The Role of Inorganic Nitrite in the Transport of Nitric Oxide in Health and Heart Failure

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The Role of Inorganic Nitrite in the Transport of Nitric Oxide in Health and Heart Failure

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Declaration and Statements

Declaration

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature.

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Statement 1

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Statement of Contribution to Research

Professor Frenneaux and I conceived and designed all the studies.

Execution

I performed all of the recruitment and organisation of the appointments involved in the study. I performed assessment of forearm blood flow, radionuclide plethymography and radionuclide ventriculography studies in the University of Birmingham. Biochemical analysis was performed by Dr Phil James and his team at the University of Cardiff, Wales.

The *in vitro* work described in chapter 2 was performed by Alexandra Milsom, University of Cardiff, Wales.

The systemic studies described in chapter 6 were designed and organised by myself with the normoxic studies being executed by myself and the hypoxic studies by Dr Sayqa Arif.

The studies in chapter 7 were performed in collaboration with Dr Jules Ormerod – The initial washout studies were designed and performed by myself. Myoglobin knockout mice were obtained by me. The transfection and restitution studies in this chapter were performed by Dr Ormerod.

Analysis

All data were collated and analysed by myself, Phil Thomas, Simon Anderson, Mohammed Nasimizadeh and Kunal Chudasama.

Power Calculations, Statistical Analysis and Recruitment

Statistical Analyses

Clinical characteristics of subjects versus controls were compared utilizing non-paired t-tests for normally distributed parameters, and Wilcoxon tests for skewed data.

Serial changes in parameters such as FBF, FVV, pH and concentrations of NO_2^- and protein-bound NO were compared by 2-way ANOVA with repeated measures, utilizing Dunnett's t-test to assess for significance of changes at individual time points. Logit transformation of data was utilized to detect possible disparity of concentration-response relationships between groups of subjects in chapter 3.

All results are expressed as mean+/- SEM unless otherwise stated.

Power Calculations

The human studies were designed to provide 90% power to detect a 15% increase in FBF and a 20% increase in FVV within groups and a 20% difference in FBF and FVV between groups at a significance level 0.05. Calculations were based on mean and standard deviation data from previous studies and published literature.

Subject Recruitment

Healthy volunteers were recruited to act as 'control subjects' by way of advertisements placed around Birmingham University and at the Birmingham Blood Transfusion Centre.

Chronic Heart Failure patients were recruited from a specialised advanced heart failure and cardiomyopathy clinic run by Professor Frenneaux at the University Hospital Birmingham.

No study was performed more than once on an individual subject.

Subjects in each study/chapter were recruited specifically to that study and as such a new cohort was used each time.

Where studies involved more than one limb, subjects were allocated on a random basis.

Publications

This thesis for the Doctoral Degree PhD in cardiovascular medicine is based on the following publication listed below:

Original research

- Maher AR, Milsom AB, Gunaruwan P, Abozguia K, Ahmed I, Weaver RA, Thomas P, Ashrafian H, Born GV, James PE, Frenneaux MP. *Hypoxic modulation of exogenous nitrite-induced vasodilation in humans. **Circulation***. 2008 Feb 5;117 (5):670-7

- Abdul R. Maher, Julian O. M. Ormerod, Sayqa Arif, Houman Ashrafian and Michael P. Frenneaux **Xanthine Oxidoreductase and Endothelial Nitric Oxide Synthase Do Not Contribute To Vascular Relaxation by Nitrite in Man.** Submitted to the Clinical Science

- Abdul R Maher, Saiqa Arif, Houman Ashrafian, Prasad Gunaruwan, Khalid Abozguia, Ibrar Ahmed, Rebekah A Weaver,Ullasini Naidoo, Philip Thomas, Simon Anderson, Philip E James & Michael P Frenneaux. **Nitrite Retains Its Vascular Effects in Chronic Heart Failure.** Currently being considered by The British Journal of Pharmacology

- Julian O.M. Ormerod[#], Houman Ashrafian[#], Abdul Maher[#], Sayqa Arif, Violetta Steeples, Gustav V.R. Born, Stuart Egginton, Martin Feelisch, Hugh Watkins, Michael P. Frenneaux. **The Role of Vascular Myoglobin in Nitrite mediated blood vessel relaxation.** **Cardiovasc Res.** 2011 Feb 15;89(3):560-5. Epub 2010 Oct 1.

