly being used for the preservation, optimisation and reconditioning of donor lungs before transplantation [178, 179]. However, a significant limitation of EVLP is pulmonary oedema [181] and it is possible that periods of hypoxic perfusion and ventilation could help to reduce oedemagenesis and improve lung function during EVLP of donor lungs. Further studies are warranted to investigate the potential benefits of hypoxic ventilation and/or perfusion in reducing oedema and preserving lung function during EVLP of donor lungs in clinical lung transplantation.

In studies investigating the effects of hypoxia on the pulmonary veins an interesting and serendipitous discovery was that hypoxia and reoxygenation precipitated pulsatile activity in the vessels. It is thought that this activity represents an intrinsic automaticity in the veins and is arrhythmogenic in nature. Future studies are planned to further investigate this possibility and it is hoped that *in vitro* human pulmonary veins will prove to be a simple and effective model for investigating arrhythmic activity in the pulmonary veins and could facilitate the future development of new and effective treatments for atrial fibrillation.

In Chapter 5 of this thesis the effects of ET-1 on the human pulmonary vasculature were investigated in isolated human pulmonary arteries and veins and in *ex vivo* human lungs with a view to identifying the role of ET-1 in mediating the effects of hypoxia. In this study it was found that that the effects of ET-1 were the opposite to the effects of hypoxia. In isolated pulmonary arteries and veins ET-1 caused a dose dependent constriction whereas hypoxia dilated the vessels and reversed the effect of ET-1 and in isolated lungs hypoxia attenuated oedema formation whereas ET-1 caused

an increase in pulmonary vascular permeability suggesting that ET-1 does not have a role in the effects of hypoxia on the human pulmonary vasculature. However there were a number of novel and significant findings to arise from the studies in this chapter. It was shown for the first time that ET-1 causes an increase in vascular permeability in the human lung and further studies are therefore warranted to investigate the mechanisms of ET-1 induced increases in pulmonary vascular permeability which could lead to the development of effective treatments for permeability oedema. It was also found that ET-1 is a more potent and effective vasoconstrictor in human pulmonary veins than in human pulmonary arteries. The fact that the pulmonary veins are more vasoactive than the pulmonary arteries suggests that the pulmonary veins could play a more significant role in the pathogenesis of pulmonary hypertension than was previously believed. Furthermore, previous animal studies [212, 219] have shown that contraction of the pulmonary veins is predominantly mediated by activation of the ET_B receptor. These findings could have significant clinical implications for the selection of appropriate endothelin antagonists, and in the future development of therapeutics, for the treatment of pulmonary hypertension. If it is the case that the vasopressor effect of ET-1 in the lung is predominantly due to a venoconstriction, mediated by activation of the ET_B receptors, then selective ET_A receptor antagonists could increase the risk of oedema formation by causing a preferential dilation of the pulmonary arterial system without reducing pulmonary venous resistance. The use of dual ET_A/ET_B or even selective ET_B receptor antagonists would therefore be indicated. Further studies are currently underway to characterise the ET receptors responsible for the vasoconstrictor effects of ET-1 isolated human pulmonary arteries and veins. It is hoped that the results from these studies will

provide rationale for a more informed selection of pharmacotherapeutics in the treatment of pulmonary hypertension and facilitate the development of more effective treatments for pulmonary vascular disease in the future.

In this thesis it has been shown for the first time in *ex vivo* human lungs and isolated human bronchi that hypoxia causes a robust and reversible relaxation of human airways. It was also found, in isolated human bronchi, that changing oxygen tensions caused a rapid, robust and reversible, concentration-dependent change in bronchial tone. This finding challenges the currently accepted belief, that the local effects of oxygen on bronchial tone are merely a vestigial response and have no physiological role, and suggests that changes in airway tone in response to changing oxygen concentrations could play an important homeostatic role in perfusion-ventilation matching by equalising the distribution of oxygen in the airways and alveoli. The finding that hyperoxia causes a robust bronchoconstriction has significant implications for the use of oxygen therapy; for example, in constrictive airway diseases, such as COPD and asthma, an acute bronchoconstriction on the initiation of oxygen therapy could have serious clinical consequences and is in need of further investigation.

In isolated human bronchi it was found that the maximum magnitude of hypoxic bronchodilation (HBD) was greater than the maximum magnitude of relaxation to the full β-adrenoceptor agonist isoprenaline. An understanding of the mechanism of HBD therefore represented a significant target for the development of novel and effective bronchodilator therapies and further studies were undertaken to determine the mechanism of HBD. In Chapter 7 the role of the epithelium and a number of known intracellular signalling pathways in the mechanism of HBD was investigated and it was

found that the epithelium, nitric oxide, carbon monoxide and the β-adrenergic system are not involved in the mechanism of HBD. However, in this study a role for the cGMP and cAMP signalling pathways could not be ruled out. In chapter 8 the role of the potassium channels and intra and extracellular calcium in the mechanism of HBD was investigated. In this study it was shown that the potassium channels are not involved in the mechanism of HBD and that changes in intracellular or extracellular calcium do not affect HBD. However, the finding in this study that relaxation of the bronchi was significantly prolonged after contraction to KCl compared to MCh suggested that HBD could be mediated by a mechanism involving desensitisation of the contractile apparatus which was further supported by findings in Chapter 9.

The ability of different cells in the body to sense and respond to changes in oxygen concentrations is an essential homeostatic mechanism to match oxygen supply with demand. The mechanisms mediating oxygen sensing and signalling has been the subject of intense investigation and a number of different hypothesis have been proposed which have mostly suggested a central role for an effect on mitochondrial oxidative phosphorylation which is signalled by a change in the intracellular ATP/ADP ratio or by ROS. However, the exact mechanisms mediating oxygen sensing remain to be resolved and are the subject of much debate (reviewed by Ward [58] and Evans *et al*. [59]). The putative role of the mitochondria as oxygen sensors and the signalling role of ATP and reactive oxygen species in the effects of hypoxia in the bronchi were investigated in Chapter 9. It was found that ATP, the mitochondria and ROS are probably not involved in oxygen sensing or signalling in the human bronchi. However, a significant finding in this study was that diphenyleneiodonium (DPI) completely abolished HBD from resting tension and active tension to KCl via a mechanism not

involving inhibition of NADPH oxidase or complex I of the electron transport chain (which DPI has previously been shown to inhibit). It is suggested that the fact that DPI completely abolished contraction to MCh and attenuated contraction to KCl, and abolished HBD from active tension to KCl, adds further evidence to support the hypothesis that HBD is mediated by an effect on the calcium sensitisation mechanism. This suggestion is further supported by studies which have shown that hypoxic pulmonary vasoconstriction is mediated by activation of the calcium sensitisation mechanism [60, 61] and it would seem reasonable to suggest that a relaxant response to hypoxia could also be mediated via the same pathway. If this is indeed the case then the mechanism of HBD would not be subject to the limitations of desensitisation which occurs with the chronic use of GPCR agonists and is an attractive therapeutic target for the development of novel bronchodilator therapies.

The hypothesis that hypoxia could be causing relaxation of the airway smooth muscle by inhibiting the cellular contractile apparatus and/or the calcium sensitisation mechanism adds further support for the suggestion that hypoxia could be attenuating oedemagenesis by inhibiting the contractile response in pulmonary vascular endothelial cells. The contractile status of vascular endothelial cells, which is determined by the level of MLC phosphorylation [176, 277] and possibly calcium sensitisation [278, 279], has a central role in determining endothelial barrier function. If hypoxia does indeed inhibit the fundamental contractile mechanism and/or the calcium sensitisation mechanism then it would seem reasonable to suggest that hypoxia could cause relaxation of pulmonary vascular ECs which augments endothelial barrier function and attenuates oedemagenesis. Further studies are planned to

investigate the effect of hypoxia on the contractile status and barrier function of human pulmonary vascular endothelial cells at a molecular and functional level.

A number of previous studies by Ken Olson's group from Indiana have provided compelling evidence that H_2S could act as an oxygen sensor [130, 287, 291] and in this thesis the putative role of H_2S as an O_2 sensor was investigated in the human pulmonary vasculature and bronchi. The results from this study concluded that H_2S does not act as an O_2 sensor in the human lung. However, the finding in this study that the absolute magnitude of effects mediated by H_2S and hypoxia was very similar and that in the human bronchi relaxation to H_2S was completely abolished by DPI suggests that the effects of H_2S and hypoxia could be mediated via the same mechanism. Furthermore, in this study it was shown that H_2S is a very effective vasodilator and bronchodilator and could therefore have significant therapeutic potential in the treatment of human pulmonary disease such as pulmonary hypertension and constrictive airway diseases such as COPD and asthma.

Studies in this thesis used a number of *ex vivo* human lung tissue models to investigate the effects of hypoxia (and changing oxygen concentrations) on the human pulmonary vasculature and airways. It is recognised that there are a number of significant limitations in the use of human tissue preparations which should be considered when interpreting the results from studies in this thesis. In general there is an intrinsic inter-individual variability in the responses of human tissue preparations (largely due to the innate genetic diversity of the population from which samples are obtained) which is often apparent in the variability of results obtained. A number of other factors could also influence the responses of human lung tissue preparations, for example; age of

the donor (the age range of patients was 40-80 years), sex of the donor (there is growing evidence that there are significant differences in the responses of tissue preparations from males and females [293, 294]), smoking history, asthma status, medication, existing comorbidities and the perioperative anaesthetic regime used. A significant failure in the *in vitro* human pulmonary vessel and human bronchi studies in this thesis was that demographic and clinical data about the donor patients was not collected. In the experimental design of most *in vitro* experiments performed in this thesis standard control responses to contractile and relaxant agonists were obtained before starting experimental protocols. If donor patient data had been collected it would have been possible to explore the effects of different demographic and clinical parameters on baseline responses. For example, in human bronchi it would have been interesting to determine if sex, age, smoking history, asthma status and β-agonist therapy (duration and dose) had an effect on cholinergic contraction and relaxation to isoprenaline. Similarly, in pulmonary vessels it would have been interesting to determine if age, sex, systemic hypertension or antihypertensive medications had an effect on pulmonary vascular responses. It is also possible that various factors could have affected experimental results which should have been considered in the interpretation of the results. In future experiments demographic and clinical data about the donor patients should be collected to enable a comparison of baseline responses between different patient cohorts.

In addition to an inherent variability in physiological and pharmacological responses a specific limitation of the isolated human lung model used for studies in this thesis was the presence of a tumour which was of unknown size and type. It is possible that the tumour which was present in all of the *ex vivo* lung preparations used could have

released pharmacologically active agents and cytokines which altered vascular and airway responses. Further studies are needed to determine if different types of tumour do release biologically active agents which could affect the results obtained from isolated perfused lung studies which use lung specimens resected for cancer.

Conversely, it could be argued that the limitations of the human lung models used in this thesis represent significant advantages over animal models. Tissue for animal experiments is generally obtained from young, healthy, single sex animals with a pure genetic lineage which have been raised in a clean and controlled environment and as such results obtained from animal studies are unlikely to be truly representative of effects in a diverse human population. Furthermore, it is now recognised that significant species differences exist in the physiological and pharmacological responses of the pulmonary vasculature and airways which emphasises the need for studies in human tissue preparations in the development of effective human therapeutics.

A number of further studies using human tissue preparations have been planned to build on the studies described in this thesis;

- To further investigate the nature of the phasic activity observed in the human pulmonary veins with a view to developing a model for investigating arrhythmic activity and the effects of antiarrhythmic agents in human pulmonary veins.
- To further investigate the relative importance of the pulmonary veins in the regulation of human pulmonary vascular resistance.
- To characterise the endothelin receptor mediating contraction of human pulmonary veins.

- To investigate the effect of hypoxia on the contractile status and barrier function of human pulmonary vascular endothelial cells at a molecular and functional level.
- To explore the clinical potential of pulmonary hypoxia in attenuating oedemagenesis during cardiac surgery and ECMO therapy with cardiopulmonary bypass and during *ex vivo* lung perfusion for donor organ optimisation in clinical lung transplantation.
- To investigate the therapeutic potential of H_2S in the treatment of human pulmonary disease.
- To determine the mechanism of hypoxic bronchodilation which would represent a significant therapeutic target in the development of novel and effective bronchodilators which are not subject to the limitations of desensitisation.

Appendix I: Drugs Used

Drug stock solutions: DMSO; Dimethyl sulfoxide.

Drug	Pharmacological Action	Supplier	Molar Mass (g/mol)	Mass (mg)	Solvent	Solvent Volume (ml)	Stock solution [mM]
Acetylcholine chloride (ACh)	Cholinergic agonist	Sigma-Aldrich	181.66	120	water	6.61	100
Acetyl-β-methylcholine chloride (Methacholine) (MCh)	Cholinergic agonist	Sigma-Aldrich	195.69	100	water	5.11	100
Adenosine 5'-triphosphate disodium salt (ATP)	P2 purinoceptor agonist.	Tocris	551.14	50	water	0.91	100
Adrenaline	α and β adrenoceptor agonist	Hamein Pharma. Ltd.	183.2	1	water	1	5.49
Amiodarone hydrochloride	Broad-spectrum ion channel blocker; blocks late I_{Na}, I_{Ca}, I_{Kr}, I_{Ks} + β blocker	OI Sciences Ltd	681.77	150	Benzyl Alcohol	3	73.34

Drug stock solutions continued.

Drug	Pharmacological Action	Supplier	Molar Mass (g/mol)	Mass (mg)	Solvent	Solvent Volume (ml)	Stock solution [mM]
4-Aminopyridine (4-AP)	Non-selective voltage-dependent K+ channel blocker	Tocris	94.12	100	water	10.62	100
Apamin	Selective inhibitor of small- conductance Ca^{2+}-activated K^+-channels	Tocris	2027.34	1	10% DMSO	4.93	0.1
2-APB	IP_3 receptor antagonist, TRP channel modulator	Tocris	225.1	10	DMSO	0.6	75
Carbonyl cyanide 4-(triflouromethoxy)phenylhydrazone (FCCP)	Uncouples oxidative phosphorylation in mitochondria	Tocris	255.97	10	DMSO	3.91	10

Drug stock solutions continued.

Drug	Pharmacological Action	Supplier	Molar Mass (g/mol)	Mass (mg)	Solvent	Solvent Volume (ml)	Stock solution [mM]
Diphenyleneiodonium chloride (DPI)	NADH reductase and oxidase inhibitor = ETC complex I inhibitor. Also inhibits K^+, Ca^{2+} channels?	Tocris	314.55	10	DMSO	3.18	10
DL-Propargylglycine	Selective inhibitor of cystathione γ-lyase (CSE)-responsible for H_2S production	Sigma-Aldrich	113.11	250	water	4.42	500
Endothelin-1(ET-1) (human)	$ET_{A/B}$ agonist	American Peptide Company	2492.2	3	water	12.04	0.1

Drug stock solutions continued.

Drug	Pharmacological Action	Supplier	Molar Mass (g/mol)	Mass (mg)	Solvent	Solvent Volume (ml)	Stock solution [mM]
Glibenclamide	ATP-dependent K^+ Channel and CFTR Cl^- channel blocker	Tocris	494	100	DMSO	4.05	50
HC-030031	Selective TRPA1 antagonist	Gift from Dr LR Sadofsky			DMSO		10
Heparin	Antithrombin III activation/Anticoagulant	CP Pharmaceuticals			water		5000 iu/ml
Hydrogen peroxide	ROS	Sigma-Aldrich	34.02	3% w/v	water	56.68µl in 5ml	10

Drug stock solutions continued.

Drug	Pharmacological Action	Supplier	Molar Mass (g/mol)	Mass (mg)	Solvent	Solvent Volume (ml)	Stock solution [mM]
Hydroxylamine hydrochloride	Non selective inhibitor of cystathione γ-lyase (CSE) and cystathione β-synthase (CBS) via inhibition of pyridoxyl-5'-phosphate	Sigma-Aldrich	69.49	50	water	7.20	100
Iberiotoxin	Selective blocker of high conductance $Ca2^{+}$ - activated K^{+} channels	Tocris	4230	0.1	10% DMSO	0.95	0.03
Indomethacin	Cyclooxygenase (COX) inhibitor	Tocris	362.29	100	ethanol	5.52	50
Isoprenaline (isoproterenol) hydrochloride	Selective β-adrenoreceptor agonist	Tocris	247.72	100	water	4.04	100

Drug stock solutions continued.

Drug	Pharmacological Action	Supplier	Molar Mass (g/mol)	Mass (mg)	Solvent	Solvent Volume (ml)	Stock solution [mM]
KT 5720	Selective inhibitor of Protein Kinase A	Tocris	537.61	0.1	DMSO	1.86	0.1
KT 5823	Selective inhibitor of Protein Kinase G	Tocris	495.53	0.1	DMSO	2.02	0.1
Labetalol hydrochloride	Non selective adrenergic antagonist	UCB Pharmaceuticals	364.87	100	water	20.00	14
L-NAME	NO synthase inhibitor	Tocris	269.69	100	water	3.71	100
LY 83583	Generates superoxide	Sigma-Aldrich	250.25	5	ethanol	2.00	1
Methylthioninium chloride (Methylene Blue)	Guanylate cyclase inhibitor	Martindale Pharmaceuticals	319.85	1% w/v	water		31

Drug stock solutions continued.

Drug	Pharmacological Action	Supplier	Molar Mass (g/mol)	Mass (mg)	Solvent	Solvent Volume (ml)	Stock solution [mM]
Nifedipine	L-type calcium channel blocker	Tocris	346.34	100	DMSO	2.89	100
Noradrenaline	adrenoreceptor agonist	Aguettant Ltd	169.18	1	water	1.00	6
Ouabain	Na^+/K^+ ATPase inhibitor	Tocris	719.77	100	water	13.89	10
Papaverine	antispasmodic, vasodilator, non-selective phosphodiesterase inhibitor	Huddersfield Royal Infirmary P.M.U.	339.39	40	water	1.00	118

Drug stock solutions continued.

Drug	Pharmacological Action	Supplier	Molar Mass (g/mol)	Mass (mg)	Solvent	Solvent Volume (ml)	Stock solution [mM]
Potassium Chloride (KCl)	Membrane depolarisation	BDH	74.55	29820	water	200.00	2000
Pyrrolidinedithiocarbamate ammonium (PDTC)	Enhances clearance (augments catalytic degradation) of H_2O_2 + Inhibitor of nuclear Factor -kB	Tocris	164.3	50	DMSO	3.04	100
Rotenone	Mitochondrial ETC inhibitor (complex I)	Tocris	394.42	50	DMSO	1.27	100

Drug stock solutions continued.

Drug	**Pharmacological Action**	**Supplier**	**Molar Mass (g/mol)**	**Mass (mg)**	**Solvent**	**Solvent Volume (ml)**	**Stock solution [mM]**
Rp-8-Bromoguanosine-3',5'-cyclic monophosphorothioate sodium salt (Rp-8-Br-cGMPs)	Competitive cGMP analogue	Santa Cruz Biotechnology	462.2	1	water	2.16	1
Ryanodine	Inhibits Ca^{2+} release from sarcoplasmic reticulum	Tocris	493.55	1	ethanol	2.03	1
Sitaxsentan sodium	Selective ET_A receptor antagonist	Pfizer	476.89	100	water	20.97	10
Sodium hydrosulphide hydrate	H_2S donor	Sigma-Aldrich	56.06	100	water	5.95	300
Sodium Nitroprusside (SNP)	Nitric oxide donor	Mayne Pharma Plc	261.92	50	water	7.64	25

Drug stock solutions continued.

Drug	Pharmacological Action	Supplier	Molar Mass (g/mol)	Mass (mg)	Solvent	Solvent Volume (ml)	Stock solution [mM]
Tetraethylammonium (TEA)	Non- selective Kv channel antagonist	Tocris	165.2	50	water	3.03	100
Tempol (1-Oxyl-2,2,6,6-tetramethyl-4-hydroxypiperidine)	Superoxide scavenger	Tocris	172.24	100	DMSO	5.81	100
Thapsigargin	inhibitor of sarco-endoplasmic reticulum Ca^{2+} -ATPase	Tocris	650.76	1	DMSO	1.54	1
Tin protoporphyrin IX dichloride (Sn-PP)	Haem oxygenase inhibitor	Tocris	750.25	10	DMSO	2.67	5
Tram-34	Selective $Kca_{3.1}$ Channel antagonist	Donation from the University of Leicester	344.84	15.5	DMSO	4.50	10

Drug stock solutions continued.

Drug	Pharmacological Action	Supplier	Molar Mass (g/mol)	Mass (mg)	Solvent	Solvent Volume (ml)	Stock solution [mM]
Triphenylphosphine oxide (TPPO)	Putative TRPM5 receptor antagonist	Sigma-Aldrich	278.28	100	ethanol	3.59	100
Y-27632	Inhibits Rho-associated protein kinase p160ROCK +PKC, cAMP dependent PC, MLCK	Tocris	329.27	1	water	3.04	1

Appendix II: Raw Data

Chapter 3: Effects of Hypoxia on the Human Pulmonary Vasculature: *In Vitro* Human Pulmonary Arteries and Veins

3.3.1.1 Raw data: Relaxation of isolated human pulmonary arteries to 1 µM Acetylcholine

	Resting Tension (gf)		Δ Resting Tension (gf)	Δ Resting Tension (%)
Prep	**Pre ACh**	**Post ACh**		
PA9.1	2.02	1.79	-0.23	-11.39
PA9.2	1.44	1.04	-0.40	-27.78
PA9.3	1.53	1.28	-0.25	-16.34
PA9.4	1.52	1.13	-0.39	-25.66
PA11.1	1.14	0.95	-0.19	-16.67
PA13.2	2.03	1.88	-0.15	-7.39
PA13.4	2.00	1.77	-0.23	-11.50
Mean	**1.67**	**1.41**	**-0.26**	**-16.67**
SD	**0.35**	**0.40**	**0.10**	**7.58**
SEM	**0.13**	**0.15**	**0.04**	**2.87**

3.3.1.1 Raw data: Contraction of isolated human pulmonary arteries to 1 µM Acetylcholine

	Resting Tension (gf)		Δ Resting Tension (gf)	Δ Resting Tension (%)
Prep	**Pre ACh**	**Post ACh**		
PA10.1	1.71	2.1	0.39	22.81
PA10.2	1.98	2.23	0.25	12.63
PA10.3	1.45	1.67	0.22	15.17
PA10.4	1.46	1.76	0.30	20.55
PA12.2	1.57	1.63	0.06	3.82
Mean	**1.63**	**1.88**	**0.24**	**15.00**
SD	**0.22**	**0.27**	**0.12**	**7.46**
SEM	**0.10**	**0.12**	**0.05**	**3.33**

3.3.1.1. Raw data: Effect of hypoxia on resting tension in isolated human pulmonary Arteries								
	Resting Tension (gf)			Δ Resting Tension (gf)		Δ Resting Tension (%)		Time to relaxation (s)
Prep	Pre-Hypoxia	Hypoxia	Re-oxygenation	Hypoxia	Re-oxygenation	Hypoxia	Re-oxygenation	
PA3.1	1.30	1.16	1.11	-0.14	-0.19	-10.77	-14.62	1320
PA9.1	2.23	1.58	1.54	-0.65	-0.69	-29.15	-30.94	6180
PA9.2	1.27	0.67	0.60	-0.60	-0.67	-47.24	-52.76	5280
PA9.3	1.25	0.71	0.65	-0.54	-0.60	-43.20	-48.00	5160
PA9.4	1.20	0.75	0.70	-0.45	-0.50	-37.50	-41.67	6000
Mean	**1.45**	**0.97**	**0.92**	**-0.48**	**-0.53**	**-33.57**	**-37.60**	**4788**
SD	**0.44**	**0.39**	**0.40**	**0.20**	**0.20**	**14.44**	**15.22**	**1988**
SEM	**0.22**	**0.20**	**0.20**	**0.10**	**0.10**	**7.22**	**7.61**	**994**
***p* vs. pre-hypoxia via Wilcoxon signed-rank test**		**0.04**	**0.04**					

3.3.1.2. Raw data: Effect of hypoxia on active tension to noradrenaline in isolated human pulmonary arteries												
	Isometric Tension (gf)				Active Tension (gf)			Δ Active Tension (gf)		Δ Active Tension (%)		
Prep	Baseline	Post Contraction	Hypoxia	Re-oxygenation	Pre-Hypoxia	Hypoxia	Re-oxygenation	Hypoxia	Re-oxygenation	Hypoxia	Re-oxygenation	Time to relaxation (s)
PA3.1	1.10	1.34	1.20	1.16	0.24	0.10	0.06	-0.14	-0.18	-58.33	-75.00	600
PA9.1	1.52	2.21	1.97	1.83	0.69	0.45	0.31	-0.24	-0.38	-34.78	-55.07	1620
PA9.2	0.60	0.96	0.91	0.87	0.36	0.31	0.27	-0.05	-0.09	-13.89	-25.00	1320
PA9.3	0.64	1.06	0.97	0.96	0.42	0.33	0.32	-0.09	-0.10	-21.43	-23.81	1440
PA9.4	0.70	1.16	0.94	0.87	0.46	0.24	0.17	-0.22	-0.29	-47.83	-63.04	1380
PA10.1	1.75	2.25	1.79	1.70	0.5	0.04	-0.05	-0.46	-0.55	-92.00	-110.00	1140
PA10.2	1.74	2.65	2.12	1.50	0.91	0.38	-0.24	-0.53	-1.15	-58.24	-126.37	540
PA10.3	1.39	1.79	1.46	1.30	0.40	0.07	-0.09	-0.33	-0.49	-82.50	-122.50	1320
PA10.4	1.34	1.83	1.56	1.48	0.49	0.22	0.14	-0.27	-0.35	-55.10	-71.43	960
Mean	**1.20**	**1.69**	**1.44**	**1.30**	**0.50**	**0.24**	**0.10**	**-0.26**	**-0.40**	**-51.57**	**-74.69**	**1147**
SD	**0.46**	**0.60**	**0.46**	**0.36**	**0.20**	**0.14**	**0.19**	**0.16**	**0.32**	**25.78**	**38.39**	**375**
SEM	**0.15**	**0.20**	**0.15**	**0.12**	**0.07**	**0.05**	**0.06**	**0.05**	**0.11**	**8.59**	**12.80**	**125**
***P* vs. Pre-hypoxia via paired samples, 2-tail, t- test**			**0.001**	**0.006**		**0.001**	**0.006**					

