Nitrite Uptake and Metabolism in Human Erythrocytes – A Source of Vascular Nitric Oxide?

By

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This thesis is dedicated to my family, my parents, Caroline & George, my brother, Ross and my girlfriend Karin.

And is written in loving memory of my little sister Lorna Helen Rose and my Grandad, Gerald Fred Tarr.

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COMMONLY USED ABBREVIATIONS

2,3-DPG	2,3-Diphosphoglycerate
3Cs	Copper chloride/cysteine/carbon monoxide
AA	Arachidonic acid
ANOVA	Analysis of variance
AO	Aldehyde oxidase
ATP	Adenosine triphosphate
Ca^{2+}	Calcium
CaM	Calmodulin
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine 3'-5'monophosphate
CO	Carbon monoxide
CO_2	Carbon dioxide
COX	Cyclooxygenase
DMSO	Dimethyl sulphoxide
EC_{50}	Concentration required to achieve 50% effect
EDRF	Endothelium derived relaxing factor
EDTA	Ethelene diamine tetra acetic
EPO	Erythropoietin
EPR	Electron paramagnetic resonance
ETC	Electron transport chain
FAD	Flavin-adenine dinucleotide
Fe^{2+}	Ferrous iron
Fe^{3+}	Ferric iron
FiO_2	Fraction of inspired oxygen
FMN	Flavin mononucleotide
g	Gravity
GSNO	*S*-nitrosoglutathione
Hb	Haemoglobin
HbA	Adult haemoglobin

HbNO	Iron nitrosyl haemoglobin
HbSNO	S-nitrosohaemoglobin
HCl	Hydrochloric acid
Hg	Mercury
HIF-1α	Hypoxia inducible factor-1α
HPLC	High performance liquid chromatography
I_2	Iodine
I_3^-	Tri-iodide
K^+	Potassium
$K_3Fe^{III}(CN)_6$	Potassium ferricyanide
L-NMMA	N^G-methyl-L-arginine
N_2O_3	Dinitrogen trioxide
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NO	Nitric oxide
NO^-	Nitroxyl
NO^+	Nitrosonium
NO_2^-	Nitrite
NO_2	Nitrogen dioxide
NO_2^*	Nitrogen dioxide (in excited state)
NO_3^-	Nitrate
NOA	Nitric oxide analyser
NOS	Nitric oxide synthase
NOx	Nitrate and nitrite
O_2^-	Super oxide
O_2	Oxygen
O_3	Ozone
ODQ	1H-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one
$ONOO^-$	Peroxynitrite
P_{50}	Haemoglobin's 50% oxygen saturation
PE	Phenylepherine

pO_2	Oxygen partial pressure
RNNO	*N*-nitrosamine
RSNO	*S*-nitrosothiol
sGC	Soluble guanylate cyclase
VCl_3	Vanadium chloride
XO	Xanthine oxidase

1 General Introduction

The general introduction of this thesis is intended to introduce the reader to important background information that is relevant to the findings presented in subsequent chapters. Each individual results chapter contains an introduction and discussion that will cover more specific areas and address findings in the literature to date.

1.1 Oxygen Delivery

The primary function of the cardiovascular and pulmonary systems is to produce a flow of oxygenated blood in sufficient supply to maintain aerobic metabolism in all organs and tissues. The system is required to be energy efficient but also receptive to changes in cellular metabolic demand. In order to function both at rest and during demand the system must also match oxygen allocation to metabolic requirements at a localised level. Once blood enters an area that requires substrates, oxygen should have the capacity to efficiently move from the blood, across the vessel wall and into the tissue.

Global oxygen delivery (Do_2, product of cardiac output and arterial oxygen content) under normal, resting conditions is more than adequate to meet metabolic demands/oxygen consumption (Vo_2) (Figure 1.1). At a tissue level, oxygen delivery is governed by two processes, convective and diffusive oxygen transport. Convective transport can be described as the bulk movement of oxygen in the blood, encompassing changes in cardiac output and the mechanisms that regulate flow in the microcirculation. Diffusive oxygen transport simply refers to the movement of oxygen from the blood into tissue, down the capillary-intracellular oxygen tension (Po_2) gradient and is governed by arterial oxygen tension (Pao_2) [1].

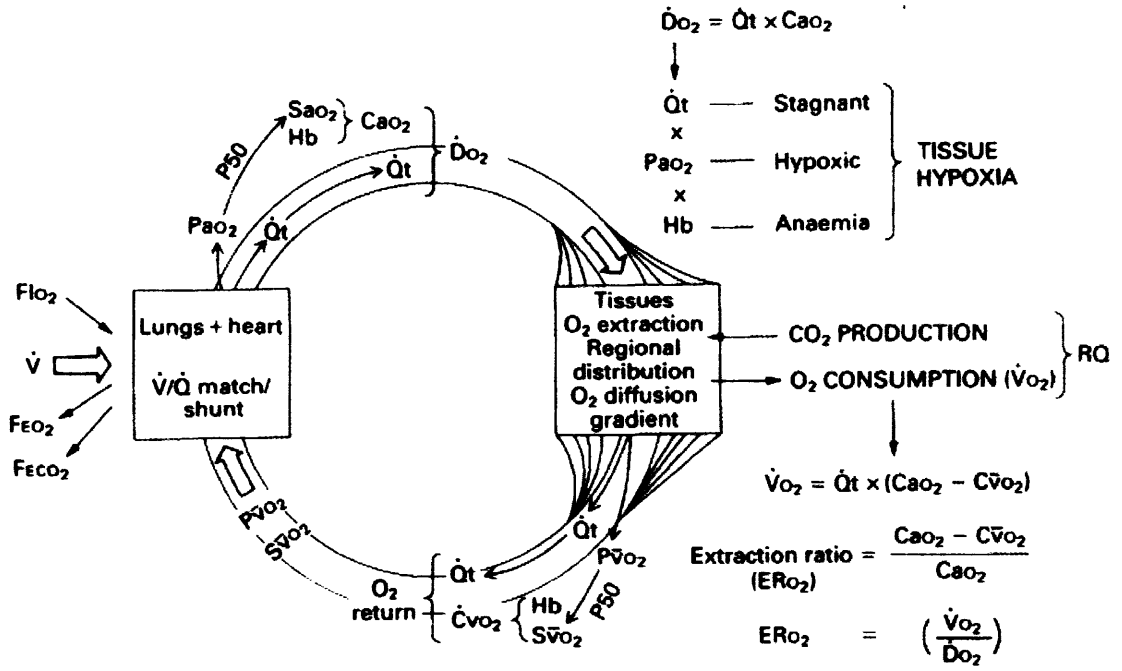

Figure 1.1. Diagrammatic representation of oxygen transport in the body. Note that oxygen delivery (DO_2) is dependent upon cardiac output (Qt), the arterial partial pressure of oxygen (PaO_2) and the concentration of haemoglobin (Hb). A reduction in any of these parameters can lead to tissue hypoxia. Abbreviations: V-ventilation; FIO_2/FEO_2-fractional inspired/and mixed expired oxygen concentration; $FECO_2$-fractional mixed expired carbon dioxide concentration; Qt-cardiac output; PaO_2/PvO_2-partial pressure of arterial/venous oxygen tension; SaO_2, SvO_2- saturation of arterial/venous blood; CaO_2/CvO_2-oxygen content of arterial/mixed venous blood; Hb-haemoglobin concentration; RQ-respiratory quotient; DO_2-oxygen delivery; p50-the pO_2 at which haemoglobin is 50% saturated. Adapted from [1].

1.2 Oxygen Sensing in the Body

In order to accurately match oxygen delivery to demand, the body requires methods of sensing the oxygen level in the blood stream and hence the tissue. This 'sensing' is generally believed to be carried out by a structure known as the carotid body.

1.2.1 The Carotid Body

The carotid body represents the primary oxygen sensing site in the vasculature, located at the bifurcation of the carotid artery. The carotid body is formed from chemoreceptor cells and is sensitive to changes in pO_2 and to a lesser extent carbon dioxide/pH and glucose of the blood that continually passes it [2, 3]. Within the carotid body there are two key cell types, glial-like sustentacular (type II) cells which represent 15-20% of cells and have processes that surround the more abundant, neuron-like glomus (type I) cells [4]. Glomus cells are thought to be the primary cell type involved in oxygen sensing, as they are chemoreceptive and release neurotransmitters such as ATP, dopamine and acetylcholine, in a calcium-dependent manner [5]. As pO_2 levels fall, the firing of sensory nerves from the carotid body increases, enhancing the ventilatory reflex through the respiratory centre to match ventilation on a second to second basis [6]. The oxygen sensing and signalling pathways of the carotid body are not fully understood, however, a number of candidate sensors and mechanisms have been proposed and will be covered in more detail below.

1.2.1.1 The Mitochondria

Mitochondria normally rely on oxygen to synthesise ATP via oxidative phosphorylation, with oxygen acting as the final acceptor of the electron transport chain. In hypoxia, this process cannot occur and so cells switch to anaerobic energy generation via glycolysis. Hypoxia also brings about other mitochondrial alterations that include changes in reactive oxygen species level (ROS), cytochrome oxidase activity and ion channel opening [7]. Inhibitors of the electron transport chain have been shown to change the oxygen sensitivity of glomus cells, mimicking hypoxia [8]. However, long term exposure to these inhibitors abolishes the glomus cell hypoxia sensitivity when measured by neural discharge [9]. The mechanism by which these mitochondrial changes result in increased afferent firing and the level at which other pathways (e.g. hypoxia inducible factor (HIF) [10]) contribute is unclear. However, agents that uncouple oxidative phosphorylation have been shown to alter calcium levels [11] which

is known to control neurotransmitter release from these cells. Although mitochondria are clearly sensitive to changes in oxygen, it seems unlikely that cytochrome oxidase is responsible for 'continuous' monitoring of oxygen level over a broad range of concentrations given that it has a K_m for oxygen of ~0.1 μmol/L [12] and so the oxygen level would need to fall significantly before the enzyme would stop binding oxygen and have reduced function.

1.2.1.2 The Membrane

The increase in cytosolic calcium that triggers transmitter release is thought to be partly mediated by calcium entry through voltage-gated calcium channels in response to membrane depolarisation [13]. The membrane potential of glomus cells is largely controlled by various, different potassium channels [14]. It was first shown that these channels were oxygen sensitive in the late 1980s [15]; under hypoxic conditions the channels are inhibited leading to membrane depolarisation, which causes subsequent changes in intracellular calcium level via other membrane channels. Given that calcium is thought to control transmitter release, this could provide an alternative oxygen sensitive mechanism.

1.2.1.3 Non-Mitochondrial Enzymes

NADPH oxidase is a haem containing enzyme expressed in glomus cells and is postulated to act as an oxygen sensor [16]. NADPH oxidase is known to generate reactive oxygen species such as superoxide which can in turn form hydrogen peroxide (H_2O_2) which can potentially regulate membrane potential via interaction with potassium channels. The generation of these species is governed by oxygen, so in hypoxia hydrogen peroxide production is lower and therefore the open probability of the potassium channels decreases, resulting in depolarisation. In support of this theory, it has also been shown that administration of hydrogen peroxide will inhibit carotid body sensory discharge [17].

It is unlikely that any of these proposed mechanisms are mutually exclusive and so varying contributions from each of these distinct pathways is likely. Although there are a number of different suggested 'oxygen sensor' pathways in the carotid body, many of them demonstrate a role for haem-containing enzymes. The carotid body is representative of acute oxygen sensing in the body but there are also chronic physiological responses that are activated by changes in oxygen level.

1.2.1.4 Hypoxia Inducible Factor

The identification of the transcription factor, hypoxia inducible factor 1 (HIF-1) was brought about by the discovery of a genetic response element that controlled erythropoietin [18]. HIF-1 has now been shown to regulate over seventy distinct genes that mediate many adaptive physiological responses to hypoxia including angiogenesis, altered glycolysis and erythropoiesis [19].

HIF-1 is a heterodimeric protein comprised of a constitutively expressed β subunit and an oxygen regulated α subunit [20]. HIF-1α is hydroxylated by prolyl hydroxylases, with oxygen representing a rate-limiting substrate in the process, under physiological conditions [21]. Hydroxylation is required for HIF-1α to interact with the VHL protein forming the recognition component for E3 ubiquitin-ligase [22], binding of VHL results in ubiquitination and degradation by a 26S proteasome [23, 24]. When cells become hypoxic, the hydroxylation process is limited and HIF-1α accumulates before translocation to the nucleus where it dimerises with HIF-1β and binds to DNA in genes with specific nucleotide sequences (Figure 1.2). The hydroxylation process also requires co-factors such as reduced iron, ascorbate and 2-oxoglutarate. The requirement for these co-factors closely links the process with both the redox state of the cell and energy homeostasis.

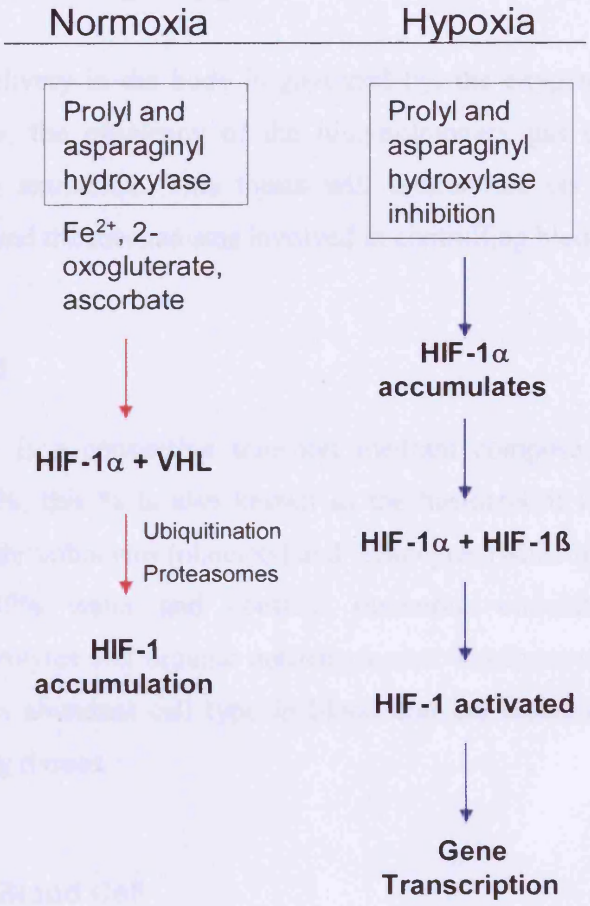

Figure 1.2. The inhibition and activation pathway of HIF-1 in normoxia and hypoxia respectively. Based on original schematic from [7]

HIF-1α is also hydroxylated by factor inhibiting HIF-1 (FIH-1), which prevents interaction with co-activators CBP and P300 [25, 26]. The co-activators control interactions with RNA polymerase II and other transcription factors. In hypoxia, FIH-1 is limited and hydroxylation is decreased, which allows the co-activators to interact and alter target gene expression [27, 28]. FIH-1 also interacts with VHL, linking transcriptional activity with protein stability [29]. These interactions allow control that is both rapid and graded, depending on the period and extent of hypoxic exposure.

1.3 Factors Governing Oxygen Delivery

Oxygen delivery in the body is governed by, the oxygen carrying capacity of blood, blood flow, the efficiency of the lung/pulmonary gas exchange system and changes in tissue extraction. This thesis will concentrate on the oxygen carrying capacity of blood and the mechanisms involved in controlling blood flow.

1.4 The Blood

The blood is a convective transport medium composed of plasma (~55%), erythrocytes (~44%, this % is also known as the haematocrit level) and Buffy Coat (~1%, containing thrombocytes (platelets) and leukocytes (white blood cells)). Plasma is composed of ~80% water and contains numerous circulating proteins, lipids, lipoproteins, electrolytes and organic nutrients/waste. Erythrocytes, or red blood cells, represent the most abundant cell type in blood and are essential for the delivery of oxygen to respiring tissues.

1.4.1 The Red Blood Cell

The pool of erythrocytes that circulate throughout the body represent an essential and complex tissue for homeostatic control. In addition to carrying both a substrate and the metabolic waste products of respiration, erythrocytes interact with other tissues to a considerable extent. In a healthy human with ~5L blood, erythrocytes number approximately 2.5 $x10^{13}$ cells, a quantity sufficient to meet the requirements of aerobic respiration. The lifespan of an erythrocyte is approximately 100 to 120 days, with a daily loss of ~0.8% to 1.0% of the total circulating mass. To compensate for this loss, the body has a normal production capacity of ~$2.5x10^{11}$ cells per day in the bone marrow [30].

1.4.1.1 Erythrocyte Development

The total erythrocyte mass requires precise regulation, the mass must be sufficient for adequate oxygen delivery without levels being excessively elevated as to impede blood flow. Levels of circulating erythrocytes are governed by an oxygen sensitive feedback loop which utilises erythropoietin as a signalling messenger. Erythropoietin (EPO) is a glycoprotein, largely produced in the kidney by interstitial fibroblasts [31]. Once systemic, erythropoietin acts upon haemopoietic stem cells found in the bone marrow via specific cell surface receptors [32].

Early haematopoietic progenitors differentiate into burst-forming unit–erythroid cells. These burst-forming unit–erythroid cells then further differentiate into colony-forming unit–erythroid cells and are dependent on EPO for survival [33]. With continued EPO stimulation, the differentiation pathway moves to erythroblasts, which in turn enucleate to form reticulocytes and after a few days reticulocytes show loss of reticulin, resulting in mature erythrocytes. Once the maturation process enters the later phases, both reticulocytes and erythrocytes cease to express the EPO receptor and so are unresponsive to further changes in EPO levels [34].

EPO has a terminal half-life of approximately 5 hours [35]. A number of factors can affect EPO levels including iron availability, nutritional status, disease, environmental conditions, and genetic factors (congenital polycythemias). A directly proportional relationship between erythrocyte production and plasma EPO level does exist. However, a significant change in EPO plasma levels only results in a modest increase in the rate of erythropoiesis, therefore it is time of elevated exposure which is important, not maximum plasma level achieved. Despite this modest increase in rate, the downstream effects tend to be prolonged due to the difference in half-life between EPO and erythrocytes (hrs vs. days). It has been demonstrated that only 30 minutes hypoxia is required to elevate EPO levels [35], thus a short exposure to elevated EPO levels will result in prolonged increases in erythrocyte levels.

EPO has been demonstrated to directly relax vascular tissue which has been shown to be dependent upon both the presence of endothelium and the operation of NOS [36]. Transgenic mice, engineered to over-express EPO show increases in eNOS expression, nitric oxide synthesis and nitric oxide inducible relaxation. Despite having an increased hematocrit (up to 80%), these mice did not exhibit any of the accompanying effects of increased hematocrit, e.g. hypertension or myocardial infarction [37]. In addition, the effect of EPO upon endothelial upregulation of eNOS and nitric oxide production has been shown to be enhanced at lower oxygen tensions [38]. In contrast, nitric oxide has been shown to suppress haematopoiesis in human cells, representing a possible 'feed forward' mechanism [39]. Haemoglobin mRNA/protein levels have also been shown to be altered by nitric oxide through a cGMP dependent mechanism in human erythroid progenitor cells [40].

1.4.1.2 Erythrocyte Geometric and Rheologic Properties

Erythrocytes are biconcave in shape and smaller than most other human cells, with a diameter of approx. 6-8 microns (Figure 1.3).

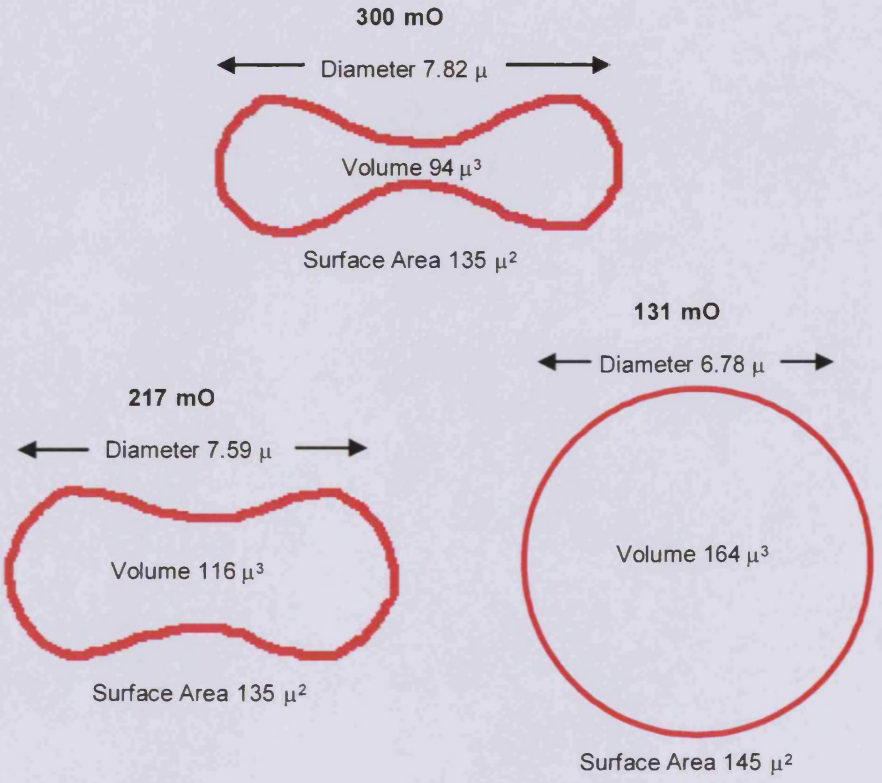

Figure 1.3. Geometrical data of the erythrocyte, measured whilst suspended in buffers with differing osmolarity values. For reference, normal, isotonic serum tonicity is approximately 270-300 milliosmoles (mOsm). Based on original figure from [41].

The study of blood rheology involves two key aspects; firstly, blood flow and secondly the deformation of blood cells. Blood rheology is important in the cardiovascular system because in addition to vascular tone it is the other key determinant of vascular resistance. In large vessels, flow is more likely to be altered by the concentration of erythrocytes. It is in the microcirculation that erythrocyte deformability, adherence to endothelium and aggregation play a significant role.

Erythrocyte rheology/deformability can be measured either as a bulk suspension of cells or in individual cells. Deformability can be calculated under shear stress (ektacytometry), by erythrocyte filterability or analysis of individual cells using a micropipette [42-44]. The key determinants of erythrocyte deformability are cell geometry (e.g. the cells surface area to volume ratio), cytoplasmic viscosity and viscoelastic properties of the erythrocyte membrane.

It has been shown that the viscosity of a haemoglobin solution increases exponentially when the concentration exceeds 32g/dL [45]. Similarly, when the mean corpuscular haemoglobin concentration (MCHC) is increased, intracellular viscosity increases and thus deformability of the erythrocyte decreases [46]. Cytoplasmic viscosity can also change as a function of temperature and alterations in osmotic balance that lead to water loss from the cell [47].

The viscous and elastic properties of the membrane are largely governed by the amount and organisation of cytoskeletal proteins. The interactions of these proteins with other proteins and the lipid bilayer are of particular significance [48]. Impairment of viscoelasticity has been shown to correlate with levels of glycosylated haemoglobin in diabetics, suggesting a role for cytoskeleton-haemoglobin interaction in deformability [49]. Erythrocytes which are senescent, abnormally shaped or damaged may be less deformable and are selectively removed from the circulation.

Nitric oxide donor drug addition to erythrocytes improves deformability in a dose-dependent manner [50]. The exact mechanism controlling this effect remains unknown, although it appears to be in part modulated by cGMP. When sGC is blocked, erythrocyte deformability is impaired, however, this effect can be reversed in a dose-dependent manner by nitric oxide donors, suggesting the participation of alternative pathways [50]. It is quite likely that the nitric oxide pathway may involve changes in phosphorylation of the cytoskeleton [51]. In this respect, endothelin-1 has been demonstrated to alter erythrocyte deformability though a pathway regulated by protein

kinase C (PKC) [52]. These findings are of particular interest given that erythrocytes may express a functional eNOS [53].

1.4.1.3 The Erythrocyte Membrane

The erythrocyte membrane is composed of 3 main elements; membrane lipids, membrane proteins and cytoskeletal proteins. The structural core of the erythrocyte membrane is formed primarily from cholesterol and phospholipid molecules. There are hundreds of distinct phospholipids within the membrane, differing with respect to the polar head group, the carbon back bone and the length and level of unsaturation of the acyl groups [54]. This diversity in chain length allows the lipids to be packed into the bilayer in a coordinated fashion, dictating protein-lipid interactions. The bilayer that is formed is very dynamic, with organised and rapid movement of lipids/embedded proteins, which gives rise to domains that are rich in specific lipid or protein species [55]. These domains or 'rafts' often have specialist functions such as signal transduction, with different components working collectively to convey a signal.

A large number of erythrocyte transmembrane proteins have been characterised. Broadly, they fall into groups involved in transport, adhesion and cell-cell interactions, signalling receptors and blood group antigens, for a comprehensive analysis of the proteome refer to reference [56]. Of specific interest to the current project, are the anion exchanger AE1/band 3 protein and the macrocomplex which can be formed with other proteins, in particular, the rhesus (Rh) proteins.

AE1 is an integral membrane protein which is found abundantly in the erythrocyte membrane (around 10^6 copies per cell) [57]. AE1 can be separated into its membrane-spanning domains and its cytoplasmic domains. The 12 membrane spanning sections of AE1 catalyse the exchange of anions (Cl^- and HCO_3^-) in and out of the erythrocyte during gas exchange [58]. Carbonic anhydrase II hydrates carbon dioxide to HCO_3^- and H^+, HCO_3^- then leaves the cell via AE1 in exchange for Cl^-, leaving H^+ to bind to haemoglobin and promote the release of oxygen [57]. This process not only

allows the efficient transport of carbon dioxide (as HCO_3^- in the plasma) but also enhances oxygen delivery in the tissue. It has also been recognised that the membrane spanning domain of AE1 may signal for the removal of senescent erythrocytes from the circulating pool [59].

The C-terminal cytoplasmic domain binds to carbonic anhydrase II and forms a metabolon to channel HCO_3^- , facilitating the combined functions of both the enzyme and channel [60]. The N-terminus binds to a number of proteins such as haemoglobin [61] and glycolytic enzymes [62-64], but its main function is to anchor the erythrocyte membrane to the cytoskeleton. Band 3 exists as both dimers and tetramers, in tetrameric form the protein binds to ankyrin [65] and protein 4.2 [66] to secure the membrane to the cytoskeleton.

Part of the AE1 macrocomplex is formed by the Rh proteins, these Rh polypetides and Rh associated glycoproteins (RhAG) are thought to act as gas channels, involved in the transport of CO_2, O_2 and interestingly the solo or co-transport of nitric oxide [57]. Despite the highly diffusible nature of these gases, the co-localisation of these proteins and their ability to act as channels may aid the efficient transport of gases across the membrane within the time course of a transitory, circulatory cycle.

1.4.2 Haemoglobin

Haemoglobin represents one of the most significant and genetically conserved proteins in the human body. Haemoglobin functions to transiently bind/release oxygen in the circulation and its ability to perform this function so well is due to its specific structure.

1.4.2.1 Haemoglobin Structure

Adult haemoglobin (HbA) represents the most important oxygen carrying protein in the human body. HbA is a heterotetrameric metalloprotein (64,500Da), consisting of two α and two β-globin subunits each bound to haem. During terminal erythropoiesis huge amounts of HbA are synthesised, constituting around 95% of the total mature erythrocyte proteome, a concentration of approximately 35g/dl [67].

The subunits that form HbA adopt a 3-dimensional structure known as the globin fold. The fold comprises a number of specially arranged helices that form a pocket, which both encloses and binds to a haem prosthetic group (a planar porphyrin structure with an iron centre). The haem is linked to the polypeptide subunit via a covalent bond formed between a nitrogen atom of the proximal histidine (F8His) and the haem iron (Figure 1.4d) [68]. The iron found in the haem group is capable of binding diatomic gases e.g. molecular oxygen, carbon monoxide and nitric oxide, when it is in the ferrous state (2+ oxidation state). Diatomic gases bind to the distal side of the haem plane, opposite the proximal histidine, forming a hexa-coordinate iron structure [68].

1.4.2.2 Haemoglobin Structure-Function

The intricate conformational changes that occur in the haemoglobin protein structure during its binding and releasing of ligands has given rise to a significant amount of research into the protein structure-function relationship.

Haemoglobin exists as two classic quaternary structures which are designated R (relaxed) and T (tense) (Figure 1.4a). Perutz and colleagues identified these exact structures using X-ray crystallography and their observations generated the concept that the molecular structure of a protein can determine its biological function [69]. In vivo, as each successive oxygen binds, haemoglobin conformation moves through a number of states resulting in a transition from T-state to R-state. Within the haemoglobin structure there are a number of specific structures that are important to this transition that will be covered below.

The $\alpha_1\beta_1$ and $\alpha_2\beta_2$ interfaces in HbA contain many hydrogen bonds and are thought to be relatively fixed regardless of changes in quaternary structure [70]. In contrast, the $\alpha_2\beta_1$ and $\alpha_1\beta_2$ interfaces are subject to notable changes during allosteric movement between R and T states. Each helix within a subunit is designated a letter, the β-chain for example has eight helices, A through to H. Regions in the $\alpha_1\beta_2$ interface in proximity to the pivot point (FG corner of the α chain and the C helix of the β chain) stay in contact and are known as the 'hinge' [68]. The point most distant from the pivot undergoes the largest shift during a conformational change and is known as the 'switch region'. During a 'switch', $\beta_2$97His is displaced to the greatest extent by the turning of the C helix (α_1) from a position between α_1Pro44 and α_1Thr41 to a groove between α_1Thr38 and α_1Thr41 (Figure 1.4C) [68]. All of these movements result in a physical change in haemoglobin conformation that also has different biochemical properties compared to its non-ligand state.

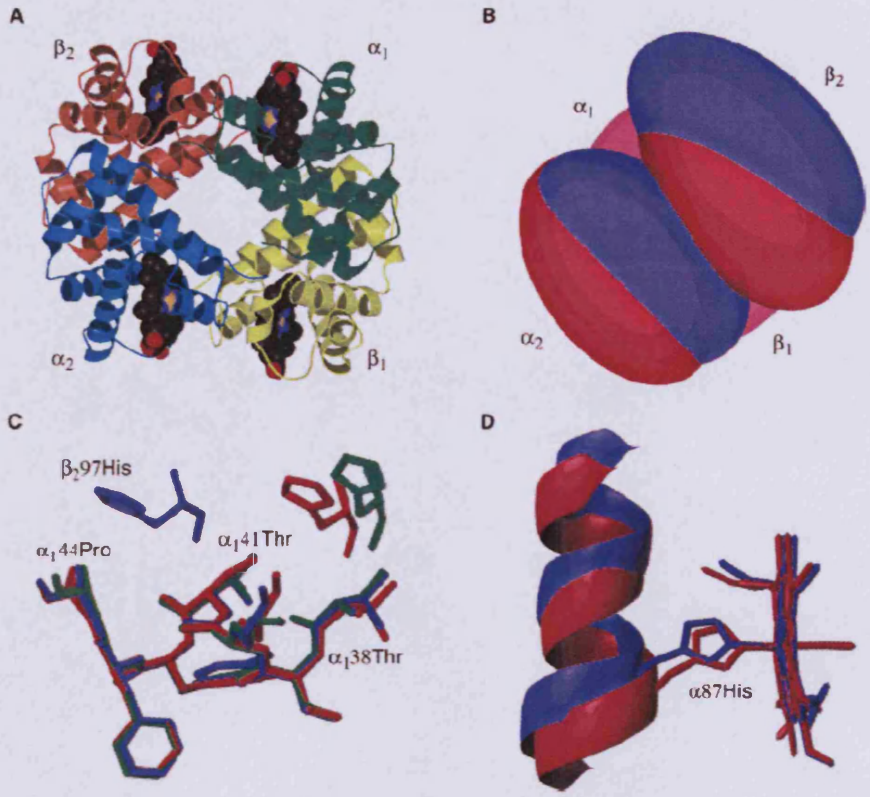

Figure 1.4. Illustrations of Hb A structure. (A) Deoxy-Hb A, viewed along the 2-fold symmetry axis, showing the water-filled central cavity. (B) Illustration of the reorientation of αβ dimers in the T-to-R transition. The $\alpha_1\beta_1$ dimers of the two conformations, shown in violet toward the rear of the figure, have been superimposed at the $\alpha_1\beta_1$ interface, whilst the α_2 and β_2 subunits of deoxy and carbonmonoxy-Hb are shown in blue and red, respectively. (C) Switch region of the $\alpha_1\beta_2$ interface of Hb A in the T, R, and R2 conformations, shown in blue, red, and green, respectively. Here, the backbone atoms of residues 38–44 in the α_1 subunit have been superimposed, to illustrate the relative motion of $\beta_2$97His. (D) The α-subunit hemes, proximal histidines, and F helices of deoxy-Hb (blue) and carbonmonoxy-Hb (red), with hemes superimposed. Reproduced from [68].

In addition to the obvious physical differences between the R and T conformational states, it has been long postulated that the sequence of changes in quaternary structure when moving from one state to another are responsible for the varied oxygen affinities of the states. It was always presumed that T state had low affinity and R state high. Haemoglobin's relationship with oxygen is altered by both conformational changes in its protein structure due to substrate (oxygen) binding

(homotropic allostery) and by effectors that interact with other sites on the protein (heterotropic allostery).

1.4.2.3 Homotropic Allostery

The proposal of co-operative oxygen binding by haemoglobin is attributed to a Danish Physician, the late Christian Bohr. It was the sigmoidal nature of the haemoglobin oxygen binding curve that stimulated Bohr to generate this concept [71].

At that time it was generally believed that haemoglobin only contained one haem, it was not until 1925 that Adair showed that there were four haem groups present in haemoglobin [72]. Once this discovery had been made it became accepted that the binding of the first oxygen must enhance that of the second and successive oxygen binding events. Despite these findings, it was still unknown if the mechanism responsible was via electronic interaction between haem groups or changes in the carrier protein. A key breakthrough came when Haurowitz demonstrated a change in crystal structure upon oxygenation [73], suggesting that a change in conformation was responsible. As more detailed structural information became available, it was clear that, given the relatively large distance between oxygen binding sites, co-operativity must be conveyed through the protein structure. In 1970, Perutz, armed with detailed structural information, proposed novel stereochemical mechanisms to explain the phenomenon [69]. Perutz noted that specific inter-subunit salt bridges and out-of-plane haem iron position was present in the T, but not R state [69]. He speculated that when the haem iron shifts from out-of-plane (with respect to the pyrol nitrogen atoms of haem) to in-plane this induces displacement of the proximal F-helix and subsequent helix rearrangements. These concerted structural changes take place during the T to R transition in addition to changes in porphyrin sterochemistry, globin structure and at the subunit interfaces [74]. Given that R state haemoglobin oxygen affinity resembles that of free haem subunits, it can be said that it is the structural constraints that stabilise the T-state and alter affinity to govern co-operativity [74].

Recently, a new hypothesis has been proposed in which variations in HbA affinity for oxygen are more likely to be governed by tertiary structural dynamics than overall quaternary structure changes [75]. This area of research has received an inordinate amount of attention and there are many variations in models to explain the co-operativity phenomenon. As yet a consensus on the different states of haemoglobin and how they are changed by allosteric effectors is yet to be reached, for a current review of the topic see [75].

1.4.2.4 Heterotropic Allostery

In addition to the substrate affinity relationship seen in haemoglobin, there are also sites (other than the oxygen binding pocket) located on haemoglobin where alternative allosteric regulators can bind and influence oxygen affinity. Principally, these regulators are respiratory metabolites which aid in the matching of substrate supply to metabolic demand. It should be noted that binding characteristics can also be regulated by non-chemical entities, including temperature which can alter oxygen offloading.

1.4.2.4.1 Carbon Dioxide

The efficient transport of carbon dioxide (CO_2) is often said to be of equal importance to that of oxygen. CO_2 is transported in three main forms; dissolved as CO_2, as bicarbonate or bound as carbamate (to the amino termini of the globin chains, also known as carbaminohaemoglobin).

CO_2 diffuses out of respiring tissues into the blood stream, where it enters the erythrocyte (Figure 1.5). Erythrocytes are significantly more permeable to CO_2 than other cells [76], largely due to specific gaseous exchange channels e.g. AE1 and Rhesus proteins (previously discussed in section 1.4.1.3, page 12). Once inside the erythrocyte, CO_2 is converted to bicarbonate (HCO_3^-) and a proton (H^+) by the enzyme, carbonic anhydrase (CA). Since the initial discovery of CA by Meldrum and Roughton in 1933 [77], a number of CA isoezymes have been found, CA II being particularly abundant in

erythrocytes with high specific activity (10 times that of CA I) [78]. Once formed bicarbonate is then free to leave the erythrocyte in exchange for chloride ions, in what is commonly referred to as the 'Chloride shift'. As erythrocytes enter the lung the reverse process occurs, bicarbonate is converted to carbonic acid (H_2CO_3) which rapidly dehydrates to water and CO_2, at the same time haemoglobin is re-oxygenated and CO_2 stored as carbamate is released. The liberated CO_2 then diffuses freely into the lung for excretion, summarised in figure 1.5. This system is not only key to transport homeostasis but also plays a notable role in the buffering of blood pH.

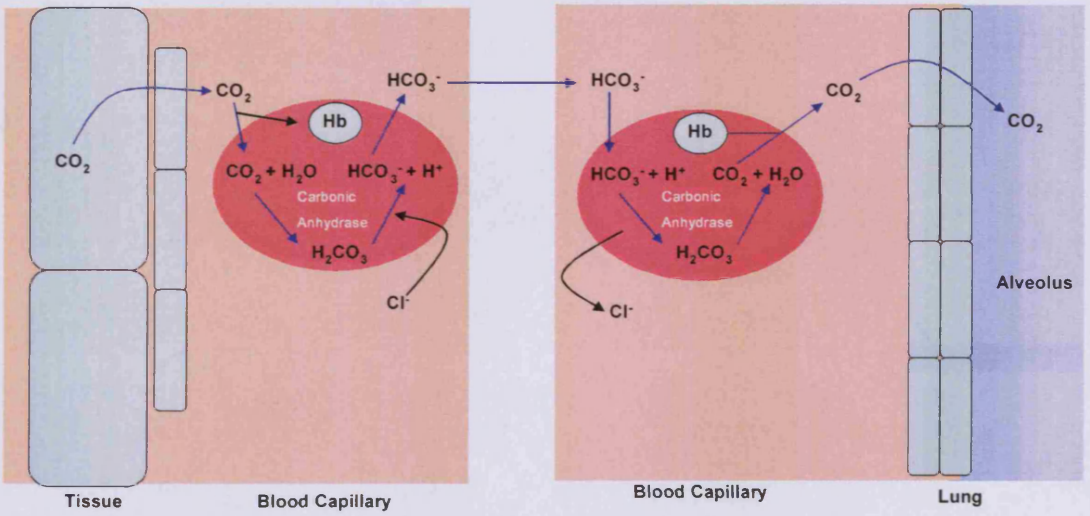

Figure 1.5. Carbon dioxide transport in the blood stream.

1.4.2.4.2 The Bohr Effect

In addition to demonstrating the sigmoidal curve generated by the interaction of haemoglobin with oxygen, Bohr, with colleagues Hasselbalch and Krogh demonstrated that the binding curve was sensitive to changes in both CO_2 and pH (H^+) [79]. Although carbon dioxide can alter haemoglobin oxygen affinity independently of pH (during formation of carboxy-haemoglobin), it is the changes in CO_2 and concomitant changes in pH that occur during CO_2 transport that produce the 'Bohr effect'. An increase in CO_2 and subsequent reduction in pH shifts the binding curve to the right, increasing the p50

(oxygen tension required to achieve 50% saturation)(Figure 1.6). At a molecular level, this can be explained in terms of haemoglobin conformational state. As haemoglobin shifts from the R-state to the T-state the pK_a of specific amino acids change (detailed in Figure 1.7), this change makes them more likely to interact with Bohr protons. When these protons bind (forming haemoglobinic acid) they stabilise the T-state and delay transition back to the R-state [80]. The physiological relevance of this shift becomes apparent in respiring tissues, where CO_2 levels are high. Haemoglobin encounters an area of low O_2/high CO_2 and is more likely to off load oxygen and less likely to re-bind, aiding adequate oxygen delivery. The converse is also true with respect to the effects of oxygen on CO_2/H^+, known as the 'Haldane effect' [81].

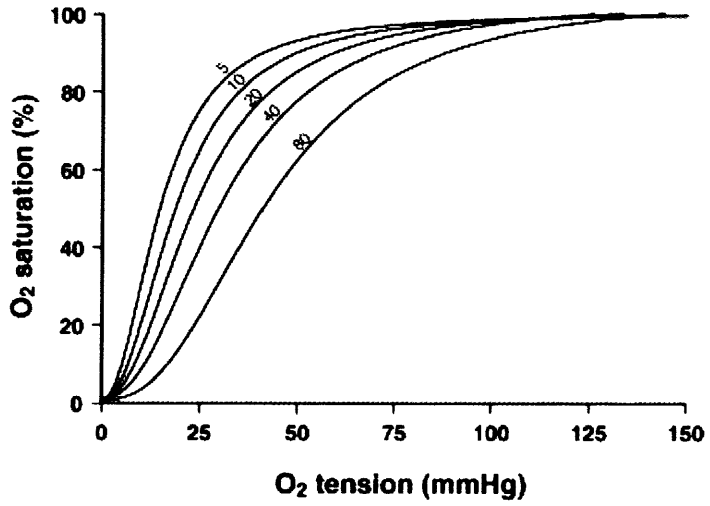

Figure 1.6. Influence of CO_2 on O_2 equilibrium curves of blood at 38 °C. Numerical values above each curve represent the CO_2 tension in mmHg. Reproduced from [80].

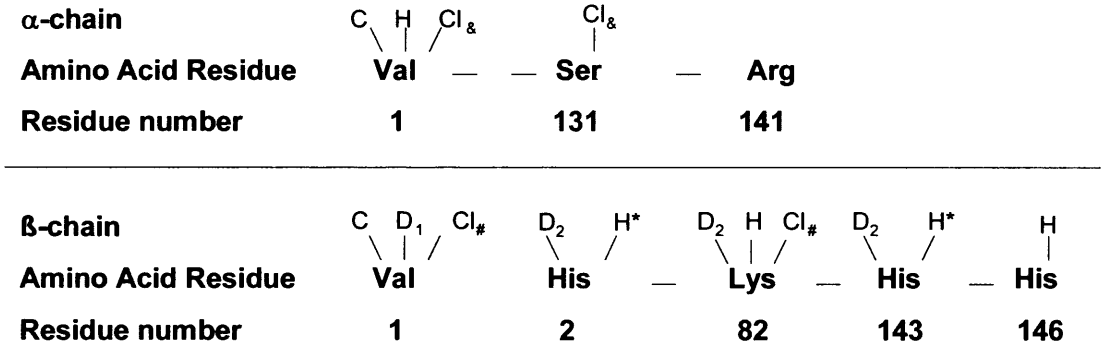

Figure 1.7. Schematic representation of the major amino acid residues of the α and β chains of human Hb considered to be involved in binding CO_2 (C), protons (H), chloride ion (Cl) and DPG (D). D_1-binding to one β chain; D_2-binding to both β chains; & #-Shared chloride binding sites on α and β chains, respectively; H*-proton binding in the presence of DPG. Based on original table from [82].

1.4.2.4.3 2,3-DPG

2,3-Diphosphoglycerate (2,3-DPG or 2,3-BPG) is an organic phosphate intermediate formed during glycolysis. The intraerythrocytic 2,3-DPG concentration is approximately equal to the molar concentration of tetrameric haemoglobin (5mM)[83], although the amount of free 2,3-DPG is dependent on haemoglobin saturation and can fall as low as 0.5mM in deoxygenated erythrocytes [84]. It was simultaneously shown by Chanutin & Curnish [85] and Benesch & Benesch [86] that organic phosphates could act as allosteric modulators of haemoglobin. 2,3-DPG binds to histidine, valine or lysine residues in the β-chains of HbA, forming electrostatic cross-links with residues situated in the central cavity between the two subunits (Figure 1.7) [87]. In R-state oxyhaemoglobin this cavity is too small for 2,3-DPG to enter, however, in T-state binding is very favourable. Through binding to T-state, 2,3-DPG shifts the T-R equilibrium toward T, shifting the binding curve to the right, which indicates an enhancement of oxygen offloading with reduced re-capture. Exposure to high altitude has been demonstrated to increase intraerythrocytic 2,3-DPG levels which may be necessary for sufficient oxygen delivery, particularly at a capillary level [88].

1.4.2.5 Other Allosteric Modulators

Of interest to the current thesis, nitric oxide bound to the 93 position cysteine residue of the β chain of haemoglobin (HbSNO) has been shown to produce a rightward shift in the binding curve, enhancing offloading [89, 90]. Glycosylation of haemoglobin has also been shown to cause a slight rightward shift in the oxygen binding curve [91]. Haemoglobin glycosylation in healthy individuals is ~5% while in diabetic patients can range up to 15-20% in some cases. Given these relatively high percentages, the effects of glycosylation on haemoglobin functionality could potentially be quite dramatic.

It is also well documented that the oxygen binding curve of foetal haemoglobin (HbF) is leftward shifted [92]. These altered binding characteristics aid in the transport of oxygen from the maternal bloodstream across the placenta. In most species the characteristics of HbF disappear during the postnatal period. Interestingly, HbF does not polymerise like sickle haemoglobin (HbS) and so augmentation of HbF levels is now being considered as a therapeutic option in sickle cell disease [93].

1.5 Blood Flow

Blood flow at a local level is governed by cardiac output/perfusion pressure and vascular resistance or tone.

Mathematically, laminar flow in a tube can be described by Darcy's law, where flow (F) is dependent upon the pressure gradient (ΔP) and resistance (R) [94].

$$F = \frac{\Delta P}{R} \quad (1)$$

To apply this simple equation to the biological setting the components governing resistance must be taken into account, in particular viscosity. The Hagen-Poisseuille equation describes this relationship, resistance (R), (v) Viscosity, (L) length of tube and (r) radius.

$$R = (\frac{\nu L}{r^4})(\frac{8}{\pi}) \quad (2)$$

Combining equations 1 and 2 gives equation 3. The important thing to note is that the relationship between flow and radius is to the fourth power, therefore a 2-fold change in radius will give a 16-fold change in flow for example.

$$F \propto \frac{\Delta P . r^4}{v . L} \quad (3)$$

In reality, this is only an approximation, as blood does not behave as a Newtonian fluid i.e. viscosity varies with respect to flow and shear rate, the reader is directed to an interesting paper regarding the complexities of the topic by Sirs [95].

1.6 Cardiac Output

Cardiac output is the product of heart rate and stroke volume. A key factor in the control of heart rate is the autonomic nervous system; an increase in sympathetic stimulation to the heart releases noradrenaline which acts via β-adrenoceptors and results in an increase in heart rate [96]. Stroke volume is governed by myocardial contractility (also influenced by the autonomic nervous system), pre-load and after-load [97]. Pre-load is essentially the pressure stretching of the ventricle after passive filling and atrial systole. Pre-load is often equated to end-diastolic volume of the left ventricle and is largely dependent upon venous return and ventricular distensibility [97]. After-load is in essence the pressure that the ventricle must generate in order to contract, i.e. the force required to overcome aortic pressure, open the aortic valve and eject blood and is often referred to as end-systolic pressure. After-load is heavily influenced by peripheral resistance as this tends to be a major factor in determining blood pressure [97].

1.7 Vascular Resistance

Vascular resistance represents the resistive forces that must be overcome to produce flow within a vessel. The basic anatomy and biological factors that govern vascular resistance are covered below.

1.7.1 Vessel Structure and the Vascular Tree

The vascular tree comprises a number of vessels which are distinct at many levels. In physical terms, arteries are more muscular whereas veins tend to be more compliant and contain valves to prevent retrograde flow (Figure 1.8). The response of these different vessels to environment, local and systemic mediators also varies hugely [98]. It is important to note that despite the various physical differences, all vessels are lined with endothelial cells that are essential in controlling tone and maintaining the health of the vessel. Veins generally don't receive the same research attention as arteries

have in the past, but the fact that approximately 70% of the total blood volume is found within the venous circuit, with the majority contained in the small veins and venules [99], highlight their importance. Given that these vessels represent the primary capacitance region of the body, minor changes in venous tone can impact significantly on venous return and thus cardiac output [100], stressing the significance of active vascular tone control in veins.

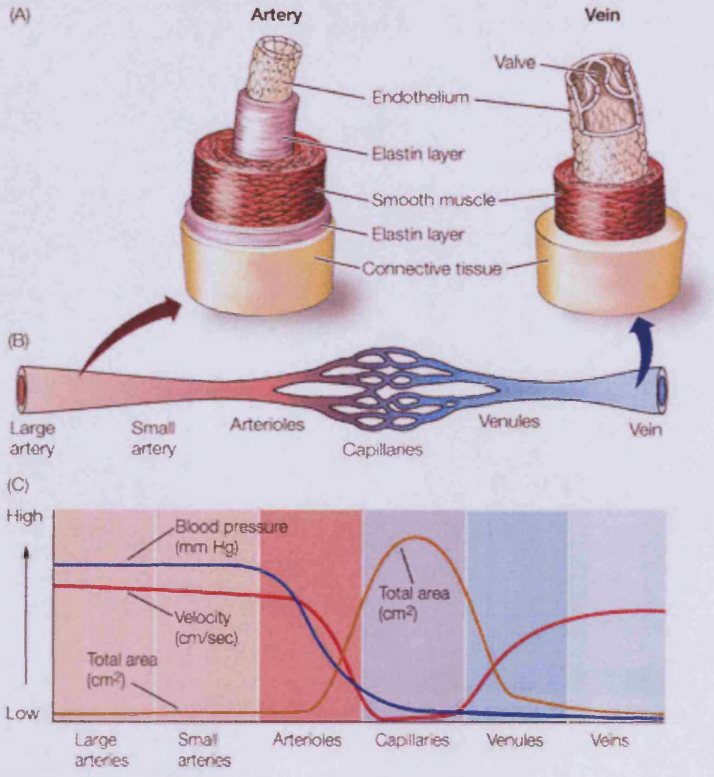

Figure 1.8. The structure (A), arrangement (B) and characteristics (C) of arteries and veins. Adapted from [101].

1.7.2 Control of Vascular Tone

The regulated control of vascular tone is so implicit that it occurs via many different pathways at both a local and whole body level (figure 1.9). The fact that so many different physiological agents and systems influence blood vessel tone could suggest significant redundancy. However, because blood vessels play such an important role in maintaining flow, the presence of multiple pathways allows for compensatory upregulation in the event of a problem. The origins, control and mechanism of some of these influences are described below.

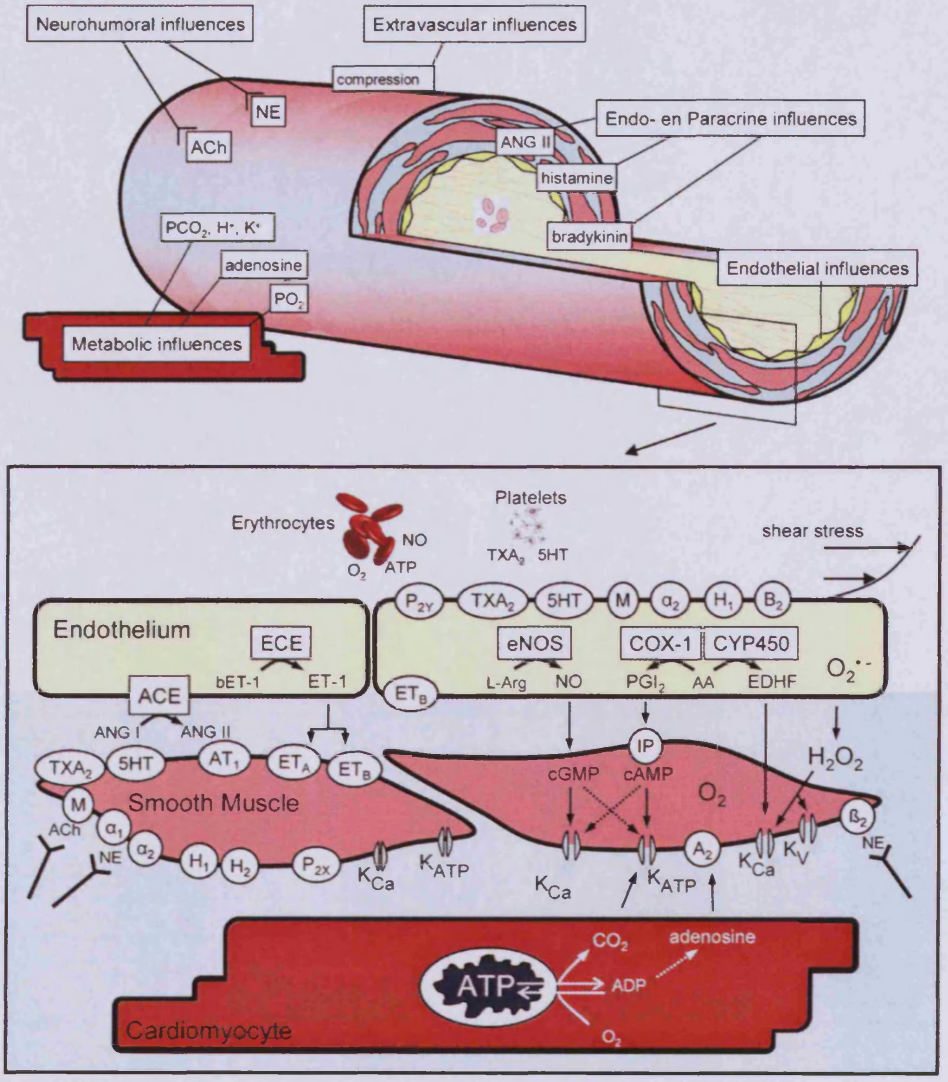

Figure 1.9. Schematic drawing of an arteriole and the various influences that determine vasomotor tone. Abbreviations: PO_2-oxygen tension; TxA_2-thromboxane A_2 (receptor); 5HT-5-hydroxytryptamine (receptor); P_{2X} and P_{2Y}, purinergic receptor subtypes 2X and 2Y that mediate ATP-induced vasoconstriction and vasodilation, respectively; ACh-acetylcholine; M-muscarinic receptor; H_1 and H_2, histamine receptors type 1 and 2; B_2-bradykinin receptor subtype 2; ANG I and ANG II, angiotensin I and II; AT_1-angiotensin II receptor subtype 1; ET-endothelin; ET_A and ET_B, endothelin receptor subtypes A and B; A_2-adenosine receptor subtype 2; ß$_2$-ß$_2$-adrenergic receptor; α_1 and α_2, α-adrenergic receptors; NO-nitric oxide; eNOS-endothelial nitric oxide synthase; PGI_2-prostacyclin; IP-prostacyclin receptor; COX-1-cyclooxygenase-1; EDHF-endothelium-derived hyperpolarizing factor; CYP_{450}-cytochrome P_{450}; K_{Ca}- calcium-sensitive K^+ channel; K_{ATP}-ATP-sensitive K^+ channel; K_V-voltage-sensitive K^+ channel; AA-arachidonic acid; L-Arg-L-arginine; $O_2^{\cdot}$-, superoxide. Receptors and enzymes are indicated by an oval or rectangle, respectively. Adapted from [102].

1.7.2.1 Neuronal Input and Output

The autonomic nervous system (ANS) comprises both afferent and efferent neurons which connect the central nervous system with visceral effectors. The ANS has two branches, the sympathetic and parasympathetic (Figure 1.10).

1.7.2.1.1 Afferent Signals

The nucleus tractus solitarius (NTS), found in the dorsomedial region of the medulla oblongata, receives thousands of afferent input signals each second [103]. Ascending information from baroreceptors represents one of the most significant inputs, providing tonic and phasic information that can modulate sympathetic outflow to splanchnic and muscle vasculature to produce adjustment on a second-to-second basis [104, 105].

A number of functionally different arterial baroreceptors have been found in the aorta and carotid sinus, they are pressure sensitive and relay information to the vasomotor centre via the vagus and glossopharyngeal nerves respectively [106]. Additional inputs are also received from cardiopulmonary mechanoreceptors [107], chemoreceptors that are sensitive to changes in blood/cerebral spinal fluid (CSF) metabolic metabolites e.g. pCO_2 [108] and pulmonary stretch receptors that respond to changes in lung volume [109].

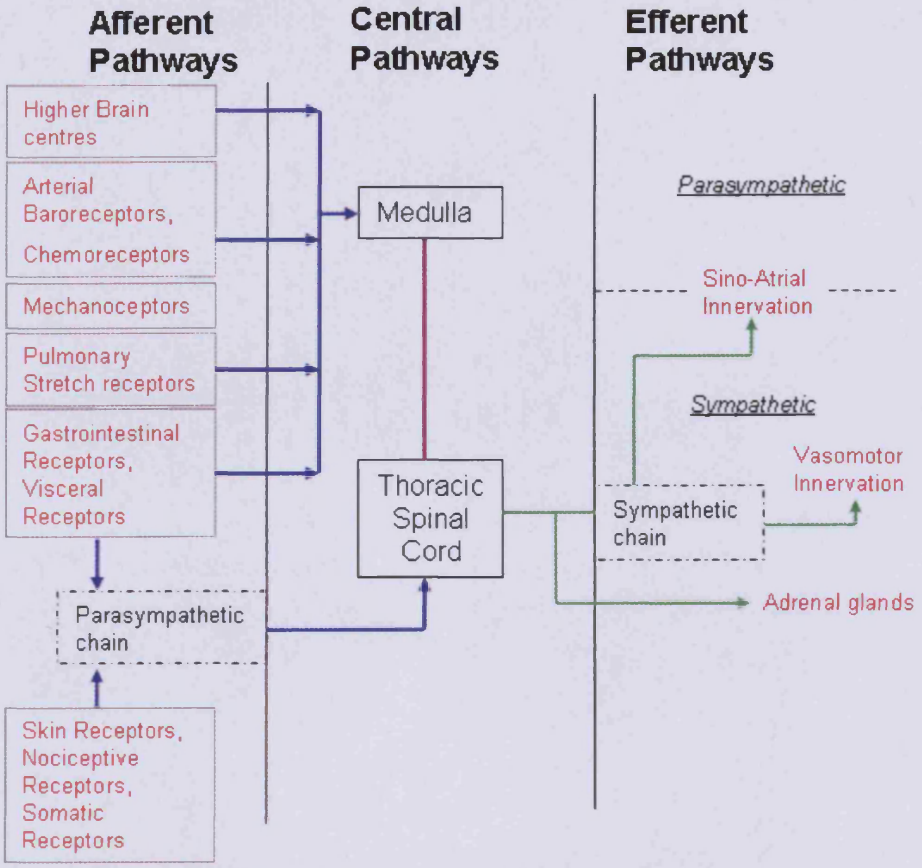

Figure 1.10. A schematic of the autonomic nervous system. Peripheral vascular function is regulated by the afferent input, central integration, and efferent outflow of the sympathetic nervous system. Afferent pathways from baroreceptors, pulmonary stretch receptors, cardiac and respiratory mechanoreceptors, and gastrointestinal and visceral are primarily vagal fibers that receptors feed information to the CNS. The central integration of the vagal afferent input into the NTS (nucleus tractus solitaruis) is responsible for initiating the reflex control of the circulation via complex patterns of sympathetic outflow that are modulated by other neural inputs, peptides and amino acids collectively termed "neuromodulators." Efferent pathways alter cardiovascular parameters through; initiating hormone release, modulating the heart and innervating vessels directly. Diagram based on original from [103].

1.7.2.1.2 Efferent Signals

Efferent signals that control the circulation come from both the sympathetic and parasympathetic branches of the ANS. Parasympathetic innervation plays a more minor role, working in opposition to sympathetic drive in the control of the heart and to a lesser extent in vessels [110]. The sympathetic efferent neurons can be separated into three groups; barosensitive, thermosensitive and glucosensitive. The thermosensitive efferents are activated in response to temperature change or emotional stimuli [111], while glucosensitive efferents are activated by hypoglycaemia or exercise [112]; neither, however, play a considerable role in cardiovascular control. It is the baroreceptor efferents that appear to have the most significant role. Found in numerous tissues and organs, these efferents are highly active (sympathetic tone) even under basal conditions [113]. Baroreceptor efferents are thought to have roles in both the long and short term regulation of vascular tone and blood pressure [114]. Interestingly, it has been demonstrated that nitric oxide and thiol-bound nitric oxide compounds (SNOs) can modulate the activity of these baroreceptors *in vivo* [115]. In the majority of cases, ANS vessel innervation is mediated by noradrenaline and acetylcholine [116]. Activation of β-adrenergic receptors on vascular smooth muscle results in vasodilatation, whereas α_1 and to a lesser extent α_2 activation produces vasoconstriction [117]. The parasympathetic stimulation of vascular smooth muscle muscarinic receptors can also produce vasoconstriction [118]. Endothelial cells have both α and β adrenoceptors; stimulation of β receptors produces vasodilatation through the release of nitric oxide [117]. Endothelial cells also produce vasodilatation when stimulated by the primary parasympathetic neurotransmitter, acetylcholine [118].