Abstracts

- Maher AR, Gunaruwan P, Abozguia K, Palin TP, James PE, and Frenneaux MP. *Sodium Nitrite is a Potent Venodilator*. The 56th Annual American College of Cardiology (ACC) scientific sessions, March 2007, New Orleans, USA

- Maher AR, Thomas P, Anderson S, Abozguia, Ahmed I, Weaver R, James P, Frenneaux MP. Intra-arterial causes an enduring Vasodilation in Man. The 57th Annual American College of Cardiology (ACC) scientific sessions, March 2008, Chicago, USA

- Maher AR, Gunaruwan P, Abozguia K, Palin , James P, Frenneaux MP. Venoselectivity of Nitrite: Evidence favouring Hypoxic release of NO. The Annual Scientific Conference of The British Cardiovascular Society (BCS) June 2007, Glasgow, UK

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Abstract

The potential for nitric oxide (NO) metabolites (e.g. inorganic nitrite) to act as stable stores of "Transported Nitric Oxide" has excited huge interest due to the substantial potential therapeutic avenues. The prospect developing of a "silver bullet" that could target areas most in need of vasodilatation, by releasing NO in areas of hypoxia and ischaemia, could prove a massive advance in the treatment of vascular disease.

In this thesis I examine the effects of nitrite infusion in both hypoxia and normoxia. I examine the effects both in health and heart failure, and investigate the potential roles of Nitric Oxide Synthase (NOS) and Xanthine Oxidase (XO) in mediating the reduction of nitrite.

We found, and were the first to report in man, that intra-arterial infusions of nitrite had little effect upon the vasculature in high oxygen tension environments but led to significant vasodilatation during hypoxaemia. We found that patients suffering with Chronic Heart Failure responded differently to nitrite infusion to healthy controls, possibly as a result of differences in redox-stress. In healthy volunteers, at rest, neither NOS nor XO appeared to play a significant role in nitrite induced vasodilatation in normoxia and mild hypoxia.

We found that vascular myoglobin contributes to the reduction of nitrite to nitric oxide and may play a role in prolonging the vasodilatation induced by nitrite infusion.

Chapter 1 - Introduction

Transported Nitric Oxide and Hypoxic Vasodilatation

– Evolving concepts and Contending theories.

1.1 Introduction

The ability of the body to match perfusion to the demands of a particular tissue bed is highly conserved and involves an extremely efficient system for harmonizing delivery of blood to the local requirements. This phenomenon has been well described and studied. There remained, however, a 'missing link' in this mechanism which led to the development and description of a new paradigm – that of 'Transported Nitric Oxide'. It is this paradigm that I have investigated in the studies presented in this thesis.

This new concept excited huge interest due to the substantial therapeutic avenues that were opened. The potential for the development of a "silver bullet" that could target areas most in need of vasodilatation could prove a massive advance in the treatment of vascular disease.

In this chapter I will aim to review the current literature in this field and to examine the current understanding of the mechanisms that underpin this phenomenon. In particular I will focus on the potential roles of inorganic nitrite and haemoglobin bound Nitric oxide in mediating this effect and the therapeutic opportunities that are being investigated. With this background presented I will then describe the series of studies that I have performed and discuss how these studies will advance our understanding of nitrite physiology.

1.2 Hypoxic Vasodilatation

The ability of vessels and more specifically, arterioles to vasodilate as a consequence of downstream tissue hypoxia is a phenomenon that has been recognised for over a century[1]. Traditionally this was felt to be due to the release of local mediators as a result of increased metabolic activity[2]. These mediators include potassium, adenosine and prostacyclin[1]. Hypoxic vasodilatation still occurs, however, even after the blockade of these 'traditional' mediators[3]. It has been postulated that NO may be transported in the bloodstream to areas of low oxygen tension where it is released causing vasodilatation[4, 5].

