THE IMPACTS OF INFLAMMATORY MEDIATORS AND HYPOXIA UPON VASCULAR REACTIVITY: A MODEL FOR COPD?

By

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ABSTRACT

Chronic obstructive pulmonary disease (COPD) is characterised with a poorly reversible airflow limitation. It is a major public health challenge in many developed countries. Cardiovascular complication is a common co-morbidity in COPD and is linked to hypoxia and inflammation. Arterial stiffness is also correlated with emphysema severity in COPD patients, with a suggestion that this might be related to systemic inflammation.

In this thesis, we have investigated the influence of inflammatory mediators and hypoxia on vascular dysfunction in an in vivo and in vitro rat model. In addition, we looked to identify a potential interaction of hypoxia and inflammation upon arterial stiffness of alpha-1 antitrypsin deficient (AATD) subjects.

We found that TNF–α (2-6hrs) had a significant effect upon isolated rat pulmonary artery dilatation in response to carbachol and sodium nitroprusside. Unlike tempol, allopurinol has completely restored the carbachol response whereas none of the antioxidants had any effects on sodium nitroprusside response. Thus suggesting that TNF–α impaired carbachol response via xanthine oxidase pathway. Hypoxia applied for 1-2 weeks in vivo then acute (1hr) TNF-α challenge (in vitro) had no effect upon vascular reactivity. However, when combined acute hypoxia and TNF–α simultaneously (in vitro, 1hr) pulmonary artery vasodilatation was impaired. These data suggest that prolong inflammation or combination of acute inflammation and hypoxia can have a combined and detrimental effect upon vascular reactivity and so provide a potential model for studying these exacerbations in vitro.

Also, our results have shown that the serum TNF–α level of AATD subjects correlated positively and significantly with arterial stiffness as measured by pulse wave velocity (PWV). Multivariate regression analysis revealed that TNF–α is not an independent predictor of PWV, unlike age and systolic pressure. However, the model that entered age, sex, Forced Expiratory Volume in 1 second, systolic pressure and gas transfer factor TLCO and TNF–α as the independent variables was found to effectively predict PWV (independent variable). There was no significant interaction observed between hypoxia and inflammation to modulate arterial stiffness.

Dedication

This thesis is dedicated my late beloved father **Alhagi Kawsu Ibrahim Gassama** who continued to inspire me beyond the heavens to achieve the goals he once had for me and to my dear mum **Hajja Matida Sillah** who has always been my source of inspiration throughout my academic struggle.

Also, I would like to dedicate this achievement to my inspirational uncles; Kebba Sillah and Karali Sillah who had both answered the call of God during my write-up stage. May the mercy of Allah be with you and all deceased.

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Chapter 1

1 INTRODUCTION

1.1 Chronic obstructive pulmonary disease and its impact on healthcare

Chronic obstructive pulmonary disease (COPD) is characterised by poorly reversible airflow obstruction (1-5) and is associated with abnormal inflammation in the lungs (3, 6). This phenomenon arises due to an irregular inflammatory response to harmful substances or gases, mainly cigarette smoking (7). The World Health Organisation has reported that COPD is the fourth leading cause of mortality in the world and by 2025 is projected to become the third (8-10). Therefore, COPD presents a major challenge towards the public health sectors in both developed and developing countries. In the UK alone, it is estimated that COPD affects 3 million people (1, 11) and accounts for 1 in 8 hospitalisations. In the USA, COPD is the fourth leading cause of death(12). The BOLD study 2011, reported prevalence of Gold Stage II or higher in 22% of men and 17% of women in South Africa (13). Also, between five cities in the southern cone of Latin America, COPD prevalence ranges from 8% in Mexico City to 20% in Montevideo (5). Millions of people are affected by COPD throughout the world and many may die prematurely from the disease or consequent to its complications; this problem is anticipated to increase in the years to come due to persistent exposure to COPD risk factors as well as the aging population (5, 8, 10, 13-16).

The economic cost of COPD on the UK economy is substantial. According to the National Institute for Health and Clinical Excellence (NICE) (17) the total annual cost of COPD to the NHS is projected to be over £800 million for direct healthcare costs, which is equivalent to £1.3 million per 100,000 people. This stresses the significance

of undertaking research to investigate to understand the disease in order to identify new potential cost effective remedies.

1.1.1 Diagnosis of COPD

COPD is diagnosed by spirometry, which measures airflow obstruction by means of forced expiratory volume in 1 second (FEV_1) and forced vital capacity (FVC) and measures of reversibility of these after bronchodilating drugs (7, 18) . Reduction in the ratio of FEV_1 to FVC defines the existence of airflow limitation whereas a decrease in FEV_1 is used to determine disease severity (19) . Almost all global bodies and national guidelines accept the use of spirometry based diagnosis of COPD (1, 11, 12, 20-22) although definitions vary (**Table 1.1**).

The Global Initiative for Chronic Obstructive Lung Disease (GOLD) was the earliest agreement or declaration, produced in 2001 (23). This was based on the use of a fixed $FEV_1/FVC < 0.7$ as well as a fixed FEV_1 to determine its severity (20). This definition does not account for the differences in population, age or sex; therefore it is prone to misclassifying the diagnosis. Using a fixed $FEV_1/FVC <0.7$ can over-diagnose COPD in elderly patients especially among non-smokers (24-28).

In 2004, the American Thoracic Society (29) and European Respiratory Society (ERS) published a joint statement to promote the implementation of the lower limit of normal (LLN) rather than a fixed $FEV_1/FVC < 0.7$ to diagnose COPD (4). LLN is defined as "the lower fifth percentile of the frequency distribution of a reference population, to define pulmonary function abnormalities" (19, 24) and can be determined by deducting 1.64 times the standard deviation from the mean or expected value(19). There is still an on-going debate to determine which of these definitions is more accurate. More recently far greater characterisation of patients

has been proposed as a means of categorising COPD patients into groups whose clinical features or outcomes differ (30, 31). Additional tests might be undertaken that include computed tomographic (CT) (32) scanning of the chest to evaluate the severity and distribution of emphysema, gas transfer measurement, assessment of co-morbid disease, exercise capacity or rapidity of decline in FEV1 over time (16, 30, 31, 33-36). Exercise capacity can be measured by six minute walk test (34, 37) or endurance shuttle test(37) and contributes to prognosis when assessed as part of the BODE (body mass index, airway obstruction, dyspnoea and exercise tolerance) index (33) .

Common co-morbidities in COPD include cardiovascular disease, muscle dysfunction, osteoporosis, anxiety and depression (2, 38-42) –their prevalence, relationship to lung disease in COPD and pathophysiology has been reviewed elsewhere (43). Patients with COPD may also experience episodes where their condition worsens, termed exacerbations. Their frequency is also recognised as an important feature of disease (44). Exacerbations are defined as 'an event in the natural course of the disease characterised by a change in the patient's baseline dyspnoea, cough and/or sputum beyond day-to-day variability sufficient to necessitate a change in management (4).

	NICE 2004[2]	**ATS/ERS 2004[1]**	**GOLD 2011[51]**	**BTS/NICE 2010[9]**
FEV_1 % predicted	FEV1/FVC <0.7	<LLN	<LLN	FEV1/FVC <0.7
≥80		Mild	Stage I: Mild	Stage I: Mild
50-79	Mild	Moderate	Stage II: Moderate	Stage II: Moderate
30-49	Moderate	Severe	Stage III: Severe	Stage III: Severe
<30	Severe	Very severe	Stage IV: Very severe	Stage IV: Very severe

Table 1.1.1: **Classification & severity of COPD disease**

1.1.2 Classification of COPD

COPD is often an umbrella term denoted to respiratory diseases typified by airway obstruction(45) and comprises of a number of overlapping phenotypes(46). These can include chronic bronchitis, emphysema, bronchiectasis, bronchiolitis or a combination of these(46, 47). It is a destructive inflammatory process causing partially reversible airflow limitation that defines COPD (48). The inflammation is not solely confined to the lungs and there are often systemic consequences(49) (see 1.1.3). The pathologies of COPD are briefly detailed below.

Chronic bronchitis is typified by inflammation of the bronchial tubes (tubes that carry air to the lungs) which might cause the individual to develop a persistent cough. In COPD, chronic bronchitis involves various clinical manifestation that consist of accelerated decline in lung function, higher risk of air flow limitation (mainly in smokers), susceptibility to lower tract infection, increased frequency of exacerbation and overall increase in mortality (50). Chronic bronchitis is mainly caused due to hypersecretion and excessive production of mucus by the goblet cells. This phenomenon is responsible for worsening airflow limitation of the small airways and epithelial remodeling(50). In addition, the surface tension of the airways might be altered due the accumulation of mucus thereby making the making it airway surface susceptible to collapse (50).

Emphysema is a lung condition that is associated with shortness of breath. It is characterised with damage to air sacs in the lungs (alveoli). Progressively, the inner walls of the air sacs weaken and rupture leading to formation of enlarged air spaces rather than several small normal ones. Consequently, the surface area of the respiratory alveoli is reduced thus minimising the amount of oxygen transferred into

bloodstream. People with emphysema also struggle to exhale the air that gets trapped in the lungs thereby precluding entry of fresh oxygen rich air.

Current research evidence suggest that the majority of people with emphysema also suffer from chronic bronchitis, hence small airway pathology was associated with poor clinical outcome, worsen mortality and reduced augmentation of the lung function post lung volume reduction surgery (50). In fact, unlike the classic COPD spectrum definition that classified individuals into chronic bronchitis at one end and the emphysema on another, it is established that majority of COPD patients found themselves somewhere in the between.

Moreover, bronchiectasis is associated with a permanent expansion of bronchi and bronchioles that consequently cause airway obstruction due to abnormal mucus secretion similar to what is observed in chronic bronchitis. However, evaluation and treatment are different between the two disease conditions(51). A study Arram & Elrakhawy (51) has reported that about 48% (33 of 69) of COPD population they had studied were found to have both bronchiectasis and chronic bronchitis. Further investigation using higher resolution CT scan suggests that bronchiectasis with chronic bronchitis might exhibit severe airway dysfunction, frequent exacerbation and increased bacterial colonization (51). Smoking is the main cause of COPD though there is specific genetic condition that might make individuals more susceptible to developing emphysema. The genetic contribution to COPD is detailed in sections 2.1 & 3.1 Treatment may slow the progression of COPD, but it can't reverse the damage.

1.1.3 Role of inflammation in COPD

COPD is characterised by chronic airway inflammation, which represents a key component of disease development and pathogenesis (3). Studies of bronchoalveolar lavage have reported elevated inflammatory cells, such as neutrophils, in sputum from COPD patients compared with controls (52). Keatings *et al* (52) also found significant levels of inflammatory cytokines such as interleukin–8 (IL-8) and tumour necrosis factor–alpha (TNF–α) in sputum from COPD patients compared with healthy smokers and non-smoking controls, suggesting these are implicated in COPD (52). Numerous other studies have confirmed these findings (6, 53-58), although significant day to day variability occurs (58).

Furthermore the relationship of pulmonary to systemic inflammation is not yet clear. Several studies have reported elevation of various inflammatory markers such as TNF–α, IL–8, IL–6 and C–reactive protein (CRP) in the serum (59-61) of patients with stable COPD suggesting that inflammation is not confined to the lungs. Levels of inflammatory mediators might also increase in COPD patients during exacerbations, which in turn is associated with cardiovascular events (62) suggesting that systemic inflammation promotes advancement of cardiovascular diseases (63). In COPD this is thought to occur by various mechanisms including endothelial dysfunction (64), arterial stiffness (65) and increased platelet activation (66).

Erythropoiesis (EPO) is also influenced by pro-inflammatory mediators such as IL–1α, IL–1β, IL–6, IL–10, TNF–α and interferon-gamma (IFNγ). The main stimulus for EPO production is the fall of tissue PO_2, therefore increased production was observed under hypoxic condition in the kidney and other organs such as the liver and brain (minor amounts). Thus low red blood cells count and high EPO levels

signals a cellular hypoxia (67). During inflammation, EPO production by kidney and liver cells may be reduced and increased hepcidin production occurs, which down-regulates iron efflux from body stores, thereby minimizing the iron level for erythropoiesis and impairing haemoglobin secretion and bone marrow reaction to erythropoietin (68-71). This mechanism has not directly been linked to COPD, however, inflammation and hypoxia were implicated in the increased production of hepcidin by the liver, and thus it might be relevant to explore the association of this mechanism with COPD. It will principally be important in finding alternative therapies for aneamic COPD patients as previous studies showed increased levels of pro-inflammatory cytokines (IL-10, IFN-y, CRP, TNF–α) and erythropoietin in anaemic COPD patients compared to non-anaemic controls (54, 72). The prevalence of aneamia in patients with COPD varies from 7.5% to 33% (73).

1.1.4 Hypoxia and use oxygen therapy in COPD

COPD patients with severe hypoxaemia at rest may receive long term oxygen therapy (LTOT), defined as oxygen use for ≥12 hours/day, and is indicated when PaO_2 <7.3kPa (55mmHg) at rest when well, or <8.0kPa in the presence of cor pulmonale (74, 75). LTOT improves their daily activity, and reduces mortality (22, 74-77). Ambulatory oxygen is defined as the use of supplemental oxygen during exercise and activities of daily living[41]. Ambulatory oxygen therapy is often used for patients on LTOT during exercise, or for non LTOT users who achieve some subjective and/or objective benefit from oxygen during exercise (1, 22, 76, 77). The latter group of COPD patients, who are not severely hypoxaemic at rest, might experience considerable breathlessness, due to hypoxia, on exertion. However, there is no conclusive evidence to justify the use of ambulatory oxygen in patients not on LTOT. Consequently, international guidelines differ, and clinical practice

varies. One of the main treatments to improve symptoms in COPD is pulmonary rehabilitation (35, 78, 79), which includes exercises to improve fitness; ambulatory oxygen might also be used under these circumstances, although again the evidence base has not been thoroughly explored, or consistent in its results.

Findings from various studies have revealed potential contribution of tissue hypoxia in the development of skeletal muscle disease in COPD (80). These might be summarised in the following points. Chronic hypoxia inhibited protein synthesis in muscle cells which caused net loss of amino acids and limited production of myosin heavy chain isoforms (81). Also, muscle mass loss was reported in healthy individuals at high altitude (82, 83). In addition, skeletal muscle derived from patients with COPD and chronic respiratory failure exhibits structural (decrease of type I fibres) and functional (upregulation of mitochondrial cytochrome oxidase) alterations proportional to the severity of arterial hypoxaemia. If tissue hypoxia plays a pathogenic role, domiciliary oxygen therapy may have a beneficial effect upon muscle disease in COPD.

1.1.5 Genetic susceptibility to COPD: Role of Alpha-1-antitrypsin deficiency

Alpha-1-antitrypsin deficiency (AATD) is broadly recognised as the sole genetic factor that makes the subjects susceptible to COPD. Based on a disease registry in the West Midlands, it is estimated that 670 people in England have emphysema caused by AATD (84). In North America, the prevalence of AATD is about 1 in 3000–5000 people (85). Globally, it was eestimated that 1-5% of patients with diagnosed COPD have AATD (85, 86).

AATD was first described in 1963 by Laurel and Eriksson. They have recognised alpha-1 (α1) band were missing on protein electrophoresis of human serum (87). Generally, when serum isolated from healthy individuals undergoes electrophoresis,

separates the serum proteins into 5 main bands: alpha-1-globulins, alpha-2-globulins, beta globulins, gamma globulins and albumin. However, the major composition of alpha-1-antitrypsin is the alpha-1-globulins, hence the absence of this band results in AATD (87). Interestingly, the absence of alpha-1-globulins band was associated with early onset of emphysema thus suggesting a potential role for AATD in the disease pathogenesis. Alpha-1-antitrypsin (AAT) is regarded as an acute phase glycoprotein which is mainly synthesised by hepatocytes then released into the plasma (88). In addition, tiny amount of AAT is generated from alveolar macrophages, circulating monocytes and lung epithelial cells (89-91).

1.1.5.1 Clinical features

Previous studies have suggested that AATD was associated with disease pathogenesis of COPD (such as emphysema, chronic bronchitis, and bronchiectasis), neonatal jaundice, vasculitis, panniculitis and liver cirrhosis (92)**.** While AATD is a susceptibility factor to COPD phenotypes including emphysema, the condition does not lead all patients to develop the pulmonary disease. The difference in the clinical phenotype might be due to variations in environmental factors (for example; cigarette smoking) that might be predisposed by other genetic factors. Subjects were mostly diagnosed with AATD post pulmonary or liver disease investigation. However, this process might be subjective to the local and national common practices, especially where a neonatal screening is available (93). For example; in countries that implement screening programme might diagnose subjects at birth, rather than when the disease symptoms were observed. Most of the symptoms of AATD are non-specific; therefore, subjects might experience variety of symptoms such as cough, wheeze and chest infection(93). These experiences might

interfere with the disease diagnosis thus delaying the diagnosis of the condition shown by many by nearly 6 years from the onset of the symptoms (94). The typical presentation of AATD is with progressive dyspnoea, plus or minus chronic bronchitis, comparable to typical COPD and despite younger age and less smoke exposure (92). The regression analysis of UK AATD population reveals that features of emphysema may occur in late teenage years (93), whereas usual COPD presentation occurs at middle age (at approximately 45 years).

The current guidelines in the UK suggest testing for AATD only in subjects below the age of 45 or in people with disease severity inconsistent with their level of exposure to cigarette smoke (95). Typically, cigarette smoke exposure is estimated by pack years. One pack year is equal to one pack of cigarette per day (i.e 20 sticks per day). Majority of COPD subjects had 10 years or more exposure to cigarette smoke whereas the AATD subjects had far less exposure (92).

The lung function tests AATD mostly indicate feature typical to COPD; for example airflow obstruction, plus or minus as small gas transfer and elevated static lung volumes. In addition, it common to observe in AATD subjects, changes mostly associated asthma such as post bronchodilator reversibility (96). This has been shown in the initial assessment of NHLBI AATD registry, where reversibility post bronchodilator administration stands at 12% of baseline FEV1 in 28% of the subjects (96). Besides, normal lung function might be observed mainly both asymptomatic subjects though is not entirety. Exposure to smoking is probably the most essential determinant of lung function as variation was reported between smokers and non-smokers in late teen years (~ 18 years), as well as a significant association between smoking and lung function decline in adults, with the average decline per year in FEV1 from 70mL to 47mL in current smokers and never smoked respectively (97).

Other features such as male sex, low body mass index, frequent exacerbations and severity of upper zone emphysema were all recorded as alternative determinants to rapidly declining lung function (98).

Currently, high-resolution computerised tomography (HRCT) scanning is commonly used in the assessment of COPD and AATD as a direct consequence of improved interpretation of the images obtained with this the technique (99). The scan can be useful to diagnose emphysema and bronchiectasis, as well as their distribution and severity levels (99, 100). It has been shown that emphysema distribution in AATD has a relationship to the lung function (101). AATD subject predominantly develop lower zone emphysema, whereas in COPD, upper zone disease predominates. In addition, it has been reported that bronchiectasis occurs in the majority of AATD subjects mainly together with emphysema (100). There are specific test for assessment of AATD that involves quantifying the serum level of AAT levels (< 11μM regarded as pathophyiologically significant), phenotyping technique (protein electrophoresis) or genotyping to identify common diseases linked with variants such as Z allele (100).

1.2 Vascular Wall Composition and Classification of arteries

Generally, the vascular wall comprises of three main layers. This includes the tunica intima, tunica media and tunica externa, and these are referred sometimes as the internal, middle and outer layers respectively (102). Locating in the intima, are the endothelial cells that release of essential mediators such as neurotransmitter, hormones and vasoactive substances to regulate vascular function (103). The vascular tone and blood pressure are maintained primarily through the actions of the endothelium and smooth muscle cells. The main role of the arteries is to supply oxygenated blood to all organs with high pressure; however, pulmonary artery

delivers deoxygenated blood to the lungs. Based on the size, anatomical feature, location and functionality, the arteries were divided conducting arteries, macrovasculature (conduit arteries) and microvasculature (resistance) (104, 105).

The conducting arteries are rich in elastic tissues that give the vessels flexibility to expand and recoil in order to respond to changes in blood pressure. Examples of conducting arteries include the aorta, large pulmonary and carotid arteries (105, 106). Conducting arteries subdivide to form conduit arteries such as small muscular pulmonary arteries, femoral, radial, and brachial arteries that are specialised in regulating the flow of blood to specific organs and areas of the body (107). Conduit arteries further divide to form resistance branches of arteries that comprise mainly of smooth muscle cells with extensive networks of sympathetic nerves. The resistance arteries constrict and dilate in response to sympathetic activities thereby regulating the flow of blood to tissues (105, 108).

1.3 Regulation of Vascular Tone

Vascular tone denotes the general contractile state of a vessel or vasculature region (109). It is a term commonly used to characterise the degree of constriction experienced by a blood vessel relative to its maximally dilated state. Under basal conditions, arterial and venous vessels manifest a level of smooth muscle vasoconstriction, which influences the vessels diameter and tone. Vascular tone is determined by opposing vasoconstrictor and vasodilator stimuli acting on the blood vessels (110).

1.3.1 Vascular Smooth Muscle Contraction

The wall of blood vessels contain layers of smooth muscle cells, similar to the wall lining of a variety of organs such as stomach, intestine, airways and uterus (111). Generally, contraction causes shortening of the smooth muscle cells that result in

changes in the diameter of the vessel. These changes cause the lumen contents to be pushed along the vessel thus allowing it to regulate flow. The contractile state of the smooth muscle cells are controlled by neural innervation from the autonomic nervous system, hormones (paracrine or autocrine) and local chemical stimuli (111).

1.3.2 Mechanism of contraction

The smooth muscle cell contraction is mainly controlled via the induction of mechanical (stretch) and receptor that cause the contractile proteins (myosin and actin) to be stimulated. The myosin and actin interaction that results from the phosphorylation of myosin light chain (MLC) by myosin light chain kinases (MLC Kinase) is an essential step for any contraction to happen. ATP generated via myosin ATPase activity is utilised as fuel to cycling of the myosin cross-bridges and actin contraction. Therefore, MLC phosphorylation state is used as a determinant of the contractile activity of a vascular smooth muscle. When there is no receptor or stretch stimulation, the phosphorylation state of the MLC remains low, a phenomenon referred as smooth muscle tone (111).

1.3.3 Contraction of Smooth Muscle is Ca^{2+} dependent

Ca^{2+}-mediated alterations in the thick filaments results in the initiation of contraction of the smooth muscle cells (111). The smooth muscle reacts to the stimulation of a variety of external stimuli (including vasoconstrictor agonists), by increasing $[Ca^{2+}]_{i.}$ Sarcoplasmic reticulum and receptor-operated Ca^{2+} channels both contribute to the elevation of $[Ca^{2+}]_i$ via release of Ca^{2+} from intracellular stores and influx of Ca^{2+} from the extracellular space into the cytosol respectively (111, 112). The free calcium forms a complex with a special calcium binding protein called calmodulin, which triggers stimulation of MLC kinase that subsequently activate LCM (111) (**Figure 1.1**).

Moreover, upon binding to a heterotrimeric G protein coupled receptor, vasoconstrictor agonists (such as norepinephrine, thromboxane A2, angiotensin II, endothelin and vasopressin) induces Gα subunit dissociation (in the case of a contractile Gq-coupled receptor the Gαq unit) and activation of phospholipase Cβ (PLCβ) enzyme (112). Activated phospholipase C, catalyse phosphatidylinositol 4, 5-bisphosphate (membrane lipid) to generate the second messengers, inositol trisphosphate (IP_3) and diacylglycerol (DG). IP_3 activates IP_3 receptors (IP_3R) on the sarcoplasmic reticulum to induce a rapid release of Ca^{2+} into the cytosol, whereas DAG activates receptor operated channels (ROC), possibly formed of transient receptor potential proteins such as TRPC6, which mediate Na^+ and Ca^{2+} entry (112). In addition, cluster of IP_3R cause localized elevation of Ca^{2+} that subsequently stimulate ryanodine receptors to trigger Ca^{2+} induced Ca^{2+} release (CICR), thereby potentiating the rise in Ca^{2+} and leading to regenerative Ca^{2+} oscillations (113, 114). Increased level of cytosolic Ca^{2+} initiate smooth muscle contraction through formation of Ca^{2+}-calmodulin and subsequent activation of myosin light chain kinase (MLCK), resulting in the phosphorylation of the 20 kDa regulatory myosin light chain (MLC) and cross bridge cycling. The extent of MLC phosphorylation is proportional to force, and is determined by the balance of activity between MLCK and the MLC-dephosphorylating enzyme myosin light chain phosphatase (MLCP) (112). Also, activation of PKC increases the myofilament force sensitivity to $[Ca^{2+}]i$ and MLC phosphorylation, and maintains vascular smooth muscle contraction (115). In addition, mechanical activity of the smooth muscle cells results in membrane depolarisation that leads to the opening of voltage-operated L-type Ca^{2+} channels to increase intracellular free Ca^{2+} level (111).

1.3.4 GPCR-induced Rho/ROCK mediated Ca^{2+} sensitization

Increased MLC phosphorylation can also occur as a consequence of Ca^{2+}-sensitization, which involves inhibition of MLC phosphatase (MLCP). Rho/Rho kinase and PKC mediated signaling pathways mainly control the activity of MLCP (112). This Ca^{2+} independent pathway modulate the Ca^{2+}/camodulin activation of MLCK and the subsequent phosphorylation of MLC through inhibition of MLCP that results in augmented phosphorylation of MLC and subsequent increase in the level of smooth muscle contraction for any given elevation of cytosolic [Ca^{2+}] (112, 116). This phenomenon is known as Ca^{2+} sensitization (116, 117). It is suggested the mechanism might involves monomeric G-protein RhoA and activation of its main downstream effector Rho kinase (ROCK) though is not clarified (112).

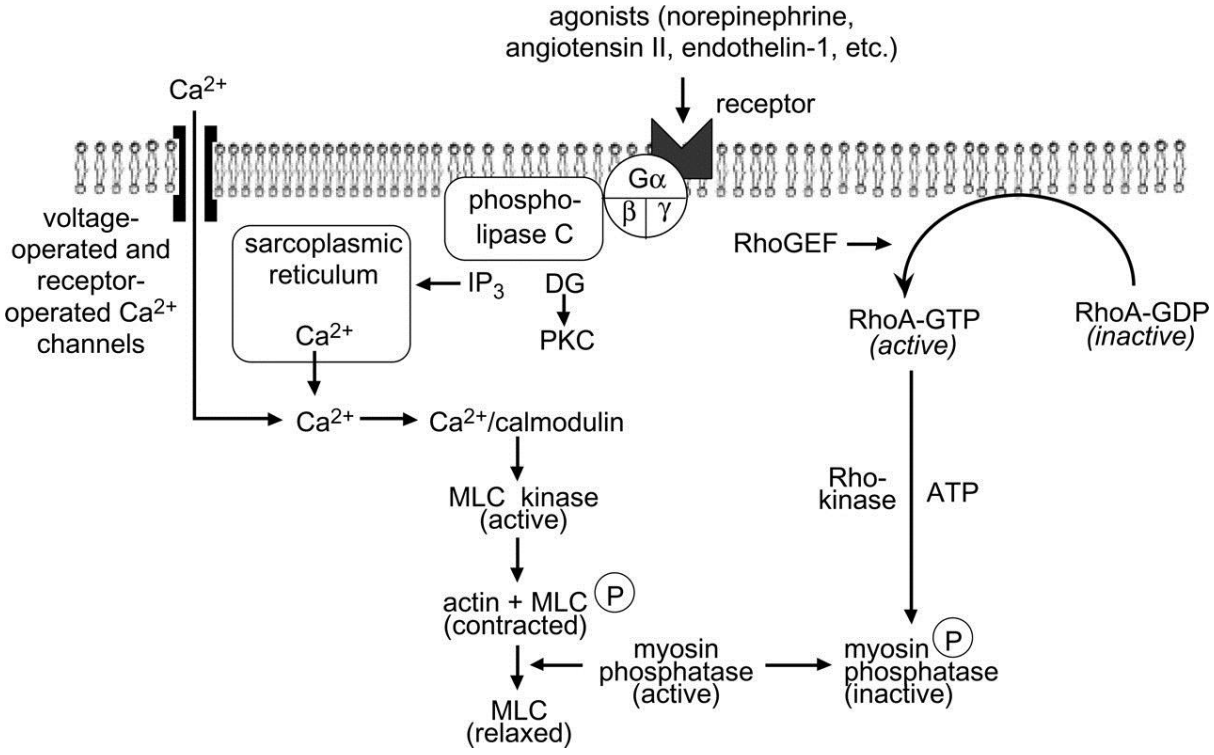


Figure 1.1: Regulation of smooth muscle contraction. Various agonists (neurotransmitters, hormones, etc.) bind to specific receptors to activate contraction in smooth muscle to increase phospholipase C activity via coupling through a G protein. Phospholipase C produces two potent second messengers from the membrane lipid phosphatidylinositol 4,5-bisphosphate: diacylglycerol (DG) and inositol 1,4,5-trisphosphate (IP_3). IP_3 binds to specific receptors on the sarcoplasmic reticulum, causing release of activator calcium (Ca^{2+}). DG along with Ca^{2+} activates PKC, which phosphorylates specific target proteins. Activator Ca^{2+} binds to calmodulin, leading to activation of myosin light chain kinase (MLC kinase). This kinase phosphorylates the myosin light chain, and, in

conjunction with actin, cross-bridge cycling occurs, initiating shortening of the smooth muscle cell leading to contraction. Rho kinase inhibits myosin phosphatase activity to maintain the contractile state via Ca^{2+}-sensitizing mechanism. Though the mechanism is not fully elucidated, this involve activation of Rho A by nucleotide exchange factor (RhoGEF) and migration of RhoA to the plasma membrane, which subsequently result in increased Rho kinase leading to inhibition of myosin phosphatase. Adapted from (111).

1.4 Endothelium-Derived Vasoactive Substances

In the early 1980s, Robert Furchgott observed that acetylcholine released a substance that produced vascular relaxation, but only when the endothelium was intact (118, 119). This observation opened this field of research and eventually led to his receiving a Nobel Prize. Initially, Furchgott called this substance endothelium-derived relaxing factor (EDRF), but by the mid-1980 he and others identified this substance as nitric oxide (NO) (109, 110). Furchgott and Zawadzki (120) studied the obvious inconsistency that acetylcholine could not always generate vasodilatation in vitro. They conducted investigations to unravel this mystery which led them to the finding that blood vessels isolated with intact endothelium consistently elicited acetylcholine–mediated vasodilatation. In addition, rubbing the intimal surface of segments of rabbit aorta vessel in order to remove the endothelium prevented the observed acetylcholine–mediated vasodilatation response. Moreover, Furchgott and Zawadzki (120) has shown that Ach acts on the muscarinic receptors found on the endothelium cells, which stimulates release of a substance(s) that causes vasodilatation of the vascular smooth muscle. At this point, the significance of endothelial cells in vascular biology became recognised, hence, the earliest demonstration of the notion that endothelial cells generated mediators that has the potential to modulate vascular function (120). This has been the cornerstone of several investigations undertaken from the 80s to uncover substances that affect vascular tone (121, 122). Therefore, numerous mediators of vascular tone were

identified and suggested to act in a similar manner to acetylcholine(121). The findings suggest that endothelium perform a critical role in the regulation of vascular function in both disease and health conditions. It is well documented that the endothelial cells synthesises and release a host of heterogeneous vasoactive substances that are essential in cardiovascular homeostasis and this process is controlled by well-organized local, systemic responses or interaction between the two in complex mechanisms (110). Some of this explained below:

Vasoactive mediators synthesised or released from endothelial cells might be divided broadly into two main groups; the *endothelium-derived relaxing factors* (EDRFs) and *endothelium-derived contracting factors* (EDCFs)(123). Examples of EDRFs include nitric oxide (NO), prostacyclin (PGI_2), whereas, EDCFs include endothelin, angiotensin, thrombin, superoxide anion (110, 122, 124). The third group referred to as the *endothelium-dependent hyperpolarizing factor* (EDHF) is also known to be involved in the maintenance of vascular tone especially in the absence or blockage of EDRFs (125). We have used a synthetic mimetic of Ach called Carbachol (Carbamoylcholine chloride) in our experiments.

EDRFs and EDCFs might act via variety of mechanisms. These range from specific receptors to second messenger systems that consequentially modulate the smooth muscle cell response. The endothelium-derived mediators might act in autocrine (directly on endothelium cells themselves) or paracrine (indirectly on the vascular smooth muscle cells) manner. Hence, it is important to be able distinguish the features of the various endothelium-derived factors which are generated endogenously and might produce various responses in different vascular beds. This is due to the complex nature of the communication that exists between these

mediators because the observed physiological processes were not entirely due to a single vasoactive substance. Relevant endothelium-derived vasoactive substances such as NO will be discussed more broadly in the next sections.

1.5 Nitric Oxide

1.5.1 Nitric Oxide plays a variety of roles in different systems

A variety of cell types such as vascular endothelium cells, smooth muscle cells, leukocytes, platelets and neurones synthesise and release NO. In addition, nitric oxide has been implicated in a variety of conditions thus playing different roles. These include regulation of immunity (126), inflammation (127), apoptosis (128), central nervous system signaling (129, 130). However, the most prominent role of NO is the regulation of vascular tone and blood pressure (131).

In the cardiovascular systems, nitric oxide plays a major role as a signaling messenger (132). NO contributes to several significant biological functions within the cardiovascular physiology beside its key role as an endothelium-derived relaxing factor. NO promote maintenance of the vascular integrity by inhibiting platelet aggregation (133, 134), platelet adhesion, leucocyte–endothelium adhesion (135-137) and vascular smooth muscle proliferation (138), as well as promotes platelet disaggregation (139). These help maintain anti-atherogenic state of the endothelium under non-pathological or resting conditions (140). Within an *In vivo* context, NO exerts these beneficial effects continually, under normal condition, as NO is constitutively released from the vascular endothelium cell via shear stress as result of the blood flow (141). The physiological range of shear flow is from 2 – 10 dyne/cm^2. In addition, shear-stress and pulsatile stretching of the vascular wall induce an uninterrupted release of NO which is essential in the generation of tonic

NO-induced vasodilation, which subsequently encourages local blood flow and maintenance of low peripheral vascular resistance (142).

1.5.2 NO synthesis

As earlier stated, the release of NO from the endothelium was and the its subsequent effect on the vascular tone was first reported by Furchgott in the 80's (118-120). The actual identity of this gaseous relaxing molecule; NO, was not uncovered until couple of years following the initial discovery of the activity (143). The road to the discovery of NO was reviewed by elsewhere by Moncada and Higgs (144). Currently, the pathway of NO synthesis is widely recognised in the literature. The amino acid L-arginine is the natural precursor for NO synthesis. In the presence of co-substrates NADPH and oxygen molecule (O_2), L-arginine get oxidised in a reaction catalysed by nitric oxide synthase (NOS) to generate L-citrulline and NO (145) (**Figure 1.2**). The NO produced can go on to perform variety of function locally and regionally within the vasculature such as bringing about vascular relaxation as detailed in the schematic diagram (**Figure 1.3**). Fleming and Busse (142) have established that other redox-active cofactors such as terahydrobiopterin (BH4), heme, calmodulin (CaM), Flavin mononucleotide (FMD) and flavin adenone dinucleotide (FAD) are prerequisite for NOS catalysis.

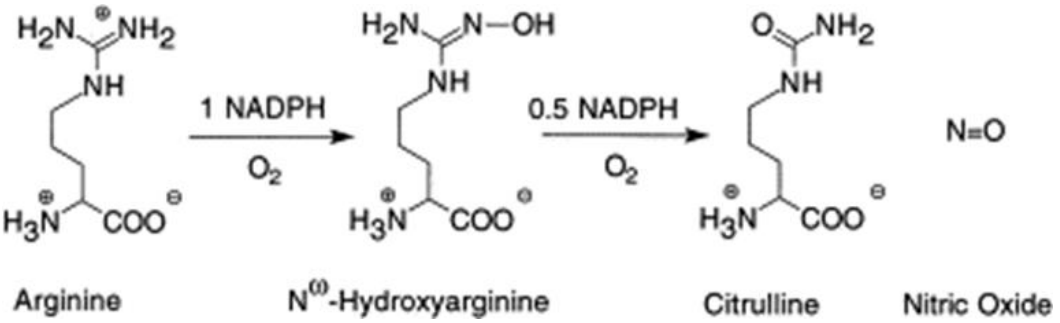


Figure 1.2: Enzyme–catalysed nitric oxide (NO) synthesis from L-arginine. Hydroxylation of l-arginine produces N-hydroxy-l-arginine as an intermediate. The second step converts N-hydroxy-l-arginine to generate NO and citrulline as products. Adapted from (145)

1.5.3 Isoforms of nitric oxide synthase and activation

NO is a free radical produced by NOS in two step five-electron oxidation of the terminal nitrogen of l-arginine as detailed above. There are three isoforms of NOS that have been characterised. These include neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS) (125) and these are referred sometimes as NOS I, NOS II and NOS III respectively. In addition, nNOS and eNOS are called as constitutive NOS (cNOS) (125, 146). As we can probably tell from their names, the three isoform of NOS are derived from different genes and might be regulated by variety of signaling pathways. eNOS and nNOS are constitutively expressed in cells that are mainly regulated by the Ca^{2+} influx which subsequently binds with calmodulin (145). However, iNOS expression is mainly stimulated by inflammatory cytokine (such as TNF–α, LPS, IL–1β, IL–2 and so on) and once generated, iNOS remain stable at normal intracellular Ca^{2+} concentration $[Ca^{2+}]_i$, thus iNOS is suggested to be Ca^{2+} independent (145).

Endothelial NOS generates NO, a process that is critical in cardiovascular homeostasis. It is well established within the literature that eNOS activation was Ca^{2+}/calmodulin-dependent due the fact numerous agonist activated the enzyme only when the intracellular free calcium concentration $[Ca^{2+}]_i$ was increased(147, 148). Interestingly, shear stress was initially considered the most fundamental physiological element required for constant production of NO as viscous drag mediated phosphorylation of eNOS to modulate its enzymatic activity (141, 149). Stefanie et al (150) revealed that serine/threonine protein kinase Akt/ protein kinase

B (PKB) activated eNOS that resulted into increased generation of NO. However, reduced serine phosphorylation and lack of eNOS activation was observed with the blockage of phosphatidylinositol-3-OH kinase/Akt pathway or mutation of the Akt site on eNOS protein (at serine 1177). Moreover the eNOS activity and sensitivity to Ca^{2+} was altered by Serine 1177 phosphorylation analogue was used that generated optimal enzymatic activity at below physiological intracellular calcium concentration $[Ca^{2+}]_i$. This observation confirms that the Akt activation of eNOS is Ca^{2+} independent (150).

1.5.4 Actions of NO

The enzymatic action of nitric oxide synthase converts amino acid L-arginine to NO in the endothelial cells. NO is a non-polar molecule that can diffuse freely across the cell membrane (151). Therefore, NO rapidly diffuses from the endothelium into the adjacent vascular smooth muscle cells. It binds and activates soluble enzyme guanylyl cyclase (GC) which then dephosphorylate guanosine-5'-triphosphate (GTP) to cyclic guanosine monophosphate (cGMP) thus increasing the level of cGMP. Subsequently, cGMP serves as second messenger that signal smooth muscle relaxation. The relaxation of smooth muscle cells could be induced by cGMP via several mechanisms such as by increasing the level of intracellular cGMP, which oppose calcium entry into the cell. This is achieved variety of mechanisms, knowledge of which is ever evolving. However, they include inhibition of voltage gated Ca^{2+} channels thus reducing $[Ca^{2+}]_I$ and activation of large conductance potassium (BK) channels (that results into hyperpolarisation and relaxation) and induction of a cGMP–dependent protein kinase G (PKG) leading to stimulation of myosin light chain (MLC) phosphatase, an enzyme which dephosphorylates MLC leading to smooth muscle relaxation (110, 152). Hence, the activation of cGMP-

dependent PKG is the main mechanism through which NO acts to stimulate relaxation in vascular smooth muscle.

Rembold et al. has shown that elevated cGMP levels might cause reduction of vascular tone through phosphorylation of heat shock protein (hsp) – 20 that binds with thin actin filaments thereby inhibiting cross-bridge formation or cycling. The cellular mechanisms response of cGMP was reviewed by Francis et al (152). They suggested that mechanism NO stimulates vascular relaxation were unlikely to be due to a single pathway (152). However, together the pathways might be interacting to effect changes on the vascular tone, although evidence may indicate relative significance of variety of pathways locally or regionally (152). Biosynthesis of NO and generalised action is summarised below (**Figure 1.3**). This *in-vivo* vascular tone regulation mechanism is amenable to investigation *ex-vivo*.

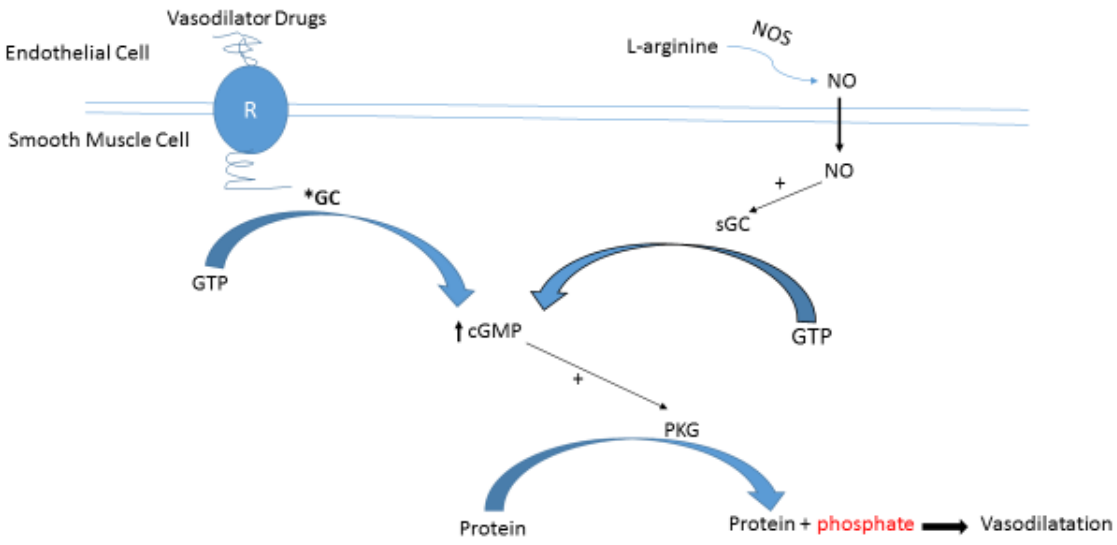


Figure 1.3: NO synthesis and generalised action on VSM relaxation. The enzymatic action of nitric oxide synthase converts amino acid L-arginine to NO in the endothelial cells. NO rapidly diffuse into the VSM to activate guanylyl cyclase (GC) which then dephosphorylate Guanosine-5-triphosphate (GTP) to cyclic

Guanosine monophosphate (cGMP). Some vasodilator drugs can acts via receptor bound GC to increase CGMP level. The build-up of cGMP then activate PKG to phosphorylate a relevant protein to bring about relaxation ((110). *GC= Receptor bound GC, R=Receptor.

1.5.5 Physiological roles of NO in cardiovascular systems

The physiological role of endogenously generated NO has been one the fastest growing areas of biomedical research (153). The evidence gathered suggests a prominent role for this tiny molecule in a variety of physiological function that included regulation of vascular tone and proliferation of mesenchymal cells (154). Enormous efforts have been rendered in finding the role of NO in pulmonary circulation, both in the normal state and during pulmonary hypertension (153).

In cardiac cells, NO regulates cardiac contractility (155). In addition, availability of sufficient amount of NO is critical to maintain normal vascular physiology to face challenges of reduced NO bioavailability, endothelial dysfunction, which might predispose to atherosclerotic disease (139, 156). Hence, NO signaling deficiency has been associated with atherosclerosis, diabetes mellitus, congestive heart failure, hypertension, hypercholesterolemia, thrombosis and stroke (139, 156-158). The important of NO in the physiology and pathogenesis of disease can be shown through genetic manipulation of enzymes that generate NO in mice or studies where NO generation has been nullified. Specific inhibition of NO generation by L-arginine analogues such as N^G-monomethyl-L-arginine (L-NMMA) or via genetic knock-out mice, demonstrated a significant elevation of central blood pressure in subjects with sepsis. Moreover, NO has been shown to diminish action of several vasoconstrictors such as noradrenaline, angiotensin II and vasopressin, as well as inhibited response of endothelin (159, 160). Therefore it can be suggested that balance between

vasoconstrictor and vasodilator substances is a cornerstone of maintenance of homeostasis in the vascular system (161).

1.6 Reactive oxygen species & chemical characteristics

Reactive oxygen species refers to a group of molecules that encompasses the free radicals that are naturally produced as by-products of oxygen metabolism as well as non-radical species. Most ROS are free radicals (such as superoxide anion ($O_2^{-\cdot}$), hydroxyl racial ($HO^{\cdot}$) and lipid radicals) (157), due to the presence of unpaired electrons (162). Other reactive oxygen species, such as hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), hypochlorous acid (HOCl) ozone (O_3), and lipid peroxide (LOOH), are non-free radical species. However, they do possess oxidizing capabilities that promote oxidant stress.

Several studies have reported ROS as an intermediate that acts as second messenger in signaling pathways. Superoxide and hydrogen peroxide the commonest ROS involved in cell signaling pathways (162). Electrically charged $O_2^{-\cdot}$ is highly reactive and is rapidly dismutated into H_2O_2, while the enzyme, catalase, decomposes H_2O_2 to water and oxygen (162, 163). $O_2^{-\cdot}$ exist in small concentration (picomolar–nanomolar range) within the cells and has limited ability to readily diffuse thus acts as intracellular messenger, whereas, the increased cellular levels of H_2O_2 (high nanomolar-micromolar range) which can readily diffuse across the hydrophobic cellular membranes to act as intra- and inter-cellular second messenger (163). Through the process of the generation of ROS within the cell, a variety of other free radicals are generated via radical chain reactions (164). For example, reactions between radicals and polyunsaturated fatty acids within cell membrane may result peroxyl radical that attack the side chains of adjacent fatty acids to initiate production of other lipid radicals. Lipid radicals produced in this chain reaction might be stored

in the cell membrane and might subsequently cause cellular function abnormalities such as leakage of the plasmolemma and dysfunction of membrane-bound receptors (164).

There are several sources of ROS in mammalian cells. These include the mitochondrial respiration, arachidonic acid pathway enzymes lipoxygenase and cyclooxygenase, cytochrome p450s, xanthine oxidase, NADH/NADPH oxidases, NO synthase, peroxidases, and other hemoproteins (157). ROS generation sites exist in the lungs, endothelial cells, alveolar macrophage, eosinophils and alveolar epithelial cells. Moreover, in the pulmonary vasculature, ROS is mainly formed by complexes within the cell membrane (Nicotinamide Adenine Dinucleotide Phosphate (NADPH) – oxidase), cellular organelles (such as peroxisomes and mitochondria) and cytoplasm (Xanthine Oxidase). Besides, limited levels of eNOS co-factors or substrates such as L-arginine and BH_4 can result to uncoupled eNOS, thus producing a shift from NO generation to ROS production(165). Several mechanisms were recognised with ROS production in hypoxia associated PH and these involves but not limited to dysfunctional mitochondria, uncoupled NOS, xanthine oxidase and NADPH oxidase (NOX). However, in the vasculature, NADPH and mitochondria were reported to be the main source of ROS production (166, 167), hence this will covered in detail in the next sub-sections.

1.6.1 NADPH Oxidase

Investigating the source of ROS in the homogenates of endothelial and vascular smooth muscle cells has revealed that NADH is the main substrate feeding the $O2^{-\cdot}$ generation. Also, NADPH generated smaller proportion of $O2^{-\cdot}$ (168). Variety of factor such as cytokines, hormones and mechanical forces were suggested to have a role in the regulation of the vascular NADH/NADPH oxidase during pathogenesis

of vascular disease (157). These have been demonstrated by the increased activity of vascular ROS production and NADH/NADPH oxidase activity in vascular smooth muscle cell that was stimulated by angiotensin II, thrombin, platelet-derived growth factor, tumor growth factor-α, and lactosylceramide (169-172). Furthermore, when human umbilical endothelial cells (HUVEC) were subjected to a unidirectional laminar shear stress, this caused transient increase of NADH-dependent $O2^{-\cdot}$ production, contrary to the observed sustained elevation in oxidase activity when oscillatory shear stress was utilised (173). All these evidence demonstrate the role of NADH/NADPH oxidase in ROS generation. In cultured rat vascular smooth muscle cells stimulated with angiotensin II resulted in $O2^{-\cdot}$ production due to increased activity NADH/NADPH oxidase (170). Likewise, a sudden rise in $O2^{-\cdot}$ generation and NADH/NADPH oxidase activity was demonstrated in chronic angiotensin II infused hypertensive rats (174).

The NOX system is a transmembrane mutimeric protein structure that catalyses an electron reduction of O_2 to generate $O2^{-\cdot}$ by utilising NADH/NADPH as a donor(168). There are seven catalytic homologs (such as NOX1–5 and Duox1–2) that make up the NADPH oxidase family. However, the pulmonary vascular smooth muscle cell expresses NOX1, NOX2, NOX4 (166) and NOX5 (162). Each of these NOX isoforms has specific adapter sub-units that are essential for the enzymatic action of the complexes. This is demonstrated by the protypic NOX isoform, NOX2 that constitutively combined with $p22^{phox}$ (175-177) in the membrane and without the latter adapter sub-unit, NOX2 becomes unstable (178). NOX 2 is stimulated on binding to the cytosolic $p47^{phox}$ (organiser sub-unit) which gets phosphorylated and promote recruitment of other sub-units such as the $p67^{phox}$ (activator sub-unit) and $p40^{phox}$ subunits into the NOX2 complex (179). The last step of the complex

formation is when a small GTPase RAC1 directly binds to the NOX2 and consequently interact with $p67^{phox}$ thus activating the sub-unit (180, 181). Subsequently, the assembled NOX2 complex has the ability to produce $O2^{-\cdot}$ by donating an electron to molecular O_2 in the luminal or extracellular space from the NADPH in the cytosol (182). NOX1 activation is different from NOX2 as its activation requires interaction with cytosolic sub-units $p47^{phox}$, $p67^{phox}$, NADPH oxidase organiser 1 (NOXO1) and NADPH activator 1 (NOXA1) accordingly (183). NOX4 and NOX 5 are constitutively active and do not rely on interaction with the cytosolic homologues such as $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and GTPase Rac1 (162, 166) (184, 185), although NOX4 requires interaction with the membrane sub-unit $p22^{phox}$ (162).

Moreover, NADPH oxidase might be stimulated due to various stimuli growth factors (PDGF, EGF and TGF-1β), inflammatory mediators/cytokines (such as TNF-α, IL-1 and platelet aggregation factors), metabolic factors (for example; hyperglycemia, hyperinsulinemia, free fatty acids, advanced glycation end products, mechanical factors (cyclic stretch, laminar and oscillatory-shear stress) and G protein-coupled receptor agonist (such as serotonin, thrombin, bradykinin, ET-1 and Ang II) (162, 186-188). Contrastingly, statins, esradiol and peroxisome proliferator-activated receptor (PPAR) agonist diminish activation of NADPH oxidase (189, 190). Superoxide derived from NADPH was revealed to chiefly contribute in vascular dysfunction associated with a variety of animal models of PH including hypoxia-induced PH (162, 191).