1.7.2.2 Metabolic Messengers

Vessel tone is perhaps unsurprisingly affected by metabolic factors in the blood stream or within the tissues themselves. The existence of this relationship gives rise to matching of supply (through alterations of flow) to demand at a very localised level.

1.7.2.2.1 Carbon Dioxide

Carbon dioxide, or hypercapnic acidosis is know to cause vasodilatation [119]. However, the mechanism of action is incompletely understood and it is unclear whether carbon dioxide itself or the accompanying change in pH (or both, via alternative mechanisms) is responsible. Although it is generally perceived that the pH change is most likely the key mediator [120], there is evidence to suggest that differing mechanisms involving both carbon dioxide and H^+ could be involved [121]. Carbon dioxide has also been demonstrated to potentiate the effects of nitric oxide in cerebral arteries [122].

1.7.2.2.2 Adenosine

Adenosine is a by-product of normal metabolism generated via two distinct pathways that use either AMP (adenosine monophosphate) or S-adenosylhomocysteine [123]. Due to this metabolic link, adenosine release increases during cell stress, as seen with hypoxia, for example [124]. Once released, adenosine acts as a vasorelaxant and aids to increase flow to areas of oxygen demand. A number of adenosine receptors have been cloned and have been shown to be G-protein coupled, operating via phospholipases and/or adenylate cyclase [125, 126]. In addition to its direct vascular effects, adenosine can also influence the cardiovascular system via platelets, mast cells and the autonomic nervous system. Interestingly, adenosine has also been shown to promote nitric oxide release in vascular tissue [127].

1.7.2.2.3 Oxygen

The effects of pO_2 on vascular contractility have been a point of discussion for many years [128, 129]. It is clear that most vascular tissue relaxes under hypoxic conditions; conversely the same is true of constriction in hyperoxic conditions, however the mechanism still remains unclear. As with most rules, there is an exception; the pulmonary vasculature behaves in reverse, constricting during hypoxic exposure [130]. Although the mechanism of hypoxic vasodilatation has not been truly defined, there are several mechanistic theories that have been broadly separated into two groups: those that involve energy limitation and those associated with a disruption in excitation-contraction coupling/relaxation mechanism [131]. Our laboratory has previously published a finding that could explain this phenomenon [132]. At moderately low oxygen conditions (~5%) a condition arises where NAD(P)H oxidase generation of superoxide is limited, but nitric oxide synthase production is not (eNOS Km for oxygen ~6-9μM). This produces a situation over a narrow oxygen concentration range where, despite the same level of production, the relative abundance of nitric oxide is increased due to decreased superoxide scavenging.

1.7.2.3 Endocrine and Paracrine

As previously mentioned, the control of vessel tone occurs at both local and systemic/global levels. Those substances which are known to alter vessel tone are also released and act in this way i.e. some act in an endocrine or paracrine fashion whilst others may do both.

1.7.2.3.1 Peptides

A vast number of signalling peptides exist in the circulation and carry out a number of vascular effects. Primarily, there are those that produce vasoconstriction (Angiotensin II (AT-II), antidiuretic hormone (ADH), Neuropeptide Y (NPY) and endothelin (ET)) and those that produce vasodilatation (atrial natriuretic peptide (ANP), vasoactive intestinal peptide (VIP), substance P (SP), Neurotesin, cacitonin gene-related

peptide (CGRP) and the kinins). This field is represented by a huge body of research that cannot be covered in detail here, therefore detail on some specific signalling peptides that are of importance in vascular regulation will be expanded upon.

1.7.2.3.1.1 Bradykinin

Bradykinin is a product of the kallikrein-kinin system (Figure 1.11) and is generated in blood by converting enzymes found in or on the surface of vascular cells. The kinins have broad effects that are mediated through two distinct G-protein coupled receptors (B_1 and B_2), these include roles in; cardiovascular and renal control, inflammation and pain [133]. Both receptors are principally linked to phospholipase C activation, which in turn causes an increase in intracellular calcium from inositol 1,4,5-trisphosphate (IP_3)-mobilised stores [133]. Circulatory actions of the kinins generally arise from enzymatic processes which are calcium dependent, in endothelial cells both nitric oxide and prostacyclin production are stimulated [134]. Bradykinin has also been shown to initiate endothelium derived hyperpolarising factor (EDHF) in the human forearm [135]. In addition to potent vasodilator effects, bradykinin has actions upon the heart, altering both heart rate and myocardial contractility [136].

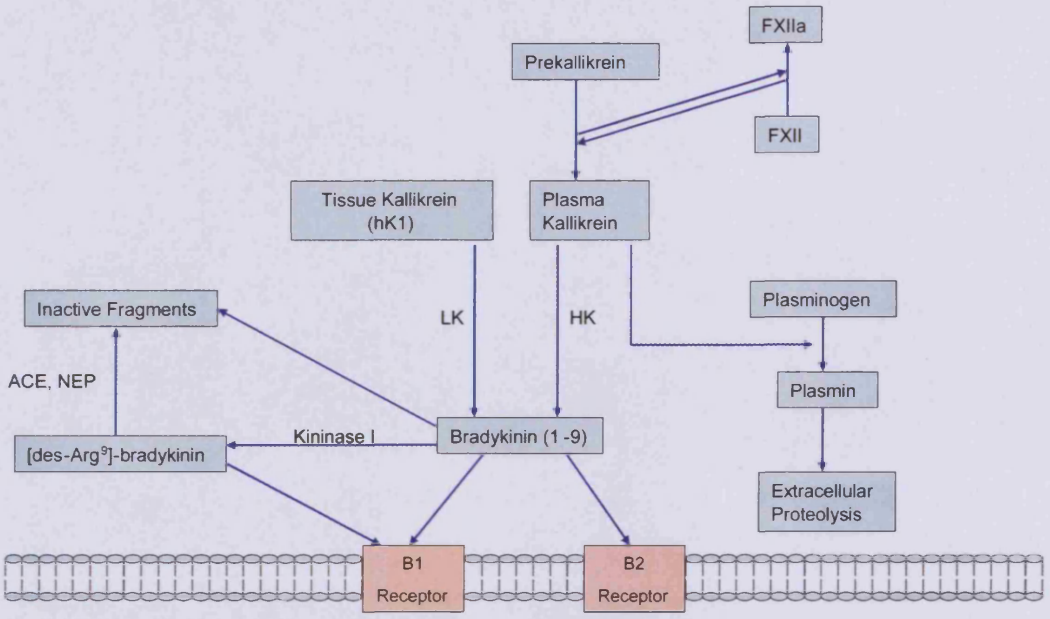

Figure 1.11. The kallikrein–kinin system. Abbreviations: ACE-angiotensin-converting enzyme, B1-rec-bradykinin 1 receptor, B2-rec-bradykinin 2 receptor, FXII-factor XII, FXIIa-factor XIIa, HK-high-molecular-weight kininogen, LK-low-molecular-weight kininogen, NEP-neural endopeptidase. Based on original schematic from [137].

1.7.2.3.1.2 Endothelins

Endothelin is a 21 amino acid residue protein that is released by endothelial cells and was discovered in the late 1980s [138, 139]. Endothelin is usually cleaved from a precursor protein and exists as three different isoforms, Endothelin 1, 2 and 3, each having more or less potency at different receptor subtypes [140]. In mammalian cells, the endothelins have two cell surface receptor subtypes, ET_A and ET_B, both operate through multiple G-protein coupled pathways (Figure 1.12) [141]. Key signalling mechanisms appear to be through phospholipases, IP_3 and changes in intracellular calcium levels [142]. Different receptor subtypes are found in varying quantities in a number of different cell types, suggesting that endothelins may participate in a variety of control mechanisms [143]. In vascular tissue, ET_B is found on the endothelium where it produces vasorelaxation through stimulation of both nitric oxide and prostacyclin production. Conversely, ET_A is found in significant quantities in vascular smooth

muscle where upon stimulation it produces vasoconstriction (Figure 1.12). Endothelin-1 is released by the endothelium in response to humoral factors and shear stress and is preferentially released at the basal surface of the cell directly onto the smooth muscle beneath, the subsequent effect on vascular tone is dependent on both local concentration and receptor density/subtype distribution [144]. The relative contribution of endothelin in the control of vascular tone is known to be different, depending on the circulatory system studied [145]. Sustained exposure to shear stress reduces endothelin-1 production and is likely to be mediated by increased nitric oxide production and increased levels of cyclic guanosine monophosphate (cGMP) [146].

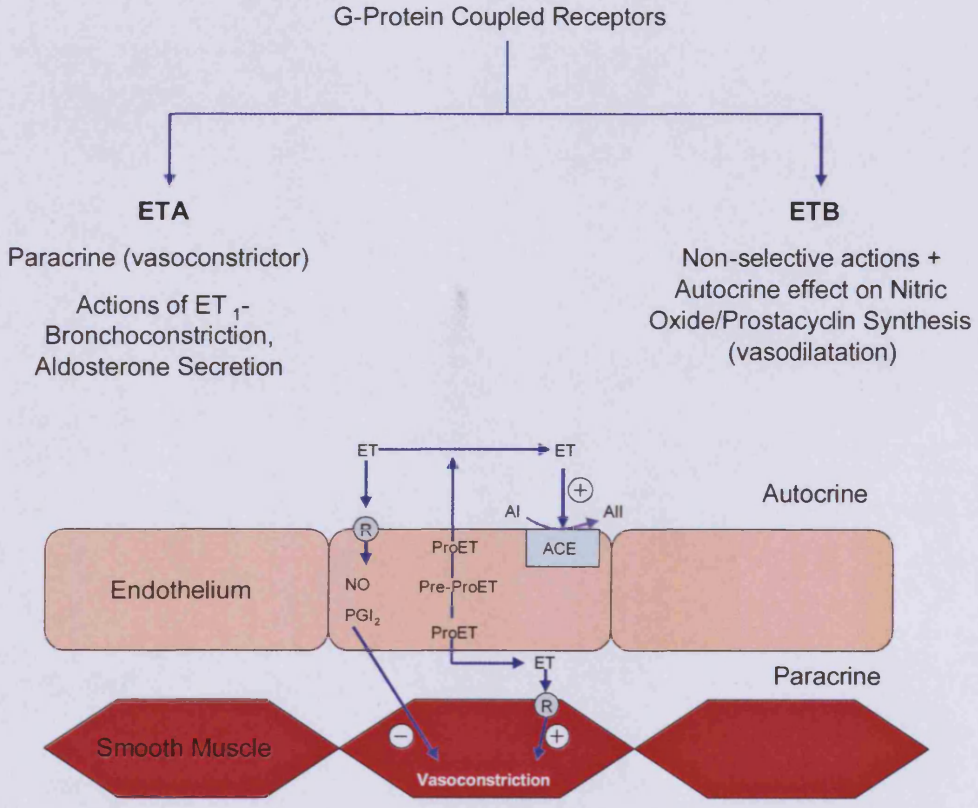

Figure 1.12. Several G-protein coupled endothelin receptors have been identified but they are generally grouped into ETA and ETB according to their main functions (top section of diagram). The autocrine (vasodilatator, via nitric oxide, PGI_2) and paracrine (vasoconstrictor) effects of ET-1 on vascular smooth muscle cells (bottom of diagram). In addition, ET-1 is also thought to play a role in the activation angiotensin converting enzyme (ACE). Based on original schematic from [147].

1.7.2.3.2 The Renin-Angiotensin System

When the body detects a loss of blood volume or a drop in blood pressure, the kidneys are stimulated to release renin. Renin is an aspartyl protease that cleaves angiotensin to angiotensin I. Angiotensin I is then further cleaved by angiotensin converting enzyme (ACE, found on the endothelial cell surface) to active angiotensin II (AT-II), summarised in figure 1.13 [148]. In addition to the well-know chronic effects of AT-II on fluid homeostasis (modulating sodium/water reabsorption in the kidneys, the thirst centre in the central nervous system and adrenal gland aldosterone release), it also has both direct and indirect effects upon blood vessel tone. AT-II directly stimulates angiotensin II type I receptor (AT-IR) present in the vasculature to cause vasoconstriction and produces changes indirectly by increasing sympathetic tone and antidiuretic hormone (ADH, also produces vasoconstriction) release [148]. Angiotensin II type II receptors (AT-IIR) are also known to exist and thought to mediate vasodilatation [149], however it is AT-I that is highly expressed in adult cardiovascular tissue [150].

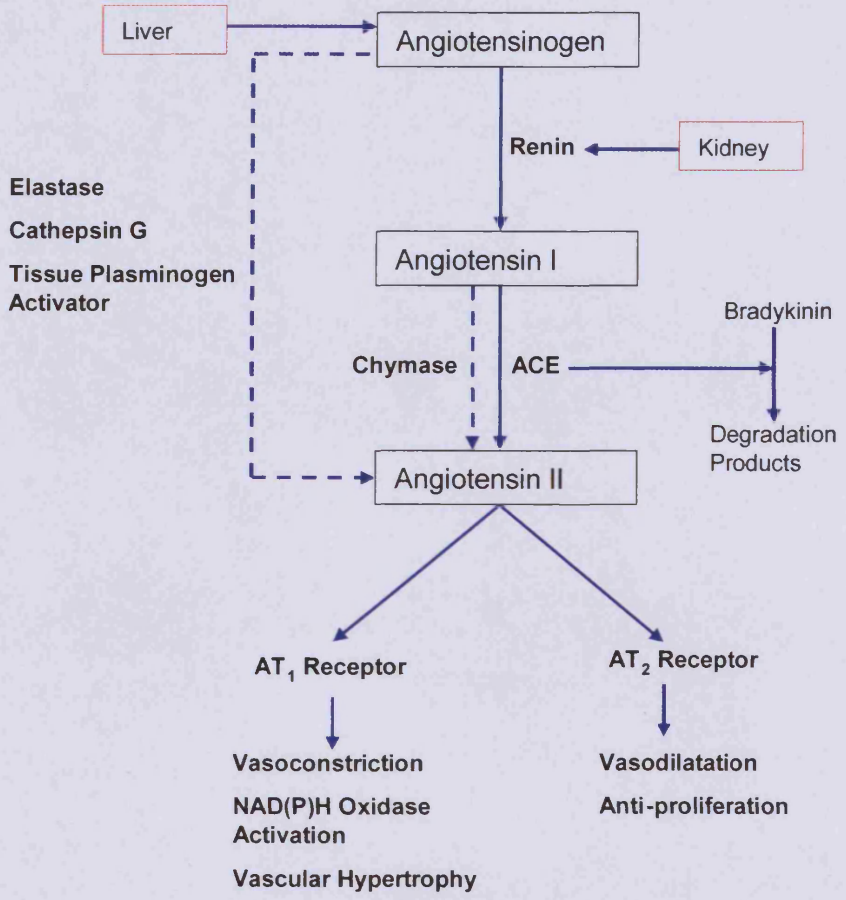

Figure 1.13. Schematic illustration of the renin–angiotensin system including tissue sites, enzymes, and intermediates, as well as functional impacts on blood vessels. Based on original schematic from [151].

1.7.2.4 Endothelium Derived Mediators of Relaxation

The endothelium, adjacent to the smooth muscle and blood flow is perfectly placed to sense changes in smooth muscle or blood and make a corresponding alteration to address said change. Although the endothelium does produce vasoconstrictors e.g. endothelin and thromboxane A_2, it is the vasodilators that it produces that are essential for normal vessel health and control, particularly in respect to the altered patency observed in cardiovascular disease states.

1.7.2.4.1 Eicosanoids

The eicosanoid family encompasses a number of different lipid signalling molecules that include prostaglandins (PGs), thromboxanes (TXs), leukotrienes (LTs) and hydroxyeicosatetraenoic acids (HETEs). All members of this family are biosynthetically derived from the same primary pathway. A change in intracellular calcium (Ca^{2+}) brought about by physical stimuli, chemical stimuli (e.g. hormones, autacoids) or receptor operated pathways (e.g. cytokines) leads to activation of phospholipases, predominantly C or A_2 [152]. These phospholipases cleave phospholipids from the membrane; in humans arachidonic acid is the most abundant phospholipid precursor. Subsequently, unesterified arachidonic acid (AA) is rapidly converted to unstable intermediates by either lipoxygenases (LOXs) or prostaglandin H synthases (also known as the cyclooxygenases (COXs)) [153]. Further conversion by specific enzymes gives rise to final products that have biological activity (Figure 1.14).

Two isozymes of COX are known to exist: COX-1, which is encoded by a constitutively expressed gene and COX-2 which is encoded by an immediate response gene [154]. The majority of cells contain COX-1, whereas only a number of nucleated cells can increase COX-2 levels upon inflammatory/mitogenic stimuli [155]. Homology of key residues in the catalytic domains in the two isoforms gives rise to very similar rates of catalytic activity [156]. Both isoforms contain two distinct catalytic domains. Firstly, an oxygenase domain that oxygenates and cyclises AA to form an endoperoxide known as PGG_2, which in turn enters the peroxidase domain (thought to operate through

the enzyme's haem centre) that converts PGG_2 to PGH_2 by reducing an OOH group at position 15 [157]. The cyclooxygenase site of COX-1 is more flexible than that of COX-2, giving rise to differences in substrate binding [158], this difference is thought to contribute in the development of more isoform specific antagonists [159].

The biological roles of the eicosanoids are far reaching, with key functions in inflammatory regulation and a number of vascular effects on both vessels and blood constituents (e.g. platelets, inflammatory cells) (briefly described in figure 1.14). In the vasculature, both isoforms of COX have been shown to be localised close to the luminal surface of endothelial cells [160]. Close to the interface between vessel wall and blood, the enzymes are perfectly positioned to generate mediators that effect both platelets and vascular smooth muscle. The eicosanoids of particular interest in vascular terms are prostacyclin (PGI_2) and thromboxane A_2 ($(TX)A_2$).

Prostacyclin is converted from PGH_2 by prostacyclin synthase (PGIS), which is found to be co-localised with COX [161]. Once generated, prostacyclin can exert both potent vasodilator and anti-thrombotic activities. In vascular smooth muscle, prostacyclin exerts its effect through a specific G-protein linked receptor (IP), which upon stimulation results in relaxation that is thought to be mediated through increases in cAMP and changes in potassium currents [162-164].

Thromboxane A_2 is generated from PGH_2 by thromboxane synthase (Figure 1.14) and is produced by a number of cell types, principally activated platelets [165]. In vascular terms thromboxane A_2 is important because it has potent actions in contracting smooth muscle [166] and is thought to have underlying involvement in hypertension [167]. The thromboxane A_2 receptor is a G-protein coupled receptor that has signal transduction pathways involving cyclic AMP and the IP_3 (inositol 1,4,5-trisphosphate)/ DAG (diacylglycerol) pathway that operates via modulation of intracellular calcium [168].

A growing number of publications have shown nitric oxide (endogenous and exogenous) to increase COX activity in a number of tissues, including vascular tissue [169-175]. The exact mechanism of the relationship remains to be elucidated, however it has been suggested that nitric oxide may interact with the haem found in COX to alter function in some way [176]. Other work suggests that when nitric oxide reacts with superoxide (O_2^-) to from peroxynitrite ($ONOO^-$), it can nitrate a tyrosine residue of prostacyclin synthase and produce inhibition through steric hindrance [177]. Ironically, the same process (specific tyrosine nitration) is thought to activate COX [178].

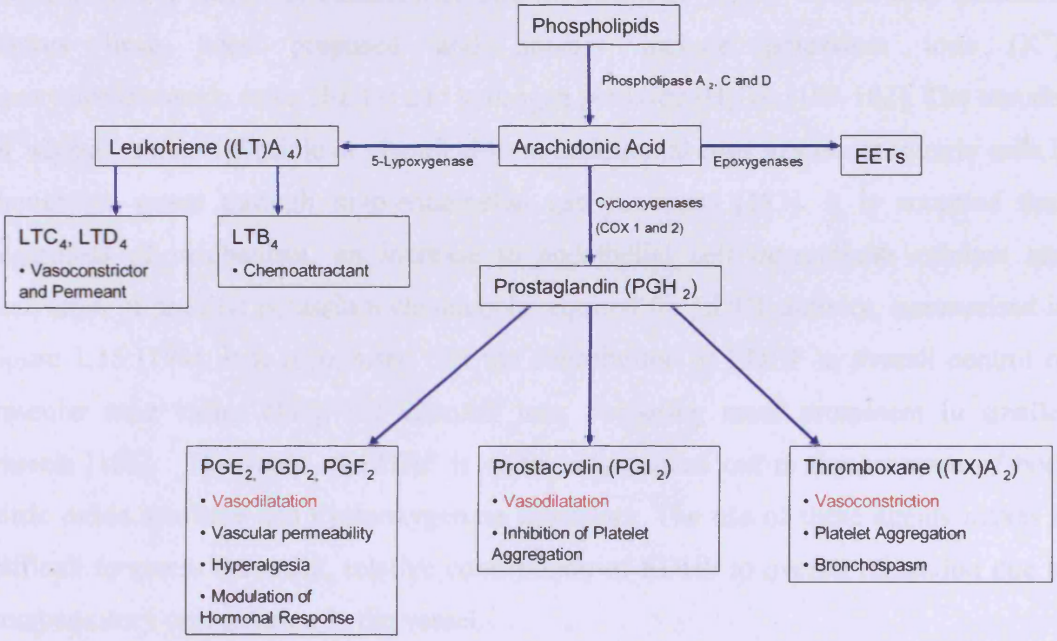

Figure 1.14. Biosynthesis of the eicosanoids. Cell stimulation is followed by activation of phospholipases which liberates arachidonic acid from the membrane, which in turn is converted to unstable intermediates by distinct oxygenases. $(LT)A_4$ and $(PG)H_2$ are then further metabolised into different bioactive products by specific enzymes. Based on original schematic from [179].

1.7.2.4.2 EDHF

In addition to the classic transmitters released by the endothelium, another 'factor' exists to control vessel tone that is more elusive. Termed 'endothelium derived hyperpolarising factor' or EDHF this phenomenon is receiving increasing research interest. EDHF appears to be triggered by a number of endothelial stimuli and ultimately results in smooth muscle hyperpolarisation and subsequently relaxation. The mechanisms that commence and propagate EDHF remain unclear, although some aspects of the phenomenon are understood. EDHF activity is thought to be reliant on either diffusible factors or contact-mediated mechanisms (Figure 1.15). Many diffusible factors have been proposed and notably include potassium ions (K^+), epoxyeicosatrienoic acids (EETs) and hydrogen peroxide (H_2O_2) [180-182]. The transfer of 'signal,' either diffusible or electrical from endothelial cells to smooth muscle cells is thought to occur through myo-endothelial gap junctions [183]. It is accepted that, regardless of mechanism, an increase in endothelial cell intracellular calcium and activation of specific potassium channels is required for EDHF activity, summarised in figure 1.15 [184]. It is recognised that the contribution of EDHF to overall control of vascular tone varies along the vascular tree, becoming more prominent in smaller vessels [185]. The study of EDHF is commonly carried out in the presence of both nitric oxide synthase and cyclooxygenase inhibitors. The use of these agents makes it difficult to assess the exact, relative contribution of EDHF to overall relaxation due to compensatory upregulation in the vessel.

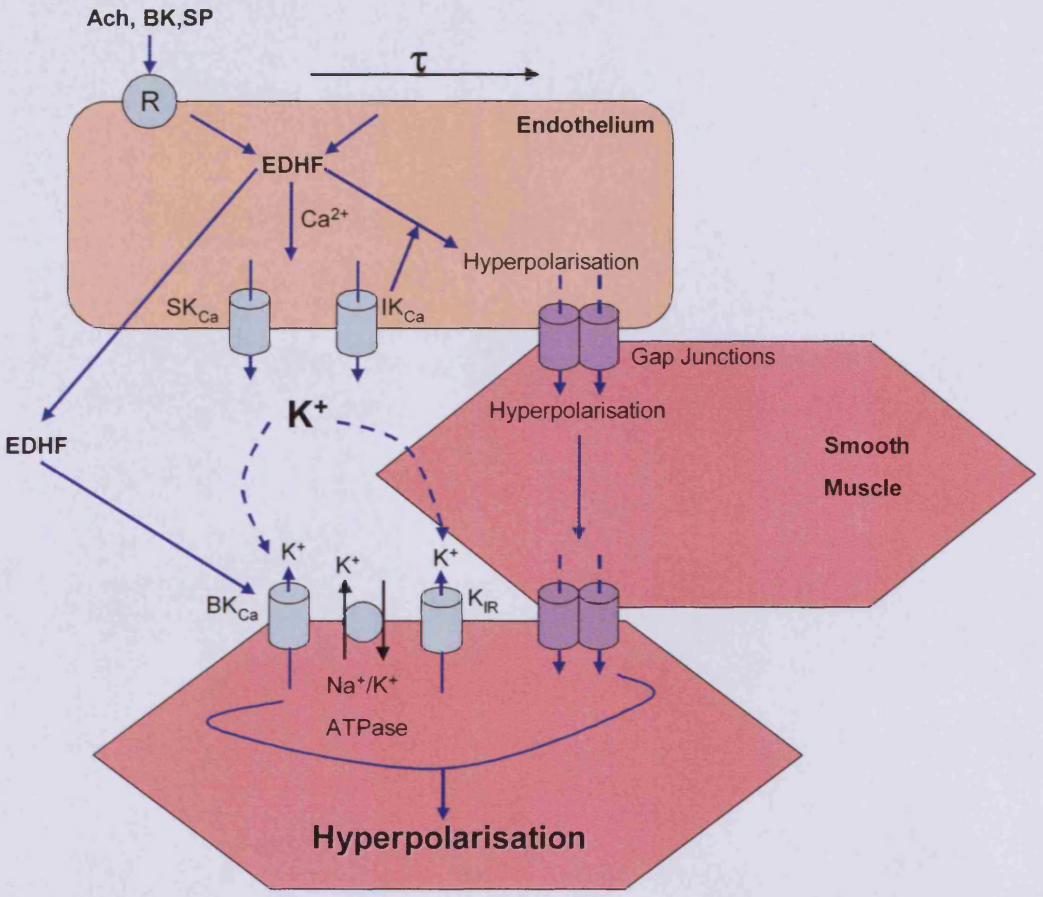

Figure 1.15. Schematic description of the main hyperpolarizing mechanisms mediated by EDHF. The binding of acetylcholine (Ach), bradykinin (BK) and substance P (SP) to their endothelial receptors and the increase in wall shear stress(s) (τ) promote EDHF. EDHF can then hyperpolarize the smooth muscle cells via three principal pathways. 1) EDHF can passively diffuse from the endothelium to activate calcium-activated potassium (K_{Ca}) channels of large conductance (BK_{Ca}) located on the smooth muscle cells thereby promoting the release of K^+ and membrane hyperpolarization. 2) EDHF can act in an autocrine manner to facilitate the activation of the endothelial K_{Ca} channels, both small (SK_{Ca}) and intermediate (IK_{Ca}) conductance channels that release K+ and produce hyperpolarization of the endothelial cells, which is in turn transmitted to the underlying smooth muscle via gap junctions. 3) K^+ released from the endothelial SK_{Ca} and IK_{Ca} channels into the myoendothelial space activates the Na^+/K^+ ATPase and the inward rectifying potassium channels (KIR) located on the smooth muscle cells promoting the release of K^+ and subsequent hyperpolarization of these cells. Smooth muscle cell hyperpolarization decreases the open-state of voltage-gate Ca^{2+} channels, lowering cytosolic Ca^{2+} and thereby provoking vasorelaxation. Based on original schematic from [186].

1.8 Nitric Oxide

Perhaps the most widely studied and understood molecule involved in the control of vascular tone is nitric oxide or endothelium derived relaxing factor (EDRF). The aims of this thesis focus on both nitric oxide and its metabolites in terms of their biochemistry and physiologically relevant bioactivity. Different biological aspects and chemistry of nitric oxide and its metabolic products are covered in detail below.

1.8.1 Nitric Oxide Chemical Identity

Nitric oxide (NO), or nitrogen monoxide, is composed of a single nitrogen atom and a single oxygen atom. The simple covalent interaction between these atoms gives rise to a very chemically active diatomic free radical. It is this chemistry which dictates the actions of nitric oxide in both simple chemical and biological systems [50].

At the heart of nitric oxide chemistry is the nitrogen oxidation state (Figure 1.16). The capacity of nitrogen to form compounds in a range of oxidation states, from -3 to +5, gives rise to a considerable and varied chemistry [187]. Nitric oxide is often only considered in its free radical form ($NO^{\bullet}$), however there are two key redox-activated species of particular interest, the nitroxyl anion (NO^{-}) and the nitrosonium cation (NO^{+}), formed by reduction and oxidation of nitric oxide respectively.

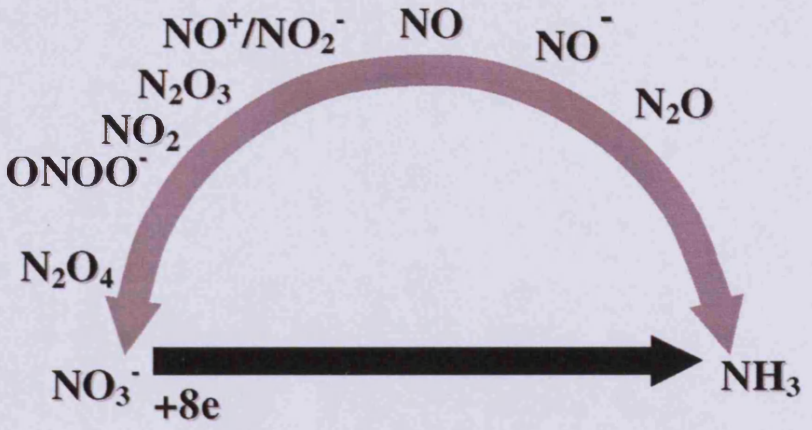

Figure 1.16. The spectrum of nitrogen redox species, extending from fully oxidised (nitrate) to fully reduced (ammonia). The positioning of nitric oxide, nitrosonium, and nitroxyl at the top of the arc indicates that these species are relatively easily oxidised or reduced. The presence of the higher oxides on the left of the arc indicates that reduction of these species back to free radical nitric oxide is relatively difficult and that they are more easily converted to fully oxidized nitrate. Based on original schematic from [188].

1.8.2 Normal *in vivo* Production

The production and release of nitric oxide by the endothelium can be induced by both agonists (such bradykinin or acetylcholine [189]) but also by physical stimuli such as shear stress [190]. The mechanisms that control the production and release of nitric oxide traditionally centre on the specialised enzymes that produce it.

1.8.2.1 Nitric Oxide Synthase (NOS)

There are three main isoforms of nitric oxide synthase, the first to be isolated and purified was neuronal NOS (nNOS or NOS I) [191], this was quickly followed by the isolation and cloning of inducible NOS (iNOS or NOS II) [192] and endothelial NOS (eNOS or NOS III) [193, 194]. Since this initial classification, a number of additional, specific splice-variants have now been identified [195]. nNOS and eNOS are constitutively expressed and are activated by interaction with calcium calmodulin.

Conversely, iNOS is inducible in expression and is activated even at resting cellular calcium levels [196]. Structural differences in the different isoforms can be seen in figure 1.17 below.

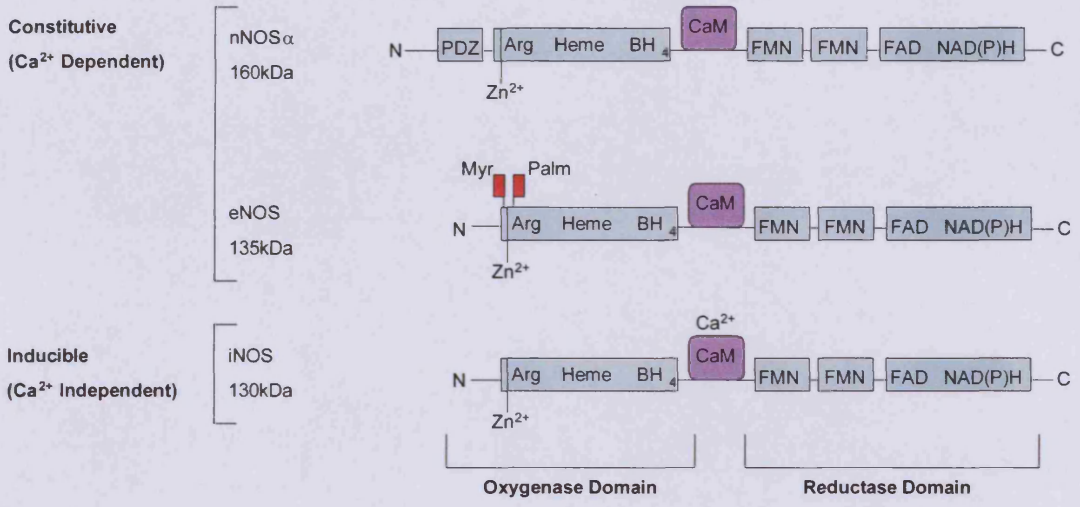

Figure 1.17. A schematic showing the structural arrangement of the three different isoforms of nitric oxide synthase. Note the myristoylation (Myr) and palmitoylation (Palm) sites of eNOS. Based on original schematic from [197].

The NOS enzymes are haem-based oxido-reductases that catalyse the conversion of the amino acid L-arginine and molecular oxygen to L-citrulline and nitric oxide. The NOS family of enzymes contain both an oxygenase and a reductase domain and have been shown to share close homology with the cytochrome P-450 reductase family of enzymes [198].

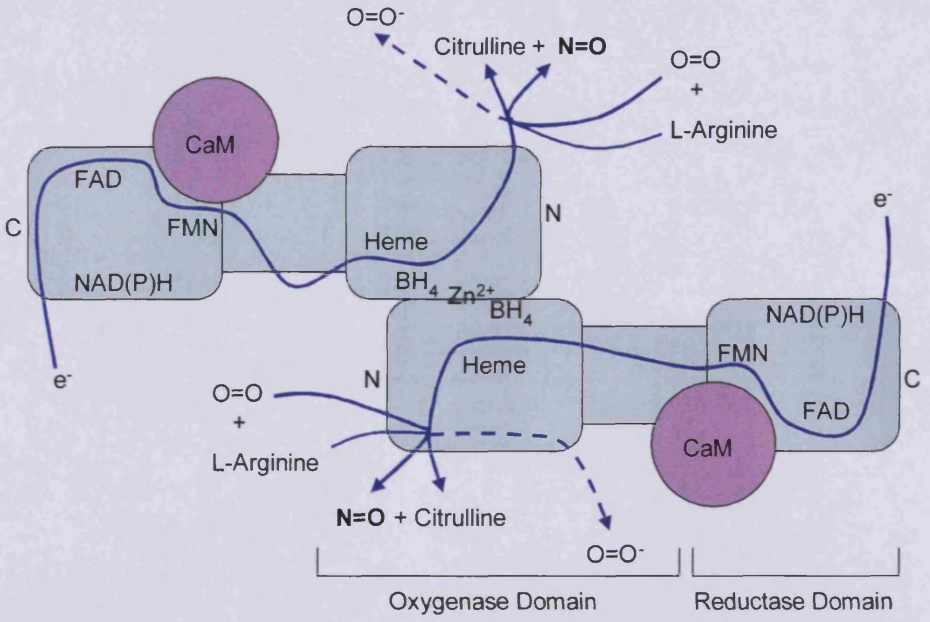

Figure 1.18. Schematic of the functional NOS dimer. A functional NOS dimer requires calmodulin (CaM) and a number of cofactors and substrates. Note the overall oxo-reductase reaction catalyzed in the presence of sufficient (solid lines), or limiting (dashed lines) substrate and/or BH_4 (tetrahydrobiopterin). e- stands for reducing equivalent. Based on original schematic from [197].

In order to operate, NOS must first form a homodimer through the N-terminal interface (Figure 1.18) [199]. This dimerisation is stabilised by the co-factors 6(R)-5,6,7,8-tetrahydrobiopterin (BH_4) (which works as a stabiliser in addition to having a catalytic capacity) and the metallic element, zinc (Zn^{2+}) [200, 201]. The C-terminus (reductase domain) of the enzyme shuttles electrons from NADPH (nicotinamide adenine dinucleotide phosphate) to the co-factors, FMN (flavin mononucleotide) and FMD (flavin adenine dinucleotide), which are in turn passed to the iron within the N-terminus, oxygenase domain protoporphorin IX haem [202]. Acting as an electron acceptor, the haem becomes reduced, enabling the binding of molecular oxygen which in turn leads to oxidation of the guanido nitrogen of L-arginine to form the stable intermediate N^G-hydroxy-L-arginine [203]. A subsequent molecule of oxygen is then recruited to convert this intermediate into L-citrulline and Nitric oxide [204] (figure 1.19). Scenarios can arise where substrates/co-factors are limited. This can lead to

'uncoupling' of the enzyme and generation of oxygen free radicals such as superoxide (O_2^-), peroxynitrite ($ONOO^-$) and hydrogen peroxide (H_2O_2) [205] (figures 1.18 and 1.19, dotted lines).

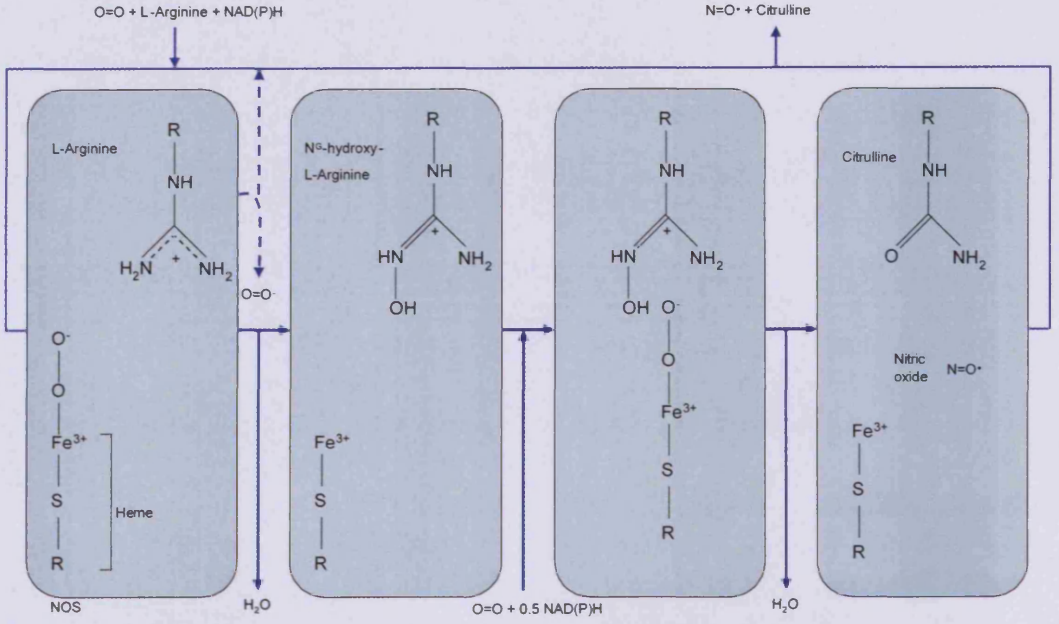

Figure 1.19. Schematic depicting the reactions at the N-terminus, NOS oxygenase Catalytic site. Simplified reaction mechanism in the presence of sufficient (solid lines), or limiting (dashed lines) substrate and/or critical cofactors. Based on original schematic from [197].

1.8.2.1.1 eNOS

eNOS is modified at both co- and post-translational levels by myristoylation and palmitoylation respectively, these modifications function to target eNOS to caveolae (Figure 1.17) [206, 207]. Caveolae are lipid raft micro-domains that form invaginations in the plasma membrane and contain specific functional proteins known as the caveolins [208]. eNOS interacts with the scaffolding domain of caveolin-1, blocking the binding of calmodulin and inhibiting the enzyme [209]. Upon stimulation (e.g. by shear stress or by an agonist) intracellular calcium increases, eNOS is released from caveolin-1 and the inhibitory state of eNOS is reversed by interaction with the calcium/calmodulin complex or Akt-induced phosphorylation at specific sites [210, 211]. It is also thought that heat

shock protein 90 (Hsp-90) may also be involved in the allosteric modulation of eNOS [212]. By targeting eNOS to the plasma membrane and through its interaction with proteins within the caveolae, the enzyme is perfectly placed to adapt quickly to changes in shear stress or receptor interaction. Nitric oxide is thought at some level, to control its own production, in the short-term by inhibitory interactions with eNOS (possibly through haem binding) [213] and in the long-term via inhibition of eNOS transcription [214].

1.8.3 Nitric Oxide Signal Transduction.

The solubility of nitric oxide in water is relatively low (~1.9 x 10^{-6} mol cm^{-3} atm^{-1} at 25°C) [215]. Given the highly reactive nature of the molecule and relatively short distances it can diffuse in biological media [216], its low solubility can be advantageous. Being hydrophobic in nature allows the molecule to easily pass out of one cell i.e. the endothelium, and enter another to exert its actions i.e. into smooth muscle to produce vasorelaxation.

1.8.3.1 Smooth Muscle

When nitric oxide diffuses into smooth muscle cells, it activates the soluble form of guanylate cyclase (sGC) (Figure 1.20). sGC catalyses the conversion of guanosine triphosphate (GTP) to the second messenger, cyclic guanosine 3,5,-monphosphate (cGMP) [217]. As levels of intracellular cGMP rise a number of cGMP-dependent kinases (e.g. PKGs) become activated [218]. These activated kinases phosphorylate a number of key target proteins including potassium channels, IRAG (inositol trisphosphate (IP_3) receptor associated cGMP kinase substrate) which inhibits IP_3 mediated calcium release and phospholamban, which stimulates SERCA (sarco/endoplasmic reticulum Ca^{2+}-ATPase) to sequester cytosolic calcium [219-222] (Figure 1.20). All of these signalling events serve to reduce intracellular calcium, which reduces the formation of calcium-calmodulin-myosin light chain kinase complex (MLCK) and in turn decreases phosphorylation of serine residues in the myosin

regulatory light chain (MLC), inhibiting vasoconstriction [223]. PKG directly and cGMP (via unknown mechanisms) are also known to activate myosin light chain phosphatase (MLCP) which de-phosphorylates MLC, resulting in enhanced vasorelaxation [224, 225]. The level of cGMP and hence the level of kinase activation is controlled by phosphodiesterases (PDEs) that break down cGMP. These enzymes have received much recent attention with the discovery that novel, pharmaceutical PDE inhibitors can be used in the treatment of erectile dysfunction [226].

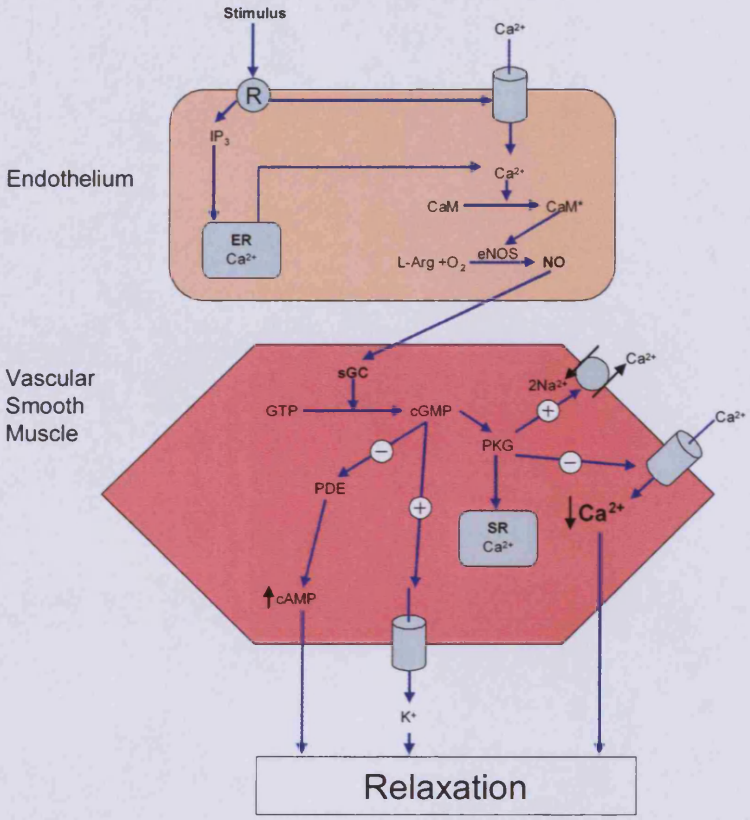

Figure 1.20. Schematic showing the production of nitric oxide in endothelial cells and the mechanism of action of this mediator on vascular smooth muscle mediated by guanylyl cyclase. The activation of endothelial receptors produces an increase in calcium entry via channels, and results in a greater concentration of inositol triphosphate (IP_3) in these cells. IP_3 subsequently releases calcium from the endoplasmic reticulum (ER). This increase in intracellular calcium in the endothelium activates calmodulin (CaM), which in turn activates nitric oxide synthase (NOS). Nitric oxide (NO) is then produced from its precursor, the amino acid L-arginine (L-Arg). The nitric oxide that is synthesised then diffuses into the smooth muscle where it activates soluble guanylyl cyclase (GC), forming cyclic guanosine monophosphate (cGMP) from the guanosine triphosphate (GTP). cGMP activates various protein kinases (eg. PKG), causing a lowering of intercellular Ca^{2+} levels, inhibits PDEs to increase cAMP and can also activate KC channels. All these effects of cGMP produce relaxation of vascular smooth musle. Based on an original schematic from [227].

1.8.3.1.1 Soluble Guanylate Cyclase

Soluble guanylate cyclase was first purified in the 1980s [228] and was subsequently shown to contain haem and to be nitric oxide sensitive [229, 230]. The enzyme is a large αβ heterodimer: haem binds to the N-terminus of the β-chain, while the catalytic domain is comprised of elements from both the α and β chains and is located in the C-terminal region of the molecule [231]. The traditional model for activation of sGC proposes that nitric oxide must bind to the haem and disunite the bond formed between the haem iron and the proximal histidine of the porphyrin structure, altering the protein conformation and increasing catalytic activity [232, 233]. However, more recent studies suggest that the process has two steps that are both nitric oxide dependent [234]. Two theories have been put forward to explain the mechanism of this phenomenon. The first suggests that a second nitric oxide molecule could bind to the haem iron to give an NO-Fe-NO complex, further altering the protein structure [235]. The theory is supported by evidence that shows that even when 80% of sGC haem has nitric oxide bound, the enzyme exhibits only 10% full activity [235]. This suggests there is a low activity form of sGC that is spectrally indistinguishable from the active NO-Fe penta co-ordinate complex. The second theory suggests a non-haem nitric oxide binding site to be responsible. The model is based on the observation that the rate that nitric oxide dissociates from sGC haem is slower than the rate of deactivation [236]. The deactivation of sGC is thought to either occur through simple dissociation of nitric oxide that subsequently reacts with intracellular globins e.g. myoglobin [237], or the iron is oxidised to Fe (III) with the formation of nitrate [238]. Interestingly, the dissociation of nitric oxide from sGC has been shown to be 50 times faster in the presence of substrates (GTP and magnesium ions); this dissociation rate (0.04 s^{-1}) is the fastest reported dissociation of nitric oxide from a ferrous haem protein and the finding gives insight into the control of this enzyme [239].

1.8.4 Other Actions of Nitric Oxide

The signalling actions of nitric oxide are not limited to relaxation of smooth muscle or functions in vascular health, they involve diverse roles from neurotransmission to inhibition of platelet aggregation. Given the magnitude and complexity of the signalling roles in question, the next section of this thesis will concentrate on those that have relevance to the vasculature.

1.8.4.1 Platelets

Nitric oxide generated by the endothelium and by platelets [240] has been shown to have a number of effects upon platelet responses. These include inhibition of aggregation [241], adhesion [242] and recruitment [243]. Although platelet aggregation is a vital process in the prevention of bleeding, disorders or over-stimulation of this process can result in unwanted thrombosis [244]. As in smooth muscle, nitric oxide signalling in platelets is via cGMP production, leading to a reduction in intracellular calcium [245, 246]. The cGMP-dependent reduction in intracellular calcium is also associated with SERCA activation and phosphorylation of phospholamban, as in smooth muscle [247]. As calcium levels fall, it is thought that the association between platelets and fibrinogen decreases and the formation of active glycoprotein IIb/IIIa is inhibited [248, 249]. cGMP increases cAMP levels by inhibiting its breakdown by PDEs, producing enhanced cGMP-like activity [250]. In addition, cGMP also inhibits thrombin mediated activation of phosphoinositide 3-kinase (PI3-K) which normally promotes the active form of the glycoprotein IIb/IIIa complex [251].

1.8.4.2 Cell Proliferation and Angiogenesis

Nitric oxide is intimately involved in the control of angiogenesis via its ability to alter both cell death signalling and promote cellular proliferation with angiogenesis attenuated in the absence of nitric oxide [252]. Nitric oxide has been shown to inhibit the proliferation of vascular smooth muscle [253] and is proposed to operate in this capacity through both cGMP dependent and independent mechanisms. Through cGMP dependent mechanisms, nitric oxide increases levels of cAMP and activates protein kinase A [254]. PKA reduces intracellular calcium levels which counteracts high calcium fluctuations that are known to promote proliferation [255]. Independently of cGMP, nitric oxide inhibits the production of polyamines required for DNA synthesis through blocking of critical enzymes, ornithine decarboxylase and arginase [256].
Nitric oxide is also capable of preventing apoptosis and promoting proliferation of endothelial cells [257, 258].

1.8.4.3 Mitochondria

Nitric oxide has been shown to interact with a number of metal-centred enzymes of the electron transport chain (ETC), including Complexes I, II, III and cytochrome c oxidase (complex IV) [259-261]. The effects of nitric oxide on the system can be both reversible and irreversible and appear to be dependent upon concentration [262]. Cytochrome c oxidase is the final enzyme complex of the ETC which catalyses the reduction of oxygen to water. Cytochrome c oxidase is inhibited by nitric oxide due to nitric oxide binding to the haem oxygen site of the enzyme at physiological concentrations [263]. The inhibition of cytochrome c oxidase causes a reduction in flux through the ETC and a block of cellular respiration [264]. The sensitivity of this mechanism to changes in nitric oxide to oxygen ratio has been proposed to provide a potential link between oxygen sensing and respiration [264]. One scenario in which this reversible/transient mechanism could beneficially alter physiology is in smooth muscle, where a depletion of oxygen and consequent change in respiration/nitric oxide bioavailability could result in vasodilatation [265].

1.8.4.4 Protein Modification

In addition to classic signalling pathways, nitric oxide is now believed to signal through modification of specific proteins, at specific sites. A biologically relevant example of nitric oxide mediated protein modification is S-nitrosylation of cysteine residues, which was first shown by Stamler *et al* in 1992 [266]. Unusually, the covalent bond formed between nitric oxide and the sulphur of a thiol group is readily reversible (i.e. could infer an 'on' and 'off' mechanism) and in light of the fact that nitric oxide can easily pass from one thiol group to another (transnitrosation) there is a suggestion that S-nitrosothiol complexes could have roles in cellular signalling [267]. Since the original observation by Stamler *et al* [266], hundreds of target proteins and processes that can be modulated by S-nitrosylation have been identified, these include regulation of vascular enzymes such as eNOS [268, 269] and modification of mitochondrial proteins to alter respiration [270], ion channels [271], plasma proteins (e.g. factor XIII [272]) and signalling proteins [273].

1.8.4.5 Inflammation

Nitric oxide also plays a number of essential and wide ranging roles in the inflammatory response. The release of inflammatory cytokines causes the activation and upregulation of inducible nitric oxide synthase (iNOS) in a wide range of cell types [274]. The amount of nitric oxide generated in this response is often considerable when compared to the average turn over in endothelium, however, the role that nitric oxide plays is very much dependent upon the site of synthesis, local targets and local concentration [274]. Nitric oxide is belived to play key signalling roles via the regulation of the transcription factor NF-κB [275, 276], which can control processes such as ECAM (cell surface glycoproteins involved in inflammatory cell adhesion, rolling and extravsation) expression on the endothelial cell surface [277]. Nitric oxide is also important in first-line defence providing cytotoxicity against invading parasites, bacteria and viruses [278, 279].

Although nitric oxide is clearly essential in the immune response, in scenarios where production is high (as in sepsis [280]), the ability of nitric oxide to act at multiple targets, act as an oxidant or antioxidant and form other highly reactive species (e.g. peroxynitrite) can potentially be detrimental.

1.9 Nitric Oxide Metabolism in Biological Media

1.9.1 Blood

A proportion of nitric oxide generated by the endothelium inevitably enters the lumen of the blood vessel where it interacts with blood. The metabolism of nitric oxide in this setting varies depending on whether the molecule interacts with plasma constituents or the erythrocytes.

1.9.1.1 Plasma

Nitric oxide metabolism in plasma, as in most aqueous solutions, primarily proceeds via the autoxidation route where nitric oxide undergoes reaction with molecular oxygen to yield NO_2[A] [281, 282].

$2NO^{\bullet} + O_2 \rightarrow 2NO_2^{\bullet}$ [A]

Once formed, NO_2 can undergo further reaction with nitric oxide to form the N-oxide N_2O_3[B] [283] or can dimerise to form N_2O_4[C] [284].

$NO_2^{\bullet} + NO^{\bullet} \leftrightarrow N_2O_3$ [B]

$2NO_2^{\bullet} \leftrightarrow N_2O_4$ [C]

In turn, these products may be hydrolysed to yield nitrite (NO_2^-)[D] [285] and nitrate (NO_3^-)[E] [283], respectively.

$N_2O_3 + H_2O \rightarrow 2HNO_2 \rightarrow 2NO_2^- + 2H^+$ [D]

$N_2O_4 + H_2O \rightarrow NO_3^- + NO_2^- + 2H^+$ [E]

The autoxidation reaction of nitric oxide is second order with respect to nitric oxide concentration and third order overall [286]. Under normal conditions, where the oxygen concentration is much higher than the nitric oxide concentration, the half-life is inversely dependent upon concentration and so the reaction proceeds slowly. This could explain how nitric oxide appears to have sufficient longevity to induce signalling within the immediate area of generation. In areas of high nitric oxide concentration i.e. the micro-environment surrounding activated macrophages [287], the converse is also true and the half-life decreases dramatically.

Both nitric oxide itself and the higher N-oxides (e.g. N_2O_3[B] and N_2O_4[C]) that it forms are effective nitrosating agents and can interact with nucleophilic centres (-S, -N, -O, -C) on plasma proteins (e.g. albumin) to generate a number of species [288]. One of the key nucleophilic groups found on proteins are the thiol (R-S) groups, which are readily nitrosated via several proposed mechanisms. Nitric oxide itself will react with both thiols and thiol radicals[F] to generate nitrosothiols (RSNOs) and nitrosothiol radicals respectively [289]. In the case of direct reaction where a RSNO radical is formed, the RSNO is stabilised by electron abstraction by another molecule, or by protonation to form the RSNOH radical [290]. Generally, the formation of RSNO compounds is thought to proceed via formation of the nitrosonium ion (NO^+) formed via interaction with metals (M) often contained in haem proteins [291][G][H], dinitrosyl iron complexes[I] [292] or via higher N-oxides that have 'NO^+-like' activity[J][K][L] [293]. In areas of low pH, it is also possible for nitrite to generate NO^+ and thus RSNOs[M] [294].

$NO^{\bullet} + RS^{\bullet} \rightarrow RSNO$ [F]

$NO^{\bullet} + M \rightarrow MNO \rightarrow M(reduced) + NO^+$ [G]

$NO^+ + RS^- \rightarrow RSNO$ [H]

[I]

$N_2O_3 + RSH \rightarrow RSNO + H^+ + NO_2^-$ [J]

$N_2O_4 + RSH \rightarrow RSNO + H^+ + NO_3^-$ [K]

$ONOOH^- + RS \rightarrow RSNO + HOO^-$ [L]

$NO_2^- + H^+ + RSH \rightarrow RSNO + H_2O$ [M]

Nitric oxide present in plasma also undergoes reactions with oxygen derived free radicals such as superoxide (O_2^-) [295], hydrogen peroxide (H_2O_2) [296] and the hydroxyl radical ($^{\bullet}OH$)[297]. *In vivo*, there are many sources of superoxide, including the electron transport chain and enzymes such as xanthine oxidase and NAD(P)H oxidase [298]. Nitric oxide reacts extremely rapidly with superoxide (6-10 x 10^9 $M^{-1}s^{-1}$, near diffusion limited [299]) to form peroxynitrite ($ONOO^-$)[N]. Once formed, peroxynitrite can be protonated[O] which leads to rapid decomposition to nitrate[P]. When excess nitric oxide is present, peroxynitrite will undergo further reaction to form nitrite[Q].

$O_2^- + NO^{\bullet} \rightarrow ONOO^-$ [N]

$ONOO^- + H^+ \rightarrow ONOOH$ [O]

$ONOOH \rightarrow OH^{\bullet} + NO_2^{\bullet} \rightarrow NO_3^- + H^+$ [P]

$ONOO^- + NO^{\bullet} \rightarrow 2NO_2^-$ [Q]

Peroxynitrite is a potent oxidant and can have deleterious effects upon many biological molecules including lipids, nucleic acids and proteins [300]. To control the levels of these oxidative species and thus control nitric oxide bioavailability, the body has a number of clearance mechanisms. Superoxide dismutases (SOD), represent a class of enzymes that utilise metal cofactors (e.g. copper, zinc, iron, nickel and manganese), to help scavenge superoxide and catalyse its conversion to hydrogen peroxide [301]. Hydrogen peroxide is then catalysed to water and oxygen by catalase/glutathione peroxidase or to hypochlorous acid by myeloperoxidase [302]. Both reactive oxygen (e.g. superoxide) and nitrogen species (e.g. peroxynitrite) have been heavily implicated in vascular disease states, with over-production of these species closely linked to endothelial dysfunction [303].

1.9.1.2 Erythrocytes

Although nitric oxide can react with erythrocyte membrane components, it is the reactions with the haemoglobin contained within the erythrocytes that are more likely to govern its metabolism. Nitric oxide will react very rapidly with both oxygenated (R-state) and deoxygenated (T-state) haemoglobin ($\sim 10^7$ $M^{-1}s^{-1}$)[304], however the metabolites formed in each case are different. When nitric oxide reacts with oxyhaemoglobin it forms nitrate and met haemoglobin i.e. the oxidation state of the haem iron goes from 2+ to 3+ in the reaction[R] [305]. Methaemoglobin levels in healthy individuals are around 1-3% [306] and are kept at this level by NADH-cytochrome b_5 reductase and soluble electron carrier cytochrome b_5, which converts met haemoglobin back to deoxyhaemoglobin within the erythrocyte [307].

$$Hb^{(Fe2+)}O_2 + NO\bullet \rightarrow metHb^{(Fe3+)} + NO_3^- \qquad [R]$$

The similarities in diatomic spatial structure between nitric oxide and molecular oxygen denote similar bonding patterns. Like molecular oxygen, nitric oxide rapidly

bonds in the same binding pocket of T-state haemoglobin to form iron nitrosyl-haemoglobin (HbNO)[S] [305].

$Hb^{(Fe2+)} + NO\bullet \rightarrow HbNO$ [S]

The dissociation of nitric oxide from HbNO is very slow $\sim 10^{-5}s^{-1}$ [308]. However, *in vivo*, where partial nitrosylation can occur HbNO is subject to different metabolism. Stamler and colleagues have demonstrated that partially nitrosylated haemoglobin can, upon oxygenation, release nitric oxide through haemoglobin conformational changes where it subsequently binds to conserved cysteine residues in the 93 position of β haemoglobin, forming HbSNO[T] [309]. At physiologically relevant NO:Hb ratios, they report an 80% yield of HbSNO upon oxygenation [309]. More recently, Stamler and colleagues have reported that a similar reaction can occur upon oxygenation of nitrite/deoxyhaemoglobin mixes [310].

