

The Significance of a Nitric Oxide Reserve and its Utilisation in the Human Circulation

by

Mr Stephen Colin Rogers

B.Sc. (Hons) Exercise and Sports Science, University of Exeter
M.Sc. Exercise Physiology, Liverpool John Moores University

Submitted for

Philosophiae Doctor degree

Department of Cardiology
Wales Heart Research Institute
School of Medicine
Heath Park
Cardiff University
Cardiff
CF14 4XN

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*This thesis is dedicated to my family, Graham Rogers and Sue Rogers and
Gerald Rogers.*

*And to the memory of my mother
Ann Rogers*

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British Heart Foundation

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SUMMARY

Historically nitric oxide (NO) was viewed as playing a purely paracrine role in the regulation of vascular tone, acting within the vicinity of its release. Reactions of NO in blood were consequently assumed only to scavenge and inactivate NO, thus regulating localised vasodilatation. However, this view of NO metabolites changed following the emergence of an endocrine hypothesis proposing that blood borne NO metabolites might conserve bioactivity allowing for the storage, transport and potential release of NO far from its location of synthesis. Today an endocrine role of blood borne NO metabolites is now widely accepted, although the species accounting for the vascular regulatory function still remains in contention. This current status mainly reflects controversies and difficulties associated with both the measurement and the handling of biological samples containing dilute metabolite concentrations.

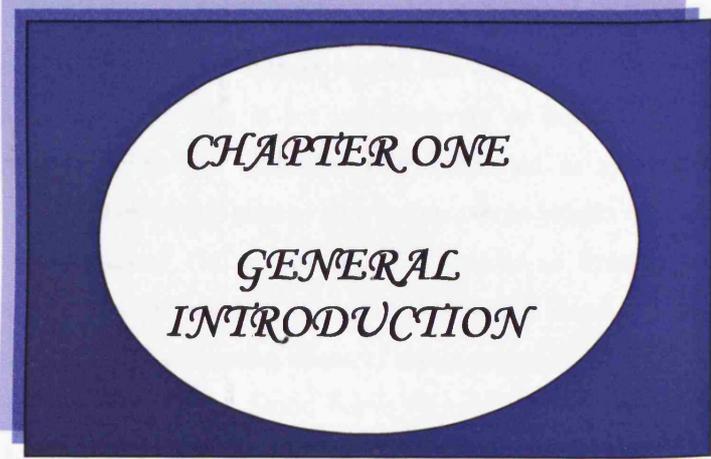
The original aim of this thesis was to analyse the two main NO metabolite hypotheses (i.e., SNO-Hb and nitrite hypotheses) to provide a more comprehensive understanding of their potential significance in the human circulation. The rationale was based on making biochemical measurements in combination with parallel assessments of physiological changes in the human vasculature. Initially the focus was on developing methods capable of measuring baseline levels of NO metabolites in human blood. During this work a major confounding factor was identified influencing the measurement of NO attached to red blood cells/haemoglobin, namely the auto-capture of NO by cell free haemoglobin in the reaction chamber. A means was developed to overcome this via the modification of one of the most commonly used assay reagents in the NO metabolite field. Subsequently this modified reagent was used along with several other methods to analyse NO metabolites in human blood. Ultimately however, levels of SNO-Hb measured at baseline were on the border of methodological sensitivity, although haemoglobin-bound NO was clearly distinguishable (and found to increase at lower oxygen). Consequently my work focussed on the potential of nitrite versus haemoglobin-bound NO per se to act as endocrine NO metabolites in the human vasculature. Nitrite metabolism in arterial and venous human whole blood was analysed whilst the vasodilatory properties of nitrite were investigated to assess whether this species could account for the relaxation response observed from native red blood cells under hypoxic conditions. Finally for the first time ever NO metabolites were simultaneously measured across the coronary and pulmonary vascular beds in the human circulation to address the potential for NO metabolites (including nitrite) to regulate vascular tone at baseline and under conditions of increased oxygen demand.

ABBREVIATIONS

3Cs	Copper chloride/cysteine/carbon monoxide
Ag/AgCl	Silver/silver chloride
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
BH ₄	Tetrahydrobiopterin
Ca ²⁺	Calcium
CaM	Calmodulin
cAMP	Cyclic adenine monophosphate
cGMP	Cyclic guanosine 3'-5' monophosphate
CO ₂	Carbon dioxide
Cu/ZnSOD	Copper/Zinc superoxide dismutase
Cu ⁺	Copper
CuCl/CSH	Copper chloride/cysteine
DAN	2,3-diaminonaphthalene
DTPA	Diethylenetriaminepentaacetate
DMSO	Dimethyl sulphoxide
EC ₅₀	Concentration of 50% effect
ecSOD	Extracellular superoxide dismutase
EDRF	Endothelium derived relaxing factor
EDTA	Ethelene diamine tetra acetic
EPR	Electron paramagnetic resonance
FAD	Flavin-adenine dinucleotide
Fe ²⁺	Ferrous iron
Fe ³⁺	Ferric iron
FMN	Flavin mononucleotide
<i>g</i>	Gravity
GSNO	<i>S</i> -nitrosoglutathione
HbNO	Iron nitrosyl haemoglobin
HCl	Hydrochloric acid
Hg	Mercury
HPLC	High performance liquid chromatography

I ₂	Iodine
I ₃ ⁻	Tri-iodide
K ⁺	Potassium
K ₃ Fe ^{III} (CN) ₆	Potassium ferricyanide
KCN	Potassium cyanide
KI	Potassium iodide
L-NMMA	N ^G -methyl-L-arginine
MLCK	Myosin light chain kinase
M-NO	Metal-nitrosyl adduct
MnSOD	Magnesium superoxide dismutase
N ₂ O 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
NO _x	Nitrate and nitrite
N-oxides	Nitrogen oxides
O ₂	Oxygen
O ₃	Ozone
ODQ	1H-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one
ONOO ⁻	Peroxynitrite
ONOOH	Peroxynitrous acid
P ₅₀	Haemoglobin's 50% oxygen saturation
PE	Phenylephrine
pO ₂	Oxygen partial pressure
RSNO	S-nitrosothiol
RNNO	N-nitrosamine

sGC	Soluble guanylate cyclase
SNO-Hb	S-nitrosohaemoglobin
VCl ₃	Vanadium chloride
ΔNO	Nitric oxide difference



CHAPTER ONE

*GENERAL
INTRODUCTION*

1.0 Structure and Summary of Chapters

The structure of this thesis follows a well established template. The general introduction offers a comprehensive background detailing issues of relevance to the rest of this work. The second chapter outlines precise methodological protocols and materials used. Each of the following chapters subsequently combines results and discussion. The first of these, chapter three, is slightly unconventional in that it is devoted to work undertaken investigating and developing methods for the measurement of blood borne NO metabolite species. Within this chapter each area thought to contribute to the wide range of metabolite values reported in the literature is addressed along with the experiments performed to determine the final methodology used throughout this thesis. The three further results and discussion chapters all focus on the potential of nitrite and red blood cells to act independently or dependently as endocrine NO metabolite(s) to elicit vasodilatation. Chapter four offers an *in vitro* comparison of the metabolism of nitrite and NO in human arterial and venous whole blood. Chapter five utilises the *ex vivo* organ chamber bioassay (in normoxia and hypoxia) to investigate the vasodilator properties of nitrite alone and the possible role of nitrite in red blood cell induced relaxation. Chapter six (the final results and discussion chapter) brings together the work from the previous chapters in an *in vivo* investigation of blood borne NO metabolites across the coronary and pulmonary vascular beds in the human circulation under conditions of changing oxygen demand. The final chapter (chapter seven) attempts to summarise the results obtained in the substantive chapters three to six, drawing together conclusions and highlighting perspectives for future research.

1.1 Chemistry of NO

Nitric oxide (NO), also called nitrogen monoxide, exists as the result of a covalent interaction between oxygen and nitrogen. This simple diatomic gaseous free radical has a rich and varied chemistry much of which is determined by its electron structure [1].

1.1.1 Electron Structure

Eleven of the fifteen electrons which make up NO are valence electrons (electrons in the outer atomic shell). Six are derived from oxygen and five from nitrogen. These electrons pair up forming three fully occupied bonding orbitals, which leaves one unpaired electron in the highest occupied molecular orbital (2p anti-bonding orbital) [2] (Figure 1.1). Along with its ability to exist independently this unpaired electron characterises NO as a free radical.

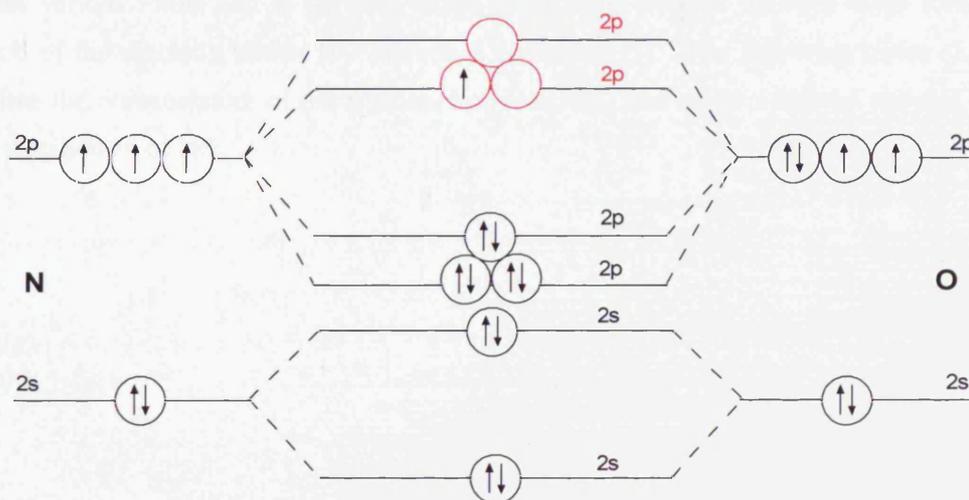


Figure 1.1: Molecular orbital diagram for NO in its ground state illustrating the arrangement of the 11 valence electrons. The single 2s and three 2p atomic orbitals of nitrogen and oxygen combine to form 3 fully occupied bonding orbitals with the unpaired electron in the antibonding p orbital. Adapted from [1]

NO can act as a reducing agent by losing the single unpaired electron or an oxidising agent by gaining up to five electrons in the free anti-bonding p orbitals. Of particular relevance in

the chemistry, reactivity and metabolism of NO are its one electron oxidation and reduction forming the nitrosonium cation (NO^+) and nitroxyl anion (NO^-) respectively [3] (Figure 1.2).



Figure 1.2: Representation of the one electron oxidation and reduction of NO forming the nitrosonium cation (NO^+) and the nitroxyl anion (NO^-) respectively. Adapted and reproduced with kind permission of Alexandra Milsom.

1.1.2 Oxides of Nitrogen and Oxygen

Nitrogen (like oxygen) is able to exist in a variety of oxidation states. This capacity to adopt various forms and to perform redox reactions to transfer between these forms explains much of the diversity within NO biological chemistry [3]. The following tables (1.1 and 1.2) review the nomenclature of nitrogen oxides (*N*-oxides) and oxygen derived radicals relevant to the metabolism of NO.

Table 1.1: Nitrogen oxides relevant to NO metabolism *in vitro* and *in vivo*, listed according to the oxidation state of nitrogen [4].

Chemical name	Molecular formula	Oxidation state of nitrogen
Ammonia/Ammonium ion	$\text{NH}_3/\text{NH}_4^+$	-3
Hydrazine	N_2H_4	-2
Hydroxylamine	NH_2OH	-1
Nitrogen	N_2	0
Nitrous oxide	N_2O	+1
Hyponitrous acid/nitroxyl anion	HNO/NO^-	+1
Nitric oxide	NO	+2
Dinitrogen dioxide	N_2O_2	+2
Nitrosonium cation	NO^+	+3
Dinitrogen trioxide	N_2O_3	+3
Peroxynitrous acid/peroxynitrite	$\text{ONOOH}/\text{ONOO}^-$	+3
Nitrous acid/nitrite	$\text{HNO}_2/\text{NO}_2^-$	+3
Nitrogen dioxide	NO_2	+4
Dinitrogen tetroxide	N_2O_4	+4
Dinitrogen pentoxide	N_2O_5	+5
Peroxynitric acid/ peroxynitrate	$\text{O}_2\text{NOOH}/\text{O}_2\text{NOO}^-$	+5
Nitric acid/nitrate	$\text{HNO}_3/\text{NO}_3^-$	+5

Table 1.2: Oxygen derived species relevant to NO metabolism *in vitro* and *in vivo*, listed according to the oxidation state of oxygen [4].

Chemical name	Molecular formula	Oxidation state of oxygen
Hydroxyl	OH	-3
Superoxide anion	O_2^-	-2
Hydrogen peroxide/Peroxide anion	$\text{H}_2\text{O}_2/\text{O}_2^{2-}$	-1
Oxygen	O_2	0

1.2 Reactivity of NO

Each of the various *N*-oxides has its own particular reactivity and can react with a number of biological molecules in a potentially bewildering array of reactions [5]. In terms of NO, NO⁺ and NO⁻ the main biological reactions are outlined in figure 1.3. These reactions however can be simplified into direct and indirect reactions [6, 7].

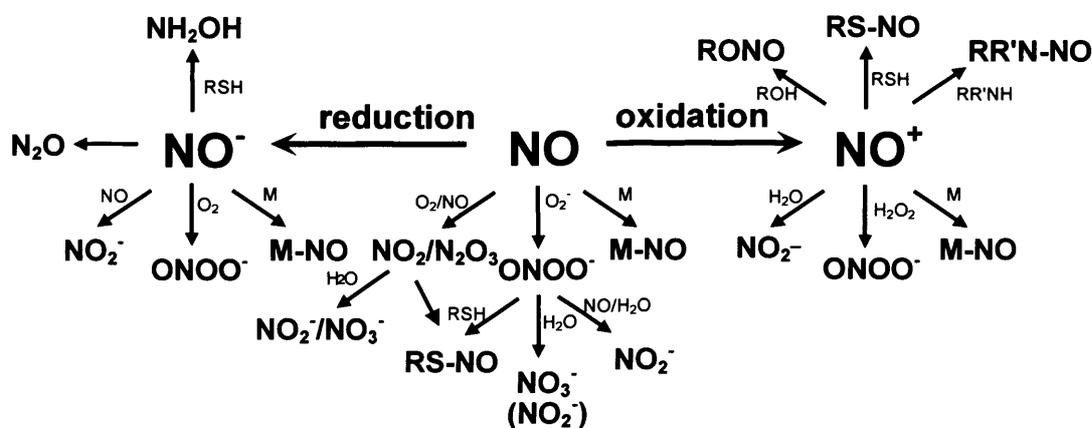


Figure 1.3: Reactivity of nitric oxide (NO), nitrosonium (NO⁺) and nitroxyl (NO⁻). O₂ = molecular oxygen, O₂⁻ = super oxide, ONOO⁻ = peroxynitrite, M = metal centre, M-NO = metal nitrosyl adduct, NO₂ = nitrogen dioxide, N₂O₃ = dinitrogen trioxide, NO₂⁻ = nitrite, NO₃⁻ = nitrate, N₂O = dinitrogen oxide, RSH = thiol, RS-NO = nitrosothiol, NH₂OH = hydroxylamine, ROH = primary amine, RONO = nitrosamine, RR'NH = secondary amine, RR'N-NO = nitrosamine, H₂O = water, H₂O₂ = hydrogen peroxide. Adapted from [8].

1.2.1 Direct

The direct reactions of NO in biological systems are with oxygen, free radical species (such as superoxide) and metal containing centres. The respective products of these reactions are *N*-oxides (NO₂, N₂O₃), peroxynitrite (ONOO⁻) and metal-nitrosyl adducts (M-NO).

1.2.2 Indirect

The products of the direct reactions can potentially undergo further indirect reactions, for example hydration reactions or reactions at nucleophilic centres, forming higher *N*-oxides (NO₂⁻, NO₃⁻) or S-nitrosothiols (RSNO) respectively.

1.2.3 Classification of Reactions

The reactions of NO and its redox related forms can be classified as follows:

Table 1.3: Definitions and species involved in the different reactions of NO and its interrelated redox forms.

Reaction	Definition	Involves
Oxidation	Reaction with oxygen	NO and related <i>N</i> -oxides
Nitrosylation	Reaction involving the covalent attachment of NO (without change in the formal charge of the substrate) [9]. NO based modification of protein function analogous to phosphorylation.	Metal centres, Protein thiol side chains
Nitrosation	Reaction involving modification by NO ⁺ [9].	Nucleophilic centres of nitrogen (e.g., amines), sulphur (e.g., thiols), oxygen (e.g., superoxide) and carbon (e.g., tyrosine)
Trans-nitrosation	The passing of NO ⁺ between thiol groups	
Nitration	Reactions in which a nitro group (-NO ₂) is added to or substituted in a molecule	Aromatic amino acids including tyrosine

All of these reactions are subject to reaction rates and are dependent upon the concentration of reactants and the rate of competing reactions including the respective concentration of their reactants. This complexity has been simplified into a concept known as the reaction target area principle [10], introduced into the NO field by Beckman [11]. According to this principle, the relative amount of a compound (e.g., NO) that reacts with its respective targets can be determined by multiplying the reaction rate and the concentration of the target. Therefore, although the reaction of NO with superoxide is high ($\sim 10^{10} \mu\text{M/s}$), the physiological concentration of superoxide is low ($\sim 0.1\text{-}1\text{ nM}$) [11]. On the other hand, the reaction of NO with a metal centre is much lower ($\sim 10^7 \mu\text{M/s}$), but the concentration of metal centres is much higher ($\sim 10 \mu\text{M}$). This makes the reactive exposure for metal centres tenfold higher than that for superoxide ($10^8 \nu 10^7$), which means that the former reaction is more likely to occur *in vivo*. The various products from the reactions of NO have the potential to be either toxic or protective depending on the biological environment in which the reactions take place.

1.3 Production of NO

1.3.1 Nitric Oxide Synthases

NO is produced by a family of enzymes known as the nitric oxide synthases (NOS). To date three distinct NOS isoforms have been identified [12, 13], all of which differ in their structure, localisation, regulation and inhibitor sensitivities (Table 1.4).

Table 1.4: Properties of the different isoforms of NOS. NADPH = nicotinamide adenine dinucleotide phosphate, FAD = flavin adenine dinucleotide, FMN = flavin mononucleotide, BH₄ = tetrahydrobiopterin, pmoles = picomoles, nmoles = nanomoles. Reproduced with kind permission of Alexandra Milsom.

	NOS Isoform		
	Type I	Type II	Type III
Other designation	nNOS/ncNOS	iNOS	eNOS/ecNOS
Substrates	L-arginine, oxygen, NADPH	L-arginine, oxygen, NADPH	L-arginine, oxygen, NADPH
Co-factors	FAD, FMN, BH ₄	FAD, FMN, BH ₄	FAD, FMN, BH ₄
Prosthetic groups	Haem, Calmodulin	Haem, Calmodulin	Haem, Calmodulin
Expression	Constitutive	Inducible	Constitutive
Inducible by calcium	Yes	No	Yes
Levels of NO produced	Pico moles	Nano moles	Pico moles
Cellular localisation	Central and peripheral neurones, skeletal muscle, epithelial cells	Macrophages, endothelial cells, hepatocytes, vascular smooth muscle cells, lung and gut epithelial cells	Vascular endothelial cells, neurones, epithelial cells, heart, skeletal muscle
Subcellular localisation	Mainly cytosolic	Cytosolic	Membrane bound
Major function	Neuronal messenger, synaptic plasticity	Immuno-cytotoxicity	Relaxation of vascular smooth muscle

The NOS enzymes were originally named according to the cell types in which they were first located (neuronal, immune and endothelial) these isoforms have since been re-classified (type I, II and III respectively) due to identification elsewhere [13, 14].

1.3.1.1 Structure

Active (coupled) NOS forms a homodimer made up of two identical enzyme subunits (Figure 1.4). Each subunit exhibits a bi-domain structure consisting of an N terminal oxygenase domain and a C terminal reductase domain. The reductase domain contains binding sites for NADPH, flavin mononucleotide (FMN), flavin-adenine dinucleotide (FAD) and is linked through calmodulin (CaM) to an oxygenase domain with binding sites for haem, 6(R)-5,6,7,8-tetrahydrobiopterin (BH₄) and L-arginine [15].

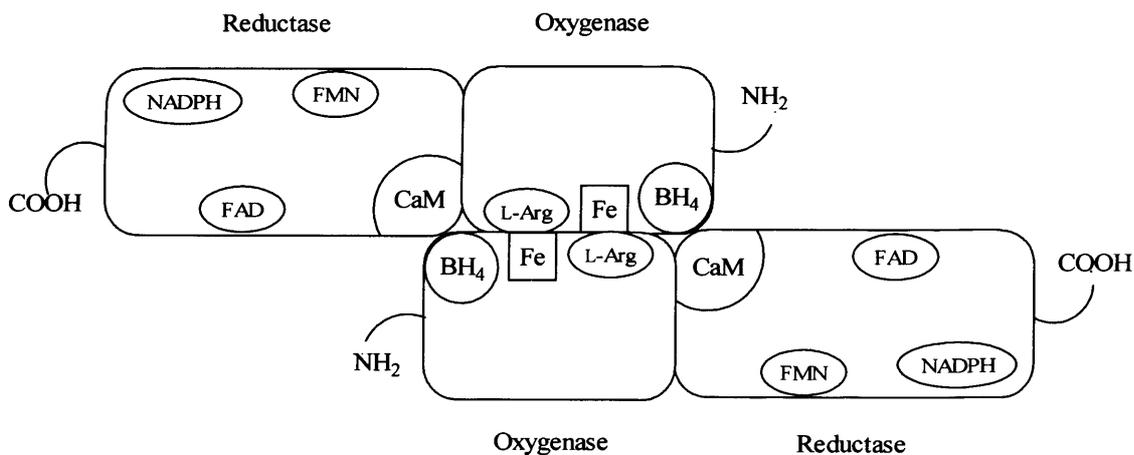


Figure 1.4: Scheme of the domain structure of the NOS dimer showing cofactor and substrate binding sites. Reproduced from [16].

1.3.1.2 NO Formation

It still remains in question whether NOS directly synthesises NO, some other reactive nitrogen species (e.g. NO⁻) [17, 18], or a combination of both depending on the conditions [19,

20]. For the direct production of NO, NOS has to catalyse a five-electron oxidation of the terminal guanidine nitrogen atom on the amino acid L-arginine [13] (Figure 1.5).



Figure 1.5: Reaction stoichiometry for NOS production of NO in its free radical form.

The reaction is thought to occur in two phases, the first of which involves electrons from the two electron donor NADPH being transferred through the reductase domain of one NOS subunit (via the flavins) to the one electron acceptor haem in the oxygenase domain of the other subunit. This reduces the haem iron in the catalytic centre enabling it to bind and activate oxygen. During the second phase the activated oxygen is inserted into L-arginine forming the intermediate *N*^ω-hydroxy-L-arginine from which NO and citrulline are generated by oxygenation [21] (Figure 1.6). Under certain situations such as limiting substrate or co-factor availability, the NOS enzyme becomes uncoupled resulting in the production of superoxide and hydrogen peroxide [22].

1.3.2 Other Sources of NO

Although the NOS enzymes have been viewed as the classical pathway of NO generation in mammals, it is now clear that several alternative routes also exist *in vivo* by which the *N*-oxides nitrite (and under certain conditions nitrate) and the oxidative bi-products of NO in blood can be reduced back to NO. In the case of nitrite, these include non-enzymatic acidic reduction [23], reduction by deoxyhaemoglobin in blood [24-27], reaction with xanthine oxidoreductase [28, 29] and enzymes of the mitochondrial respiratory chain [30]. This means that dietary sources of nitrite (and nitrate), i.e., meat, vegetables and drinking water, could potentially contribute to the bio-available blood borne pool of NO *in vivo* [31].

1.4 Vascular NO

1.4.1 Vascular Anatomy

Blood vessel anatomy changes throughout the cardiovascular tree reflecting specificity of function (Figure 1.6 and Figure 1.7). In general, blood vessels consists of a hollow centre (the lumen) surrounded by three main structural layers, the intima, media and externa. The intima, or inner layer, is comprised of a single layer of endothelial cells, a basement membrane (sub-endothelial layer) and elastic tissue called the internal elastic lamina. The media which surrounds the intima is composed primarily of smooth muscle cell layers, the number of which range from ~ 25 in large arteries to 1 or 2 in veins. Finally, the outermost layer of the vascular wall (the externa) consists largely of collagenous tissue [32].

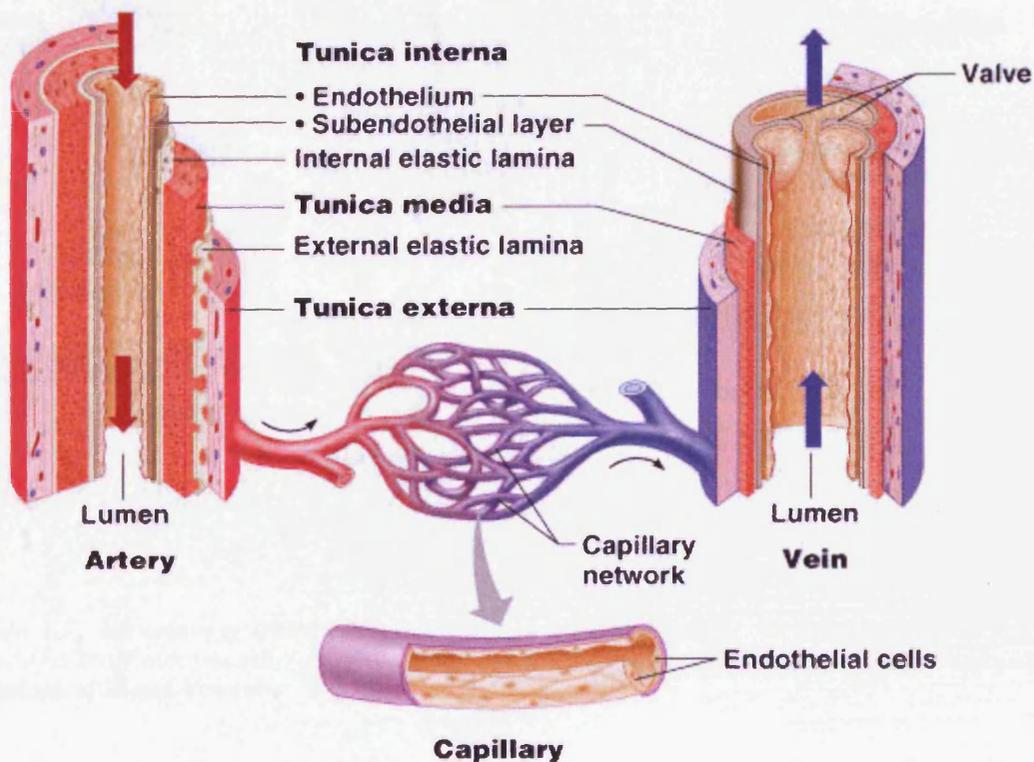


Figure 1.6: Structure of large blood vessels and capillaries. Reproduced from [http://www.med.howard.edu/physio.biophys/MILLIS%20HOME%20PAGE_files/Biomed/19PPT_lect/262,4,Generalized Structure of Blood Vessels](http://www.med.howard.edu/physio.biophys/MILLIS%20HOME%20PAGE_files/Biomed/19PPT_lect/262,4,Generalized%20Structure%20of%20Blood%20Vessels).

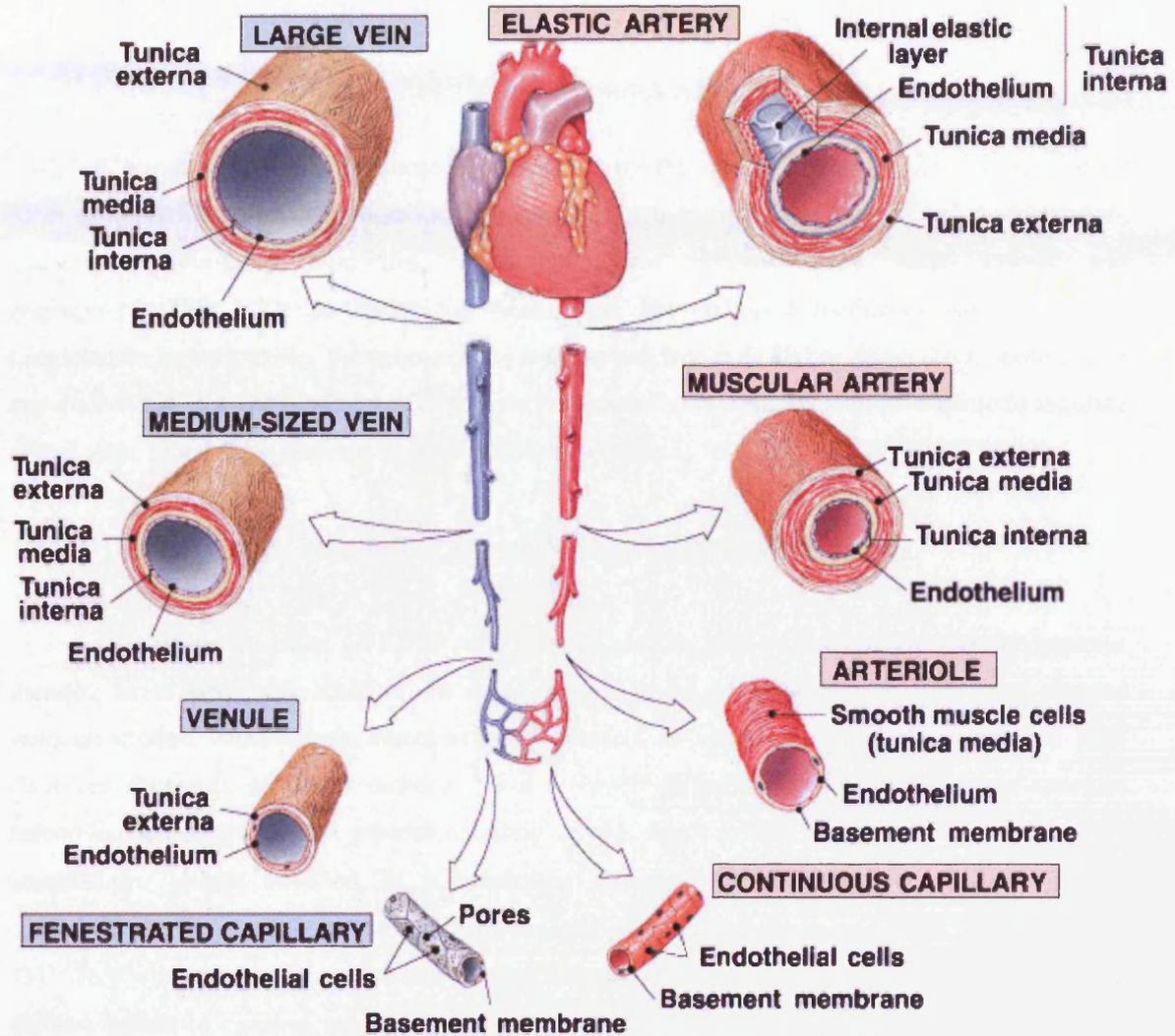


Figure 1.7: Structure of blood vessels throughout the circulation. Reproduced from [http://facstaff.bloomu.edu/gwassmer/ap2summer2003/lecturenotes/259,4,Histological Structure of Blood Vessels](http://facstaff.bloomu.edu/gwassmer/ap2summer2003/lecturenotes/259,4,Histological%20Structure%20of%20Blood%20Vessels).

1.4.2 Endothelium

The vascular endothelium is made up of a continuous single layer of cells which line the inner surface of the blood and lymphatic vessels. Originally thought of as a relatively inert barrier

between the underlying smooth muscle tissue and the circulating blood, our understanding of its function has changed dramatically over the last 25 years.

1.4.2.1 Endothelial Function

Various substances produced and released by the endothelium play critical regulatory roles in maintaining vascular homeostasis; regulating smooth muscle tone, vessel anticoagulant and anti-thrombogenic properties, inflammation and immunity and vessel growth and angiogenesis [33]. Of particular significance are the chemical mediators which constrict (angiotensin II, endothelin, thromboxane A₂ and oxygen free radicals) or dilate (NO, prostacyclin and endothelial derived hyperpolarising factor) the underlying vascular smooth muscle to regulate vessel tone, blood flow and thus oxygen delivery to tissue.

1.4.2.2 Discovery of the Endothelial Derived Relaxing Factor (EDRF) and NO

The phenomenon of an EDRF was first reported in 1980 with the answer to the apparent paradox as to why acetylcholine, an agent known to be a vasodilator *in vivo* often caused vasoconstriction when administered to blood vessels *in vitro*. Experiments performed with dissected segments of rabbit thoracic aorta mounted on an apparatus for studying vascular responses demonstrated that careful handling of the tissue which preserved the blood vessel endothelium always resulted in acetylcholine having relaxant properties (following pre-constriction with norepinephrine) whereas removal of the endothelium eradicated this action [34, 35]. This work consequently led to a race to discover the chemical identity of the endothelium derived substance causing vasodilatation. Although experimental evidence was mounting from several laboratories that EDRF possessed pharmacological, biochemical and chemical properties that were similar to those for NO, it was not until seven years later that two groups simultaneously published definitive studies characterising and identifying EDRF as NO [36, 37]._Following the discovery of EDRF and its identity as NO, what remained in question was how NO exerted its physiological effects on cells. However, the search for “NO receptor(s)” had been going on concurrently. Ultimately it was this work, which identified the signalling pathway of NO via soluble guanylate cyclase (sGC) and cyclic guanosine 3':5'-monophosphate (GMP) which is what finally brought the whole NO story together [38-40].

1.4.2.3 Endothelium Derived NO

Endothelial derived NO is synthesised by type III NOS which binds to the plasma membrane in pockets called caveolae [41, 42]. Here it interacts with the transmembrane proteins caveolin-1 and -3, which catalytically inactivate the enzyme. Activation of endothelial cells however results in an increase in intra-cellular free calcium triggering calmodulin binding to NOS which displaces caveolin resulting in enzyme activation and NO production [43] (Figure 1.8).

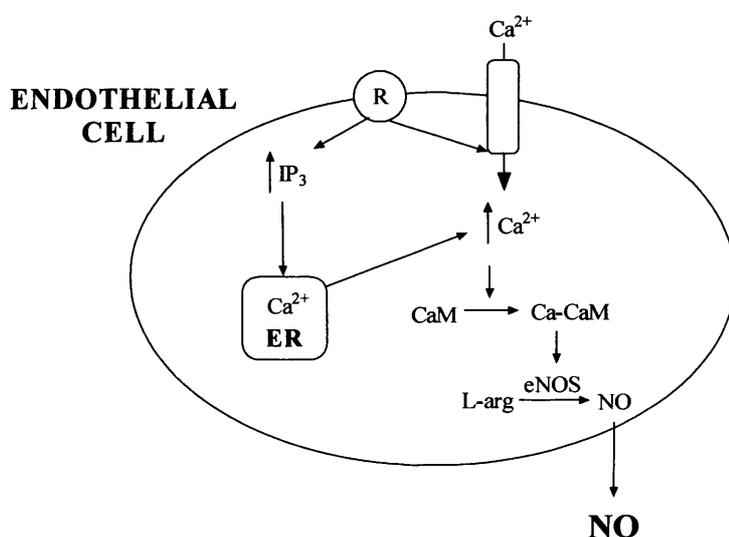


Figure 1.8: Production of nitric oxide by endothelial cells. Activation of endothelial receptors (R) by agonists (e.g., acetylcholine and bradykinin) and the effect of shear stress results in an increase in the intra-cellular calcium concentration via extra cellular calcium influx and inositol triphosphate (IP_3) stimulated release of calcium from the endoplasmic reticulum (ER). Calcium activates calmodulin (CaM) which in turn activates nitric oxide synthase (NOS) producing nitric oxide (NO) from its amino acid pre-cursor (L-arg). Adapted from [44].

Under baseline conditions endothelial derived NO accounts for 25–30% of human blood flow providing constant vasodilator tone against sympathetic vasoconstriction [45]. This production of NO can be enhanced by increasing both expression and activity of the type III NOS enzyme. This occurs in response to several factors including shear stress (i.e., the frictional force applied tangentially across the surface of the endothelium resulting from the pulsatile flow of

blood), the binding of agonists to endothelial receptors (e.g., acetylcholine, bradykinin, ATP) and hypoxia.

1.4.2.4 Diffusion of Endothelium Derived NO

The movement of NO is mainly controlled by free diffusion, which means the net movement of molecules occurs only if a concentration gradient develops. Micro sensor measurements made under physiological conditions have given NO a diffusion coefficient of $3300 \mu\text{m}^2 \text{s}^{-1}$ with the diffusion range approximating 150-300 μm for a time of 4-15 seconds [46, 47]. This distance however does not necessarily correspond to a straight line trajectory since the movement is totally random. Predictions suggest that for a cell of radius 4-15 μm it would take one NO molecule in the order of 0.002-0.03 seconds to escape [47]. Theoretically it has been calculated that NO can diffuse more rapidly out of the endothelial cell than the rate at which it reacted with targets within it [48, 49]. However, this depends entirely on the concentration of targets within and around the cell and their intrinsic rates of reaction with NO.

1.5 Metabolic Fate of Endothelium Derived NO

The metabolic fate of endothelium derived NO is dependent on its concentration, diffusibility and the surrounding concentration of other bio-reactants. Two important routes of endothelial derived NO metabolism follow its diffusion to the vascular smooth muscle (where it binds to soluble guanylate cyclase in the cytosol to activate the second messenger cGMP) or to the blood vessel lumen (where it rapidly reacts with blood constituents).

1.5.1 Regulation of Vascular Smooth Muscle Tone

Like many of the biological actions of NO, relaxation of vascular smooth muscle is mediated through the activation of soluble guanylate cyclase (sGC). This enzyme converts guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP) [50]. It remains to be fully elucidated how the second messenger cGMP initiates smooth muscle relaxations; however the increase in cGMP activates cGMP dependent protein kinases which reduce the intra-cellular calcium concentration (via a variety of different mechanisms) and decrease calcium-calmodulin stimulation of myosin light chain kinase (MLCK). This in turn decreases the phosphorylation of myosin light chains (the contractile proteins) thereby decreasing smooth muscle tension development causing vasodilatation [51]. cGMP might also lead to myosin light chain dephosphorylation via the activation of myosin light chain phosphatase, lower phosphodiesterase (PDE) levels thereby increasing the concentration of cyclic adenosine monophosphate (cAMP) and activate potassium (K^+) channels, all of which stimulate vascular smooth muscle relaxation [51] (Figure 1.9).

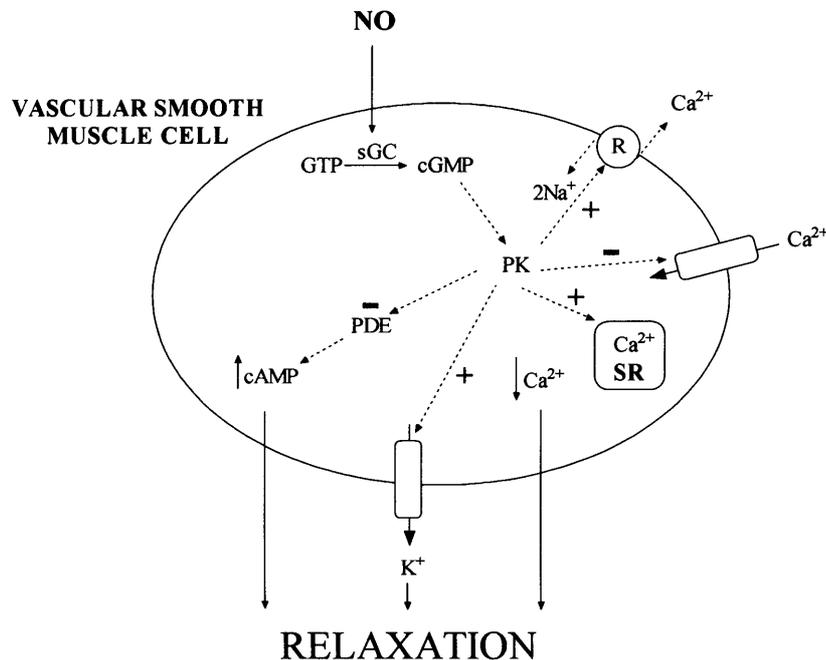


Figure 1.9: Mechanism of action of nitric oxide on vascular smooth muscle, mediated by guanylyl cyclase. Nitric oxide (NO); soluble guanylate cyclase (sGC); guanosin triphosphate (GTP); cyclic guanosine monophosphate (cGMP); protein kinases (PK); sodium (Na⁺); calcium (Ca⁺); receptor (R); sarcoplasmic reticulum (SR); potassium (K⁺); phosphodiesterase (PDE); cyclic adenosine monophosphate (cAMP). Adapted from [44].

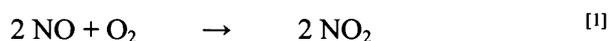
1.5.2 Metabolism of NO in Blood

The mode and rate of NO metabolism in blood is dependent on the micro-environmental conditions into which NO is released. Important factors include the concentration of NO itself and surrounding conditions such as the pO₂, the pH, the type and amount of high energy free radicals (e.g., hydroxyl radical, carbon-, oxygen- and nitrogen-centred radicals), the concentration of thiols and the presence of other traps such as haem proteins (e.g., guanylate cyclase, haemoglobin, catalase, xanthine oxidase or superoxide dismutase) [4, 52].

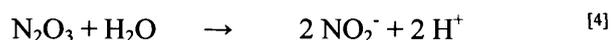
1.5.2.1 Plasma

In plasma the major decomposition pathways of NO involve reactions with molecular oxygen and oxygen derived free radicals. The reaction of NO with molecular oxygen is known as

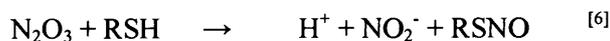
the autoxidation reaction ^[1]. This reaction produces the highly toxic NO₂ which can either dimerise to form N₂O₄ ^[2] or couple with an un-reacted NO to form N₂O₃ ^[3]. The autoxidation reaction is second order with respect to NO and first order with respect to oxygen, i.e., third order overall, involving two NO molecules and one oxygen [5, 53]. Consequently the reaction is relatively slow at the very low physiological NO concentrations observed in health. However, this reaction might assume more relevance within certain micro-environments, e.g., surrounding NOS itself, within hydrophobic lipid membranes where the local concentration of NO and oxygen is greatly increased, or under pathophysiological conditions where the NO concentration is raised, e.g., in the immediate vicinity of activated macrophages and neutrophils [54].



Unlike NO which does not undergo any hydration reaction [55] *N*-oxides can be further hydrolysed to produce nitrite ^[4] with smaller amounts of nitrate ^[5].



N-oxides are potent nitrosating agents ^[6], especially at nucleophilic centres of which thiols are among the most reactive [56]. Amines are also potential targets for nitrosation, however physiologic conditions favour the formation of thiol adducts owing to the propensity for amines to exist in their un-reactive protonated forms [52]. Potential targets for nitrosation in plasma include the proteins albumin and glutathione and low molecular weight thiols including cysteine. These species, collectively referred to as plasma *S*-nitrosothiols (RSNOs), may also be formed by radical-radical reactions ^[7] [11]. Once the thiol group is nitrosated it is possible for the NO to be exchanged between other thiol groups by a process known as trans-nitrosation (see table 1.3).



NO also reacts with oxygen derived free radical species including superoxide, hydrogen peroxide and hydroxyl radicals, all of which are produced constitutively by mammalian cells. Superoxide which results from the one-electron reduction of molecular oxygen is formed by various enzyme systems including NADPH-oxidase, xanthine oxidase, NOS and the mitochondrial electron transport chain. It reacts at almost diffusion controlled rates with NO ($6.7 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$) forming the potent oxidant peroxynitrite (ONOO^-) [8] [57]. ONOO^- reacts with virtually all classes of biomolecules including proteins, lipids and DNA. ONOO^- can further react with NO to produce nitrite [9] [58]. However, ONOO^- decays rapidly once protonated forming peroxynitrous acid (ONOOH) [10] and eventually nitrate [11] [57]:



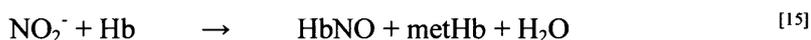
Enzymatic defence mechanisms *in vivo* prevent the accumulation of reactive oxygen species. These include the enzyme superoxide dismutase, of which there are three main forms; Cu/ZnSOD localised in the cytosol, MnSOD found in the mitochondria and ecSOD located in the extracellular space. These enzymes dismutate the superoxide anion with a very high reaction constant ($2.9 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ [59]) into hydrogen peroxide and oxygen. Hydrogen peroxide in turn can be converted to hypochlorous acid (HOCl) in the presence of chloride anion by myeloperoxidase or into water and oxygen by the actions of catalase and/or glutathione peroxidase.

1.5.2.2 Haemoglobin/Erythrocytes

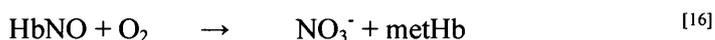
The principle metabolic pathway for the reaction of endogenous NO in blood is with the haem group of oxy- and deoxyhaemoglobin. Both these reactions have relatively high reaction rates ($\sim 10^7 \text{ M}^{-1} \text{ s}^{-1}$) [60, 61]. The reaction between NO and oxygenated haemoglobin (often referred to as the oxidation reaction) forms nitrate and methaemoglobin [12]. NO also reacts with deoxygenated haemoglobin or deoxygenated haem subunits on partially oxygenated haemoglobin (the addition reaction) to form iron nitrosyl haemoglobin (HbNO) [13].



It has been suggested that nitrite is also able to cross the erythrocyte membrane and can react with oxygenated haemoglobin to form methaemoglobin and nitrate [14] or deoxygenated haemoglobin to form HbNO and methaemoglobin [15]. In terms of apportionment of NO to these reactions, this is likely to be limited in comparison to the oxidation or addition reactions because of the rate limiting step of nitrite formation.



HbNO via its reaction with oxygen [16] and erythrocytic nitrite via a reaction with HbNO [17] can potentially also be broken down to methaemoglobin and nitrate.



One further reaction of NO with haemoglobin is the nitrosation of an amino acid present on the β chain of haemoglobin at position 93 to form *S*-nitrosohaemoglobin (SNO-Hb). The formation of this RSNO is not fully understood but has been postulated to result from the transfer of NO from β -subunits of HbNO to the β -93 cysteine residue following conformational change of haemoglobin from deoxygenated to oxygenated state, with either oxygen or ferrihaem serving as the electron acceptor [62].

1.5.2.3 Whole Blood

NO metabolite formation in whole blood is dependent on the concentration of NO, haemoglobin, oxygen, free radicals and thiols. Assuming all substrates are present without significant oxidative stress, free NO would mainly be metabolised to nitrate following its reaction with oxygenated haemoglobin, especially given the relatively high oxygen saturation of haemoglobin even in the venous circulation. Any free haems should form HbNO, however the oxygenation status of blood will determine whether or not this is metabolised to nitrate [16]. Nitrite should also be formed following the reaction of NO with dissolved oxygen, along with a small proportion of *S*-nitrosothiols [63].

A comparison of exogenous NO and nitrite metabolism in human arterial and venous whole blood utilising up to date NO metabolite measurement methodology forms the basis of Chapter 4.

1.6 Role of NO Metabolites in the Circulation

1.6.1 NO Inactivation by Haemoglobin

Haemoglobin was historically thought to interact with NO solely in a manner that would eliminate its biological activity, forming metabolites unable to activate soluble guanylate cyclase [50]. It was therefore questioned how NO could function to regulate vascular tone given the rapid association rate between haemoglobin and NO ($\sim 10^7 \text{M}^{-1}\text{s}^{-1}$) and the relatively high concentration of haemoglobin in blood ($\sim 2.5\text{mM}$) [49, 60, 64]. It was thought that NO would be drawn intraluminally rather than diffusing to the underlying vascular smooth muscle. However, it emerged that the rate of NO scavenging by erythrocytes was almost three orders of magnitude lower than that of an equivalent concentration of cell-free haemoglobin [65, 66] as a result of diffusional barriers presented by the erythrocyte membrane, the sub membrane protein matrix and an unstirred layer around the red blood cell [65, 67]. Furthermore, laminar blood flow was found to create an 'erythrocyte free zone' adjacent to the vessel lumen (the size of which was proportional to vessel diameter) [66] further reducing the quantity of NO diffusing intra luminally.