1.6.2 Mitochondrial dysfunction

The mitochondrial dysfunction is associated with ROS production and this has been noted in the pathophysiology of variety diseases including PH (192, 193). In the cardiovascular system, mitochondria are one of the main sources of ROS generation

(166, 167). ROS production in the mitochondria likely takes place in the electron transport chain (ETC) which involves the Flavin site, complex I (ubiquinone reducing site), complex II (Flavin site) and complex III (ubiquinol oxidizing site) (194). However, complexes I and III were reported to be responsible for majority of ROS produced by the mitochondria (195). Tissue heterogeneity of complexes is a useful determinant of the individual roles of complexes in ROS generation as suggested due to findings from the following studies. In neonatal pulmonary artery myocytes exposed to hypoxia, inhibition of complex III blunted ROS generation (196), whereas complex II was shown to generate most of the mitochondrial ROS produced by hypoxic mouse lung (197). In addition, increased activity of complex II resulted in predominantly complex II derived ROS production in monocrotaline-induced rat models of PH (198). Moreover, PASMC exposed to hypoxia showed elevated ROS production in the mitochondrial inter-membrane space and reduced ROS in the mitochondrial matrix thereby suggesting that ROS production is differentially regulated in the mitochondrial compartments (199). In hypoxia induced pulmonary hypertension, ROS synthesised from the mitochondria caused elevated $[Ca^{2+}]_{i,}$ depolarisation or hyperpolarisation of the membrane potential of mitochondria, PASMC constriction and growth leading to pulmonary vascular remodeling (200-202). Also, hypoxia exposure caused mitochondrial derived ROS to triggered proliferation of PASMC which was later considered to be due to opening of $K^{+}{}_{ATP}$ channels, elevated ROS production that consequently led to stimulation of redox sensitive transcription factors (AP-1) (203). Contrastingly, others have showed pulmonary hypertension and moderate hypoxia gave rise to a hyperpolarised the mitochondrial membrane potential, reduced mitochondrial derided ROS production

and attenuated K^+ current thereby causing Ca^{2+} influx, depolarised plasma membrane and PASMC contraction (204).

It estimated that about 3% of electron flux via the METC is constantly utilized within the mitochondria to generate $O2^{-\cdot}$ (205). Manganese superoxide dismutase (MnSOD) or SOD2 converts $O2^{-\cdot}$ within the mitochondria matrix to H_2O_2 that is subsequently broken down to H_2O by glutathione peroxidase (GPX) (205) (**Figure 1.4**). Copper and zinc superoxide dismutase (Cu-Zn-SOD) or SOD1 breaks down the $O2^{-\cdot}$ within the intermembrane space which then get scavenged and recycled by cytochrome c or transmitted through voltage-dependent anion channels (found on the outer mitochondrial membrane) into the cytosol (206, 207).

Moreover, a variety of antioxidant protective mechanisms reside with the cytosol which includes SOD1, catalase, GPX, GSH and scavengers (such as heme or sulphur-containing protein residue) (208, 209). Superoxide is a major precursor for variety of other ROS and radicals including peroxynitrite (which results from the reaction between NO and $O2^{-\cdot}$) and has the ability to decrease reducing equivalents such as the oxidation of GSH to GSSG. Superoxide is readily reactive and mainly membrane impermeable. However, superoxide can travel via anion channels and is quickly dismuted by SOD to the more stable peroxide (205). Therefore, superoxide and SOD levels are used as a significant marker to define cellular oxidant stress and redox state (210) .

Catalase and/or GPX together with glutathione (GSH) break down H_2O_2 to oxygen and water (**Figure 1.4**). In addition, H_2O_2 can be decomposed by transitional metals like Fe(II) to form a more sensitive OH in a reaction commonly known as Fenton reaction. H_2O_2 has a higher stability, portability and permeability compared to $O2^{-\cdot}$ thus peroxide was considered as the most probable ROS to mediate HPV, though

some researchers proposed that superoxide might cause direct activation of Rho kinase (205, 211).

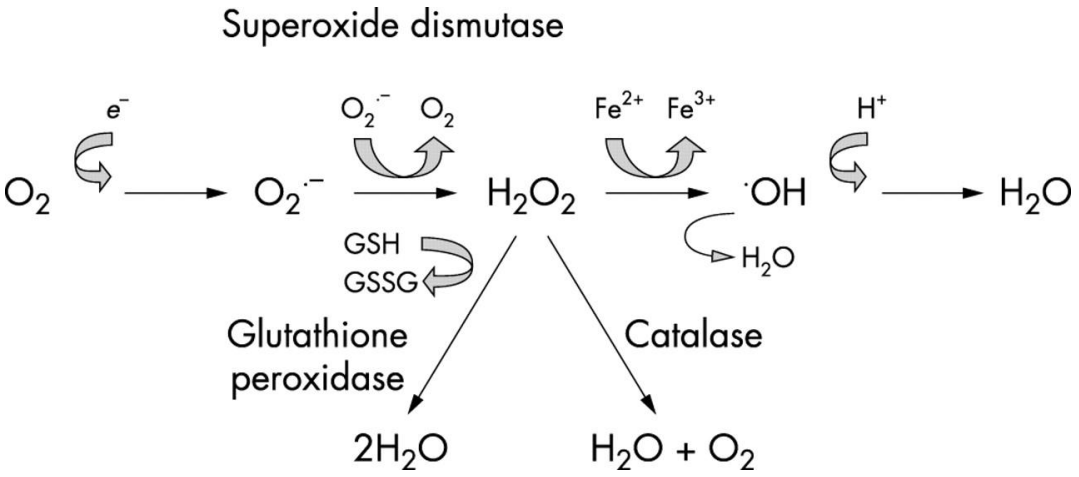


Figure 1.4: Generation of reactive oxygen species: Molecular oxygen (O_2) reacts with an impaired electron (e^-) to form the superoxide anion ($O_2^{\cdot-}$). The enzyme superoxide dismutase converts superoxide anion to hydrogen peroxide (H_2O_2). Hydrogen peroxide undergoes spontaneous conversion to the highly reactive hydroxyl radical ($^{\cdot}OH$). Alternatively, it can be detoxified via either glutathione peroxidase or catalase to water (H_2O) and oxygen (GSH, reduced glutathione; GSSG, oxidised glutathione) adapted from (164).

1.6.3 Xanthine Oxidase

In the process of purine metabolism, xanthine oxidase (XO) is an oxidoreductase enzyme that catalyses the oxidation of hypoxanthine and xanthine. XO can exist in two interchangeable forms; either as xanthine oxidase or xanthine dehydrogenase. The former reacts with molecular oxygen leading to the generation of ROS (superoxide anion; $O2^{-\cdot}$ and hydrogen peroxide; H_2O_2), whereas the latter reduces NAD^+ (157).

A soluble cytokine; interferon (IFN)–γ, can increase the expression of XO and its activity in the endothelial cells (212). In spontaneously hypertensive rats, it has been shown that XO generated $O2^{-\cdot}$ that consequently reduced NO bioavailability,

however, when SOD was added, this significacntly reduced blood pressure in hypertensive rats. In addition, XO inhibitor; oxypurinol reduced blood pressure in the same rats thus suggestion a role for XO in the pathogenesis (213). This coupled with the prevention of the initially elevated free radical levels in the microcirculation of SHRs by XO inhibitor (214).

1.6.4 Endothelial Nitric Oxide Synthase

Endothelial NOS is known as a cytochrome p450 reductase-like enzyme that catalyses Flavin-mediated electron transport from the donor NADPH to a prosthetic heme group. In order to bind near the heme group to transfer electrons to guanidino nitrogen of L-arginine to generate NO, eNOS require BH_4 (157). However, in the absence of BH_4 oxidation or L-arginine, eNOS will generate $O2^{-\cdot}$ (and H_2O_2) thus contributing to the ROS formation. This observation is commonly referred to as NOS uncoupling and several studies have indicated this phenomenon (215, 216). Extensive evidence has suggested that eNOS can become uncoupled in various pathophysiological conditions *in vivo*. This has been shown in the aorta of stroke-prone spontaneously hypertensive rat, where $O2^{-\cdot}$ generation was increased that was reversed by exposure to L-NAME or removal of the endothelium (217).

Similarly, it was suggested that increased $O2^{-\cdot}$ generation observed in aorta of mice due to deoxycorticosterone acetate–salt induced hypertension, might have come due to eNOS, as the effect was prevented by L-NAME treatment, endothelium denudation and was not observed in eNOS deficient mice with deoxycorticosterone acetate–salt hypertension. Moreover, endothelium-dependent vascular relaxation has been impaired in rat made insulin resistant via feeding on high fructose and this effect was normalised by BH_4 supplementation (218). Besides, in chronic smokers,

intra-arterial infusion of BH_4 demonstrated an improved endothelium-dependent vasodilatation. These, therefore, suggest that depletion of BH_4 might play a role in converting eNOS to superoxide anion (157).

Moreover, eNOS uncoupling in the endothelium might stimulate oxidative stress and endothelial dysfunction via at least three mechanisms. Firstly, instead of reacting with NO, free radicals might attack other cellular targets due reduction of enzymatic production of NO. Finally, it is likely that eNOS become partially uncoupled, thereby forming both $O2^{-\cdot}$ and $NO^{\cdot}$ simultaneously. This provides a platform for eNOS to form peroxynitrite thereby leading to increase in oxidative stress (157).

1.7 Role of ROS in endothelial dysfunction

Endothelium dysfunction mainly referred to damage of endothelium-dependent relaxation due to due to loss of NO bioavailability in the vascular wall (219). However, other defined endothelium dysfunction, as the impairment of basic endothelium function such as changes of the anti-coagulant and anti-inflammatory characteristics due to pathological conditions, which eventually cause damage with vascular growth and irregular vascular remodeling (220). The reduction of NO bioavailability might be due to diminished level eNOS (221), absence of co-factors or substrate for eNOS (215), changes to cellular signaling, consequent to inappropriate activation of eNOS (222) and rapid degradation of NO by ROS (157). Early studies have demonstrated that EDRF might be deactivated by superoxide anion, a phenomenon that was prevented by the presence of superoxide dismutase (SOD) (223). The EDRF molecules is currently known as NO, thus the chemical interactions are widely documented. NO reacts rapidly with superoxide anion (with a reaction rate

of $6.7x10^{9}$ mol/L^{-1}), which is almost 3 times quicker than the reaction rate between superoxide and SOD (224). Because of this rapid reaction rate, superoxide anion always has a potential to react with some NO with the cells and extracellular space. Therefore, endogenously generated anti-oxidant can help circumvent this interface under physiological conditions. Whereas in disease conditions, inhibitory measure is changed thereby allowing superoxide to react freely with NO to form peroxynitrite thus minimising bioavailability of NO to the vascular relaxation (157).

Moreover, it has been demonstrated in several animal models that impairment of endothelium-dependent vasodilatation is linked increased degradation of NO by ROS. This has been shown to be implicated in animal models of hypertension, diabetes, cigarette smoking and heart failure (225-227).

1.8 Cardiovascular disease in COPD

COPD is associated with a relative risk of ischaemic heart disease, stroke and sudden death almost double that of healthy individuals, even after accounting for smoke exposure (43). Patients with COPD exhibit increased systemic arterial stiffness (42, 65), which relates to severity of lung disease (228), and could predict subsequent cardiovascular events. Whether systemic vascular disease is present in early COPD has not been well studied, although it is intriguing that therapies traditionally thought to target systemic atherosclerosis may influence outcome in COPD. A reduction in exacerbations in patients with COPD has been seen in several observational studies using statins (229-231), as well as reductions in FEV1 decline (230) and mortality, particularly when combined with ACE inhibitors (231).

It is also, well known that the pulmonary vasculature may be affected in COPD. Pulmonary vascular remodeling, in the form of intimal hyperplasia, is regarded as the main driver of pulmonary hypertension (due to lung disease), and secondary right heart failure in COPD. This occurs even in mild COPD (232), and in smokers with normal lung function (233) and may be driven by inflammation. This contrasts with the long held belief that pulmonary hypertension in COPD is a consequence of hypoxia. Consistent with this observation, LTOT rarely normalizes pulmonary pressures and does not impact on cardiac output in COPD (234). Consequently there has been interest in drugs initially developed to target vascular remodeling in primary pulmonary hypertension in usual COPD. These include endothelial receptor antagonists and phosphodiesterase-5 inhibitors. Thus far, clinical trials have been small, few in number and largely negative (235).

There is also growing evidence that interactions between vascular endothelium and smooth muscle are a major contributor to regulation of local pulmonary vasculature (236). Together these are linked to the development of pulmonary hypertension and chronic heart failure (237). Pulmonary arterial endothelial dysfunction occurs in COPD (238, 239) and in chronic hypoxic rats (240, 241). Endothelial dysfunction contributes to a reduction in secretion of vasodilators such as NO and prostacyclin that leads to vascular remodeling (236, 242). This in combination with endothelial damage and dominant production of vasoconstrictors favours pro-coagulants and vascular obstruction. The abnormal vasoconstriction following alterations in pulmonary vascular function that comes about in both endothelium and vascular smooth muscle are thought not to be dependent on variations in blood vessel structure (243), rather they are dependent on production of vasoactive mediators either locally by vascular endothelium, airway epithelium, vascular smooth muscles

or nerve fibres, or distributed globally via the blood (236, 244). This may be investigated *ex vivo* by determining the effects of acute *vs.* chronic hypoxia on development of vascular tone, and by assessing the influence of putative inflammatory markers on vascular reactivity.

1.9 Pulmonary Hypertension in COPD

1.9.1 Pathophysiology of pulmonary hypertension

Pulmonary hypertension refers to a group of heterogeneous clinical disorders that share a similar pathological characteristic. According to World Health Organisation (WHO), pulmonary hypertension is categorised into pulmonary arterial hypertension (group I), pulmonary venous hypertension (group II), Pulmonary hypertension associated with hypoxia (group III), Pulmonary hypertension associated with chronic thrombotic disease (group IV) and pulmonary hypertension with unclear multifactorial mechanisms (group V) (162, 245). Pulmonary hypertension is a progressive disease characterised by increase in pulmonary vascular resistance, a mean pulmonary arterial pressure (PAP) >25 mmHg at rest or >30 mmHg during exercise (246). Histological studies have demonstrated that Pulmonary hypertension is generally associated with small muscular peripheral arteries, the media of the muscular arteries undergoing hypertrophy, lack of small pre-capillary arteries, development of neointima and in the advance stages of the disease, endothelial proliferate to form plexiform lesion, which is an abnormal channel on the vessel lumen and in the adventitia. These vascular changes together are known as pulmonary vascular remodeling (162).

This PhD is focused on hypoxia-induced pulmonary hypertension (PH) which also involves a diverse group of diseases sharing the common feature of chronic hypoxia-

induced pulmonary vascular remodeling (247). Generally, hypoxic PH is characterised with mild to moderate pulmonary vascular remodeling that is mostly reversible, as opposed to the WHO group 1 pulmonary arterial hypertension (PAH) (247). However, the burden of significant morbidity and mortality remain as PH worsens. Pulmonary hypertension is a well-recognised complication of chronic hypoxic lung diseases, which are among the most common causes of mortality and disability worldwide (248). PH development independently predicts reduced life expectancy (248). Recently, Stenmark and colleagues (247) suggested that a subset hypoxia induced PH might develop irreversible severe pulmonary vascular remodeling similar to WHO group I PAH.

Moreover, in COPD, long-term oxygen therapy might improves pulmonary hypertension and greatly increases the chance of survival and quality of life, though only a partial correction of alveolar hypoxia and PH was observed (248). Increased understanding of the regulation vascular smooth muscle tone suggest a role for chronic vasoconstriction in the pathogenesis of hypoxic PH (248, 249) (**Figure 1.5**) and potential for less contribution of structural changes as earlier thought (248). It was illustrated in **Figure 1.5** that acute hypoxia can rapidly induced HPV within 15 minutes that was sustained for hours and when this continues over a long term can lead into development of pulmonary hypertension.

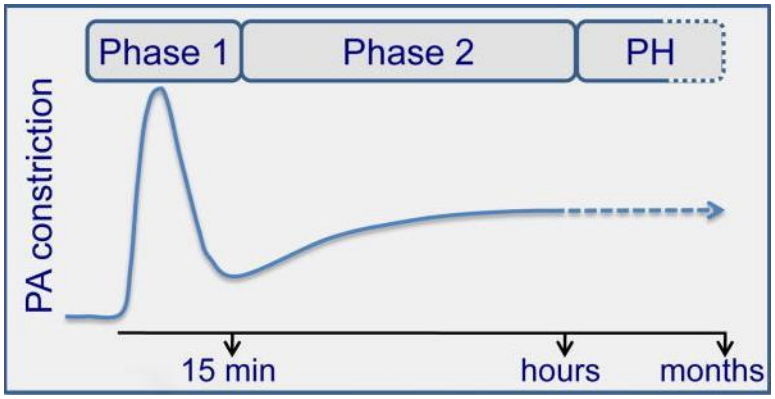


Figure 1.5: Hypoxic Pulmonary Vasoconstriction Demonstrates acute and chronic hypoxic vasoconstriction of pulmonary arteries with the latter leading to pulmonary hypertension. Adapted from (249)

Evidence from an existing vasodilator trial revealed that hypoxic PH was ameliorated and systemic oxygen delivery improved in carefully selected patients (248). However, systemic hypotensive reaction limited influenced of the doses applied (248). To overcome this challenge, McLoughlin's group recently suggested that blockage of vasoconstrictor pathways selective for the pulmonary circulation might reduce hypoxic PH without causing systemic hypotension, and thus provide potential targets for novel therapeutic strategies. Moreover, it has been suggested that in all cases of hypoxia-related vascular remodeling and PH, inflammation, particularly persistent inflammation may play a role (247). This will be discussed in detail in later sections.

1.9.1 Cardiovascular consequence of hypoxic PH: Right heart failure in COPD

Heart failure is a complicated clinical disorder that limits efficiency of the heart due to damage to its ability to pump blood. It is mostly caused by left ventricular systolic dysfunction, coronary artery disease or pulmonary hypertension (250-252). In UK nearly 1 million people are diagnosed with heart failure (253). Left heart failure does not have a direct association with COPD. Therefore, the focus is on right-sided heart failure which has a direct link with COPD, and impacts upon oxygen prescription to patients. Right-sided heart failure (also known as cor pulmonale) is common in severe COPD patients. In severe COPD, PO_2 falls, the walls of the main blood vessels, pulmonary arteries in the lungs change, the pressure inside them rises and is known as pulmonary hypertension. The heart's right ventricle pumps blood through the PAs into the lungs. Pulmonary hypertension puts excess strain on the

right ventricle, which over time becomes stretched and dilated, and fails to pump blood effectively. Right-sided heart failure causes fluid to accumulate in the legs and abdomen, a condition known as oedema. There is an increased risk of deep vein thrombosis or blood clot with leg swelling that can travel to the lung to cause pulmonary embolism.

1.9.2 Role of reactive oxygen species in chronic hypoxia-induced inflammation and pulmonary hypertension

Reactive oxygen species (ROS), including superoxides ($O_2^{\cdot -}$), contribute to chronic hypoxic pulmonary hypertension; hypoxia increases their production in the pulmonary artery (254-260). Despite some ambiguity regarding the production of ROS within the hypoxic pulmonary artery, it is known that NADPH oxidases, mitochondrial electron transport chain, cytochrome p450, nitric oxide synthase, and xanthine oxidase contribute to their generation (162, 261). Endothelium, SMC, neutrophils, macrophages, and epithelial cells may all contribute to ROS generation in hypoxic pulmonary circulation. However, vascular adventitia is particularly interesting as this compartment is affected quicker and greater than any other compartment; and remains the source of ROS that contribute to"outside-in" effects on pulmonary vasoconstriction and vascular remodeling (262-264). NADPH oxidase (NOX) isoform 1, 2 and 4 are understood to contribute the most towards ROS generation within vascular adventitia (262, 265-267) (**Figure 1.6**). ROS production from local adventitia fibroblasts promotes mononuclear cell/macrophage recruitment. This generates more ROS, probably via inflammatory cell NOX and xanthine oxidase (268); affecting not only the local compartment's redox state but also that in other compartments (262, 269) (**Figure 1.6**). Superoxide ($O_2^{\cdot -}$) act rapidly; they can modulate targets directly or impact cell signaling pathways indirectly following their

enzymatic dismutation to hydrogen peroxide. They therefore can modulate the phenotype of a number of cells by affecting proliferation, migration, differentiation, and matrix production (269). ROS also act as signaling molecules and they are known to target HIF, NF-$_{\kappa}$B, Nrf2, MAPK, K^+ channels, and even BMPR2 signaling (162, 262, 269). Xanthine oxidoreductase (XOR), another source of ROS in pulmonary circulation also contributes towards hypoxic pulmonary hypertension and inflammation (255, 262, 270). Although how exactly does xanthine oxidase contribute to hypoxic pulmonary hypertension remains undefined, its activation in leukocytes under hypoxia appears to be important factor in mediating vascular dysfunction (**Figure 1.6**). Studies show that XOR regulates leukocyte adhesion in vivo (271-273) and its inhibition protects airways in COPD (274), ischemia reperfusion injury (275), acute lung injury (276), as well as in other respiratory disorders or conditions that exhibit a hypoxic-inflammatory component (277). More recently, XOR is understood to promote inflammation of pulmonary mononuclear phagocytes thought to have effects on hypoxia-inducible factor (HIF)-1α (278). During hypoxic conditions, HIF 1 and 2 heterodimeric transcription factors that comprised of HIF-1α and HIF-2α accordingly, forms a dimer with a constitutively expressed β subunit (279). The dimerised subunits then bind to hypoxic response element located within the promoter regions of the target genes. Several factors (including oxygen level, growth factors, oncogene activation and tumour suppressor limitations) control expression of HIF-α protein. The α-subunits of the protein are degraded by von Hippel-Lindau (VHL) ubiquitin E3 ligase complex under normal oxygen tension, whereas hypoxia prevent hydroxylation thereby allowing the stabilization of the HIF-α heterodimer and subsequently causing transcriptional activation of targeted genes (279-281).

Moreover, HIF-2α degradation by intermittent hypoxia is understood to be mediated by XOR (and not the NOX)-derived ROS (282). XOR has also been identified as an important molecular signature of sepsis-induced systemic inflammation in many organs – including lungs (283). Importantly, microenvironment of cigarette smoke is understood to promote epigenetic modifications via XOR (a source of both ROS and reactive nitrogen species) promoted DNA double-strand breaks and H2AX histone activation (284). Moreover, XOR-derived ROS also play a significant role in the epigenetic changes of inflammation-induced colorectal cancer (285). These studies strongly support the role of XOR (i.e., XOR-generated ROS) as an important inflammatory mediator and perhaps it's likely involvement in chronic hypoxic pulmonary vascular remodeling.

The presence and localization of high expression of a dominant antioxidant enzyme, extracellular superoxide dismutase (SOD 3), in vascular adventitia supports the importance of ROS in regulation of vascular structure / function under hypoxic conditions (286, 287). Hypoxic and pulmonary hypertension models show that the overexpression of SOD in the lungs protects against medial and intimal wall remodeling (259, 288-290). Conversely, hypoxia induced pulmonary hypertension is exaggerated in SOD1 knockout mice (291).Collectively, these studies show that that there is good evidence for hypoxia-induced ROS generation; these molecules play a crucial role in the activation / regulation of signaling pathways that are involved in chronic hypoxia-induced pulmonary vascular remodeling and pulmonary hypertension.

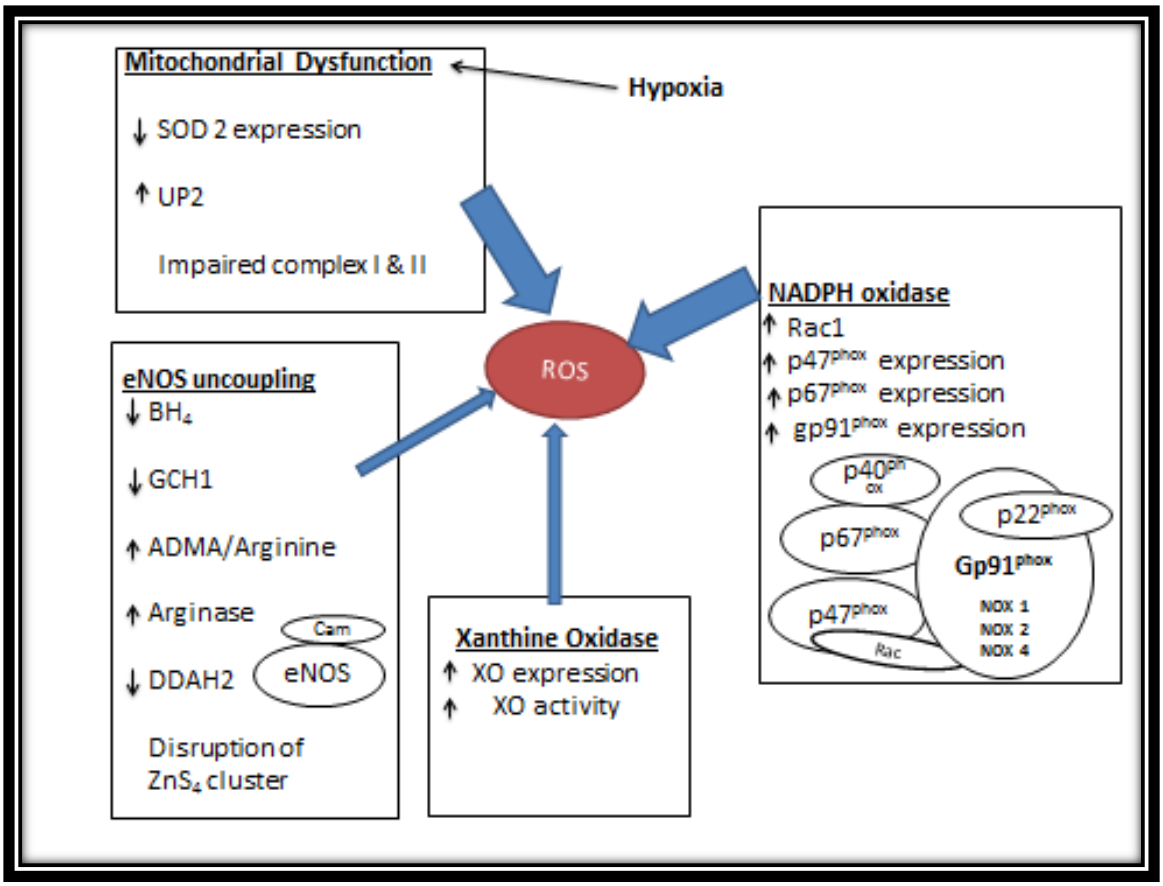


Figure 1.6: Reactive oxygen species generation in pulmonary hypertension -The major sources of ROS in the vasculature include uncoupled eNOS, mitochondrial dysfunction, NADPH oxidase, and XO. In PH, all of these sources contribute to the development of oxidative stress. Endothelial NOS uncoupling is mediated by limited L-arginine availability, as a result of increased degradation by arginase upregulation and attenuated synthesis by the downregulation of ASS and ASL, the enzymes responsible for the conversion of L-citrulline to L-arginine. Moreover, a sustained increase in ADMA levels, due to a decrease in DDAH2 activity, competes with L-arginine for binding to eNOS. In addition, in PH, eNOS function is impaired by a decrease in BH4, an essential co-factor for NO generation. GCH1, the rate-limiting enzyme in BH4 biosynthesis, is ubiquinated and targeted for degradation by Hsp70/CHIP. Therefore, the low GCH1 levels, limit the production of BH4. Finally, The disruption of the zinc tetrathiolate (ZnS4) cluster by oxidative attack disrupts the eNOS dimer, which is accompanied by decreased NO generation and increased ROS production. Further, in PH, several markers of mitochondrial dysfunction are observed, including increased levels of uncoupling protein-2 (UCP-2), decreased levels of the mitochondrial antioxidant, superoxide dismutase-2 (SOD2), and the impaired function of complexes I, II, and III of the respiratory chain. Interestingly, ADMA appears to promote these changes in the mitochondria, and also augment mitochondrial ROS generation and decrease ATP production. In addition, several subunits of NADPH oxidase, including p47phox, p67phox, gp91phox, and Rac1, are upregulated in PH, increasing ROS production in the vasculature. Increased levels and activity of XO also contribute to oxidative stress and vascular dysfunction in the early stages of PH. Adapted from (162).

1.10 Effects of hypoxia on pulmonary vascular constriction

Hypoxia refers to supply of oxygen to cells, tissues and organs below normal physiological levels. In COPD especially in emphysema, airway obstruction can

reduce the amount of oxygen present in the blood (hypoxemia) thereby limiting the supply of oxygen that reaches the cells and tissues of the body thus leading to hypoxia. Interestingly, in the pulmonary circulation moderate hypoxia results to an acute physiological response characterised with a profound vasoconstriction and consequent increase in pulmonary vascular resistance. This response is commonly referred to as hypoxic pulmonary vasoconstriction (HPV), which is unique to the pulmonary vasculature; whereas the systemic circulation mainly demonstrates vasodilatation during hypoxia. von Euler and Liljestrand in 1946 were the first to define HPV and it remain the only known vascular feedback control mechanism in the lung (292).

1.10.1 Characteristics of HPV

Bradford & Dean first observed HPV in 1894 and they referred to it an “active constriction of the pulmonary vessels” in dogs (293, 294). However, it was not until 1946 when von Euler and Liljestrand revealed comprehensive information about HPV, hence, they were credited with the discovery (295). They observed that the pulmonary and systemic circulation in cats responded differently to hypoxia and suggested HPV’s contribution to ventilation perfusion (V/Q) matching. Motley *et al* (296) reported that 10% oxygen doubled pulmonary vascular resistance (PVR) in humans (296). Hypoxia acutely increased PVR from 1.2 ± 0.3 to 2.9 ± 0.3 Wood units in healthy controls whereas the systemic vascular resistance decreased accordingly (296, 297). HPV plateaus within 2 hours and thereafter PVR remains constant for the duration of hypoxic ventilation of 8 hours (297, 298).

Investigation by Marshall and Marshall has revealed that alveolar PO_2 (PAO_2) provides the main stimulus for HPV but not the mixed venous PO_2 (PvO_2) (299). They reported that PAO_2 had a greater effect on HPV compared to PvO_2 due to O_2

exchange between alveolar gas and blood in small PA. Hence the pair and others concluded that mixed venous PO_2 is not strong enough to trigger HPV, whereas the small muscularized resistance arteries adjacent to the alveoli that experienced alveolar PO_2 were mostly strong enough to exhibit HPV (300-302).

1.10.2 Potential Mechanisms of HPV

In mature lungs, HPV mainly occurs within the small pulmonary arteries. The phenomenon was conserved in isolated perfused lungs as well as in pulmonary artery sections dissected from the lungs (303-305). Hence, the mechanisms that mediate HPV must be intrinsic to the pulmonary system and not directly dependent on circulating neuronal feedbacks and humoral modulators (306). However, various mediators secreted the cells within the pulmonary vasculature might play key role in initiation of HPV (306). Therefore, the fact that hypoxia induced vasoconstrictor agonists release from perfused lungs that reportedly generated similar response to hypoxia in isolated pulmonary vessels and showed selectivity for pulmonary over mesenteric arteries in rats (307). It is yet to be established whether this substance is critical for HPV initiation. Over the years, several other potential vasoconstrictors were tested but none proved to be essential for HPV. The specific site of HPV initiation is not confirmed, even though, there is a consensus that it occurs within the vasculature. Therefore, many vascular cells such as endothelial and vascular smooth muscle cells were investigated for their possible contributions (306). There remain different thoughts on whether endothelial or vascular smooth muscle cells mediate the action hypoxia to effect release of vasoactive substances to cause the constriction, thus it was suggested that HPV might involve several factors to generate optimum response (303, 308). A review conducted by Sylvester *et al* (205) concluded from isolated lung studies that the increase in pulmonary pressure as a

response to hypoxia was due to vasoconstriction and that HPV does not rely on neural or humoral stimuli from outside the lungs. This findings were in accordance with the views of von Euler and Liljestrand (295) that pulmonary vasoconstriction was due to local mechanism triggered by hypoxia leading to regulation of the local ventilation-perfusion relationship (205).

Sylvester *et al* (205) postulated two general hypotheses as potential mechanisms of HPV. Firstly, through indirect activation and/or deactivation vasoconstrictor and vasodilator responses respectively by hypoxia leads to pulmonary vasoconstriction thus subsequently causing contraction of pulmonary vascular smooth muscle. Secondly, hypoxia induces pulmonary vasoconstriction via direct contractile effect on pulmonary vascular smooth muscle (205). To address the first hypothesis, several researchers conducted studies on intact animals and isolated lungs and their results revealed numerous endogenous substances that elevated pulmonary vasomotor tone, however, none proved be a unique mediator of HPV (309, 310).

As far as the second hypothesis is concerned, a study by Bergofsky and Holtzman (311) showed that hypoxia raised intracellular [Na^+] and reversibly decreased intracellular [K^+] in pulmonary artery segments stripped of adventitia and intima though did not affect a similarly treated pulmonary veins or systemic artery segments. These changes were attributed to the depolarisation of pulmonary artery smooth muscle as a consequent of direct effects of hypoxia treatment on the cells. Further evidence, by McMurtry *et al* (312) revealed that blockers of the L-type voltage-operated Ca^{2+} channels (found in the pulmonary artery smooth muscle) significantly inhibited the pulmonary artery pressure increased observed with hypoxia in isolated lungs. This coupled with other findings by Madden *et al* (313, 314) that hypoxia exposure was associated with both constriction and depolarisation in small

pulmonary arteries in cats, supported the earlier reports by Bergofsky and Holtzman. Other studies have later confirmed the central role of pulmonary artery smooth muscle cell (PASMC) in HPV, as they have showed hypoxia to be responsible for both depolarisation and contraction in isolated PASMC thus concluding that the essential sensor, transducer and effector mechanisms of HPV are enclosed within the pulmonary arterial myocyte (205, 304, 315, 316).

1.10.3 Modulation of pulmonary artery smooth muscle cell contractility by oxygen

In the absence of the endothelium, hypoxia resulted into enhanced vasoconstriction thus suggesting that the endothelium might not be essential for HPV (305). Madden *et al* (304) then Sham *et al* (317), have shown that in the absence of other cell types, hypoxia induced vasoconstriction in isolated pulmonary artery myocytes (205, 304). Bakhramov *et al* (318) suggested that this observation might be due increased intracellular calcium concentration $[Ca^{2+}]_i$ as hypoxia was associated with rise in $[Ca^{2+}]_I$ in isolated pulmonary artery myocytes (306). These evidences together swayed the direction towards the pulmonary artery smooth muscle cells as the ultimate sensor of hypoxic stimulus to propagate HPV, although the precise mechanism PASMC senses O_2 is not well understood (306). Moreover, various mechanisms were investigated, which included an inhibitory action of hypoxia on transmembrane potassium channels, initiation of store-operated Ca^{2+} entry (capacitative Ca^{2+} entry), stimulation of the release of Ca^{2+} release from the sarcoplasmic reticulum (40) and the contraction generating proteins sensitisation to Ca^{2+}, as a possible O_2 sensing pathways in pulmonary vasculature (306).

The fact that hypoxia caused vasoconstriction in isolated PASMC confirmed the notion that sensor, transduction, and effector mechanisms of HPV are present in these cells (205). Broadly two methods were utilised to measure contraction in

PASMC. These first one involves the use of flexible polymerized polydimethyl siloxane membrane for cell growth and once cells were attached to the membrane, contraction is assessed by determining the level of wrinkling and/or distortion. However, it was shown that the main or branch pulmonary arteries isolated from fetal calf myocytes exposed to normoxic conditions (PO_2 equivalent to 145 mmHg) developed minor wrinkling thus suggesting basal contractility (315). Moreover, the reduction in PO_2 from 20–30 mmHg caused a significant increase in the level of wrinkling observed and this was reversed by re-oxygenation. Similar experiments conducted in PASMC indicated that hypoxia resulted in phosphorylation of myosin light chains comparable to active contraction (205).

The other method used to measure contraction in PASMC uses microscopic field images or a reticule in the eyepiece of the microscope to quantify cell length. Hypoxia was shown to cause decrease in cell length in distal pulmonary arteries isolated from cats or pigs (304, 317), though the influence of hypoxia on proximal arteries remained inconclusive as both unchanged and decreased in cell length were observed (304, 317, 319). Hypoxia contraction in the distal pulmonary arteries was attributed to myosin phosphorylation (304). This has been reviewed in greater details by Sylvester *et al* (205). Hypoxia caused reduction PASMC length similar to what was observed with KCL, norepinephrine, phenylephrine, $PGF_{2\alpha}$ and 5–HT (304, 317, 319). The decrease of the initial PASMC length due to hypoxia ranges between 9–24% and was maximal between 5–30 minutes (304, 317, 319).

1.10.4 Mediation of HPV

It is widely established that mechanism intrinsic to PASMC such as O_2 sensing, signal transduction and smooth muscle vasoconstriction were essential in the mediation of HPV response (205).

Several potential candidates have been identified as the O_2 sensor that trigger HPV thus it was challenging to identify a particular O_2-sensor responsible for the hypoxic response. Different O_2 sensitive mechanisms responding to varied ranges of PO_2 have been identified and potentially serving a variety on function (320-323).

Oxygen and proteins interact via biochemical reactions using O_2 as substrate or a reversible binding of molecular O_2 resulting into allosteric modifications of the protein that enable the initiation of the signaling cascade (example, heme-ligand association) (**Figure 1.7**). The former category could occur with or without the initial heme-binding phase. The other category of the interaction between proteins and oxygen is sub-divided into bioenergetic hypoxia (due to inadequate PO_2 levels to preserve usual cytochrome aa3 function), high-energy phosphates and redox state of pyridine nucleotides and metabolic hypoxia that happens due to inadequate PO_2 to preserve the usual function of O_2-dependent enzymes different from cytochrome aa3 (205).

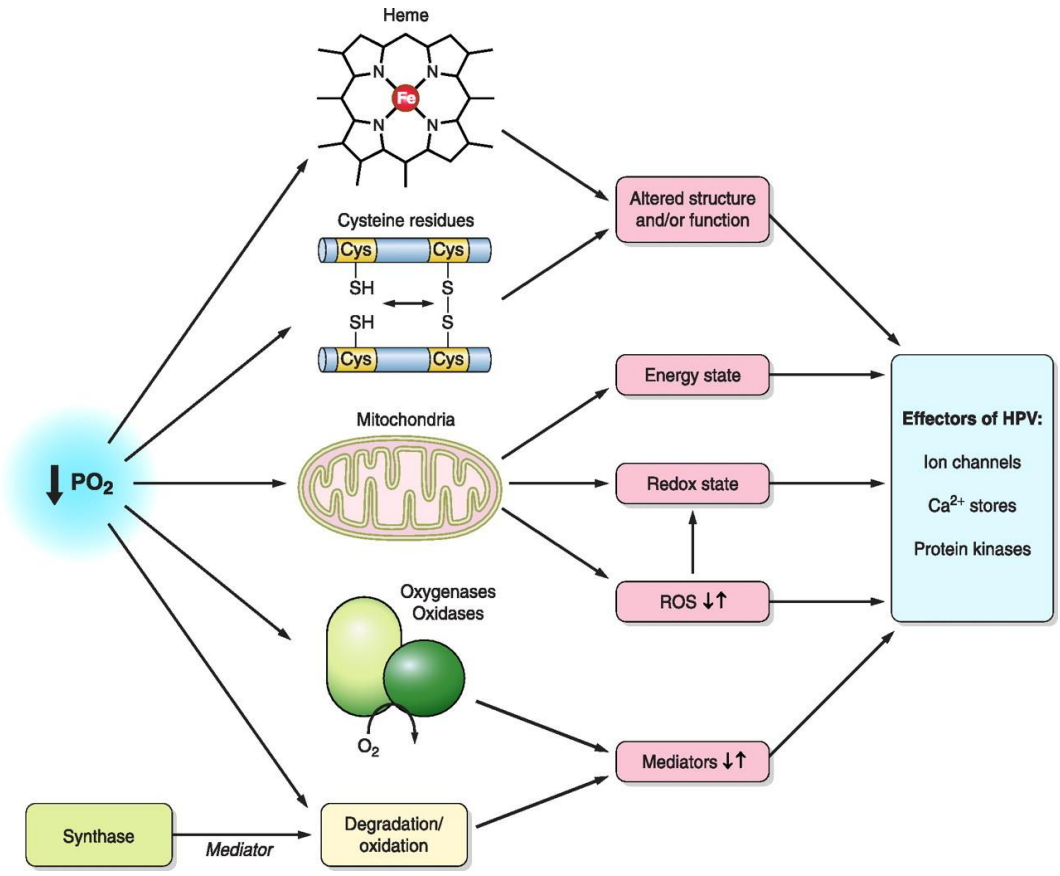


Figure 1.7 Shows potential O_2 sensing pathways. Biochemical interactions between O_2 and proteins to initiate signaling cascade leading activation of HPV effectors. ROS, reactive oxygen species. (205)

Oxygen-sensitive systems were broadly sub-divided into, direct modification of protein residues, heme and oxygenase. Oxygen and redox states has the ability to alter sulfur-containing protein residues directly and reversibly to produce disulfide bonds or sulfoxide which leads to modification of the protein structure thus alter function(205). Heme is a typical O_2 binding molecule and consists of four pyrrole rings joined to a central iron atom. The iron on binding to O_2 get reduced or converted from ferrous (Fe[II]) to ferric (Fe[III]). The heme proteins such as mitochondrial cytochromes, NADPH oxidase, cytochrome *-P450*, NO synthase, guanylate cyclase, catalase, cystathionine β-synthase, and heme oxygenase are notably implicated in oxygen sensing. Moreover, oxygenases were characterized as

an oxidoreductase enzyme that speed up reversible reduction-oxidation (redox) reactions, where one substrate acts as an electron or H^+ donor and is oxidized, and the other acts as acceptor and become reduced. The O_2 sensing systems including relevant examples has been extensively been reviewed by Sylvester et al (205).

1.10.4.1 Mitochondria

Elevation of intracellular [Ca^{2+}] is a precondition for HPV as confirmed by studies in isolated PASMC and endothelium-denuded pulmonary arteries exposed to hypoxia. The studies confirmed that the major O_2 sensor definitely located within the PASMC (205). Therefore the sensor is required to be triggered by a suitable range of PO_2 that is probably dependent on rate and site of O_2 consumption of a cell, as well as, the obstruction of the O_2 diffusion to those particular sites (320). Mitochondria are the most important O_2 consumption sites within the cell (320) and serve as signaling organelles and energy production (324, 325). Mitochondria-dependent factor such as [ATP], energy state, redox state, and ROS generation can be influenced by low PO_2 thus hypothetically leading to HPV. Mitochondria were shown to control Ca^{2+} level of the sarcoplasmic reticulum (SR) in vascular smooth muscle (326, 327). Several investigators have reported that blockage of oxidative phosphorylation had a strong and selective influence on HPV (202, 205, 207, 328-332). In addition, depletion of mitochondrial DNA by treating PASMC with ethidium bromide rendered the cells non responsive to hypoxia (207, 333) and this might be attributed to absence of a working mitochondrial electron transport chain (METC). It suggested that METC is an essential precondition for O_2 sensing in PASMC, carotid body type1 and neonatal chromaffin cells (320, 322, 334-336). Hence, mitochondria are apparent contenders for the O_2 sensor in HPV. See the review by Sylvester *et al* (205) for comprehensive

details on mitochondria and O_2 sensing in HPV with evidences drawn from cellular and molecular studies.

1.10.4.2 Redox State

The equilibrium between cytosolic oxidizing and reducing counterparts give rise to the cellular redox state and this includes the main redox couples such as the reduced and oxidized glutathione (GSH/GSSG) and NADH and NAD^+ as well as the equilibrium between ROS generation and degradation. Mitochondrial tricarboxylic acid (TCA) and METC and the key source and sink respectively for reducing equivalents of NADH. In PASMC and carotid body glomus cells, exposure to hypoxia resulted into more reduced state due to elevated ratio of GSH/GSSG and NADH/NAD^+ (332, 337-339).

Studies have reported that blockage of oxidative phosphorylation generated similar effect as hypoxia on pulmonary vessels (205, 340) and showed oxidant mediated reversal of HPV (341). This was the basis for the Redox Hypothesis of HPV which suggested that the inhibition of oxidative phosphorylation due to hypoxia resulted into further reduced cytosolic redox state and lowered the ROS content. This subsequently triggered a cascade of events including inhibition of K^+ current (I_K) leading to depolarization of PASMC, influx of CA^{2+} through the voltage-gated CA^{2+} channels and vasoconstriction (**Figure 1.8A**).

1.10.4.3 ROS

Reactive oxygen species, sources and characteristics was detailed in section 1.6. The most prominent ROS are $O_2^{\cdot -}$, $RO_2^{\cdot}$ and $^{\cdot}OH$ (208). ROS is originally considered a harmful byproduct of metabolism. However, ROS are currently viewed as a signaling molecule with a variety of functions in several regulatory mechanisms in vascular smooth muscle and endothelium (205, 342). Hence, in PASMC, ROS was

noted as hypoxic signal leading the way for several pathways such Ca^{2+} release from ryanodine-sensitive stores (343, 344) promote production of cADPR (345), stimulation of AMP kinase (346) and activation of Rho kinase-mediated Ca^{2+} sensitization (347), src kinase (348), stimulate Ca^{2+} entry via nonselective cation and TRP channels (349, 350) and other signaling pathways (351) involved in HPV (205).

Research evidences have suggested that superoxide and peroxide as the major ROS species involved HPV mediation (205). However, significant disagreements remained of the origin of the ROS species and effector mechanisms and whether they are increased, reduced or have any specific association (334, 352, 353). As opposed to the expectation due to Redox Hypothesis (since O_2 was the substrate for ROS formation), hypoxia caused increased in mitochondrial ROS synthesis in a variety of cells types including PASMC (202, 205, 207, 328, 354, 355) (**Figure 1.8B**).

1.10.4.4 Energy state

Cellular functions utilise readily available ATP as source of energy. Though more ATP were generated by mitochondrial oxidative phosphorylation than glycolysis, surprisingly, only about 30% of ATP was derived from glycolysis during normoxia compared to 65% of ATP production by glycolysis during hypoxia (205, 356). Hypoxia augmented glycolysis and increased smooth muscle ATP synthesis partly due enhanced glucose uptake (205, 355).

Phosphocreatine (PCr) is a tiny diffusible molecule that plays a crucial role in energy transfer between sites. PCr is critical for moderately immobile ATP and ADP and energy utilisation. Creatine kinase uses ATP to phosphorylate creatine to phosphocreatine that then transferred into the sites of energy utilisation and the reaction is reversed (Lohman reaction: $ATP + Cr \leftrightarrow PCr + ADP + H^+$). Moreover,

adenylate kinase reaction ensures high-energy phosphate transfer from one ADP to another whilst retaining the ATP concentration. During hypoxia, the collective influence of PCr, adenylate kinase and glycolysis in the presence of glucose, contribute to conserve the PASMC [ATP] and maintain optimum contractile activity in the event of lack of working METC (202, 207, 260, 328, 329, 355).

Furthermore, phosphorylation potential ([ATP]/[ADP][P_i]) and AMP/ATP and AMP-activated kinase are utilised index of energy states. Phosphorylation potential plays an important role in the control of oxidative phosphorylation, membrane transport, meberane transport, actin myosin cross-bridge cycling thereby affecting vascular reactivity (205, 357-359). During metabolic tensions such as hypoxia or lack of glucose, elevation of AMP/ATP levels trigger AMP-activated kinase (AMPK) to increase synthesis and reduce utilisation of ATP which ensure a conserved ADP/ATP ratio to circumvent the challenges(205). Inhibition of AMPK with compound C (a non-selective AMPK inhibitor) prevented HPV in rat pulmonary arteries and reduced intracellular Ca^{2+} concentration in human PASMC (205, 360, 361). This phenomenon is consistent with the hypothesis (**Figure 1.8C**), though the exact mechanisms remains unclear.

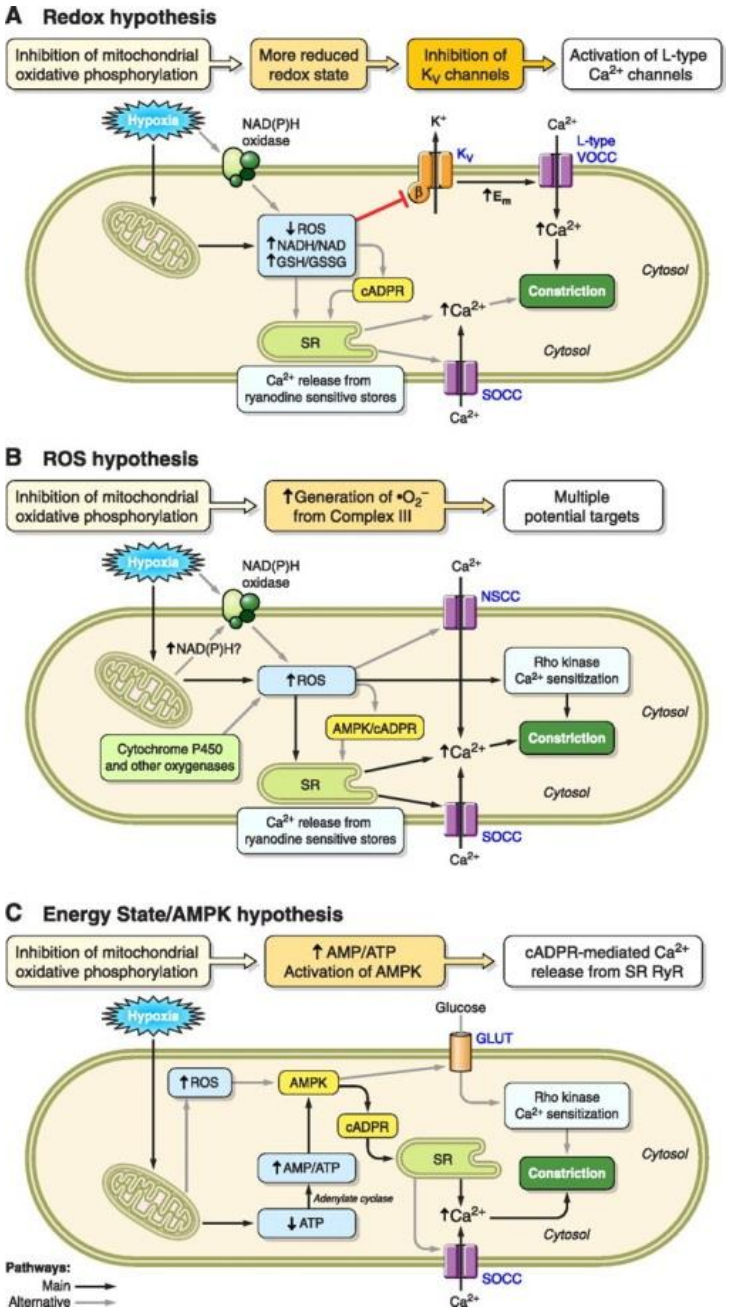


Figure 1.8: Diagrams illustrating potential mechanisms that demonstrate how hypoxia gives rise to constriction in pulmonary arterial smooth muscle, as suggested by the Redox (*A*), ROS (*B*), and energy state/AMP kinase (*C*) hypotheses. Black and gray arrows represent the main and alternative pathways 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 (205).