3.3.1.2. Raw data: Effect of hypoxia on active tension to ET-1 in isolated human pulmonary arteries												
	Isometric Tension (gf)				Active Tension (gf)			Δ Active Tension (gf)		Δ Active Tension (%)		
Prep	**Baseline**	**Post Contraction**	**Hypoxia**	**Re-oxygenation**	**Pre-Hypoxia**	**Hypoxia**	**Re-oxygenation**	**Hypoxia**	**Re-oxygenation**	**Hypoxia**	**Re-oxygenation**	**Time to relaxation (s)**
PA11.1	0.95	1.43	1.18	1.14	0.48	0.23	0.19	-0.25	-0.04	-52.08	-8.33	720
PA11.2	1.11	1.56	1.33	1.28	0.45	0.22	0.17	-0.23	-0.05	-51.11	-11.11	420
PA12.1	1.36	1.49	1.44	1.39	0.13	0.08	0.03	-0.05	-0.05	-38.46	-38.46	900
PA12.2	1.46	1.66	1.61	1.62	0.20	0.15	0.16	-0.05	0.01	-25.00	5.00	960
PA12.3	1.42	1.58	1.54	1.48	0.16	0.12	0.06	-0.04	-0.06	-25.00	-37.50	1320
PA13.1	1.06	1.94	1.03	1.07	0.88	-0.03	0.01	-0.91	0.04	-103.41	4.55	2100
PA13.2	2.51	2.79	2.12	2.00	0.28	-0.39	-0.51	-0.67	-0.12	-239.29	-42.86	3360
PA13.3	1.62	1.89	1.43	1.37	0.27	-0.19	-0.25	-0.46	-0.06	-170.37	-22.22	3120
PA13.4	2.18	2.27	1.65	1.54	0.09	-0.53	-0.64	-0.62	-0.11	-688.89	-122.22	3540
Mean	**1.52**	**1.85**	**1.48**	**1.43**	**0.33**	**-0.04**	**-0.09**	**-0.36**	**-0.05**	**-154.85**	**-30.35**	**1827**
SD	**0.52**	**0.44**	**0.31**	**0.28**	**0.25**	**0.27**	**0.31**	**0.32**	**0.05**	**213.25**	**38.89**	**1230**
SEM	**0.17**	**0.15**	**0.10**	**0.09**	**0.08**	**0.09**	**0.10**	**0.11**	**0.02**	**71.08**	**12.96**	**410**
***p* vs. pre-hypoxia via Wilcoxon signed- rank test**			**0.008**	**0.008**		**0.008**	**0.008**					

3.3.2.1. Raw data: Effect of hypoxia on resting tension in isolated human pulmonary veins								
	Resting Tension (gf)			Δ Resting Tension (gf)		Δ Resting Tension (%)		
Prep	Pre-Hypoxia	Hypoxia	Reoxygenation	Hypoxia	Reoxygenation	Hypoxia	Reoxygenation	Time to relaxation (s)
PV1.2	0.88	0.79	0.79	-0.09	-0.09	-10.23	-10.23	660
PV1.3	0.58	0.63	0.69	0.05	0.11	8.62	18.97	840
PV1.4	0.43	0.42	0.46	-0.01	0.03	-2.33	6.98	780
PV2.2	1.19	1.21	1.25	0.02	0.06	1.68	5.04	2640
PV2.3	0.48	0.49	0.56	0.01	0.08	2.08	16.67	
PV2.4	0.89	0.61	0.72	-0.28	-0.17	-31.46	-19.10	2700
PV3.2	1.93	1.85	1.86	-0.08	-0.07	-4.15	-3.63	900
PV3.3	1.43	1.45	1.47	0.02	0.04	1.40	2.80	
PV4.1	2.41	1.52	2.67	-0.89	0.26	-36.93	10.79	1440
PV4.2	1.46	0.86	1.40	-0.60	-0.06	-41.10	-4.11	1440
PV4.3	1.40	1.26	1.39	-0.14	-0.01	-10.00	-0.71	1680
PV5.1	2.33	2.1	2.37	-0.23	0.04	-9.87	1.72	1800
PV5.2	2.54	2.19	3.14	-0.35	0.60	-13.78	23.62	1200
PV6.1	1.78	1.35	1.67	-0.43	-0.11	-24.16	-6.18	1620
PV6.2	1.40	1.21	1.40	-0.19	0.00	-13.57	0.00	180
PV6.3	1.48	1.36	1.42	-0.12	-0.06	-8.11	-4.05	2340
Mean	**1.41**	**1.21**	**1.45**	**-0.21**	**0.04**	**-11.99**	**2.41**	**1444**
SD	**0.67**	**0.55**	**0.76**	**0.26**	**0.18**	**14.54**	**11.13**	**754**
SEM	**0.17**	**0.14**	**0.19**	**0.06**	**0.05**	**3.64**	**2.78**	**189**
***P* vs. Pre-hypoxia via Wilcoxon signed-rank test**		**0.005**	**0.80**					

3.3.2.2. Raw data: Effect of hypoxia on active tension in isolated human pulmonary veins contracted to ET-1												
	Isometric Tension (gf)				Active Tension (gf)			Δ Active Tension (gf)		Δ Active Tension (%)		Time to relaxation (s)
Prep	Baseline	Post Contraction	Hypoxia	Re-oxygenation	Pre-Hypoxia	Hypoxia	Re-oxygenation	Hypoxia	Re-oxygenation	Hypoxia	Re-oxygenation	
PV2.2	1.06	2.34	1.40	1.53	1.28	0.34	0.47	-0.94	-0.81	-73.44	-63.28	2400
PV2.3	0.49	0.82	0.65	0.65	0.33	0.16	0.16	-0.17	-0.17	-51.52	-51.52	2280
PV2.4	0.53	1.44	0.84	1.07	0.91	0.31	0.54	-0.60	-0.37	-65.93	-40.66	1920
PV3.2	1.61	3.54	2.90	2.81	1.93	1.29	1.20	-0.64	-0.73	-33.16	-37.82	2460
PV3.3	1.47	1.74	1.62	1.64	0.27	0.15	0.17	-0.12	-0.10	-44.44	-37.04	1980
PV6.1	1.66	4.03	2.16	2.17	2.37	0.50	0.51	-1.87	-1.86	-78.90	-78.48	3180
PV6.2	1.39	3.95	2.62	2.82	2.56	1.23	1.43	-1.33	-1.13	-51.95	-44.14	2700
PV6.3	1.41	1.75	1.61	1.63	0.34	0.20	0.22	-0.14	-0.12	-41.18	-35.29	1740
Mean	**1.20**	**2.45**	**1.73**	**1.79**	**1.25**	**0.52**	**0.59**	**-0.73**	**-0.66**	**-55.07**	**-48.53**	**2333**
SD	**0.46**	**1.23**	**0.80**	**0.77**	**0.94**	**0.47**	**0.48**	**0.63**	**0.61**	**16.18**	**15.24**	**466**
SEM	**0.16**	**0.39**	**0.25**	**0.24**	**0.30**	**0.15**	**0.15**	**0.20**	**0.19**	**5.12**	**4.82**	**147**
***p* vs. pre-hypoxia via Wilcoxon signed- rank test**			**0.005**	**0.005**		**0.01**	**0.01**					

4.3.1 Raw data for *ex vivo* human lungs: Effect of hypoxia on mean perfusion pressure (PP)									
Time:	**Baseline**	**10 min post hypoxia**		**20 min post hypoxia**		**50 min post hypoxia**		**Room air**	
Prep	**Mean PP (mmHg)**	**Mean PP (mmHg)**	**Change in mean PP (mmHg)**	**Mean PP (mmHg)**	**Change in mean PP (mmHg)**	**Mean PP (mmHg)**	**Change in mean PP (mmHg)**	**Mean PP Room Air (mmHg)**	**Change in mean PP (mmHg)**
Hull 15	18.09	18.85	0.76	18.72	0.63			17.40	-0.69
Hull 15 (h)	13.82	12.90	-0.93	13.16	-0.66	12.60	-1.22		
Hull 17 (h)	17.95	19.05	1.11					20.37	2.43
Hull 21	14.46	12.84	-1.62	13.07	-1.39	13.56	-0.90	16.44	1.98
Hull 25	14.10	14.00	-0.10	13.77	-0.33	13.61	-0.50	13.74	-0.36
Hull 30	34.08	33.76	-0.32	33.61	-0.47	33.60	-0.48	33.76	-0.33
Hull 31	5.96	4.80	-1.16	4.73	-1.23			5.47	-0.48
Hull 32	26.80	23.52	-3.27	21.91	-4.89	20.49	-6.31		
Hull 33	14.49	13.54	-0.95	12.86	-1.63				
Hull 34	16.02	15.58	-0.44	14.87	-1.15	14.13	-1.89	13.33	-2.69
Mean	**17.58**	**16.88**	**-0.69**	**16.30**	**-1.24**	**18.00**	**-1.88**	**17.22**	**-0.02**
SD	**7.75**	**7.72**	**1.24**	**8.00**	**1.53**	**8.16**	**2.23**	**8.65**	**1.73**
SEM	**2.45**	**2.44**	**0.39**	**2.67**	**0.51**	**3.33**	**0.91**	**3.27**	**0.65**
n	**10**	**10**	**10**	**9**	**9**	**6**	**6**	**7**	**7**
Paired t-test vs. baseline		**0.11**		**0.04**		**0.09**		**0.98**	
Wilcoxon signed-rank test vs. pre hypoxia		**0.09**		**0.02**		**0.03**		**0.61**	

4.3.2 Raw data for ex-vivo human lungs: Rate of weight gain (% of initial Wt. min^{-1}) pre hypoxia, during hypoxia and on reoxygenation										
		Pre Hypoxia			Hypoxia			Reoxygenation		
Prep	Initial weight (g)	Weight (g) -30 min	Weight (g) Pre hypoxia	Rate of weight gain (%/min) Pre Hypoxia	Hypoxic Time (min)	Weight (g) post hypoxia	Rate of weight gain (%/min) Hypoxia	Reoxygenation Time (min)	Weight (g) post reoxygenation	Rate of weight gain (%/min) Reoxygenation
Hull 15	218	212	294	1.25	35	376	1.07	30	411	0.54
Hull 15 (h)	398	413	462	0.41	178	612	0.21	12	618	0.13
Hull 17 (h)	364	364	408	0.40	17	427	0.31	15	481	0.99
Hull 21	418	744	737	-0.06	62	759	0.08	24	769	0.10
Hull 25	187	187	209	0.39	76	250	0.29	14	259	0.34
Hull 28	272	278	396	1.45	87	513	0.49	15	513	0.00
Hull 30	271	299	347	0.59	91	369	0.09	13	373	0.11
Hull 31	500	529	548	0.13	30	567	0.13	15	586	0.25
Hull 32	345	415	434	0.18	77	418	-0.06			
Hull 33	260	358	373	0.19	48	386	0.10			
Hull 34	455	572	764	1.41	56	774	0.04	15	774	0.00
Hull 41	306	452	713	2.84	60	876	0.89			
Mean	**333**	**402**	**474**	**0.77**	**68**	**527**	**0.30**	**17**	**532**	**0.27**
SD	**97**	**159**	**180**	**0.83**	**41**	**194**	**0.35**	**6**	**174**	**0.32**
SEM	**28**	**46**	**52**	**0.24**	**12**	**56**	**0.10**	**2**	**50**	**0.09**
Paired t-test vs. Pre hypoxia							**0.03**			**0.13**
Paired t-test vs. Hypoxia										**0.82**
Wilcoxon signed-rank test vs. pre hypoxia							**0.01**			**0.17**
Wilcoxon signed-rank test vs. Hypoxia										**0.95**

5.3.1.2 Raw data: Patient and specimen data with experimental parameters for *ex vivo* human lung experiments to determine the effect of ET-1 on capillary filtration coefficient. Tv; tidal volume, BPM; breaths per minute.

	Patient		Specimen		Experimental Parameters				
Prep	**Sex**	**age (years)**	**Lobe**	**Weight (g)**	**Perfusate Flow (ml/min)**	**Tv (ml)**	**BPM**	**Weight (g) T 20min**	**Rate of Weight Gain (%/min)**
ET9	M	81	Right Upper Lobe	275	200	200	10	310	0.64
ET10	F	74	Left Lower Lobe	218	200	100	10	253	0.80
ET12	M	62	Left Upper Lobe	315	100	200	10	433	1.87
ET13	M	75	Right Upper Lobe	411	100	100	10	535	1.51
ET14	M	69	Right Lower Lobe	282	200	200	10	513	4.10
ET15	F	73	Right Lung	717	200	300	10	943	1.58
ET16	M	71	Right Lower Lobe	296	200	100	10	334	0.64
ET17	F	79	Right Upper Lobe	-	100	100	10	-	PAP >50mmHg
ET18	F	57	Right Lower Lobe	266	100	100	10	288	0.41
ET19	F	28	Left Lower Lobe	180	100	100	10	263	2.31
ET20	M	62	Left Lung	1060	350	200	10	1228	0.79
Mean		**66**		**402**	**168**	**155**	**10**	**510**	**1**
SD		**15**		**276**	**78**	**69**	**0**	**326**	**1**
SEM		**4**		**83**	**24**	**21**	**0**	**98**	**0**

5.3.1.2.2 Raw data: Pulmonary vascular permeability Pre ET-1. .Ppc = capillary filtration pressure; Pa = pulmonary artery pressure; Pv = pulmonary venous pressure; Kf,c = capillary filtration coefficient (%/min/mmHg).

Pre ET-1

	Pre challenge				Post challenge				Ppc Pre challenge			Ppc Post challenge				
Prep	**Wt (g)**	**Wt. -5min (g)**	**Wt. pre challenge (g)**	**Wt. gain (%/min)**	**Wt. +5min (g)**	**Wt. +10min (g)**	**Wt. gain (%/min)**	**Rate of Wt. gain Post-Pre (%/min)**	**Pa (mmHg)**	**Pv (mmHg)**	**Ppc (mmHg)**	**Pa (mmHg)**	**Pv (mmHg)**	**Ppc (mmHg)**	**Δ Ppc (mmHg)**	**Kfc Pre ET-1**
ET9	275	595	604	0.65	608	623	1.09	0.44	17.74	13.2	15.47	17.19	21.53	19.36	3.89	0.11
ET12	315	637	627	-0.63	675	685	0.63	1.27	35.93	-25.31	5.31	35.99	3.11	19.55	14.24	0.09
ET13	411	715	725	0.49	756	760	0.19	-0.29	24.15	6.66	15.41	28.02	14.33	21.18	5.77	-0.05
ET16	296	392	401	0.61	459	513	3.65	3.04	21.98	-17.01	2.49	28.37	5.71	17.04	14.56	0.21
ET18	266	316	332	1.20	373	389	1.20	0.00	12.27	2.1	7.19	14.32	8.76	11.54	4.36	0.00
ET20	1060	1161	1172	0.21	1203	1206	0.06	-0.15	9.78	3	6.39	9.24	6.67	7.96	1.57	-0.10
Mean	**437**	**636**	**644**	**0.42**	**679**	**696**	**1.14**	**0.72**	**20.31**	**-2.89**	**8.71**	**22.19**	**10.02**	**16.10**	**7.40**	**0.04**
SD	**310**	**298**	**298**	**0.61**	**292**	**282**	**1.31**	**1.27**	**9.42**	**14.91**	**5.45**	**10.17**	**6.79**	**5.22**	**5.59**	**0.11**
SEM	**126**	**122**	**122**	**0.25**	**119**	**115**	**0.54**	**0.52**	**3.85**	**6.09**	**2.23**	**4.15**	**2.77**	**2.13**	**2.28**	**0.05**

5.3.1.2.2: Raw data: Effect of hypoxia on pulmonary vascular permeability, Post ET-1. Ppc = capillary filtration pressure; Pa = pulmonary artery pressure; Pv = pulmonary venous pressure; Kf,c = capillary filtration coefficient

Post ET-1

		Pre ET-1			Post ET-1				Ppc Pre ET-1			Ppc Post ET-1				
Prep	**Weight (g)**	**Wt. - 5min (g)**	**Wt. pre ET-1 (g)**	**Wt. gain (%/min)**	**Wt +5min post ET-1 (g)**	**Wt. + 10min (g)**	**Wt gain post ET-1 (%/min)**	**Rate of Wt. gain Post-Pre (%/min)**	**Pa (mmHg)**	**Pv (mmHg)**	**Ppc (mmHg)**	**Pa (mmHg)**	**Pv (mmHg)**	**Ppc (mmHg)**	**Δ Ppc (mmHg)**	**Kfc Post ET-1**
ET9	275	797	810	0.95	826	848	1.60	0.65	25.27	1.79	13.53	27.78	25.35	26.57	13.04	0.05
ET12	315	627	637	0.63	710	739	1.84	1.21	20.88	-6.1	7.39	23.82	0.32	12.07	4.68	0.26
ET13	411	772	779	0.34	785	779	-0.29	-0.63	31.83	17.72	24.78	31.10	17.32	24.21	-0.56	1.12
ET16	296	704	752	3.24	783	866	5.61	2.36	28.01	6.4	17.21	29.62	4.49	17.06	-0.15	-15.8
ET18	266	440	459	1.43	506	551	3.38	1.95	13.84	7.9	10.87	18.39	13.42	15.91	5.04	0.39
ET20	1060	1205	1201	-0.08	1209	1211	0.04	0.11	9.31	4.64	6.98	10.81	8.60	9.71	2.73	0.04
Mean	**437**	**758**	**773**	**1.09**	**803**	**832**	**2.03**	**0.94**	**21.52**	**5.39**	**13.46**	**23.59**	**11.58**	**17.59**	**4.13**	**-2.32**
SD	**310**	**254**	**246**	**1.17**	**229**	**217**	**2.20**	**1.13**	**8.61**	**7.81**	**6.75**	**7.76**	**9.08**	**6.64**	**4.95**	**6.60**
SEM	**126**	**104**	**100**	**0.48**	**94**	**89**	**0.90**	**0.46**	**3.52**	**3.19**	**2.75**	**3.17**	**3.71**	**2.71**	**2.02**	**2.69**

5.3.1.2.1, 5.3.1.2.2 Raw data: Pre and post ET-1; Rate of weight gain and Kfc excluding anomalous results

Prep	Rate of Wt gain pre ET-1 (%/min)	Rate of Wt. gain post ET-1 (%/min)	Kfc (%/min/mmHg) Pre ET-1	Kfc (%/min/mmHg) Post ET-1
ET9	0.95	1.60	0.11	0.05
ET12	0.63	1.84	0.09	0.26
ET13	0.34	-0.29	-	-
ET16	3.24	5.61	-	-
ET18	1.43	3.38	0.00	0.39
ET20	-0.08	0.04	-0.10	0.04
Mean	1.09	2.03	0.03	0.18
SD	1.17	2.20	0.10	0.17
SEM	0.48	0.90	0.05	0.08
Statistics; *p* values				
paired t-test vs. pre ET-1		0.10		0.18

5.3.2 Raw data: Dose dependent effect of ET-1 on isometric tension in isolated human pulmonary arteries and veins.

	Isometric tension (gf): Veins										
	Base	[ET-1 M]									
Prep	line	1E-12	3E-12	1E-11	3E-11	1E-10	3E-10	1E-09	3E-09	1E-08	3E-08
PV2.2	1.06					1.21	1.68	2.04	2.34		
PV2.3	0.49					0.57	0.71	0.83	0.82		
PV2.4	0.53					0.71	1.18	1.34	1.44		
PV3.1	0.87	0.88	0.88	0.88	0.9	0.98	1.05	1.33	1.44	1.5	1.47
PV3.2	1.61	1.61	1.58	1.59	1.64	1.91	2.28	2.86	3.27	3.55	3.53
PV3.3	1.47	1.46	1.48	1.47	1.53	1.55	1.57	1.67	1.72	1.76	1.74
PV6.1	1.66			1.63	1.7	1.97	2.19	3.2	3.69	4.06	3.99
PV6.2	1.39			1.47	1.48	1.94	2.22	3.43	3.77	3.99	3.89
PV6.3	1.41			1.43	1.45	1.59	1.68	1.79	1.81	1.78	
Mean	**1.17**	**1.32**	**1.31**	**1.41**	**1.45**	**1.38**	**1.62**	**2.05**	**2.26**	**2.77**	**2.92**
SD	**0.45**	**0.39**	**0.38**	**0.27**	**0.29**	**0.54**	**0.56**	**0.91**	**1.08**	**1.21**	**1.22**
SEM	**0.15**	**0.22**	**0.22**	**0.11**	**0.12**	**0.18**	**0.19**	**0.30**	**0.36**	**0.50**	**0.55**
	Isometric tension (gf): Arteries										
	Base	[ET-1] [M]									
Prep	line	1E-12	3E-12	1E-11	3E-11	1E-10	3E-10	1E-09	3E-09	1E-08	3E-08
PA11.1	0.95	0.95	0.93	0.89	0.86	0.91	0.96	1.43	1.64	1.42	
PA11.2	1.11	1.11	1.08	1.05	1.02	1.01	1.03	1.32	1.53	1.56	
PA12.1	1.36			1.36	1.35	1.37	1.37	1.4	1.43	1.48	1.49
PA12.2	1.46			1.48	1.49	1.51	1.52	1.58	1.6	1.63	1.66
PA12.3	1.42			1.4	1.4	1.39	1.4	1.43	1.49	1.6	1.58
PA13.1	1.06			1.04	1.01	0.99	0.98	1.17	1.7	2.06	1.92
PA13.2	2.51			2.41	2.45	2.32	2.3	2.46	2.45	2.71	2.81
PA13.3	1.62			1.6	1.57	1.54	1.52	1.64	1.92	1.96	
PA13.4	2.18			2.26	2.22	2.1	2.11	2.35	2.34	2.29	
Mean	**1.52**	**1.03**	**1.01**	**1.50**	**1.49**	**1.46**	**1.47**	**1.64**	**1.79**	**1.86**	**1.89**
SD	**0.52**	**0.11**	**0.11**	**0.53**	**0.54**	**0.49**	**0.48**	**0.45**	**0.37**	**0.43**	**0.54**
SEM	**0.17**	**0.08**	**0.07**	**0.18**	**0.18**	**0.16**	**0.16**	**0.15**	**0.12**	**0.14**	**0.24**
A vs. 2-tail, unpaired t-test, para-metric data, unequal variance	**0.14**	**0.33**	**0.29**	**0.68**	**0.87**	**0.75**	**0.54**	**0.25**	**0.25**	**0.13**	**0.14**

5.3.2 Raw data: Dose dependent effect of ET-1 on isolated human pulmonary arteries and veins. Active tension (gf).

		Active Tension (gf): Veins										
		log [ET-1] [M]										
Prep	**Base line**	**-12.0**	**-11.5**	**-11.0**	**-10.5**	**-10.0**	**-9.5**	**-9.0**	**-8.5**	**-8.0**	**-7.5**	**EC_{50} [nM]**
PV2.2	1.06					0.15	0.62	0.98	1.28			0.33
PV2.3	0.49					0.08	0.22	0.34	0.33			0.53
PV2.4	0.53					0.18	0.65	0.81	0.91			0.22
PV3.1	0.87	0.01	0.01	0.01	0.03	0.11	0.18	0.46	0.57	0.63	0.60	0.65
PV3.2	1.61	0.00	-0.03	-0.02	0.03	0.30	0.67	1.25	1.66	1.94	1.92	0.65
PV3.3	1.47	-0.01	0.01	0.00	0.06	0.08	0.10	0.20	0.25	0.29	0.27	0.65
PV6.1	1.66			-0.03	0.04	0.31	0.53	1.54	2.03	2.40	2.33	0.76
PV6.2	1.39			0.08	0.09	0.55	0.83	2.04	2.38	2.60	2.50	0.56
PV6.3	1.41			0.02	0.04	0.18	0.27	0.38	0.40	0.37		0.14
Mean	**1.17**	**0.00**	**0.00**	**0.01**	**0.05**	**0.22**	**0.45**	**0.89**	**1.09**	**1.37**	**1.52**	**0.50**
SD	**0.45**	**0.01**	**0.02**	**0.04**	**0.02**	**0.15**	**0.26**	**0.62**	**0.79**	**1.06**	**1.02**	**0.22**
SEM	**0.15**	**0.01**	**0.01**	**0.02**	**0.01**	**0.05**	**0.09**	**0.21**	**0.26**	**0.43**	**0.46**	**0.07**
		Active Tension (gf): Arteries										
		log [ET-1] [M]										
Prep	**Base line**	**-12.0**	**-11.5**	**-11.0**	**-10.5**	**-10.0**	**-9.5**	**-9.0**	**-8.5**	**-8.0**	**-7.5**	**EC_{50} [nM]**
PA11.1	0.95	0.00	-0.02	-0.06	-0.09	-0.04	0.01	0.48	0.69	0.47		0.79
PA11.2	1.11	0.00	-0.03	-0.06	-0.09	-0.1	-0.08	0.21	0.42	0.45		1.15
PA12.1	1.36			0.00	-0.01	0.01	0.01	0.04	0.07	0.12	0.13	3.00
PA12.2	1.46			0.02	0.03	0.05	0.06	0.12	0.14	0.17	0.20	0.80
PA12.3	1.42			-0.02	-0.02	-0.03	-0.02	0.01	0.07	0.18	0.16	4.20
PA13.1	1.06			-0.02	-0.05	-0.07	-0.08	0.11	0.64	1.00	0.86	2.46
PA13.2	2.51			-0.10	-0.06	-0.19	-0.21	-0.05	-0.06	0.20	0.30	8.80
PA13.3	1.62			-0.02	-0.05	-0.08	-0.10	0.02	0.30	0.34		2.77
PA13.4	2.18			0.08	0.04	-0.08	-0.07	0.17	0.16	0.11		0.75
Mean	**1.52**	**0.00**	**-0.03**	**-0.02**	**-0.03**	**-0.06**	**-0.05**	**0.12**	**0.27**	**0.34**	**0.33**	**2.75**
SD	**0.52**	**0.00**	**0.01**	**0.05**	**0.05**	**0.07**	**0.08**	**0.16**	**0.26**	**0.28**	**0.30**	**2.58**
SEM	**0.17**	**0.00**	**0.01**	**0.02**	**0.02**	**0.02**	**0.03**	**0.05**	**0.09**	**0.09**	**0.14**	**0.86**
Statistical analysis												
***P*. Artery vs. vein. 2-tail, unpaired t-test, unequal variance.**	**0.14**	**1.00**	**0.24**	**0.23**	**0.00**	**0.00**	**0.00**	**0.01**	**0.01**	**0.06**	**0.06**	**0.03**