1.6.2 Paracrine Regulator

Originally NO was viewed as playing a purely paracrine role in the regulation of vascular tone, acting within the vicinity of its release [68]. Those factors limiting the rate of reaction between NO and haemoglobin were generally considered to allow the local concentration of NO adjacent to the endothelial cells to increase sufficiently to provide a diffusional gradient for NO to activate soluble guanylate cyclase in vascular smooth muscle. Reactions of NO in blood were consequently assumed only to scavenge and inactivate NO limiting its availability to elicit vasodilatation (Figure 1.10).

1.6.3 Endocrine Regulator

In 1992 the endocrine hypothesis emerged with the proposal that blood borne NO metabolites might conserve bioactivity allowing for the storage, transport and potential release of NO far from its location of synthesis [69]. This hypothesis was made on the back of the discovery that endogenously produced NO circulated in human plasma primarily complexed as an S-nitrosothiol, namely S-nitrosoalbumin [63]. This was further extended to include a reactive

thiol of haemoglobin (Cys β 93) which was demonstrated to undergo nitrosylation and sustain NO bio-activity under oxygenated conditions and release of this NO under low oxygen conditions [70].

Although there is increasing acceptance of the endocrine model (the principle of which has been demonstrated in NO inhalation studies [71] and following infusions of aqueous solutions of authentic NO [72]) heated debate surrounds the nature of NO species responsible for this action and its mechanisms of formation and subsequent release. In addition to SNO-albumin and SNO-haemoglobin, other putative candidates for endocrine NO species include iron-nitrosylated haemoglobin [71, 73], N-nitrosated proteins [74, 75], nitrated lipids [76, 77], and the anion nitrite [24, 26-28, 78, 79].

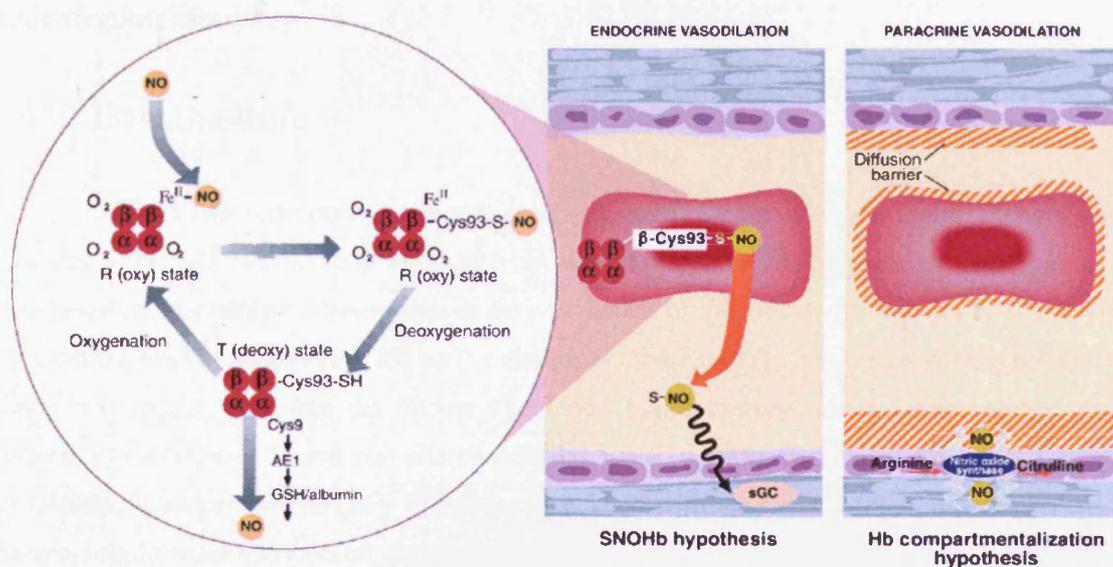


Figure 1.10: Paracrine and endocrine models for the role of haemoglobin in modulating NO bioactivity. Adapted from [80]

1.6.3.1 SNO-Hb Hypothesis

In 1996 an active role for red blood cells in the transport and delivery of NO was proposed following the discovery that haemoglobin itself sustained S-nitrosylation [70]. The binding and stability of NO at different sites on the haemoglobin molecule was shown to be dependent on haemoglobin quaternary conformation [70, 81, 82]. It was proposed that haem bound NO on the β haemoglobin chain (β HbNO), favoured in T-state (deoxygenated) haemoglobin, was transferred to the β chain cysteine 93 (SNO-Hb) in the R-state (oxygenated) haemoglobin during haemoglobin oxygenation [83]. Furthermore, deoxygenation destabilised this cysteine bound NO facilitating transfer to low molecular weight and membrane associated thiols [84] resulting in an increase in the availability of NO for vasodilatation in the microcirculation (Figure 1.10). This novel proposal consequently provided a mechanism for the delivery of relatively unstable NO produced in the larger arteries to the smaller blood vessels which regulate blood flow.

1.6.3.2 Nitrite Hypothesis

In 2003 two independent research groups demonstrated that nitrite might potentially act as a vasoactive NO metabolite in the human circulation [26, 27]. The rationale for studying nitrite was based on mechanisms demonstrating the conversion of this metabolite species to NO *in vivo* by xanthine oxidoreductase [28, 29] and/or disproportionation [23]. It was therefore hypothesised that nitrite might vasodilate the human circulation under exercise stress. Surprisingly intra-arterial nitrite infusion caused vasodilatation in the human forearm circulation even at rest at near physiological concentrations [26]. This was associated with the rapid formation of iron-nitrosyl haemoglobin across the forearm circulation implying a novel mechanism of nitrite bioactivation based on a simple reaction of nitrite with deoxyhaemoglobin as described by Doyle and colleagues [25].

Subsequently it has been demonstrated that vasodilatation via this mechanism occurs maximally as haemoglobin unloads oxygen to 50% saturation where there is the optimal balance between the availability of deoxyhaems for nitrite binding (maximal for fully deoxygenated or T-state haemoglobin) and the ability of the haem to donate an electron to nitrite (which is maximal for R-state haemoglobin) [85] (Figure 1.11).

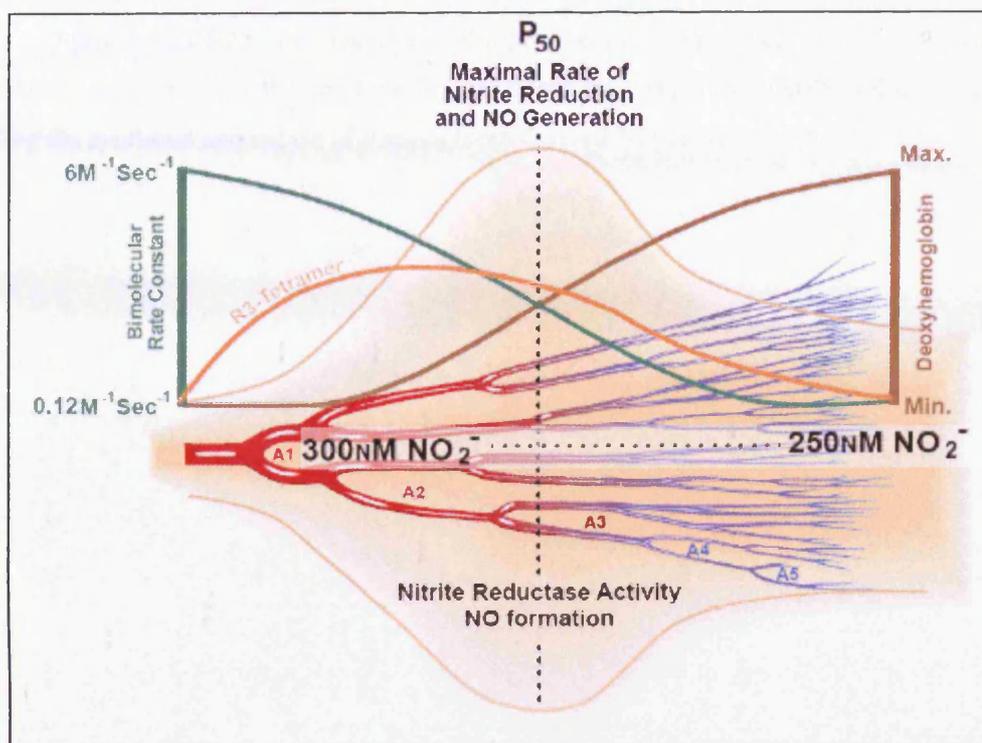


Figure 1.11: Nitrite reductase equilibrium along the A1 to A5 arterioles demonstrating that the maximal rate of nitrite reduction to NO occurs at the P_{50} . Reproduced from [86].

1.6.3.3 Need for Further Investigation

Similarities between the SNO-Hb and nitrite hypotheses are quite striking. Both imply that 1) an NO equivalent is involved, 2) it is transported in blood, 3) delivery of the NO moiety is linked to haemoglobin, 4) is allosterically mediated and also 5) involves a thiol based mechanism [87]. Despite these commonalities disagreements have abounded between groups supporting either hypothesis. These have related to the concentrations of metabolite species measured in the human circulation, the presence or not of A-V gradients, the methodologies used to measure metabolites and so on. Perhaps the most pressing issue related to both hypotheses is the means by which the NO equivalent is transferred from the red blood cell to the vessel wall [88]. In terms of the SNO-Hb hypothesis Pawloski et al. [84] have demonstrated in human erythrocytes that SNO-Hb is associated predominantly with the red blood cell membrane and principally with cysteine residues in the haemoglobin-binding cytoplasmic domain of the anion exchanger AE1 (Band 3 protein).

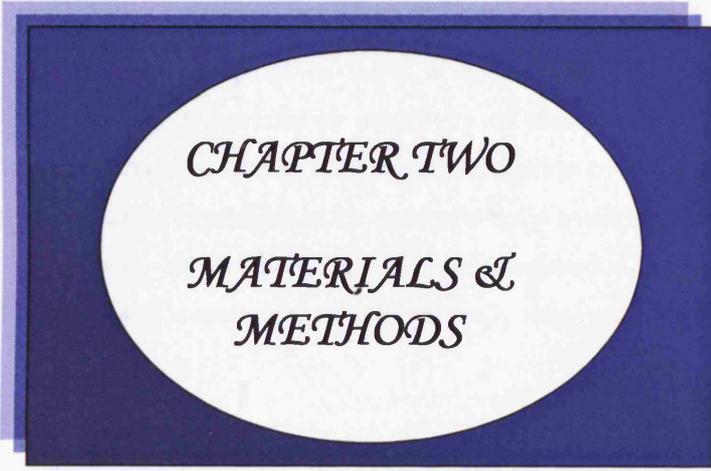
Interaction with AE1 promotes the deoxygenated structure in SNO-haemoglobin, which serves NO group transfer to the membrane. Furthermore, deoxygenation results in the release of vasodilatory activity from this membrane, providing an oxygen-regulated cellular mechanism coupling the synthesis and export of haemoglobin-derived NO bioactivity [84].

1.7 **Thesis Aims**

The work of this thesis will provide a clearer understanding of the physiological relevance of blood borne NO metabolites in the human circulation. This will be undertaken on several levels; methodological investigation into the measurement of these species, examination of the metabolism/apportionment of the exogenous addition of nitrite and NO to healthy human arterial and venous whole blood; exploration into the vascular activity of these species in relation to red blood cells and an in vivo investigation using a human model to assess NO metabolites at rest and during increased oxygen demand across the coronary and pulmonary circulations.

1.7.1 **Specific Aims**

- To develop methods capable of measuring baseline levels of blood borne NO metabolites in healthy human subjects. The chemical reagents used to liberate NO from metabolites will be investigated along with biological sample handling protocols to minimise contamination and the loss of less stable NO metabolite species.
- To determine baseline concentrations of the major blood borne NO metabolite species and assess the apportionment of nitrite and NO between the various metabolic pathways in arterial and venous human blood.
- To investigate the regulation of vascular tone by native red blood cells and nitrite in combination and alone at different oxygen tensions to elucidate whether nitrite plays a role in the red blood cell relaxation response observed under hypoxic conditions.
- To examine NO metabolites across the coronary and pulmonary circulations in healthy individuals at rest and under conditions of increased tissue oxygen demand (elicited by rapid atrial pacing) under normal conditions and following systemic NO synthesis inhibition to determine the physiological relevance of the NO metabolite reserve in the human circulation.



CHAPTER TWO

*MATERIALS &
METHODS*

2.0 NO Measurement Techniques

A range of techniques have been developed to measure NO metabolites based on a number of different principles.

2.0.1 Electrodes

NO electrodes can be used in the aqueous or gas phase. Both set ups require NO release from biological samples prior to NO detection by the electrode. In the gas phase, once the NO is released it is carried in an inert gas stream (e.g., nitrogen) to the electrode surface. The electrode itself comprises a platinum wire 'working' electrode (anode) and Ag/AgCl wire as the counter-electrode (cathode). A constant potential of 0.9V is applied, which provides the highest sensitivity for NO [89] and also determines its selectivity of measurement (for NO). Direct current is subsequently measured from the electrochemical oxidation of NO gas on the platinum 'working' electrode (anode) surface resulting in the generation of a small redox current [90]. The redox current flowing between the working and reference electrodes is proportional to the concentration of NO oxidised and is measured amperometrically using an NO meter.

2.0.2 Chemiluminescence

Chemiluminescence involves the emission of light (luminescence) as a result of a chemical reaction. To use this technique to measure NO from biological samples a method is required to release the NO from the sample. Once released the NO is carried in an inert gas stream (e.g., nitrogen) to the nitric oxide analyser (NOA) where it can be quantified following its chemiluminescent reaction with ozone (O_3). This reaction forms nitrogen dioxide (NO_2), a proportion of which arises in an electronically excited state (NO_2^*). In the excited state the electrons are unstable and they dissipate energy as they regain their original stable 'ground' state [91]. The excess energy of NO_2^* is released as a photon ($h\nu$; h = Plank's constant, ν = frequency of light) emitted in the red and near-infrared region of the spectrum (~ 640 - 3000 nm) with maximum intensity at ~ 1100 nm. A photomultiplier is used to amplify the signal and detect the emitted light, which is sensitive to wavelengths below 900 nm [92]. Nevertheless, the amount of light emitted by the $NO + O_3$ reaction in the 640-900 nm range is still sufficient to make chemiluminescence one of the most sensitive NO measurement techniques available. Provided O_3

is present in excess and reaction conditions are held constant the intensity of light emitted is directly proportional to NO concentration [91].

2.0.3 Fluorimetry

Fluorimeters belong to a class of instruments called luminometers, which are essentially light detectors. Fluorometry is a method for detecting and measuring fluorescence in compounds using ultraviolet light to stimulate a compound to emit visible light. In NO measurement specific probes are used that upon reaction with NO produce a product that fluoresces following excitation at a set wavelength.

2.0.4 Colourimetry

Colourimetry is a technique for measuring the concentration of a solution by comparison of colours. Absorbance is an example of colourimetry. For a set wavelength, absorbance is determined by comparing the intensity of light which irradiates a sample (incident light) to the intensity of light exiting a sample (transmitted light). This method has previously been used throughout the NO metabolite research field to measure nitrite and nitrate (following its conversion to nitrite) in plasma samples (Griess Reaction) and in this thesis has been used to determine haemoglobin concentration in blood samples.

2.1 Biochemical Measurement Protocols

2.1.1 Biological Sample Collection

Unless otherwise stated all blood samples were collected in a syringe and then immediately injected into EDTA vacutainers. These were centrifuged at 600g (with time and temperature experiment specific). The red cell fraction and plasma were immediately separated (taking care to remove the 'buffy' coat) and either measured fresh or snap frozen in liquid nitrogen and stored at - 80°C for subsequent analysis. Upon analysis frozen red blood cell and plasma samples were thawed in a water bath at 37°C for 3 minutes.

Table 2.0: Summary table of techniques used to measure NO metabolites.

Metabolite(s) Measured	NO Measurement Technique	Assay/Reagent	Sample Pretreatment
Plasma NOx	Fluorescence	DAN Assay	Various
	Electrode	Vanadium Chloride	None
	Chemiluminescence	Vanadium Chloride	None
Plasma NO (excluding nitrate)	Electrode	Tri-iodide	None
	Chemiluminescence	Tri-iodide	None
Plasma protein-bound NO	Electrode	Tri-iodide	Sulphanilamide
	Chemiluminescence	Tri-iodide	Sulphanilamide
Red blood cell NO	Electrode	Modified tri-iodide	Water dilution
	Chemiluminescence	Modified tri-iodide	Water dilution
Red blood cell Hb-bound NO	Electrode	Modified tri-iodide	Sulphanilamide
	Chemiluminescence	Modified tri-iodide	Sulphanilamide

2.1.2 Plasma NOx (Nitrate and Nitrite) by Fluorescence

This assay, based on a previously described method [93] works via the N-nitrosation of 2,3-diaminonaphthalene (DAN), forming the fluorescent product 1-(H)-naphthotriazole (Figure 2.0). Nitrite is converted to NO^+ under acidic conditions and this reacts rapidly with DAN. To measure nitrate it has to first be converted to nitrite by a nitrate reductase. Fluorescence was measured using excitation and emission wavelengths of 365nm and 450nm, respectively.

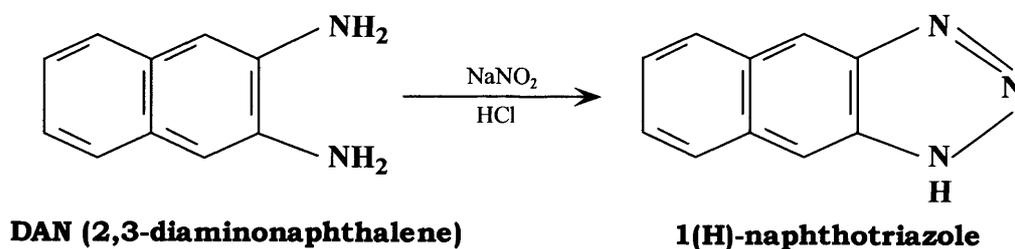


Figure 2.0: Reaction of 2,3-diaminonaphthalene (DAN) forming the fluorescent product 1-(H)-naphthotriazole. Reproduced from [93].

2.1.2.1 Chemicals, Reagents and Equipment

Vivaspin eppendorf filters (500 μ l; 10,000M_r cut-off) from VivaScience, UK and white opaque 96-well plates from Dynatech Laboratories, Inc. (Chantilly, VA) were used to optimise measurement of fluorescent intensity. Reagents were prepared at room temperature in HPLC grade water to reduce background nitrite. For composition of reagents and buffer see Table 2.1.

2.1.2.2 Protocol

Plasma samples were filtered to remove contaminating plasma proteins and remaining haemoglobin.

Table 2.1: Composition of reagents for the flurometric analysis of NO_x. All solutions were prepared fresh on the day of the experiment.

Sodium phosphate buffer	Stock solution 1.4M (8.06 grams Na ₂ HPO ₄ , 2.08 grams NaH ₂ PO ₄ ·2H ₂ O in 50ml water). Working solution 14mM. Dilution of stock solution in water
NADPH	Stock solution 30 μ M (0.3 grams in 1ml buffer). Diluted with buffer to 10 μ M working solution.
Glucose-6-phosphate	Stock solution 50mM (in buffer). Diluted in buffer to a 5mM working solution
Glucose-6-phosphate dehydrogenase	Stock solution 100 units/ml (in buffer). Diluted in buffer to 1.6 units/ml working solution
Nitrate reductase	Stock solution 5 units/ml (in buffer). Diluted in buffer to 0.8 units/ml working solution
Enzyme mixture	Equal quantities of sodium phosphate buffer, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and nitrate reductase.
HCl	Diluted in water to a 0.62M working solution.
DAN	Stock solution 0.5mg ml ⁻¹ (in 0.62 HCl). Diluted with 0.62 HCl to 0.05mg ml ⁻¹ working solution and stirred for 30 min before use. Both solutions protected from light.
NaOH	Diluted in water to a 2.8N working solution.

10 μ l of 10 μ M NADPH was added to all 50 μ l samples in a 96 well plate. For nitrate measurement, 40 μ l of an enzyme mixture containing 5mM glucose-6-phosphate, 1.6 units/ml glucose-6-phosphate dehydrogenase and 0.8 units/ml nitrate reductase in 14mM sodium phosphate buffer was added. For nitrite measurement the relevant duplicate samples received

40 μ l 14mM sodium phosphate buffer only. Samples were incubated for 1 hour at 37°C. After incubation 10 μ l of 0.05mg ml⁻¹ DAN in 0.62M HCl was added with a further incubation for 10 minutes at room temperature protected from light. The reaction was halted by adding 5 μ l of 2.8N NaOH solution, which also optimises fluorphore. Fluorescence was measured after 10 minutes using a Perkin Elmer luminescence spectrophotometer.

2.1.2.3 Calibration

Calibration was achieved using a fresh stock solution of sodium nitrate made up on the day of the experiment as a 10mM stock solution (to increase accuracy) and further diluted to a concentration range of 0.1 - 50 μ M. The standard curve of sodium nitrate is linear to 100 μ M. When compared against a standard curve of sodium nitrite, the nitrate recovery (i.e., efficiency of the nitrate reductase) was ~ 88% at 100 μ M as demonstrated from our laboratory in Figure 2.1.

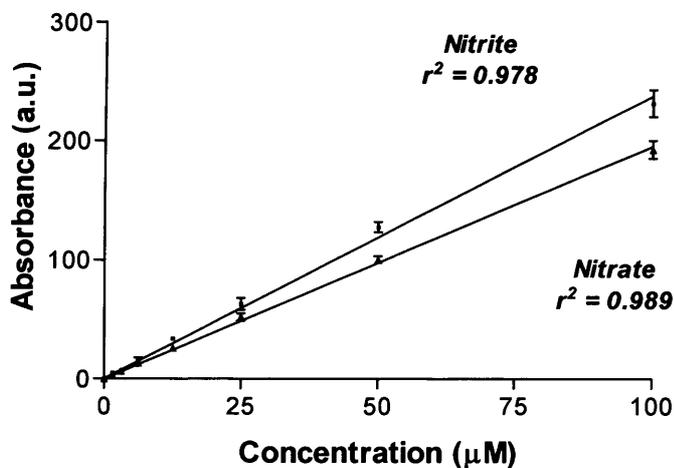


Figure 2.1: Standard curve of sodium nitrite (Top line) and sodium nitrate (Bottom line). Data are presented as mean \pm SEM ($n = 10$).

2.1.2.4 Potential Confounding Factors

- NADP/NADPH can interfere with fluorescence. However, standard curves of nitrite performed with and without NADPH showed this effect to be negligible (< 5%).

- The anti-coagulant heparin has an inhibitory effect on nitrate reductase activity; therefore care must be taken to sample blood using an anti-coagulant such as EDTA.
- Azide, commonly used as a preservative in some filters, inhibits the enzymatic reduction of nitrate to nitrite. In this assay the vivaspin filters were washed three times with HPLC grade water to remove sodium azide from the membranes prior to use.
- Other compounds known to interfere with this assay are phenol red, plasma proteins, haemoglobin and dithiothreitol.

2.1.3 Plasma NO_x by Electrode/Ozone Based Chemiluminescence

Vanadium III chloride in hydrochloric acid reduces nitrate, nitrite and protein-bound NO back to NO which can be detected in the gas phase using an NO electrode or ozone based chemiluminescence.

2.1.3.1 Chemicals, Reagents and Equipment

A saturated solution of VCl₃ in 0.8M HCl was prepared fresh (0.785 grams of VCl₃; 20ml water; 80ml of 1M HCl). The solution was left to stir for 10 minutes before being filtered prior to use (once filtered the solution appeared turquoise blue). The reagent (8ml plus 20µl anti-foam) was placed in a glass purge vessel.

Oxygen-free nitrogen gas (100% Nitrogen) was bubbled through the reagent mix, which was heated to 85°C (± 1°C) in a water bath on a thermostatically controlled hotplate. A chemical trap containing 25ml of 1N sodium hydroxide (16g NaOH/400ml HPLC grade water) was placed between the reagent vessel and the NO analyser (Sievers NOA 280i, Analytix, UK) or electrode (ami 700µm NO electrode Harvard apparatus, USA) to prevent damage to the NOA or electrode from hydrochloric acid vapour. The carrier gas (oxygen free nitrogen) was maintained at a constant flow rate by two adjacent flow meters (Figure 2.2).

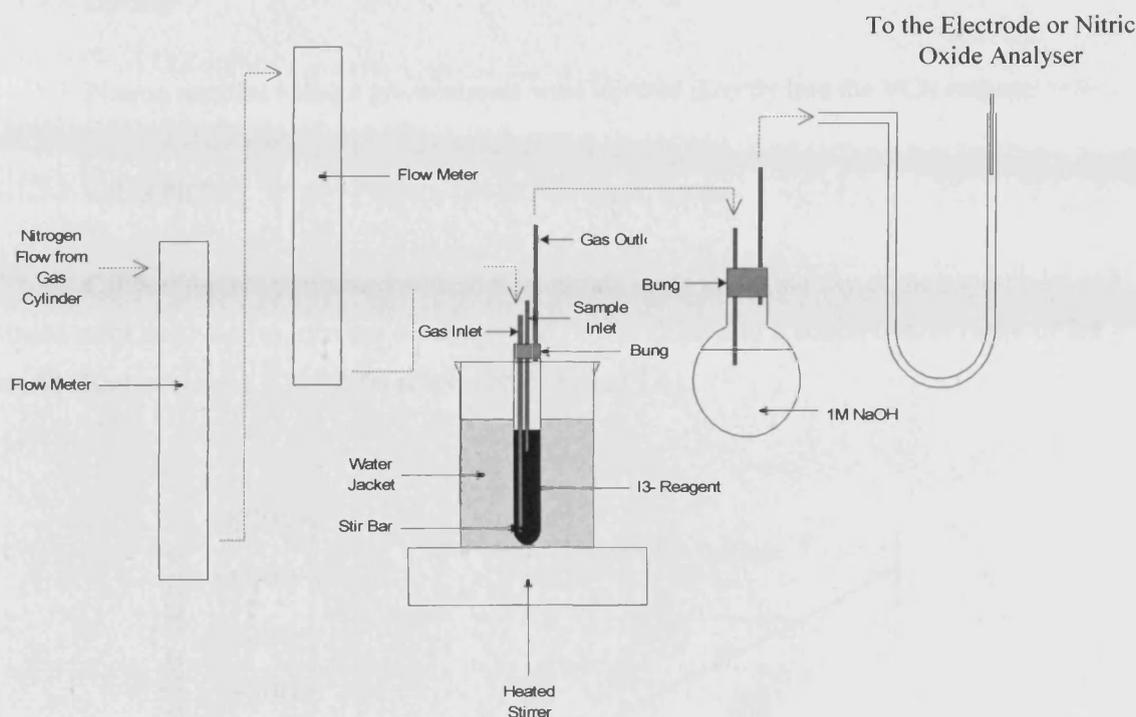


Figure 2.2: Experimental set-up for electrode and ozone based chemiluminescence determination of NO metabolites. The original injection system is demonstrated.

Throughout this thesis (as will become evident in the following chapter) a number of developments were made to measurement protocols. During this process a new reaction cell and injection system was developed. In the early experiments an NO electrode was used. With this system samples were injected into a reaction cell injection port through a three way tap. Consequently plastic 1ml syringes were used. This changed with the development of a custom made glassware system comprising a replaceable rubber septum (Jones Chromatography, UK), which allowed the introduction of samples with a Hamilton gas-tight micro-syringe. Please refer to chapter 3 section 3.3.1 for full details of these developments.

The release of NO was digitally recorded from the electrode using commercially available data acquisition and analysis software (Duo 18™, World Precision Instruments) or from the NOA using the associated Liquid program. All signals were subsequently transferred to Origin version 7.0, where they were smoothed using adjacent averaging (to improve the signal to noise) and subsequently measured using a peak analysis package (Origin 7.0).

2.1.3.2 Protocol

Plasma samples without pre-treatment were injected directly into the VCl_3 reagent.

2.1.3.3 Calibration

Calibration was performed with sodium nitrate made up on the day of the experiment as a 10mM stock solution (to increase accuracy) and further diluted to a concentration range of 2.5 - 40 μM (50 μl injections; 125 - 2000 pmoles NO) (Figure 2.3).

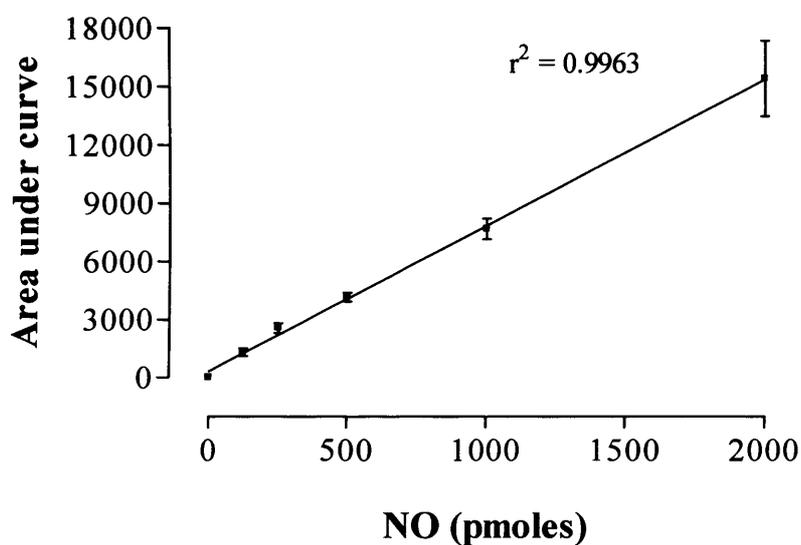


Figure 2.3: Standard curve of sodium nitrate in vanadium chloride. Data are presented as mean \pm SEM ($n = 6$).

2.1.3.4 Potential Confounding Factors

- At times fresh reagent added to the reaction vessel produced peaks even when no injection was made, as a consequence of the slow conversion of materials in the reagent to species that react with ozone. These signals disappeared with continued purging of the reagent.

- Unless anti-foam was added to the reagent plasma addition caused severe foaming. Only ~ 400µl of plasma could be added to the reagent before foaming occurred (even with added anti-foam)

2.1.4 Plasma NO and Plasma Protein-bound NO by Electrode/Ozone Based Chemiluminescence

The original tri-iodide reagent was used to measure plasma nitrite and plasma protein-bound NO (for reasons highlighted in chapter 3; section 3.2.2.5).

2.1.4.1 Chemicals, Reagents and Equipment

A stock solution of tri-iodide reagent (90ml) was prepared fresh each day (70ml Glacial acetic acid, 650mg iodine, 20ml HPLC grade nitrite free water, 1 gram potassium iodide) [94]. This solution was left to mix for 30 minutes prior to use. The same set up was used as for plasma NO_x determination by ozone based chemiluminescence (Figure 2.2) the only difference being the temperature of the reaction cell was maintained at 50°C (± 1°C).

2.1.4.2 Protocol

Plasma samples without pre-treatment were injected directly into the tri-iodide reagent to measure combined plasma nitrite and plasma protein-bound NO. A duplicate sample was treated with a 10% volume of acidified sulphanilamide (500mg sulphanilamide in 10ml of 1N HCl; resulting in a final 0.5% sulphanilamide and 0.1N HCl) and incubated for 15 minutes in the dark before injection into tri-iodide to specifically measure plasma protein-bound NO (Figure 2.4). The acid with the sulphanilamide converts nitrite to NO⁺ which then reacts with the sulphanilamide forming a stable diazonium salt that goes undetected in the tri-iodide reagent [95]. The difference between the two signals was taken to represent plasma nitrite. Following the two plasma injections (i.e., without and with sulphanilamide) the reagent was replaced.

2.1.4.3 Calibration

Calibration was performed with sodium nitrite made up on the day of the experiment as a 10mM stock solution (to increase accuracy) and further diluted to a concentration range of 62.5 -

1000nM (100 μ l injections; 6.25 - 100pmoles NO) for chemiluminescence protocol and 60 - 960nM (900 μ l injections; 54 - 864pmoles NO) for the electrode protocol (please refer to section 3.2.1.1; Figure 3.0 for an electrode standard curve and section 3.2.1.2; Figure 3.3 for an ozone based chemiluminescence standard curve).

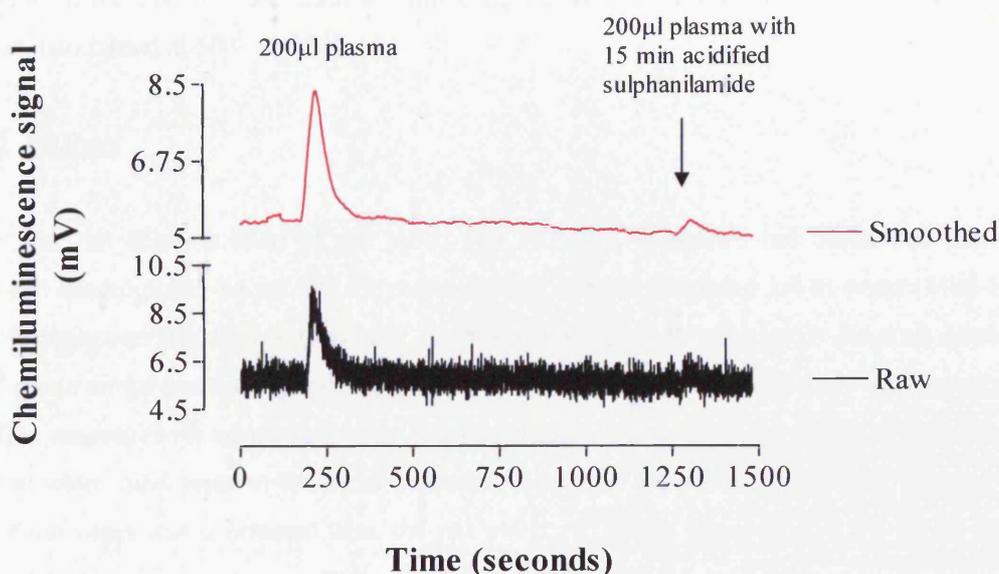


Figure 2.4: Representative trace of plasma measurement protocol. Combined plasma nitrite and protein-bound NO measurement followed by just plasma protein-bound NO measured in tri-iodide. Undiluted plasma (200 μ L) followed by plasma incubated with acidified sulphanilamide for 15 minutes in the dark (200 μ L)

2.1.5 Red Blood Cell NO and Haemoglobin-bound NO by Electrode/Ozone Based Chemiluminescence

A modified tri-iodide reagent with added potassium ferricyanide was used to measure red blood cell associated nitrite and haemoglobin bound NO. This was developed in order to prevent the auto-capture of NO released within the reagent mix by cell free haem (please refer to chapter 3; section 3.2.2).

2.1.5.1 Chemicals, Reagents and Equipment

A stock solution of tri-iodide reagent (90ml) was prepared fresh each day (as outlined above) along with a 250mM stock solution of potassium ferricyanide (823mg in 10ml HPLC water; kept on ice). The same set up was used as for plasma NO_x determination by ozone based chemiluminescence (Figure 2.2) the only differences being that 7.2ml of tri-iodide reagent was added to the reaction vessel along with 800µl of potassium ferricyanide - just prior to being connected to the system - and 20µl of anti-foam). Furthermore the temperature of the reaction cell was maintained at 50°C (± 1°C).

2.1.5.2 Protocol

For the determination of red blood cell NO (i.e., combined red blood cell associated nitrite and haemoglobin-bound NO) the red blood cell sample was lysed 1:4 in water (Mini-Plasco water for injection BP; Braun, Germany - found to give the smallest signals from all diluents or HPLC grade nitrite free water) and 200µl of the lysate was injected immediately into the modified tri-iodide reagent (with added potassium ferricyanide). As a control for potential contaminating nitrite in water (and prior to the blood injection) 160µl of the water diluent was injected. The signal from water was subtracted from the red blood cell lysate signal to give red blood cell NO (Figure 2.5).

To determine red blood cell haemoglobin-bound NO alone, red blood cells were lysed 1:4 in water to which a 10% volume of acidified sulphanilamide was added (to remove nitrite). The sample was then incubated in the dark for 15 minutes before 200µl of the lysate was injected. Prior to the blood injection 160µl of the sulphanilamidated water in which the sample was lysed was injected. This never gave a signal because the nitrite in the water is removed by the sulphanilamide. Pre-treatment of the water in this manner also prevented any possible contamination of the blood sample via the addition of exogenous nitrite (Figure 2.6). The difference between the two signals (i.e., red blood cell NO and red blood cell haemoglobin-bound NO) was taken to represent red blood cell associated nitrite. This terminology reflects the fact that the nitrite might only be associated with the red blood cell membrane and not come from within the red blood cell.

Following each duplicate blood injection (i.e., without and with sulphanilamide) the reagent was replaced.

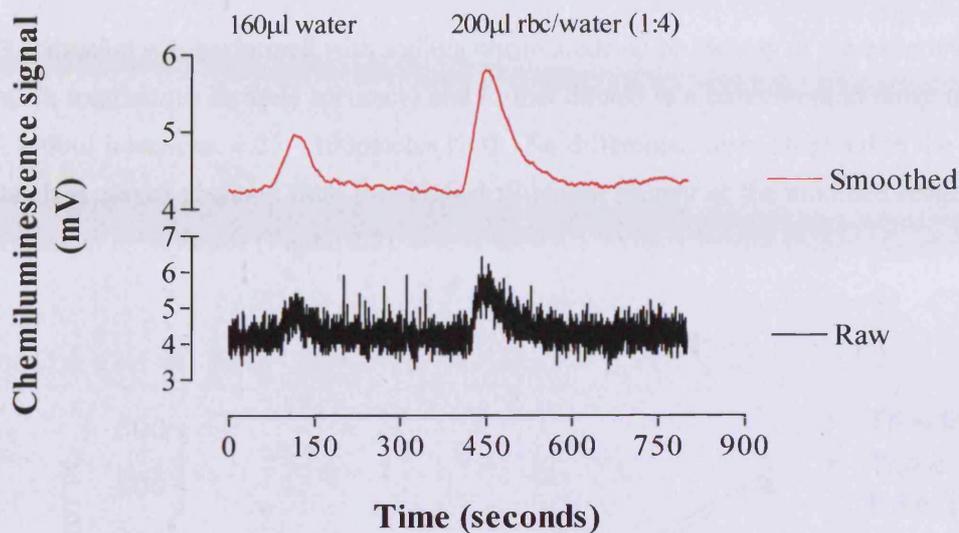


Figure 2.5: Representative trace of red blood cell NO measurement (i.e., combined red blood cell haemoglobin-bound NO and red blood cell associated nitrite) in tri-iodide with added $K_3Fe^{III}(CN)_6$. Water injection (160 μ L) followed by red blood cells lysed 1:4 in water (200 μ L).

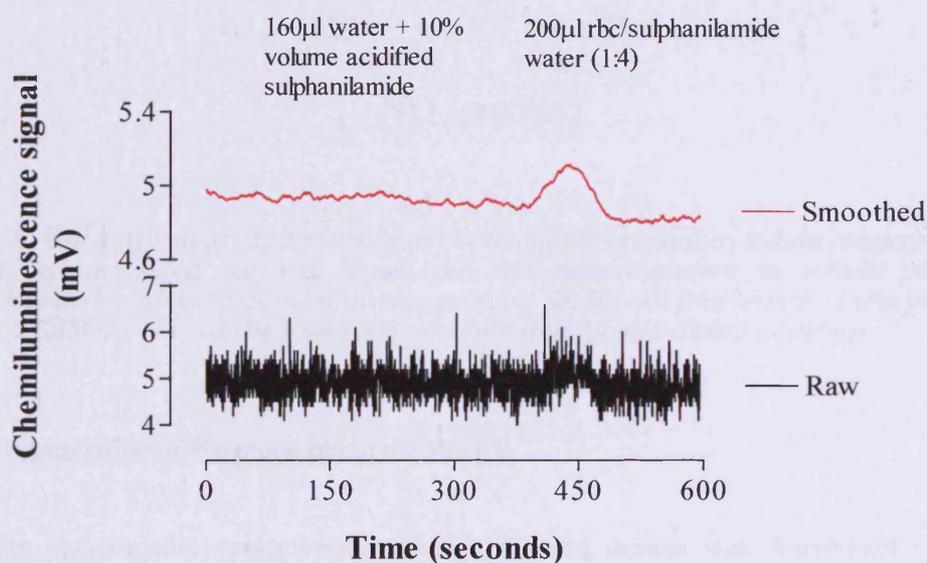


Figure 2.6: Representative trace of haemoglobin-bound NO measurement in tri-iodide with added $K_3Fe^{III}(CN)_6$. Sulphanilamided water injection (160 μ L) followed by red blood cells lysed 1:4 in sulphanilamided water (200 μ L) after 15 minute incubation in the dark.

2.1.5.3 Calibration

Calibration was performed with sodium nitrite made up on the day of the experiment as a 10mM stock solution (to increase accuracy) and further diluted to a concentration range of 62.5 - 1000nM (100µl injections; 6.25 - 100pmoles NO). No differences were observed in the sodium nitrite standard curves obtained from the original tri-iodide reagent or the modified reagent with added potassium ferricyanide (Figure 2.7).

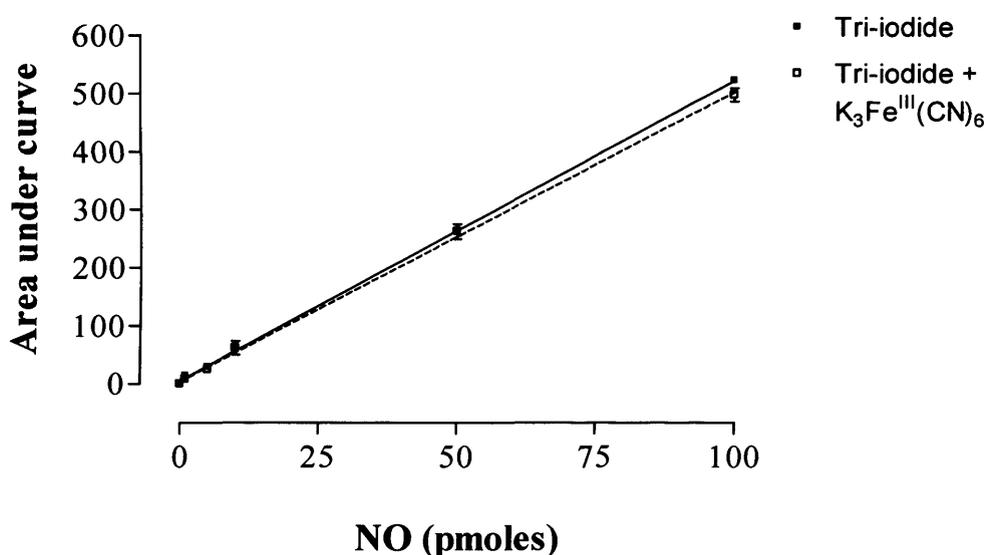


Figure 2.7: Comparison of nitrite standard curves in the original tri-iodide reagent and the modified reagent used for red blood cell NO measurements to which potassium ferricyanide was added (to prevent auto-capture of NO by cell free haem). Data presented as mean \pm SEM ($n = 6$). At the lower NO amounts data points directly overlap.

2.1.5.4 Concentration of Haemoglobin in the Sample

The haemoglobin concentration of each injected sample was determined using the cyanomethaemoglobin method [96]. The reagent, comprising KCN (0.77mM), K₃Fe^{III}(CN)₆ (0.61mM), potassium dihydrophosphate (1.03mM) and 100µl Triton X was made up each month and tested to ensure that it produced a blank signal compared with water and also maintained a

neutral pH. 40µl of sample was added to the reagent and following a ten minute incubation period the absorbance was measured at 540nm using the stock solution on its own as a blank.

2.2 **Bioassays**

2.2.1 **Organ Chamber Bioassay**

Male, New Zealand white rabbits (~ 2-2.5kg) were terminally anaesthetised with an intravenous injection of sodium pentobarbitone (0.75 ml/kg). A transverse incision was made into the abdominal cavity using blunt-ended scissors. The diaphragm was then cut and the rib cage opened to allow the thoracic aorta to be exposed. Taking care not to stretch the tissue, the aorta was excised and placed directly into fresh Krebs buffer (Table 2.2). The aorta was then cleaned of fat, blood and connective tissue before being cut into 2mm wide rings on a purpose built apparatus using equidistant upturned razor blades.

Experiments were performed with endothelium denuded and intact rings. To denude the vessels, the luminal surface was gently rubbed with a rounded wooden applicator. The aortic rings were subsequently mounted in 8ml tissue baths containing 5ml Krebs buffer and gassed with 95% O₂ / 5% CO₂ at 37°C (Figure 2.7). A resting tension of 2.1-2.3g was set. After a one hour equilibration period during which time the tension was reset (to allow for stretch induced relaxation) all rings were exposed to phenylephrine (PE, 1µM) to induce constriction. When a steady plateau was reached acetylcholine (10µM) was added to check the viability of the endothelium (functional endothelium was assumed by the presence of at least a 50% relaxation response; vessels in which this was not achieved were excluded from analysis). Following this the rings were washed re-equilibrated and repeat PE constrictions performed until a reproducible constriction was achieved. Various experiments were subsequently performed with differing protocols (Chapter 5).

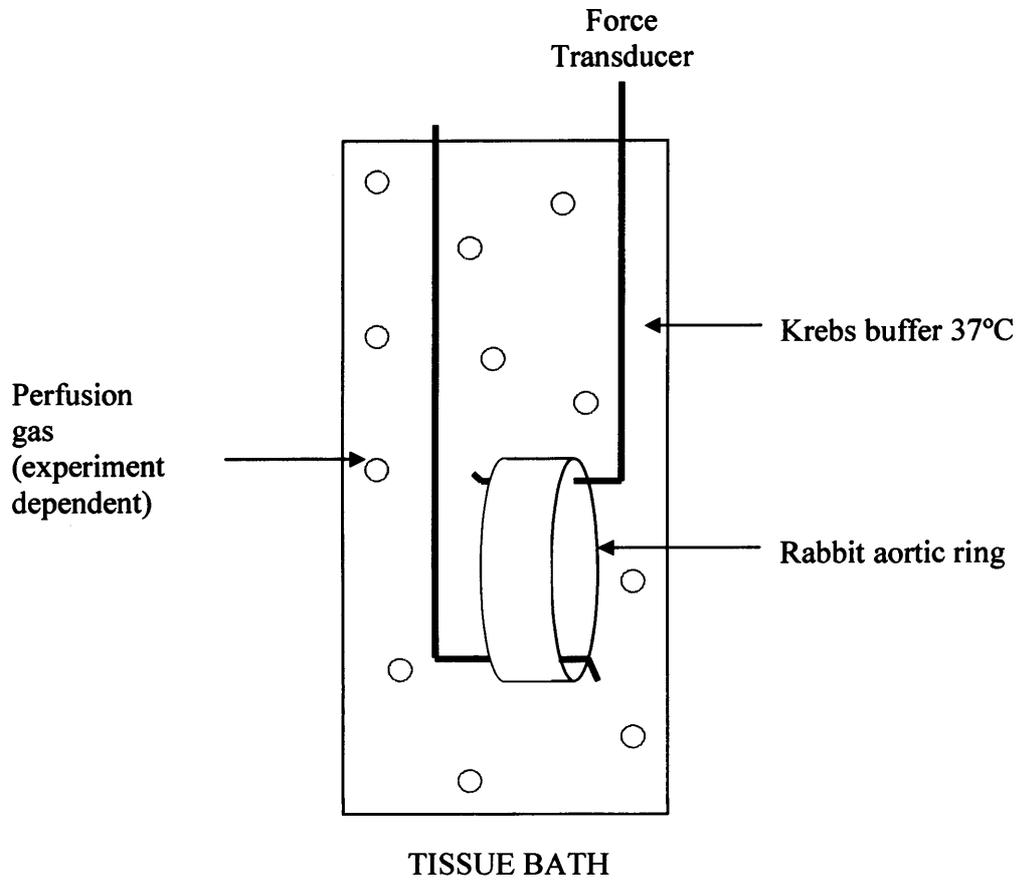


Figure 2.7: Schematic diagram of the isometric tension recording apparatus. Aortic rings are mounted on stainless steel hooks in 8ml tissue baths containing Krebs buffer (gassed) at 37°C and set to a resting tension between 2.1 - 2.3 grams.

Table 2.2: Composition of Krebs Buffer.