1.3 The Paracrine Role of Nitric Oxide (NO) and NO generation

Since the discovery of nitric oxide (NO) as EDRF in the 1980's [6, 7] NO has emerged as an important cellular messenger molecule and has been implicated as a physiological and pathophysiological molecule in the cardiovascular, immune and nervous system [8]. NO is produced by various types of cells and is thought to be associated in a wide range of disease processes, exerting both beneficial and detrimental effects at the cellular and vascular levels[9]

Vascular NO is released from the endothelium in response to shear stress or agonists, such as acetylcholine, and is formed from L-arginine.. The NO thus formed diffuses across to the vascular smooth muscle inducing vascular relaxation. The process is mediated by a family of complex enzymes: the Nitric Oxide Synthases (NOS)[10]. Several co-factors are required for the unusual five electron oxidation of one electron of the L-arginine guanidino group. [11] These cofactors include Nicotinamide adenine dinucleotide phosphate **(**NADPH), flavin adenine dinucleotide (FAD), Flavin mononucleotide (FMN) and Tetrahydrobiopterin (BH4). [12]

$$\text{L-arginine} + n\,\text{NADPH} + n\,H^{+} + m\,O_2 \rightarrow \text{citrulline} + \text{nitric oxide} + n\,NADP^{+}$$

NOS are very well conserved being found in all eukaryotes. NOS was first described is 1989[13] and are not ATP dependent. Three isoforms of NOS have been identified. Endothelial (eNOS), inducible (iNOS) and neuronal (nNOS). This family of enzymes catalyses the conversion of L–arginine to L-citrulline forming NO in the process. A distinction was initially made between iNOS and eNOS/nNOS. iNOS was felt to be 'induced' in response to inflammatory and immunomodulatory stimuli whereas eNOS and nNOS were felt to be 'constitutively' expressed. It has since become apparent that the gene expression of both eNOS and nNOS can also be affected by stimuli such as haemodynamic shear stress (in the case of eNOS). This nomenclature is now used to reflect the tissue of origin for the original DNA and protein isolates. Although eNOS was originally isolated and

cloned from vascular endothelium its expression has been described in blood platelets, cardiac myocytes and hippocampal neurons.

iNOS and nNOS are soluble and found predominantly in the cytosol, while eNOS is membrane associated.

nNOS was first isolated in the mammalian brain[14] but its role in cardiac function has since been described. nNOS activity is primarily regulated by increases in intracellular Ca2+, which activate nNOS through calmodulin binding [15]. The biological actions of NO rely upon the association of nNOS with specific protein complexes. These physical interactions with nNOS allow the integration of NO signalling into distinct transduction cascades.

In health eNOS has been found to reside primarily in the sarcolemma caveolae and endothelial cells whereas nNOS has been found to be associated with the ryanodine receptor (RyR) in the sarcoplasmic reticulum[16]. These specific locations may play a role in excitation-contraction coupling. In heart failure this relationship is altered. Damy and colleagues[17] examined the expression and localisation of NOS in health and heart failure. The myocardium of explanted hearts from patients who had undergone cardiac transplantation was compared with individuals who had died from head trauma or intracranial bleeds. The myocardium from patients with heart failure displayed increased nNOS mRNA and protein expression. The activity was associated with a translocation of nNOS to the sarcolemma via interactions with caveolin 3. This increased nNOS activity appeared to counterbalance a decrease in the activity and expression of eNOS[17].

NO generated by eNOS diffuses into adjacent vascular smooth muscle cells where NO activates guanylate cyclase, which induces smooth muscle relaxation by a variety of

mechanisms. Firstly, increased intracellular cGMP inhibits calcium entry into the cell, and decreases intracellular calcium concentrations. Secondly, activation of K^+ channels leads to hyperpolarization and relaxation. cGMP also stimulates a cGMP-dependent protein kinase (cGK) that activates myosin light chain phosphatase, the enzyme that dephosphorylates myosin light chains, which leads to smooth muscle relaxation.[18]

cGK also phosphorylates specific target proteins such as the 46/50-kDa vasodilator-stimulated phosphoprotein (VASP), the IP_3receptor and associated proteins, phospholamban, rapB1and the myosin binding subunit of myosin phosphatise[19, 20].

In platelets, vascular smooth muscle cells, endothelial cells, and fibroblasts, VASP is concentrated at and associated with actin microfilaments, focal adhesions, and cell-cell contacts [21, 22] VASP appears to be an important modulator of microfilaments and regulates spatially confined actin polymerization[23].