$HbNO + O_2 \rightarrow oxyHbSNO + 1e^- + 1H^+$ [T]

Metabolic products of other nitric oxide reactions e.g. nitrite will also undergo reactions with both oxy and deoxyhaemoglobin, although at considerably slower rates than that of native nitric oxide [311].Nitrite reacts with oxyhaemoglobin in a process that is thought to be autocatalytic and forms nitrate[U] [312].

$4Hb^{(Fe2+)}O_2 + 4NO_2^- + 4H^+ \rightarrow 4Hb^+ + O_2 + 2H_2O + 4NO_3^-$ [U]

The reaction between deoxyhaemoglobin and nitrite is more complex and involves several component reactions[V][W][X][Y]. The reaction is though to proceed via the formation of nitrous acid (pH dependent) and result in the final products of HbNO and nitrate in a stoichiometric ratio of 1:1 [313]. Nagababu *et al* also propose that the nitric oxide formed in this reaction could exists as an unstable met-haemoglobin adduct ($Hb(II)NO^+ \leftrightarrow Hb(III)NO$) which, in the presence of excess nitric oxide, is thought to decompose to HbNO and the nitrosonium ion (NO^+) [314]. However, spectral and

reaction rate data suggests that this intermediate is transient and unlikely to accumulate [315].

$H^+ + NO_2^- \rightarrow HONO$ [V]

$Hb + HONO \rightarrow met\ Hb + NO + OH^-$ [W]

$Hb + NO \rightarrow HbNO$ [X]

$Hb + ONO^- \rightarrow metHb + `NO_2^{2-}$' [Y]

1.9.1.3 Tissue

In tissues, nitric oxide is likely to participate in free radical reactions as previously discussed in plasma, but perhaps more importantly will bind to metal-centred proteins. The adducts formed with these proteins are usually oxidised to finally form nitrate, which will inevitably leach into the bloodstream [238]. Nitric oxide will also form nitrated lipids through reaction with lipid molecules such as those found in the membrane. Theses molecule have recently started to receive attention after it was recognised that they can act as potent signalling molecules, particularly in inflammatory processes [316]. A summary of the reductive and oxidative reactions of nitric oxide can be seen in figure 1.21.

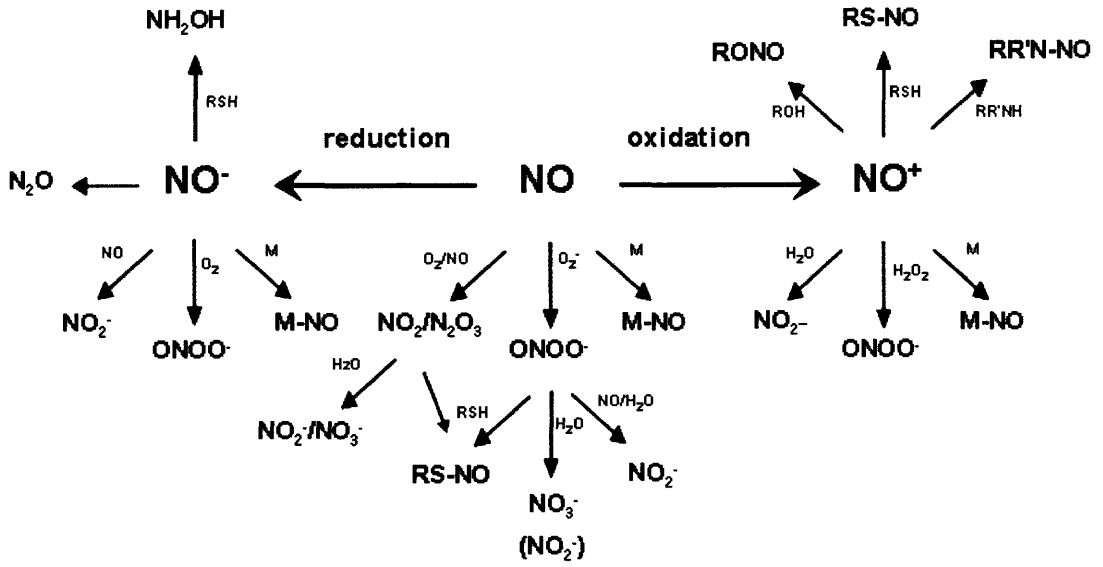

Figure 1.21. A schematic summary of the reactions of nitric oxide. M-metal, RSH-Thiol, RSNO-nitrosothiol, N_2O-dinitrogen oxide, NH_2OH-hydroxylamine, ROH-primary amine, RONO-nitrosamine, RR'NH-Secondary Amine. Based on original schematic from [317].

1.9.2 Excretion

Nitric oxide and its metabolites are known to leave the body via a number of routes. Nitric oxide itself can be exhaled, while its metabolites, namely nitrate, can be excreted via the bowel or principally via the urinary tract [318]. Other products are thought to be metabolised by the liver, through the urea cycle for excretion as nitrate in urine [318].

1.10 Biological Activity of Nitric Oxide Metabolites.

Haemoglobin has historically only been considered in terms of its ability to quench the biological activity of nitric oxide. In light of the rapid rate of reaction between the two and the high concentration of haemoglobin present in the blood stream, it has often been questioned as to how it is possible for nitric oxide to exert its effects *in vivo* given proximity of the reactants in question. Several factors that are thought to enhance nitric oxide longevity have been proposed within the vessel setting. Firstly, haemoglobin contained within erythrocytes does not react as quickly with nitric oxide as free haem [319], this is thought to be due to membrane impedance [320] or an 'unstirred' layer around the erythrocyte that acts to limit diffusion [321, 322]. In addition, it has been proposed that erythrocytes subjected to laminar flow in the vessel lumen do not travel in a uniform arrangement [323]. Erythrocytes travel in the area of fastest flow, down the centre of the lumen, this creates a 'red blood cell free zone' adjacent to the vessel wall and allows nitric oxide to accumulate on the luminal side of the endothelium (undisturbed by erythrocytes) thereby creating a diffusion gradient toward the smooth muscle. However, this phenomenon is unlikely to occur in narrow, resistance vessels, other mechanisms of nitric oxide 'protection' and other regulators of vascular tone (e.g. EDHF) are likely to take precedence in these vessels. Despite these protective mechanisms, nitric oxide has always been considered a paracrine regulator acting within a short diffusion distance. However, recognition that some of the metabolic waste products of nitric oxide could themselves be bioactive has given rise to an entirely new field of research. The relative stability of these metabolites meant that nitric oxide (as its metabolites) could be preserved and be delivered in an endocrine fashion to distal sites.

Original proposals for the preservation of bioactive nitric oxide came from the Stamler laboratory in 1992 [266]. The group discovered that endogenous nitric oxide could form RSNO compounds with thiol containing plasma proteins, principally albumin [324]. The proposal underwent further development after the finding that specific cysteine residues in the β-chains of haemoglobin could also be nitrosylated and

convey biological activity [325]. Since these findings, a number of other candidate metabolites that could conserve nitric oxide bioactivity have been proposed including HbNO [326], the nitrite anion [327] and nitrated lipids [328]. The majority of suggested metabolites/mechanisms are centred around two components, erythrocytes and oxygen. Native erythrocytes have been shown to transiently relax vascular tissue, but only upon introduction to a hypoxic tissue environment [329]. Collectively, these findings have given rise to two main theories regarding the mechanism of conserved nitric oxide bioactivity and erythrocytes, which will be covered in detail below.

1.10.1 The Hb-SNO Theory

After the discovery that the highly conserved β93 cysteine residue of haemoglobin could be nitrosylated (HbSNO) and produce biological effects [325], Stamler and colleagues developed a novel concept in which the vasodilator properties of HbSNO could be matched to oxygen demand in the respiratory cycle (Figure 1.22) [329]. The theory relies upon the conformational state of haemoglobin (i.e. oxygen saturation) and the manner in which this changes over a respiratory cycle.

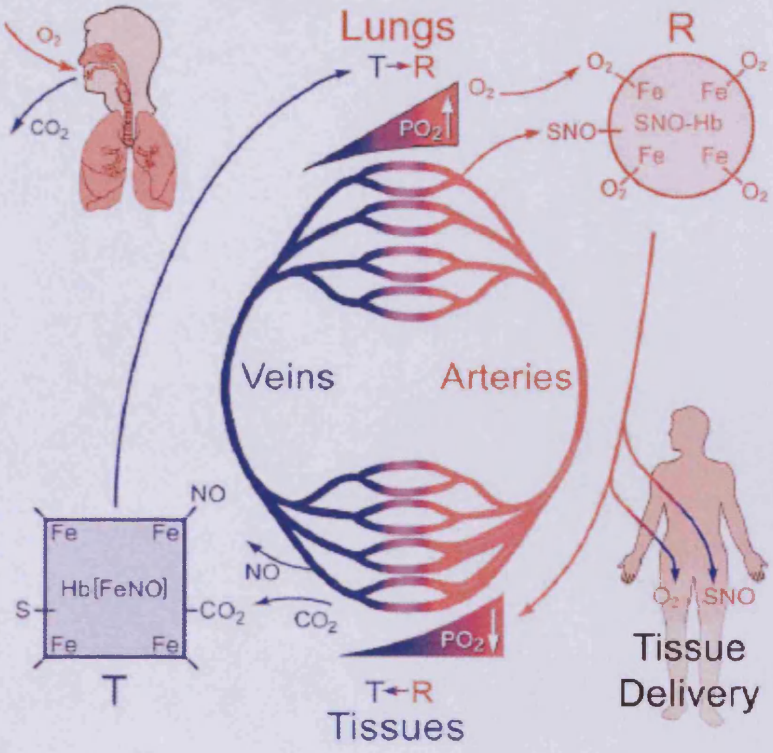

Figure 1.22. Schematic representation of the 'SNO theory' depicting the human respiratory cycle involving 3 key gases, NO, CO_2 and O_2. The SNO theory suggests that O_2 and CO_2 are the allosteric effectors that govern the equilibrium between the R-state and T-state structures of Haemoglobin, which in turn dictates whether nitric oxide bioactivity is dispensed to dilate blood vessels or sequestered thus constricting them. In theory this process has the ability to couple blood flow to metabolic demand. Reproduced from [329].

In the venous circulation, haemoglobin is only partially oxygenated and so the haems that are not carrying oxygen can potentially react with nitric oxide to form HbNO. When erythrocytes carrying partially nitrosylated haem enter the pulmonary circulation it is believed that, through oxygen binding and conformational changes of the haemoglobin protein, nitric oxide is displaced from the oxygen binding pocket and subsequently reacts with the nearby thiol of the β chain, generating R-state, HbSNO [309].

The re-oxygenated blood carrying HbSNO then passes through the arterial system to tissues where oxygen is in demand. Here, the reverse process occurs. As oxygen is offloaded, the haemoglobin conformation changes from R to T-state (HbSNO being less stable in the T-state) and so SNO is also offloaded, thereby concomitantly delivering both oxygen and SNO to the tissue. The dependency of β93 cysteine reactivity upon haemoglobin conformation has been known for sometime [330] and applies in this situation. One key question of the SNO theory is, how does nitric oxide escape from the erythrocyte avoiding recapture by haemoglobin? The proposed mechanism involves transnitrosation, through being transferred from the thiol group of one protein to another the nitric oxide is effectively protected. The favoured candidate protein, AE1, also known as membrane protein band III was originally proposed by Pawloski *et al* (Figure 1.23) [331]. AE1 is the perfect candidate, positioned close to the membrane for subsequent transfer, in addition to the cytosolic N-terminus interacting with T-state haemoglobin to lower oxygen affinity, thereby enhancing oxygen/SNO offloading. The physiological importance of this interaction can be seen with the use of DIDS (4,4'-diisothiocyanostilbene-2,2'-disulfonic acid, an AE1 channel blocker), inhibition by DIDS has been shown to prevent HbSNO vasodilatation of hypoxic aortic rings [331]. The SNO theory is also supported by *in vivo* evidence showing an arterial to venous gradient of HbSNO concentration, suggesting consumption across the tissue bed [332].

Overall, the principle role of erythrocytes is to deliver oxygen, which is largely governed by blood flow [332]. Therefore the SNO theory suggests that erythrocytes could have an intrinsic capacity to enhance their primary function.

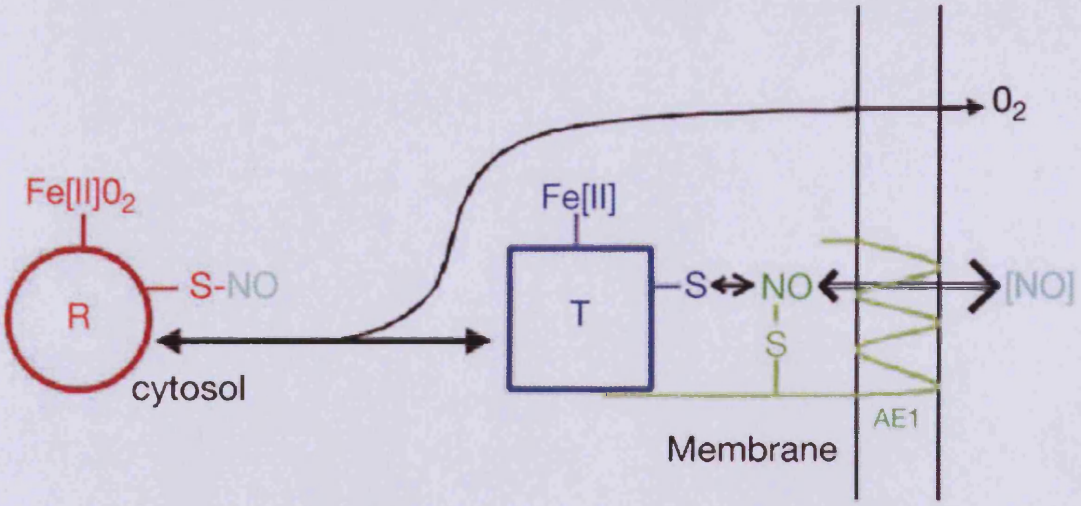

Figure 1.23. How does nitric oxide liberated from HbSNO leave the red cell? The cytoplasmic domain of AE1 preferentially binds T-state Hb and thus promotes the R-state to T-state transition of SNO–Hb, which effects transfer of nitric oxide groups to the membrane. SNO on AE1 is then exported from this site to thiols outside the erythrocyte e.g. plasma proteins. Thus, the transition from high to low p_{O2} would be transduced by the interaction of Hb and AE1 into increased export by RBCs of nitric oxide-related bioactivity and decreased scavenging of endothelial-derived nitric oxide. Adapted from [331].

1.10.2 The Nitrite Reduction Theory

The vasoactive properties of the nitrite anion have been known for sometime [333]. However, until recently the biological activity of nitrite has never been considered in terms of its position as a circulating metabolite of nitric oxide. Early work demonstrated that nitrite could be reduced to nitric oxide in a biological setting by oxidoreductase enzymes, such as xanthine oxidase [334], disproportionation [335] and deoxyhaemoglobin[W] [336]. These observations, coupled with the fact that an artery to vein gradient of nitrite had been shown to exist [327], prompted research into the activation of circulating nitrite *in vivo* [337]. Overall, this research propagated a novel idea that linked these facts, with deoxyhaemoglobin nitrite reduction at the heart of the concept. The next major step in the nitrite field was a proposal by the Gladwin laboratory, associating the nitrite reduction properties of deoxyhaemoglobin to allosteric control [313, 338]. This allosteric control proposal gave the research applicability to the physiological setting and, like the SNO theory, could explain a potential mechanism for matching vasodilatation/flow to tissue oxygen demand.

Haemoglobin, through a process termed 'R-state autocatalysis,' is suggested to operate maximally as a nitrite reductase at approximately 50% oxygen saturation (P_{50}) (Figure 1.23a), which is based upon several observations. It is possible for deoxyhaem within haemoglobin to have allosteric character of both R-state and T-state depending upon the ligand status of the 3 other haem groups. Nitrite has been show to react differently with these two conformations, nitrite reductase activity being higher in the so called R-state deoxyhaem (6 $M^{-1}s^{-1}$ vs. 0.12 $M^{-1}s^{-1}$, R vs. T) [338]. The mechanism is only based upon these two rate constants. However, within a haemoglobin tetramer, a global rate constant exists which is dictated by the individual haem groups within it. These observations have been confirmed by experiments utilising a gel matrix to lock haemoglobin in specific allosteric configurations whilst monitoring reaction kinetics [339]. As nitrite reacts with haem in the T-state tetramer, the rate of reaction decreases as deoxyhaem is consumed. This is balanced by an increasing rate of reaction with R-state deoxyhaem within the tetramer, which results from, and is stabilised by

metHb/HbNO formation [338]. It is likely that the increasing rate of nitrite reduction in the R-state is in some part due to its lower reduction potential i.e. the R-state is more likely to donate electrons [340]. As levels of metHb and HbNO increase in the tetramer the haemoglobin molecule shifts from T-state to R-state. Therefore, a balance point exists (occurring around 50% saturation/when haemoglobin is in the R_2/R_3 tetramer states), where there is sufficient T-state haemoglobin to bind nitrite but also enough R-state haemoglobin to efficiently catalyse nitrite reduction. This work has all been performed with high nitrite concentrations using *in vitro* preparations starting with purely T-state haem. *In vivo,* this scenario is unlikely to occur, so how does the theory translate?

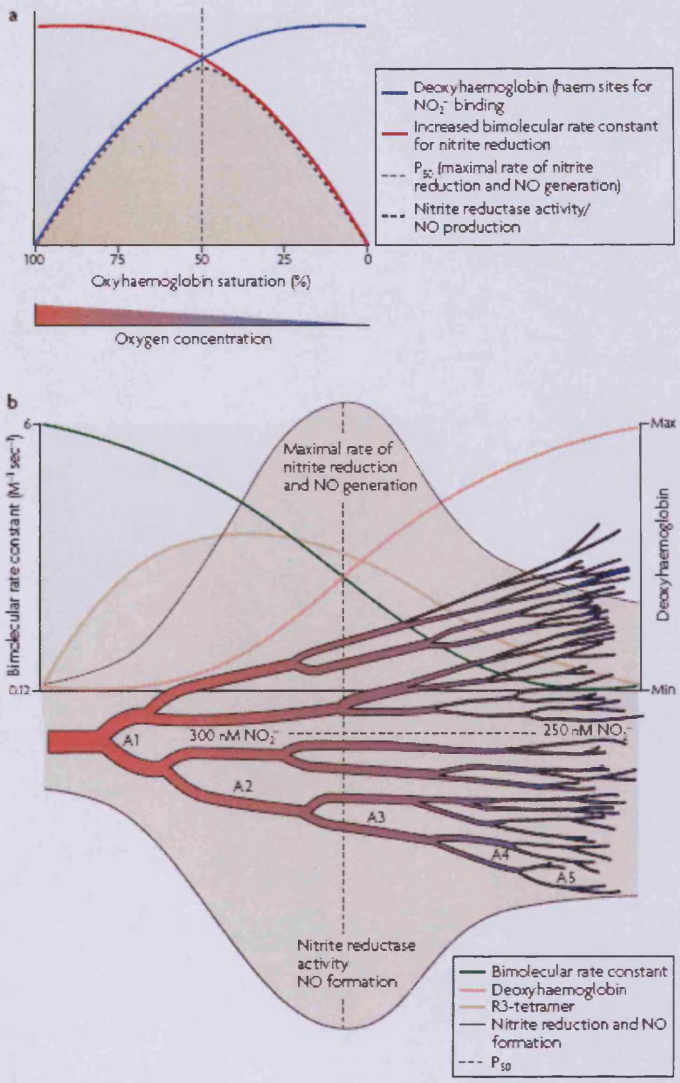

Figure 1.23. R-state or allosteric autocatalysis of nitrite reduction by haemoglobin. A) The rate at which haemoglobin converts nitrite into nitric oxide (NO) is maximal when it is 50% saturated (P_{50}). This effect is mechanistically determined by two factors, the availability of deoxyhaems (reaction substrate) to bind nitrite, which is maximal in T-state or deoxygenated haemoglobin, and the amount of R-state or oxygenated haemoglobin tetramer, which increases the intrinsic reactivity of the haem with nitrite. B) In the mammalian circulation, the allosteric state of haemoglobin tetramers is modulated primarily by oxygen ligation such that the *in vivo,* intrinsic reactivity is therefore dictated by oxygen binding to other haems on the same tetramer. This model therefore predicts that the most effective tetrameric nitrite reductase would be the R-state haemoglobin that rapidly deoxygenates during arterial to capillary transit. The R3 and R2 tetramers would therefore be the most effective nitrite reductases. A1–A5 reflects the arteriolar size, which decreases at bifurcation points along the vascular tree. Reproduced from [311].

In vivo, it is believed that oxygen ligation, as opposed to metHb/HbNO, acts as the R-state stabiliser (Figure 1.23b) [341]. Therefore, in venous deoxygenated blood maximum reactant levels (high T-state) exist, while in arterial blood the most effective nitrite reductase, (R-state), exists. Again, this gives rise to a balance point, suggesting that nitrite reductase activity will be maximal in the rapidly deoxygenating arterial blood as it crosses the tissue bed. At this point in the vascular tree, it is assumed that the haemoglobin tetramer will pass from R_4 to R_3 and R_2 (maximal nitrite reduction) before flipping to the T-states, T_2 and T_1 [338]. This notion is supported by work demonstrating that nitrite has maximal effect upon nitric oxide-mediated signalling, vasoactivty and mitochondrial inhibition when haemoglobin is 50% saturated [342]. Questions as to how nitric oxide can be formed and leave the erythrocyte to exert an effect, within the time-course of one arterial to venous transit, and if a saturation near 50% is regularly achieved in the circulation under normal conditions are still points of debate [343]. In addition to haemoglobin, other proteins with nitrite reductase activity are under investigation, such as the previously mentioned oxidoreductase enzymes and myoglobin found in muscle [344].

1.11 Nitric Oxide Metabolites and Hypoxic Pathologies.

In addition to nitric oxide and haemoglobin, the other key factor involved in the mechanism of nitric oxide metabolite actions is a change in oxygen level. *In vivo*, under normal conditions, oxygen levels drop over a respiratory cycle, however, dramatic changes in oxygen level only tend to occur under conditions of serious vascular pathophysiology. Long term vascular pathology is associated with endothelial dysfunction and a resultant reduction in so called 'nitric oxide bioavailability'. The mechanisms of endothelial dysfunction remain unclear, although there is apparent evidence for a link with atherosclerosis [345], increases in reactive oxygen species leading to nitric oxide scavenging and radical damage [346] and altered eNOS levels and functionality [347, 348]. Endothelial dysfunction and the accompanying reduction in nitric oxide bioavailabilty are detrimental to the overall health of the vessel and inhibit the vessel from fully dilating. The inability to accurately control vessel tone can lead to a reduction in blood flow and oxygen supply that can be devastating, particularly in high demand organs such as the heart, brain and kidneys. Persistent disease can lead to narrowing of arteries, such as the coronary arteries of the heart, causing tissue hypoxia and angina in the case of the heart. As the health of the vessel deteriorates, atherosclerotic plaques can become unstable and if ruptured they initiate a clotting cascade that blocks the vessel, resulting in infarction. If and when a clot is cleared, the vessel and the tissue which it supplies is then subjected to further damage by a process know as 'ischaemia-reperfusion injury'[349].

Nitric oxide metabolites have been shown to be beneficial in a number of hypoxic/ischaemic environments found in the liver [350], the heart [344], the brain [351] and the kidney [352]. It is these potential therapeutic benefits that warrant further research into the basic interactions of nitric oxide and its metabolites and the mechanisms by which they convey physiologically relevant, bioactivity.

1.12 Nitric Oxide/Nitric Oxide Metabolite Detection in Biological Samples/Systems

There has long been an interest in the measurement of free nitric oxide, however its reactive nature and short half-life are the source of a number of practical limitations in terms of real time detection. A practical solution to this problem is the measurement of the more stable metabolites of nitric oxide. The measurement of nitric oxide metabolites is now common, although disagreements over methodology and measured levels still ensue. As detection methods have developed and sensitivity improved, reported blood levels have slowly decreased to a consensus between laboratories of the level expected in certain media. A review of methodologies and their development is covered briefly below with an emphasis on those most commonly and currently used within the field.

1.12.1 Nitric Oxide

The measurement of free nitric oxide can essentially be split into two different forms of methodology, those that monitor generation in real time and those that are cumulative. The real time study of nitric oxide is largely based around the use of electrochemical methods, principally electrodes. These electrodes are designed to be specific for nitric oxide and allow real time and *in situ* measurement of *in vivo* and *in vitro* nitric oxide generating systems [353]. Although electrodes represent the most advanced method of real time nitric oxide measurement, particularly *in situ*, they do have a number of practical limitations. Electrodes often generate non-specific signals and are sensitive to a number of environmental factors, such a temperature and movement of the surrounding media, the latter being a major problem for the intra vascular measurement of nitric oxide *in vivo* where blood flow is constantly changing.

Cumulative nitric oxide measurement methods exploit the free radical nature of nitric oxide and its ability to form adducts that have paramagnetic properties. In this way nitric oxide levels can be assessed via the interaction with specific 'spin traps' that bind

nitric oxide to give altered paramagnetic properties [354]. These paramagnetic species have unique signals that can be visualised and quantified with electron paramagnetic resonance spectroscopy (EPR). The generation of unique signals makes EPR measurement of nitric oxide adducts very specific, however the technique does lack sensitivity at lower concentrations and signals represent an accumulation of adduct rather than a real time level.

It is also possible to image and measure nitric oxide using fluorescent probes. 4,5-diaminofluorescein (DAF) is one of the most widely used probes, however this probe like many others suffers from a lack of specificity [355]. For this reason, fluorescent probes are generally no longer used for quantification of nitric oxide.

1.12.2 Nitrite and Nitrate

Nitrite and nitrate represent the most commonly measured metabolic products of nitric oxide. Although more stable than free nitric oxide, the measurement of nitric oxide metabolites does have its own caveats. Firstly, metabolites are present in complex biological media; secondly, many similar metabolites are present in one sample; thirdly, levels found in biological samples can be in the low nanomolar range; fourthly, many chemicals, solutions and equipment contain contamination and lastly, metabolite species can change from one to another, particularly in the presence of oxygen.

Early methodology quantified nitrite and nitrate using light spectroscopy in combination with the Griess reagent (N-1-naphthyl-ethylenediamine dihydrochloride, sulphanilamide and phosphoric acid) in which nitrite forms a coloured azo dye [356]. However, the need for a more precise measurement lead to the development of high performance liquid chromatography (HPLC) based methodologies [357]. Despite being more sensitive, these methods do have more general issues, principally pertaining to the over processing of the sample. Due to the continued risk of contamination and metabolite stability, methods that employ minimum sample processing are optimal. The current gold-standard for the measurement of nitric oxide metabolite species, where

minimum processing can be used, is ozone based chemiluminescence (OBC). OBC measures nitric oxide directly, therefore OBC is always linked to a reductive process that will cleave or reduce nitric oxide metabolites back to free nitric oxide that can be carried away by a flow of inert gas for measurement [358]. Initial chemical cleavage reagents were formed from potassium iodide and acid, however it was recognised that the reductive power of this reagent was variable but could however be stabilised by the addition of excess iodide to the reagent [359], giving rise to the most commonly used cleavage reagent, tri-iodide. In addition to the measurement of nitrite, tri-iodide has also been validated for the measurement of nitrosothiol compounds (SNOs) [360]. In order to measure nitrate, a cleavage reagent with increased reductive power is required, the most commonly used reagent for this purpose is vanadium III chloride in acid at high temperature [361], which cleaves nitrate, nitrite and RSNO/RNNO compounds back to nitric oxide.

1.12.3 Haemoglobin-Bound Products

The vast majority of research regarding nitric oxide metabolites centres on circulating levels in blood. Nitric oxide metabolites are present in the largest quantity in the plasma fraction of whole blood but they also enter erythrocytes and directly interact with haemoglobin, forming HbNO and HbSNO as previously discussed (section 1.9.1.2, page 59).

HbNO levels can be assessed using light based spectrophotometry [313] and EPR (electro-paramagnetic resonance spectroscopy) [362]. However, despite the excellent specificity, sensitivity at lower concentrations, such as those found in biological samples, is an issue with these methods. OBC linked methodologies are widely used to measure erythrocyte nitric oxide metabolites and both chemical and non-chemical cleavage methods have been developed. Photolysis is the most commonly used non-chemical method of reducing HbNO and RSNO compounds (including HbSNO) [325]. Photolysis does represent a simple method in terms of chemistry but the need for sample preparation, expense of equipment, limited number of species measured, reduced

ease of use and questions over specificity [359] have prevented photolysis from being extensively used in many laboratories for the measurement of metabolites.

Chemical reductive methodologies have also under gone development to make them more suitable for erythrocyte nitric oxide metabolite quantification. One key problem with tri-iodide reduction was a phenomenon known as auto-capture. When nitric oxide is liberated in the reductive mixture it can be re-bound by free haemoglobin in the purge vessel and cause signal broadening and error in metabolite concentration quantification. To combat, this problem three different ideas have been put into practice. The first is an alternative reductive agent containing copper and cysteine that is utilised with carbon monoxide used as a purging carrier gas at high pressure to block haemoglobin from binding nitric oxide [363]. This method is limited to the measurement of RSNO compounds and in addition to its increased health and safety risk, our laboratory have not found it to be efficient in preventing auto capture. The second method involves the addition of a stabilisation solution to blood samples that largely contains hexacyanoferrate, thought to bind haemoglobin and prevent further reactions before the cleavage in tri-iodide [364]. Unfortunately, the addition of stabilisation solution results in the need to process samples heavily, introducing possibility of contamination and can also cause the inter-conversion of metabolite species. The third and final method, based on hexacyanoferrate inhibition, was developed in our laboratory [365]. Hexacyanoferrate is added to the tri-iodide cleavage reagent and blocks haemoglobin re-capture of nitric oxide during the cleavage process, negating the need for sample processing and improving recovery of all cleavable species. This method and its validation are covered in more detail in the general methodology of this thesis, in chapter 2.

Arriving at a consensus point with regard to specific, reproducible methodology and standardised protocols is the result of many years of work by a number of people within our laboratory. I was fortunate enough to work with Dr Stephen Rogers and Dr Afshin Khlalatbari, when this body of work was in its infancy, who both dedicated the work of their doctorates to establishing these methods. Work by Dr Rogers regarding the

development of modified tri-iodide [365] and more recent progress in our laboratory have formed the basis of a text book chapter that details the practical aspects of measuring nitric oxide metabolites in biological media and the reasoning behind the developments that have been made. In possession of these developed methodologies, it was possible to investigate nitric oxide metabolites in different model systems. These investigations formed the primary aims of this thesis.

1.13 Thesis Aims

This thesis aims to provide a clearer understanding of the relationship between nitric oxide, its metabolites and haemoglobin contained within erythrocytes. In addition, the physiological contributions of these metabolites will be assessed and their mechanisms of action both at a simple biochemical level and at a more complex level in human subjects will be challenged. Overall, this thesis aims to consider the significant role of oxygen in modulating these mechanisms and physiological outcomes. The literature to date has two distinct ideas regarding the physiological relevance and mechanism of action with regard to nitric oxide metabolites, which has led to publications that are misrepresented, misinterpreted and polar in view. The current work endeavours to be subjective and critical, taking into account all aspects of published work in the field and to carry out studies under physiologically relevant conditions.

1.13.1 Specific Aims

- To investigate the effect of oxygen upon the metabolism of nitric oxide and its metabolites in blood. In addition, novel methodologies will be developed to consider the movement of these species with respect to blood constituents and oxygen.

- To determine the acute mechanisms of action by which nitrite affects vascular tissue, assessing sources of nitrite reduction and the contribution of altered oxygen level. The chronic effects of nitrite in the vasculature will be reviewed by analysis of gene activation.

- To examine the erythrocyte-induced hypoxic vasodilatation phenomenon in detail and to provide novel evidence regarding the nature of the vasorelaxant responsible and the mechanism by which it influences hypoxic vascular tissue.

- To study the effect of nitrite supplementation on both physiology and biochemistry in a human model of hypoxia. The metabolism of nitrite and the mechanisms by which it exerts its effects in human vasculature will also be explored.

2 General Methods

2.1 Blood Collection and Processing

2.1.1 Phlebotomy

Subjects gave consent for a sample to be drawn, all procedures were carried out in line with Cardiff and Vale NHS trust phlebotomy guidance/training and were approved by a local ethics committee. Blood samples were collected by venepuncture of the antecubital veins (median cubital and cephalic veins) using a syringe and butterfly needle. Samples were then decanted into 4ml K3E EDTA vacutainers (Greiner Bio-One) to prevent clotting and maintain current oxygen saturation.

2.1.2 Blood Sample Processing

Blood samples, unless otherwise stated, were centrifuged at 2000g for 5 minutes at 4°C. Usually, the plasma and erythrocyte fractions were immediately separated, while the Buffy coat was discarded. Fractionated samples for future analysis were then directly snap-frozen in liquid nitrogen followed by storage at -80°C. Frozen samples, when required for analysis were rapidly thawed in a water bath at 37°C and immediately analysed.

2.2 Altering Blood Oxygen Saturation

To alter the saturation of erythrocytes, centrifuged erythrocyte samples were first diluted (usually ~1 in 3, to achieve a final haemoglobin concentration of 10g/dl) in phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl and 10 mM phosphate buffer solution, pH 7.4 at 25°C). The reconstituted mixture was then loaded into a thin film rotating tonometer as in figure 2.1. The tonometer is purged with different gas mixes that interact with the thin film of erythrocytes created upon rotation to rapidly achieve the desired saturation. The system can be sealed to maintain a specific saturation and samples can be taken for blood gas analysis. The tonometer utilised for work in this thesis was developed by Dr Doctor of the Department of Paediatrics and Biochemistry & Molecular Biophysics, Washington University in St Louis whose laboratory has previously published established methodology for use of the tonometer [363].

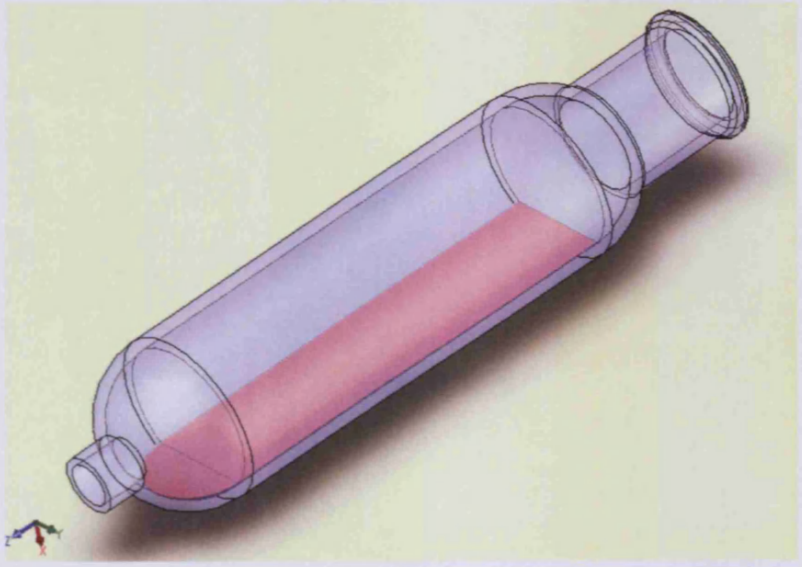

Figure 2.1. Diagrammatic representation of the thin film rotating tonometer (approximately 200mm in length). As the tonometer rotates a thin film of suspended erythrocytes is formed around the surface, this creates a large surface area for rapid exchange of gases, mimicking a circulatory scenario. Picture drawn with the aid of Ross Pinder.

2.3 Ozone Based Chemiluminescence

Ozone based chemiluminescence (OBC) is a measurement technique that exploits the luminescent nature of specific chemical reactions. The nitric oxide analyser (Sievers NOA 270, Boulder, CO, USA) utilises OBC for quantification of nitric oxide via its reaction with ozone (O_3).

The NOA has a specialised reaction cell where a constant amount of ozone is generated, the nitric oxide sample in a flow of inert gas is drawn into the reaction cell where it reacts with ozone to form 'excited' $NO_2^{*[Z]}$. This form of NO_2 is unstable and so, as electrons in the excited state move back to ground state, they dissipate energy in the form of a photon ($h\nu$, h-Plank's constant ν-frequency)[AA] (in the red-infrared ~640-3000nm, with peak at ~1100nm, [366]).

$$NO + O_3 \rightarrow NO_2^* + O_2 \qquad [Z]$$

$$NO_2^* \rightarrow NO_2 + h\nu \qquad [AA]$$

One face of the reaction cell is a red cut-off lens, which filters the light energy before it enters a photomultiplier tube (PMT), preventing the detection of light at other wavelengths, generated by unwanted reactions. The PMT is formed of a number of sensitive surfaces, as a photon hits the first, photosensitive surface an electron is emitted which in turn hits the second, electron-sensitive surface to release several electrons, as the process continues down the tube the signal is amplified and can be measured as a potential difference across the tube in millivolts (mV). Therefore, the change in potential difference is directly proportional to the number of photons entering it and thus the amount of nitric oxide entering the reaction cell.

2.3.1 Cleavage Reagents

The measurement of nitric oxide (and its metabolites) in the gas phase via OBC requires cleavage from its parent compound(s) or conversion to its radical form. A number of chemical cleavage reagents have been developed for the measurement of specific metabolites (summarised in table 2.1) and will be discussed in the following sections.

Table 2.1. A summary of cleavage reagents used with ozone based chemiluminescence and the species that they are capable of measuring.

	Tri-iodide	Vanadium chloride
Reagent temperature	50 (±1)°C	85 (±1)°C
Species cleaved or reduced	NO_2^-, RSNO, RNNO, RONO, RMNO (where M is a metal)	NO_3^-, NO_2^-, RSNO, RNNO, RONO, RMNO (where M is a metal)

The cleavage reagent used is loaded into a glass purge vessel with injection port (custom developed in our laboratory) that is purged with inert gas. The gas carries the gas phase products formed in the purge vessel to the NOA and also aids homogenous mixing of the reagents (Figure 2.2). The reagent is also heated (see Table 2.1) to increase reaction rate and provide clear sample peaks. 20µl of anti-foam 204 organic is added to the cleavage reagent in the purge vessel to prevent foaming upon injection of samples containing protein. It should be noted that all reagents are freshly prepared on the day of use. A sodium hydroxide trap (1N) and solvent filter is placed between the refluxing reagents in the purge vessel and the NOA to prevent hot acidic vapours or liquids from entering the NOA.

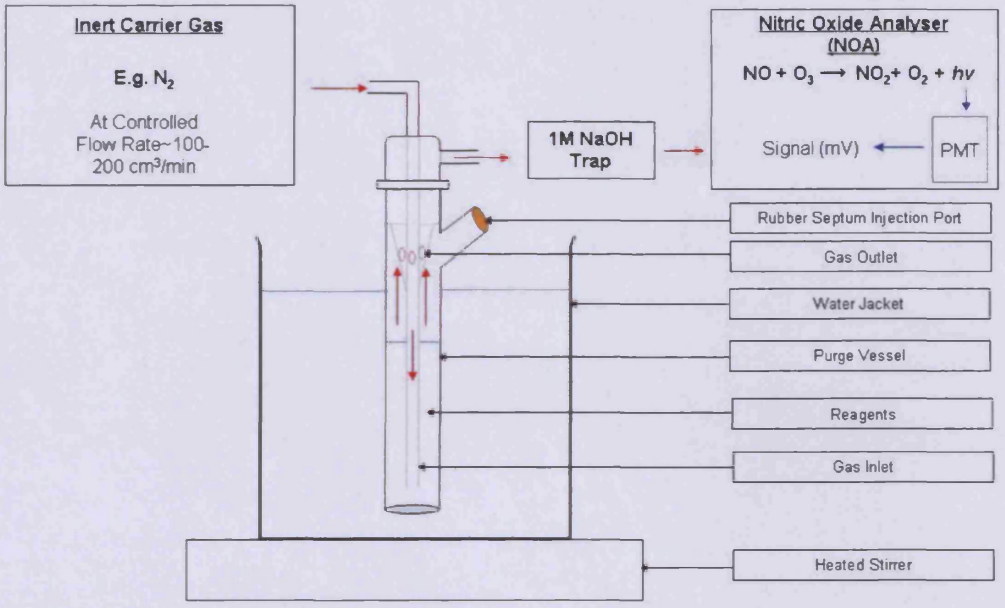

Figure 2.2. A schematic of the purge vessel apparatus utilised for tri-iodide and modified tri-iodide reagent measurements.

2.3.1.1 Tri-Iodide and Modified Tri-Iodide Reagents

Originally studies utilised a simple mixture of potassium iodide (KI) and glacial acetic acid as a cleavage reagent, however the reductive power of the mix was variable, so the yield of nitric oxide from a given sample was not consistent. The requirement for a reagent with long-term stability that could measure a number of biological nitric oxide species lead to the development of 'tri-iodide' reagent [359]. The tri-iodide reagent is essentially the same as the KI/acid mix, apart from the addition of iodine crystals to saturate the solution and promote the generation of large amounts of the reductive I_3^- molecule.

- To make the reagent, 1g of potassium iodide was first dissolved into 20ml of HPLC grade water to give mixture 1. In addition, a second mixture was formed, comprising 650mg of iodine crystals and 70ml of glacial acetic acid [367]. Once fully dissolved mixture 1 is added to mixture 2 in a fume hood and stirred for 30 minutes prior to use.

The measurement of haemoglobin-bound nitric oxide presents its own difficulties. When a sample containing haemoglobin is injected into the reaction vessel, a process known as auto-capture occurs where nitric oxide released from one haem is immediately scavenged by another. In principle this should only serve to reduce peak height and broaden signal but not alter the overall area under curve (AUC). However, our laboratory has previously found that AUC is significantly underestimated in the presence of haem, particularly when nitric oxide to haem ratios are low as is the case with human erythrocyte samples [365]. This observation led our laboratory to develop a novel cleavage reagent for use with samples that contained haem, known as 'modified tri-iodide' [365]. Modified tri-iodide contains the usual tri-iodide mix but also has potassium ferricyanide ($K_3Fe^{III}(CN)_6$)(250mM added 1 in 10 to tri-iodide) added to the purge vessel. Potassium ferricyanide reacts with haem injected into the purge vessel, preventing it from undergoing further reactions with free nitric oxide thus preventing auto-capture (Figure 2.2). Another solution to this problem proposed by the Doctor group involves purging the vessel with high pressure carbon monoxide as a carrier gas to also bind and block haem groups. However this method does have a number of health and safety considerations [363].

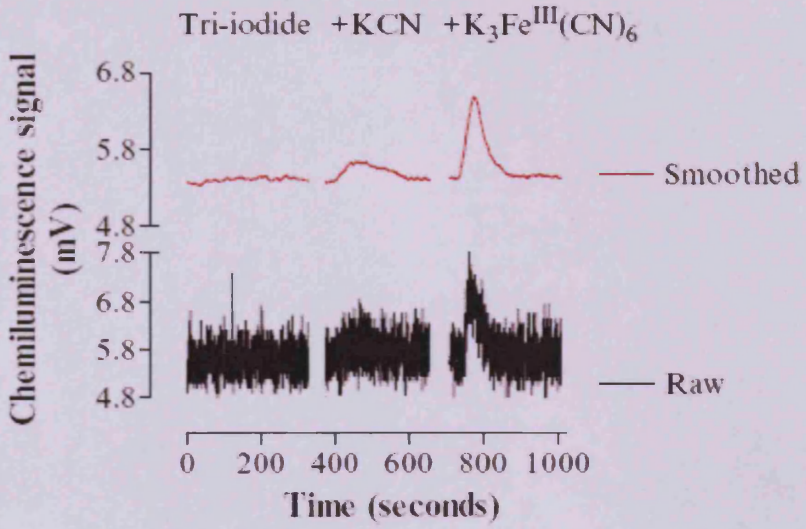

Figure 2.2. The measurement of red blood cell lysate in tri-iodide and modified tri-iodide reagent mixtures. Example traces of raw chemiluminescence signals and the same data smoothed for analysis with Origin (version 7.0) are shown. Injection of haem based sample into the classic tri-iodide mix does not give a signal. Addition of potassium cyanide improves the signal, however, the addition potassium ferricyanide ($K_3Fe^{III}(CN)_6$) (to form modified tri-iodide) restores the signal in full. Reproduced with the kind permission of Dr. Stephen Rogers.

2.3.1.2 Vanadium III Chloride Reagent

The vanadium chloride reagent is capable of measuring all of the species that tri-iodide can, but due to its increased reductive power can also reduce nitrate back to nitric oxide for measurement.

- The vanadium chloride reagent is prepared by creating a saturated solution of VCl_3 (0.785g VCl_3, 20ml HPLC grade water and 80ml of 1M hydrochloric acid). Once the solution is formed it is mixed for 10 minutes before being filtered to give the final solution that is blue/turquoise in appearance.

The high temperatures required for the vanadium III chloride assay have led us to develop a specific glassware set-up. This set-up employs a Liebig condenser which prevents loss of reagent and acid vapour damage under reflux. This glassware also allows for larger reagent volumes and so can cope with multiple sample injections.

Figure 2.3. Schematic diagram of the glassware developed for refluxing vanadium III chloride reagent.

2.3.2 Pre-Treatments

Most biological samples contain a number of nitric oxide species, in order to measure these individually it is sometimes necessary to employ a pre-treatment to remove species that are not of interest. Measurement of nitric oxide species in our laboratory involves the use of two key pre-treatments that will be covered in further detail below and are summarised in table 2.2.

Table 2.2. Summary of pre-treatments used to establish levels of specific species in biological samples.

Treatment	Concentration	Use
Acidified sulphanilamide	290 m*M* stock (500 mg in 10 mL of 1 *M* HCl) Add 1 part acidified sulfanilamide to 9 parts sample	Renders nitrite undetectable by OBC. Requires minimum 15-min incubation in the dark
Mercuric chloride	50 m*M* stock (67.9 mg in 5 mL of HPLC grade water). Add 1 part to 9 parts sample	Cleaves NO from RSNO compounds

2.3.2.1 Acidified Sulphanilamide

Acidified sulphanilamide is used to render nitrite undetectable by OBC (Figure 2.3). Firstly, in an acidic environment nitrite forms the nitrosonium cation[AB]. The nitrosonium cation then interacts with sulphanilamide[AC] and ultimately forms a diazonium salt[AD] that is undetectable in tri-iodide/modified tri-iodide or vanadium linked OBC. The sample is usually incubated for 15 minutes, in the dark (to preserve any RSNO is the sample).

$$NO_2^- + H^+ \leftrightarrow NO^+ + OH^- \leftrightarrow HONO \qquad [AB]$$

$$NO^+ + Ar\text{-}NH_2 \rightarrow Ar\text{-}N_2+ + H_2O \qquad [AC]$$

$$Ar' + Ar\text{-}N_2^+ \rightarrow Ar\text{-}N{\equiv}N\text{-}Ar^- \qquad [AD]$$

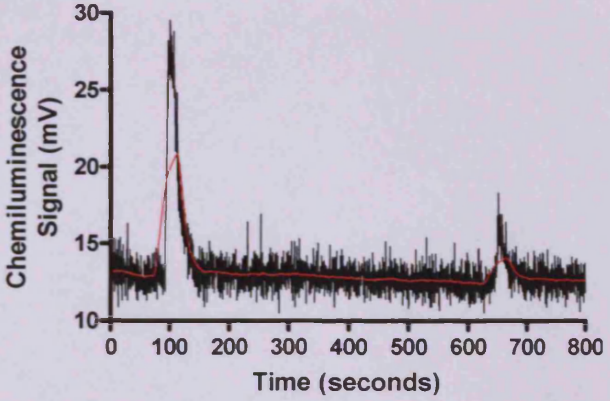

Figure 2.4. Raw (Black) and smoothed (red) chemiluminescence trace from injection of plasma (80 seconds) and injection of plasma incubated with acidified sulphanilamide for 15 minutes (625 seconds). The plasma signal is largely composed of nitrite which is rendered undetectable by acidified sulphanilamide, the remaining signal, seen at 625 seconds, is attributable to RSNO/RNNOs which are thought to be stable in the acidic environment.

2.3.2.2 Mercury Chloride

Mercury chloride can be used to selectively cleave nitric oxide from RSNO compounds to generate the nitrosonium cation[AE]. If acidified sulphanilamide is also present then this will feed into reactions [AC] and [AD], rendering the nitric oxide from the RSNO undetectable by OBC. The sample is usually incubated for 15 minutes.

$$2RSNO + Hg_2^{+} \rightarrow Hg(RS)_2 + 2NO^{+} \qquad [AE]$$

2.3.3 Validation

2.3.3.1 Calibration

To account for day-to-day variation within the assay, a standard curve was performed daily (Figure 2.5A). The standards that were used encompass the range of concentrations observed in biological samples which is of particular importance at the lower end. All standards were prepared fresh daily, nitrite standards ranged from 62.5nM to 1000nM (200µl injections) (Figure 2.5B), while nitrate standards ranged from 6.25µM to 100µM (20µl injections) (Figure 2.5D). The presence of potassium ferricyanide in the tri-iodide mix (modified tri-iodide) did not alter calibration (Figure 2.5C). The accuracy of the calibration is high (see Figures 2.5B, C and D) and the relationship between area under curve and concentration was always linear in the tested range.

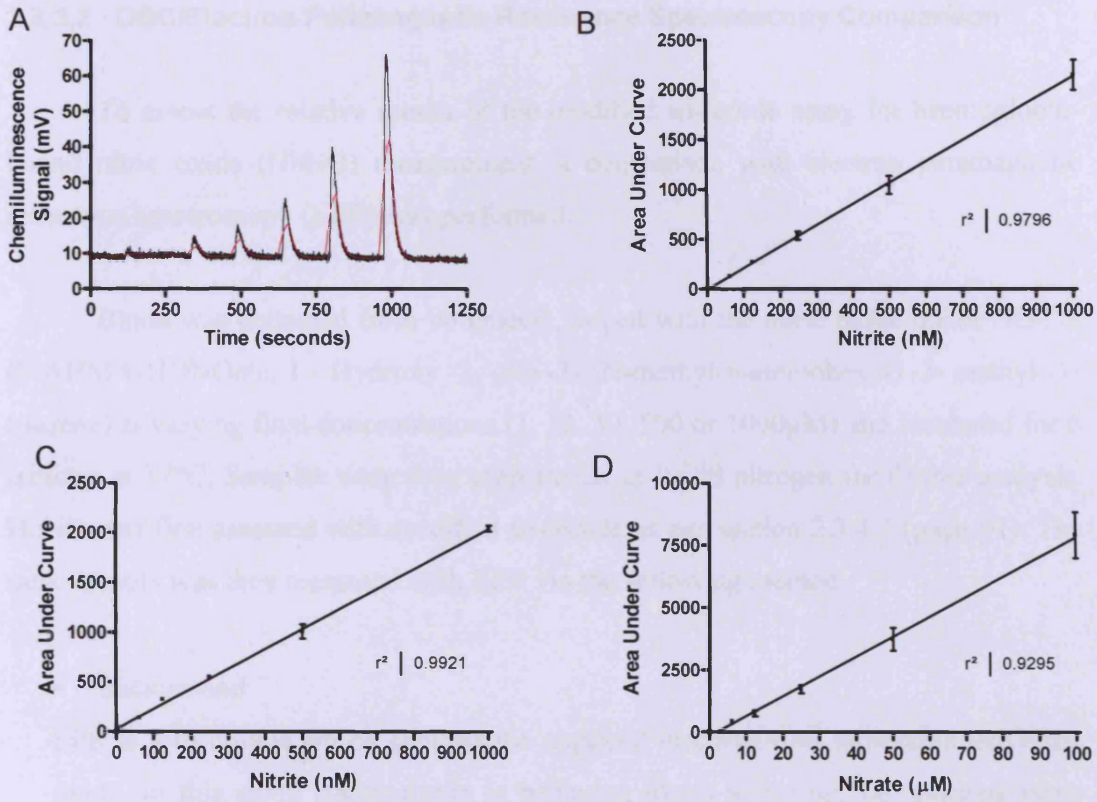

Figure 2.5. Calibration of OBC. A) Raw trace (black) and smoothed signal (red) of nitrite standards (water, 62.5, 125, 250, 500 and 1000nM) into tri-iodide cleavage reagent. B) Nitrite standard curve in tri-iodide. C) Nitrite standard curve in modified tri-iodide. D) Nitrate standards (water, 6.25, 12.5, 25, 50, 100μM) injected into vanadium III chloride reagent. (n=4 in all cases, each one performed on a different day).

Our laboratory have previously reported the coefficient of variance to be 7% intra-assay and 10% inter-assay [368] for the tri-iodide assay, the inter-assay variance largely arising from day to day NOA variability, which is accounted for by daily calibration.

2.3.3.2 OBC/Electron Paramagnetic Resonance Spectroscopy Comparison

To assess the relative merits of the modified tri-iodide assay for haemoglobin-bound nitric oxide (HbNO) measurement, a comparison with electron paramagnetic resonance spectroscopy (EPR) was performed.

Blood was collected from volunteers, doped with the nitric oxide donor NOC-9 (MAHMA-NONOate; 1 - Hydroxy -2- oxo -3- (N-methyl-6-aminohexyl) -3- methyl -1-triazene) at varying final concentrations (1, 10, 50, 500 or 1000μM) and incubated for 6 minutes at 37°C. Samples were then snap frozen in liquid nitrogen for further analysis. HbNO was first assessed with modified tri-iodide as per section 2.3.4.2 (page 97). The same sample was then measured with EPR via the following method.

- Background

EPR is a technique which exploits the unpaired electron(s) of molecules like nitric oxide. In this study haemoglobin is behaving like a spin trap, i.e. trapping nitric oxide from further reaction and influencing the outer valance electron of nitric oxide. By manipulating the environment around HbNO with EPR (increasing energy in the sample with microwaves at a fixed frequency and altering the electron's magnetic moment by changing the external magnetic field), an energy absorption spectrum can be generated (Figure 2.6). This spectrum is very specific to the species being studied and is very clear as most chemicals and matrices that would cause noise in other assays do not contain unpaired electrons and are therefore undetectable. The HbNO spectrum (Figure 2.6) is composed of a number of elements but of interest to measurement are the three peaks in the centre of the spectrum. These peaks are attributable to nitric oxide bound to the α subunits of deoxyhaemoglobin and are known as 'α or alpha, hyperfine'.

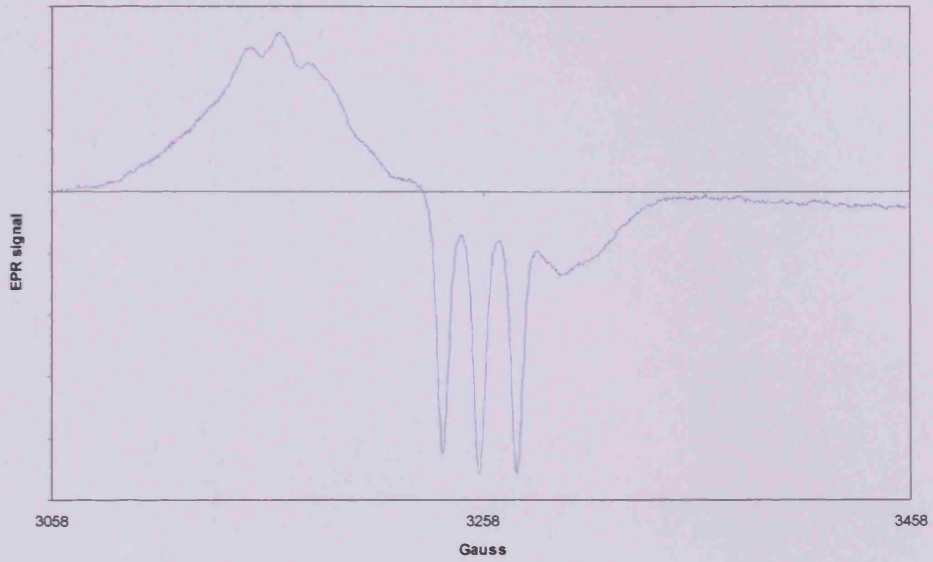

Figure 2.6. Example of HbNO EPR spectrum generated by the addition of nitric oxide (equimolar to haem concentration) to deoxygenated whole blood.

- Method

Samples were loaded into a liquid nitrogen filled insulated EPR Dewar and placed into the EPR machine cavity. EPR spectra were recorded using a Varian 104 X-band EPR spectrometer. Typical instrument settings were as follows, gain 4 x 10^2 x10 – 10x 10^3 x10, time constant 0.25 seconds, Magnetic field strength Gauss 3238, scan width 500 Gauss, scan time 240 seconds, power 10dB. Using specific EPR software, alpha hyperfine peak height and area under curve (area under total spectrum) were measured.

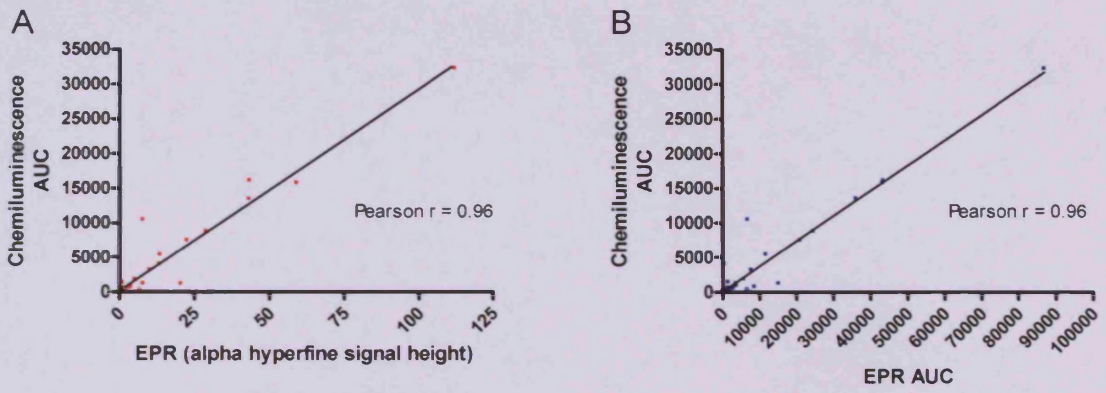

Figure 2.7. Blood doped with NOC-9 nitric oxide donor (1, 10, 50, 500 or 1000μM) and incubated for 6 minutes at 37°C. A) Correlation of AUC measured by modified tri-iodide method vs. alpha hyperfine signal height measured by EPR ($P<0.0001$), n=40. B) Correlation of AUC measured by modified tri-iodide method vs. AUC of signal generated by EPR measurement ($P<0.0001$), n=40.

The two techniques show good agreement for HbNO measurement with both aspects of the EPR measurement (α-hyperfine and AUC) highly significant and linear in relationship (Figure 2.7). The Pearson correlation coefficients are 0.96 ($P<0.0001$) and 0.96 ($P<0.0001$) for EPR alpha hyperfine and EPR AUC measurements respectively.

2.3.4 Protocols

At the beginning of each analysis session all reagents and standards are freshly prepared. The NOA is given time to achieve operating temperature before calibration is commenced, which is completed on a daily basis. The following protocols have been previously validated in our laboratory [365, 369] and are summarised in figure 2.8.