Chemical	Concentration (mM)	Grams/L
NaCl	138	6.38
KCl	5.3	0.2
KH ₂ PO ₄	1.2	0.16
MgSO ₄ ·7H ₂ O	1.2	0.3
NaHCO ₃	15	2.1
Glucose	24	1.98
CaCl ₂	1.5	0.22

2.3 NO Donors

In a controlled manner these compounds generate and release NO or *N*-oxides that may be converted to NO. Structural differences between various compound classes promote diversity in NO formation, release kinetics and resulting chemistry (reviewed in [97]). The following commercially available compounds were used in this thesis; the diazeniumdiolate MAHMA NONOate (noc-9), the *S*-nitrosothiol *S*-nitrosoglutathione (GSNO) and inorganic sodium nitrate and sodium nitrite.

2.3.1 NONOates

The NONOates are adducts of NO and nucleophiles. The generation of NO from these donors is dependent on pH, temperature and the chemical nature of the nucleophile. Under strong alkaline conditions NONOates are relatively stable however rapid decomposition occurs as the pH value approaches the physiological range (pH ~ 7.4) with NO release being almost instantaneous under acidic conditions. Noc-9 was selected because of its predictable rate of NO release which is unaffected by biological reactants [98] (Figure 2.8). At 37°C and pH 7.4, noc-9 has a half-life ($t_{1/2}$) of 1.5 to 2 minutes. Decomposition follows first-order kinetics yielding 2 moles NO per mole of the donor. By-products of NO release are NO^+ , NO^- , nitrite and the free nucleophile $(\text{C}_2\text{H}_5)_3\text{NH}_3^+$ (reviewed in [99]).

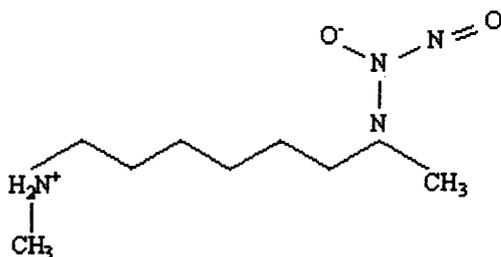


Figure 2.8: Chemical structure of MHAMA NONOate (noc-9).

Pre-weighed (5mg) vials of noc-9 powder were stored in the freezer (-20 °C). Fresh vials were prepared on a daily basis with the addition of one millilitre of 0.1M NaOH (prepared using HPLC grade water) to the 5mg of powder (24mM stock). Stock solutions were stored on ice (4°C) and protected from light. For experimental protocols in which noc-9 was added to biological matrices final dilutions were prepared in assay buffer immediately prior to application.

2.3.2 S-nitrosoglutathione (GSNO)

Nitrosation of sulphur residues of thiols and proteins results in the formation of *S*-nitrosothiols. These endogenously produced compounds can release NO, NO⁺, or NO⁻ depending upon the reaction conditions and process of decomposition. The stability of *S*-nitrosothiols in solution varies as a function of temperature, pH, nucleophiles, redox-active species, the trace metal content of the incubation solution and exposure to light.

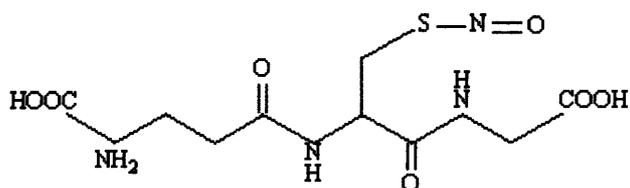


Figure 2.9: Chemical structure of *S*-nitrosoglutathione (GSNO).

Pre-weighed (25mg) vials of GSNO were stored in the freezer (-20 °C) protected from light. Fresh vials of 10mM stock (25mg in 7.44ml 100µM DTPA; DTPA 15.7mg/400ml HPLC water; used to increase the stability of the compound). Aliquots of the stock were stored in light protected vials in the freezer (-20 °C). Once thawed, vials were stored on ice (4°C). For experimental protocols in which GSNO was added to biological matrices, final dilutions were prepared in assay buffer immediately prior to application.

2.3.3 Sodium Nitrate/Nitrite

The stability of the inorganic sodium nitrate and sodium nitrite makes them ideal standards for the calibration of assays. These donors only release NO or related *N*-oxide species following chemical reduction. Stock solutions (10mM) were made up daily in HPLC water (69mg/100ml sodium nitrite; 84.9mg/100ml sodium nitrate).

2.4 Chemical Inhibitors

Chemical inhibitors not only allow the relevance of different signalling pathways to be determined, they also provide identification of specific pathways via which compounds work.

2.4.1 Inhibition of NOS

The biosynthesis of NO by NOS may be inhibited by naturally occurring or pharmacologically administered compounds. The most widely known NOS inhibitors are the L-arginine analogues, formed via the methylation of the amino acid L-arginine (Figure 2.10). Inhibitors, such as L-NMMA compete for the L-arginine binding site which means that they are non selective for the different NOS isoforms. However, L-NMMA preferentially inhibits Type III > Type I > Type II NOS isoforms [13].

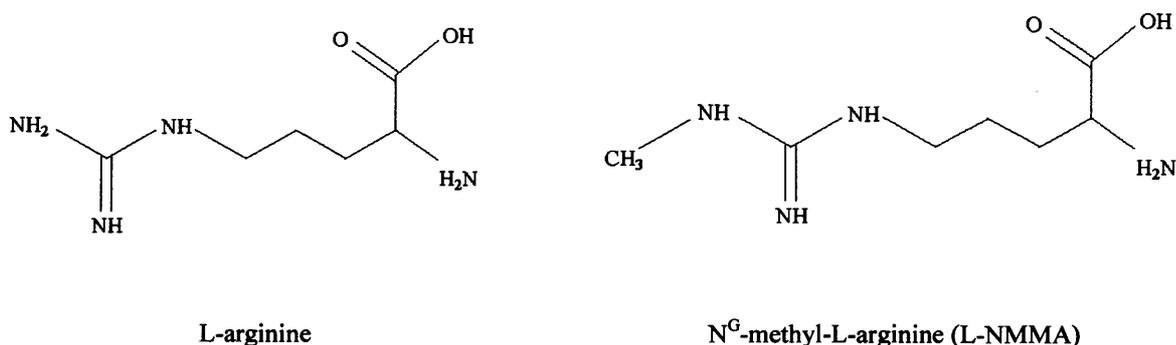


Figure 2.10: Chemical structures of L-arginine and the active site NOS inhibitor N^G-methyl-L-arginine (L-NMMA).

The selectivity of L-NMMA is determined by its interaction with the substrate binding site of the NOS enzyme [100]. In this thesis L-NMMA was only used in clinical protocols. Specific details are outlined in the relevant chapter (Chapter 6).

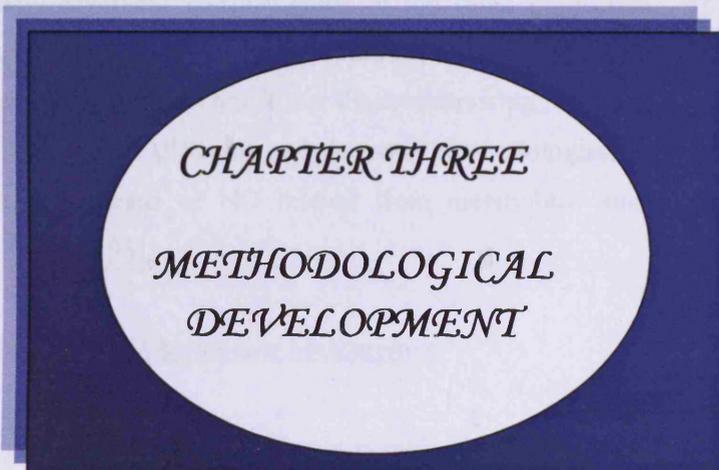
2.4.2 Inhibition of Soluble Guanylate Cyclase

Compounds have been developed that irreversibly oxidise prosthetic haem groups. 1H-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) is an example of such a compound which selectively inhibits guanylyl cyclase thus preventing NO mediated vascular smooth muscle relaxation. Pre-weighed (50mg) vials of ODQ were stored in the freezer (-20 °C). Fresh vials were prepared monthly with the addition of 2.67ml DMSO to the 50mg of powder (100mM stock). Prior to addition, final dilutions were prepared in assay buffer.

2.5 Chemicals and Other Agents

Table 2.11: Chemicals and agents used and sources.

Product	Source
<u>NO Donors</u>	
MHAMA NONOate (Noc-9)	Alexis Bio-chemicals UK
S-nitrosoglutathione (GSNO)	Alexis Bio-chemicals UK
Sodium Nitrite (NaNO ₂)	Sigma Aldrich UK
Sodium Nitrate (NaNO ₃)	Sigma Aldrich UK
Sodium Nitroprusside (SNP)	Sigma Aldrich UK
3-nitro-L-tyrosine	Sigma Aldrich UK
<u>Chemical Inhibitors</u>	
N ^G -methyl-L-arginine (L-NMMA)	Clinalfa, Germany
1H-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one (ODQ)	Sigma Aldrich, UK
<u>Reagent Chemicals</u>	
HPLC Grade Water	Fisher Scientific UK
Glacial Acetic Acid	Fisher Scientific UK
Hydrochloric Acid	Fisher Scientific UK
Potassium Iodide (KI)	Sigma Aldrich UK
Iodine (I ₂)	Sigma Aldrich UK
Cuprous Chloride (CuCl)	Sigma Aldrich UK
L-Cysteine (CSH)	Sigma Aldrich UK
Sodium Hydroxide (NaOH)	Fisher Scientific UK
Potassium Hexacyanoferrate (K ₃ Fe ^{III} (CN) ₆)	Sigma Aldrich UK
Potassium Cyanide (KCN)	Sigma Aldrich UK
Sulphanilamide	Sigma Aldrich UK
Mercury Chloride (HgCl ₂)	Sigma Aldrich UK
2,3-diaminonaphthalene (DAN)	Fisher Scientific UK
Diethylenetriaminepentaacetate (DTPA)	Sigma Aldrich UK
Phenylephrine (PE)	Sigma Aldrich UK
Acetylcholine (Ach)	Sigma Aldrich UK
Antifoam 204 Organic	Sigma Aldrich UK
Triton X	Sigma Aldrich UK
Vanadium Chloride (VCl ₃)	Sigma Aldrich UK
Dimethyl Sulfoxide (DMSO)	Fisher Scientific UK
Sodium Hydrogen Phosphate (Na ₂ HPO ₄)	Sigma Aldrich UK
Sodium Dihydrogen Phosphate (NaH ₂ PO ₄)	Sigma Aldrich UK
Glucose-6-phosphate	Sigma Aldrich UK
Glucose-6-phosphate dehydrogenase	Sigma Aldrich UK
NADPH	Sigma Aldrich UK
Nitrate Reductase	Sigma Aldrich UK
<u>Krebs</u>	
Sodium Chloride (NaCl)	Fisher Scientific UK
Potassium Chloride (KCl)	Fisher Scientific UK
Potassium Dihydrogen Orthophosphate (KH ₂ PO ₄)	Fisher Scientific UK
Magnesium Sulphate (MgSO ₄ ·7H ₂ O)	Fisher Scientific UK
Sodium Hydrogen Carbonate (NaHCO ₃)	Fisher Scientific UK
Glucose	Fisher Scientific UK
Calcium Chloride (CaCl ₂)	Fisher Scientific UK



CHAPTER THREE
METHODOLOGICAL
DEVELOPMENT

3.0 Introduction

The chemical reactivity of free NO makes its detection in biological systems extremely challenging. Equally as demanding are measurements of NO metabolites in biological matrices due to the diversity of species, dilute concentrations (at the lower end of sensitivity of many techniques), various stabilities, complex interactions and reactivity. The wide range of reported baseline human NO metabolite concentrations in the literature lay testament to these difficulties.

The NO metabolite research field is somewhat unique with regard to the limited agreement over measurement methodologies. A variety of analytical approaches have been utilised yielding vastly divergent measurements of the same product(s). This has led to unreliable conclusions being made which have unfortunately served to confuse rather than enlighten. Of late general consensus has it that these contrasting reports of metabolite values have resulted from the use of different measurement methodologies, that is; various NO measurement techniques, means of NO release from metabolites and biological sample handling protocols [9, 101-103].

3.0.1 General Principle of NO Metabolite Measurement

All metabolite measurement techniques combine a method of detecting NO (or a related redox species or reaction product of NO) with a means of releasing NO from the biological metabolite to allow its detection (or subsequent reaction prior to detection).

3.0.2 Variability Introduced by NO Measurement Techniques

Numerous techniques including spectrophotometry [104, 105], fluorimetry [106-108], electron paramagnetic resonance (EPR) spectroscopy [104], chemiluminescence [26, 69, 72, 73, 78, 83, 109-114] and amperometric electrodes [115] have been utilised for NO metabolite measurement (Table 1). Whilst each technique has its own significant individual advantages, all suffer from the usual limitations including lack of sensitivity or specificity or interference by factors such as proteins and suspended materials commonly present within biological media.

Table 3.0: Baseline human NO metabolite concentrations determined with different measurement techniques. Values reported as nM concentrations. ^a = serum; ^b = below detection limit; ^c = foetal blood samples; OBC = ozone based chemiluminescence; HPLC = high performance liquid chromatography; GC-MS = gas chromatography mass spectrometry; HgCl₂ = mercury chloride; KI = potassium iodide; 3Cs = cupric chloride/cysteine/carbon monoxide; CuCl = cupric chloride; CSH = cysteine.

Metabolite			NO measurement technique	Means of NO release	Ref
SNO-Hb		Plasma RSNO			
Arterial	Venous	Venous			
		8000 ± 3000	Fluorimetry	HgCl ₂	[106]
		7190 ± 5730	OBC	Photolysis	[63]
6500 ± 1925	7175 ± 1875		Electrode	Tri-iodide	[115]
		5930 ± 750	Spectrophotometry	HgCl ₂	[104]
3625 ± 1650 ^c	5470 ± 3025 ^c		Spectrophotometry	HgCl ₂	[105]
		4200 ± 1000	Fluorimetry	Photolysis	[116]
2500 ± 1000	300 ± 700		OBC	Photolysis	[83]
		930 ± 360 ^a	OBC	CuCl/CSH	[109]
		450 ± 450	Fluorimetry	HgCl ₂	[107]
250-560	45-325		OBC	3Cs	[114]
		250 ± 200	OBC	KI/Acetic acid	[110]
		220 ± 190	HPLC	Griess	[117]
		156 ± 64	GC-MS	HgCl ₂	[118]
161 ± 42	142 ± 29	63 ± 13 ^a	OBC	Tri-iodide	[78]
		62 ± 24	HPLC	Griess	[108]
110 ± 40	130 ± 60		OBC	Tri-iodide	[73]
	< 100 ^b		Fluorimetry	HgCl ₂	[108]
46 ± 17	69 ± 11		OBC	Tri-iodide	[111]
< 50 ^b	< 50 ^b		OBC	Tri-iodide	[26]
		40 ± 7	OBC	CuCl/CSH	[72]
		28 ± 7	OBC	Tri-iodide	[95]
		15 ± 6	OBC	Tri-iodide	[112]
< 1 ^b	< 1 ^b	7.2 ± 1.1 ^a	OBC	Tri-iodide	[113]

3.0.3 Variability Introduced by Means of NO Release

Much of the early work on NO metabolite measurement was performed utilising UV-light induced photolysis to liberate NO (specifically *S*-nitrosothiols; RSNOs) [63, 70]. It has since been demonstrated under extremely high energy conditions that photolysis can release NO from nitrosyl haems, nitrite and from nitrate in the presence of reduced thiol [119], which might explain why such high levels of *S*-nitrosoalbumin and SNO-Hb were originally reported [63, 70]. More recently chemical reduction has become a more favoured method of cleaving NO from biological matrices. Several chemical reagents have been developed, the majority of which reduce NO related species back to NO gas in strong acid [27]. However, as the

reducing properties of these solutions vary greatly so does the efficiency of reduction and the selectivity of metabolite species [120]. This complexity is reflected in the fact that for many of the chemical reagents the exact metabolite species detected and the reaction processes involved still remain to be fully elucidated.

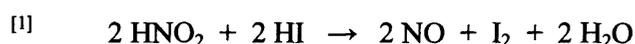
Even where the same NO measurement technique has been used as in the case of ozone based chemiluminescence, the various means of NO release have induced variance in the results obtained (Table 3.1).

Table 3.1: Baseline concentrations of human NO metabolites measured with the same technique (ozone based chemiluminescence) but different means of NO release from metabolites. All values reported as nM concentrations. ^a = serum; ^b = below the detection limit; ^c = foetal blood sample; 3Cs = cupric chloride/cysteine/carbon monoxide; CuCl = cupric chloride; CSH = cysteine.

Metabolite					Means of NO release	Ref
SNO-Hb		HbNO		Plasma RSNO		
Arterial	Venous	Arterial	Venous	Venous		
				7190 ± 5730	Photolysis	[63]
2500 ± 1000	300 ± 700	2500 ± 500	5000 ± 1250		Photolysis	[83]
				930 ± 360 ^a	CuCl/CSH	[109]
250-560	45-325				3Cs	[114]
				250 ± 200	KI/Acetic acid	[110]
161 ± 42	142 ± 29			63 ± 13 ^a	Tri-iodide	[78]
110 ± 40	130 ± 60	150 ± 80	160 ± 50		Tri-iodide	[73]
		81 ± 36	103 ± 32		Acetic acid/ Ferricyanide	[27]
46 ± 17	69 ± 11				Tri-iodide	[111]
< 50 ^b	< 50 ^b	< 50 ^b	< 50 ^b		Tri-iodide	[26]
				40 ± 7	CuCl/CSH	[72]
				28 ± 7	Tri-iodide	[95]
				15 ± 6	Tri-iodide	[112]
< 1 ^b	< 1 ^b	< 1 ^b	< 1 ^b	7.2 ± 1.1 ^a	Tri-iodide	[113]

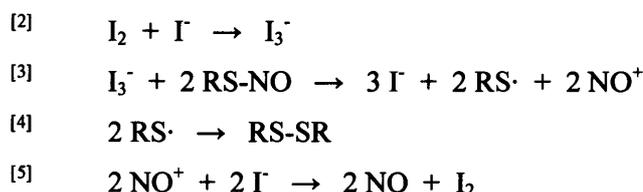
3.0.3.1 Tri-iodide

Tri-iodide is possibly the most widely used chemical reductive reagent in the NO metabolite literature [26, 73, 78, 95, 111-113]. It was developed from a reaction mix comprising potassium iodide (KI) at acidic pH which was used to reduce nitrite to NO via the formation of nitrous acid [121].^[1]



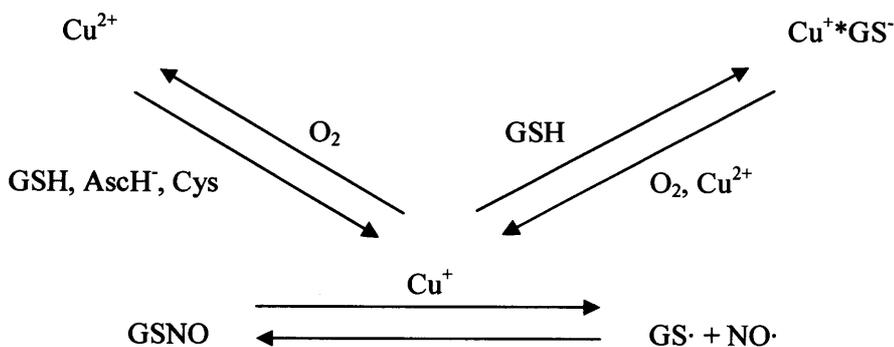
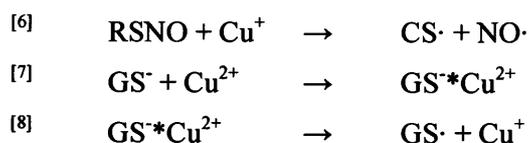
Uncontrolled formation of free iodine (I_2) from the reaction ^[1] resulted in poor reproducibility of the NO yield from RSNOs. This issue was resolved with the addition of crystalline iodine to the reagent which controlled the amount of free iodine in solution and thus tri-iodide formation, resulting in the stoichiometric release of NO from RSNOs [121].

Reported NO yields of the various metabolite species with tri-iodide are ~ 100% for low molecular weight RSNOs, ~ 78% for SNO-albumin, ~ 81% for HbNO and ~ 67-105% for RNNO compounds [74, 120]. However, whilst the reactions involved in the cleavage of RSNO have previously been outlined ^{[2]-[5]} [121], the underlying reaction mechanisms for the release of NO from RNNO/nitrosyl haem species still remain in question.



3.0.3.2 Cuprous Chloride/Cysteine

Another chemical reagent used in conjunction with ozone-based chemiluminescence comprises cuprous chloride and cysteine (CuCl/CSH). This was developed to specifically measure RSNOs, exploiting trans-nitrosation reactions between biological SNOs and the liberation of NO from nitrosothiols in the presence of Cu^+ ^[6], with the re-reduction of Cu^{2+} by CSH ^[7-8] [109, 122, 123].



The neutral pH of the reagent ensures that nitrite, nitrate, and 3-nitrotyrosine are not detected, thus maintaining specificity for RSNO. NO yields from this reagent are reportedly stoichiometric for low mass SNOs; however, the yield from SNO-albumin is low (~ 33.9%), probably reflecting a sheltering of the SNO bond inhibiting trans-nitrosation to cysteine [109].

3.1 Aims

In order to address the significance of a NO reserve in the circulation it was imperative to use techniques and develop assays with the capability of measuring baseline human NO metabolite concentrations. This work was undertaken to:

- Identify the most sensitive NO measurement technique within our laboratory
- Investigate the chemical reagents used to liberate NO from metabolites
- Develop biological sample handling protocols to minimise contamination and loss of less stable metabolite species

3.2 Results

The first objective was to analyse NO measurement techniques within the laboratory.

3.2.1 Established Techniques used in the Laboratory

At the outset of this thesis several established NO measurement techniques were employed in our laboratory. For red blood cell metabolites an NO electrode was used in conjunction with the tri-iodide chemical reagent to measure total haemoglobin bound NO, HbNO and SNO-Hb. EPR spectroscopy was also used for HbNO quantification. Plasma RSNO measurements were made with the spectrophotometric Saville assay, whilst nitrite and NOx (nitrite plus nitrate) measurements were made with a fluorometric assay. Amongst all these methods, only the electrode had the sensitivity to measure the lower levels of baseline red blood cell and plasma NO metabolite concentrations reported in the literature. The fluorescence assay was sensitive enough for NOx measurements due to the presence of $\mu\text{mol/L}$ concentrations of nitrate in plasma. However, baseline levels of the other metabolite species were below the sensitivity of the corresponding NO measurement techniques. Consequently these were only useful for the analysis of in vitro chemistry conducted at elevated analyte concentrations.

3.2.1.1 Assessment of Amperometric NO Electrode

In this set up the tri-iodide reagent was used to liberate NO from the sample. Released NO was subsequently carried in a stream of nitrogen gas to the NO electrode where it was detected following its electrochemical oxidation on the electrode surface.

Standard curves indicated that the NO electrode had the sensitivity to detect baseline levels of red blood cell and plasma NO metabolites reported in the literature (Figure 3.0). However the electrode was challenging to work with because of its sensitivity to gas flow and temperature changes which meant it took ~ 2 hours at the start of the day and ~ 15 -20 minutes each time the assay reagent was replaced to reach a steady baseline. Furthermore signals often did not return to baseline so peak height had to be used to determine NO concentration. Despite the fact that an excellent correlation could be obtained between peak height and NO concentration across the range of a nitrite standard curve, this did not hold for red blood cell samples in part due to the broadening of the signals in the presence of haemoglobin (see Effect of Haemoglobin in the Reaction Chamber on NO Detection in Tri-iodide, section 3.2.2.1).

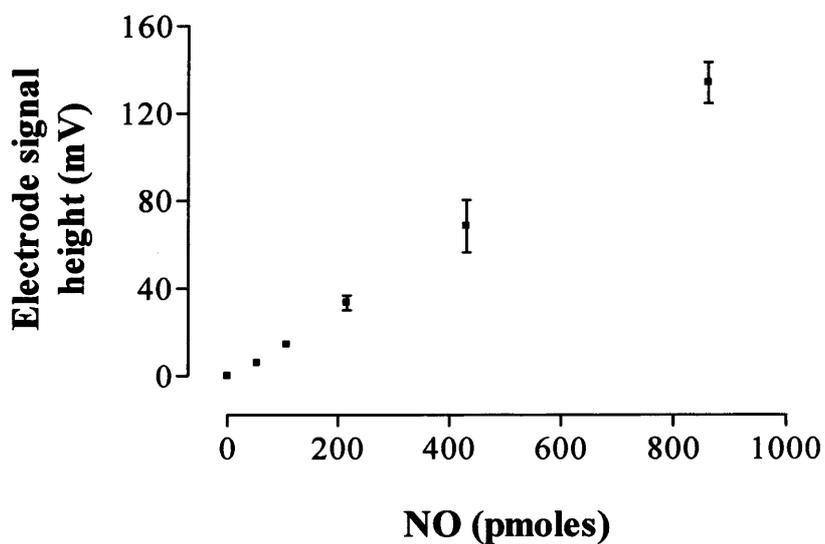
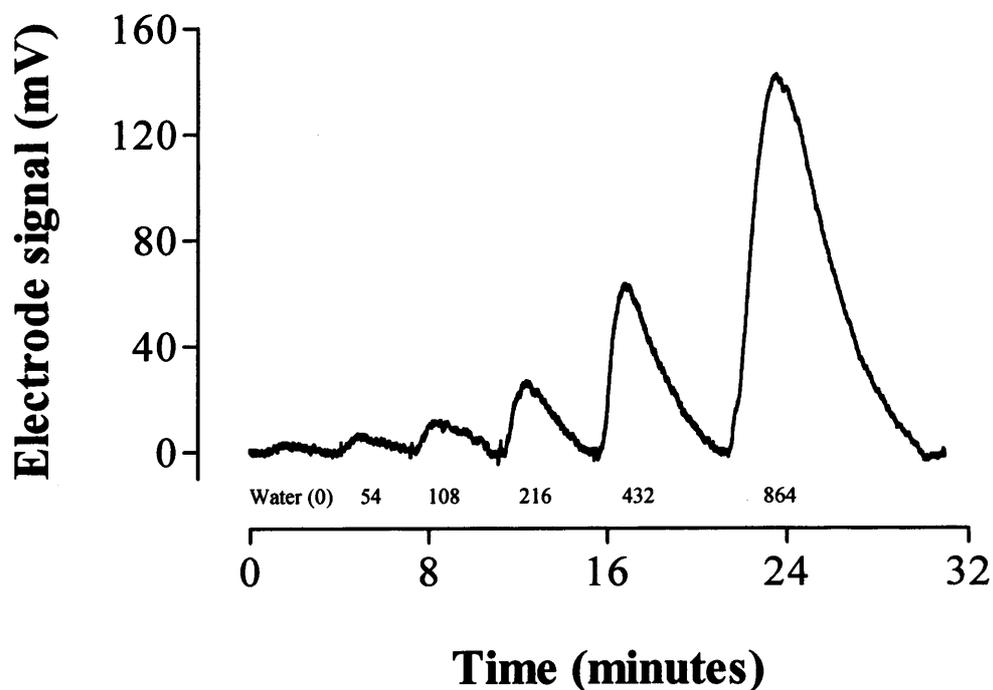


Figure 3.0: Standard curve of nitrite injected into tri-iodide (8ml) in conjunction with the NO electrode in the gas phase (Top). Correlation between NO injected and signal height (Bottom). Below each peak on the trace is the amount of nitrite injected in picomoles. Values presented as mean \pm SEM ($n = 3$ curves on separate days).

Further anomalies also persisted with the electrode:

1. The first injection of the day (water for the standard curve) was always significantly higher than subsequent water injections (Figure 3.1).
2. The first injection of red blood cell sample (lysed red blood cell or haemoglobin from a Sephadex G25 separation column) into fresh assay reagent consistently gave a signal whilst repeat injections into the same reagent rarely did (see, Effect of Haemoglobin in the Reaction Chamber on NO Detection in Tri-iodide, section 3.2.2.1).

These experiments outlined were performed with a custom made reaction cell comprising an injection port with a three-way tap, venflon needle (allowing sample injection to the bottom of the reagent), gas inlet (through which oxygen-free nitrogen gas flowed to the bottom of the reagent) and gas outlet allowing flow to the NO electrode via a sodium hydroxide (NaOH) trap. Samples were introduced into the tri-iodide reagent using a plastic syringe which interlocked with the three way tap (Figure 3.2 - Left).

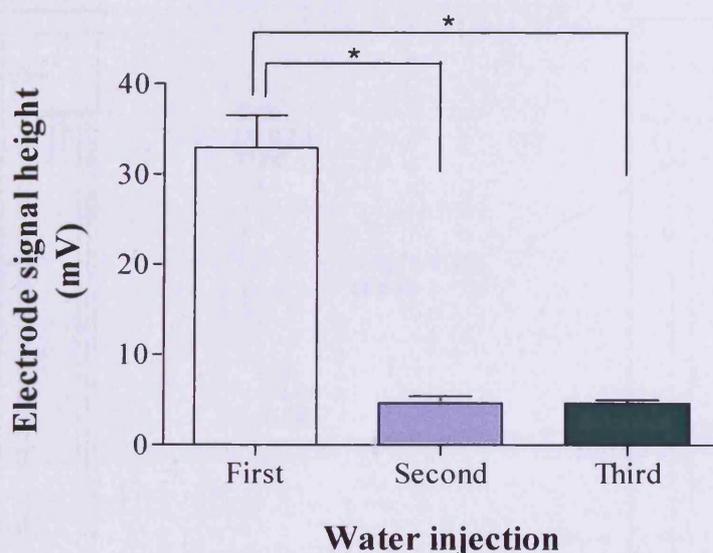


Figure 3.1: Signal heights from the first, second and third water injections (400 μ L) into tri-iodide reagent (8ml) using the NO electrode in the gas phase. ($n = 5$ experiments; Repeat measures ANOVA * $p < 0.001$).

The dead space within the venflon needle inevitably caused the introduction of air prior to the sample, which was ultimately found to produce an electrode signal. Therefore every initial injection comprised a mixture of air and sample and each subsequent injection was diluted equivalently by the fluid left in the needle. The signal from air was found to equate to the difference between the first and second water injections into fresh tri-iodide reagent. In terms of blood measurements, the three way tap and venflon injection needle were cleaned and dried after each injection. This meant that all blood measurements comprised the air signal leading to an over-estimation of NO levels. This explains in part why $\mu\text{mol/L}$ whole blood concentrations of SNO-Hb and HbNO were reported from our laboratory at that time [115]. Interestingly these values were in close agreement with reported levels from other groups using totally different NO measurement techniques and means of NO release [83, 105].

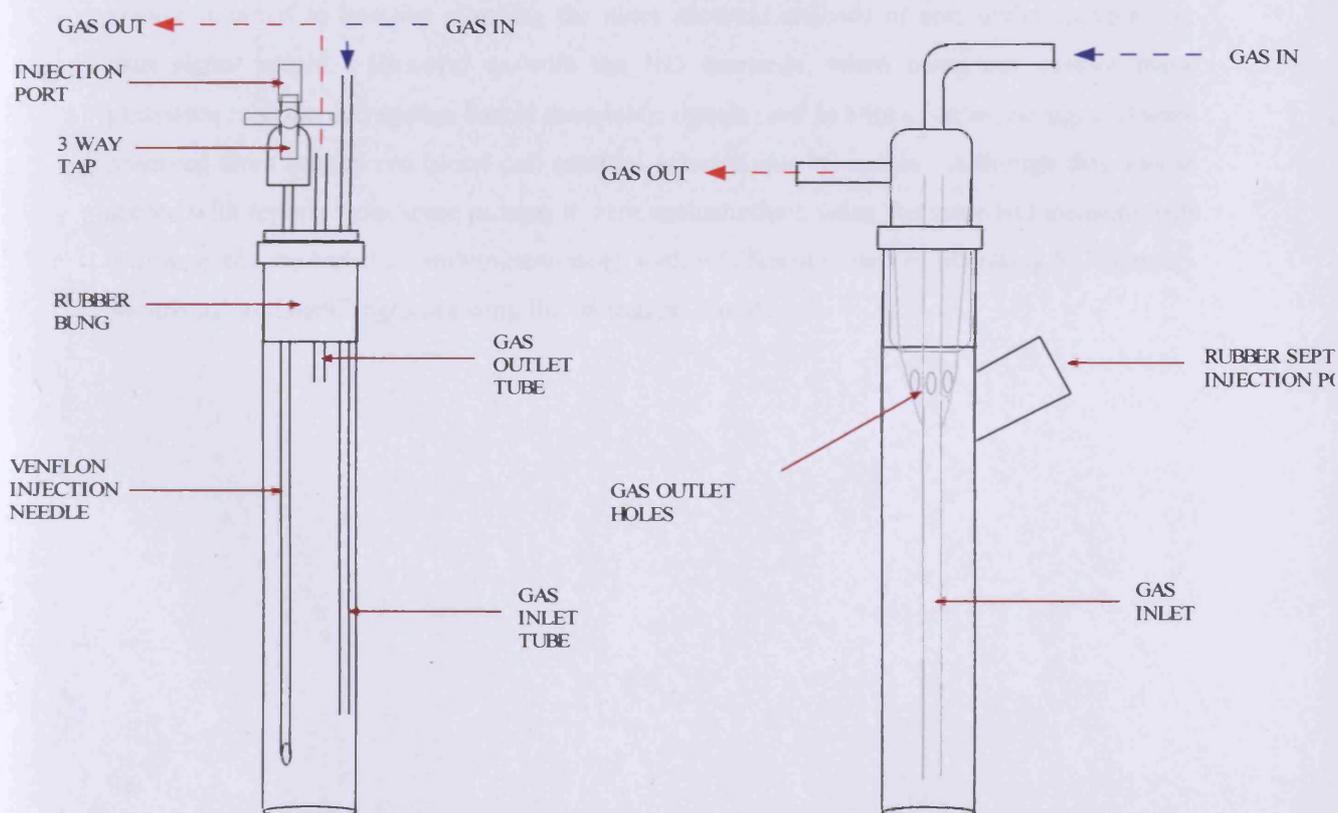


Figure 3.2: The original injection system (Left) and the custom made glassware reaction cell system (Right) used with the NO electrode and ozone based chemiluminescence.

The issue of the air injection was subsequently overcome with the development of a custom made glassware reaction cell system designed with a rubber septum injection port. This allowed the introduction of samples with a gas tight Hamilton syringe, preventing the injection of air and interference from previous sample injections (Figure 3.2 - Right). Utilising the same electrode set up with the custom made glassware system no signals from human blood samples were obtained. However, this was in accord with the findings from other groups in the literature at that time using ozone based chemiluminescence [26, 113].

3.2.1.2 Assessment of Ozone Based Chemiluminescence

Based on the results already outlined, our laboratory invested in a Sievers Ozone based chemiluminescence Nitric Oxide Analyser (NOA). The data obtained with the NO electrode was compared under the same conditions with that derived from the NOA. The NOA was found to be ~ 10 times more sensitive than the electrode (Figure 3.3) and also more practical in terms of the time taken to set up and measure samples. Furthermore signals always returned to baseline allowing the more accurate analysis of area under curve rather than signal height. However as with the NO electrode, when using our custom made glassware reaction cell system barely detectable signals (and in a lot of cases, no signals) were observed from human red blood cell samples injected into tri-iodide. Although this was in accord with reports from some groups, it went against others using the same NO measurement technique (ozone based chemiluminescence), with a different means of liberating NO (namely photolysis) and handling/processing the biological sample.

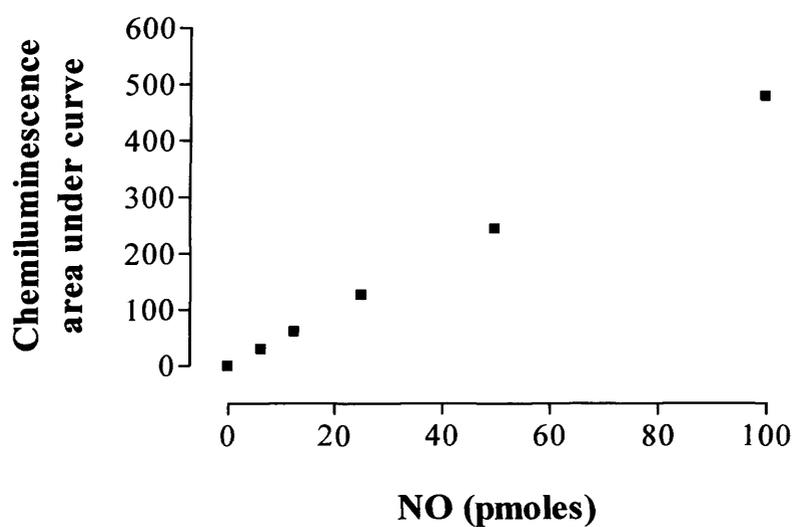
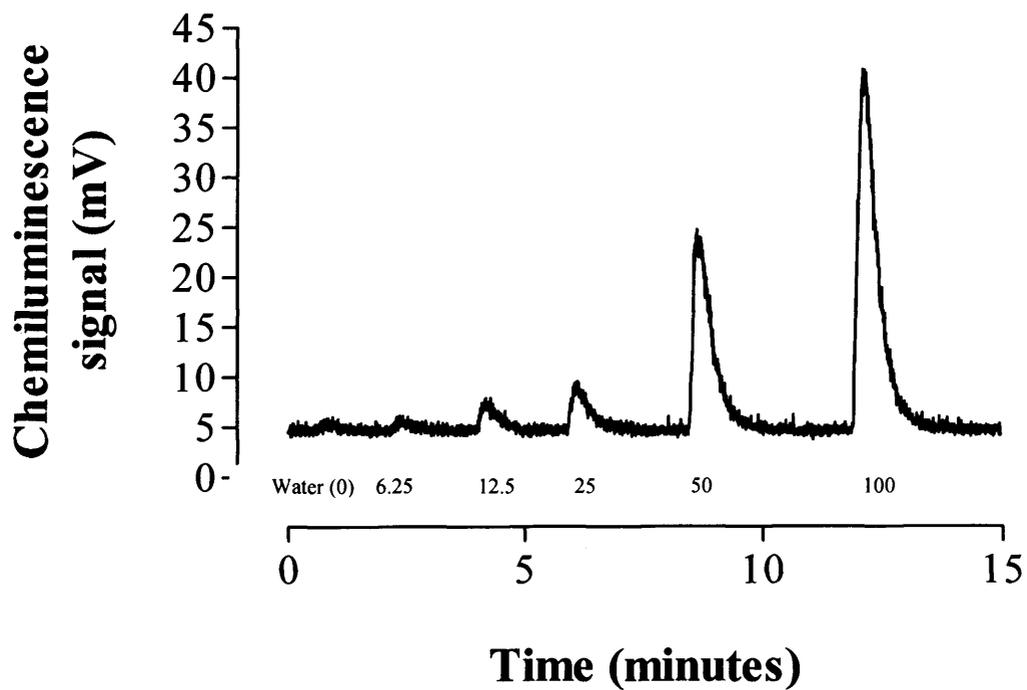


Figure 3.3: Standard curve of nitrite injected into tri-iodide (8ml) in conjunction with ozone based chemiluminescence. Correlation between NO injected and area under curve (Bottom). Below each peak on the trace is the amount of nitrite injected in picomoles. Values presented as mean \pm SEM ($n = 8$ curves on separate days).

3.2.2 Analysis of NO Release from Metabolites

In order to ascertain why barely detectable/no signals were obtained from red blood cell samples injected into tri-iodide, the chemical reagent itself was analysed along with its related methodology. At the same time the CuCl/CSH assay was also assessed as it was intended to use this reagent to provide a means by which to directly measure SNO-Hb and compare metabolite values obtained with tri-iodide. **The specific aim was to identify potential confounding factors that could influence the sensitivities of these chemical assays.** This involved an investigation into the effect of:

- Haemoglobin in the reaction chamber on NO detection
- Plasma in the reaction chamber on NO detection
- Biological sample handling on the recovery of NO from human blood

3.2.2.1 Effect of Haemoglobin in the Reaction Chamber on NO Detection in Tri-iodide

The NO donors sodium nitrite (NaNO_2^-) and *S*-nitrosoglutathione (GSNO) were injected into tri-iodide in the presence/absence of haemoglobin in the reaction chamber. The chemiluminescence signal heights from NaNO_2^- (Figure 3.4) and GSNO (Figure 3.5) were found to be inversely related to the concentration of haem in the reaction chamber. The relationship of reduced signal height with increasing addition of haem was similar whether red blood cell lysate or haemoglobin was added to the reagent [124].

Haem auto-capture of NO in the tri-iodide reagent had previously been acknowledged and postulated to occur as a result of the rapid ($\sim 10^7 \text{M}^{-1} \text{s}^{-1}$) re-capture of metabolite derived NO by cell free haem in the reaction chamber inhibiting its passage in the carrier gas to the NO analyser [120]. Due to the reagent being held under anaerobic conditions (purged with oxygen free nitrogen gas) the resulting interaction between cell free haem and NO was thought most likely to form HbNO. As tri-iodide reportedly cleaved this species, the effect was suggested only to broaden the NO signals from red blood cell samples [120]. This held true with relatively high NO addition (100 pmoles) as signal height was diminished across a range of haem concentrations, but area under curve analysis remained unaffected (Figure 3.6 - Top). However, with lower amounts of NO (10 pmoles) more in line with that anticipated from a processed red cell sample, both signal height and area under curve diminished (Figure 3.6 - Bottom), i.e., where haem was in vast excess of free NO, auto-capture was of much greater relevance with practical detection being severely hampered. The onset of this phenomenon occurred at an NO:haem ratios of $\sim 1:50,000$, equating to an NO:Hb ratio of $\sim 1:12,500$.

This data not only emphasised the need to change reagent between blood injections when utilising tri-iodide (which was already being undertaken to prevent excessive foaming of the reagent) it also provided a potential explanation to why, after the first blood injection using our electrode system, subsequent injections into the same reagent rarely produced a signal.

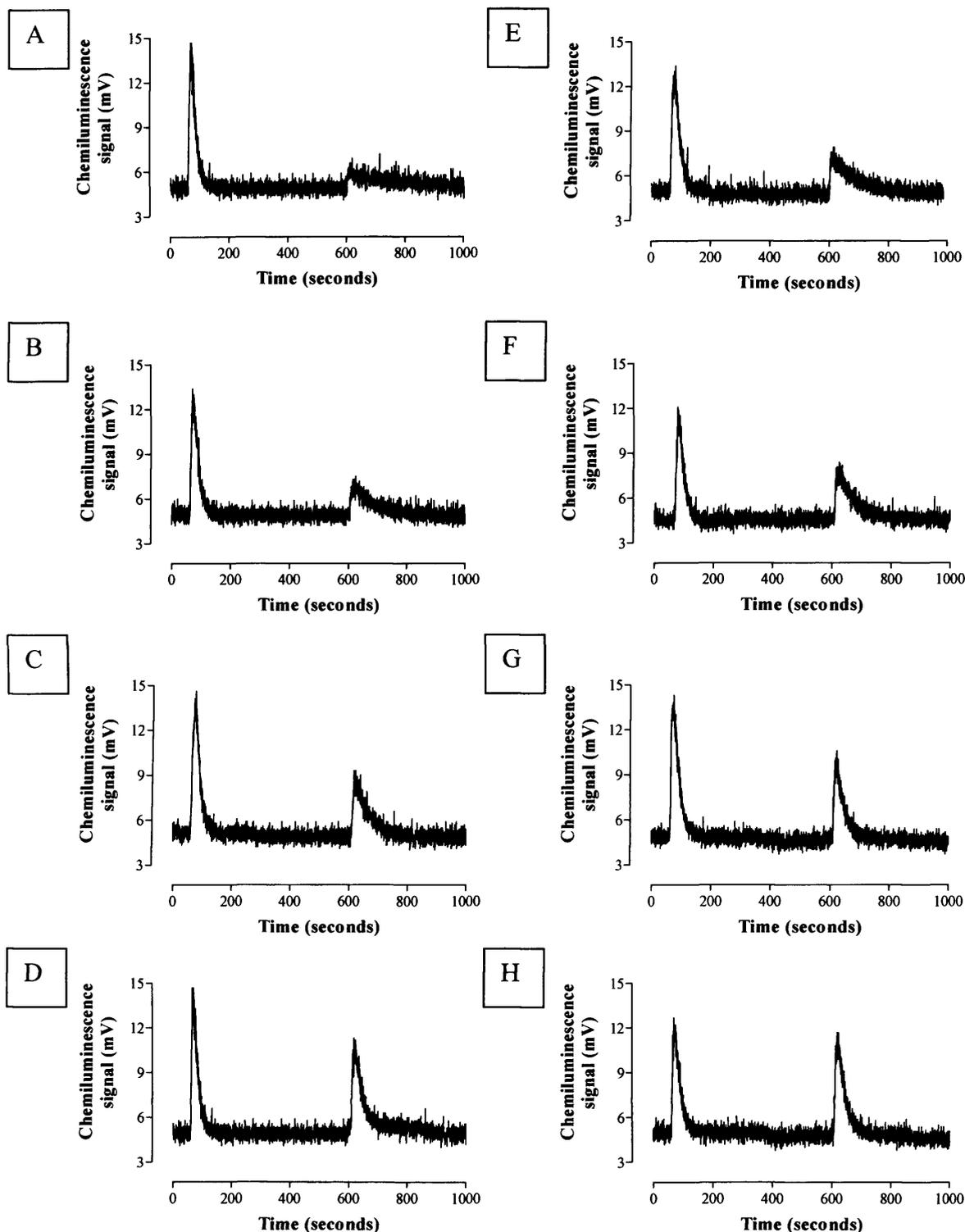


Figure 3.4: Haem NO scavenging from nitrite in the tri-iodide reagent. Raw chemiluminescence signals observed from repeat 25pmole nitrite injections before and after the addition of lysed red blood cells (Left column) 1ml (A) 250 μ l (B) 50 μ l (C) 25 μ l (D) - final haem concentrations ~ 20-660 μ M; haemoglobin (Right column) 1ml (E) 250 μ l (F) 50 μ l (G) 25 μ l (H) - final haem concentrations ~ 5-160 μ M. Lysed red blood cells (A-D) and haemoglobin (E-H) were added at 300 seconds.