1.10.5 Role of the endothelium in oxygen sensing

Many investigators have revealed that endothelium is essential for HPV due to inhibition of the response in its absence that was reported in a variety of species (303, 308). However, other studies have shown HPV to be independent on the endothelium as endothelium removal did not alter the response and even augmented it at times. This conflicting outcome could not be linked to the difference between species as both endothelium dependence and independence were shown in the same species (362-365). Though the variation could be attributed to variation in experimental protocol applied, size of the vessel utilized (306). HPV is likely to result, if the action of hypoxia on the vessel caused minimised release of vasodilators and increased release of constrictors. Besides, there is no consensus on the direct involvement of endothelium-derived nitric oxide in HPV due to inconsistence response seen. Studies have exhibited that hypoxia can induced or inhibited release of NO, which might indicate that HPV is not dependent on its release. Other endothelium-derived vasodilators such as prostanoids were also investigated at, but again, no consistent result as HPV showed both dependence and independence on prostacyclin release.

Therefore, based on the evidences so far, endothelium cell or its mediators could not be regarded as essential mediator of HPV but it would be ideal to consider it as a modulator of the response.

1.11 Cytokines and endothelial dysfunction

Pro-inflammatory cytokines elevation has been associated with endothelial dysfunction in different cardiovascular diseases such as congestive heart failure, atherosclerosis, septic shock, diabetes, hypertension and aging (366-371). Cytokine might influence impairment of the endothelium-dependent relaxation there by

precluding the endothelium response to circulating autacoids or hormones. Therefore, this makes the vessel susceptible to spasm, thrombosis and/or atherogenesis (372). The effect of pro-inflammatory cytokines was investigated in both conductance and resistance arteries in both hum and animals (372).

In *vivo* studies of human forearm resistance artery revealed that exposure to TNF–α for 1 hour has resulted into increased baseline vascular resistance due to elevation of baseline vasoconstrictor prostanoids bioavailability and diminished NO bioavailabilty (373). Moreover, exposing isolated rat aorta to IL–1β treatment for 1 hour had increased the vasoconstrictor activity of angiotension II via mechanisms that include prostaglandin thromboxane A_2 (374).

Different investigators studied variety of exposure times to various cytokines to investigate their effects on endothelium-dependent vasodilatation in isolated mesenteric resistance arteries and the results yield was not conclusive (372). A previous study has demonstrated that 3 days exposure of rats to IL–1β and lL–6 *in vivo,* caused impairment of the acetylcholine mediated relaxation of perfusion pressure, on rat isolated mesenteric vascular bed (252). The mesenteric arteries were incubated in organ culture for 14 hours with IL–1β and this treatment nearly eliminated the acetylcholine mediated vasodilatation, perhaps due to enhanced superoxide anion generation (375). However, mesenteric arteries incubation with IL–1β (100pM) for 30 minutes had not altered acetylcholine mediated relaxation, though TNF–α (1nM) treatment for the same period caused impairment of acetylcholine and bradykinin response but not on the sodium nitroprusside mediated endothelium-independent relaxation (376).

Hence, it could be suggested that the observation might be due to the effectiveness of TNF–α to substantially elevate production of superoxide anion and consequentially reducing nitric oxide expression (376). Moreover, Nedeljkovic *et al* (377) has reported that TNF–α resulted into sustained superoxide anion production due via activating NADPH oxidase. Similarly, Hernanz *et al* (378) has shown that isolated rat middle cerebral arteries exposure to lipopolysaccharide (LPS – 10 µg/ml for 1–5 h) *in vitro,* has significantly blunted bradykinin mediated endothelium-dependent vasodilatation, via mechanism including increased generation of nitric oxide and superoxide anion from inducible nitric oxide synthase (iNOS) and potentially due to cyclooxygenase-2 (COX-2) respectively.

Cytokines might exert their influence on endothelial function via variety of signaling mechanisms. Hence, TNF–α has the ability to cause damage to endothelial nitric oxide synthase (eNOS) mRNA (367). Besides, it has been shown that cytokines and LPS might stimulate expression iNOS in endothelial cells and vascular smooth muscle cells and this inevitably induce elevated baseline NO level thereby contributing to impairment of endothelium-dependent vasodilatation (379, 380).

1.12 Definition of Arterial Stiffness, effect on haemodynamic and mechanisms

Practically, arterial stiffness causes changes in the mechanical behaviours of the structural characteristics of the artery wall thus effecting alterations in the wall geometric properties and wall tension (381). The material properties were defined in term of the proportion of strain (deformation due to an applied force per unit area (stress)". It is part of the innate mechanical characteristics for arteries that their walls become stiffer as results of distending pressure (381). This phenomenon might be because rise in the proportion of stiffer collagen fibres being recruited with increased

distension (381). It has been reported that arterial stiffness might act as a primary determinant of vascular impedance, thereby influencing arterial pressure and flow relationship (381).

The basic mechanism behind this stress/strain relationship include complex interplay between the structural properties of connective tissue and cell signaling pathways that changes the inherent and collective function of elastin, collagen, proteoglycans and glycoproteins of the extra-cellular matrix of the artery wall (381). A number of reviews have been conducted with regard to the mechanism of arterial stiffness with the suggestion that depending on the extent of the stimulus, the specific mechanisms can interact by way of positive or negative feedback pathways (381).

1.12.1 Factors regulating arterial stiffness

Blood pressure is an important factor that has a complex interplay with arterial wall properties. The PWV varies along the arterial segment and this is based on distending pressure mainly represented by the mean arterial pressure (MAP). Increase in MAP cause equivalent circumferential pressure increase with greater recruitment of inelastic collagen fibre and this might lead to higher stiffness measured, hence MAP status most be considered during PWV measurement (382). In addition, a longitudinal study showed that progression of arterial stiffness could be determined by increased blood pressure (383).

Other factors that affect arterial stiffness include structural characteristics of the artery wall that determine arterial elasticity through vascular smooth muscle tone. Therefore factors associated with arterial stiffness can be considered in terms of structural and functional changes to the endothelium or the medial layer affecting smooth muscle cells, the elastic lamina or the connective matrix (382).

1.12.2 Functional changes associated with arterial stiffness

When the smooth muscle tone is altered, endothelial function affects arterial stiffness although in humans the impact is more marked in the smaller arteries than in the aorta which has a smaller percentage of smooth muscle cells within the media (382). Schmitt et al (384) demonstrated that iliac artery stiffness in human was affected by nitric oxide modulation. They infused glyceryl trinitrate and highest dose of N^G-monomethyl-l-arginine via catheter and both significantly reduced velocity by 31.43±5.80% (mean±SEM; $P<0.01$; $P=0.02$ for comparison) and 27.25±8.20% ($P=0.001$; $P=0.02$ for comparison) respectively and there was no change in mean arterial blood pressure throughout the studies. These results suggest that at rest, local NO production modulates human iliac artery dispensibility and that exogenous NO increases arterial distensibility (384). In addition, administration of endothelin-1 via infusion caused elevation of PWV, whereas an endothelin-1 blocker prevented this effect (385, 386). Endothelial function has been associated with aortic PWV in healthy humans and in addition to acute modulation of vessel tone this may be a mechanism by which cardiovascular risk factors cause chronic changes to the vessel wall (382).

1.13 Hypothesis & Aims from this PhD

This PhD will examine the mechanisms behind cardiovascular disease co-morbidities associated with COPD. Specifically, the relationship between inflammation and hypoxia in modulating the pathogenesis of vascular changes associated with the disease will be explored using animal model.

A. Humans

To quantify inflammatory mediators in the serum samples of alpha-1-antitrypsin deficient (AATD) subjects and investigate the relationship of inflammation with arterial stiffness (PWV, measured from carotid artery or aorta), hypoxia and lung function test parameters measured. Interaction between inflammation and hypoxia in modulating arterial stiffness in AATD will be investigated.

B. Rats

The influence of acute and chronic inflammation of vascular (pulmonary and systemic) reactivity in vitro will be investigated. Also, effects of both acute and chronic hypoxia will be explored. The combination effects of inflammation and chronic hypoxia or intermittent hypoxia on pulmonary and systemic arteries will be investigated. Finally, stiffness assessment will be completed on isolated systemic (iliac) arteries.

Chapter 2

2 METHODS & MATERIAL

2.1 Clinical assessment of AATD patients

2.1.1 Subject selection

Subjects were recruited from the UK national registry for AATD, which is which is located in Birmingham, UK. The registry is under is overseen by ADAPT (Antitrypsin Deficiency and Assessment Programme for Treatment). This programme was initiated in 1996 with the funding provided by a non-commercial grant from Bayer Biological (USA). Referral of the subject to the registry comes through health care professionals which includes chest physicians and general practitioners. Occasionally, subjects might be identified through family screening of those related to someone with AATD. ADAPT has obtained ethical approval to carry out clinical and genetic studies on registered participants (South Birmingham Local Ethics Committee 3359 and 3359a). At registration, participants have given an informed, written consent and this consent form has been provided as an **Appendix I**. The diagnosis of all subjects registered with ADAPT was confirmed by genotyping (Heredilab, Salt Lake City, USA). A total of 181 subjects with a stored serum sample suitable for cytokine quantification were included in this thesis. The procedures described in this section are those for all subjects attending ADAPT.

Clinical data was collected by all research fellows and nurses in the department and I have conducted cytokine assays on the samples and analysed the data.

2.1.2 Demographic data collection

All relevant basic demographic information such as gender, date of birth, height, weight and home address were documented. In addition, full smoking history, including amount smoked per day, age started and age quit. The information is used

to pack year exposure that is calculated by dividing the number cigarettes per day by 20 and multiplying by number of years smoked. A full clinical history was collected at the baseline assessment, which included the symptoms required to diagnose chronic bronchitis (387) and frequency of exacerbations (388). Subjects were classified as either index case or non-index cases based of on whether AATD was diagnosed after investigation of their symptoms or via family screening respectively. This information about classification was available for all subjects; however, it is significant solely in familial replication dataset. Therefore, no emphasis will be placed on the classification in this thesis.

2.1.3 Lung function

Trained respiratory physiologists within the Lung Investigation Unit at University Hospitals Birmingham, UK conducted all the all the lung function measurement and compared to predicted values derived from standard reference equations for Caucasian adults. Spirometry was performed before and after nebulised short acting bronchodilators were provided as a baseline measurement (389). An increase of more than 200ml and greater than 12% predicted of FEV1 is regard as a positive bronchodilator reversibility (390). This definition was applied to calculate for all subjects on this basis of the raw data held in AADAPT databases (391). At subsequent annual visits spirometry was performed only after bronchodilatation, such that changes in lung function over time were derived from post-bronchodilator values. Annual follow up has been offered throughout the period that ADAPT has been established, such that those registered in the early years of the programme may have up to 12 years data available, with correspondingly fewer years for those registered later.

Static lung volume measurements including total lung capacity (TLC) and residual volume (RV) were assessed using helium dilution (392). Gas transfer measurements (DLCO) were obtained by the single breath carbon monoxide method(392). Together these values were used to calculate the uptake of gas per unit of effective alveolar volume (KCO). All these values were determined only after bronchodilatation.

2.1.4 ADAPT databases

The ADAPT programme comprise 2 clinical databases, the older of the two was used to collect data from 1996, the newer one started in 2000 to improve both the quality and quantity of data collected. There is also a microbiological database and a laboratory database containing information on the location and type of stored samples. Data was extracted from all four sources, and unified with a project database, set up in Access 2000 and employing referential integrity to link all data types to individuals. Additional data from CT scan reports was extracted from medical notes and intranet based hospital records.

2.2 Enzyme-linked immunosorbent assay (ELISA)

This technique was used to measure the level of human TNF–α, IL–8 and IL–1β levels. We illustrate the method for TNF–α which basically the same principle for measuring the expression IL–8 and IL–1β cytokine.

2.2.1 Reagent Preparation

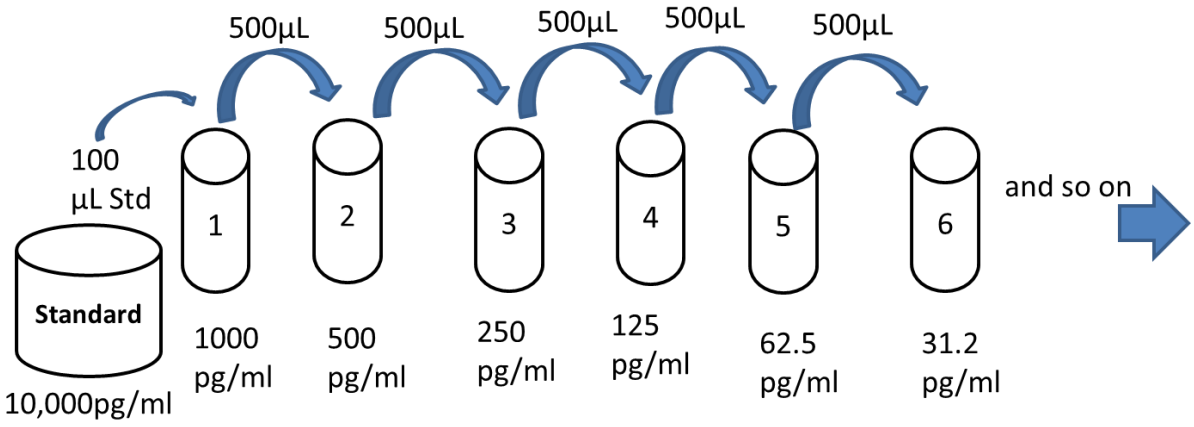
All reagents were allowed to warm in order to reach the room temperature before use. The Wash Buffer diluted in deionised or distilled water in a ratio of 1:25 then mixed gently until the crystals were completely dissolved (for example, 20mL Wash Buffer in 500mL of distilled water).

2.2.2 TNF-α and Standard

TNF–α was reconstituted in deionised or distilled water, according to the manufacturer label on the vial to generated a stock solution of 10,000 pg/ml. The standard stock was kept on gentle agitation prior to making the dilutions for 15 minutes.

2.2.3 Serial Dilutions

Suitable pipette was used to transfer 900µL of Calibrator Diluent RD6-35 into tube 1 and 100µL of a known standard to make up 1000 pg/ml tube. 500µL of Calibrator Diluent RD6-35 was added into tube 2–6 and so on. Take 500µl from tube 1 and into the next tube, mix thoroughly then repeat the step with next tube and so on. The appropriate Calibrator Diluent serves as the zero standard (0 pg/mL).



2.2.4 Assay procedure

Once all reagents and standards are prepared according to the instructions mentioned in the above section, 50µL of Assay Diluent RD1F was added into each well of the 96 well plates. The diluent mixture should be properly mixed prior to use

then 200µL of the standard, sample or control per well (in duplicates) was added and cover with adhesive strips. The plate was kept at room temperature for 2 hours. An example plate layout is shown Table 2.2.1**.**

	1	2	3	4	5	6	7	8	9	10	11	12
A	0 pg/ml	0 pg/ml	P.S 1	P.S 1	P.S 9	P.S 9	P.S 17	P.S 17	P.S 25	P.S 25	P.S 33	P.S 33
B	Std 1	Std 1	P.S 2	P.S 2	P.S 10	P.S 10	P.S 18	P.S 18	P.S 26	P.S 26	P.S 34	P.S 34
C	Std 2	Std 2	P.S 3	P.S 3	P.S 11	P.S 11	P.S 19	P.S 19	P.S 27	P.S 27	P.S 35	P.S 35
D	Std 3	Std 3	P.S 4	P.S 4	P.S 12	P.S 12	P.S 20	P.S 20	P.S 28	P.S 28	P.S 36	P.S 36
E	Std 4	Std 4	P.S 5	P.S 5	P.S 13	P.S 13	P.S 21	P.S 21	P.S 29	P.S 29	P.S 37	P.S 37
F	Std 5	Std 5	P.S 6	P.S 6	P.S 14	P.S 14	P.S 22	P.S 22	P.S 30	P.S 30	P.S 38	P.S 38
G	Std 6	Std 6	P.S 7	P.S 7	P.S 15	P.S 15	P.S 23	P.S 23	P.S 31	P.S 31	P.S 39	P.S 39
H	Std 7	Std 7	P.S 8	P.S 8	P.S 16	P.S 16	P.S 24	P.S 24	P.S 32	P.S 32	P.S 40	P.S 40

Table 2.2.1: illustration of ELISA plate **with standard and samples duplicates.**
Std= Standard, P.S = Patient Sample

All liquid was removed from the plate and washed with the wash buffer (400µL) four times. We made sure all liquid was removed by inverting the plate and blotting against a clean tissue paper. Then 200µL of TNF-α Conjugate was placed into each well and the plate was covered with a clean adhesive strip. After 2 hours at room temperature, the washing and aspiration steps were repeated. The substrate solution was then prepared by mixing together equal volumes of Reagent A and B, 15 minute prior to use and ensuring sunlight did not come into contact with the solution. Then 200µL of substrate was added into each well and allowed the plate to stand for 20 minutes, before adding 50µL of Stop Solution into each well. The reaction between the substrate and the stop solution will cause the colour in the well to changes from blue to yellow. The microplate reader used to read the plate and the reader was set

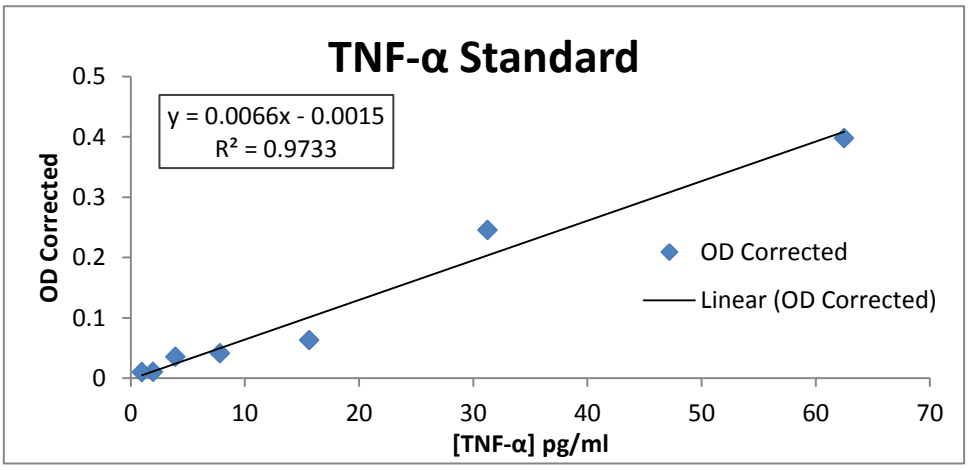
at 450nm, to quantify the level of expression of the target cytokine in each well, which was measured as optical density (OD).

2.2.5 Calculation of the results

The average of the zero standards was subtracted from the average optical density (OD) readings for each standard, sample or control. Standard curve was constructed with the OD on the y-axis versus the known concentration of TNF–α, IL–8 or IL–1β (standard) on the x-axis. Line of best fit was generated by regression analysis. The equation the line of best fit is used to calculate the concentration of the samples from their obtained OD (Figure 2.1 **A & B**).

A

[TNF-α STND] pg/ml	OD1	OD2	Mean OD	OD Corrected
62.5	0.567	0.416	0.4915	0.3975
31.25	0.456	0.223	0.3395	0.2455
15.625	0.163	0.151	0.157	0.063
7.8125	0.139	0.132	0.1355	0.0415
3.90625	0.145	0.114	0.1295	0.0355
1.953125	0.105	0.104	0.1045	0.0105
0.976563	0.108	0.1	0.104	0.01
0	0.09	0.098	0.094	0



B

[IL-8 stnd]pg/ml	OD1	OD2	Mean OD	OD Corrected
62.5	0.2	0.199	0.1995	0.130
31.25	0.131	0.133	0.132	0.063
15.625	0.104	0.101	0.1025	0.033
7.8125	0.083	0.083	0.083	0.014
3.90625	0.082	0.082	0.082	0.013
1.953125	0.086	0.095	0.0905	0.021
0.976563	0.113	0.079	0.079	0.009
0	0.068	0.071	0.0695	0.000

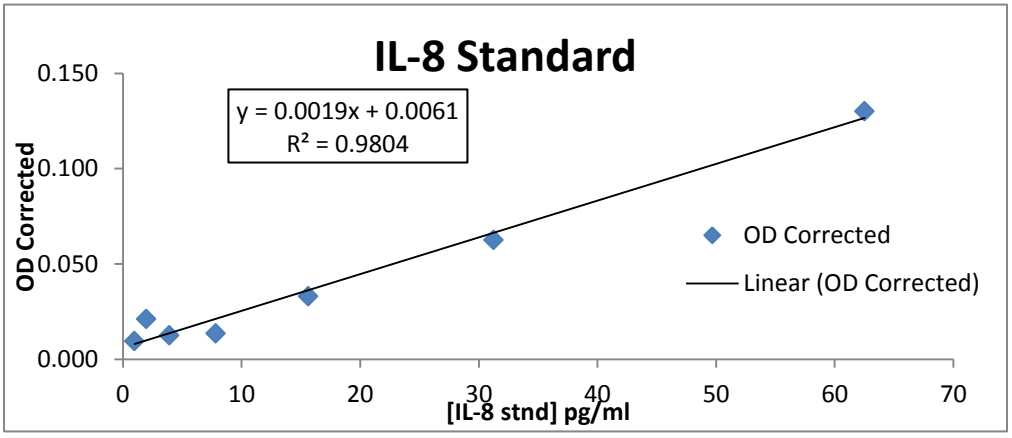


Figure 2.1 A & B: Illustrate the process of calculating the concentration of the samples from their obtained O.D and standard equation. A) TNF-α & **B)** IL–8

2.3 Bronchoalveolar lavage

2.3.1 BAL Collection

2.3.2 Reagents/Equipment

PBS with 0.05% EDTA

PBS with 2% formaldehyde

10% formal saline

PBS

2.3.3 BAL proceedure

Rats were exposed to chronic hypoxia (2 weeks at 12% O_2). The animals had ad libitum access to food and water at all times. At the completion of the two weeks, rats were euthanized by anaesthetic overdose (Sodium pentobarbital (Euthatal) 200mg/kg, intraperitoneal injection) and then cardiac puncture was performed. Once the rat was confirmed dead due to non-responsiveness toe pinching and lack of heartbeat and respiration, the chest cavity was open to expose lungs. The trachea was uncovered by carefully teasing away the lymph node and surrounding tissues. A loop suture was made under trachea (this is not tied at this point). This followed by making a small incision into the trachea. A cannula was inserted then tied in place with the suture. The lungs was gently inflated with 5ml BPS with 0.05% EDTA in a syringe and flushed twice. The syringe containing BAL was removed, while leaving the cannula in place.

2.3.4 Processing BAL Fluid

The BAL fluid collected in the syringe was placed in Eppendorf tubes. The tubes containing BAL fluid was spin at high speed to pellet the cells. The aliquot BAL

supernatant was stored at -20°C to be used for cytokine array assay and the cells re-suspended in 200-1000µl PBS for cytospin assay.

2.4 Proteome Profiler Array – Panel A – Rat Cytokine Array Assay

2.4.1 Reagents Supplied

Rat Cytokine Array Panel A,

Detection Antibody Cocktail,

1X Wash Buffer and

Chemi Reagent Mix

2.4.2 Array Procedure

All reagents were allowed to come to room temperature while samples are kept on ice. Glover was worn throughout to avoid contamination. All samples and reagents were prepared according to the manufacturer's guidelines (R&D Systems). Use the block buffer; Array Buffer 6 and pipette 2ml into each of the 4-Well Multi-dish to be used. Flat tip tweezers were used to remove a remove a membrane from between the protective sheet and placed in a well of 4-Well Multi-dish with the number on the membrane facing upwards. The blue dye from the spots on the membrane will disappear upon contact with the Array Buffer 6, however the capture antibody are retained in their specific locations. This was incubated for 1 hour on a rocking platform shaker.

The BAL samples were prepared according to the manufacturer's instruction while waiting. In a tube, 1mL of the BAL Sample, 0.5ml of Array Buffer 4 and 15µL of Detection Antibody Cocktail prepared were mixed and incubated at room temperature for 1 hour. Then the Array Buffer 6 was removed and washed from the

well of the 4-Well Multi-dish and samples/antibody prepared was added. The 4-Well Multi-dish was covered with a lid and incubated at 2–8°C overnight on a rocking platform shaker. Then, the membrane were then removed and placed into individual plastic containers with 20mL of 1X Wash Buffer. The 4-Well Multi-dish was rinsed with deionized or distilled water and dried thoroughly for future use. The membrane was washed and repeated three times with 1X Wash Buffer on a rocking platform shaker, 10 minute each. Prepare Streptavidin-HRP in Array Buffer as instructed by manufacturer. Remove each membrane from wash container carefully and let drain excess buffer from the membrane. Place the membrane in the 4-Well Multi-dish containing the diluted Streptavidin-HRP, cover the well with the lid and incubate for 30 minutes at room temperature on a platform shaker. Then the each membrane was washed as described earlier. The membrane then removed from the wash container and drained excess Wash Buffer using a paper towel. Each membrane was then placed on a plastic sheet protector with identification number facing upwards. 1mL of the prepared Chemi Reagent Mix was placed evenly to cover each membrane fully. The membrane was carefully cover with the top sheet of the plastic protector, air bubbles removed and incubated for 1 minute. The top plastic sheet was removed and excess Chemi Reagent Mix blotted off. The membrane was left on the bottom plastic sheet protector and covered gently with a plastic wrap completely around the membrane and the bottom plastic protector, making sure air bubbles smoothed out. The membrane with the identification number was placed in an autoradiography cassette that is not used with radioactive isotope detection. Then the membrane was exposed to X-ray film with multiple exposure times between 1-10 minutes.

2.4.3 Data Analysis

The signal observed on developed film was identified by placing a transparency overlay template and aligning it with the pairs of reference in the three corners of each array. Each array can capture up to 30 different cytokines or chemokines as indicated by the results. Pixel densities on developed X-ray film can be collected and analysed using a transmission-mode scanner and imageJ analysis software. The average signal (pixel density) of the pair of duplicate spots representing each cytokine was determined and the average background signal was subtracted from each average signal and corresponding signal on different arrays was used to determine relative change between samples.

2.5 Myography

2.5.1 Principles of the multi-chamber wire myograph system

The multi-chamber myograph (Model 610M, Danish Myo Technology A/S, Aahrus, Denmark) is used for multiple assessment of vessel reactivity (Figure 2.2 & Figure 2.3). The four chambers are arranged in parallel in a single unit and are good for high throughput experiments, offering the potential to compare vessel responses to a wide range of pharmacological compounds. Individual chambers have inbuilt valves to control oxygenation, automatic suction and selectable force ranges.

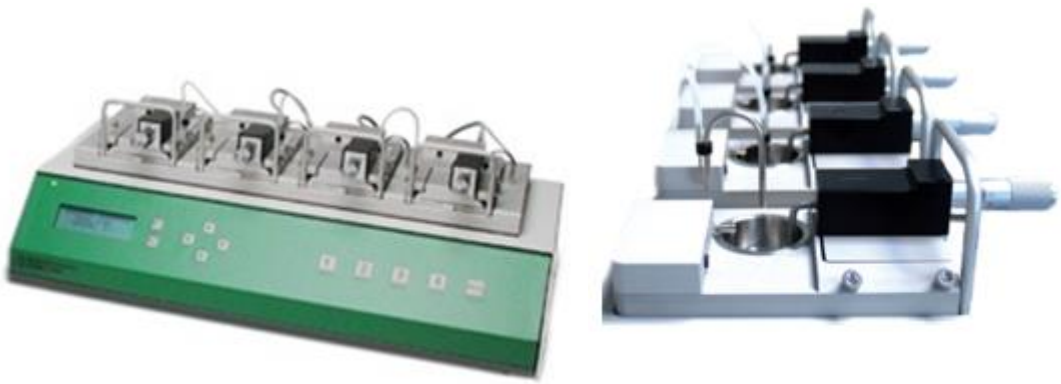


Figure 2.2: *The interface of myograph (left top) and individual loading chambers (bottom right) (Adapted from AD Instruments manual).*

Vessel segments can be maintained under physiological conditions, in an isometric state, with any changes to basal tension being accurately recorded with a high degree of sensitivity. In addition, the basal tension of each vessel segment can be determined through a normalisation process (2.5.6). The vessel segment (~2mm) is mounted on myograph with two wires inserted through it, tightened at the transducer and micrometer ends, respectively. The tension is measured by parallel wires attached to the transducer, and micrometer used to set initial tension. The responses were recorded by the PowerLab acquisition system and assessed using a LabChart software.

2.5.2 Animals

Wistar Rats (250–300g) were used. Charles River, Laboratories International, Margate, UK, supplied all the rats used in the experiments. Rats were housed in the biomedical services unit (University of Birmingham) maintained in a controlled environment. Rat chow and water were available ad libitum.

CH animals were housed in a normobaric hypoxic chamber (Oxycycler, model A84XOV, Biospherix) with an ambient oxygen concentration controlled at 12% oxygen (6kPa), the equivalent to an altitude of 4000m, for a period of 1 or 2 week(s) prior to the experiment. Testing and handling of the animals were conducted in accordance with the Animals (Scientific Procedures) Act 1986.

2.5.3 Tissue isolation and preparation for myograph

The rats were killed by an overdose of anaesthesia (induced in an airtight induction chamber using 4% isoflurane in medical O_2 administered at flow rate of 1.5–3

mL/min) with cervical dislocation, as approved by the UK Home Office. Immediately after death, the chest cavity was opened, the lungs were dissected then placed in ice-cold Krebs solution (393) containing (mM): 118 NaCl, 24 $NaHCO_3$, 0.435 NaH_2PO_4, 1 $MgSO_4$, 4 KCl, 5.56 glucose and 1.8 $CaCl_2$, and the lobes were separated.

2.5.4 Dissection of small pulmonary arteries from rat lungs

The dissection protocol and guideline used for pulmonary artery was informally received from Dr Vlad Snetkov at King's College London, the guideline followed were established method used by Professor JPT Ward and his group at Kings College London and many other researchers around the world apply similar techniques.

Rat lung is divided into several lobes and pulmonary arteries could provide appropriate size of the pulmonary segments required for these experiments. The large lobe can provide at least 4 arteries and the small ones can provide one each.

First, I ensured the microscope is set accordingly so that sharp clear image could be obtained. Fine dissecting forceps and small dissecting scissors were used for the dissection. A lobe from the lungs was placed in cold kreb solution on a Sylgard-filled dissecting dish. Excess blood was washed away from the surface of the lobs. The lungs is organised in such a way that arteries, veins and veins run in parallel. The lobes were oriented on the dissecting dish in such a way that the main vein can be seen entering the upper surface of the lobe. The selected lobe was pinned in an appropriate orientation with 3 or 4 small needles which are position at the edges.

Keeping the forceps and the scissors straight, the main airway was opened up and then located it severed proximal end with the lower blade of the scissors and cutting

horizontally and longitudinally through the upper surface. The remains of this longitudinal cut airway were then carefully removed without damaging the arteries underneath. Once the main airway was opened, excess blood was removed out this need to be washed away. Following the side branches of the main airway by cutting through it will lead to the pulmonary artery segments of interest in our experiment. This sits just below the side branches of the main airways. Small PAs of second to third order branching were dissected according to established protocols (393). Under a dissecting microscope excess fat and connective tissue were stripped off the PAs (53) and cut into small (2mm) segments ready to be mounted on the myograph (Figure 2.3).

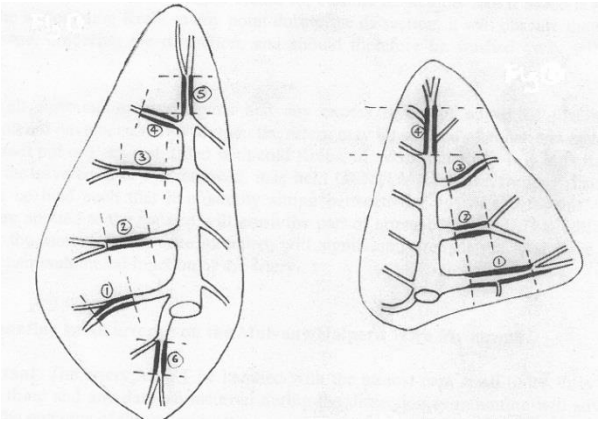


Figure 2.3: ***Diagrammatical representation of different lobes of rat lungs showing visible pulmonary arteries. The sections marked with darker line are the similar sized PA branches ideal for use our experiments.***

In order to assess the reactivity of systemic vessels, I also dissected iliac artery (IA). Following the location of the spine, cotton buds were used to gently tease away other organs to reveal the abdominal aorta in association with the veins. Further clearing revealed branching of the abdominal aorta into the common IAs. Fine forceps were used to clear away other tissue, using blunt dissection to separate the IA from the vein and perivascular nerves. Fine spring-bow scissors were then used to cut the IA distally, with care not to stretch the vessels during clearing from the proximal end, and placed straightway in ice-cold Krebs solution. The IA was freed

from excess adventitia under the microscope in a dissecting dish placed on ice, and cut into small vessel segments.

Segments of vessel types were mounted in a temperature controlled multi myograph system at 37°C and gassed continuously with 95% air and 5% CO_2 (pH 7.35) depending on the experimental protocol. When mounting rings of vessel segments into each of the four channels of the myograph, the chambers were filled with 5ml ice-cold Krebs solution. Then small wires (20µm diameter) were inserted through each vessel lumen, with care taken not to damage the endothelial lining, and tensioned by screws at the transducer end, then a second wire tightened at the micrometer gauge end. The wires going through the vessel were kept straight and parallel to the myograph jaws. Once all vessels in the myograph bath reached the target temperature, the PAs and IAs were normalised to an equivalent pulmonary pressure of 25mmHg and systemic pressure of 100mmHg, respectively. After normalisation, the vessel rings were left to equilibrate at baseline tension for 45mins to permit the vessels to stabilise. The overall set up of the experiment is shown diagrammatically in Figure 2.4.

Also, at the beginning of my research I was using physiological salt solution (as mentioned above) gassed continuously with 95% O_2 and 5% CO_2 (pH 7.35). However, due to undesired effects associated with the use of too much oxygen and best practices in field, we decided to replace this with 95% air and 5% CO_2. This became the gas used for majority of our experiment. Therefore, unless otherwise stated, consider the gas used as 95% air and 5% CO_2.

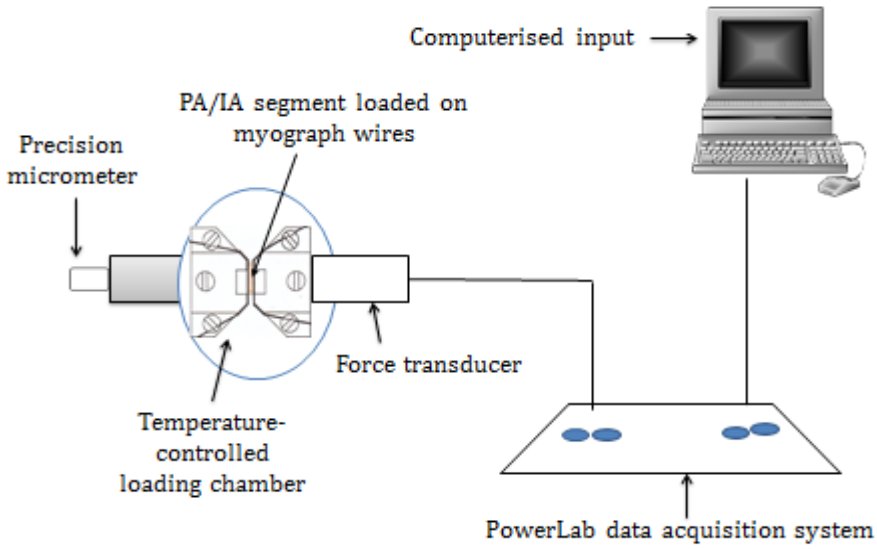


Figure 2.4: ***Schematic representing of the components used in the practical quantitative measure of vascular reactivity. The responses of a functional tissue are translated into an electrical signal which can be processed and subsequently analysed.***

2.5.5 Calibration

To achieve acceptable level of accuracy in the recording, the wire myograph was subjected to a weight calibration prior to carrying out any of the experimental protocols (and subsequently re-calibrated each month throughout the study). This synchronisation procedure allows for the correct interpretation of the output voltage signal derived from the myograph, and the conversion of this signal, via PowerLab (AD Instruments, Oxford, UK), into a measure of force in LabChart.

2.5.5.1 Internal myograph calibration

The signal developed during the myograph calibration is subsequently converted into the correct units of force measurement for calibration of the LabChart system. Calibration of the myograph was accomplished via a weight calibration kit, consisting of calibration-bridge, balance and a 2g weight. All 4 of the chamber units were calibrated individually and in sequence before set-up, whereby the 2g weight was

used to calibrate the force transducer with a standard mass equivalent to a force of 20mN.

The principle of this calibration is that as the 2g weight is placed on the horizontal "pan" arm of the balance, the vertical "transducer" arm exerts a force on the mounting pin that mimics the contractile force of a tissue section. The unit is weight calibrated to a force of 9.81mN, which is derived from the relationship between the weight, the relative lengths and 90^{o} angle of the balance arms, and the standard acceleration of gravity (9.81 m/s^{2}).

2.5.5.2 Calibration with LabChart

A 2-point units conversion can be performed from the degree of change in amplitude of the LabChart trace before and after the calibration weight is added, enabling the output voltage to be translated into mN. These 2 points are selected from regions of the trace either side of the weight addition, where the signal is constant and devoid of any artefacts. The successful calibration is achieved when the voltage signals are set to corresponding tension values of 0mN and 9.82mN (see Figure 2.5).

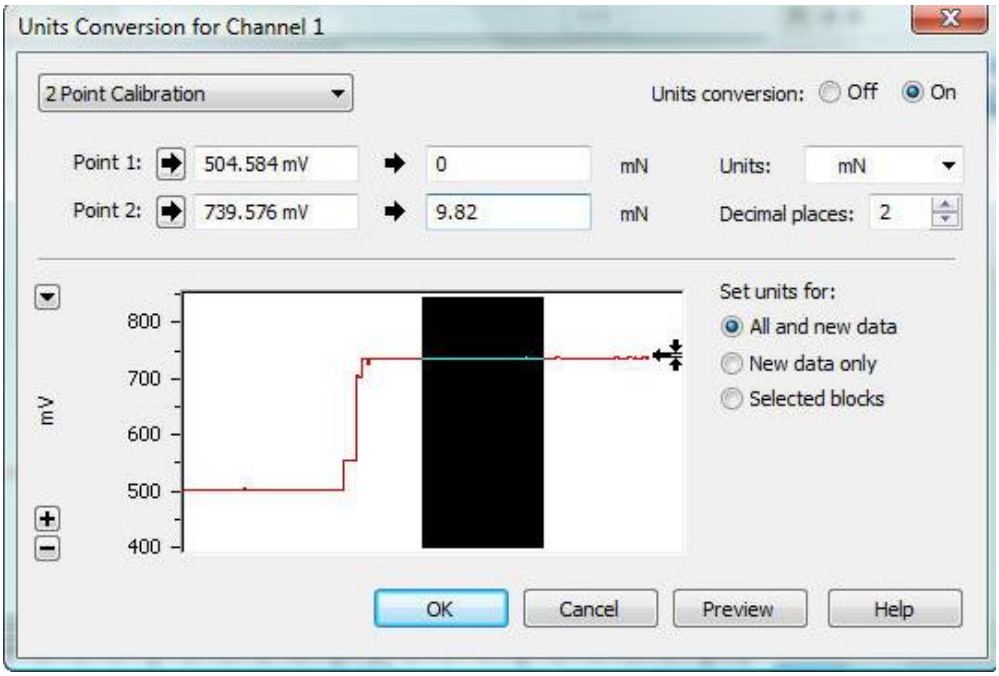
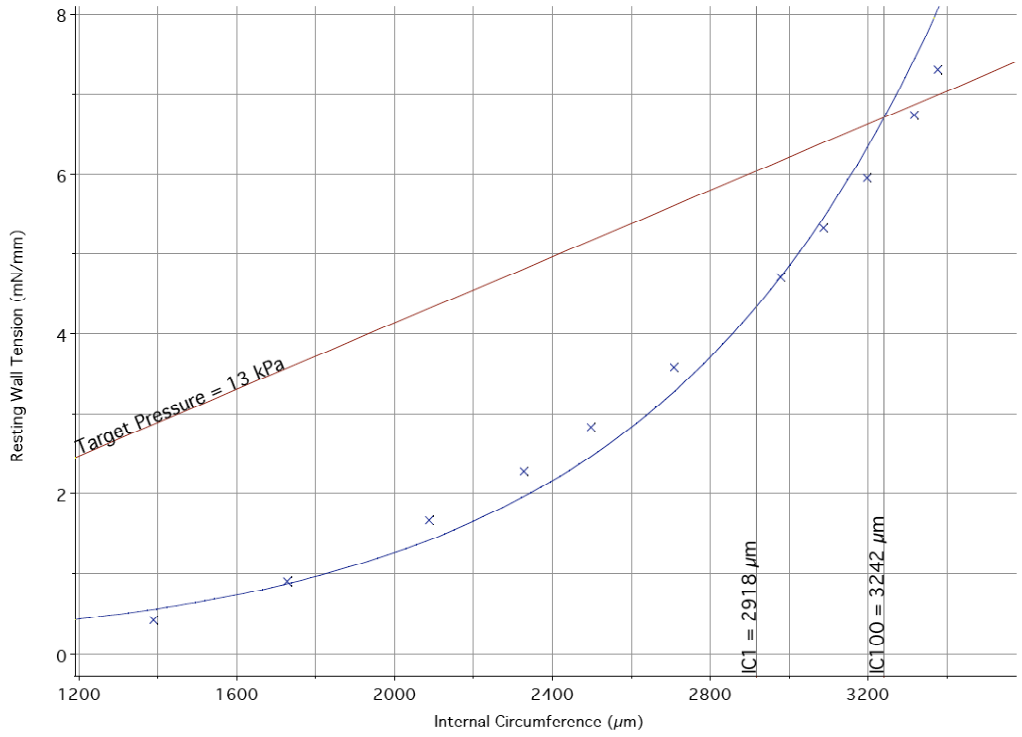


Figure 2.5: *Typical LabChart trace for the 2-point conversion. (Taken from AD Instruments manual).*

2.5.6 Normalisation

As well as calibration of the myograph, standardisation is also achieved by normalising the tissue preparation after loading each of the segments. This procedure is vital as it determines the basal tension of the vessel, by which any active responses can be accurately identified and quantified. Considering that there is variation in the elastic properties between vessels, as well as between individual vessel segments, by normalising the internal circumference of each segment via the same mathematical relationship, observed responses can be compared.

The normalisation procedure used is based on the typical transmural pressure found within the specific vessel segment. The pressure is set at 25mmHg and 100mmHg for pulmonary and iliac arteries respectively. The internal circumference (IC_{100}) that a relaxed vessel has at this pressure is calculated by a series of stepwise distensions (in micrometer) and the measurements are correlated with the force generated (mN). This provides values for the internal circumference (μm) and vascular wall tension (mN), which are plotted against each other to produce an exponential curve. This curve will cross that extrapolated isobar corresponding to the transmural pressure (100mmHg), and via the Laplace relationship the site of intersection can be used to derive the value for IC_{100} (see **Figure 2.6**).



Result: Micrometer X1 = 7005

Figure 2.6*: Extrapolated normalisation curve following the step-wise distension procedure. The intersection with the transmural pressure isobar reveals the value for* IC_{100}*, from which the normalised internal circumference can be obtained. Adjusting the myograph to the calculated micrometer denomination allows the baseline vessel tension to be set.*

XI = the value extrapolated as the basal tension to set that particular channel

The normalised internal circumference and the corresponding micrometer values are then calculated through the multiplication of IC_{100} by a specific factor. This factor represents the proportion of the internal circumference which would generate the maximal tension; for small rat arteries the factor used is 0.9. By adjusting the micrometer on each unit according to the calculated values (XI value on **Figure 2.6**), each vessel segment is tuned to its set normalised tension for equilibration (see Figure 2.7). After normalisation and leaving the vessel rings to equilibrate at baseline tension for about 45 minutes, the chambers were regularly washed out and the buffer solution replaced with a fresh one.

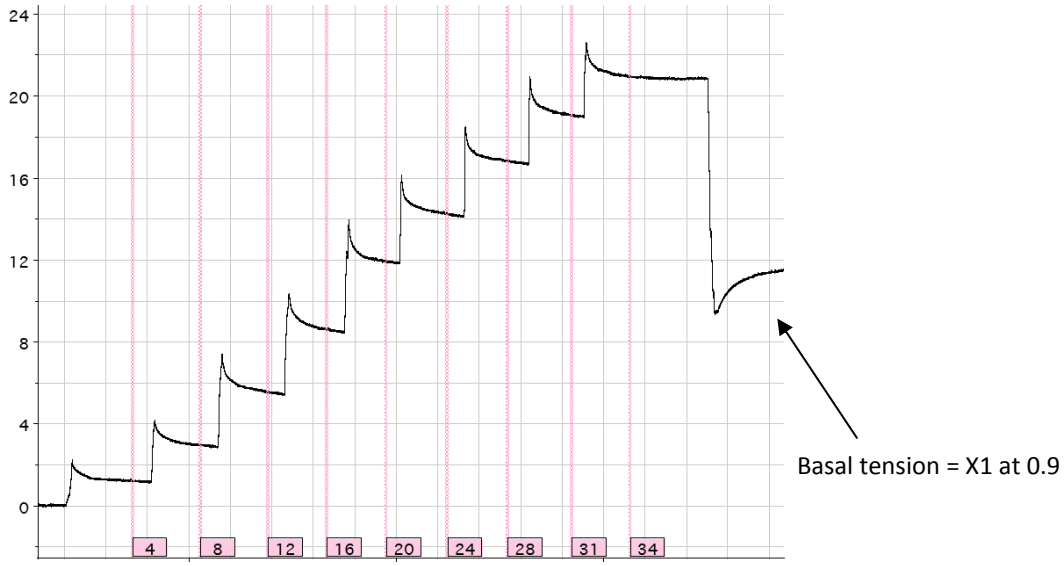


Figure 2.7: ***Primary trace displaying each distension step during normalisation. Vessel segments are increasingly stretched and the micrometer values logged to calculate the normalised basal tension.***

The above normalisation steps have always worked well for the iliac vessels used in this project. However, for the small pulmonary arteries used in the experiments, it was not reproducible as the baseline line tension had to be set manually at times in order for the agonist response to be observed. This was noticed at the very beginning of the research project. After several attempt to troubleshooting we then decided to adopt a well-established manual protocol of setting baseline tension for pulmonary arteries at about 3mN used by Jeremy Ward and his group at King's College, London. The latter method worked well with PA vessels as expected and generated consistent reproducible data.

2.5.7 Drug treatment to stimulate vascular response

The contractile function of vessel segments were tested against pharmacological agents by direct administration to each chamber containing 5ml oxygenated buffer solution at 37^{o}C. In order to check vessel viability and to normalise the series elastic component, vessels were exposed to physiological salt solution (393) containing 80mM high K^{+} (PSS, using isotonic replacement of NaCl by KCl). This also acts as a reference contraction to compare other responses from vasoconstrictor and

dilatators agents to be used. KCl should produce maximum electrogenic, non-agonist mediated constrictor tone by the vessels. This is because K^+ causes a depolarisation and contraction through activation of ion channels, and does not rely on receptors on the vascular smooth muscle. The response from other agents can be expressed as percentage of KCl vasoconstriction to compare vascular reactivity from the same tissue, and also between control and treated groups. This helps eliminate discrepancies given the biological variation in baseline tension between vessel rings. Once the KCl response has peaked, the chambers were emptied and washed a few times to allow the tension return to its baseline position.

Then the vessel segment would be treated with a range of drug concentrations (10^{-11} M to 10^{-5}M) using phenylephrine (PE) to construct a cumulative dose-response curve for vasoconstriction. For the thromboxane A2 agonist, U46619, the dose used ranges from 20nM to 1000nM. The agonist is washed off and then stimulated with EC_{80} of PE or U46619 (R & D Systems) concentration, and increasing doses of carbachol–CC or sodium nitroprusside–SNP (R & D Systems) was added to observe endothelium-dependent and independent relaxation respectively. This helps to confirm integrity of the endothelium to ensure it was not damaged during dissection, mounting or application of tension to vessels. It also confirms the vasoconstrictor effect is actually from the treatment not due to endothelial damage. The drug treatments used are summarized in Table 2.5.1 below.

Tube#	[Stock]	Volume fom the stock (uL)	Final Volume (mL)	Final Concentration	[Log]
7	1 nM	50	5	10 pM (10^{-11}m)	-11
6	10 nM	50	5	100 pM (10^{-10}m)	-10
5	100 nM (0.1uM)	50	5	1 nM (10^{-9}m)	-9
		150	5	3 nM ($3*10^{-9}$m)	-8.5

4	1 uM	50	5	10 nM (10^{-8}m)	-8
		150	5	30 nM ($3*10^{-8}$m)	-7.5
3	10 uM	50	5	100 nM (10^{-7}m)	-7
		150	5	300 nM ($3*10^{-7}$m)	-6.5
2	100 uM	50	5	1 uM (10-6m)	-6
		150	5	3 uM ($3*10^{-6}$m)	-5.5
1	1 mM	50	5	10 uM (10^{-5}m)	-5

Table 2.5.1: Shows a summary of the vasoconstrictor/vasodilator drugs added to individual chambers of myograph bath.

Through the course of my project we have changed vasoconstrictor from phenylephrine due to inconsistency observed with vasoconstriction response generated, especially in pulmonary arteries, which might be attributed least stability of the agonist or minimal sensitivity of PAs to the vasoconstrictor. For example, at times the freshly made aliquot of phenylephrine might induce response and when the same does is used again after 30 minute later on the same vessel, might not induce a response, or the freshly made aliquot of phenylephrine generating no response. However, when another vasoconstrictor (such as U46619) was used on the same vessel, vasoconstriction was observed. Therefore, we used U46619, a synthetic analogue of thromboxane A2 agonist, which provided a more consist results.

2.5.8 Inflammatory stimulus

Tissue isolation and preparation for myograph were conducted as described in section 2.5.3. Pulmonary and systemic vessels were then exposed to 80mM KCl, treated with either a vasoconstrictor or vasodilator agent and one of each vessel pair was incubated with tumour necrosis factor alpha (TNFα; 1000U/mL or IL–8; 50ng/mL

or IL–1β; 20 ng/mL – R&D System) for 1 hour. The extended inflammatory challenge involve incubation of pulmonary artery with TNF–α for 2 or 6hr in Dulbecco's Modified Eagle's medium (DMEM – Sigma-Aldrich) Subsequently, the initial stimulation with vasoconstrictor or vasodilator drugs was repeated, then finally 80mM KCl added at the end to ensure the vessels were still viable.

2.5.9 Hypoxia Treatment in vitro

2.5.9.1 Concurrent Acute Hypoxia and TNF–α Treatment in vitro

Pulmonary artery vessels were isolated, mounted myograph, normalised as described and baseline tension was set. After stabilisation of the vessel, KCL response was perform to ensure viability of the vessel and then hypoxia was induced on the myograph bath by bubbling the Kreb Solution containing the vessel with 95% N_2 and 5% CO_2 for the duration of the protocol (1 hour). Then agonist response was tested (see results in 6.3.6).