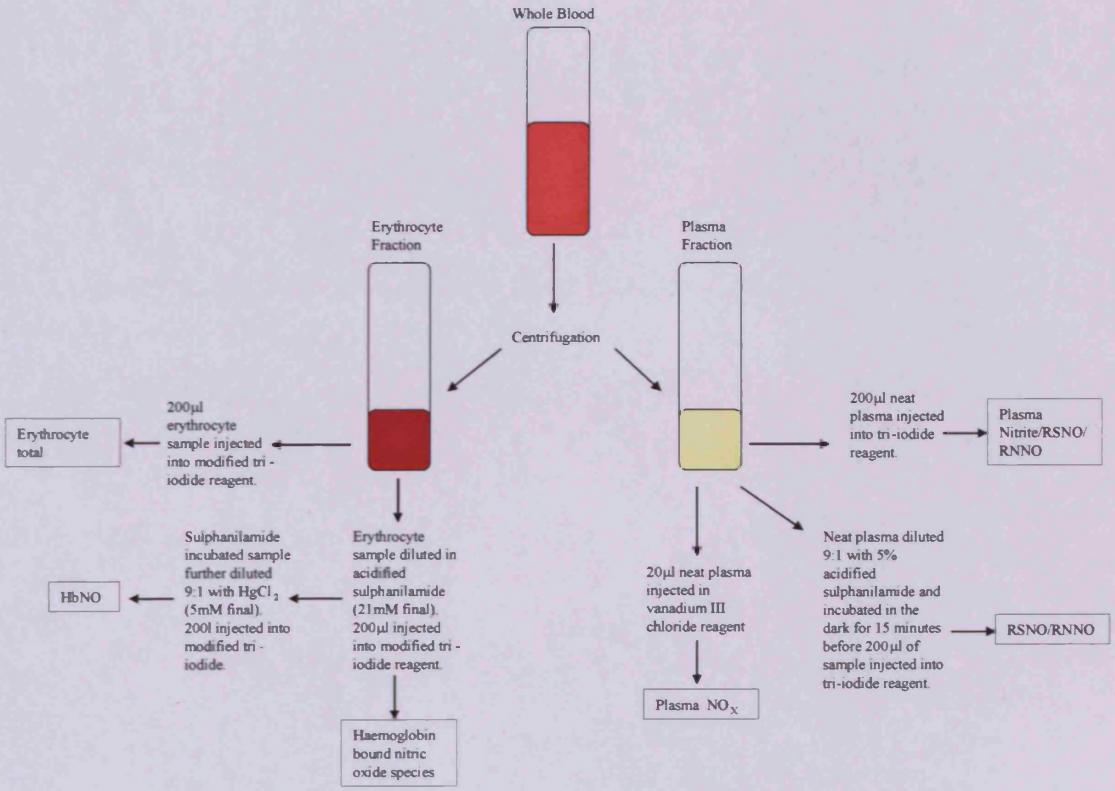

Figure 2.8. Summary of nitric oxide metabolite measurement protocols for blood borne species.

2.3.4.1 Measurement of Plasma/Supernatant Nitric Oxide Species

200µl of neat plasma was injected into 5ml of refluxing tri-iodide reagent using a gas tight Hamilton syringe to give a total plasma signal. A duplicate plasma sample was diluted 9:1 with 5% acidified sulphanilamide (29mM final) to remove nitrite. This sample was then incubated for 15 minutes in the dark and then 200µl injected to provide a measure of plasma protein bound nitric oxide species (RSNO and RNNO). The difference between total injection and RSNO/RNNO injection was taken to represent plasma nitrite.

In a separate experiment, a further 20µl of neat plasma was injected into 30ml of refluxing vanadium III chloride reagent using a gas tight Hamilton syringe to give a total plasma NO_X measurement. The difference between the total plasma measurement in tri-iodide and the total plasma NO_X in vanadium III chloride reagent was taken to represent plasma nitrate concentration.

2.3.4.2 Measurement of Erythrocyte Associated Nitric oxide Species

200µl of neat erythrocytes were injected into 8ml (7.2ml tri-iodide and 0.8ml potassium ferricyanide) of modified tri-iodide using a gas-tight Hamilton syringe to obtain a total erythrocyte measurement. A duplicate red blood cell sample was lysed 1:4 in an acidified sulphanilamide solution (21 mM final) to remove nitrite. This sample was then incubated for 15 minutes in the dark and then 200µl injected into a fresh 8ml of modified tri-iodide to provide a measure of haemoglobin-bound nitric oxide (including all sulphanilamide stable species). An aliquot of this sample was also further diluted 9:1 with $HgCl_2$ (5mM final) and incubated in the dark for 15 minutes, selectively cleaving nitric oxide from Hb-SNO and injected into modified tri-iodide to provide a measure for iron nitrosyl haemoglobin (HbNO).

There is a difference between the total erythrocyte and sulphanilamide signals that is often taken to represent 'erythrocyte associated nitrite'. The precise source of this signal is unknown but is most likely from contaminating plasma, as it is impossible to remove all plasma even with high speed centrifugation. This thesis acknowledges the presence of this nitric oxide metabolite derived signal but because of the questionable nature of this measurement it will not be utilised to form conclusions. Therefore, validated methodologies for haemoglobin bound species will be used to assess erythrocyte nitric oxide interaction.

2.3.5 Methodological Considerations

2.3.5.1 NOA Sensitivity

The NOA reaction cell is very sensitive to changes in pressure. Reaction cell pressure is governed by vacuum, in flow of purge gas and ozone generation. The NOA maintains the reaction cell under vacuum to evacuate unwanted oxygen and other reactive gases that may alter nitric oxide 'detectability' and to draw the sample into the reaction cell. In addition, the NOA is also constantly purged by an inert gas (e.g. nitrogen or argon). This must be kept at a constant flow rate as changes in flow will also alter both baseline and sensitivity. To maintain flow, the set-up in our laboratory employs sensitive flow meters to regulate in-flow at the optimum rate.

The other variable that alters machine sensitivity is temperature. The PMT has a 'dark current' which is background activity in the tube and is known to be temperature dependent. The reaction of nitric oxide and ozone is also temperature dependent. For these reasons, the Sievers NOA used in our laboratory is internally cooled to -20°C to reduce background signal and increase sensitivity. The equipment is also set up in a temperature controlled room to aid consistency.

2.3.5.2 Contamination

Nitrite represents the single most significant cause of unwanted OBC signal. Despite careful laboratory practice, nitrite can contaminate samples from a number of sources:

1. *Buffers and water.* Even the purer, high-quality reagents and water contain nitrite contamination. HPLC-grade water (Fisher) or water for injection (Braun) were found to contain the least contamination in our laboratory's experience. However, once open, these waters also accumulate further nitrite contamination over time. This contamination is unavoidable, although it can be minimized by good practices and accounted for by inclusion of a water injection in the standards used for calibration, which are made up and tested on a daily basis.

2. *Plasticware, glassware, and apparatus.* All disposable and reusable apparatus will contain considerable nitrite contamination that can be minimized by repeat washing. Plasticware in particular can vary vastly depending on both source and batch. The use of filters and columns in this respect should be avoided. Glassware and syringes should be cleaned extensively (e.g., overnight in glass cleaner, followed by extensive rinsing with water). For blood collection, EDTA vacutainers were shown to contain minimum contamination.

3. *Hamilton syringes.* Syringes that are in regular use should be acid washed followed by repeat neutral washes on a regular basis, as residue builds up in both the needle and in the barrel of the syringe.

4. *Chemicals.* All reagents contain a degree of nitrite contamination, however the use of calibration and careful practice can remove or reduce their impact.

2.3.5.3 Freezing Time

The effect of long-term freezing has previously been assessed in our laboratory. The results demonstrate that although total nitric oxide moiety does not change the apportionment between species can change over time. Some species such as RSNO/RNNO in plasma (Figure 2.9) and haemoglobin bound species (Figure 2.10) are not stable over time and are oxidised to nitrite (Figures 2.9 and 2.10 upper panels in both cases). To prevent this factor from confounding results, all samples in all studies were measured within 1 week of collection.

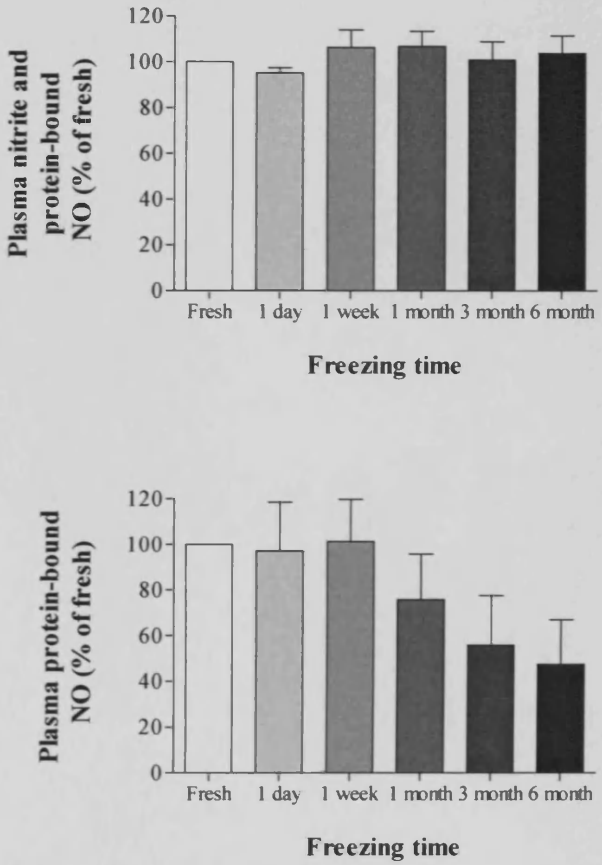

Figure 2.9. The effect of sample freezing on plasma nitrite and protein-bound nitric oxide levels (upper panel) and plasma protein-bound nitric oxide (lower panel) measured using tri-iodide in conjunction with ozone based chemiluminescence. Fresh represents snap frozen and immediately measured. Values presented as mean ± SEM (n = 5). This work was carried out by Dr. Stephen Rogers and is reproduced from [370], with his kind permission.

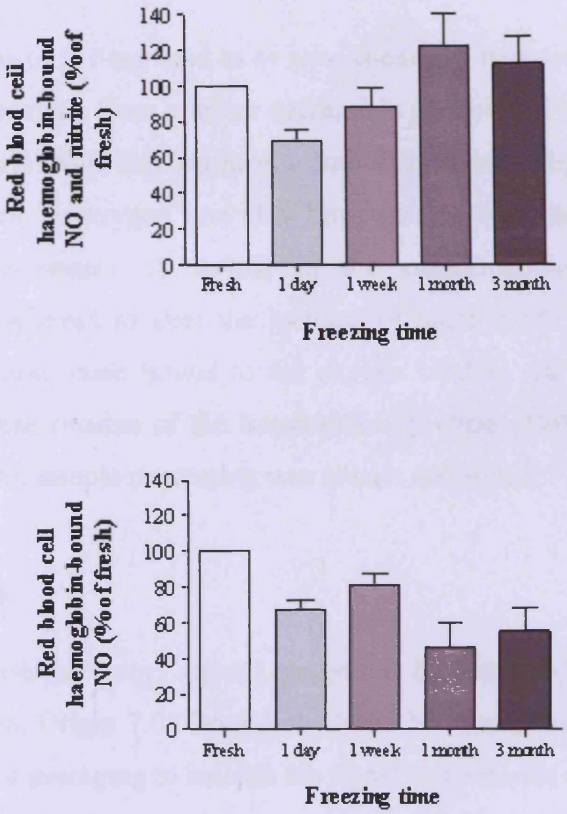

Figure 2.10. The effect of sample freezing on red blood cell associated nitrite and haemoglobin-bound nitric oxide (upper panel) and red blood cell haemoglobin-bound nitric oxide (lower panel) measured using modified tri-iodide in conjunction with ozone based chemiluminescence. Fresh represents snap frozen and immediately measured. Values presented as mean ± SEM (n = 5). This work was carried out by Dr. Stephen Rogers and is reproduced from [370] with his kind permission.

2.3.5.4 Changing pH/Oxygen

Alterations of pH can lead to *in vitro* chemistry between nitric oxide metabolite species with the sample. Even a minor decrease in pH below 7 is enough to reduce some nitrite back to nitric oxide that can form a number of species depending on the sample in question. Changes in oxygen are also important in the case of RBC nitric oxide metabolite measurements. A switch in the conformational/oxygenation state of haemoglobin is believed to alter the location of nitric oxide within the haemoglobin structure (e.g., nitric oxide bound to the oxygen binding site [HbNO] or nitric oxide bound to a cysteine residue of the haemoglobin β chain [HbSNO]). In this respect, a policy of minimum sample processing was always employed.

2.3.6 Analysis

To increase accuracy, signals generated by the NOA are reproduced in the graphical program, Origin 7.0 (OriginLab Corp., Northampton, MA, USA), this allows 150 point adjacent averaging to smooth the signal and analysis of area under curve (with a 'peak analysis' add-on). Area under curve is much more sensitive to changes than peak height, which can vary dramatically depending upon the presence of different matrices (e.g. haemoglobin) in the reaction cell, see section 2.3.1.1 (page 84).

2.4 Spectrophotometry

To asses the level of oxygen saturation in some erythrocyte or haemoglobin samples a UV/visible light absorbance spectrum was taken using a spectrophotometer (6705 UV/Vis Spectrophotometer, Jenway, UK) (Figure 2.11). Oxygenated haemoglobin gives a characteristic doublet in the 500-600nm range while deoxygenated haemoglobin only produces a single peak over the same wavelengths (Figure 2.11). A sample containing both oxygenated and deoxygenated haemoglobin will give a spectrum that is a combination of the two and proportions of each can be determined by comparing sample data with reference base-spectra of pure, individual species using a least-squares method [371]. The level of methaemoglobin can also be monitored with this method with characteristic changes at ~630nm [372].

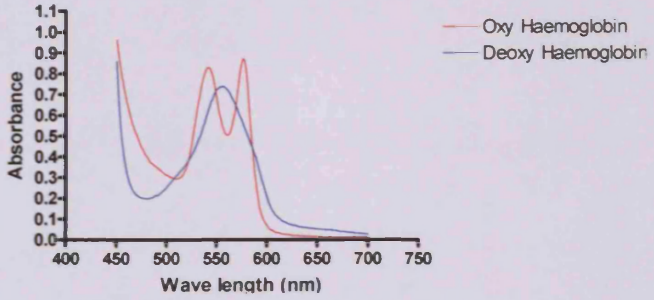

Figure 2.11. The light absorbance spectra of fully oxygenated (red) and full deoxygenated (blue) haemoglobin in the 450-700nm range.

2.5 Myography

2.5.1 Test System

Male New Zealand White rabbits (2–2.5 kg) were terminally anaesthetised with sodium pentobarbitone (120 mg/kg, intravenously). The rib cage was then opened and the aorta was carefully excised and placed in fresh Krebs buffer (NaCl 109.0 mM, KCl 5.36mM, KH_2PO_4 1.17mM, $MgSO_4.7H_2O$ 1.21mM, $NaHCO_3$ 24.99mM, $C_6H_{12}O_6$ 10.99mM, $CaCl_2.2H_2O$ 1.49mM). The aorta was then cleaned of minor vessels and adipose tissue and cut into 8 equally divided 2mm rings, discarding the tissue most distal and proximal to the heart (aortic arch and start of abdominal aorta). For experiments where endothelium-denuded rings were required, a wooden applicator was used to gently remove the endothelium. Rings were mounted on matched stainless steel hooks for isometric tension recording in 8ml baths containing 5ml of Krebs' buffer, 37°C. Output transducer (AD instruments) signals were amplified (Powerlab 8Sp/octal bridge (AD Instruments)) and converted for visualisation on 'Chart for Windows' (Version 4.1.2, AD Instruments). Aortic rings were maintained at 2g resting tension in all experiments. For each experiment, eight aortic ring preparations were run in parallel, n = 1 represents data averaged from 2 individual aortic ring preparations given identical treatment. % relaxation is the induced decrease in gram tension as a percentage of the maximum phenylephrine induced contraction.

2.5.2 Experimental Protocol

After a one hour equilibration period, ring tension was reset (allowing for stretch-induced relaxation). All tissue was then exposed to phenylephrine (PE, 1μM) (Sigma), once constriction had reached a plateau, acetylcholine (10 μM) (Sigma) was added to demonstrate endothelium viability/absence. To accept vascular rings for further testing, the tissue was expected to relax a minimum of 50% to acetylcholine when the endothelium was intact, and show no relaxation, or a small constriction, to acetylcholine in endothelium denuded vessels. The preconditioning process was then repeated until consistent PE induced tensions were achieved. Preconditioning was carried out at 95% O_2/5% CO_2.

Hypoxic experiments were carried out at ~1% O_2 (gassed with 95% N_2/5% CO_2) as utilised previously in our laboratory [91]. The tissue was kept under hypoxic conditions for 10 minutes before exposure to PE (3μM, used to achieve the same gram tension observed at 95% O_2, ensuring a true % relaxation as opposed to a contractile artefact). All pharmacological blocking agents were pre-incubated at respective concentrations and time periods. Hypoxia always caused a degree of relaxation in PE pre-constricted aortic rings, above that seen in normoxia.

The slow and continuous relaxation of isolated vessels during hypoxia, as observed in the controls of all our experiments, is an established phenomenon. The precise cause of the background vasodilatation remains unknown. Both energy limitation and interruption of excitation contraction coupling have been suggested as possible causes [131]. In our model, hypoxia induced relaxation proceeds to the same extent independently of endothelium/NOS (denudation/L-NMMA data). It is important to note that the smooth muscle cells are not simply dying in hypoxia. After a period of hypoxia, tissue can be re-equilibrated in oxygenated buffer and show comparable contraction and relaxation to exogenous agents.

2.6 Chemicals and Specialist Items

Table 2.3. Chemicals and agents utilised and sources.

Product	Source
Krebs Buffer	
Sodium Chloride (NaCl)	Fisher scientific UK
Potassium Chloride (KCl)	Fisher scientific UK
Potassium Dihydrogen Orthophosphate (KH_2PO_4)	Fisher scientific UK
Magnesium Sulphate ($MgSO_4.7H_2O$)	Fisher scientific UK
Sodium Hydrogen Carbonate ($NaHCO_3$)	Fisher scientific UK
Glucose ($C_6H_{12}O_6$)	Fisher scientific UK
Calcium Chloride ($CaCl_2$)	Fisher scientific UK
Reagent Chemicals	
HPLC Grade Water	Fisher scientific UK
Glacial Acetic Acid	Fisher scientific UK
Hydrochloric Acid (HCL, 1M)	Fisher scientific UK
Sodium Hydroxide (NaOH, 10N)	Fisher scientific UK
Dimethyl Sulphoxide (DMSO)	Fisher scientific UK
Potassium Iodide (KI)	Sigma Aldrich UK
Iodine (I_2)	Sigma Aldrich UK
Potassium Hexacyanoferrate ($K_3Fe^{III}(CN)_6$)	Sigma Aldrich UK
Sulphanilamide	Sigma Aldrich UK
Mercury Chloride ($HgCl_2$)	Sigma Aldrich UK
Phenylephrine (PE)	Sigma Aldrich UK
Acetylcholine (Ach)	Sigma Aldrich UK
Anti-Foam 204 Organic	Sigma Aldrich UK
Vanadium Chloride (VCl_3)	Sigma Aldrich UK
Phosphate Buffered saline (PBS) Tablets	Sigma Aldrich UK
Chemical Inhibitors and Agents	
2,3-Diphosphoglycerate (2,3-DPG)	Sigma Aldrich UK (Fluka BioChemica)
Carbon Monoxide (CO) Gas (90%)	BOC UK
95% N2/5% CO_2 Speciality Gas Mix	BOC UK
1H [1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ)	Alexis Bio-Chemicals UK
Carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (C-PTIO)	Alexis Bio-Chemicals UK
U-51605	Cayman Chemicals USA
Indomethacin	Sigma Aldrich UK
Raloxifene	Sigma Aldrich UK
Oxypurinol	Sigma Aldrich UK
L-NG-Monomethyl Arginine (L-NMMA)	Sigma Aldrich UK
NO Donors	
Sodium Nitrite ($NaNO_2$)(Laboratory Grade)	Sigma Aldrich UK
Sodium Nitrate ($NaNO_3$)	Sigma Aldrich UK
Spermine NONOate	Alexis Bio-Chemicals UK
MAHMA NONOate (NOC-9)	Alexis Bio-Chemicals UK
Clinical Agents	
Sodium Nitrite (Infusion Grade)	Martin Dale UK
Saline (Infusion Grade)	Baxter Healthcare UK

3 The Metabolism of Nitric Oxide and its Metabolites in Blood, the Influence of Oxygen

3.1 Introduction

3.1.1 Nitric Oxide Metabolism in Blood

Nitric oxide derived from the endothelium ultimately enters the blood-stream either directly as free nitric oxide or as one of a number of metabolites. The suggestion that metabolites of nitric oxide could behave as stores of potentially bioactive nitric oxide (see section 1.10, page 63) implies that the dynamic relationship between these species and blood constituents is a very important one. The metabolic fate of nitric oxide (and its metabolites that are subject to further metabolism) is dependent upon a number of factors, such as the concentration of nitric oxide and other bio-reactants but also the ability of the surrounding environment (particularly *in vivo*) to alter the 'reactivity-state' of said bio-reactants. Ultimately, the study of this relationship has resulted in much interest in the measured levels of nitric oxide metabolites and the dynamic changes between them. The methods used and information yielded from these measurements has come under scrutiny [373]. However, with a conversant approach to interpretation, metabolite levels can be very informative. In this respect, it should be noted that nitric oxide itself has been demonstrated to have an EC_{50} of ~10nM in vascular ring preparations [374] therefore, at levels that could often be considered negligible as far as methodological limitations are concerned, nitric oxide is particularly potent.

3.1.2 The Translocation of Nitric Oxide and its Metabolites in Blood

In order for both nitric oxide and its metabolites to have biological function, it is essential that they have the ability to progress from the site of synthesis to the site of action. In the vessel, movement is governed by factors such as: reaction with bio-reactants, rate of diffusion in diverse media, cellular uptake, trans-cellular transport and the flow of blood within the lumen [323, 375]. One particular area that has emerged, is the study of nitric oxide movement into erythrocytes. Studying the movement of such a reactive molecule is inherently complex and most studies have relied upon 'stop flow' or competition experiments which present their own difficulties [320, 376].

In contrast, the relative stability of nitric oxide metabolites reduces the complexity of studying their movement. However, practically completing this is not without complication. Proposition of the nitrite reduction theory (see section 1.10.2, page 68) has resulted in increased interest in the movement of nitrite particularly with respect to erythrocytes. One aspect of the theory that has been debated in the literature surrounds the ability of nitrite to enter an erythrocyte, be reduced to nitric oxide and subsequently escape from the erythrocyte to alter biological function during transit across a single vascular bed [343].

3.1.3 Aims

This component of work was undertaken to:

- Establish the apportionment of nitric oxide metabolites at baseline in human blood samples.

- Determine the effect of oxygen level upon metabolite apportionment.

- Assess the species formed when exogenous nitric oxide is added to human blood.

- Develop specific methodology for real time assessment of nitric oxide entry into erythrocytes and investigate the effect of haemoglobin oxygen saturation upon nitric oxide uptake.

- Consider the interaction of nitrite in the physiological concentration range with erythrocytes and how this interaction is altered by oxygen.

3.2 Specific Protocols

3.2.1 Baseline Nitric Oxide Metabolite Measurement

Baseline samples were taken from consenting male patients undergoing angiography at the University Hospital of Wales. Patients receiving acute or chronic nitrate (i.e. GTN, ISDN) treatment were excluded from the study. Arterial blood samples were taken via a catheter inserted into the radial artery and venous samples were taken via a catheter inserted into the antecubital vein. Immediately after collection, samples underwent blood gas analysis (OSM3, Radiometer, Copenhagen) and were centrifuged and processed as per section 2.1.2 (page 80). Plasma and erythrocyte fractions were subsequently analysed via ozone based chemiluminescence as per sections 2.3.4.1 (page 96) and 2.3.4.2 (page 97) respectively.

3.2.2 Exogenous Nitric Oxide Addition to Erythrocytes

Aliquots of the arterial and venous samples taken in section 3.2.1 (page 110) were also exposed to exogenous nitric oxide addition. The nitric oxide donor MAHMA NONOate (NOC-9, $t_{1/2}$=2.7 minutes, [377]) was added to all samples to achieve a final concentration of 425nM. The NOC-9 molecule is capable of liberating two moles of nitric oxide for every mole of parent compound, giving a final nitric oxide concentration of 850nM. Samples were incubated at 37°C for 30 minutes to allow reactions to complete before high speed centrifugation (10,000*g* for 2 minutes). Plasma and erythrocyte fractions were immediately separated, snap frozen and stored at -80°C for future analysis. Rapidly thawed plasma and erythrocyte fractions were subsequently analysed via ozone based chemiluminescence as per sections 2.3.4.1 (page 96) and 2.3.4.2 (page 97) respectively.

3.2.3 A Real Time Nitric Oxide Reaction Model

This model utilises ozone-based chemiluminescence, however, it is employed almost in reverse to usual cleavage reagent methodology (section 2.3, page 82). Instead of a cleavage reagent, the purge vessel (figure 2.2) is filled with 5ml of PBS, maintained at 25°C and purged with nitrogen. The nitric oxide donor, spermine NONOate (capable of liberating two moles of nitric oxide for every mole of parent compound) is injected into the purge vessel to achieve a final concentration of 38μM (i.e. 76μM nitric oxide). Unlike NOC-9, spermine NONOate has a relatively long half-life (230 minutes at 22°C, pH 7.4 in phosphate buffered saline [378]). Upon injection this results in an initial peak that is followed by a relatively stable, slowly falling, nitric oxide generation level that represents the balance between nitric oxide generation by spermine NONOate in the purge vessel and the removal of nitric oxide by the nitrogen carrier gas (Figure 3.1).

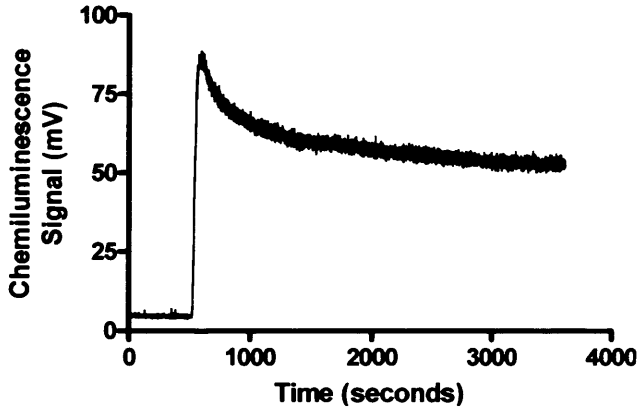

Figure 3.1 Example trace of Spermine NONOate (38μM) injection into PBS at 25°C. An initial spike in signal is followed by a stable nitric oxide generation baseline that drops slowly over the course of hours.

Once the baseline is stable, the model can be utilised to study any chemical or biological sample that behaves as a nitric oxide 'sink'. The model therefore lends itself nicely to studying the interaction of haemoglobin/erythrocytes and nitric oxide.

When a nitric oxide scavenging entity is injected into the system, the signal drops before returning to baseline upon completion of the reaction. This process generates an area below baseline that can be calculated and taken to represent the total

amount of nitric oxide consumed by the entity in relative terms. The gradient of the initial signal decay from baseline can also be utilised to calculate the relative initial rate of reaction between nitric oxide and the molecule of interest.

3.2.3.1 Nitric Oxide Interaction with Haemoglobin and Erythrocytes

Erythrocyte samples were collected from healthy volunteers and processed as previously described (Section 2.1, page 80). Erythrocytes were diluted ~1 in 300 into either PBS to study whole cells or HPLC grade water in order to study lysed cells and adjusted to give a final concentration of 0.1g/L.

To alter oxygenation, samples were gently purged with nitrogen or oxygen gas in a sealed cuvette. The degree of oxygenation was confirmed by light spectroscopy as per section 2.4 (page 104). Methaemoglobin levels were monitored at 630nm, but did not change significantly between samples.

10µl of whole or lysed erythrocyte samples were injected giving a final (in purge vessel) haemoglobin concentration of 0.2×10^{-3} g/L. Samples were left to run in full i.e. until the signal had clearly returned to the nitric oxide generation baseline. The nitrogen purge flow rate was maintained at $200cm^3$/min in all experiments using an accurate gas flow meter.

3.2.4 Nitrite Interaction with Erythrocytes

Erythrocyte samples were collected from consenting healthy volunteers within the Wales Heart Research Institute as stated in section 2.1 (page 80). Post centrifugation (2000g for 5 minutes) separated erythrocytes were reconstituted in phosphate buffered saline (PBS) to give a final haemoglobin concentration of 10g/dL and haematocrit of ~35%, the mix was then placed in a thin film rotating tonometer as described in section 2.2 (page 81). A nitrogen or 95% oxygen purge was utilised to alter the haemoglobin saturation to either ~50% or ~100% respectively, saturation was confirmed using a blood gas analyser (OSM3, Radiometer, Copenhagen). The mix (50 or 100% saturation) was then separated into aliquots and nitrite of varying concentrations (150, 250, 450 and 850nM final concentrations respectively) was added and incubated at 37°C. For rate analysis, samples were taken at 5 min intervals (up to 20 minutes and again at 30 minutes) and centrifuged at high speed (10,000*g* for 2 minutes) to quench the reaction, the supernatant and erythrocyte fractions were immediately separated, snap frozen and stored at -80°C for future analysis. Rapidly thawed supernatant and erythrocyte fractions were subsequently analysed via ozone-based chemiluminescence as per sections 2.3.4.1 (page 96) and 2.3.4.2 (page 97), respectively.

3.3 Statistical Analysis

For group comparisons the student t-test was utilised, either paired or unpaired where appropriate. To assess correlations, the Pearson's correlation coefficient was used. Data are presented as means and error bars represent the standard error of the mean (SEM). A P value of less than 0.05 was taken to be significant, individual significance levels are indicated within the figure legends.

3.4 Results

3.4.1 Apportionment of Nitric Oxide in Blood

To assess the relative amount of different nitric oxide metabolite species in blood and the effect of haemoglobin oxygen saturation upon apportionment of metabolites both matched arterial and venous blood samples were collected and analysed.

3.4.1.1 Sample Characteristics

The haemoglobin concentrations of arterial and venous blood samples were 15.4±0.7 g/dL and 16.0±0.6 g/dL respectively, a paired t-test revealed no significant difference between the sample groups. Haemoglobin oxygen saturation in arterial blood measured 97.4±0.5 % which was significantly higher ($P<0.01$) than the venous sample that measured 67.2±4.2 %.

3.4.1.2 Baseline Arterial and Venous samples

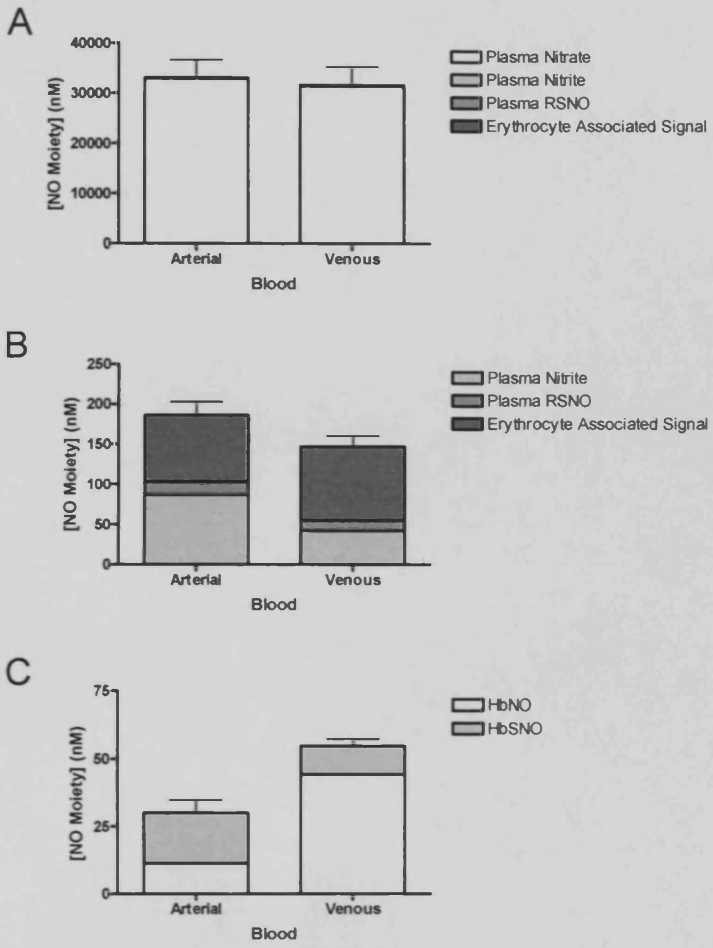

Figure 3.1. Apportionment of nitric oxide species in; A) Whole blood B) Whole blood with plasma nitrate level not shown and C) Erythrocytes bound to haemoglobin. No significant difference in total levels (A) between Arterial and Venous samples. n=4 in all cases.

The vast majority of nitric oxide moiety in the blood circulates as nitrate in both arterial and venous vasculature (Figure 3.1A). If nitrate is excluded, the remaining metabolites (nitrite, RSNO and erythrocyte associated signal) account for less than one percent of the total metabolite pool (Figure 3.1B). Interestingly, the total amount of nitric oxide moiety does not change in arterial vs. venous blood (Figure 3.1A). However, apportionment between different species does change from artery to vein e.g. haemoglobin bound species and plasma nitrite (Figures 3.1 and 3.2).

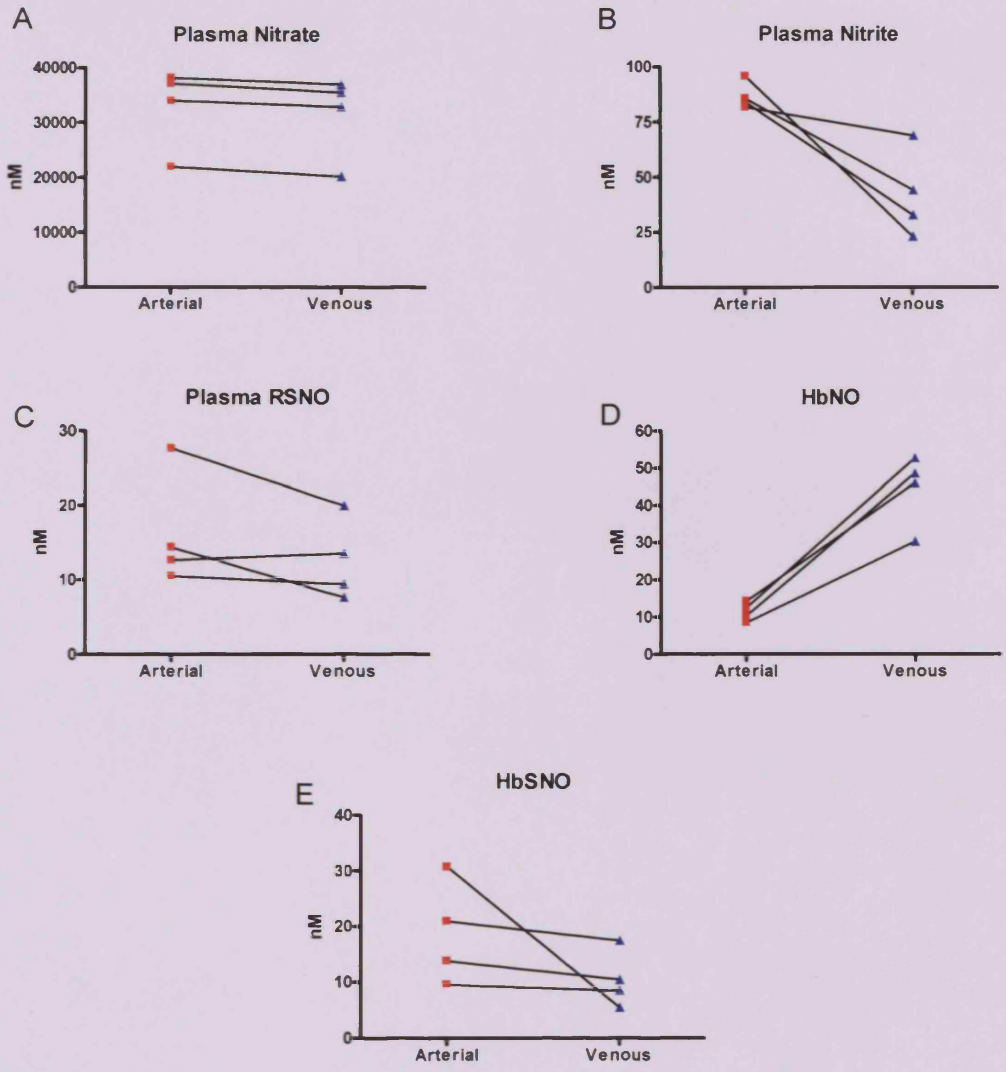

Figure 3.2. Changes in arterial and venous apportionment of nitric oxide metabolites in each of the individuals tested A) Plasma nitrate is significantly lower in venous blood compared to matched arterial samples ($P<0.001$) B) A significant ($P<0.05$) gradient in plasma nitrite level exists from artery to vein C) Plasma RSNO, no significant change from arterial to venous D) Nitric oxide bound to the oxygen binding pocket of haemoglobin increases significantly ($P<0.001$) from artery to vein E) HbSNO levels show a trend which is higher in arterial samples but is not significantly different from matched venous samples. n=4 in all cases.

The transit from artery to vein and associated change in oxygen level alters the apportionment of nitric oxide metabolites. The clearest trends appear to be in plasma nitrite and HbNO, where plasma nitrite levels decrease from artery to vein, in contrast to HbNO levels which increase by approximately the same amount across the same vascular bed (Figure 3.2B and D).

3.4.1.3 Exogenous Nitric Oxide Addition to Arterial and Venous Blood

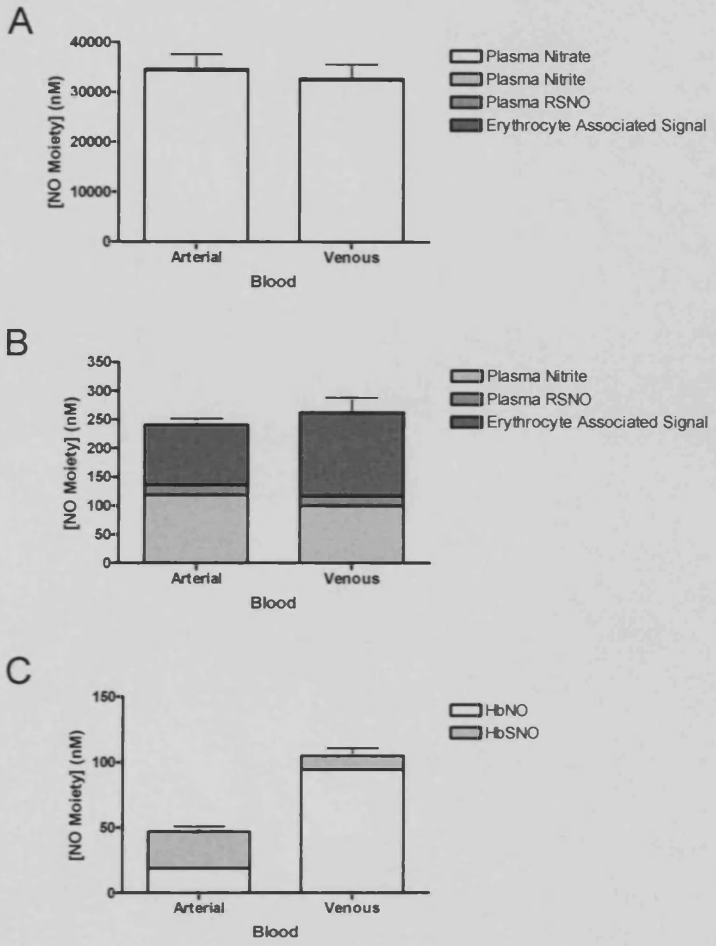

Figure 3.3. Apportionment of nitric oxide species post NOC-9 (425nM) addition in; A) Whole blood B) Whole blood with plasma nitrate level not shown and C) Erythrocytes bound to haemoglobin. No significant difference in total levels (A) between Arterial and Venous samples. n=4 in all cases.

The total increase in nitric oxide moiety was the same in arterial and venous blood (Figure 3.3A) with an overall nitric oxide recovery level of 154.4%. This recovery level is very acceptable given that 850nM nitric oxide equivalent was added to a ~ 30,000nM pool of nitric oxide metabolites. However, it should be noted that the aim of this study was to asses the apportionment of nitric oxide in relation to blood saturation, not the addition of nitric oxide *per se*. The metabolism of exogenously added nitric oxide to specific species appears to be oxygen dependent.

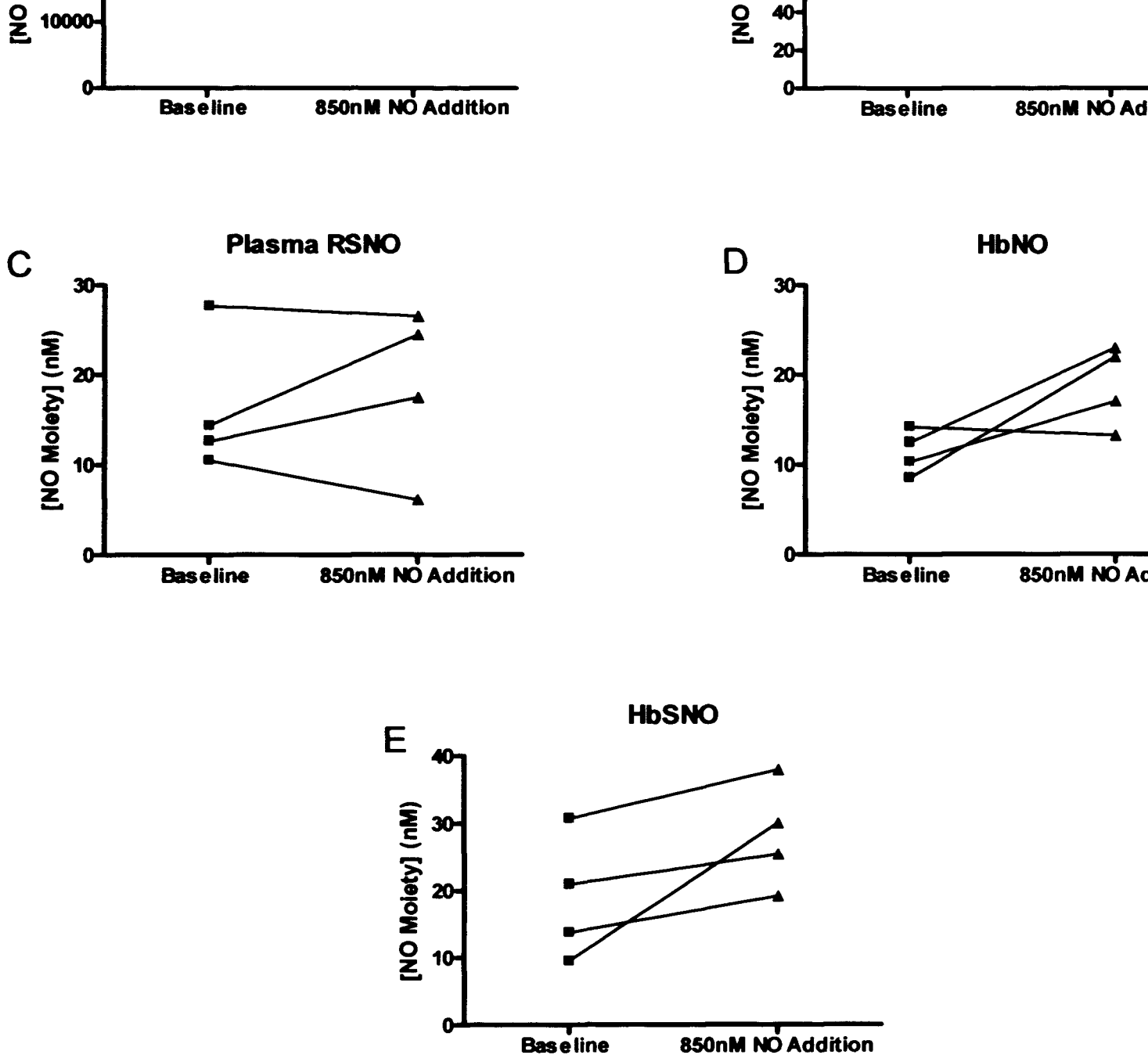

Figure 3.4. The metabolism of NOC-9 (425nM) in arterial blood samples. A) Plasma nitrate leve Plasma nitrite levels increase significantly upon NOC-9 addition ($P<0.05$) C) Plasma RSNO leve HbNO levels E) HbSNO levels appear to increase in all samples, however levels achieved ar significantly different from baseline. n=4 in all cases.

In arterial blood most species appeared to be slightly elevated, however it is the observed increases in plasma nitrite ($P<0.05$) and nitrate that account for most of the exogenously added nitric oxide (Figure 3.4). In venous blood, plasma nitrite levels are also elevated ($P<0.05$) (Figure 3.5B), however, there is also a notable increase in HbNO level (Figure 3.5D).

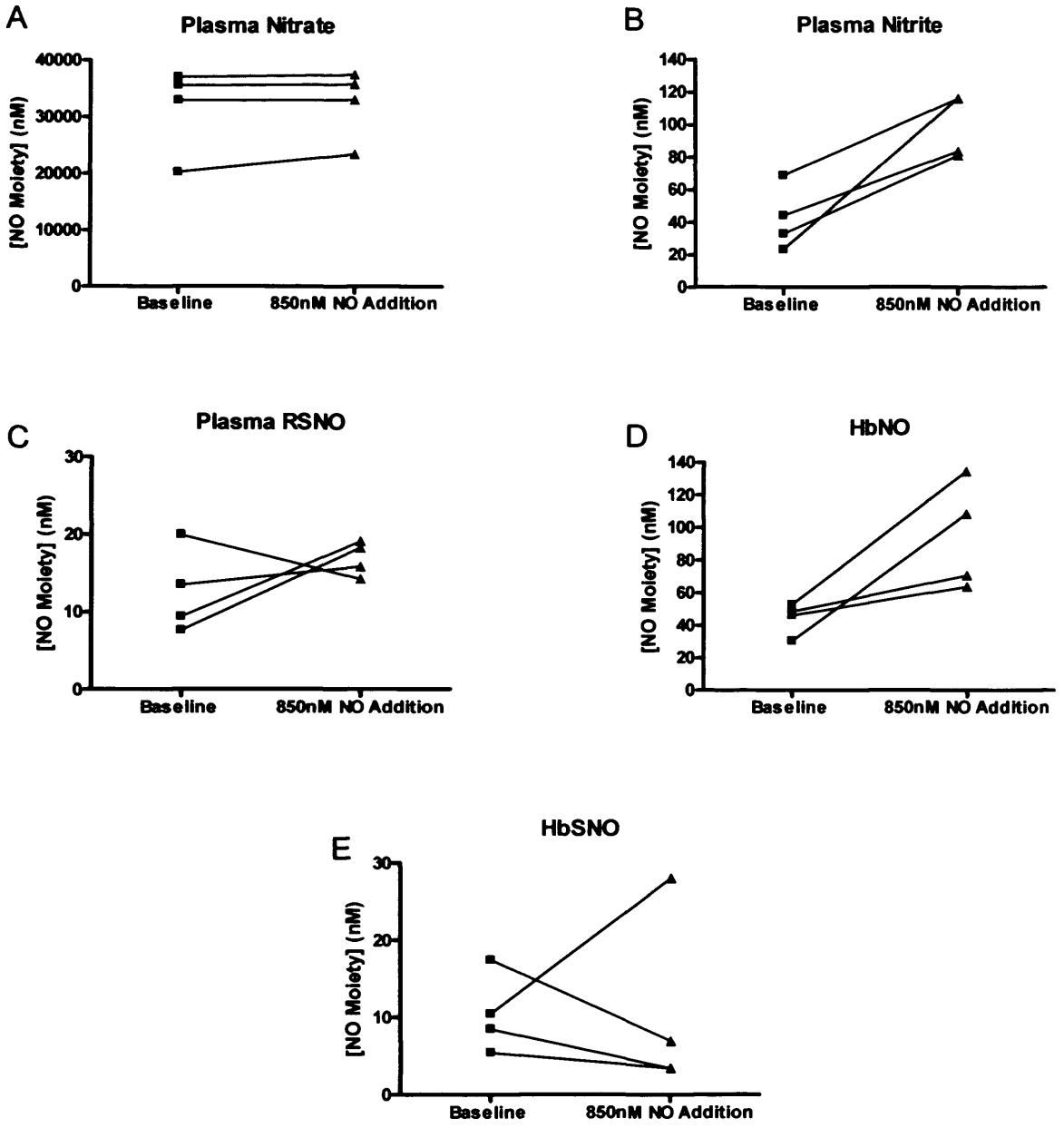

Figure 3.5. The metabolism of NOC-9 (425nM) in venous blood samples. A) Plasma nitrate levels B) Plasma nitrite levels increase significantly upon NOC-9 addition ($P<0.05$) C) Plasma RSNO levels D) HbNO levels appear to increase in all samples, however levels achieved are not significantly different from baseline E) HbSNO levels. n=4 in all cases.

3.4.2 Real Time Nitric Oxide Reaction Model

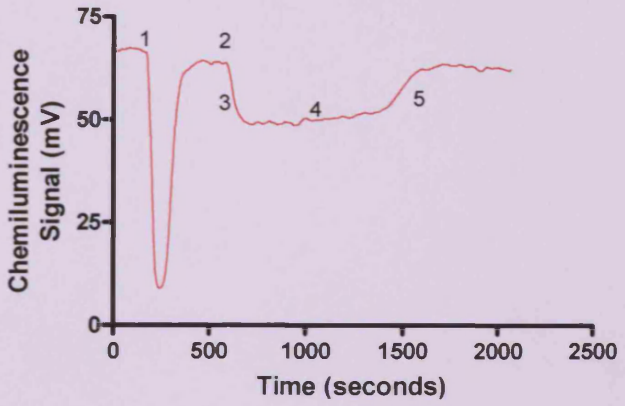

Figure 3.5. Smoothed trace showing injection of a lysed erythrocyte sample (1) and injection of the same but whole erythrocyte sample (2). The whole erythrocyte signal is comprised of a number of components, an initial uptake or decay phase (3), a plateau or steady state phase (4) and a return to baseline phase (5).

The injection of whole and lysed erythrocytes into the model produces visually distinct signals (Figure 3.5). Injection of lysed erythrocytes produces a signal with rapid decay to a low detected nitric oxide level and completes its reaction within a few minutes. Conversely, injection of whole erythrocytes produces a signal with a more modest decay to a level that is much closer to the nitric oxide level detected before erythrocyte injection, with the reaction completing over a longer period of ~20 minutes.

In order to test the suitability of the model for erythrocyte/haemoglobin testing, a number of validation experiments were undertaken to establish the characteristics of the model. The nitric oxide baseline level was found to be directly dependent upon the flow rate of the purging nitrogen gas, for this reason the nitrogen flow in all experiments was kept at a constant rate (Figure 3.6A). The relative amount of nitric oxide consumed upon injection of haemoglobin was found to be inversely proportional to the % met haemoglobin content (Figure 3.6B). All test samples were monitored for changes in methaemoglobin, however the manipulations used in this chapter did not alter levels significantly. The amount of haemoglobin injected was found to have a linear relationship with the relative amount of nitric oxide consumed (Figure 3.6C),

demonstrating that the model is suitable of erythrocyte/haemoglobin testing. In addition, the relative total amount of nitric oxide consumed was found to be independent of the extracellular nitric oxide concentration (Figure 3.6D).

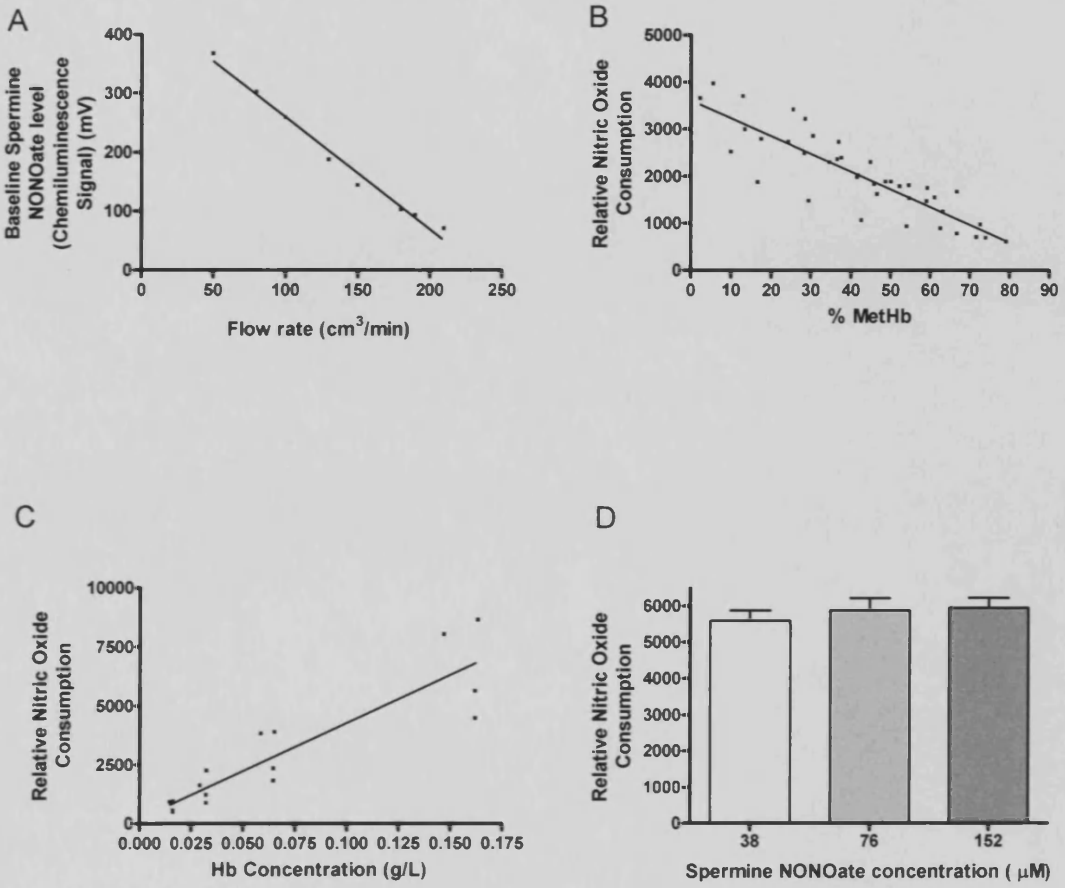

Figure 3.6. Real time nitric oxide reaction model characteristics A) The effect of nitrogen purge flow rate on the baseline line of spermine NONOate (R^2=0.984, Pearson's correlation coefficient= -0.992, P<0.0001, n=8). B) The effect of met haemoglobin on the relative amount of nitric oxide consumed by lysed red blood cells (0.1g/L) (R^2=0.754, Pearson's correlation coefficient= -0.868, P<0.0001, n=40). Methaemoglobin samples were generated over time by incubation of oxyhaemoglobin with high concentration nitrite C) The effect of haemoglobin concentration upon the relative amount of nitric oxide consumed by lysed red blood cells (R^2=0.815, Pearson's correlation coefficient=0.903, P<0.0001, n=16). D) The effect of spermine NONOate concentration on the relative amount of nitric oxide consumed by lysed red blood cells (0.1g/L), no significant difference.

The starting nitric oxide baseline level was not significantly different between all groups used for cross comparison (oxy vs. deoxy and whole vs. lysed). This is important as the baseline level is indicative of the extracellular nitric oxide concentration in the purge vessel and although this will not alter total nitric oxide consumption, it could impact upon the rate of uptake.

The initial rate of nitric oxide consumption is significantly slower in whole cells when compared to their lysed counterparts (Figure 3.7A and B). The initial rate of oxygenated whole cells is approximately 20 times slower than its lysed counterpart, while in deoxygenated cells it is approximately 10 times. Independent of oxygenation level, whole cells consistently consume more nitric oxide than when they are lysed (Figure 3.7C and D).

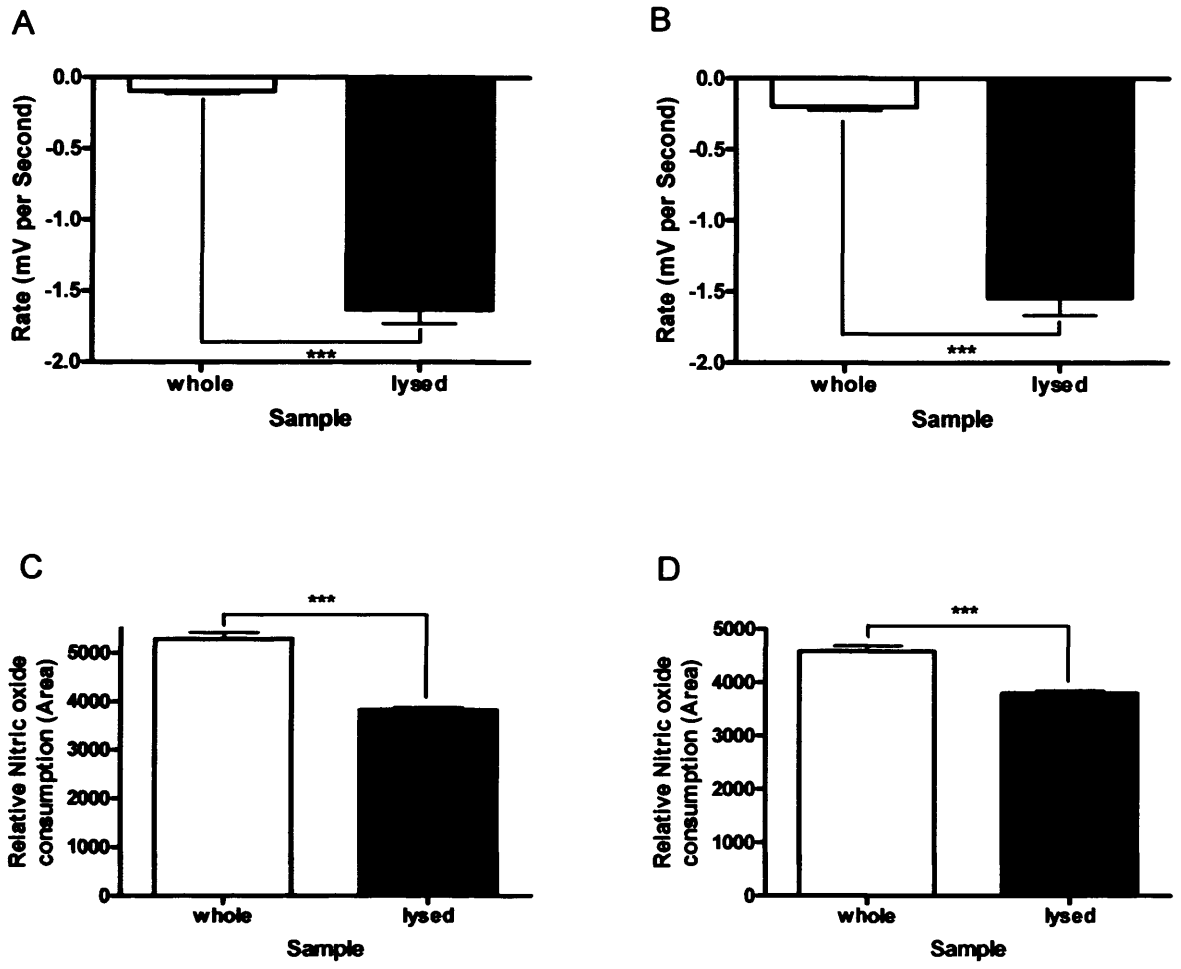

Figure 3.7. A) Initial rate of nitric oxide consumption measured as a drop from the spermine NONOate generated baseline by both whole and lysed oxygenated cells ($P<0.001$). B) Initial rate of nitric oxide consumption by both whole and lysed deoxygenated cells ($P<0.001$). C) The relative amount of nitric oxide consumed by both whole and lysed oxygenated cells ($P<0.001$). D) The relative amount of nitric oxide consumed by both whole and lysed deoxygenated cells ($P<0.001$). Haemoglobin was normalised to 0.1g/L in all cases. n=8, met haemoglobin remained unchanged between samples.

The initial rate of nitric oxide uptake in whole deoxygenated erythrocytes is more than double that of oxygenated erythrocytes (Figure 3.8A). Once the same cells are lysed the relationship is lost with both deoxygenated and oxygenated lysed cells displaying the same initial rate (Figure 3.8B). A similar pattern occurs with respect to the relative amount of nitric oxide consumed. Whole oxygenated erythrocytes consume significantly more nitric oxide than the same cells in a deoxygenated state (Figure 3.8C). Again, this relationship is not present if the cells are lysed (Figure 3.8D).