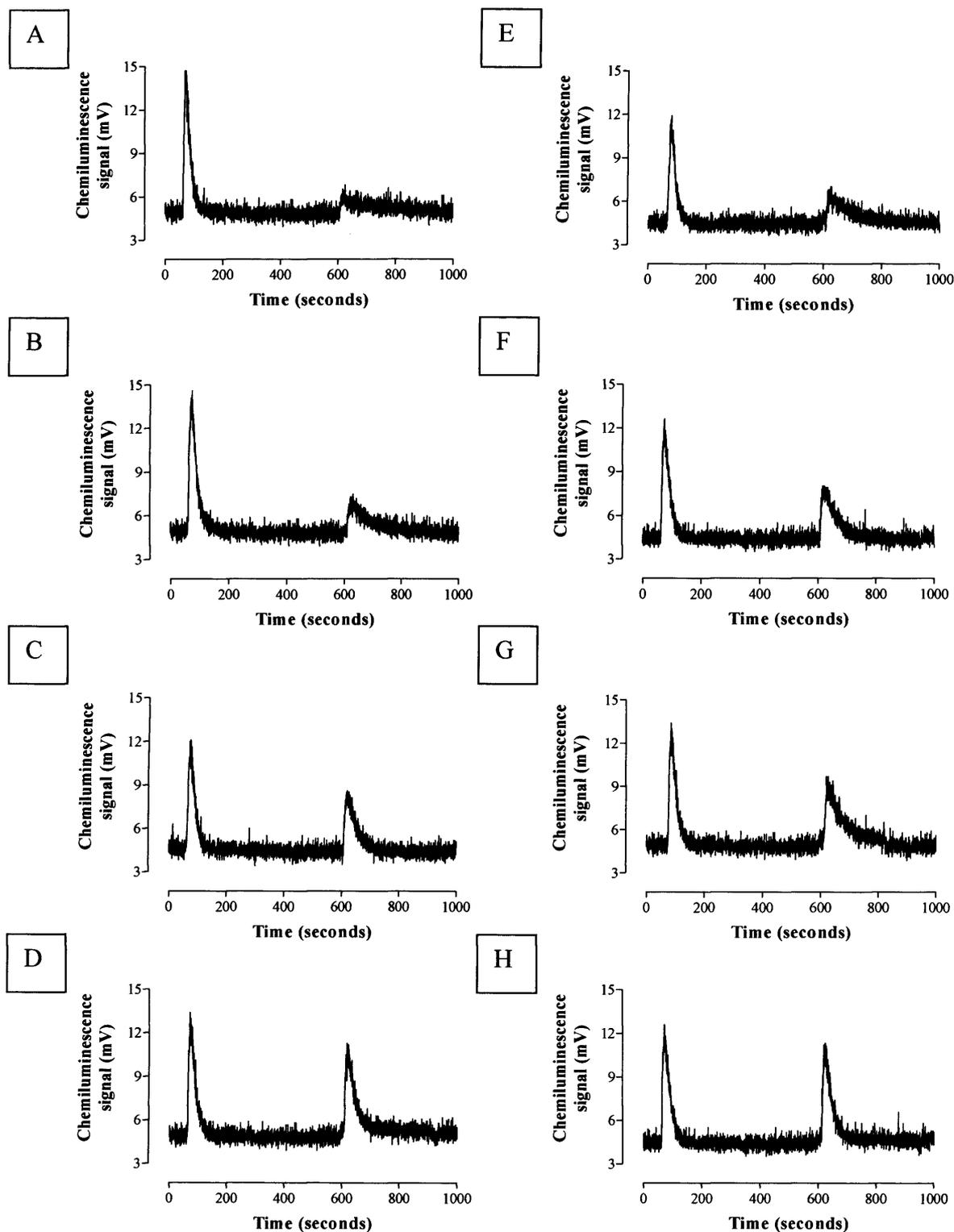


Figure 3.5: Haem NO scavenging from GSNO in the tri-iodide reagent. Raw chemiluminescence signals observed from repeat 50pmole GSNO injections before and after the addition of lysed red blood cells (Left column) 1ml (A) 250 μ l (B) 50 μ l (C) 25 μ l (D) - final haem concentrations \sim 20-660 μ M; haemoglobin (Right column) 1ml (E) 250 μ l (F) 50 μ l (G) 25 μ l (H) - final haem concentrations \sim 5-160 μ M. Lysed red blood cells (A-D) and haemoglobin (E-H) were added at 300 seconds.

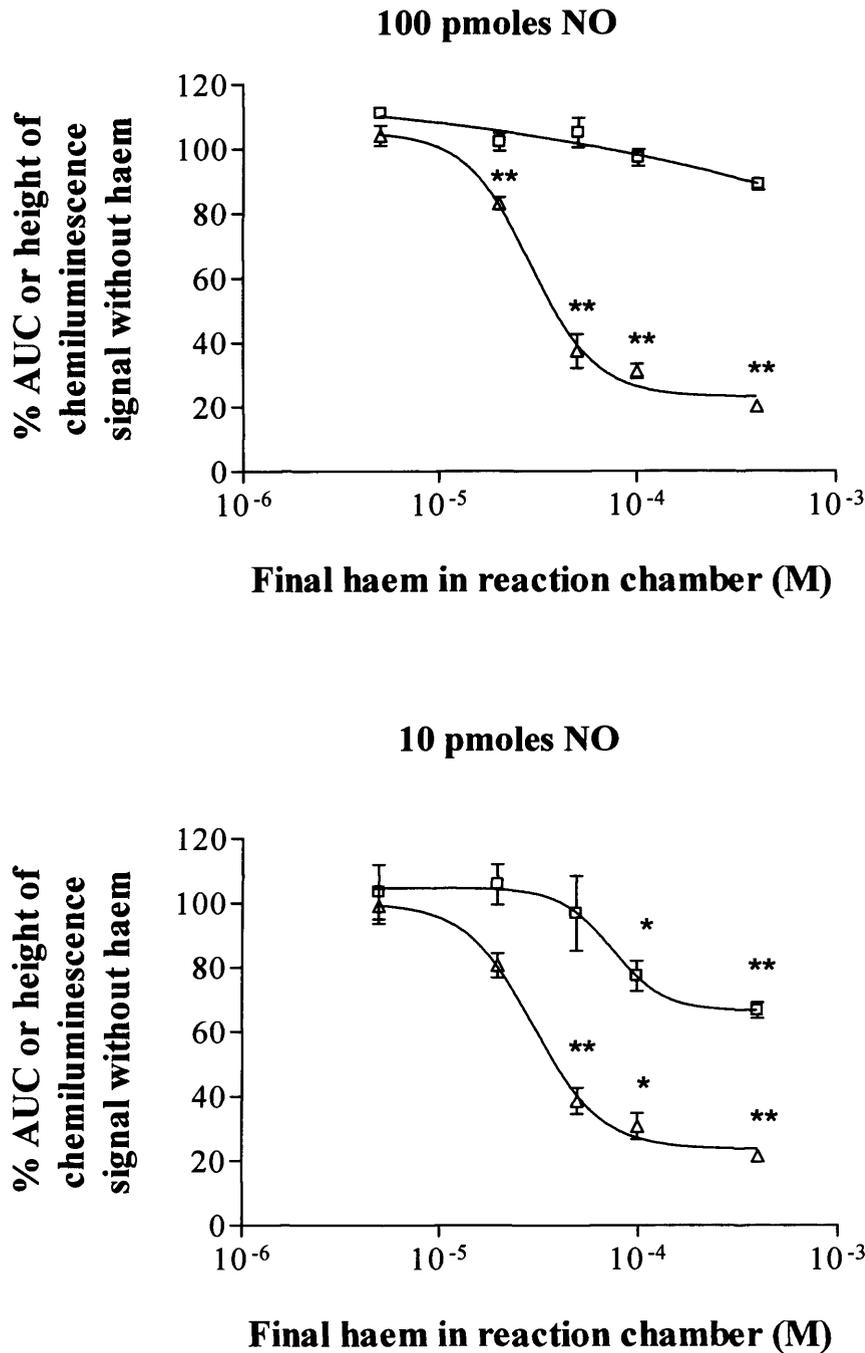


Figure 3.6: Dose dependent haem NO auto-capture. Effect of haem NO scavenging in tri-iodide on signal height and area under curve analysis in the presence of 100 pmole (Top) and 10 pmole (Bottom) NO. Squares represent area under curve analysis, triangles represent signal height. Values are presented as mean \pm SEM ($n = 4-7$ for each concentration). * $p < 0.05$, ** $p < 0.01$ in relation to 100% values; One way ANOVA.

3.2.2.2 Overcoming Auto-capture of NO by Haemoglobin in Tri-iodide

In order to prevent auto-capture of NO by deoxygenated cell free haem in the reaction chamber a method had to be developed to slow the reaction between the two. It was rationalised that adding potassium ferricyanide ($K_3Fe^{III}(CN)_6$) to the original tri-iodide reagent would oxidise haem from its ferrous Fe(II) to its ferric Fe(III) form producing methaemoglobin that would bind NO less rapidly ($\sim 10^3 M^{-1} s^{-1}$) [61] and thus be a less potent scavenger, allowing NO time to escape re-capture [124].

Different means of inactivating the haem within the tri-iodide reagent were assessed utilising reagents containing potassium cyanide (KCN), $K_3Fe^{III}(CN)_6$ and both cyanides together. It was found that the addition of $K_3Fe^{III}(CN)_6$ worked best to inhibit NO auto-capture (Figure 3.7 and 3.8).

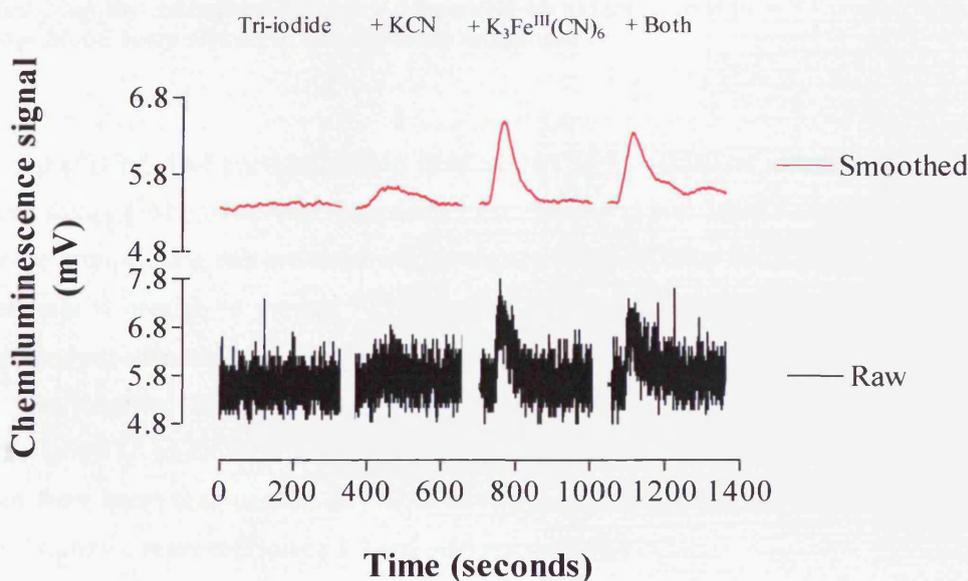


Figure 3.7: Measurement of red blood cell lysate in different reaction mixtures. Reagents include tri-iodide alone; tri-iodide plus potassium cyanide (KCN); tri-iodide plus potassium ferricyanide ($K_3Fe^{III}(CN)_6$) and tri-iodide plus both cyanides. Example traces of chemiluminescence signals and the same data smoothed for analysis with Origin version 7.0.

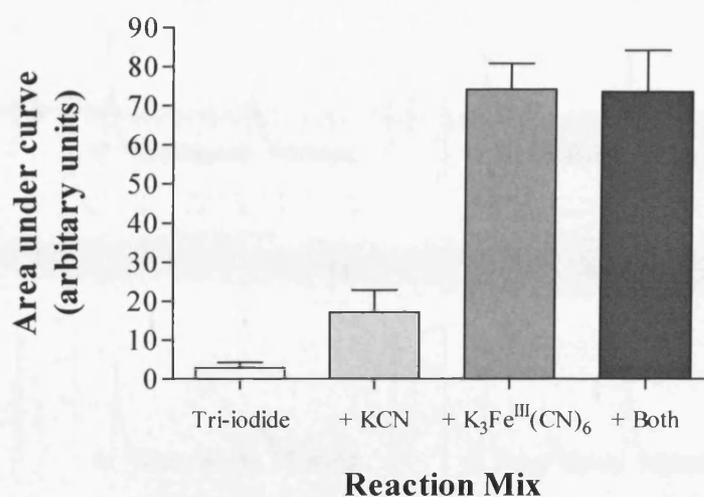


Figure 3.8: Measurement of red blood cell lysate in different reaction mixtures. Reagents include tri-iodide alone; tri-iodide plus potassium cyanide (KCN); tri-iodide plus potassium ferricyanide ($K_3Fe^{III}(CN)_6$) and tri-iodide plus both cyanides. Area under curve values obtained from the smoothed data are presented as mean \pm SEM ($n = 3$ - frozen aliquots of the same blood were tested in the different reagents).

$K_3Fe^{III}(CN)_6$ had previously been used as part of a cocktail of chemicals added to red blood cell samples when collected, postulated to selectively cleave HbNO and/or stabilise SNO-Hb. Those groups using this pre-treatment have since suggested that the $K_3Fe^{III}(CN)_6$ within the chemical mix is enough to prevent NO rebinding to haem [125], however this has yet to be demonstrated experimentally.

The modified tri-iodide reagent with added $K_3Fe^{III}(CN)_6$ was tested with NO donor standards ($NaNO_2^-$ or GSNO) in the presence/absence of haemoglobin. The broader signals observed from haem auto-capture of NO in tri-iodide were inhibited following the addition of $K_3Fe^{III}(CN)_6$ to the reagent (Figures 3.9 and 3.10 respectively) [124].

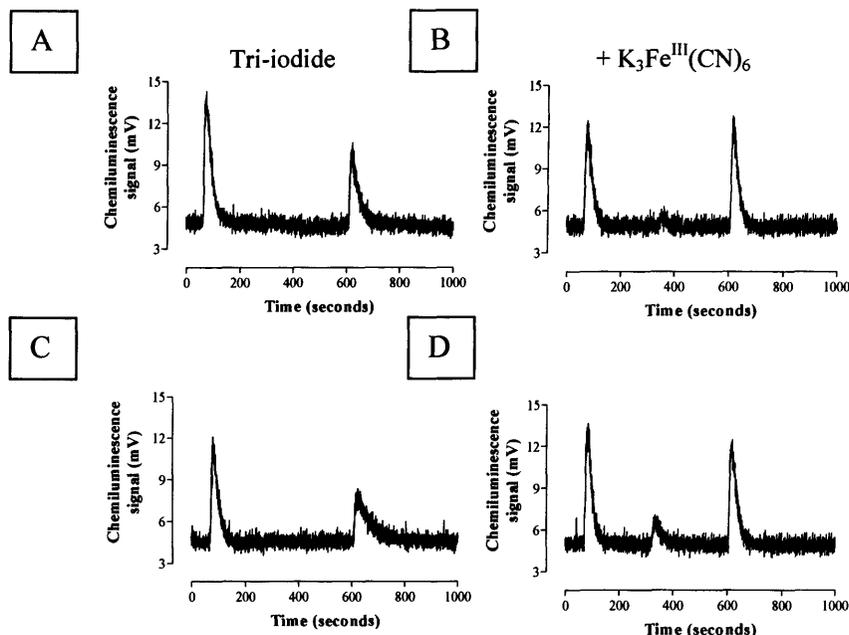


Figure 3.9: Inhibition of haem NO scavenging from nitrite in tri-iodide with added $K_3Fe^{III}(CN)_6$. Raw chemiluminescence signals from repeat 25 pmole nitrite injections before/after addition of haemoglobin (A and B) $\sim 10\mu M$ haem final; (C and D) $\sim 50\mu M$ haem final. Timing of the haemoglobin addition at 300 seconds.

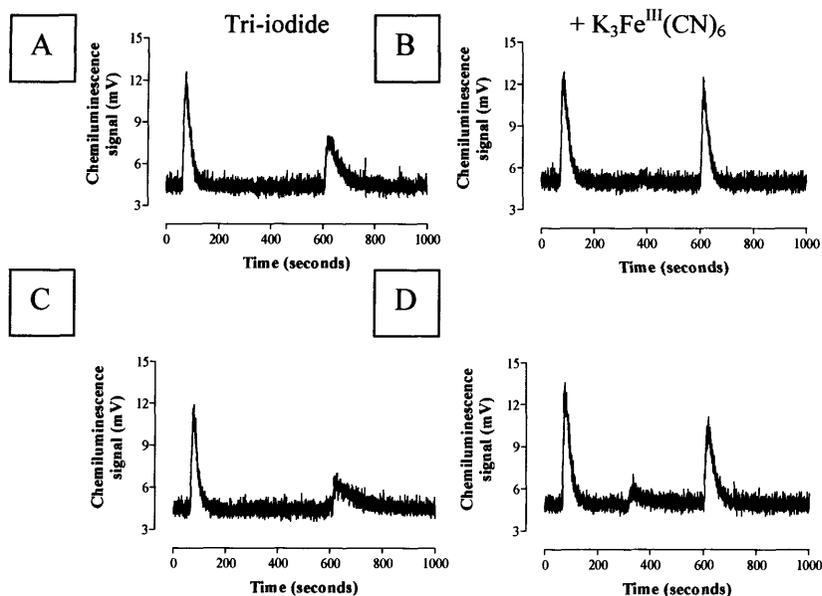


Figure 3.10: Inhibition of haem NO scavenging with GSNO in tri-iodide with added $K_3Fe^{III}(CN)_6$. Raw chemiluminescence signals from repeat 50 pmole GSNO injections before/after addition of lysed red blood cells (A and B) $\sim 40\mu M$ haem final; (C and D) $\sim 200\mu M$ haem final. Timing of lysed red blood cell addition at 300 seconds.

Signals from red blood cell lysate and haemoglobin were compared in both reagents. Nothing was detected in the original tri-iodide reagent (Figures 3.9A and C, 3.10A and C - at 300 seconds) however signals were observed in the modified reagent mix (Figures 3.9B and D, 3.10B and D - at 300 seconds). Also red blood cell-bound NO measurements were compared from lysed red blood cell samples (one part red blood cells to four parts EDTA made up with HPLC grade water) injected into both reagents. Whereas the chemiluminescence signal was indistinguishable in the original reagent, a clear signal was observed in the modified mix (3 repeat experiments using different frozen aliquots of the same red blood cell sample) (Figure 3.11) [124].

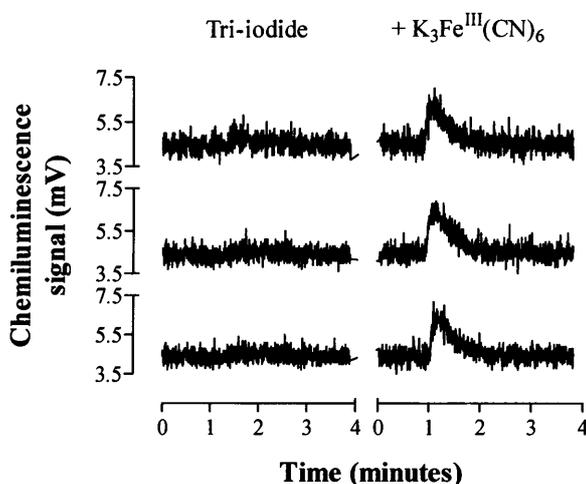


Figure 3.11: Red blood cell-bound NO measurement in the original and modified tri-iodide reagent with added $K_3Fe^{III}(CN)_6$. Raw chemiluminescence signals observed from 200 μ l injections of lysed red blood cells 1:4 in EDTA (HPLC grade water). Signals in tri-iodide (Left) and signals in tri-iodide plus potassium ferricyanide (Right). All injections were at one minute.

3.2.2.3 NO Species Detected in the Modified Tri-iodide Reagent

It was considered a possibility that the larger signals observed with the modified tri-iodide reagent might have arisen from more complete NO release from products already detected in the original tri-iodide reagent or the release of NO related products essentially inert in the original reagent. The reductive potential of the reagents under standard conditions were analysed by testing several major NO species including nitrate, nitrite, GSNO, N-nitrotyrosine, the iron nitrosyl sodium nitroprusside (SNP) and also plasma. There was no difference between the two

reagents in the yields obtained from any of these species. Essentially nitrite, GSNO and plasma were returned 100% of theoretical, whilst nitrate, N-nitrotyrosine and SNP returned < 0.1% of theoretical. Others have since reported that the yields obtained from the biological RSNOs S-nitrosoalbumin and SNO-Hb are the same in both assays, with the only difference being a more complete recovery of nitrosylhaemoglobin (HbNO) in the modified reagent (100% of theoretical in comparison to a 60-80% yield in the original reagent) [126]. It has also been demonstrated that injections of nitro-linoleic and nitro-oleic acid standards yield very similar recoveries of < 1% in either reaction mixture [126].

These results demonstrate that the cleavage and reductive potential of the original and modified tri-iodide reagents are very similar. Consequently the clearer signals observed in the modified reagent result in part to the improved detection of HbNO and also the inhibition of NO auto-capture by cell free haem.

3.2.2.4 Effect of Haemoglobin in the Reaction Chamber on NO Detection in CuCl/CSH

Having developed the modified tri-iodide reagent (to be used with minimal sample pre-treatment) to obtain a measure of total red blood cell NO, it was intended to use the CuCl/CSH reagent to provide a direct measure of SNO-Hb. Consequently the CuCl/CSH reagent was analysed in the same manner as tri-iodide. The NO donor GSNO was injected into the RSNO specific CuCl/CSH reagent in the presence/absence of haemoglobin in the reaction chamber. The chemiluminescence signal heights and area under curve analysis from GSNO were found to be inversely related to the concentration of haem in the reaction chamber. These relationships with increasing addition of haem were similar whether red blood cell lysate or haemoglobin was added to the reagent.

Haem auto-capture of NO in the CuCl/CSH reagent was even more avid than in tri-iodide (compare x axis scales in figures 3.6 and 3.13). This probably reflects the fact that S-nitrosothiol decomposition in the CuCl/CSH reagent releases NO in its radical form (reaction ^[6] page 53) which would be more avidly scavenged and secondly free haem is not denatured under the neutral conditions of the CuCl/CSH reagent and is potentially a more potent scavenger than in tri-iodide.

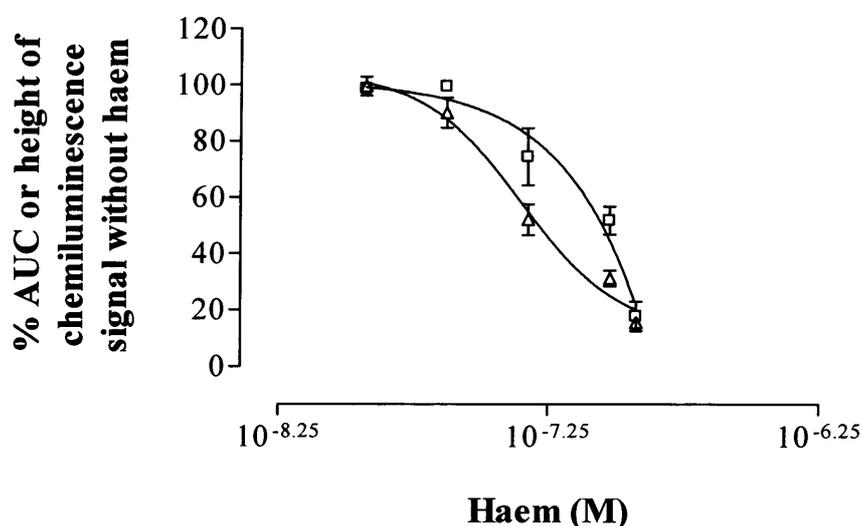


Figure 3.13: Dose dependent effect of haem NO auto-capture in the CuCl/CSH reagent. The effect of haem NO scavenging on signal height and area under curve analysis in the presence of 50 pmole GSNO. Squares represent area under curve analysis, triangles represent signal height. Values are presented as mean \pm SEM ($n = 4$ for each concentration).

With increasing haem addition not only did signal height but also the area under curve declined. This suggested that the species created by the auto-capture of NO by haem was HbNO which went undetected in the RSNO specific CuCl/CSH reagent. Consequently greater haem in the reaction chamber resulted in less NO escaping auto-capture and thus smaller signals, both in terms of signal height and area.

3.2.2.5 Overcoming Auto-capture of NO by Haemoglobin in CuCl/CSH

At the same time that the auto-capture effect of haemoglobin within the tri-iodide reagent was observed within our laboratory, the same effect was reported independently by another group with regard to the CuCl/CSH reagent [114]. To overcome this effect in the CuCl/CSH reagent the authors changed the carrier gas within their chemiluminescence set up to carbon monoxide (CO). By saturating the reagent with CO, free haem was inactivated via the production of carboxyhaemoglobin [114].

The 3Cs assay (CuCl/CSH/CO) was set up within our laboratory as outlined in the literature. However, instead of mixing CO with helium as in the original method a pre-mixed cylinder containing 90% CO balanced with nitrogen was used. Unfortunately we could not achieve the same inhibitory effect of CO on haem. The effectiveness of CO gas to inhibit auto-capture was tested both at 20 μ M haem final concentration in the reaction chamber (to represent the final haem concentration widely reported by the majority of laboratories [94, 111, 120]; Figure 3.14) and 1 μ M haem final (Figure 3.15). Although an inhibitory effect was observed with CO, this was only ~ 40% across all doses of GSNO with 20 μ M final haem concentration and ~ 65% across all doses of GSNO with the 1 μ M final haem.

In order to solve this issue a visit was arranged to the laboratory where the 3Cs assay was developed (Charlottesville, VA). Working with the assay it became apparent that for CO to fully inhibit haem auto-capture the reagent cell (where the CuCl/CSH reagent was housed within the system) had to be maintained at positive pressure; to produce a high enough concentration of CO in the reagent (Figure 3.16 Bottom). As was the case with our system in the UK, even with the original set up in the USA 100% CO at normal NOA system pressure only inhibited ~ 50% of auto-capture with a 1 μ M final haem concentration (compare Figures 3.15 Bottom and 3.16 Top).

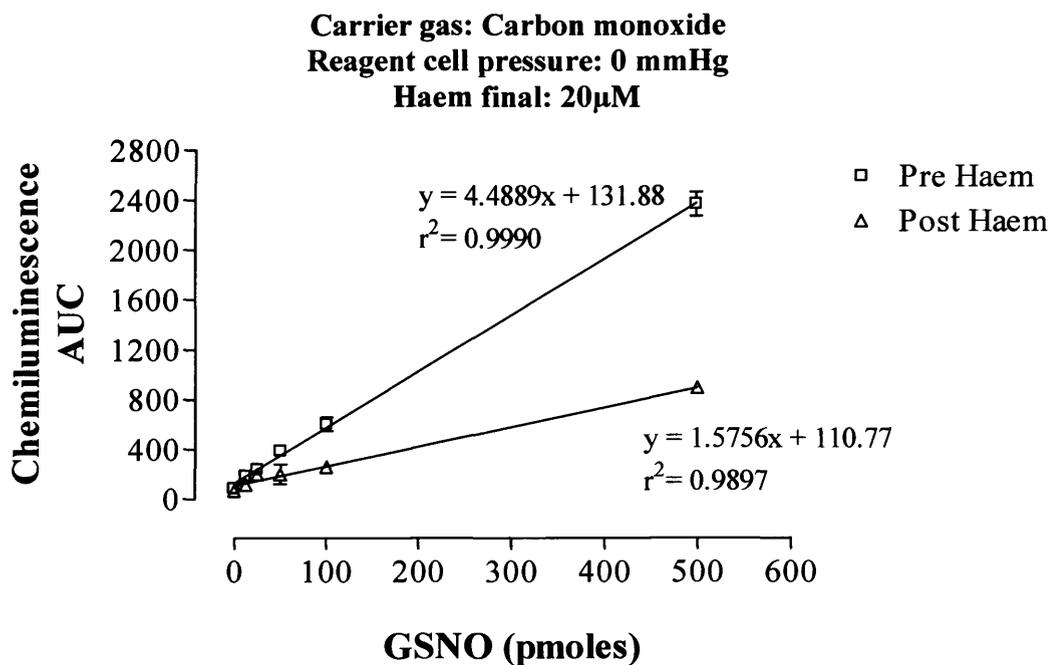
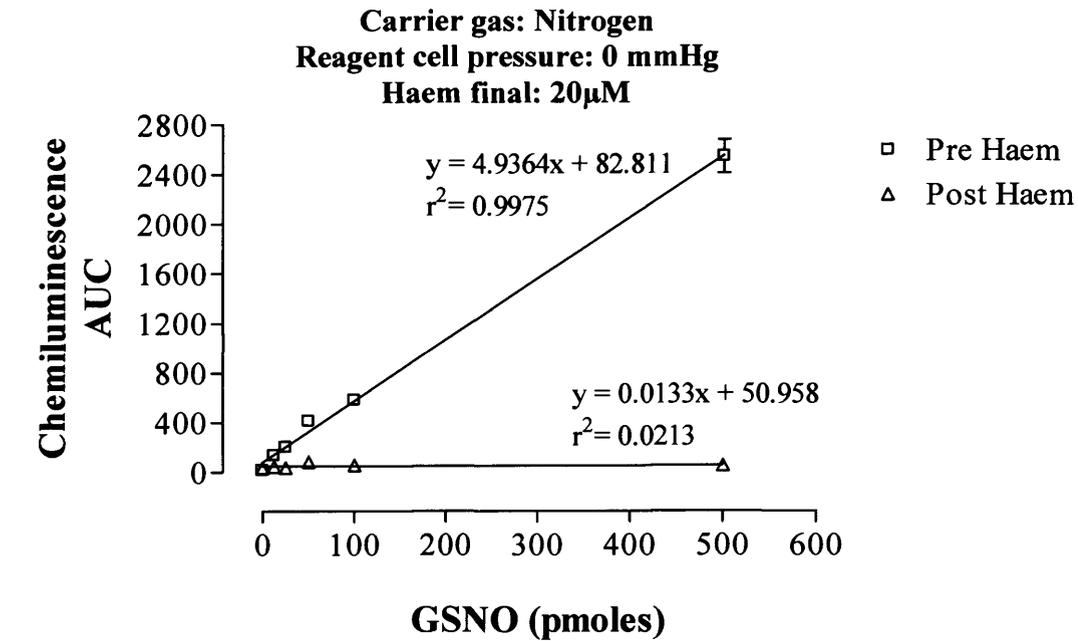


Figure 3.14: Inhibitory effect of carbon monoxide on NO auto-capture by cell free haem in the CuCl/CSH reagent. Purge gas flow of nitrogen (Top) or carbon monoxide (Bottom) in our ozone based chemiluminescence set up; 20µM final haem concentration. Values presented as mean ± SEM (n = 1-2 for each concentration).

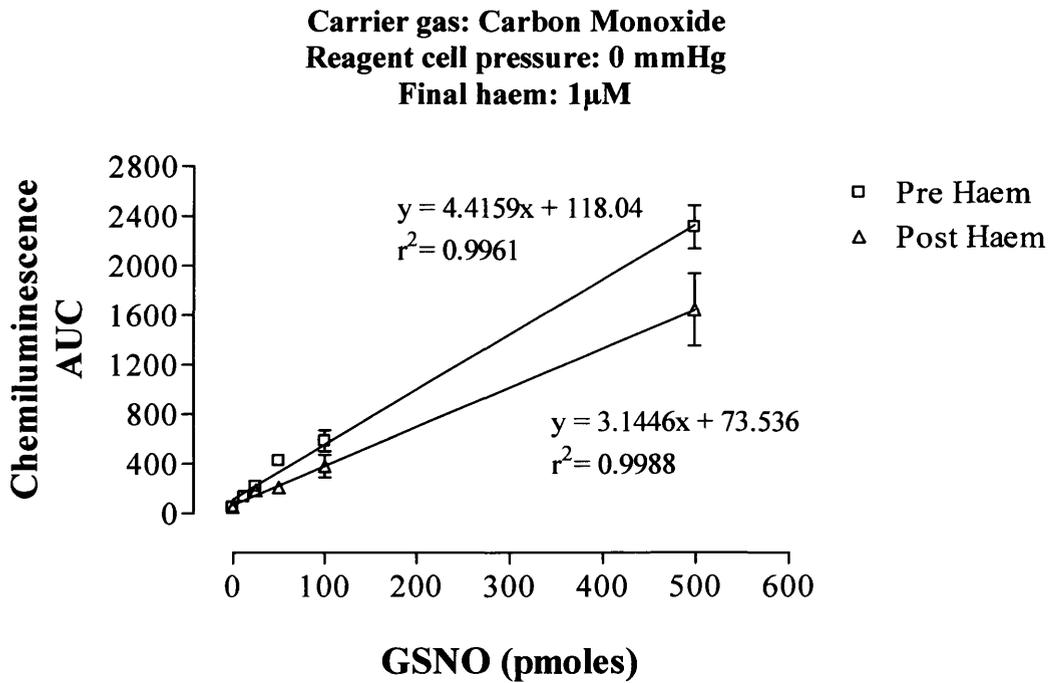
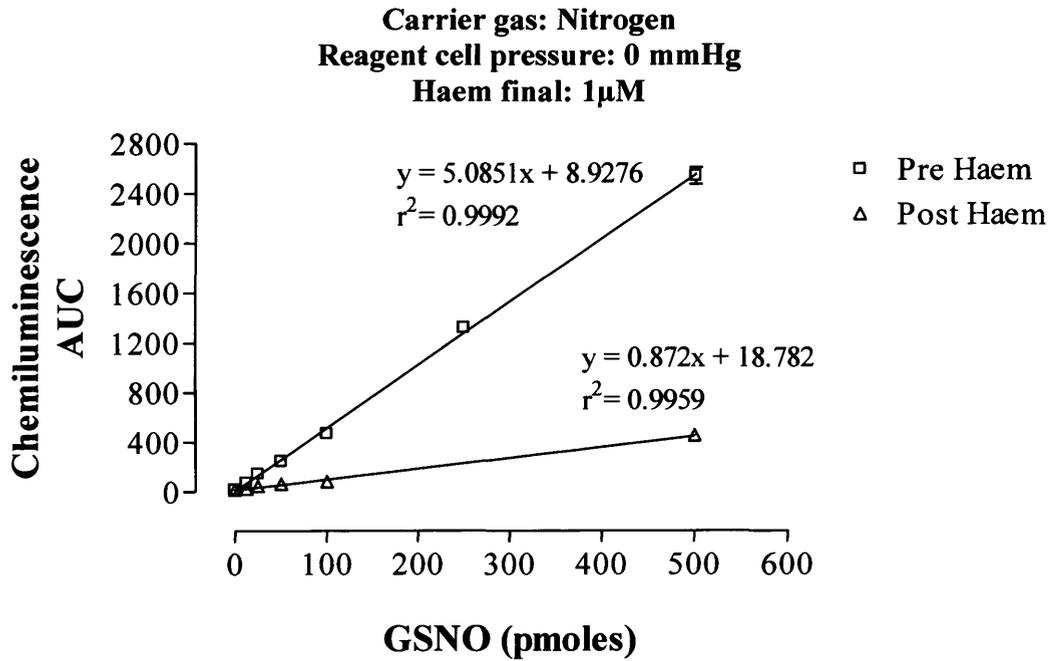


Figure 3.15: Inhibitory effect of carbon monoxide on NO auto-capture by cell free haem in the CuCl/CSH reagent. Purge gas flow of nitrogen (Top) or carbon monoxide (Bottom) in our ozone based chemiluminescence set up; 1µM final haem concentration. Values presented as mean ± SEM (n = 1-2 for each concentration).

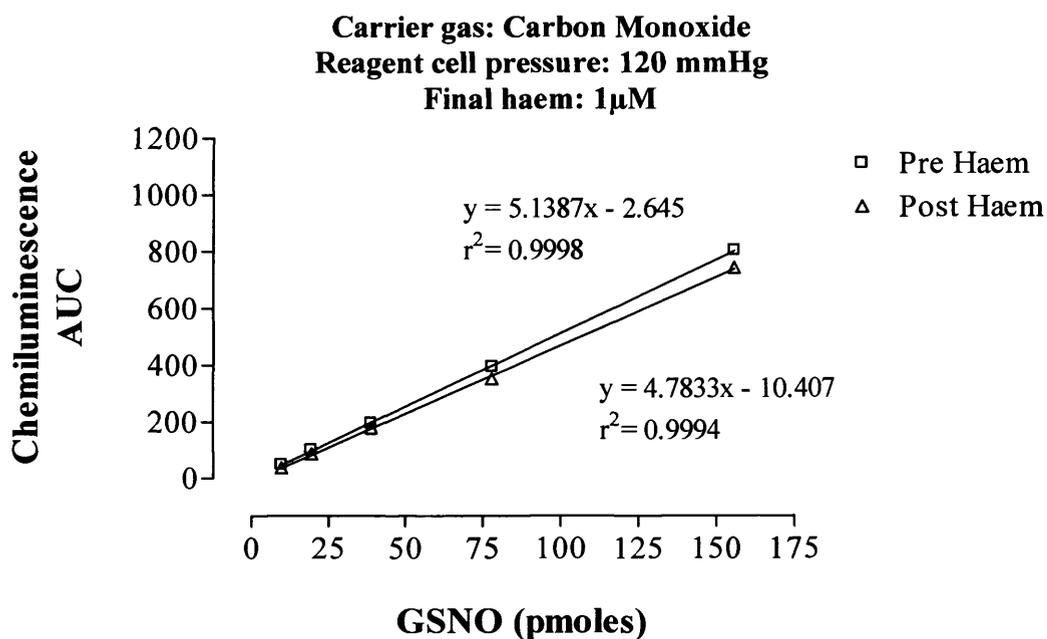
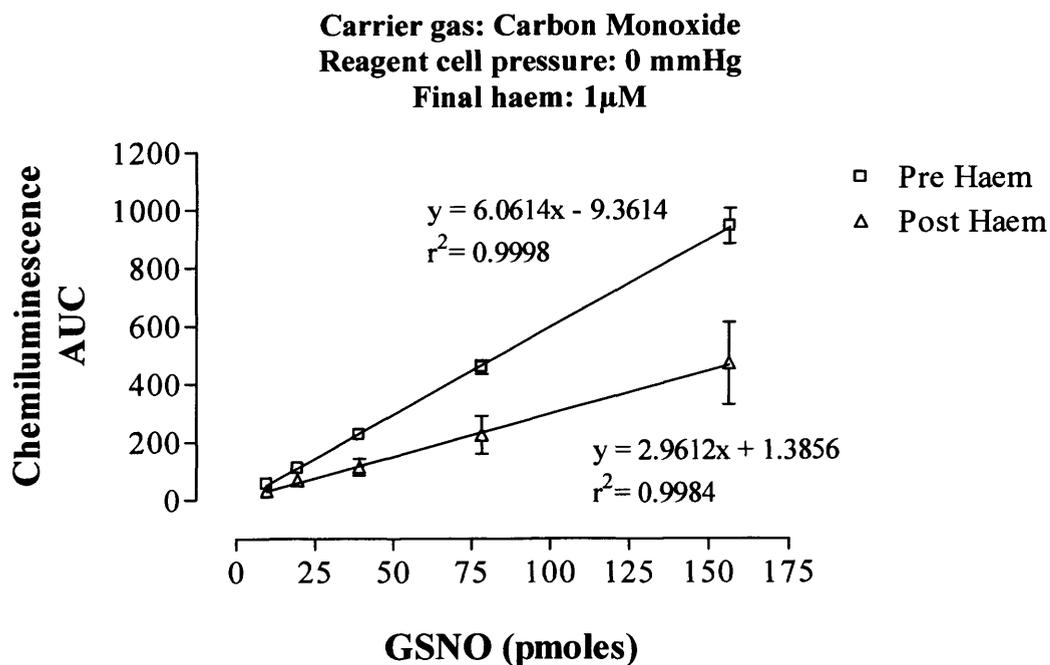


Figure 3.16: Inhibitory effect of carbon monoxide on NO auto-capture by cell free haem in the CuCl/CSH reagent. Purge gas flow of CO with normal system reaction cell pressure (Top) or a positive pressure of 120 mmHg (Bottom) in the Charlottesville set up; 1µM final haem concentration. Values presented as mean ± SEM (n = 3 for each concentration).

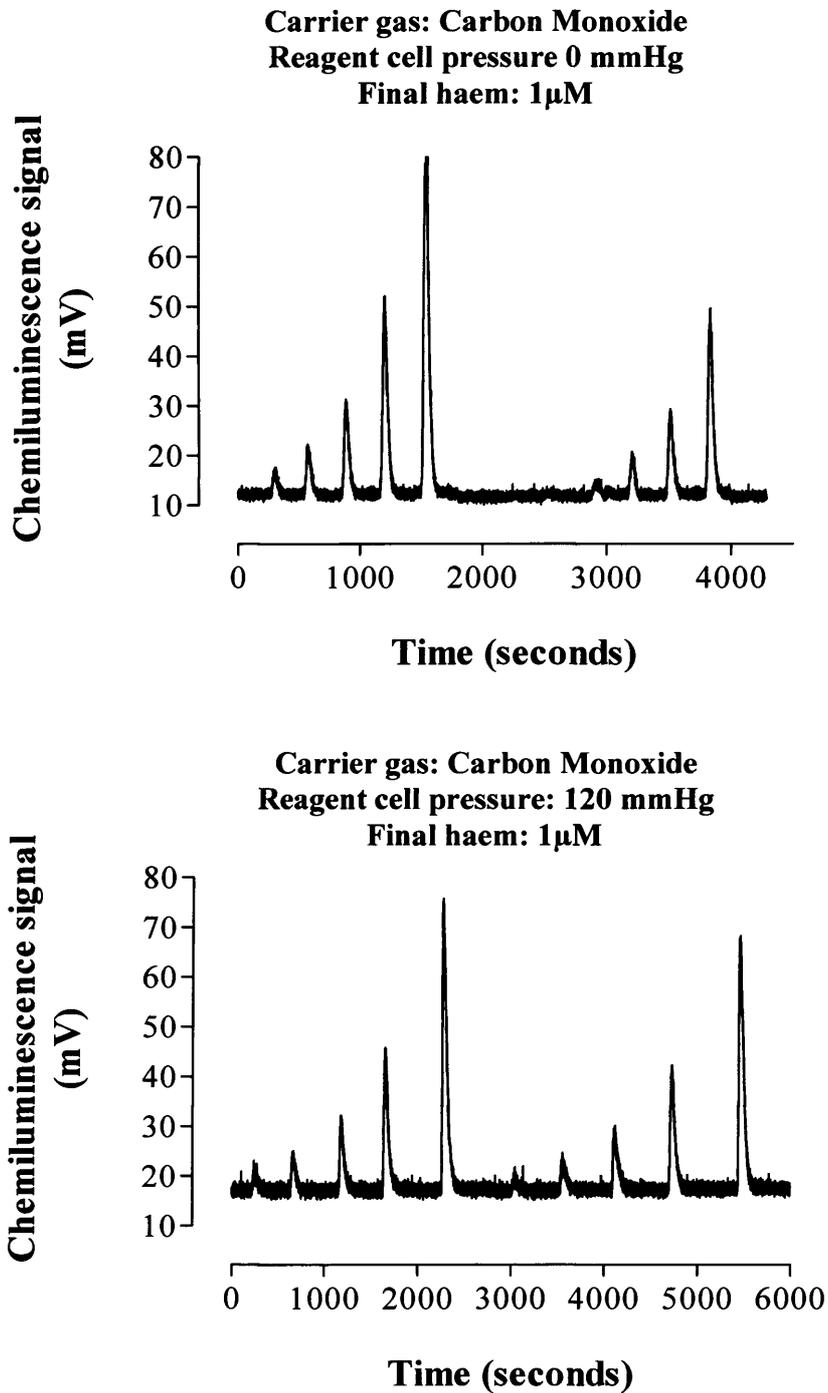


Figure 3.17: Example traces of the auto-capture effect in the CuCl/CSH reagent purged with CO at normal system reaction cell pressure (Top) or a positive pressure of 120 mmHg (Bottom) in the ozone based chemiluminescence set up in Charlottesville; 1 μ M final haem concentration.

A higher reagent cell pressure was also tested (~ 240 mmHg) but this did not improve recovery further. In addition, the signal from GSNO in the absence of haem decreased as CO pressure increased. This was attributed to the CO pressure slightly increasing the pressure in the reaction cell (where the reaction between NO and O₃ occurs within the NOA itself) affecting the sensitivity of the machine.

Unfortunately our custom made glassware reagent cell system in the UK could not run at the pressures required for CO to work at its best.

3.2.2.6 Effect of Plasma in the Reaction Chamber on NO Detection in Tri-iodide

Experiments were performed with the addition of the NO donors NaNO_2^- and GSNO into tri-iodide in the presence/absence of plasma. Chemiluminescence signals from NaNO_2^- and GSNO were unaffected by the presence of plasma in the tri-iodide mix (Figure 3.12), demonstrating the limited capacity of plasma in tri-iodide to auto-capture NO and also providing evidence of limited red blood cell lysis as a result of our blood sampling protocol [124]. As plasma added to the tri-iodide was not demonstrated to auto-capture NO, $\text{K}_3\text{Fe}^{\text{III}}(\text{CN})_6$ was not added to the reagent for plasma measurements.

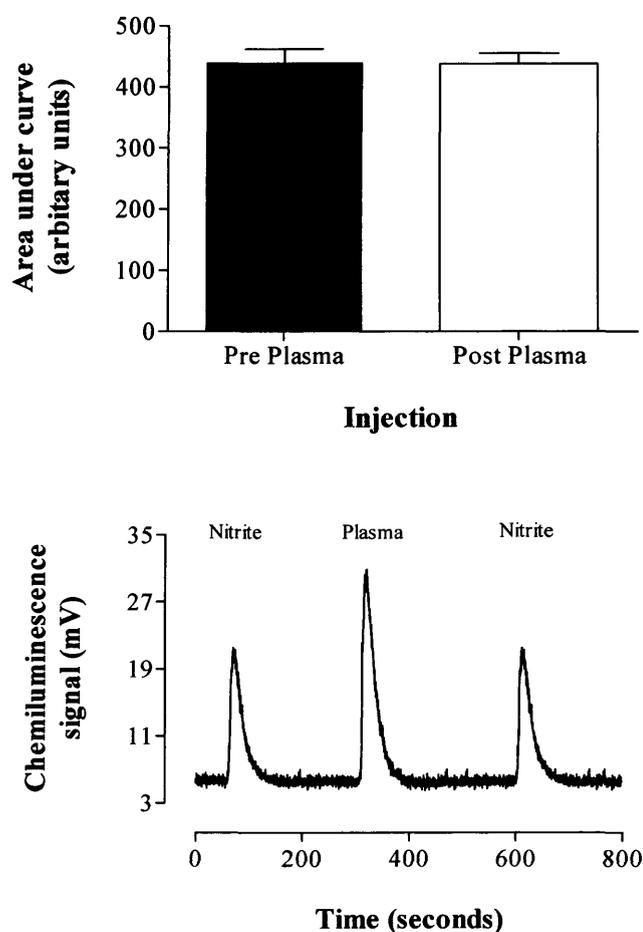


Figure 3.12: Absence of NO scavenging by plasma in the tri-iodide reagent. Area under curve values presented as mean \pm SEM ($n = 4$) (Top). Example trace of chemiluminescence signals observed from repeat 25pmole sodium nitrite injections before and after (100 and 600 seconds respectively) a 200 μ l plasma injection (Bottom).

3.2.3 Analysis of Biological Sample Handling Protocols

Various biological sample handling protocols have been developed in conjunction with the different measurement techniques and processes of releasing NO from metabolites. The idea behind these are several fold; to 'stabilise' individual metabolite species via the blocking of potential reactants [94, 125], prevent artificial ex vivo metabolite formation [94] and allow differentiation between individual metabolite species through elimination of all but the species of interest [94, 95, 121]. However, it is through these processes that much of the variability in metabolite measurement is thought to derive as a result of the addition of contaminant [113, 127], loss of less stable metabolite species [27], destruction of the physiological sample and disturbance of the NO metabolite equilibrium/homeostasis [101, 103].

Much of the earlier work on NO metabolite measurement within our laboratory involved the utilisation of stabilisation solutions and chemical pre-treatment processes. However upon working so closely on the methodologies the pitfalls associated with these processes became more evident which led to the aim of developing methodologies which minimised these processes.

3.2.3.1 Acidified Sulphanilamide

Acidified sulphanilamide sample pre-treatment was developed as a means to eliminate nitrite from biological samples. This was an important development for the tri-iodide assay, due to the abundance of this species (readily reduced to NO in this reagent) in most biological samples. The pre-treatment process involved the addition of a 10% volume of acidified sulphanilamide to a biological sample followed by a short incubation period. During this process nitrite forms NO^+ under the acidic conditions, which binds to sulphanilamide forming a stable diazonium salt that is not converted to NO in the original tri-iodide reagent [95].

Preliminary experiments demonstrated the ability of acidified sulphanilamide to remove large quantities of free nitrite in solution (up to $100\mu\text{M}$) almost instantaneously in the original tri-iodide reagent and in our modified tri-iodide with added $\text{K}_3\text{Fe}^{\text{III}}(\text{CN})_6$ (data not shown). Similar experiments were performed with plasma and lysed red blood cell samples to identify the incubation times necessary for the removal of added nitrite from biological samples (data not shown). Complete removal of added nitrite was found to take up to 15 minutes, which is in line with reports from other laboratories [127].

3.2.3.2 Stabilisation Solutions

Stabilisation solutions were originally developed in order to prevent the natural decay of metabolite species in biological matrices. The majority of these solutions include $K_3Fe^{III}(CN)_6$ to oxidise species that potentially react with the metabolite(s) of interest. In addition, KCN is often included to redox inactivate haem. However, the powders of these chemicals (even obtained to the highest purity) contain nitrite, as mentioned in passing by others [94]. This contamination can be observed upon the injection of these chemicals into tri-iodide, which following pre-treatment with sulphanilamide eliminates the signal (data not shown). If samples are incubated with chemicals which introduce nitrite contamination, this can result in the artificial formation of metabolite species *in vitro* [127]. One further issue with the chemical pre-treatment of samples is the fact that it is difficult to ascertain that all chemicals have been removed prior to sample injection. This is normally assumed following the passing of samples through a sizing column (Sephadex G25).