2.5.9.2 Effects of acute TNF–α Treatment on HPV

Pulmonary artery segments were mounted on the myograph, baseline tension set and vessels were allowed to become stable. Then KCL response tested. HPV was generated by bubbling the Kreb Solution containing the vessel with 95% N_2 and 5% CO_2, post pretone generation with prostaglandin F2α (3–7μM). Then the vessels were incubated with/without TNF–α for hour then HPV generation was repeated (see raw HPV trace in **Figure 6.3.5**).

The second HPV values was taken as percentage of the first HPV generated for phase 1 and 2 respectively and results plotted as bar chart (this was not shown and results was insignificant). We have also calculated HPV phase 1 and 2 as percentage of high K^+ (% High K^+ [80 mM]), averaged the results for all vessels and plotted as a bar chart (Mean ±SEM) (see results in 6.3.5).

2.5.10 Stiffness Measurement Protocol

In this protocol, w attempted to measure vascular stiffness in systemic vessels (iliac) isolated from rats to compare with the pulse wave velocity measured in human alpha-1-antitrypsin deficient subjects. Unfortunately, we had no access to pressure myography, therefore we had to develop a manual method to help us derive some form of stiffness measure.

Vessels were mounted on a 4-channel myograph then applied a series of stepwise distensions (μm) and the measurements are correlated with the force generated (mN), similar to the normalisation step observed in earlier section (2.5.6). The values for the corresponding distension and tension extrapolated from LabChart trace (Figure 2.8). These values were plotted as an exponential curve graph as in a sample (Figure 2.9).

IC100 was identified from the graph and from this, the IC89 and IC91 were calculated for the circumference and diameter of the vessel. The circumference at IC89 and IC91 were calculated by multiplying IC100 by either 89 or 91 and divided by 100 (IC100 x IC89 or 91/100). The diameter calculated by dividing the circumference at IC89 and IC91 by π (3.142) respectively. The changed in circumference and diameter were then calculated by subtracting IC89 from IC91. This value is denoted as delta X–axis for the circumference and diameter.

Then we used the known X values at IC89 and IC91 (calculated above) in the exponential curve equation to calculate the Y values at IC89 and IC100 respectively and subsequently subtracted Y at IC89 from Y at IC100 to derive change in the Y-axis.

The slope of the change in Y divided by change in X, was used derive circumference (mN/um) and the change in Y was divided by the change in X/π (calculated above) to derive the diameter (mN/um).

We then differentiated the exponential equation ($y = 0.3038e^{0.1421x}$; R^2=0.981) and used this differential equation [(0.3038*0.1421)*(EXP(0.1421*IC90)] to again find the circumference (mN/μm) which gave the same answer as the slope calculation. Then the circumference (mN/μm) was divided by (3.14) to generate the diameter (mN/μm).

These steps were repeated for each of the four channels or different treatments and the experiments repeated to collect replicates. The replicates of calculated IC90, circumference (mN/μm) and diameter (mN/μm) were plotted as bar chart to provide an idea about stiffness of the vessel.

Please note all values, graph and trace provided were arbitrary and were used for demonstration purpose only.

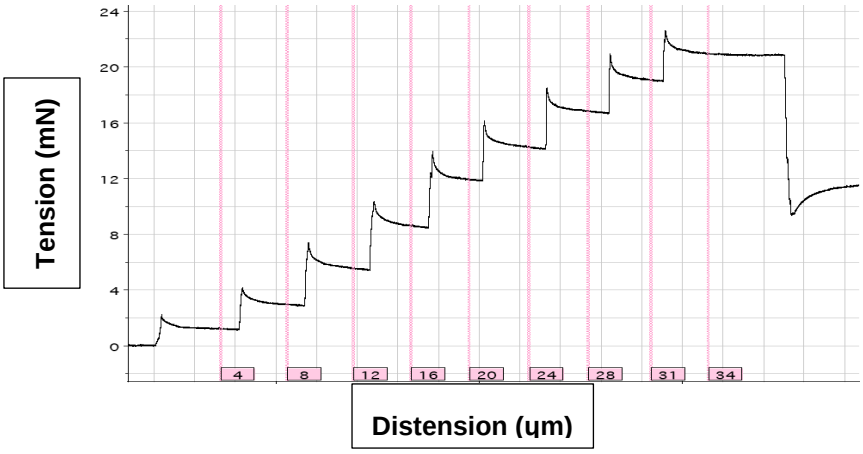


Figure 2.8: Demonstrates a sample step by step distension trace of a vessel

A

Distension (uM)	Tension (mN)
0.2	0.28
1	0.3
3	0.5
5	0.55
9	1.6
14	1.9
15	3.5
19	4.2
25	9.3
28	15.8

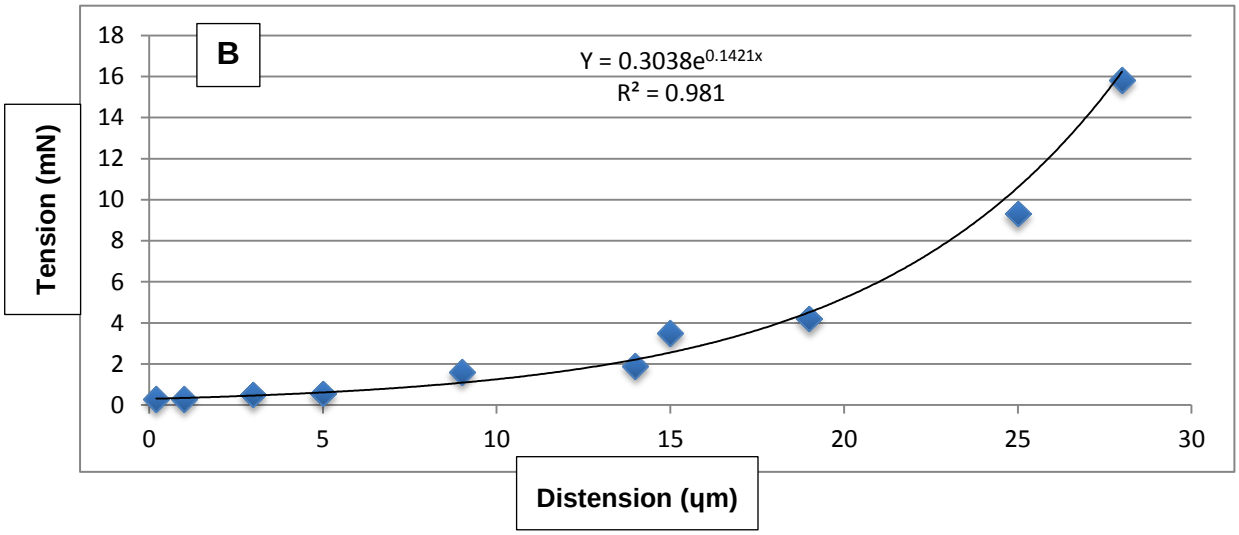


Figure 2.9: A) showing the data extrapolated from distension trace (B) exponential curve of the data. Note: **All values were arbitrary.**

Chapter 3

3 DO HYPOXIA AND INFLAMMATION INTERACT TO EFFECT CHANGES IN ARTERIAL STIFFNESS IN PATIENTS WITH α1-ANTITRYPSIN DEFICIENCY?

3.1 Chapter Introduction and Overview

According to the World Health Organisation (8), COPD is a global public health problem and affects millions of people each year. Generally COPD refers to diseases such as emphysema and chronic bronchitis. Emphysema arises as a result of damage to air sacs in the lungs leading to shortness of breath during activity as an early symptom of the disease, a phenomenon that can later be observed at rest as the disease worsens.

Smoking and environmental factors are the major causes of emphysema. However, there is growing evidence that genetic factors such as Alpha-1 Antitrypsin Deficiency (AATD) can predispose to the development of emphysema. AATD is considered a rare hereditary disorder, though still significantly underdiagnosed (394-396), even though AATD is among the commonest autosomal genetic disorders found in human (397). AATD was first reported to increase the risk of developing emphysema in 1963 (398), as well as a number of other phenomena, such as liver cirrhosis (391). People with AATD do not have sufficient amount of the protein known as alpha-1 antitrypsin in their blood, a problem which arises due to polymerisation of AAT in most cases (399)

Alpha-1 antitrypsin is one of the key proteins found within the blood which is mainly manufactured by the liver cells and is then transported into the blood stream as a protective mechanism against inflammation. This is achieved by preventing the effects of enzymes such as elastase which usually accompany leucocytes that

protect delicate lung tissues by eliminating bacteria and neutralising small particles inhaled into the lungs. Once elastase completes its function, it is neutralised by alpha-1 antitrypsin. Hence, in the absence of alpha-1 antitrypsin, elastase concentrations can continue to build up thereby leading to alveolar damage.

The normal genotype is usually described as PiMM, due to the nature of the protein movement on phenotyping (medium mobility). This results in a normal level of alpha-1 antitrypsin in the blood stream. An extensive number of genetic variants of alpha1-antitrypsin have so far been identified, there could be more than 100 resultant variants of the protein (397, 400). The predominant genetic variants linked with AATD are Z (Glu342Lys) and S (Glu264Val) mutations, triggered by substitution of a single amino acid; glutamic acid at position 342 and 264 with lysine and valine respectively (395). It is possible for some individuals to inherit a single abnormal gene (e.g. genotype such as PiMZ) which increases the risk of developing emphysema in a smoking population from 20% (among smoker with normal allele) to 50% (smokers with PiMZ genetic variant) (401).

Cardiovascular morbidity and mortality is prevalent among COPD patients. This observation seems to be independent of common risk factors such as smoking, gender and age (402-404). A reasonable mechanistic link is the existence of a chronic inflammation in the both the lung and cardiovascular system (228, 404, 405), which was reported to be linked with endothelial dysfunction (405, 406). In addition, it was suggested that the phenomenon might be associated with loss of elastin (65, 228, 407) and subsequent vascular calcification (408). It has been suggested that persistent low-grade systemic inflammation might serve as the pathophysiological link between COPD and subsequent development of cardiovascular disease (409).

Moreover, several inflammatory markers including C–reactive protein (403, 404) are found to be elevated in COPD patients. There is enormous evidence suggesting a role for systemic inflammation in COPD. This due to increased level of inflammatory cytokines and acute phase proteins found in stable COPD subjects and the fact that exacerbation was associated with both pulmonary and systemic inflammation. This has been extensively discussed in the introduction section. Moreover, the role of hypoxia in COPD and subsequent cardiovascular complications such as pulmonary hypertension has been detailed in the introduction section of the thesis. The schematic diagram (Figure 3.1: schematic diagram showing the potential pathophysiology of COPD that leads to pulmonary hypertension. It highlights the role of contribution of inflammation, hypoxia and genetic factors in the disease pathogenesis. Adapted from (410)) summarises the potential contribution of inflammation due to smoke exposure and hypoxia COPD pathogenesis.

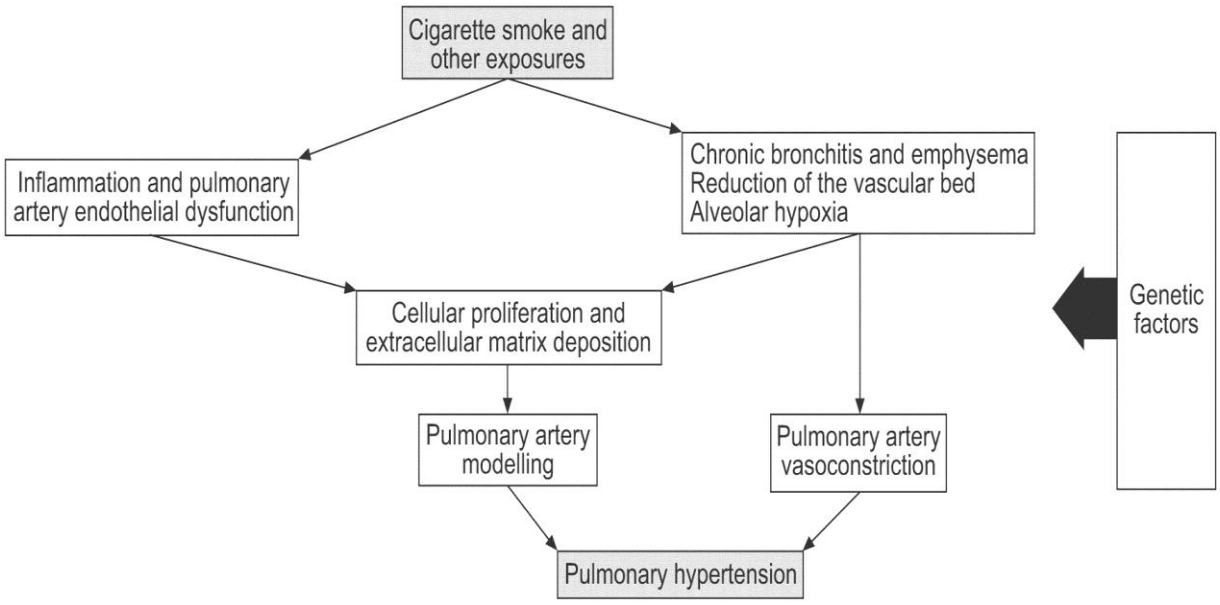


Figure 3.1: schematic diagram showing the potential pathophysiology of COPD that leads to pulmonary hypertension. It highlights the role of contribution of inflammation, hypoxia and genetic factors in the disease pathogenesis. Adapted from (410)

Also, arterial stiffness (65, 228, 407), and thoracic aortic calcification (408) as well as impaired flow mediated vasodilation (406) were markedly elevated in COPD patients. These associations are independent of smoking status and correlated inversely with lung function as well as being directly connected to the extent of emphysema as measured by quantitative CT scanning (228, 402, 404, 408). This suggests that there is a direct link between the two pathologies.

Arterial stiffness is a potentially adjustable characteristic with an added predictive value beyond what can be generated by using traditional cardiac risk factors (411-413). Therefore, arterial stiffness has been the target of pharmacologic and exercise interventions in patients with COPD (414, 415). These studies have suggested a possible advantage with inhaled treatments and exercise. However, the benefit with pharmacologic intervention seems to be limited to those patients with a substantial augmentation in arterial stiffness (415). Hence, any process that could identify specific subjects who may potentially benefit from an intervention will add a significant value to phenotyping and targeting therapy (416).

Pulse wave velocity (PWV) is the gold standard for measuring arterial stiffness and this can be measured from the aorta or carotid artery (417). Arterial stiffness was shown to be increased in COPD patients (42, 418) and it can be used as an independent predictor of cardiovascular episodes and mortality in COPD patients (419) and is positively associated with level of specific systemic inflammatory

makers (such as C–reactive protein level) found in healthy controls (420) and wider patient populations (421, 422). This is evident in the study that has shown a weak link between arterial stiffness and interleukin–6 as well as with soluble tumour necrosis factor receptor 1 (sTNFR1) but not with sTNFR2 (418).

Finally, various interventional studies have investigated the influence of pharmacological intervention and exercise on arterial stiffness in COPD patients. However, these studies were focused on those patients with more elevated levels of arterial stiffness (416). In addition, some studies have looked at the predictors of increased arterial stiffness in patient with moderate to severe COPD (416).

In this study, we aimed to identify predictors of increased arterial stiffness in an AATD cohort, who are genetically predisposed to develop COPD.

Finally, it is established in the literature that both hypoxia and inflammation play a critical role in COPD disease prognosis and progression as well as in the pathophysiology associated with cardiovascular diseases. Therefore, we speculated that there might be an interaction between hypoxia and inflammation and this might be useful to predict the risk of cardiovascular disease in COPD.

The aims of the investigations in this chapter are summarised below:

In a cohort of patients with AATD:

I. We aimed to identify the variables that related to:

- **i.** arterial stiffness measure (PWV)
- **ii.** the inflammatory markers (TNF–α, IL–8 & IL–1β)
- **iii.** oxygen level (PO_2)

II. To identify predictors of arterial stiffness in AATD patient

III. To evaluate interaction between inflammatory markers and hypoxia

3.2 Methods

3.2.1 Arterial Stiffness

All the physiological measurements were conducted by a qualified respiratory clinical scientist at the Queen Elizabeth Hospital Birmingham and not by the author of this thesis. The author analysed the data and performed assays on the serum samples collected from the patients to measure cytokine levels as well as conducted animal.experiments disscussed in chapter 2 to complement the human data. The patient selection or recruitment, demographic and method used were disscused in Section 2.1. The arterial stiffness measure PWV, was done on the aorta or carotid artery thus the use of a systemic vessel in rats to compare the effects. Iliac artery experimenets reported in **Chapter 4** were conducted prior to human study data been made available. It would have been more suitable to use corotid artery to assess stiffness in rats, however, it was not practically feasible to complete the task. Nonetheless, as we will be comparing a systemic vessel to another, and this makes a reasonable sense.

3.2.2 ELISA

ELISA was used to quantify level of cytokine in the serum samples of AATD subjects. Levels of TNF–α, IL–8 and IL–1β were measured. Detailed explanation is given in the method section of the thesis (2.2).

3.2.3 Statistics

Variables were initially tested for normality this determine which univariate analysis is conducted. Pearson correlation was used to analyse parametric variables, whereas Spearman's correlation was used for non-parametric variable. Variables of interest and that one found to be associated with arterial stiffness at univariate level were included in the regression model. We have adopted significant when *p value* is less than or equal to 0.05.

3.3 Results

3.3.1 Baseline Demographic Characteristic within the Patient Population

The demographic data shows the composition of men and women at 66 and 34 percents respectively (**Error! Reference source not found.**). Showing that there were about twice as many male participants in the study compared to female participants. All the different variants of alpha-1 antitrypsin deificiency (such as MMalton, Mprocida, Z null and Z phenotypes) were displayed by the participants. The Z variant constituted of more than 92 percent of the cases, whereas the other phenotypes were much less or rarely observed.

71 percent of the patient population were ex-smokers, 24 percent were non-smokers and 5 percent were current smokers (Table 3.3.1). Index case and non-index case was used to refer to the way the patients were diagnosed with alpha-1 antitrypsin deficiency. Index case referes to the that patients were discovered because a close family member was diagnosed with the disease and thus they were diagnosed because a family member was ill. However, the index case refers to the fact that the patients were diagnosed with disease because they were ill themselves. Predominant cases of alpha-1 antitrypsin deficiency was discovered as non index 2,

hence more than three-quarter of the cases were diagnosed because the patients were ill.

The mean age between male and female categories were similar with respect to smoking status. Non-smokers were an average age of 60 years in both male and female groups, ex-smokers were about 58 years in both categories and current smokers had a mean age of about 45 years (Table 3.3.1)**.**

The alpha-1 antitrypsin deficiency phenotypic variation between male and female showed a similar pattern as the Z phenotype was predominant in both catergories. However, count for Z phenotype among males was almost double that of the Z phenotypes observed among female patients.

The minimum and maximum ages of patients were 24 and 79 years respectively. The mean age of the patient population was 55.7±0.79. The minimum and maximum BMI were 17 and 41 respectively and the mean BMI was 25.9±0.37. The blood PO_2 ranged from 5.5 to 12.6 (mmHg) and the mean 8.97±0.12, whereas the minimum and maximum PWV were 6 and 15 respectively. The mean TNF– α and IL–8 levels were 3.5±0.28 (pg/mL) and 24.8±2.23 (pg/mL) respectively.

Variables	N	Range	Min	Max	Mean	Std. Error	Std. Deviation
Age (Yrs)	176	55.68	24.15	79.83	55.6634	0.78754	10.44791
Height (m)	176	0.41	1.51	1.92	1.7198	0.00679	0.09006
Weight (Kg)	176	81.00	42.00	123.00	76.7886	1.20532	15.99043
BMI	176	24.12	16.56	40.68	25.9114	0.36807	4.88305
Pack Years	126	57.90	0.10	58.00	17.3424	1.11073	12.46791
pH	156	1.95	7.35	9.30	7.4578	0.01225	0.15301
PO_2	156	11.02	5.5	12.62	8.9672	0.11545	1.44202
TNF–α (pg/mL)	149	23.56	-4.92	18.64	3.5347	0.27857	3.40036
IL–8 (pg/mL)	142	203.68	-8.21	195.47	24.8334	2.22528	26.51723
PWV	168	8.92	6.04	14.96	9.0941	0.12969	1.68104

Table 3.3.1: Shows Descriptive statistics of the various relevant variables used in this chapter PO_2 = Partial pressure of Oxygen, BMI= Body Mass Index, TNF–α = Tumour Necrosis Factor Alpha, IL –8 = Interleukin 8, PWV = Pulse Wave Velocity.

3.3.2 Normality Test of Variables

All relevant variables were explored to check for parametric or non-parametric distribution of the data and whether to reject or accept the null hypothesis that the data is significantly non-normal for each variable. Demographic variables such as Age, Weight, Height, BMI, St George's (SG) quality of life score such as SG total, SG Impact and SG Symptoms were found to be significantly normal as k–s

statistics were ≤0.1 whereas pack years and SG activities were significantly non-normal as k-s statistics were ≥ 1 (see Appendix 2).

The test of normality of blood gases and cardiovascular variable indicated that BE, TLC, TLCO, RV, Stroke Volume, Systolic Pressure, Cardiac Output, Pulse Pressure, Aortic Pulse Pressure, Mean Arterial Pressure, Total Peripheral Resistance, and augmentation index were all significantly normal as k–s statistics were ≤ 0.1. On the other hand, the partial pressure of oxygen in the blood (PO_2), partial pressure of carbon dioxide in the blood (PCO_2), hydrogen carbonate (HCO_3), pH, KCO were found to be significantly non–normal as k-s statistics were ≥ 1.

The Test of normality indicated that Interleukin–1–Beta (IL–1β), Interleukin–8, Tumour Necrosis Factor Alpha (TNF–α), C–Reactive Protein (CRP) were all significantly non–normal because the k–s statistic were ≥ 1; whereas the Cholesterol was significantly normal due to the k–s statistic being ≤ 1.

When a normality test was performed on the spirometry variables, it was could be concluded that forced expiratory volume in 1 second (FEV1), forced expiratory volume ratio (FEV1/FVC ratio) and forced expiratory volume percent predicted were all found to be significantly non–normal because k–s statistics were ≥ 1. However, forced vital capacity (FVC) and forced vital capacity percent predicted (FVC % Predicted) were normal

3.3.3 Univariate Analysis

Once all variables were tested for normality Univariate analysis was performed on the variables by conducting correlation between relevant variables using the Pearson's correlation test for parametric and Spearman's test for non-parametric data as described previously (Appendix 3). Our data has shown that pulse wave

velocity (PWV) significantly correlated with age, sex, TNF–α, FEV1, FEV1 % predicted, FEV1/FVC ratio, systolic pressure, pulse pressure, aortic pulse pressure, cardiac output, total peripheral resistance, TLCO and TLCO % predicted (Table 3.3.2**)**. In contrast, there was no significant correlation between PWV and BMI, IL–8, IL–1β, FVC, PO_2 and PCO_2 because their *p–values* were all greater than 0.05 (Table 3.3.2Table 3.3.2).

From the our data, PO_2 has significantly correlated with FEV1, FEV1 %, FEV1/FCV ratio, FVC, FVC % predicted, TLCO, TLCO % Predicted, Pulse pressure and aortic pulse pressure. However, PO_2 did not indicate any strong correlation with TNF–α, IL–8, IL–1β, PCO_2 and systolic pressure (Table 3.3.2).

3.3.4 Correlation of pulse wave velocity against measured variables

	Variables	R	p Value
PWV	Age	0.655	**< 0.0001***
	Sex	N/A	**0.010***
	BMI	-0.079	0.315
	TNF-α	0.178	**0.037***
	IL-8	0.091	0.299
	IL-1β	-0.018	0.916
	FEV1	-0.248	**0.001***
	FEV1 Percent Predicted	-0.174	**0.026***
	FVC	-0.086	0.274
	FEV1 ratio	-0.240	**0.002***
	Systolic Pressure	0.433	**< 0.0001***
	Pulse Pressure	0.34	**< 0.0001***
	Aotic Pulse pressure	0.352	**<0.0001***
	Cardiac Output	0.317	**< 0.0001***
	Total Peripheral Resistance	-0.156	**0.043***
	TLCO	-0.366	**< 0.0001***
	TLCO Percent Predicted	-0.315	**< 0.0001***
	PO_2	-0.101	0.226
	PCO_2	-0.013	0.876
PO_2	FEV1	0.462	**< 0.0001***
	FEV1 Percent Predicted	0.469	**< 0.0001***
	FEV1 ratio	0.419	**< 0.0001***
	FVC	0.17	**0.036***
	FVC Percent Predicted	0.225	**0.005***
	TNF-α	-0.053	0.558
	IL-8	-0.036	0.698
	IL-1β	0.088	0.600
	TLCO	0.426	**< 0.0001***
	TLCO Percent Predicted	0.422	**< 0.0001***
	PCO2	-0.092	0.262
	Systolic Pressure	0.091	0.263
	Pulse Pressure	0.224	**0.005***
	Aotic Pulse Pressure	0.214	**0.008***

Table 3.3.2: Correlation of pulse wave velocity against measured variables

3.3.5 Correlation TNF–α and IL–8 against measured variables

Univariate analysis had indicated that IL–8 strongly correlated with pack years, whereas it did not indicate any correlation with FEV1 % predicted, FEV1 ratio, KCO, KCO % predicted, TLCO, TLCO % predicted, age, systolic pressure, aotic pulse pressure or SG total (Table 3.3.3). TNF-α had earlier shown to be correlated with PWV and has been shown to be significantly correlated with total peripheral resistance. On the other hand, TNF-α, through univariate analysis, did not show a correlation with any other variable it was compared with such as FEV1, FEV1 % predicted, FEV1 ratio, total peripheral resistance, KCO, KCO % predicted TLCO and age (Table 3.3.3).

	Variables (units)	R	p Value
IL-8	FEV1	0.044	0.603
	FEV1 Percent Predicted	0.127	0.134
	FEV1 ratio	0.099	0.245
	Augmentation Index	0.054	0.526
	Total Peripheral Resistance	-0.075	0.373
	KCO	0.064	0.373
	KCO Percent Predicted	0.04	0.64
	TLCO	-0.029	0.733
	TLCO Percent Predicted	0.037	0.666
	Age	0.083	0.33
	Pack Year	-0.18	**0.038***
	Systolic Pressure	-0.087	0.305
	Aotic Pulse Press	-0.049	0.566
	SG total	-0.081	0.349
TNF-α	FEV1	-0.016	0.851
	FEV1 Percent Predicted	0.049	0.557
	FEV1 ratio	-0.002	0.982
	Augmentation Index	0.027	0.742
	Total Peripheral Resistance	-0.205	**0.012***
	KCO	-0.067	0.423
	KCO Percent Predicted	-0.06	0.471
	TLCO	-0.102	0.224
	TLCO Percent Predicted	-0.038	0.644
	Age	0.136	0.1
	Pack Year	-0.002	0.983

Systolic Pressure	0.059	0.478
Aotic Pulse Press	0.133	0.106
SG total	0.01	0.91

Table 3.3.3: Correlation TNF-α and IL – 8 against measured variables

3.3.6 Multivariate Analysis

3.3.7 Multiple Linear Regression Analysis

For this stage of analyses, variable were categorised into dependent and independent variables. The variable of interest that we hypothesised might be predicted by the independent variables (PWV) was entered as the dependent variable. Variables that indicated significance correlation with PWV and/or PO_2 and TNF-α at univariate level were included in the regression model. The variables entered as independent variable included; age, gender, PO_2, TNF–α, FEV1, systolic pressure and TLCO. Age, gender, FEV1, systolic pressure, and TLCO were included because they had shown a strong correlation with PWV. TLCO is also used as a determinant for emphysema. TNF–α was included as independent variable because it significantly correlated with PWV. All above–mentioned target independent variables were entered into the regression model.

From the model summary of the regression analysis, the adjusted r square (adjusted r^2) represent the proportion of the total variability in dependent variable, PWV that is explained by the independent variables in the model. Our data shows that from the adjusted r^2 values that age (r^2 = 0.351), can predict 35% of the total variation in PWV. Age and gender (r^2 = 0.366) can explain about 37% of the total variation in PWV. Age, gender and TNF–α (r^2 =0.371) can predict 37% of the total variation in PWV. Age, gender, TNF–α and systolic pressure (r^2 = 0.470) can explain 47% of the total variation observed in PWV. Age, gender, TNF–α, systolic pressure and FEV1 (r^2 =0.480) can explain 48% of the total variation in PWV. Age, gender, TNF-α, systolic pressure, FEV1 and PO_2 (r^2 =0.480) can explain 48% of the total variation in

PWV. Finally the independent variables entered into the multivariate linear regression model; age, gender, TNF-α, systolic pressure, FEV1, PO_2 and TLCO (r^2 =0.495) can explain about 50% of the variation observed in our dependent variable; PWV (

Table 3.3.4).

The ANOVA table of the linear regression analysis indicates how well the regression model predicts the dependent variable and whether this was significant or not. The p values shown indicate statistical significance of the regression model that was run. If the p value is ≤ 0.05, this indicates that, statistically, it is acceptable to reject the null hypothesis of the F statistic of the regression (

Table 3.3.5). The null hypothesis of the F statistic of the regression is that the regression model has no explanatory power which means all the co–efficient of the independent variables are zero, which is the same as saying none of the independent variables help to predict the dependent variable (meaning the model is useless).

A variety of regression models were tested to ascertain which parameters explain the greatest degree of variance in PWV. Overall, the multivariate regression model that entered TNF–α, systolic pressure, FEV1, PO_2 and TLCO was found to statistically significantly predicts PWV (F=20.3, *p value* =0.0001).

Model	R	R Square	Adjusted R Square	Change Statistics		
				R Square Change	F Change	Sig. F Change
1	0.592[a]	0.351	0.345	0.351	61.655	0.0001
2	0.605[b]	0.366	0.355	0.015	2.706	0.103
3	0.609[c]	0.371	0.354	0.005	0.919	0.340
4	0.686[d]	0.470	0.451	0.099	20.730	0.0001
5	0.693[e]	0.480	0.456	0.010	2.070	0.153
6	0.693[f]	0.480	0.451	0.000	0.000	0.983
7	0.703[g]	0.495	0.462	0.015	3.117	0.080

Table 3.3.4: Model summary of the multivariate regression

a, b, c, d, e, f, g and h gives details of the stepwise addition of the independent variables, which represent PWV (dependent variable), age, sex, TNF-α, systolic pressure, FEV1, PO_2 and TLCO respectively. The variable were added into the multivariate regression mdoel using step-wise manner so that impact of each individual variable could be established as well as the model as a whole.

ANOVA[a]						
Model		Sum of Squares	Df	Mean Square	F	Sig.
1	Regression	109.977	1	109.977	61.655	< 0.0001[b]
	Residual	203.348	114	1.784		
	Total	313.325	115			
2	Regression	114.733	2	57.367	32.642	< 0.0001[c]
	Residual	198.592	113	1.757		
	Total	313.325	115			
3	Regression	116.349	3	38.783	22.052	< 0.0001[d]
	Residual	196.976	112	1.759		
	Total	313.325	115			
4	Regression	147.346	4	36.836	24.635	< 0.0001[e]
	Residual	165.979	111	1.495		
	Total	313.325	115			
5	Regression	150.411	5	30.082	20.312	< 0.0001[f]
	Residual	162.914	110	1.481		
	Total	313.325	115			
6	Regression	150.412	6	25.069	16.773	< 0.0001[g]
	Residual	162.913	109	1.495		
	Total	313.325	115			
7	Regression	154.982	7	22.140	15.101	< 0.0001[h]
	Residual	158.343	108	1.466		
	Total	313.325	115			
a. Dependent Variable: PWV						
b. Predictors: (Constant), Age						
c. Predictors: (Constant), Age, Sex						
d. Predictors: (Constant), Age, Sex, TNF						
e. Predictors: (Constant), Age, Sex, TNF, Systolic Pressure						
f. Predictors: (Constant), Age, Sex, TNF, Systolic Pressure, FEV1						
g. Predictors: (Constant), Age, Sex, TNF, Systolic Pressure, FEV1, PO_2						
h. Predictors: (Constant), Age, Sex, TNF, Systolic Pressure, FEV1, PO_2, TLCO						

Table 3.3.5 Shows the ANOVA table, which reports how well the regression equation fits the data (i.e., predicts the dependent variable) as reported above. Significance: p value ≤ 0.05

Then we come to the co-efficient section of the multivariate linear regression outcome (Table 3.3.6). The co-efficient indicates the relationship between an independent variable and the dependent variable; hence, the co-efficient is the most important outcome of the multivariate linear regression. First we have looked at the t-statistic whether there is enough evidence to reject the null hypothesis. The null hypothesis of the t-statistic states that “the co-efficient of an independent variable is zero. The same as saying that particular independent variable does not help predict the dependent variable. If the p value of the t-statistic ≤ 0.05, we reject the null hypothesis. Accordingly, when we reject the null hypothesis for the t-statistic, this means that particular independent variable has predictive ability for the dependent variable. Once it is established that the independent variable has predictive ability we can look at the unstandardized co-efficient of the independent variable, making note of the whether the direction of association (positive or negative) makes sense. In addition, based on the unstandardized co-efficient value, we can deduce level of increase or increase of the dependent variable by a particular independent variable.

From my data, in the regression model 1, the t–statistic was found to be significant for age. In the regression model 2, age was observed to be significant whereas gender was not. In the regression model 3, the t–statistic for age was found to be significant whereas gender, and TNF–α were not significant. The regression model 4 indicates that age and systolic pressure were significant whilst gender and TNF–α did not show significance. Again in model 5, age and systolic pressure were observed to generate a significant t–statistics whereas other independent variables entered (gender, TNF–α, FEV1 did not show significance. Moreover, in the regression model 6, the t–statistic for age and systolic pressure were found to be

significant whereas gender, TNF–α, FEV1 and PO_2. And in the overall model in 7, the statistic of gender, TNF–α, FEV1, PO_2 and TLCO were all found to be non–significant (Table 3.3.6).

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	3.426	0.748		4.578	<0.001
	Age	0.102	0.013	0.592	7.852	<0.001
2	(Constant)	4.057	0.836		4.852	<0.001
	Age	0.101	0.013	0.587	7.838	<0.001
	Sex	-0.463	0.281	-0.123	-1.645	.103
3	(Constant)	4.079	0.837		4.875	<0.001
	Age	0.099	0.013	0.576	7.590	<0.001
	Sex	-0.494	0.283	-0.132	-1.743	0.084
	TNF	0.036	0.037	0.073	0.958	0.340
4	(Constant)	0.356	1.124		0.316	0.752
	Age	0.086	0.012	0.500	6.952	<0.001
	Sex	-0.448	0.261	-0.119	-1.713	.090
	TNF	0.036	0.034	0.074	1.049	0.297
	Systolic Pressure	0.031	0.007	0.324	4.553	<0.001
5	(Constant)	0.928	1.187		0.781	0.436
	Age	0.081	0.013	0.471	6.321	<0.001
	Sex	-0.512	0.264	-0.137	-1.941	0.055
	TNF	0.040	0.034	0.083	1.179	0.241
	Systolic Pressure	0.032	0.007	0.332	4.674	<0.001
	FEV1	-0.180	0.125	-0.104	-1.439	0.153
6	(Constant)	0.912	1.389		0.657	0.513
	Age	0.081	0.013	0.471	6.292	<0.001
	Sex	-0.514	0.274	-0.137	-1.873	.064
	TNF	0.040	0.035	0.083	1.168	0.245
	Systolic Pressure	0.032	0.007	0.332	4.618	<0.001
	FEV1	-0.182	0.149	-0.105	-1.220	0.225
	PO_2	0.003	0.116	0.002	0.022	0.983
7	(Constant)	1.698	1.445		1.175	.243
	Age	0.071	0.014	.413	5.086	.000
	Sex	-0.580	0.274	-.155	-2.116	.037

	TNF	0.035	0.034	.073	1.029	.306
	SystolicPressure	0.035	0.007	.359	4.931	.000
	FEV1	0.096	0.216	.056	.447	.656
	PO_2	-.001	.115	-.001	-.013	.990
	TLCO	-.185	.105	-.222	-1.766	.080

Table 3.3.6: Co-efficient of the multivariate analysis.

3.3.8 Analyses of Interaction between inflammation & hypoxia variables

Our data has indicated that there no interaction between inflammation and hypoxia to modulate arterial stiffness biologically.

3.4 Chapter discussion

3.4.1 Summary of key findings

1) At the univariate level, our data showed that:

    a. PWV positively correlated with age, TNF–α, systolic pressure, pulse pressure and CO. PWV negatively correlated with FEV1, FEV1 % Predicted, FEV1/FVC ratio, total peripheral resistance, TLCO and TLCO % predicted. PWV did not correlate with IL–8, IL–1β and PO_2.

    b. IL–8 and TNF–α correlated negatively with pack years and total peripheral resistance respectively.

    c. PO_2 positively correlated with FEV1, FEV1 % predicted, FEV1/FVC ratio, ,FVC, FVC % predicted, TLCO, TLCO % predicted, pulse pressure and aortic pulse pressure and PO_2 did not correlate with TNF–α, IL–8 and IL–1β.

2) The multivariate linear regression indicated that:

    a. When age, sex, TNF–α, systolic pressure, FEV1, PO_2 and TLCO were entered as independent variables, these variables accounted for 49.5 percent of the variability observed in the dependent variable; PWV.

    b. Age and systolic pressure significantly predict PWV, whereas TNF–α, FEV1, PO_2 and TLCO did not significantly predict PWV. From the ANOVA outcome of the regression, the independent variables that were entered into the model (TNF–α, systolic pressure, FEV1, PO_2 and TLCO), together significantly predict PWV.

    c. From the co-efficient outcome, only age, sex and systolic pressure were found to effectively predict PWV.

PWV

Pulse wave pressure refers to the propagation of pressure along arteries and is linked to the inherent elasticity of the arterial wall (423). Pulse wave velocity is a measure of arterial stiffness and serves as an independent predictor of cardiovascular events and mortality (423). Undeniably, cardiovascular complications is a main cause of death in several diseases including COPD (409). Among COPD patients, it has been shown that arterial stiffness is increased in a greater proportion of COPD subjects compared with healthy controls (409). Pulse wave velocity is known to be increased in stiffer arteries; a phenomenon known as arterial stiffness. This mean the artery wall has reduced capability to expand and contract in response to stimuli or changes in pressure (423). The main novel finding from the present study is that in alpha1 anti-trypsin deficient patients, pulse wave velocity is associated with the inflammatory marker, TNF-α. Similarly, another study has revealed a weak link between arterial stiffness and Interleukin–6 and soluble tumour necrosis factor receptor 1 (sTNFR1) in patient with COPD (418). However, the same study suggested no link between pulse wave velocity and soluble tumour necrosis factor receptor 2 (sTNFR2) (418). Yasmin *et al*. have also shown that in healthy individuals, pulse wave velocity was associated with another inflammatory maker, CRP(420). However, we did not observe any association between pulse wave velocity and CRP. This might be due to the limited number of subjects with completed CRP readings; hence the sample size might not be sufficient to generate any statistically significant association. However, we have had more subjects with completed reading for TNF–α thus we have observed a significant correlation with PWV.

Contrary to our findings and substantive evidence from the literature indicating an association between various systemic inflammatory markers and arterial stiffness, Lowie and colleagues suggested that arterial stiffness in COPD subjects had no association with systemic inflammation (409). This group might have based their conclusion on various reasons. Firstly, Lowie *et al* (409). suggest that aortic PWV did not correlate with CRP, IL–6, IL–8 and sTNFR2 in COPD patients. However, aortic PWV did correlate positively with sTNFR1 but this was obliterated after controlling for the co-founding factors such as age, mean blood pressure, sex and BMI (409). Secondly, most of the evidence in the literature suggesting association between arterial stiffness and inflammation has been either conducted in healthy controls or other diseases that might not reflect the phenomenon observed in subjects with COPD (420, 421). Nonetheless, arterial stiffness was suggested to be independently associated with emphysema severity in patients with COPD (228). Thirdly, according Lowie et al. a quarter of 213 eligible COPD patients were excluded from their investigation due to unsuccessful aortic PWV measurement. These patients with unsuccessful aortic PWV measurement were found to have significantly elevated BMI, fat free mass index, triglycerides, glucose and diffusion capacity of the lung for carbon monoxide (DLCO) compared to those where aortic PWV measurements were obtained. Therefore, this might render their results inconclusive as far as a COPD population is concerned.

Regardless of the difference of opinion pertaining to the association between arterial stiffness and inflammation, we have established here that TNF–α has a strong correlation with arterial stiffness. Also, it can be deduced from our results that arterial stiffness is not related to PO_2 as this was a key objective question we aimed to clarify in this chapter. This has not been demonstrated prior this observation.

Our findings also have demonstrated a strong positive correlation between arterial stiffness with haemodynamic measurements such as systolic pressure, pulse pressure and cardiac output. This evidence was expected given the known relationship between arterial stiffness and cardiac events, such as myocardial infarction (MI) (424), and the similar profile observed for blood pressure and MI risk (425). An elevation of variables such as cardiac output and pulse pressure will certainly increase the blood pressure (as per blood pressure equation: BP= Cardiac Output (CO) x Total Peripheral Resistance, and CO= Stroke Volume x Heart rate). Hence, an increase in arterial stiffness will directly or indirectly result into increased systemic blood pressure physiologically, and we would expect that modulation of blood pressure will change arterial stiffness. Consistent with this observation that the mechanism of stiffness might be driven by blood pressure, Dudenbostel and Glasser (426) on their recently concluded review of antihypertensive trials, suggested that ACE inhibitors, calcium channel blockers and mineralocorticoid receptor antagonists showed a beneficial effect in reducing arterial stiffness and central blood pressure. However, some beta blockers such as atenolol might induce reverse effect (426).

Moreover, our data has shown a strong negative correlation between arterial stiffness and FEV1, FEV1 percent predicted, TLCO and TLCO percent predicted. This means that an increase in arterial stiffness could result in a decrease of the pulmonary function and blood gas variables stated–above and vice versa.

PO_2 refers to the partial pressure of oxygen and this reflects the capacity of dissolved oxygen gas in the blood. In addition, PO_2 is a primary measure of the effectiveness of the lungs in pulling oxygen into the blood stream from the atmosphere. Our findings have shown a strong positive correlation between PO_2 and FEV1, FEV1 %

predicted, FEV1/FVC ratio, FVC, FVC % predicted, TLCO, TLCO % predicted, pulse pressure, aortic pulse pressure.

In addition, TNF–α and IL–8 showed a strong positive association with total peripheral resistance and pack years respectively. However, the inflammatory markers did not show any correlation between with PO_2.

Multivariate Regression Analyses

Moreover, we have performed further investigation of our data by conducting multivariate regression. Given the objectives of our investigations, we have carefully included among the independent variables, variables that have been shown to be associated with PWV from our univariate analyses or the literature (416). Therefore, we have included age, gender, TNF–α, FEV1, systolic pressure and TLCO as independent variables and PWV as the dependent variable. These six independent variables were entered into the multivariate regression model. Evidence from the analyses suggest that the independent variables together account for nearly 50% of the total variability observed in pulse wave velocity, the dependent variable. In addition, our findings have shown that age, sex, TNF–α and systolic pressure collectively can significantly predict pulse wave velocity.

These findings were validated by the ANOVA outcome of the regression analyses. The ANOVA of the regression suggested that our model has a significant explanatory power to predict the dependent variable. Hence, the co-efficient of the independent variables was not zero and thus can predict pulse wave velocity. The co-efficient outcome suggests that individually, age, gender and systolic pressure can significantly predict pulse wave velocity. Similarly, previous studies have shown age, gender, mean blood pressure to be independently associated with PWV in healthy individuals (420) and COPD subjects (409). However, our findings have

suggested that TNF–α, PO_2, FEV1 and TLCO were not independently associated with PWV in patients with alpha-1 anti-trypsin deficiency. This might be due difference in population used.

Alternatively, in another multivariate regression model, this time we replaced an independent variable TLCO with total peripheral resistance (data not shown). This was done to investigate further how our model might be affected by inclusion of a different independent variable. This based on the evidence in the literature suggesting an association between peripheral resistance and vascular stiffness physiologically. This model included independent variables such as age, gender, TNF–α, FEV1, systolic pressure and total peripheral resistance, was found to account for 48.8% of the total variability observed in pulse wave velocity. Again, this regression model has shown that age, sex, TNF–α and systolic pressure collectively can significantly predict pulse wave velocity. The ANOVA outcome of the multivariate regression suggests that our model fit the purpose because all the independent variables demonstrated a significant explanatory power to predict PWV. In addition, the co-efficients have shown that age and systolic pressure were independently associated with PWV in AATD subjects. Further supporting our initial evidence from earlier model discussed. However, the independent variables in this latter model account for (49%) slightly less of the total variability in PWV compared to former model (50%) discussed. This variation might be due to collinearity as lower tolerance was observed in the latter model when some of the lung function variables were added, though the tolerance and VIF were still within accepted limits.

Finally, to summarise our findings have shown a correlation between PWV and TNF–α in AATD. However, TNF–α is not an independent predictor of PWV, whereas age and systolic pressure were. The findings suggest that arterial stiffness might be

related to the inflammatory mediators and that systemic inflammation could contribute to arterial stiffening. Although, in this study, we did not investigate the possible mechanism by which inflammation might promote arterial stiffness, some studies have indicated that endothelial-derived nitric oxide is essential the regulating and modulating the function of systemic artery stiffness in vivo (427, 428). Hence, endothelial dysfunction might provide a clue as a potential mechanism linking arterial stiffness and inflammation. Further longitudinal studies whilst investigating an array of inflammatory mediators might be required to confirm the findings and to establish association between inflammation and arterial stiffness.

3.4.2 Limitation of the investigations included in this chapter

I. Only three inflammatory mediators where considered and this might not give a clear representative influence of systemic inflammation on arterial stiffness.

II. It was not possible to obtain measurements for all inflammatory mediators the whole cohort. Therefore, it was not possible to include some of the inflammatory cytokines in the regression model due to missing values.

III. The number of subjects with completed data points for the measured physiological variables also varied between subjects, as some variables had missing data points. These ultimately will affect the regression analysis. The regression analysis only took into account the completed data sets of the variables entered.

IV. The ELISA kit might not be specific enough to measure the tiny quantity of the cytokine found in this serum samples. However, other techniques has there down sides too, thus it is well established in literature other group using ELISA to quantify cytokine levels in serum samples.

Chapter 4

4 INFLUENCE OF INFLAMMATION AND CHRONIC HYPOXIA ON VASCULAR REACTIVITY AND STIFFNESS OF ISOLATED ILIAC ARTERIES

4.1 Introduction

An involvement of inflammatory cytokines or mediator in vascular dysfunction is widely documented in the literature (125, 429, 430). Tumour necrosis factor alpha (TNF–α) and Interleukin–1beta (IL–1β) were suggested to act as a pro-coagulant and pro-inflammatory stimulus on the vascular endothelium (376, 431). The role of TNF–α in vascular dysfunction has been discussed extensively in the thesis introduction section. See Figure 4.1 for the summary of TNF–α involvement in vascular dysfunction and possible mechanisms.

Moreover, inflammatory mediators were noted be elevated in various disease conditions and this increase has been associated with increased oxidative stress that might modulate vascular dysfunction (430, 432-435). Inflammatory mediators might be contributing to the alteration of the endothelium function through several mechanisms. Dresler and Horning (436) have suggested that pro-inflammatory cytokines might alter the viability of endothelial nitric oxide synthase (eNOS) mRNA (435). In addition, pro-inflammatory cytokines such as IL–1β might cause increased production of superoxide anion within the arteries (376, 437), which has a high tendency to react rapidly with NO thus generating peroxynitrite (435, 438). The reaction of the superoxide anion with NO decreases bioavailability of NO, therefore leading to the impairment of the endothelium-dependent relaxation (157). Similarly, it was suggested that peroxynitrite might cause nitration of protein tyrosine residues, which could limit the function of the enzymes associated with endothelium relaxation (435). Elevation of cytokine levels was observed in a variety of cardiovascular pathologies like arterial stiffness, hypertension, diabetes, atherosclerosis and sepsis

where endothelial dysfunction was suggested to be a key contributor (435). Hence, peripheral vascular resistance disorders are common to various cardiovascular diseases. Therefore, it is important to investigate contribution of pro-inflammatory mediators to analyse effects of cytokines on endothelium dependent vasodilatation in systemic arteries (372). It is widely known that cytokines stimulate vasoconstriction and either cause fall or rise of vasoconstrictor responses generated by variety of agonists (372). This might depend on the disparity in the local mediators released and responsiveness of different vascular beds. Besides, deficiency of endothelium-dependent relaxation was shown in different vessel types, conduit and resistance arteries treated with pro-inflammatory mediators (372).

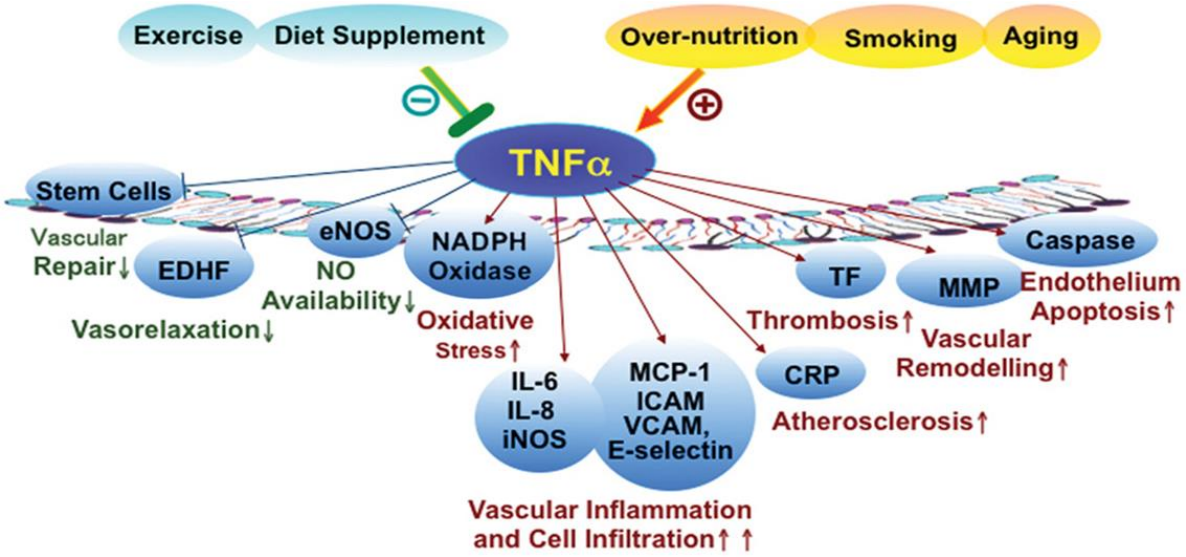


Figure 4.1: Role of TNF-α in vascular dysfunction. Even though numerous risk factors, such as physical inactivity, smoking and over-nutrition, appear to contribute to the development of vascular dysfunction, normal aging is also an independent factor in the aetiology of cardiovascular diseases. There is evidence, however, that those seemingly diverse processes converge on modulating TNF-α signaling to lead to the generation of dysfunctional endothelium and the onset of vascular diseases. TNF-α induces the gene expression of various inflammatory cytokines and chemokines, either dependently or independently of the activation of transcriptional factors, such as NF-κB and AP-1 (activator protein 1). This TNF-α-mediated signaling initiates and accelerates atherogenesis, thrombosis, vascular remodeling, vascular inflammation, endothelium apoptosis, vascular oxidative stress and impaired NO bioavailability, which contribute to the blunted vascular function. Dietary supplements and exercise favourably reduce the risk of vascular dysfunction by inhibiting TNF-α production and (or) TNF-α-mediated signaling. Risk factors in orange those factors that converge on TNF-α to induce vascular dysfunction. Factors in green denote those that protect against vascular damage mediated by TNF-α expression and signaling. TNF-α-induced pathophysiological conditions related to vascular function are shown in blue. Both vascular risk factors and protective factors affect the regulation of vascular functions by modulating TNF-α production and downstream signaling. MCP-1, monocyte chemoattractant protein-1; MMP, matrix metalloproteinase; TF, tissue factor. From Zhang et al. (125)

On the other hand, findings from exposure of systemic vasculature to chronic hypoxia generated conflicting outcome (439). Endothelial NOS expression and NO-dependent vascular relaxation activity were noted to be elevated in sheep uterine arteries and fetal guinea pig hearts due to chronic hypoxia treatment (440, 441). Contrastingly, prolonged chronic hypoxia downregulated eNOS protein expression and reduced NO-dependent vasodilatation in isolated rat aorta (442). Similarly, CH has been linked to the increased vasoconstriction observed in the guniea pig middle cerebral arteries and this alteration was due to diminished NO expression (443). These contradictory findings might be due to a variety in species, vascular bed or different specific characteristic variation between conduit and resistance vessels (439).