In the vasculature the exact function of VASP remains to be ascertained. Three cGK phosphorylation sites on VASP have been identified *in vitro* and in intact platelets: serine 157, serine 239, and threonine 278. [24, 25]

NO also diffuses into the vessel lumen where this highly reactive radical readily forms various NO metabolites. Whereas these 'metabolites' were previously considered 'metabolic dead-ends', they are now the focus of considerable attention.

The understanding of the role of NO in human biology has evolved from it being considered a simple vasodilator to being seen as an important and complex cellular signalling molecule with a vast array of biological functions.

NO appears to play an important role in modulating ischaemia-reperfusion injury(IRI). Jones and colleagues showed that eNOS deficient mice have increased infarct size following myocardial IRI [26]. In the light of this, numerous strategies and various exogenous pharmacological agents designed to manipulate NO have been examined. Exogenous administration of NO donors (e.g DETA-NO and SNAP) prior to, during or at reperfusion reduces infarct size and endothelial dysfunction[27].

The role of NO in the setting of acute ischaemia appears to be a double edged sword. While the studies quoted above support a protective role for NO when released at relatively low levels, higher concentrations have been shown to be harmful.

During severe ischaemia and infarction, the generation of superoxide and nitric oxide in endothelial cells results in the release of inflammatory mediators (e.g. platelet-activating factor, tumour necrosis factor). This increases the synthesis of adhesion molecules that mediate leukocyte-endothelial cell adhesion. The release of these inflammatory mediators, following reperfusion, appears to activate endothelial cells in remote organs that are not exposed to the initial ischaemic insult. The remote response to ischaemia-reperfusion results in leukocyte-dependent microvascular injury that is often seen in multiple organ failure.[28]

How exactly NO protects against IRI remains unclear and several mechanisms have been proposed e.g. inhibition of influx through L-type calcium channels, activation of sarcolemmal and mitochondrial KATP channels, antioxidant effects, activation of cyclooxygenase- 2 with production of cytoprotective prostanoids, and inhibition of proapoptotic proteins[29]. Currently, the role of NO in inhibiting cytochrome *c* oxidase on the electron transport chain thereby decreasing mitochondrial respiration and limiting oxygen consumption is an area of intense research and could hold important information about how NO exerts a beneficial effect following IRI.

Furthermore, NO also plays an important role in maintaining and encouraging blood flow by decreasing platelet aggregation and adhesion which may contribute to the protective effect of NO in IRI [30, 31].

In this context NO generated in an ischaemic environment can inhibit cytochrome *c* oxidase on the electron transport chain thereby decreasing mitochondrial respiration and limiting oxygen consumption. This then leads to decreased cellular damage.

NO also plays an important role in maintaining and encouraging blood flow by decreasing platelet aggregation and adhesion.

1.4 NO Metabolites

There has been extensive investigation into the relative importance of different NO stores (described below) and of the physiological importance (or otherwise) of NO released from these stores. NO is highly reactive and either rapidly exerts its physiological effect upon the underlying vascular smooth muscle or reacts to form NO metabolites such as HbNO, Nitrite and Nitrate. (Figure 1.1)