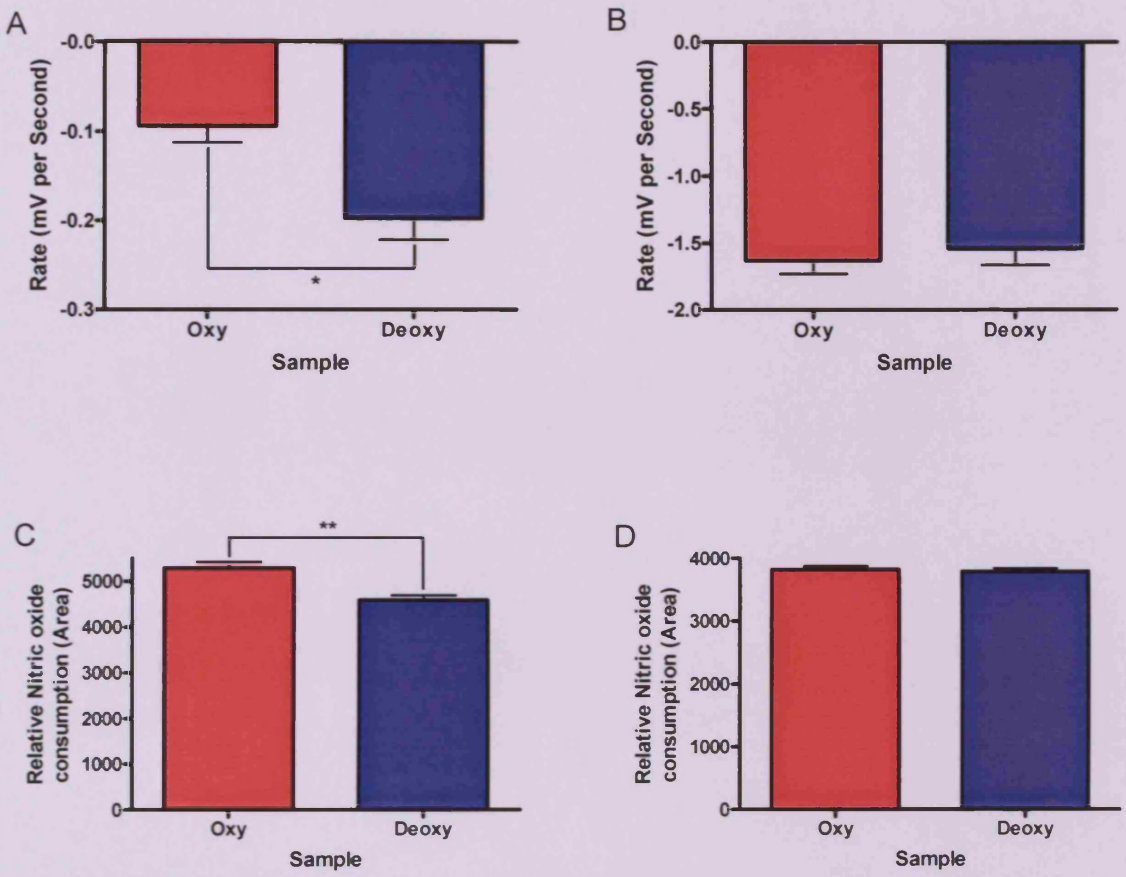

Figure 3.8. A) Initial rate of nitric oxide consumption, measured as a drop from the spermine NONOate-generated baseline in whole oxygenated and deoxygenated cells ($P<0.05$). B) Initial rate of nitric oxide consumption in lysed oxygenated and deoxygenated cells (i.e. free haemoglobin). C) The relative amount of nitric oxide consumed by injection of whole oxygenated and deoxygenated cells into the test system, ($P<0.01$). D) The relative amount of nitric oxide consumed by injection of lysed oxygenated and deoxygenated cells into the test system. Haemoglobin was normalised to 0.1g/L in all cases. n=8, met haemoglobin remained unchanged between samples.

cells, nitrite uptake generally increased as extracellular nitrite concentration incr
However, at lower extracellular concentrations (150nM and 250nM) in the s
containing fully oxygenated cells, the extracellular nitrite level did not fall signifi
from the starting value over thirty minutes. The same was also true for the pa
oxygenated erythrocyte sample but only at the lowest extracellular nitrite concent
(150nM). Nitrite uptake also increased as haemoglobin oxygen saturation decrease
relationship only becomes significant at the higher extracellular nitrite concentrati
450nM and 850nM (oxygenated vs. partially oxygenated, $P<0.05$ and P
respectively), tested after thirty minutes of incubation.

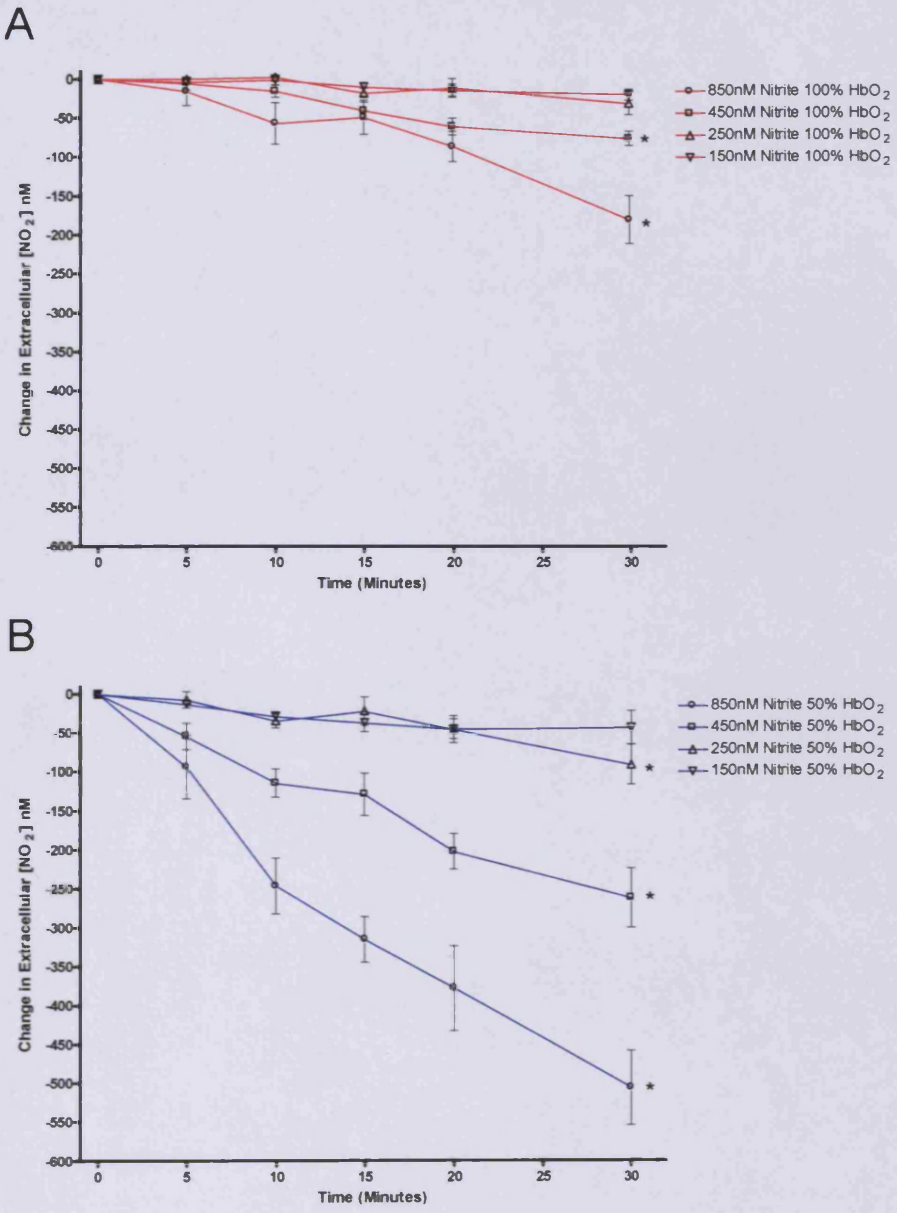

Figure 3.9. A) Nitrite consumption by erythrocytes at ~100% (98.45 ± 0.15%) oxygen saturation. No significant difference between 0 and 30 time points for 150 and 250nM nitrite, $P<0.05$ 0 vs. 30 time points for 450 and 850nM. B) Nitrite consumption by erythrocytes at 50% (51.04 ± 0.83%) oxygen saturation. No significant difference between 0 and 30 time points for 150nM nitrite, $P<0.05$ 0 vs. 30 time points for 250, 450 and 850nM. Change in extracellular nitrite concentration after 30 minutes is significantly different at 450 and 850nm starting concentration ($P<0.05$ and $P<0.01$ respectively), oxygenated vs. partially oxygenated. Starting concentrations were measured at 852.6 ± 33.1nM, 475.2 ± 26.2nM, 244.4 ± 29.9nM and 177.0 ± 19.0nM respectively. Haemoglobin was normalised to 10g/dL (10.07g/dL ±0.08). n=4 for all groups, all measurements were made in duplicate and averaged.

The overall rate of nitrite uptake, particularly in the physiological range (~50-250nM, see section 3.5.2 (page 133)), in relative terms is not very rapid (Table 3.1). This is compounded by high haemoglobin oxygen saturation, where entry to fully oxygenated samples at lower concentrations is almost negligible. At all extracellular nitrite concentrations the overall rate is approximately three times faster in partially oxygenated compared to the corresponding fully oxygenated samples.

Table 3.1. Nitrite consumption rate by erythrocytes *ex vivo* at 50% (51.04 ± 0.83%) and 100% (98.45 ± 0.15%) oxygen saturation. Samples were taken every 5 minutes over 30 minutes from different starting concentrations. Starting concentrations were measured at 852.6 ± 33.08nM, 475.2 ± 26.23nM, 244.4 ± 29.91nM and 177.0 ± 18.99nM respectively. Haemoglobin was normalised to 10g/dL (10.07g/dL ±0.08). Data are expressed as nitrite consumption, nM per minute. n=4 for all groups, all measurements were made in duplicate and averaged.

Nitrite Consumption Rate over 30 mins ($nM\ min^{-1}$) ±SEM	**Approx. Starting Nitrite Concentration: 850nM**	**450nM**	**250nM**	**150nM**
50 % Saturation	17.76 ±1.93	8.72 ±1.27	3.03 ±0.86	1.43 ±0.72
100 % Saturation	6.01 ±1.03	2.52 ±0.30	1.03 ±0.48	0.65 ±0.23

3.5 Discussion

3.5.1 Nitric Oxide Apportionment in Blood

Understanding the way in which nitric oxide is apportioned in blood as individual metabolite species is essential for both the application and interpretation of nitric oxide metabolite biology. Many early studies based hypotheses and theories for experimental findings upon changes in specific metabolite levels. In these studies, species levels (particularly haemoglobin bound species) were reported to be relatively high, in the micro-molar range [329]. Over time, reported levels have slowly come down and consensus between the majority of laboratories exists [369, 379]. However questions surrounding the niceties of measurement methodology still ensue [373]. Nevertheless, it should be remembered, especially given the relative potency of nitric oxide, that amount measured does not always infer physiological relevance. The observed decrease in reported levels is most likely due to improvements in methodology and a greater awareness of confounding factors such as nitrite contamination. The methodologies utilised in this thesis have reported levels (~50-250nM, ~30μM nitrate) of individual metabolite species that are in agreement with the values published in more recent, aforementioned publications [379].

The finding that concentration gradients of specific nitric oxide metabolites exist in the circulation when passing from artery to vein (A-V) has been the stimulus of much research [337]. The premise of this research relies heavily upon the assumption that, when a decrease in a specific metabolite (e.g. plasma nitrite) is observed it must have been converted back to free, biologically active nitric oxide. In agreement with one aspect of this research, this chapter has also demonstrated a concentration gradient of specific species in human arterial and venous blood samples. Specifically, plasma nitrite decreases significantly across the vascular bed. However, the results of this chapter and other publications [369] do not support the finding that free nitric oxide is produced. The explanation lies in the oxygen dependent re-apportionment of species. The results of this chapter show, regardless of haemoglobin saturation, that the total amount of

nitric oxide moiety does not rapidly change, i.e. although levels of specific metabolites change with respect to saturation, the total nitric oxide 'pool' remains constant. Therefore, studying the change of just one specific metabolite is not sufficient to appreciate the dynamic interplay that occurs within a physiological system. In this respect, the results presented in this chapter show a decrease in plasma nitrite A-V that is matched by a concomitant increase in HbNO, redressing the balance of the total nitric oxide pool. The concept of re-apportionment is complex in the *in vivo* setting where, in addition to changes in oxygen level, there are both multiple 'sources' and 'sinks' of nitric oxide throughout the vascular tree.

As this chapter reveals, the apportionment of metabolites in blood is ultimately dependent upon haemoglobin saturation. However, overall apportionment is also a balance between nitric oxide production, consumption and the inter-conversion between metabolite species. The measurement of nitric oxide metabolites is therefore reflective of all of these processes and should be interpreted with this in mind. It is therefore unlikely that the measurement of just one metabolite species will be sufficient to gain understanding with regard to an overall physiological process (e.g. endothelial nitric oxide synthase activity) as has been used in previous studies [380, 381]. Indeed, our own laboratory have demonstrated that inhibition of nitric oxide synthase at baseline in human subjects effects vessel tone but does not alter nitric oxide metabolite levels in the short term [369].

The addition of exogenous nitric oxide to arterial and venous whole blood gives an insight into the way in which blood metabolises nitric oxide *in vivo*. In arterial blood, small increases were seen in most species, however increases in plasma nitrate and a significant increase in plasma nitrite accounted for the majority of the metabolised nitric oxide. The observed increase in plasma nitrate occurred in all samples, but, as a result of the study having a low number of samples, was not found to be statistically significant. Metabolism to these oxidised species corresponds to the relative quantity of oxygen in the sample. In the parallel venous samples plasma nitrite again increased significantly, in addition, the only other species to increase in all venous samples was HbNO. The

observed increase in HbNO is to be expected in the venous sample given the increased availability of haemoglobin (that is not carrying oxygen) to participate in a binding reaction with nitric oxide. The fact that plasma nitrite also increased significantly in the venous sample could possibly reflect an inability of nitric oxide to rapidly enter the red blood cell and so free nitric oxide could be oxidised by dissolved oxygen in the plasma before having the opportunity to enter. Interestingly, in both arterial and venous blood, exogenously added nitric oxide was metabolised to species that are thought be biologically active, thereby 'preserving' so called nitric oxide activity within the blood.

As with baseline samples, the apportionment of exogenously added nitric oxide was dependent upon oxygen level. In broad terms, nitric oxide added to oxygenated, arterial blood is more likely to undergo oxidation reactions. The same is somewhat true for partially oxygenated, venous blood, however, haem groups in the sample that do not have oxygen bound can potentially form HbNO. In this chapter, the same amount of exogenous nitric oxide was added to both arterial and venous samples i.e. production was constant and the species that were formed differed depending on oxygen level, highlighting the difficulties in interpreting A-V gradients of specific species from a more complex system where production etc can be variable.

The addition of nitric oxide elevated the total metabolite pool by the same amount in both arterial and venous samples. The slightly elevated recovery of exogenously added nitric oxide could either reflect nitrite contamination in NOC-9 (as occurs with most chemical agents) or systematic error introduced during measurement (particularly likely with respect to nitrate measurement given its abundance over other species). However, given that the amount of exogenous nitric oxide added only accounted for ~2.5% of the total metabolite pool, this recovery level is very acceptable.

3.5.2 Real Time Reaction of Nitric Oxide with Erythrocytes and Haemoglobin

The bioavailability of nitric oxide within a vessel is largely governed by the concentration of and rate of reaction with other bio-reactants. Haemoglobin is thought to be one of the most significant 'inactivators' of nitric oxide, due to its high rate of reaction. However, despite this high rate and the fact that the vessel lumen contains a considerable amount of haemoglobin, nitric oxide is still able to carry out its normal function.

In the early 1990s there was a focus of research on the simulation of nitric oxide diffusion in the vessel setting, with prominent work from Jack Lancaster [375, 382]. Lancaster's early models suggested that the efficient scavenging of nitric oxide by haemoglobin would prevent it from entering vascular smooth muscle, igniting claims that EDRF was not in fact nitric oxide. As ideas progressed, the assumptions that had been made in early modelling work were disputed and previously described phenomena regarding blood flow were now being considered in this context. Notably, publications by Butler *et al* and Vaughn *et al* described a concept that has come to be known as the 'red blood cell free zone' [322, 383]. This work builds on observations of blood flow in a vessel like structure or tube [384], in simple terms, blood flowing in a tube is composed of two elements; an erythrocyte core flowing down the centre of the lumen and a peripheral plasma layer adjacent to the periphery or endothelium. It is this erythrocyte free plasma layer that has special relevance to nitric oxide diffusion and bioactivity. This creates an area where nitric oxide can exist without being scavenged by erythrocytes and allows the molecule to accumulate. This accumulation creates a concentration gradient that favours diffusion toward vascular smooth muscle where nitric oxide acts to produce vasodilatation. The Vaughn group went on to demonstrate that intravascular flow reduces nitric oxide scavenging by erythrocytes in an isolated vessel model [323]. This theory goes some way to explaining the ability of nitric oxide to function in the vessel setting, but other mechanisms are expected play a role. More recent research and the work of this chapter have started to focus on the direct interaction of nitric oxide with erythrocytes.

The preliminary finding of the model developed in this chapter demonstrates that whole and lysed erythrocytes generate visually distinct signals with regard to nitric oxide uptake. To further explore this finding, assessments of initial rate of uptake and total relative nitric oxide consumption were performed.

The measured initial uptake rate is significantly slower (~10-20 times) in whole erythrocytes compared to the corresponding lysed sample. The encapsulation of haemoglobin, as in the case of whole erythrocytes, clearly retards the rate of reaction with nitric oxide, suggesting an additional mechanism by which nitric oxide may be protected in the vessel setting. This property of whole erythrocytes has been investigated by other research groups, leading to a number of proposed theories that explain the mechanism of limited nitric oxide entry. The Vaughn and Liao group published on the topic, suggesting that erythrocytes possess an intrinsic barrier to nitric oxide consumption [320]. The mechanisms involved are thought to stem from a resistance to transmembrane diffusion or a limited intracellular diffusion mechanism. A competition model was utilised in these experiments, which was thought to create a homogenous reaction without extracellular diffusion limitation. The Lancaster group countered this work, suggesting that the model was inappropriate (in terms of extracellular diffusion limitation) and that it is in fact the 'unstirred layer' thought to surround erythrocytes that is the main diffusion barrier, not the membrane [321]. A recent publication utilising phospholipid vesicles filled with haemoglobin has also provided an alternative mechanism that does not implicate the membrane *per se* [385]. The work suggests that as nitric oxide enters the vesicle and reacts with haemoglobin close to the interior membrane surface it forms a diffusion barrier, this is thought to retard the reaction with haemoglobin in the core of the vesicle and decrease overall reaction rate. As with a number of studies of this type, a 'stopped-flow' analysis was utilised. In this method, the reactants are rapidly mixed and then the products formed (used as surrogate marker instead of actual nitric oxide level) are continually measured. In contrast to the model developed in this chapter, the extracellular nitric oxide level is changing over the course of the stopped-flow experiment and could potentially influence the measured rate. In

this regard, the difference in initial rate between whole and lysed cells was calculated to be ~10-20 times in this chapter, while some reported values have been as much ~500 times [320], this could reflect differences in methodology and rate assessment, however, irrespective of magnitude, the measured rate is always slower in whole cell preparations.

The results of this chapter do not fully confirm nor refute any of these diffusion limitation mechanisms but do provide novel findings that support certain aspects of these mechanisms. The model developed in this chapter utilises a reaction vessel that is bubbled to provide a homogenous reaction mixture, theoretically, limitations of extracellular diffusion should therefore not play a significant role. In this respect, a diffusion limitation in whole erythrocytes is still observed, suggesting that mechanisms intrinsic to the cell do play a role. Results obtained in oxygenated and deoxygenated cells potentially imply a role for intra-erythrocyte diffusion limitation which will be considered in the later part of this discussion section.

The overall uptake of nitric oxide was significantly higher in whole cells compared to the corresponding lysed samples; the potential reasons for this are two fold: In its whole form, the cell is more likely to be able to process the influx of nitric oxide. For example, the cell will have the potential to convert methaemoglobin back to haemoglobin via methaemoglobin reductase and associated co-factors, allowing further nitric oxide consumption, a process that is unlikely to occur in the lysed sample. Secondly, and perhaps more probable, the whole erythrocyte, regardless of containing haemoglobin could act as a 'reservoir' due to the relative partitioning of nitric oxide into one media vs. another. Nitric oxide, like oxygen, has previously been shown to be ~4 times more soluble in liposomes relative to water [386].

As is becoming apparent, the biochemistry of nitric oxide is heavily influenced by the presence of oxygen in the environment. To this end, the influence of haemoglobin saturation upon the nitric oxide uptake model was tested. In whole cell samples, the initial uptake rate was significantly faster (~2 times) in deoxygenated cells compared to the same cells in an oxygenated state. Given that there is no difference between

deoxygenated and oxygenated lysed samples, this implies something intrinsic to the cell as opposed to a difference in the rate of reaction between nitric oxide and haemoglobin. The increased uptake by deoxygenated cells could be due to haemoglobin membrane interaction. Deoxygenated, but not oxygenated, haemoglobin is known to bind to proteins (such as AE1) on the interior of the plasma membrane [387]. This positions the haemoglobin perfectly, close to the membrane and channels which are thought to facilitate nitric oxide entry [83], where it can rapidly react with nitric oxide entering the cell. In support of this, the uptake of nitric oxide by erythrocytes has been shown to be influenced by altering the degree of AE1-cytoskeleton interaction [388]. The nitric oxide permeability of the erythrocyte membrane is also thought to be considerably greater in deoxygenated cells [389]. These data suggest that differences in diffusion rate (transmembrane and intraerythrocytic) may be a significant determinant in the difference in whole and lysed initial uptake rates.

The overall amount of nitric oxide uptake was significantly higher in oxygenated whole cells compared to deoxygenated cells. Again, this relationship was absent in the equivalent lysed samples. Oxygenated haemoglobin in the whole erythrocyte can potentially consume two molecules of nitric oxide compared to deoxygenated haem that has the ability to only bind one. It is possible for the first nitric oxide to react with the oxygen bound to the haemoglobin forming nitrate and resultant methaemoglobin, the methaemoglobin is then reduced back to haemoglobin (by methaemoglobin reductase in the whole cell) which can participate in a second nitric oxide reaction forming HbNO. This reaction is unlikely to completely cope with very rapid methaemoglobin formation in this model, however it may account for the increased consumption observed by oxygenated whole cells.

3.5.3 The Uptake of Nitrite by Erythrocytes

Gladwin and colleagues propose that nitrite may behave as a biologically active source of nitric oxide that is activated by haemoglobin, maximally around the 50% saturation point (see section 1.10.2, page 68). For this process to be biologically active *in vivo*, nitrite must enter erythrocytes in sufficient quantities, react with haemoglobin and release free nitric oxide over the course of a circulatory cycle. The mechanism by which free nitric oxide is able to escape re-capture by haemoglobin within the erythrocyte continues to be heavily questioned [343], however, the assessment of nitrite entry to the erythrocyte has been somewhat neglected.

Wider research to date would suggest that the anion exchanger (AE1) could be a likely candidate for nitrite entry into the red blood cell. Early work by Shingles *et al* demonstrated inhibition of HCO_3^-/NO_2^- exchange across the erythrocyte membrane with the AE1 channel blocker DIDS (4,4'-diisothiocyanato-2,2'-stilbenedisulfonate) [390]. However, more recent publications have been unable to demonstrate an effect of DIDS [391, 392]. To further explore the possible role of carrier-mediated transport, Jensen attempted to block transport with phloretin (a general inhibitor of facilitated diffusion), but again found no influence [391]. Because of the charged nature of the nitrite anion it cannot simply diffuse across the membrane like nitric oxide. Other possible routes of entry are via diffusion through channels (conductive transport) or diffusion as HNO_2. Although HNO_2 can simply diffuse across the lipid bilayer it has a pKa of ~3.3 [391] and so is unlikely to form to any great extent at normal extracellular pH. In addition, intracellular pH is normally lower than extracellular and therefore an outward diffusion gradient for HNO_2 would exist. However, nitrite would be consumed by haemoglobin inside the cell and so could potentially reverse this gradient. In summary, a likely candidate is conduction channel diffusion. The results of this chapter show concentration dependency and the generally low uptake rates in the physiological nitrite range that implies diffusion to be responsible, rather than an active uptake process. In this regard, previous studies have suggested that nitrite entry to erythrocytes is relatively rapid. However, the extracellular concentrations of nitrite utilised are often in the

millimolar range [391, 392]. The rates observed and mechanistic assumptions derived at these nitrite concentrations clearly do not translate to mechanisms occurring at physiological levels. The extrapolation of findings, generated under non-physiological conditions, to the physiological setting is a problem that is prominent within the nitric oxide field. In order to study a system such as nitric oxide metabolism it is often necessary to introduce species to alter baseline equilibrium. There is not a problem with this research approach, however it is essential to interpret findings in context.

A number of publications have previously suggested that nitrite uptake by erythrocytes under normal conditions is independent of oxygenation [391, 393]. The results presented here find that partially oxygenated samples have a higher uptake rate compared to oxygenated samples, particularly at elevated extracellular nitrite concentrations. This observed difference in uptake cannot be explained in terms of the rate of reaction between haemoglobin and nitrite, as oxyhaemoglobin is reported to react ~10 times faster than deoxy [394]. However, the removal of nitrite by haemoglobin is still likely to contribute to the force that drives uptake. As with the interaction seen between erythrocytes and nitric oxide, an intrinsic interaction between haemoglobin and the cell membrane may have a function. Deoxygenated haemoglobin is known to interact with channels and the cytoskeleton on the interior membrane surface [387] and so potentially could alter the entry of nitrite by association. The diffusion of nitrite through membrane channels may also be altered by membrane potential. Changes in oxygenation are known to alter the membrane potential through mechanisms such as the Haldane effect (see section 1.4.2.4, page 18) [391].

The sluggish rate of nitrite uptake by erythrocytes (particularly in an oxygenated state) at physiologically relevant extracellular nitrite concentrations does not favour a mechanism by which nitrite, through an erythrocyte/haemoglobin mechanism, effects vessel tone under basal conditions. The overall importance of the findings in this chapter section will be put in context with respect to *in vivo* data that is presented and discussed in chapter 6.

3.6 Summary and Conclusions

- The apportionment of nitric oxide and its metabolites in blood is dependent upon the degree of haemoglobin oxygen saturation, creating arterio-venous gradients of specific species. However, the total nitric oxide metabolite pool remains relatively constant with decreases in one group of species being compensated for by increases in another. Apart from nitrate (which is in the μM range), measured values ranging from ~10-250nM are in agreement with recent publications.

- The metabolism or species formed when exogenous nitric oxide is added to arterial and venous blood differs. In oxygenated blood, nitric oxide is more likely to undergo oxidation reactions to form nitrite and nitrate. In venous blood, the increased availability of haemoglobin (i.e. with no oxygen bound) leads to the production of HbNO. In both cases, some of the species formed are thought to be biologically active and may potentially preserve nitric oxide.

- The rate of initial uptake of nitric oxide by erythrocytes is ~10-20 times slower than the reaction of free haemoglobin (lysed cells) with nitric oxide. The exact mechanisms of this retarded uptake remain unknown, but the phenomenon may serve to increase nitric oxide longevity *in vivo*. In whole erythrocytes, the initial uptake rate of nitric oxide is significantly faster (~2 times) in deoxygenated cells, most likely resulting from an interaction of deoxyhaemoglobin and the erythrocyte membrane/membrane proteins.

- The uptake of nitrite by erythrocytes is concentration-dependent and does not occur at a rapid rate when extracellular nitrite is in the physiological (i.e. plasma concentration) range. The rate of uptake is also influenced by haemoglobin oxygen saturation with partially oxygenated (SaO_2 50%) cells taking up more nitrite, particularly at high extracellular nitrite concentrations.

4 Nitrite Induced Vasorelaxation, the Influence of Oxygen

4.1 Introduction

The nitrite anion (NO_2^-) represents an interesting constituent of the nitrogen oxide(s) (NO_x) family in pharmacological terms. The real-time study of nitric oxide has always posed a problem due to its highly reactive nature: nitrite, although significantly less potent than nitric oxide, has been utilised as a more stable and practical solution to this experimental problem. However, nitrite has its own caveats, most notably contamination in *in vitro* systems and alternative sources *in vivo* (e.g. diet) where levels can be oxygen dependent. Nitrite itself has been known to be vasoactive for a number of years [395]. Although relatively less potent than pharmaceutical nitrovasodilators, interest in its mode of action has recently revived after recognition that it may represent a circulating source of bioavailable 'NO-like' activity (see section 1.10.2, page 68) and may have an important role clinically [98].

Nitrite was previously thought to be a relatively inert nitric oxide metabolite, although current studies now allow us to appreciate the dynamic 'interplay' between the metabolites of nitric oxide and their potential as physiological effectors (see chapter 3 and [369]). The biological effects of nitrite are thought to be predominantly mediated via its reduction back to nitric oxide. Therefore, the mechanisms by which nitrite is reduced to nitric oxide has become a principal area of interest within the nitrite field. To date, research has largely focused upon the reduction of nitrite by haemoglobin, a mechanism that is thought to match blood flow and oxygen demand (see section 1.10.2, page 68). However, this mechanism has not been demonstrated within the *in vivo* setting and the original *ex vivo* vessel work demonstrating enhanced nitrite induced vasodilatation in hypoxia in the presence of haemoglobin [337] is yet to be repeated [315, 396]. Several issues remain with this concept, including the effective entry of nitrite into erythrocytes at physiological concentrations (see chapters 3 and 6), and subsequent escape of bioavailable nitric oxide from haemoglobin recapture. In addition to haemoglobin reduction, an alternative mechanism of nitrite reduction involving

oxidoreductase enzymes has also been suggested [397]. The proposed mechanism suggests that the principal route of nitrite reduction occurs in the vascular tissue via enzymatic reduction as opposed to in the blood stream via haemoglobin reduction. The main enzymes thought to be involved are xanthine oxidase (XO) and aldehyde oxidase (AO) [397], however it has been suggested that nitric oxide synthase [398], myoglobin [344] and mitochondrial enzymes [399] also have the capacity to reduce nitrite.

Despite the considerable amount of speculative work surrounding nitrite as both a circulating vasoactive moiety and a potential therapeutic, very little work regarding its downstream mechanism of action has been completed, particularly in hypoxic vessels, where it is though to be most effectual. Dalsgaard and colleagues have presented some interesting mechanistic findings from rat aortic rings in hypoxia [396]. The work finds that in the presence of cyclooxygenase and nitric oxide synthase inhibitors, nitrite appears to operate via soluble guanylate cyclase. The authors also fail to show a role for haemoglobin, mitochondrial bc_1 complex or xanthine oxidase in the reduction of nitrite and suggest that a mechanism involving the formation of s-nitrosothiols within smooth muscle could be involved. More recently, the Zweier group assessed the mechanism of nitrite-induced vasodilatation, also in hypoxic rat aortic rings [400]. As with the previous publication, this work was unable to demonstrate the involvement of xanthine oxidase or the mitochondrial transport chain, in addition to nitric oxide synthase and cytochrome P450, in the reduction of nitrite. They also found that nitrite induced vasorelaxation could be inhibited by ODQ (1H[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one). ODQ is commonly used to inhibit soluble guanylate cyclase, but will bind to a number of haem based proteins. To complement the vessel-based work, the authors also completed some nitric oxide generation measurements from aortic tissue using a nitric oxide-sensitive electrode to consider the involvement of haem-based enzymes in nitrite reduction. The generation of nitric oxide from the aortic tissue was inhibited by both heating of the tissue (to denature proteins) and ODQ. The results suggest that haem-based tissue enzymes are responsible for nitrite reduction. In the case of soluble guanylate cyclase, the enzyme could potentially be acting as both a nitrite reductase and catalyser of cGMP production to affect vasorelaxation.

levels both directly and indirectly. Within the cell, nitric oxide is capable of modifyin proteins via thiol, tyrosine and metal groups, like phosphorylation, these modification can have huge influences upon the functionality of the protein. In the short term thes modifications can influence receptor sensitivity and enzyme or channel activity fo example. However, if the protein is a transcription factor, then exposure to nitric oxid can have long lasting effects via alteration of gene expression (for a comprehensiv review of nitric oxide signalling particularly with regard to gene expression see [401] Given these facts, it is important to consider the additional effects of nitrite, not onl from a clinical dosing or chronic administration point of view, but also in terms of th potential mechanisms that could be activated *in vivo* by basal nitrite levels, particularl during a hypoxic insult. Publications in this area are limited but some studies have bee completed. One study that utilised a rodent model has demonstrated that twenty fou hours after high dose nitrite infusion, aortic heme oxygenase-1 and heart heat shoc protein 70 expression (at a protein level) had been altered, despite plasma nitrit returning to basal levels at the same time point [402].

vasodilatation in hypoxia. In addition, the broader effects of nitrite in vascular tissu will also be investigated in this chapter in order to gain crucial understanding about t acute effects of nitrite exposure at a cellular level. Specifically this chapter aims to;

- Assess the effect of vascular tissue oxygenation upon nitrite-depende vasodilatation.

- Investigate the dependency of nitrite-induced vasodilatation upon the formati of nitric oxide and elucidate the primary routes of nitrite reduction with hypoxic vascular tissue.

- Establish the down-stream mechanisms involved in nitrite-mediat vasodilatation in hypoxia via the selective blockade of key enzymes/steps witl vascular signalling cascades.

- Examine the acute effects of nitrite exposure upon the expression of speci genes in normoxic and hypoxic vascular tissue.

4.2 Specific Protocols

4.2.1 Myography

Rabbit aortic rings were harvested, mounted and conditioned as per section 2.5 (page 104). For experiments requiring endothelium-denuded tissue, the lumen of the ring was gently rubbed with a wooden applicator and absence of endothelium was then tested during the experimental protocol by acetylcholine challenge. In hypoxic experiments, tissue was always exposed to 10 minutes of hypoxia before phenylephrine induced pre-constriction (detailed in section 2.5.2, page 105). The final concentration of nitrite utilised in all experiments was 10μM, an effective concentration previously established in our laboratory *in vitro* [98], but also a concentration that is achievable *in vivo*, in plasma with pharmacological nitrite dosing [98, 379, 403].

The slow and continuous relaxation of isolated vessels during hypoxia, as observed in the controls of all our experiments, is an established phenomenon. The precise cause of vasodilatation remains unknown. Both energy limitation and interruption of excitation contraction coupling have been suggested as possible causes [131]. In our model, hypoxia-induced relaxation is independent of the endothelium or NOS (denudation/L-NMMA data). It is important to note that the smooth muscle cells are not simply dying in hypoxia. After a period of hypoxia, tissue can be re-equilibrated in oxygenated buffer and show comparable contraction and relaxation to exogenously added agents. Of related interest, our laboratory has previously shown that relaxation induced in arteries and veins by nitric oxide donors (such as GSNO) and nitrite is enhanced under hypoxic compared to normoxic conditions [91, 98].

All pharmacological inhibitors were incubated for thirty minutes under normoxic conditions before switching to hypoxic conditions for experimental testing. Concentrations of pharmacological inhibitors used were selected after the completion of concentration curves (see results section of this chapter) or were based upon values used in the current literature, CPTIO [404], U-51605 [405], raloxifene and oxypurinol [397],

L-NMMA [398] (for values see table 4.1). Pharmacological inhibitors were all dissolved in dimethyl sulphoxide (DMSO) apart from oxypurinol, which was dissolved in 1N sodium hydroxide (NaOH) and CPTIO and L-NMMA, which were dissolved in Krebs' buffer. Control experiments to test the effect of vehicle solutions upon vascular response to nitrite were completed and showed no demonstrable effect.

Table 4.1. Concentrations and actions of pharmacological inhibitors utilised. These represent final in-bath concentrations required or established for maximum effect on nitrite-induced vasodilatation.

Agent	Action	Final Concentration
ODQ	Soluble Guanylate Cyclase (sGC) Inhibitor	10 μM
Indomethacin	Cyclooxygenase Inhibitor (COX)	5 μM
CPTIO	Nitric Oxide Scavenger	1 mM
U-51605	Prostacyclin Synthase Inhibitor (PGISi)	10 μM
Raloxifene	Aldehyde Oxidase Inhibitor	50 nM
Oxypurinol	Xanthine Oxidase Inhibitor	100 μM
L-NMMA	eNOS Inhibitor	300 μM

Maximum relaxation was measured twenty minutes post nitrite addition and expressed as percentage of the maximum constriction to phenylephrine. The twenty minute time point is used to reflect total nitrite-induced relaxation, as vasorelaxation to nitrite was observed to reach a sustained plateau and have had its maximum effect within this time. When using pharmacological blockers, percentage inhibition is always with respect to the appropriate control tissue/agent control as some inhibition agents alter basal tone alone.

4.2.2 Gene Expression Study

4.2.2.1 Sample Generation

To study the effects of oxygen level and nitrite treatment upon the transcription of specific genes, equal sized aortic rings were prepared and mounted in myograph tissue baths as per the vascular reactivity studies. The phenylephrine conditioning phase of the vascular reactivity protocol was omitted and all experiments were carried out at two grams of resting tension. Each pair of aortic rings were designated to one of four treatment groups, those incubated in normoxia in the presence of 10μM nitrite or saline (0.9% w/v) and those exposed to hypoxia in the presence of 10μM nitrite or saline (0.9% w/v). All groups were exposed to the specific treatment (nitrite, saline, hypoxia or normoxia) for thirty minutes before aortic rings were carefully demounted and rapidly snap frozen in liquid nitrogen. Thirty minutes of hypoxic exposure was chosen as it corresponds to the vascular reactivity studies of this chapter and an exposure of this duration has previously been shown to alter gene transcription in endothelial cells [406].

4.2.2.2 Total RNA Extraction

Samples were homogenised on ice in TRIzol® reagent (Invitrogen) to extract and stabilise RNA from the sample. RNA was then purified from the homogenate using a Purelink RNA mini kit (Invitrogen) according to the manufacturer's instructions. Once purified, the yield of RNA was measured using a Nanodrop (Thermo Scientific) spectrophotometer reading absorbance at 260nm, with RNA integrity assessed from the ratio of 260/280 nm absorbance ratio.

4.2.2.3 cDNA Generation

A cDNA library was constructed from RNA samples using a high capacity cDNA reverse transcription kit (Applied Biosystems), after normalisation for initial sample

RNA content. The thermocycling conditions utilised were 25°C for 10 mins, 37°C for 120 mins, 85°C for 5 mins samples were then maintained at 4°C before storage at -80°C.

4.2.2.4 Real Time Polymerase Chain Reaction Assay

COX-2 (cyclooxygenase-2), eNOS (endothelial nitric oxide synthase), GAPDH (glyceraldehyde 3-phosphate dehydrogenase) and HIF-1α (hypoxia inducible factor-1 alpha) mRNA expressions were all analysed on an Applied Biosystems 7500 real-time polymerase chain reaction (PCR) system using platinum SYBR green qPCR supermix UDG (Invitrogen). All rabbit primer sequences (table 4.2) were obtained from literature [407-409] and were constructed by Sigma Aldrich. PCR conditions were 50°C for 2 mins, 95°C for 10 mins, 40 cycles for 15 s at 95°C and 1 min at 60°C, followed by a melt curve (95°C for 15 s), ramp to 60°C for 1 min and 95°C for 20 s. Conditions were taken from those optimised in the literature [407].

Estimates of cDNA abundance were assessed from the portion of the curve for which the plot of the log input amount versus the cycle threshold (C_T) differences resulted in a slope of approximately zero, which indicated that the amplicon efficiencies were approximately equal. The relative quantification of the target genes was calculated using the formula $2^{-\Delta\Delta CT}$, in which ΔC_T equals the difference between C_T values for both target gene and GAPDH.

Table 4.2. Specific polymerase chain reaction primer nucleiec acid sequences.

HIF-1 alpha sense	GGATATAAATTTGGCAATGCTTCCAT
HIF-1 alpha anti-sense	GAGTGCAGGGTCAGCACTACTTC
GAPDH sense	ACTTTGTGAAGCTCATTTCCTGGTA
GAPDH anti-sense	GTGGTTTGAGGGCTCTTACTCCTT
COX-2 sense	GAGATGATCTACCCGCCTCA
COX-2 anti-sense	GATTCATAGGGCTTCAGC
eNOS sense	GCTGCGCCAGGCTCTCACCTTC
eNOS anti-sense	GGCTGCAGCCCTTTGCTCTCAA

4.2.3 Statistical Analysis

Data was compared using a one way-ANOVA with a Bonferroni's multiple comparison *post hoc* test. All analysis was carried out using Graphpad Prism software (version 4.0). n=1 represents data averaged from a pair of matched aortic rings in both vascular reactivity and genetic studies. Data showing time course profiles were compared statistically over the entire profile. The 20 minute time point was chosen for a single time point comparison of total relaxation induced.

4.3 Results

4.3.1 The Effect of Oxygen

To establish the effect of oxygen upon vascular constriction in the aforementioned aortic ring model, vessels were subjected to a phenylephrine concentration response at both 95 and ~1% oxygen. Vessels constricted with phenylephrine at 95% oxygen constricted to a maximum of 8.21 grams, the tension in these vessels did significantly decrease thereafter (over 20 minutes) by a mean value of 1.08 grams (Figure 4.1A). Vessels constricted at ~1% oxygen also lost a significant amount of tension over the 20 minute investigation period with the mean loss in tension (3.08 grams) considerably more than that observed at 95% oxygen (Figure 4.1B). The phenylephrine EC_{50} at 95% oxygen was significantly less from that at 1% oxygen ($P<0.002$), indicating that a higher concentration of phenylephrine is required to achieve 50% of the maximum effect at 1% oxygen.

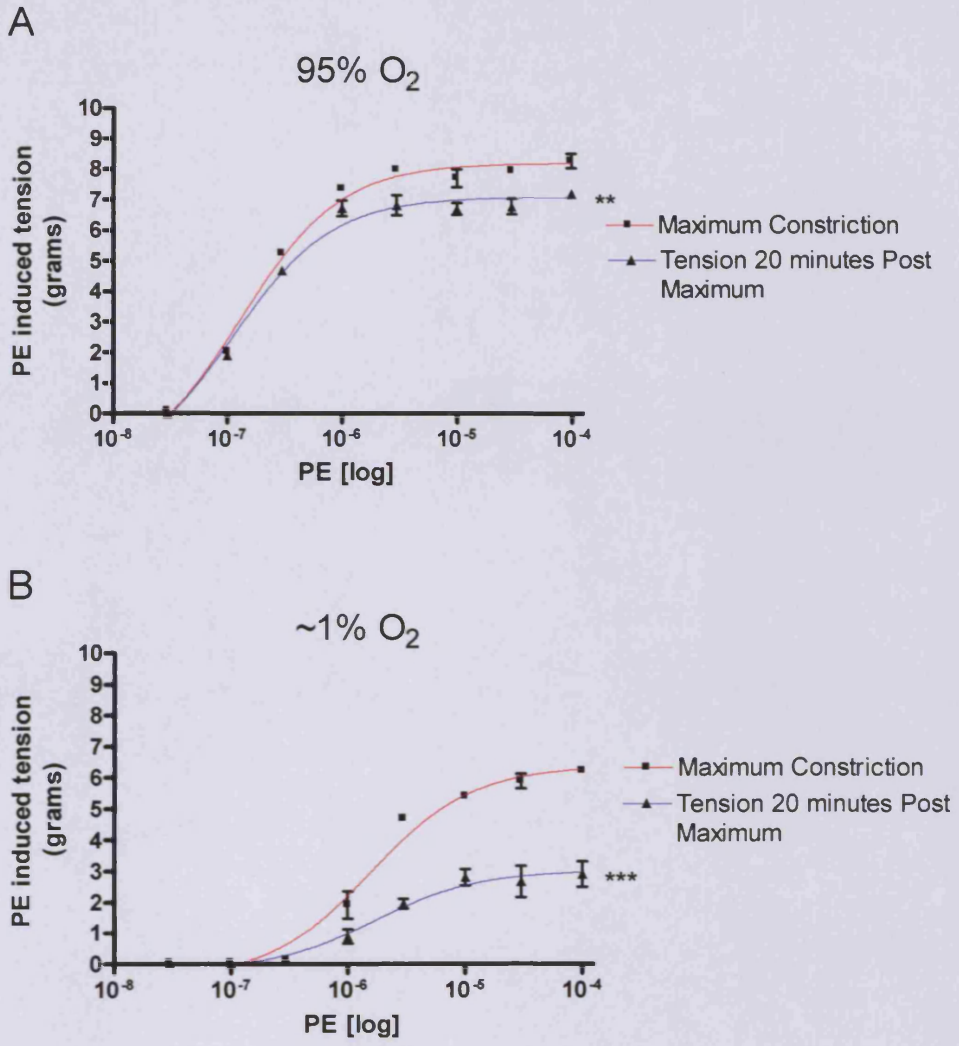

Figure 4.1. Phenylephrine concentration response curves at maximum constriction and 20 minutes post maximum constriction. A) Response at 95% oxygen, R_{max} and EC_{50} at maximum, 8.21 ± 0.10 grams and 133 ± 6.7 nM respectively, R_{max} 20 minutes most maximum constriction 7.10 ± 0.11 grams. $R_{max\ is}$ significantly different between maximum and 20 minutes post maximum (**P<0.01) B) Response at ~1% oxygen, R_{max} and EC_{50} at maximum, 6.38 ± 0.18 grams and 1560 ± 289.9 nM respectively, R_{max} 20 minutes most maximum constriction 3.02 ± 0.19 grams. $R_{max\ is}$ significantly different between maximum and 20 minutes post maximum (***P<0.001). EC_{50} at maximum is significantly different between 95% oxygen and 1% oxygen (P=0.0023). (n=4 in all cases).

Our laboratory has previously found that the responsiveness of vascular tissue in this model to S-nitrosoglutathione (GSNO) is increased when the tissue is subjected to hypoxic conditions [91]. To establish if the model would react in the same way to nitrite exposure, nitrite vasorelaxation experiments were carried out at both 95 and 1% oxygen. As is the case with GSNO, the same concentration of nitrite induced significantly more vasorelaxation in hypoxic tissue compared to normoxic tissue ($P<0.0001$) (Figure 4.2).

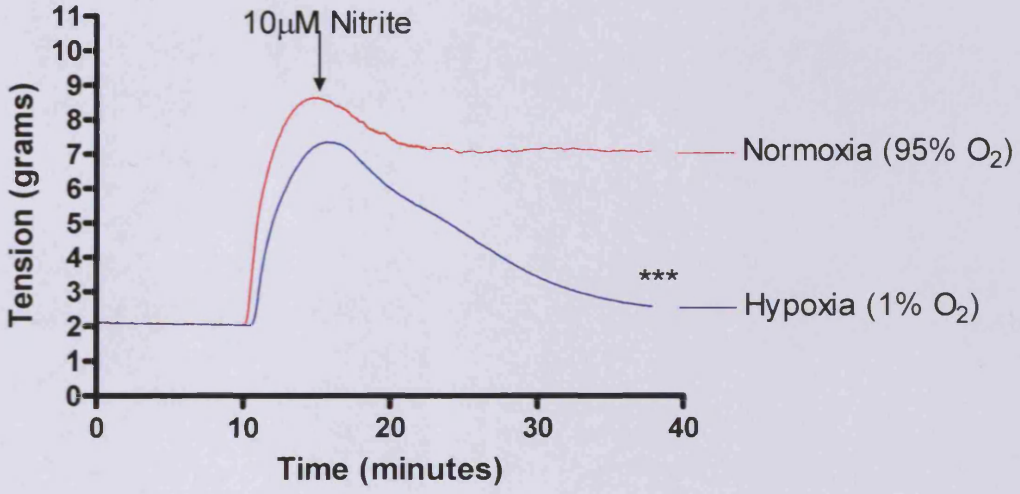

Figure 4.2. Raw trace of nitrite induced vasorelaxation. Aortic rings were pre-constricted with phenylephrine (1 x10^{-6}M final at 95% and 3 x 10^{-6}M final at 1% oxygen) before addition of nitrite (10μM final). Nitrite induced significantly more relaxation in hypoxia (1% oxygen) versus normoxia (95% oxygen) (***$P<0.0001$) (n=4).

4.3.2 Pharmacological Inhibitors

The two key inhibitors utilised within this chapter, ODQ and indomethacin were subjected to concentration-effect testing to establish the appropriate concentration for maximum effect (Figure 4.3). ODQ gave maximum effect at a final concentration of 10μM, incubation with higher concentrations reduced inhibition, possibly due to toxicity at these elevated concentrations (Figure 4.3A). Indomethacin in the concentration range of 1-10μM had a similar inhibitor effect (Figure 4.3B), therefore a concentration of 5μM was chosen for experimentation.

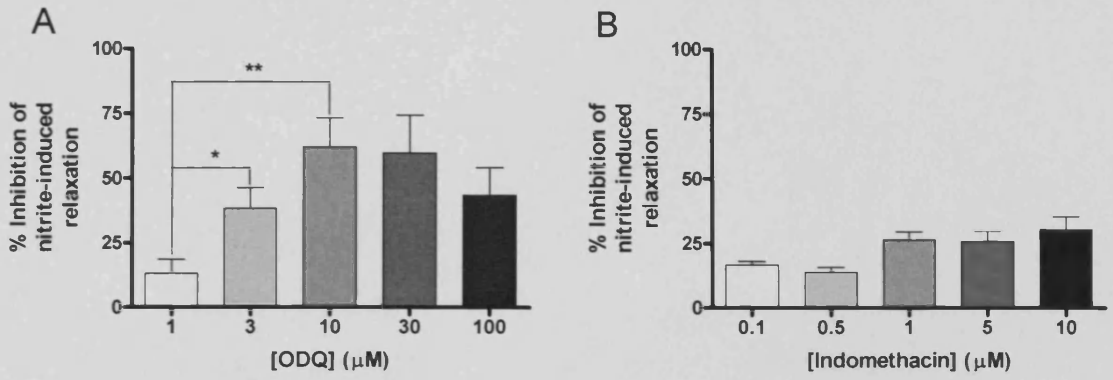

Figure 4.3. % inhibition of nitrite-induced vascular relaxation, an inhibitor concentration-effect analysis. A) Inhibition by ODQ over a concentration range, a concentration of 10μM ODQ gave maximum effect (1μM versus 10μM, **P<0.01). A concentration of 100μM was found to have a reduced effect, most likely due to toxicity. B) Inhibition by indomethacin over a concentration range. No significant difference was found between the effects of 1, 5 and 10μM indomethacin, therefore 5μM was chosen for experimental testing. (n=4 in all cases).

4.3.3 Downstream Mechanisms in Endothelial Denuded and Intact Vessels

To investigate the role of the endothelium in the downstream mechanisms of nitrite-induced vasodilatation, both endothelium-intact and -denuded vessels were assessed. Nitrite consistently induced significant relaxation in hypoxic blood vessels, regardless of the presence or absence of endothelium (Figures 4.4A and 4.6A). It should be noted that the time-course of this relaxation is minutes rather than seconds as is seen with more potent nitric oxide donors, native nitric oxide or RBC-induced hypoxic vasodilatations (see Chapter 5).

4.3.3.1 Endothelium Denuded Vessels

In vessels that are denuded of endothelium, ODQ (10μM) blockade of soluble guanylate cyclase completely inhibited the nitrite induced relaxation ($P<0.0001$, comparison of entire profile versus appropriate control) (Figure 4.4C). This result was unaffected by the addition indomethacin (5μM) ($P<0.0001$, comparison of entire profile versus appropriate control) (Figure 4.4D). Incubation with indomethacin alone had no inhibitory effect upon the nitrite-induced vasodilatation (Figure 4.4B).

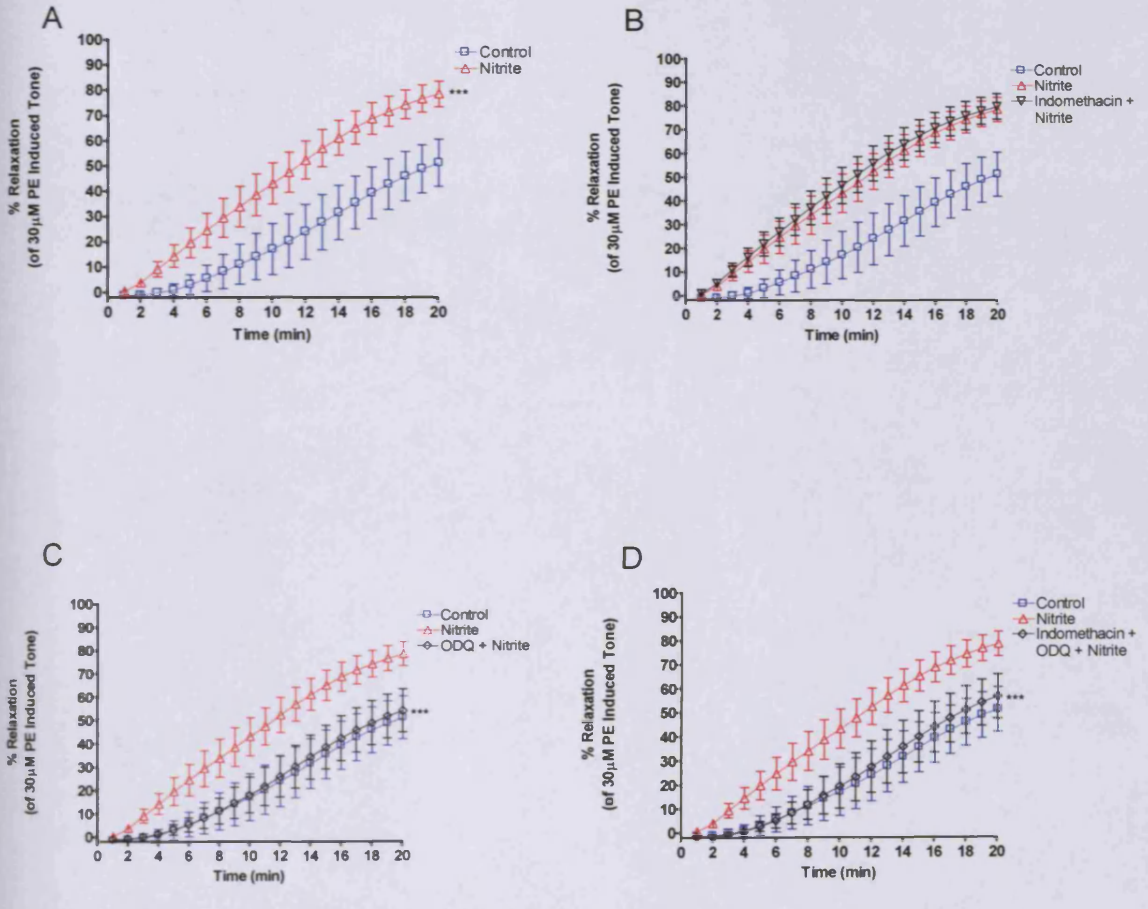

Figure 4.4. 20 minute relaxation profiles of endothelium denuded aortic rings. A) Nitrite (10μM) produces a marked relaxation compared to control (***$P<0.001$). B) No inhibition of nitrite induced relaxation by Indomethacin (5μM). C) Complete inhibition of nitrite induced relaxation by ODQ (10μM) (***$P<0.001$) compared to Nitrite alone. D) Complete inhibition of nitrite induced relaxation by indomethacin (5μM) and ODQ (10μM) in combination (***$P<0.001$) compared to Nitrite alone. (n=5 in all cases).

To simplfy comparisons between groups, the twenty minute time point was chosen, with maximum response to nitrite typically occurring within this time. A comparison at the twenty minute time point in endothelium denuded vessels (Figure 4.5) confirms the findings observed over the entire relaxation profile, with ODQ incubation completely inhibiting the response to nitrite.

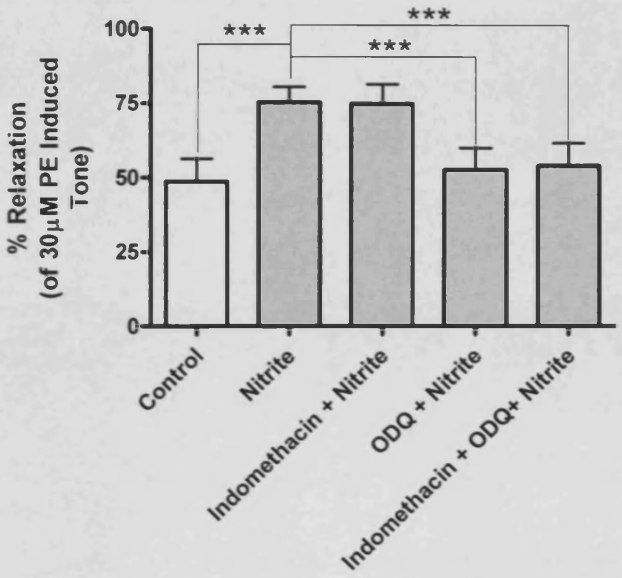

Figure 4.5. Maximum relaxation induced by nitrite at the 20 minute time point in endothelium denuded aortic rings. Nitrite produces a marked relaxation compared to control (***P<0.001) which is completely inhibited ODQ (10μM) (***P<0.001) compared to Nitrite alone. Indomethacin produced no inhibition in denuded vessels. (n=5 in all cases).

to nitrite (Figure 4.6D). If the profiles of vasodilatation are studied in more detail, some interesting dynamics can be observed. The inhibition by ODQ appears to be having maximum effect during the first six minutes, before gradually declining (Figure 4.6C). Conversely, inhibition by indomethacin appears to be continual throughout (Figure 4.6B). Regardless of these dynamics, when used in combination, the two agents produce complete inhibition throughout (Figure 4.6D).

Interestingly, the use of COX inhibition in combination with a nitric oxide scavenger also completely inhibited the response (Figure 4.8). Utilising a prostacyclin synthase inhibitor (PGISi) (that in itself altered baseline tension) we were able to demonstrate that prostacyclin was not responsible for the COX-mediated effects (Figure 4.8).

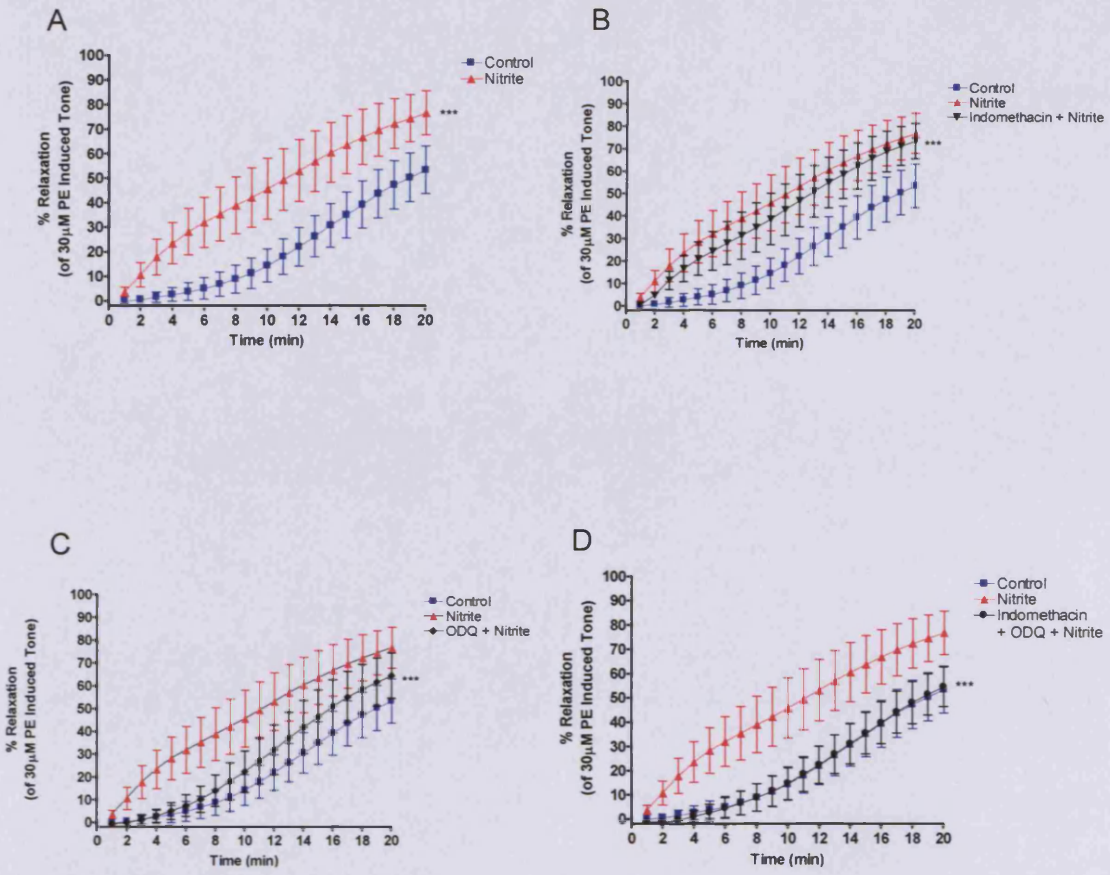

Figure 4.6. 20 minute relaxation profiles of endothelium intact aortic rings. A) Nitrite (10μM) produces a marked relaxation compared to control (***P<0.001). B) Partial inhibition of nitrite induced relaxation by Indomethacin (5μM). (***P<0.001) significantly different from nitrite alone over the course of the profile C) Substantial inhibition of nitrite induced relaxation by ODQ (10μM) (***P<0.001) compared to nitrite alone. D) Complete inhibition of nitrite induced relaxation by indomethacin (5μM) and ODQ (10μM) in combination (***P<0.001) compared to Nitrite alone. ODQ relaxation is significantly different from indomethacin and ODQ in combination (P<0.001). (n=5 in all cases).