3.2.3.3 Freezing Samples

When obtaining blood samples from a clinical setting it is often not possible to analyse them immediately. Consequently samples have to be frozen. Where this was necessary samples were frozen in liquid nitrogen and subsequently stored at $-80^{\circ}C$ until analysis.

The effect of sample freezing on NO metabolite stability was analysed in red blood cell and plasma samples. Whole blood was centrifuged at 600 g for 5 minutes followed by immediate separation of plasma and red blood cells. One sample was measured immediately (fresh) and the rest were frozen in 300 μ l aliquots for future analysis at time points up to 3 months for red blood cell samples and 6 months for plasma samples. Upon analysis frozen samples were thawed at $37^{\circ}C$ for three minutes prior to undergoing the set protocols (red blood cell protocol; Chapter 2 Section 2.1.5 and plasma protocol; Chapter 2 Section 2.1.4).

Freeze thawing (i.e., fresh v 1 day frozen) had no effect on any of the measurements. Combined red blood cell haemoglobin-bound NO/red blood cell associated nitrite and combined plasma nitrite/protein-bound NO were not affected over the total period of storage. However, a trend was observed towards lower levels of plasma protein-bound NO over the six months and red blood cell haemoglobin-bound NO diminished significantly after one month of freezing. As the total signals were not affected it was understood that over time the plasma protein-bound NO and red blood cell haemoglobin-bound NO most likely lost NO to form nitrite in the sample.

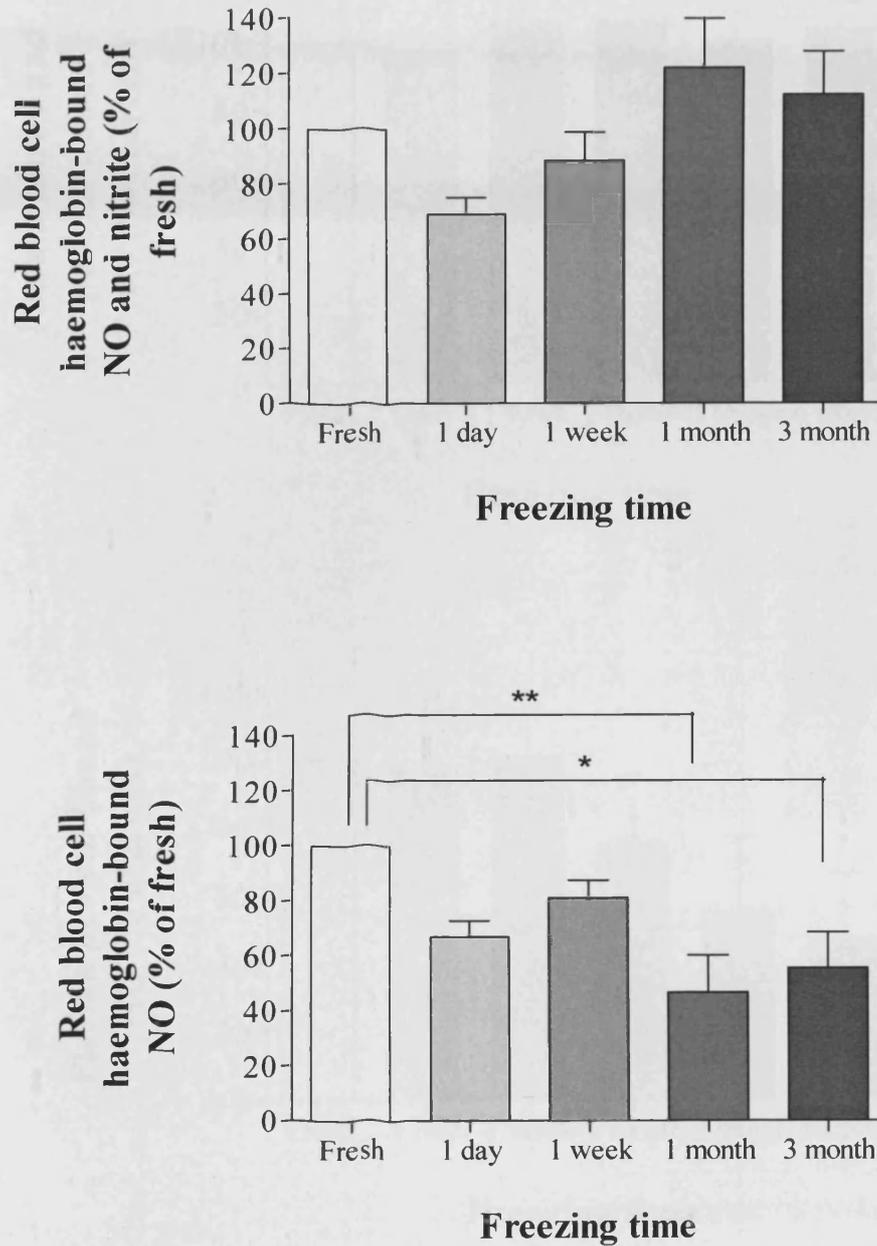


Figure 3.18: Effect of sample freezing on red blood cell associated nitrite and haemoglobin-bound NO (Top) and red blood cell haemoglobin-bound NO (Bottom) measured using tri-iodide with added $K_3Fe^{III}(CN)_6$ in conjunction with chemiluminescence. Values presented as mean \pm SEM ($n = 5$); * $p < 0.05$; ** $p < 0.01$ Repeat measures ANOVA.

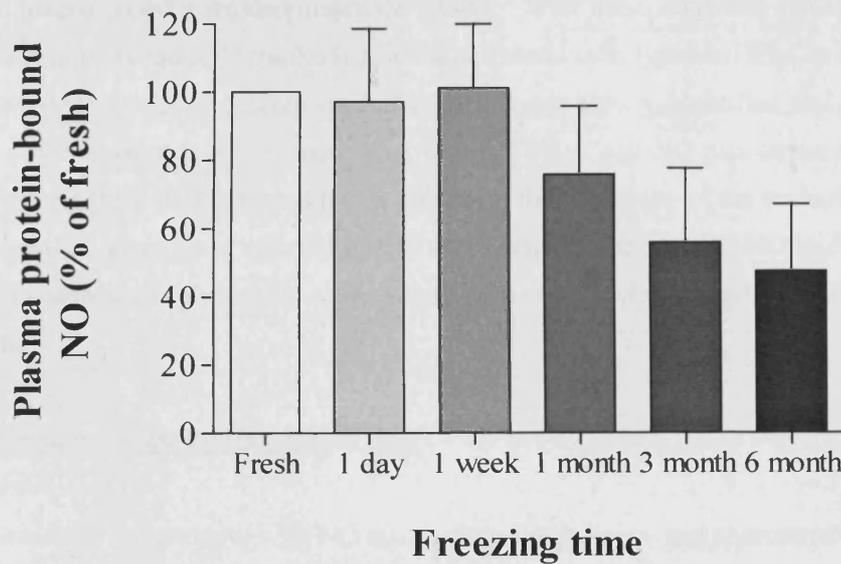
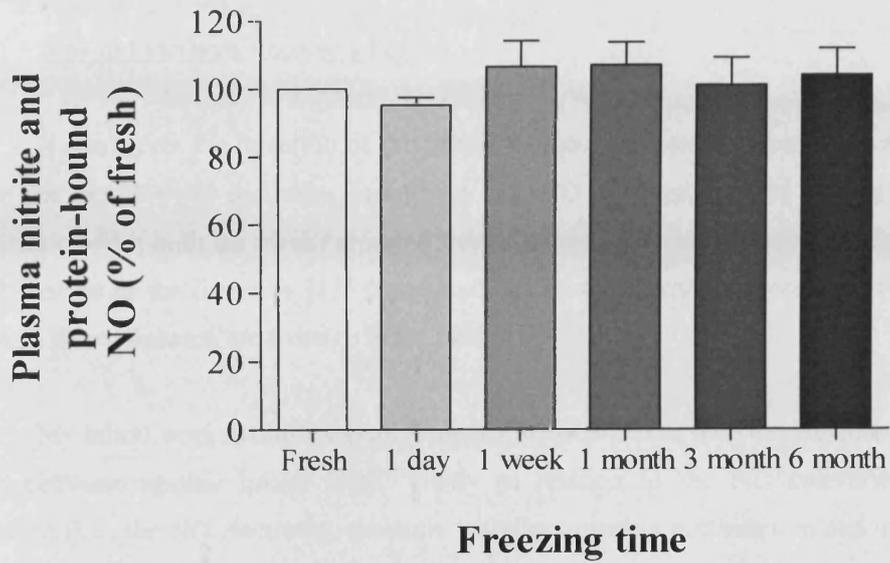


Figure 3.19: Effect of sample freezing on plasma nitrite and protein-bound nitric oxide levels (Top) and plasma protein-bound NO (Bottom) measured using tri-iodide in conjunction with chemiluminescence. Values presented as mean \pm SEM ($n = 5$).

3.3 Discussion

3.3.1 Why did Methods Become a Focus?

It was never the intention of this thesis to focus so intently on methodology. However given the scrutiny of methods throughout the NO metabolite field during this thesis in combination with both the results reported from our laboratory at the time (which were at the top end of values in the literature [115]) and findings from my early experiments (outlined in this chapter), there appeared little choice in the matter.

My initial work identified several methodological issues with the means of measuring red blood cell/haemoglobin bound NO. Firstly in relation to the NO measurement technique employed (i.e., the NO electrode), secondly with the injection port/reaction cell utilised with this system and finally with the means of introducing the sample for analysis. As a result of these findings a new reaction cell was developed, a cleaner means of introducing the sample was employed (gas tight Hamilton syringes) and an investment was made in a new NO measurement technique (ozone based chemiluminescence NOA). With these advances the signals observed from untreated red blood cell samples (i.e., fresh red blood cells lysed in HPLC water) declined to barely detectable levels as sources of contaminant were slowly identified and eliminated and awareness of the potential pitfalls associated with red blood cell NO measurement became more evident. During these developments it was noted that the sensitivity of the tri-iodide reagent (that had been used all along) was affected by cell free haem auto-capture of NO in the reaction cell. This was investigated as a potential confounding factor influencing the detection of red blood cell derived NO.

3.3.2 Measuring Total Red Blood Cell NO

Numerous experiments with NO standards in the presence and absence of haemoglobin in the reaction chamber were performed to demonstrate auto-capture of NO by cell free haem. To overcome this issue it was rationalised that the addition of ferricyanide to the reagent would oxidise haem from its ferrous Fe(II) to its ferric Fe(III) form creating a less potent scavenger; therefore allowing NO to escape the reaction chamber and be detected by the NOA. It was also hypothesised that the addition of ferricyanide to tri-iodide might improve the yield of NO from HbNO, facilitating the release of haem NO via the oxidation of HbNO to nitrite [27]. The

improved detection of HbNO from ~ 60-80% in the original reagent to ~ 100% in the modified reagent was later demonstrated by others [126]. Clearer signals observed with the modified tri-iodide reagent have consequently been attributed in part to the improved detection of HbNO but more so to the inhibition of NO auto-capture in the reaction cell.

One point of contention raised in the literature regarding NO auto-capture in tri-iodide relates to the concentration of haem utilised in experiments to demonstrate this phenomenon [126]. The haem concentration alone however provides no insight into this issue. It is the ratio of NO:haem which is the important parameter. As the data herein reveals, at physiological NO:haem ratios auto-capture is an issue in the original tri-iodide reagent.

3.3.3 Measuring Individual Red Blood Cell Species

Having developed a pre-treatment free means to measure baseline levels of total red blood cell NO in human blood samples, the next objective was to split the signal into its component parts and identify specific sources of red blood cell NO. Previously this had been achieved in our laboratory utilising various chemical pre-treatment processes outlined in the literature [73, 94]. However, the artificial formation of metabolites *in vitro* was a major concern. This had been demonstrated by others following the incubation of biological samples with nmol/L levels of nitrite; equivalent to the trace amounts of nitrite observed in chemicals or from filter/column diluent [127]. Another concern regarding pre-treatments was the loss of less stable metabolite species as a result of sample handling time prior to measurement [27, 101, 103]. **For these reasons a decision was made to try and avoid all stabilisation solutions and chemical pre-treatment protocols.** Instead, other means of differentiating individual metabolite species were explored, including the 3Cs assay.

3Cs was identified as the most promising means to measure SNO-Hb because it provided a direct measure, it was specific for RSNO (with nitrite remaining undetected due to the neutral pH of the reagent ~ pH 6.5) which in turn meant that no sample pre-treatment was required. Unfortunately in our laboratory CO was only partially effective at inhibiting auto-capture of NO by cell free haem in the reaction chamber. During a visit to the laboratory where the 3Cs assay was developed, it was discovered that to achieve the greatest inhibition of haem by CO, the reaction cell had to be maintained at pressure to produce a high enough concentration of CO in the CuCl/CSH reagent. Unfortunately it was not possible to run our reaction cell system at the pressures required. Furthermore even at pressure CO was found to be ineffective at blocking just

1 μ M final haem in the reaction chamber, well below the amount of haem thought necessary to observe a SNO-Hb signal from a baseline human blood sample. Consequently other means of determining SNO-Hb were assessed.

An attempt was made to measure SNO-Hb in the modified tri-iodide reagent using previous pre-treatment protocols. This involved the incubation of blood samples with and without HgCl₂ (to cleave and convert SNO to nitrite) in conjunction with acidified sulphanilamide (to remove nitrite) [94]. A dozen baseline red blood cell samples were analysed (6 arterial/6 venous), seven of which produced negative SNO-Hb values, i.e., the red blood cell samples treated with HgCl₂ followed by dilution in sulphanilamided water 1:4 (representing HbNO) produced larger signals than the red blood cell samples just diluted in sulphanilamided water 1:4 (a combined measure of SNO-Hb and HbNO) (Figure 3.20).

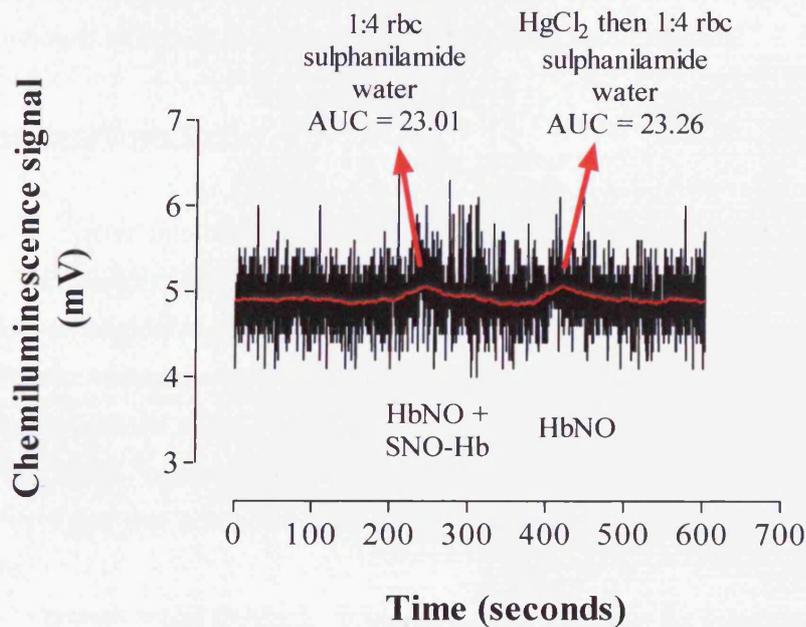


Figure 3.20: Representative trace demonstrating the measurement SNO-Hb using previously published sample pre-treatment protocols. The red blood cell sample was either diluted 1:4 in sulphanilamided water for 15 minutes (to measure HbNO + SNO-Hb) or pre-treated with HgCl₂ (5mM final concentration) to convert SNO to nitrite for 3 minutes before being diluted 1:4 in sulphanilamided water for 15 minutes (to measure just HbNO). Note the second peak has a larger area under curve (AUC) which results in a negative value for SNO-Hb.

From these findings it was decided not to attempt to split the haemoglobin-bound NO signal into its constitutive elements (HbNO and SNO-Hb) but to report a total red blood cell NO measurement and a separate haemoglobin-bound NO measurement. The only way to obtain the haemoglobin-bound NO signal involved the use of acidified sulphanilamide. The subtraction of haemoglobin-bound NO from the total red blood cell NO provided a measure of nitrite in the sample, which throughout this thesis has been termed “red blood cell associated nitrite”. This reflects the fact that nitrite might not necessarily be found within the red blood cell, only associated to the red blood cell membrane. Finally it is acknowledged herein that the treatment of biological samples with acid is far from ideal. However, whilst it has been implied that metabolite species could be destroyed following such pre-treatment [103], to date no experimental evidence demonstrating this has been published [126].

The concentrations of red blood cell NO and the proportions of haemoglobin-bound and red blood cell associated nitrite are examined in more detail in the following chapter, however it is important to note that with the methodology outlined, red blood cell NO and more specifically haemoglobin-bound NO could be detected in baseline human blood samples.

3.3.4 Measuring Plasma Species

Plasma injected into the tri-iodide reagent was found not to affect the signal from nitrite or GSNO. This demonstrated the limited capacity of plasma in tri-iodide to scavenge NO. Consequently, the original tri-iodide reagent was used to measure plasma samples. In order to obtain a difference measurement, plasma samples were incubated with acidified sulphanilamide for 15 minutes to remove nitrite. The remaining sulphanilamide stable signal has been termed throughout this thesis ‘plasma protein-bound NO’ (incorporating species such as RSNO and RNNO). This signal was subtracted from an undiluted plasma signal to provide a measure of plasma nitrite.

Ideally protein-bound standards should be used to determine the concentration of protein-bound NO metabolites. However, throughout this thesis concentrations of protein-bound NO were determined from a standard curve of nitrite. This was undertaken for two reasons, firstly because the majority of NO observed from plasma samples was found to come from nitrite and secondly due to the difficulties associated with making a physiologically representative protein-bound NO standard.

3.3.5 Storage of Blood Samples

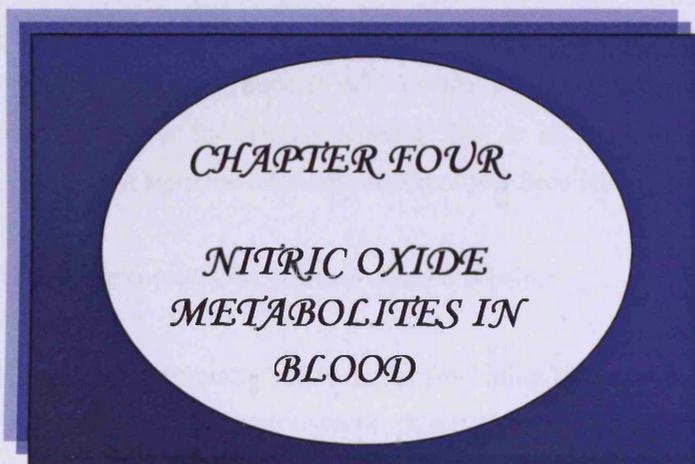
The data in this chapter demonstrates that total NO signal in modified tri-iodide for red blood cell measurements and the original tri-iodide for plasma measurement remains stable following the freeze/thaw cycle and storage of samples in the freezer, so long as the sample is thawed only once. Due to the fact that red blood cell lysate usually exhibits lower signal:noise (compared to plasma signals), greater variability is observed. Whilst the total signal remained constant, haemoglobin-bound NO from red blood cell samples and plasma protein-bound NO declined over time. However, the fact that the total signal remained constant suggests that NO lost from these species most likely formed nitrite. As a result of these findings all red blood cell samples were analysed within one week of collection and all plasma samples within one month.

3.3.6 Conclusion

Progress in the understanding of NO biology hinges on the accuracy and specificity of methods used to measure NO metabolites. This work has added to this knowledge base emphasizing the need for all assays of red blood cell or haemoglobin-bound NO to incorporate a means to prevent free haem rebinding NO in the reaction chamber. Other future goals should address the development of assays which measure only the species of interest in order to minimise the need for sample processing/pre-treatment which will allow maintenance of sample integrity thus limiting the possibility of contamination, loss of less stable metabolite species and disruption to the NO metabolite equilibrium. Only when the same answers are achieved from multiple NO measurement methodologies can we be satisfied that we have the true levels of NO metabolites in human blood.

SUMMARY

- When used in the gas phase the NO electrode is hyper sensitive to changes in gas flow and temperature and it also produces a signal upon the injection of air. Signals observed often don't return to baseline so peak height has to be used to determine NO concentration. Chemiluminescence is not only a more sensitive NO measurement technique but it is also more practical in terms of time taken to set up and measure samples.
- Assays used to determine red blood cell/haemoglobin metabolites must incorporate a means of preventing NO auto-capture by cell free haem in the reaction chamber. This issue affects both the tri-iodide and CuCl/CSH reagents (and most probably other methodologies). It can be overcome via the addition of ferricyanide to tri-iodide or by changing the purge gas through the chemiluminescence system to CO. However, in order for CO to work at its best the reaction cell has to be maintained under pressure to obtain a high enough concentration of CO in solution.
- Samples can be snap-frozen and stored for analysis as long as they are processed within a short time frame. The freeze thaw process has no effect on metabolite stability. However, whilst total red blood cell and plasma signal remains constant over time in the freezer, red blood cell haemoglobin-bound NO and plasma protein-bound NO are lost, most probably to nitrite.



CHAPTER FOUR
NITRIC OXIDE
METABOLITES IN
BLOOD

4.0 Introduction

Endothelium derived NO produced by type III NOS which diffuses into the bloodstream is rapidly metabolised [52]. The reactions and products formed are dependent on the concentration of both NO itself and other bio-reactants. Erythrocytes are known to represent a major sink for NO as a result of the following reaction pathways:

- Co-oxidation reaction of NO with oxyhaemoglobin to form methaemoglobin and nitrate, recognised as the major inactivation pathway of NO *in vivo* [4, 128].
- Nitrosylation of deoxyhaemoglobin or deoxygenated haem groups on partially oxygenated haemoglobin to form iron-nitrosyl haemoglobin (HbNO).
- Nitrosation of specific thiol groups (Cys⁹³) on the β -haemoglobin chains to form S-nitrosohaemoglobin, either by direct reaction of NO or of a higher oxidation product (NO₂, N₂O₃ or NO₂⁻) or from the inter-conversion of NO from HbNO.

NO also interacts with plasma constituents, the main reactions being:

- Oxidation of NO via the formation of N-oxides (including NO₂ and N₂O₃) to nitrite and nitrate. Nitrite in plasma reacts with erythrocytic oxygenated haemoglobin to form nitrate and methaemoglobin [52].
- Nitrosation of plasma protein thiol and amine groups forming S-nitrosothiols (RSNO) and N-nitrosamines (RNNO) respectively. N-oxides formed from the reaction of NO with oxygen are potent nitrosating agents.

4.1 Aims

This work was undertaken to:

- Determine baseline concentrations of the major blood borne NO metabolite species in arterial and venous human blood utilising the most up to date and sensitive methodologies available.
- Investigate routes of metabolism of nitrite (because of its association as a potential bioactive metabolite store) and NO (representing the physiological situation) in human whole blood.

- Assess the effect of oxygen (which regulates nitrite bioactivity) on pathways of metabolism via analysis in arterial and venous whole blood.

4.2 Subjects

Due to the semi-invasive requirements of the study, samples were obtained from eleven subjects undergoing electrophysiology (EP) testing in the cardiology department of St Peters Hospital Chertsey; a procedure diagnostic for paroxysmal arrhythmias. Although all subjects had no past history of ischemic heart disease, smoking, diabetes mellitus, hypertension (blood pressure > 140/90mmHg) or hypercholesterolemia (total cholesterol > 5.2mmol/L), potential underlying pathologies might have induced greater variance than a control population. All patients on nitrite or nitrate therapy were excluded from this study. All individuals underwent an 8 hour fast prior to blood taking during which time they were allowed water. Subjects gave fully informed written consent approved by the relevant local research ethics committee.

4.3 Protocol

Parallel blood samples were drawn from the femoral artery and femoral vein into 20ml syringes. Blood was transferred immediately after blood gas analysis (Hemoximeter OSM3, Radiometer) into four or five 4ml ice chilled EDTA vacutainers. Nothing was added to one vacutainer (to give baseline measurement), saline only was added to another (as NO donor addition was administered in saline) and different concentrations of noc-9 ($t_{1/2} = 1.5-2$ minutes at 37°C) or sodium nitrite ($t_{1/2}$ in blood = 1.5 minutes at 37°C [52]) (Alexis, UK) were added to the rest to provide final concentrations of 0, ~ 2.4, ~ 4.7 and ~ 9.4 μ M NO. All samples were incubated in a water bath at 37°C for 6 minutes before being transferred to ice and centrifuged at 600g for 5 minutes at 4°C. Both plasma and the red cell fractions were separated, snap frozen in liquid nitrogen and stored at -80°C for subsequent analysis.

Within this study it must be highlighted that baseline samples were treated in exactly the same manner as all other samples, to which NO was added. The effect of the incubation period must therefore be considered in relation to the baseline NO metabolite measurements. Furthermore, although bloods were maintained within vacutainers throughout the majority of the study, ultimately samples were transferred into eppendorf tubes in room air. Others have previously demonstrated the effect of blood sample processing in room air compared to a glove box where the native sample pO_2 is maintained [83].

4.4 Determination of NO Metabolites

Plasma NO_x was measured by ozone based chemiluminescence in combination with the vanadium chloride reagent as detailed in chapter 2; section 2.1.3. Plasma nitrite and plasma protein-bound NO was measured by ozone based chemiluminescence using the tri-iodide reagent as detailed in chapter 2; section 2.1.4. Haemoglobin-bound NO and red blood cell associated nitrite were quantified by ozone based chemiluminescence using our modified tri-iodide reagent as detailed in chapter 2; section 2.1.5.

4.5 Statistical Analysis

All data are presented as mean \pm SEM. A two tailed p value of less than 0.05 was considered statistically significant. Differences between group means (noc-9 versus nitrite additions) were compared by unpaired student *t* test. Differences between means within each group (arterial versus venous blood) were compared by paired student *t* test. Bivariate correlations were performed using Pearson's coefficient.

4.6 Results

4.6.1 Baseline Measurements

Baseline NO metabolite levels were measured in five subjects, two male and three female, aged 53 ± 7 years (Table 4.1). Mean arterial and venous HbO₂ (%) were $95.8 \pm 1.7\%$ and $60.1 \pm 8.2\%$ respectively.

*Table 4.1: Baseline NO metabolite concentrations in human arterial and venous blood (n = 5). Values are presented as mean \pm SEM. Star(s) denote where mean is significantly different from the arterial sample; * $p < 0.05$.*

Metabolite Species	Arterial	Venous
Plasma Nitrate	$37.3 \pm 13.5 \mu\text{M}$	$33.4 \pm 11.2 \mu\text{M}$
Plasma Nitrite	$200.7 \pm 76.6 \text{ nM}$	$95.1 \pm 22.4 \text{ nM}$
Plasma Protein-bound NO	$10.5 \pm 5.9 \text{ nM}$	$27.4 \pm 15.6 \text{ nM}$
RBC Associated Nitrite	$43.2 \pm 26.3 \text{ nM}$	$17.0 \pm 10.4 \text{ nM}$
RBC Hb-bound NO	$27.5 \pm 8.9 \text{ nM}$	$43.3 \pm 12.2 \text{ nM}^*$

4.6.1.1 Plasma NO Metabolites

In both arterial and venous blood, plasma nitrate made up by far the largest component of the total NO metabolite pool (> 99%). This reflects its in vivo half life (~ 5-8 hours) and various sources of formation, both NOS dependent (e.g., type III NOS NO metabolism) and independent (e.g., diet, saliva). Plasma nitrite formed the second largest component of the total metabolite pool (< 0.55 %), with the rest being made up of protein-bound NO (including RSNOs and N-nitrosamines) forming < 0.1% of all NO metabolites (Figure 4.1).

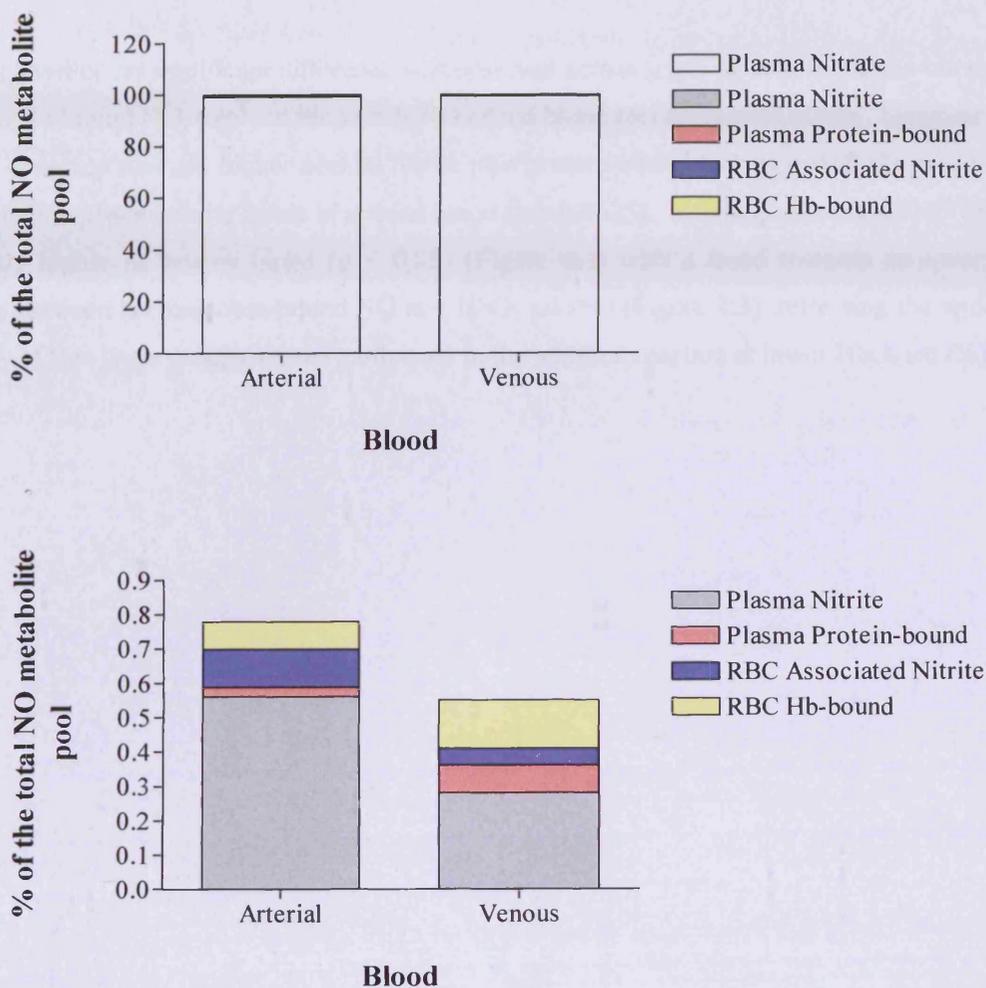


Figure 4.1: Baseline proportions of all NO metabolite species including nitrate (Top) and excluding nitrate (Bottom) as a percentage of the total NO metabolite pool in arterial and venous blood.

4.6.1.2 Red Blood Cell NO Metabolites

Red blood cell NO metabolites (haemoglobin-bound NO and red blood cell associated nitrite) made up < 0.25% of the total NO metabolite pool in both arterial and venous blood (Figure 4.1).

4.6.1.3 Relationship of Baseline Metabolite Apportionment to Oxygen

At baseline no significant difference was observed across artery to vein in plasma nitrate, plasma protein-bound NO, total red blood cell NO or red blood cell associated nitrite. However a trend was observed towards higher plasma nitrite plus protein-bound NO ($p = 0.0952$) possibly reflecting higher plasma nitrite levels in arterial blood ($p = 0.0625$). Haemoglobin-bound NO was significantly higher in venous blood ($p < 0.05$) (Figure 4.2) with a trend towards an inverse correlation between haemoglobin-bound NO and HbO₂ sat (%) (Figure 4.3), reflecting the wider availability of free haem groups able to participate in the addition reaction at lower HbO₂ sat (%).

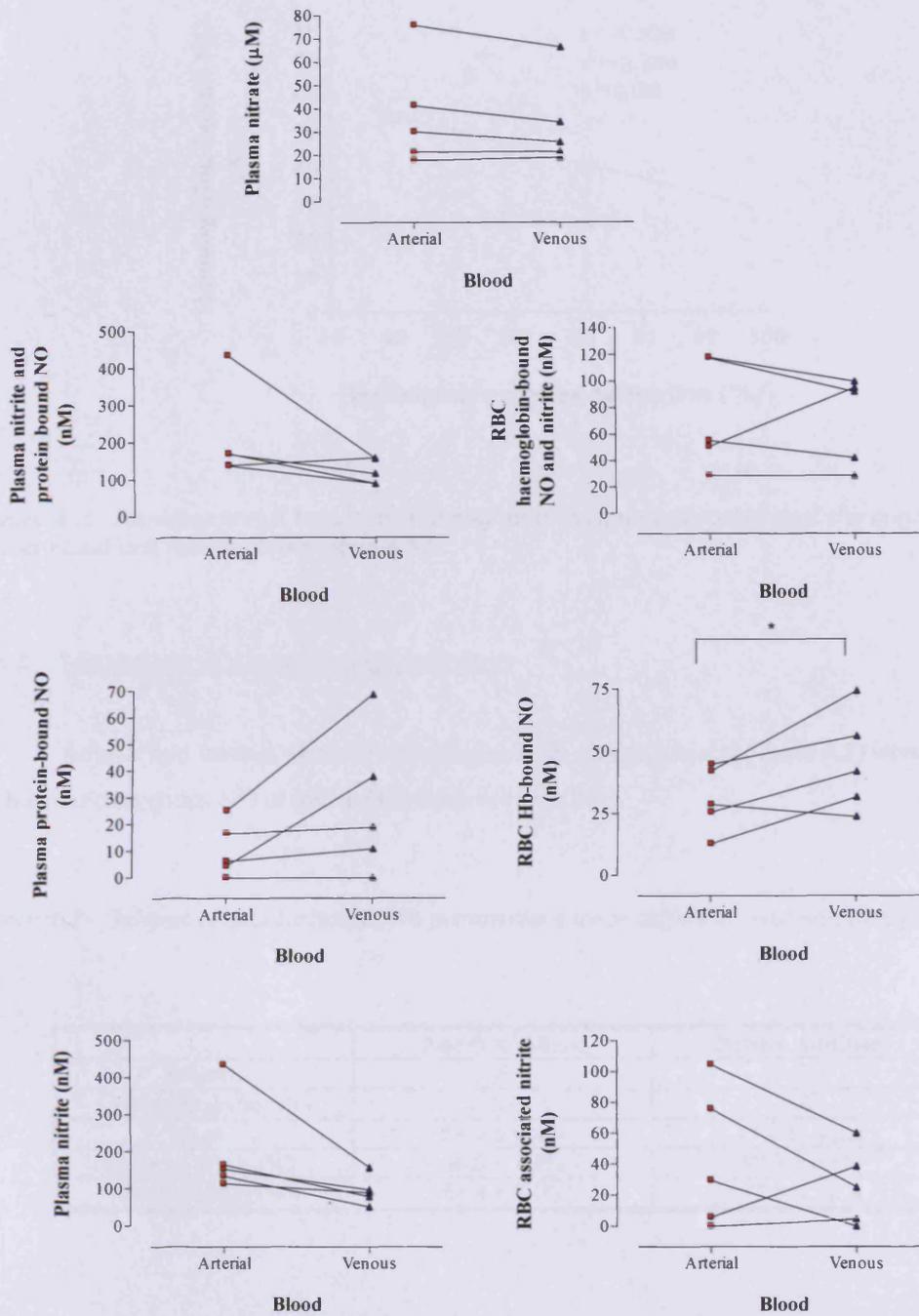


Figure 4.2: Baseline arterial and venous concentrations of NO metabolites. Individual data points plotted. * $p < 0.05$.

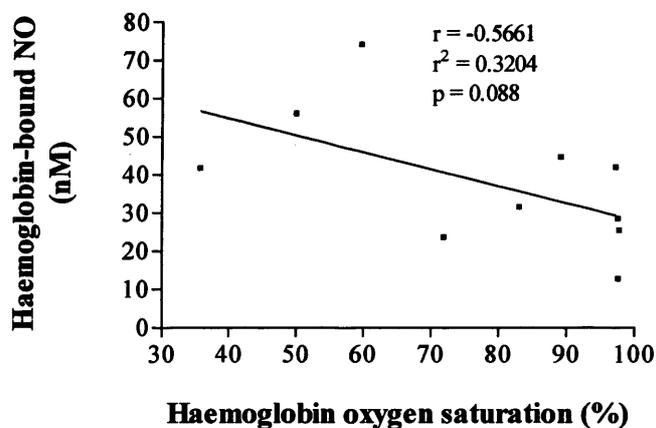


Figure 4.3: Baseline trend between haemoglobin oxygen saturation and the concentration of red blood cell haemoglobin-bound NO.

4.6.2 Metabolism of Exogenous NO and Nitrite

Arterial and venous whole blood samples from eleven subjects (Table 4.2) were incubated with either exogenous NO or nitrite (administered in saline).

Table 4.2: Subject characteristics. No parameters were different between groups.

	Noc-9 Addition	Nitrite Addition
Subjects	6	5
Gender (male/female)	3/3	3/2
Age	54 ± 6 years	47 ± 11 years
Arterial HbO ₂ (%)	96.0 ± 1.4%	97.3 ± 0.3%
Venous HbO ₂ (%)	62.4 ± 7.1%	72.8 ± 5.2%

4.6.2.1 NO/Nitrite Addition - Comparison to Baseline

The addition of saline on its own had no significant effect on the concentrations of metabolites or the apportionment of NO between species (data not shown). In arterial blood the

addition of exogenous NO resulted in the non nitrate portion of the total metabolite pool increasing ~ 7% and ~ 39% following nitrite addition (Figure 4.4). In venous blood the corresponding figures were ~ 10% with NO addition and ~ 30% with nitrite addition (Figure 4.5). This demonstrates reactions competing with the oxidation reaction, resulting in the formation of potentially bio-available forms of NO. The higher levels of nitrate free signal following nitrite addition mainly reflected the accumulation of plasma nitrite.

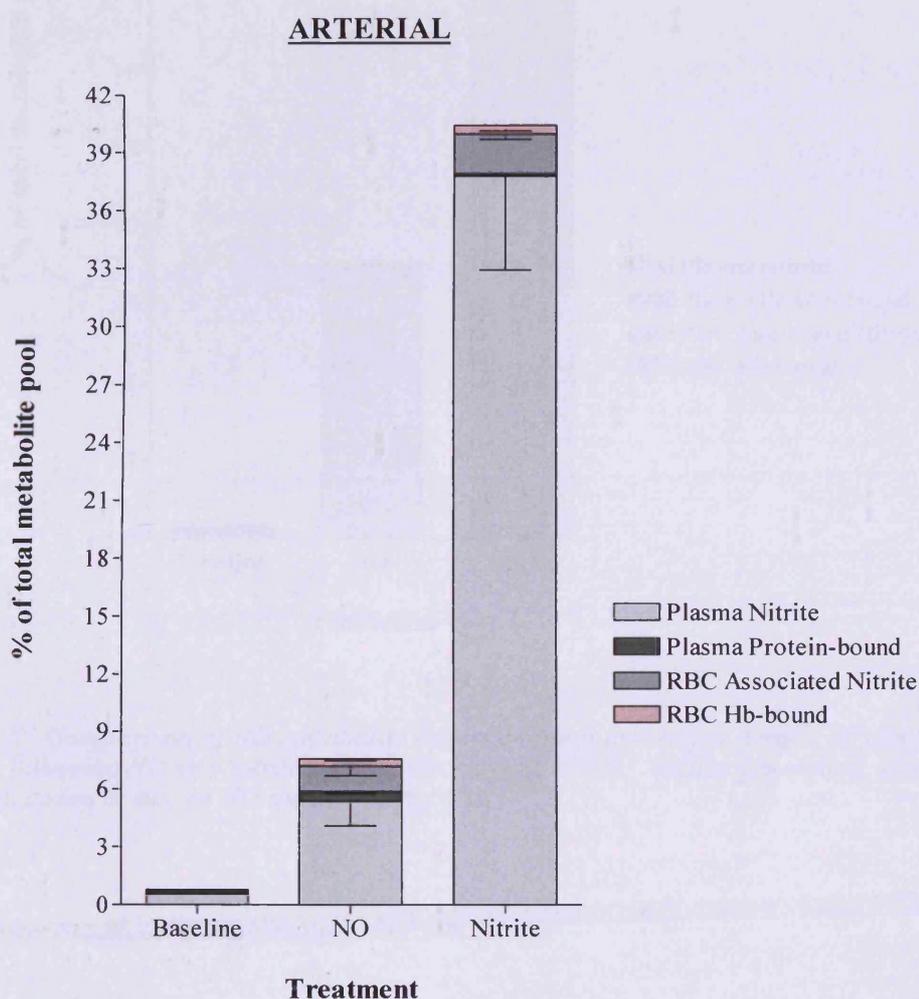


Figure 4.4: Comparison of NO metabolite apportionment (averaged across all doses) from baseline following NO and nitrite addition to **arterial** blood. Values presented as mean across all doses of added NO and nitrite \pm SEM.

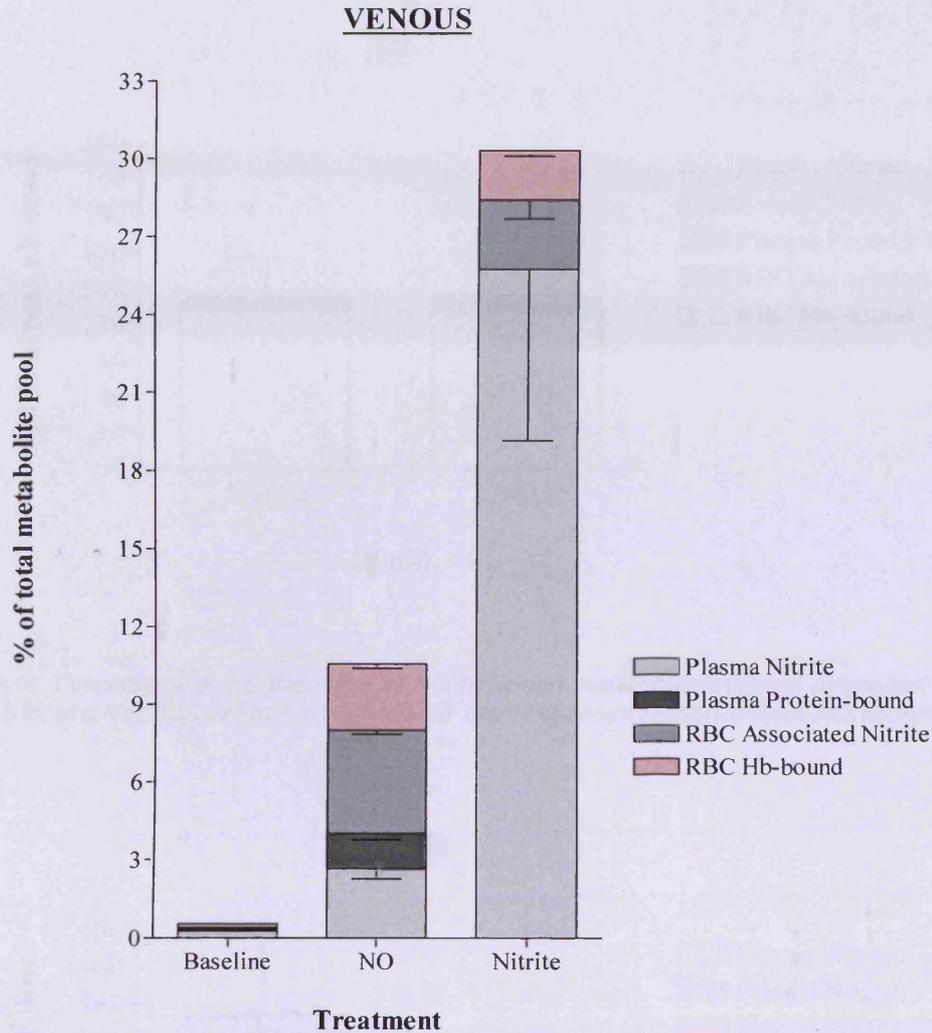


Figure 4.5: Comparison of NO metabolite apportionment (averaged across all doses) from baseline following NO and nitrite addition to **venous** blood. Values presented as mean across all doses of added NO and nitrite \pm SEM.

4.6.2.2 Recovery of Exogenously Added NO and Nitrite

An average of $94.5 \pm 21.7\%$ and $96.3 \pm 23.3\%$ of total added NO (Figure 4.6) and $135.5 \pm 41.8\%$ and $100.5 \pm 22.1\%$ of total added nitrite (Figure 4.7) was recovered across all doses in arterial and venous blood respectively. This suggests that the metabolite species measured within this study represent not only the main metabolic pathways for NO and nitrite but also the main metabolites in human blood.

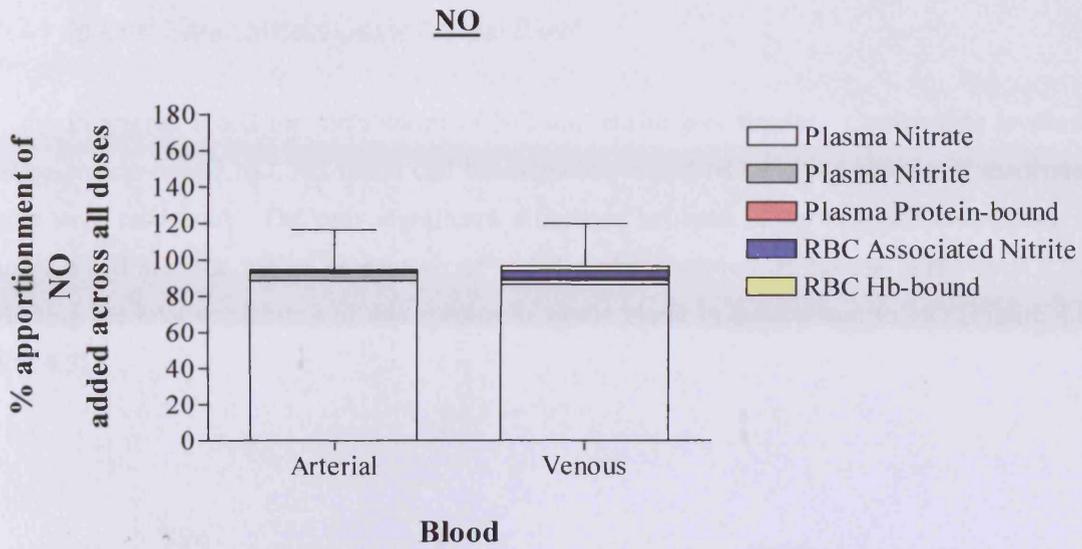


Figure 4.6: Percentage apportionment of NO between metabolite species averaged across all doses in arterial and venous blood. Error bars represent SEM of total NO return.

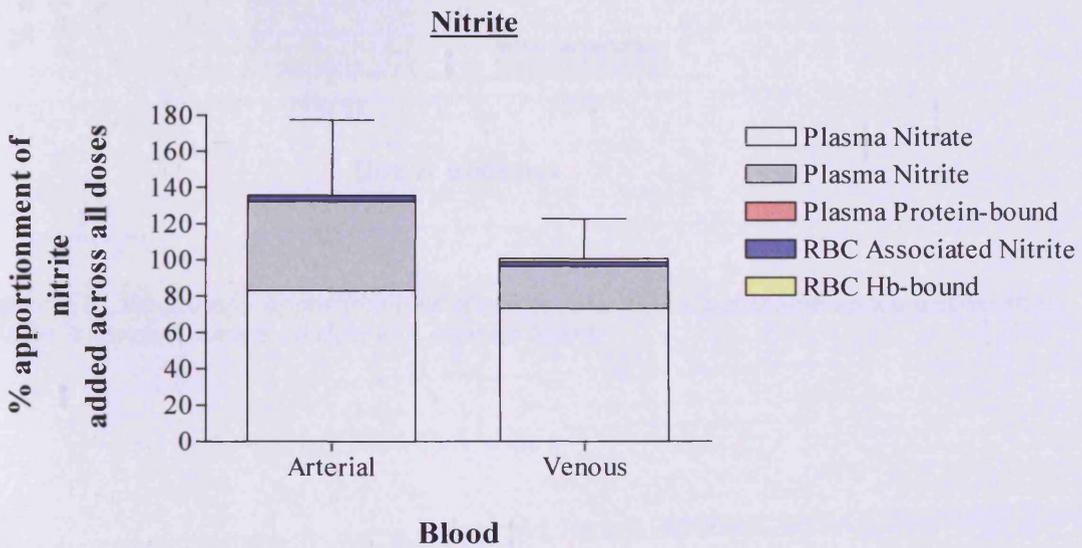


Figure 4.7: Percentage apportionment of nitrite between metabolite species averaged across all doses in arterial and venous blood. Error bars represent SEM of total NO return.