Furthermore, the concurrent association of elevated cytokine level and endothelial dysfunction with different cardiovascular diseases suggests a potential connection between inflammation and cardiovascular pathologies. Similarly, though inconsistent findings were reported, chronic hypoxia was suggested to be associated with vascular dysfunction and several cardiovascular diseases. Therefore, an understanding of the interaction between hypoxia and inflammation might unravel mechanism involved in vascular dysfunction.

The Aims and Objectives from this chapter are to investigate the effects of:

1. inflammatory mediators on iliac artery vasoconstriction
2. inflammatory mediators on iliac artery vasodilatation
3. chronic hypoxia (1 week) plus inflammatory mediators on iliac artery vasoconstriction
4. chronic hypoxia (1 week) ± inflammatory mediators on iliac artery vasodilatation

5. inflammation on arterial stiffness of isolated iliac artery segments and finally compare the influence of hypoxia and inflammation in both the human study (**in chapter 3**) and animal model used here.

4.2 Methods

4.2.1 Effects of TNF–α on phenylephrine or thromboxane A_2 agonist mediated vasoconstriction in isolated rat iliac arteries

As described in the method section **2.5**, isolated iliac artery was mounted on the myograph and incubated with TNF–α for 1 hour then the cumulative concentration curve vasoconstrictor was constructed. Results displayed in **4.3.1** & **4.3.2.** The percentage maximum was calculated by dividing each of the individual dose responses by the response of the highest does of the vasoconstrictor and multiplied by 100.

4.2.2 Effects of TNF–α on carbachol mediated vasodilatation in isolated rat iliac arteries

In this section iliac vessels were pre-contracted with phenylephrine and then cumulative dose-response curve of carbachol was tested. The percentage maximum was calculated by dividing each of the individual dose responses of the vasodilator by the response of the pretone (vasoconstrictor) and multiplied by 100. The result is shown in **section 4.3.3.**

4.2.3 Influence of chronic hypoxia ±TNF–α on carbachol mediated endothelium-dependent vasodilatation in isolated rat iliac arteries

In this section, animal incubated in chronic hypoxia for 1 week were exposed to 12% O_2 for week. Then the iliac artery isolated and challenged with the cytokine in vitro.

4.2.4 Influence of TNF–α and anti-oxidants on vasoreactivity and stiffness of isolated rat iliac artery segments

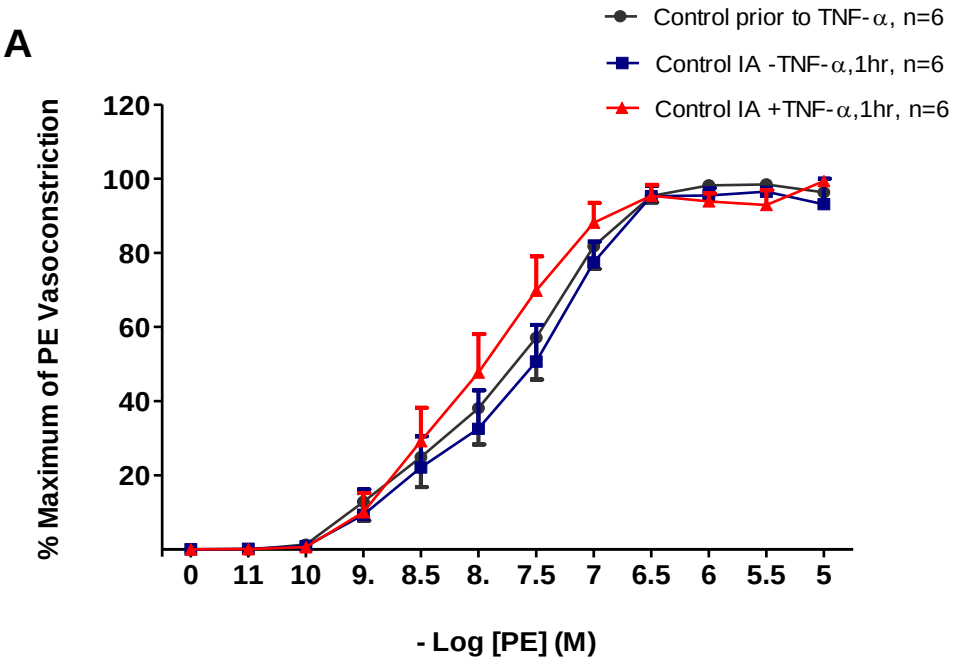
In this section, iliac artery was subject to TNF–α treatment for 6 hour in DMEM. Then the vessel was mounted on myograph. We used 6 hour incubation as 1 hour incubation with the cytokine did not show any effect. However, after several modifications of the protocol, 6 hours showed some changes vasodilatation in pulmonary arteries (to be discussed in later chapters) and we were in encouraged to follow similar method with the systemic vessel. The vessels were normalised and allowed stabilise then high K^+ response tested twice. U46619 was then used to generate pretone followed by either 1uM CC or SNP. We expressed the vasodilator response as percentage of the pretone response. (see results in **4.3.5**).

4.3 Results

4.3.1 Effects of TNF–α on phenylephrine mediated vasoconstriction in isolated rat iliac arteries

Our data from healthy rats did not show any significant difference in the maximum percentage of the α–adrenergic agonist mediated vasoconstriction by phenylephrine. The average of the percentage of maximum PE vasoconstriction were 50.35±12.14% (Mean±SEM), 47.78±11.99% and 52.27±12.16% for the Control Prior to TNF–α (quality control), Control–TNF–α (time control) and TNF–α treated respectively (**Figure 4.3.1A**, n=6).

Similarly, there was no significant difference in the ratio of tension to the individual iliac artery weight (mN)/mg) generated by PE between the treated vessels and controls. The values for control prior to TNF–α, Control–TNF–α and Control+TNF–α were 4.264±1.060 (mN/mg), 4.430±1.098 and 4.391±1.010 respectively (**Figure 4.3.1B**).


A
Control prior to TNF-α, n=6
Control IA -TNF-α,1hr, n=6
Control IA +TNF-α,1hr, n=6
% Maximum of PE Vasoconstriction
120
100
80
60
40
20
0
11
10
9.
8.5
8.
7.5
7
6.5
6
5.5
5
- Log [PE] (M)

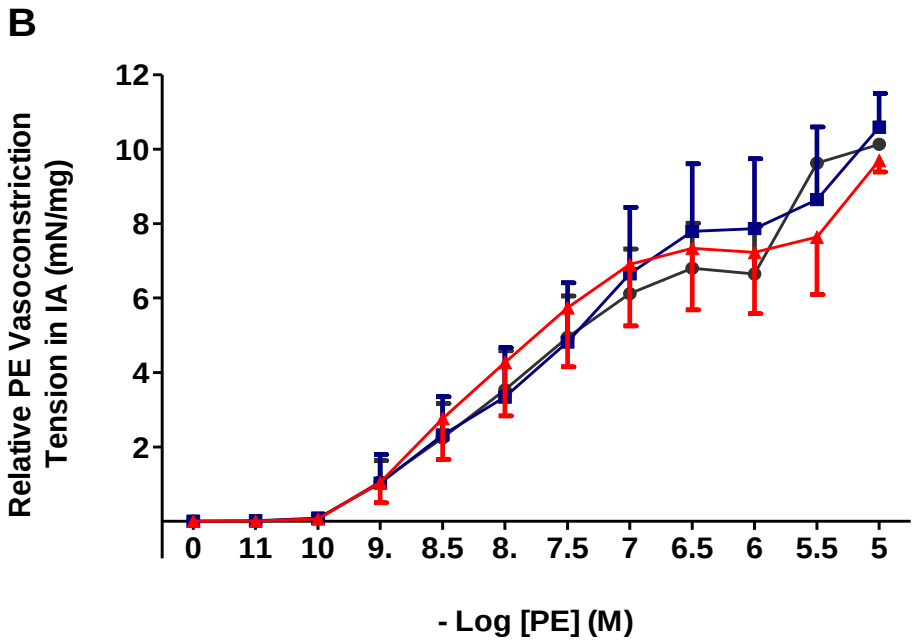

B
Relative PE Vasoconstriction Tension in IA (mN/mg)
12
10
8
6
4
2
0
11
10
9.
8.5
8.
7.5
7
6.5
6
5.5
5
- Log [PE] (M)

Figure 4.2: Vasoconstriction by phenylephrine prior and post TNF-α treatment in isolated rat iliac arteries. The vessels segments were equilibrated and normalised then allowed to be stabilised for 45 minutes. Vessel viability was tested with KCL (80mM) and a control cumulative dose-response curves (10-11 – 10-5M) were generated for phenylephrine. Then the vessels were incubated with or without TNF-a (1000 U/L) for 1 hour then cumulative dose-response curves (10-11 – 10-5M) were generated for phenylephrine response in IA ± TNF-a. Percentage maximum was calculated by taking response as percentage of the maximum PE response. The relative tension was generated by dividing individual PE tension by weight of the vessel.
A) Shows percentage maximum of the PE and
B) Shows ratio PE tension by weight of the vessel.
P > 0.05, n=6

4.3.2 Effects of TNF–α on thromboxane A2 agonist mediated vasoconstriction in isolated rat iliac arteries

Our data from iliac arteries isolated from healthy Wistar rats demonstrate that there is no significant difference in the maximum percentage of the thromboxane A_2 agonist mediated vasoconstriction by U46619. The average percentage of maximum vasoconstriction for control vessel minus TNF–α was 44.08±12.49 (Mean±SEM) and for TNF–α treated vessel was 42.49±12.44 (**Figure 4.3.2**, n=6).

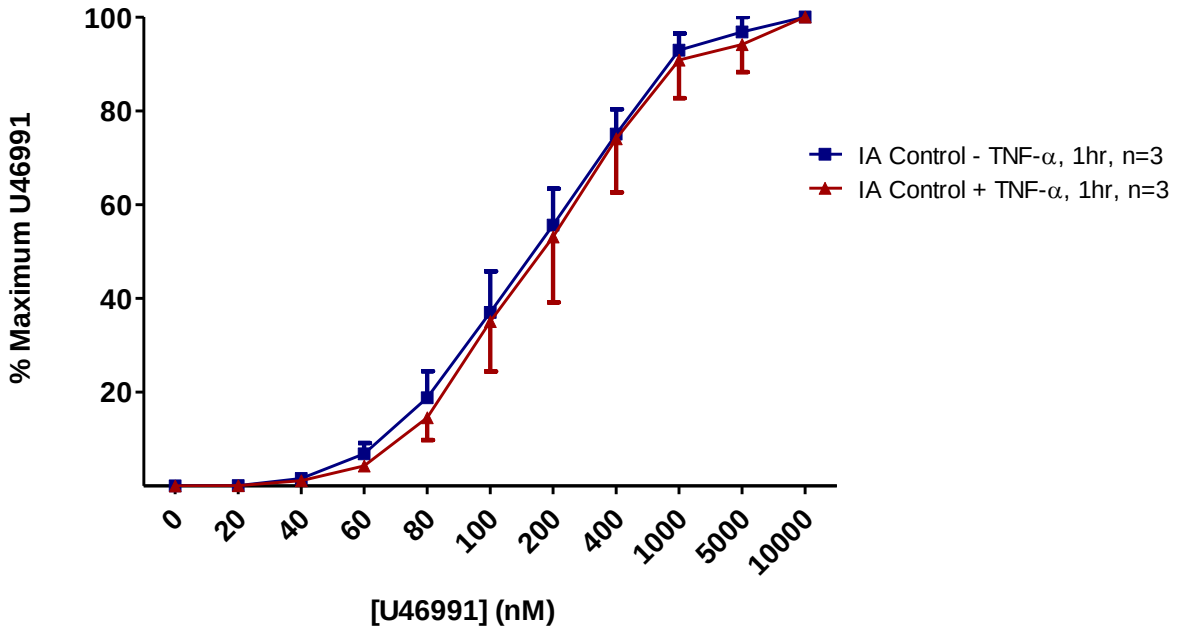


Figure 4.3: Vasoconstriction by thromboxane A2 agonist (U46619) ±TNF-α treatment in isolated rat iliac arteries. The vessels segments were equilibrated and normalised then allowed to be stabilised for 45 minutes. Vessel viability was tested with KCL (80mM) and the control cumulative dose-response curves (0 – 10mM) were generated for U46619. Then the vessels were incubated ± TNF-α (1000 U/L) for 1 hour then a cumulative dose-response curves (0 – 10mM) were repeated for U46619 ± TNF- α. Percentage maximum was calculated by taking each vasoconstrictor response as percentage of the highest U46619 dose response. **P > 0.05, n=3**

4.3.3 Effects of TNF–α on carbachol mediated vasodilatation in isolated rat iliac arteries

The cumulative dose response curve of CC demonstrated a typical vasodilator response. Control Prior to TNF–α treatment, control without TNF–α and showed a maximum vasodilation of 35.10±6.01%, 23.27±7.09%, 28.86±6.04% (Mean ±SEM) respectively **(Figure 4.3.3A)**. When the dose response curves were compared using one-way *ANOVA*, the data showed a significant difference between the TNF–α treated vessels Vs the quality control and time control (**Figure 4.3.3A,** *p value* <0.0001, n=7). The post hoc test (Bonferroni) did not indicate any significant difference between individual doses used. The results suggest that TNF–α increased endothelium-dependent vasodilation of pulmonary arteries isolated from healthy rats.

In contrast, there was no significant difference in the ratio of tension to the individual iliac artery weight [Tension/Weight; (mN)/(mg)] generated by endothelium-dependent vasodilator; CC, between the TNF–α treated (4.58±0.58), control prior to TNF–α (5.48±0.53) and Control–TNF–α (5.29±0.46) PA segments (**Figure 4.3.3B)**.

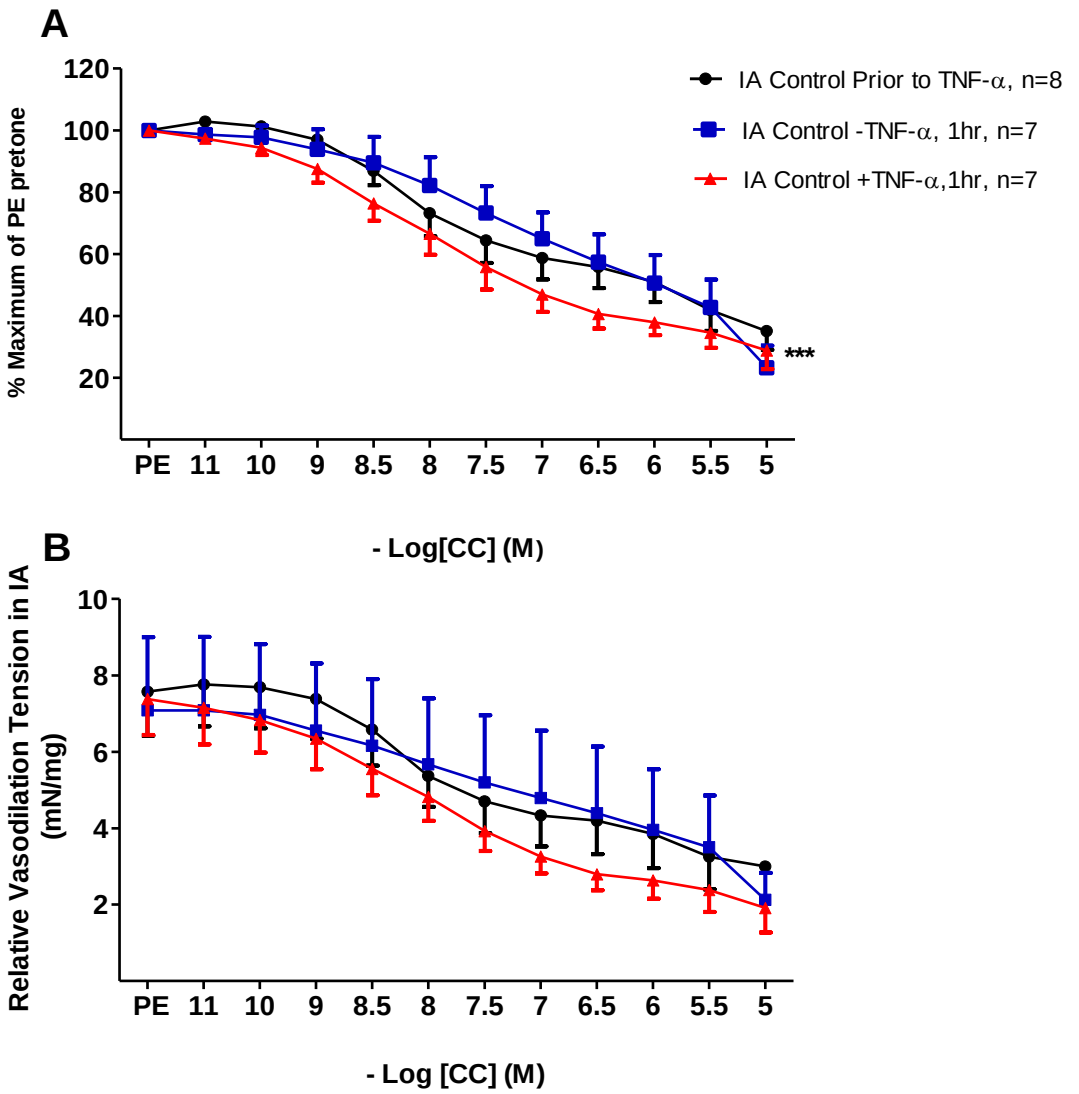


Figure 4.4: Shows a cumulative dose-response of vasodilatation mediated by Carbachol prior and post TNF-α treatment in isolated rat iliac arteries. The vessels segments were equilibrated and normalised then allowed to be stabilised for 45 minutes. Vessel viability was tested using KCL (80mM). Then phenylephrine (10^{-4}M PE) was used to generate a pretone followed by a control cumulative dose-response curves (10^{-11} – 10^{-5}M) for Carbachol. Then the vessels were incubated with ± TNF-α (1000 U/L) for 1 hour and subsequently a cumulative dose-response curves (10^{-11} – 10^{-5}M) were generated for Carbachol ± TNF-α.
A) Shows percentage maximum of the U46619 and
B) Shows ratio U46619 tension by weight of the vessel.
*** Vs Control Prior & Vs Control -TNF-a **p value< 0.0001, n=7**

4.3.4 Influence of chronic hypoxia ±TNF–α on carbachol mediated endothelium-dependent vasodilatation in isolated rat iliac arteries

This preliminary data on chronic hypoxia (CH) rats did not show any significant difference in the maximum percentage of the endothelial dependent vasodilation by CC (CH prior to TNF–α = 55.15±10.05%, CH**–**TNF–α=70.23±4.90% and CH**+**TNF–

α=62.43±2.82%; **Figure 4.3.4C:** p value >0.05, **n=3**). The statistic shows there was no significant effect for both chronic hypoxia and acute inflammation on systemic vascular endothelial-dependent relaxation.

The mean tension generated within this experiment did not differ much between the treated and control groups CH prior to TNF–α =6.25±0.46 (mN), CH**–**TNF–α =6.96±0.44 (mN) and CH**+**TNF–α =5.89±0.51 (mN) **Figure 4.3.4B)**

Similarly, CH ±TNF–α had no significant difference on the pretone generated by PE prior the cumulative dose-response of CC. The mean±SEM of PE generated were 9.23±0.59(mN), 9.27±1.26(mN), 8.27±0.58(mN) for CH prior to TNF–α, CH **–**TNF–α and CH **+**TNF–α respectively (**Figure 4.3.4A**)

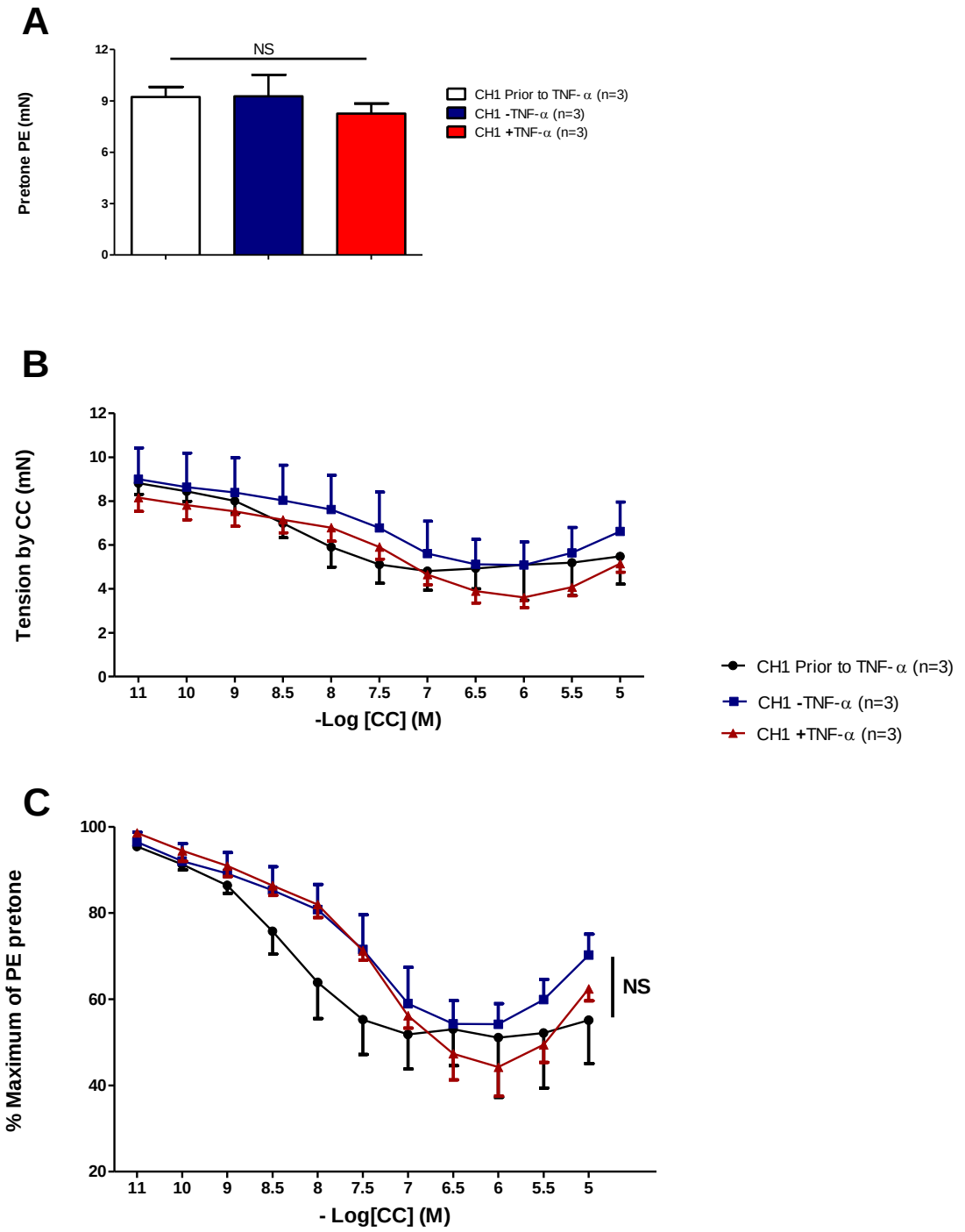


Figure 4.5: Shows influence of hypoxia ±TNF-α on iliac artery reactivity. Iliac arteries isolated from CH (1 week at 12% O_2) rats and the vessels subjected to TNF-α for 1 hour in vitro. This treatment had no significant effect on either constrictor or dilator response by PE and CC respectively. The increase at higher dose in C might be due the fact PE acting on the smooth muscle to cause contraction.
p value > 0.05 n=3

4.3.5 Influence of TNF–α and anti-oxidants on vasoreactivity and **stiffness of isolated rat iliac artery segments**

Iliac arteries isolated from healthy Wistar rats were initially subjected to incubation in DMEM ± TNF–α (1000U/L) **AND** ±Allopurinol (1mM) **OR** Tempol (1μM) for 6 hours. Then the vessels were mounted on myograph in a PSS solution (95% air, 5% CO_2, 37^{o}C) and the protocol completed as discussed. Our results has shown that there was no significant difference on the response if high K^+ (80mM) of the control iliac vessel segments versus TNF–α alone and versus TNF–α plus allopurinol OR tempol (**Figure 4.3.5**). In addition, neither TNF–α nor Allopurinol and Tempol had any significant effects on thromboxane A_2 mediated pretone generated prior to vasodilator treatment (**Figure 4.3.6**). In addition, TNF–α plus Allopurinol or Tempol treatment did not alter CC mediated vasodilatations in isolated iliac artery vessels (**Figure 4.3.7**). Contrastingly, the results have indicated that SNP mediated vasodilatations in isolated iliac artery vessels were significantly affected by TNF–α and the anti-oxidants (**Figure 4.3.8**).

From our data, we could deduce that incubation of iliac arteries with TNF–α (for 6 hours) significantly reduced the internal circumference at 90% distended. This effect seems to disappear in the iliac vessels were treated with allopurinol or tempol (**Figure 4.3.9**). However, treatment of iliac arteries with TNF–α plus allopurinol or tempol had not altered the iliac artery vessel diameter or circumference (**Figure 4.3.10**). Therefore, suggesting that TNF–α had not increase stiffness in systemic iliac vessel isolated from rats.

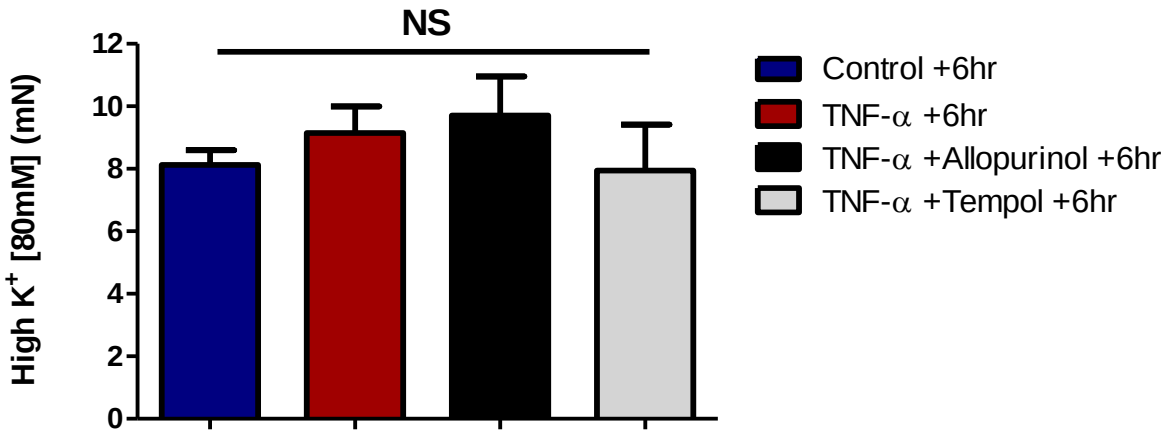


Figure 4.6: Shows the response of isolated iliac arteries to 80mM KCL (mN) Challenge. Isolated iliac artery vessels were incubated ± TNF-a (1000 U/l) AND/OR ± Allopurinol (1mM) or Tempol (1uM) for 6 hours.
P value > 0.05 , n = 6

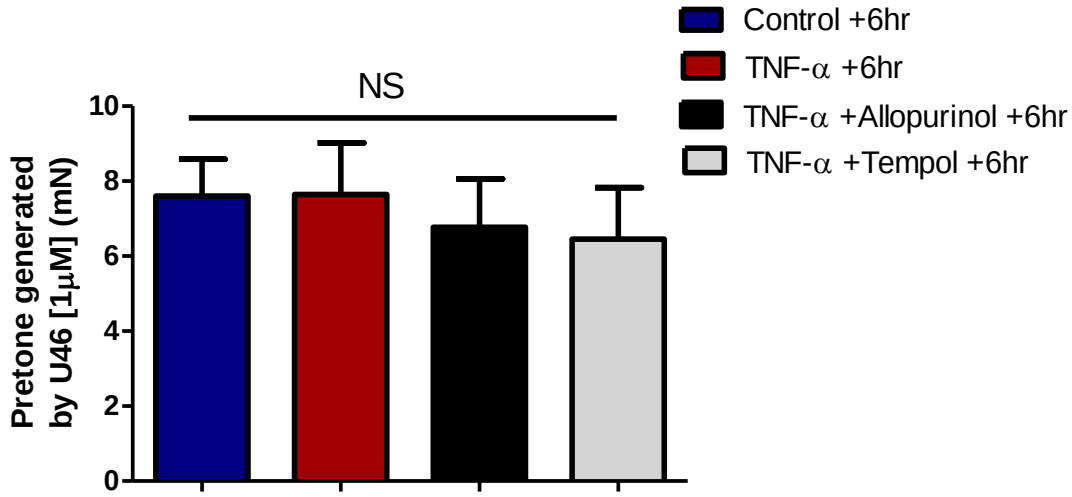


Figure 4.7: Vasoconstriction by U46619 post TNF-α treatment in isolated iliac arteries. The vessels segments were incubated ± TNF-a with/without Allopurinol (1mM) or Tempol (1uM) for 6 hours.
P > 0.05, n=6

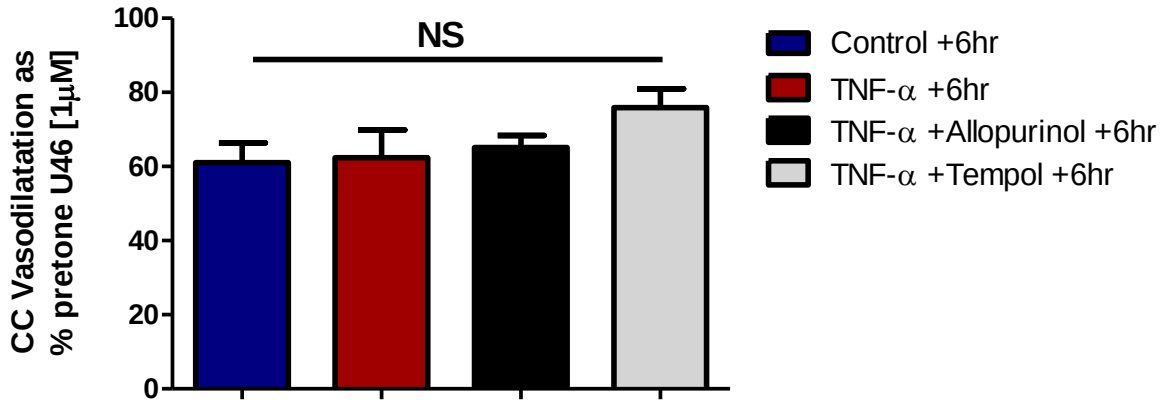


Figure 4.8: Show vasodilatation mediated by CC (1uM) after iliac arteries incubation ± TNF-α, AND/OR ± anti-oxidant for 6hrs. The bars represent conditions as follows: Blue bar indicates control response after 6hrs; Red bar represents TNF-α (1000U/l) response alone after 6hrs; Black bar represents TNF-α (1000U/l) + Allopurinol (1mM) after 6hrs; Light grey bar represent TNF-α (1000U/l) + Tempol (1μM) after 6hrs. **There was no significant difference between any of the categories, p value > 0.05, n = 6**

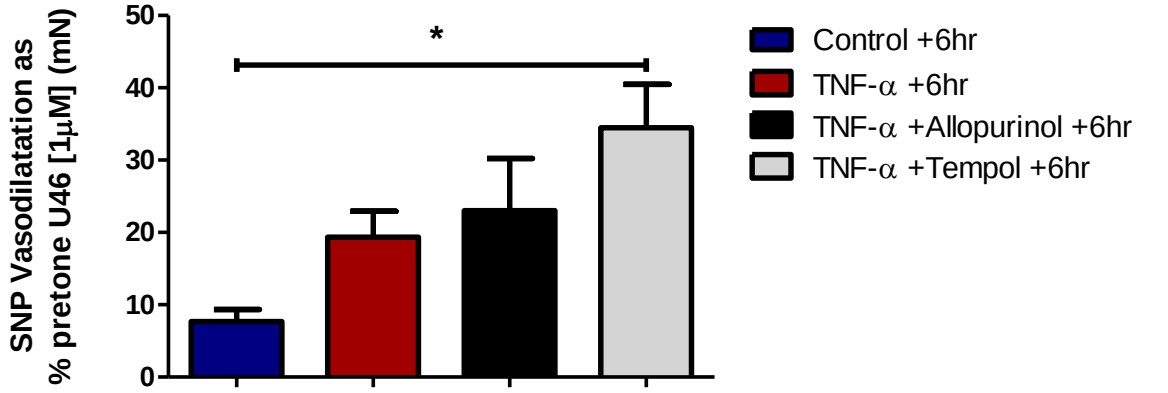


Figure 4.9: Show vasodilatation mediated by SNP [1uM] post iliac arteries incubation ± TNF-α, AND/OR ± anti-oxidant for 6hrs. The bars represent conditions as follows: Blue bar indicates control response after 6hrs; Red bar represents TNF-a (1000U/l) response alone after 6hrs; Black bar represents TNF- α (1000U/l) + Allopurinol (1mM) after 6hrs; Light grey bar represent TNF-α (1000U/l) + Tempol (1mM) after 6hrs.
*** Control Vs +TNF- α , TNF-α +Allopurinol and TNF-α +Tempol response, p value < 0.05; n = 6**

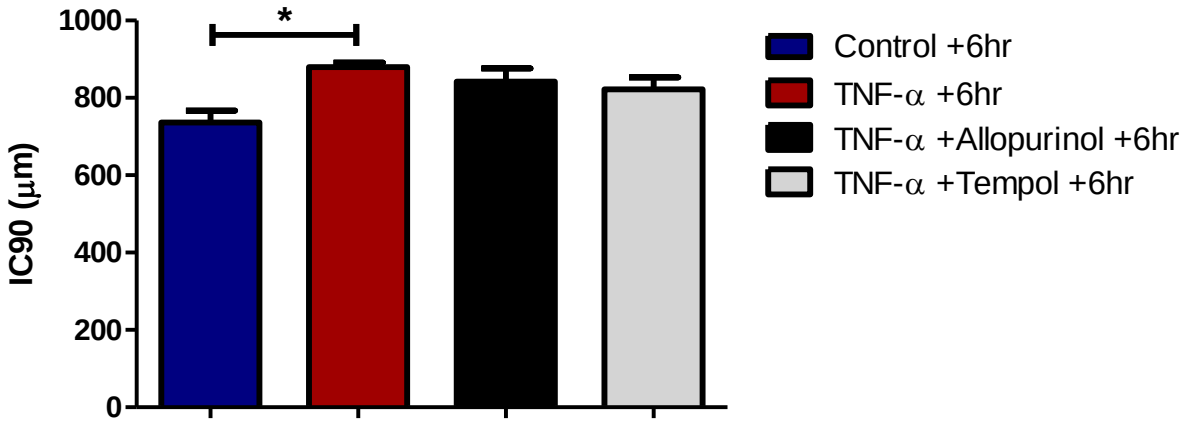


Figure 4.10: Show IC90 of iliac arteries post incubation ± TNF-α, AND/OR ± Allopurinol/Tempol for 6hrs. The bars represent conditions as follows: Blue bar indicates control response after 6hrs; Red bar represents TNF-a (1000U/l) response alone after 6hrs; Black bar represents TNF-α (1000U/l) + Allopurinol (1mM) after 6hrs and Light grey bar represents TNF-α (1000U/l) + Tempol (1mM) after 6hrs. IC90 was derived from the IC100 for each category which was then averaged to draw this graph. No significant difference between the control and TNF-α treated category, **p value < 0.05, n = 6.**
IC90= Internal Circumference of the vessel at 90 percent
IC100= Internal Circumference of the vessel at 100 percent

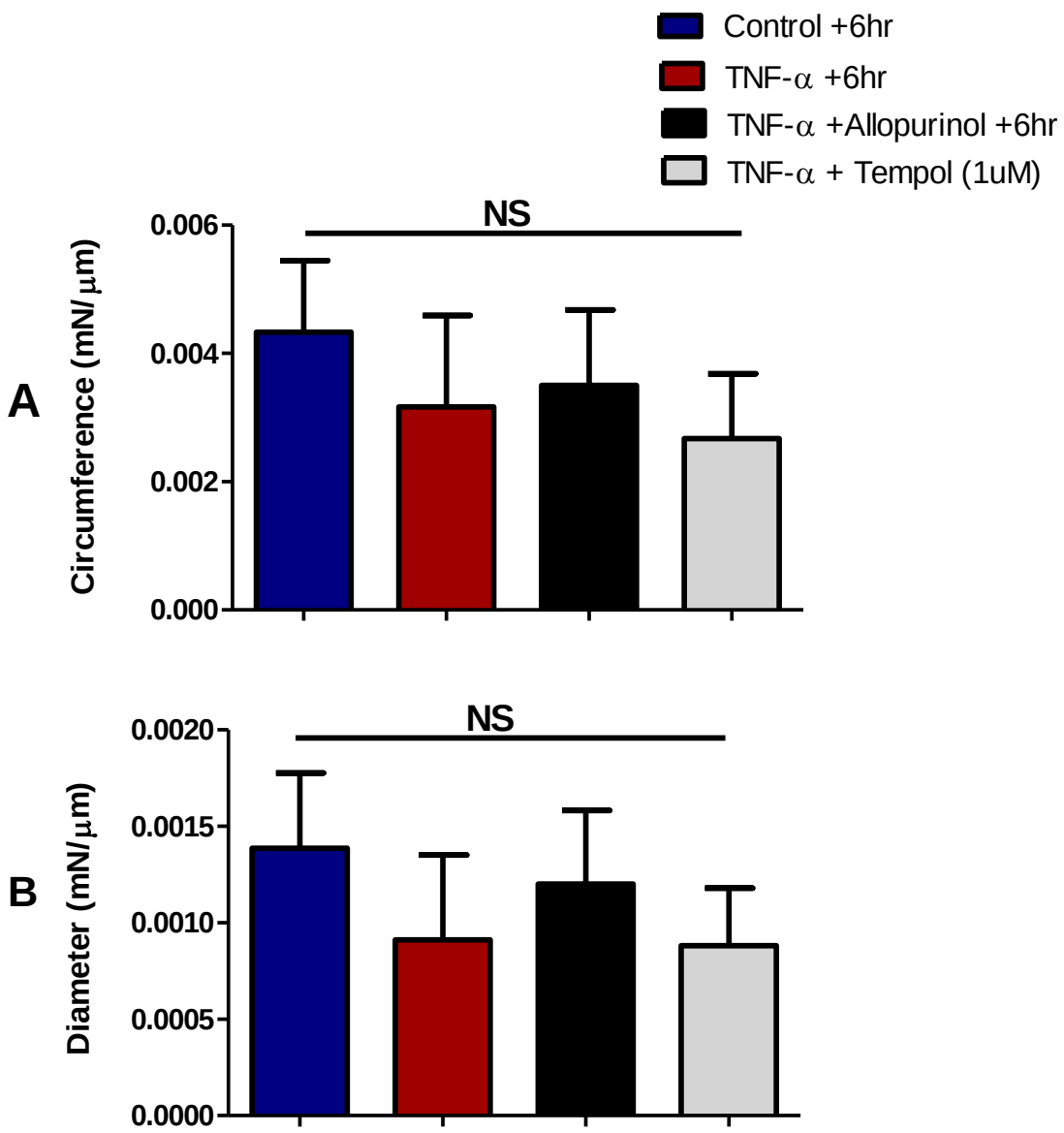


Figure 4.11: Shows circumference and diameter of Iliac arteries post incubation ± TNF-α, AND/OR ± Allopurinol/Tempol for 6hrs. The bars represent conditions as follows: Blue bar indicates control response after 6hrs; Red bar represents TNF-a (1000U/l) response alone after 6hrs; Black bar represents TNF-a (1000U/l) + Allopurinol (1mM) after 6hrs and Light grey bar represents TNF-a (1000U/l) + Tempol (1μM) after 6hrs. IC90 was derived from the IC100 for each treatment category, the normalisation values graphed and curve drawn.
The iliac artery (A) circumference and (B) Diameter were deduced from the equation. There was no significant difference between any of the treatment categories. p value > 0.05, n = 6.

4.4 Discussion

Contribution of cytokines to the instigation and development of vascular disease is widely documented in literature (125, 444-447). This has been extensively discussed in the thesis introduction, and to some extent in the introduction of this chapter. Elevated levels of inflammatory mediators such as cytokines (e.g IL–6 and TNF–α), cell adhesion molecules (e.g soluble ICAM-1) and downstream acute phase reactants (e.g CRP), were shown to be associated with increased risk of cardiovascular disease (65, 405, 409, 444, 447-450). TNF–α is a primary cytokine expressed in injured vasculature and plays multiple roles in regulating the production platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), fibroblast growth factor, adhesion molecules and other cytokines, and directly modulate vascular smooth muscle cell growth and migration. Also, a variety of cytokines such as IL–1β, IL–6 and TGF–β contribute to the pathogenesis of specific vascular disease (444).

4.4.1 Summary of the findings

a) **Influence of TNF–α on vasoconstrictor response on isolated iliac arteries**

   Both phenylephrine and U46619 mediated vasoconstriction was not altered by TNF–α treatment

b) **Influence of TNF–α on vasodilator response on isolated iliac arteries**

   TNF–α had significantly increased endothelium-dependent vasodilatation mediation by CC in iliac arteries. Hence, there was a significant difference between the CC-cumulative dose response curves of the TNF–α treated vessel compared to the untreated controls (**Figure 4.3.3**).

However, there was no significant difference in the ratio of the tension generated to weight of vessel (mN/mg) between the TNF–α treated vessel and the controls (**Figure 4.3.3**). This suggests that TNF–α had not affected the specific tension of the systemic vessel. However, measuring the exact weight of the tiny vessels was a struggled and values obtained were not consistent. Several attempted was made to resolve the matter but it persisted. Therefore, we ceased to undertake vessel weight measurement in the latter experiments due to inconsistency.

c) Effect of hypoxia & TNF–α on iliac artery reactivity of isolated iliac arteries

The combination treatment of rats with chronic hypoxia for one week then subject the isolated iliac vessel in vitro to TNF–α for an hour, had neither altered endothelium-dependent vasodilatation mediated by CC, nor did it affect PE pretone generated by isolated iliac arteries. Besides, chronic hypoxia and TNF–α did not affect the specific tension generated by CC.

d) Effect of TNF–α & anti-oxidants on vascular reactivity and stiffness on isolated iliac arteries

The IC90 was significantly altered by TNF–α which has not been reversed by neither allopurinol nor tempol treatment. However, the circumference and diameter of the isolated arteries were not significantly affected by TNF–α plus Allopurinol or Tempol treatment. Stiffness is a measure of the instantaneous slope of the pressure–volume relationship (ΔP/ΔV)(382). Due to lack of pressure myograph, we were unable to measure arterial stiffness directly. Therefore, we used manual methods to calculate the diameter and circumference of the iliac vessel both of which will have an

influence on the propagation of pressure and/or volume travelling along a vessel segment to provide us glimpse of stiffness.

Moreover, incubation of iliac arteries with TNF–α plus Allopurinol or Tempol had a significant effect on endothelium-independent vasodilatation (**Figure 4.3.8**). However, TNF–α plus Allopurinol or Tempol treatments had no significant effect on high K^+ and thromboxane A_2 agonist responses and endothelium-dependent vasodilatation.

4.4.2 Short term TNF – α on iliac arteries

In this study, a short term incubation of iliac artery segments in TNF–α for 1 hour had no significant effect on α-adrenergic mediated vasoconstriction by phenylephrine. Similarly, thromboxane A_2 agonist mediated vasoconstriction was not affected by acute TNF–α treatment in isolated rat iliac artery segments. Previous investigations have reported that inflammatory mediators were associated with vascular dysfunction (125, 164, 247), therefore, we expected that TNF–α treatment would at least increase the vasoconstriction of systemic iliac artery. It is possible that the short exposure period of cytokine treatment conducted was not sufficient to alter the mechanisms responsible for vascular contraction in this systemic vessel.

However, *in vivo* studies of human forearm resistance artery revealed that exposure to TNF–α for 1 hour has resulted into increased baseline vascular resistance due to elevation of baseline vasoconstrictor prostanoids bioavailability and diminished NO bioavailability (373). Moreover, exposing isolated rat aorta to IL–1β treatment for 1 hour had increased the vasoconstrictor activity of angiotension II via mechanisms that include prostaglandin H_2/TxA_2 (374).

Moreover, incubation of iliac artery pre-contracted with TNF–α for 1 hour caused a significant increase of endothelial dependent vasodilation mediated CC compared to controls. This has not been shown in iliac artery prior to this observation. The association between inflammatory mediators and vascular dysfunction has been widely recognised in the literature. This result confirms that TNF–α might reduce endothelial function in systemic iliac arteries isolated from Wistar rats. Similarly, Wimalasundera *et al.* (376) has demonstrated that in mesenteric arteries pre-contracted with PE, TNF–α caused impairment of acetylcholine mediated vasodilatation by significantly shifting the dose-response curve of acetylcholine to the right. In addition, TNF–α induced impairment of bradykinin mediated relaxation by significantly reducing the concentration-response curve (376). However, TNF–α did not affect endothelium-independent vasodilatation mediated by SNP (376). In this study we did not look at the SNP mediated vasodilation in iliac artery. However, our current results with the endothelium-dependent vasodilation agrees with Wimalasundera *et al's (376)* observation in systemic arteries. Again, the same group (376) showed that in mesenteric arteries pre-constricted with high K^+ (80mM), TNF–α significantly reduced vasodilatation of acetylcholine but not SNP (376).

Contrary to our findings, Gillham *et al* (451), showed that incubation of pre-constricted systemic omental arteries (isolated from healthy humans) with TNF–α (1nM) for 1 or 2 hours had not affected endothelium-dependent vasodilatation mediated by bradykinin. However, TNF–α had significantly reduced endothelium-dependent vasodilatation when NO/prostacyclin or endothelial-derived hyperpolarising factor were blocked with L-NNA and indomethacin incubation respectively (451).

4.4.3 Treatment with chronic hypoxia (1 week) with short term TNF–α (in vitro)

Healthy Wistar rats were exposed to chronic hypoxia (12% O_2 for 1 week, in normobaric chamber). The systemic iliac vessels isolated from rats were then subjected to TNF–α treatment for 1 hour *in vitro*. TNF–α plus hypoxia exposure did not affect high K^+ and vasoconstrictor response in iliac artery, suggesting that CH had not augmented the cytokine response**.** The combined hypoxia and TNF–α treatment has not been done before in a systemic vasculature such as iliac artery. Chronic hypoxia (12% O_2 for 1 week) with or without TNF–α (1hour) had not altered α–adrenergic mediated pretone by phenylephrine. This suggests that chronic hypoxia and acute inflammatory challenge do not influence vasoconstriction in isolated iliac artery from rats. There too much variability within the data probably due low power of the experiment, hence this was just a preliminary data.

Furthermore, chronic hypoxia and acute inflammatory challenge had not affected endothelium-dependent vasodilatation by CC in iliac arteries isolated from healthy Wistar rats. Previous publications on the subject of exposure of systemic vasculature to chronic hypoxia generated conflicting outcomes (439). For instance, exposure to hypoxia resulted in an elevation of endothelial nitric oxide synthase (eNOS) and increased NO-dependent vasodilatory effects in sheep uterine arteries(440) and fetal guinea pig hearts (439, 441). However, exposure to a prolonged hypoxia caused a reduction of eNOS expression and NO-dependent vasodilatation in rat aorta (442). Similarly, chronic hypoxia has induced increase contractility of the middle cerebral arteries due to reduced generation of NO in guinea pigs (452). These varying observations reveal the distinct nature of different vascular beds or variation between conduit and resistance vessels. Gonzales *et al (453)* has suggested that following chronic hypoxia exposure, resistance mesenteric arteries exhibited blunted vascular

reactivity similar to those demonstrated by intact animals. The variability of our chronic hypoxia plus or minus acute inflammatory challenge data might be due to limited number of animal used for the experiments. Contrary to earlier thought, we found that hypoxia and inflammation have no interaction to induce vascular endothelium.

Furthermore, Pro-inflammatory cytokines have the potential to exert a rapid vasoactive influence on the blood vessels possibly via the receptors found on the endothelium and the smooth muscle cells of the vessel (372). Interestingly, Inversen *et al*.(454) and Van der Poll and lowry *et al*. (455), have demonstrated the presence of receptors for TNF–α and IL–1β on the endothelium and the smooth muscle cells. Moreover, it has been shown that increased concentrations of TNF–α, IL–6 and IL–10 stimulated vasoconstriction in distal human internal mammary arteries (454). The vasoconstrictor response was shown to be mediated by endothelin-A (ET_A), thus it might be endothelium dependent. Contrary to this finding, TNF–α and/or IL–1β had no effect on pre-contracted resistance arteries isolated from human (456). In rat skeletal muscle arterioles incubated with IL–1β and IL–6 for 1 hour resulted in generation of strong vasodilator response *in vivo* although it did not produce the same effect *in vitro* (457, 458).

4.4.4 Prolong exposure to TNF–α on iliac arteries

Additionally, our findings discovered that incubation of isolated rat iliac arteries in DMEM with or without TNF–α AND/OR allopurinol/tempol for 6 hours had not altered high K^+ and thromboxane A2 agonist response in iliac artery. In addition, the long-term incubation of iliac arteries with TNF–α plus allopurinol OR tempol had not caused any significant alteration of the endothelium-dependent vasodilatation by CC. However, TNF–α seemed to affect endothelium-independent vasodilatation mediated

by SNP and the effect was not affected by neither allopurinol nor tempol. Contrastingly, Jiménez-Altayó *et al* (435) reported that long-term incubation of mesenteric arteries with IL–1β significantly blunted both endothelium-dependent and endothelium-independent relaxation mediated by acetylcholine (Ach) and sodium nitroprusside (SNP) respectively. In agreement to our data, we have both observed impairment of endothelium-independent vasodilatation by SNP. The group also observed increased production of superoxide anion ($O2^{-\cdot}$), nitrite, inducible nitric-oxide synthase (iNOS) and xanthine oxidase, all of which are known to contribute to vascular dysfunction (435). Interestingly, IL–1β treatment of mesenteric arteries had not altered expression levels of NOS and soluble guanylate cyclase proteins (435). These varying observations might be due divergent properties between conduit and resistance arteries or the distinctive features of some vascular beds (439).