At the twenty minute time point, nitrite-induced relaxation is partially inhibited by indomethacin, although the result is not significant (Figure 4.7). ODQ does produce a significant inhibition (P<0.001). However, it does not generate the complete inhibition seen in endothelium-denuded vessels (Figure 4.5). When both ODQ and indomethacin are used in combination, a complete inhibition of nitrite-induced vasodilatation is observed after twenty minutes.

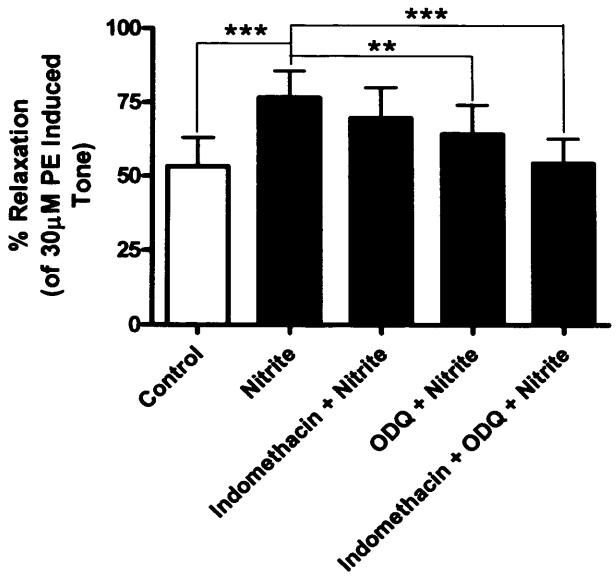

Figure 4.7. Maximum relaxation induced by nitrite (10μM) at the 20 minute time point in endothelium intact aortic rings. Nitrite produces a marked relaxation compared to control (***P<0.001) which is inhibited in part by both indomethacin (5μM) and ODQ (10μM) (P<0.01) respectively and completely inhibited by indomethacin (5μM) and ODQ (10μM) in combination (***P<0.001) compared to Nitrite alone. (n=5 in all cases).

4.3.3.3 Nitric Oxide Dependency and Sources of Nitrite Reduction

In light of previous research, it is likely that nitric oxide could represent a significant intermediate in the downstream actions of nitrite. Utilising a nitric oxide specific scavenger, (CPTIO), the relative contribution of nitric oxide was assessed. In endothelium intact vessels, the actions of nitrite did not appear to be solely dependent upon its reduction back to nitric oxide, incubation with CPTIO was capable of abolishing the nitrite-induced vascular response in part, but not completely (Figure 4.8). Interestingly, inhibition of soluble guanylate cyclase in combination with a nitric oxide specific scavenger was capable of increased inhibition compared to the individual agents alone, but was not able to produce a full inhibition (Figure 4.8).

Given that nitric oxide clearly plays a significant role in the mechanism of nitrite-induced vasorelaxation, sources of nitrite reduction were examined. Three tissue based enzymes, xanthine oxidase, aldehyde oxidase and nitric oxide synthase were considered, using specific pharmacological inhibitors. Aldehyde oxidase was found to be responsible for a significant amount of nitrite reduction, while inhibition of xanthine oxidase and nitric oxide synthase fail to have an effect (Figure 4.8). Interestingly, L-NMMA did not appear to alter the basal hypoxia induced relaxation in control tissue (data not shown).

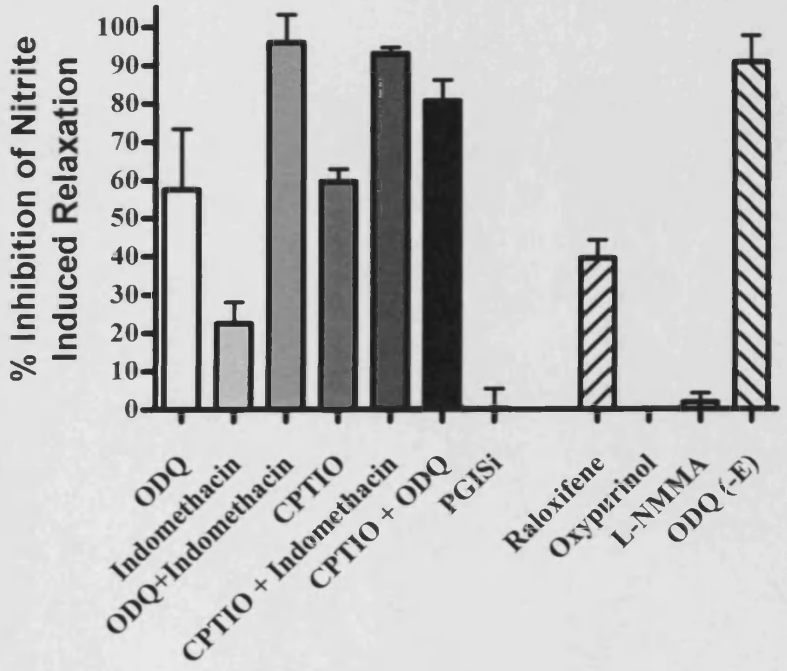

Figure 4.8. % inhibition of nitrite vasorelaxation in endothelial intact vessels. Inhibitor concentrations used, ODQ 10μM, CPTIO 1mM, Raloxifene 50nM, Oxypurinol 100μM, PGI synthase inhibitor 10μM, L-NMMA 300μM. (n=4 or 5).

4.3.4 The Effect of Acute Hypoxia/Nitrite Exposure upon Gene Expression

To investigate the wider actions of nitrite in the acute exposure setting, the effect of nitrite upon the expression of specific genes was assessed in normoxia and hypoxia. A thirty minute exposure to hypoxia increased relative expression levels of both HIF-1α and COX-2 ($P<0.001$) (Figure 4.9A and B). The expression of COX-2 was inhibited by nitrite in both normoxia and hypoxia, however, the study was not powered to confirm this statistically. The acute exposure to nitrite and a change in oxygen level did not alter the expression of endothelial nitric oxide synthase (Figure 4.9C).

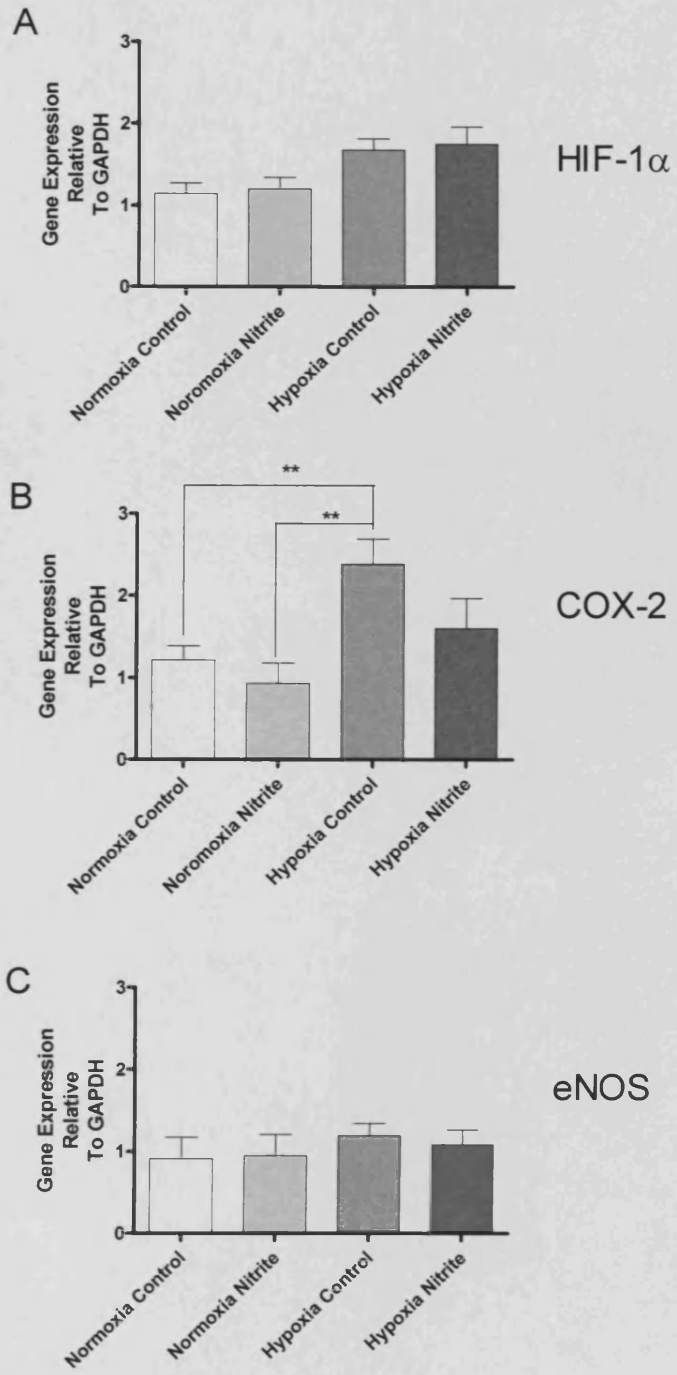

Figure 4.9. The effect of acute nitrite exposure upon specific gene expression. A) HIF-1α expression levels, exposure to hypoxia increases expression of HIF-1α, however the increase is not statistically significant. B) COX-2 expression levels, exposure to hypoxia significantly increased expression of COX-2 in the hypoxic control group ($P<0.001$ versus normoxia nitrite and normoxia control groups) but not in the hypoxia nitrite group. C) eNOS expression levels, no significant changes in expression between groups. n=4 in all cases.

4.4 Discussion

Nitrite is capable of exerting direct vasodilatory effects that are enhanced in the hypoxic vessel setting. An enhancement in activity of other nitric oxide donors under hypoxic conditions has previously been reported [91], however the mechanism behind the observed difference is unknown. It is possible that soluble guanylate cyclase (sGC) is more responsive to the same nitric oxide stimulus in hypoxia or that down stream signalling is perhaps more sensitive. In this respect, an experiment involving the measurement of cGMP production over a range of oxygen tensions after stimulation with known sGC activators could provide important information. The increased activity of nitrite in hypoxia represents an interesting and potentially exploitable aspect of its pharmacology, consequently, insight into the mechanistic background of this pharmacology is therefore of great value.

It has long been assumed that the vascular actions of nitrite in hypoxia were reliant upon the formation of nitric oxide as an intermediate, with stimulation of the classic downstream pathways. This chapter is the first to provide evidence that nitrite (under hypoxic conditions at physiological pH) is also capable of exerting physiological effects independently of a free nitric oxide intermediate and via the operation of multiple signalling pathways.

4.4.1 Component Pathways

The signalling pathways that are stimulated by nitrite in hypoxic vessels appear to be dependent upon the cell types present in the vessel. In endothelium denuded vessels, the actions of nitrite are largely, if not completely mediated via a mechanism involving sGC. In contrast, despite the same level of overall vasodilatory response to nitrite, the actions of nitrite in endothelium intact vessels are mediated via multiple pathways. In these vessels, nitrite at a pharmacological concentration operates via two distinct pathways, one controlled by nitric oxide/sGC and the other through COX,

although the effect appears to be predominantly signalled through the former. Figure 4.10 depicts a summary of the potential signalling mechanisms involved.

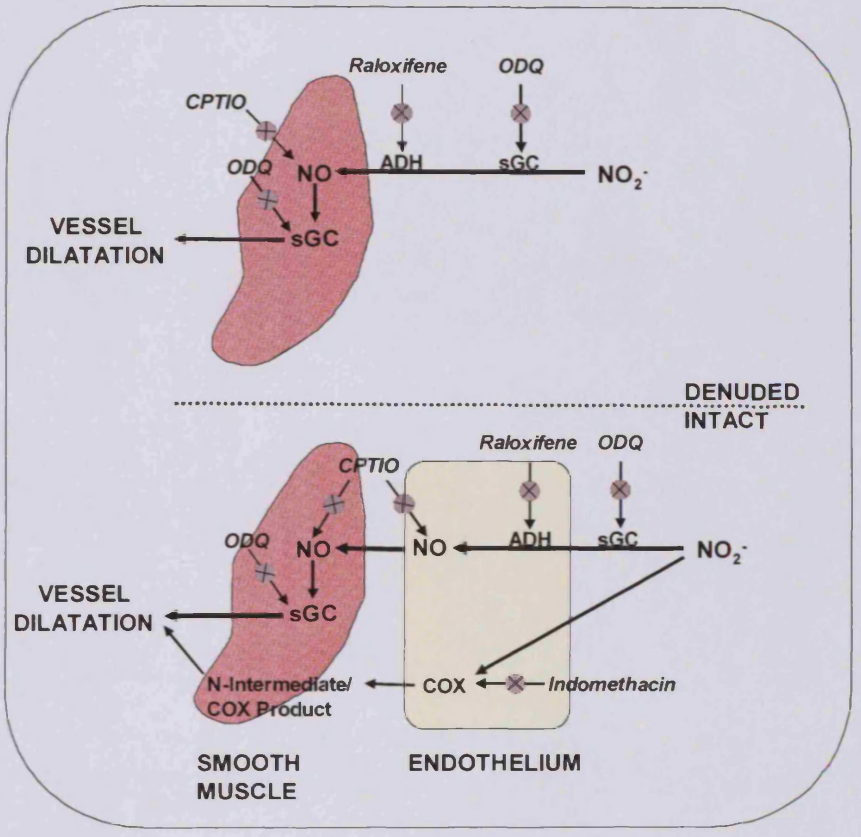

Figure 4.10. Potential pathways that control the nitrite mediated relaxation of hypoxic vessels. Upper section of diagram depicts denuded tissue where sGC appears to predominantly control relaxation. Lower section of diagram attempts to show possible pathways in endothelium intact vessels.

Having found that the COX pathway appears to operate independently of nitric oxide as an intermediate, this interesting aspect of the signalling was investigated further. COX is known to be essential for the generation of prostacyclin, a key COX product responsible for vasodilatation in the control of vessel tone (see section 1.7.2.4, page 38). To explore the relative contribution of prostacyclin as opposed other COX-derived eicosanoids, prostacyclin synthase, which occurs distally to COX in the prostaglandin biosynthetic pathway, was inhibited using a specific agent. Inhibition of

prostacyclin synthase did alter the vessel baseline tension, but failed to show an effect upon the nitrite-induced hypoxic vasodilatation. This suggests either a possible role for other COX-derived eicosanoids such as PGE2, also known to cause vasodilatation [410] or an enzymatic function of COX, possible involving the haem component of the enzyme, which could convert nitrite to an alterative bioactive nitrogen species. In relation, endothelial COX has been demonstrated to be capable of free radical species generation under hypoxic conditions [411]. Given that some COX products are vasoconstrictors, it is also possible that nitrite may have a negative effect upon COX that could cause an inhibition of constriction, as opposed to an enhancement of relaxation. Previous studies that have investigated nitrite vasodilatation have failed to recognize these differential signalling pathways, principally due to the pre-incubation of tissue with indomethacin and nitric oxide synthase inhibitors [396].

Although the nitrite-induced vasodilatation requires twenty minutes to reach completion, it is interesting to note the differences in profile between inhibition of sGC and COX over the full time course. The inhibition of COX by indomethacin appears to be uniform throughout the profile, whereas sGC inhibition (with ODQ) is appreciable in the first part of the profile, but appears to tail off over the twenty minute period. It is possible that a sGC mediated mechanism takes precedence during the initial part of the nitrite response, especially given the longevity of nitric oxide which is most likely the source of activation. The constant inhibition produced by COX inhibition is more suggestive of nitrite having a negative effect upon constriction as opposed to a positive effect upon relaxation. It would seem that these pathways could be mutually exclusive, however crosstalk between them cannot be ruled out. It has been demonstrated that nitric oxide can regulate COX, both increasing activity via a cGMP independent mechanism [170] or inhibiting activity. The effect of nitric oxide upon COX is also thought to be dependent upon the COX isoform in question, i.e. differential effects are observed with COX-1 compared to COX-2 [412]. In terms of these two nitrite stimulated pathways, it is important to remember that inhibition of one may simply cause up-regulation of another, making the relative contribution of each difficult to estimate. This difference in profile also reinforces the idea that nitrite may operate via

pathways that are separate both physically and with respect to time. However, irrespective of the different inhibition profiles, the combination of sGC and COX inhibition abolished the response to nitrite throughout the entire profile.

4.4.2 Nitric Oxide Dependency

Inhibition of sGC or nitric oxide scavenging (using CPTIO), individually or in combination, was not able to abolish the vascular response to nitrite in hypoxia. However, the combination of the two agents was additive, but the results did display more variability when compared to other agents. CPTIO is a competitive nitric oxide scavenger, therefore it is possible that some of the nitric oxide generated may not be scavenged. This nitric oxide does not appear to be acting through sGC and would need to be generated in very close proximity to the enzyme it activates. This idea could fit a model in which an enzyme acts as a source of nitrite reduction and is simultaneously stimulated by the generated nitric oxide. This mechanism could be operational in the case of COX, for example, where the haem group of the enzyme could both reduce nitrite and bind nitric oxide. It has been suggested that nitrite may be capable of generating species such as nitrosylated products without the requirement for free nitric oxide as an intermediate [393]. It is possible that these products may be responsible, in part, for the actions of nitrite in the *ex vivo* tissue model of this chapter, interestingly, the data would suggest that these products operate through or are generated by a COX mediated mechanism.

4.4.3 Sources of NO_2^- Reduction

In spite of the presence of different mechanistic pathways, nitric oxide is the signalling intermediate that appears to be largely responsible for the vascular actions of nitrite. Nitrite must be reduced to produce nitrite oxide and this process can occur via several routes in a biological setting, most notably via enzymatic reduction. Haemoglobin has been touted as an important nitrite reductase in the *in vivo* setting (see section 1.10.2 (page 68) and chapter 6), however the current chapter is concerned with

the direct actions of nitrite in vascular tissue and those mechanisms that are independent of haemoglobin.

The importance of tissue-based oxidoreductase enzymes in the vascular actions of nitrite has recently become apparent, with both xanthine oxidase and aldehyde oxidase being identified as key reductive enzymes [397]. For normal function, these oxidoreductase enzymes rely upon the presence of oxygen. In hypoxia, these enzymes are capable of reducing alternative substrates such as nitrite [397]. The results of this chapter confirm the involvement of aldehyde oxidase in nitrite-induced hypoxic vasodilatation, but fail to show a dependence upon xanthine oxidase. This is in agreement with published reports, showing a more significant role for aldehyde oxidase in nitrite reduction to nitric oxide at a tissue level [397]. However, a reductase role for xanthine oxidase cannot be completely discounted because although oxypurinol blocks the molybdenum site, the flavin site of the enzyme remains free and could be functionally active [413]. Other studies have suggested that endothelial nitric oxide synthase may also function as a reductase [398], however, the results of this chapter do not demonstrate a role for the enzyme in nitrite reduction. As is potentially the case with COX, it is possible that sGC may be acting as both a nitrite reductase and be stimulated by the subsequently produced nitric oxide. In respect to this point Alzawahra *et al* have shown that ODQ prevents the reduction of nitrite in vascular tissue, suggesting that sGC could behave as a potential reductase [400].

4.4.4 Vascular Gene Expression

In addition to its effects as a vasodilator, the results of this chapter demonstrate that nitrite has additional biological effects at the gene expression level. When aortic tissue was acutely exposed to hypoxia, the expected increase in HIF-1α expression level was observed (see section 1.2.1.4, page 5). However, the result did not reach statistical significance, which, could be attributable to the short hypoxic exposure time and the requirement for repeated testing. Interestingly, hypoxic exposure also resulted in a significant increase in COX-2 expression, which was blunted by the simultaneous

exposure to nitrite. However, because the study was under powered the effect of nitrite was not found to be significantly different. Nitrite was also able to cause a degree of inhibition on COX-2 expression under normoxic conditions, supporting the result observed in hypoxia.

Hypoxic exposure has previously been demonstrated to increase the expression of COX-2 in arterial smooth muscle cells [414]. Although, the effects of nitrite upon COX-2 expression have not previously been studied, studies with nitric oxide suggest that nitric oxide augments, rather than inhibits COX-2 expression [415]. This would suggest that the inhibitory effects of nitrite upon COX-2 expression observed here do not operate via a nitric oxide intermediate or that the aortic tissue utilised in this chapter responds differently to those previously studied. The majority of studies have focussed on gene regulation in inflammatory cells, rather than vascular cells, which are stimulated with nitric oxide at considerably higher concentrations than those that could be achieved with the concentration of nitrite used here. It is therefore possible that the effects of acute, lower nitrite concentrations on certain gene expression, may differ from those seen when relatively higher concentration of nitric oxide donors are utilised.

COX-2 is generally thought to be involved in processes that are pro-inflammatory [416]. The results presented here would suggest that nitrite could be acting as an anti-inflammatory agent in the hypoxic vasculature. This effect of nitrite would complement its actions as a vasodilator and anti-platelet agent [417], thereby enhancing its actions as an anti-inflammatory agent.

The final gene studied was endothelial nitric oxide synthase (eNOS). The thirty minute exposure to both hypoxia and/or nitrite did not significantly alter expression levels within aortic tissue measured. Previous studies have found that hypoxic exposure can increase eNOS mRNA expression in human umbilical vein endothelial cells (HUVECs). However, the hypoxic stimulus utilised was more chronic [418] than the one used here. It should also be remembered that the tissue sampled in the current model

was taken from the complete vessel and therefore, contained both smooth muscle and endothelial cells, with the former being the most abundant tissue present.

It is possible that nitrite may have the potential to protect hypoxic tissue areas via a number of mechanisms, above and beyond a simple increase in blood flow. Although the changes observed in gene expression are unlikely to have significant effects at a protein level during this acute exposure, the results of this study do suggest that even a short term exposure to increased nitrite levels, particularly in hypoxia, could impact upon the vasculature in the long term.

4.4.5 Implications for *in vivo* Conditions

The findings of this chapter are directly applicable to conditions in which plasma nitrite is elevated pharmacologically or by diet. A common misconception in the literature is the extrapolation from studies that are performed by infusing nitrite at elevated doses to nitrite as a circulating store of nitric oxide under basal physiological conditions (200~400 nM nitrite). It must be recognised, therefore, that the component mechanisms of nitrite-induced relaxation at higher doses may not be operational at normal levels, and this is difficult to confirm *in vitro* and *in vivo* largely because of assay limitations. Given the interest in nitrite as a therapeutic agent, the mechanistic findings of this chapter are of importance. The experimental model utilised is haemoglobin independent and demonstrates that nitrite alone directly induces vessel relaxation. However, these results cannot discount a role for deoxyhaemoglobin *in vivo*; indeed, it is possible that blood-borne nitric oxide species derived from nitrite during an infusion could contribute to the modulation of long-term vessel tone [310].

Although a relatively weak vasodilator compared to pharmacological nitrodilators, the fact that nitrite clearly dilates hypoxic vessels preferentially implies it could act as a targeted vasodilator without causing global changes in haemodynamics (e.g. the fall in blood pressure seen with nitrodilators). The results of this chapter provide novel findings regarding the vasodilator mechanisms of nitrite that now need to

be confirmed *in vivo* following nitrite infusion. It is clear that nitrite has the potential to alter vessel tone, however the fact that it can also alter signalling at a genetic level after an acute exposure suggests it may have the potential to impact on the overall health of the vessel in addition to affecting vascular tone.

4.5 Summary and Conclusions

The results of this chapter demonstrate that nitrite has a varied and complex pharmacology, with the ability to influence vascular physiology via different mechanisms and within different time frames. The effects of nitrite are clearly oxygen dependent, hypoxia appears to increase both the activation of nitrite but also the sensitivity of vascular tissue to the nitrite derived products that are formed. Specifically this chapter has demonstrated that:

- The vasodilatory properties of nitrite are enhanced in hypoxic vascular tissue

- Nitrite-mediated relaxation proceeds via different mechanisms depending upon the presence or absence of endothelium. In denuded vessels, a soluble guanylate cyclase-mediated mechanism prevails, while in intact vessels this mechanism is complemented by an alternative mechanism operating via cyclooxygenase.

- The actions of nitrite are in part mediated via a nitric oxide intermediate. To form nitric oxide, nitrite must be reduced and the current model would suggest that the enzyme, aldehyde oxidase is primarily responsible.

- An acute exposure to hypoxia is capable of altering gene expression. Specifically, nitrite has the potential to able to inhibit the hypoxia induced increase in COX-2 expression but requires further investigation.

5 Erythrocyte Induced Vasorelaxation: the Influence of Oxygen

5.1 Introduction

The phenomenon of whole erythrocytes producing vasorelaxation under hypoxic conditions represents an interesting field of research that has been investigated for a number of years, but the vasorelaxant species and mechanism behind its actions are yet to be confirmed. Over this time, a number of different mechanistic theories to explain this phenomenon have been published and the area still represents a matter of contention. A background to the three most prominent suggestions and current understanding will be covered below.

5.1.1 The HbSNO Theory

The Stamler group had previously demonstrated the vasoactive properties of nitric oxide groups attached to proteins via cysteine residues e.g. albumin-SNO [266, 324]. The freely reactive nature of the cysteine residue in the 93 position of the β-haemoglobin chain (not compromised by steric hindrance) and the fact that its reactivity was modulated by haemoglobin allostery had been known for nearly 5 decades [330, 419]. However, it was not until 1996 that the Stamler laboratory suggested that nitric oxide bound to the β93 cysteine residue of haemoglobin may act as a functional regulator of vessel tone and oxygen delivery [325]. The finding that arterial to venous gradients in HbSNO and HbNO levels existed, gave rise to a novel mechanism for HbSNO cycling [332]. Stamler *et al* proposed that nitric oxide that reacted with partially oxygenated haemoglobin (forming HbNO) in the venous circuit was displaced upon re-oxygenation in the lung where it was likely to react with proximity thiols, forming HbSNO. In the tissue, the reverse process is thought to occur, as haemoglobin is deoxygenated, nitric oxide from HbSNO (thought to be less stable on T-state haemoglobin [420]) is released. The released nitric oxide is believed to leave the

erythrocyte through a series of transnitrosation reactions, passing from one protein to another, AE1 is thought to represent the principal protein involved [331]. AE1 contains available cysteine residues and is located perfectly in the membrane to interact with extracellular proteins and with haemoglobin through an intracellular binding site. Given that deoxyhaemoglobin is know to preferentially bind AE1 [387] it suggests that nitric oxide from HbSNO could transnitrosate residues of AE1 upon deoxygenation. This transfer method would protect nitric oxide from being re-captured by haemoglobin, allowing it to not only leave that individual erythrocyte but to also preserve nitric oxide bioactivity in blood. In support of this mechanism, a number of publications have shown extracellular thiols to potentiate erythrocyte induced relaxation [325, 421]. In addition, the Doctor laboratory have also demonstrated that when isolated erythrocytes are deoxygenated in the presence of extracellular N-acetylcysteine (NAC), NAC-SNO levels increase in proportion to desaturation [422].

In contrast, there is also evidence which disputes a role for HbSNO in erythrocyte-mediated vasorelaxation. The first publication relates to the formation of HbSNO, suggesting that oxygenation of partially nitrosylated haemoglobin (HbNO) does not alter HbNO levels and so cannot be forming HbSNO [423]. However, this data only disagrees with the HbSNO cycle theory and does not exclude a role for HbSNO in the regulation of vascular tone. Perhaps the most significant evidence to date was that published by the Patel laboratory using a β-cysteine 93 amino acid substituted mouse model [424]. The data demonstrate that erythrocytes from mice producing human haemoglobin with the β-cysteine 93 substituted for an alanine residue can still induce a hypoxic vasodilatation. This publication also shows that the vasorelaxant response to erythrocytes, both native and alanine mutants, can be inhibited with L-NMMA (eNOS inhibitor). However, there are a number of caveats to this work. Despite substitution of the β-cysteine 93 residue, the overall erythrocyte SNO content remained unchanged, suggesting that other nitrosothiols (e.g. GSNO or membrane RSNOs) could still be responsible for the observed vasoactivity. The investigators also utilise an exercise stress test to investigate their mouse model. In this respect it should be appreciated that exercise *per se* is not a model of hypoxia, in exercise the increased oxygen demand of

vessels which exposed to hypoxia i.e. it constricts instead of dilating [423].

Although, the relationship between HbSNO content and haemoglob conformation [363] and the necessity of low tissue oxygenation for erythrocy vasodilatation [331] have been shown, the role of haemoglobin conformation in th release of a vasoactive moiety has not been fully explored.

5.1.2 The Haemoglobin Nitrite Reduction Theory

The second proposed mechanism involves the reduction of nitrite b haemoglobin under hypoxic conditions. There have been a number of publications this area, largely from the Gladwin and Patel laboratories [337, 342, 426], all of whic essentially propose the same mechanism. Early publications demonstrate that th relaxant properties of nitrite are enhanced in the presence of erythrocytes c haemoglobin and that deoxygenation of haemoglobin enhances its ability to reduc nitrite to free nitric oxide [337]. However, the demonstration of enhanced relaxation b erythrocytes and nitrite in combination, reported in the original work by Cosby *et al*, yet to be repeated. As time has passed, more recent research papers from this grou paint a slightly different picture. Firstly, published results do not show an enhancemer by haemoglobin but rather a lack of inhibition (haemoglobin usually scavenging nitri oxide to inhibit relaxation rather than augmenting it), with relaxation identical unde hypoxia regardless of the presence of haemoglobin [426]. Secondly, later publication have completely shifted the focus away from *in vitro* vessel work and have been largel based around speculative *in vivo* mechanisms, where haemoglobin is thought to ac maximally as a nitrite reductase around the p50 of haemoglobin, see section 1.10. (page 68). As a suggested mechanism of erythrocyte-induced hypoxic relaxation, th nitrite reduction theory remains heavily questioned, especially given that nitrite-derive

relaxations occur over a much longer time frame than the transient relaxation observed from native erythrocytes (minutes vs. seconds) and that free nitric oxide is unlikely to escape re-capture by haemoglobin [343]. Several mechanisms for the escape of free nitric oxide (produced from haemoglobin nitrite reduction) from the erythrocyte have been put forward, including the suggestion that intermediates such as dinitrogen trioxide (N_2O_3) could form SNO compounds [424, 427] or as a result of changes in haemoglobin reactivity/membrane association [428].

One idea that has linked elements of this hypothesis to the HbSNO hypothesis is the observation that haemoglobin can act as an HbSNO synthase, utilising nitrite as a substrate [310]. However, although it is feasible that this may occur in the circulation where haemoglobin allostery is constantly changing, it is unlikely to work in an isolated system and still ultimately suggests that an SNO is responsible.

5.1.3 The Adenosine Triphosphate Theory

The final mechanism is one that involves adenosine triphosphate (ATP) and, despite receiving less attention than the previously described mechanisms, is the longest standing of the three. It was initially shown by Bergfeld and Forrester that, under hypoxia/hypercapnia, erythrocytes would release ATP [429]. Dietrich *et al* developed this observation into a mechanism in which erythrocytes are thought to act as oxygen sensors and affect vessel tone to meet oxygen demand [430]. It is believed that when erythrocytes encounter an area of low oxygen, they off load both oxygen and ATP. The ATP is then thought to stimulate P2Y receptors on the endothelium and stimulate the production of nitric oxide and prostacyclin [431]. The theory has been tested in isolated vessel perfusion models, where it was found that when erythrocytes were introduced into hypoxic vessels, both changes in vessel diameter and increases in ATP were observed [432, 433].

While it is plausible that this mechanism could play a role *in vivo*, there is evidence that does not support it as a mechanism of erythrocyte-induced hypoxic

vasodilatation. Principally, it would seem that this mechanism is reliant upon the endothelium to produce vasorelaxant substances and the erythrocyte mediated vasodilatation is known to proceed in the absence of endothelium/nitric oxide synthase [421].

5.1.4 Current Understanding

Although the exact identity of the vasorelaxant released from erythrocytes and the mechanism by which it is released remain to be elucidated, there are a number of known observations (many published from our laboratory) that should be considered with respect to the results of this chapter. They are:

- Low tissue oxygenation appears to be essential for erythrocyte induced vasorelaxation [331].

- Erythrocyte addition to hypoxic tissue produces a transient relaxation that is followed by a constriction [91].

- The mechanism of vasorelaxation is mediated via soluble guanylate cyclase and is inhibited by ODQ [91, 421].

- The vasodilatation mechanism is independent of both the endothelium and nitric oxide synthases, demonstrated by denudation and the use of L-NMMA/genetic elimination [91, 421].

- The vasorelaxant response of erythrocytes from diabetic patients is impaired, most likely due to haemoglobin glycation [91].

5.1.5 Aims

The true identity of the vasorelaxant released from erythrocytes remains unknown, however, investigating the mechanisms which govern its movement and release could be essential in identifying the molecule responsible. The work of this chapter was undertaken to perform these investigations and specifically aimed to:

- Assess the haemoglobin allostery dependence of the erythrocyte mediated hypoxic vasorelaxation.

- Gain understanding with regard to the transport and consumption of the relaxant entity.

- Consider novel vasorelaxant species that may be responsible.

- Provide evidence that may aid in identifying the exact nature of the relaxant entity.

5.2 Specific Protocols

5.2.1 Myography

The myography system was set up daily as per section 2.5 (page 104). All erythrocyte experiments were carried out under the hypoxic protocol at ~1% oxygen (see section 2.5.2, page 105). To induce relaxation, 20µl of prepared erythrocytes were injected into the bath at peak constriction using gel loading pipette tips for efficient delivery.

5.2.2 Blood Preparation

For all protocols, fresh blood samples were collected by venepucture of the antecubital vein from healthy subjects immediately prior to requirement. Samples were centrifuged (1200*g*, 5mins, 4°C) and the plasma and buffy coat were removed as per section 2.1 (page 80).

5.2.3 Erythrocyte Manipulation

Erythrocytes were diluted (approx. 1 in 10 to give 3g/dL haemoglobin) in isotonic saline (0.9% w/v, Fresenius Kabi) before loading into the thin film rotating tonometer (Section 2.2, page 81). Different gas mixtures were then utilised to obtain samples over a range of haemoglobin oxygen saturations for subsequent testing. A sub group of samples were subjected to oxygen cycling to assess the effect of repeat oxygenation on relaxation (protocol shown in Figure 5.1). To identify whether the vasorelaxant moiety was simply passed between the erythrocyte and the extracellular milieu upon de-oxygenation and re-oxygenation, erythrocyte samples taken to low saturation had the extracellular milieu removed prior to re-oxygenation by repeated washing with fresh oxygenated saline, saturation and haemoglobin content were then reconfirmed (Figure 5.1). All blood gas analysis was carried out using a blood gas

analyser (OSM 3 Hemoximer, Radiometer, Copenhagen). All samples underwent a final centrifugation step to remove saline before addition to the tissue bath.

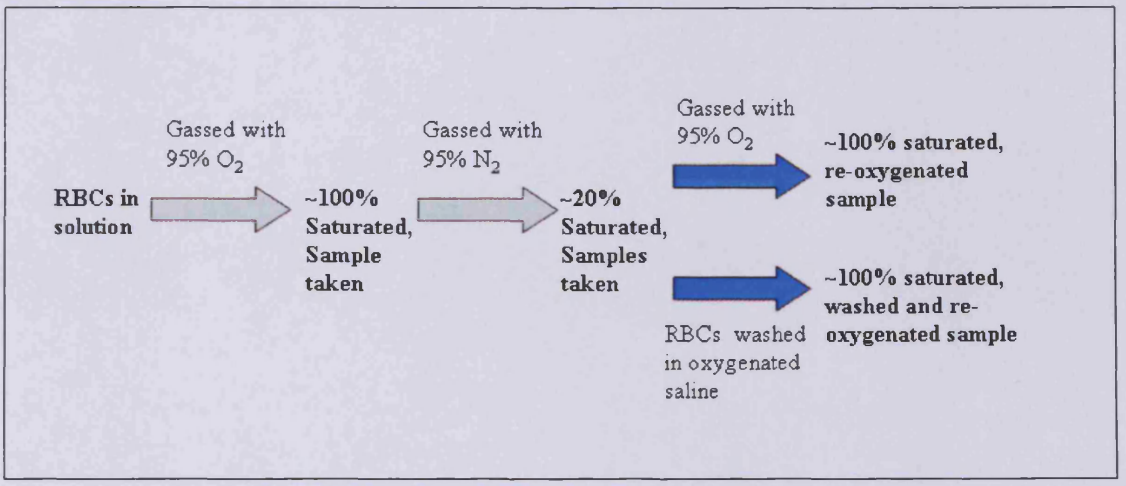

Figure 5.1. A schematic representation of erythrocyte haemoglobin oxygen saturation cycling and the samples generated for vasoactivity assessment.

5.2.4 2,3-DPG

2,3-Diphosphoglycerate (2,3-DPG or 2,3-BPG) is an organic phosphate intermediate formed during glycolysis. The intraerythrocytic 2,3-DPG concentration is approximately equal to the molar concentration of tetrameric haemoglobin (5mM)[83], although the amount of free 2,3-DPG is dependent on haemoglobin saturation and can fall as low as 0.5mM in deoxygenated erythrocytes [84]. To assess the effect of 2,3-DPG on the erythrocyte mediated hypoxic dilatation, 2,3-DPG was added to the bath (prior to commencing the hypoxic protocol) at a final concentration of 20μM, equimolar to the final haemoglobin concentration upon erythrocyte addition to the bath (average haemoglobin concentration =32.73g/dl ± 0.79 equivalent to 20.2μM haemoglobin in bath concentration), mimicking the *in vivo* ratio. In contrast to previous experiments, the erythrocyte samples were lysed before addition to allow 2,3-DPG to interact with the haemoglobin (confirmed by centrifugation). All erythrocyte samples were adjusted to ~100% haemoglobin saturation before addition to the bath.

5.2.5 Carbon Monoxide

An initial assessment of the vasorelaxant properties of carbon monoxide (CO) in hypoxia and normoxia was made using the carbon monoxide releasing molecule, CORM-A1 ($Na_2[H_3BCO_2]$, t1/2 = 21 minutes, [434]), a kind gift from Dr Roberto Motterlini, Northwick Park Institute for Medical Research, London. CORM-A1 was added to the appropriate tissue baths (not in the presence of erythrocytes) at a final concentration of 100μM, although this concentration appears relatively high, it compensates for CORM-A1 being a relatively slow CO releasing molecule. Maximum relaxation in this experiment was recorded at the 5 minute time point.

To investigate the role of carboxyhaemoglobin (HbCO), erythrocytes were loaded into the thin film rotation tonometer as previously described (section 2.2, page 81), deoxygenated to desired level and exposed to a 90% carbon monoxide gas mixture to achieve a range of haemoglobin carbon monoxide saturation levels. Vasoreactivity of erythrocyte samples was then evaluated as per section 5.2.1 (page 176).

5.2.6 Oxygen

In addition to nitric oxide and carbon monoxide, oxygen is also a diatomic gas that could interact in a similar way with tissue and can also be bound to haemoglobin in abundance. To test the actions of oxygen independently of haemoglobin upon hypoxic vascular rings, aliquots of saline (0.9% w/v) containing different concentrations of oxygen were introduced to pre-constricted hypoxic tissue rings (see section 2.5.2, page 105). Saline was gassed for 30 minutes with 95% oxygen, 21% oxygen (equivalent to atmospheric levels) or nitrogen to produce three different oxygen content samples. 100μl of each sample was introduced to separate tissue baths, an increased volume was used to attempt to adjust the amount of oxygen added to the bath to within the range of to that contained within a 20μl sample of erythrocytes. To assess potential pathways involved, a subsection of tissue was pre-incubated with ODQ (10μM final) to inhibit soluble guanylate cyclase before the addition of 100μl saline gassed with 95% oxygen.

5.2.7 Analysis and Statistical Analysis

Erythrocyte-induced relaxation was calculated by taking the actual maximum relaxation and expressing it as a percentage of the maximum phenylephrine induced constriction (Figure 5.2). Similarly, erythrocyte induced constriction was calculated by taking the actual maximum constriction (with respect to the maximum phenylephrine induced constriction) and expressing it as a percentage of the maximum phenylephrine induced constriction (Figure 5.2).

Testing between groups was performed using either the student t-test (either paired or unpaired where appropriate) or an analysis of variance (ANOVA) with an appropriate *post hoc* test. To assess correlations, the Pearson's correlation coefficient was used. Data are presented as means and error bars represent the standard error of the mean (SEM). A P value of less than 0.05 was taken to be significant, individual significance levels are indicated within the figure legends. Generally, n=1 represents data averaged from a matched pair of aortic rings.

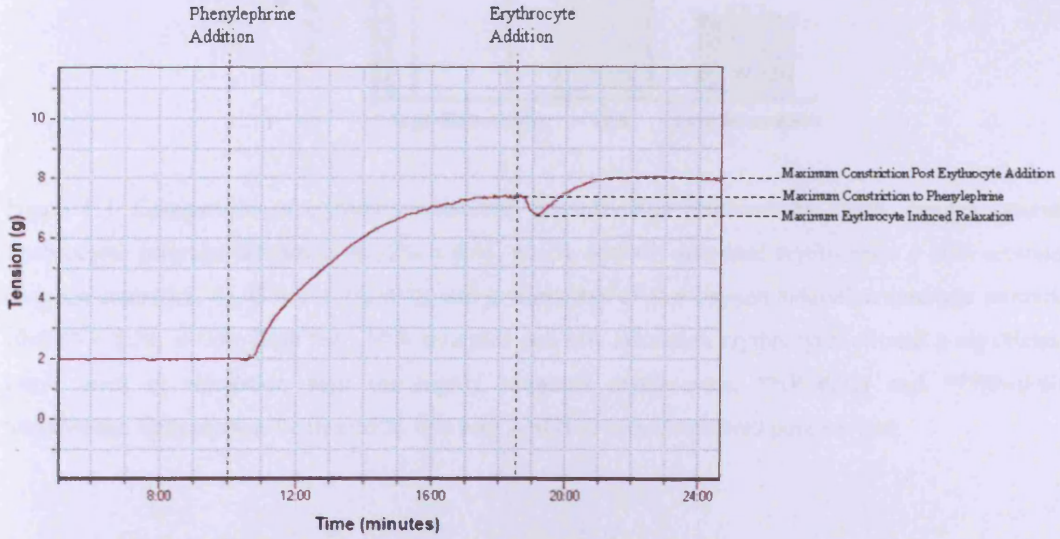

Figure 5.2. A representative raw trace of erythrocyte induced vasorelaxation after erythrocyte addition to pre-constricted hypoxic tissue. Significant time points and tension changes are appropriately marked.

5.3 Results

5.3.1 The Effect of Haemoglobin Allostery on Erythrocyte Induced Vasorelaxation

To establish the dependency of the erythrocyte induced vasorelaxation upon haemoglobin allostery, the vasorelaxant properties of a number of erythrocyte samples with varying haemoglobin oxygen saturations were tested. Erythrocytes containing haemoglobin held in the R-state by oxygen bound to all haem groups (~100% HbO_2, high saturation) elicited significantly more relaxation than partially oxygenated samples (a mixture of R and T-state haemoglobin) that were ~50% and ~20% saturated (low saturation) (Figure 5.3).

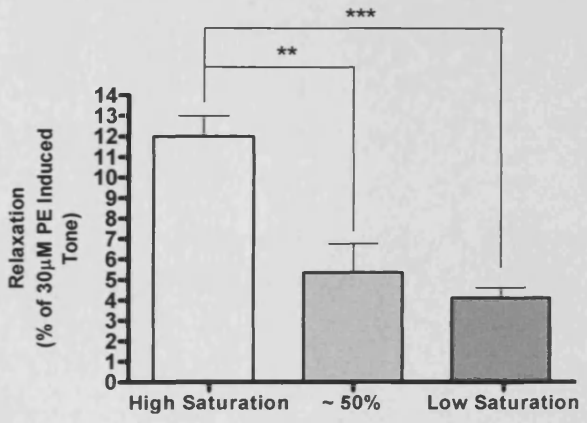

Figure 5.3. Comparison of erythrocyte induced vasorelaxation produced by highly oxygen saturated erythrocytes (average saturation 98.22% ± 0.45, n=13), partially saturated erythrocytes (~50% saturated) (average saturation 51.43% ± 6.16, n=4) and erythrocytes of low oxygen saturation (average saturation 20.40% ± 5.28, n=13). Both the ~50% saturated and low saturation erythrocytes elicited a significantly lower level of relaxation than the highly saturated erythrocytes, **($P<0.01$) and ***($P<0.001$) respectively. Groups were compared by one way ANOVA with Bonferroni post *hoc* test.

When a range of maximum erythrocyte induced relaxation data is correlated against the corresponding haemoglobin oxygen saturation, a positive linear relationship is observed (Figure 5.4). This observation directly links haemoglobin allostery (controlled by oxygen saturation in this case) to the maximum erythrocyte induced relaxation.

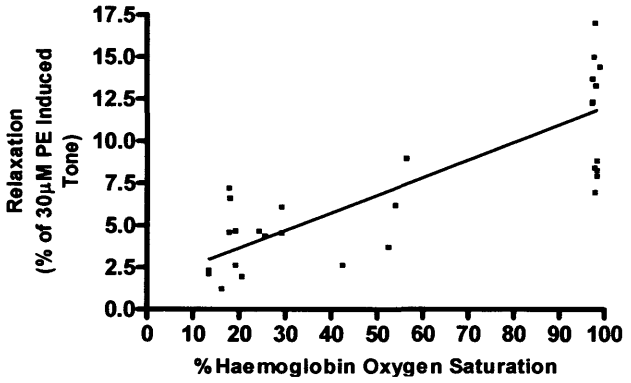

Figure 5.4. Correlation of erythrocyte induced vasorelaxation vs. % erythrocyte haemoglobin oxygen saturation. A significant positive correlation (Pearson's correlation coefficient $r=0.82$, $p<0.0001$) exists between the two variables. ($n=30$, $R^2= 0.66$).

5.3.2 The Effect of Haemoglobin Oxygen Saturation Cycling on the Erythrocyte Induced Relaxation

To ascertain whether or not the same erythrocyte sample could achieve repeat relaxations a preliminary experiment utilising fully oxygenated erythrocytes was performed (Figure 5.5). Erythrocytes were first added to one hypoxic bath to induce relaxation (oxygenated erythrocytes) then, because of the isotonic nature of the Krebs' buffer these cells remained whole and so were recovered, re-oxygenated and introduced into a second bath to asses repeatability of relaxation (repeat oxygenated erythrocytes). Although this preliminary work demonstrated that the same erythrocyte sample could perform repeat relaxations it was technically difficult to control so a tonometer based cycling protocol was developed to essentially achieve the conditions.

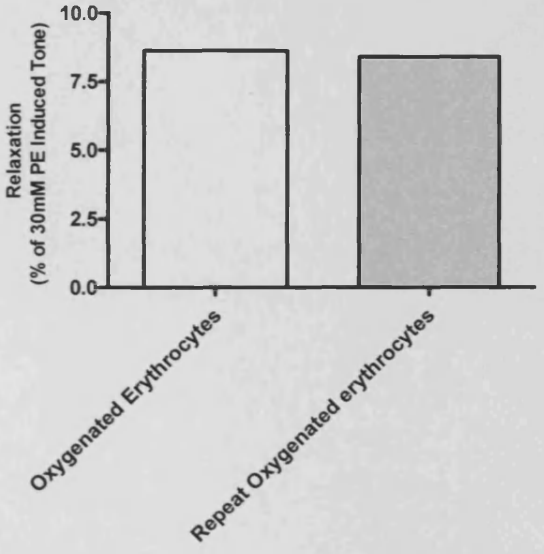

Figure 5.5. Preliminary experiment where erythrocytes were utilised to induce relaxation (oxygenated erythrocytes) before being recovered, re-oxygenated and used to perform a repeat relaxation (repeat oxygenated erythrocytes). n=2.

The cycling of haemoglobin oxygenation (oxy to deoxy, thought to enhance vasorelaxant offloading, followed by a further deoxy to oxy cycle before addition to bath) using the tonometer protocol did alter the maximum relaxation (a mean relaxation of 12.00% ± 3.6 vs. 8.28% ± 2.3) induced by erythrocytes (Figure 5.6).

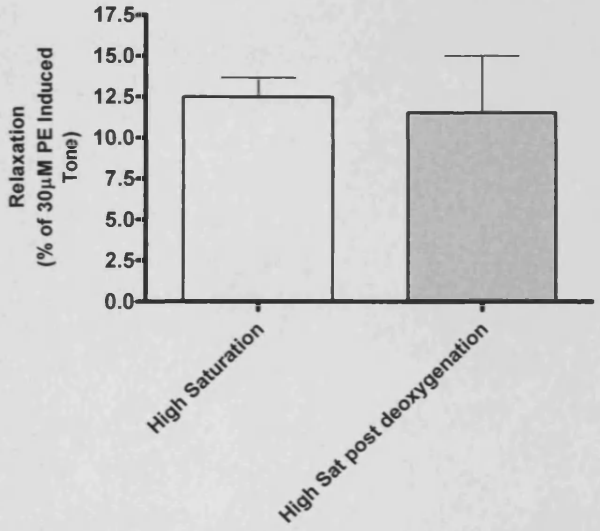

Figure 5.6. The effect of haemoglobin oxygenation cycling. Erythrocyte induced vasorelaxation produced by highly oxygen saturated red cells (average saturation 98.22% ± 0.45, n=15) compared to highly saturated red cells which were previously taken down to a deoxygenated saturation of ~ 20% (20.68% ± 1.66) before being re-oxygenated (average saturation 97.70% ± 0.39, n=5). No significant difference exists between the two groups.

5.3.3 Potential Cross-Membrane Transfer of the Vasorelaxant

The ability of the erythrocyte to produce repeated relaxation prompted the investigation of the movement or recycling of the vasorelaxant in and out of the cell. Deoxygenation could simply release the vasorelaxant out of the cell, which is subsequently taken back up upon re-oxygenation. To explore this potential mechanism, erythrocytes were deoxygenated to a low saturation upon which the extracellular milieu was removed before re-oxygenation in fresh saline. Erythrocytes that were deoxygenated, followed by removal of the extracellular milieu and re-oxygenation induced a mean relaxation of 13.0% ± 1.9 compared to erythrocytes taken immediately at high saturation 12.0% ± 3.6 (Figure 5.7). The removal of the initial extracellular milieu and replenishment with fresh saline had no statistically significant effect on erythrocyte-induced vasorelaxation.

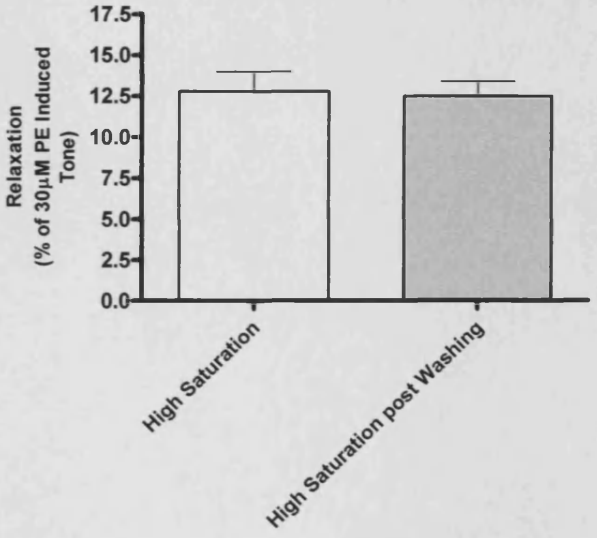

Figure 5.7. Potential movement of the vasorelaxant. Erythrocyte induced vasorelaxations produced by highly saturated red cells (average saturation 98.2% ± 0.5, n=13) compared to highly saturated red cells which were previously taken down to a deoxygenated saturation of ~ 20% (22.4% ± 1.7) then centrifuged at 1200*g* before the extracellular solution was removed, erythrocytes were then washed 3 times in oxygenated saline to increase the haemoglobin oxygen saturation up to ~100% (average saturation 98.05% ± 0.65, n=4). No significant difference exists between the two groups.

5.3.4 The Effect of 2,3-DPG

To further investigate the role of haemoglobin allostery in the erythrocyte induced vasorelaxation, the effect that the allosteric modulator 2,3-DPG had (see section 1.4.2.4, page 18) upon maximum relaxation was considered. Because the access of 2,3-DPG to whole cells is limited, lysed erythrocyte samples had to be utilised. However, this did not appear to alter the ability of the sample to induce vasorelaxation. The addition of 2,3-DPG to the bath before erythrocyte addition significantly increased the maximum relaxation achieved when compared to the respective control group (Figure 5.8).

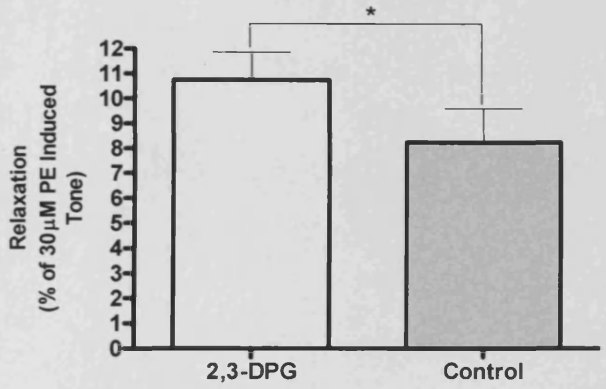

Figure 5.8. The effect of 2,3-DPG on the erythrocyte induced vasorelaxation. Lysed erythrocytes were added to either control baths or baths containing 20µM 2,3-DPG. The maximum vasorelaxation generated by erythrocytes in the presence of 2,3-DPG was significantly *(P= 0.03) higher than the control group. Average haemoglobin saturation (96.9% ± 0.9). n=4.

One aspect of the erythrocyte vasorelaxant response that has received less attention is the vasoconstriction that is observed post relaxation. The magnitude of this post relaxation vasoconstriction was assessed in the 2,3-DPG treat group vs. the control group and was found to be higher in the 2,3-DPG group although the difference was not statistically significant (Figure 5.9).

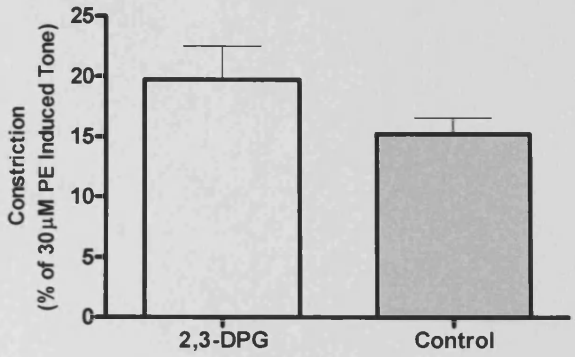

Figure 5.9. The effect of 2,3-DPG on the erythrocyte induced vasoconstriction. Lysed erythrocytes were added to either control baths or baths containing 20µM 2,3-DPG. The maximum vasoconstriction generated (post relaxation) by erythrocytes in the presence of 2,3-DPG was higher than the control group, although this difference was not found to be statistically significant. Average haemoglobin saturation (96.28% ± 0.933). n=4.

5.3.5 Carbon Monoxide

The identity of the vasorelaxant released from erythrocytes that is responsible for inducing the vascular activity, despite several proposed candidates, remains to be confirmed. In this respect it is important to consider other possible candidates, in the case of this chapter, carbon monoxide. Carbon monoxide, released from the carbon monoxide releasing compound, CORM-A1 was capable of inducing a significant relaxation in hypoxic tissue compared to both the hypoxic control and the normoxic CORM-A1 treated counterpart (Figure 5.10).

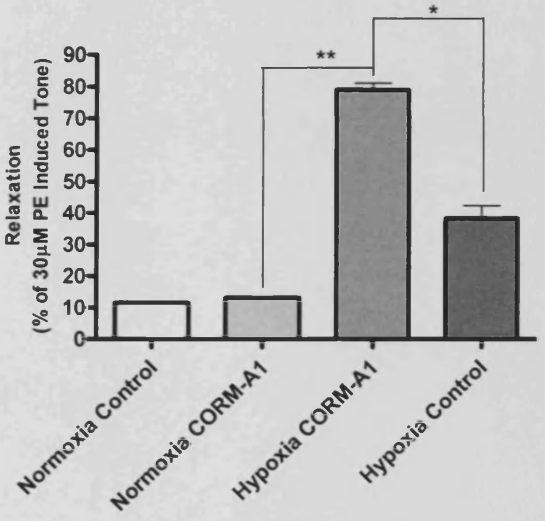

Figure 5.10. Carbon monoxide induced vasorelaxation in hypoxia and normoxia after 5 minutes exposure to 100μM carbon monoxide releasing molecule, CORM-A1. CORM-A1 induced significantly more relaxation in hypoxia compared to normoxia ** (P=0.001) and compared to the relevant hypoxia control *(P=0.012). n=4.

In preliminary experiments, a stream of high concentration carbon monoxide gas was utilised to generate erythrocytes containing 100% HbCO to assess the vasorelaxant properties of HbCO. 100% HbCO was found to completely inhibit the ability of the erythrocyte to vasodilate hypoxic tissue (data not shown). Given the observed allosteric control of this phenomenon, erythrocytes partially saturated with HbCO (~22% HbCO) were generated and assessed. The partial saturation of erythrocytes with carbon monoxide reduced the maximum vasorelaxation achieved, however, due to the power of the study the result was not found to be statistically significant (Figure 5.11).

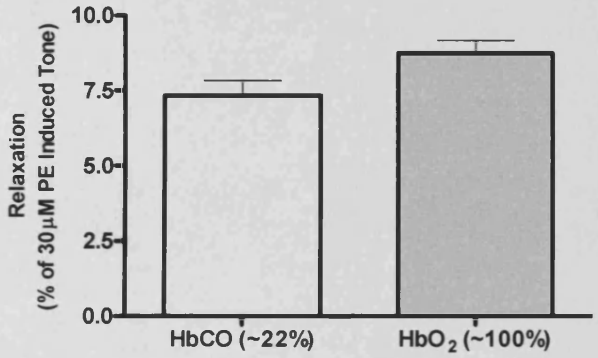

Figure 5.11. The effect of partial carbon monoxide saturation upon the erythrocyte induced vasorelaxation. Partially saturating the haemoglobin of erythrocytes with carbon monoxide (22.00% HbCO, 78% HbO_2) reduced vasorelaxation when compared to erythrocytes with fully oxygenated haemoglobin (97.75%), however the study was not powered to show this difference statistically. Post relaxation erythrocytes partially saturated with carbon monoxide where recovered and re-tested for HbCO levels and still exhibited 22.0% saturation. n=4.

5.3.6 Oxygen

As a potential candidate for the vasorelaxant released from erythrocytes, the vasorelaxant properties of oxygen was assessed on hypoxic tissue, independently of haemoglobin. The addition of oxygenated saline (gassed with 95% oxygen) was capable of inducing a significant relaxation (figure 5.12A, $P<0.001$) that is comparable to the relaxation induced by oxygenated, native erythrocytes (Figure 5.13). The degree of relaxation observed from saline addition was directly proportional to the oxygen content of sample (Figure 5.12B). Pre-incubation of the vascular tissue with ODQ completely inhibited the relaxation response to oxygenated saline (gassed with 95% oxygen) but was unable to inhibit the post-vasorelaxation vasoconstriction as seen with native erythrocyte samples.