4.6.2.3 NO and Nitrite Metabolism in Arterial Blood

In arterial blood the metabolism of NO and nitrite was similar. Comparable levels of plasma-protein-bound NO, red blood cell haemoglobin-bound NO and red blood cell associated nitrite were recovered. The only significant difference between nitrite and NO metabolism in arterial blood was the higher percentage of added nitrite observed as plasma nitrite ($p < 0.01$) reflecting the relative stability of this species in whole blood in comparison to NO (Figure 4.8; Table 4.3).

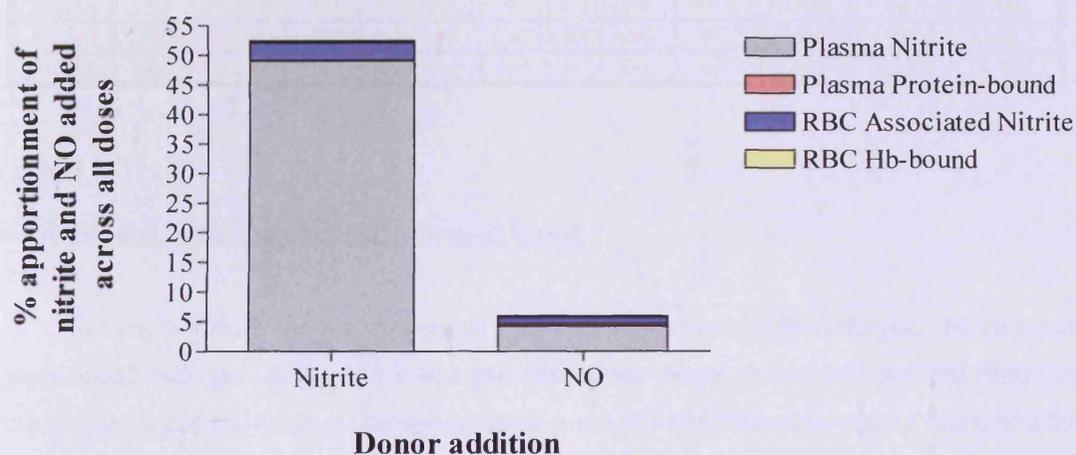


Figure 4.8: Percentage apportionment of nitrite and NO to metabolite species other than nitrate, averaged across all doses in arterial blood.

Table 4.3: NO metabolite concentrations in arterial blood following addition of nitrite (Top) and NO (Bottom). Values presented as mean \pm SEM. Star(s) denote where mean is significantly different from the NO addition; ** $p < 0.01$.

Nitrite Final	Plasma NO ₃ ⁻	Plasma NO ₂ ⁻	Plasma Protein-bound	RBC Hb-bound	RBC associated NO ₂ ⁻
Saline	20.2 \pm 3 μ M	54.3 \pm 21 nM**	19.3 \pm 5 nM	18.8 \pm 1 nM	11.6 \pm 4 nM
2.4 μ M	23.3 \pm 4 μ M	1.3 \pm 0.2 μ M**	21.1 \pm 9 nM	23.8 \pm 4 nM	99.8 \pm 29 nM
4.7 μ M	23.2 \pm 5 μ M	2.5 \pm 0.2 μ M**	34.0 \pm 12 nM	32.3 \pm 3 nM	133.8 \pm 36 nM
9.4 μ M	26.6 \pm 2 μ M	4.9 \pm 0.5 μ M**	23.9 \pm 7 nM	48.2 \pm 8 nM	310.6 \pm 106 nM

NO Final	Plasma NO ₃ ⁻	Plasma NO ₂ ⁻	Plasma Protein-bound	RBC Hb-bound	RBC associated NO ₂ ⁻
Saline	31.6 \pm 10 μ M	184.4 \pm 32 nM	27.4 \pm 16 nM	34.1 \pm 8 nM	27.3 \pm 11 nM
2.4 μ M	35.3 \pm 10 μ M	296.1 \pm 39 nM	42.3 \pm 10 nM	43.1 \pm 10 nM	43.1 \pm 15 nM
4.7 μ M	34.4 \pm 8 μ M	409.8 \pm 33 nM	47.4 \pm 13 nM	47.1 \pm 6 nM	92.8 \pm 9 nM
9.4 μ M	38.2 \pm 10 μ M	591.3 \pm 77 nM	55.1 \pm 17 nM	67.7 \pm 5 nM	123.4 \pm 17 nM

4.6.2.4 NO and Nitrite Metabolism in Venous Blood

In venous blood, the metabolism of NO and nitrite were very different. More plasma protein bound NO ($p < 0.01$), red blood cell associated nitrite ($p < 0.05$) and red blood cell haemoglobin-bound NO (only at the highest dose; $p < 0.05$) was formed in venous blood to which NO was added (Figure 4.9; Table 4.4). However, a higher percentage of plasma nitrite was observed following nitrite addition ($p < 0.01$) reflecting the relative stability of exogenously added nitrite in whole blood even at lower oxygen tensions.

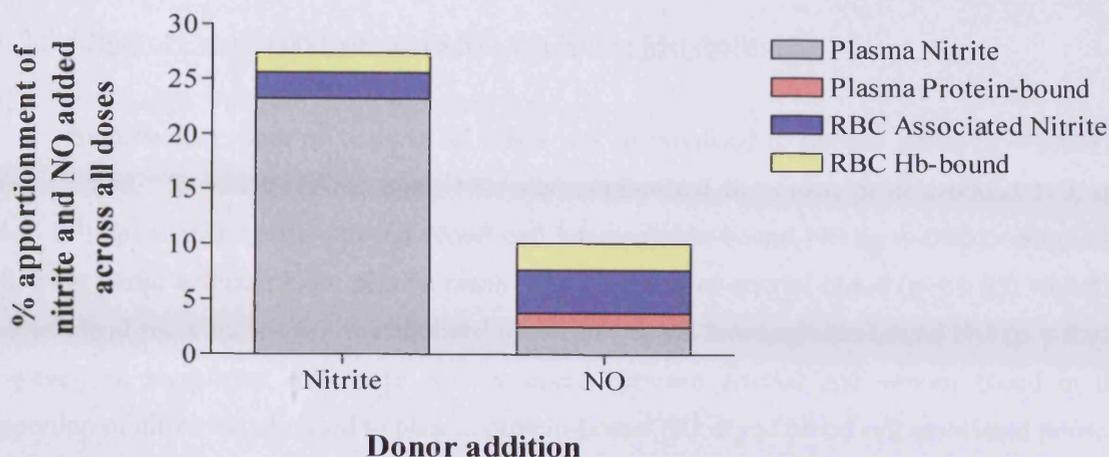


Figure 4.9: Percentage apportionment of nitrite and NO to metabolite species other than nitrate, averaged across all doses in venous blood.

Table 4.4: NO metabolite concentrations in venous whole blood following addition of nitrite (Top) and NO (Bottom). Values are presented as mean \pm SEM. Star(s) denote where mean is significantly different from the NO addition; * $p < 0.05$, ** $p < 0.01$.

Nitrite Final	Plasma NO_3^-	Plasma NO_2^-	Plasma Protein-bound	RBC Hb-bound	RBC associated NO_2^-
Saline	21.1 \pm 3 μM	42.0 \pm 10 nM**	28.6 \pm 9 nM	18.0 \pm 5 nM **	9.6 \pm 5 nM
2.4 μM	23.4 \pm 3 μM	0.6 \pm 0.04 μM **	21.9 \pm 4 nM**	71.5 \pm 15 nM	64.2 \pm 15 nM*
4.7 μM	25.5 \pm 3 μM	1.2 \pm 0.1 μM **	20.5 \pm 8 nM**	110.3 \pm 21 nM	124.4 \pm 22 nM**
9.4 μM	24.5 \pm 5 μM *	2.5 \pm 0.3 μM **	21.7 \pm 7 nM**	167.8 \pm 32 nM*	274.9 \pm 42 nM*

NO Final	Plasma NO_3^-	Plasma NO_2^-	Plasma Protein-bound	RBC Hb-bound	RBC associated NO_2^-
Saline	31.3 \pm 10 μM	123.4 \pm 17 nM	14.3 \pm 5 nM	39.0 \pm 4 nM	24.8 \pm 11 nM
2.4 μM	33.3 \pm 9 μM	232.3 \pm 25 nM	50.9 \pm 7 nM	96.3 \pm 9 nM	110.6 \pm 13 nM
4.7 μM	35.4 \pm 8 μM	329.2 \pm 36 nM	85.7 \pm 14 nM	171.6 \pm 24 nM	222.6 \pm 24 nM
9.4 μM	40.8 \pm 10 μM	410.8 \pm 55 nM	104.2 \pm 16 nM	277.5 \pm 36 nM	430.7 \pm 70 nM

4.6.2.5 Effect of Oxygen on Exogenous NO and Nitrite Metabolism

Significantly more NO across all doses was metabolised to plasma nitrite ($p < 0.05$) in arterial blood. In venous blood more NO was metabolised to plasma protein-bound NO, red blood cell associated nitrite and red blood cell haemoglobin-bound NO ($p < 0.05$). Similarly, following nitrite addition more plasma nitrite was observed in arterial blood ($p < 0.05$) whilst in venous blood more nitrite was metabolised to red blood cell haemoglobin-bound NO ($p < 0.05$). However, no significant difference was observed between arterial and venous blood in the proportion of nitrite metabolised to plasma protein-bound NO or red blood cell associated nitrite.

Across all added doses of NO and nitrite (but not the saline addition) positive correlations were observed between plasma nitrite and HbO₂ sat (%) (Figure 4.10) and inverse correlations between haemoglobin-bound NO and HbO₂ sat (%) (Figure 4.11). However, upon NO addition an inverse correlation was observed between red blood cell associated nitrite and HbO₂ sat (%), but no correlation was observed between these parameters with nitrite addition (Figure 4.12).

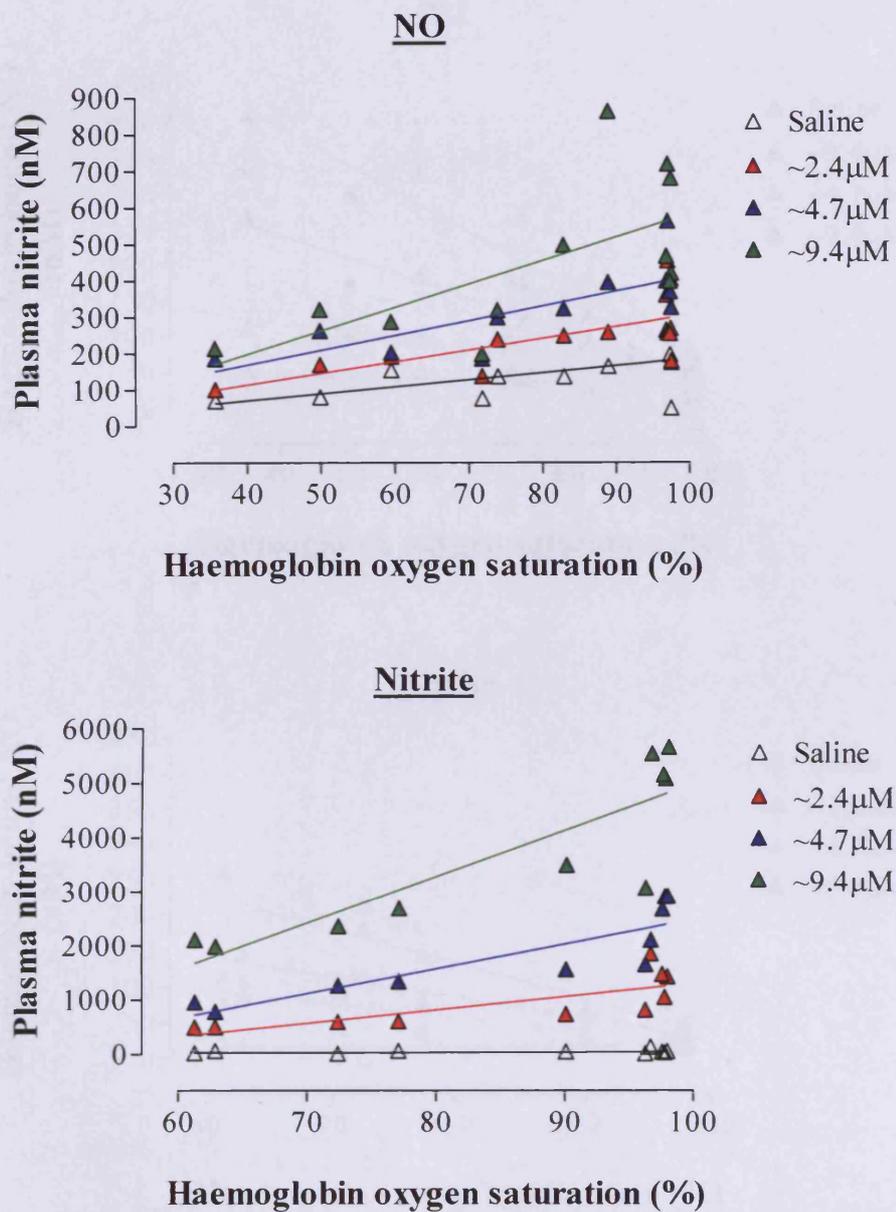


Figure 4.10: Relationship between plasma nitrite and haemoglobin oxygen saturation following the addition of saline, 2.5, 5 and 10 μ M NO (Top) and nitrite (Bottom) to whole arterial and venous blood. NO addition (Top) saline $r = 0.46$; $p = 0.13$; 2.5 μ M $r = 0.64$; $p = 0.02$; 5 μ M $r = 0.79$; $p = 0.002$; 10 μ M $r = 0.65$; $p = 0.02$. Nitrite addition (Bottom) saline $r = 0.19$; $p = 0.60$; 2.5 μ M $r = 0.77$; $p = 0.009$; 5 μ M $r = 0.87$; $p = 0.001$; 10 μ M $r = 0.87$; $p = 0.001$.

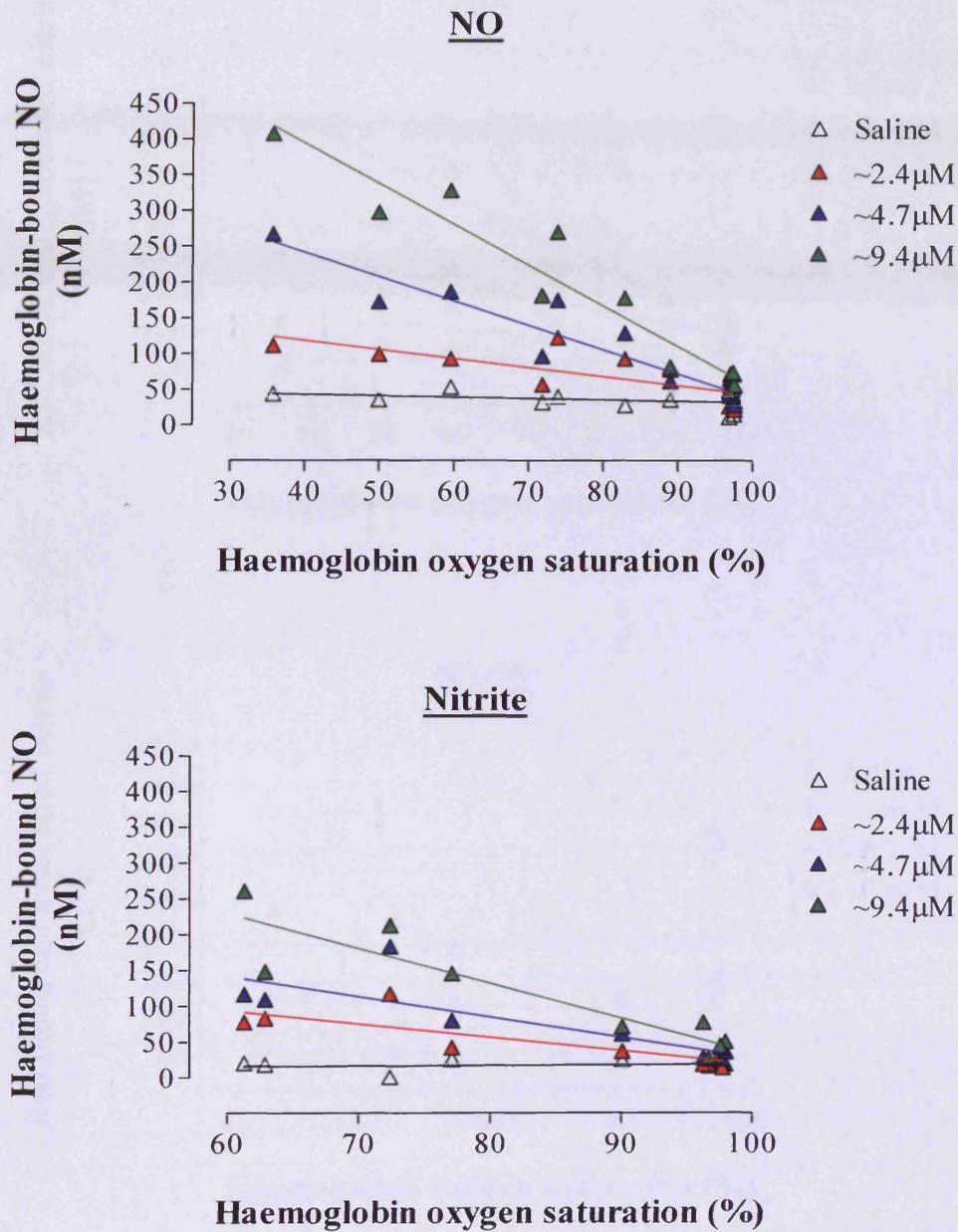


Figure 4.11: Relationship between haemoglobin-bound NO and haemoglobin oxygen saturation following the addition of saline, 2.5, 5 and 10 μ M NO (Top) and nitrite (Bottom) to whole arterial and venous blood. NO addition (Top) saline $r = -0.27$; $p = 0.39$; 2.5 μ M $r = -0.75$; $p = 0.005$; 5 μ M $r = -0.94$; $p < 0.0001$; 10 μ M $r = -0.96$; $p < 0.0001$. Nitrite addition (Bottom) saline $r = 0.18$; $p = 0.63$; 2.5 μ M $r = -0.83$; $p = 0.003$; 5 μ M $r = -0.83$; $p = 0.003$; 10 μ M $r = -0.92$; $p = 0.0002$.

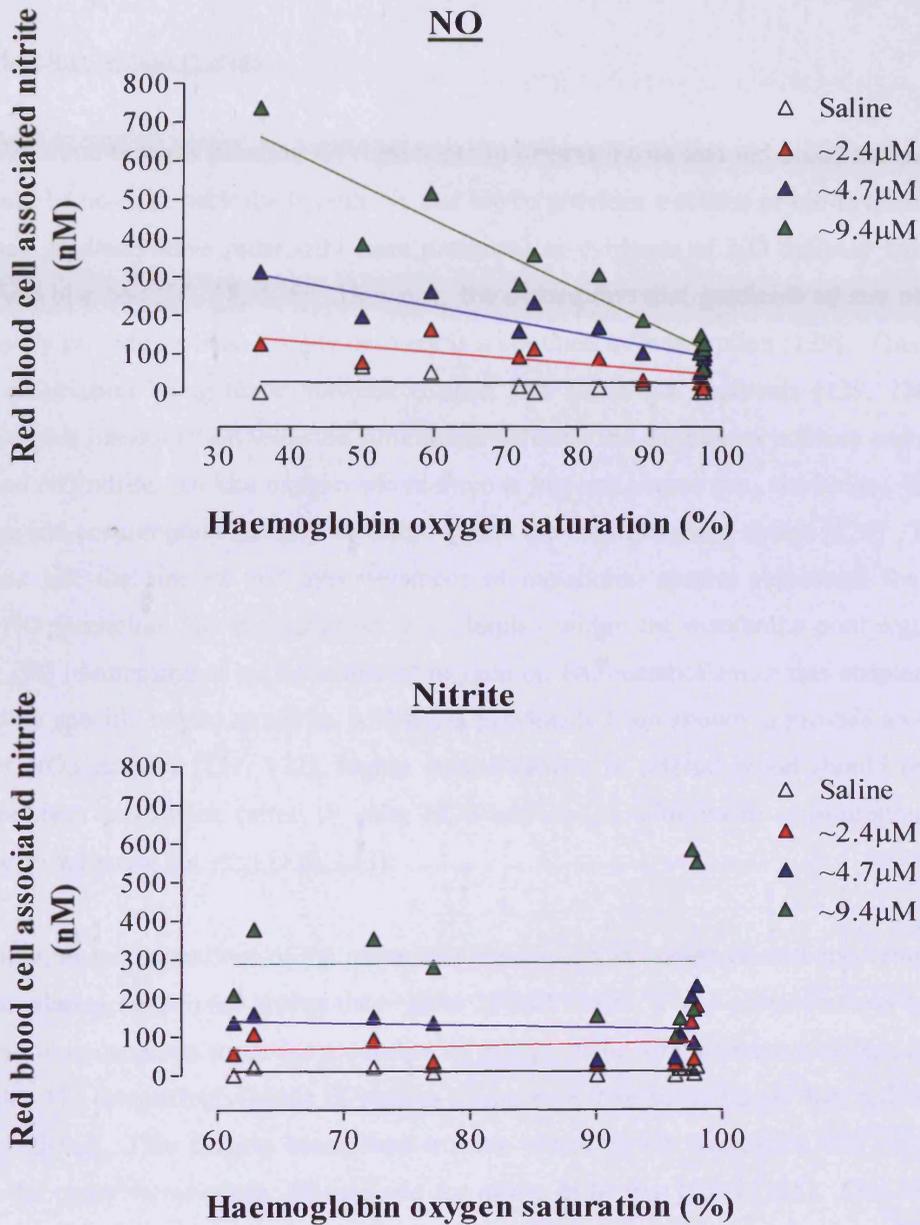


Figure 4.12: Relationship between red blood cell-bound nitrite and haemoglobin oxygen saturation following the addition of saline, 2.5, 5 and 10µM NO (Top) and nitrite (Bottom) to whole arterial and venous blood. NO addition (Top) saline $r = -0.04$; $p = 0.90$; 2.5µM $r = -0.67$; $p = 0.02$; 5µM $r = -0.90$; $p < 0.0001$; 10µM $r = -0.95$; $p < 0.0001$. Nitrite addition (Bottom) no correlations observed.

4.7 **Discussion**

4.7.1 **Baseline Measurements**

The trend towards baseline A-V gradients in plasma nitrite and red blood cell associated nitrite could be taken to back the hypothesis that nitrite provides a source of bio-available NO *in vivo*. Such gradients have previously been presented as evidence of NO delivery from nitrite across a vascular bed [26, 78, 125]. However, the assumption that gradients of any metabolite species solely provide an index of NO delivery is a common misconception [129]. This is based upon an association being made between oxygen and NO/nitrite gradients [129, 130]. The accuracy of this interpretation relies on similarities between the circulatory sources and sinks for oxygen and NO/nitrite. Unlike oxygen where there is just one source (i.e., the lungs), NO/nitrite production and consumption are dispersed throughout the cardiovascular circuit [129]. Therefore at any one site the amount and apportionment of metabolite species represents the balance between NO formation, NO consumption and interplay within the metabolite pool regulated by HbO₂ sat (%) (demonstrated by the effect of oxygen on NO metabolism in this chapter, section 4.7.3). With specific regard to nitrite, which has previously been shown to provide an excellent marker of NOS activity [131, 132], higher concentrations in arterial blood should reflect the balance between production (artery > vein; NOS activity) in addition to consumption (vein > artery; nitrite reductase activity) [130, 133].

Baseline concentrations of the major NO metabolites in human arterial and venous blood (other than plasma nitrate) fell within the ~ 10 to 200nM range. These concentrations are in line with recent measurements made by a number of groups using similar methodologies (see table 3.0; chapter 3). Interestingly levels of plasma nitrite were five times larger than red blood cell associated nitrite. This finding contradicts a more recent report suggesting that erythrocytes represent the major intravascular storage site for nitrite in human blood [125]. One significant difference between these two studies concerns the biological sample handling protocols used. Herein sample pre-treatment was kept to a minimum with only the dilution of red blood cells either in water or sulphanilamidated water prior to measurement. In the work of Dejam et al. [125] whole blood or red blood cells were mixed with a ferricyanide-based 'stabilization-solution' (5:1 vol:vol; designed to limit *ex vivo* nitrite reactions) containing ferricyanide, NEM and NP-40. In addition to this samples were de-proteinated with methanol (1:1 vol:vol) before injection into tri-

iodide. Interestingly, no mention is made of the control for the addition of contamination via these chemicals and reagents.

The baseline NO metabolite values reported herein are generally much lower than those upon which the original metabolite theories were founded (μM concentrations of SNO albumin [63] and SNO haemoglobin [70]). This follows a recent trend in the literature towards lower reported metabolite concentrations measured by a number of groups, mainly reflecting greater awareness of potential contaminants and more widespread use of similar methodologies (i.e., tri-iodide reductive ozone based chemiluminescence). However, lower reported metabolite concentrations have no bearing on the physiological relevance of these species. Within NO biology amount does not reflect importance [103]. If this were so NO itself would be regarded as inconsequential being that it cannot be directly measured in blood or tissue. So long as enough related NO moiety is available to regulate physiological processes (e.g., to activate soluble guanylate cyclase; $EC_{50} \sim 20\text{nM}$ [134]) actual bio-chemically measured levels of baseline metabolite species tell us little about their significance.

4.7.2 NO and Nitrite Metabolism

NO and nitrite were metabolised in a similar manner in arterial blood. The major difference appeared to be the relative stability of exogenously added nitrite in arterial whole blood, reflected by its accumulation in the plasma compartment. Identifying why a major proportion of added nitrite remained in the plasma compartment was not possible because no other species declined to account for this, as reflected in the fact that the overall return of NO across all nitrite additions to arterial blood was $\sim 135\%$ (Figure 4.7). This excessive return of NO in itself could potentially come from red blood cell nitrate (a metabolite species not measured throughout this thesis as no current methodology exists) leaching from the red blood cells into plasma (where nitrate has been measured in this study). Nitrate can be measured in plasma but not in red blood cells due to the effect of haemoglobin auto-capturing NO (previously demonstrated in Chapter 3 in the tri-iodide and CuCl/CSH reagents) and the fact that within the nitrate assays no method currently exists to prevent this process. In venous blood on average across all added doses more NO than nitrite was metabolised to plasma nitrate, plasma protein-bound NO, red blood cell haemoglobin bound NO and red blood cell associated nitrite. However, once more a large proportion of added nitrite remained in the plasma compartment. Taken together these results in both arterial and venous blood imply a difference in the ability of NO and

nitrite to cross the red blood cell membrane, with nitrite consequently being protected from the red blood cell associated reactions (i.e., the oxidation and addition reactions). Whilst the charge neutrality of NO is assumed to facilitate its free diffusibility across cell membranes [135] it is possible that the negative charge of nitrite restricts entry into the red blood cell. As yet the mechanisms involved in nitrite uptake by human red blood cells remain to be resolved, however the anion exchanger AE1 appears to have been ruled out in this process [125, 136].

Although exogenous NO/nitrite added to whole blood was predominantly metabolised via the oxidation reaction, other reactions were shown to compete with this (even in arterial blood) resulting in the conservation rather than the consumption of NO. This was demonstrated by the non nitrate percentage of the total metabolite pool increasing in comparison to baseline ~ 7-10% following NO addition and ~ 30-39% with nitrite addition (mainly reflecting nitrite in the plasma compartment) in whole arterial and venous blood. These findings are in agreement with others who have previously demonstrated under physiologically relevant experimental conditions preferential reaction mechanisms forming metabolites which preserve rather than destroy NO bioactivity [137].

4.7.3 Effect of Oxygen on NO/Nitrite Metabolism

The fact that set amounts of NO and nitrite were metabolised differently in arterial and venous blood demonstrates the role that oxygen plays in the regulation of the metabolite pool. This adds to the complexity of analysing and interpreting A-V NO metabolite gradients as it implies that even were the production and consumption of NO to be the same *in vivo* across the vascular circuit from artery to vein (which we know it is not) different A-V NO metabolite profiles would be observed.

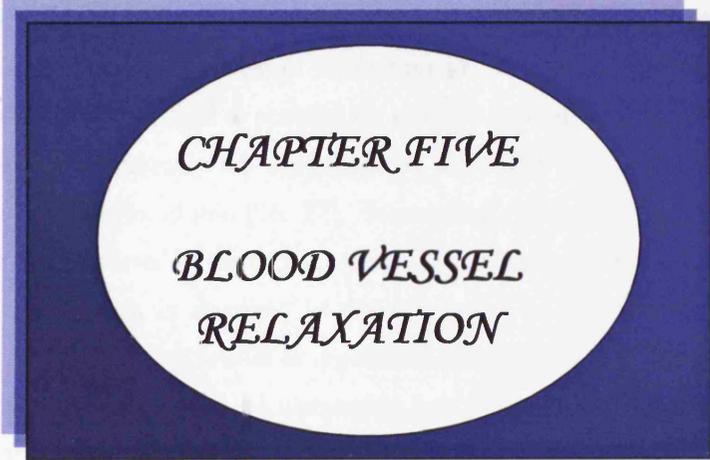
Levels of plasma nitrite were significantly greater in arterial blood following the addition of both NO and nitrite. With NO addition plasma nitrite levels reflected the balance between nitrite formation and metabolism. Conversely following nitrite addition plasma nitrite levels solely reflected metabolism, which was enhanced in venous blood as evidenced by the lower concentration of nitrite remaining in the plasma compartment. The only metabolite species to increase in venous blood alone following nitrite addition was haemoglobin-bound NO, implicating the reduction of nitrite by deoxyhaemoglobin as the mechanism accounting for venous nitrite loss from the plasma compartment. From this reaction either iron-nitrosyl

haemoglobin or as more recently suggested (via the same reaction mechanism) SNO-Hb [138] could have formed.

Following NO addition to whole blood an inverse correlation was observed between red blood cell associated nitrite and HbO₂ sat (%), however no correlation was observed following nitrite addition. This reflects the fact that red blood cell associated nitrite accumulated to a similar extent in both arterial and venous blood upon the addition of nitrite, however much lower levels of red blood cell associated nitrite were observed in arterial blood following the addition of NO.

SUMMARY

- Baseline concentrations of all NO metabolite species other than plasma nitrate (which itself is in the ~ 20-50 μ M range) fall within the ~ 10 to 200nM range. Apart from the level of red blood cell associated nitrite, these concentrations are in line with recent measurements made by a number of groups using similar methodologies.
- NO and nitrite were metabolised similarly in arterial blood, the only difference being greater plasma nitrite accumulation following nitrite addition. However, in venous blood across all added doses a greater percentage of NO than nitrite was metabolised to plasma nitrate, plasma protein-bound NO, red blood cell haemoglobin-bound NO and red blood cell associated nitrite. Taken together these results in arterial and venous blood possibly reflect the differing ability of NO and nitrite to cross the red blood cell membrane.
- The different metabolism of set amounts of NO/nitrite in arterial and venous blood demonstrate the regulatory function of oxygen in determining the relative proportion of species within the NO metabolite pool. This adds to the complexity of analysing and interpreting A-V NO metabolite gradients.



CHAPTER FIVE
BLOOD VESSEL
RELAXATION

5.0 Introduction

The vascular regulatory properties of blood transported NO are generally agreed to be enhanced in hypoxia [26, 45, 103, 129] potentially accounting for the hypoxic vasodilator response *in vivo* (the relaxation of vascular smooth muscle in regions of low tissue pO₂) which acts to enhance blood flow to match oxygen delivery with metabolic demand [139, 140]. What elicits the hypoxic vasodilator response still remains to be conclusively proven. Several diverse NO independent and NO dependent hypotheses have emerged to explain this phenomenon [86, 129, 141, 142]. More recently studies have identified that the release of NO bioactivity from red blood cells plays a part in this process [26, 70, 83, 114, 115, 143, 144]. In these models haemoglobin is proposed to act as the hypoxic sensor coupling decreasing oxygen tension to increased vessel dilatation via the release or formation of NO related moiety. Two different metabolite species have been proposed to account for this NO mediate response; the *S*-nitrosothiol SNO-Hb [70, 83] and more recently the anion nitrite, following its conversion to NO via the reductase activity of deoxyhaemoglobin [26, 27]. Interestingly both these hypotheses imply that 1) an NO equivalent is transported in the blood, 2) NO delivery occurs in regions of hypoxia, 3) a haemoglobin based mechanism is involved in the regulation of the bioactive equivalent and therefore 4) that red cells have the potential to regulate vascular tone [87]. These similarities lend support to the idea of linkage between the metabolite hypotheses, previously outlined following the suggestion that the nitrite reductase reaction of deoxyhaemoglobin contributes to SNO-Hb formation [83, 133, 138]. Specifically this chapter focuses on the potential role that nitrite plays in red blood cell mediated regulation of vascular tone.

The biological properties of nitrite have been studied for over a century [145]. Early organ chamber work demonstrated nitrite to be orders of magnitude less potent than many bioactive substances [146], with *in vitro* levels required to elicit a response typically a hundred fold greater (hundred- μ M to mM range) than circulating concentrations measured *in vivo* (< 1 μ M) [147]. These findings in combination with an *in vivo* study which demonstrated no effect of arterially infused nitrite in the human forearm circulation [131] all but ruled out nitrite as a potential source of bioactive NO in the human circulation. However, more recently reports of A-V nitrite gradients *in vivo* [78, 125, 148, 149] in combination with demonstrated vasodilator activity of nitrite in the human forearm circulation at near physiological concentrations [26] have reignited interest in this compound and specifically the nitrite-reductase reaction of haemoglobin [24-27] which has been postulated, via the release of free NO, to independently mediate hypoxic vasodilatation.

5.1 Aims

This work was undertaken to:

- Investigate the effect of oxygen on red blood cell mediated regulation of vascular tone.
- Analyse the direct vasoactive properties of nitrite at different oxygen tensions.
- Verify whether nitrite enhances red blood cell induced vasodilatation.

5.2 Protocol

5.2.1 Aortic Ring Preparation

The thoracic aorta of male New Zealand white rabbits were dissected and prepared as described in chapter 2 section 2.2. Experiments were performed with endothelium denuded and intact rings. To denude the vessels, the luminal surface was gently rubbed with a rounded wooden applicator. The aortic rings were subsequently mounted in 8ml tissue baths containing 5ml Krebs buffer and gassed with 95% O₂ / 5% CO₂ at 37°C. A resting tension of 2.1-2.3g was set. After a one hour equilibration period during which time the tension was reset (to allow for stretch induced relaxation) all rings were exposed to phenylephrine (PE, 1µM) to induce constriction. When a steady plateau was reached acetylcholine (10µM) was added to check the viability of the endothelium. Following this the rings were washed re-equilibrated and repeat PE constrictions performed until a reproducible constriction was achieved.

5.2.2 Blood Samples

Blood samples (from healthy volunteers within the laboratory) were drawn from the antecubital vein and immediately transferred into 4ml ice chilled EDTA vacutainer(s). For experiments with native red blood cells, samples were spun at 600g for 5 minutes at 4°C. The plasma and buffy coat were removed and discarded and the red cells subsequently washed in an equal volume of saline and spun at 2000g for 5 minutes at room temperature. Saline was removed and 20µl of the red blood cell pellet was injected into the 5ml bath (final haemoglobin concentration 20µM; 80µM haem; final haematocrit 0.4%). For incubation experiments with noc-9 and nitrite, saline only (500µl) was added to one vacutainer (as NO donor addition was administered in saline) and different concentrations of noc-9 ($t_{1/2} = 1.5$ minutes at 37°C) or

sodium nitrite ($t_{1/2} = 1.5$ minutes at 37°C [52]) were added to the other vacutainers to provide final concentrations of ~ 2.4 and $\sim 9.4\mu\text{M}$ NO. All samples were incubated in a water bath at 37°C for 6 minutes before being transferred to ice and centrifuged at $600g$ for 5 minutes at 4°C . The plasma and buffy coat were removed and discarded and the red cells subsequently washed three times in an equal volume of saline and spun at $2000g$ for 5 minutes at room temperature. Saline was removed and replaced following each washing. After the final spin the saline was removed and $20\mu\text{l}$ of the red blood cell pellet was injected into the 5ml bath.

5.2.3 Oxygen Tension

Experiments were performed at different oxygen tensions by varying the oxygen (O_2) perfusing the Krebs buffer bathing the tissues. Vessel tension experiments at low O_2 were performed using 0% O_2 (i.e., 95% N_2 , 5% CO_2 gas mix) and at high O_2 using 95% O_2 (i.e., 95% O_2 , 5% CO_2 gas mix). All gases contained 5% CO_2 , essential for maintaining pH in bicarbonate buffered systems [150]. Due to O_2 back-diffusion from the atmosphere, solution O_2 tensions do not reflect perfused gas tensions. Previously in our laboratory the O_2 tension of the gassed buffer at 0% O_2 had been measured with an O_2 electrode and was found to equate to $1.0 \pm 0.3\%$ (approximate tissue pO_2 of ~ 8 mmHg) [144]. At 95% O_2 the measured O_2 tension equated to an approximate tissue pO_2 of 700 mmHg. Throughout this chapter all experiments performed with 0% O_2 are termed $\sim 1\%$ (representing the measured O_2 percentage in the tissue bath and experienced by the tissue) and 95% O_2 are termed 95%. All tissue equilibration was performed at 95% O_2 . For experiments at $\sim 1\%$ vessels were subjected to 10 minutes at 0% O_2 prior to contraction with PE.

In organ chamber bioassays the standard protocol for assessment of vasoactivity involves a pre-constriction of blood vessels to a preset initial tone. As O_2 affects the constrictor tone, different doses of PE had to be used to elicit the same gram tension (i.e., more PE at lower O_2 ; as demonstrated in this chapter). Therefore a 1×10^{-6} mol/L final PE concentration was used in all experiments performed at 95% O_2 (representing $\sim 70\%$ maximal tension) and a 3×10^{-6} mol/L final PE concentration in all experiments performed at $\sim 1\%$ O_2 . Upon reaching a constriction plateau (i.e., maximal tension) blood, nitrite or both were added to the rings (see individual experiments). Relaxations (and additional constrictions) were calculated as the change in tension relative to sub-maximal constriction (as a percentage). In some experiments ODQ (1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one; 10^{-5} mol/L; Alexis) was added to baths (30 minutes prior to PE) to inhibit soluble guanylate cyclase (sGC) mediated relaxation of smooth muscle.

5.3 Statistical Analysis

All data are presented as mean \pm SEM or as original traces. A two tailed p value of less than 0.05 was considered statistically significant. Differences between group means were compared by unpaired student *t* test, whilst differences between means within each group were compared by paired student *t* test. A one way ANOVA (Bonferrini post hoc test) was used for comparisons featuring more than two groups.

5.4 **Results**

5.4.1 Oxygen Dependent Responsiveness of Aortic Rings

To demonstrate the O₂ dependent responsiveness of aortic rings, the same final concentration of PE (1 x 10⁻⁶ M final) was added to rings at 95% and ~ 1% O₂. The maximal tension observed was significantly higher in the rings maintained at 95% (Figures 5.0 and 5.1). It was found that the final concentration of PE required to elicit the same gram tension observed at 95% with vessels maintained at ~ 1% was 3 x 10⁻⁶M PE (Figures 5.0 and 5.1). Therefore in the following experiments the lower concentration of PE was used in all experiments performed at 95% O₂ and the higher PE concentration at experiments performed at ~ 1% to invoke similar vessel gram tension at the different oxygen tensions.

5.4.2 Native Human Red Blood Cell Responses

Venous red blood cells added to PE constricted endothelium intact rings perfused at 95% O₂ elicited a marked vasoconstriction, resulting from scavenging of endothelial derived NO by haemoglobin. However, at ~ 1% a prompt and transient relaxation response was observed, followed by vasoconstriction (Figures 5.2 and 5.3). In experiments performed with denuded vessels (to remove the influence of the vascular endothelium) the vasoconstrictor response at 95% was abolished due to the removal of vessel NO production. However, the vasodilator response was not significantly different to that observed in endothelium intact vessels, highlighting the endothelium independence of the relaxation response (data not shown).

To inhibit soluble guanylate cyclase mediated relaxation of smooth muscle, ODQ was added to two baths in each of these native red blood cell experiments. The addition of ODQ had no effect on vessel constriction, however the relaxation response from venous red blood cells at ~ 1% was abolished demonstrating sGC involvement in the red blood cell relaxation response (Figure 5.3).

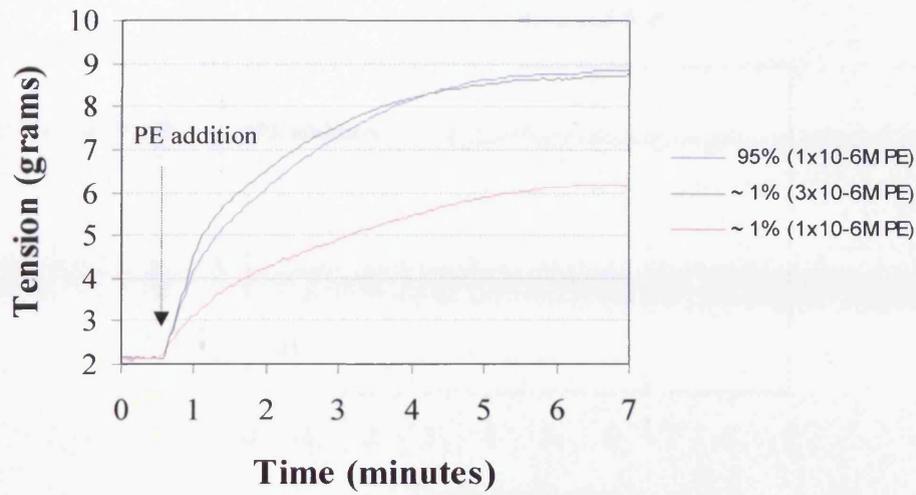


Figure 5.0: Raw aortic ring traces demonstrating the different maximal tension observed at 95% and ~ 1% oxygen using (1×10^{-6} M final) PE concentration. The same gram tension observed at 95% was achieved following the addition of (3×10^{-6} M final) at ~ 1% oxygen.

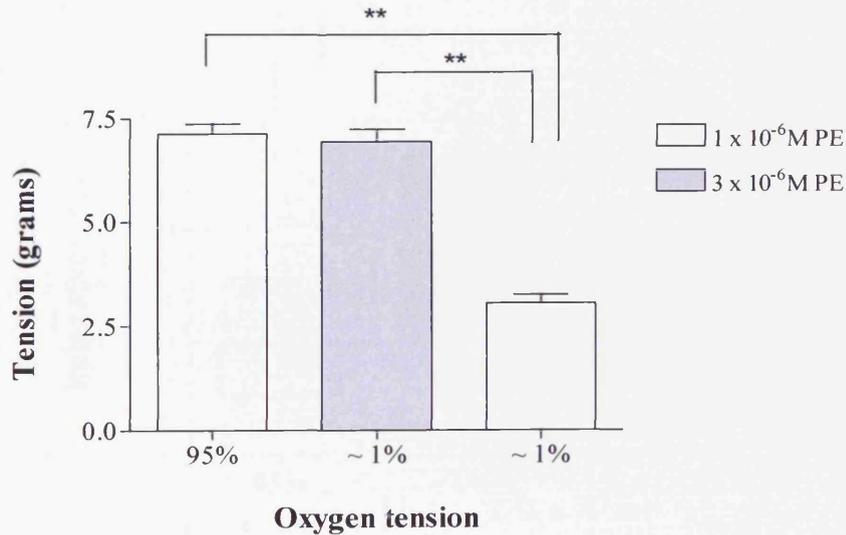


Figure 5.1: Maximal phenylephrine induced tension in endothelium intact aortic rings perfused at 95% oxygen (PE = 1×10^{-6} M final) and ~ 1% oxygen (PE = 1×10^{-6} M and 3×10^{-6} M PE final). Values presented as mean \pm SEM (n = 5). ** $p < 0.01$; One way ANOVA with Bonferroni post hoc test.

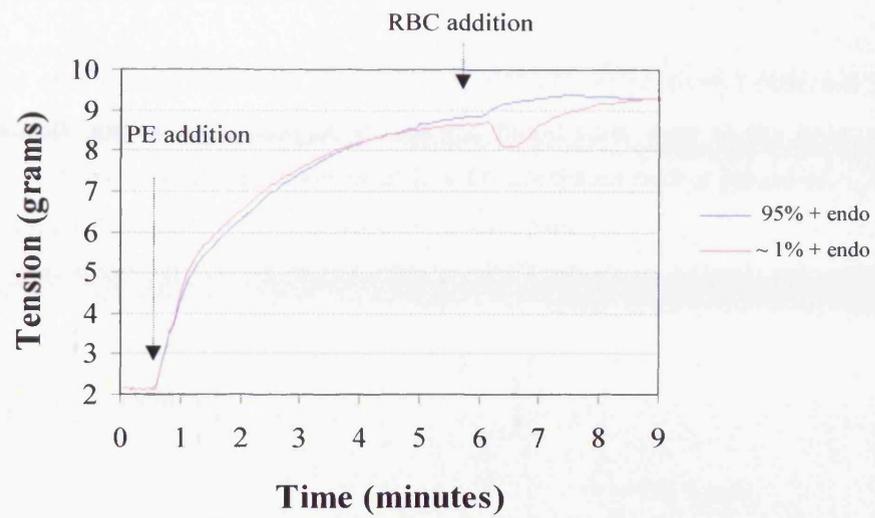


Figure 5.2: Raw aortic ring traces following the addition of PE and venous red blood cells to aortic rings perfused at 95 and ~ 1% oxygen with intact endothelium.

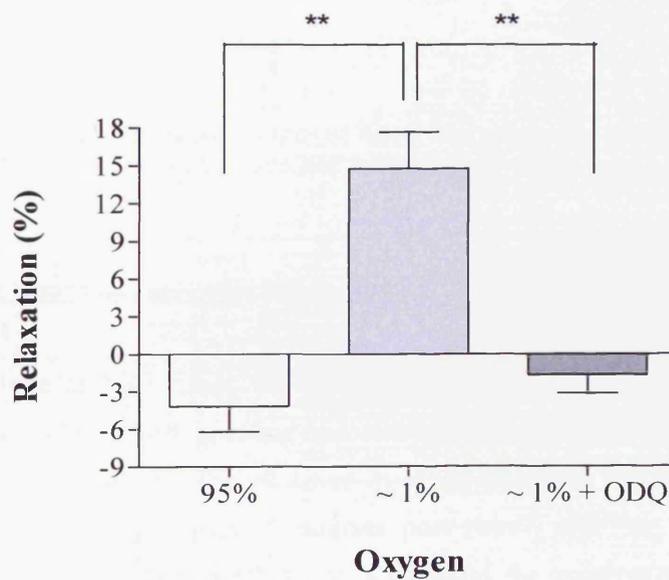


Figure 5.3: Venous red blood cell response in endothelium intact aortic rings perfused at 95% and ~ 1% oxygen and at ~ 1% oxygen in the presence of ODQ. Values presented as mean \pm SEM (n = 5) ** p < 0.01; One way ANOVA (Bonferroni post hoc test).

5.4.3 Immediacy of Red Blood Cell Response

Relaxation and constriction responses (at ~ 1% and 95% O₂ respectively) observed in endothelium intact vessels upon the addition of venous red blood cells were in the order of seconds (Figure 5.4). In total, relaxation responses at low O₂ continued over a period of ~ 20 seconds until the vasodilatory capacity of the red blood cells was spent.