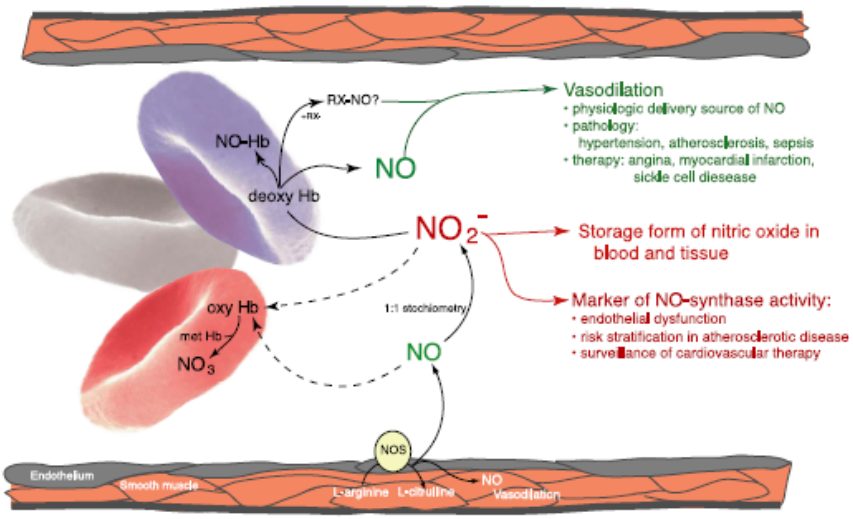


Figure 1.1 - NO metabolites in plasma and RBCs

NO has a very high affinity for heme groups[32] and reacts with oxyhaemoglobin to form methaemoglobin and nitrate. NO reacts with thiol (SH) containing amino acids, peptides and proteins to from S-nitrosothiols. These S-nitrosothiols include S-nitroso-albumin, S-nitroso-haemoglobin, S-nitroso-L-cysteine and S-nitroso-L-glutathione amongst others. There has been significant interest in these S-nitrosothiols, some holding that these metabolites act as the main store of transported NO and in particular it has been postulated that NO bound to haemoglobin (Hb) may act as this reservoir of NO[33]. Moreover, it has been suggested by Stamler and colleagues that the Hb molecule acts as both oxygen "sensor", in that it undergoes a conformational change when deoxygenated, as well as a NO reservoir[34]. Although Hb does not act as a sensor in the traditional sense its structural response to abmient oxygen tension has led to it being described in these terms. It is proposed that this conformational change allows the release of NO from Hb and hence delivery to the hypoxic tissue by this allosteric mechanism. In this model, as blood passes through the pulmonary circulation NO binds to deoxyhaemoglobin to form Hb-Fe(II)NO. During oxygenation NO is transferred to the highly conserved ß chain cysteine 93 residue of Hb to produce *S*-nitrosylhemoglobin (SNO-Hb). In hypoxaemic conditions the conformational change that Hb undergoes results in NO being transferred and some released in the process leading to vasodilatation[35]. (Figure 1.2)

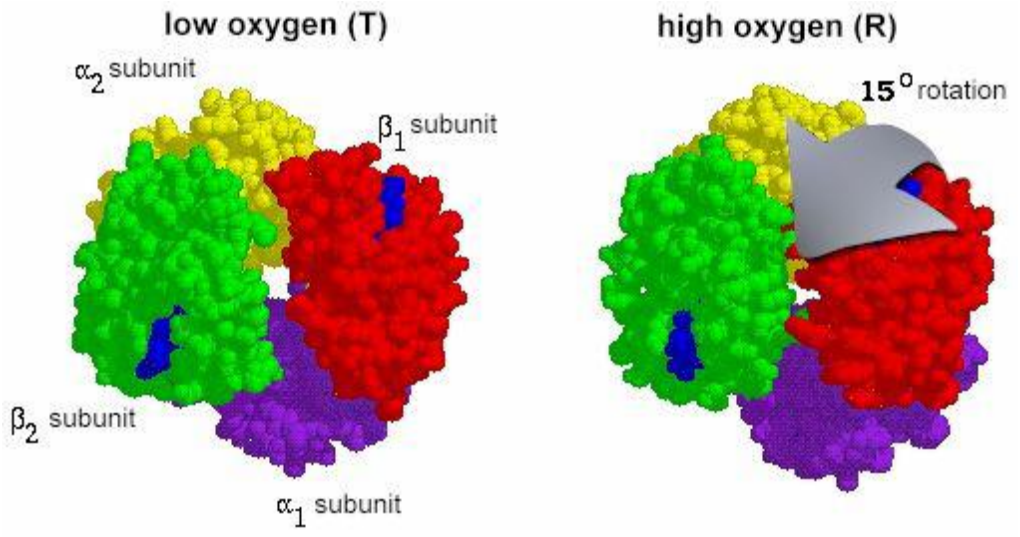


Figure 1.2 – Hb structure in high and low oxygen tension environments

1.5 Limitations of the HbSNO theory

In this model of NO transport, haemoglobin acts as both sensor of ambient oxygen tension and carrier molecule for the delivered NO. While inherently attractive this model has potential limitations. It has been suggested that the concentration of HbSNO *in vivo* is too small to produce a significant physiological effect[36]. This has been countered by the argument that while the levels may be relatively small this does not preclude a physiologically important role since the minute, picomolar, concentrations of NO allow a critically important role to be played by this key molecule.[37]

Differences exist over the relative concentration of HbSNO across the circulation. Proponents of the HbSNO model reported arterio-venous gradients of HbSNO which was felt to be supportive of a role for haemoglobin in NO transport. [4] Others, however, have failed to reproduce these findings. [38] These differences are confounded by the different techniques used by the various groups to determine *in vivo* HbSNO concentrations.