5.3.2 Raw data: Dose dependent effect of ET-1 on isolated human pulmonary arteries and veins. % Change in active tension from baseline.										
	% Δ in Active tension from baseline: Veins									
	log [ET-1] [M]									
Prep	**-12.0**	**-11.5**	**-11.0**	**-10.5**	**-10.0**	**-9.5**	**-9.0**	**-8.5**	**-8.0**	**-7.5**
PV2.2					14.15	58.49	92.45	120.75		
PV2.3					16.33	44.90	69.39	67.35		
PV2.4					33.96	122.64	152.83	171.70		
PV3.1	1.15	1.15	1.15	3.45	12.64	20.69	52.87	65.52	72.41	68.97
PV3.2	0.00	-1.86	-1.24	1.86	18.63	41.61	77.64	103.11	120.50	119.25
PV3.3	-0.68	0.68	0.00	4.08	5.44	6.80	13.61	17.01	19.73	18.37
PV6.1			-1.81	2.41	18.67	31.93	92.77	122.29	144.58	140.36
PV6.2			5.76	6.47	39.57	59.71	146.76	171.22	187.05	179.86
PV6.3			1.42	2.84	12.77	19.15	26.95	28.37	26.24	
Mean	**0.16**	**-0.01**	**0.88**	**3.52**	**19.13**	**45.10**	**80.59**	**96.37**	**95.08**	**105.36**
SD	**0.92**	**1.62**	**2.71**	**1.64**	**10.84**	**34.15**	**47.64**	**56.24**	**67.06**	**62.97**
SEM	**0.53**	**0.94**	**1.11**	**0.67**	**3.61**	**11.38**	**15.88**	**18.75**	**27.38**	**28.16**
	% Δ in Active tension from baseline: Arteries									
	log [ET-1] [M]									
Prep	**-12.0**	**-11.5**	**-11.0**	**-10.5**	**-10.0**	**-9.5**	**-9.0**	**-8.5**	**-8.0**	**-7.5**
PA11.1	0	-2.11	-6.32	-9.47	-4.21	1.05	50.53	72.63	49.47	
PA11.2	0	-2.70	-5.41	-8.11	-9.01	-7.21	18.92	37.84	40.54	
PA12.1			0.00	-0.74	0.74	0.74	2.94	5.15	8.82	9.56
PA12.2			1.37	2.05	3.42	4.11	8.22	9.59	11.64	13.70
PA12.3			-1.41	-1.41	-2.11	-1.41	0.70	4.93	12.68	11.27
PA13.1			-1.89	-4.72	-6.60	-7.55	10.38	60.38	94.34	81.13
PA13.2			-3.98	-2.39	-7.57	-8.37	-1.99	-2.39	7.97	11.95
PA13.3			-1.23	-3.09	-4.94	-6.17	1.23	18.52	20.99	
PA13.4			3.67	1.83	-3.67	-3.21	7.80	7.34	5.05	
Mean	**0.00**	**-2.40**	**-1.69**	**-2.89**	**-3.77**	**-3.11**	**10.97**	**23.78**	**27.94**	**25.52**
SD	**0.00**	**0.42**	**3.20**	**4.00**	**3.97**	**4.48**	**16.12**	**26.95**	**29.30**	**31.12**
SEM	**0.00**	**0.30**	**1.07**	**1.33**	**1.32**	**1.49**	**5.37**	**8.98**	**9.77**	**13.92**
Artery vs. vein *p* via unpaired t-test, para-metric data, unequal variance.	**0.80**	**0.12**	**0.12**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.06**	**0.05**

5.3.2 Raw data: Estimated EC_{50} and pEC_{50} for ET-1 in individual isolated human pulmonary artery and vein preparations

Veins				Arteries		
Prep	**EC_{50} [nM]**	**pEC_{50}**		**Prep**	**EC_{50} [nM]**	**pEC_{50}**
PV2.2	0.33	9.48		**PA11.1**	0.79	9.10
PV2.3	0.53	9.27		**PA11.2**	1.15	8.94
PV2.4	0.22	9.67		**PA12.1**	3.00	8.52
PV3.1	0.65	9.18		**PA12.2**	0.80	9.10
PV3.2	0.65	9.18		**PA12.3**	4.20	8.38
PV3.3	0.65	9.19		**PA13.1**	2.46	8.61
PV6.1	0.76	9.12		**PA13.2**	8.80	8.06
PV6.2	0.56	9.25		**PA13.3**	2.77	8.56
PV6.3	0.14	9.85		**PA13.4**	0.75	9.12
Mean	**0.50**	**9.36**		**Mean**	**2.75**	**8.71**
SD	**0.22**	**0.26**		**SD**	**2.58**	**0.38**
SEM	**0.07**	**0.09**		**SEM**	**0.86**	**0.13**
Artery vs. vein *p* via 2-tail, unpaired t-test for parametric data, unequal variance.					**0.031**	**0.001**

6.3.1.1 Raw data: *Ex Vivo* lungs: Patient and specimen data with experimental parameters						
	Patient		**Specimen**		**Experimental Parameters**	
Prep	**Sex**	**age (years)**	**Lobe**	**Weight (g)**	**Tv (ml)**	**BPM**
Hull 15	m	79	LUL	190	100	10
Hull 15 (h)	m	65	RUL	398	100	10
Hull 17 (h)	m	75	RLL	310	100	10
Hull 21	m	61	RUL	348	100	10
Hull 25	m	53	LUL	177	100	10
Hull 28	m	73	RUL	244	100	10
Hull 30	m	76	LLL	265	100	10
Hull 31	m	55	LLung	480	200	10
Hull 32	m	75	RUL	330	100	10
Hull 33	m	51	RUL	240	100	10
Hull 34	m	79	RUL	421	100	10
Hull 41	m	79	LUL	261	100	10
Mean		**68**		**305**		
SD		**11**		**94**		
SEM		**3**		**27**		

6.3.1.1 Raw data: Ex Vivo Lungs: Hypoxic Times and mean change in peak airway pressure					
Lung	**Hypoxic Time (min)**	**Peak AP (mmHg) Pre hypoxia**	**Peak AP (mmHg) Post hypoxia**	**% change**	**Time to maximum dilation (min)**
hull 15	35	21.00	16.00	-24	30
hull 15 H	178	23.00	17.00	-26	5
hull17 H	17	26.00	22.50	-13	5
hull 21	62	23.30	16.50	-29	9
hull 25	76	8.40	6.60	-21	4
hull 28	87	14.00	10.40	-26	9
hull 30	91	25.60	20.90	-18	22
hull 31	30	15.30	7.10	-54	18
hull 32	77	15.10	10.50	-30	6
hull 33	48	12.20	12.20	0	
hull 34	56	14.40	11.60	-19	3
hull 41	60	21.00	21.00	0	
Mean	**68**	**18.28**	**14.36**	**-22**	**11**
SD	**41**	**5.74**	**5.42**	**14**	**9**
2-tailed t-test, pre vs. post hypoxia		**0.0002**			

6.3.1.1 Raw data: Effect of hypoxia on perfusate pO_2 , *ex vivo* human lungs				
Prep	**Baseline pO_2 (KPa)**	**pO_2 (KPa) 20min post hypoxia**	**pO_2 (KPa) 50min post hypoxia**	**pO_2 (KPa) post Room Air**
Hull 15	20.3	15.5		
Hull 15 (h)		15.2		
Hull 21	20.7	5.2	3.3	21.2
Hull 25	20.4	5.3	4.8	
Hull 30	19.5	3.5	5.2	22.7
Hull 31	20	5.2		18.1
Hull 32	19.9	4.8	5.3	
Hull 33	19.3	3.9		
Hull 34	20.3	6.7	5.7	
Mean	**20.1**	**7.3**	**4.9**	**20.7**
SD	**0.5**	**4.7**	**0.9**	**2.4**
SEM	**0.2**	**1.6**	**0.4**	**1.4**
n	**8**	**9**	**5**	**3**
Statistics				
Paired t-test vs. baseline		**1.50499E-05**	**1.13888E-05**	**0.72324064**
Wilcoxon signed-rank test vs. pre hypoxia		**0.012**	**0.042**	**0.593**

6.3.1.1 Raw data for *ex vivo* human lungs: Effect of hypoxia on Mean Airway Pressure (AP)									
Time	**Base Line**	**10 min Post Hypoxia**		**20 min Post Hypoxia**		**50 min Post Hypoxia**		**Room Air**	
Prep	**Mean AP Pre Hypoxia (mmHg)**	**Mean AP 10 min Post Hypoxia (mmHg)**	**Change in Mean AP 10 min Post Hypoxia (mmHg)**	**Mean AP 20 min Post Hypoxia (mmHg)**	**Change in Mean AP 20 min post hypoxia (mmHg)**	**Mean AP 50 min Post Hypoxia (mmHg)**	**Change in Mean AP 50 min post hypoxia (mmHg)**	**Mean AP 10 min Post Room Air (mmHg)**	**Change in Mean AP 10 min post room air (mmHg)**
Hull 15	9.40	8.99	-0.42	8.43	-0.98			10.07	0.66
Hull 15 (h)	8.26	6.79	-1.47	6.94	-1.32	6.81	-1.45		
Hull 17 (h)	9.01	8.55	-0.46					9.32	0.32
Hull 21	9.14	6.54	-2.60	6.15	-2.99	6.21	-2.93	8.15	-0.99
Hull 25	4.23	3.58	-0.65	3.61	-0.62	3.68	-0.55	4.10	-0.13
Hull 28	5.42	4.21	-1.21	3.98	-1.43	3.34	-2.08	4.96	-0.46
Hull 30	8.59	7.54	-1.05	7.20	-1.39	6.78	-1.81	7.75	-0.83
Hull 31	5.58	3.10	-2.48	2.85	-2.73			5.36	-0.22
Hull 32	5.11	4.33	-0.78	4.21	-0.89	3.62	-1.49		
Hull 33	4.56	4.43	-0.14	4.38	-0.19				
Hull 34	6.01	5.54	-0.47	5.45	-0.56	5.28	-0.73	5.68	-0.33
Hull 41	8.33	8.29	-0.04	8.41	0.08	8.88	0.55		
Mean	**6.97**	**5.99**	**-0.98**	**5.60**	**-1.18**	**5.57**	**-1.31**	**6.92**	**-0.25**
SD	**1.98**	**2.06**	**0.84**	**1.95**	**0.96**	**1.96**	**1.06**	**2.19**	**0.55**
SEM	**0.57**	**0.60**	**0.24**	**0.59**	**0.29**	**0.69**	**0.38**	**0.78**	**0.19**
n	**12**	**12**	**12**	**11**	**11**	**8**	**8**	**8**	**8**
Paired t-test (2-tail) vs. baseline		**0.0019**		**0.0022**		**0.0101**		**0.2409**	

6.3.1.1 Raw data for *ex vivo* human lungs: Effect of hypoxia on Peak Airway Pressure (AP)									
Time	**Base Line**	**10 min Post Hypoxia**		**20 min Post Hypoxia**		**50 min Post Hypoxia**		**Room Air**	
Prep	**Peak AP Pre Hypoxia (mmHg)**	**Peak AP 10 min Post Hypoxia (mmHg)**	**Change in Peak AP 10 min post hypoxia (mmHg)**	**Peak AP 20 min Post Hypoxia (mmHg)**	**Change in Peak AP 20 min post hypoxia (mmHg)**	**Peak AP 50 min Post Hypoxia (mmHg)**	**Change in Peak AP 50 min post hypoxia (mmHg)**	**Peak AP 10 min Post Room Air (mmHg)**	**Change in Peak AP 10 min post room air (mmHg)**
Hull 15	30.00	26.92	-3.08	24.87	-5.13			31.54	1.54
Hull 15 (h)	23.08	18.72	-4.36	18.97	-4.10	18.72	-4.36		
Hull 17 (h)	26.50	23.00	-3.50					26.25	-0.25
Hull 21	26.07	17.29	-8.77	16.29	-9.77	16.29	-9.77	23.56	-2.51
Hull 25	10.20	8.67	-1.53	8.16	-2.04	7.14	-3.06	8.67	-1.53
Hull 28	14.25	11.45	-2.80	9.67	-4.58	8.14	-6.11	12.72	-1.53
Hull 30	26.53	22.96	-3.57	22.45	-4.08	22.96	-3.57	22.96	-3.57
Hull 31	17.09	10.46	-6.63	7.65	-9.44			16.07	-1.02
Hull 32	16.41	13.33	-3.08	13.08	-3.33	11.03	-5.38		
Hull 33	13.27	12.24	-1.02	11.99	-1.28				
Hull 34	16.71	13.11	-3.60	12.60	-4.11	11.83	-4.88	13.62	-3.08
Hull 41	21.63	21.60	-0.03	21.46	-0.17	23.37	1.74		
Mean	**20.14**	**16.65**	**-3.50**	**15.20**	**-4.37**	**14.93**	**-4.43**	**19.42**	**-1.49**
SD	**6.33**	**5.93**	**2.36**	**5.99**	**2.99**	**6.36**	**3.23**	**7.82**	**1.64**
SEM	**1.83**	**1.71**	**0.68**	**1.81**	**0.90**	**2.25**	**1.14**	**2.77**	**0.58**
n	**12**	**12**	**12**	**11**	**11**	**8**	**8**	**8**	**8**
Paired t-test (2-tail) vs. baseline		**0.0003**		**0.0007**		**0.0061**		**0.04**	

6.3.1.2 Raw data for *ex vivo* human lungs: Effect of normoxic, hypoxic and hyperoxic ventilation in 5% CO_2 on peak airway pressure

Ventilation Gas	**Room Air**		**Air: 5% CO_2**				**95%N_2: 5% CO_2**				**95%O_2: 5% CO_2**			
	Peak Airway Pressure (mmHg)		**Peak Airway Pressure (mmHg)**			**pO_2 (KPa)**	**Peak Airway Pressure (mmHg)**			**pO_2 (KPa)**	**Peak Airway Pressure (mmHg)**			**pO_2 (KPa)**
	Baseline	pO_2 (KPa)	Pre	Post	Change		Pre	Post	Change		Pre	Post	Change	
IL 1	32	22.8	32	32	0	22.7	32	32	0	17.1	32	33	1	47.4
IL2	18	23.2	18	17	-1	20.6	22	19.5	-2.5	20.3	22.5	22.5	0	23.3
Mean	**25**	**23**	**25**	**24.5**	**-0.5**	**21.65**	**27**	**25.75**	**-1.25**	**18.7**	**27.25**	**27.75**	**0.5**	**35.35**
SD	**9.90**	**0.28**	**9.90**	**10.61**	**0.71**	**1.48**	**7.07**	**8.84**	**1.77**	**2.26**	**6.72**	**7.42**	**0.71**	**17.04**

6.3.1.3 Raw data for *ex vivo* human lungs: Effect of independent changes in perfusate and ventilation pO_2 on airway pressure

	Ventilation/Perfusate															
	Normoxia/hypoxia				**Hypoxia/hypoxia**				**Normoxia/hyperoxia**				**Hyperoxia/hyperoxia**			
	Peak Airway Pressure (mmHg)				**Peak Airway Pressure (mmHg)**				**Peak Airway Pressure (mmHg)**				**Peak Airway Pressure (mmHg)**			
Prep	**Pre**	**Post**	**Change**	**Perfusate pO_2 (KPa)**	**Pre**	**Post**	**Change**	**Perfusate pO_2 (KPa)**	**Pre**	**Post**	**Change**	**Perfusate pO_2 (KPa)**	**Pre**	**Post**	**Change**	**Perfusate pO_2 (KPa)**
IL 2	22	21	-1	5.7	21	19	-2	16	19	19.5	0.5	50	19.5	20	0.5	

6.3.2.1: Raw data for *in vitro* human bronchi: HBD from Baseline					
Prep	**Baseline pre hypoxia (gf)**	**Baseline post hypoxia**	**Hypoxic Dilation from baseline (gf)**	**Hypoxic Dilation from baseline (% Change)**	**Time to HBD plateau (min)**
B29.2	1.88	1.35	-0.53	-28.19	6.00
B31.1	2.68	1.39	-1.29	-48.13	17.00
B31.2	2.84	0.96	-1.88	-66.20	8.00
B33.1	1.43	1.01	-0.42	-29.37	7.00
B33.2	1.78	0.87	-0.91	-51.12	9.00
B44.1	2.63	2.02	-0.61	-23.19	31.00
B44.2	2.12	1.78	-0.34	-16.04	15.00
B45.1	2.13	1.01	-1.12	-52.58	19.00
B45.2	2.40	0.58	-1.82	-75.83	16.00
B50.1	2.95	0.95	-2.00	-67.80	35.00
B50.2	2.95	0.58	-2.37	-80.34	35.00
B53	2.43	1.10	-1.33	-54.73	12.00
B56.1	2.36	0.65	-1.71	-72.46	11.00
B56.2	3.22	0.87	-2.35	-72.98	13.00
B57.1	2.97	0.65	-2.32	-78.11	10.00
B59.1	2.27	2.04	-0.23	-10.13	18.00
B59.2	2.27	0.72	-1.55	-68.28	15.00
B62.1	2.14	0.99	-1.15	-53.74	12.00
B62.2	2.65	1.83	-0.82	-30.94	12.00
B64.1	2.12	1.30	-0.82	-38.68	12.00
B64.2	2.12	0.98	-1.14	-53.77	11.00
B65.1	2.08	0.67	-1.41	-67.79	27.00
B65.2	2.12	0.58	-1.54	-72.64	27.00

6.3.2.1 Continued					
B66.1	2.65	1.35	-1.30	-49.06	18.00
B66.2	2.16	0.82	-1.34	-62.04	16.00
B67.1	2.92	0.75	-2.17	-74.32	18.00
B67.2	2.93	1.15	-1.78	-60.75	18.00
B68.1	1.93	0.80	-1.13	-58.55	28.00
B68.2	2.02	1.11	-0.91	-45.05	20.00
B69.2	1.74	0.63	-1.11	-63.79	20.00
B70.1	1.41	0.88	-0.53	-37.59	20.00
B71.2	2.40	1.54	-0.86	-35.83	17.00
B72.1	2.15	2.00	-0.15	-6.98	11.00
B72.2	2.12	1.54	-0.58	-27.36	12.00
B76.1	2.10	1.91	-0.19	-9.05	25.00
B76.2	2.02	0.58	-1.44	-71.29	17.00
B76.3	1.90	1.00	-0.90	-47.37	21.00
B76.4	2.00	0.98	-1.02	-51.00	26.00
B79.1	2.25	0.38	-1.87	-83.11	8.00
B79.2	1.67	0.57	-1.10	-65.87	14.00
B79.3	2.39	0.66	-1.73	-72.38	12.00
B79.4	2.34	1.15	-1.19	-50.85	12.00
B81.1	2.00	1.70	-0.30	-15.00	11.00
B81.2	1.83	1.54	-0.29	-15.85	10.00
B81.3	1.72	1.00	-0.72	-41.86	11.00
B81.4	1.88	1.54	-0.34	-18.09	14.00
B84.1	2.34	0.67	-1.67	-71.37	12.00
B84.2	1.54	0.67	-0.87	-56.49	10.00

6.3.2.1 Continued					
B84.3	1.85	1.13	-0.72	-38.92	8.00
B84.4	1.78	0.67	-1.11	-62.36	15.00
Mean	**2.21**	**1.07**	**-1.14**	**-50.10**	**16.04**
SD	**0.42**	**0.44**	**0.60**	**21.27**	**7.02**
SEM	**0.06**	**0.06**	**0.09**	**3.01**	**0.99**
Statistics					
Paired t-test vs. Base Line		**6.1E-18**			

6.3.2.2 Raw data for *in vitro* human bronchi: HBD and Isoprenaline dilation From Baseline											
	Hypoxia					Isoprenaline					
Prep	Baseline pre hypoxia (gf)	Baseline post hypoxia (gf)	HBD from baseline (gf)	HBD from baseline (% Change)	Time to HBD plateau (min)	Baseline pre Iso (gf)	Baseline post Iso (gf)	Iso Dilation (gf)	Iso dilation from Baseline (% Change)	Time to Iso plateau from Baseline (s)	Time to Iso plateau from Baseline (min)
B31.1	2.68	1.39	-1.29	-48.13	16.00	2.51	1.31	-1.20	-47.81	45	0.75
B31.2	2.84	0.96	-1.88	-66.20	7.00	2.88	0.87	-2.01	-69.79	45	0.75
B33.1	1.43	1.01	-0.42	-29.37	7.00	1.67	1.43	-0.24	-14.37	300	5.00
B33.2	1.78	0.87	-0.91	-51.12	9.00	2.27	1.01	-1.26	-55.51	180	3.00
B79.1	2.25	0.38	-1.87	-83.11	8.00	3.01	0.40	-2.61	-86.71	240	4.00
B79.2	1.67	0.57	-1.10	-65.87	14.00	2.87	0.53	-2.34	-81.53	240	4.00
B79.3	2.39	0.66	-1.73	-72.38	12.00	2.41	0.54	-1.87	-77.59	60	1.00
B79.4	2.34	1.15	-1.19	-50.85	12.00	3.09	0.81	-2.28	-73.79	120	2.00
Mean	**2.17**	**0.87**	**-1.30**	**-58.38**	**10.63**	**2.59**	**0.86**	**-1.73**	**-63.39**	**154**	**2.56**
SD	**0.50**	**0.33**	**0.51**	**16.82**	**3.38**	**0.47**	**0.37**	**0.78**	**23.70**	**100.38**	**1.67**
SEM	**0.18**	**0.12**	**0.18**	**5.95**	**1.19**	**0.17**	**0.13**	**0.28**	**8.38**	**35.49**	**0.59**
Statistics											
Parameters		**HBD from BL:**			**HBD Time vs. Iso Time:**		**Iso from BL:**		**Base Line HBD vs. Iso:**		**Magnitude (gf) of HBD vs. Iso:**
Paired t-test		**0.0002**			**0.0011**		**0.0004**		**0.8895**		**0.0587**

6.3.2.3 Raw data: HBD and Isoprenaline relaxation post contraction to MCh												
Prep	**Baseline pre MCh (gf)**	**Max MCh Plateau (gf)**	**MCh contraction (gf)**	**HBD BL post MCh (gf)**	**HBD post MCh (gf)**	**HBD post MCh (% of MCh)**	**Time to HBD plateau post MCh (min)**	**Iso BL post MCh (gf)**	**Iso dilation (gf)**	**Iso dilation (% of MCh)**	**Time to Iso plateau post MCh (s)**	**Time to Iso plateau post MCh (min)**
B31.1	2.06	3.82	1.76	1.77	-2.05	-116	16.0	2.40	-1.42	-81	200	3.33
B31.2	1.68	3.80	2.12	1.39	-2.41	-114	21.0	2.50	-1.30	-61	155	2.58
B37.1	2.02	2.65	0.63	1.92	-0.73	-116	35.0	1.87	-0.78	-124	120	2
B37.2	2.12	3.24	1.12	1.27	-1.97	-176	35.0	1.87	-1.37	-122	60	1
B38.1	1.60	9.39	7.79	1.00	-8.39	-108	45.0	1.05	-8.34	-107	300	5
B38.2	1.54	7.60	6.06	0.91	-6.69	-110	45.0	1.01	-6.59	-109	180	3
B39.1	1.30	9.10	7.80	1.58	-7.52	-96	19.0	5.81	-3.29	-42	720	12
B39.2	0.87	6.87	6.00	1.25	-5.62	-94	17.0	4.23	-2.64	-44	840	14
B40.1 sp 39	1.77	11.24	9.47	1.87	-9.37	-99	74.0	5.10	-6.14	-65	420	7
B40.2 sp 39	1.97	7.02	5.05	1.73	-5.29	-105	50.0	3.51	-3.51	-70	300	5
B41.1	2.36	12.49	10.13	2.85	-9.64	-95	30.0	4.37	-8.12	-80	360	6
B41.2	2.26	6.97	4.71	1.25	-5.72	-121	24.0	3.85	-3.12	-66	120	2
B43.1	2.02	5.30	3.28	1.28	-4.02	-123	35.0	1.41	-3.89	-119	180	3
B43.2	1.20	4.52	3.32	0.91	-3.61	-109	40.0	1.11	-3.41	-103	240	4
B44.1	1.87	5.83	3.96	2.04	-3.79	-96	50.0	3.77	-2.06	-52	240	4
B44.2	2.02	4.09	2.07	1.83	-2.26	-109	34.0	2.21	-1.88	-91	180	3
B45.1	1.28	6.34	5.06	1.39	-4.95	-98	44.0	3.10	-3.24	-64	240	4
B45.2	0.82	5.53	4.71	0.72	-4.81	-102	44.0	2.00	-3.53	-75	420	7
B50.1	1.45	6.35	4.90	2.00	-4.35	-89	32.0	1.39	-4.96	-101	360	6
B50.2	1.10	4.10	3.00	1.16	-2.94	-98	21.0	0.87	-3.23	-108	540	9