Arterial stiffness has been long considered a significant measure of pulse pressure and recently it has been recognised as a determinant of several cardiovascular diseases (381). Several epidemiological investigations of morbidity and mortality with cardiovascular diseases have led to the emergence of arterial stiffness as an independent predictor of cardiovascular risk (381). In this chapter, we had deduced arterial stiffness measure from the internal circumference at 90 percent of optimum tension of individual vessels from the step-by-step micrometre tension increment to derive diameter and circumference of the vessel (protocol explained in **2.5.10**). Our results have shown that neither TNF–α nor Allopurinol or Tempol had any significant effect on IC90, diameter and circumference; hence the stiffness was not altered in these setting. Contrary to our observation, previous studies has suggested a link between decreased endothelial-dependent production of NO and the elevation of arterial stiffness *in vivo* in the iliac artery of humans and sheep (381, 459, 460).

Similarly, McEniery *et al* (461) has shown endothelin-1 caused increased stiffness in common iliac artery. However, this effect was abolished when endothelin-A receptor was blocked. The group measured PWV by using the foot-to-foot methodology to measure two pressure waveforms simultaneously recorded with a high-fidelity, dual pressure-sensing catheter placed in the common iliac artery (461).

Among the original aim of the present study was, first, to determine the effect of short-term and long-term incubation with TNF–α on endothelium-dependent relaxation of rat iliac arteries and, second, to analyse the mechanisms involved in the effect of TNF–α thoroughly. However, due to predominantly negative data achieved, the exact mechanisms underlying the impaired vascular activity in systemic arteries remain unresolved and there is no current scientific consensus (125). Therefore, further investigation is required to explore the possible role of TNF–α or cytokine mediated effects on the systemic vasculature.

Chapter 5

5 EFFECTS OF INFLAMMATION ON VASCULAR REACTIVITY IN ISOLATED PULMONARY ARTERIES: A MODEL FOR VASCULAR DISEASES ASSOCIATED WITH COPD?

5.1 Introduction

Endothelial dysfunction and inflammation is associated with cardiovascular complications in several disease conditions including COPD (52, 372, 435). COPD is characterised by chronic airway inflammation, which represents a key component of disease development and pathogenesis (3). Studies of bronchoalveolar lavage have reported elevated inflammatory cells, such as neutrophils, in sputum from COPD patients compared with controls (52). Keatings *et al* (52) also found significant levels of inflammatory cytokines such as interleukin–8 (IL–8) and tumour necrosis factor–alpha (TNF–α) in sputum from COPD patients compared with healthy smokers and non-smoking controls, suggesting these are implicated in COPD (52). Numerous other studies have confirmed these findings (6, 53-58), although significant day to day variability occurs (58).

Furthermore, the relationship of pulmonary to systemic inflammation is not yet clear. Several studies have reported elevation of various inflammatory markers such as TNF–α, IL–8, IL–6 and CRP in the serum (59-61) of patients with stable COPD suggesting that inflammation is not confined to the lungs. The level of inflammation might be aggravated during exacerbation events in COPD patients thereby promoting cardiovascular events (62). This suggests that systemic inflammation promotes advancement of cardiovascular diseases(63). In COPD this is thought to occur by various mechanisms including endothelial dysfunction (64), arterial stiffness (65) and increased platelet activation (66).

Previous animal studies have established that inflammatory mediators and hypoxia promote endothelial damage causing an imbalance between vasodilator and vasoconstrictor intermediaries, thus altering vascular tone (462). These lead to increased smooth muscle cell proliferation and vasoconstriction as well as vascular remodeling and subsequently result in an increased pulmonary arterial pressure[1, 2]. Progressively, cardiovascular complications occur including pulmonary hypertension associated with chronic obstructive pulmonary disease (**Figure 5.1)**.

IL–8 is also known as CXCL8 is a chemoattractant inflammatory cytokine mainly expressed by macrophage, endothelial cells, monocytes and T cells possibly via NF-κB activity. IL–8 acts through its receptors (IL–8RA & IL–8RB) present on neutrophils, T cells and monocytes to induce pro-inflammatory activity, chemotaxis, leucocyte arrest and atherogenic activities (444). The first line of the body defence mechanism during tissue injury is neutrophil. Infected or injured tissue trigger increased expression of IL–8, which then stimulate neutrophils to travel to the injured site. IL–8 targets selective IL–8RA and IL–8RB receptors located on the surface of neutrophils that cause activation of the receptors leading secretion of variety of chemotactic mediators and subsequent local inflammation (463). Therefore, neutrophils and IL–8 play a critical role in the initial pro-inflammatory response following endothelial damage, which promote the invasion of other inflammatory cells such as monocytes, macrophages and T cells in the setting of acute vascular injury (463).

The exact mechanism for the involvement of pro-inflammatory cytokines such as TNF–α, IL–1β and IL–8 and other that were found to be elevated in COPD patient in the pathogenesis of cardiovascular disease is unknown. However, in systemic vessels, it is suggested that TNF-α acts through inhibition of enzymatic activity of

eNOS thereby minimising NO production and promote removal of NO by increasing NADPH-dependent superoxide production to react with NO to form peroxynitrite (125). As a result, TNF–α decreases the bioavailability of NO that could then reduce vasodilatation of the vascular smooth muscle cell (125). Thereby causing imbalance of between vasoconstrictors and vasodilator that potentially could be damaging to the endothelium. This will mostly tilt the favour towards increased vasoconstrictor activities, which if prolonged could result in increased blood pressure and subsequently hypertension.

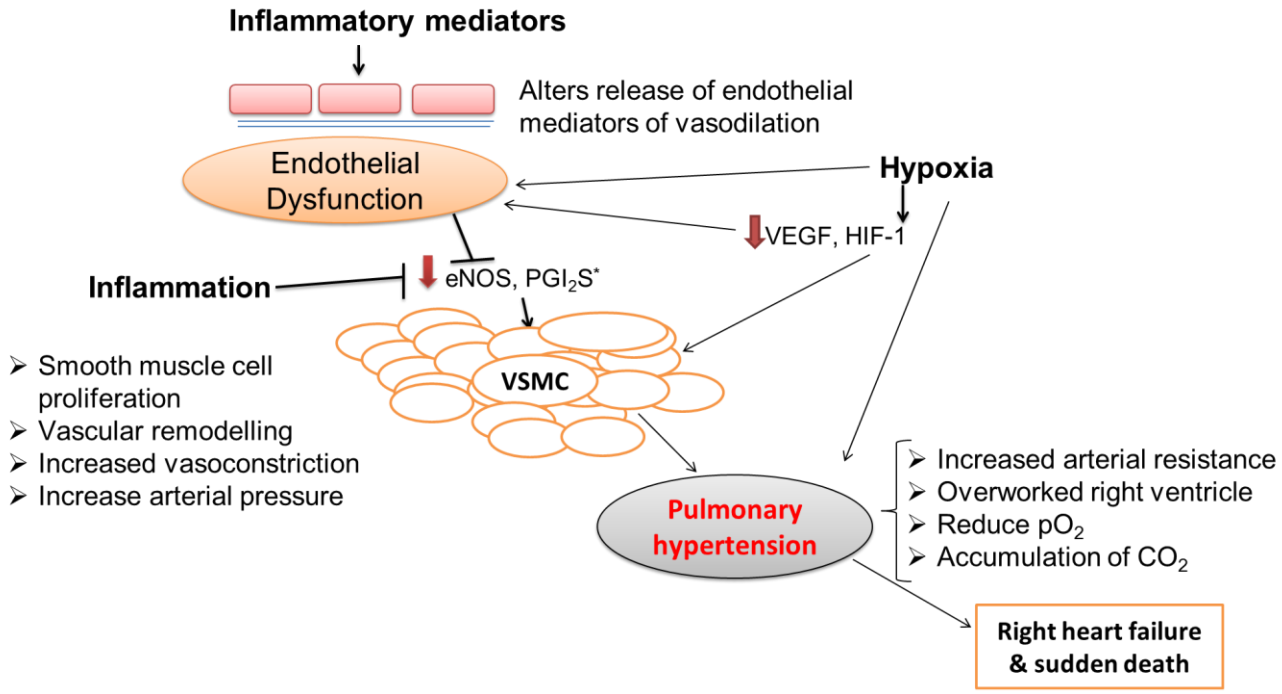


Figure 5.1*: Shows diagrammatic representation of the mechanism of inflammation on vascular diseases.* Cigarette smoke components or inflammation damages endothelial cells and causes endothelial dysfunction. This results in imbalance between vasoactive and growth factors that promote proliferation of SMC. Hypoxia, inflammation, and shear stress amplify and perpetuate these effects, further contributing to the development of pulmonary hypertension. Inflammatory mediators promote vascular endothelial damage which alters vascular tone. (Adapted and modified from (429).

eNOS= endothelial-derived nitric oxide,
PGIS= prostaglandin I_2 Synthase
HIF= Hypoxia-induced factor 1
VEGF= Vascular endothelial growth factor

5.1.1 The aims of the investigations in this chapter are:

1) To investigate effects of TNF–α on phenylephrine mediated vasoconstriction in PA
2) To investigate effect of TNF–α and IL–8 on thromboxane A2 agonist, U46619 mediated vasoconstriction in PA
3) To investigate effects of TNF–α and IL–8 on CC and SNP mediated vasodilatation in PA

We hypothesised that TNF–α and IL–8 contributes to pulmonary vascular endothelial dysfunction which impairs mechanisms that effect vasodilatation.

5.2 Method

.In these set of experiments, Wistar rats (as described in 2.5.2) were euthanized by overdose of anaesthesia with cervical dislocation. Pulmonary artery vessels were isolated cleared from any adventive tissue and then cut into small segment (~2mm in length). The myograph was normalised and vessels were allowed to stabilise for about three quarter of an hour (2.5.6).

A cumulative dose response of a vasoconstrictor phenylephrine (PE) or U46691 (U46) or a pretone generated by PE or U46 (EC_{80}) followed by a cumulative dose response of a vasodilator CC, SNP. Then a pro-inflammatory cytokine TNF-α or IL-8 or IL-1β (1000U/ml, 50ng/ml, 20 or 50ng/ml respectively) added for 1 hour and vasoconstrictor or dilator response re-assessed.

5.2.1 Effect of TNF-α on Phenylephrine on vasoconstriction of rat pulmonary artery

In the following experiments, pulmonary arteries from healthy Wistar rats were exposed to phenylephrine (PE) with or without TNF-α to investigate the effects of inflammation on vasoconstriction. The results are presented as a percentage of

maximum PE response (%Max of PE) to determine sensitivity and relative tension to compare potency of specific tension (mN tension/mg vessel wet weight). We have compared three conditions;

a) PE response prior to TNF-α treatment (Control prior to TNF–α),
b) PE response after TNF-α treatment, without TNF-α for 1 hour (**–**TNF–α, Time Control)
c) PE response after TNF-α treatment, with TNF-α for 1 hour (**+**TNF–α, treated)

The vessels were subjected to an increasing concentration of PE until the dose-response curve plateau. The tension generated (mN) was used to calculate percentage maximum (% Max) by dividing the each tension by the maximum tension (after subtracting baseline values) and multiplying by 100. The percentage maximum of PE was plotted against individual PE concentration (expressed as –[Log]M) and specific tension (mN/mg) was used to compare drug potency. As discussed in the previous chapter (4.2.1), we achieved inconsistent data with vessel weighing which remained unresolved. Therefore we only completed this protocol for only the early studies conducted and later ceased weight the vessels due to inconsistency.

5.2.2 Effects of TNF–α on U46619–mediated Vasoconstriction of Rat Pulmonary Artery

The pulmonary vessels were subjected to an increasing dose concentration of U46619 (20–1000nM) until the dose-response curve plateau. The tension generated (mN) were used to calculate percentage maximum (% Max) by dividing the each tension by the maximum tension (after subtracting baseline values) and multiplying by 100. The percentage maximum of U46619 was plotted against individual U46619 dose concentration in the myograph chamber. (see results in **section 5.3.2)**.

5.2.3 Effects of TNF–α on Vasodilatation Mediated by Carbachol in Rat PA

In the following experiments, isolated rat PA vessels were exposed to cumulative doses of carbachol (CC; a stable analogue of acetylcholine) to investigate their vasodilatation and effect of TNF–α on the response. Subsequent to inducing vasoconstriction by PE [at EC80 = 300nM ($3*10^{-7}$m)], the vessels were treated with increasing CC doses. **Figure 5.3.3**, represents vasodilator response ±TNF–α. After normalising for baseline, the tension generated by individual doses of CC was divided by the maximum tension generated by PE pretone and multiplied by 100 to calculate percentage maximum response (%Max) then plotted the %Max against CC concentration (-[Log]M). See section **5.3.3** for the results. The EC_{80} quoted above was the PE pretone used for all isolated PA vessels throughout this thesis.

5.2.4 Effects of IL–8 on vasodilatation mediated by Carbachol in rat PA

Result from this section, isolated rat PA vessels were exposed to cumulative doses of carbachol (CC; a stable analogue of acetylcholine) to investigate their vasodilatation and effect of interleukin–8 (IL–8) on the response. Subsequent to inducing pre-contraction with U46619 (400nM), the vessels were treated with increasing CC doses. We used U46619 instead of PE as the latter was shown to generate inconsistent results as described in chapter 2 (section 2.5.7). Figure **5.3.4**, represents vasodilator response ±IL–8. After normalising for baseline, the tension generated by individual doses of CC was divided by the maximum tension generated by U46619 and multiplied by 100 to calculate percentage maximum response (%Max) then plotted as %Max versus Log of CC concentrations (-[Log]M). See **section 5.3.4** for the results.

5.2.5 Effects of IL–8 on vasodilatation mediated by SNP in rat PA

In this section, isolated rat PA vessels were exposed to cumulative doses of Sodium Nitroprusside (SNP) to investigate influence of interleukin–8 (IL–8) on endothelium-independent vasodilatation of pulmonary arteries. In PA vessels, pretone was generated with U46619 (400nM) which was followed by cumulative dose of SNP addition to generate a dose dependent curve. **Figure 5.3.5**, represents vasodilator response ±IL–8. After normalising for baseline, the tension generated by individual doses of CC was divided by the maximum tension generated by U46619 and multiplied by 100 to calculate percentage maximum response (%Max) then plotted as %Max against CC concentration (-[Log]M).

5.2.6 Effects of 2 or 6 hours TNF-α treatment on pulmonary artery vascular responses

In the following experiments, isolated rat PA vessels were first incubated in DMEM medium with or without TNF–α for 2 or 6 hours. The vessels were then mounted on the myograph. The TNF–α treated and untreated pulmonary vessel segments in the myograph were then subjected to high K^+ (80mM) to check pulmonary vessels viability. This was followed with pretone generation by U46619 (400nM) that was then exposed to cumulative doses of either CC or SNP to effect relaxation. This was done to investigate the short-term and prolonged effects of TNF–α on vasodilatation response as well as on KCL and pretone (U46619) responses. The results for short-term and long-term exposure to TNF–α treatment are shown in sections **5.3.6** and **5.3.7** respectively.

5.2.7 Influence of anti-oxidant on TNF–α mediated reduction of relaxation in PA

In these set of experiments, isolated rat PA vessels were first incubated in DMEM medium in four catergories: Control, Control+TNF–α, TNF–α+allopurinol and TNF–

α+tempol, in a normoxic chamber (95% O_2, 5% CO_2, 37^0C) for 6 hours. The vessels were then mounted on the myograph and normalised (PSS, 95% air, 5% CO_2, 37^0C). The TNF–α treated and untreated pulmonary vessels in the myograph were then subjected to KCL (80mM) to check viability of the vessels. This was followed with pretone generation by U46619 (400nM) that was then exposed to cumulative doses CC or SNP mediated relaxation. The percentage maximum of CC or SNP vasodilatation was calculated for the control, control+TNF–α, TNF–α+allopurinol and TNF–α+tempol and their cumulative dose response curve were constructed. This was done to investigate influence of antioxidants on TNF–α mediated inhibition of CC or SNP–mediated vasodilatation.

5.2.8 Statistic

GraphPad (Prism 5) was used for analysis and plotting graphs. KaleidaGraph and Prism 5 were used to calculate EC50 of agonists. We used two-way ANOVA or One-way ANOVA depending on the number of the groups to be compared. At times, t-test (unpaired) has been used. The statistical tests were followed by Bonferroni's multiple comparison post hoc tests. Significance was taken at p value ≤ 0.05.

5.3 Results

5.3.1 Effect of TNF-α on Phenylephrine on vasoconstriction of rat pulmonary artery

PAs were activated at lower PE doses and continue to increase gradually until it reached maximum at -[Log] M=7.5; equivalent to 30nM, at where the dose-response curves began to decline. There was no significant different in the dose-response curve of PE between the TNF-α treated and untreated controls (**Figure 5.3.1**, p> 0.05). From **Figure 5.3.1**, we have shown that there was a significant difference in

the relative tension of PE–mediated vasoconstriction between TNF–α treated and untreated control (*p value* < *0.05*). Though there was a significant difference in the relative tension (tension due to agonist/weight of vessel (mN/mg)) of PE–mediated vasoconstriction, we have not included the results as the data appear to be inconsistent due to difficulty measuring the weight of tiny pulmonary artery segments.

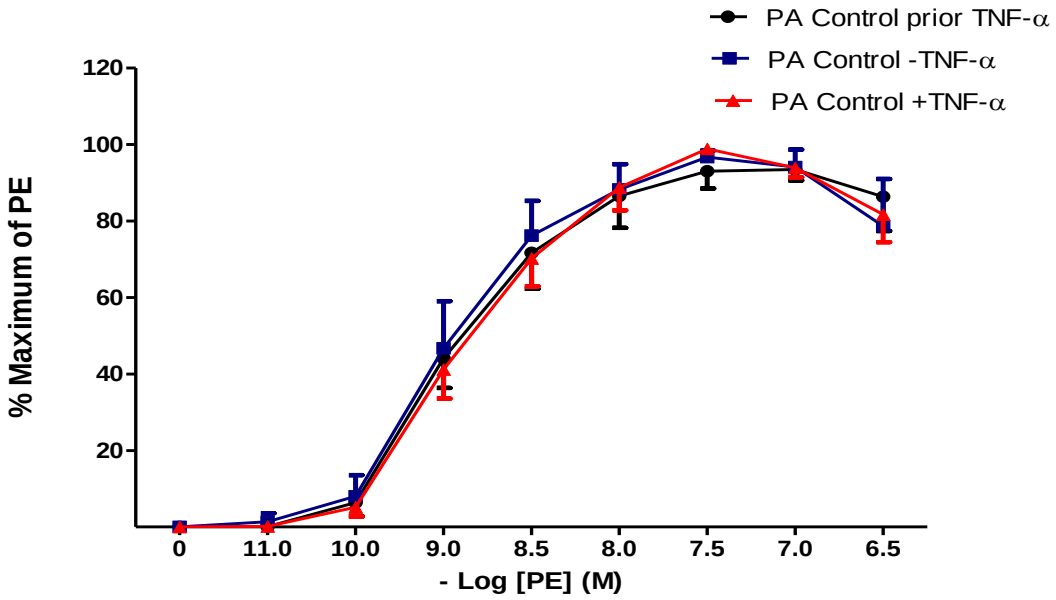


Figure 5.2 : Vasoconstriction by phenylephrine (PE) prior and post TNF-α treatment in isolated rat pulmonary arteries. The vessels segments were equilibrated and normalised then allowed to be stabilised for 45 minutes. Vessel viability was tested with high K^+ (80mM) and the control cumulative dose-response curves (10^{-11} – 10^{-5}M) were generated for PE. Then the vessels were incubated with or without TNF-α (1000 U/L) for 1 hour and then cumulative dose-response curves (10^{-11} – 10^{-5}M) for PE response repeated in PA ± TNF-α. Percentage maximum was calculated by taking each PE dose response as percentage of the maximum PE response. Higher dose of PE might be acting on cause relaxation in PA. p > 0.05, n=6.

5.3.2 Effect of TNF-α on U46619 vasoconstriction of rat pulmonary artery

U46619 activated vasoconstriction of pulmonary arteries at lower doses and continued to increase with increasing of the dose until the response plateau at 1000nM (of U46). Our findings have shown that in all vessels regardless of the

treatment conditions, responded similarly to the U46619 exposure. Hence, there was no significant different in the dose-response curve of U46619 between the TNF–α treated and untreated control pulmonary arteries as well as between control PA prior to TNF–α treatment and control PA without TNF–α (**Figure 5.3.2** *p value > 0.05*).

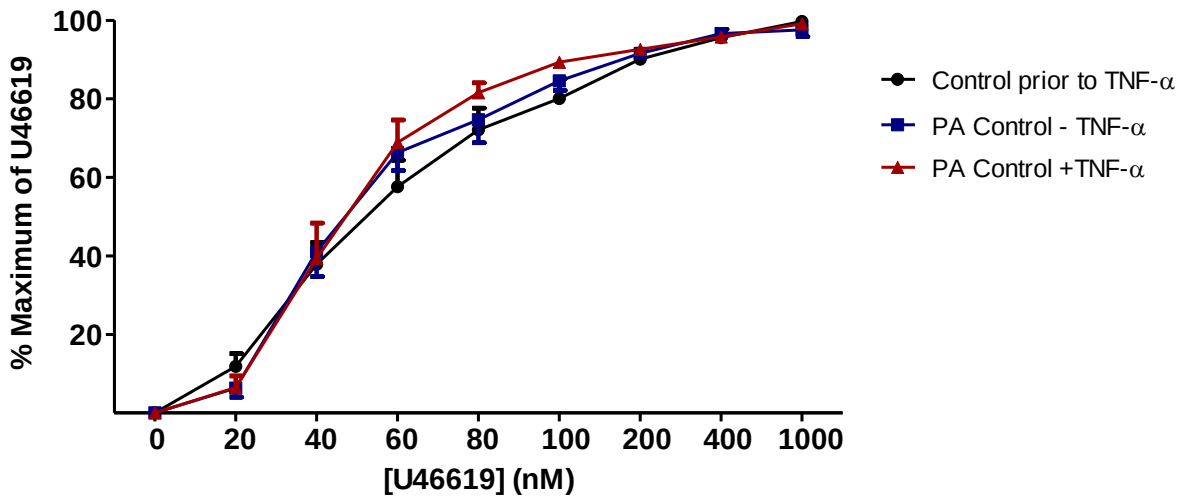


Figure 5.3: Vasoconstriction by thromboxane A2 agonist (U46619) prior and post TNF-α treatment in isolated rat pulmonary arteries. The vessels segments were equilibrated and normalised then allowed to be stabilised for 45 minutes. Vessel viability was tested with KCL (80mM) and the control cumulative dose-response curves (20 -1000nM) were generated for U46619. Then the vessels were incubated ± TNF-α (1000 U/L) for 1 hour then a cumulative dose-response curves (20 -1000nM) were repeated for U46619 ± TNF-a. Percentage maximum was calculated by taking response as percentage of the maximum U46619 response. The relative tension was generated by dividing individual U46619 tension by weight of the vessel segment. p value > 0.05, n =6

5.3.3 Effects of TNF-α on vasodilatation mediated by Carbachol in rat PA

As cumulative CC concentrations mediated vasodilatation of PA vessels from all the conditions as they all demonstrated graded relaxation. At the highest dose applied they were both reduced by more than 40%. There was a significance difference in CC–mediated vasodilatation in PA between the control prior to TNF–α addition,

compared to time control without TNF–α (p value < 0.05), as well between the control prior to TNF–α treatment and TNF–α treated (p value < 0.05). However, there was no significant difference between the TNF–α treated and time control without TNF–α (*p value* > 0.05) (**Fig. 5.3.3**).

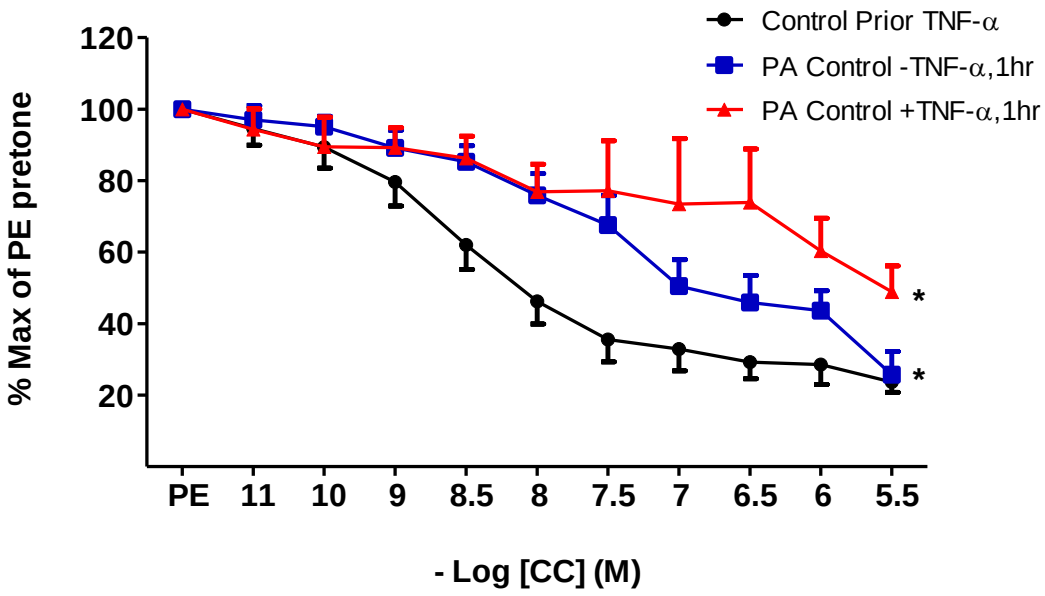


Figure 5.4: Shows a cumulative dose-response of vasodilatation mediated by Carbachol prior and post TNF-α treatment in isolated rat pulmonary arteries. The vessels segments were equilibrated and normalised then allowed to be stabilised for 45 minutes. Vessel viability was tested with high K^+ (80mM). Then phenylephrine ($3*10^{-7}$M equivalent to 300nM) was used to generate a pretone followed by a control cumulative dose-response curves (10^{-11} – 10^{-5}M) for Carbachol. Then the vessels were incubated ± TNF-α (1000 U/L) for 1 hour and subsequently cumulative dose-response curves (10^{-11} – 10^{-5}M) were repeated for Carbachol ±TNF-α.

*** Control Prior TNF-α Vs Time control and TNF-α treated, p < 0.05, n=7**

TNF-α treated Vs Time control, p > 0.05, n=7

5.3.4 Effects of IL –8 on vasodilatation mediated by Carbachol in rat PA

As cumulative CC concentrations mediated vasodilatation of PA vessels from both the IL–8 treated and untreated conditions as they both induced gradual vasoconstriction. However, the vessels were not greatly relaxed even at highest dose applied, both relaxed by just about 30%. IL–8 had no significant effect on the 46991 pretone and KCL responses. Our results showed that there was a significance

difference in CC–mediated vasodilatation in PA between the IL–8 treated compared to control without IL–8 (p value < 0.05) (**Fig. 5.3.4**).

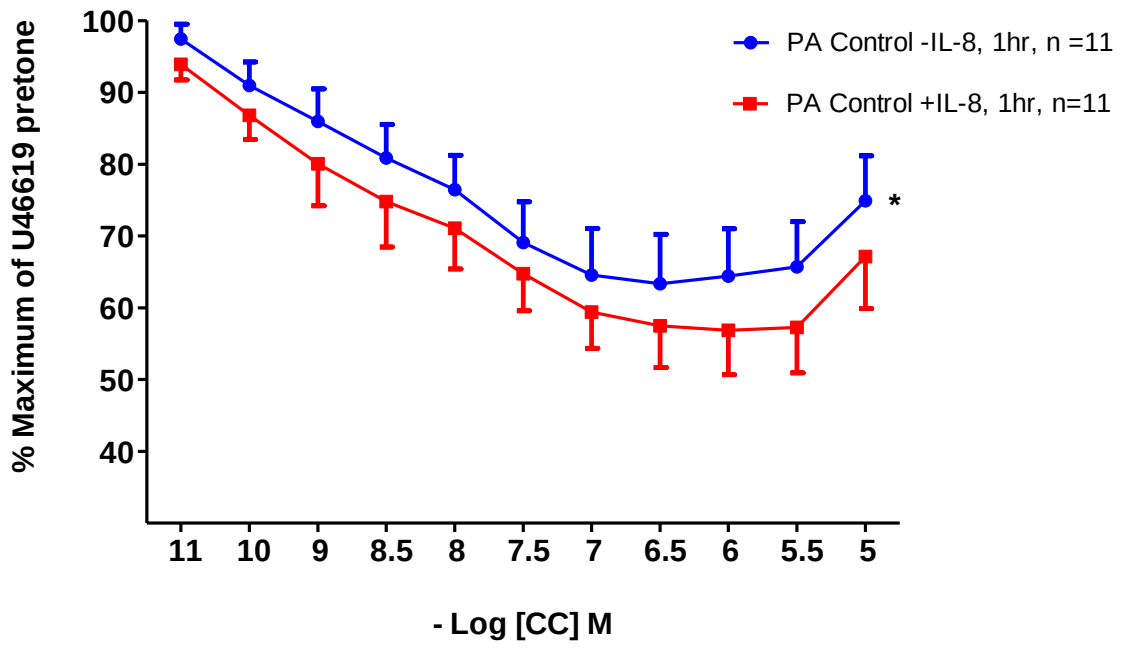


Figure 5.5: Shows a cumulative dose-response of vasodilatation mediated by CC post IL-8 treatment in isolated rat pulmonary arteries. The vessels segments were equilibrated and normalised then allowed to be stabilised for 45 minutes. Vessel viability was tested with high K^+ (80mM). Then the vessels were incubated ±IL-8 (50nM) for 1 hour and subsequently U46619 (400nM) was used to generate a pretone followed by a cumulative dose-response curves (10^{-11}–10^{-5}M) for CC ±IL-8. Percentage maximum was calculated by taking individual CC dose responses as percentage of the U46619 pretone. The log of the CC-mediated vasodilatation expressed as % maximum of U46619 plotted against the log of the [CC]M.
***Control vs IL-8 treated, p < 0.05, n=11**

5.3.5 Effects of IL–8 on vasodilatation mediated by SNP in rat PA

In this section, isolated rat PA vessels were exposed to cumulative doses of Sodium Nitroprusside (SNP) to investigate influence of interleukin–8 (IL–8) on endothelium-independent vasodilatation of pulmonary arteries. In PA vessels, pretone was generated with U46619 (400nM) which was followed by cumulative dose of SNP addition to generate a dose dependent curve. **Figure 5.3.5**, represents vasodilator response ±IL–8. After normalising for baseline, the tension generated by individual doses of CC was divided by the maximum tension generated by U46619 and

multiplied by 100 to calculate percentage maximum response (%Max) then plotted as %Max against CC concentration (-[Log]M).

As cumulative SNP concentrations mediated vasodilatation of PA vessels from both the IL–8 treated and untreated conditions as they both demonstrated gradual vasoconstriction. At highest dose applied, in both conditions were relaxed by 50–55 %.

We have observed a significance difference in SNP–mediated vasodilatation in PA between the IL–8 treated compared to control without IL–8 (p value < 0.05) (**Figure 5.3.5**). Again, IL–8 had no significant effect on the U46619 pretone and KCL responses.

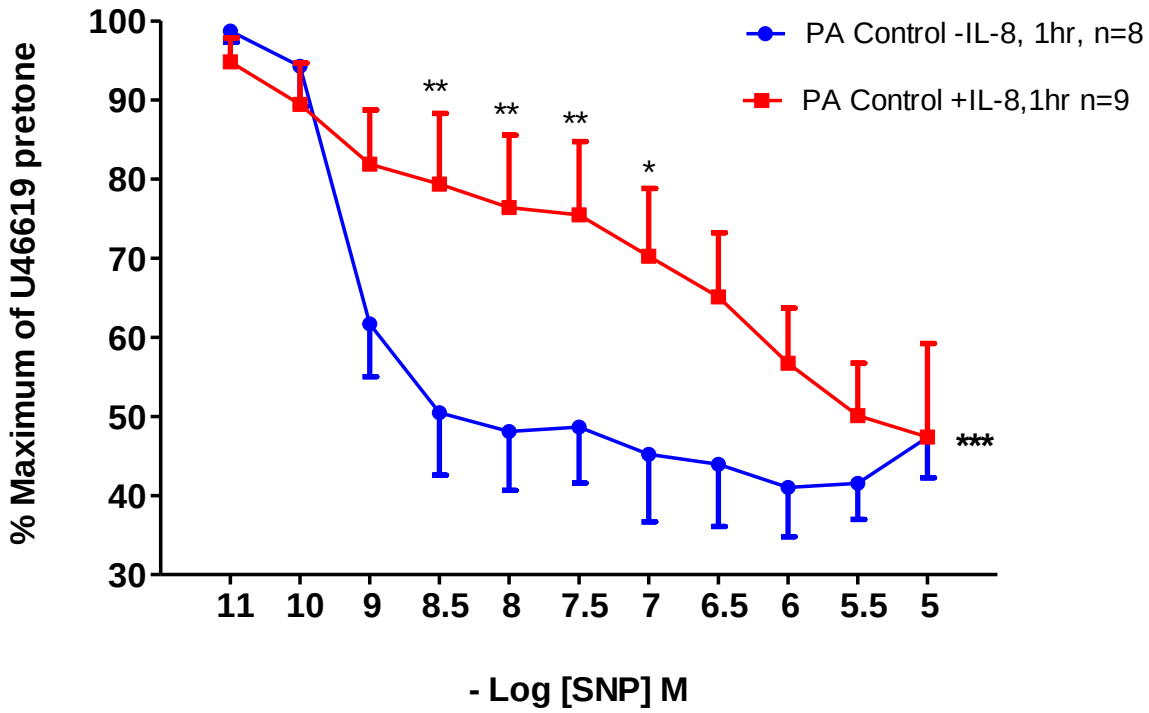


Figure 5.6: Shows a cumulative dose-response of vasodilatation mediated by SNP post IL-8 treatment in isolated rat pulmonary arteries. The vessels segments were equilibrated and normalised then allowed to be stabilised for 45 minutes. Vessel viability was tested with high K^+ (80mM). Then the vessels were incubated ±IL-8 (50nM) for 1 hour and subsequently U46619 (400nM) was used to generate a pretone followed by a cumulative dose-response curves (10^{-11}–10^{-5}M) for SNP ±IL-8. Percentage maximum was calculated by taking

individual SNP dose responses as percentage of the U46619 pretone. The log of the SNP-mediated vasodilatation expressed as % maximum of U46619 plotted against the log of the [SNP]M.
***** Control vs IL-8 treated, p <0.0001, n=8**

5.3.6 Effects of 2 hours TNF-α treatment on pulmonary artery vascular responses

In Figure 5.3.6 A & B, there was no significant effect of TNF-α on KCL response and the U46619 generated pretone (p value > 0.05). Our finding indicated that, there was a significant difference in both CC and SNP mediated vasodilatation (**Figure 5.3.6 C & D).** However, There was no significant difference in EC50 dose for both CC and SNP regardless of whether the vessels were treated with TNF-α or not (**Figure 5.3.6 E & F)**.

5.3.7 Effects of 6 hours TNF-α treatment on pulmonary artery vascular responses

In Figure 5.3.7 A & B, there was no significant effect of TNF-α on KCL response and the U46619 generated pretone (p value > 0.05). Our finding indicated that, there was a significant difference in both CC and SNP mediated vasodilatation (**Figure 5.3.7 C & D).** Though, there was no significant difference in EC50 dose for both CC and SNP regardless of whether the vessels were treated with TNF-α or not (**Figure 5.3.7 E & F)**.

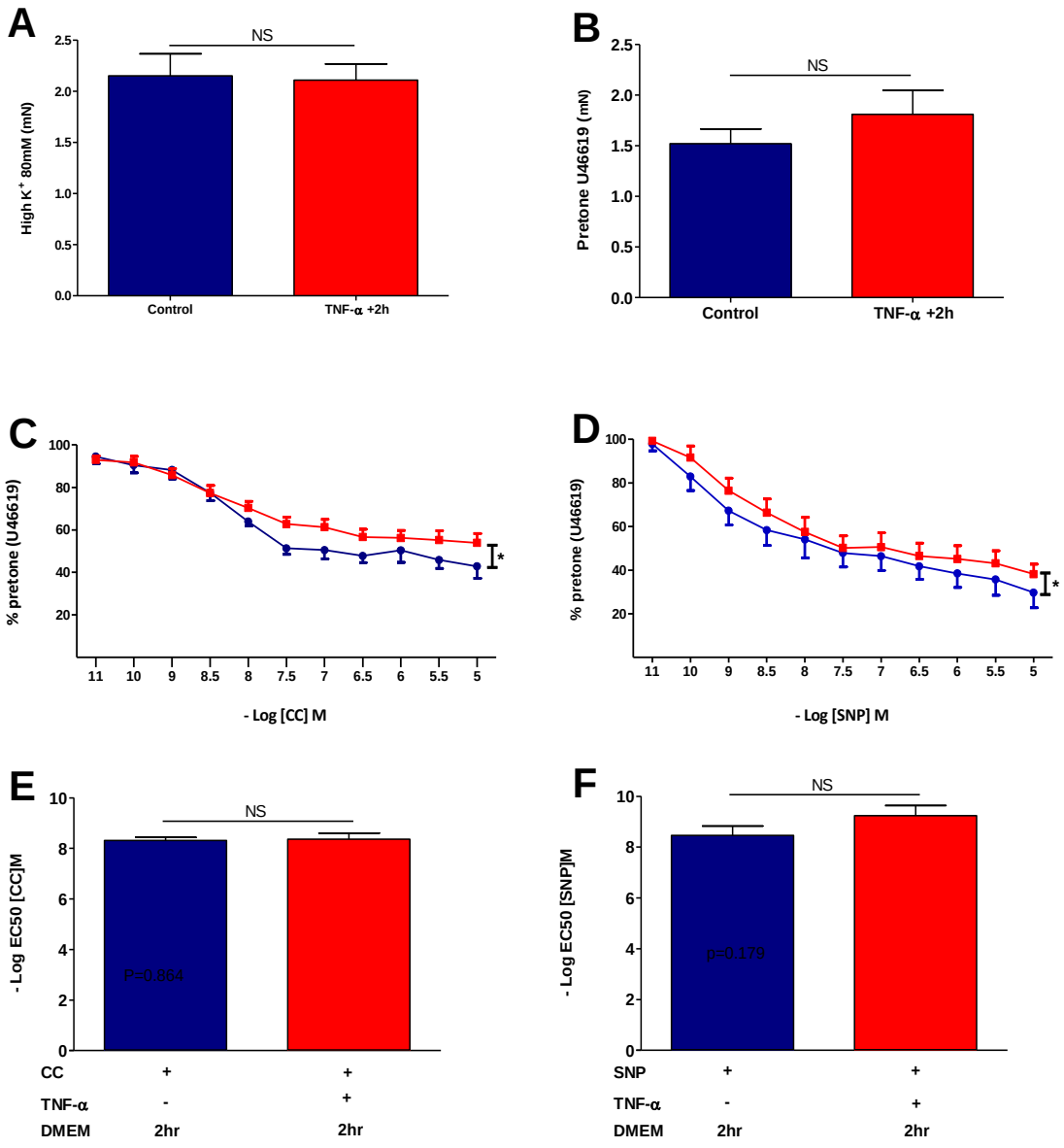


Figure 5.7: Shows cumulative dose-responses of CC or SNP-mediated vasodilatation post TNF-α treatments in isolated rat pulmonary arteries. The isolated vessels segments were first incubated in DMEM medium ± TNF-α (1000 U/L) for 2 hours then mounted on the myograph. The myograph was equilibrated and normalised and the vessels were allowed to be stabilised for 45 minutes. Vessel viability was tested with high K^+ (80mM). Then U46619 (400nM) was used to generate a pretone followed by a cumulative dose-response curves (10^{-11} – 10^{-5}M) for CC and SNP (± TNF-α). Percentage maximum was calculated by taking CC or SNP response as percentage of the U46619 pretone.

A) High K^+(80mM) response (mN) **p >0.05, n=10**

B) Pretone generated by U46619 (400nM) (mN) **p >0.05, n=10**

C) Shows percentage maximum of CC-mediated vasodilatation **p < 0.05*, n=10**

D) Shows percentage maximum of SNP-mediated vasodilatation **p < 0.05*, n=10**

E) Log EC50 of CC ±TNF-α, 2hours **p >0.05, n=10**

F) Log EC50 of SNP ±TNF-α, 2hours **p >0.05, n=10**

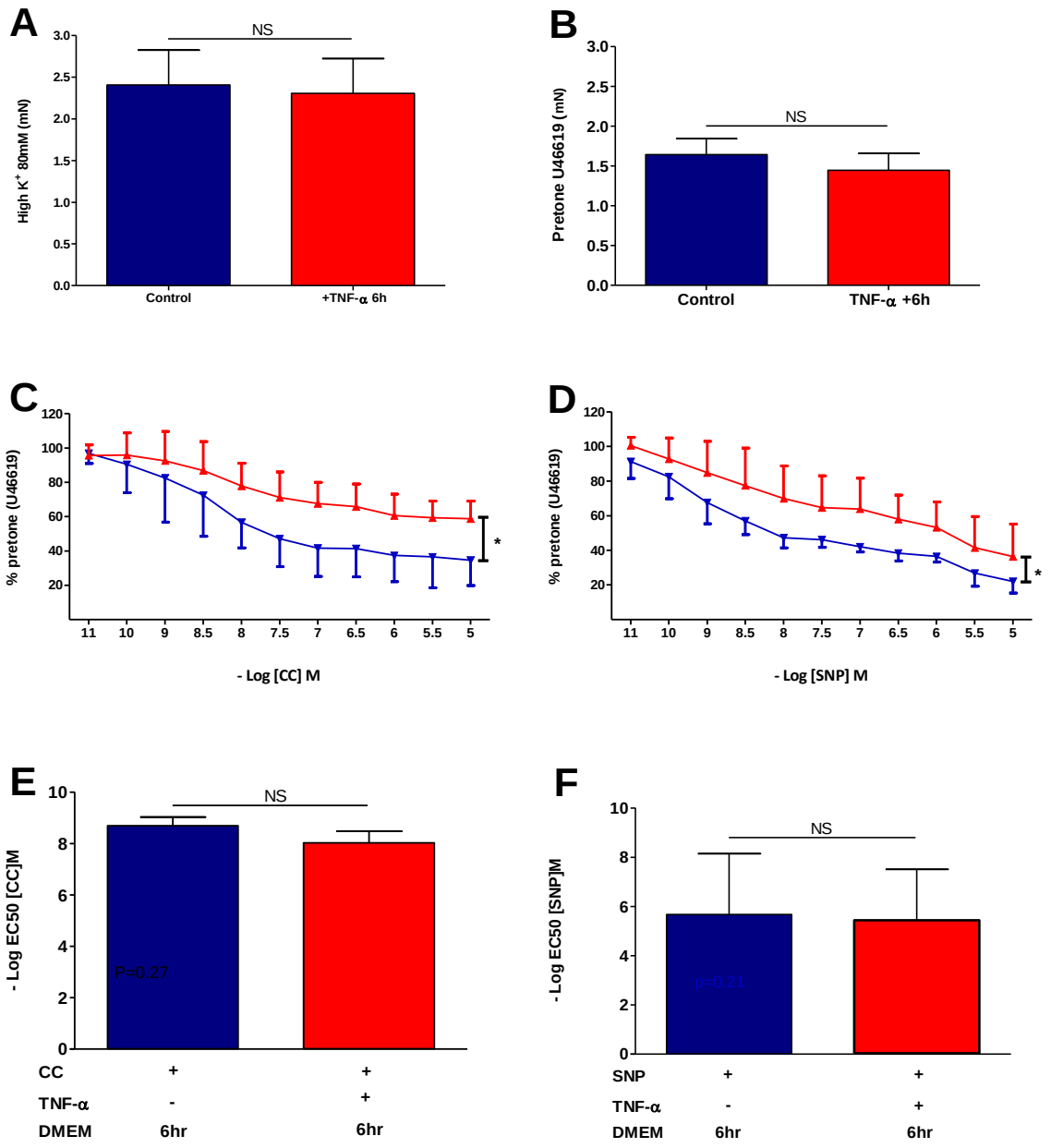


Figure 5.8: Shows a cumulative dose-response of vasodilatation mediated by CC or SNP post TNF-α treatments in isolated rat pulmonary arteries. The isolated vessels segments were incubated in DMEM medium ± TNF-α (1000 U/L) for 6 hours then mounted on the myograph. The myograph was equilibrated and normalised and the vessels were allowed to be stabilised for 45 minutes. Vessel viability was tested with high K^+ (80mM). Then U46991 (400nM) was used to generate a pretone followed by a cumulative dose-response curves (10^{-11} – 10^{-5}M) for CC and SNP (± TNF-α, 6hours). Percentage maximum was calculated by taking CC or SNP response as percentage of the U46991 pretone. **Prism 5 was used to analyse the data and to plot graphs while KaleidaGraph was used to calculate the EC50 of the agonists.**

A) High K^+ (80mM) response (mN) **p > 0.05, n=7**

B) Pretone generated by U46991 (400nM) (mN) **p > 0.05, n=7**

C) Shows percentage maximum of CC mediated vasodilatation **p < 0.05, n=7**

D) Shows percentage maximum of SNP mediated vasodilatation **p < 0.05, n=7**

E) Log EC50 of CC ±TNF-α, 6hours **p > 0.05, n=7**

F) Log EC50 of SNP ±TNF-α, 6hours **p > 0.05, n=7**

5.3.8 Influence of anti-oxidant on TNF–α reduction of relaxation in PA

5.3.8.1 TNF–α ± Allupurinol or Tempol did not affect constrictor responses in PA

Our results had shown that treatment of pulmonary artery with TNF–α alone, TNF–α ± Allupurinol OR Tempol had no significant difference between the treated groups and control on the KCL reponse (80mM). In addition, there was significant effect on the pretone generated by U46619 (**Figure 5.3.8 A & B**). This indicates that neither TNF–α, nor antioxidant treatment significantly affected constrictor response in pulmonary arteries.

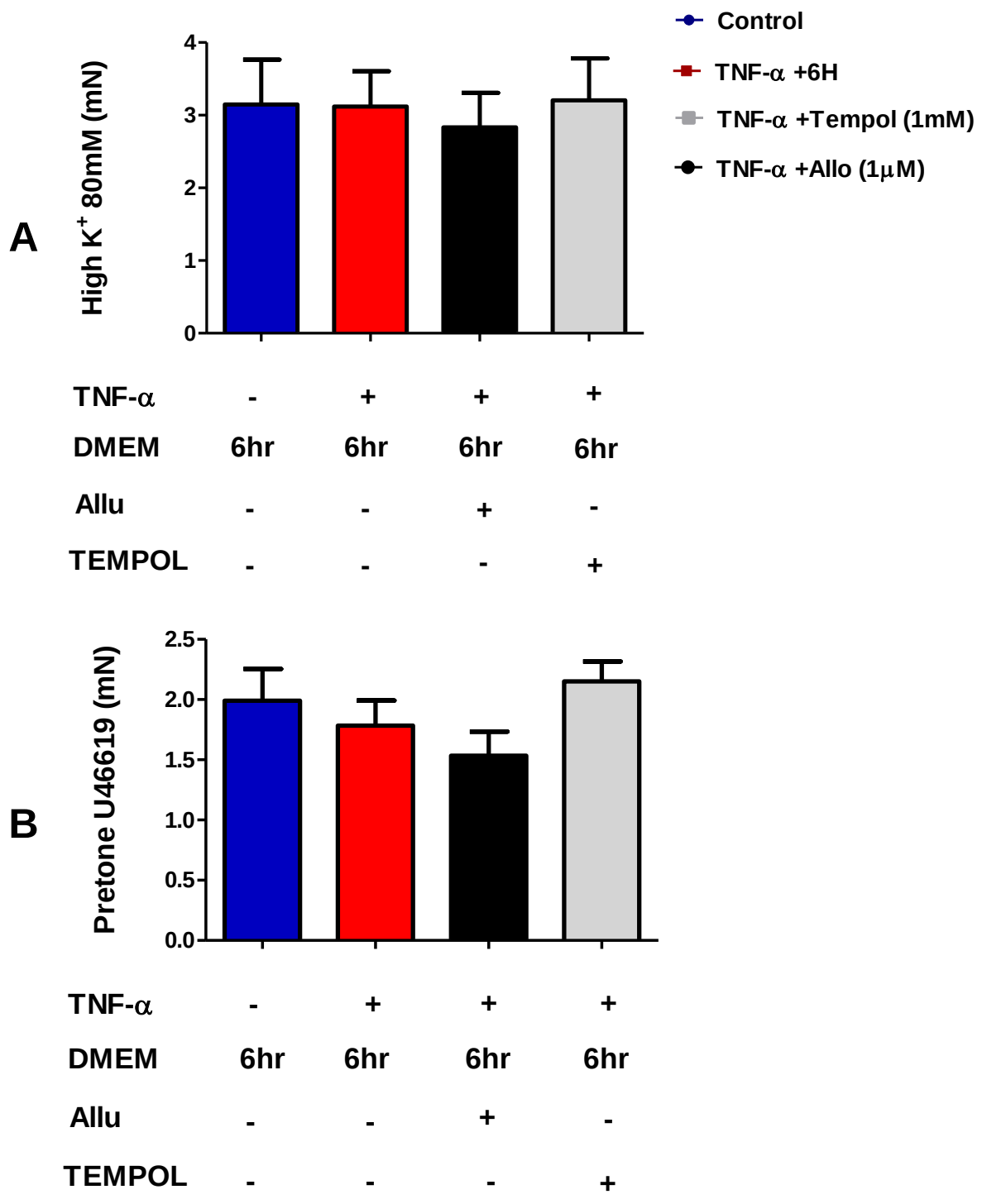


Figure 5.9: Shows vasoconstriction of PA incubated with TNF-α in DMEM for 6 hours. The isolated vessels segments were first incubated in DMEM medium ± TNF-α (1000 U/L) AND ± allupurinol OR tempol for 6 hours then mounted on the myograph. High K+ response tested then U46619 pretone generated.
A) High K^+ (80mM) response (mN)
B) U46619 Pretone (mN) $p > 0.05$, n=6

5.3.8.2 Allopurinol reversed TNF–α reduction of CC–mediated relaxation in PA

Statistical test indicated that there was a significant difference between control group and TNF–α treated group as well as TNF–α+tempol category. However, there was no significance difference between the control category and TNF–α+allopurinol group. In addition, there was a significant difference between TNF–α treated group versus TNF–α+allopurinol category. The results suggest that treatment PA with TNF–α for 6 hours, again, significantly blunted CC–mediated vasodilatation. Incubation of PA with TNF–α+tempol did not alter the effect of TNF–α whereas TNF–α+allopurinol treatment completely reversed the effect of TNF–α on the pulmonary vessel (**Figure 5.3.9**).