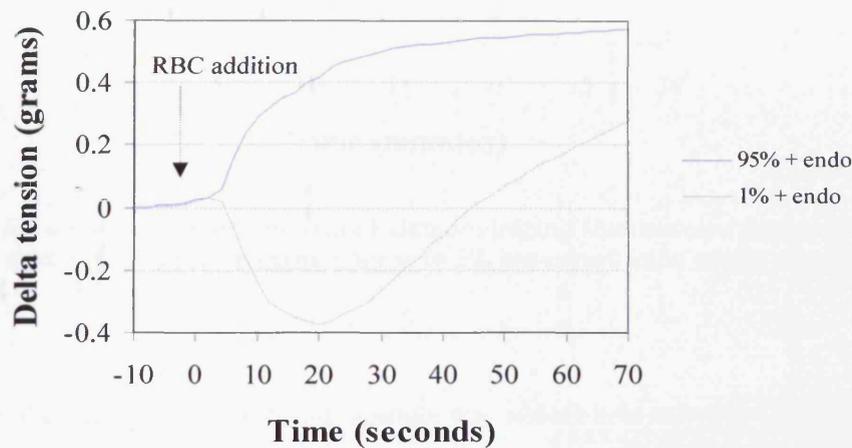


Figure 5.4: Raw aortic ring traces demonstrating the response of endothelium intact aortic rings at 95 and ~ 1% oxygen to venous red blood cells.

5.4.4 Slow and Long Lasting Nitrite Relaxations

Nitrite alone at high doses relaxed PE pre-constricted aortic rings. However, this response was slow and long lasting, acting over tens of minutes (Figure 5.5). With no comparable immediate relaxation response to that observed from red blood cell samples, the measurement of nitrite induced relaxation was taken 5 minutes post nitrite addition. In those experiments performed with red blood cells in combination with nitrite, the transient red blood cell relaxation was measured along with the vessel response after 5 minutes.

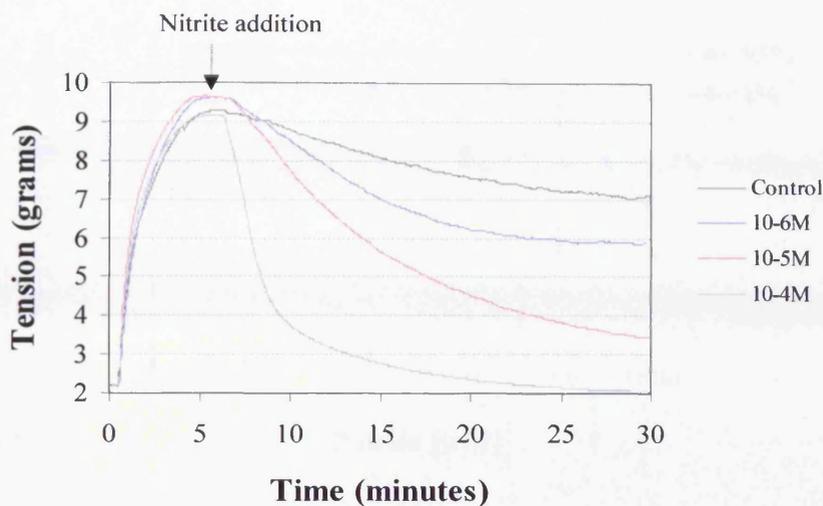


Figure 5.5: Raw aortic ring tension traces demonstrating the relaxant properties of nitrite ($1\mu\text{M}$; $10\mu\text{M}$ and $100\mu\text{M}$ final concentrations) in PE pre-constricted aortic rings without endothelium at $\sim 1\%$ oxygen.

Internal control vessels (to which nothing was added) held at $\sim 1\%$ O_2 over an extended period demonstrated a relaxation response following PE constriction (Figures 5.5) which was not observed in vessels maintained at 95% O_2 (data not shown). Controls were therefore run with each nitrite experiment at $\sim 1\%$ to account for the oxygen dependent loss in vessel tension

5.4.5 Influence of Oxygen on Nitrite Relaxation

Nitrite relaxation was enhanced at low O_2 tension in both endothelium denuded (Figure 5.6) and endothelium intact vessels (data not shown). This follows the same trend observed for a number of NO donors which have been demonstrated to show enhanced responsiveness in vessels maintained at low oxygen tensions [144, 151].

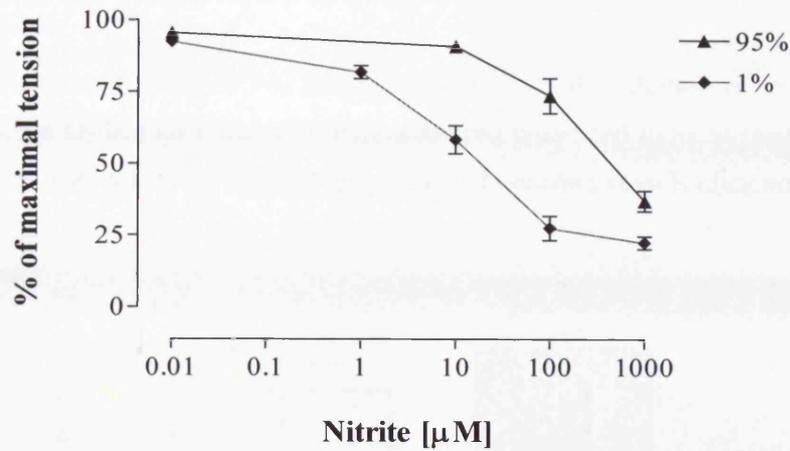


Figure 5.6: Percentage of maximal tension following the addition of different nitrite doses in endothelium denuded aortic rings at 95% and ~ 1% oxygen. Values presented as mean \pm SEM ($n = 1-7$ for each concentration).

5.4.6 Inhibition of Nitrite Relaxation by Endothelium

Nitrite induced relaxation at ~ 1% O₂ was greater in endothelium denuded than in intact rings (Figure 5.7), implying that the endothelium provides a barrier to nitrite induced relaxation.

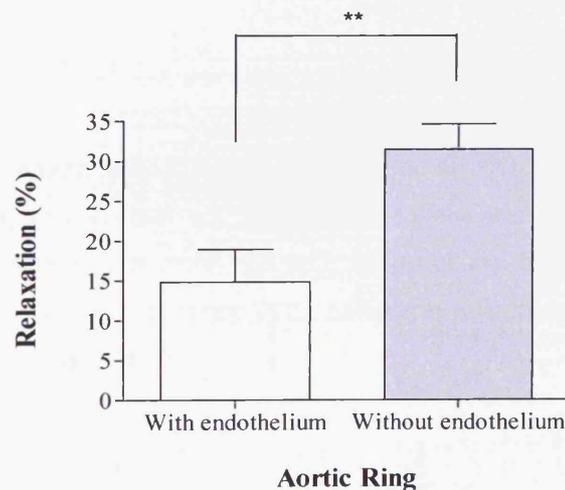


Figure 5.7: Percentage relaxation of 10^{-5} M nitrite in endothelium intact and denuded aortic rings at ~ 1% oxygen. Values presented as mean \pm SEM ($n = 7$ with endothelium and 20 without endothelium). ** $p < 0.01$; Student's paired t test.

5.4.7 Effect of Nitrite in Combination with Red Blood Cells

The addition of nitrite (10^{-5}M) immediately prior to the addition of red blood cells to aortic rings at $\sim 1\%$ O_2 had no effect on the transient red blood cell induced relaxation response. This was the same in endothelium intact (Figure 5.8) and denuded vessels (data not shown).

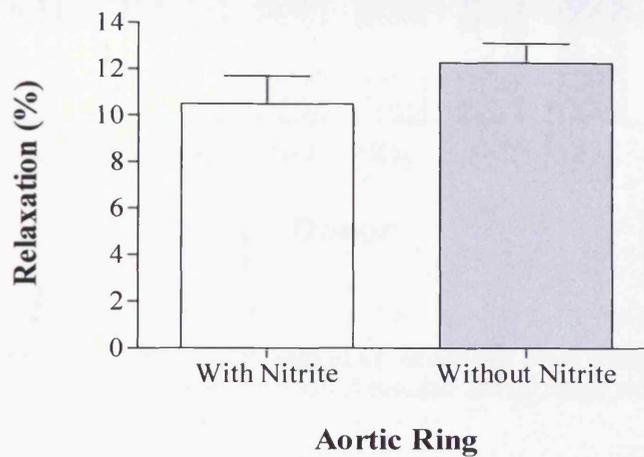


Figure 5.8: Percentage red blood cell relaxation response in the presence or absence of added exogenous nitrite in endothelium intact aortic rings at $\sim 1\%$ oxygen. Values presented as mean \pm SEM ($n = 4$).

5.4.8 Effect of Red Blood Cell Pre-incubation with Nitrite and NO

Incubation of venous whole blood with nitrite or NO resulted in a dose dependent increase in relaxation response observed. At each dose there was no difference in the relaxation observed between red blood cells incubated with nitrite or red blood cells incubated with NO (Figure 5.9). Also there was no difference in the long term relaxation response as measured after five minutes (data not shown).

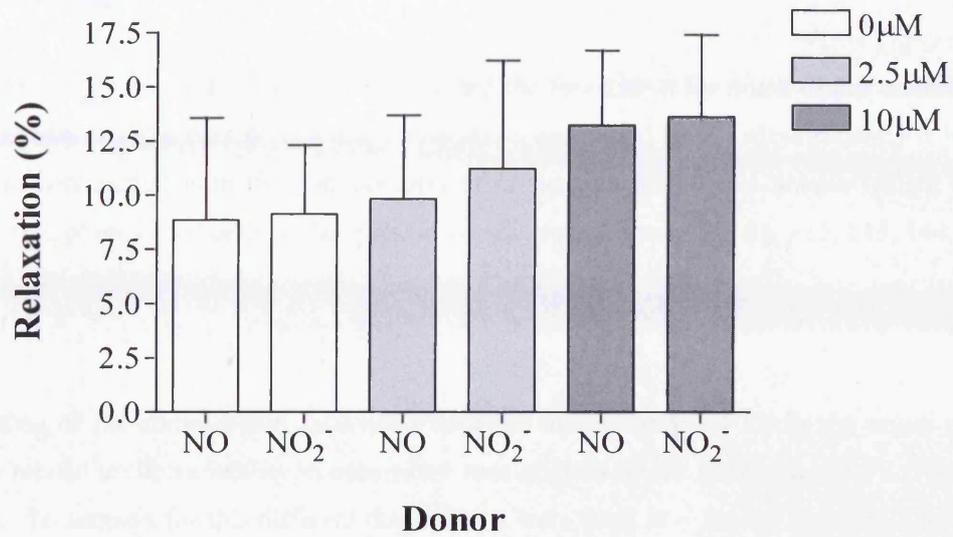


Figure 5.9: Percentage red blood cell relaxation response from whole blood samples incubated with NO or nitrite in endothelium denuded aortic rings at ~ 1% oxygen. Values presented as mean \pm SEM ($n = 3$).

5.5 Discussion

The organ chamber bioassay has provided the foundation for much of our understanding of the mechanisms that control vascular homeostasis and blood flow. More recently it has been utilised in conjunction with the manipulation of O₂ tension to allow a unique insight into the potential role of red blood cells in the hypoxic vasodilator response [70, 83, 115, 143, 144, 150].

5.5.1 Effect of Oxygen on Vascular Tone

One of the complexities associated with the manipulation of O₂ in the organ chamber bioassay related to the variability in constrictor tone elicited by the same dose of PE (Figures 5.0 and 5.1). To account for this different doses of PE were used at ~ 1% O₂ and 95% O₂ to obtain the same gram tension. This ensured that all relaxants worked against the same constrictor force and within the same tension range of the vessel. Although the percentage change in tension relative to sub-maximal constriction can be used to compare responses from vessels with different maximal constrictions, these calculations assume a linear responsiveness of the vessel across its whole tension range, which has been questioned by some [130].

Rabbit aorta maintained at low oxygen tension (~ 1% O₂) for extended periods demonstrated a loss of PE induced constriction. This O₂ dependent response (not observed in vessels maintained at 95% O₂) could reflect the inhibition of cytochrome c oxidase/mitochondrial respiration and adenosine triphosphate (ATP) production, upon which vascular contraction/dilation ultimately depends [150]. Loss of constrictor tone was only observed in the nitrite experiments which required aortic tissue to be left at low O₂ for long periods (due to the slow relaxation response). For this reason the relatively short five minute time point was taken to analyse all nitrite relaxations. To account for the loss of tension, all experiments performed at low O₂ included a control (to which nothing was added) that was subsequently subtracted from all relaxation responses.

Conventionally, experiments performed to assess the effect of hypoxia on relaxation responses have involved comparisons at fixed pO₂s. More recently a novel experimental protocol has been developed to specifically address the question at what pO₂ vessels start to dilate in response to different treatments [150]. This involves the comparison of relaxation in vessels exposed to a progressively declining pO₂. In light of the variability associated with constriction

responses in vessels at maintained at set O₂ tensions it is difficult to comprehend how reliably interpretable data could be obtained from experiments in which the rate of decline in O₂ might potentially vary from chamber to chamber. More importantly the rate and extent of relaxation is not linked to chamber pO₂ but rather pO₂ in the muscle interior which could potentially vary from strip to strip as a function of muscle thickness, adventitial content and deoxygenation rate [130].

5.5.2 Red Blood Cell Mediated Regulation of Vascular Tone

In endothelium intact vessels at 95% O₂, red blood cells elicited marked vasoconstriction, representing the scavenging of endothelial produced NO. At ~ 1% O₂ representative of the pO₂ observed within the cardiovascular system (e.g., coronary venous pO₂ ~ 10-20mmHg) a prompt vasorelaxation was observed followed by a delayed vasoconstriction once the endogenous NO bioactivity had been spent. These responses demonstrated the capacity of native red blood cells to contribute to both hyperoxic vasoconstriction and hypoxic vasodilatation, as previously demonstrated by others [83].

In endothelium denuded vessels the response at ~ 1% was similar to that observed in endothelium intact vessels, establishing that the red blood cell relaxation response was endothelium independent. Furthermore the inhibition of sGC (by ODQ) totally eradicated the red blood cell relaxation response proving that the observed effect was sGC dependent. These findings which are in line with reports from other laboratories [83, 143] provide a strong case that the release of NO bioactivity from red blood cells plays some part in mediating hypoxic vasodilatation. In order to further investigate the potential role of nitrite in this vasodilator response its relaxation properties were investigated further.

5.5.3 Direct Vasoactive Properties of Nitrite at Different Oxygen Tensions

Nitrite alone was demonstrated to relax thoracic aorta in a dose dependent manner at 95% and 1% O₂. Interestingly, at near physiological nitrite concentrations (< 1µM) a relaxation response was observed under hypoxic conditions. However, this response was totally different to the relaxation observed from red blood cells. Whilst red blood cells induced a transient relaxation at low O₂ that was almost instantaneous following the addition of cells to the organ chamber, nitrite alone produced a much slower (over a period of minutes) and prolonged relaxation. Slow relaxations as observed from nitrite are incompatible with the mediation of hypoxic vasodilatation

which occurs within the capillary transit time, i.e., is a second or less at rest and a fraction of that during exercise [152].

The relaxation of nitrite alone was much greater at lower oxygen tension ($EC_{25} \sim 1\mu\text{M}$ at $\sim 1\% \text{O}_2$; $\sim 100\mu\text{M}$ at $95\% \text{O}_2$). A similar enhanced responsiveness of vessels to non oxygen dependent nitrovasodilators under hypoxic conditions has previously been demonstrated [144, 151]. This phenomenon was suggested to bring into question the allosteric based mechanism for red blood cell (and specifically SNO-Hb) mediated vasodilatation. This issue was resolved in a series of experiments performed in our laboratory which demonstrated that hypoxic hyperresponsiveness of vessels alone does not account for the potentiation of red blood cell induced relaxations observed at lower O_2 [144]. This was deduced by calculating the fold enhancement of relaxation for GSNO (2.6 ± 0.8 -fold; across a range of concentrations) and red blood cells (7.6 ± 1.7 -fold) at $\sim 1\%$ compared with $95\% \text{O}_2$. Even accounting for the enhanced vessel responsiveness (i.e., 2.6 fold) the red blood cell response was still ~ 5 fold greater at lower O_2 . In this work the nitrite relaxation response was ~ 3.6 fold greater at $\sim 1\% \text{O}_2$ compared to $95\% \text{O}_2$.

Interestingly nitrite relaxation was greater in endothelium denuded than intact rings. This suggests that the endothelium acts as a barrier to nitrite induced relaxation. It might be that elements within the endothelium react with nitrite, or that endothelial cells in some way repel nitrite, limiting its capacity to activate sGC and mediate relaxation. This lends further support (in addition to the data in chapter 4 demonstrating the accumulation of exogenously added nitrite in the plasma compartment following its addition to whole blood) to the theory that nitrite is in some way inhibited from crossing cell membranes. The fact that the nitrite relaxation response was greater in endothelium denuded vessels also highlights potential implications for individuals with damaged endothelium whereby nitrite might be able to preferentially act upon the damaged regions.

5.5.4 Does Nitrite Enhance Red Blood Cell Induced Relaxation?

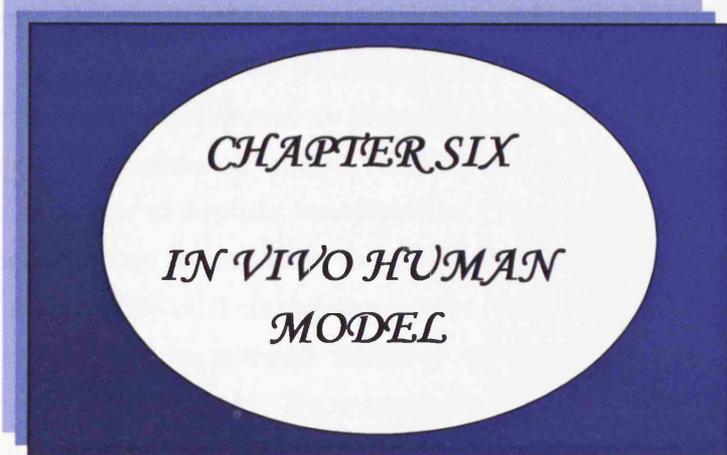
The transient relaxation response observed from red blood cells alone was no different when exogenous nitrite was added to the organ chamber immediately prior to the red cells. The fact that vasodilatory activity was not enhanced in the presence of nitrite implies that the reduction of nitrite by deoxyhaemoglobin at low O_2 results in very little (if any) free NO

formation. Whilst these observations imply that extra-cellular nitrite does not play a role in red blood cell mediated relaxation response they do not rule out the fact that intra-erythrocytic nitrite could account for the rapid burst of relaxation observed from native red blood cells.

To address this issue red blood cells were isolated from whole blood that had been incubated with NO or nitrite (following the same protocol outlined in chapter 4). Red blood cells that had been doped with NO or nitrite demonstrated increased transient vasodilator activity at ~ 1%. The increased relaxation response was dose dependent with both the NO and nitrite additions and there was no difference in the relaxation response between the NO and nitrite dosed samples. In the previous chapter it was demonstrated that bloods loaded with nitrite had a significantly less red blood cell associated nitrite compared to those incubated with NO, whilst red blood cell haemoglobin bound NO accumulated to a similar extent. As the same response was observed from both NO and nitrite loaded red blood cells this implies that nitrite was not the source of the vasorelaxant response, but it does suggest that haemoglobin-bound NO might have been.

SUMMARY

- In endothelium intact vessels red blood cells elicited marked vasoconstriction at high oxygen (95% O₂) and prompt vasorelaxation at low oxygen (~ 1% O₂). These responses demonstrate the capacity of native red blood cells to contribute to both hyperoxic vasoconstriction and hypoxic vasodilatation.
- Nitrite alone relaxed thoracic aorta in a dose dependent manner at 95% and 1% O₂, however the relaxation response was much greater at lower oxygen tension, demonstrating the enhanced responsiveness of vessels at low O₂ to nitrovasodilators. However, the relaxation response from nitrite was much slower and more prolonged than that observed from native red blood cells.
- The addition of exogenous nitrite to the organ chamber prior to the addition of red blood cells did not change the transient red blood cell relaxation response observed. This implies that the reduction of nitrite by deoxyhaemoglobin at low O₂ results in very little (if any) free NO formation.
- In the previous chapter it was demonstrated that bloods loaded with nitrite had a significantly less red blood cell associated nitrite compared to those incubated with NO, whilst red blood cell haemoglobin bound NO accumulated to a similar extent. As the same response was observed from both NO and nitrite loaded red blood cells this implies that nitrite was not the source of the vasorelaxant response, but it does suggest that haemoglobin-bound NO might have been.



CHAPTER SIX
IN VIVO HUMAN
MODEL

6.0 Introduction

The high energy consumption of the myocardium is reflected in its oxygen extraction. Even at rest ~ 75% of arterially delivered oxygen is extracted by the heart compared to ~ 30-40% by skeletal muscle. As a consequence of the limited extraction reserve greater myocardial oxygen demand (during exercise or rapid atrial pacing) has to principally be met by an increase in coronary blood flow. This is thought to be mediated primarily by local metabolic vasodilators. Several potential candidates have been analysed including adenosine, K^+_{ATP} channels, lactate, phosphate, potassium, prostaglandins and endothelial NO, none of which has been found to adequately account for the regulatory response [140]. However, the potential role of NO metabolites has not previously been addressed.

The theory of a blood derived NO reserve of circulating metabolites conserving bioactivity has been subject to much interest. At present it is generally agreed that the NO reserve can function in an endocrine manner to regulate vascular tone under conditions of low oxygen, acting as a potential mediator of hypoxic vasodilatation. The NO species involved has been subject to intense debate. Two models have developed each emphasizing the significance of different metabolites; the ability of *S*-nitrosohaemoglobin (SNO-Hb) within red blood cells to provide bio-available NO and the potential formation of NO from nitrite mediated by the reductive potential of deoxyhaemoglobin. These metabolite species are proposed to be under the regulatory control of haemoglobin, which acts as the oxygen dependent sensor eliciting the vasodilator response.

The heart was identified as an ideal model system in which to assess NO metabolites. Given the large oxygen gradient it was possible even at rest to obtain blood samples from different sites within the coronary circulation at the extremes of HbO₂ sat (%). Furthermore, it was possible to manipulate myocardial oxygen demand via rapid atrial pacing whilst simultaneously obtaining blood samples.

6.1 Aims

The main aims of this work were to:

- Identify changes in the NO metabolite pool across the coronary circulation at rest and under conditions of increased oxygen demand

- Assess whether oxygen drives apportion between NO metabolites *in vivo* and induces a shift in this balance across a single vascular bed.
- Determine the function of endothelial NOS and the NO metabolite pool in the regulation of vascular tone at rest and under conditions of increased oxygen demand.

6.2 Subjects

Seven otherwise healthy subjects (3 men and 4 women, mean age 49 ± 15 years) undergoing electrophysiology (EP) testing for paroxysmal supraventricular arrhythmias were studied. All subjects were in sinus rhythm with no past history of ischemic heart disease, smoking, diabetes mellitus, hypertension (blood pressure $> 140/90$ mmHg) or hypercholesterolemia (total cholesterol > 5.2 mmol/L). All gave fully informed written consent approved by the relevant local research ethics committee.

6.3 Protocol

Prior to catheterisation subjects underwent an 8 hour fast during which time they were allowed water. Diagnostic left heart catheterisation was performed via per cutaneous right femoral approach. A left heart catheter was positioned in the left main coronary artery and two further catheters were introduced via the right femoral vein and positioned in the coronary sinus and pulmonary artery. A bipolar pacing wire was also placed into the right atrium via the right femoral vein. The position of the sampling catheters was confirmed with contrast injection.

At the outset of the protocol aminophylline (Phoenix Pharma Ltd) was infused to block adenosine receptors. A loading dose of 5mg/kg was administered via the antecubital vein over a period of 20 minutes, followed by a maintenance dose of 500 μ g/kg/hour. Once baseline haemodynamic parameters had been maintained for 5 minutes, peripheral blood pressure was measured and blood samples (10ml syringe) were obtained from the left main coronary artery (LMCA), coronary sinus (CS) and pulmonary artery (PA). In terms of analysis LMCA-CS difference was taken to reflect “cross heart” and PA-LMCA “cross lung”. Strictly “cross lung” should be measured as PA to pulmonary vein (PV) however it is practically difficult to obtain PV samples from normal human subjects.

Blood samples were transferred after blood gas analysis (OSM3 Hemoximeter, Radiometer) into 6ml gas tight EDTA vacutainers. These were centrifuged at 600g for 10 minutes at room temperature. The red cell fraction and plasma were immediately separated, snap

frozen in liquid nitrogen and stored at -80°C for subsequent analysis. Following baseline sampling rapid atrial pacing at 65% and then 85% of maximal heart rate (calculated as 220 minus the subject's age) was commenced. During each pacing interval blood was sampled (for blood gas and metabolite analysis) and an angiogram was taken. After discontinuation of pacing and confirmation that blood pressure had returned to levels observed before pacing, L-NMMA (Clinalfa) was infused intravenously to block systemic synthesis of NO from type III NOS. A loading dose of 5mg/kg was administered over seven minutes followed by a maintenance dose of $50\mu\text{g}/\text{kg}/\text{min}$. Blood pressure was measured for comparison without L-NMMA and the whole protocol was repeated with the continued infusion of L-NMMA (Figure 6.0).

LMCA vessel luminal diameter was later determined at baseline and each pacing interval in the absence and presence of L-NMMA by quantitative analysis of the angiograms using an automated edge detection computer analysis system (Philips QCA software).

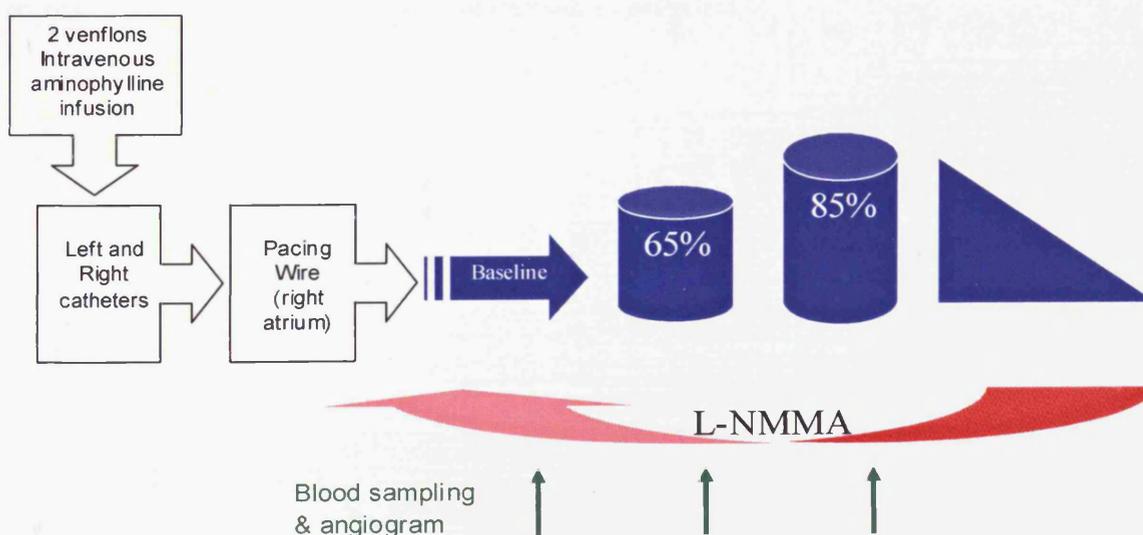


Figure 6.0. Schematic of the pacing protocol. Green arrows demonstrate timing of blood sampling and angiograms. These were performed without and then with L-NMMA at baseline and then during rapid atrial pacing at 65% and 85% of maximum heart rate.

6.4 Determination of NO Metabolites

Plasma NO_x was measured by fluorimetry as detailed in chapter 2; section 2.1.2. Plasma nitrite and plasma protein-bound NO metabolite species were measured by ozone based chemiluminescence using the tri-iodide reagent as detailed in chapter 2; section 2.1.4. Red blood cell haemoglobin-bound NO and red blood cell associated nitrite were quantified by ozone based chemiluminescence using our modified tri-iodide reagent as detailed in chapter 2; section 2.1.5.

6.5 Statistical Analysis

Data are presented as individual data points (with mean) or mean values \pm SEM. A two-tailed p value of less than 0.05 was considered statistically significant. Differences between means (arterial/venous; baseline/L-NMMA) were compared by paired student *t* test. A repeat measures one way ANOVA was used to compare differences between pacing increments. Bivariate correlations were performed using Pearson's coefficient.

6.6 Results

6.6.1 Baseline

6.6.1.1 Blood Oxygenation

Cross heart and cross lung baseline HbO₂ sat (%) and blood oxygen content (ml/dl) are illustrated in figure 6.1. The cross heart oxygen gradient reflects the large oxygen extraction across the coronary circulation. Upon the mixing of CS blood with blood from the systemic circulation (to give rise to PA blood) HbO₂ sat (%) and oxygen content increase to reflect near systemic levels, whilst across the lung blood is once more fully oxygenated (> 95% HbO₂ sat).

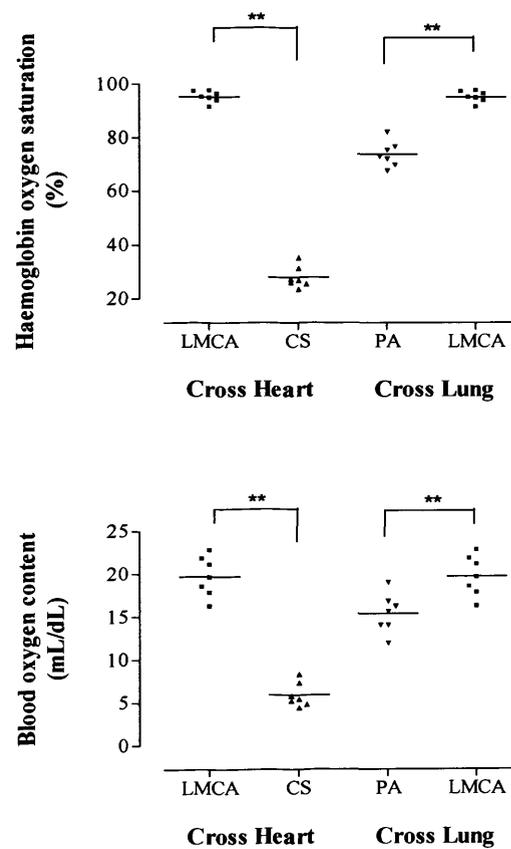


Figure 6.1: Baseline haemoglobin oxygen saturation and oxygen content across the heart and lungs. Individual data points presented with mean. ** $p < 0.01$: Student's paired t test.

6.6.1.2 NO Metabolites

Across the heart there was no significant net loss or gain of NO from the total metabolite pool ($p = 0.2381$) (i.e., plasma nitrate, plasma nitrite, plasma protein-bound NO, red blood cell haemoglobin-bound NO and red blood cell associated nitrite). However, a significant loss was observed across the lungs ($p = 0.0145$) mainly reflecting a decrease in plasma nitrate ($p = 0.0142$). Excluding plasma nitrate, combined red blood cell and plasma NO remained unchanged across both the coronary ($p = 0.6973$) and pulmonary ($p = 0.4054$) circuits (Figure 6.2).

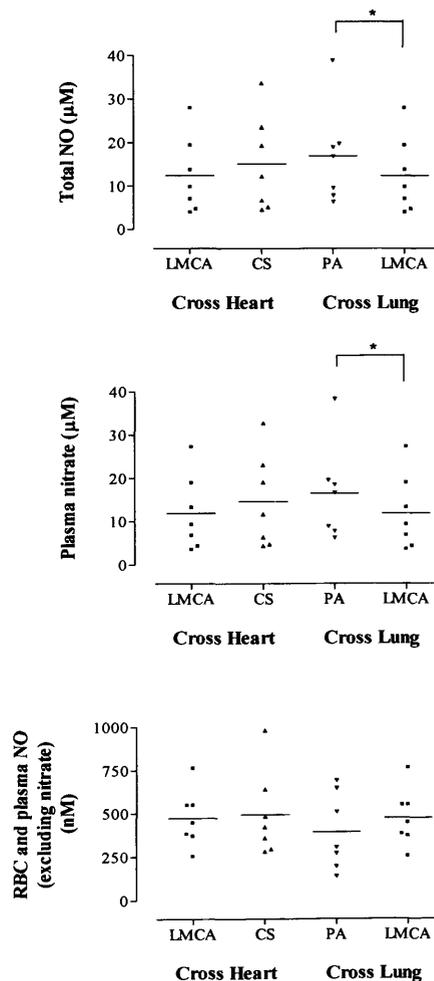


Figure 6.2: Total metabolite pool, plasma nitrate and combined red blood cell and plasma NO (excluding nitrate) at baseline. Individual data points presented with mean. * $p < 0.05$; Student's paired t test.

Red blood cell NO (haemoglobin-bound NO and red blood cell associated nitrite) increased across the heart ($p = 0.0359$) mainly representing a gain in haemoglobin-bound NO ($p = 0.0313$). Mirroring this change was a trend towards a decrease in red blood cell associated nitrite ($p = 0.0938$) and plasma NO ($p = 0.0011$) largely reflecting the potential loss of plasma nitrite ($p = 0.1250$). The gain in total red blood cell NO and haemoglobin-bound NO across the heart was partially reversed on mixing of systemic venous return with CS blood (to give rise to PA blood). Across the lung the effects observed across the heart were completely reversed, with an increase in plasma NO ($p = 0.0748$), plasma nitrite ($p = 0.0235$) and red blood cell associated nitrite ($p = 0.0260$) and a decrease in haemoglobin-bound NO ($p = 0.0241$) (Figure 6.3).

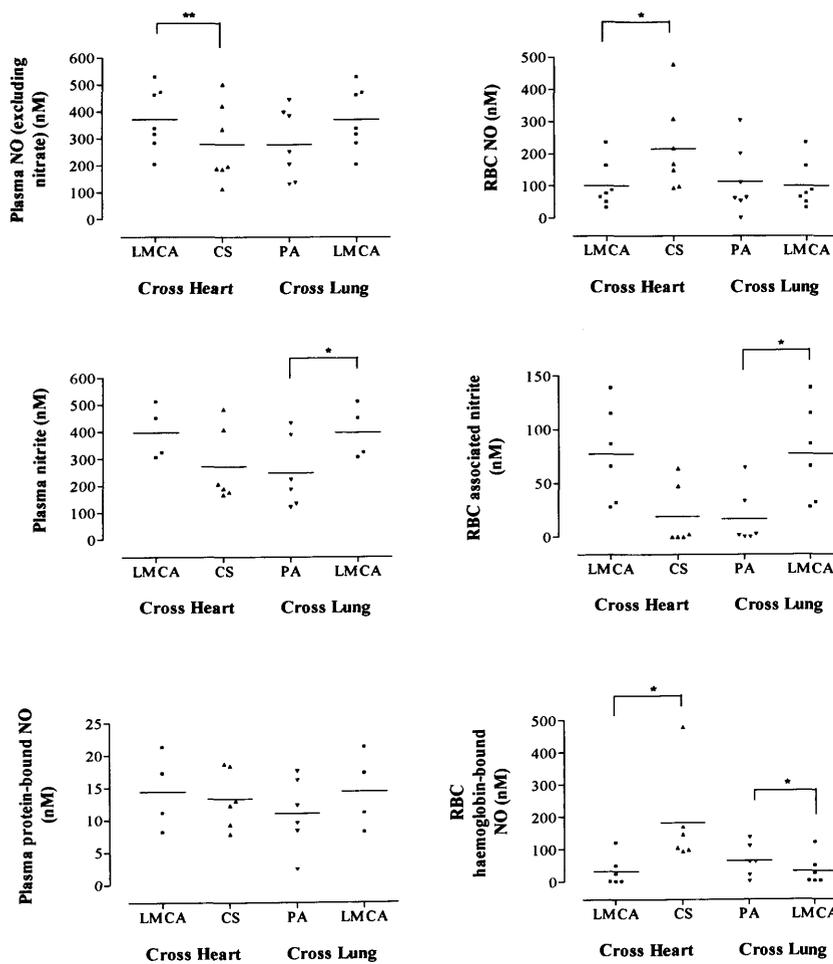


Figure 6.3: Baseline red blood cell and plasma NO metabolites. Individual data points presented with mean. * $p < 0.05$; ** $p < 0.01$; Student's paired t test.

6.6.1.3 Correlation of NO Metabolites with Oxygen

At baseline a trend was observed towards a direct correlation between red blood cell associated nitrite and HbO₂ sat (%) and an inverse correlation between haemoglobin-bound NO and HbO₂ sat (%) across all sites sampled, suggestive of potential interchange between these species as a function of oxygen.

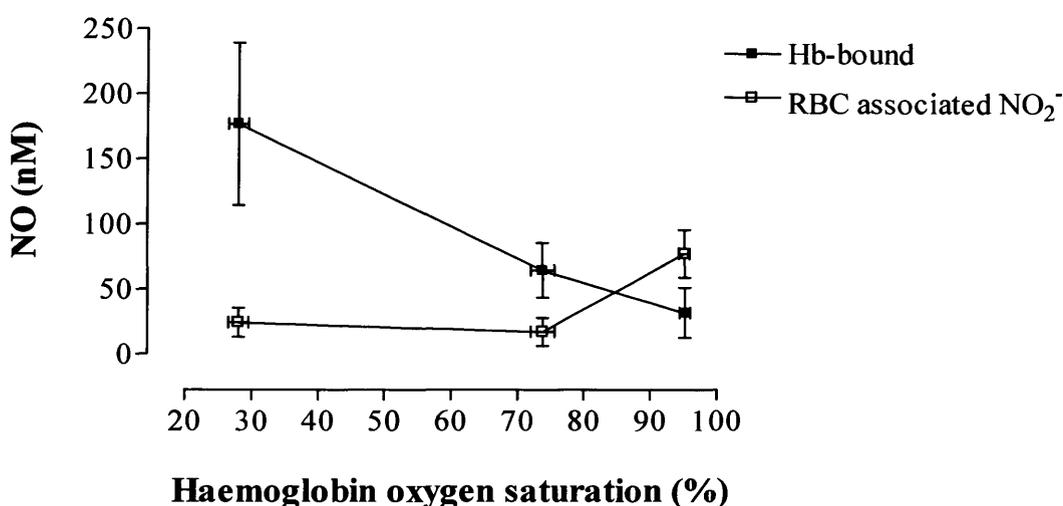


Figure 6.4: Relationship between haemoglobin-bound NO, red blood cell associated nitrite and haemoglobin oxygen saturation (%). Values presented as mean \pm SEM. Correlation $r = -0.6138$ ($p = 0.0067$) for haemoglobin-bound NO; $r = 0.4243$ ($p = 0.0792$) for red blood cell associated nitrite.

6.6.1.4 Baseline with L-NMMA

The administration of L-NMMA systemically did not change the cross heart HbO₂ sat (%) oxygen gradient (67.4 ± 1.2 to 66.7 ± 1.8 %). However PA HbO₂ sat (%) decreased significantly ($p = 0.0329$; 74.2 ± 1.6 to 70.4 ± 1.8 %) reflecting increased systemic oxygen extraction. Coronary artery diameter also decreased significantly whilst systolic, diastolic and mean arterial blood pressure increased. The decrease in coronary artery diameter in combination with the increase in blood pressure confirms NOS inhibition (Figure 6.5 and Table 6.0).

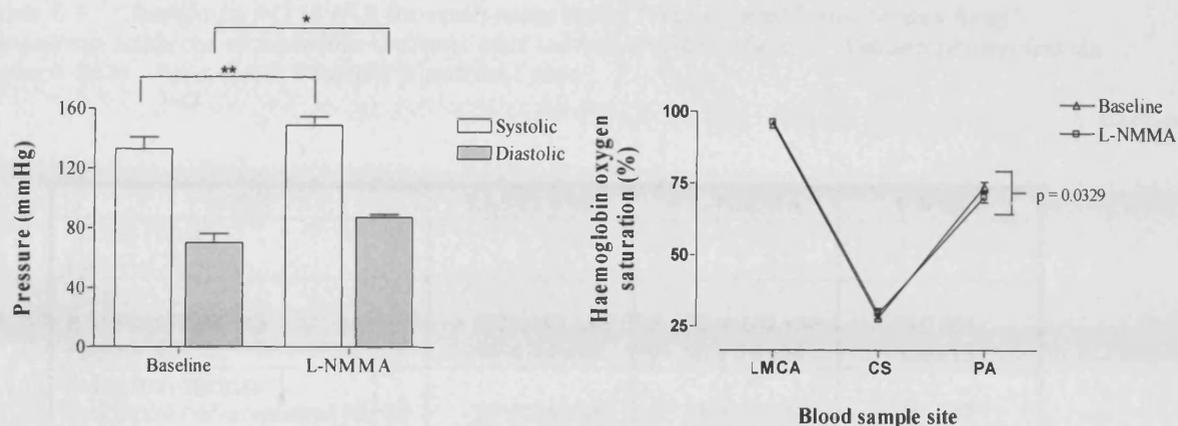


Figure 6.5: Systolic and diastolic blood pressure and haemoglobin oxygen saturation at baseline and following systemic NOS inhibition. Infusion of L-NMMA increased systolic and diastolic blood pressure (by ~ 11 and 20% respectively) and oxygen extraction from the blood in the systemic circulation by ~5%. Data presented as mean \pm SEM. * $p < 0.05$; ** $p < 0.01$; Student's paired t test.

Table 6.0: Physiological data for subjects comparing baseline to L-NMMA infusion. Data presented as mean \pm SEM. * $p < 0.05$; ** $p < 0.01$; Student's paired t test.

	BASELINE	+ L-NMMA	P Value
Mean Arterial Pressure (mmHg)	91 \pm 6	108 \pm 3	0.0054 **
Coronary Artery Diameter (mm)			
Proximal	3.30 \pm 0.35	3.14 \pm 0.31	0.0655
Mid	2.52 \pm 0.38	2.40 \pm 0.37	0.0297 *
Distal	2.08 \pm 0.40	1.99 \pm 0.35	0.3308

The apportionment of NO between metabolite species in samples taken during L-NMMA infusion was similar to that observed at baseline, with “cross heart” and “cross lung” trends essentially unaffected. The only significant difference was a smaller loss of plasma nitrite across the heart following L-NMMA infusion (Table 6.1).

Table 6.1: Change in NO (Δ NO) for each metabolite “cross heart” and “cross lung” comparing subjects at baseline without and with L-NMMA infusion. Values presented as mean \pm SEM. * $p < 0.05$; Student’s paired t test.

Δ NO	BASELINE	+ L-NMMA	P Value
“Cross Heart” LMCA-CS			
Totals			
Red blood cell NO	+ 115 \pm 43 nM	+ 172 \pm 108 nM	0.7104
Plasma NO	- 96 \pm 14 nM	- 50 \pm 19 nM	0.0813
Individual Species			
Red blood cell associated nitrite	- 59 \pm 24 nM	+ 28 \pm 82 nM	0.5887
Red blood cell Hb-bound NO	+ 150 \pm 40 nM	+ 144 \pm 75 nM	0.1807
Plasma nitrate	+ 2.4 \pm 1.8 μ M	+ 2.9 \pm 0.6 μ M	0.9335
Plasma nitrite	- 78 \pm 25 nM	- 48 \pm 27 nM	0.0458 *
Plasma protein-bound NO	- 1.1 \pm 2.4 nM	- 0.8 \pm 1.0 nM	0.7302
“Cross Lung” PA-LMCA			
Totals			
Red blood cell NO	- 12 \pm 44 nM	+ 54 \pm 45 nM	0.6200
Plasma NO	+ 93 \pm 44 nM	+ 55 \pm 48 nM	0.3823
Individual Species			
Red blood cell associated nitrite	+ 61 \pm 15 nM	+ 58 \pm 43 nM	0.9075
Red blood cell Hb-bound NO	- 33 \pm 9 nM	- 3.4 \pm 14 nM	0.1391
Plasma nitrate	- 4.0 \pm 1.4 μ M	- 3.7 \pm 1.1 μ M	0.6833
Plasma nitrite	+ 102 \pm 18 nM	+ 107 \pm 56 nM	0.9589
Plasma protein-bound NO	+ 4.6 \pm 3.4 nM	+ 0.7 \pm 2.1 nM	0.6149

6.6.2 Pacing

6.6.2.1 Blood Oxygenation

Pacing the heart had no effect on LMCA or PA HbO₂ sat (%). However CS HbO₂ sat (%) increased significantly from baseline (Figure 6.6). This was reflected in a trend towards a decline in the cross heart HbO₂ sat (%) gradient (Figure 6.7).

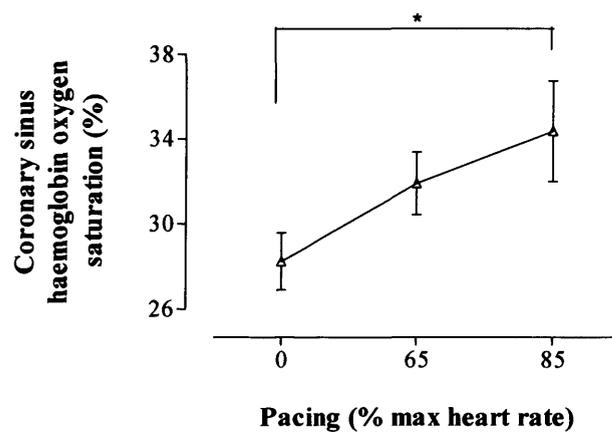


Figure 6.6: Coronary sinus haemoglobin oxygen saturation with pacing. Values presented as mean \pm SEM. * $p < 0.05$; One way repeat measures ANOVA.

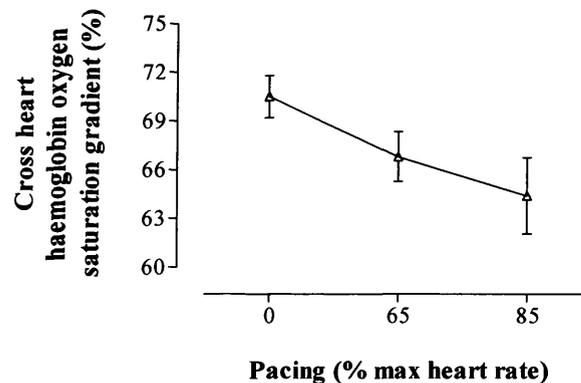


Figure 6.7: Cross heart haemoglobin oxygen saturation gradient with pacing. Values presented as mean \pm SEM.

6.6.2.2 Blood Vessel Diameter

Pacing the heart had no significant effect on coronary arterial diameter (proximal, mid or distal) although there were trends towards an increase in the mid and distal portions (Figure 6.8).

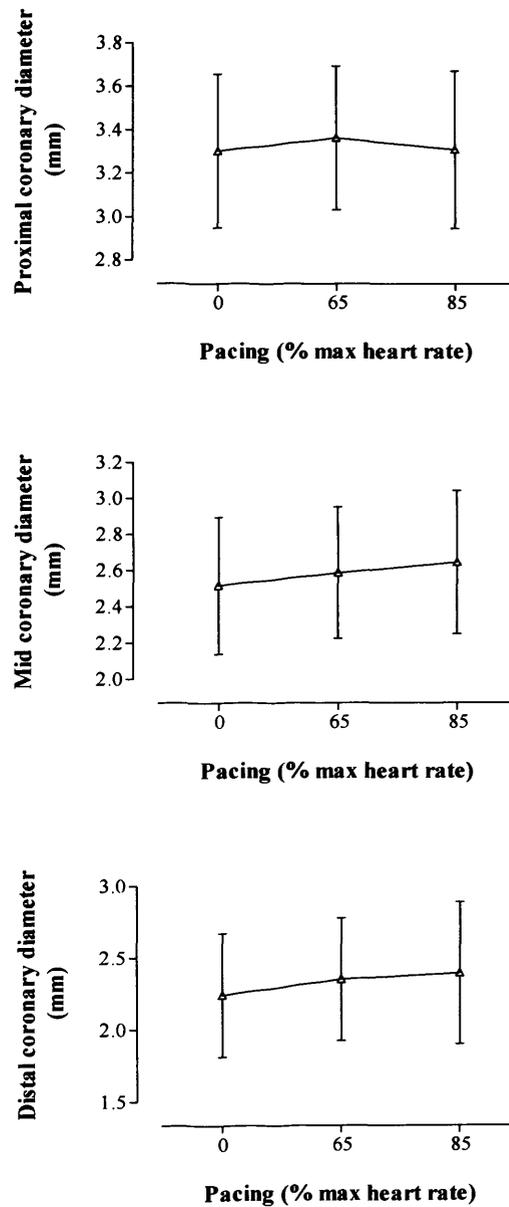


Figure 6.8: Coronary artery diameter with pacing. Values presented as mean \pm SEM.