6.3.2.3 Cont.												
B53	2.38	5.00	2.62	1.41	-3.59	-137	26.0	1.27	-3.73	-142	240	4
B56.1	2.90	5.05	2.15	0.88	-4.17	-194	17.0	1.01	-4.04	-188	240	4
B56.2	3.75	6.11	2.36	1.30	-4.81	-204	17.0	1.49	-4.62	-196	180	3
B57.1	2.63	7.16	4.53	1.00	-6.16	-136	15.0	4.28	-2.88	-64	420	7
B59.1	2.13	10.60	8.47	2.99	-7.61	-90	21.0	2.15	-8.45	-100	180	3
B59.2	2.36	4.05	1.69	0.96	-3.09	-183	13.0	2.94	-1.11	-66	300	5
B62.1	1.43	4.24	2.81	1.22	-3.02	-107	53.0	1.66	-2.58	-92	240	4
B62.2	1.88	5.40	3.52	2.12	-3.28	-93	50.0	2.84	-2.56	-73	360	6
B64.1	2.10	4.14	2.04	1.30	-2.84	-139	16.0	1.12	-3.02	-148	30	0.5
B64.2	2.40	7.88	5.48	2.12	-5.76	-105	16.0	3.36	-4.52	-82	120	2
B66.1	2.56	4.94	2.38	1.64	-3.30	-139	43.0	1.20	-3.74	-157	420	7
B70.1	0.86	6.00	5.14	1.60	-4.40	-86	23.0	1.16	-4.84	-94	100	1.67
B72.1	2.02	5.85	3.83	2.36	-3.49	-91	40.0	4.27	-1.58	-41	180	3
B72.2	1.59	5.38	3.79	1.92	-3.46	-91	35.0	3.75	-1.63	-43	120	2
Mean	**1.89**	**6.12**	**4.23**	**1.56**	**-4.56**	**-117**	**32.24**	**2.53**	**-3.59**	**-93.19**	**273.68**	**4.56**
SD	**0.62**	**2.30**	**2.37**	**0.54**	**2.13**	**31**	**14.46**	**1.36**	**2.02**	**39.32**	**174.91**	**2.92**
SEM	**0.11**	**0.41**	**0.42**	**0.10**	**0.38**	**5**	**2.56**	**0.24**	**0.36**	**6.95**	**30.92**	**0.52**
Statistics												
Parameters		**HBD post MCh:**			**HBD Time vs. Iso Time post MCh:**		**Iso post MCh:**		**Base Line HBD vs. Iso post MCh:**		**Magnitude (gf) of HBD vs. Iso post MCh:**	
Paired t-test		**0.0000**			**0.0000**		**0.0000**		**0.0001**		**0.0001**	

6.3.2.4 Raw data: Concentration dependent effect of O_2 on baseline tension												
	Baseline (gf)				Change (gf)				% Change from Baseline			
	$\%O_2$				$\%O_2$				$\%O_2$			
Prep	**95**	**40**	**21**	**0**	**95**	**40**	**21**	**0**	**95**	**40**	**21**	**0**
B79.1a	1.7	0.97	0.67	0.44	0	-0.73	-1.03	-1.26	0	-42.94	-60.59	-74.12
B79.2a	0.96	0.81	0.43	0.43	0	-0.15	-0.53	-0.53	0	-15.63	-55.21	-55.21
B79.3a	0.61	0.57	0.57	0.57	0	-0.04	-0.04	-0.04	0	-6.56	-6.56	-6.56
B79.4a	1.3	1.09	1.04	0.79	0	-0.21	-0.26	-0.51	0	-16.15	-20.00	-39.23
B80.1a	2.29	1.98	1.62	0.99	0	-0.31	-0.67	-1.3	0	-13.54	-29.26	-56.77
B80.2a	2.6	1.64	0.92	0.48	0	-0.96	-1.68	-2.12	0	-36.92	-64.62	-81.54
B80.3a	2.5	2.12	1.54	1.13	0	-0.38	-0.96	-1.37	0	-15.20	-38.40	-54.80
B80.4a	2.52	1.81	1.53	1.2	0	-0.71	-0.99	-1.32	0	-28.17	-39.29	-52.38
Mean	**1.81**	**1.37**	**1.04**	**0.75**	**0.00**	**-0.44**	**-0.77**	**-1.06**	**0**	**-21.89**	**-39.24**	**-52.58**
SD	**0.78**	**0.58**	**0.47**	**0.32**	**0.00**	**0.33**	**0.51**	**0.66**	**0**	**12.71**	**20.33**	**22.77**

6.3.2.4 Raw data: Concentration dependent effect of O_2 on cholinergic contraction															
				Baseline (gf)				Change (gf)				% of Max MCh			
				$\%O_2$				$\%O_2$				$\%O_2$			
Prep	**Pre MCh**	**post MCh**	**MCh response (gf)**	**95**	**40**	**21**	**0**	**95**	**40**	**21**	**0**	**95**	**40**	**21**	**0**
B79.1a	1.18	4.95	3.77	4.95	3.35	1.94	0.48	0	-1.6	-3.01	-4.47	0	-42.44	-79.84	-118.57
B79.2a	0.57	3.25	2.68	3.25	2.39	1.44	0.67	0	-0.86	-1.81	-2.58	0	-32.09	-67.54	-96.27
B79.3a	0.63	3.02	2.39	3.02	2.69	2.21	0.72	0	-0.33	-0.81	-2.3	0	-13.81	-33.89	-96.23
B79.4a	1.04	3.81	2.77	3.81	3.51	2.26	1.11	0	-0.3	-1.55	-2.7	0	-10.83	-55.96	-97.47
B80.1a	2.44	5.61	3.17	5.61	4.61	3.53	1.2	0	-1	-2.08	-4.41	0	-31.55	-65.62	-139.12
B80.2a	2.12	6.22	4.1	6.22	4.24	2.89	1.35	0	-1.98	-3.33	-4.87	0	-48.29	-81.22	-118.78
B80.3a	1.99	5.71	3.72	5.71	4.24	2.91	1.81	0	-1.47	-2.8	-3.9	0	-39.52	-75.27	-104.84
B80.4a	2.11	4.28	2.17	4.28	3.25	2.56	1.98	0	-1.03	-1.72	-2.3	0	-47.47	-79.26	-105.99
B85.1a	2.41	4.6	2.19	4.6	3.48	1.91	0.94	0	-1.12	-2.69	-3.66	0	-51.14	-122.83	-167.12
B85.2a	1.98	4.1	2.12	4.1	2.17	1.3	0.72	0	-1.93	-2.8	-3.38	0	-91.04	-132.08	-159.43
B85.4a	2.2	4.41	2.21	4.41	2.95	2.48	1.9	0	-1.46	-1.93	-2.51	0	-66.06	-87.33	-113.57
Mean	**1.70**	**4.54**	**2.84**	**4.54**	**3.35**	**2.31**	**1.17**	**0.00**	**-1.19**	**-2.23**	**-3.37**	**0.00**	**-43.11**	**-80.08**	**-119.76**
SD	**0.70**	**1.01**	**0.73**	**1.01**	**0.78**	**0.66**	**0.53**	**0.00**	**0.57**	**0.76**	**0.95**	**0.00**	**22.59**	**27.73**	**24.99**

6.3.2.5 Raw data: Effect of Hypercapnia on resting tension and cholinergic contraction											
Prep	**Exposure**	**Baseline pre hypercapnia (gf)**	**Baseline post hypercapnia (gf)**	**Hypercapnic Dilation from baseline (gf)**	**Hypercapnic Dilation from baseline (% Change)**	**Baseline pre MCh(gf)**	**Max MCh Plateau**	**MCh contraction (gf)**	**BL post Hypercapnia (gf)**	**Hypercapnic dilation (gf)**	**Hypercapnic dilation % of MCh**
B58.1.1	**Hypercapnia**	1.71	1.58	-0.13	-7.60	2.91	5.35	2.44	4.8	-0.55	-22.54
B58.2.1	**Hypercapnia**	1.73	1.39	-0.34	-19.65	1.54	3.27	1.73	2.98	-0.29	-16.76
B58.1.2	**Hypercapnia**	1.58	1.52	-0.06	-3.80						
B58.2.2	**Hypercapnia**	1.73	1.3	-0.43	-24.86						
B58.1.3	**Hypercapnia**	1.66	1.6	-0.06	-3.61						
B58.2.3	**Hypercapnia**	1.68	1.39	-0.29	-17.26						
Mean		**1.68**	**1.46**	**-0.22**	**-12.80**	**2.23**	**4.3**	**2.09**	**3.89**	**-0.42**	**-19.65**
SD		**0.06**	**0.12**	**0.16**	**9.00**	**0.97**	**1.5**	**0.50**	**1.29**	**0.18**	**4.09**
t-test (paired, 1-tail) vs. baseline tension:					**0.009**						**0.10**

6.3.2.5 Raw data: Effect of hypercapnia on HBD from resting tension									
		Normocapnic				Hypercapnic			
Prep	Exposure	Baseline pre hypoxia (gf)	Baseline post hypoxia (gf)	Hypoxic Dilation from baseline (gf)	Hypoxic Dilation from baseline (% Change)	Baseline pre hypoxia (gf)	Baseline post hypoxia (gf)	Hypoxic Dilation from baseline (gf)	Hypoxic Dilation from baseline (% Change)
B58.1	**Hypercapnia**	1.66	1.24	-0.42	-25.30	1.62	1.26	-0.36	-22.22
B58.2	**Hypercapnia**	1.59	1.15	-0.44	-27.67	1.3	1.15	-0.15	-11.54
	Mean	**1.63**	**1.20**	**-0.43**	**-26.49**	**1.46**	**1.21**	**-0.26**	**-16.88**
	SD	**0.05**	**0.06**	**0.01**	**1.68**	**0.23**	**0.08**	**0.15**	**7.55**
	t-test (Paired, 2-tail) Normocapnic vs. Hypercapnic HBD:							**0.24**	**0.22**

6.3.2.5 Raw data: Effect of hypercapnia on HBD from Cholinergic contraction												
	Normocapnic						Hypercapnic					
Prep	Baseline pre MCh(gf)	Max MCh Plateau	MCh contraction (gf)	BL post Hypoxia (gf)	HBD (gf)	Hypercapnic dilation % of MCh	Baseline pre MCh(gf)	Max MCh Plateau	MCh contraction (gf)	BL post Hypoxia (gf)	HBD (gf)	Hypercapnic dilation % of MCh
B58.1	1.73	5.85	4.12	2	-3.85	-93	1.73	5.85	4.12	1.9	-3.95	-95.87
B58.2	1.49	3.37	1.88	1.25	-2.12	-113	1.49	3.37	1.88	1.15	-2.22	-118.09
Mean	**1.61**	**4.61**	**3**	**1.63**	**-2.99**	**-103**	**1.61**	**4.61**	**3**	**1.525**	**-3.09**	**-106.98**
SD	**0.17**	**1.75**	**1.58**	**0.53**	**1.22**	**13.66**	**0.17**	**1.75**	**1.58**	**0.53**	**1.22**	**15.71**
t-test (Paired, 2-tail) Normocapnic vs Hypercapnic HBD:											**0.94**	**0.23**

Chapter 7: Mechanism of Hypoxic Bronchodilation: Role of the Epithelium, G-Protein Coupled Receptors and Intracellular Signalling Pathways

7.3.1 Raw Data: Effect of removing the epithelium on HBD from resting tension (experimental preps)										
Experimental	**Epithelium**					**Epithelium Denuded**				
Prep	**Baseline pre hypoxia (gf)**	**Baseline post hypoxia (gf)**	**Hypoxic Dilation from baseline (gf)**	**Hypoxic Dilation from baseline (% Change)**	**Time to HBD plateau (min)**	**Baseline pre hypoxia (gf)**	**Baseline post hypoxia (gf)**	**Hypoxic Dilation from baseline (gf)**	**Hypoxic Dilation from baseline (% Change)**	**Time to HBD plateau (min)**
B87.1	1.81	1.69	-0.12	-6.63	17	1.14	0.98	-0.16	-14.04	25
B87.2	1.71	1.57	-0.14	-8.19	10	1.40	1.25	-0.15	-10.71	23
B89.1	1.86	1.06	-0.80	-43.01	12	0.98	0.82	-0.16	-16.33	12
B89.2	1.76	1.36	-0.40	-22.73	8	1.60	1.34	-0.26	-16.25	10
Mean	**1.79**	**1.42**	**-0.37**	**-20.14**	**12**	**1.28**	**1.10**	**-0.18**	**-14.33**	**18**
SD	**0.06**	**0.28**	**0.32**	**16.88**	**4**	**0.27**	**0.24**	**0.05**	**2.64**	**8**
SEM	**0.03**	**0.14**	**0.16**	**8.44**	**2**	**0.14**	**0.12**	**0.03**	**1.32**	**4**
Statistics										
pre vs. post denudation; *p* via paired t-test (2-tail)						**0.05**		**0.33**	**0.50**	**0.05**

7.3.1 Raw Data: Effect of removing the epithelium on HBD from resting tension (Control preps)										
Controls	**Pre Control**					**Post Control**				
Prep	**Baseline pre hypoxia (gf)**	**Baseline post hypoxia (gf)**	**Hypoxic Dilation from baseline (gf)**	**Hypoxic Dilation from baseline (% Change)**	**Time to HBD plateau (min)**	**Baseline pre hypoxia (gf)**	**Baseline post hypoxia (gf)**	**Hypoxic Dilation from baseline (gf)**	**Hypoxic Dilation from baseline (% Change)**	**Time to HBD plateau (min)**
B87.3	1.85	1.81	-0.04	-2.16	20	1.68	1.44	-0.24	-14.29	17
B87.4	1.96	1.85	-0.11	-5.61	26	2.07	1.89	-0.18	-8.70	30
B89.3	1.99	1.28	-0.71	-35.68	6	1.20	0.96	-0.24	-20.00	10
B89.4	1.68	0.76	-0.92	-54.76	14	1.46	0.77	-0.69	-47.26	22
Mean	**1.87**	**1.43**	**-0.45**	**-24.55**	**17**	**1.60**	**1.27**	**-0.34**	**-22.56**	**20**
SD	**0.14**	**0.51**	**0.44**	**25.14**	**9**	**0.37**	**0.50**	**0.24**	**17.10**	**8**
SEM	**0.07**	**0.26**	**0.22**	**12.57**	**4**	**0.18**	**0.25**	**0.12**	**8.55**	**4**
Statistics										
pre vs. post; *p* via paired t-test (2-tail)						**0.25**		**0.53**	**0.76**	**0.25**
Denuded vs. control *p* via t-test (2- tail, unpaired)						**0.21**		**0.25**	**0.38**	**0.71**

7.3.1 Raw Data: Effect of removing the epithelium on relaxation to 10 µM isoprenaline from resting tension (experimental preps)										
Experimental	**Epithelium**					**Epithelium Denuded**				
Prep	**Baseline pre Iso (gf)**	**Baseline post iso (gf)**	**Iso Dilation from baseline (gf)**	**Iso Dilation from baseline (% Change)**	**Time to Iso plateau (min)**	**Baseline pre Iso (gf)**	**Baseline post Iso (gf)**	**Iso Dilation from baseline (gf)**	**Iso Dilation from baseline (% Change)**	**Time to Iso plateau (min)**
B87.1	1.84	1.57	-0.27	-14.67	1	1.11	0.84	-0.27	-24.32	1
B87.2	1.74	1.38	-0.36	-20.69	2	1.36	1.11	-0.25	-18.38	1
B89.1	1.63	1.05	-0.58	-35.58	2	1.23	0.75	-0.48	-39.02	2
B89.2	1.60	1.34	-0.26	-16.25	1	1.52	1.27	-0.25	-16.45	1
Mean	**1.70**	**1.34**	**-0.37**	**-21.80**	**2**	**1.31**	**0.99**	**-0.31**	**-24.54**	**1**
SD	**0.11**	**0.21**	**0.15**	**9.54**	**1**	**0.18**	**0.24**	**0.11**	**10.22**	**1**
SEM	**0.05**	**0.11**	**0.07**	**4.77**	**0**	**0.09**	**0.12**	**0.06**	**5.11**	**0**
Statistics										
pre vs. post denudation; *p* via paired t-test (2-tail)						**0.06**		**0.15**	**0.37**	**0.39**

7.3.1 Raw Data: Effect of removing the epithelium on relaxation to 10 µM isoprenaline from resting tension (control preps)										
Controls	**Pre Control**					**Post Control**				
Prep	**Baseline pre Iso (gf)**	**Baseline post iso (gf)**	**Iso Dilation from baseline (gf)**	**Iso Dilation from baseline (% Change)**	**Time to Iso plateau (min)**	**Baseline pre Iso (gf)**	**Baseline post Iso (gf)**	**Iso Dilation from baseline (gf)**	**Iso Dilation from baseline (% Change)**	**Time to Iso plateau (min)**
B87.3	1.83	1.75	-0.08	-4.37	3	1.71	1.29	-0.42	-24.56	2
B87.4	2.01	1.85	-0.16	-7.96	3	2.10	1.81	-0.29	-13.81	2
B89.3	1.83	1.21	-0.62	-33.88	1	1.41	0.89	-0.52	-36.88	1
B89.4	1.52	0.69	-0.83	-54.61	2	1.91	0.57	-1.34	-70.16	4
Mean	**1.80**	**1.38**	**-0.42**	**-25.20**	**2**	**1.78**	**1.14**	**-0.64**	**-36.35**	**2**
SD	**0.20**	**0.54**	**0.36**	**23.60**	**1**	**0.29**	**0.54**	**0.47**	**24.43**	**1**
SEM	**0.10**	**0.27**	**0.18**	**11.80**	**0**	**0.15**	**0.27**	**0.24**	**12.21**	**1**
Statistics										
pre vs. post; *p* via paired t-test						**0.94**		**0.19**	**0.07**	**1.00**
Denuded vs. control; *p* via t-test (2- tail, unpaired)						**0.03**		**0.22**	**0.41**	**0.19**

7.3.1 Raw Data: Effect of removing the epithelium on HBD post contraction to 1 mM MCh (experimental preps)

Experimental	Epithelium							Epithelium Denuded								
Prep	Baseline pre MCh (gf)	Max MCh Plateau (gf)	MCh contraction (gf)	HBD BL post MCh (gf)	HBD post MCh (gf)	HBD post MCh (% of MCh)	Time to HBD plateau post MCh (min)	Baseline pre MCh (gf)	Max MCh Plateau (gf)	MCh contraction (gf)	HBD BL post MCh (gf)	HBD post MCh (gf)	HBD post MCh (% of MCh)	Δ in HBD vs. Pre	% Change vs. Pre	Time to HBD plateau post MCh (min)
B87.1	1.73	3.10	1.37	1.41	-1.69	-123	13	0.99	2.72	1.73	1.55	-1.17	-68	-56	45	15
B87.2	1.84	3.89	2.05	1.66	-2.23	-109	12	1.20	3.78	2.58	1.75	-2.03	-79	-30	28	12
B89.1	1.09	3.09	2.00	1.11	-1.98	-99	17	0.69	2.83	2.14	1.18	-1.65	-77	-22	22	15
B89.2	1.47	3.09	1.62	1.45	-1.64	-101	12	1.31	2.01	0.70	1.56	-0.45	-64	-37	36	10
Mean	**1.53**	**3.29**	**1.76**	**1.41**	**-1.89**	**-108**	**14**	**1.05**	**2.84**	**1.79**	**1.51**	**-1.33**	**-72**	**-36**	**33**	**13**
SD	**0.33**	**0.40**	**0.32**	**0.23**	**0.27**	**11**	**2**	**0.27**	**0.73**	**0.80**	**0.24**	**0.68**	**7**	**14**	**10**	**2**
SEM	**0.17**	**0.20**	**0.16**	**0.11**	**0.14**	**6**	**1**	**0.14**	**0.36**	**0.40**	**0.12**	**0.34**	**4**	**7**	**5**	**1**
Statistics																
post vs. pre denudation; *p* via paired t-test								**0.03**		**0.94**		**0.08**	**0.02**			**0.64**

7.3.1 Raw Data: Effect of removing the epithelium on HBD post contraction to 1 mM MCh (control preps)																
Controls	**Pre-Control**							**Post-Control**								
Prep	**Baseline pre MCh (gf)**	**Max MCh Plateau (gf)**	**MCh contraction (gf)**	**HBD BL post MCh (gf)**	**HBD post MCh (gf)**	**HBD post MCh (% of MCh)**	**Time to HBD plateau post MCh (min)**	**Baseline pre MCh (gf)**	**Max MCh Plateau (gf)**	**MCh contraction (gf)**	**HBD BL post MCh (gf)**	**HBD post MCh (gf)**	**HBD post MCh (% of MCh)**	**Δ in HBD vs. Pre**	**% Change vs. Pre**	**Time to HBD plateau post MCh (min)**
B87.3	1.81	2.29	0.48	1.76	-0.53	-110	27	1.31	1.99	0.68	1.40	-0.59	-87	-24	21	14
B87.4	2.32	3.62	1.30	2.24	-1.38	-106	35	1.90	3.20	1.30	2.09	-1.11	-85	-21	20	17
B89.3	1.20	2.12	0.92	1.12	-1.00	-109	13	0.86	1.97	1.11	1.04	-0.93	-84	-25	23	14
B89.4	1.02	4.50	3.48	1.03	-3.47	-100	22	0.57	2.63	2.06	0.90	-1.73	-84	-16	16	20
Mean	**1.59**	**3.13**	**1.55**	**1.54**	**-1.60**	**-106**	**24**	**1.16**	**2.45**	**1.29**	**1.36**	**-1.09**	**-85**	**-21**	**20**	**16**
SD	**0.59**	**1.13**	**1.33**	**0.57**	**1.30**	**5**	**9**	**0.58**	**0.59**	**0.58**	**0.53**	**0.48**	**1**	**4**	**3**	**3**
SEM	**0.30**	**0.57**	**0.67**	**0.29**	**0.65**	**2**	**5**	**0.29**	**0.29**	**0.29**	**0.27**	**0.24**	**1**	**2**	**2**	**1**
Statistics																
post vs. pre; *p* via paired t-test (2-tail)								**0.001**		**0.56**		**0.31**	**0.002**			**0.17**
Denuded vs. control; *p* via t-test (2-tail, unpaired)						**0.77**		**0.74**		**0.35**		**0.59**	**0.01**	**0.09**	**0.05**	**0.14**

7.3.1 Raw Data: Effect of removing the epithelium on relaxation to 10 µM Isoprenaline from active tension to MCh (experimental preps).														
Experimental	**Epithelium**							**Epithelium Denuded**						
Prep	**Baseline pre MCh (gf)**	**Max MCh Plateau (gf)**	**MCh contraction (gf)**	**Iso BL post MCh (gf)**	**Iso relaxation post MCh (gf)**	**Iso relaxation (% of MCh)**	**Time to Iso plateau post MCh (min)**	**Baseline pre MCh (gf)**	**Max MCh Plateau (gf)**	**MCh contraction (gf)**	**Iso BL post MCh (gf)**	**Iso relaxation post MCh (gf)**	**Iso relaxation (% of MCh)**	**Time to Iso plateau post MCh (min)**
B87.1	1.73	3.10	1.37	1.30	-1.80	-131	3	0.99	2.72	1.73	0.99	-1.73	-100	2
B87.2	1.84	3.89	2.05	1.93	-1.96	-96	3	1.20	3.78	2.58	1.54	-2.24	-87	2
B89.1	1.09	3.09	2.00	1.93	-1.16	-58	3	0.69	2.83	2.14	1.69	-1.14	-53	2
B89.2	1.47	3.09	1.62	1.46	-1.63	-101	2	1.31	2.01	0.70	1.34	-0.67	-96	1
Mean	**1.53**	**3.29**	**1.76**	**1.66**	**-1.64**	**-96**	**3**	**1.05**	**2.84**	**1.79**	**1.39**	**-1.45**	**-84**	**2**
SD	**0.33**	**0.40**	**0.32**	**0.32**	**0.35**	**30**	**1**	**0.27**	**0.73**	**0.80**	**0.30**	**0.68**	**21**	**1**
SEM	**0.17**	**0.20**	**0.16**	**0.16**	**0.17**	**15**	**0**	**0.14**	**0.36**	**0.40**	**0.15**	**0.34**	**11**	**0**
Statistics														
pre vs. post denudation; *p* via paired t-test (2-tail)												**0.52**	**0.15**	

7.3.1 Raw Data: Effect of removing the epithelium on relaxation to 10 μM Isoprenaline from active tension to MCh (control preps).														
Controls	**Pre-Control**							**Post-Control**						
Prep	**Baseline pre MCh (gf)**	**Max MCh Plateau (gf)**	**MCh contraction (gf)**	**Iso BL post MCh (gf)**	**Iso relaxation post MCh (gf)**	**Iso relaxation (% of MCh)**	**Time to Iso plateau post MCh (min)**	**Baseline pre MCh (gf)**	**Max MCh Plateau (gf)**	**MCh contraction (gf)**	**Iso BL post MCh (gf)**	**Iso relaxation post MCh (gf)**	**Iso relaxation (% of MCh)**	**Time to Iso plateau post MCh (min)**
B87.3	1.81	2.29	0.48	1.87	-0.42	-88	4	1.31	1.99	0.68	1.29	-0.70	-103	2
B87.4	2.32	3.62	1.30	2.47	-1.15	-88	3	1.90	3.20	1.30	1.93	-1.27	-98	3
B89.3	1.20	2.12	0.92	1.62	-0.50	-54	1	0.86	1.97	1.11	1.48	-0.49	-44	1
B89.4	1.02	4.50	3.48	1.64	-2.86	-82	3	0.57	2.63	2.06	1.22	-1.41	-68	6
Mean	**1.59**	**3.13**	**1.55**	**1.90**	**-1.23**	**-78**	**3**	**1.16**	**2.45**	**1.29**	**1.48**	**-0.97**	**-78**	**3**
SD	**0.59**	**1.13**	**1.33**	**0.40**	**1.13**	**16**	**1**	**0.58**	**0.59**	**0.58**	**0.32**	**0.44**	**27**	**2**
SEM	**0.30**	**0.57**	**0.67**	**0.20**	**0.57**	**8**	**1**	**0.29**	**0.29**	**0.29**	**0.16**	**0.22**	**14**	**1**
Statistics														
pre vs. post; *p* via paired t-test												**0.55**	**0.98**	**0.82**
Denuded vs. control; *p* via t-test (2-tail, unpaired)												**0.29**	**0.76**	**0.30**

7.3.2 Raw data: Maximum relaxation to hypoxia and SNP from active tension															
	HBD							SNP							
Prep	BL pre MCh (gf)	MCh Plateau (gf)	MCh (gf)	HBD BL post MCh (gf)	HBD post MCh (gf)	HBD post MCh (% MCh)	Time to HBD (min)	BL pre MCh (gf)	MCh Plateau (gf)	MCh (gf)	SNP BL post MCh (gf)	SNP Relaxation post MCh (gf)	SNP Relaxation post MCh (% MCh)	Time to relaxation (min)	% Difference (SNP vs. HBD)
B37.1	2.03	2.66	0.63	1.92	-0.74	-117	29	1.22	1.77	0.55	1.72	-0.05	-9	1	8
B37.2	2.11	3.25	1.14	1.26	-1.99	-175	34	1.61	2.44	0.83	2.34	-0.1	-12	2	7
B38.1	1.55	9.37	7.82	1.07	-8.3	-106	40	0.42	2.45	2.03	1.88	-0.57	-28	7	26
B38.2	1.5	7.66	6.16	0.95	-6.71	-109	40	0.52	2.91	2.39	1.56	-1.35	-56	4	52
B57.2	2.85	7.51	4.66	1.37	-6.14	-132	16	2.85	7.51	4.66	3.82	-3.69	-79	1	60
Mean	**2.01**	**6.09**	**4.08**	**1.31**	**-4.78**	**-128**	**31.8**	**1.32**	**3.42**	**2.09**	**2.26**	**-1.15**	**-37**	**3.0**	**31**
SD	**0.54**	**2.96**	**3.13**	**0.38**	**3.24**	**28**	**10.0**	**0.99**	**2.32**	**1.63**	**0.92**	**1.51**	**30**	**2.5**	**25**
t-test (paired, 2-tail) HBD vs. SNP													**0.01**		