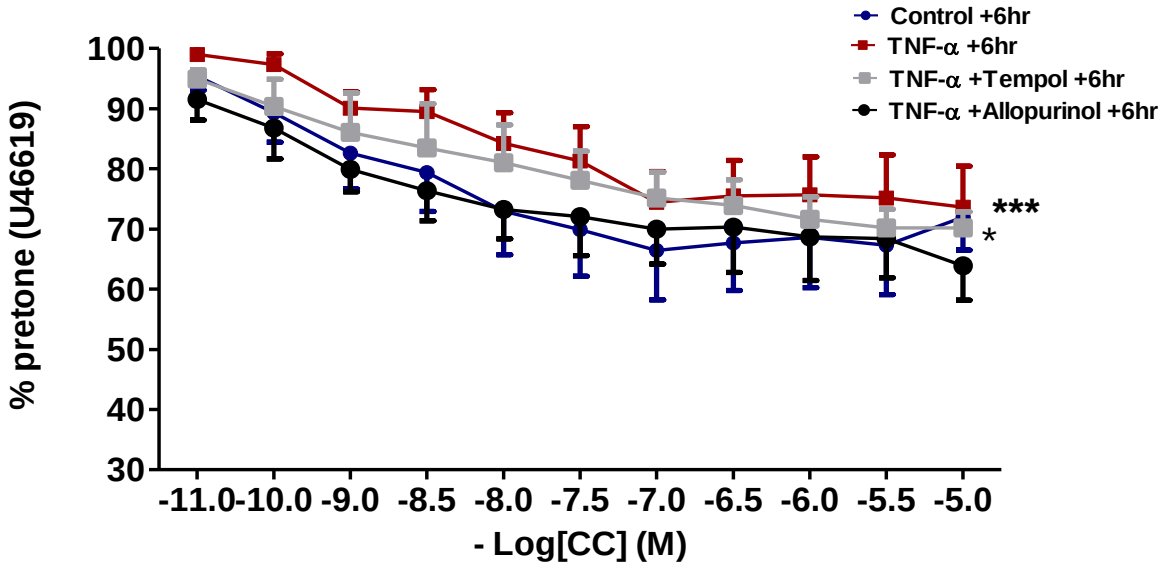


Figure 5.10: Shows a cumulative dose-response of vasodilatation mediated by CC post PA incubation with TNF-α plus allopurinol or tempol in DMEM for 6 hours. The isolated vessels segments were first incubated in DMEM medium ± TNF-α (1000 U/L) AND allopurinol (1mM) OR tempol (1mM) for 6 hours then mounted on the myograph. The myograph was equilibrated and normalised and the vessels were allowed to be stabilised for 45 minutes. Vessel viability was tested with KCL (80mM). Then U46619 (400nM) was used to generate a pretone followed by a cumulative dose-response curves (10^{-11} – 10^{-5}M) for CC (of all treatment groups). Percentage maximum was calculated by taking CC dose responses as percentage of the U46619 pretone.

***** Control Vs TNF-α treated, p <0.0001, n=6**

***Control Vs TNF-α+Tempol, p > 0.05, n=6**

TNF-α +allopurinol Vs TNF-α treated, p > 0.05, n=6

5.3.8.3 *Allopurinol & Tempol did not alter TNF–α reduction of SNP–mediated relaxation in PA*

In these set of results, isolated rat PA vessels were first incubated in DMEM medium ±TNF–α AND allopurinol OR tempol for 6 hours. We used the same protocol as explained in the last section (**5.3.8.2**). This was done to investigate influence of antioxidants on TNF–α mediated inhibition of SNP–mediated vasodilatation.

The statistical analyses we have applied, suggest that there were significant differences between the control compared to the TNF–α, TNF–α+allopurinol and TNF–α+tempol treated groups. Besides, there was no difference between the treated groups when compared to each other. These results indicated that incubation of PA with TNF–α plus allopurinol OR tempol had not altered TNF–α–mediated inhibition of pulmonary relaxation by SNP (**Figure 5.3.10**).

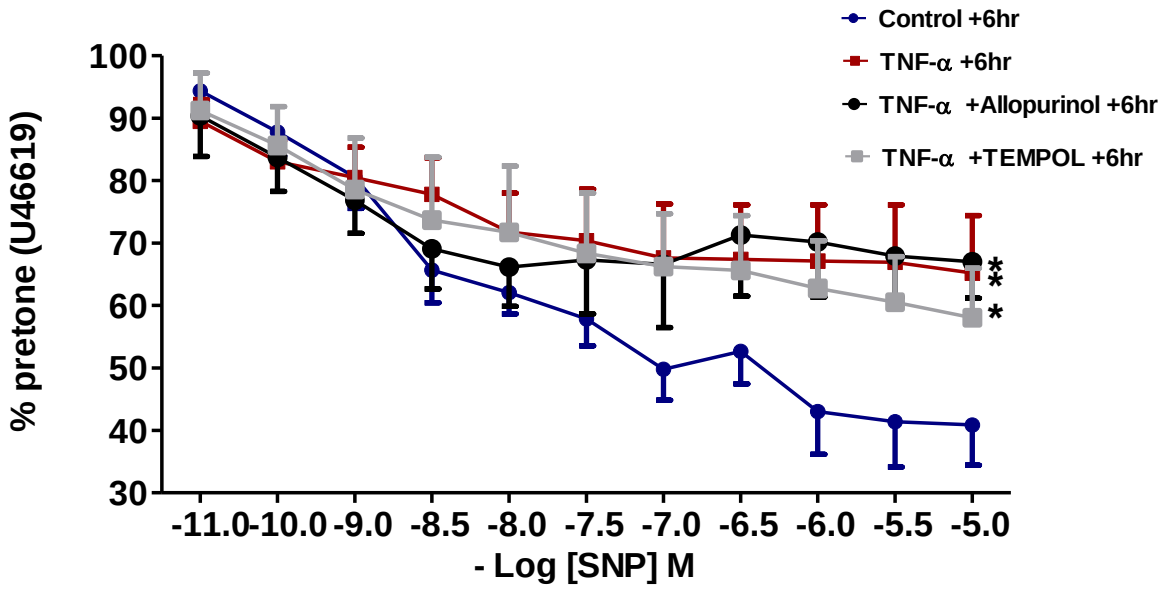


Figure 5.11: Shows cumulative dose-response of vasodilatation mediated by SNP post PA incubation with TNF-α plus allopurinol or tempol in DMEM for 6 hours. The isolated vessels segments were first incubated in DMEM medium ± TNF-α (1000 U/L) AND allopurinol (1mM) OR tempol (1mM) for 6 hours then mounted on the myograph. The myograph was equilibrated and normalised and the vessels were allowed to be stabilised for 45 minutes. Vessel viability was tested with KCL (80mM). Then U46619 (400nM) was used to generate a pretone followed by a cumulative dose-response curves (10^{-11} – 10^{-5}M) for SNP (of all treatment groups). Percentage maximum was calculated by taking SNP dose responses as percentage of the U46619 pretone.
*** Control Vs TNF-α treated, TNF-α +allopurinol and TNF-α+tempol, p > 0.05, n=4**

5.4 Chapter discussion

5.4.1 Summary of key findings

1. Effects treatment with TNF–α OR IL–8 (1 hour) on vasoconstriction of pulmonary arteries isolated from healthy Wistar rats:

   TNF-α treatment (for 1 hour) had no significant effect on phenylephrine and U46619 mediated vasoconstriction of pulmonary arteries.

2. Effects treatment with TNF–α or IL–8 (1 hour) on vasodilatation of pulmonary arteries isolated from healthy Wistar rats:

   a) TNF-α (1hour) treatment on CC-mediated vasodilatation had no significant effect on CC–mediated vasodilatation in PA compared to the time control without TNF–α (p value > 0.05).

   b) IL–8 treatment (for 1 hour) of isolated PAs had a significant difference CC–mediated vasodilatation (p-value = 0.0043).

   c) IL–8 treatment (for 1 hour) of isolated PAs had a significant difference on SNP–mediated vasodilatation.

3. Effects of pre-incubation of isolated healthy pulmonary arteries in DMEM ±TNF-α for 2 hours

   a) TNF–α significantly reduced both CC and SNP mediated vasodilatation isolated PAs, however, had no effect on the EC50 obtained.

   b) TNF–α did not affect the vasoconstrictor responses by KCL & U46619

4. Effects of pre-incubation of isolated healthy pulmonary arteries in DMEM ±TNF-α for 6 hours

   a) Prolonged TNF–α incubation of isolated PAs significantly reduced CC and SNP mediated vasodilatation. Again TNF–α had no effect on the EC50s.

b) TNF-α did not affect the vasoconstrictor responses of KCL and U46619

5. Effects of pre-incubation of isolated healthy pulmonary arteries in DMEM ±TNF–α ± Allopurinol OR Tempol for 6 hours

a) Allopurinol completely reversed TNF–α inhibition of CC–mediated relaxation in pulmonary artery.

b) Tempol did not significantly affect TNF–α inhibition of CC–mediated relaxation in pulmonary artery.

c) Neither Allopurinol, nor Tempol had any significant effect on TNF–α inhibition of SNP–mediated relaxation in pulmonary artery

d) Neither Allopurinol, nor Tempol had any significant effect on the pulmonary artery vasoconstrictor response by KCL and U46619.

5.5 Discussion

The endothelium is regarded as the functional barrier that is located between the blood vessel and the blood stream (125, 233, 367, 372, 429). Endothelial cells are known to play various functions within the vasculature ranging from fibrinolysis, coagulation, maintenance of vascular tone to growth and immune response (125). In a healthy vascular environment, these functions are regulated via several factors including endothelium dependent relaxing factor, endothelium-dependent contracting factor and endothelium-dependent hyperpolarizing factor. When these factors fail to regulate the vascular function, it could lead to vascular endothelial dysfunction which is associated with a plethora of diseases including COPD and atherosclerosis (125). Inflammation and endothelial dysfunction contribute to the pathogenesis and development of pulmonary hypertension (42, 125, 233, 367, 372, 445-447). In COPD

patients increased level of various cytokines were discovered in both the serum and the sputum (52, 68, 464) and was discussed in chapter 1. The presence of these cytokines was associated with increased arterial stiffness and poor disease prognosis (447, 449, 450). The mechanism for this link between inflammation and endothelial dysfunction and subsequently right heart failure is not clearly known, and therefore, we used animal models in an attempt to further elucidate part of this association.

In this chapter, the effects of the vasoconstrictor; phenylephrine (an α-1 receptor agonist) and vasodilator; Carbachol (a cholinomimetic drug that binds and activates the acetylcholine receptor) on the vascular reactivity of pulmonary vessels freshly isolated from healthy Wistar rats was investigated. In addition, the effects of inflammatory cytokines such as TNF–α and IL–8 on the PE and CC responses were assessed.

It has been shown that two major excitation-contraction coupling mechanisms contribute in regulating pulmonary vascular tone. Hence, in this chapter, we have addressed agonist-mediated pulmonary contraction (pharmacomechanical coupling induced by PE and CC) and depolarisation-mediated contraction (electromechanical coupling induced by high potassium solution) (465).

In this chapter, the results obtained for pulmonary artery sensitivity to phenylephrine were similar to previous studies (465-467). Our results demonstrated that there is no significant difference between the sensitivity of acute (1 hour) TNF–α treated and untreated pulmonary arteries to phenylephrine vasoconstrictor. The effect of TNF–α on pulmonary artery vasoconstriction mediated by phenylephrine has not been

previously reported in the literature. The other studies that have investigated similar protocol were focused on vasodilatation (435, 451). Therefore, we have established here that short TNF–α challenge does not significantly affect α–1 receptor agonist response. Similarly, our findings also indicated that short term challenge of TNF–α on pulmonary artery did not significantly affect thromboxane A_2 agonist response. There was no significant different in vasoconstriction mediated by U46619 between TNF–α treated and controls.

This finding might be physiologically relevant given that, when blood vessels constrict, the flow of blood is restricted or decreased, thus, retaining body heat and increasing vascular resistance. Therefore, if TNF–α treatment results into increased vasoconstriction in pulmonary arteries, this will caused localised decline in blood flow that might subsequently diminish oxygen to the tissues. Thus the observed lack of significant of short term TNF–α treatment on vasoconstrictor response of pulmonary artery certainly conform to vascular physiology.

Moreover, PAs treated with TNF–α and untreated controls showed a similar sensitivity to endothelial-dependent NO release. Our findings suggest that there was a significant difference between TNF–α treated PAs compared to Control PAs prior to TNF–α treatment. This observation might suggest that TNF–α influenced endothelial–dependent vasodilatation mediated by CC in PA. However, when we compared control groups, control PA prior to TNF–α and control PA without TNF–α, there was a significant difference between them even though both control were without the influence of TNF–α activity. One of the controls was used as a quality control and the other as time–control for the treatment group, hence, it was not anticipated to observe any significance or variation between the two controls. This

might suggest that the earlier supposed effect of TNF–α observed is due another factor. Hence, the difference might be due to time delay factors AND/OR TNF –α, which might be explained by the fact that this was the earliest result obtained when operator was still learning the techniques. In addition, there was no significant difference between TNF–α treated PAs and time–control PAs samples without TNF–α, though pulmonary vascular vasodilatation was reduced in TNF–α treated vessels. This observation further strengthens the view that the effect observed in this particular case might not be due to TNF–α alone. Therefore, we are not going to merely conclude the effect of TNF–α on endothelial–dependent vasodilatation mediated by CC, based on the current observation alone, as further investigation is required to verify the findings.

Similarly, a previous studies have shown that TNF–α incubation did not affect endothelial–dependent relaxation in omental arteries, a systemic small arteries obtained from healthy pregnant women (451). However, when the capacity for vasodilatation is halted then TNF–α incubation promote endothelial damage (451).

Since we were not able to confirm the effect of TNF–α on endothelial–dependent relaxation of PAs, we decided to investigate the effect of another inflammatory mediator, IL–8 on pulmonary vascular reactivity. The PAs presented similar in vitro sensitivity to endothelial NO release. Our findings have shown that IL–8 significantly affected vasodilatation of PAs. However, the observation is contrary to our hypothesis as we expected IL–8 to blunt the NO mediated relaxation in PA compared the control without IL–8. Interestingly we have observed increased vasodilatation in PAs treated with IL–8 compared to control. Additional investigation might be required to ascertain this phenomenal manifestation. In addition, we have

investigated effects of IL–8 on endothelial-independent vasodilatation mediated by sodium nitroprusside. Our findings have demonstrated a significant reduction in endothelial–independent vasodilatation in IL–8 treated vessels compared to the control. This is a novel finding and has not shown in the literature previously.

Furthermore, we have extended PA incubation in TNF–α to two hours, to investigate whether this might augment the effect observed in one hour incubation in the same cytokine. Interestingly, unlike in the 1 hour treatment, two hours TNF–α incubation of pulmonary artery segments, has significantly diminished both endothelial–dependent and endothelial–independent vasodilatation mediated by CC and SNP respectively. This means that both endothelial and smooth muscle cell mediated relaxations were altered by the incubation of PAs in TNF–α for two hours. Similarly, an extend incubation of PAs in TNF–α for 6 hours has further attenuated both endothelial–dependent and endothelial–independent vasodilatation mediated by CC and SNP respectively. The long term incubation period of PAs in TNF–α has almost doubled the difference between the TNF–α treated vessel and untreated ones.

Though the extended and long term incubation of PA segments in TNF–α (for 2 and 6 hours respectively) has altered vasodilatation in healthy pulmonary vasculature, the same treatments had no significant effect on neither the U46619; thromboxane A2 agonist response nor on the KCL response, hence, vasoconstriction or contraction of PAs was not altered by TNF–α treatment. In addition, both 2 and 6 hour TNF–α treatment had not caused a significant change in pEC50 of both CC and SNP mediated vasodilatations.

From this chapter, we have established that inflammatory mediators such as TNF–α predominantly reduced both endothelial–dependent and endothelial–independent vasodilatation in pulmonary arteries. Interestingly, this is the first time, to report such an observation in this sort of protocol in PA. From the literature, is known that inflammation can be triggered by infection, trauma, hypoxia at times immunological processes (372) and research evidence has suggested a strong association between inflammation and the risk of developing cardiovascular diseases (367, 369, 371). Cytokines mainly interact together in synergy to initiate of the inflammatory cascade that triggers expression of intermediary factors (369). Hence, cytokines has the ability to express cytokine receptors and other cytokines and chemokines to promote the inflammatory cascade. Besides, inflammatory mediators can trigger the expression of various enzymes, like the inducible NO synthase (iNOS) and cyclooxygenase–2 (COX–2). The induction of these enzymes can lead to the production of mediators that can act at the vascular bed which can promote inflammatory response (372).

Several lines of indirect evidence suggest that, in COPD patients, vascular changes result from endothelial dysfunction and NO insufficiency. First, endothelium–dependent vasodilatation is impaired in patients with COPD (468). Second, inhibition of NO synthase potentiates the vasoconstrictor effect of hypoxia (469), suggesting that NO serves to prevent excessive hypoxic vasoconstriction, which has been linked to the pathogenesis of cor pulmonale in COPD (470). Third, structural changes in pulmonary arteries of patients with COPD resemble those observed in response to endothelial denudation and vascular injury, i.e., after angioplasty (471).

We have shown that thromboxane A_2 agonist (U46619) pre-constricted PAs derived from healthy Wister rats have demonstrated both agonist–mediated endothelial–

dependent and endothelial–independent vasodilatation. This observed relaxation was mediated by NO and potentially EDHF–dependent pathways. This observation is normal to be expected in healthy animals, because the rats will ensure vascular relaxation is optimum thus will utilise all available mechanisms (451). When the pulmonary arteries were pre–incubated with TNF–α for either 2 or 6 hours, there was a significant change in the overall profile of both the endothelial–dependent and endothelial–independent relaxation.

In the same way, Hughes *et al* (376), has shown that TNF–α pre-incubation caused inhibition of both acetylcholine and bradykinin mediated relaxation in rat mesenteric arteries. However, their results showed that TNF–α treatment did not alter non-endothelial dependent relaxation by SNP in rat mesenteric arteries (376). In addition, Gillham *et al* (451)., has shown that TNF–α incubation for 1 hour caused no alteration to the endothelial–dependent vasodilation in omental arteries obtained from healthy pregnant women. Our current study revealed a similar observation as reported earlier in this chapter.

In contrast, a similar study protocol reported no changes in endothelial–dependent vasodilatation profile in healthy omental arteries incubated with TNF–α for 2 hours (451), though they did not test the long term (6 hours) incubation period adopted in our study. The difference might be due to the fact that vessels obtained from rats and human do display diverse endothelial responses to TNF–α treatment even under similar condition.

Hughes *et al* (376) has also shown that the endothelial–independent vasodilation (mediated by NO donor; sodium nitroprusside) of the mesenteric arteries was not affected after incubation with TNF–α. This is contrary to the observation we have revealed in this study as both 2 and 6 hour incubation of pulmonary arteries with

TNF–α had a significant reduction on vascular relaxation. It may be that systemic and pulmonary vessels isolated from rats might exhibit different endothelial sensitivities to TNF–α. Hughes and colleagues suggested that the attenuation of the endothelial–dependent vasodilatation observed with acetylcholine and bradykinin might be as consequence of TNF–α lowering the synthesis or release of NO from the endothelial cells (376, 451). We assume that this suggestion might also apply to our investigations reported in this chapter on rat pulmonary arteries. Our results showed a greater proportion of pulmonary vascular relaxation was not affected by TNF–α incubation thus allowing normal vascular relaxation. However, other pathways that effect relaxation in the vasculature such as EDHF might be play a role in maintaining both the endothelial–dependent and independent vasodilatation of vessels incubated with TNF–α (451). Therefore, this issue might be addressed with the usage of EDHF pharmacological inhibitors.

These data support the hypothesis that TNF–α might worsen pre-existing endothelial dysfunction as well as in intact pulmonary vessels. This is due to the observation cytokine affected both endothelial and non–endothelial mediated vasodilatation which suggest that plasma TNF–α level could trigger endothelial dysfunction. Future studies should focus on identifying the mechanisms by which TNF–α exert its inhibitory effect on the pulmonary vascular relaxation.

Investigating the mechanism of TNF–α inhibition of vasodilatation in pulmonary arteries, we have used Allopurinol; a *xanthine oxidase inhibitor* and Tempol; a membrane–permeable radical scavenger. Interestingly, Allopurinol has completely reversed TNF–α inhibition of CC–mediated relaxation in pulmonary artery, though Tempol did not significantly affect TNF–α inhibition of CC–mediated relaxation in pulmonary artery.

A meta-analysis conducted by Kanbay *et al* (472). on endothelial function suggested that Allopurinol had significantly increased endothelium–dependent vasodilatation in human subjects. All the studies included in this study assessed effect of allopurinol on endothelial–dependent which was evaluated using flow–mediated dilatation (FMD) and forearm blood flow (FBF) response to acetylcholine or flow-dependent flow assessment (472). In contrast, Kanbay and colleagues (472) indicated that Allopurinol has no significant effect on the endothelium–independent vasodilation mediated by SNP. Interestingly, our current findings though from different species and set-up, demonstrated a similar observation.

The result suggests that TNF–α inhibition of CC–mediated relaxation in pulmonary artery was via *Xanthine Oxidase* pathways. *Xanthine Oxidase* is a form of *Xanthine oxidoreductase;* an enzyme that generates reactive oxygen species. The enzyme can catalyze the oxidation of hypoxanthine to *xanthin* (473) . *Aslo, it can further* catalyze the oxidation of *xanthine* to uric acid (473). XO was shown to be elevated in the epithelial lining fluid of COPD patients compared to healthy controls (434). It was suggested that XO activity is mainly regulated by its gene expression (434). Another study has indicated that pro-inflammatory cytokines; TNF–α, IL–1β and IFT–γ significantly stimulated XOR gene expression in rat alveolar macrophages (474), bovine renal epithelial cells (475) and human mammary epithelial cells (476). In addition, Wright *et al*.(477) has shown that infusion of IL–1 and IFT–γ in a rat model, resulted into elevation XO activity in the lungs. Considering these evidence, it appears that pro-inflammatory cytokines might play a significant role in enhancement of the XO gene expression in COPD airways (434). Furthermore, Komaki *et al* (434), found a strong positive association between the levels of TNF–α or IL–1β and XO activity, hence, an added evidence for the proposition.

On the other hand, neither Allopurinol, nor Tempol significantly affected TNF–α inhibition of SNP–mediated endothelial–independent relaxation in pulmonary artery. Moreover, like TNF–α, Allopurinol and Tempol had no effect on the pulmonary vasoconstrictor responses mediated by U46619 and KCL.

Our data have shown that inflammation might negatively affect No mediated vascular relaxation in pulmonary artery isolated from healthy rats. This finding once verified could open probable or potential therapeutic target for treatment of pulmonary hypertension associated with COPD. It might be possible to further investigate NO compared to non-NO mediated effects on vessels and investigate whether specific manipulation of NO or cGMP signal transduction system might be beneficial in patients with COPD. Further experiments would need to test the contribution of NO by comparing NO donor responses with those of endothelium-derived NO release, and blockade of NOS activity.

5.5.1 Limitation of the study

The limitation of myography was discussed in earlier chapters. The same applies in this chapter. Temperatures of the individual myograph chambers are controlled collectively, but we noticed that not all chambers were heated at the same rate and sometimes a slight difference in the bath temperature was observed between chambers. Different vessels types, vasoconstrictors, vasodilators and cytokines were therefore randomly selected to be mounted on various myograph chambers to avoid any predisposition. In addition, the chambers were allowed to reach optimum temperature (37^0C) before any protocol was started and the equipment was calibrated frequently to maintain consistency of the data generated. This limitation did not seem to affect data generated, as the results were consistent for individual vessels types.

When TNF-α or IL–8 was used to treat the pulmonary vessels, this does not mean that the inflammatory mediator remained in the myograph bath throughout the protocol. The vessel was incubated with the inflammatory mediator either on the myograph bath for 1 hour or in petri dish in DMEM for 2 or 6 hour in normoxic chambers (then mounted on the myograph). In addition, the saline solution in the myograph does get replaced with fresh warm solution regularly between different stages of the protocol as the vessels get washed. This process might cause the vessels to become desensitised from the effect of the inflammatory mediator, though we have not noticed any major different between treated and untreated vessels due to this activity. We have ensured to keep our protocols short as possible to ensure this has not become an issue for our experiment.

There is a chance of mechanical damage to the pulmonary arteries during the dissection and isolation process, however, careful proven steps reported in past literature was applied to ensure the vessel integrity was maintained and damage to the isolated vessels minimised or prevented (249, 393, 478, 479). In addition, high K^+ challenge was performed to check vessel viability and only PA vessel that generated 1mN or more were used for the experiments.

The values obtained for weighing individual PA vessels segments post completion of the experimental protocol were not consistent. At times, no reading was obtained and even when obtained sometimes it does not look to be the right value. Hence, it was difficult getting reliable weight value for the tiny PA vessel segments used in this experiment. We have used alternative weight machines the same issue persisted and reliable data could not be achieved. Therefore, we have excluded these data from the analyses due to inconsistence and unpredictable weight values obtained. Also, the vessel weight measurement was not conducted for latter experiments.

CHAPTER 6

6 INFLUENCE OF CHRONIC AND/OR ACUTE HYPOXIA AND PRO-INFLAMMATORY CYTOKINES ON PULMONARY VASCULAR REACTIVITY

6.1 Introduction

Chronic exposure to alveolar hypoxia is known to cause to constriction in pulmonary arteries. Prolong exposure to hypoxia can cause luminal narrowing of the pulmonary vasculature which progressively results in increased pulmonary arterial pressure, as a consequence of both structural remodeling of the pulmonary vasculature and sustained active vasoconstriction of pulmonary arterial smooth muscle (205, 480, 481). Oka *et al.* (240) have shown that exposure to simulated high altitude (17 000 ft) for 3–4 weeks significantly induced right ventricular hypertrophy which has increased the right ventricular (RV) ratio over the left ventricular plus septum (LV+S) ratio from 0.32 to 0.58 in low altitude and chronic hypoxic rats respectively (240) . In addition, high altitude resulted in elevation of Ppa from 19mmHg to 41 mmHg (240, 468). Intravenous infusion of acetylcholine in patient with COPD stimulated a rapid decline of mean Ppa from 31 mmHg to 28 mmHg, as well as breathing in NO, caused reduction in Ppa in a concentration–dependent manner (468).

Chronic hypoxia is known as a common trigger for development of clinical pulmonary hypertension (482). Prior to causing structural and functional vascular reactivity damage, chronic hypoxia was suggested to promote various inflammatory challenges specific to the pulmonary vasculature that included perivascular inflammatory cell infiltration and elevation of the expression of various inflammatory mediators, such as cytokines, chemokines and differentiation factors (482, 483). In addition, hypoxia stimulated increase of adhesion molecules such as intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1. These

adhesion molecules perform a critical role in mononuclear cell recruitment under hypoxia in lung tissue (482, 483). Madjdpour *et al* (484) has reported elevated level of macrophages in the bronchoalveolar fluid of rats exposed to 10% oxygen for a period between 1 and 8 hours as well increased mRNA levels of TNF-α, macrophage inflammatory protein (MIP)-1β, and monocyte chemoattractant protein (MCP)-1 within the intial 2 hours of being in the hypoxic environment (484). In addition, they have showed increased mRNA levels of hypoxia-inducible factor (HIF)-1α and ICAM-1 between 1 and 6h. The mechanism of chronic hypoxia interaction with inflammation is not well defined. Hence, further investigations are required to elucidate the mechanism underlying the observed association.

Moderate physiological hypoxia (~20–60 mmHg PO_2) is known to cause constriction in pulmonary arteries, whilst leading to vasodilatation in systemic arteries and this observation is commonly referred as to hypoxic pulmonary vasoconstriction (HPV) (205, 249, 485). This physiological difference between the pulmonary and systemic circulations plays an important role within the body. HPV is crucial for the regulation and maintenance of the ventilation–perfusion ratio in the event of localised hypoxia (485). On the other hand, global hypoxia in disease conditions might cause a damaging effect of elevated total pulmonary vascular resistance and thus increase load on the right side of the heart (249, 485). It is credible that both local hypoxia and global hypoxia might play key role at various stages in chronic lung diseases such as COPD (486). The role of inflammation on vascular dysfunction has been extensively covered in the previous chapters. Inflammation was found to be associated with COPD as well cardiovascular event associated the disease pathogenesis (233, 429, 487, 488). In addition, both hypoxia and inflammation were recognised as possible culprits in promoting vascular dysfunction associated with COPD (233, 486, 487).

Endothelial dysfunction is a key feature of PH and this might be detrimental to the pulmonary vascular tone due to reduced vasodilator mediators and anti-proliferative factors (488). These coupled with increased vasoconstrictors and proliferative factors in long term could promote cardiovascular complications.

Therefore, it is useful to investigate how inflammation might influence HPV in order to identify probable mechanisms that might be utilised in future therapeutic development.

We have previously discussed the influence of inflammation alone on pulmonary vascular reactivity (see Chapter 5). In this chapter, we will be investigating the combined effects of hypoxia and inflammation together on vascular reactivity.

The aims of the investigations in this chapter are summarised below:

a) Effects of chronic hypoxia (1 or 2 weeks at 12% oxygen) and inflammation
    i. To investigate effects of chronic hypoxia AND TNF–α OR IL–8 on pulmonary artery vasoconstriction mediated by phenylephrine or U46619 thromboxane A2 agonist in PA
    ii. To investigate effect of chronic hypoxia AND TNF–α OR IL–8 on CC and SNP mediated vasodilatation in PA
b) Effects of chronic hypoxia (2 weeks) on cytokine expression in rat
    i. Quantify level of inflammatory mediator in the bronchoalveolar lavage of hypoxic rats
    ii. Identify possible link between elevated pro-inflammatory mediator and chronic hypoxia
c) Effects of acute inflammation on HPV

Investigate influence of TNF–α (1 hour) on HPV phase 1 & 2

d) Effects of concurrent short term hypoxia AND inflammation (1 hour) on vascular reactivity

   i. Investigate effects of hypoxia AND TNF–α (1 hour) in vitro treatment on vasoconstriction mediated by U46619.

   ii. Investigate effects of hypoxia AND TNF–α (1 hour) in vitro treatment on both endothelium-dependent and endothelium-independent vasodilatation mediated by CC and SNP respectively.

We hypothesised that hypoxia (short term OR long term) plus TNF-α AND/OR IL–8 will promote pulmonary vascular endothelial dysfunction which impairs mechanisms modulating vasodilatation as well as impact on vasoconstriction.

6.2 Methods

6.2.1 Animal, treatments, tissue isolation, preparation, drug & cytokine treatment for myograph

Detailed description provided in **chapter 2**. Male Wistar rats (~250–300g) were exposed to 12% O_2 for either 1 or 2 week(s). Upon the completion of this protocol, rats were killed by an overdose of anaesthesia with cervical dislocation, as approved by the UK Home Office. Immediately after death, the chest cavity was opened, the lungs were dissected then placed in ice-cold Krebs solution (393).

Pulmonary arteries were dissected, isolate and mounted on myograph in a PSS solution (95% air, 5% CO_2, 37^{o}C) (355, 393). The vessel was allowed to equilibrate for 45 minutes then treated with a pretone generated by PE or U46 (using EC_{80} from the cumulative dose response curve of the vasoconstrictor followed) by a cumulative

dose response of a vasodilator CC, SNP. Then a pro-inflammatory cytokine TNF-α or IL-8 (1000U/ml, 50ng/ml respectively) added for 1 hour and vasoconstrictor or dilator response re-assessed.

6.2.2 HPV & TNF–α treatment

Healthy Wister rats (~250 – 300g) were anaesthetised with sodium pentobarbitone (55 mg kg−1, intraperitoneal injection) and killed by cervical dislocation (355). PA vessels was isolated and mounted on myograph in a PSS solution (95% N_2, 5% CO_2, 37^oC) (355, 393). High K^+ response tested. Prostaglandin F2α (3 – 7μM) (393, 479, 489) used to generate pretone then control HPV generated twice before the vessels were treated with TNF–α for 1 hour. Then the HPV challenge was repeated to test hypoxia treatement effects. We used the second control HPV generated and HPV post TNF-α to assess effects of the inflammatory challenge. HPV phase 1 and 2 were taken as percentage of the high K^+ response (see results **6.3.5**).

6.2.3 Concurrent Short term hypoxia AND TNF–α treatment

Helathy Wister rats were killed by an overdose of anaesthesia with cervical dislocation. PA vessels was isolated and moutnted on myograph in a PSS solution (95% air, 5% CO_2, 37^oC). High K^+ response tested. Then vessel incubated with TNF–α in PSS solution (95% N_2, 5% CO_2, 37^oC) for 1 hour. Then vasodilator CC and SNP were tested. Responses were plotted as percentage of the U46619 pretone (see results in **6.3.6**).

6.3 Results

6.3.1 Chronic Hypoxia Induced RV Hypertrophy in Rats

In three categories of rats, there was a significant difference in the right ventricle ratio to left ventricle plus septum ratio between the healthy controls and chronic hypoxia exposed rats for 1 and 2 week(s) (**Figure 6.3.1**, *p value* < 0.001). Also there

was a significant difference in the right ventricle ratio to left ventricle plus septum ratio between the chronic hypoxia exposed rats for 1 week and two weeks accordingly (**Figure 6.3.1**, *p value* < 0.01). This suggests that chronic hypoxia induced right ventricular hypertrophy in rats which is augmented by the longer duration the animal remained in the hypoxic environment.

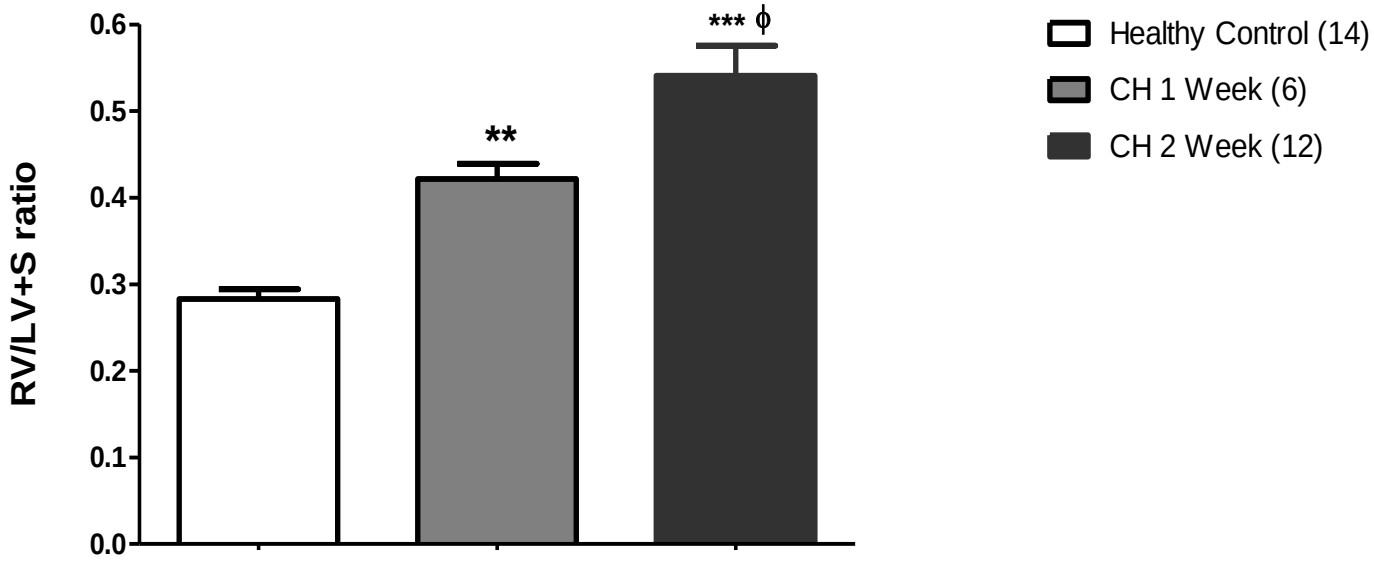


Figure 6.1: Shows effects of chronic hypoxia on right ventricular hypertrophy. Rats were divided into three groups: healthy controls, chronic hypoxia for 1 week and chronic hypoxia 2 weeks. The hypoxia groups were subjected to fixed 12% oxygen for either 1 or 2 week(s) whereas healthy control remained at room air.
*** Vs** Control
ϕ Vs CH 1 Week
CH =chronic hypoxia**, RV=** right ventricular**, LV+S=** left ventricular + Septum

6.3.2 Influence of Chronic Hypoxia for 1 Week (IL–8 for 1hr) on Isolated PA Vasoconstriction and Vasodilatation

Exposing rats to chronic hypoxia (CH) for 1 week followed by treatment with or without IL–8 for 1 hour (±IL–8, 1hr) in the myograph chamber did not cause any significant difference in the thromboxane A_2 agonist generated pretone. This implies that IL–8 had no significant effect on the U46619 mediated vasoconstriction in rats subjected to chronic hypoxia for 1 week (**Figure 6.3.2,** *p value* > 0.05).

Our results showed that CH for 1 week ±IL–8 (for 1hr) did not significantly affect CC–mediated endothelial vasodilatation in rats (**Figure 6.3.3 A & B,** *p value* >0.05). In addition, it has been shown that neither CH treatment for 1 week nor IL–8 exposure for 1 hour in vitro, had any significant effect on the SNP–mediated endothelial independent vasodilatation in rats (**Figure 6.3.4 A & B,** *p value* > 0.05).

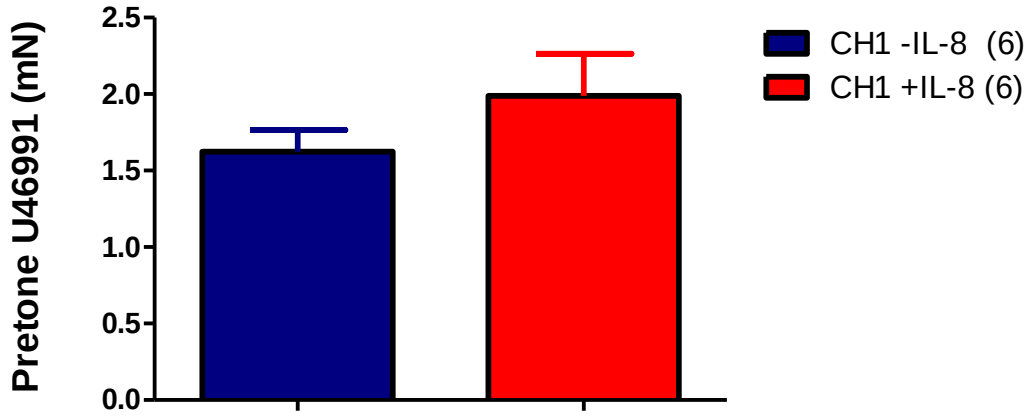


Figure 6.2: Demonstrates influence of hypoxia (1 week at 12% O_2) ±IL–8. Rats exposed to hypoxia (12%) for 1 week. PA vessels were isolated and mounted on myograph then subjected to IL-8 treatment for 1 hour and pretone was generated with U46619 (400nM). IL-8 and hypoxia had no significant effect on the pretone, p value >0.05; n=6.

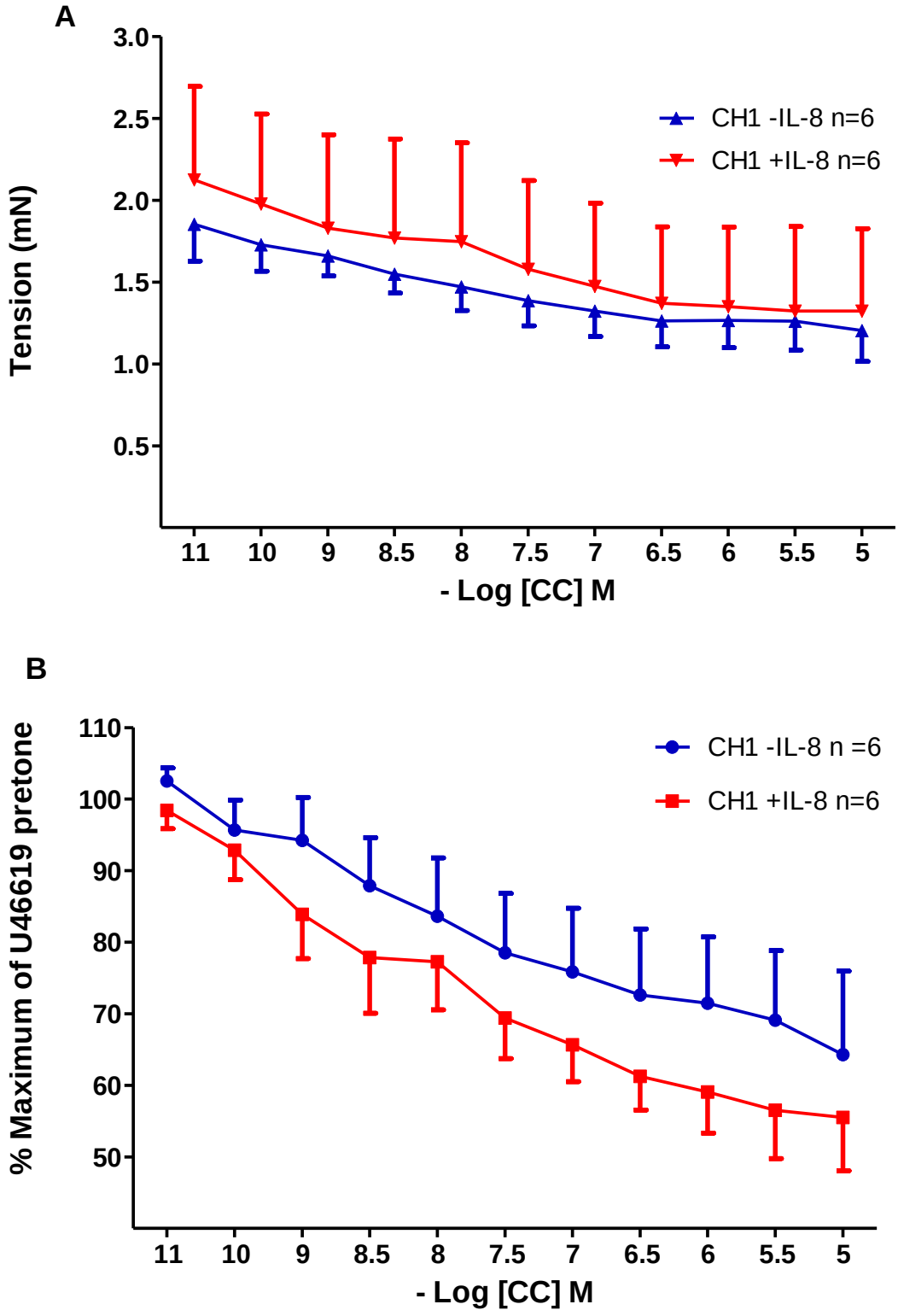


Figure 6.3: **Shows effects of CH ±IL–8 on CC-mediated vasodilatation in isolated PAs.** Rats exposed to hypoxia (12% O_2) for 1 week. PA vessels were isolated and mounted on myograph and then subjected to IL-8 treatment for 1 hour and pretone was generated with U46619 (400nM) followed by CC-mediated vasodilation. CC dose responses taken as percentage of the pretone. **CH1**=chronic hypoxia for 1 week.

A) Shows absolute tension by CC-mediated vasodilatation

B) Shows CC-mediated vasodilatation (as %Max of pretone, U46619) p value >0.05, n=6.

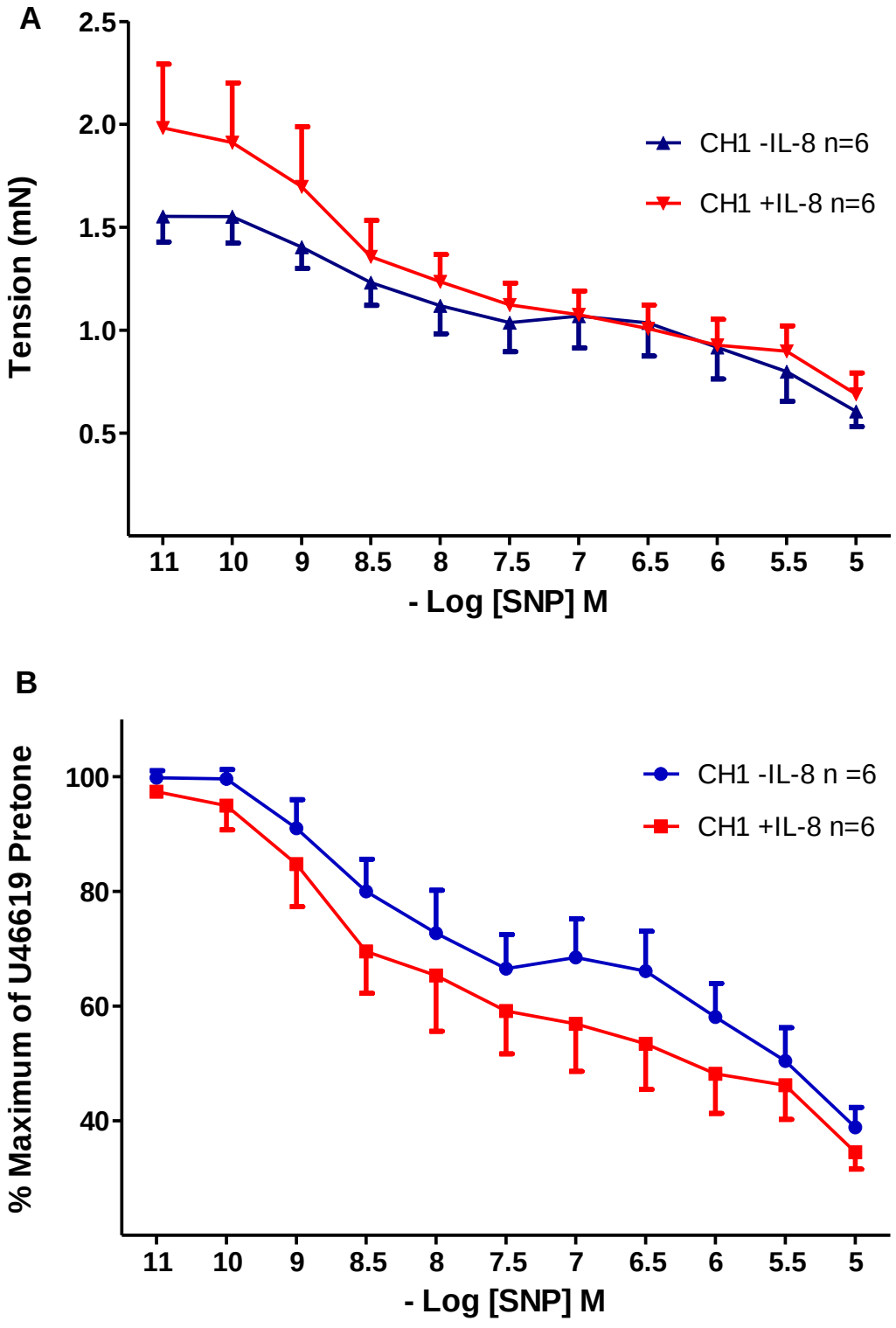


Figure 6.4: **Shows effects of CH ±IL–8 on SNP-mediated vasodilatation in isolated PAs.** Rats exposed to hypoxia (12% O_2) for 1 week. PA vessels were isolated and mounted on myograph and then subjected to IL-8 treatment for 1 hour and pretone was generated with U46619 (400nM) followed by CC-mediated vasodilation. CC dose responses taken as percentage of the pretone. **CH1**=chronic hypoxia for 1 week.

A) Shows absolute tension by SNP-mediated vasodilatation

B) Shows SNP-mediated vasodilatation (as %Max of pretone, U46619) p value >0.05; n=6

6.3.3 Influence of Chronic Hypoxia for 2 Weeks (± TNF-α or IL-8 for 1hr) on Isolated PA Vasoconstriction and Vasodilatation

We have also exposed rats to 12% oxygen for 2 weeks then rats euthanized and pulmonary arteries isolated and mounted on myograph. Our results showed that CH treatment for 2 week ±TNF–α exposure for 2 hours did not significantly affect CC–mediated endothelial vasodilatation in rats (**Figure 6.3.5,** *p value* >0.05). Also, our data has shown that neither CH treatment for 2 weeks nor ±TNF–α exposure for 1 hour in vitro, had any significant effect on the SNP–mediated endothelial independent vasodilatation in rats (**Figure 6.3.6,** *p value* > 0.05). We have also shown ±TNF–α did not alter KCL or U46619 mediated vasoconstrictions in 2 weeks CH exposed rats (Figure not shown).

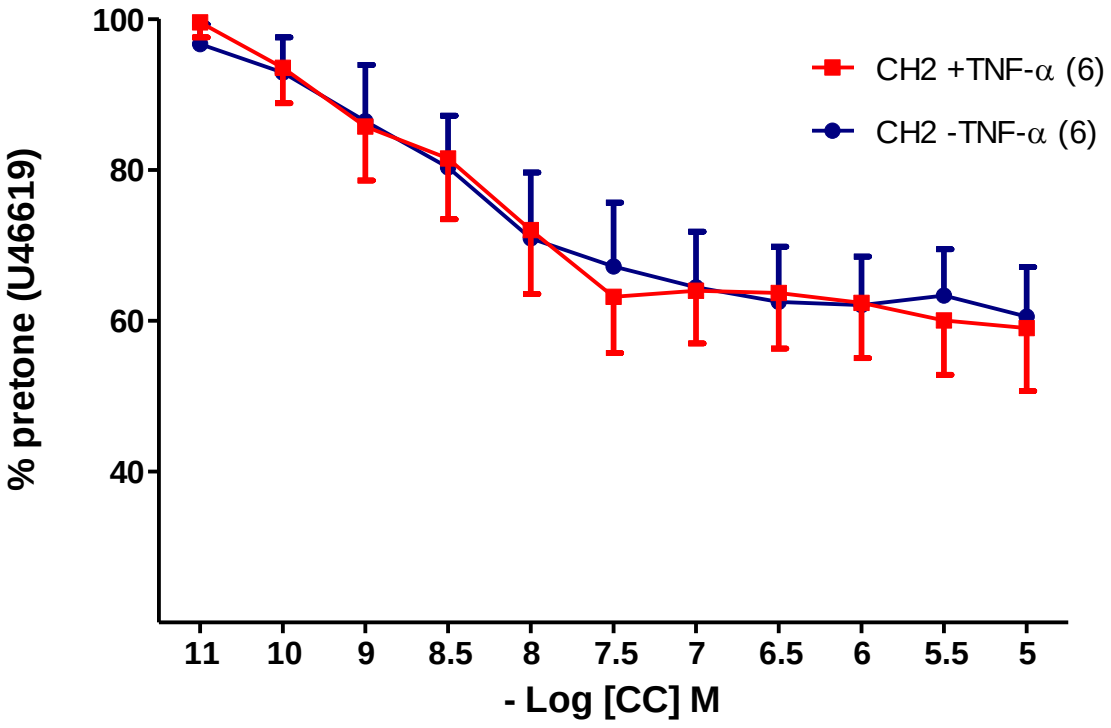


Figure 6.5: Shows CC-mediated vasodilatation in rats exposed to chronic hypoxia (2 weeks) ± TNF-a (in vitro). Rats were exposed to hypoxia (12% O_2) for 2 weeks. PA vessels were isolated and mounted on myograph and then subjected to IL-8 treatment for 1 hour and pretone was generated with U46619 (400nM) followed by CC-mediated vasodilation. CC dose responses taken as percentage of the pretone. Neither CH nor TNF-α had any significant effect on vasodilatation. **CH2**=chronic hypoxia for 2 weeks.