1.6 Descriptors of Oxygen Levels

In order to avoid misunderstandings that may arise from imprecise use of terminology it may be helpful to clarify basic terms at this point. Hypoxia refers to reduced oxygen levels in tissues whereas hypoxaemia refers to reduced circulating arterial free oxygen levels. Alternatively hypoxia could be defined as a failure of normal tissue oxygenation and hypoxaemia as a failure of normal blood oxygenation.

By way of nomenclature normoxaemia is defined as a pO2 in the range of 77mmHg to 100mmHg.[39] Free oxygen levels in excess of this range is defined as hyperoxaemia and conversely levels below this range are classified as hypoxaemic.

At a tissue level oxygen levels vary from 15mmHg in perivenous tissue to just under 100mmHg in periarterial tissue. Mitochondrial respiration is severely compromised by oxygen levels below 1.5mmHg and as such anoxia is defined as the range 0-1.5mmHg and hypoxia as 1.5 – 15mmHg.[39]

1.7 Inorganic Nitrite 'Rediscovery', Historical perspective

NOx species have been used for over a century in clinical medicine[40]. While the enthusiasm of chemists such as Alfred Nobel led to the discovery of the destructive powers of TNT the same curiosity has led to the use of the substances in the treatment of myocardial ischaemia. So successful was this discovery that patients continue to benefit from these anti-anginal properties to this very day. In 1844 amyl nitrite was synthesised by Antoine Balard and its effects subsequently studied by the English Chemist Fredrick Guthrie. When inhaled it was noted that amyl nitrite produced facial flushing, throbbing of neck arteries and tachycardia[40]. Based on these findings physicians used amyl nitrite successfully to treat angina pectoris prior to the development of glyceryltrinitrate (GTN). GTN superseded organic nitrite primarily due to its longer duration of action. Nitrite was subsequently placed back on the chemist's shelf and it has remained there until recent years. Interest has however been reignited as the concept of 'transported nitric oxide' has developed.

The role of *in*organic nitrite in normal physiology, however, continues to be the subject of hot debate. Although it is well recognised as a relatively weak direct dilator of arteries in normoxia, the potential for circulating nitrite in plasma to act as a reservoir of vasoactive NO that can be released when tissues are stressed by ischaemia/ hypoxaemia continues to be investigated.

1.8 The "Nitrite Theory"

This proposed model focuses on the nitrite anion as the main reservoir of 'transported NO'. For many years nitrite was considered to be a metabolic 'dead end' of NO oxidation since its vasodilatory properties were observed to be modest at most[41] or at best a metastable intermediate in the formation of nitrate from NO radicals, a cascade that was felt to be irreversible. Lauer and colleagues infused sodium nitrite into the brachial arteries of healthy volunteers at physiological doses (0.01–36 μmol/min) and observed no changes in forearm blood flow (FBF), indicating a lack of vascular dilatation[42]. Most of the early work, however, was performed *in vitro* and in normoxic conditions[41]. Interestingly in 1998 Furchgott noted in his Nobel lecture that under acidic conditions nitrite potently relaxed vascular rings. More recently *in vivo* work has rekindled interest in the nitrite anion. Studies by Gladwin and co-workers found that, in contrast to other NO metabolites, there are significant gradients of nitrite across the forearm vascular bed, particularly during exercise[43]. Intra arterial nitrite retained its vasodilatory effect after inhibition of nitric oxide synthase (with L-NMMA) and forearm exercise, from which a physiological role for the nitrite anion was suggested[5]. In contrast to the findings of Lauer and colleagues, more recent work has found nitrite to be capable of increasing forearm blood flow when infused into the brachial artery at physiologically relevant doses[44, 45]. Intrabrachial infusions of nitrite at concentrations as low as 0.07μg/kg/min, leading to a rise in plasma nitrite levels from 300nM to 350nM, have been reported to lead to a modest but significant increase in Forearm Blood Flow in healthy human volunteers. [46]