7.3.3.1 Raw data: Effect of receptor antagonists and enzyme inhibitors on resting baseline tension

Prep	Drug [µM]	Baseline post wash	Post-Antagonist		Change in baseline (%)
			Baseline post antagonist	Change in baseline (gf)	
B29.2	**L-NAME [1000]**	1.35	1.27	-0.08	-6
B92.2	**L-NAME [1000]**	0.56	0.76	0.2	36
	Mean	**0.96**	**1.02**	**0.06**	**15**
	SD	**0.56**	**0.36**	**0.20**	**29**
	t-test (paired, 2-tail)				**0.74**
B34.1	**Labetalol [100]**	2.25	2.17	-0.08	-4
B58.1	**Sn-PP [100]**	1.04	0.85	-0.19	-18
B58.2	**Sn-PP [100]**	2.04	1.4	-0.64	-31
	Mean	**1.54**	**1.13**	**-0.42**	**-25**
	SD	**0.71**	**0.39**	**0.32**	**9**
	t-test (paired, 2-tail)				**0.32**

7.3.3.1 Raw data: Effects of receptor antagonists and enzyme inhibitors on cholinergic contraction								
		Pre-Antagonist			Post-Antagonist			
Prep	Drug [μM]	BL pre MCh (gf)	MCh Plateau (gf)	MCh Active Tension (gf)	BL pre MCh (gf)	MCh Plateau (gf)	MCh Active Tension (gf)	% Change in MCh vs. pre antagonist
B34.1	**Labetalol [100]**	2.14	4.44	2.3	2.18	4.07	1.89	-18
B30.1	**Sitaxsentan [100]**	4.75	7.01	2.26	4.75	7.01	2.26	0
B33.1	**Indomethacin [10]**	2.53	4.75	2.22	2.58	4.79	2.21	0
B33.2	**Indomethacin [10]**	2.24	6.27	4.03	2.66	6.87	4.21	4
	Mean	**2.39**	**5.51**	**3.13**	2.62	**5.83**	**3.21**	**2**
	SD	**0.21**	**1.07**	**1.28**	**0.06**	**1.47**	**1.41**	**3**
	t-test (paired, 2-tail)						**0.54**	
B29.2	**L-NAME [1000]**	1.51	2.49	0.98	1.27	1.94	0.67	-32
B92.2	**L-NAME [1000]**	0.94	3.15	2.21	0.76	3.62	2.86	29
	Mean	**1.23**	**2.82**	**1.60**	1.02	2.78	1.77	-1
	SD	**0.40**	**0.47**	**0.87**	**0.36**	**1.19**	**1.55**	**43**
	t-test (paired, 2-tail)						**0.78**	
B58.1	**Sn-PP [100]**	1.43	4.86	3.43	1.2	3.08	1.88	-45
B58.2	**Sn-PP [100]**	2.01	3.89	1.88	1.05	2.93	1.88	0
	Mean	**1.72**	**4.38**	**2.66**	**1.13**	**3.01**	**1.88**	**-23**
	SD	**0.41**	**0.69**	**1.10**	**0**	**0.11**	**0**	**32**
	t-test (paired, 2-tail)						**1**	

7.3.3.1 Raw data: Effects of receptor antagonists and enzyme inhibitors on HBD and β-adrenergic relaxation from active tension															
		Pre-Antagonist						Post-Antagonist							
Prep	Drug [μM]	MCh (gf)	HBD BL post MCh (gf)	HBD post MCh (gf)	HBD post MCh (% MCh)	Post Iso BL (gf)	Iso dilation (% MCh)	MCh (gf)	HBD BL post MCh (gf)	HBD post MCh (gf)	HBD post MCh (% MCh)	% change vs. pre antag	Post Iso BL (gf)	Iso dilation (% MCh)	% change vs. pre antag
B34.1	**Labetalol [100]**	2.30	1.9	-2.54	-110	2.8	-71	1.89	1.99	-2.08	-110	0	4.02	-3	-96
B33.1	**Indomethacin [10]**	2.22	1.59	-3.16	-142			2.21	1.61	-3.18	-144	1			
B33.2	**Indomethacin [10]**	4.03	1.65	-4.62	-115			4.21	1.73	-5.14	-122	6			
	Mean	**3.13**	**1.62**	**-3.89**	**-128**			**3.21**	**1.67**	**-4.16**	**-133**	**4**			
	SD	**1.28**	**0.04**	**1.03**	**20**			**1.41**	**0.08**	**1.39**	**15**	**4**			
	t-test (paired, 2-tail)							**0.54**			**0.37**				
B29.2	**L-NAME [1000]**	0.98	1.17	-1.32	-135			0.67	1.20	-0.74	-110	-18			
B92.2	**L-NAME [1000]**	2.21	0.92	-2.23	-101	1.14	-91	2.86	1.31	-2.31	-81	-20	0.85	-97	6
	Mean	**1.60**	**1.05**	**-1.78**	**-118**	**1.14**	**-91**	**1.77**	**1.26**	**-1.53**	**-96**	**-19**	**0.85**	**-97**	**6**
	SD	**0.87**	**0.18**	**0.64**	**24**			**1.55**	**0.08**	**1.11**	**21**	**1**			
	t-test (paired, 2-tail)							**0.78**			**0.06**				
B58.1	**Sn-PP [100]**	3.43	1.48	-3.38	-99	0.94	-114	1.88	1.07	-2.01	-107	8	0.66	-129	13
B58.2	**Sn-PP [100]**	1.88	1.30	-2.59	-138	1.54	-125	1.88	1.56	-1.37	-73	-47	0.85	-111	-11
	Mean	**2.66**	**1.39**	**-2.99**	**-118**	**1.24**	**-120**	**1.88**	**1.32**	**-1.69**	**-90**	**-19**	**0.76**	**-120**	**1**
	SD	**1.10**	**0.13**	**0.56**	**28**	**0.42**	**8**	**0**	**0.35**	**0.45**	**24**	**39**	**0.13**	**13**	**17**
	t-test (paired, 2-tail)							**0.5**			**0.58**				

7.3.3.3.2 Raw data: Effects of intracellular antagonists on HBD from resting tension										
		Pre- Antagonist				Post-Antagonist				
Prep	Drug [µM]	Baseline pre hypoxia (gf)	Baseline post hypoxia	Hypoxic Dilation from baseline (gf)	Hypoxic Dilation from baseline (% Change)	Baseline post antagonist	Baseline post hypoxia	Hypoxic Dilation from baseline (gf)	Hypoxic Dilation from baseline (% Change)	% Change vs pre antagonist
B88.1	**2-APB [50]**	1.53	1.01	-0.52	-34	1.29	1.10	-0.19	-15	-63
B88.2	**2-APB [50]**	1.04	0.64	-0.40	-38	1.24	0.78	-0.46	-37	15
	Mean	**1.29**	**0.83**	**-0.46**	**-36**	**1.27**	**0.94**	**-0.33**	**-26**	**-24**
	SD	**0.35**	**0.26**	**0.08**	**3.16**	**0.04**	**0.23**	**0.19**	**16**	**55**
	t-test (paired, 2-tail)									**0.45**
B57.1	**KT 5720 [0.2]**	2.88	0.67	-2.21	-77	2.36	0.83	-1.53	-65	-31
B57.2	**KT 5823 [0.4]**	2.52	0.92	-1.60	-63	2.7	1.17	-1.53	-57	-4
B30.1	**Methylene Blue [50]**	2.55	1.68	-0.87	-34	2.57	1.79	-0.78	-30	-10
B30.2	**Methylene Blue [50]**	2.94	1.64	-1.3	-44	2.88	1.65	-1.23	-43	-5
	Mean	**2.75**	**1.66**	**-1.09**	**-39**	**2.73**	**1.72**	**-1.01**	**-37**	**-8**
	SD	**0.28**	**0.03**	**0.30**	**7**	**0.22**	**0.10**	**0.32**	**9**	**4**
	t-test (paired, 2-tail)									**0.26**
B56.1	**Ryanodine [0.01]**	2.35	0.64	-1.71	-73	1.71	0.93	-0.78	-46	-54
B56.2	**Ryanodine [10]**	3.22	0.94	-2.28	-71	2.48	1.15	-1.33	-54	-42

7.3.3.2 Raw data: Effect of intracellular antagonists on resting baseline tension					
			Post-Antagonist		
Prep	Drug [µM]	Baseline post wash	Baseline post antagonist	Change in baseline (gf)	Change in baseline (%)
B88.1	**2-APB [50]**	1.56	1.29	-0.27	-17
B88.2	**2-APB [50]**	2.25	1.24	-1.01	-45
	Mean	**1.91**	**1.27**	**-0.64**	**-31**
	SD	**0.49**	**0.04**	**0.52**	**20**
	t-test (paired, 2-tail)			**0.17**	
B57.1	**KT 5720 [0.2]**	2.36	2.81	0.45	19
B57.2	**KT 5823 [0.4]**	2.7	3	0.3	11
B30.1	**Methylene Blue [50]**	2.44	2.57	0.13	5
B30.2	**Methylene Blue [50]**	3.03	2.88	-0.15	-5
	Mean	**2.74**	**2.73**	**-0.01**	**0**
	SD	**0.42**	**0.22**	**0.20**	**7**
	t-test (paired, 2-tail)			**0.48**	
B38.1	**Rp-8-Br-cGMPs [10]**	0.48	0.43	-0.05	-10
B38.2	**Rp-8-Br-cGMPs [10]**	0.53	0.52	-0.01	-2
B36.1	**Rp-8-Br-cGMPs [10]**	1.49	1.52	0.03	2
	Mean	**0.83**	**0.82**	**-0.01**	**-3**
	SD	**0.57**	**0.61**	**0.04**	**6**
	t-test (paired, 2-tail)			**0.71**	
B56.1	**Ryanodine [0.01]**	1.30	1.71	0.41	32
B56.2	**Ryanodine [10]**	2.76	2.48	-0.28	-10

7.3.3.2 Raw data: Effects of intracellular antagonists on cholinergic contraction								
		Pre-Antagonist			Post-Antagonist			
Prep	Drug [μM]	BL pre MCh (gf)	MCh Plateau (gf)	MCh Active Tension (gf)	BL pre MCh (gf)	MCh Plateau (gf)	MCh Active Tension (gf)	% Change in MCh vs. pre antagonist
B88.1	**2-APB [50]**	1.43	2.10	0.67	1.22	1.65	0.43	-36
B88.2	**2-APB [50]**	0.97	3.13	2.16	0.69	2.31	1.62	-25
	Mean	**1.20**	**2.62**	**1.42**	**0.96**	**1.98**	**1.03**	**-30**
	SD	**0.33**	**0.73**	**1.05**	**0.37**	**0.47**	**0.84**	**8**
	t-test (paired, 2-tail)					**0.18**	**0.24**	
B57.1	**KT 5720 [0.2]**	2.62	7.14	4.52	2.58	7.35	4.77	6
B57.2	**KT 5823 [0.4]**	2.37	7.25	4.88	2.78	7.51	4.73	-3
B30.1	**Methylene Blue [50]**	4.75	7.02	2.27	2.31	3.32	1.01	-56
B30.2	**Methylene Blue [50]**	3.69	5.69	2.00	2.58	2.94	0.36	-82
	Mean	**4.22**	**6.36**	**2.14**	**2.45**	**3.13**	**0.69**	**-69**
	SD	**0.75**	**0.94**	**0.19**	**0.19**	**0.27**	**0.46**	**19**
	t-test (paired, 2-tail)					**0.10**	**0.08**	
B38.1	**Rp-8-Br-cGMPs [10]**	1.54	9.39	7.85	0.43	3.24	2.81	-64
B38.2	**Rp-8-Br-cGMPs [10]**	1.50	7.69	6.19	0.52	3.05	2.53	-59
B36.1	**Rp-8-Br-cGMPs [10]**	1.27	2.09	0.82	1.52	2.52	1.00	22
	Mean	**1.44**	**6.39**	**4.95**	**0.82**	**2.94**	**2.11**	**-34**
	SD	**0.15**	**3.82**	**3.67**	**0.61**	**0.37**	**0.97**	**48**
	t-test (paired, 2-tail)						**0.21**	
B56.1	**Ryanodine [0.01]**	2.91	5.08	2.17	1.72	3.91	2.19	1
B56.2	**Ryanodine [10]**	3.74	6.18	2.44	2.37	4.03	1.66	-32

7.3.3.2 Raw data: Effects of intracellular antagonists on HBD, β-adrenergic relaxation and relaxation to SNP from active tension															
		Pre-Antagonist						Post-Antagonist							
Prep	Drug [µM]	MCh (gf)	HBD BL post MCh (gf)	HBD post MCh (gf)	HBD post MCh (% MCh)	Post Iso/SNP BL (gf)	Iso/SNP dilation (% MCh)	MCh (gf)	HBD BL post MCh (gf)	HBD post MCh (gf)	HBD post MCh (% MCh)	% change vs. pre antag	Post Iso/SNP BL (gf)	Iso/SNP dilation (% MCh)	% change vs. pre antag
B88.1	**2-APB [50]**	0.67	1.26	-0.84	-125			0.43	1.24	-0.41	-95	-24			
B88.2	**2-APB [50]**	2.16	1.60	-1.53	-71			1.62	1.04	-1.27	-78	11			
	Mean	**1.42**	**1.43**	**-1.19**	**-98**			**1.03**	**1.14**	**-0.84**	**-87**	**-7**			
	SD	**1.05**	**0.24**	**0.49**	**39**			**0.84**	**0.14**	**0.61**	**12**	**24**			
	t-test (paired, 2-tail)							**0.24**	**0.48**	**0.16**	**0.66**				
B57.1	**KT 5720 [0.2]**	4.52	1.01	-6.13	-136	4.27	-63	4.77	1.29	-6.06	**-127**	**-6**	**3.09**	-89	41
B57.2	**KT 5823 [0.4]**	4.88	1.26	-5.99	-123	5.44	-37	4.73	1.37	-6.14	-130	6	4.18	-70	90
B30.1	**Methylene Blue [50]**	2.27	2.05	-4.97	-219			1.01	1.88	-1.44	-143	**-35**			
B30.2	**Methylene Blue [50]**	2.00	1.89	-3.80	-190			0.36	1.78	-1.16	-322	70			
	Mean	**2.14**	**1.97**	**-4.39**	**-204**			**0.69**	**1.83**	**-1.30**	**-232**	**17**			
	SD	**0.19**	**0.11**	**0.83**	**20**			**0.46**	**0.07**	**0.20**	**127**	**74**			
	t-test (paired, 2-tail)							**0.08**	**0.14**	**0.18**	**0.84**				

7.3.3.2 Raw data: Effects of intracellular antagonists on HBD, β-adrenergic relaxation and relaxation to SNP from active tension. Cont.															
		Pre-Antagonist						Post-Antagonist							
Prep	Drug [µM]	MCh (gf)	HBD BL post MCh (gf)	HBD post MCh (gf)	HBD post MCh (% MCh)	Post Iso/SNP BL (gf)	Iso/SNP dilation (% MCh)	MCh (gf)	HBD BL post MCh (gf)	HBD post MCh (gf)	HBD post MCh (% MCh)	% change vs. pre antag	Post Iso/SNP BL (gf)	Iso/SNP dilation (% MCh)	% change vs. pre antag
B38.1	**Rp-8-Br-cGMPs [10]**	7.85	1.08	-8.31	-106	1.89	-27	2.81	0.34	-2.90	-103	**-3**	**1.59**	**-59**	**119**
B38.2	**Rp-8-Br-cGMPs [10]**	6.19	0.96	-6.73	-109	1.77	-49	2.53	0.44	-2.61	-103	-5	1.35	-67	37
B36.1	**Rp-8-Br-cGMPs [10]**	0.82	1.41	-0.68	-83			1.00	1.23	-1.29	-129	56			
	Mean	**4.95**	**1.15**	**-5.24**	**-99**	**1.83**	**-38**	**2.11**	**0.67**	**-2.27**	**-112**	**16**	**1.47**	**-63**	**78**
	SD	**3.67**	**0.23**	**4.03**	**14**	**0.08**	**16**	**0.97**	**0.49**	**0.86**	15	**34**	**0.17**	**6**	**58**
	t-test (paired, 2-tail)							**0.21**							**0.17**
B56.1	**Ryanodine [0.01]**	2.17	0.88	-4.20	-194	0.99	-188	2.19	1.31	-2.60	-119	-39	0.80	-142	-25
B56.2	**Ryanodine [10]**	2.44	1.38	-4.80	-197	1.53	-191	1.66	1.74	-2.29	-138	-30	0.93	-187	-2

Chapter 8: Mechanism of Hypoxic Bronchodilation: Role of the Potassium Channels and Intra and Extra-Cellular Calcium

8.3.1 Raw data: HBD and Isoprenaline relaxation data post contraction to MCh [1mM] and KCl [100mM]

Prep	Baseline pre MCh (gf)	Max MCh Plateau (gf)	MCh contraction (gf)	HBD BL post MCh	Time to HBD plateau post MCh (min)	HBD post MCh (gf)	HBD (% MCh)	Iso BL post MCh	Iso dilation post MCh (gf)	Iso dilation (% MCh)	Time to Iso plateau post MCh (s)
B39.1	1.30	9.10	7.80	1.58	19	-7.52	-96	5.81	-3.29	-42	720
B39.2	0.87	6.87	6.00	1.25	17	-5.62	-94	4.23	-2.64	-44	840
B48	6.20	12.30	6.10	2.26	40	-10.04	-165	5.35	-6.95	-114	270
B53	2.38	5.00	2.62	1.41	26	-3.59	-137	1.27	-3.73	-142	240
B82.1	1.90	4.50	2.60	1.40	50	-3.10	-119				
B82.2	1.16	3.33	2.17	1.30	20	-2.03	-94				
B83.1	0.95	3.44	2.49	0.97	31	-2.47	-99	1.09	-2.35	-94	180
B83.2	2.17	3.23	1.06	0.82	33	-2.41	-227	0.72	-2.51	-237	270
Mean	**2.12**	**5.97**	**3.86**	**1.37**	**30**	**-4.60**	**-129**	**3.08**	**-3.58**	**-112**	**420**
SD	**1.74**	**3.27**	**2.41**	**0.43**	**11**	**2.89**	**47**	**2.31**	**1.73**	**73**	**283**
SEM	**0.62**	**1.15**	**0.85**	**0.15**	**4**	**1.02**	**17**	**0.82**	**0.61**	**26**	**100**

8.3.1 Continued. Raw data: HBD and Isoprenaline relaxation data post contraction to MCh [1mM] and KCl [100mM]											
Prep	**Baseline pre KCl (100mM) (gf)**	**Max KCl Plateau (gf)**	**KCl contraction (gf)**	**HBD BL post KCl**	**Time to HBD plateau post KCl (min)**	**HBD post KCl (gf)**	**HBD (% KCl)**	**Iso BL post KCl**	**Iso dilation post KCl (gf)**	**Iso dilation (% KCl)**	**Time to Iso plateau post KCl (s)**
B39.1	1.50	8.15	6.65	2.46	120	-5.69	-86	6.24	-1.91	-29	300
B39.2	1.00	9.13	8.13	1.87	120	-7.26	-89	6.25	-2.88	-35	450
B48	3.26	12.43	9.17	3.01	157	-9.42	-103	8.64	-3.79	-41	360
B53	1.25	6.30	5.05	3.04	90	-3.26	-65	3.02	-3.28	-65	270
B82.1	2.75	4.25	1.50	2.31	20	-1.94	-129				
B82.2	1.59	3.76	2.17	1.59	87	-2.17	-100				
B83.1	2.46	3.76	1.30	2.04	76	-1.72	-132	2.96	-0.80	-62	330
B83.2	0.53	3.04	2.51	1.69	41	-1.35	-54	2.46	-0.58	-23	360
Mean	**1.79**	**6.35**	**4.56**	**2.25**	**89**	**-4.10**	**-95**	**4.93**	**-2.21**	**-43**	**345**
SD	**0.94**	**3.31**	**3.13**	**0.56**	**44**	**3.00**	**28**	**2.48**	**1.33**	**17**	**62**
SEM	**0.33**	**1.17**	**1.11**	**0.20**	**16**	**1.06**	**10**	**0.88**	**0.47**	**6**	**22**
Statistics	**Magnitude of KCl vs MCh**		**Magnitude of iso dilation Post MCh /KCl (%)**		**Time to max iso dilation post MCh/KCl**		**Magnitude of HBD post MCh/KCl (%)**		**HBD time post MCh/KCl**		
***P* via paired t-test (2-tail)**	**0.30**		**0.08**		**0.51**		**0.19**		**0.01**		

8.3.2.1 Raw data: Effect of Ca^{2+} free buffer on cholinergic contraction and relaxation to hypoxia and isoprenaline												
	Controls											
Prep	BL pre MCh (gf)	MCh Plateau (gf)	MCh contraction (gf)	HBD BL post MCh (gf)	HBD post MCh (gf)	HBD post MCh (%)	Post Iso BL (gf)	Iso dilation (% MCh)				
B59.1	2.12	10.72	8.6	3.12	-7.6	-88	2.19	-99.19				
B59.2	2.4	4.12	1.72	0.92	-3.2	-186	2.98	-66.28				
Mean	**2.26**	**7.42**	**5.16**	**2.02**	**-5.40**	**-137**	**2.59**	**-82.73**				
SD	**0.20**	**4.67**	**4.86**	**1.56**	**3.11**	**69**	**0.56**	**23.27**				
	Calcium free											
Prep	BL pre MCh (gf)	MCh Plateau (gf)	MCh contraction (gf)	% Change in MCh control vs. Ca^{2+} free	HBD BL post MCh (gf)	HBD post MCh (gf)	HBD post MCh (%)	% change: control vs. Ca^{2+} free	Post Iso BL (gf)	Iso dilation (%)	% change: control vs. Ca^{2+} free	
B59.1	1.88	2.37	0.49	-94	1.95	-0.42	-86	-3	1.85	-106	7	
B59.2	1.41	3.05	1.64	-5	0.84	-2.21	-135	-28	1.6	-88	33	
Mean		**2.71**	**1.07**	**-49**	**1.40**	**-1.32**	**-110**	**-15**	**1.73**	**-97**	**20**	
SD		**0.48**	**0.81**	**63**	**0.78**	**1.27**	**35**	**17**	**0.18**	**13**	**19**	

8.3.2.1 Raw data: Contraction of bronchi to 80 mM $CaCl_2$							
Prep	BL pre $CaCl_2$ (gf)	$CaCl_2$ Plateau (gf)	$CaCl_2$ active tension (gf)	HBD BL post $CaCl_2$ (gf)	HBD post $CaCl_2$ (gf)	HBD post $CaCl_2$ (% $CaCl_2$)	$CaCl_2$ active tension: % of control MCh
B59.1	1.86	3.72	1.86	3.18	-0.54	-29	22
B59.2	1.36	3.25	1.89	1.64	-1.61	-85	110
Mean	**1.61**	**3.49**	**1.88**	**2.41**	**-1.08**	**-57**	**66**
SD	**0.35**	**0.33**	**0.02**	**1.09**	**0.76**	**40**	**62**

8.3.2.2 Raw data: HBD and iso relaxation post contraction of bronchi to 10 µM thapsigargin																	
	Pre thapsigargin								Post thapsigargin								
Prep	**BL pre MCh (gf)**	**MCh Plateau (gf)**	**MCh (gf)**	**HBD BL post MCh (gf)**	**HBD post MCh (gf)**	**HBD post MCh (% MCh)**	**Post Iso BL (gf)**	**Iso dilation (% MCh)**	**BL pre Thap (gf)**	**Thap Plateau (gf)**	**Active tension to Thap (gf)**	**% of max MCh**	**HBD BL post Thap (gf)**	**HBD post Thap (gf)**	**HBD post Thap (%)**	**Iso BL (gf)**	**Iso relaxation (%)**
B90.2	0.41	4.84	4.43	1.00	-3.84	-87	0.97	-87	0.5	4.76	4.26	96	0.65	-4.11	-96	0.51	-100
B91.1	1.93	2.75	0.82	1.40	-1.35	-165	1.12	-199	1.74	2.31	0.57	70	1.29	-1.02	-179	0.89	-249
B91.3	**1.56**	**6.02**	**4.46**	**2.44**	**-3.58**	**-80**	**1.03**	**-112**	**1.22**	**5.27**	**4.05**	**91**	**1.78**	**-3.49**	**-86**	**0.99**	**-106**
Mean	**1.30**	**4.54**	**3.24**	**1.61**	**-2.92**	**-111**	**1.04**	**-133**	**1.15**	**4.11**	**2.96**	**85**	**1.24**	**-2.87**	**-121**	**0.80**	**-152**
SD	**0.79**	**1.66**	**2.09**	**0.74**	**1.37**	**47**	**0.08**	**59**	**0.62**	**1.58**	**2.07**	**14**	**0.57**	**1.63**	**51**	**0.25**	**85**
Paired t-test (2-tail) vs. pre thapsigargin										**0.07**	**0.06**				**0.05**		**0.37**