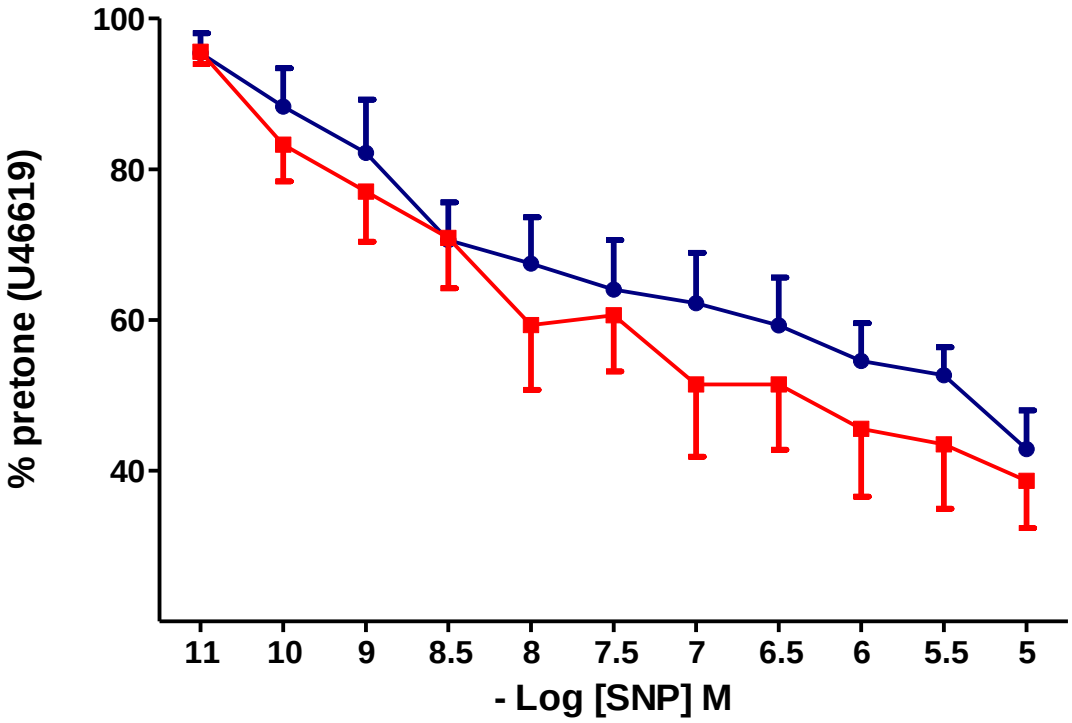


Figure 6.6: Shows SNP-mediated vasodilatation in rats exposed to chronic hypoxia (2 weeks) ± TNF-α (in vitro). Rats were exposed to hypoxia (12% O_2) for 2 weeks. PA vessels were isolated and mounted on myograph and then subjected to IL-8 treatment for 1 hour and pretone was generated with U46619 (400nM) followed by CC-mediated vasodilation. CC dose responses taken as percentage of the pretone. Neither CH nor TNF-α had any significant effect on vasodilatation. **CH2**=chronic hypoxia for 2 weeks.

6.3.4 Influence of Chronic Hypoxia on Cytokine Profile of Bronchoalveolar Lavage in Rats

In another experimental set up, 12 rats were divided into two groups of 6. One group were placed in a hypoxic chamber set at 12% oxygen for two weeks and the other remained at room air for two weeks. AT the end of the week, the rats were euthanized by overdose of Euthatal® (pentobarbital sodium, 200 mg ml^{-1}, Merial Animal Health Ltd), and bronchoalveolar lavage collected. Then assayed to quantify cytokine level levels and compare the control with hypoxia treated animals detailed in the methods (section **2.3**). The results have shown that chronic hypoxia significantly down regulated the level of expression of cytokines such as CINC –1, CINC–2ab, sICAM–1, LIX, L–Selectin and Thymus (**Figure 6.3.7A, B & C**)

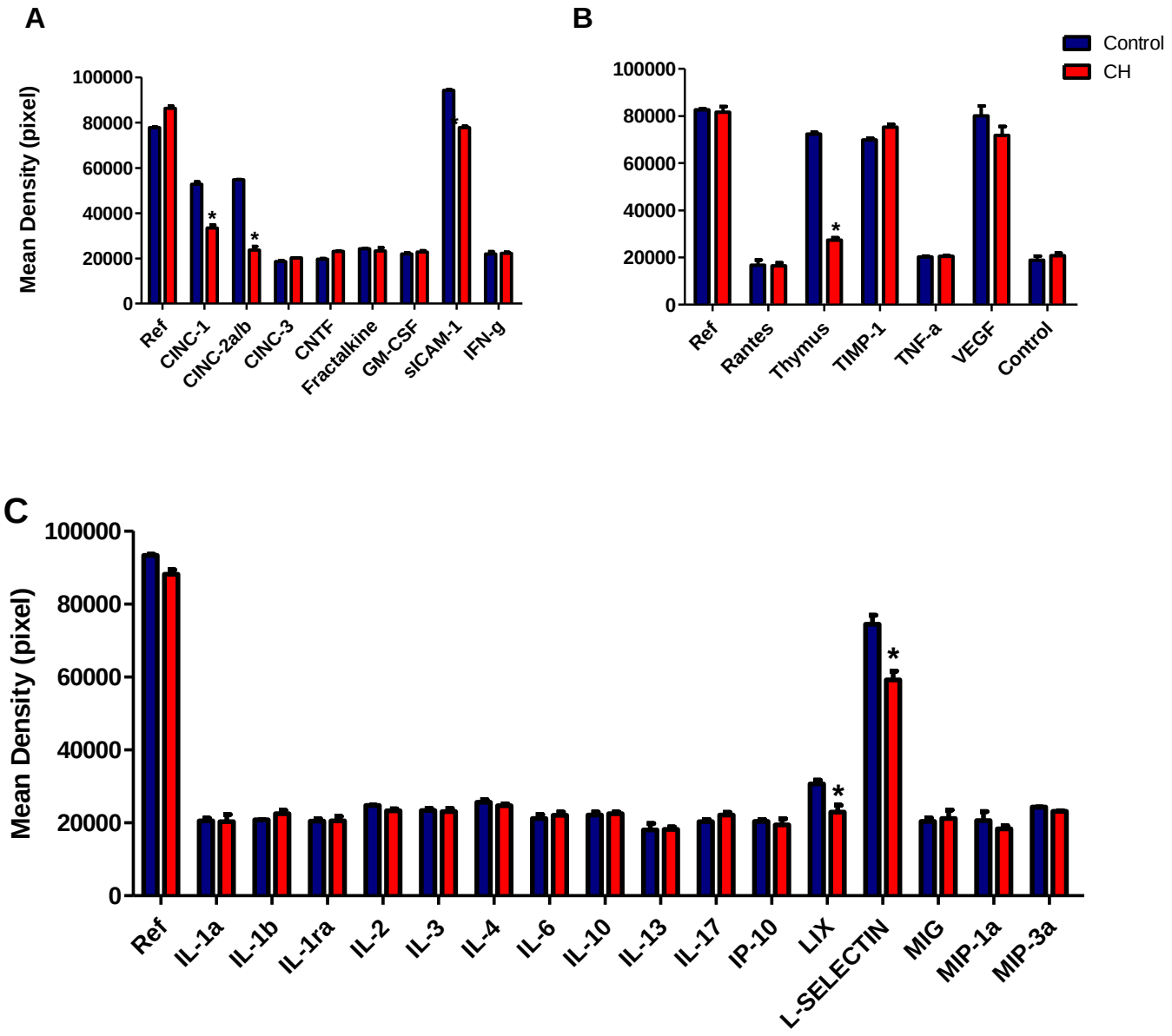
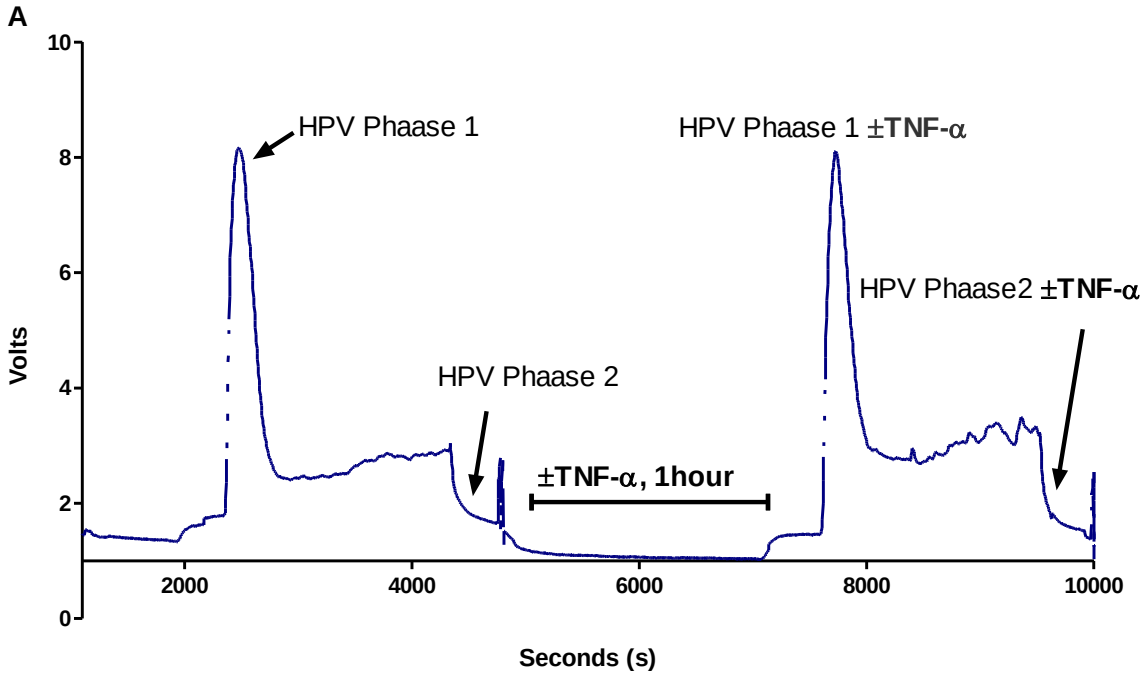


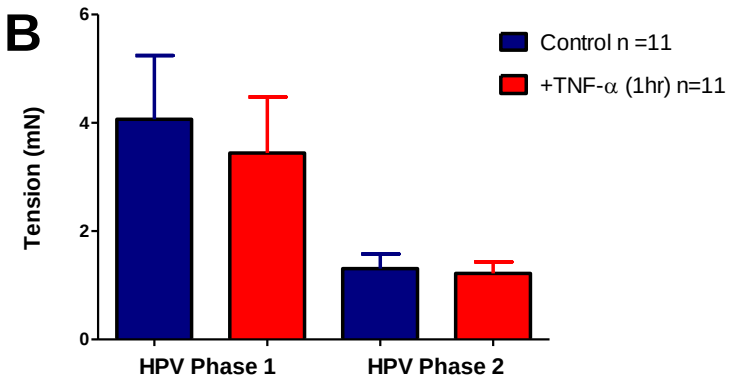
Figure 6.7: Shows influence of hypoxia on rat cytokine/chemokine (x29). Rats were exposed to hypoxia (12% O_2) for 2 weeks then euthanized with overdose of sodium pentobarbital. Bronchoalveolar lavage collected from the rats and Proteome Profiler Rat Cytokine Array Kit, Panel A (that measure level 29 different cytokine antibodies) was used to quantify level of cytokines and chemokines. **Hypoxia significantly down regulated CINC –1, CINC–2ab, sICAM–1, LIX, L–Selectin and Thymus.** ***p value < 0.01, n=6***
CH=chronic hypoxia for 2 weeks.

6.3.5 Effects of Acute TNF–α Treatment on HPV of Isolated PAs

Incubation of PA with TNF–α for 1 hour did not alter the raw tension (mN) generated in HPV phase 1 and 2 as there was no significant difference between the control and

the treated vessels (**Figure 6.3.8A**). Similarly, the results have shown that treatment with TNF–α has not caused any significant effect on the HPV phase 1, though constriction in the TNF–α treated vessel was less. Similarly the same vein, there was no significant different in HPV phase 2 vasodilatation observed between TNF–α treated and controls (**Figure 6.3.8B**).


A
Volts
10
8
6
4
2
0
HPV Phaase 1
HPV Phaase 1 ±TNF-α
HPV Phaase2 ±TNF-α
HPV Phaase 2
±TNF-α, 1hour
2000
4000
6000
8000
10000
Seconds (s)


B
Tension (mN)
6
4
2
0
Control n =11
+TNF-α (1hr) n=11
HPV Phase 1
HPV Phase 2

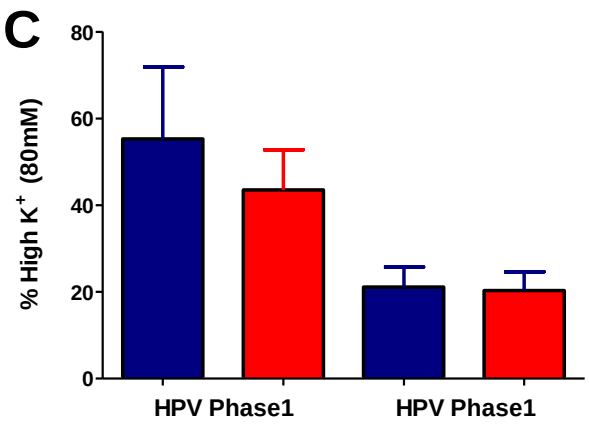

C
% High K+ (80mM)
80
60
40
20
0
HPV Phase1
HPV Phase1

Figure 6.8 A&B: **Shows the influence of acute TNF-α treatment on hypoxic pulmonary vasoconstriction**. Isolated rats PAs were mounted on two channel myograph. High K^+ response performed and then HPV generated twice prior to TNF-α exposure for 1 hour (one chamber) and the HPV repeated (TNF-α treated & control). HPV phase 1 and 2 were expressed as percentage of the high K^+ (80mM) for both the control and TNF-α treated.

A) Sample Raw trace of HPV

B) Shows raw tension of HPV phase 1 and 2 ±TNF- α, *p values* >0.05

C) Shows HPV phase 1 and 2 ±TNF-α, *p value* >0.05

6.3.6 Effects of Concurrent Hypoxia ± Acute TNF-α Treatment on Isolated PAs in Vitro

Myograph mounted PA vessels isolated from healthy control rats were subjected to hypoxia (95% N2, 5% CO2, 37^{o}C) plus TNF –α at the same time for 1 hour. This treatment had no effect on the pretone generated (**Figure 6.3.9A**). However, hypoxia plus TNF–α together had significant effect on both endothelium-dependent and endothelium-independent vasodilatations mediated by CC and SNP respectively (**Figure 6.3.9B & C**).

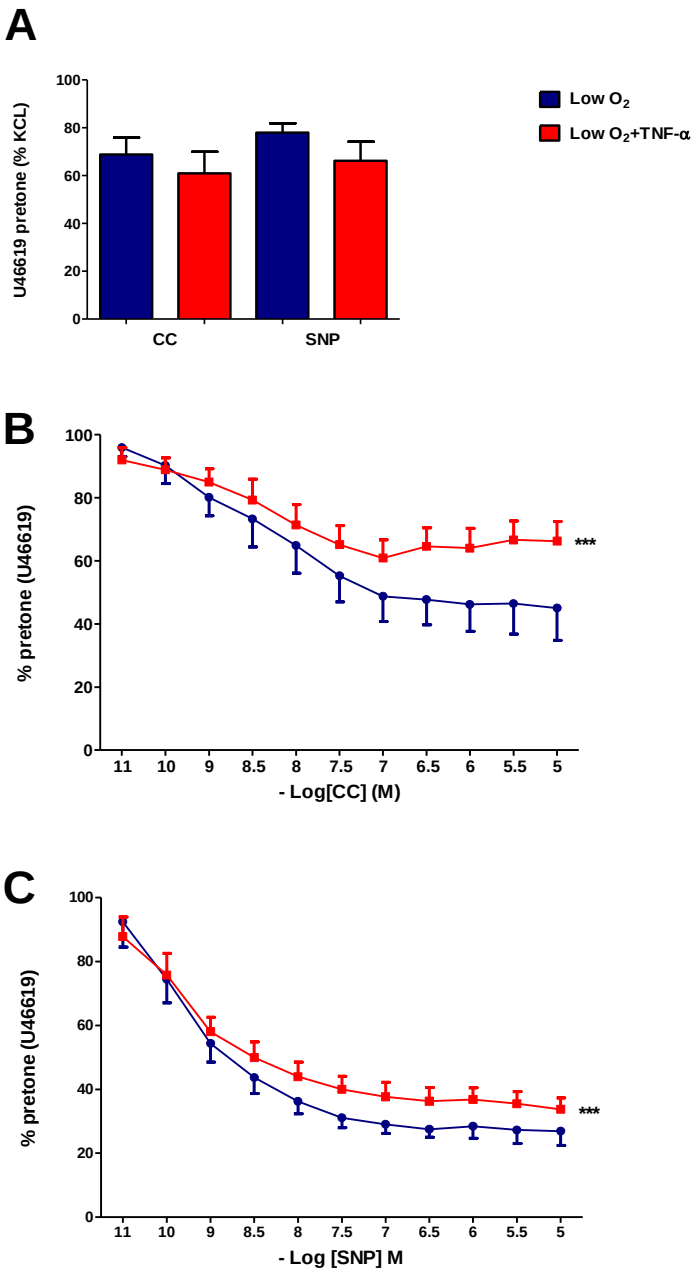


Figure 6.9 A-C: Shows concurrent effects of hypoxia AND TNF–α on isolated rat PAs (*in vitro*). PAs were mounted on myograph. High K^+ (80mM) response tested. Vessels exposed to hypoxia (95% N_2, 5% CO_2, 37°C) plus or minus TNF-α for 1 hour simultaneously then U46619 pretone and cumulative dose response curve generated for CC and SNP for hypoxia ±TNF-α.

A) U46619-mediated vasoconstriction expressed as percentage of high K^+ (*p value* > 0.05, CC n=7, SNP n=6)

B) CC mediated vasodilatation expressed as percentage of U46619 pretone (*p value* <0.0001, n=7)

C) SNP mediated vasodilatation expressed as percentage of U46619 pretone (*p value* <0.0001, n=6)

6.4 Chapter Discussion

6.4.1 Summary of Key Findings

6.4.1.1 Right Ventricular Hypertrophy

There was a significant difference in RV hypertrophy between healthy control, 1-week hypoxia and 2 weeks hypoxia thus indicating that the healthy rats have adapted to hypoxic medium.

6.4.1.2 Myograph data of 1 week chronically hypoxic rats

1. CH1 ±TNF–α had no effect on a selective α1-adrenergic receptor agonist mediated vasoconstriction in pulmonary arteries. Both percentage maximum of vasoconstriction and specific tension data suggested no significant difference the animal were treated with CH with or without TNF–α.
2. Similarly, CH1 ± IL–8 OR TNF–α had no significant effect on thromboxane A_2 receptor agonist mediated vasoconstriction in pulmonary artery segments.
3. Following hypoxia treatment for 1 week and PE pretone, ±TNF–α had no significant effects on either PE vasoconstriction or CC vasodilatation in PAs (data not shown). There was no significant difference in specific tension due to TNF–α in PAs.
4. Following hypoxia treatment and U46619 pretone generation, both TNF–α and IL–8 had no effect on CC or SNP mediated vasodilatation in PAs.

6.4.1.3 Myograph results of 2 weeks chronically hypoxic rats

1. Hypoxia treatment of rats (for 2 weeks) had not significantly altered CC–mediated endothelial dependent or SNP–mediated endothelial independent vasodilatations in isolated rat PAs.

2. TNF–α (1 hour) had not significantly augmented chronic hypoxia to effect changes in CC–mediated endothelial dependent vasodilatation and SNP–mediated endothelial independent vasodilatation in isolated rat PA segments.
3. Neither CH treatment for 2 weeks nor TNF–α (1 hour) addition had any significant effects on thromboxane A_2 agonist mediated vasoconstriction in isolated PAs.

6.4.1.4 *Bronchoalveolar lavage of 2 weeks chronically hypoxic rats*

Chronic hypoxia treatment for 2 weeks has significantly down regulated the level of expression of cytokines such as: CINC–1, CINC–2ab, sICAM–1, LIX, L–Selectin and Thymus.

6.4.1.5 *Acute inflammatory challenge on HPV*

HPV ±TNF-α treatment (1 hour) had no significant effect on HPV phase 1 and phase 2, suggesting that acute inflammation had no effects on HPV.

6.4.1.6 *Concurrent acute hypoxia and inflammation on pulmonary vascular reactivity*

Hypoxia treatment of isolated pulmonary arteries ±TNF–α has altered both the endothelium–dependent and endothelium–independent vasodilatations mediated by CC and SNP respectively.

6.5 Discussion

Our findings from the chapter have confirmed that chronic hypoxia induces a right ventricular hypertrophy. This has been shown in literature and in different species (483). Minamino *et al* (483) have shown that the ratio of the right ventricle weight to left ventricle plus septum weight [RV/(LV + S)] to be significantly increased with 48 hours up to 3 weeks of exposure to hypoxia (8–12% O2 environment) in healthy mice. The increase was in the RV hypertrophy was exhibited in a time dependent

manner and attained plateau at 2 weeks of hypoxia (483). This RV to LV plus septum ratio was used to verify development of RV hypertrophy and adaptation of animal to hypoxia (483). Right ventricular hypertrophy suggests pulmonary hypertension that develops due to right ventricle pressure overload (483). Our findings have revealed that chronic hypoxia for both 1 and 2 weeks had no significant effects on the both the high K^+ response and thromboxane A_2 mediated vasoconstriction. It would not be expected that chronic hypoxia would alter high K^+ response; however, we expected that hypoxia might augment pulmonary vasoconstriction in this model. IL–8 or TNF–α addition slightly increased the vasoconstriction to chronic hypoxia but the effect was not statistically significant.

Moreover, chronic hypoxia for 1 or 2 weeks and with or without IL – 8 or TNF–α (for 1 hour) had no significant effect on neither the endothelium dependent vasodilatation, nor on the endothelium independent vasodilatation mediated by CC and SNP respectively. Adnot *et al* (240). have shown that acetylcholine caused reduction of pulmonary artery pressure in lungs excised from rats exposed to hypoxia for 1 week (240). However, this vasodilatory effect of was not observed in the lungs of the rats exposed to hypoxia for 3 weeks, hence a continuous rise of pulmonary artery pressure was noted. This group further showed that the absence of both the receptor mediated (acetylcholine) and non-receptor (ionophore A23187) mediated endothelium dependent vasodilatation in rats exposed to 3 weeks hypoxia was reversed when the animal were allowed to recover at room air for 48 hour (240).

In contrast to our findings, a recent publication has shown that rats exposed to 2 weeks hypobaric hypoxia developed pulmonary hypertension as results indicated a significant increase of the mean pulmonary artery pressure in chronic hypoxic rats

compared to the controls (490). This difference from our results seen might be attributed to difference in the experimental setup as well as use of hypobaric hypoxic chamber in their study rather than the normobaric hypoxic chamber used in our study. This might be due to the fact that both hypobaric pressure at normal air and oxygen- poor air at normal pressure can stimulate to animals exposed the environment. These treatments have the tendency to cause muscularization of resistance vessels that are hypersensitive to limited or low oxygen levels (491). The muscularization of pulmonary arteries or arterioles due exposure to hypoxia and vasoconstriction is a process that can be reversed with the restoration of normal oxygen levels as demonstrated by several studies (240, 490, 491). Hence, the phenomenon might act as an adaptive measure to increased pulmonary artery pressure (491). It came as surprise that 2 weeks chronic hypoxia exposure of rats did not significantly alter vasodilatation in our current experiment. Whether this was due to insufficient length of exposure to hypoxia or recovery of rats once the animal were taken out of hypoxic chamber into room air could not be elucidated. Though, we suggested it might be due the former proposition. Interestingly, development of hypoxaemia in patients with COPD is indeed associated with vascular changes, including medial hypertrophy of muscular pulmonary arteries, as well as migration and proliferation of vascular smooth muscle cells into normally non-muscular vessels of the pulmonary circulation (470).

Chronic hypoxia has been known to upregulate the expression of pro-inflammatory cytokines (492). Lam *et al.* (493) has reported that chronic hypoxia stimulated increased expression of pro-inflammatory cytokines such as interleukin (IL)–1 receptor, IL–6 receptor, IL–1β, IL–6 and TNF–α in carotid body of rats exposed to chronic hypoxia (493)

Previous studies have revealed the extensive contribution of inflammatory mediators to vascular dysfunction in both chronic lung disease in humans and animal models (3, 42, 52, 63, 68, 125, 372, 376, 409, 429, 430, 449, 450, 494-496). This has been discussed in **chapter 5** in the introduction and discussion sections. In this chapter, as mentioned at beginning, we aimed to investigate influence of inflammation and hypoxia on pulmonary vascular reactivity of rats in vitro. This was essential to exclude other factors that might be influencing the response in vivo. Hence, it might reduce confounding factors. Surprisingly, just like chronic hypoxia for 1 or 2 week(s) had no significant effect on vascular dilatation, addition of TNF–α or IL–8 (for 1 hour), did not induce any significant alteration on the pulmonary vascular relaxation. Though it came out negative, this is the first time the interaction of hypoxia and inflammation had been tested on isolated pulmonary artery vasodilatation in vitro. In addition, chronic hypoxia with or without TNF–α or IL–8 had no significant effect on the pretone generated by thromboxane A_2 or KCl response.

A synchronised contribution of several mediators such cytokines, chemokine and adhesion molecules are responsible for the regulation of lung inflammatory response (484). Leucocyte migration via the endothelium is facilitated by the activities of cell adhesion molecules like selectins, integrins and immunoglobulin gene super family (484, 497). In addition, cytokines and chemokines play a key role in leukocyte chemoattractant process and coordination of the complex cellular interaction involved in the cell migration. Besides, chemokines are also known to mediate various stages of migration process of leukocyte within the endothelium (484, 497).

Continuing on the investigation of the interaction of hypoxia and inflammation to cause vascular dysfunction, we had subjected rats to chronic hypoxia for two weeks and then collected bronchoalveolar lavage from the rats and subsequently measured

cytokine levels. The outcome of our investigations indicated that there was a significant difference between the chemokine levels found between the normoxic control and normobaric hypoxic chamber treated rats. Rat cytokine-induced neutrophil chemoattractant-1 (CINC-1) protein and cytokine-induced neutrophil chemoattractant-2-alpha/beta (CINC-2-αβ) were significantly downregulated in normobaric hypoxic chamber treated compared to the normoxic control rats. These CINC proteins as their names signify play a critical role in neutrophil chemotaxis process and neutrophils comprise of major proportion of leukocyte (white blood cells) blood and probably the first leukocyte to respond to infection or inflammation within the body. Hence, decrease of these CINC chemokines in chronic hypoxic environment might diminish the potential of the body to fight against and infection and inflammation thus allowing inflammation to progress without a proper check.

Also, the current study has showed a significant decrease of soluble intercellular adhesion molecule (sICAM)–1, lipopolysaccharide-induced CXC chemokine (LIX) and L–Selectin; adhesion molecule, in the BAL of rats subjected to chronic hypoxia compared to the control. Soluble ICAM-1 is the form of ICAM-1 (CD54) in the circulation which might be either be constitutively expressed or could be stimulated on the cell surface of various cell line (498). ICAM-1 structurally is classed among the immunoglobulin superfamily and predominantly acts as counter receptor for the lymphocyte function-associated antigen (LFA-1) thus the interaction between ICAM-1 and LFA-1 enables leukocyte adhesion and migration along the endothelium (498). LIX is a pro-inflammatory chemokine which plays an important role in neutrophil movement along the endothelium during leukocyte migration. In addition, L-selectin is known to be accountable for constitutive regulation of lymphocyte movement to the lymph nodes and payer’s patches as well as lead lymphocytes and neutrophils to

the location of inflammation (499). Together, blocking these pro-inflammatory chemokines and adhesion molecules might influence inflammatory events due to the impaired production of mediators we have reported here (499). For example; L-selectin deficient mice (CD62L−/−) offers a valuable alternative to investigate CD62L contribution within the inflammatory settings in comparison to the L-selectin sufficient mice. Among the host of dysfunction observed in the L-selectin deficient mice was reduced T cell proliferation and cytokine expression (499). Therefore, the knockout mice data suggests that L-selectin plays a crucial role in trafficking the leukocytes to the location of the inflammation (499, 500). Moreover, our current investigation has shown diminished level thymus production in the BAL of rats exposed to chronic hypoxia compared to the control. Thymus is a dedicated primary lymphoid organ of the immune system and within it, T lymphocytes develop is mature. This development and maturation of the T cells is critical for the maintenance of the adaptive immune system that enables the body to identify and fight foreign bodies.

Furthermore, the impact of TNF–α on HPV was assessed in this chapter. In patients with lung disease and those who had to use one-lung ventilation, HPV fixes mismatch in lung ventilation-perfusion ratio (485, 501). This is brought about through the reflex constriction of pulmonary vascular smooth muscle cell's reaction to diminished local partial pressure of oxygen (PO_2) (501). Our results demonstrated similar pattern to the bi-phasic pattern reported in literature (501). However, we have not observed any significant effect of TNF–α on HPV as both phase1 and phase 2 were not affected by the treatment. Regardless of lack of observing a desired outcome, this phenomenon has not been reported in the literature before. It appears as if there might have been an effect, however, the results were variable and the different has not reached significance. It might be sensible to carefully repeat the

experiment to confirm our findings. Investigating HPV is complex process due variety of associated biological mechanisms and this can cause between studies discrepancies in whole animals, isolated lungs, vessels or cells (205, 501). It was suggested that data derived from animal investigations of HPV contain substantial variability between species as well as the length of hypoxia period thus making it challenging to compare different studies even from the same species (501). In addition, investigating HPV in humans is also challenging due to complication associated of measuring the pulmonary arterial pressure (PAP), the impact of pathology and drugs (501).

In another group of animals, we have shown that concurrent treatment of isolated PA vessel with acute hypoxia plus TNF–α has significantly altered pulmonary vasodilatation. Both endothelium-dependent and endothelium-independent vasodilatations were affected by the inflammatory mediator. This is the first time such an observation has been reported in pulmonary vessels. As hypothesised, we suggest that there might be an interaction between hypoxia and inflammation to induce vascular dysfunction in the pulmonary artery. Thought some of the earlier results obtained not indicate this link, with a slight changes of experimental setup (by simultaneously treating PA segments with hypoxia and inflammation), we are able to demonstrate this notable association. Future studies should investigate the mechanisms involved in this interaction between hypoxia and inflammation leading to pulmonary vascular dysfunction. Hence, it will increase our understanding regarding pulmonary artery dysfunction and open new therapeutic avenues to be tested especially where chronic lung disease is concerned.

6.5.1 Limitations

1. No test conducted to check whether manipulation during experiment have caused endothelial damage. Though the process was handled with care n caution to ensure vessel integrity
2. Some of the pulmonary arteries isolated from CH1 rat did not respond to phenylephrine as pretone though the same phenylephrine stock stimulated a pretone in systemic iliac arteries isolated from the same CH1 rats. This was rather unexpected, however, the experiment was repeated with U46619 thromboxane A_2 agonist as pretone and pulmonary vessels responded.
3. The temperature of the myograph chambers might not be properly regulated as expected (as discussed in detail in **chapter 5**)**.** The chambers were allowed to reach optimum temperature (37^0C) before any protocol is started and the machine was calibrated once every month to maintain consistency of the data generated. This limitation does not seem to affect data generated, as the results were consistent for individual vessels types.
4. The effect of hypoxia on the animal might seem to disappear once the rats were out of the hypoxic chamber. In order to ensure the animal did not recover before the protocols were completed. We ensure the protocols were short and the times for dissection and isolation process were kept short. Besides, it has been shown that rats recovered from hypoxic treatment after 48 hour recovery period and our protocol time is less than 3 hours.
5. All the other limitations mentioned in **chapter 5 are relevant to the results in this chapter.**

Chapter 7

7 General Discussion

Inflammation and hypoxia can both play a crucial role in vascular dysfunction that is associated with the pathogenesis of a variety of vascular, cardiovascular and respiratory disorders such as arterial stiffness, hypertension, myocardial infarction, stroke, COPD and many others. This thesis explored the role of inflammation and hypoxia on rat vascular reactivity as a model of vascular complications associated with COPD. Additionally, we examined the effects of hypoxia and inflammation on arterial stiffness in AATD subjects genetically predisposed subjects to COPD.

7.1 Do Hypoxia and Inflammation Interact to Effect Changes in Arterial Stiffness in Patients with α1–antitrypsin Deficiency and on Isolated Rat Iliac Artery Vascular Reactivity?

Stiffening of arteries can occur as a consequence of aging and/or arteriosclerosis and increased stiffness of the arteries can determine cardiac work rate. Arterial stiffness is therefore a potentially adjustable factor to predict cardiovascular events. Hence, increased arterial stiffness has been linked to increased risk of cardiovascular events such as stroke and myocardial infarction, the two leading cause of mortality in developed countries. Increased arterial stiffness was also suggested to be elevated in COPD patients. However, this increase was not dependent on the smoking status but did correlate inversely with lung function and directly linked to emphysema severity. Therefore, arterial stiffness is a target of pharmacological and exercise intervention in COPD. PWV is the gold standard for measuring arterial stiffness and this can be measured from the aorta or carotid artery (417).

We have shown (see **Chapter 3)** that arterial stiffness positively correlated with age, systolic pressure, pulse pressure and CO. However, arterial stiffness was inversely correlated to FEV1, FEV1 percent predicted, total peripheral resistance, TLCO and TLCO percent predicted. Although early studies were focused on diastolic pressure, current practise recognises that systolic and pulse (systolic minus diastolic) pressures contribute a greater role, particularly with evolving age. Thus, for decades blood pressure has been known as a vital risk factor for coronary, cerebrovascular and cardiovascular diseases (106, 420, 424, 502). Our current findings and those of others suggest that the systolic pressure is influenced by arterial stiffness and might advance continually with age (420). This illustrates that our observation is in line with the knowledge currently available. The fact that our current data reported strong positive correlation with haemodynamic parameters such as systolic pressure, pulse pressure and cardiac as expected, confirmed a known relationship, given the role arterial stiffness play as an independent predictor of cardiovascular events and mortality in disease such as myocardial infarction (424). A similar contour was observed for myocardial infarction and blood pressure (420, 425). Building on these associations, an increased arterial stiffness could potentially cause high blood pressure, directly or indirectly. Therefore, we suggest that pharmacological modulation of the blood pressure might alter arterial stiffness. Interestingly, in a recently concluded review of hypertensive trials by Dudenbostel and Glasser (426), suggested that angiotensin converting enzyme inhibitors, calcium blockers and mineralocorticoid receptor antagonists significantly reduced arterial stiffness and central blood pressure. However, some beta blockers such as atenolol might stimulate an opposite effect. Dudenbostel and Glasser (426) have also, suggested that most of studies on beta blockers have examined the effects of atenolol and

there is insufficient data available regarding the effects of vasodilating beta blockers (426).

More importantly, we have found arterial stiffness to be positively correlated with TNF–α. This was a novel finding, as it has not been demonstrated before with this patient population. This suggests that there is an association between arterial stiffness and TNF–α that support our hypothesis. Similar to this observation, Sabit et al (418) have demonstrated a weak correlation between arterial stiffness and sTNFR1 and IL–6, although no link was shown between PWV and sTNFR2 in COPD patients. However, the same study suggested no link between PWV and soluble tumour necrosis factor receptor 2 (sTNFR2) (418). Yasmin *et al*. have also shown that in healthy individuals, pulse wave velocity was associated with another inflammatory maker, CRP (420). CRP is mainly used in clinical investigations as a measure of inflammation in the blood. These findings coupled with our own suggest a role for inflammation in arterial stiffness. Moreover, we found that there is no correlation between arterial stiffness and IL–8 and IL–1β. However, both TNF–α and IL–8 were found to be inversely correlated with total peripheral resistance and pack years respectively.

Contrary to our findings, evidence from the literature indicates an association between various systemic inflammatory markers and arterial stiffness. Vanfleteren et al. (409), have suggested that arterial stiffness in COPD subjects had no association with systemic inflammation. This alternative finding has been extensively discussed previously (see **Chapter 3**). However, according to Vanfleteren *et al*.(409), a quarter of 213 eligible COPD patients were excluded from their investigation due to unsuccessful aortic PWV measurement. These patients with unsuccessful aortic

PWV measurement were found to have significantly elevated BMI, fat free mass index, triglycerides, glucose and diffusion capacity of the lung for carbon monoxide (DLCO) compared to those where aortic PWV measurements were obtained. This might therefore render their results inconclusive as far as a COPD population is concerned.

We investigated the impact of decreases in PO_2, to understand the role of hypoxia in COPD where the need for use of long-term oxygen therapy and/or ambulatory oxygen in often seen in this subset of patients. Contrary to our expectation, we established that there was no correlation between arterial stiffness and PO_2, No previous literature had shown any relationship (positive or negative) prior to this demonstration. Interestingly, and unexpectedly, PO_2 correlated proportionally with almost all lung function measure (such as FEV1, FEV1 percent predicted, FEV1/FVC ratio, FVC, FVC percent predicted), lung gas transfer factors (such TLCO and TLCO percent predicted) and some cardiovascular measures (such as pulse pressure, and aortic pulse pressure).

In our multivariate linear regression model the entered independent variables included age, sex, TNF–α, PO_2, systolic pressure, FEV1 and TLCO, whereas the PWV was added as dependent variable. The overall outcome showed that these variables together can account for about 50% of variability observed in the dependent variable. Individually, among the dependent variables, only age and systolic pressure could significantly predict PWV. However, the regression analysis suggested that our model had a significant explanatory power to predict the dependent variable. Hence, the co-efficient of the independent variables was not zero and thus the model can predict PWV. Other models were explored to

investigate which variable predict PWV best. However, these models accounted for less variability in the dependent variable than the one reported above.

We analysed for a possible biological interaction between TNF–α and PO_2. This was to account for what might happen in vivo. Our analyses indicated that there was no interaction between TNF–α and PO_2.

Following incubation of isolated systemic iliac artery vessels with TNF–α for 1 hour, we found that TNF–α significantly altered vascular relaxation in isolated iliac artery. This has not been shown in iliac artery prior to this observation. Elevation of the levels of pro-inflammatory cytokines (such as TNF–α) has been associated with endothelial dysfunction in different cardiovascular diseases such as congestive heart failure, atherosclerosis, septic shock, diabetes, hypertension and aging (366-371). The inflammatory mediators might influence impairment of the endothelium-dependent relaxation there by precluding the endothelium response to circulating autacoids or hormones. Therefore, this makes the vessel susceptible to spasm, thrombosis and/or atherogenesis (372).

So far our data has confirmed that link between inflammation and systemic vascular related disease pathogenesis in both human and rats. TNF–α was associated with arterial stiffness as well as endothelial dysfunction in AATD patients and healthy iliac arteries isolated from rats. Hence, our human and animal data were in agreement with regard to TNF–α role in vascular disease pathogenesis. The association between inflammatory mediators and vascular dysfunction has been widely recognised in the literature. This result confirms that TNF–α might influence endothelial function in systemic iliac arteries isolated from Wistar rats. Similarly, Wimalasundera *et al.* (376) has demonstrated that in mesenteric arteries pre-

contracted with PE, TNF–α caused impairment of acetylcholine mediated vasodilatation by significantly shifting the dose-response curve of acetylcholine to the right. In addition, TNF–α induced impairment of bradykinin mediated relaxation by significantly reducing the concentration-response curve (376).

Furthermore, combination treatment of isolated iliac arteries to chronic hypoxia (1 week at 12% O_2) and TNF–α (1 hr in vitro) had not augmented the effect observed with TNF–α alone. Again, though contrary to our expectations, the human and animal data were in accordance. This was due to fact that we have not observed an interaction between hypoxia and inflammation in both settings. However, the animal data on chronic hypoxia might be regarded as a preliminary due to limited sample size.

7.2 Effects of Inflammation and Hypoxia on Vascular Reactivity in Isolated Pulmonary Arteries: A Model for Vascular Diseases Associated With COPD?

7.2.1 Short-term inflammatory challenge ±hypoxia (acute chronic)

The current investigations revealed no effect of short-term TNF–α (1 hr) treatment on pulmonary vasoconstriction and vasodilatation. The vasoconstriction responses of phenylephrine and U46619, as well as the CC-mediated vasodilatation were not altered by the TNF–α challenge. On the other hand, IL–8 treatment for 1 hour had a significant effect on both the endothelium-dependent and endothelium-independent vascular relaxation mediated by CC and SNP respectively. This suggests that short-term exposure to IL–8 might play a role in endothelial dysfunction in pulmonary arteries. Similarly, Gillham *et al* (451) has shown that TNF–α incubation for 1 hour caused no alteration to the endothelial–dependent vasodilation in omental arteries obtained from healthy pregnant women.

Moreover, combination of chronic hypoxia for 1 or 2 week(s) plus short term TNF–α treatment of pulmonary artery segments *in vitro* had affected neither vasoconstriction nor vasodilation in isolated pulmonary arteries. Similarly, IL–8 treatment (1 hr) did not affect vasodilatation in rats exposed to chronic hypoxia for 1 week. In addition, CH plus TNF–α or lL–8 did not alter the pretone generated by PE (selective α1-adrenergic receptor agonist) or U46619 (thromboxane A_2 agonist). Therefore, this suggests that hypoxia (1 or 2 weeks at 12% O_2) and inflammatory mediators had no influence on the vasoconstrictor response of pulmonary vessels, at least in this experimental setting. A recently concluded review by Pugliese et al (247) suggested that hypoxia has the potential to start inflammation and stimulate a significant effect on local pulmonary vascular cells. They also postulated that the pathological changes induced in an hypoxic PH animal model (up to 5 weeks exposure) and many types of PH in human generally can resolve rapidly with seemingly relative normoxia (247). It is probable that these suggestions might be applicable to our CH experiments as we have observed adaptation of the rats to hypoxia with increased RV hypertrophy but no effect on vascular reactivity.

In our study of the bronchoalveolar lavage of rats exposed to chronic hypoxia for two weeks, we found a significant reduction in the expression levels of some cytokines found. These include CIN –1, CINC–2ab, sICAM–1, Lix, L–selectin and thymus. The role of these cytokines or chemokines in the vascular function was discussed in **Chapter 6** (6.5). Our results indicated that CINC-1, CINC-2 and Lix as that are neutrophil chemoattractant were reduced in the CH rats compared to the controls suggesting a possible delay to neutrophil response to the site of infection or inflammation under chronic hypoxia conditions. Also there level of adhesion molecules such as sICAM-1 was reduced in chronic hypoxic rats that lead to

reduced leukocyte adhesion and migration along the endothelium. In addition, T cell development and maturation might be affected in chronic hypoxic condition due to reduced level thymus observed in CH rats.

Concurrently subjecting isolated rat pulmonary artery segments to acute hypoxia and TNF–α (1 hr) in vitro significantly altered both endothelium-dependent and endothelium-independent vascular relaxation. However, TNF–α (1hr) treatment had no impact on hypoxic pulmonary vasoconstriction in isolated pulmonary arteries from healthy Wistar rats. TNF–α had no significant effect on either the phase 1 or phase 2 of the HPV.

It is important to note that development of hypoxaemia in patients with COPD is associated with vascular changes, including medial hypertrophy of muscular pulmonary arteries, as well as migration and proliferation of vascular smooth muscle cells into normally non-muscular vessels of the pulmonary circulation (470) and impaired NO production promotes in situ thrombosis, which can also worsen pulmonary hypertension. In addition to its hemodynamic effects, NO might contribute a pivotal role in preventing pulmonary vascular remodeling and enlargement of the right ventricle. At least in vitro, NO regulates many functions of vascular smooth muscle cells that modulate intimal thickening of pulmonary arteries, including vascular smooth muscle cell proliferation, migration, apoptosis, and extracellular matrix formation (503, 504). NO also modulates platelet adhesion and aggregation, leukocyte recruitment and activation, and cytokine-induced endothelial cell activation (503, 504). Experimental studies also suggest that continuous inhalation of NO prevents hypoxic vasoconstriction and reduces pulmonary arterial remodeling and RV hypertrophy in rats exposed to chronic hypoxia (505, 506).

7.2.2 Prolonged inflammatory challenge

Isolated pulmonary vessels were incubated with TNF–α for 2 and 6 hours then vascular reactivity was measured with wire myography. The finding from this study showed that prolonged TNF–α (2 and 6 hrs) exposure had significantly reduced both endothelium-dependent and endothelium-independent vasodilation in the pulmonary artery segments mediated by CC and SNP respectively with the effect being augmented by the 6 hour exposure. These results suggest that TNF–α exposure might result in vascular dysfunction in rat pulmonary arteries. Our studies illustrated that TNF–α exposure impaired both endothelial and pulmonary artery smooth muscle cell NO-mediated relaxations. Similarly, TNF–α was shown to inhibit relaxation mediated by acetylcholine and bradykinin, although SNP mediated vasodilatation was not affected (376). This was discussed in detail in **Chapter 5** (**5.4**). In contrast, a similar study protocol reported no changes in endothelial–dependent vasodilatation profile in healthy omental arteries incubated with TNF–α for 2 hours (451).

7.2.3 Investigating Potential Mechanism involved in TNF–α mediated vascular dysfunction

Investigating the mechanism of TNF–α inhibition of vasodilatation in pulmonary arteries, we used allopurinol, a xanthine oxidase inhibitor and Tempol, a membrane–permeable radical scavenger. Interestingly, allopurinol completely reversed TNF–α inhibition of CC–mediated relaxation in pulmonary artery, though Tempol did not significantly affect TNF–α inhibition of CC–mediated relaxation in pulmonary artery. Our result suggests that TNF–α impaired endothelial-mediated relaxation in isolated PAs via XO mediated increase of ROS. Similarly, Kanbay et al (472) reported that allopurinol had significantly increased endothelium–dependent vasodilatation in human subjects. All the studies included in this study assessed effect of allopurinol on endothelial–dependent vasodilatation which was evaluated using flow–mediated

dilatation (FMD) and forearm blood flow (FBF) response to acetylcholine or flow-dependent flow assessment (472). Interestingly, our current findings though from different species and set-up, demonstrated a similar observation. The result suggested that TNF–α inhibition of CC–mediated relaxation in pulmonary artery was via xanthine Oxidase pathways. Xanthine oxidase is a form of xanthine oxidoreductase, an enzyme that generates reactive oxygen species. The enzyme can catalyze the oxidation of hypoxanthine to xanthin (473) . Also, it can further catalyze the oxidation of xanthine to uric acid (473). XO was shown to be elevated in the epithelial lining fluid of COPD patients compared to healthy controls (434). Another study has indicated that pro-inflammatory cytokines; TNF–α, IL–1β and IFT–γ significantly stimulated XOR gene expression in rat alveolar macrophages (474), bovine renal epithelial cells (475) and human mammary epithelial cells (476). In addition, Wright *et al*.(477) showed that infusion of IL–1 and IFT–γ in a rat model resulted in elevation of XO activity in the lungs. Considering these evidences, it appears that pro-inflammatory cytokines might play a significant role in enhancement of the XO gene expression in COPD airways (434). Furthermore, Komaki et al (434), found a strong positive association between the levels of TNF–α or IL–1β and XO activity.

On the other hand, we have showed that neither allopurinol, nor tempol significantly affected TNF–α inhibition of SNP–mediated endothelial–independent relaxation in pulmonary artery. SNP is an NO donor and acts on PASMC to mediate relaxation. The fact that SNP-mediated relaxation was impaired in isolated rat PAs as a results of TNF–α treatment, suggest that the inflammatory cytokine might impair PASMC mechanism that bring about the NO-mediated vasodilatation. Also, Kanbay and colleagues (472) indicated that allopurinol had no significant effect on the

endothelium–independent vasodilation mediated by SNP. Our results combined with other suggest that the TNF–α impairment of the endothelium-independent vasodilatation is not dependent on increase in ROS generation. Therefore, the impairment might be induced through other mechanisms (apart from the ROS mediated pathways that are normally inhibited by tempol or allopurinol) such as impairment to cGMP activation or downstream signaling component that mediates vasodilatation with the smooth muscle cells. Further research might be required to unravel this potential mechanism.

7.3 Conclusion & Future Research

7.3.1 Human

Our findings have shown a correlation between PWV and TNF–α in AATD. However, TNF–α did not independently predict PWV, whereas age and systolic pressure has the ability to independently predict PWV. The findings suggest that arterial stiffness might be related to the inflammatory mediators and that systemic inflammation could contribute to arterial stiffening. Further longitudinal studies whilst investigating an array of inflammatory mediators might be required to confirm the findings and to establish association between inflammation and arterial stiffness.

7.3.2 Animal

These data support the hypothesis that TNF–α might worsen pre-existing endothelial dysfunction as well as in intact pulmonary vessels. This is due to the observation that cytokine affected both endothelial and non–endothelial mediated vasodilatations which suggest that plasma TNF–α level could trigger endothelial dysfunction. Future studies should focus on identifying the mechanisms by which TNF–α exert its inhibitory effect on the pulmonary vascular relaxation. There was some evidence that

IL–8 might be involved in mediating endothelial dysfunction in the pulmonary arteries from rats. Due to the fact that many cytokines act in synergy with other cytokines, a possible combinational cytokine challenge might be useful in understanding the pulmonary vascular dysfunction.

To answer the question posed by the title of this thesis, namely, is our combined inflammatory and hypoxic insult an appropriate model for COPD-associated vascular disease, the answer must be conditional. Therefore, within the limits of our protocols we did see evidence of cardiac remodeling, indicative of a hypoxic response as well as evidence of cytokine-mediated effects on vascular dysfunction that was reversed by antioxidant. Contrary to our expectation, we saw little, if any, combinational effects of the two stimuli acting together when the hypoxia was delivered in vivo for 1 or 2 weeks. Thus, vascular function was not worsened by in vivo hypoxia when challenged subsequently in vitro with cytokine. However, when hypoxia was delivered in vitro for just one hour in combination with cytokine (TNF-α), we did observe vascular dysfunction that was due a reduced vasodilatory capacity. On the face of it, this suggests that a purely in vitro model of combinational hypoxia and cytokine might have advantage over in vivo hypoxia as a model of COPD. However, we did not examine combinational hypoxia plus inflammation in vivo and our in vitro findings suggest that this may be a better model for future studies.

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Publications during my PhD

Jounal Article

Ejiofor SI BS, Gassamma B, Turner AM. Ambulatory Oxygen for Exercise-Induced Desaturation and Dyspnea in Chronic Obstructive Pulmonary Disease (COPD): Systematic Review and Meta-Analysis. Chronic Obstr Pulm Dis (Miami). 2016;3(1):419-34.

ABSTRACTS/Posters

Gassama A, Ray C, Turner A, Egginton S, Kumar P. Influence of TNF-α on vasodilation of isolated rat pulmonary artery rings (1106.17). The FASEB Journal. 2014;28(1 Supplement).

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