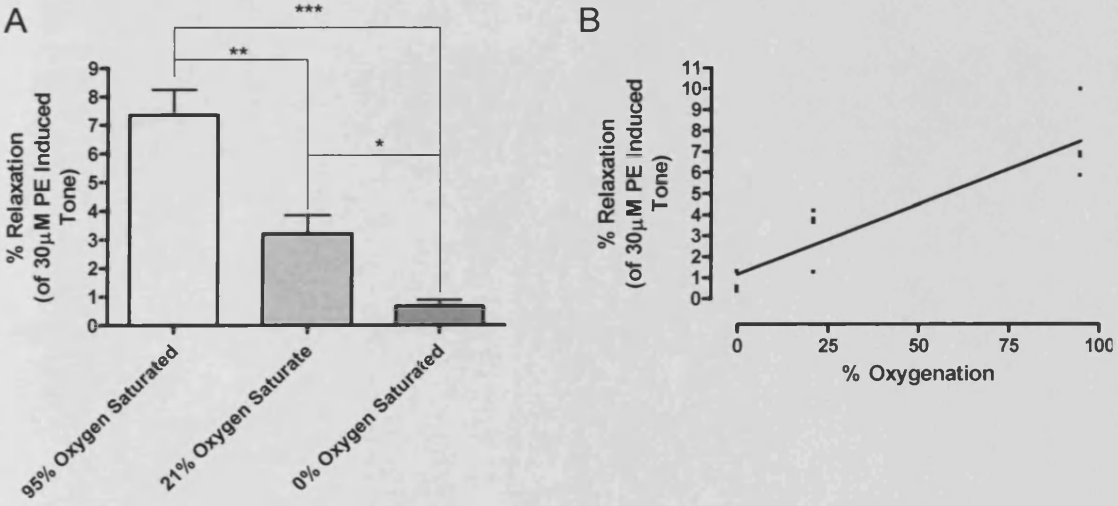

Figure 5.12. % relaxation induced by saline samples with varying oxygen content. A) 95% oxygen saturated saline induced significantly more relaxation than samples gassed with 21% or 0% oxygen ($P<0.01$ and $P<0.001$, respectively). n=4 B) A correlation of relaxation against % oxygenation of saline samples added. The two variables correlate significantly and positively ($P<0.0001$, Pearson's r value=0.91, n= 12 pairs).

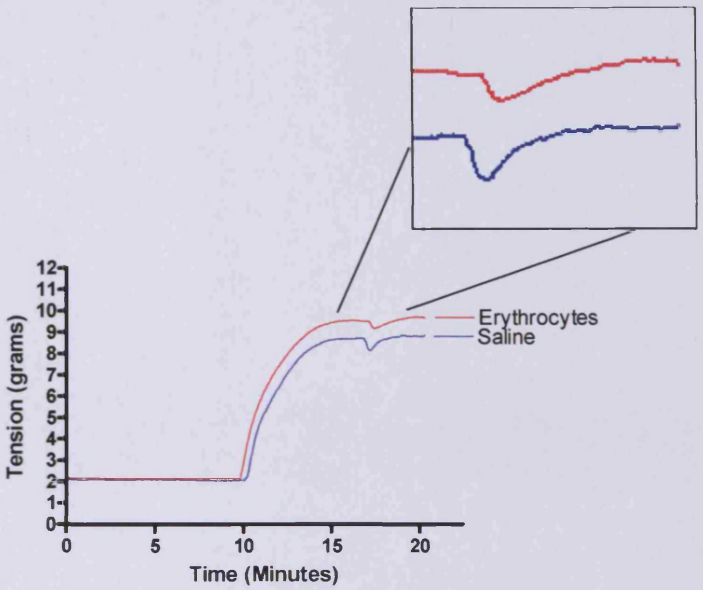

Figure 5.13. example traces taken from experiments where either 20µl of ~100% oxygen saturated erythrocytes or 100µl of saline gassed with 95% oxygen were added to pre-constricted hypoxic vascular rings.

5.3.7 Vasorelaxation vs. Vasoconstriction

The addition of erythrocytes to the hypoxic tissue bath initially induces vasorelaxation that is directly followed by vasoconstriction (Figure 5.2). To consider the relationship between these two events, the maximum percentage vasorelaxation induced by a sample was correlated against the corresponding maximum percentage (post vasorelaxation) vasoconstriction (Figure 5.14). A significant, positive correlation exists between the two variables that appears to go through the origin (0,0) (Figure 5.14).

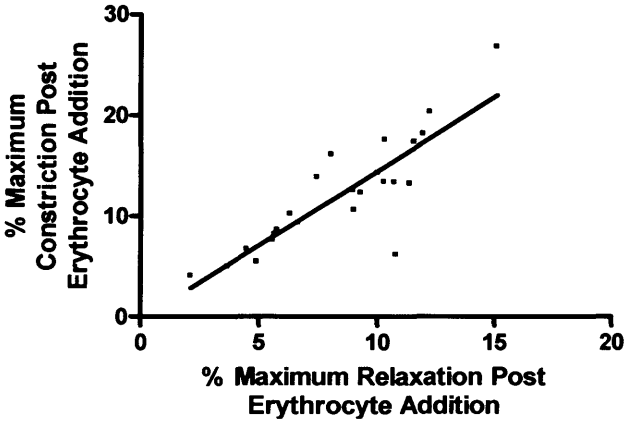

Figure 5.14. A correlation of maximum erythrocyte induced relaxation vs. the corresponding erythrocyte induced constriction. A positive and significant (Pearson's r=0.85, ***P<0.0001) relationship exists between the two variables. Measurements were taken from a variety of experiments to cover a range of relaxation/constriction levels. (r^2 =0.73, n=24).

5.4 Discussion

The ability of native human erythrocytes to induce relaxation in the hypoxic vessel bioassay is well known [331]. This *ex vivo* response, demonstrated to be at least in part mediated via soluble guanylate cyclase [421], has been attributed to several mediators from within the erythrocyte, including SNO-Hb [331], ATP [432] and/or nitrite [337]. Regardless of the mediator involved, it is clear that changes in oxygen level play a significant role in the mechanism with respect to both the erythrocyte and the tissue.

5.4.1 Allosteric Modulation

A number of studies have proposed that the mechanism behind the erythrocyte-induced vasorelaxation is under the control of haemoglobin allostery, this suggestion is based largely on data collected from erythrocyte relaxations in tissue baths at varying oxygen levels [331] and saturation-dependent changes in erythrocyte HbSNO content/nitrite reductase activity [337, 363]. This chapter contains the first study to present data on the direct relationship between haemoglobin conformational state and erythrocyte induced relaxation in a hypoxic tissue environment. The findings demonstrate that the erythrocyte induced vasorelaxation is dependent on and directly proportional to haemoglobin allosteric state, in this case controlled by oxygen saturation. Release of the vasoactive moiety is therefore reliant on haemoglobin 'switching' conformation from R-state to T-state. The results of this chapter show that the relationship between haemoglobin conformation and relaxation is linear, favouring a mechanism by which the vasoactive moiety is offloaded in response to progressive deoxygenation. This supports the HbSNO theory where the release of nitric oxide from HbSNO is thought to be linked to haemoglobin allostery, but disagrees with some aspects of the nitrite reduction theory. Although the original investigations into nitrite reduction by haemoglobin suggested deoxy (T-state) haemoglobin was responsible for generating nitric oxide [337], more recent publications from the field have suggested the reduction process to be optimal at the point where haemoglobin is 50% saturated (p50)

[342]. If this mechanism was in operation one would expect a more Gaussian type distribution between haemoglobin oxygen saturation and erythrocyte induced vasorelaxation with a maxima at 50% saturation, not the linear relationship observed.

Previous studies have specified the requirement for a lowered tissue oxygen level in order to produce a vasorelaxant response from erythrocytes [91, 421]. Although the responsiveness of tissue to the same nitric oxide-based stimulus is known to be enhanced in hypoxia [98] it is possible that 'tissue hypoxia' *per se* is not required, but rather that an environment (hypoxic or otherwise) to drive the transition of haemoglobin from R to T-state. Unfortunately, this scenario would be unlikely to ever arise *in vivo* and would be technically demanding to create *ex vivo*, restricting the ability to study this mechanism. One could speculate that it may be feasible to create a 'donor and recipient' type set-up, akin to that developed by Loewi [435] in the discovery of acetylcholine, where one frog heart was stimulated via the vagus nerve and washed, the washings were simultaneously dripped onto the heart of a second frog to produce an effect. In this format, erythrocytes contained in a semi-permeable container could be rapidly deoxygenated in buffer contained in the first compartment, the buffer would then be directly passed over oxygenated tissue in a second compartment, to assess the presence and activity of a vasorelaxant. This experiment could also address whether or not erythrocytes need to be in direct contact with the tissue to induce vasorelaxation and determine if tissue hypoxia is a requirement.

To further investigate the haemoglobin conformational control of the release mechanism, the allosteric modulator 2,3-DPG was investigated. 2,3-DPG is known to shift the oxygen binding curve to the right i.e. it enhances the offloading of oxygen and helps to prevent oxygen re-binding to deoxygenated haem in the tissue (see section 1.4.2.4, page 18). The addition of 2,3-DPG to the bath at a final concentration to match final haemoglobin concentration was found to increase the maximum vasorelaxation induced by lysed erythrocytes. This increase in maximum vasorelaxation could be expected given the positive allosteric actions of 2,3-DPG on 'offloading'. In contrast, the vasoconstriction that occurred post relaxation was found to be unaffected. The

vasoconstriction has previously been attributed to haemoglobin scavenging of endogenous nitric oxide generated by the endothelium [331]. As in the case of oxygen re-binding, one would predict 2,3-DPG to aid in the prevention of nitric oxide binding, however no difference was observed (if anything constriction was increased) suggesting that scavenging of nitric oxide is not responsible for the post relaxation vasoconstriction. If the erythrocyte induced vasorelaxation was to play a role in the control of vascular tone *in vivo* it is likely that it may be most beneficial in tissue areas suffering from vascular disease. In this respect, 2,3-DPG levels have been found to be significantly increased in diabetic patients, but only in those showing vascular complications [436]. In addition to the obvious oxygen offloading advantages, it is possible that this adaptive augmentation of 2,3-DPG levels in some diabetics could also enhance the release of erythrocyte derived vasorelaxant.

In the assessment of 2,3-DPG, lysed erythrocyte samples were utilised to allow 2,3-DPG access to the haemoglobin. Erythrocyte lysate was found to still have the ability to generate vasorelaxation in the model used in this chapter, which carries a number of implications. Firstly, it suggests that the mechanism controlling the vasorelaxation can operate independently of the erythrocyte membrane or outside the whole cell scenario, suggesting that the vasorelaxant is associated with haemoglobin and can work in isolation from the cell. Secondly, this evidence does not support a role for the ATP release theory as this theory would appear to only function in a whole cell situation.

5.4.2 Movement and Recycling

It is apparent that allosteric changes in haemoglobin are essential for vasorelaxant release, but it is unclear as to how much is released upon each hypoxic exposure or how and if the store can be reloaded. To investigate this, erythrocytes were subjected to repeat oxygenation cycles and vasorelaxations. The results presented in this chapter demonstrate that erythrocytes are capable of producing repeat vasorelaxations (post deoxygenation) that are not significantly different from initial vasorelaxations.

This suggests that erythrocytes contain a considerable (potentially replenishable) store of vasorelaxant which is either not released in its entirety during an individual hypoxic exposure or is replenished in some way upon re-oxygenation. This raises the question as to why only a proportion of vasorelaxant would be released upon each hypoxic exposure? In the case of the HbSNO theory it is proposed that the haemoglobin transition from R-state to T-state that destabilises the thiol bound nitric oxide resulting in release [363], it is therefore viable that not all HbSNO groups are destabilised and so not released or that nitric oxide released from one thiol subsequently reacts with a another thiol (on haemoglobin or in the membrane) and so is also not released from the erythrocyte. This could explain the ability of erythrocytes to produce repeated relaxations, indicative of a vasorelaxant 'store', however, it is interesting that the repeat vasorelaxations are comparable to initial vasorelaxations, suggesting that the store is not depleting.

The reproducibility of erythrocyte vasodilatation, despite repeat hypoxic exposure, could suggest release followed by re-uptake. To address this, experiments were conducted where erythrocytes were deoxygenated, had the extracellular buffer removed and then re-oxygenated in fresh buffer. In theory this should prevent the vasorelaxant from being passed out of the cell and then taken back up upon re-oxygenation. However the results obtained showed no difference in relaxation between control samples and those washed in fresh buffer. The vasorelaxant is therefore not simply passed back and forth between the intra and extra cellular compartments. With respect to the HbSNO theory, it is possible that nitric oxide could be released from haemoglobin and bind to membrane thiols and not leave the erythrocyte, particularly as there are no extracellular 'acceptor' thiols present.

5.4.3 Other Possible Mediators

5.4.3.1 Carbon Monoxide

All evidence to date points to the release of a species that is linked to haemoglobin allostery and can bind the haem centre of sGC to produce activation. The most likely candidates are selected diatomic gases, as these both associate with haemoglobin and can activate sGC. The potential roles of nitric oxide have already been researched in depth and are still in question, essentially leaving oxygen and carbon monoxide. The decision to investigate carbon monoxide as a potential erythrocyte vasorelaxant was based on a number of published observations that supported a potential function of carbon monoxide, these observations are summarised below;

- The normal circulating level of HbCO is ~1.5% but can range up to around 10-15% in some cases, particularly in those who smoke [437]. Therefore, based on normal *in vivo* haemoglobin concentrations (~2.5mM) at ~1% HbCO there is a considerable circulating concentration of bound carbon monoxide (~25μM).

- Carbon monoxide is known to produce vasorelaxation that is in some part mediated via a sGC dependent mechanism [438].

- Encapsulation of haemoglobin by a phospholipid membrane retards the reaction of nitric oxide but not carbon monoxide [385].

- Arteriovenous gradients in HbCO levels have been found in both healthy and critically ill patients [439].

- The rate of dissociation of carbon monoxide is approximate one order of magnitude faster than that of nitric oxide (~10^{-3} vs. ~10^{-4} s^{-1}) [440, 441].

- Extracellular haem groups are capable of extracting carbon monoxide, but not nitric oxide from haemoglobin contained within erythrocytes [442].

- Carbon monoxide inhalation has been demonstrated to be protective in scenarios such as ischemia-reperfusion injury caused during organ transplantation [443].

In light of these previous observations and given the results of this chapter showing carbon monoxide to have increased activity in hypoxia (CORM-A1 results), the vasorelaxant properties of erythrocytes containing 100% HbCO were tested. The addition of carbon monoxide to full saturation was found to completely inhibit the erythrocyte induced vasorelaxation. The observed inhibition is most likely due to the stability of the fully saturated haemoglobin, holding it in the R-state and not allowing transition from R-state to T-state conformation. This finding led to the working hypothesis that carbon monoxide could potentially be released from partially carbon monoxide saturated haemoglobin (balance oxygen saturated) that may still undergo some conformational changes upon deoxygenation. In support of this hypothesis, it has previously been shown that mono-liganded HbCO has a faster dissociation rate than di-liganded intermediates and that carbon monoxide dissociates faster from α haem than β haem [444]. In order to investigate this hypothesis, erythrocyte samples containing ~22% HbCO were generated and tested for vasorelaxant properties. The partial saturation of haemoglobin with carbon monoxide resulted in a partial inhibition of the vasorelaxation induced, however, the study was not powered to demonstrate that the result was statictally significant. In accordance with the allosteric data using oxygen to control conformation it is likely that even in samples that are only partially saturated with carbon monoxide, haemoglobin is held in the R-state, therefore insufficient R to T-state transition occurs to release the full amount of vasorelaxant and produce maximum vasorelaxation. It can therefore be concluded from these results that carbon monoxide bound to haemoglobin is not the intra-erythrocyte species responsible for vasorelaxation of hypoxic tissue and that the use of agents that hold haemoglobin in the R-state inhibit conformation change and similarly inhibit vasorelaxation.

5.4.3.2 Oxygen

Having concluded that carbon monoxide was not responsible for the vasorelaxant properties of erythrocytes, the role of oxygen itself was considered. Oxygenated saline was capable of inducing vasorelaxation in hypoxic tissue independently of haemoglobin. The vasorelaxation observed was comparable to that seen with oxygenated erythrocytes and also displayed some post-addition vasoconstriction, suggesting that oxygen could be the vasorelaxant released from erythrocytes. In support of this notion, the oxygenated saline mediated relaxation was also soluble guanylate cyclase dependent as is the case with that mediated by erythrocytes. In addition, the degree of vasorelaxation also directly correlated to the oxygen content of the saline added, which is comparable to the direct correlation between erythrocyte induced vasorelaxation and haemoglobin oxygen saturation. In this respect, our laboratory has previously shown that the concentration of oxygen in the tissue bath increases upon the addition of oxygenated erythrocytes that coincides with changes in vessel tone [91], in agreement with a role for oxygen within the mechanism. These results do not discount a role for HbSNO but would suggest that oxygen could be the primary vasorelaxant released. It is interesting to note that the presence of HbSNO shifts the haemoglobin binding curve to the right, which would result in increased oxygen offloading and may explain the link between erythrocyte induced vasorelaxation and HbSNO content [421].

During the recording of maximum vasorelaxation, data concerning the corresponding post-relaxation vasoconstriction was also recorded during many experiments. These data were subsequently used to create a correlation between the two variables, maximum vasorelaxation against matched, maximum vasoconstriction. The correlation is highly significant and the relationship observed between the two is a positive, linear one, demonstrating that one variable is closely related to the other. Using linear regression analysis, the data appears to go through the origin, suggesting that if a given sample were to generate no vasorelaxation, then no post relaxation vasoconstriction would be observed. This finding cannot be reconciled with the proposal

that the vasoconstriction is produced by haemoglobin scavenging of endogenous nitric oxide [331] as this should occur independently of vasorelaxation. After the completion of the work contained in this chapter the Stamler group produced new findings and proposals with regards to the mechanism of vasoconstriction that occurs post relaxation [421]. The publication demonstrates that a post relaxation vasoconstriction still occurs even when the vessel is denuded of endothelium and that the vasoconstriction is in some part inhibited by ODQ [421]. It is proposed that the vasoconstriction could therefore be mediated to some extent by the vasorelaxant entity through a cGMP mediated mechanism or in the case of HbSNO, an SNO mediated contractile mechanism [421]. A dual vasorelaxant/vasoconstrictor mechanism would agree with the correlation between vasorelaxation and constriction presented in this chapter.

In light of the fact that oxygen could be the vasorelaxant moiety released by erythrocytes, can the direct relationship between vasorelaxation and vasoconstriction be explained by oxygen dependent mechanisms? In terms of the vasorelaxation there are two potential mechanistic roles for oxygen: firstly, in hypoxia it is feasible that soluble guanylate cyclase is more sensitive to binding agonists (such as diatomic gases) so although less potent than nitric oxide, oxygen could activate soluble guanylate cyclase. Secondly, the relaxation mechanism could involve endothelial nitric oxide synthase, in hypoxia the enzyme will not efficiently produce nitric oxide, but a transient burst of oxygen to the tissue may be sufficient for the enzyme to briefly produce some nitric oxide to produce a brief relaxation. Although it is possible that nitric oxide synthase could play some role, the fact that erythrocytes can induce relaxation in endothelium denuded hypoxic vessels [421] would suggest it not to be the primary pathway involved. It is clear from the results of this chapter that hypoxic tissue will not vasoconstrict to the same extent as normoxic tissue given the same phenylephrine stimulus, suggesting that contractile mechanisms are limited by hypoxia. It is possible that a burst of oxygen could allow mitochondria to aerobically produce ATP, providing the energy required to generate further constriction to the phenylephrine still present in the bath. This suggestion could be fitting in a temporal sense as the process of ATP generation could take longer than say the direct stimulation of soluble guanylate cyclase, lending support

to a mechanism where transient vasorelaxation precedes a vasoconstriction that is related in magnitude.

As the post vasoconstriction is obviously a key element of the erythrocyte-induced vasorelaxation it is important to consider the net change in vessel tone that is induced by erythrocytes. In the *ex vivo* experiments of this chapter, the percentage maximum vasoconstriction was comparable, if not higher than the percentage maximum vasorelaxation. However, this is only maximum vasorelaxation/constriction and therefore doesn't describe the change in vessel tone with respect to time. Nevertheless, it does in some part suggest that within this model there is no overall change in vessel tone. The idea of a 'stalemate' situation becomes more complex if one assesses it within the *in vivo* type setting where there is blood flow. If the vasoconstriction mechanism is a secondary result of the vasorelaxant released it will generate both vasorelaxation and constriction within the same specific tissue area, conversely, if it is caused by the presence of erythrocytes/haemoglobin in a specific state then in a situation of flow the vasoconstriction could effect tissue distal to the site of relaxation. The distinction between these mechanisms is an important one because if the latter were true, then due to the rapid transit of erythrocytes through the circulation it could be possible to induce vasorelaxation in the arterioles/capillaries with the vasoconstrictor mechanism acting further down the vascular tree e.g. in the veins, where it may not be so influential.

Overall, it is plausible that this mechanism, in addition to many others, could serve to adjust basal tone with respect to oxygen demand. However, it should be noted that the majority of erythrocyte-induced vasorelaxations presented in this chapter were produced by erythrocytes deoxygenated from 100% oxygen saturation down to below ~10%. This dramatic change in oxygen saturation does not usually occur *in vivo* with normal arterial to venous transitions being ~100% to ~75% saturation. Therefore offloading of vasorelaxant under normal conditions would be minimal and would occur in smaller vessels that are unlikely to be capable of influencing flow to any great extent. Dramatic differences in saturation are more likely to occur in hypoxic tissue, usually caused by a restriction in blood flow. In this situation offloading of vasorelaxant to

increase blood supply to the area would be ideal. However, for the mechanism to work some blood flow is required to continually deliver 'fresh' erythrocytes with R-state haemoglobin that are capable of changing conformation and thus inducing the required vasorelaxation.

5.5 Summary and Conclusions

The results of this chapter have not conclusively elucidated the identity of the vasorelaxant released from erythrocytes but do provide significant and persuasive evidence that oxygen is the primary vasorelaxant involved. The findings do not support significant roles for either the nitrite or ATP based theories and cannot discount a role for HbSNO at some level. The results presented in this chapter do reveal some interesting characteristics with regard to the mechanism of erythrocyte induced vasorelaxation and provide supporting evidence to suggest that oxygen is the principal vasorelaxant involved, these are summarised below.

- The conformational shift in haemoglobin protein structure from R-state to T-state (that is controlled by oxygen saturation both in the reported experiments and *in vivo*) appears to be necessary for the induction of vasorelaxation. Modulators of haemoglobin allostery, such as 2,3-DPG, are capable of altering this mechanism.

- The relaxant species released from the erythrocyte must be capable of avoiding re-capture by or reaction with haemoglobin in the local region of release.

- The vasorelaxant is not simply passed in and out of the erythrocyte during oxygenation cycles.

- Erythrocytes contain a store of vasorelaxant that is either not released in its entirety upon a single deoxygenation event or is potentially replenishable and provided the cells are re-oxygenated are capable of repeat relaxations.

- Vasorelaxation induced by erythrocytes in a hypoxic environment is accompanied by a post relaxation vasoconstriction. The magnitude of this vasoconstriction is directly proportional to the preceding vasorelaxation.

Therefore, overall change in vessel tone should be considered in terms of net vasorelaxation and constriction.

- The vasorelaxant is associated with haemoglobin and can induce vasorelaxation independently of a whole cell scenario.

- Despite its apparent suitability as an erythrocyte vasorelaxant candidate, carbon monoxide or HbCO do not appear to be responsible for the vasorelaxation assessed in the current model.

- A burst of oxygen delivered to hypoxic tissue is capable of inducing a transient relaxation. The relaxation is soluble guanylate cyclase dependent and is comparable to that induced by oxygenated erythrocytes. Oxygen addition also produces a post-vasorelaxation vasoconstriction, suggesting that both vasoconstriction and vasorelaxation could be oxygen derived.

- Oxygen is likely to represent the primary vasorelaxant released from erythrocytes upon an allosteric change stimulated by hypoxia.

6 The Vasodilator Properties of the Nitrite Anion in the Human Circulatory System: the Influence of Oxygen

6.1 Introduction

The vascular actions of nitrite have been known for more than a century [395], however the development of more potent organic nitrates (e.g. glyceryl trinitrate, GTN), rendered nitrite clinically 'redundant'. Interest in nitrite as a vasodilator has resurged in recent times because of two principal reasons. Firstly, some research suggests that it may at basal plasma levels, represent a circulating, bioactive nitric oxide metabolite as apposed to a simple waste product (see section 1.10.2, page 68). Secondly, it has been proposed that nitrite, although less potent than other agents, may have some clinical potential in certain, niche settings, because it has targeted actions such as enhanced activity in hypoxic tissue beds for example [98].

At this point it is imperative to make the distinction between sources, potential mechanisms and the vascular activity of endogenous nitrite at basal plasma levels (~200nM) and at levels elevated by pharmacological nitrite administration.

Basal plasma nitrite levels are a balance between sources/production and excretion/metabolism. Although vascular nitric oxide is thought to be one of the principal sources of plasma nitrite [445], dietary influences can also play a role. In this respect, it has been demonstrated that the ingestion of high nitrate foods results in a subsequent increase in plasma nitrite level which coincides with a reduction in blood pressure and platelet inhibition, and appears to afford some protection against the damaging effects of vascular ischemia [417]. Nitrate administered orally is thought to be converted to nitrite via bacterial anaerobes in the gastrointestinal tract and it is the generated nitrite absorbed into the blood stream that is believed to be responsible for the vascular benefits observed [417]. However, it should be noted that this study is still artificially increasing plasma nitrite levels considerably above baseline. At basal plasma levels, the ability of nitrite to

alter vascular tone *in vivo* and the potential mechanism behind this action are largely theoretical. To date, the contributions of basal plasma nitrite on vascular tone have not been demonstrated *in vivo* and its biological role is inferred from observations such as arteriovenous plasma nitrite gradients [327]. Although basal plasma nitrite levels generally lie within a relatively narrow range, changes in level are thought to largely reflect endothelial function and nitric oxide production. Plasma nitrite levels have been shown to negatively correlate with the number of cardiovascular risk factors that a patient exhibits due to defective production or increased oxidative metabolism of nitric oxide [380].

A range of different pharmacological nitrite supplementation studies in animals have now been published and demonstrate numerous beneficial effects of nitrite including protection against ischemia reperfusion in a number of organs [352, 446] and positive effects in models of hypertension [447]. From a clinical perspective, one publication has also considered blood pressure and nitrite tachyphylaxis in primates, in reference to the tolerance that is observed clinically with organic nitrates [379]. The study found that, unlike organic nitrates, nitrite did not cause tachyphylaxis in primates that were administered a continuous nitrite infusion and daily boluses over two weeks.

Though there is a wealth of literature on the applications of nitrite in animal studies, this thesis is primarily concerned with studying the vasoactive properties of nitrite and its mechanisms of action with the aim of assessment in human subjects. In addition to very early work with nitrite in humans, five modern publications have assessed infusions of sodium nitrite in man ([98, 337, 379, 403, 445] summarised in table 6.1). The approach that has been taken in each of these studies is quite different making comparison between them difficult. However, there are some common findings and questions that arise from these studies collectively, including:

- Infusion of nitrite into the brachial artery, particularly at high doses (above 1μmole per minute) is capable of producing vasodilatation in the vascular bed to which it is infused.

- Increasing plasma nitrite levels with high dose nitrite significan increases levels of haemoglobin-bound nitric oxide (HbNO) in veno blood.

- Nitrite infusion has a marked effect on vein diameter in addition to effects in arteries.

- The vasodilator properties of nitrite are enhanced in hypoxic tissue beds.

- What is the mechanism of action of nitrite reduction *in vivo*? Bo haemoglobin and oxidoreductase enzymes have been suggested to redu nitrite back to nitric oxide, however, *in vivo* evidence is lacking. O publication also failed to show a role for xanthine oxidase (XO) as potential vascular nitrite reductase.

The mechanism by which nitrite produces vasodilatation *in vivo* has not yet be demonstrated. However, some mechanisms have been suggested and broadly fall into tv schools of thought. The first suggests that erythrocytes are responsible for nitri activation via the reduction of nitrite to nitric oxide by haemoglobin (see section 1.10. page 68). The second suggests that nitrite is activated at a tissue level, independently blood, via reduction to nitric oxide by oxidoreductase enzymes, a hypothesis which supported by the findings presented in this thesis in chapter 4. Aldehyde oxidase (AO has been suggested to be the principle enzyme involved [397] although xanthine oxida (XO), nitric oxide synthase and mitochondrial enzymes have also been implicated [39 399]. These enzymes are also known to more actively reduce nitrite to nitric oxide und conditions of hypoxia [413].

Table 6.1. Summary of human nitrite infusion studies

Publication	*Subjects*	*Dose regimen*	*Measurements*	*Interventions*	*Findings*	*Confounding Factors*
Lauer *et al*, 2001.	3 subjects	Intraarterial sodium nitrite was given at doses from 0.01 to 36 μmol/min.	Forearm blood flow of the infusion arm was measured by strain gauge plethysmography. Venous blood samples were also taken from the same arm.		Even at the highest dose nitrite did not alter forearm blood flow. Using estimations, the authors believe to recover 92 -97% of nitrite infused into the artery from blood taken from the corresponding vein.	Very low subject numbers and publication lacks detail with respect to protocol.
Cosby *et al*, 2006.	9 male and 9 female, 10 subjects recalled for low-dose study.	2 x 36μmol/ml infused @ 1ml/min for 5mins via the brachial artery of the study arm. Infusion 1 before exercise, infusion 2 before exercise with L - NMMA co-administration. Infusion 1 and 2 were separated by a 30min saline infusion. Total dose received 1.08mmoles. low dose studies were identical but infusions of 0.36 μmol/ml were used.	Forearm blood flow of the infusion arm was measured by strain gauge plethysmography. Arterial and venous blood samples were also taken from the infusion arm for blood gas and nitric oxide metabolite analysis.	Hand grip exercise in the infusion arm.	Both doses administered produced increases in plasma nitrite (venous levels rose to ~220 μM) and forearm blood flow. The findings were unaffected by L -NMMA administration. Nitrite infusion increased haemoglobin bound nitric oxide species and formation was inversely proportional to haemoglobin oxygen saturation.	High doses administered. Exercise was utilised as a hypoxic stimulus. The latter 9 subjects received a nitrite infusion that contained sodium bicarbonate to titrate the pH to above pH 7.0, raising questions over the pH of the initial infusion.
Dejam *et al*, 2007.	20 subjects. Average weight 71 ±7 kg.	Part A, n=5. Sodium nitrite in saline was infused @2ml/min for 5 minutes intrabrachial, at escalating doses from 0, 7, 14, 28 and 55 to 110 μg/kg/min. For reference, a dose of 28 μg/kg/min for 5 minutes at the average body weight would be equivalent to ~144 μmoles of nitrite. After dose escalation a rest period of 180 minutes was given before a dose of 28 μg/kg/min for 5 minutes. Part B, n=15. Sodium nitrite in saline was infused @2ml/min for 5 minutes, at escalating doses from 0.07, 0.14, 0.35, 0.7, 1.4, 3.5, 14 and 28 μg/kg/min, 10 subjects received a coinfusion of either oxypurinol (600μg/min) or ascorbic acid (24mg/min).	Part A. During dose escalation blood flow measurements (by strain gauge plethysmography) and blood samples were taken from the infusion arm (ipsilateral). After the rest period of 180 minutes a further infusion was given however measurements were made in the contralateral arm. Part B. Both blood flow measurements and blood samples were taken.		Part A. Escalating doses of nitrite were capable of increasing fore arm blood flow in the ipsilateral arm. Forearm blood flow in the ipsilateral arm was altered within seconds of commencing arterial nitrite infusion. An infusion of 28 μg/kg/min was capable of producing increases in forearm blood flow in the contralateral arm after a short time delay (nitrite concentration in contralateral arm ~1 μM). Part B. The lowest infusion dose (0.07 μg/kg/min) increased venous plasma nitrite to 350nM and was capable of significantly increasing arterial flow in the ipsilateral arm from 3 ± 0.3 ml/100ml/min to 3.4 ±0.3 ml/100ml/min (n=15). Neither ascorbic acid or oxypurinol altered blood flow responses.	Escalating doses were utilised with no time to plateaux between each dose. Doses used are also high, escalating up to levels significantly higher than the physiological range.

Publication	*Subjects*	*Dose regimen*	*Measurements*	*Interventions*	*Findings*	*Confounding Factors*
Maher *et al*, 2007.	40 Subjects.	Protocol A, normoxia, n=26. Sodium nitrite was infused intrabrachial at the following escalating doses 40, 100, 314, 784, nmol/min and 3.14 and 7.84 µmol/min each for 30 minutes. Protocol B, hypoxia, n=14. 7 subjects received intrabrachial nitrite (7.84 µmol/min for 20mins) in hypoxia followed by the same infusion in normoxia with a 60min saline infusion in between. A further 7 subject underwent protocol B with a 314nmol/min infusion.	Forearm venous volume was measured by radionucleotide plethysmography. Forearm arterial blood flow was measured by strain gauge plethysmography in both arms, change in flow in the study arm was corrected for flow in the contralateral arm and expressed as a ratio. Blood samples were also taken for nitric oxide metabolite assessment.	Hypoxia was induced by administratio n of 12% oxygen via a face mask containing a two-way valve.	Nitrite was capable of inducing venodilatation, which appear to be unchanged by the hypoxic intervention. In contrast, nitrite induced much greater changes in arterial flow in hypoxia compared to normoxia.	Hypoxia was administered via a mask which could introduce variability. Hypoxia itself causes vascular changes which occur over hours, in this study subjects were considered 'hypoxic' when arterial saturation had dropped to a steady 83 -88%.
Mack *et al*, 2008.	14 Sickle cell patients (9 male, 5 female).	Intra-brachial infusion of sodium nitrite at the following escalating doses 0.4, 4 and 40 µmol/ml at 1ml/min for 5 minutes.	Forearm blood flow of the infusion arm was measured by strain gauge plethysmography. Blood sample taken form venous catheters of both the infusion and contralateral arm.		Nitrite induced an increase in forearm blood flow in the study arm. Responses to nitrite in sickle cell patients were blunted compared to controls. Sodium nitrite infusion was well tolerated and did not cause hypotension or methaemoglobinaemia.	No true control subjects, control data was taken form previous studies where nitrite dose administered was different.

6.1.1 Aims

Studying nitrite at basal level *in vivo* presents many complications, as with any system it is often necessary to provoke it in some way in order to gain understanding about the way it operates. The work of chapter three presented in this thesis demonstrates that the movement and levels of circulating nitric oxide metabolites represents a dynamic equilibrium *in vivo*, this chapter aims to investigate this dynamic and its physiological consequences through increasing the circulating nitrite pool by way of a low dose infusion. Specifically this chapter aims to:

- Assess the interaction between nitrite and erythrocytes with particular respect to the mechanism of nitrite induced vasoactivity *in vivo*.

- Investigate the relationship between plasma nitrite levels and biochemical/physiological changes in the physiological concentration range.

- Consider the systemic properties and interactions of nitrite.

- Examine the influence of oxygen upon the metabolism and vasoactive properties of nitrite in the physiological concentration range.

6.2 Specific Methods and Protocols

This body of work was carried out in collaboration with Dr. Thomas Ingram. Dr Ingram was responsible for securing ethical approval, aiding in the measurement of some nitric oxide metabolites and analysing the forearm blood flow data that had been collected.

6.2.1 Subjects

18 healthy male volunteers were recruited locally and all gave informed written consent to participate. Female subjects were not included in the study because levels of nitric oxide metabolites are known to be altered by circulating estrogens [448], leading to cyclical variability which could be confounding. Other exclusion criteria included: hypertension, hypercholesterolemia, diabetes mellitus, being a current smoker, taking regular prescription drugs or having a family history of premature coronary artery disease. The experimental protocol and recruitment procedure were both approved by the local research ethics committee.

All subjects were requested to refrain from consuming alcohol, caffeine and foods with high nitrite/nitrate content (e.g. certain vegetables and red meat, which is often preserved with high concentration nitrite [449]) for the 12 hours preceding experimental testing. Detailed subject characteristics will be covered in the results section of this chapter.

6.2.2 The Environmental Chamber

To ensure accurate and consistent exposure to hypoxia all hypoxic studies were carried out in a bespoke environmental chamber (Design Environmental, Ebbw Vale, UK). The chamber is ~120M^3 and is purged with a high flow of nitrogen gas mix (via a number of diffusers that create homogenous mixing) to achieve the desired hypoxic environment (12% O_2). The high flow of gas ensures minimal changes in carbon dioxide

level (within the atmospheric range expected at sea level) and that the chamber operates at normobaric pressure. Because the chamber operates at normobaric pressure, it allows investigators to enter and exit the chamber without causing significant changes in chamber oxygen level. The chamber employs a number of highly sensitive, carefully positioned sensors to constantly monitor and affect changes in temperature, oxygen level and humidity within the chamber environment. For safety reasons, all parameters were remotely controlled via an external computer running a custom program (Contour program, Design Environmental, Ebbw Vale, UK) for control. The chamber was always run at a constant temperature (see results section) and humidity. Corresponding normoxic studies were carried out in a similar sized, temperature controlled room at normal atmospheric oxygen level (21% O_2).

6.2.3 Blood Collection and Measurement

Blood samples were collected from a catheter placed in the antecubital vein of the non-dominant arm. The same arm was also utilised for the measurement of blood flow, this procedure involved the inflation of cuffs around the arm to restrict blood flow, therefore blood samples taken at specific time points were always collected before the corresponding flow measurements were made, as cuff inflation will alter flow/haemoglobin saturation and influence nitric oxide metabolite levels. Samples were immediately analysed for blood gas readings, processed as per section 2.1.2 (page 80) and snap frozen for future analysis.

Plasma and erythrocyte samples were rapid thawed for the measurement of plasma nitrite, nitrate, RSNO/RNNO and haemoglobin bound species (HbNO and HbSNO) respectively, utilising the ozone based chemiluminescence measurement protocols detailed in section 2.3.4 (page 95). All samples were tested within one week of collection.

6.2.4 Strain Gauge Plethysmography

After an initial assessment of potential methodologies for the non-invasive measurement of forearm arterial blood flow, it became clear that strain gauge plethysmography (SGP) not only represented the most repeatable method, but was well suited to our study design.

During testing, the subject was in the seated position with the forearm resting horizontally in a support to reduce unwanted movement. To quantify arterial inflow, a cuff was first placed around the wrist and inflated to supra-systolic pressure (300mmHg), a second proximal cuff was placed around the upper arm; this cuff can be rapidly inflated using a rapid cuff inflator (E20, Hokanson) and air source (AG101, Hokanson). The proximal cuff is inflated to above venous pressure, but below arterial pressure (50mmHg), this abruptly stops venous outflow causing the volume of the limb to increase due to arterial inflow. A mercury strain gauge plethysmograph is placed between the cuffs, around the forearm at the point of largest circumference. The plethysmograph is used to measure the volume change in the limb, the rate of volume change being proportional to arterial flow rate at the moment of venous occlusion (Figure 6.1). A mercury plethysmograph is chosen that is 2cm smaller than circumference of the largest part of the forearm. The plethysmograph is connected to a EC6 interface (Hokanson, WA, USA), which is routed through an analogue to digital converter (Powerlab, AD instruments) to a laptop operating the data acquisition program, chart for windows (AD instruments). Repeat measures were taken a minimum of 6 times and the average of the 5 most repeatable measurements was taken as a final reading, expressed as ml of blood flow per 100ml of tissue, per minute (ml/100ml/min).

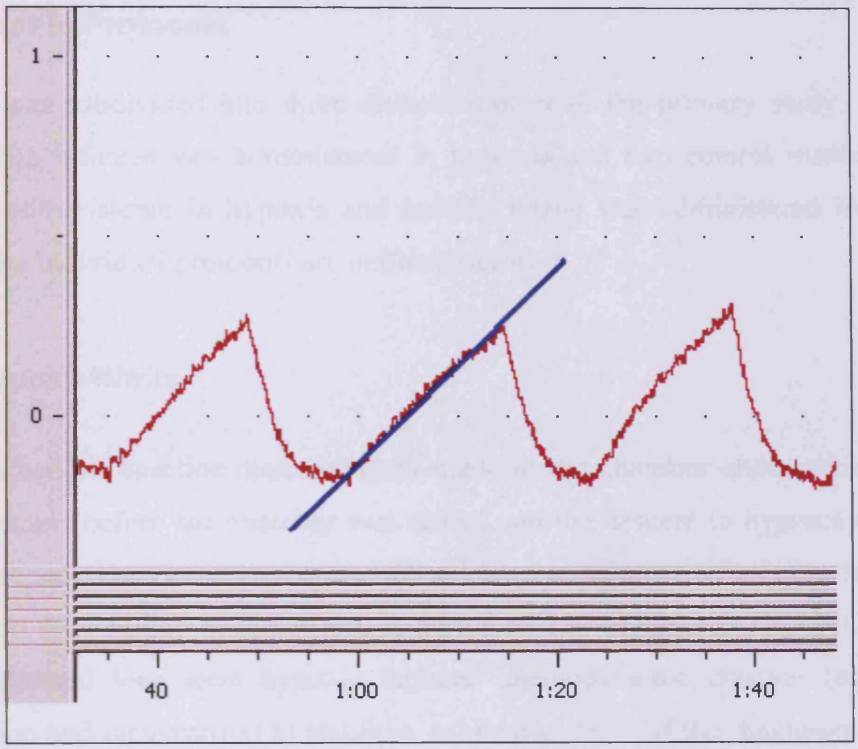

Figure 6.1. Example strain gauge plethysmography trace. The blue line indicates the section of the trace used to assess gradient which is proportional to arterial in-flow. The x-axis represents time and the y-axis, arbitrary units.

6.2.5 Specific Protocols

The study was subdivided into three distinct protocols: the primary study, in which a sodium nitrite infusion was administered in hypoxia and two control studies in which saline was administered in hypoxia and sodium nitrite was administered in normoxia. Details of the individual protocols are outlined below.

6.2.5.1 Hypoxia/Nitrite

12 subjects had all baseline measurements made in the chamber under normoxic (21% FiO_2) conditions before the chamber was sealed and the descent to hypoxia (12% FiO_2) was commenced. The chamber required 60 minutes to achieve 12% FiO_2, during which time subjects relaxed and acclimatised. A period of 120 minutes at 12% FiO_2 followed, this time allowed long term hypoxia induced haemodynamic changes (e.g. systemic vasodilatation and tachycardia) to stabilise, achieving ~90% of the maximum anticipated [450]. Subjects then received a low dose nitrite infusion (Martindale Pharmaceuticals, Brentwood, UK) (1μmol/mL @ 1mL per min for 30 minutes) to raise systemic plasma nitrite levels by approx. 100% (at peak). Blood samples and measurement of forearm blood flow were taken throughout (summarised in Figure 6.2).

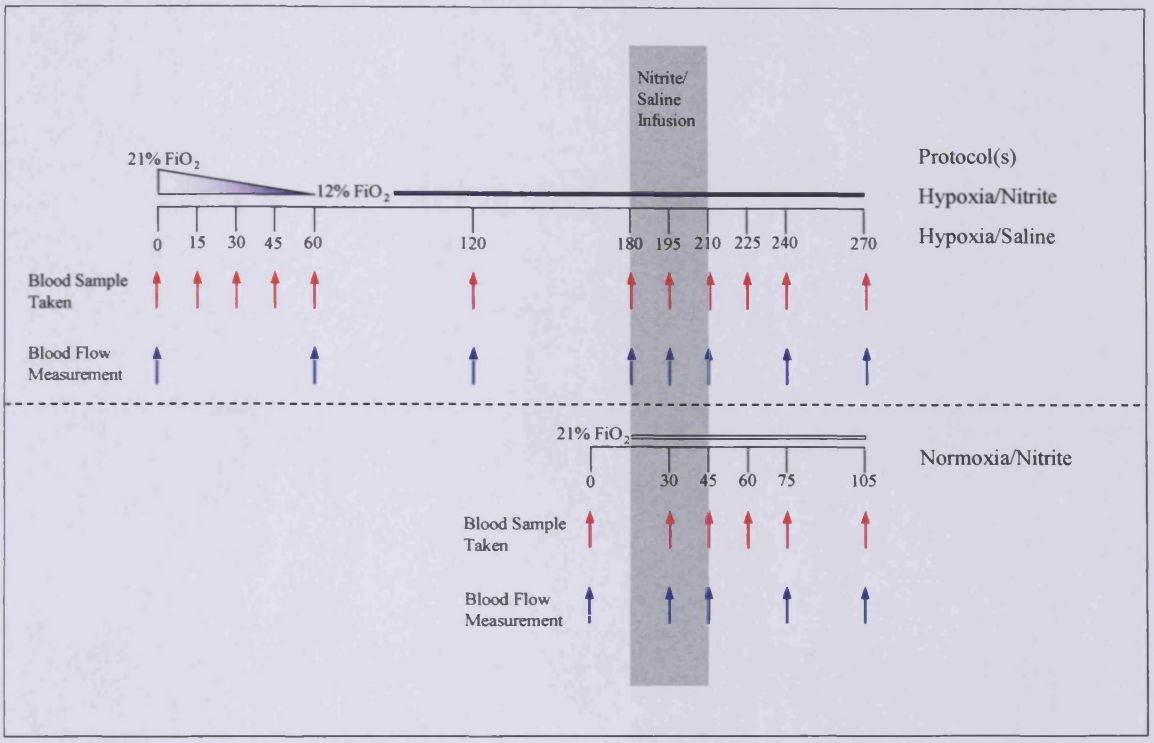

Figure 6.2. Schematic of study protocols. Upper panel shows hypoxia/nitrite and hypoxia/saline protocols, lower panel shows normoxia/nitrite protocol where the initial 3 hours of equilibration (necessary in the hypoxic protocol) is omitted. Red and blue arrows indicate time points at which blood samples and blood flow measurement were taken respectively. Grey infill denotes period of nitrite/saline infusion.

6.2.5.2 Hypoxia/Saline

An additional 6 subjects underwent the full hypoxia protocol as per 6.2.5.1 but received an equivalent volume of medical grade saline 0.9% w/v (Figure 6.2, upper panel).

6.2.5.3 Normoxia/Nitrite

6 of the subjects recruited for the hypoxia/nitrite protocol were also recruited for a second time to participate in a normoxia/nitrite protocol. Subjects essentially underwent the same protocol as in 6.2.5.1 (receiving the same dose of sodium nitrite) but the protocol was carried out in normoxia with the initial 3 hour equilibration period omitted (Figure 6.2, lower panel). Subjects had a 30 minute rest period before the measurement protocol was commenced.

6.2.6 *In vitro* Nitrite Addition to Erythrocytes

To compare the exposure of erythrocytes to nitrite via infusion in the *in vivo* setting, parameters of interest were also assessed in an *ex vivo* experimental model, designed to replicate aspects of the conditions found *in vivo*. Erythrocytes were collected from healthy volunteers as previously stated (section 2.1, page 80). Separated erythrocytes were reconstituted in PBS to give a final haemoglobin concentration of 10g/dL, the mix was then placed in a thin film rotating tonometer as previously described (section 2.2, page 81). A nitrogen purge was utilised to alter the haemoglobin saturation to ~50%, which was confirmed using a blood gas analyser (OSM3, Radiometer, Copenhagen). The mix was then separated into aliquots and nitrite of varying concentrations (up to 850nM final) was added and incubated for 30 minutes at 37°C before centrifugation (10,000*g* for 2 minutes). Erythrocyte and supernatant fractions were snap frozen immediately and stored for future analysis. Upon rapid thawing erythrocyte and supernatant fractions were assessed for nitrite and HbNO levels as per section 2.3.4 (page 95).

6.2.7 Analysis and Statistical Analysis

Testing between groups was performed using either the student t-test (either paired or unpaired where appropriate) or an analysis of variance (ANOVA) (repeat measures ANOVA where appropriate) with a Bonferroni *post hoc* test. To assess correlations the Pearson's correlation coefficient was used. Data are presented as means and error bars represent the standard error of the mean (SEM). Plasma nitrite half-life was calculated using all data from peak infusion point to 60 minutes post infusion cessation. A P value of less than 0.05 was taken to be significant, individual significance levels are indicated within the figure legends. All analysis was performed blinded. 'No significance' is abbreviated to 'NS' in some cases. The grey bar in the background of all graphs represents the 30 minute period of sodium nitrite or saline infusion.

6.3 Results

6.3.1 Subject Characteristics

General subject characteristics are detailed in table 6.2 below.

Table 6.2. Summary of subject characteristics.

	Mean	Standard Error of Mean
Age (years)	24.2	± 1.0
Height (cm)	180	± 1.7
Mass (kg)	78.6	± 2.2
BMI	24.3	± 0.6
Hb (g/dL)	16.4	±0 .3
Participants (n)		
Hypoxia/Nitrite	12	
Hypoxia/Saline	6	
Normoxia/Nitrite	6	(note, paired from hypoxia/nitrite protocol)

6.3.2 Chamber Parameters

The environmental chamber provided a controlled atmosphere, with constant temperature throughout the time course of the experiment (Figure 6.3). The chamber required 60 minutes to attain a steady 12% FiO_2 baseline (coming down from 21% O_2) and remained constant for the duration of the experiment, during which time subjects 'acclimatised' to the environment.

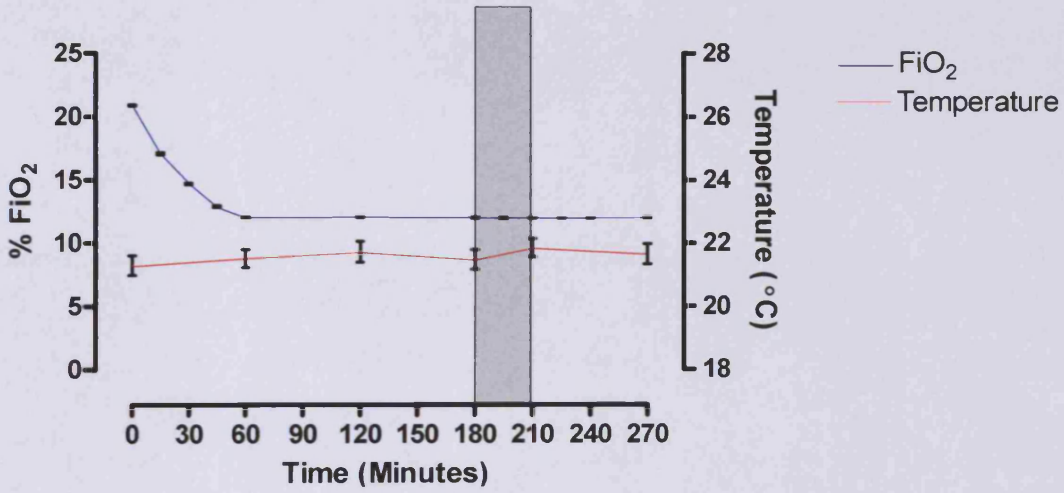

Figure 6.3. FiO_2 drops significantly until levels reach 12.0% oxygen after 60 minutes. Over the rest of the protocol, oxygen levels are maintained at 12.05 with no significant change. Time point compared by ANOVA and Bonferroni post test, n=18. Temperature level does not change significantly over the course of the protocol (mean temperature 21.6 °C ± 0.1), n=18. Shaded area represents period of nitrite/saline infusion.

6.3.3 Haemodynamic Parameters

Haemodynamic parameters were continually checked throughout all study protocols for scientific interest but also for safety i.e. to monitor subject well being. Both study protocols that were carried out in hypoxia significantly increased heart rate over the initial 3 hours of the protocol. Once a plateau rate was achieved, no significant changes were observed over the rest of the protocol, including pre-, during and post-nitrite/saline infusion (figure 6.4A and C). Heart rate in the normoxic protocol decreased significantly over the course of the entire protocol as subjects relaxed. It should be noted that heart rate remained unchanged during the infusion period (Figure 6.4E). Within the accuracy of the equipment used, blood pressure (systolic and diastolic) remained unchanged throughout all study protocols (Figure 6.4B, D and F).

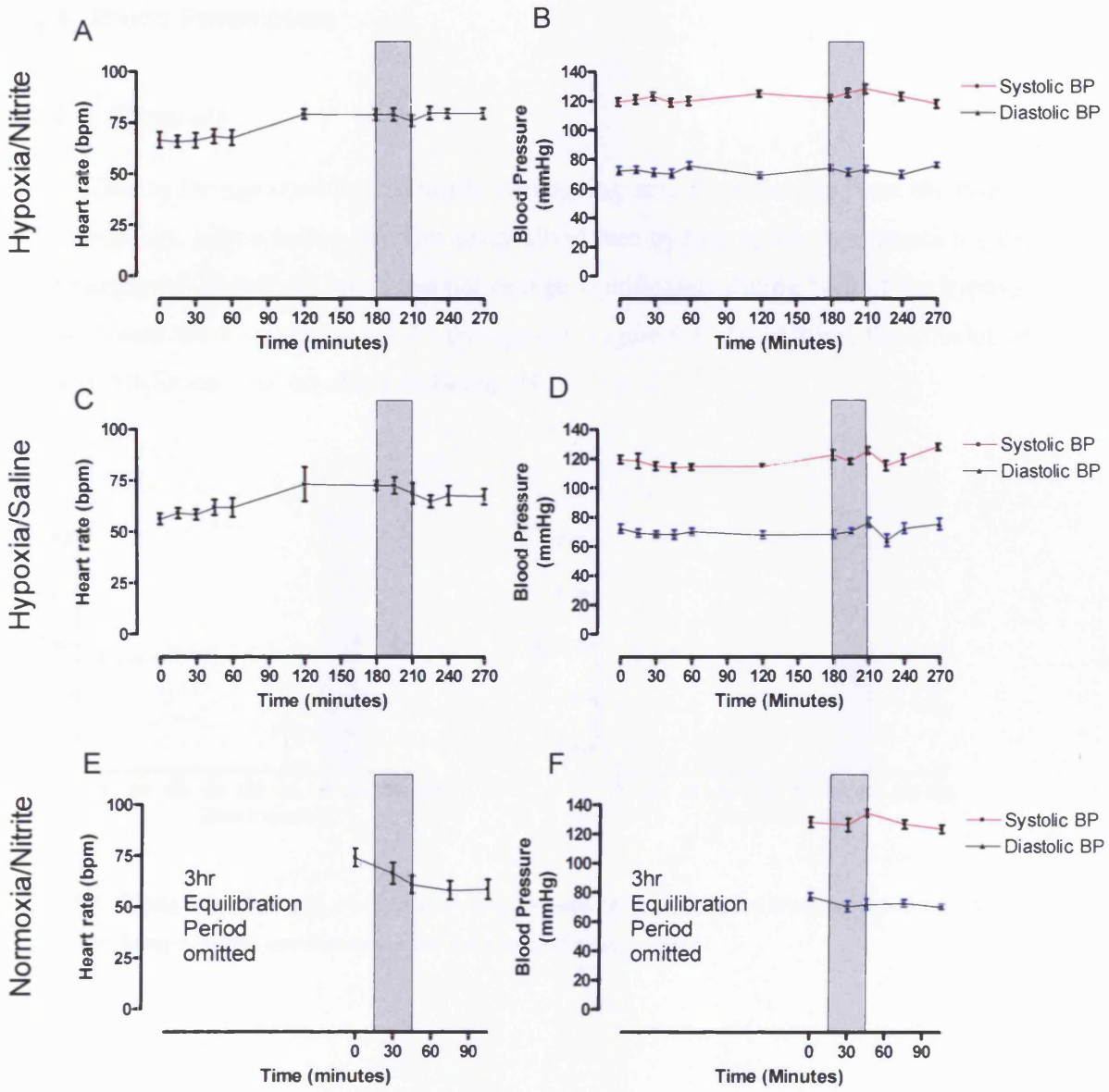

Figure 6.4. Haemodynamics. A) Hypoxia/nitrite heart rate (n=12) B) Hypoxia/nitrite blood pressure (n=12) C) Hypoxia/saline heart rate (n=6) D) Hypoxia/saline (n=6) E) Normoxia/nitrite (n=6) F) Normoxia/nitrite (n=6). Heart rate drops significantly during the normoxic protocol ($P<0.05$ 0 minutes vs. 75 and 105 minutes) but does not change significantly during infusion and increases significantly in both hypoxic protocols over the first 2 hours (hypoxia/nitrite $P<0.05$ 0 vs. 120 minutes) (hypoxia/saline $P<0.01$ 0vs. 120 minutes), no other significant changes in either protocol post 120 minutes. No significant changes in blood pressure (systolic or diastolic) during any protocol.

6.3.4 Blood Parameters

6.3.4.1 Blood pH

Due to the susceptibility of nitrite undergoing acid disproportionation, the effects of hypoxia and nitrite/saline infusion upon blood free hydrogen ion concentration (pH) were monitored. Blood pH levels did not change significantly during both of the hypoxic protocols and remained above pH 7.3 throughout (Figure 6.5). In addition, the infusion of saline or nitrite also had no effect on blood pH.

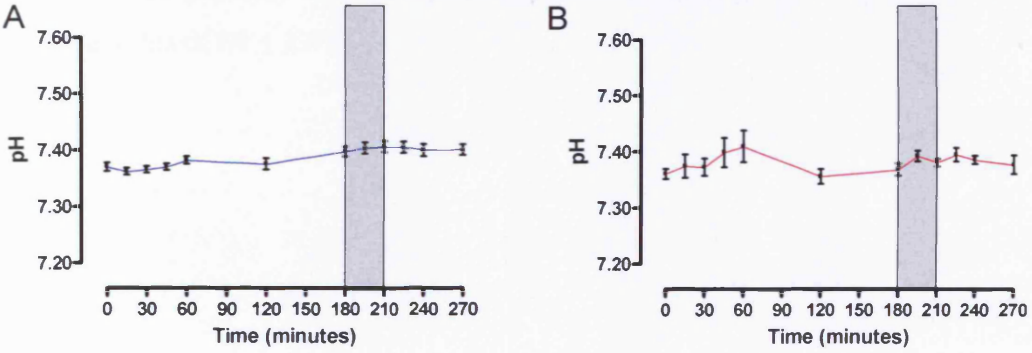

Figure 6.5. Changes in blood pH. A) Hypoxia/nitrite protocol (n=10) B) Hypoxia/saline protocol (n=4). No significant changes in pH were observed over the course of either protocol.

6.3.5 Blood Gas Analysis

All blood samples taken underwent blood gas analysis to monitor the result of hypoxic exposure. In both hypoxic protocols, venous pO_2 dropped from ~30mmHg to ~20mmHg after 60 minutes chamber exposure (Figure 6.6A and C). Once pO_2 levels had decreased (within 60 minutes), the level remained unchanged over the remaining period of the protocol. Similarly, capillary haemoglobin oxygen saturation decreased from 97.75 ± 0.2% to 87.67 ± 1.0% (0 vs. 60 minutes, $P<0.001$, minimum of 82.0 ± 2.4% at 180 minutes) during the hypoxia/nitrite protocol and from 97.83 ± 0.4% to 87.16 ± 0.6% in the hypoxia/saline protocol (Figure 6.6B and D). Venous haemoglobin saturation during the normoxia protocol did not significantly change throughout the protocol and had an average value of 69 ± 2.5%.