8.3.2.2 Raw data: HBD and iso relaxation post contraction of bronchi to 5 and 10 µM Ouabain

	Pre ouabain								Post Ouabain									
Prep	**BL pre MCh (gf)**	**MCh Plateau (gf)**	**MCh (gf)**	**HBD BL post MCh (gf)**	**HBD post MCh (gf)**	**HBD post MCh % MCh**	**Post Iso BL (gf)**	**Iso dilation (% MCh)**	**BL pre Ouabain (gf)**	**Ouabain Plateau (gf)**	**Active tension to Ouabain (gf)**	**BL Δ (%)**	**% of max MCh**	**HBD BL post Ouabain (gf)**	**HBD post Ouabain (gf)**	**HBD post Ouabain (%)**	**Iso BL (gf)**	**Iso relaxation (%)**
B50.1 [5µM]	3.56	8	4.44	3.21	-4.79	-108			2.35	5.87	3.52	150	79	1.76	-4.11	-117	2.17	-105
B51.1 [5µM]	1.51	6.38	4.87	2.03	-4.35	-89	1.39	-102	0.88	3.31	2.43	276	50	1.64	-1.67	-69	2.19	-46
Mean	**2.54**	**7.19**	**4.66**	**2.62**	**-4.57**	**-99**	**1.39**	**-102**	**1.62**	**4.59**	**2.98**	**213**	**65**	**1.70**	**-2.89**	**-93**	**2.18**	**-76**
SD	**1.45**	**1.15**	**0.30**	**0.83**	**0.31**	**13**			**1.04**	**1.81**	**0.77**	**89**	**21**	**0.08**	**1.73**	**34**	**0.01**	**42**
Paired t-test (2-tail) vs. pre ouabain											**0.06**	**0.06**						
B50.2 [10µM]	1.14	6.67	5.53	1.15	-5.52	-100	0.88	-105	0.39	4.17	3.78	969	68	2.84	-1.33	-35	1.73	-65

8.3.2.2 Raw data: Time to max HBD post MCh and post Thapsigargin

Prep	Time to max HBD post MCh (min)	Time to max HBD post Thapsigargin (min)
B90.2	49	21
B91.1	25	20
B91.3	42	36
Mean	**39**	**26**
SD	**12**	**9**
2-tail paired t-test. MCh vs. thapsigargin		**0.23**

8.3.2.2 Raw data: Time to max contraction to ouabain and time to HBD post MCh and post ouabain (5 µM)

Prep	Time to max contraction (min)	Time to max HBD post MCh (min)	Time to max HBD post Ouabain (min)
B50.1 [5µM]	90	32	35
B51.1 [5µM]	90	67	32
Mean	**90**	**50**	**34**
SD	**0**	**25**	**2**
2-tail paired t-test. MCh vs. ouabain			**0.55**

8.3.3 Raw data: Effects of potassium channel antagonists on HBD from resting tension										
		Pre- Antagonist				Post-Antagonist				
Prep	Drug [µM]	Baseline pre hypoxia (gf)	Baseline post hypoxia	Hypoxic Dilation from baseline (gf)	Hypoxic Dilation from baseline (% Change)	Baseline post antagonist	Baseline post hypoxia	Hypoxic Dilation from baseline (gf)	Hypoxic Dilation from baseline (% Change)	% Change vs pre antagonist
B66.1	**TRAM 34 [0.2]**	**2.65**	1.35	-1.30	-49	2.16	0.82	-1.34	-62	3
B66.2	**TRAM 34 [0.2]**	**2.16**	0.82	-1.34	-62	1.44	0.87	-0.57	-40	-57
B92.1	**TRAM 34 [0.2]**	**1.3**	**0.60**	**-0.70**	**-54**	**1.73**	**0.89**	**-0.84**	**-49**	**-10**
	Mean	**2.04**	**0.92**	**-1.11**	**-55**	**1.78**	**0.86**	**-0.92**	**-50**	**-7**
	SD	**0.68**	**0.39**	**0.36**	**7**	**0.36**	**0.04**	**0.39**	**11**	**31**
	t-test (paired, 2-tail)								**0.68**	**0.57**
B72.1	**TEA [1000]**	**2.15**	2.00	-0.15	-6.98	1.01	0.93	-0.08	-7.92	-46.67
B72.2	**TEA [1000]**	**2.12**	1.54	-0.58	-27.36	1.87	1.35	-0.52	-27.81	-10.34
	Mean		**1.77**	**-0.37**	**-17.17**	**1.44**	**1.14**	**-0.30**	**-17.86**	**-28.51**
	SD		**0.33**	**0.30**	**14.41**	**0.61**	**0.30**	**0.31**	**14.06**	**25.68**
	t-test (paired, 2-tail)								**0.22**	

8.3.3 Raw data: Effects of potassium and calcium antagonists on cholinergic contraction								
		Pre-Antagonist			Post-Antagonist			
Prep	Drug [µM]	BL pre MCh (gf)	MCh Plateau (gf)	MCh Contraction (gf)	BL pre MCh (gf)	MCh Plateau (gf)	MCh Contraction (gf)	% Change in MCh vs. pre antagonist
B37.1	**Nifedipine [20]**	2.02	2.65	0.63	1.28	1.83	0.55	-13
B37.2	**Nifedipine [20]**	2.12	3.24	1.12	1.17	2.12	0.95	-15
	Mean	**2.07**	**2.95**	**0.88**	**1.23**	**1.98**	**0.75**	**-14**
	SD	**0.07**	**0.42**	**0.35**	**0.08**	**0.21**	**0.28**	**2**
	t-test (paired, 2-tail)						**0.22**	
B40.1	**Iberiotoxin [0.1]**	1.77	11.24	9.47	2.50	10.25	7.75	-18
B40.2	**Iberiotoxin [0.1]**	1.97	7.02	5.05	4.00	6.78	2.78	-45
	Mean	**1.87**	**9.13**	**7.26**	3.25	8.52	5.27	-32
	SD	**0.14**	**2.98**	**3.13**	**1.06**	**2.45**	**3.51**	**19**
	t-test (paired, 2-tail)						**0.09**	
B41.1	**Apamin [0.3]**	2.36	12.49	10.13	2.36	8.97	6.61	-35
B41.2	**Apamin [0.3]**	2.26	6.97	4.71	1.35	5.48	4.13	-12
	Mean	**2.31**	**9.73**	**7.42**	1.86	7.23	5.37	-24
	SD	**0.07**	**3.90**	**3.83**	**0.71**	**2.47**	**1.75**	**16**
	t-Test (paired, 2-tail)						**0.40**	
B40.1	**Glibenclamide [0.1]**	2.5	10.25	7.75	2.06	6.20	4.14	-47
B40.2	**Glibenclamide [0.1]**	4	6.78	2.78	2.31	3.85	1.54	-45
	Mean	**3.25**	**8.52**	**5.27**	**2.19**	**5.03**	**2.84**	**-46**
	SD	**1.06**	**2.45**	**3.51**	**0.18**	**1.66**	**1.84**	**1**
	t-test (paired, 2-tail)						**0.29**	

8.3.3 Raw data: Effects of potassium and calcium antagonists on cholinergic contraction. Cont.								
		Pre-Antagonist			Post-Antagonist			
Prep	Drug [µM]	BL pre MCh (gf)	MCh Plateau (gf)	MCh Contraction (gf)	BL pre MCh (gf)	MCh Plateau (gf)	MCh Contraction (gf)	% Change in MCh vs. pre antagonist
B66.1	**TRAM 34 [0.2]**	2.58	4.94	2.36	1.89	4.39	2.50	6
B92.1	**TRAM 34 [0.2]**	1.90	5.58	3.68	4.31	2.93	-20	4.31
	Mean	**2.24**	**5.26**	**3.02**	**4.35**	**2.72**	**-7**	**4.35**
	SD	**0.48**	**0.45**	**0.93**	**0.06**	**0.30**	**19**	**0.06**
	t-test (paired, 2-tail)					**0.24**	**0.62**	
B43.1	**4-AP [100]**	**2.02**	5.30	3.28	3.55	5.26	1.71	-48
B43.2	**4-AP [100]**	**1.20**	4.52	3.32	1.72	4.04	2.32	-30
	Mean	**1.61**	**4.91**	**3.30**	**2.64**	**4.65**	**2.02**	**-39**
	SD	**0.58**	**0.55**	**0.03**	**1.29**	**0.86**	**0.43**	**13**
	t-test (paired, 2-tail)						**0.14**	

8.3.3 Raw data: Effects of potassium and calcium antagonists on cholinergic contraction. Cont.								
		Pre-Antagonist			Post-Antagonist			
Prep	Drug [µM]	BL pre MCh (gf)	MCh Plateau (gf)	MCh Contraction (gf)	BL pre MCh (gf)	MCh Plateau (gf)	MCh Contraction (gf)	% Change in MCh vs. pre antagonist
B42.1	iberiotoxin [0.1], glibenclamide [100], apamin [0.3]	0.74	3.61	2.87	0.84	6.20	5.36	87
B42.2	iberiotoxin [0.1], glibenclamide [100], apamin [0.3]	0.67	4.78	4.11	0.72	5.90	5.18	26
	Mean	0.71	4.20	3.49	0.78	6.05	5.27	56
	SD	0.05	0.83	0.88	0.08	0.21	0.13	43
	t-test (paired, 2-tail)						0.24	
B72.1	TEA [1000]	2.02	5.85	3.83	1.01	4.35	3.34	-13
B72.2	TEA [1000]	1.59	5.38	3.79	1.97	5.05	3.08	-19
	Mean	1.81	5.62	3.81	1.49	4.70	3.21	-16
	SD	0.30	0.33	0.03	0.68	0.49	0.18	4
	t-test (paired, 2-tail)						0.12	

8.3.3 Raw data: Effect of potassium and calcium channel antagonists on resting baseline tension					
			Post-Antagonist		
Prep	Drug [μM]	Baseline post wash	Baseline post antagonist	Change in baseline (gf)	Change in baseline (%)
B37.1	**Nifedipine [20]**	1.69	1.28	-0.41	-24
B37.2	**Nifedipine [20]**	2.25	1.17	-1.08	-48
	Mean	**1.97**	**1.225**	**-0.745**	**-36**
	SD	**0.40**	**0.08**	**0.47**	**17**
	t-test (paired, 1-tail)			**0.13**	
B40.1	**Iberiotoxin [0.1]**	1.9	2.5	0.6	32
B40.2	**Iberiotoxin [0.1]**	2.93	4	1.07	37
	Mean	**2.42**	**3.25**	**0.84**	**34**
	SD	**0.73**	**1.06**	**0.33**	**3**
	t-test (paired, 1-tail)			**0.09**	
B41.1	**Apamin [0.3]**	1.92	2.36	0.44	23
B41.2	**Apamin [0.3]**	1.54	1.35	-0.19	-12
	Mean	**1.73**	**1.855**	**0.125**	**5**
	SD	**0.27**	**0.71**	**0.45**	**25**
	t-test (paired, 1-tail)			**0.38**	
B40.1	**Glibenclamide [0.1]**	3	2.06	-0.94	-31
B40.2	**Glibenclamide [0.1]**	2.63	2.31	-0.32	-12
	Mean	**2.815**	**2.185**	**-0.63**	**-22**
	SD	**0.26**	**0.18**	**0.44**	**14**
	t-test (paired, 1-tail)			**0.15**	
B66.1	**TRAM 34 [0.2]**	0.73	2.16	1.43	196
B66.2	**TRAM 34 [0.2]**	0.77	1.44	0.67	87
B92.1	**TRAM-34 [0.2]**	0.71	**1.73**	**1.02**	**144**
	Mean	**0.74**	**1.78**	**1.04**	**142**
	SD	**0.03**	**0.36**	**0.38**	**54**
	t-test (paired, 1-tail)			**0.02**	
B43.1	**4-AP [100]**	1.39	3.55	2.16	155
B43.2	**4-AP [100]**	0.77	1.72	0.95	123
	Mean	**1.08**	**2.64**	**1.56**	**139**
	SD	**0.44**	**1.29**	**0.86**	**23**
	t-test (paired, 1-tail)			**0.12**	
B42.1	**iberiotoxin [0.1], glibenclamide [100], apamin [0.3]**	0.70	0.84	0.14	20
B42.2	**iberiotoxin [0.1], glibenclamide [100], apamin [0.3]**	0.60	0.72	0.12	20
	Mean	**0.65**	**0.78**	**0.13**	**20**
	SD	**0.07**	**0.08**	**0.01**	**0**
	t-test (paired, 1-tail)			**0.02**	
B72.1	**TEA [1000]**	1.82	1.01	-0.81	-45
B72.2	**TEA [1000]**	2.60	1.87	-0.73	-28
	Mean	**2.21**	**1.44**	**-0.77**	**-36**
	SD	**0.55**	**0.61**	**0.06**	**12**
	t-test (paired, 1-tail)			**0.02**	

8.3.3 Raw data: Effects of potassium and calcium antagonists on HBD and β-adrenergic relaxation from active tension															
		Pre-Antagonist						Post-Antagonist							
Prep	Drug [μM]	MCh (gf)	HBD BL post MCh (gf)	HBD post MCh (gf)	HBD post MCh (% MCh)	Post Iso BL (gf)	Iso dilation (% MCh)	MCh (gf)	HBD BL post MCh (gf)	HBD post MCh (gf)	HBD post MCh (% MCh)	% change vs. pre antag	Post Iso BL (gf)	Iso dilation (% MCh)	% change vs. pre antag
B37.1	**Nifedipine [20]**	0.63	1.92	-0.73	-116	1.87	-124	0.55	1.07	-0.76	-138	19			
B37.2	**Nifedipine [20]**	1.12	1.28	-1.96	-175	1.92	-118	0.95	1.05	-1.07	-113	-36			
	Mean	**0.88**	**1.60**	**-1.35**	**-145**	**1.90**	**-121**	**0.75**	**1.06**	**-0.92**	**-125**	**-8**			
	SD	**0.35**	**0.45**	**0.87**	**42**	**0.04**	**4**	**0.28**	**0.01**	**0.22**	**18**	**39**			
	t-test (paired, 2-tail)								**0.33**	**0.52**	**0.72**				
B40.1	**Iberiotoxin [0.1]**	9.47	1.87	-9.37	-99	5.10	-65	7.75	1.90	-8.35	-108	9	2.86	-95	47
B40.2	**Iberiotoxin [0.1]**	5.05	1.73	-5.29	-105	3.51	-70	2.78	1.63	-5.15	-185	77	2.40	-158	127
	Mean	**7.26**	**1.80**	**-7.33**	**-102**	**4.31**	**-67**	**5.27**	**1.77**	**-6.75**	**-146**	**43**	**2.63**	**-126**	**87**
	SD	**3.13**	**0.10**	**2.88**	**4**	**1.12**	**3**	**3.51**	**0.19**	**2.26**	**55**	**48**	**0.33**	**44**	**56**
	t-test (paired, 2-tail)										**0.43**			**0.29**	
B41.1	**Apamin [0.3]**	10.13	2.85	-9.64	-95	4.37	-80	6.61	3.02	-5.95	-90	-5			
B41.2	**Apamin [0.3]**	4.71	1.25	-5.72	-121	3.85	-66	4.13	1.20	-4.28	-104	-15			
	Mean	**7.42**	**2.05**	**-7.68**	**-108**	**4.11**	**-73**	**5.37**	**2.11**	**-5.12**	**-97**	**-10**			
	SD	**3.83**	**1.13**	**2.77**	**19**	**0.37**	**10**	**1.75**	**1.29**	**1.18**	**10**	**7**			
	t-test (paired, 2-tail)										**0.32**				

8.3.3 Raw data: Effects of potassium and calcium antagonists on HBD and β-adrenergic relaxation from active tension. Cont.															
		Pre-Antagonist						Post-Antagonist							
Prep	Drug [μM]	MCh (gf)	HBD BL post MCh (gf)	HBD post MCh (gf)	HBD post MCh (% MCh)	Post Iso BL (gf)	Iso dilation (% MCh)	MCh (gf)	HBD BL post MCh (gf)	HBD post MCh (gf)	HBD post MCh (% MCh)	% change vs. pre antag	Post Iso BL (gf)	Iso dilation (% MCh)	% change vs. pre antag
B66.1	**TRAM 34 [0.2]**	2.36	1.64	-3.30	-140	1.20	-158	2.50	1.76	-2.63	-105	-25	0.76	-145	-8
B92.1	**TRAM 34 [0.2]**	3.68	1.09	-4.49	-122	1.56	-109	2.93	1.97	-2.34	-80	-35	1.43	-98	-10
	Mean	**3.02**	**1.37**	**-3.90**	**-131**	**1.38**	**-134**	**2.72**	**1.87**	**-2.49**	**-93**	**-30**	**1.10**	**-122**	**-9**
	SD	**0.93**	**0.39**	**0.84**	**13**	**0.25**	**35**	**0.30**	**0.15**	**0.21**	**18**	**7**	**0.47**	**33**	**1**
	t-test (paired, 2-tail)							**0.62**	**0.41**	**0.31**	**0.06**			**0.06**	
B43.1	**4-AP [100]**	**3.28**	1.28	-4.02	-123	1.41	-119	1.71	1.71	-3.55	-208	69	1.16	-240	102
B43.2	**4-AP [100]**	**3.32**	0.91	-3.61	-109	1.11	-103	2.32	1.06	-2.98	-128	18	0.87	-137	33
	Mean	**3.30**	**1.10**	**-3.82**	**-116**	**1.26**	**-111**	**2.02**	**1.39**	**-3.27**	**-168**	**44**	**1.02**	**-188**	**68**
	SD	**0.03**	**0.26**	**0.29**	**10**	**0.21**	**11**	**0.43**	**0.46**	**0.40**	**56**	**36**	**0.21**	**73**	**49**
	t-test (paired, 2-tail)										**0.35**			**0.33**	
B40.1	**Glibenclamide [0.1]**	7.75	1.90	-8.35	-108	2.86	-95	4.14	3.18	-3.02	-73	-32	1.96	-102	7
B40.2	**Glibenclamide [0.1]**	2.78	1.63	-5.15	-185	2.40	-158	1.54	1.83	-2.02	-131	-29	1.39	-160	1
	Mean	**5.27**	**1.77**	**-6.75**	**-146**	**2.63**	**-126**	**2.84**	**2.51**	**-2.52**	**-102**	**-31**	**1.68**	**-131**	**4**
	SD	**3.51**	**0.19**	**2.26**	**55**	**0.33**	**44**	**1.84**	**0.95**	**0.71**	**41**	**2**	**0.40**	**41**	**4**
	t-test (paired, 2-tail)										**0.14**			**0.31**	

8.3.3 Raw data: Effects of potassium and calcium antagonists on HBD and β-adrenergic relaxation from active tension. Cont.																
		Pre-Antagonist						Post-Antagonist								
Prep	Drug [µM]	MCh (gf)	HBD BL post MCh (gf)	HBD post MCh (gf)	HBD post MCh (% MCh)	Post Iso BL (gf)	Iso dilation (% MCh)	MCh (gf)	HBD BL post MCh (gf)	HBD post MCh (gf)	HBD post MCh (% MCh)	% change vs. pre antag	Post Iso BL (gf)	Iso dilation (% MCh)	% change vs. pre antag	
B42.1	iberiotoxin [0.1], glibenclamide [100], apamin [0.3]	2.87	1.31	-2.30	-80	1.25	-82	5.36	1.86	-4.34	-81	1	1.10	-95	16	
B42.2	iberiotoxin [0.1], glibenclamide [100], apamin [0.3]	4.11	1.25	-3.53	-86	0.83	-96	5.18	2.26	-3.64	-70	-18	1.00	-95	-2	
	Mean	**3.49**	**1.28**	**-2.92**	**-83**	**1.04**	**-89**	**5.27**	**2.06**	**-3.99**	**-76**	**-9**	**1.05**	**-95**	**7**	
	SD	**0.88**	**0.04**	**0.87**	**4**	**0.30**	**10**	**0.13**	**0.28**	**0.49**	**8**	**14**	**0.07**	**0**	**12**	
	t-test (paired, 2-tail)										**0.53**			**0.57**		
B72.1	**TEA [1000]**	**3.83**	2.36	-3.49	-91	4.29	-41	3.34	2.06	-2.29	-69	-25	3.51	-25	-38	
B72.2	**TEA [1000]**	**3.79**	1.92	-3.46	-91	3.80	-42	3.08	2.21	-2.84	-92	1	3.70	-44	5	
	Mean	**3.81**	**2.14**	**-3.48**	**-91**	**4.05**	**-41**	**3.21**	**2.14**	**-2.57**	**-80**	**-12**	**3.61**	**-34**	**-17**	
	SD	**0.03**	**0.31**	**0.02**	**0**	**0.35**	**1**	**0.18**	**0.11**	**0.39**	**17**	**18**	**0.13**	**13**	**31**	
	t-test (paired, 2-tail)										**0.53**			**0.59**		

8.3.4 Raw data: Effect of glibenclamide [50µM] on HBD DRCs from 95% O_2 and on re-contraction from 0% O_2

DRCs HBD from 95% O_2 (% inhibition of active tension) Experimental Preps

	Pre				Post Glibenclamide [50µM]			
	% O_2				% O_2			
	95	**40**	**21**	**0**	**95**	**40**	**21**	**0**
B79.1a	0	-42	-80	-119	0	-47	-79	-113
B79.3a	0	-14	-34	-96	0	-52	-54	-83
Mean	**0**	**-28**	**-57**	**-107**	**0**	**-50**	**-66**	**-98**
SD	**0**	**20**	**32**	**16**	**0**	**3**	**18**	**21**
Paired t-test Pre vs. Post						**0.42**	**0.53**	**0.24**

DRCs HBD from 95% O_2 (% inhibition of active tension) Control Preps

	Pre				Post DMSO			
	% O_2				% O_2			
	95	**40**	**21**	**0**	**95**	**40**	**21**	**0**
B79.2a	0	-32	-68	-96	0	-53	-68	-94
B79.4a	0	-11	-56	-97	0	-42	-48	-99
Mean	**0**	**-21**	**-62**	**-97**	**0**	**-47**	**-58**	**-97**
SD	**0**	**15**	**8**	**1**	**0**	**8**	**14**	**3**
Paired t-test Pre vs. Post						**0.11**	**0.50**	**0.99**

DRCs Re-contraction from 0% O_2 (% of active tension) Experimental preps

	Pre				Post Glibenclamide [50µM]			
	% O_2				% O_2			
	95	**40**	**21**	**0**	**95**	**40**	**21**	**0**
B79.1b	-22	-49	-73	-119	-24	-44	-81	-113
B79.3b	-30	-37	-42	-96	-48	-49	-54	-83
Mean	**-26**	**-43**	**-58**	**-107**	**-36**	**-46**	**-68**	**-98**
SD	**6**	**9**	**22**	**16**	**17**	**4**	**19**	**21**
Paired t-test pre vs. post					0.42	0.77	0.14	0.24

DRCs Re-contraction from 0% O_2 (% of active tension) Control preps

	Pre				Post DMSO			
	% O_2				% O_2			
	95	**40**	**21**	**0**	**95**	**40**	**21**	**0**
B79.2b	-9	-55	-57	-96	-26	-50	-69	-94
B79.4b	-20	-40	-49	-97	-22	-34	-72	-99
Mean	**-15**	**-48**	**-53**	**-97**	**-24**	**-42**	**-71**	**-97**
SD	**8**	**11**	**6**	**1**	**3**	**11**	**2**	**3**
Paired t-test pre vs. post					**0.42**	**0.05**	**0.19**	**0.99**

8.3.5.1 Raw data: DRCs to levcromakalim																							
				Baseline (gf)						Change (gf)						% of Max MCh							
				Levcromakalim [µM]						Levcromakalim [µM]						Levcromakalim [µM]							
Prep	**BL pre MCh (gf)**	**MCh pre Lev (gf)**	**MCh E_{max} Pre Lev (gf)**	**0.1**	**0.3**	**1**	**3**	**10**	**30**	**0.1**	**0.3**	**1**	**3**	**10**	**30**	**0.1**	**0.3**	**1**	**3**	**10**	**30**	**Lev max % of HBD**	**EC_{50} [µM]**
B84.1	1.92	3.38	1.46	3.41	3.33	2.61	2.39	2.24	2.14	0.03	-0.05	-0.77	-0.99	-1.14	-1.24	2	-3	-53	-68	-78	-85	55	4.6
B84.3	2.41	3.85	1.44	3.99	3.99	3.78	3.52	3.16	3.03	0.14	0.14	-0.07	-0.33	-0.69	-0.82	10	10	-5	-23	-48	-57	27	0.9
Mean	**2.17**	**3.62**	**1.45**	**3.70**	**3.66**	**3.20**	**2.96**	**2.70**	**2.59**	**0.09**	**0.05**	**-0.42**	**-0.66**	**-0.92**	**-1.03**	**6**	**3**	**-29**	**-45**	**-63**	**-71**	41	**2.75**
SD	**0.35**	**0.33**	**0.01**	**0.41**	**0.47**	**0.83**	**0.80**	**0.65**	**0.63**	**0.08**	**0.13**	**0.49**	**0.47**	**0.32**	**0.30**	**5**	**9**	**34**	**32**	**21**	**20**	20	**2.62**
t-test (2-tail) max lev vs. HBD																						0	

8.3.5 Raw data: Magnitude of HBD pre and post levcromakalim DRC							
Prep	**BL pre MCh (gf)**	**MCh Plateau pre Lev (gf)**	**MCh E max Pre Lev (gf)**	**HBD BL Pre (gf)**	**HBD Pre (% E max)**	**HBD BL Post (gf)**	**HBD Post (% E max)**
B84.1	1.92	3.38	1.46	1.32	-141	1.11	-155
B84.3	2.41	3.85	1.44	0.99	-199	0.81	-211
Mean	**2.17**	**3.62**	**1.45**	**1.16**	**-170**	**0.96**	**-183**
SD	**0.35**	**0.33**	**0.01**	**0.23**	**41**	**0.21**	**39**
t-test (2-tail) Pre vs. post Lev						**0.05**	**0.04**

8.3.5.1 Raw data: Time to maximum HBD pre and post levcromakalim (min)		
	Time to max HBD (min)	
Prep	**Pre Levcromakalim**	**Post Levcromakalim**
B84.1	20	18
B84.3	21	22
Mean	**21**	**20**
SD	**1**	**3**