6.6.2.3 NO Metabolites

With pacing the total NO metabolite pool remained unchanged in the CS. In the PA there was no change at 65% maximum heart rate but a significant decrease from 65% to 85%. In the LMCA the total metabolite pool increased significantly at 65% maximum heart rate and remained unchanged at 85%. These patterns of metabolite interchange mainly reflect plasma nitrate (which makes up the majority of the total NO metabolite pool). Excluding nitrate, combined red blood cell and plasma NO remained unchanged across all of the sampling sites with pacing (Figure 6.9).

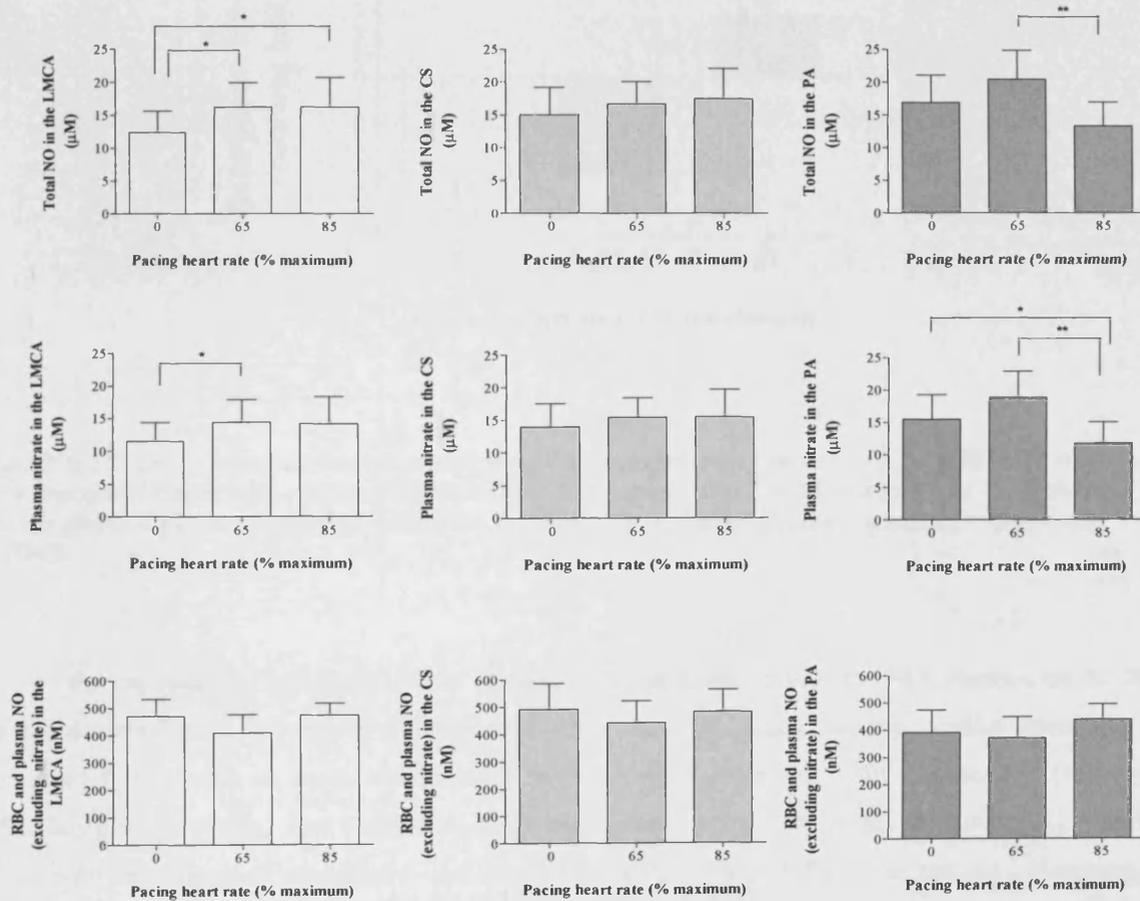


Figure 6.9: Total metabolite pool, plasma nitrate and combined red blood cell and plasma NO (excluding nitrate) with pacing. Values presented as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$; Repeat measures one way ANOVA.

Cross heart and cross lung gradients in total NO, plasma nitrate and combined red blood cell and plasma NO followed the same patterns during pacing as observed at baseline. The only difference was seen at 85% maximum heart rate where the loss of total NO and plasma nitrate observed across the lung at baseline and 65% maximum heart rate reverted to a gain at 85% maximum heart rate. This is illustrated in the delta cross lung plasma nitrate gradient with increased pacing (Figure 6.10).

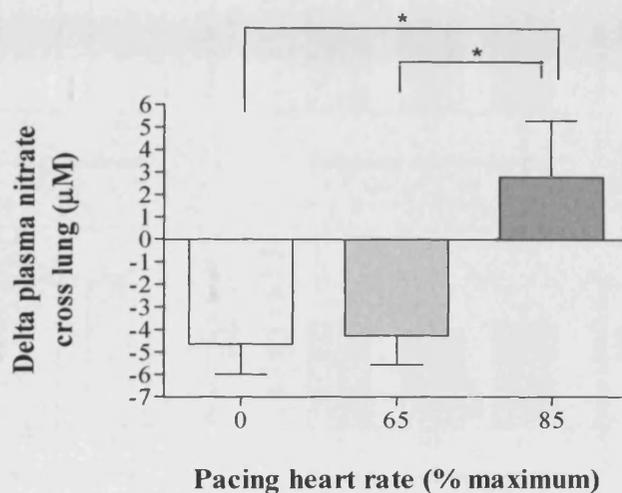


Figure 6.10: Delta plasma nitrate cross lung with increased pacing. At baseline (0) and 65% maximal heart rate plasma nitrate was lost across the lung, however, at 85% there was a gain. Values presented as mean \pm SEM. * $p < 0.05$; Repeat measures one way ANOVA.

Pacing had no significant effect on the concentrations of plasma NO, plasma nitrite or plasma protein-bound NO across the different sampling sites. Consequently, similar cross heart and cross lung trends to those observed at baseline were observed, with plasma NO (mainly reflecting plasma nitrite) lost across the heart and gained across the lungs (Figure 6.11). Red blood cell NO remained essentially unchanged in the LMCA and PA with pacing. However, there was a trend towards a decrease in red blood cell NO in the CS ($p = 0.0854$) (mainly reflecting the loss of haemoglobin-bound NO) (Figure 6.12). This change in CS haemoglobin-bound NO reflected the increase in HbO₂ sat (%) observed in the CS with pacing (Figure 6.6), which is suggestive of oxygen regulation of this metabolite species. This relationship is more clearly demonstrated in the correlation between HbO₂ sat (%) and haemoglobin-bound NO

(Figure 6.13). Similar to baseline the increase in haemoglobin-bound NO observed across the heart was matched by a loss of red blood cell nitrite and plasma NO (mainly plasma nitrite) at each pacing interval.

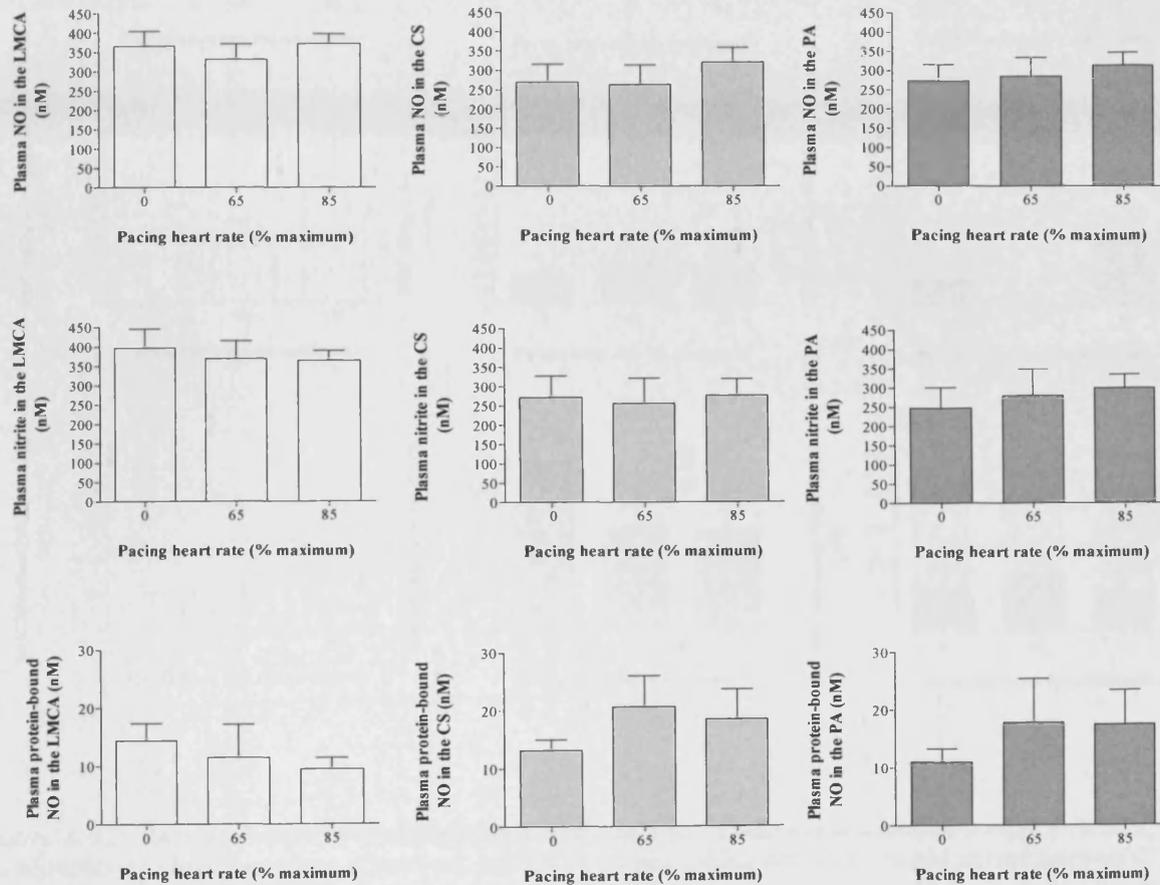


Figure 6.11: Baseline plasma NO metabolites with pacing. Data presented as mean \pm SEM. No significant changes were observed within each sampling site with rapid atrial pacing.

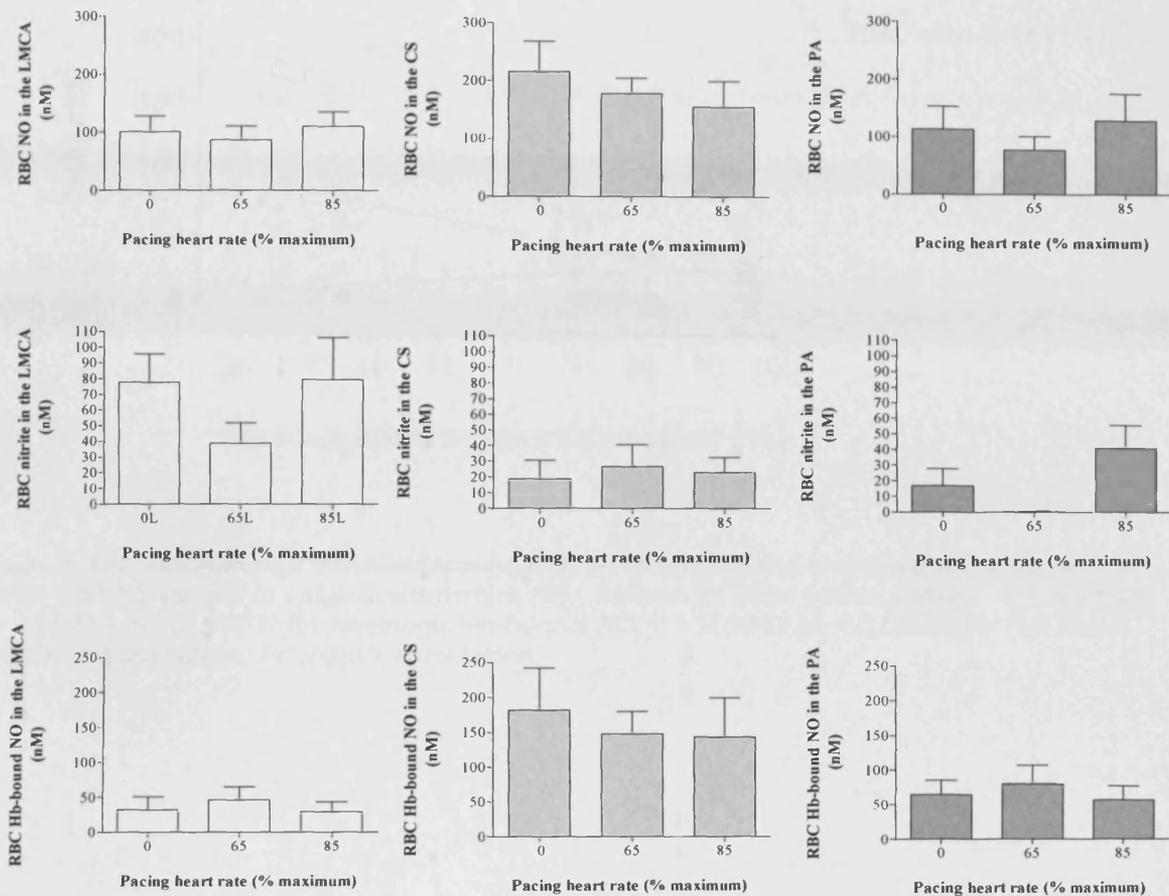


Figure 6.12: Baseline RBC NO metabolites with pacing. Data presented as mean \pm SEM. No significant changes were observed within each sampling site with rapid atrial pacing.

6.6.2.4 Correlation of NO Metabolites with Oxygen

Across all sites (including baseline and pacing samples) the data gave rise to a direct correlation between red blood cell associated nitrite and HbO_2 sat (%) ($p = 0.0288$; $r = 0.3063$) and an inverse correlation between haemoglobin-bound NO and HbO_2 sat (%) ($p < 0.0001$; $r = -0.6278$) (Figure 6.13).

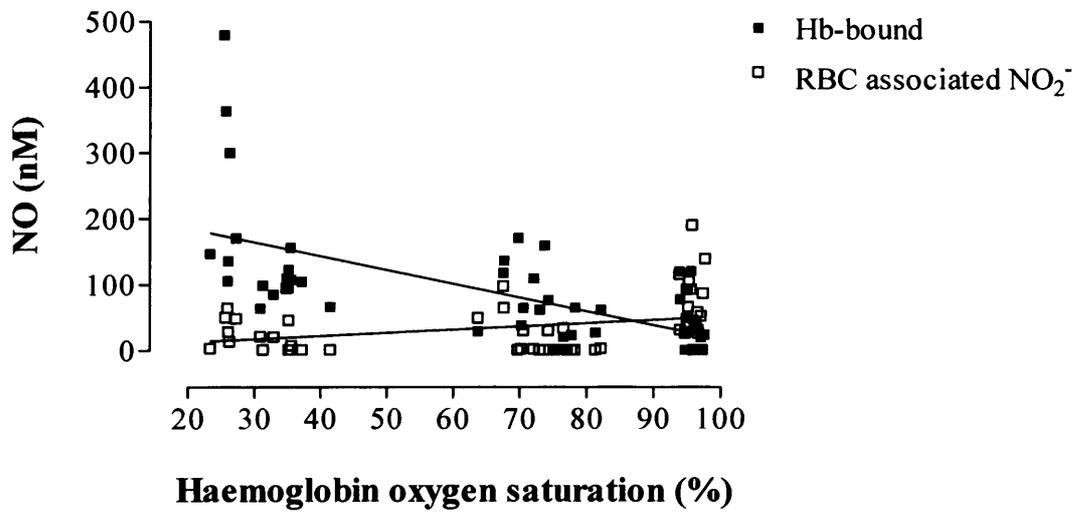


Figure 6.13: Relationship between haemoglobin-bound NO, red blood cell associated nitrite and haemoglobin oxygen saturation (%). Individual data points plotted. Correlation $r = -0.6278$ ($p < 0.0001$) for haemoglobin-bound NO; $r = 0.3063$ ($p = 0.0288$) for red blood cell associated nitrite; Pearson's correlation.

6.6.3 Pacing with L-NMMA

6.6.3.1 Blood Oxygenation

Similar trends were observed in blood oxygenation with pacing in the presence of L-NMMA as observed with pacing alone. LMCA HbO₂ sat (%) remained unchanged whilst PA HbO₂ sat (%) increased non-significantly at the first pacing interval and declined significantly at 85% maximum heart rate. CS HbO₂ sat (%) increased with each pacing interval in the same way that it increased with pacing alone (Figure 6.14). This was reflected in a significant decline in the cross heart HbO₂ sat (%) gradient (Figure 6.15). No significant differences were observed between HbO₂ sat (%) values compared at each pacing interval with pacing alone and with pacing in the presence of L-NMMA.

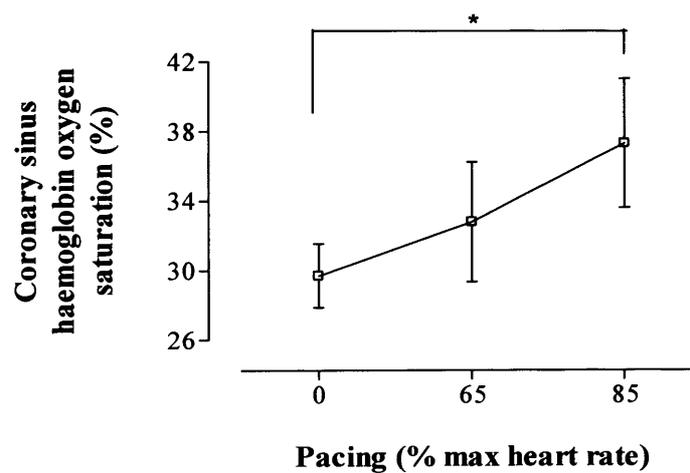


Figure 6.14: Coronary sinus haemoglobin oxygen saturation with pacing in the presence of L-NMMA. Values presented as mean \pm SEM. * $p < 0.05$; One way repeat measures ANOVA.

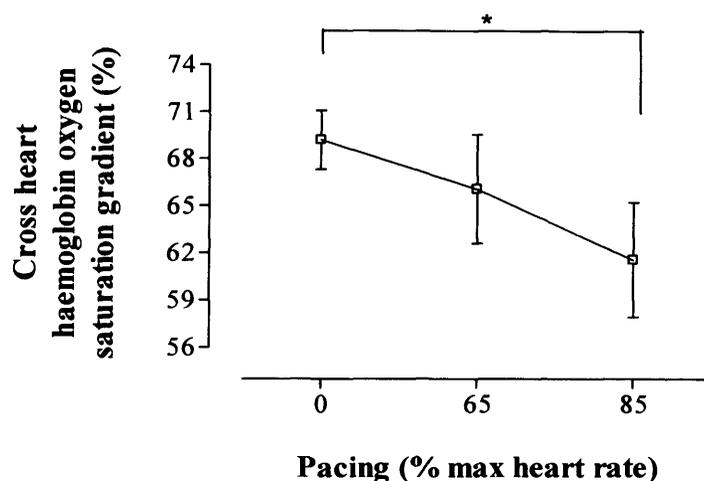


Figure 6.15: Cross heart haemoglobin oxygen saturation gradient with pacing in the presence of L-NMMA. Values presented as mean \pm SEM. * $p < 0.05$; One way repeat measures ANOVA.

6.6.3.2 Blood Vessel Diameter

In the presence of L-NMMA, pacing the heart significantly increased coronary arterial diameter at all sites (proximal, mid and distal) (Figure 6.16). Significant differences between pacing alone and pacing in the presence of L-NMMA were observed in the mid coronary artery diameter which was lower at baseline with L-NMMA ($p < 0.05$) and in the distal coronary artery diameter which was higher at maximal pacing with L-NMMA ($p < 0.01$).

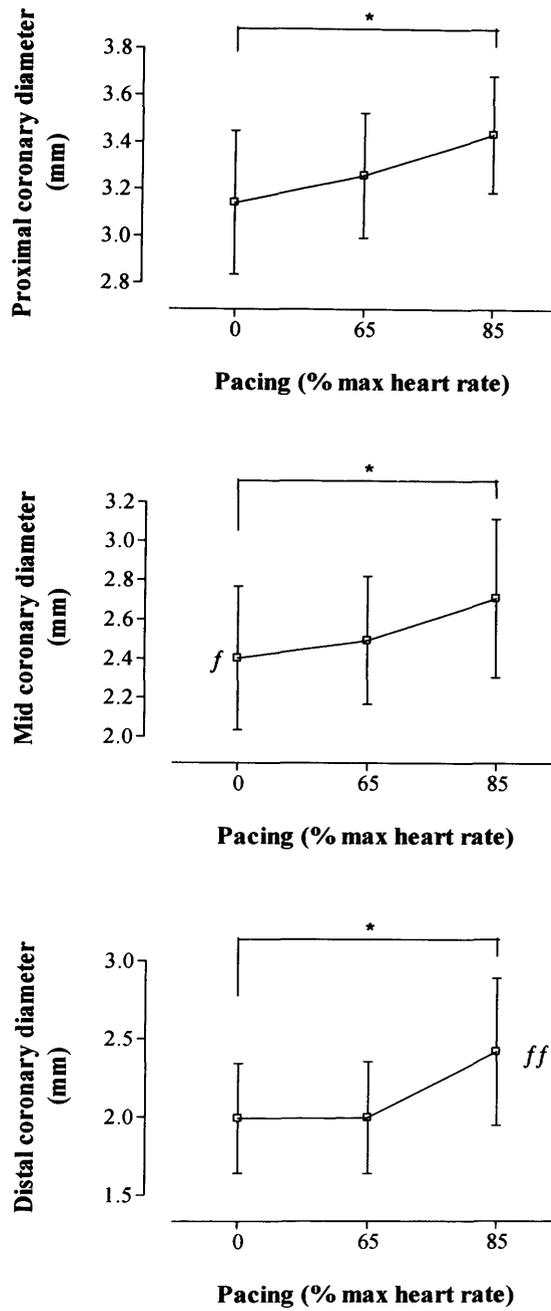


Figure 6.16: Coronary artery diameter with pacing. Values presented as mean \pm SEM. * $p < 0.05$; One way repeat measures ANOVA. Differences between pacing alone and pacing with L-NMMA; $f < 0.05$, $ff < 0.01$.

6.6.3.3 NO Metabolites

The apportionment of NO between metabolite species in samples with pacing during L-NMMA infusion was similar to that observed with pacing alone, with “cross heart” and “cross lung” trends essentially unchanged. The only significant difference was a loss of plasma nitrate across the heart following L-NMMA infusion at the highest pacing interval (Tables 6.2 and 6.3).

Table 6.2: Change in NO (Δ NO) for each metabolite “cross heart” and “cross lung” comparing 65% maximum heart rate pacing alone with pacing at the same intensity in the presence of L-NMMA. Values presented as mean \pm SEM.

Δ NO	65%	65% + L-NMMA	P Value
“Cross Heart” LMCA-CS			
<u>Totals</u>			
Red blood cell NO	+ 92 \pm 7 nM	+ 22 \pm 75 nM	0.6875
Plasma NO	- 66 \pm 14 nM	- 29 \pm 40 nM	0.3207
<u>Individual Species</u>			
Red blood cell associated nitrite	- 12 \pm 20 nM	- 66 \pm 73 nM	1.0000
Red blood cell Hb-bound NO	+ 102 \pm 18 nM	+ 88 \pm 10 nM	0.4426
Plasma nitrate	+ 1.1 \pm 0.7 μ M	- 0.01 \pm 1.8 μ M	0.8054
Plasma nitrite	- 81 \pm 29 nM	- 11 \pm 53 nM	0.3788
Plasma protein-bound NO	+ 11.6 \pm 9.6 nM	- 1.3 \pm 2.9 nM	0.5814
“Cross Lung” PA-LMCA			
<u>Totals</u>			
Red blood cell NO	+ 10 \pm 12 nM	+ 30 \pm 88 nM	0.6875
Plasma NO	+ 45 \pm 20 nM	+ 98 \pm 33 nM	0.0690
<u>Individual Species</u>			
Red blood cell associated nitrite	+ 38 \pm 12 nM	+ 38 \pm 87 nM	0.5625
Red blood cell Hb-bound NO	- 34 \pm 13 nM	- 8 \pm 6 nM	0.0740
Plasma nitrate	- 4.5 \pm 1.1 μ M	- 1.3 \pm 1.1 μ M	0.1608
Plasma nitrite	+ 39 \pm 27 nM	+ 79 \pm 35 nM	0.2352
Plasma protein-bound NO	+ 0.7 \pm 5 nM	+ 5.7 \pm 2.6 nM	0.6250

Table 6.3: Change in NO (Δ NO) for each metabolite “cross heart” and “cross lung” comparing 85% maximum heart rate pacing alone with pacing at the same intensity in the presence of L-NMMA. Values presented as mean \pm SEM, ** $p < 0.01$.

Δ NO	85%	85% + L-NMMA	P Value
“Cross Heart”			
LMCA-CS			
<u>Totals</u>			
Red blood cell NO	+ 45 \pm 39 nM	+ 53 \pm 29 nM	0.8299
Plasma NO	- 50 \pm 30 nM	- 42 \pm 29 nM	0.6842
<u>Individual Species</u>			
Red blood cell associated nitrite	- 62 \pm 28 nM	- 24 \pm 29 nM	0.8713
Red blood cell Hb-bound NO	+ 112 \pm 34 nM	+ 77 \pm 13 nM	1.0000
Plasma nitrate	+ 1.3 \pm 0.7 μ M	- 3.8 \pm 0.6 μ M	0.0028**
Plasma nitrite	- 87 \pm 31 nM	- 43 \pm 34 nM	0.2636
Plasma protein-bound NO	+ 9 \pm 4.4 nM	- 3.8 \pm 2.4 nM	0.2804
“Cross Lung”			
PA-LMCA			
<u>Totals</u>			
Red blood cell NO	- 18 \pm 49 nM	+ 19 \pm 21 nM	0.3524
Plasma NO	+ 57 \pm 14 nM	+ 21 \pm 32 nM	0.3226
<u>Individual Species</u>			
Red blood cell associated nitrite	+ 44 \pm 35 nM	+ 49 \pm 21 nM	0.3813
Red blood cell Hb-bound NO	- 26 \pm 6 nM	- 31 \pm 15 nM	0.2740
Plasma nitrate	+ 2.4 \pm 2.2 μ M	- 3.3 \pm 1.0 μ M	0.8233
Plasma nitrite	+ 63 \pm 18 nM	+ 42 \pm 26 nM	0.5630
Plasma protein-bound NO	- 8 \pm 5.2 nM	- 1.4 \pm 4.6 nM	0.0938

6.6.3.4 Correlation of NO Metabolites with Oxygen

Once more, across all sites (including baseline and pacing samples) the data gave rise to a direct correlation between red blood cell associated nitrite and HbO₂ sat (%) and an inverse correlation between haemoglobin-bound NO and HbO₂ sat (%) similar to that observed in the absence of L-NMMA (data not shown).

6.7 Discussion

6.7.1 Baseline Changes in the NO Metabolite Pool

In healthy human subjects under baseline resting conditions a significant re-apportionment of NO metabolites was observed across the coronary circulation that correlated with HbO₂ sat (%). However, no net loss or gain of NO was observed from the total metabolite pool. This NO metabolite equilibrium could be interpreted in one of two ways. Either extraction of NO by cardiac tissue was balanced by replenishment from endothelial sources giving the overall appearance of no net flux from blood metabolite stores into tissue (but potentially sufficient NO delivery to contribute to the regulation of coronary blood flow) or alternatively there was no utilisation/consumption of NO from the metabolite pool under baseline resting conditions.

Oxygen dependent increases in haemoglobin-bound NO across the coronary circulation were matched by decreases in plasma and red blood cell associated nitrite. However, with no net loss of NO from the total metabolite pool, these changes implied either the consumption of nitrite derived NO being replenished from the endothelium resulting in the formation of haemoglobin-bound NO in venous blood, or the direct exchange of NO between nitrite and haemoglobin as a function of HbO₂ sat (%), potentially via the deoxyhaemoglobin nitrite reductase reaction. Either way the significant re-apportionment of NO between metabolite species across the coronary (and pulmonary) vascular beds driven by HbO₂ sat (%) gradients highlighted the dynamic interplay between NO metabolite species throughout these vascular circuits. The metabolite changes which occurred in the brief time that blood traversed these single vascular beds lends support to the idea that NO or a related moiety could in principle be transferred from metabolite stores as a function of oxygen to potentially mediate the regulation of vascular tone.

The pattern of NO metabolite interplay observed across the coronary circulation was reversed across the pulmonary vascular circuit, reflecting the opposite oxygen gradient. This implied a role for the lungs in normalising the NO metabolite equilibrium, adjusting for systemic changes in the balance of NO metabolite species. As the lungs function to re-oxygenate blood, accounting for oxygen consumption throughout the vascular circuit, the same appears true for NO metabolites with the restoration of the NO metabolite equilibrium prior to the re-transit of blood around the vasculature.

The haemoglobin-bound NO metabolite pool has previously been demonstrated to remain constant throughout the systemic human circulation, with only the relative levels of individual haemoglobin-bound species changing as a function of oxygen tension (or more precisely HbO₂ sat (%)) [70, 81, 83]. However, never before has the relationship between haemoglobin-bound NO and oxygen tension been studied over such a substantial oxygen gradient as observed across the coronary circulation (HbO₂ sat ranging from > 95% to ~ 30%). In these measurements haemoglobin-bound NO was found to be inversely correlated with HbO₂ sat (%). Unfortunately it was not possible to assess the ratio of HbNO to SNO-Hb as a function of HbO₂ sat (%) as these individual haemoglobin-bound NO species were not differentiated for reasons outlined previously (chapter 3). Nevertheless, these results are in agreement with previous work from our laboratory demonstrating gradients in haemoglobin-bound NO metabolite species showing a clear dependence on HbO₂ sat (%). However, it must be acknowledged that this earlier work was undertaken utilising the NO electrode in combination with various sample pre-treatment protocols. Although the NO metabolite levels do not agree with values quoted herein (which are in fact in very close agreement with recent values reported by other groups using similar methodologies, showing a NO:Hb ratio ~ 0.00012 [73, 78, 111, 125]), previously observed trends with regard to the correlation with oxygen and a lack of net NO loss or gain are confirmed [115].

The concentrations of red blood cell NO metabolites measured herein within the coronary and pulmonary circulations are in very close agreement with the baseline metabolite measurements made across the femoral circulation in chapter 4. However, levels of coronary and pulmonary plasma nitrite appear much greater. With levels of plasma nitrite acting as a marker for type III NOS NO production this would infer that the heart could be a major site of NO production.

6.7.2 Effect of L-NMMA at Baseline

Systemic administration of L-NMMA resulted in a significant decrease in coronary artery diameter and a significant increase in systolic, diastolic and mean arterial blood pressure, confirming NOS inhibition. The decrease in coronary artery diameter with L-NMMA at baseline was in support of the hypothesis that endothelial derived NO functions to regulate vascular tone under baseline conditions in the human coronary microcirculation, as previously demonstrated by others [153, 154].

Surprisingly, L-NMMA had no effect on the apportionment of NO to metabolites at each sampling site. Consequently the same trends observed at baseline were observed in the presence of L-NMMA, as demonstrated by the similar cross heart and cross lung deltas (Table 6.1). This result is consistent with the re-apportionment of NO between metabolites (independent of endothelial NO) as a function of changes in oxygen across the heart.

6.7.3 Effect of Rapid Atrial Pacing

Pacing the heart significantly increased CS saturation and decreased the cross heart HbO₂ sat (%) gradient. This was somewhat of a surprise as the increase oxygen demand elicited via pacing was expected to lower CS HbO₂ sat (%). The increased oxygen demand must have been met via an increase in blood flow (given the increase in CS HbO₂ sat (%)). The increase in flow would have resulted in greater oxygen delivery but resulted in less exchange of oxygen, as a result of the reduction in blood transit time through the coronary circulation. Although pacing did not significantly alter blood vessel diameter there were trends towards an increase in the mid and distal portions of the coronary artery.

Changes in the total metabolite pool upon pacing mainly reflected a trend towards increasing plasma nitrate levels (Figure 6.8). The only decrease was observed in the PA at maximal pacing, resulting in a delta increase in plasma nitrate across the lungs (Figure 6.9). Once again the apportionment of NO to metabolites remained essentially unchanged across the heart and lungs with the same trends observed at baseline also seen during pacing. However, with increased pacing there was a trend towards a decline in red blood cell NO in the CS, mainly reflecting the loss of haemoglobin-bound NO. This change mirrored the increase in CS HbO₂ sat (%) implying the close regulation of haemoglobin-bound NO as a function of oxygen, demonstrated by the correlation in Figure 6.11.

6.7.4 Effect of L-NMMA with Rapid Atrial Pacing

Previous data in human studies have shown inconsistent results concerning the effects of NO inhibition on metabolic vasodilatation [154-156]. Whilst coronary artery diameter declined at baseline upon the administration of L-NMMA, upon pacing coronary artery diameter increased at least to the same extent as in the absence of L-NMMA. This implied that the release of

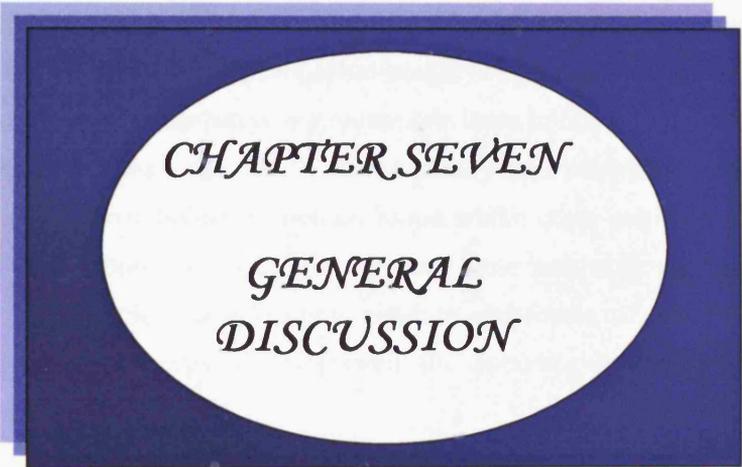
endothelial NO was not an important factor in pacing-induced arterial dilation in the human coronary circulation.

Once more, even during pacing the same trends observed during pacing without L-NMMA were observed during pacing in the presence of L-NMMA. This was demonstrated by the similar cross heart and cross lung deltas (Table 6.2). This result is consistent with the re-apportionment of NO between metabolites (independent of endothelial NO) as a function of changes in oxygen across the heart.

The only time that conclusions can be made as to whether NO metabolites regulate vascular tone is in the presence of L-NMMA, when endothelial NO is inhibited. Under these conditions for any metabolite species (such as nitrite) to function to regulate vascular tone, the total metabolite pool would have to experience a loss of NO. However, as no detectable loss of NO was observed at all in the presence of L-NMMA at baseline or during rapid atrial pacing, this would appear to rule out any function for NO metabolites in the regulation of coronary vascular tone.

SUMMARY

- Baseline - Across the coronary circulation there was no net loss of NO from the total metabolite pool, however a significant re-apportionment of NO metabolites was observed that correlated with HbO₂ sat (%).
- Baseline with L-NMMA - Coronary artery diameter decreased implying a role for endothelial NO in the maintenance of baseline vasodilator tone. The apportionment of NO to metabolites at each sampling site remained unchanged which is consistent with the re-apportionment of NO between metabolites (independent of endothelial NO) as a function of changes in oxygen across the heart.
- Pacing - CS HbO₂ sat (%) increased whilst there was a trend towards an increase in coronary artery diameter. The apportionment of NO to metabolites remained essentially unchanged across the heart and lungs with the same trends observed at baseline also seen during rapid pacing.
- Pacing with L-NMMA - Coronary artery diameter increased significantly and was no different to that observed during pacing alone implying that endothelial NO does not contribute to pacing induced vasodilatation. The same NO metabolite trends were observed as during pacing alone which is consistent with the re-apportionment of NO between metabolites (independent of endothelial NO) as a function of changes in oxygen across the heart.
- No detectable loss of NO was observed at all in the presence of L-NMMA at baseline or during rapid atrial pacing which would appear to rule out any function for NO metabolites in the regulation of coronary vascular tone.



CHAPTER SEVEN

*GENERAL
DISCUSSION*

7.0 Overview

The aims of this thesis were to develop and establish methods of measuring baseline levels of NO metabolites in human blood to enable a more comprehensive understanding of the physiological relevance of these species in the human circulation. The results herein have provided important insights into the complexities of NO metabolites/metabolism. These can be summarised as follows:

7.1 NO Metabolite Measurement

Extensive methodological work led to the identification of a potential confounding factor in all measurements of red blood cell/haemoglobin-bound NO, namely the auto-capture of NO by cell free haem. A means was developed to overcome this issue in one of the most commonly used assay reagents in the NO metabolite field. Subsequently this modified reagent was used to analyse red blood cell NO metabolites in human blood whilst other methods were employed to determine plasma NO metabolite species. At the same time protocols were also developed to limit the possibility of biological sample contamination and losses of unstable NO metabolite species. These advances subsequently improved the accuracy and sensitivity of the NO metabolite measurements made.

At the outset of this thesis it was expected from previous measurements made within our laboratory that baseline metabolite values from human blood samples would be in the $\mu\text{mol/L}$ range. Instead the measured concentrations of all metabolites species (other than nitrate) mainly fell within the $\sim 10 - 500\text{nmol/L}$ range, in line with measurements made by a number of groups using similar methodologies. Although the baseline metabolite levels measured herein were small, it is worth emphasising that:

- Red blood cell and haemoglobin-bound NO were visibly detectable in baseline human blood samples (Chapter 2)
- Intricate fluctuations in the levels of haemoglobin-bound NO, plasma nitrite and red blood cell associated nitrite under the control of oxygen were clearly measured (Chapter 3 and 6).
- Significant changes in metabolite species across the coronary and pulmonary circulation were plainly evident (Chapter 6)

- The major NO metabolite species in human blood were all accounted for, as demonstrated by the near 100% recovery of exogenously added NO and nitrite to human whole blood (Chapter 4).

Whilst the developments herein have improved the accuracy and specificity of NO metabolite measurements made within our laboratory, there are still many issues that remain to be resolved within the research field as a whole. One often cited concern relates to the exact identification of metabolite species measured by the major methodologies, including tri-iodide and photolysis (the favoured methodologies of the two main groups in this research field, which appear to yield some of the most disparate metabolite values). However, as the standards against which these measurements are made barely reflect the physiological species they are supposed to represent, there would appear little benefit to this exercise. More enlightening would be a direct comparison of measures made by tri-iodide and photolysis from duplicate blood samples.

Progress towards our understanding of NO biology hinges on the accuracy and specificity of methods used to measure NO metabolites. It is therefore imperative that closely scrutinised methods are utilised by all within this research field. Future aims should address the development of assays with the capacity to measure only the metabolite species of interest. This specificity would minimise the need for sample processing/pre-treatment thus limiting the possibility of contamination, loss of less stable metabolite species and disruption to the NO metabolite equilibrium. Only when the same metabolite concentrations can be measured by different methodologies can we be satisfied that the true levels of NO metabolites in human blood have been quantified.

7.2 NO/Nitrite Metabolism in Human Blood

NO and nitrite metabolism was analysed in human whole blood to identify and compare the major metabolic pathways of these species. Furthermore the effect of oxygen on these metabolic pathways was assessed. The results can be summarised as follows:

- NO and nitrite were metabolised in a similar manner in arterial blood, the major routes being, plasma nitrate >> plasma nitrite > red blood cell associated nitrite, with relatively little formation of haemoglobin-bound NO or plasma protein-bound NO. The major difference between NO and nitrite metabolism was the relative stability of exogenously

added nitrite in arterial whole blood reflected by its accumulation in the plasma compartment.

- In venous blood the apportionment of exogenously added NO/nitrite was more evenly distributed among the non nitrate metabolite species. However, the main pathways of metabolism remained nitrate >> nitrite > haemoglobin-bound NO > plasma protein-bound NO. Interestingly a greater percentage of exogenously added NO in comparison to nitrite was metabolised to plasma nitrate, plasma protein-bound NO, red blood cell haemoglobin-bound NO and red blood cell associated nitrite. However, once more the relative stability of nitrite was reflected in the fact that a significant proportion of exogenously added nitrite remained in the plasma compartment.
- The different metabolism of set amounts of NO/nitrite in arterial and venous blood demonstrates the regulatory function of oxygen in determining the relative proportion of species within the metabolite pool.

The fact that nitrite formed the same metabolite species as NO (although not in the same proportions) in arterial and venous human whole blood implies the ability of this metabolite species to act as a potential source from which the metabolite pool could be replenished. The relative stability of nitrite in comparison to NO (reflected in its accumulation in the plasma compartment following addition to both arterial and venous blood) would benefit its function as a potential NO reserve in the circulation. Interestingly, the apportionment of NO and nitrite to the various metabolic pathways appeared to be under the direct control of oxygen. This regulatory control adds to the complexity of analysing and interpreting A-V metabolite gradients, i.e., even where NO stays constant, any change in oxygen alters the apportionment of NO between species.

7.3 Blood Vessel Relaxation

Perhaps the most compelling evidence that NO metabolites function to regulate vascular tone is offered by organ chamber bioassay experiments. In this thesis the organ chamber bioassay was used to assess whether nitrite could account for the relaxation response observed from native red blood cells under hypoxic conditions. The results can be summarised as follows:

- In endothelium intact vessels native red blood cells were demonstrated to mediate vasoconstriction at high oxygen tension and relaxation at low oxygen tension. This implicates the red blood cell in the mediation of hyperoxic vasoconstriction and hypoxic

vasodilatation *in vivo*. The red blood cell relaxation response at low oxygen was endothelium independent and sGC dependent.

- Nitrite alone was found to relax aortic rings and this was enhanced under hypoxic conditions. However, the slow and prolonged relaxation response was totally different to the transient relaxation observed from native red blood cells.
- Addition of exogenous nitrite to the organ chambers prior to native red blood cells had no effect on the transient red blood cell relaxation response observed, implying that exogenous nitrite plays no part in this and potentially that the reduction of nitrite by deoxyhaemoglobin at very low oxygen tension results in very little (if any) free NO formation.
- Whole blood was incubated with nitrite or NO (as in chapter 4, wherein it was demonstrated that bloods loaded with nitrite contained significantly less red blood cell associated nitrite compared to those incubated with NO, whilst haemoglobin-bound NO accumulated to a similar extent). The red blood cells were then injected in the organ chamber bioassay at low oxygen tension. The same relaxation response was observed from both NO and nitrite loaded red blood cells. This would imply that nitrite was not the source of the vasorelaxant response, but that haemoglobin bound NO might have been.

These results would appear to rule out the involvement of nitrite in the relaxation response observed from native red blood cells at low pO_2 (~ 10mmHg). This is not entirely surprising as the deoxyhaemoglobin nitrite reductase reaction is proposed to function maximally at haemoglobins P_{50} . This would place its maximal activity at around ~ 30mmHg. The relative abundance of deoxygenated haem at the low oxygen tensions at which native red blood cells elicit a relaxation response would more likely scavenge any free NO (Figure 1.11). These results therefore confirm that the red blood cell hypoxic vasodilatory response is unlikely to result from direct nitrite reduction to NO. This has relevance to the *in vivo* setting where red blood cells are exposed to a range of pO_2 s across a single vascular bed (a discussion returned to below).

7.4 In Vivo Human Study

The heart was identified as an ideal model system in which to assess NO metabolites given its high energy consumption (even at rest extracting ~ 75% of arterially delivered oxygen) which provided the opportunity to obtain blood samples at the extremes of physiologically relevant HbO₂ sat (%). Also it was possible to manipulate oxygen demand via rapid atrial pacing

(whilst simultaneously obtaining blood samples). The work presented herein is subsequently the first to measure NO metabolites across the coronary and pulmonary vascular circulations in human subjects. The results can be summarised as follows:

- At rest, across the coronary circulation, there was no net loss of NO from the total metabolite pool, however a significant re-apportionment of NO metabolites was observed that correlated with HbO₂ sat (%). The intravenous infusion of L-NMMA resulted in a decrease in coronary artery diameter (along with an increase in blood pressure confirming NOS inhibition) implying a modest role for endothelial NO in the maintenance of baseline vasodilator tone. Interestingly the apportionment of NO to metabolites at each sampling site remained unchanged. This was consistent with the re-apportionment of NO between metabolites (independent of endothelial NO) as a function of changes in oxygen across the heart, without any detectable loss of NO, i.e., no utilisation/consumption of NO moiety from the metabolite pool.
- Pacing the heart resulted in a trend towards an increase in coronary artery diameter. Interestingly CS HbO₂ sat (%) also significantly increased and the cross heart HbO₂ sat (%) gradient decreased (thought to reflect the increase in blood flow through the myocardium). The apportionment of NO to metabolites remained essentially unchanged across the heart and lungs with the same trends observed at baseline also seen during pacing. The combination of pacing and L-NMMA resulted in an increase in coronary artery diameter and CS HbO₂ sat (%) whilst the cross heart HbO₂ sat (%) gradient decreased once more. The increase in coronary artery diameter was similar to that observed in the absence of L-NMMA implying no role for endothelial NO in pacing induced vasodilatation. Most interestingly, similar NO metabolite trends were observed as during pacing alone (i.e., without L-NMMA) which once more was consistent with the re-apportionment of NO between metabolites (independent of endothelial NO) as a function of changes in oxygen across the heart. Furthermore, there was no detectable loss of NO implying no utilisation/consumption of NO from the metabolite pool.

These results demonstrate no detectable loss of NO from the metabolite pool at baseline or during pacing of the heart in the absence or presence of L-NMMA. Consequently this implies that even where red blood cells are exposed to a range of pO₂s across a single vascular bed the nitrite reduction reaction unlikely contributes to the regulation of vascular tone via the release and utilisation of free NO, whereas *in vitro* and in principle, free NO may be produced maximally

from haemoglobin nitrite reduction at HbO₂ saturations around 50%. Were this to have occurred a loss of NO would have been expected across the coronary vascular bed. More consistent with my results is the re-apportionment of NO between metabolite species under the direct control of HbO₂ sat (%).

7.5 Conclusion

From my work the presence of a dynamic pool of NO metabolites in the human circulation under the close regulation of oxygen remains. Whereas the range of levels is an order of magnitude lower than anticipated at the outset of these studies, this potentially means that even very small changes might be physiologically relevant. Taken together my work highlights three important considerations for future work:

- Biochemical NO metabolite measurement methodologies should work on the principle that 'simple is best', i.e., samples should be injected with no manipulation or treatment.
- Photolysis (among other techniques) should be used to yield complementary data to that reported herein, potentially providing measures of different metabolite species.
- That NO metabolites could contribute to baseline and pathological vessel tone, only that the amounts involved would not necessarily result in A-V blood gradients.

I conclude that in the healthy human vasculature NO metabolites would appear to play only a limited role in the regulation of vascular tone. However, in various pathologies wherein differences in NO production, the structural components of blood (e.g., diabetes), or physiological parameters (e.g., decreased HbO₂ sat (%) in chronic heart disease) might change NO metabolite dynamics, this metabolite pool could potentially be of more significance. Further clinical studies which tie together biochemical NO metabolite measurements with parallel assessments of physiological changes in the vasculature offer the most promising means of advancing our understanding in this research area and it is here I will focus in the immediate future.

7.6 Publications

S.C. Rogers, A. Khalatbari, B.N. Datta, S. Ellery, V. Paul, M.P. Frenneaux, and P.E. James. NO metabolite flux across the human coronary circulation. *Cardiovasc Res.* (Submitted September 2006).

S.C. Rogers, A. Khalatbari, P.W. Gapper, M.P. Frenneaux and P.E. James. Detection of Human Red Blood Cell-bound Nitric Oxide. *J. Biol. Chem.* 280 (29):26720-26728, 2005.

S.K. Jackson, M.P. Thomas, S. Smith, M. Madhani, S.C. Rogers, and P.E. James. In vivo EPR spectroscopy: biomedical and potential diagnostic applications. *Faraday Discuss.* 126: 169-183 (2004)

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