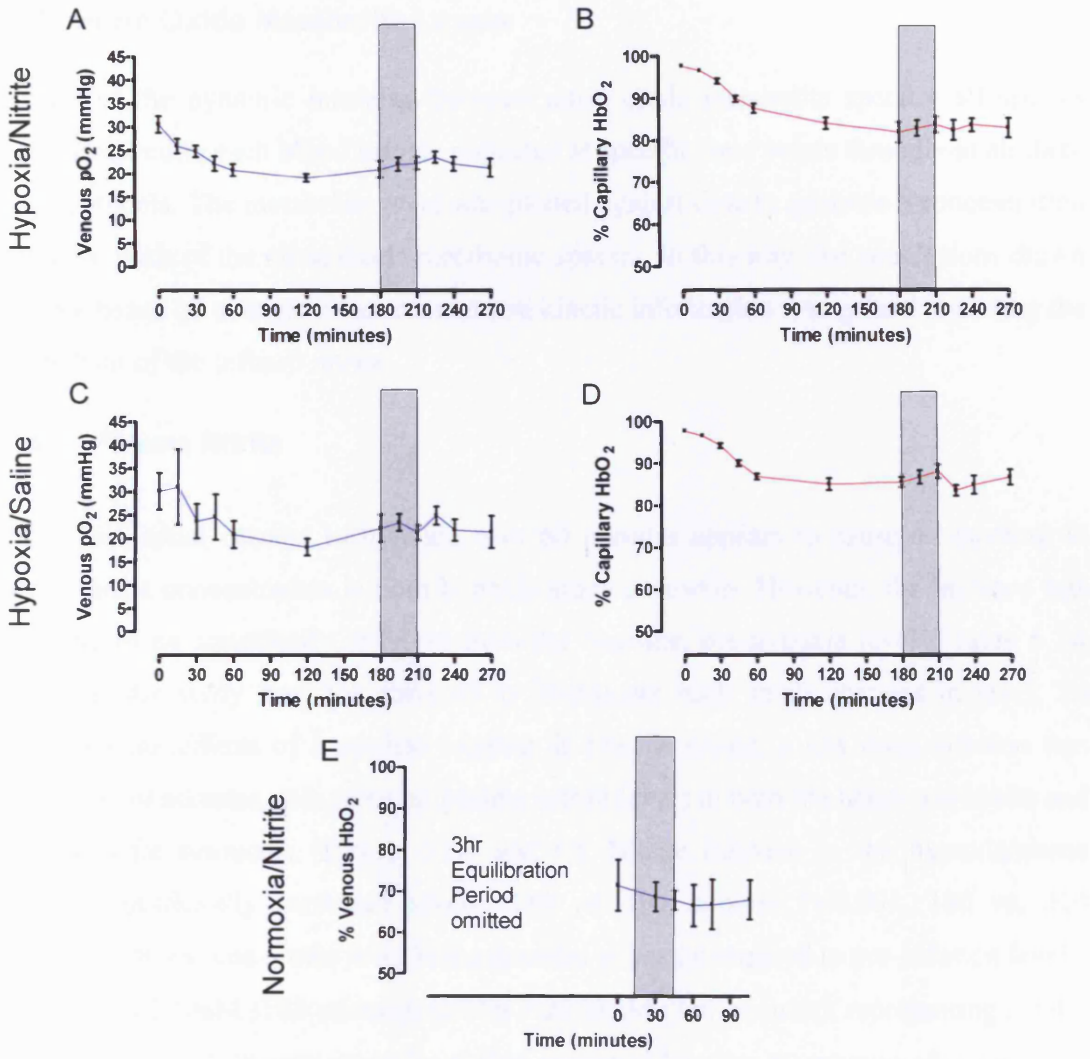

Figure 6.6. Changes in blood oxygen levels. A) Hypoxia/nitrite protocol, venous pO_2 levels (n=10). pO_2 level decreases significantly after 120 minutes and remains at this the decreased level over the remaining protocol (0 vs. 120-270 minutes $P<0.05$), B) Hypoxia/nitrite protocol, capillary haemoglobin oxygen saturation (n=12). Saturation levels decrease after 60 minutes and remain at a reduced level over the remaining protocol (0 vs. 60-270 minutes $P<0.05$) C) Hypoxia/saline protocol, venous pO_2 levels (n= 4). pO_2 level decreases significantly after 60 minutes and remains at this the decreased level over the remaining protocol (0 vs. 60-270 minutes $P<0.05$) D) Hypoxia saline protocol, capillary haemoglobin oxygen saturation (n=6). Saturation levels decrease after 45 minutes and remain at a reduced level over the remaining protocol (0 vs. 45-270 minutes $P<0.05$). E) Normoxia/nitrite protocol, venous haemoglobin oxygen saturation (n=6). No significant changes throughout protocol.

profile for each of the nitric oxide metabolite species. In this way, the conclusions dra were not based on a single measurement and kinetic information was gained regarding metabolism of the infused nitrite.

6.3.6.1 Plasma Nitrite

The initial descent to hypoxia over 60 minutes appears to cause an increase plasma nitrite concentration in both hypoxia study protocols. However, the increase v not found to be statistically different from the baseline, pre-hypoxia level (Figure 6. and C) as the study was not powered to investigate such small changes in level. investigate the effects of a modest increase in plasma nitrite, a low dose infusion v given over 30 minutes, this elevated plasma nitrite levels in both the normoxia/nitrite a hypoxia/nitrite protocols (Figure 6.7A and C). Nitrite infusion in the hypoxia/nitr protocol significantly increased levels (180 vs. 195 minutes $P<0.001$, 180 vs. 2 $P<0.001$) with plasma nitrite level being doubled at peak compared to pre-infusion leve from 191 ± 12.30nM (180 minutes) to 396 ± 26.38nM (210 minutes), representing a de change of ~205nM. Plasma nitrite level then returns to baseline 60 minutes after cessati of infusion (180 vs. 270 minutes, NS).

Plasma nitrite level in the normoxia/nitrite protocol is also increased significan with infusion (0 vs. 30 $P<0.05$ and 0 vs. 45 $P<0.001$) by an amount similar to t observed in the hypoxia/nitrite protocol, a change of 206.3 ± 36.6nM from baseline peak (0 vs. 45 minutes) (Figure 6.7C). As in the hypoxia/nitrite protocol, plasma nitr levels in the normoxia/nitrite protocol return to baseline within 60 minutes of infusi cessation (0 vs. 105 minutes, NS).

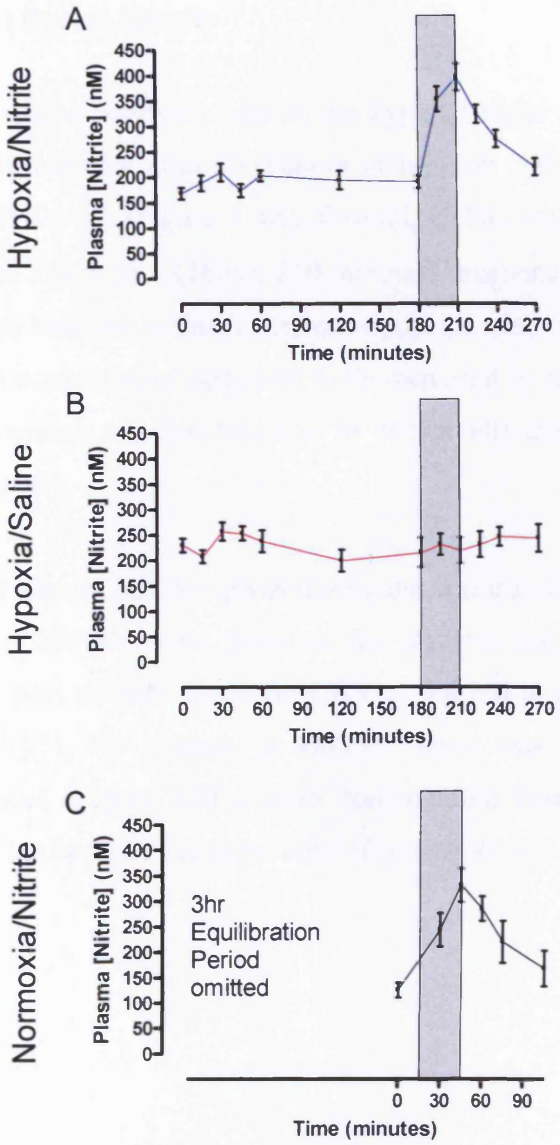

Figure 6.7. Change in plasma nitrite concentration in all three study protocols A) Hypoxia/nitrite protocol. Plasma nitrite increases significantly when infusion is given (180 vs. 195 minutes $P<0.001$, 180 vs. 210 minutes $P<0.001$) and returns to baseline 60 minutes after cessation of infusion (180 vs. 270 minutes, NS). Although the initial exposure to hypoxia itself seems to increase plasma nitrite levels the observation is not statistically significant (0 vs. 30 minutes and 0 vs. 180 minutes both NS) (n=12) B) Hypoxia/saline protocol. No significant changes throughout protocol (n=6). C) Normoxia/nitrite protocol. Plasma nitrite increase significantly with infusion (0 vs. 30 minutes $P<0.05$ and 0 vs. 45 minutes $P<0.001$) which returns to baseline within 60 minutes of infusion cessation (0 vs. 105 minutes, NS) (n=6).

6.3.6.2 Haemoglobin Bound Species

Infusion of low dose sodium nitrite in the hypoxia/nitrite protocol significantly increased HbNO levels, but only after 30 minutes of infusion (180 vs. 195 minutes NS, 180 vs. 210 minutes $P<0.001$) (Figure 6.8A). Overall, HbNO concentrations increased from 106 ± 19.2 nM to 237 ± 58.4 (180vs. 210 minutes). Interestingly, the level rapidly returned to pre-infusion baseline within 15 minutes post infusion cessation (180 vs. 225 minutes, NS). HbSNO concentration appeared to be increased by the infusion of sodium nitrite, however the increase was not found to be statistically significant (180 vs. 210 minutes, NS) (Figure 6.8B).

An identical 30 minute infusion given during the normoxia/nitrite protocol caused a moderate increase in HbNO levels, however the increase only became statistically significant 15 minutes post infusion cessation (28.9 ± 12.4 nM at time 0 vs. 96.6 ± 22.1 nM at time 60, $P<0.05$). No change in HbSNO level was observed during the normoxia/nitrite protocol (Figure 6.8F). Both haemoglobin bound species remained unchanged throughout the hypoxia/saline protocol (Figure 6.8C and D).

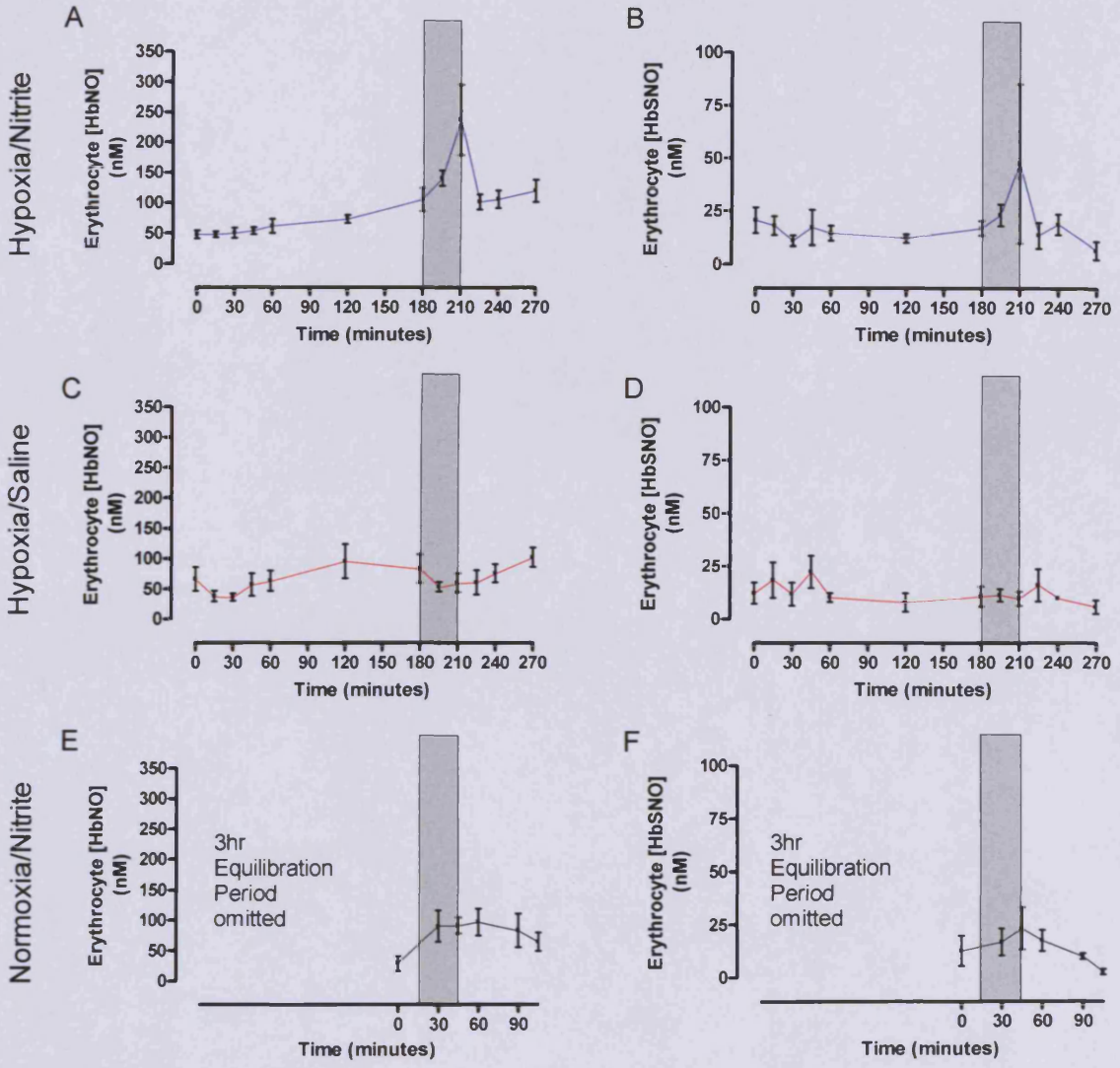

Figure 6.8. Changes in haemoglobin-bound species in all three protocol groups. A) Hypoxia/nitrite protocol, HbNO concentration levels. When nitrite infusion commences, levels increase but do not become significant until 30 minutes of infusion have been given (180 vs. 195 minutes NS, 180 vs. 210 minutes $P<0.001$) and then return to pre-infusion baseline within 15 minutes of infusion cessation (180 vs. 225 minutes NS) (n=12). B) Hypoxia/nitrite protocol, HbSNO concentration levels. Although they appear to rise during infusion, levels do not change significantly during the protocol (n=12). C) and D) Hypoxia/saline protocol, HbNO and HbSNO concentration levels. Levels of both species did not change significantly during the protocol (n= 6 in both cases). E) Normoxia/nitrite protocol, HbNO concentration levels. Levels do rise moderately but do not become significant until 15 minutes post infusion cessation (0 vs. 60 $P<0.05$) (n=6). F) Normoxia/nitrite protocol, HbSNO concentration levels. Levels do not change over the course of the protocol (n=6).

6.3.6.3 Plasma Nitrate and RSNO/RNNO

Levels of plasma nitrate did not change significantly during either of the hypoxia protocols (Figure 6.9A, C and E). Levels of plasma RSNO/RNNO did not change significantly during any of the three protocols (Figure 6.9B, D and F).

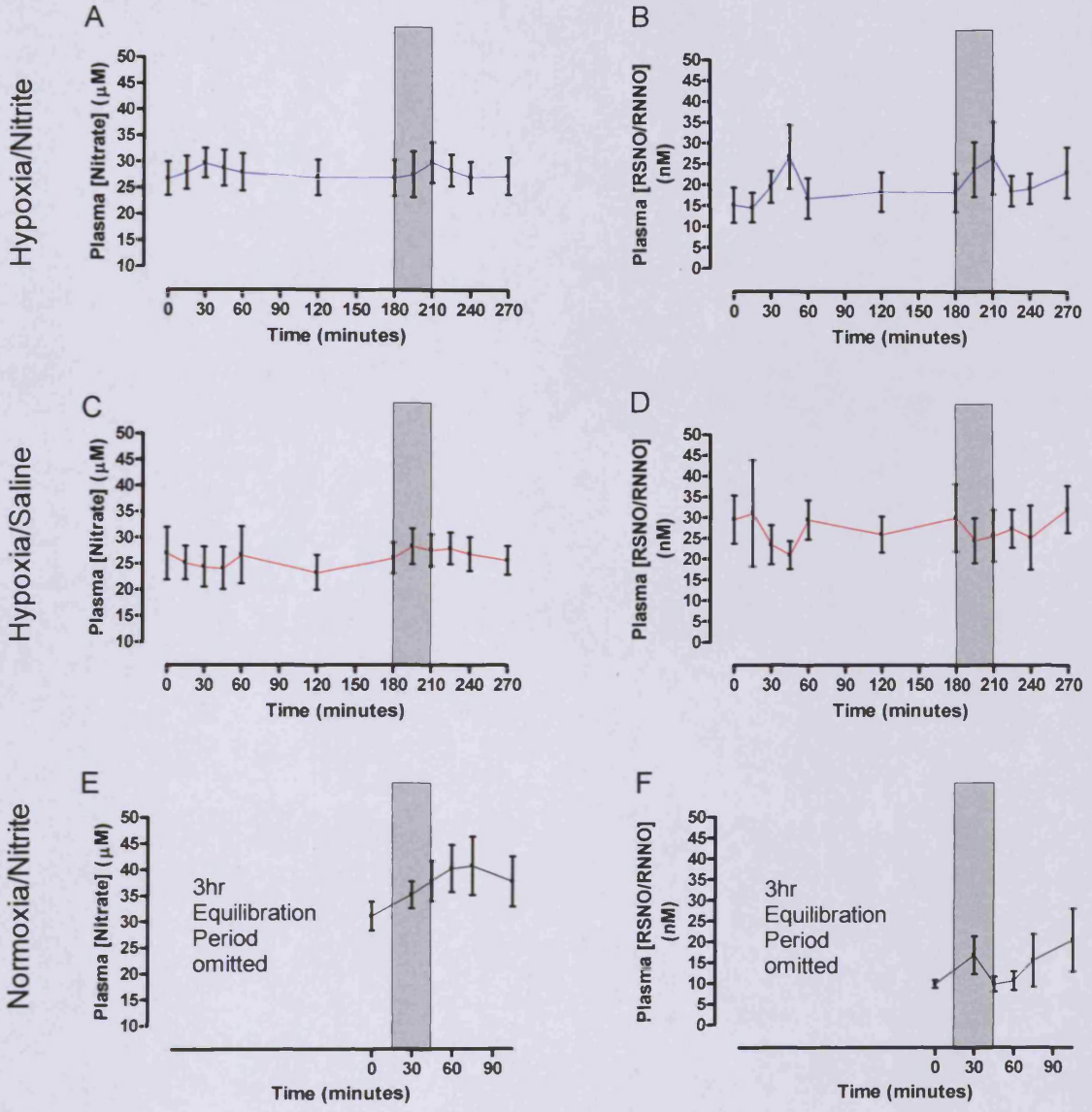

Figure 6.9. Changes in plasma nitrate and RSNO/RNNO concentration over all 3 protocols. A) Hypoxia/nitrite protocol, plasma nitrate concentration (n=12) B) Hypoxia/nitrite protocol, plasma RSNO/RNNO level (n=12) C) Hypoxia/saline protocol, plasma nitrate (n=6) D) Hypoxia/saline protocol, plasma RSNO/RNNO concentration (n=6) E) Normoxia/nitrite protcol, plasma nitrate concentration (n=6) F) Normoxia/nitrite protcol, RSNO/RNNO concentration (n=6). No statistically significant changes in nitrate or RSNO/RNNO concentration levels throughout any of the protocols.

6.3.7 Forearm Blood Flow

To assess the physiological effect of nitric oxide metabolite manipulation through nitrite infusion, forearm blood flow was measured at specific time points during all three study protocols. Infusion of sodium nitrite in the hypoxia/nitrite protocol increased forearm blood flow significantly (180 vs. 195 minutes, $p<0.05$. 180 vs. 210 minutes, $p<0.001$) (Figure 6.10A). The increased flow returned to baseline 60 minutes after cessation of infusion (180 vs. 270 NS). In both the hypoxia/saline and normoxia/nitrite protocols blood flow remained unchanged throughout (Figure 6.10B and C). Forearm blood flow was not statically different at the beginning of the protocol (0 minutes) or at the pre-infusion point (180 minutes) between groups.

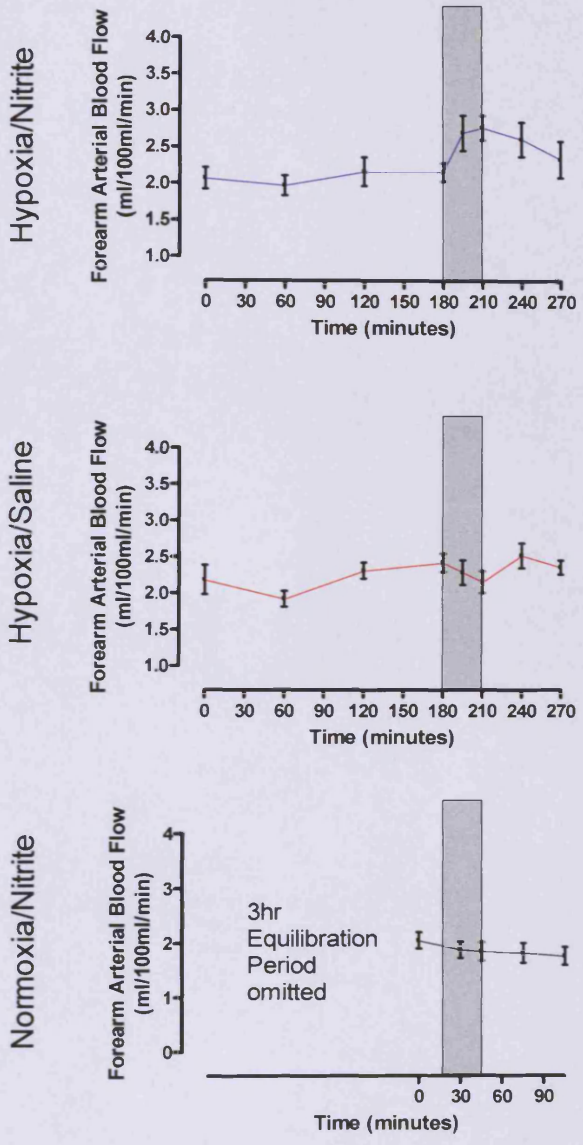

Figure 6.10. Changes in forearm arterial blood flow in all three protocols. A) Hypoxia/nitrite protocol. Forearm blood flow increased significantly during the infusion period (180 vs. 195 minutes, $P<0.05$ and 180 vs. 210 minutes, $P<0.001$) and returned to baseline 60 minutes after cessation of infusion (180 vs. 270 NS) (n=12). B) Hypoxia/saline protocol, no significant changes throughout protocol. (n=6) C) Normoxia/nitrite protocol. No significant change in forearm blood flow throughout protocol (n=6). No significant difference in forearm blood flow starting values (0 minutes) or pre-infusion (180/15 minutes) values between all groups.

6.3.8 Further Interpretation and Comparison

To compare the effect of sodium nitrite infusion on the dynamics of plasma nitrite level in hypoxia and normoxia, the profile of metabolites in each protocol was plotted (Figure 6.11). The profiles can be seen to be offset, as subjects who participated in the hypoxia protocol had a slightly elevated plasma nitrite pre-infusion value compared to their pre-hypoxia, normoxic baseline values and the levels exhibited by the normoxic controls, however a comparable hypoxia-induced elevation was also observed in those who participated in the hypoxia/saline protocol.

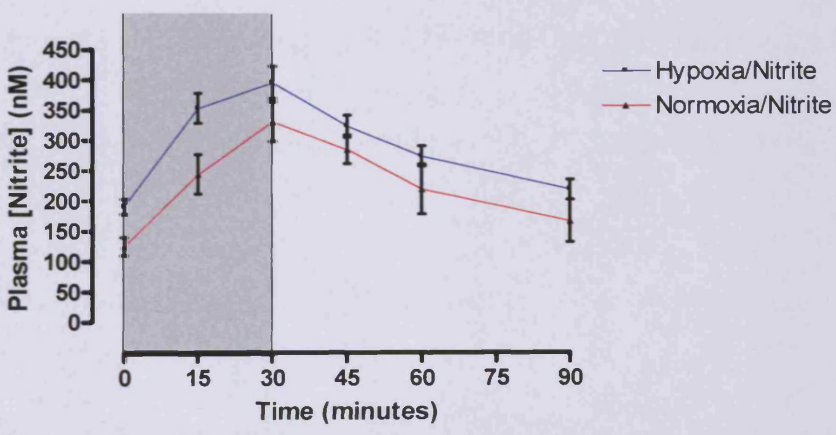

Figure 6.11. The time-corrected change in plasma nitrite level from commencement of sodium nitrite infusion to one hour post cessation of infusion in the hypoxia/nitrite and normoxia/nitrite protocols.

Interestingly, the half-life of plasma nitrite was calculated to be 21.7 ± 2.9 min in hypoxia and 21.3 ± 3.4 min in normoxia (Figure 6.12). This parallel can be seen visually by comparison of the profiles of plasma nitrite in normoxia and hypoxia that are similar, both in terms of elevation and decay (Figure 6.11).

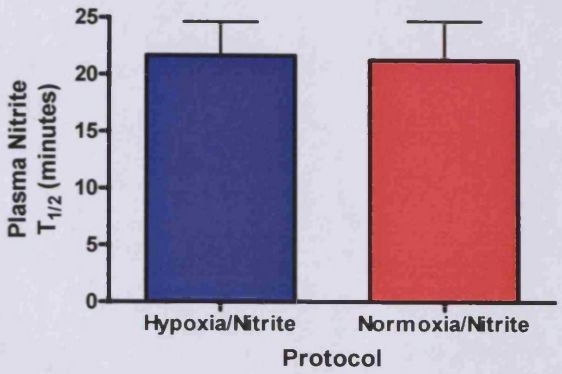

Figure 6.12. Plasma nitrite half-life post infusion cessation in both hypoxia/nitrite and normoxia/nitrite protocols. Hypoxia/nitrite average $T_{1/2}$ =21.7±2.9 minutes, (n=12). Normoxia/nitrite average $T_{1/2}$ =21.3±3.4 minutes, (n=6).

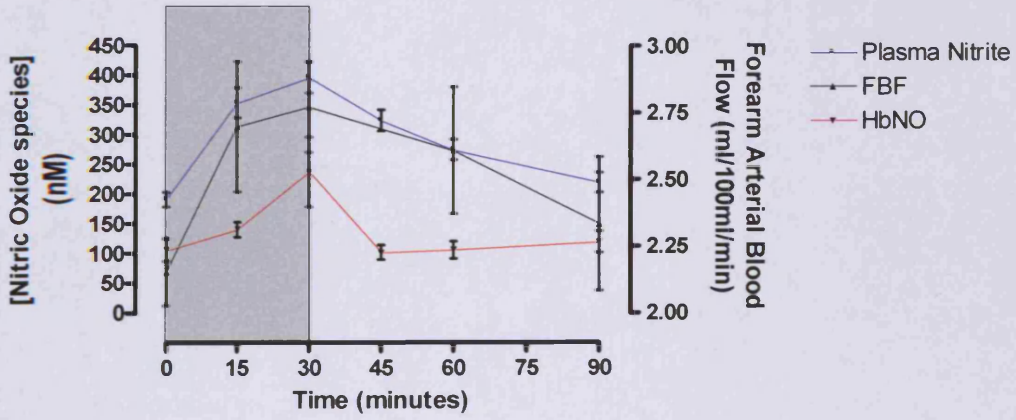

Figure 6.13. Profile of plasma nitrite, HbNO and forearm blood flow changes in the hypoxia/nitrite protocol during and 60 minutes post nitrite infusion.

To evaluate potential links between nitric oxide metabolites and altered physiology within a given protocol, the profile of change in each parameter was plotted together (Figure 6.13). In the hypoxia/nitrite protocol, the change in plasma nitrite concentration and change in forearm blood flow appear to follow a similar profile. Conversely, HbNO levels in the same protocol appear to rise in response to nitrite infusion, but only after a delay and then return to baseline upon cessation of nitrite infusion. The relationship between these parameters can be considered by correlating one against another (Figure 6.14) Plasma nitrite concentration correlates positively and significantly with forearm blood flow in the hypoxic environment (Pearson r= 0.43, $p<0.0001$) (Figure 6.14A) but not in the normoxic environment (Pearson r=0.032) (Figure 6.14C). In both the hypoxia/nitrite and the normoxia/nitrite protocols, HbNO levels did not correlate with forearm blood flow (Pearson r= 0.069, NS and-0.143, NS, respectively) (Figure 6.14B and D).

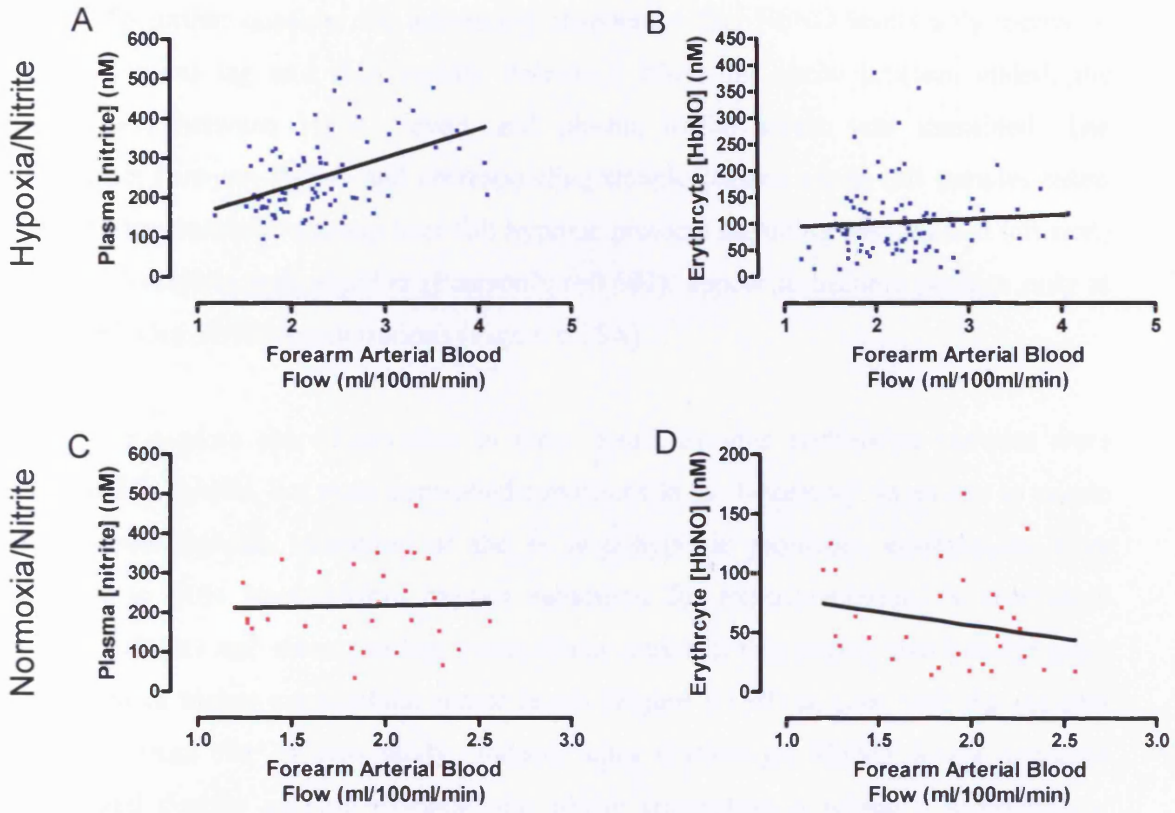

Figure 6.14. Correlations of nitric oxide metabolites with forearm blood flow in the hypoxia/nitrite and normoxia/nitrite protocols. A) Hypoxia/nitrite protocol, plasma nitrite concentration significantly correlates against forearm blood flow (Pearson's r=0.432, P<0.0001, n=93) B) Hypoxia/nitrite protocol, HbNO concentration does not correlate with forearm blood flow (Pearson's r= 0.069, NS, n=93) C) Normoxia/nitrite protocol, plasma nitrite concentration does not correlated with forearm blood flow (Pearson's r=0.032, NS, n=30) D) Normoxia/nitrite protocol, HbNO concentration does not correlated with forearm blood flow (Pearson's r=-0.215, NS, n=30).

To further consider the interesting observation that HbNO levels only increased after an initial lag and then rapidly decreased when the nitrite infusion ended, the relationship between HbNO levels and plasma nitrite levels was examined. The correlation between HbNO and corresponding sample plasma nitrite (all samples taken from the hypoxia/nitrite group over full hypoxic protocol including baseline and infusion) although correlate well together (Pearson's r=0.601), appear to become positive only at higher plasma nitrite concentrations (Figure 6.15A).

To explore this observation in more detail, *ex vivo* erythrocyte samples were subjected to similar, but more controlled conditions in the laboratory. In an aim to mimic venous haemoglobin saturation of the *in vivo* hypoxic protocols, erythrocytes were adjusted to 50% haemoglobin oxygen saturation for experimentation. A correlation between HbNO and corresponding extracellular nitrite in this setting also became more prominent at higher extracellular nitrite levels (Figure 6.15B) as seen with the samples collected from the *in vivo* study. Indeed, intra erythrocyte HbNO levels appeared unchanged despite varying extra-cellular nitrite concentration within a physiological range (50-200nM) (Figure 6.15B).

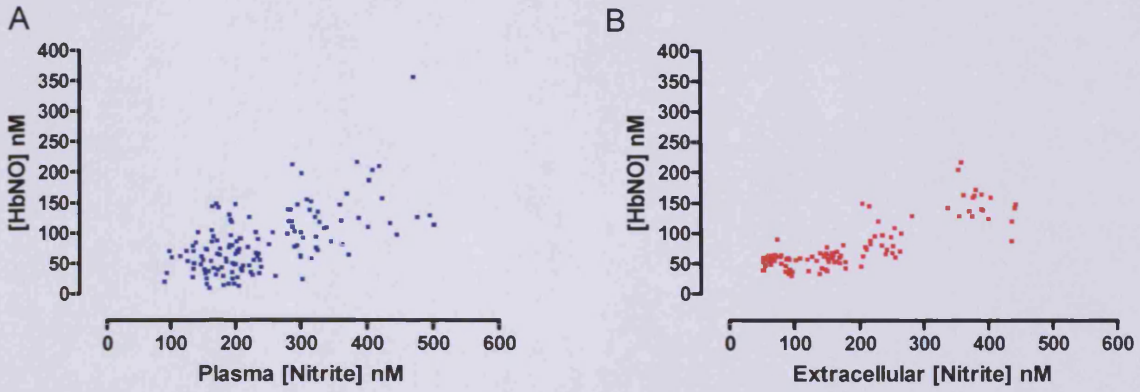

Figure 6.15. Correlations of plasma/extracellular nitrite concentration vs. corresponding HbNO concentration. A) Samples generated *in vivo* during the hypoxia/nitrite protocol (in hypoxia only) (Pearson's r=0.601, P<0.0001, n=137). B) Samples generated *ex vivo* after adjusting haemoglobin concentration to 10g/dL (10.3±0.17) and saturation to 50% (50.3±0.8) followed by incubation with varying extracellular nitrite concentrations (up to 850nM) for 30 mins at 37°C (Pearson's r=0.82, P<0.0001, n=103).

6.3.9 Forearm Blood Flow Observer Variation

To validate assessment of the strain gauge plethysmography data used to compare forearm arterial blood flow, a comparison of data analysed by one observer against a second observer (Figure 6.16A) and a comparison of two repeat analyses made by the same observer (Figure 6.16B) were made using Bland-Altman plots. The bias in both inter and intra observer measurements was minimal, however the inter-observer plot did show a trend for over estimation at higher average forearm blood flows by observer 2. All data presented in the chapter was therefore taken from observer 1, including the intra observer variability on repeat measures (Figure 6.16B), which shows very little bias.

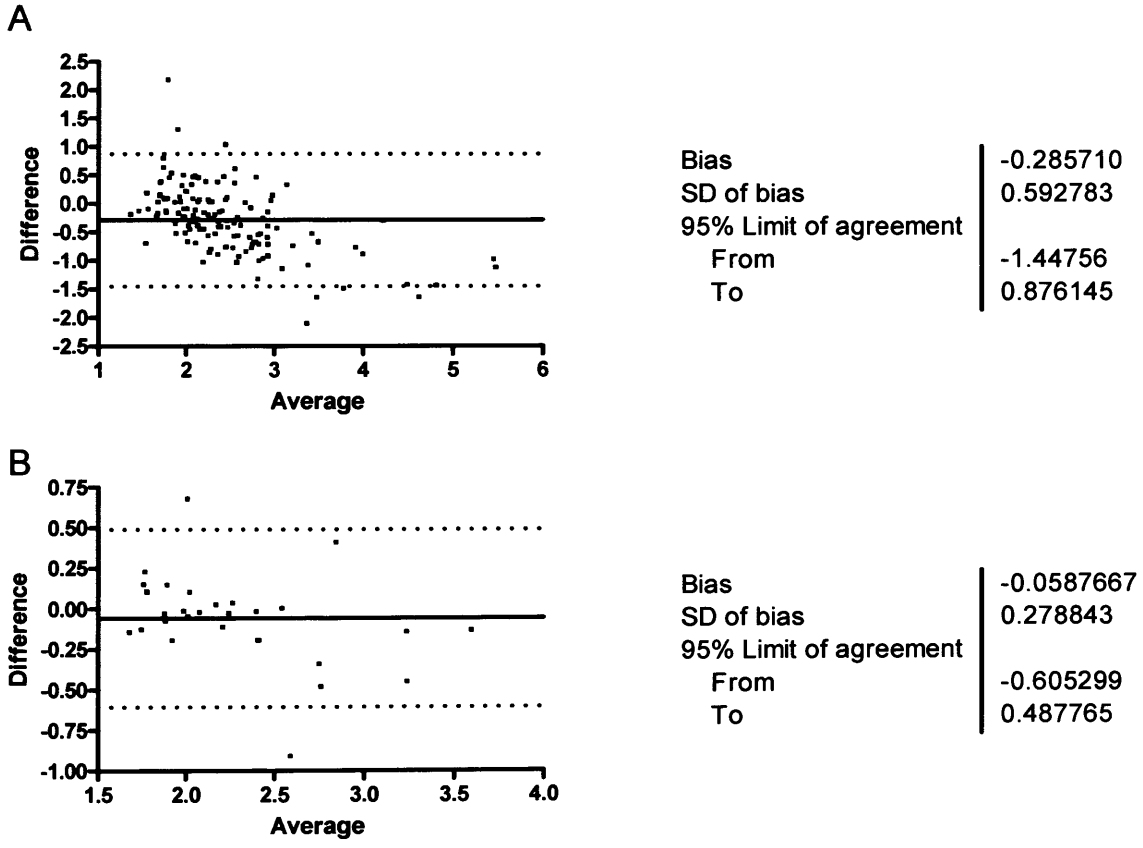

Figure 6.16. Bland-Altman plots of forearm blood flow measurements A) Inter-observer variability, bias between observer 1 and observer 2 is minimal but does show a trend at higher average blood flows, (n=140). B) Intra-observer variability, repeated measures made by observer 1, (n=30). Solid line from y-axis indicates bias level, broken lines indicate upper and lower 95% agreement limits.

effects of low dose nitrite supplementation in hypoxic human subjects and to consider mechanisms of nitrite processing at low, near basal plasma nitrite levels.

In comparison to the controlled *in vitro* experiments of a laboratory, completi study in human subjects can introduce considerable variability. The collection of accu data for this chapter required the testing protocols to be very controlled. Initially, pilot studies were completed to hone the protocols and develop data and sar collection. Efforts to accurately control variables of the experimental procedure cen on the hypoxic exposure and thus the environmental chamber. Unlike a face mask, environmental chamber provided a very effective method of producing contro hypoxia and allowed for a measured equilibration period for hypoxia indu physiological changes to take place. The importance of controlling the hypoxia expo with an equilibration period became apparent when the data was analysed. In additio the haemodynamic changes expected in hypoxia, nitric oxide metabolite levels appeared to be influenced. During the initial 60 minute descent from a FiO_2 of 21% 12% nitric oxide metabolite levels, plasma nitrite in particular appeared to transie increase before dropping to a stable, but elevated baseline prior to the infusion per Although the change did not reach statistical significance, as a result of the study being powered to assess these low level changes, a corresponding change in c metabolites suggests the result to be genuine. The exact mechanism behind observation is unclear, however it is possible that hypoxic exposure may influ vascular activity, which could occur at many signalling levels including transcription, as shown in chapter 4. In this respect, nitric oxide metabolite levels been shown to be increased in groups that inhabit high-altitude areas where atmosph oxygen levels are low compared to those living at sea level [451].

nitrite elevation and decay (half-life) was very similar in both the hypoxia/nitrite normoxia/nitrite protocols. This suggests that nitrite was processed at the same ra both cases, however, given that physiological effects are observed during hypoxia/nitrite protocol only, the route of processing or metabolism clearly mus different in the two settings.

The mechanism of nitrite-induced vasodilatation appears to be dependent on key factors: oxygen level and plasma nitrite concentration. Each of these factors wi discussed with respect to the current suggested mechanisms of activation and findin this chapter.

The activation of nitrite *in vivo* has thus far been attributed to one of two typ mechanism, those involving haemoglobin reduction [337] and those involving redu by tissue enzymes [397]. Nitrite reduction by haemoglobin *in vivo* is thought t maximal when haemoglobin is 50% saturated with oxygen [338]. Under norm conditions, the haemoglobin of circulating erythrocytes is unlikely to be de-saturat the 50% level, therefore this route of metabolism and potential source of nitric o generation is improbable. In contrast, when subjects are exposed to hypoxia, the lev inspired oxygen could potentially drop sufficiently for a proportion of 50% ox saturated haemoglobin to occur at specific points in the circulation. Consequently, mechanism of nitrite reduction could be up-rated in hypoxia and thus may pro sufficient nitric oxide to alter vessel tone, in apparent agreement with the finc presented in this chapter. However, the same is true of the oxidoreductase enz mechanism. In situations where oxygen is plentiful, oxidoreductase enzymes, suc aldehyde oxidase and xanthine oxidase will catalyse reactions between their no substrates, conversely, in hypoxic conditions these enzymes will more readily catalys

work carried out with very high dose nitrite infusion and is based on the assumption increases in HbNO levels are a marker of nitrite interaction with haemoglobin and potential reduction of nitrite to nitric oxide [337, 379]. The findings of these publicat show that plasma nitrite, HbNO and changes in blood flow all correlate well with another after high, incremental nitrite dosing.

A potential role for erythrocytes has previously been implicated from observation that plasma nitrite and HbNO correlate with one another during infus rather than from a measure of erythrocyte produced nitric oxide. The findings of chapter using low dose nitrite infusion show a clear correlation between plasma ni and forearm blood flow, but fail to find the same association with HbNO levels. In although HbNO levels did increase during infusion after a delay, levels returne baseline within 15 minutes of infusion cessation while elevated plasma nitrite levels increased forearm blood flow persisted. This is surprising, given that haemogl saturation remained low, plasma nitrite levels remained high and the results of chapt show nitrite entry to erythrocytes to be enhanced at high extracellular nitrite levels low oxygen saturation. Taken together, this suggests that haemoglobin reduc mechanisms involved with high dose nitrite may not be mirrored at low dose and imp that HbNO is not a good surrogate for haemoglobin nitrite reduction at low ni concentrations.

In support of a role for haemoglobin nitrite reduction, the same research group h suggested that erythrocytes act as circulating stores of nitrite, reporting that i erythrocyte nitrite to represent two thirds of whole blood nitrite [364]. Despite sim measured plasma nitrite levels and the use of similar methodology, our laboratory previously demonstrated a plasma nitrite to erythrocyte associated nitrite signal ratio

ferricyanide/hexacyanoferrate, commonly a potassium salt) to erythrocyte samples. T is thought to bind haemoglobin and stabilise nitric oxide metabolite species, prevent inter-conversion, so samples can be processed and stored for future analysis. Howe ferricyanide is known to cleave nitric oxide from HbNO and has been used in t capacity for quantitative HbNO assays [452]. In addition, it has previously been sho that nitric oxide cleaved from HbNO subsequently forms nitrite [367]. It therefore follo that in erythrocyte samples incubated with 'stabilisation solution', HbNO would converted to nitrite and therefore account for the exaggerated intra-erythrocyte nit levels observed by these investigators, together with expected plasma nitrite levels.

The results of Chapter 3 demonstrate that nitrite entry at physiological extracellu nitrite levels (~200nM) is a relatively slow process, particularly at high haemoglo oxygen saturation. This chapter finds both *in vivo* and *in vitro* in deoxygenated cont blood samples that HbNO formation (thought of as a marker of nitrite haemoglo interaction), does not occur appreciably when extracellular/plasma nitrite levels are lo However, as extracellular nitrite levels are augmented, the amount of HbNO does th increase in a uniform fashion. In the *in vivo* study, HbNO levels did increase dur nitrite infusion but only after a delay and returned to baseline within 15 minutes infusion cessation. The delay in formation agrees with the slow entry rate of nitrite a the rapid decrease post infusion cessation is most likely due to metabolism of HbNO haemoglobin is oxygenated across the lung [332]. It should be noted that this delay increase is not simply a methodological issue. Pre-infusion HbNO levels in hypoxia/nitrite protocol were ~100nM and chapter 3 of this thesis demonstrates that t methodology utilised is capable of distinguishing changes in HbNO at a le considerably below this.

The fact that nitrite entry into erythrocytes is slow and that HbNO levels do not appear to correlate with changes in physiology, suggests that the haemoglobin nitrite reduction mechanism does not play a significant role in the vascular effects of nitrite at low, near-basal plasma nitrite levels. It is therefore more likely that the vasoactive properties of nitrite at low dose are mediated via reduction in the tissue. This agrees with the work of chapter 4 in a isolated vessel model, where vasorelaxation was independent of haemoglobin and appeared to be considerably mediated by aldehyde oxidase. The extrapolation of findings, generated under non-physiological conditions with high dose infusion, to the physiological setting is a problem that is prominent within the nitric oxide field. The data presented in this chapter suggests this extrapolation not to be valid and, even if haemoglobin was capable of reducing nitrite to nitric oxide, its ability to escape recapture by haemoglobin to affect vessel tone is still questionable.

It is clear that circulating plasma nitrite is involved in the flux of nitric oxide moiety, whether basal levels of plasma nitrite play a role in controlling vessel tone *in vivo* remains to be demonstrated. However, the findings of this chapter would suggest that activation of nitrite in the circulation by haemoglobin is unlikely to play a significant role at these lower plasma nitrite levels. Importantly, it seems that nitrite even at lower doses does appear to be capable of altering vascular tone given a provoking environment (e.g. hypoxia). Its potential as a therapeutic agent relies upon its ability to only act in tissues where it is required. This targeted action could be of great benefit in pathologies where there are specific sites of poor blood flow or hypoxia in which nitrite could improve regional blood flow without causing dramatic changes in global haemodynamics i.e. cause hypotension.

6.5 Summary and Conclusions

The work of this chapter does not discount that circulating nitrite may interact slowly with blood and vascular tissue, contributing to the overall flux of nitric oxide metabolites. However, it does question the mechanisms by which nitrite can change vascular tone, particularly in the physiological plasma nitrite concentration range. Some interesting and perhaps unexpected findings have also been revealed through this work and specific points are summarised below.

- The infusion of low dose sodium nitrite is capable of altering arterial forearm blood flow in hypoxia but not normoxia suggesting an enhancement of nitrite vasoactivity under hypoxic conditions.

- The elevation and decay profiles of infused nitrite are similar in normoxia and hypoxia. This is unexpected, given that nitrite uptake by erythrocytes was enhanced under deoxygenated conditions (chapter 3), suggesting that metabolism of low dose nitrite by erythrocytes plays a very minor role in overall nitrite metabolism.

- In hypoxia, forearm blood flow correlates with plasma nitrite level but not with HbNO levels advocating a minimal role for erythrocytes in nitrite activation.

- Both *in vivo* and *vitro* in deoxygenated blood, intra-erythrocyte HbNO does not appear to be formed to any great extent at basal plasma nitrite levels.

- The haemoglobin-dependent mechanisms of nitrite-induced vasodilatation thought to be involved at high dose do not appear to be involved at low or basal plasma nitrite levels.

- The vasoactive properties of nitrite at low plasma nitrite levels are most likely mediated via tissue enzymes.

- Therapeutically, high dose nitrite is not required to produce a vasodilatory effect in hypoxic vasculature.

7 Overall Conclusions & Insights

The aims of this thesis were to consider nitric oxide metabolites in terms of their biochemistry and physiological actions with particular reference to the effect of oxygen upon these biological aspects. The thesis also aimed to challenge the current understanding with respect to the related mechanistic pathways involved. The results presented herein provide insight into a number of mechanisms that relate to nitric oxide metabolite bioactivity and have discovered novel aspects of signalling pathways and the molecules that control them.

It is apparent that nitric oxide metabolites represent a dynamic group of molecules that are, at some level, bioactive. Despite the vast amounts of research in the field, this work has shown me that a number of common misconceptions and extrapolations regarding the chemistry and biological activity of these metabolites exist within the literature, particularly with respect to mechanisms of action. The field has run away with some suggested mechanisms that are in essence theoretical with very little basic science foundation. The repeated publication of these suggested modes of operation has occurred so extensively and over such a lengthy period of time that one could be mistaken in thinking that they had been confirmed and are thus fact. It is therefore imperative that findings regarding any aspect of nitric oxide metabolite behaviour be considered in the context of the experimental setting utilised. In this respect, one can essentially divide the study of nitric oxide metabolites into two groups based upon the concentration of metabolites in question, i.e. assessing the biological aspects of nitric oxide metabolites at a basal level or in scenarios where levels have been artificially increased. Obviously the *in vivo* mechanisms involved in the metabolism and biological activity of metabolites do not obey this divide and most likely operate in a continuum, however, it is these settings in which the metabolite field pursues answers and therefore the context in which results must be contemplated.

At basal levels, it is unclear as to whether or not nitric oxide metabolites possess any functional roles. A huge literature exists surrounding the actions of plasma nitrite at a

this thesis that the nitric oxide metabolite pool is in a continual state of flux, with leve any one metabolite heavily influenced by both the local level of other metabolites local changes in oxygenation. These findings are in close agreement with previous v from our laboratory showing that although individual metabolite species change ac the coronary circulation, there is no global change in metabolites, just oxygen depen reapportionment. Under basal conditions, the circulating level of nitrite appears to stable and controlled, with the plasma nitrite level of subjects studied in chapte showing very little variance at baseline, for example. In relation, when erythrocytes v exposed to extracellular nitrite in the physiological range very little nitrite entered cells, however the rate of entry was found to be oxygen dependent. Nitrite does have potential to cause a number of additional side effects at a cellular level, particularl high concentration, via disruption of ionic balance or in the case of the erythroc methaemoglobinaemia. Therefore, it is perhaps not surprising that plasma levels are l within a tight range and specific cell types are resistant to extreme nitrite influx. In opinion, the field to date has focused too heavily upon the direct actions of nitric ox and its metabolites rather than the molecules, such as oxygen, that influence so m aspects of their biology.

The involvement of the erythrocyte in nitric oxide metabolite biochemistry still repres a contentious element of the nitric oxide field, particularly in regard to the functior haemoglobin. Haemoglobin has been implicated in two distinct ways, firstly as a nit reductase and secondly as a form of stored/bioactive nitric oxide, HbSNO. Both of th proposed mechanisms are dependent upon oxygenation or haemoglobin allostery generate a species that can alter vessel tone, thereby theoretically matching respirat demand with blood flow at regional level. As with many systems of nature, it ma perfect sense to link these two factors that are ultimately dependent upon one another. is the case with most good theories, these are particularly difficult to question or oppo

during the low dose nitrite infusion. Taken together, with the fact that nitrite er
erythrocytes is limited, it would suggest that although nitrite reduction by haemo
may be energetically possible, a functional role for this mechanism seems improba
should be noted, however, that these observations were gained through manipu
nitrite concentration level, although in the case of the *in vivo* study, very modestl
within the physiological range.

To date, the erythrocyte induced hypoxic vasodilatation has been largely ascribed
actions of HbSNO. However, conclusive evidence demonstrating HbSNO to b
mediator involved has thus far not been published. There is no doubt that HbSNO, l
SNO compounds, does have vasoactive properties. Nonetheless, a number of aspe
the erythrocyte induced vasorelaxation do not support a role for HbSNO. This th
the first to document significant and persuasive evidence to suggest that the media
the erythrocyte induced vasorelaxation is in fact oxygen. A role for oxygen as the pr
mediator of this phenomenon can be reconciled with the majority of current literatu
will hopefully form the basis of another 'good theory' that researchers will contir
question. These findings do not discount a role for HbSNO, but do suggest that it
the principal mediator involved. There are, however, many potential roles for thiol b
nitric oxide *in vivo*, not only from a vasodilatory point of view but from a signallin
As with many protein modifications, such as phosphorylation, the significance of
oxide thiol modification is coming to fruition. With thousands of potential target thi
a vast range of proteins from membrane receptors to transcription factors, it is clea
the potential signalling and regulatory roles of SNOs are huge and not just limited a
in the vascular setting.

Certain aspects of work piloted during the compilation of this thesis unfortunately
not be fully developed. One particular subsection of work that I believe warrants fu

investigation surrounds the application of the models developed and novel findings of this thesis to research the disease, diabetes mellitus. Diabetes mellitus represents a very interesting setting in which to study nitric oxide metabolites and erythrocytes as the disease progression is known to dramatically affect the cardiovascular system and to modify haemoglobin through glycosylation. Some initial findings are summarised below;

- Preliminary findings from the real time nitric oxide reaction model show that the haemoglobin of diabetic patients reacts with and processes nitric oxide differently, depending upon the level of haemoglobin glycosylation (HbA1c).

- Chapter 5 demonstrated that the erythrocyte induced hypoxic vasodilatation was dependent upon haemoglobin allostery. As haemoglobin oxygen binding is altered in diabetics, erythrocyte induced vasorelaxation was assessed with a range of diabetic blood samples. When tested, it appears that diabetic erythrocytes may give a reduced response in the hypoxic myography model that correlates with the degree of haemoglobin glycosylation (HbA1c). It is not clear if this is a direct haemoglobin oxygen offloading effect or if HbA1c is purely a marker of other diabetes induced changes within the erythrocyte.

- Another key aspect of this thesis has focused on the processing of nitrite by erythrocytes. Using some simple experiments where whole blood was doped with nitrite before centrifugation over a range of speeds it appears that nitrite does not partition uniformly between plasma and erythrocyte fraction when comparing control blood against blood form a diabetic patient. This could be due to differences in membrane oxidation between diabetics and controls but could have implications with regard to nitrite bioactivity. In a separate experiment using nitrite and erythrocyte ghosts, it appears that there is also a source of nitrite reduction within the erythrocyte membrane. If nitrite is more likely to associate with the erythrocyte in some cases this could provide an enhanced route for the conversion of nitrite to more biologically active species.

A direct role for nitric oxide metabolites as clinical therapeutics seems quite limited. Nitrite carries the most potential but may only show real benefit above and beyond existing treatments in very niche settings where tissue hypoxia is a key element. The true value of nitric oxide metabolites or the erythrocyte-induced hypoxic vasodilatation may ultimately come from more indirect manipulative processes. To achieve this and exploit these systems in full, it is essential that mechanisms of both molecular bioactivity and system dysfunction in disease states are fully explored. The results of this thesis contribute to the understanding with regards to mechanism, however the next step is to find a way of exploiting this novel information in the disease setting.

Overall, it is clear that the diatomic gases are not only important signalling molecules but can also dictate the responsiveness of many enzymes and tissue systems. However, it is oxygen that governs the formation, metabolism and biological activity of nitric oxide metabolites, is responsible for erythrocyte induced hypoxic vasodilatation and which ultimately dictates the health of all tissues, including those of the cardiovascular system. In this sense, it is not surprising that haemoglobin can also play an important role, but the results of this thesis would point to that being one of transportation and storage of gaseous diatoms, rather than an enzymatic converter or reductase. Nitric oxide is a very important signalling molecule that has duly received a significant amount of attention, however I conclude that it is the somewhat neglected grandparent of the family, oxygen, which provides fundamental control and is a prerequisite of signalling in this setting.

7.1 Publications

Text Book Chapters

Pinder AG, Roger SC, Khalatbari A, Ingram TE, James PE. The measurement of nitric oxide and its metabolites in biological samples by ozone based chemiluminescence. Chapter 2, Redox-Mediated Signal Transduction. Humana Press, Methods Mol Biol. 2009;476:10-27.

Peer Reviewed Publications

Pinder AG, Rogers SC, Morris KM and James PE. Haemoglobin saturation controls the red blood cell mediated hypoxic vasorelaxation. *Adv Exp Med Biol. 2009;645:13-20.*

Ingram TE, **Pinder AG**, Milsom AB, Rogers SC, Thomas DE, James PE. Blood vessel specific vasoactivity to nitrite under normoxic and hypoxic conditions. *Adv Exp Med Biol. 2009;645:21-5.*

Pinder AG, James PE. When does low oxygen become hypoxia? Implications for nitrite reduction. *Circ Res. 2009 Feb 13;104(3):e25-6.*

Pinder AG, Pittaway E, Morris K, James PE. Nitrite directly vasodilates hypoxic vasculature via nitric oxide dependent and independent pathways. *British Journal of Pharmacology, 2009 Aug;157(8):1523-30.*

Ingram TE, **Pinder AG**, Bailey DM, James PE, Fraser AG. Low-dose sodium nitrite produces sustained pulmonary vasodilation in hypoxic human subjects. *AJP, 2009 (In Press).*

Pinder AG, Ingram TE, Bailey DM, Morris K, James PE. Elevated Plasma nitrite causes vasodilation of hypoxic, but not normoxic vessels: a role for the red cell? *2009 (submitted).*

Pinder AG, Rogers SC, James PE. Oxygen mediates the erythrocyte induced vasorelaxation in hypoxic vasculature, *(manuscript in preparation).*

Published Scientific Abstracts and presented Abstracts.

Pinder AG, Rogers SC, Morris KM and James PE. Haemoglobin saturation controls the red blood cell mediated hypoxic vasorelaxation. The 35th meeting of the International Society of Oxygen Transport to Tissue. August 26-30. 2007. Uppsala, Sweden. *Adv Exp Med Biol. 2009;645:13-20.*

Ingram TE, **Pinder AG**, Bailey DM, Fraser AG, James PE. Low dose IV nitrite dilates both peripheral and central vessels in hypoxia - a potential new therapy for ischaemia. Presented at the British Cardiovascular Society Annual Scientific Conference 2-4 June 2008 Manchester. *Heart 2008; 94(S1):A83*

Ingram TE, **Pinder AG**, Milsom AB, Rogers SC, Thomas DE, James PE. Blood vessel specific vasoactivity to nitrite under normoxic and hypoxic conditions. The 35th meeting of the International Society of Oxygen Transport to Tissue. August 26-30. 2007. Uppsala, Sweden. *Adv Exp Med Biol. 2009;645:21-5.*

James PE, Maher AR, Milsom AB, Rogers SC, **Pinder AG**, Gunaruwan P, Frenneaux MP. The vascular response to exogenous nitrite in humans under normoxia and hypoxia. The 35th meeting of the International Society of Oxygen Transport to Tissue. August 26-30. 2007. Uppsala, Sweden.

Pinder AG, Pittaway E, Rogers SC, Morris K, James PE. Nitrite dependen independent hypoxic vasorelaxation: a mechanistic insight. Presented at the UK forum, London, UK, August, 2008.

Ingram TE, **Pinder AG**, Pittaway E, Fraser AG, James PE. Nitrite associated hy vasodilatation in man: The pulmonary circulation is more susceptible to the vasoc effects of nitrite than the systemic circulation. Presented at the 5th International Oxide Conference, Bregenz, Austria, August, 2008. *Nitric Oxide, Volume 19, Suppl 1, 2008, Page 38.*

Pinder AG, Ingram TE, Bailey DM, Fraser AG, James PE. Low dose systemic infusion to healthy human subjects in a hypoxic environmental chamber. Presented 5th International Nitric Oxide Conference, Bregenz, Austria, August, 2008. *Nitric O Volume 19, Supplement 1, 2008, Page 55.*

Pinder AG, Pittaway E, Morris K, James PE. Haemoglobin-independent Pathway Nitrite Induced. Vasorelaxation. Present at Festschrift Symposium for Prof. Broadley, Autonomic, Respiratory and Cardiovascular Pharmacology Sept. *Autonomic and Autocoid pharmacology, volume 29, 2009, page 105.*

2008.

Pinder AG, Pittaway E, Rogers SC, Morris K, James PE. Nitrite dependent independent hypoxic vasorelaxation: a mechanistic insight. Presented at the UK forum, London, UK, August, 2008.

Pittaway E, **Pinder AG**, Morris KM, James PE. Haemoglobin-independent hypoxic vasodilatation. Presented at the UK NO forum, London, UK, August, 2008

Pinder AG, Ingram TE, Bailey DM, Fraser AG, James PE. Low dose systemic infusion to healthy human subjects in a hypoxic environmental chamber. Presented 5th International Nitric Oxide Conference, Bregenz, Austria, August, 2008. Presented at the UK NO forum, London, UK, August, 2008.

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