9.3.2 and 9.3.3 Raw data: Effect of ETC inhibitors and ROS scavengers on resting tension					
			Post-Antagonist		
Prep	Drug [μM]	Baseline post wash	Baseline post antagonist	Change in baseline (gf)	Change in baseline (%)
B45.1	**FCCP [3]**	1.83	1.19	-0.64	-35
B45.2	**FCCP [3]**	0.75	0.49	-0.26	-35
Mean		**1.29**	**0.84**	**-0.45**	**-35**
SD		**0.76**	**0.49**	**0.27**	**0**
2-tail paired t-test		**0.25**			
B49.1	**PDTC [5]**	0.78	0.85	0.07	9
B49.2	**PDTC [10]**	0.57	0.55	-0.02	-4
B44.1	**Rotenone [1]**	1.79	1.60	-0.19	-11
B44.2	**Rotenone [1]**	1.79	1.56	-0.23	-13
Mean		**1.79**	**1.58**	**-0.21**	**-12**
SD		**0.00**	**0.03**	**0.03**	**2**
2-tail paired t-test		**0.06**			
B46.1	**Rotenone [3]**	1.31	0.98	-0.33	-25
B46.2	**Rotenone [3]**	1.96	1.22	-0.74	-38
Mean		**1.64**	**1.10**	**-0.54**	**-31**
SD		**0.46**	**0.17**	**0.29**	**9**
2-tail paired t-test		**0.23**			
B52.1	**Tempol [3000]**	1.45	1.22	-0.23	-16
B52.2	**Tempol [3000]**	1.04	0.66	-0.38	-37
Mean		**1.25**	**0.94**	**-0.31**	**-26**
SD		**0.29**	**0.40**	**0.11**	**15**
2-tail paired t-test		**0.15**			

9.3.2 and 9.3.3 Raw data: Effect of ETC inhibitors and ROS scavengers on HBD from resting tension														
		Pre- Antagonist				Post-Antagonist					Time to max HBD Pre (min)	Time HBD Post (min)	HBD time % Δ
Prep	Drug [µM]	BL pre hypoxia (gf)	Baseline post hypoxia	HBD from baseline (gf)	HBD from baseline (% Change)	Baseline post antagonist	Baseline post hypoxia	HBD from baseline (gf)	HBD from baseline (% Δ)	% Δ vs. pre antag			
B45.1	**FCCP [3]**	2.11	0.99	-1.12	-53	1.19	1.01	-0.18	-15	-72	14	7	-50
B45.2	**FCCP [3]**	2.37	0.54	-1.83	-77	0.49	0.41	-0.08	-16	-79	14	7	-50
Mean		**2.24**	**0.77**	**-1.48**	**-65**	**0.84**	**0.71**	**-0.13**	**-16**	**-75**	**14**	**7**	**-50**
SD		**0.18**	**0.32**	**0.50**	**17**	**0.49**	**0.42**	**0.07**	**1**	**5**	**0**	**0**	**0**
2-tail paired t-test		**0.21**	**0.60**	**0.19**	**0.15**								
B44.1	**Rotenone [1]**	2.61	2.01	-0.60	-23	1.60	0.98	-0.62	-39	69	32	20	-38
B44.2	**Rotenone [1]**	2.04	1.76	-0.28	-14	1.56	1.40	-0.16	-10	-25	20	10	-50
Mean		**2.33**	**1.89**	**-0.44**	**-18**	**1.58**	**1.19**	**-0.39**	**-25**	**22**	**26**	**15**	**-44**
SD		**0.40**	**0.18**	**0.23**	**7**	**0.03**	**0.30**	**0.33**	**20**	**66**	**8**	**7**	**9**
2-tail paired t-test		**0.22**	**0.29**	**0.61**	**0.64**								
B52.1	**Tempol [3000]**	1.92	1.18	-0.74	-39	1.22	0.86	-0.36	-30	-23	10	19	90
B52.2	**Tempol [3000]**	1.82	1.26	-0.56	-31	0.66	0.52	-0.14	-21	-31	10	8	-20
Mean		**1.87**	**1.22**	**-0.65**	**-35**	**0.94**	**0.69**	**-0.25**	**-25**	**-27**	**10**	**14**	**35**
SD		**0.07**	**0.06**	**0.13**	**5**	**0.40**	**0.24**	**0.16**	**6**	**5**	**0**	**8**	**78**
2-tail paired t-test		**0.15**	**0.24**	**0.03**	**0.02**								

9.3.2 Raw data: Effect of ETC inhibitors on cholinergic contraction and HBD from active tension																		
		Pre-Antagonist						Post-Antagonist								Time to max HBD Pre (min)	Time to max HBD Post (min)	% Δ
Prep	Drug [µM]	BL pre MCh (gf)	MCh Plateau (gf)	MCh (gf)	HBD BL post MCh (gf)	HBD post MCh (gf)	HBD post MCh (% MCh)	BL pre MCh (gf)	MCh Plateau (gf)	MCh (gf)	% Change in MCh vs. pre antag	HBD BL post MCh (gf)	HBD post MCh (gf)	HBD post MCh (% MCh)	% change vs pre antag			
B45.1	**FCCP [3]**	1.34	6.25	4.91	1.42	-4.83	-98	1.09	1.76	0.67	-86	1.32	-0.44	-66	-33	44	**7**	**-84**
B45.2	**FCCP [3]**	0.84	5.56	4.72	0.74	-4.82	-102	0.46	1.81	1.35	-71	0.62	-1.19	-88	-14	44	8	**-82**
Mean		**1.09**	**5.91**	**4.82**	**1.08**	**-4.83**	**-100**	**0.78**	**1.79**	**1.01**	**-79**	**0.97**	**-0.82**	**-77**	**-23**	**44**	**8**	**-83**
SD		**0.35**	**0.49**	**0.13**	**0.48**	**0.01**	**3**	**0.45**	**0.04**	**0.48**	**11**	**0.49**	**0.53**	**16**	**14**	**0**	**1**	**2**
2-tail paired t-test		**0.13**	**0.06**	**0.07**	**0.06**	**0.04**	**0.24**											
B44.1	**Rotenone [1]**	1.82	5.77	3.95	2.09	-3.68	-93	1.38	4.39	3.01	-24	2.38	-2.01	-67	-28	53	**24**	**-55**
B44.2	**Rotenone [1]**	2.01	4.08	2.07	1.83	-2.25	-109	1.58	2.37	0.79	-62	1.82	-0.55	-70	-36	35	**17**	**-51**
Mean		**1.92**	**4.93**	**3.01**	**1.96**	**-2.97**	**-101**	**1.48**	**3.38**	**1.90**	**-43**	**2.10**	**-1.28**	**-68**	**-32**	**44**	**21**	**-53**
SD		**0.13**	**1.20**	**1.33**	**0.18**	**1.01**	**11**	**0.14**	**1.43**	**1.57**	**27**	**0.40**	**1.03**	**2**	**5**	**13**	**5**	**2**
2-tail paired t-test		**0.01**	**0.07**	**0.10**	**0.52**	**0.12**	**0.12**											
B46.1	**Rotenone [3]**	1.05	2.39	1.34	1.34	-1.05	-78	0.98	1.50	0.52	-61	1.08	-0.42	-81	3	21	**15**	**-29**
B46.2	**Rotenone [3]**	1.91	3.17	1.26	1.47	-1.7	-135	1.22	2.02	0.8	-37	1.32	-0.7	-88	-35	17	14	**-18**
Mean		**1.48**	**2.78**	**1.30**	**1.41**	**-1.38**	**-107**	**1.10**	**1.76**	**0.66**	**-49**	**1.20**	**-0.56**	**-84**	**-16**	**19**	**15**	**-23**
SD		**0.61**	**0.55**	**0.06**	**0.09**	**0.46**	**40**	**0.17**	**0.37**	**0.20**	**17**	**0.17**	**0.20**	**5**	**27**	**3**	**1**	**8**
2-tail paired t-test		**0.44**	**0.08**	**0.17**	**0.17**	**0.11**	**0.53**											

9.3.3 Raw data: Effect of ROS scavengers on cholinergic contraction and HBD from active tension																		
		Pre-Antagonist						Post-Antagonist										
Prep	Drug [µM]	BL pre MCh (gf)	MCh Plateau (gf)	MCh (gf)	HBD BL post MCh (gf)	HBD post MCh (gf)	HBD post MCh (% MCh)	BL pre MCh (gf)	MCh Plateau (gf)	MCh (gf)	% Change in MCh vs. pre antag	HBD BL post MCh (gf)	HBD post MCh (gf)	HBD post MCh (% MCh)	% Δ vs pre antag	Time to max HBD Pre (min)	Time to max HBD Post (min)	% Δ
B49.1	**PDTC [5]**	1.99	6.39	4.4	1.58	-4.81	-109	0.85	3.3	2.45	-44	0.88	-2.42	-99	-10	54	**15**	**-72**
B49.2	**PDTC [10]**	2.23	4.95	2.72	1.29	-3.66	-135	0.55	4.01	3.46	27	0.71	-3.3	-95	-29	54	**15**	**-72**
B52.1	**Tempol [3000]**	1.02	5.99	4.97	1.85	-4.14	-83	1.27	2.36	1.09	-78	1.1	-1.26	-116	39	11	**19**	**73**
B52.2	**Tempol [3000]**	0.66	2.77	2.11	1.32	-1.45	-69	0.64	1.41	0.77	-64	0.67	-0.74	-96	40	11	17	**55**
Mean		**0.84**	**4.38**	**3.54**	**1.59**	**-2.80**	**-76**	**0.96**	**1.89**	**0.93**	**-71**	**0.89**	**-1.00**	**-106**	**39**	**11**	**18**	**64**
SD		**0.25**	**2.28**	**2.02**	**0.37**	**1.90**	**10**	**0.45**	**0.67**	**0.23**	**10**	**0.30**	**0.37**	**14**	**1**	**0**	**1**	**13**
2-tail paired t-test		**0.55**	**0.27**	**0.29**	**0.05**	**0.26**	**0.05**											

9.3.4.1 Raw data: Effect of DPI on resting tension (Experimental preps)					
Prep	**DPI [µM]**	**Baseline post wash**	**Baseline post DPI**	**Change in baseline (gf)**	**Change in baseline (%)**
B62.1	**300**	1.37	0.84	-0.53	-39
B63.2	**200**	0.91	0.54	-0.37	-41
B63.1	**100**	1.22	1.11	-0.11	-9
B64.1	**100**	1.12	1.03	-0.09	-8
B65.1	**100**	0.75	0.59	-0.16	-21
B68.2	**100**	1.06	1.03	-0.03	-3
B73.1	**50**	2.11	0.96	-1.15	-55
B73.2	**50**	1.32	0.49	-0.83	-63
B76.2	**30**	1.55	0.52	-1.03	-66
Mean		**1.27**	**0.79**	**-0.48**	**-34**
SD		**0.40**	**0.25**	**0.43**	**25**
2-tail paired t-test		**0.01**			

9.3.4.1 Raw data: Effect of DPI on resting tension (Control preps)					
Prep	**DPI [µM]**	**Baseline post wash**	**Baseline post 30 min**	**Change in baseline (gf)**	**Change in baseline (%)**
B62.2	**control**	1.87	1.56	-0.31	-17
B64.2	**control**	1.87	1.03	-0.84	-45
B65.2	**control**	0.50	0.46	-0.04	-8
B76.1	**control**	1.97	2.09	0.12	6
Mean		**1.55**	**1.29**	**-0.27**	**-16**
SD		**0.70**	**0.70**	**0.42**	**22**
2-tail paired t-test		**0.29**			

9.3.4.1 Raw data: Effect of DPI on HBD from resting tension														
		Pre- DPI				Post-DPI					Time to max HBD Pre (min)	Time to max HBD Post (min)	% Δ HBD time
Prep	DPI [µM]	BL pre hypoxia (gf)	BL post hypoxia (gf)	HBD from baseline (gf)	HBD from baseline (% Δ)	Baseline post DPI	Baseline post hypoxia	HBD from baseline (gf)	HBD from baseline (% Δ)	% Δ vs pre DPI			
B62.1	**300**	2.13	1.01	-1.12	-53	0.84	0.83	-0.01	-1	-98	10	A	
B63.2	**200**	1.09	0.70	-0.39	-36	0.54	0.60	0.06	11	-131	13	A	
B63.1	**100**	1.43	0.91	-0.52	-36	1.11	0.83	-0.28	-25	-31	15	15	0
B64.1	**100**	2.11	1.32	-0.79	-37	1.03	1.03	0.00	0	-100		A	
B65.1	**100**	2.08	0.68	-1.40	-67	0.59	0.60	0.01	2	-103		22	
B76.2	**30**	2.00	0.58	-1.42	-71	0.52	0.52	0.00	0	-100	17	-	
Mean		**1.81**	**0.87**	**-0.94**	**-50**	**0.77**	**0.74**	**-0.04**	**-2**	**-94**	**14**	**19**	**0.00**
SD		**0.44**	**0.27**	**0.44**	**16**	**0.26**	**0.19**	**0.12**	**12**	**33**	**3**	**5**	
2-tail paired t-test		**0.004**	**0.01**	**0.01**	**0.003**								

9.3.4.1 Raw data: Paired time matched control preps: effect of DPI on HBD from resting tension													
		Pre- DPI				Post-DPI					Time to max HBD Pre (min)	Time to max HBD Post (min)	% Δ HBD time
Prep	DPI [µM]	BL pre hypoxia (gf)	BL post hypoxia (gf)	HBD from baseline (gf)	HBD from baseline (% Δ)	Baseline post DPI	Baseline post hypoxia	HBD from baseline (gf)	HBD from baseline (% Δ)	% Δ vs pre DPI			
B62.2	**control**	2.68	1.83	-0.85	-32	1.56	0.96	-0.60	-38	21	11	14	27
B64.2	**control**	2.18	0.90	-1.28	-59	1.03	0.81	-0.22	-21	-64		8	
B65.2	**control**	2.09	0.60	-1.49	-71	0.46	0.42	-0.04	-9	-88		25	
B76.1	**control**	2.09	1.94	-0.15	-7	2.09	2.04	-0.05	-2	-67	23	-	
Mean		**2.26**	**1.32**	**-0.94**	**-42**	**1.29**	**1.06**	**-0.23**	**-18**	**-49**	**17.00**	**16**	
SD		**0.28**	**0.67**	**0.59**	**29**	**0.70**	**0.69**	**0.26**	**16**	**48**	**8.49**	**9**	
2-tail paired t-test		**0.07**	**0.31**	**0.11**	**0.22**								

9.3.4.2 Raw data: Effect of DPI on cholinergic contraction and HBD from active tension																		
		Pre-DPI						Post-DPI								Time to max HBD Pre (min)	Time to max HBD Post (min)	% Δ Time
Prep	DPI [µM]	BL pre MCh (gf)	MCh Plateau (gf)	MCh (gf)	HBD BL post MCh (gf)	HBD post MCh (gf)	HBD post MCh (% MCh)	BL pre MCh (gf)	MCh Plateau (gf)	MCh (gf)	% Δ in MCh vs. pre DPI	HBD BL post MCh (gf)	HBD post MCh (gf)	HBD post MCh (% MCh)	% Δ vs pre DPI			
B62.1	**300**	1.47	4.25	2.78	1.22	-3.03	-109	0.84	0.82	-0.02	-101	-						
B63.1	**100**	1.33	6.57	5.24	1.37	-5.20	-99	0.98	1.75	0.77	-85	-						
B63.2	**200**	1.23	6.10	4.87	0.89	-5.21	-107	0.68	0.83	0.15	-97	-						
B64.1	**100**	2.08	4.16	2.08	1.30	-2.86	-138	1.03	1.06	0.03	-99	-						
B73.1	**50**	1.84	2.92	1.08	1.03	-1.89	-175	0.96	0.96	0.00	-100	-						
B73.2	**50**	1.22	2.01	0.79	0.59	-1.42	-180	0.49	0.52	0.03	-96	-						
Mean		**1.53**	**4.34**	**2.81**	**1.07**	**-3.27**	**-135**	**0.83**	**0.99**	**0.16**	**-96**	**-**						
SD		**0.35**	**1.76**	**1.88**	**0.29**	**1.61**	**36**	**0.21**	**0.41**	**0.30**	**6**							
2-tail paired t-test		**0.0010**	**0.003**	**0.012**														

9.3.4.2 Raw data: Paired time matched control preps for; Effect of DPI on cholinergic contraction and HBD from active tension																		
Prep	**DPI [µM]**	**BL pre MCh (gf)**	**MCh Plateau (gf)**	**MCh (gf)**	**HBD BL post MCh (gf)**	**HBD post MCh (gf)**	**HBD post MCh (% MCh)**	**BL pre MCh (gf)**	**MCh Plateau (gf)**	**MCh (gf)**	**% Δ in MCh vs. pre DPI**	**HBD BL post MCh (gf)**	**HBD post MCh (gf)**	**HBD post MCh (% MCh)**	**% Δ vs pre DPI**	**Time to max HBD Pre (min)**	**Time to max HBD Post (min)**	**% Δ Time**
B62.2	**control**	1.88	5.46	3.58	2.15	-3.31	-92	1.30	3.62	2.32	-35	1.12	-2.50	-108	17	42		
B64.2	**control**	2.42	7.89	5.47	2.07	-5.82	-106	0.98	3.29	2.31	-58	1.14	-2.15	-93	-13	16	9	**-44**
B76.1	**control**	2.00	2.37	0.37	2.04	-0.33	-89	2.11	2.33	0.22	-41	2.14	-0.19	-86	-3	23	28	**22**
Mean		**2.10**	**5.24**	**3.14**	**2.09**	**-3.15**	**-96**	**1.46**	**3.08**	**1.62**	**-45**	**1.47**	**-1.61**	**-96**	**0.3**	**27**	**19**	**-11**
SD		**0.28**	**2.77**	**2.58**	**0.06**	**2.75**	**9**	**0.58**	**0.67**	**1.21**	**12**	**0.58**	**1.25**	**11**	**15**	**13**	**13**	**46**
2-tail paired t-test		**0.29**	**0.24**	**0.23**	**0.23**	**0.29**	**0.98**											

9.3.4.2 Raw data: Effect of DPI on contraction to KCl and HBD from active tension																			
		Pre-DPI						Post-DPI								Time to max HBD Pre (min)	Time to max HBD Post (min)	% Δ time
Prep	DPI [µM]	BL pre KCl (gf)	KCl Plateau (gf)	KCl (gf)	HBD BL post KCl (gf)	HBD post MCh (gf)	HBD post KCl (% KCl)	BL pre KCl (gf)	KCl Plateau (gf)	KCl (gf)	% Δ in KCl vs. pre DPI	HBD BL post KCl (gf)	HBD post KCl (gf)	HBD post KCl (% KCl)	% Δ vs pre DPI			
B65.1	**100**	1.66	3.53	1.87	1.23	-2.30	-123	0.60	1.13	0.53	-72	1.10	-0.03	-6	-95	38	-	
B68.2	**100**	1.22	7.05	5.83	2.24	-4.81	-83	1.04	1.70	0.66	-89	1.68	-0.02	-3	-96	49	-	
Mean		**1.44**	**5.29**	**3.85**	**1.74**	**-3.56**	**-103**	**0.82**	**1.42**	**0.60**	**-80**	**1.39**	**-0.02**	**-4**	**-96**	**44**		
SD		**0.31**	**2.49**	**2.80**	**0.71**	**1.77**	**29**	**0.31**	**0.40**	**0.09**	**12**	**0.41**	**0.01**	**2**	**1**	**8**		
2-tail paired t-test		**0.39**	**0.23**	**0.34**	**0.35**	**0.19**	**0.12**											

10.3.1.1 Raw data: Dose response effects of NaHS on resting tension in isolated human pulmonary arteries															
	Resting Tension (gf)					Change in Resting Tension (gf)				% Change in Resting Tension				EC_{50}	
		NaHS [mM]				NaHS [mM]				NaHS [mM]					
Prep	**0**	**0.1**	**0.3**	**1**	**3**	**0.1**	**0.3**	**1**	**3**	**0.1**	**0.3**	**1**	**3**	**(mM)**	**pEC_{50}**
PA14.1	1.87	1.93	1.87	1.81	1.71	0.06	0.00	-0.06	-0.16	3.2	0.0	-3.2	-8.6	1.43	2.48
PA14.2	1.79	1.70	1.64	1.59	1.57	-0.09	-0.15	-0.20	-0.22	-5.0	-8.4	-11.2	-12.3	0.17	3.77
PA14.3	1.60	1.57	1.54	1.50	1.47	-0.03	-0.06	-0.10	-0.13	-1.9	-3.8	-6.3	-8.1	0.41	3.39
PA14.4	2.60	2.57	2.34	2.31	2.16	-0.03	-0.26	-0.29	-0.44	-1.2	-10.0	-11.2	-16.9	0.26	3.59
PA15.1	1.84	1.70	1.48	1.41	1.40	-0.14	-0.36	-0.43	-0.44	-7.6	-19.6	-23.4	-23.9	0.18	3.74
PA15.2	1.13	1.10	1.05	1.03	1.03	-0.03	-0.08	-0.10	-0.10	-2.7	-7.1	-8.8	-8.8	0.18	3.74
PA15.3	1.28	1.29	1.26	1.27	1.27	0.01	-0.02	-0.01	-0.01	0.8	-1.6	-0.8	-0.8		
PA15.4	0.80	0.79	0.81	0.80	0.79	-0.01	0.01	0.00	-0.01	-1.3	1.3	0.0	-1.3		
Mean	**1.61**	**1.58**	**1.50**	**1.47**	**1.43**	**-0.03**	**-0.12**	**-0.15**	**-0.19**	**-1.9**	**-6.1**	**-8.1**	**-10.1**	**0.44**	**3.51**
SD	**0.55**	**0.54**	**0.48**	**0.47**	**0.42**	**0.06**	**0.13**	**0.15**	**0.17**	**3.3**	**6.8**	**7.5**	**7.70**	**0.49**	**0.36**
SEM	**0.19**	**0.19**	**0.17**	**0.16**	**0.15**	**0.02**	**0.05**	**0.05**	**0.06**	**1.17**	**2.39**	**2.67**	**2.72**	**0.20**	**0.15**
2-tail paired t-test vs. baseline resting tension		**0.17**	**0.04**	**0.03**	**0.02**										
2-tail t-test NaHS vs. Hypoxia	**0.35**				**0.71**				**0.19**				**0.43**		

10.3.1.1 Raw data: effects of hypoxia on resting tension in isolated human pulmonary arteries

	Resting Tension (gf)			
Prep	**Pre hypoxia**	**Post Hypoxia**	**Δ to Hypoxia (gf)**	**Δ to Hypoxia (%)**
PA14.1	2.03	1.98	-0.05	-2.46
PA14.2	1.92	1.71	-0.21	-10.94
PA14.3	1.42	1.34	-0.08	-5.63
PA14.4	2.04	2.04	0.00	0.00
PA15.1	1.17	0.93	-0.24	-20.51
PA15.2	1.00	0.93	-0.07	-7.00
PA15.3	1.36	1.34	-0.02	-1.47
PA15.4	1.02	0.88	-0.14	-13.73
Mean	**1.50**	**1.39**	**-0.10**	**-7.72**
SD	**0.44**	**0.47**	**0.09**	**6.98**
SEM	**0.16**	**0.17**	**0.03**	**2.47**
paired 2 tail t-test vs. resting tension		**0.01**		

10.3.1.2 Raw data: Dose response effects of NaHS on active tension to 3 nM ET-1 in isolated human pulmonary arteries																					
				Isometric Tension (gf)				Active Tension (gf)				Δ Active Tension (gf)				% Change in Active Tension					
				NaHS [mM]				NaHS [mM]				NaHS [mM]				NaHS [mM]					
Prep	BL pre-ET-1	BL post ET-1 (gf)	Active Tension to ET-1 (gf)	0.1	0.3	1	3	0.1	0.3	1	3	0.1	0.3	1	3	0.1	0.3	1	3	EC_{50} (mM)	pEC_{50}
PA14.1	1.81	1.98	0.17	1.99	1.96	1.87	1.87	0.18	0.15	0.06	0.06	0.01	-0.02	-0.11	-0.11	6	-12	-65	-65	0.57	3.24
PA14.2	1.26	1.75	0.49	1.81	1.81	1.86	1.94	0.55	0.55	0.60	0.68	0.06	0.06	0.11	0.19	12	12	22	39		
PA14.3	1.37	2.03	0.66	2.09	2.00	1.91	1.63	0.72	0.63	0.54	0.26	0.06	-0.03	-0.12	-0.40	9	-5	-18	-61	1.58	2.80
PA14.4	2.06	2.82	0.76	2.80	2.72	2.70	2.54	0.74	0.66	0.64	0.48	-0.02	-0.10	-0.12	-0.28	-3	-13	-16	-37	1.30	2.89
PA15.1	0.92	1.43	0.51	1.32	1.17	1.07	1.02	0.40	0.25	0.15	0.10	-0.11	-0.26	-0.36	-0.41	-22	-51	-71	-80	0.22	3.66
PA15.2	0.90	1.46	0.56	1.46	1.36	1.25	1.21	0.56	0.46	0.35	0.31	0.00	-0.10	-0.21	-0.25	0	-18	-38	-45	0.48	3.32
PA15.3	1.19	2.33	1.14	2.18	1.73	1.40	1.38	0.99	0.54	0.21	0.19	-0.15	-0.60	-0.93	-0.95	-13	-53	-82	-83	0.26	3.59
PA15.4	0.85	2.04	1.19	1.81	1.38	1.07	1.06	0.96	0.53	0.22	0.21	-0.23	-0.66	-0.97	-0.98	-19	-55	-82	-82	0.22	3.66
Mean	**1.30**	**1.98**	**0.69**	**1.93**	**1.77**	**1.64**	**1.58**	**0.64**	**0.47**	**0.35**	**0.29**	**-0.05**	**-0.21**	**-0.34**	**-0.40**	**-4**	**-24**	**-43**	**-52**	**0.66**	**3.31**
SD	**0.44**	**0.46**	**0.34**	**0.46**	**0.49**	**0.56**	**0.52**	**0.27**	**0.18**	**0.22**	**0.21**	**0.10**	**0.27**	**0.40**	**0.40**	**13**	**25**	**38**	**40**	**0.55**	**0.36**
SEM	**0.16**	**0.16**	**0.12**	**0.16**	**0.17**	**0.20**	**0.18**	**0.10**	**0.06**	**0.08**	**0.07**	**0.04**	**0.10**	**0.14**	**0.14**	**5**	**9**	**13**	**14**	**0.21**	**0.13**
2-tail paired t-test vs. maximum active tension								**0.24**	**0.06**	**0.05**	**0.03**										
2-tail paired t-test: NaHS vs. Hypoxia			**0.06**				**0.58**				**0.65**				**0.54**				**0.43**		

10.3.1.2 Raw data: Effect of hypoxia on active tension to 3 nM ET-1 in isolated human pulmonary arteries							
Prep	**BL pre-ET-1**	**BL post ET-1 (gf)**	**Active Tension to ET-1 (gf)**	**BL Post Hypoxia (gf)**	**Active Tension Post Hypoxia (gf)**	**Δ Active Tension to Hypoxia (gf)**	**% Δ to Hypoxia**
PA14.1	1.78	1.95	0.17	1.73	-0.05	-0.22	-129
PA14.2	1.41	1.88	0.47	1.31	-0.10	-0.57	-121
PA14.3	1.44	1.68	0.24	1.49	0.05	-0.19	-79
PA14.4	2.08	2.28	0.20	2.13	0.05	-0.15	-75
PA15.1	0.68	1.28	0.60	1.00	0.32	-0.28	-47
PA15.2	1.03	1.52	0.49	1.18	0.15	-0.34	-69
PA15.3	1.20	2.04	0.84	1.83	0.63	-0.21	-25
PA15.4	0.74	1.80	1.06	1.40	0.66	-0.40	-38
Mean	**1.30**	**1.80**	**0.50**	**1.57**	**0.28**	**-0.23**	**-59**
SD	**0.49**	**0.32**	**0.32**	**0.35**	**0.33**	**0.22**	**55**
SEM	**0.17**	**0.11**	**0.11**	**0.12**	**0.12**	**0.08**	**20**
Paired 2 tail t-test: active tension pre vs. post hypoxia					**0.02**		

10.3.2.1 Raw data: Dose response effects of NaHS and hypoxia on resting tension in isolated human pulmonary veins																			
	Resting Tension (gf)					Change in Resting Tension (gf)				% Change in Resting Tension						Hypoxia			
		NaHS [mM]				NaHS [mM]				NaHS [mM]						Resting Tension (gf)			
Prep	Baseline	0.1	0.3	1	3	0.1	0.3	1	3	0.1	0.3	1	3	EC_{50} (mM)	pEC_{50}	Pre hypoxia	Post Hypoxia	Δ to Hypoxia (gf)	% Δ to Hypoxia
PV7.1	1.16	1.22		1.21	1.12	0.06		0.05	-0.04	5		4	-3			1.25	1.29	0.04	3
PV7.2	1.10	1.13		1.13	1.11	0.03		0.03	0.01	3		3	1			1.21	1.15	-0.06	-5
PV7.3	1.46	1.47	1.41	1.32	1.24	0.01	-0.05	-0.14	-0.22	1	-3	-10	-15	0.76	3.12	1.07	0.99	-0.08	-7
PV7.4	1.50	1.51	1.47	1.47	1.39	0.01	-0.03	-0.03	-0.11	1	-2	-2	-7	1.62	2.79	1.15	1.13	-0.02	-2
PV8.1	0.35	0.39	0.36	0.32		0.04	0.01	-0.03		11	3	-9				0.56	0.37	-0.19	-34
PV8.2	1.33	1.34	1.23	0.87		0.01	-0.1	-0.46		1	-8	-35		0.56	3.25	1.61	1.22	-0.39	-24
PV8.3	1.44	1.37	1.15	0.97		-0.07	-0.29	-0.47		-5	-20	-33		0.25	3.60	0.99	1.00	0.01	1
PV8.4	0.43	0.35	0.20	0.13		-0.08	-0.23	-0.30		-19	-53	-70		0.19	3.72	1.14	0.78	-0.36	-32
Mean	**1.10**	**1.10**	**0.97**	**0.93**	**1.22**	**0.00**	**-0.12**	**-0.17**	**-0.09**	**0**	**-14**	**-19**	**-6**	**0.68**	**3.30**	**1.12**	**0.99**	**-0.13**	**-12**
SD	**0.46**	**0.47**	**0.55**	**0.47**	**0.13**	**0.05**	**0.12**	**0.21**	**0.10**	**9**	**21**	**25**	**7**	**0.58**	**0.38**	**0.29**	**0.30**	**0.17**	**15**
SEM	**0.16**	**0.16**	**0.19**	**0.17**	**0.07**	**0.02**	**0.05**	**0.08**	**0.05**	**3**	**9**	**9**	**3**	**0.26**	**0.17**	**0.10**	**0.10**	**0.06**	**5**
2-tail paired t-test vs. baseline resting tension		**0.95**	**0.06**	**0.06**	**0.17**										**2-tail paired t-test pre vs. post hypoxia**		**0.06**		
2-tail t-test NaHS vs. Hypoxia	**0.86**			**0.60**				**0.60**				**0.42**							

10.3.2.2 Raw data: Dose response effects of NaHS on active tension in isolated human pulmonary veins contracted to 1nM ET-1																					
				Isometric Tension (gf)				Active Tension (gf)				Δ Active Tension (gf)				% Change in Active Tension					
				NaHS [mM]				NaHS [mM]				NaHS [mM]				NaHS [mM]					
Prep	**BL pre-ET-1**	**Tension post ET-1 (gf)**	**Active Tension to ET-1 (gf)**	**0.1**	**0.3**	**1**	**3**	**0.1**	**0.3**	**1**	**3**	**0.1**	**0.3**	**1**	**3**	**0.1**	**0.3**	**1**	**3**	**EC_{50} (mM)**	**pEC_{50}**
PV7.1	1.04	1.20	0.16	1.16	1.19	1.19	1.18	0.12	0.15	0.15	0.14	-0.04	-0.01	-0.01	-0.02	-25	-6	-6	-13		
PV7.2	1.00	1.21	0.21	1.15	1.15	1.17	1.20	0.15	0.15	0.17	0.20	-0.06	-0.06	-0.04	-0.01	-29	-29	-19	-5	0.03	4.52
PV7.3	0.95	1.29	0.34	1.26	1.22	1.22	1.19	0.31	0.27	0.27	0.24	-0.03	-0.07	-0.07	-0.10	-9	-21	-21	-29	0.20	3.70
PV7.4	1.12	1.67	0.55	1.64	1.59	1.62	1.51	0.52	0.47	0.50	0.39	-0.03	-0.08	-0.05	-0.16	-5	-15	-9	-29	0.30	3.52
PV8.1																					
PV8.2	0.87	1.00	0.13	1.00	0.79	0.86		0.13	-0.08	-0.01		0.00	-0.21	-0.14		0	-162	-108		0.17	3.77
PV8.3	0.89	1.89	1.00	1.34	1.07	1.00		0.45	0.18	0.11		-0.55	-0.82	-0.89		-55	-82	-89		0.08	4.10
PV8.4	0.71	4.75	4.04	3.32	1.90	1.75		2.61	1.19	1.04		-1.43	-2.85	-3.00		-35	-71	-74		0.11	3.96
Mean	**0.94**	**1.86**	**0.92**	**1.55**	**1.27**	**1.26**	**1.27**	**0.61**	**0.33**	**0.32**	**0.24**	**-0.31**	**-0.59**	**-0.60**	**-0.07**	**-23**	**-55**	**-47**	**-19**	**0.15**	**3.93**
SD	**0.13**	**1.31**	**1.41**	**0.80**	**0.36**	**0.32**	**0.16**	**0.89**	**0.41**	**0.36**	**0.11**	**0.53**	**1.04**	**1.10**	**0.07**	**19**	**55**	**42**	**12**	**0.10**	**0.35**
2-tail paired t-test vs. maximum active tension								**0.18**	**0.19**	**0.20**	**0.13**										
2-tail paired t-test: NaHS vs. Hypoxia			**0.47**			**0.14**				**0.13**				**0.04**				**0.03**			

10.3.2.2 Raw data: Effect of hypoxia on active tension in isolated human pulmonary veins contracted to 1nM ET-1							
Prep	**Baseline pre-ET-1**	**Tension post ET-1 (gf)**	**Active Tension to ET-1 (gf)**	**Tension Post Hypoxia (gf)**	**Active Tension Post Hypoxia (gf)**	**Δ Active Tension to Hypoxia (gf)**	**Δ to Hypoxia (%)**
PV7.1	1.06	1.31	0.25	1.19	0.13	-0.12	-48
PV7.2	1.01	1.20	0.19	1.03	0.02	-0.17	-89
PV7.3	0.92	1.16	0.24	1.06	0.14	-0.10	-42
PV7.4	1.20	1.27	0.07	1.22	0.02	-0.05	-71
PV8.1	0.33	0.51	0.18	0.34	0.01	-0.17	-94
PV8.2	0.90	1.12	0.22	0.87	-0.03	-0.25	-114
PV8.3							
PV8.4							
Mean	**0.90**	**1.10**	**0.19**	**0.95**	**0.05**	**-0.14**	**-76**
SD	**0.30**	**0.29**	**0.07**	**0.32**	**0.07**	**0.07**	**28**
SEM	**0.12**	**0.12**	**0.03**	**0.13**	**0.03**	**0.03**	**11**
Paired 2 tail t-test: active tension pre vs. post hypoxia					**0.004**		

10.3.2.2 Raw data: Maximum change in active tension to NaHS and hypoxia in isolated human pulmonary veins				
	Maximum Δ Active Tension (gf)		**Maximum Δ Active Tension (%)**	
Prep	**NaHS**	**Hypoxia**	**NaHS**	**Hypoxia**
PV7.1	-0.04	-0.12	-25	-48
PV7.2	-0.06	-0.17	-29	-89
PV7.3	-0.10	-0.10	-29	-42
PV7.4	-0.16	-0.05	-29	-71
PV8.1		-0.17		-94
PV8.2	-0.21	-0.25	-162	-114
PV8.3	-0.89		-89	
PV8.4	-3.00		-74	
Mean	**-0.64**	**-0.14**	**-62**	**-76**
SD	**1.08**	**0.07**	**51**	**28**
2-tail t-test NaHS vs. Hypoxia		**0.56**		**0.38**

10.3.2.2 Raw data: Maximum change in active tension to NaHS and hypoxia in isolated human pulmonary veins (paired preparations)				
	Maximum Δ Active Tension (gf)		**Maximum Δ Active Tension (%)**	
Prep	**NaHS**	**Hypoxia**	**NaHS**	**Hypoxia**
PV7.1	-0.04	-0.12	-25	-48
PV7.2	-0.06	-0.17	-29	-89
PV7.3	-0.10	-0.10	-29	-42
PV7.4	-0.16	-0.05	-29	-71
PV8.2	-0.21	-0.25	-162	-114
Mean	**-0.11**	**-0.14**	**-55**	**-73**
SD	**0.07**	**0.08**	**60**	**30**
2-tail t-test NaHS vs. Hypoxia		**0.56**		**0.38**

10.3.3.1 Raw data: Dose response effects of NaHS on resting tension in isolated human bronchi

	Baseline (gf)						Change in Tension (gf)					% Change in Tension						
	NaHS [µM]						NaHS [µM]					NaHS [µM]						
Prep	**Baseline (gf)**	**1**	**10**	**100**	**300**	**1000**	**1**	**10**	**100**	**300**	**1000**	**1**	**10**	**100**	**300**	**1000**	**EC_{50} (mM)**	**pEC_{50}**
B67.1	1.28	1.07	0.95	0.88		0.67	-0.21	-0.33	-0.40		-0.61	-16	-26	-31		-48	0.01	5.00
B67.2	1.83	1.83	1.73	1.63		1.01	0.00	-0.10	-0.20		-0.82	0	-5	-11		-45	0.42	3.38
B81.1	2.02	2.00	2.00	2.04	1.92	1.83	-0.02	-0.02	0.02	-0.10	-0.19	-1	-1	1	-5	-9	0.31	3.51
B81.2	1.73	1.73	1.73	1.78	1.73	1.54	0.00	0.00	0.05	0.00	-0.19	0	0	3	0	-11	0.66	3.18
B81.3	1.54	1.54	1.52	1.54	1.47	1.00	0.00	-0.02	0.00	-0.07	-0.54	0	-1	0	-5	-35	0.60	3.22
B81.4	1.67	1.76	1.73	1.69	1.59	1.48	0.09	0.06	0.02	-0.08	-0.19	5	4	1	-5	-11	0.42	3.38
Mean	**1.68**	**1.66**	**1.61**	**1.59**	**1.68**	**1.26**	**-0.02**	**-0.07**	**-0.09**	**-0.06**	**-0.42**	**-2**	**-5**	**-6**	**-4**	**-27**	**0.40**	**3.61**
SD	**0.25**	**0.32**	**0.36**	**0.39**	**0.19**	**0.43**	**0.10**	**0.14**	**0.18**	**0.04**	**0.27**	**7**	**11**	**13**	**2**	**18**	**0.23**	**0.69**
2-tail paired t-test vs. baseline resting tension		**0.59**	**0.59**	**0.30**	**0.30**	**0.06**												

10.3.3.1 Raw data: Maximum relaxation to NaHS and hypoxia from resting tension in paired bronchi.										
	HBD					1mM NaHS				
Prep	Pre HBD Baseline (gf)	Post HBD baseline (gf)	HBD Dilation (gf)	% of Baseline	Time to Max HBD (min)	Pre NaHS Baseline (gf)	Post NaHS baseline (gf)	NaHS Dilation (gf)	% of Baseline	Time to Max NaHS dilation (min)
B67.1	2.92	0.75	-2.17	-74		1.28	0.87	-0.41	-32	
B67.2	2.93	1.15	-1.78	-61		1.83	1.01	-0.82	-45	
B68.1	1.93	0.80	-1.13	-59	27.00	1.05	0.88	-0.17	-16	13.00
B68.2	2.02	1.11	-0.91	-45	20.00	1.39	1.06	-0.33	-24	12.00
B69.2	0.97	0.63	-0.34	-35	16.00	0.87	0.63	-0.24	-28	4.00
B71.2	2.40	1.54	-0.86	-36	18.00	2.64	1.35	-1.29	-49	11.00
B81.1	2.00	1.70	-0.30	-15		2.02	1.83	-0.19	-9	
B81.2	1.83	1.54	-0.29	-16		1.73	1.54	-0.19	-11	
B81.3	1.72	1.00	-0.72	-42		1.54	1.00	-0.54	-35	
B81.4	1.88	1.54	-0.34	-18		1.67	1.48	-0.19	-11	
Mean	**2.06**	**1.18**	**-0.88**	**-40**	**20.25**	**1.60**	**1.17**	**-0.44**	**-26**	**10.00**
SD	**0.58**	**0.38**	**0.65**	**20**	**4.79**	**0.51**	**0.37**	**0.36**	**14**	**4.08**
SEM	**0.18**	**0.12**	**0.21**	**6**	**2.39**	**0.16**	**0.12**	**0.11**	**4**	**2.04**
Paired t-test HBD vs. NaHS	**0.04**	**0.75**	**0.05**	**0.03**	**0.01**					

10.3.3.2 Raw data: Dose response effects of NaHS on active tension in isolated human bronchi																	
				Tension (gf)				Δ in Active Tension (gf)				% Δ in Active Tension					
				NaHS [µM]				NaHS [µM]				NaHS [µM]					
Prep	Pre MCh BL	Post HBD/O_2 BL	Active Tension to MCh (gf)	10	100	300	1000	10	100	300	1000	10	100	300	1000	EC_{50} (mM)	pEC_{50}
B67.1	0.76	5.4	4.64	5.48	5.31	3.40	0.78	0.08	-0.09	-2.00	-4.62	2	-2	-43	-100	0.38	3.42
B67.2	1.30	5.14	3.84	5.24	4.95	3.85	1.15	0.10	-0.19	-1.29	-3.99	3	-5	-34	-104	0.48	3.32
B68.1	0.90	5.93	5.03	5.95	5.82	4.75	1.91	0.02	-0.11	-1.18	-4.02	0	-2	-23	-80	0.51	3.29
B77.1	2.04	3.91	1.87	3.89	3.83	3.81	1.49	-0.02	-0.08	-0.10	-2.42	-1	-4	-5	-129	0.66	3.18
B77.2	2.12	3.51	1.39	3.51	3.51	3.51	1.54	0.00	0.00	0.00	-1.97	0	0	0	-142	0.65	3.19
B77.3	1.87	4.99	3.12	5.19	5.37	5.14	1.32	0.20	0.38	0.15	-3.67	6	12	5	-118	0.66	3.18
B77.4	1.80	4.34	2.54	4.47	4.49	4.11	1.15	0.13	0.15	-0.23	-3.19	5	6	-9	-126	0.64	3.19
Mean	**1.54**	**4.75**	**3.20**	**4.82**	**4.75**	**4.08**	**1.33**	**0.07**	**0.01**	**-0.66**	**-3.41**	**2**	**1**	**-16**	**-114**	**0.57**	**3.25**
SD	**0.55**	**0.86**	**1.37**	**0.89**	**0.85**	**0.64**	**0.36**	**0.08**	**0.20**	**0.82**	**0.94**	**3**	**6**	**18**	**21**	**0.11**	**0.09**
2-tail paired t-test vs. BL active tension				**0.05**	**0.91**	**0.08**	**0.00**										

10.3.3.2 Raw data: Relaxation to hypoxia and NaHS from active tension in isolated human bronchi											
	HBD						1mM NaHS				
Prep	Pre MCh Baseline (gf)	Post MCh Plateau (gf)	Active tension to MCh (gf)	Baseline post HBD (gf)	HBD (gf)	HBD (% max MCh)	Post HBD/O2 baseline	Active Tension to MCh (gf)	Baseline post NaHS (gf)	NaHS dilation (gf)	NaHS % max MCh
B67.1	0.76	6.74	5.98	0.94	-5.80	-97	5.40	4.64	0.78	-4.62	-100
B67.2	1.30	7.07	5.77	1.39	-5.68	-98	5.14	3.84	1.15	-3.99	-104
B68.1	0.90	7.36	6.46	1.83	-5.53	-86	5.93	5.03	1.91	-4.02	-80
B69.2	0.53	3.91	3.38	1.11	-2.80	-83	3.09	2.56	1.16	-1.93	-75
B71.2	1.30	4.90	3.60	1.59	-3.31	-92	4.52	3.22	1.39	-3.13	-97
B76.1	2.12	2.33	0.21	2.14	-0.19	-90	2.23	0.11	1.98	-0.25	-227
B77.1	2.04	5.70	3.66	1.37	-4.33	-118	3.91	1.87	1.49	-2.42	-129
B77.2	2.12	4.90	2.78	0.91	-3.99	-144	3.51	1.39	1.54	-1.97	-142
B77.3	1.87	6.31	4.44	1.57	-4.74	-107	4.99	3.12	1.32	-3.67	-118
B77.4	1.80	6.08	4.28	1.78	-4.30	-100	4.34	2.54	1.15	-3.19	-126
B81.1	1.56	3.77	2.21	1.94	-1.83	-83	3.33	1.77	2.10	-1.23	-69
B81.2	1.49	3.95	2.46	1.88	-2.07	-84	3.57	2.08	1.69	-1.88	-90
B81.3	1.18	3.53	2.35	1.38	-2.15	-91	2.87	1.69	1.27	-1.6	-95
B81.4	1.41	3.51	2.10	1.95	-1.56	-74	2.91	1.50	1.33	-1.58	-105
Mean	**1.46**	**5.00**	**3.55**	**1.56**	**-4.07**	**-102**	**3.98**	**2.53**	**1.45**	**-2.92**	**-120**
SD	**0.50**	**1.56**	**1.73**	**0.39**	**1.68**	**18**	**1.10**	**1.34**	**0.37**	**1.30**	**43**
SEM	**0.13**	**0.42**	**0.46**	**0.10**	**0.45**	**5**	**0.29**	**0.36**	**0.10**	**0.35**	**11.58**
Paired 2-tail t-test hypoxia vs. NaHS			**3E-05**	**0.22**	**0.0003**	**0.15**					

10.3.3.5 Raw data: Effect of H2S synthesis inhibitors on resting tension					
			Post-Antagonist		
Prep	Drug [µM]	Baseline post wash	Baseline post antagonist	Change in baseline (gf)	Change in baseline (%)
B71.2	**PPG [30000]**	1.65	2.11	0.46	28
B87.1	**PPG [30000]**	0.87	1.24	0.37	43
Mean		**1.26**	**1.68**	**0.42**	**35**
SD		**0.55**	**0.62**	**0.06**	**10**
2-tail paired t-test		**0.07**			
B87.2	**HA [300]**	1.29	1.12	-0.17	-13
B87.3	**HA [300]**	1.17	1.11	-0.06	-5
B87.4	**HA [300]**	1.87	1.83	-0.04	-2
B70.1	**HA [300]**	0.70	0.54	-0.16	-23
Mean		**1.26**	**1.15**	**-0.11**	**-11**
SD		**0.48**	**0.53**	**0.07**	**9**
2-tail paired t-test		**0.05**			
B67.2	**HA [1000]**	1.89	0.90	-0.99	-52
B67.1	**PPG [10000]**	1.56	1.74	0.18	12

10.3.3.5 Raw data: Effect of H2S synthesis inhibitors on HBD from resting tension														
		Pre- Antagonist				Post-Antagonist								
Prep	Drug [μM]	Baseline pre hypoxia (gf)	Baseline post hypoxia	Hypoxic Dilation from baseline (gf)	Hypoxic Dilation from baseline (% Change)	Baseline post antagonist	Baseline post hypoxia	Hypoxic Dilation from baseline (gf)	Hypoxic Dilation from baseline (% Change)	% Change vs pre antagonist	Time to max HBD Pre (min)	Time to max HBD Post (min)	% Δ in rate
B71.2	**PPG [30000]**	2.23	1.28	-0.95	-43	2.11	1.41	-0.70	-33	-22	15	18	20
B87.1	**PPG [30000]**	1.14	0.98	-0.16	-14	1.24	1.05	-0.19	-15	9	25	15	-40
Mean		**1.69**	**1.13**	**-0.56**	**-28**	**1.68**	**1.23**	**-0.45**	**-24**	**-6**	**20**	**17**	**-10**
SD		**0.77**	**0.21**	**0.56**	**20**	**0.62**	**0.25**	**0.36**	**13**	**22**	**7**	**2**	**42**
2-tail paired t-test		**0.94**	**0.19**	**0.58**	**0.59**						**0.69**		
B87.2	**HA [300]**	1.40	1.25	-0.15	-11	1.12	1.08	-0.04	-4	-67	25	6	-76
B87.3	**HA [300]**	1.69	1.44	-0.25	-15	1.11	1.05	-0.06	-5	-63	21	6	-71
B87.4	**HA [300]**	2.07	1.89	-0.18	-9	1.83	1.68	-0.15	-8	-6	26	8	-69
B70.1	**HA [300]**	0.74	0.62	-0.12	-16	0.54	0.51	-0.03	-6	-66	22	12	-45
Mean		**1.48**	**1.30**	**-0.18**	**-13**	**1.15**	**1.08**	**-0.07**	**-6**	**-50**	**24**	**8**	**-66**
SD		**0.56**	**0.53**	**0.06**	**3**	**0.53**	**0.48**	**0.05**	**2**	**30**	**2**	**3**	**14**
2-tail paired t-test		**0.03**	**0.04**	**0.05**	**0.05**						**0.005**		
B67.2	**HA [1000]**	2.83	1.16	-1.67	-59	0.9	0.87	-0.03	-3	-94	18	NA	
B67.1	**PPG [10000]**	2.89	0.74	-2.15	-74	1.73	0.96	-0.77	-45	-40	18	14	

10.3.3.5 Raw data: Effects of H2S synthesis inhibitors on cholinergic contraction and HBD from active tension																			
		Pre-Antagonist						Post-Antagonist								Time to max HBD Pre (min)	Time to max HBD Post (min)	Change vs. control (%)
Prep	Drug [µM]	BL pre MCh (gf)	MCh Plateau (gf)	MCh (gf)	HBD BL post MCh (gf)	HBD post MCh (gf)	HBD post MCh (% MCh)	BL pre MCh (gf)	MCh Plateau (gf)	MCh (gf)	% Change in MCh vs. pre antag	HBD BL post MCh (gf)	HBD post MCh (gf)	HBD post MCh (% MCh)	% change vs pre antag			
B71.2	PPG [30000]	1.32	4.89	3.57	1.59	-3.30	-92	2.03	4.71	2.68	-25	1.77	-2.94	-110	19	15	19	**27**
B87.1	PPG [30000]	0.99	2.71	1.72	1.54	-1.17	-68	1.25	2.67	1.42	-17	1.54	-1.13	-80	17	15	15	**0**
Mean		**1.16**	**3.80**	**2.65**	**1.57**	**-2.24**	**-80**	**1.64**	**3.69**	**2.05**	**-21**	**1.66**	**-2.04**	**-95**	**18**	**15**	**17**	**13**
SD		**0.23**	**1.54**	**1.31**	**0.04**	**1.51**	**17**	**0.55**	**1.44**	**0.89**	**5**	**0.16**	**1.28**	**21**	**1**	**0**	**3**	**19**
t-test pre vs. post		**0.277**	**0.36**	**0.06**	**0.50**	**0.43**	**0.12**									**0.50**		
B87.2	HA [300]	1.20	3.77	2.57	1.74	-2.03	-79	1.11	3.09	1.98	-23	1.35	-1.74	-88	11	12	**17**	**42**
B87.3	HA [300]	1.31	1.98	0.67	1.39	-0.59	-88	1.11	1.59	0.48	-28	1.18	-0.41	-85	-3	16	**15**	**-6**
B87.4	HA [300]	1.90	3.19	1.29	2.08	-1.11	-86	1.94	2.74	0.80	-38	1.77	-0.97	-121	41	18	**17**	**-6**
B70.1	HA [300]	0.84	5.86	5.02	1.61	-4.25	-85	0.55	3.20	2.65	-47	0.87	-2.33	-88	4	19	**18**	**-5**
Mean		**1.31**	**3.70**	**2.39**	**1.71**	**-2.00**	**-84**	**1.18**	**2.66**	**1.48**	**-34**	**1.29**	**-1.36**	**-96**	**13**	**16**	**17**	**6**
SD		**0.44**	**1.62**	**1.93**	**0.29**	**1.62**	**4**	**0.57**	**0.74**	**1.01**	**11**	**0.38**	**0.84**	**17**	**19**	**3**	**1**	**24**
t-test pre vs. post		**0.154**	**0.15**	**0.74**	**0.04**	**0.24**	**0.27**									**0.76**		
B67.2	HA [1000]	1.58	7.03	5.45	1.40	-5.63	-103	0.88	1.69	0.81	-85	0.96	-0.73	-90	-13	23	**6**	
B67.1	PPG [10000]	1.12	6.75	5.63	0.94	-5.81	-103	1.62	5.71	4.09	-27	1.38	-4.33	-106	3	23	**10**	

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