1.9 Nitrite Reduction

A variety of different nitrite reductases have been identified. These include pH-dependent enzyme-independent nitrite conversion to NO,[47] deoxyhemoglobin,[48-54] myoglobin,[55-59] neuroglobin,[60] aldehyde oxidase,[61] microsomal cytochrome P-450[62], cytochrome C[63, 64], and mitochondrial aldehyde dehydrogenase[65]. However the relative importance of these different mechanisms *in vivo* remains unclear. Since the different proposed reductases exhibit tissue specificity, synergy, redundancy and sensitivity to incipient conditions[66]; and since nitrite is reduced via a number of mechanisms *ex vivo*, there are significant challenges to translating *in vitro* nitrite reducing capacity to *in vivo* physiology. For example, there has been controversy regarding the role of red blood cells (RBCs), hemoglobin (Hb) and deoxyhemoglobin in nitrite metabolism.[48-54, 67-69] Although there is a clear chemical rationale for the Fe^{II} centre of Hb to reduce nitrite, the NO scavenging capacity of the plentiful heme groups in RBCs has led to ongoing discussion regarding their likely net NO yield.[61] To reconcile this discordance, the membrane compartment hypothesis contends that RBC membrane eNOS and XOR generate NO at a distance from the RBC heme, hence the NO is free to diffuse to the vasculature.

Similarly, building on existing evidence that the vasculature has intrinsic nitrite reductase activity,[70] and that XOR and eNOS have been implicated as nitrite reductases for several years;[58, 59, 61, 68, 70-72], it has recently been reported that nitrite conversion to NO by the vasculature and/or by RBCs is attenuated by inhibition of xanthine oxido reductase (XOR) at very low oxygen tensions at pH 6.8. Furthermore, it has been shown that RBC endothelial nitric oxide synthase (eNOS) inhibition with L-NMMA diminishes nitrite bioconversion.[73] These proposals have merit as they reconcile the long held belief that RBCs yield NO with the concern that high RBC Hb concentrations would attenuate NO release through scavenging. However, while these findings may be applicable to nitrite bioconversion in the context of pronounced pathological hypoxia, the implication of these observations to the vasodilation observed with pharmacological concentrations of nitrite under normoxic and mildly hypoxic conditions is unclear.

Zuckerbraun and co-workers. have recently reported that inhaled nitrite confers protection in a rodent model of pulmonary hypertension and that this protection appears to be dependent on XOR[74].

Recently, work by our Group investigating the role of XOR and NOS in nitrite bioconversion during mild (physiological) hypoxia in healthy human volunteers has demonstrated that the role of these enzymes under these conditions appears to be minimal (chapter 4).

1.10 Limitations of the Nitrite theory

This model has been challenged by some. Luchsinger and colleagues (2005) question the interpretation of many of the findings mentioned above, in particular the relative concentration of nitrite in the resistance versus the capacitance bed [75]. The arterio-venous nitrite gradients that have been observed are interpreted to reflect decreased NOS activity coupled with increased nitrite consumption rather than a gradient created by the delivery of NO by nitrite. Moreover the authors argue that any vasodilatation that is observed with nitrite is effected via the formation of SNO-Hb which they contend is a much more potent vasodilator.

Furthermore, deoxyhaemoglobin has been proposed as a nitrite reductase. [76] However, this model has been seen as problematic. If nitrite is reduced in the red blood cells to NO then a mechanism needs to exist to enable NO to 'escape' since deoxyhaemoglobin will aggressively bind to any NO in its vicinity. Both the "membrane compartment hypothesis" and the suggestion that an 'intermediary molecule' may be generated in the reduction of nitrite, have been proposed as theories that may reconcile this apparent discrepancy. It has been proposed that this intermediary may be N_2O_3. [77] Under this proposal N_2O_3 may be formed via a series of reactions that are initiated by the reduction of nitrite to form methaemoglobin and NO. The methaemoglobin thus formed reacts with nitrite to form Hb-NO_2^-. The Hb-NO_2^- then reacts with NO to form Hb and N_2O_3. [77]

1.11 Formation of Nitrite and the role of Caeruloplasmin

The principal mechanism leading to nitrite generation was previously thought to be auto oxidation of NO[78]. While this has been demonstrated to occur *in vitro* its relevance in the physiological setting is less clear. The estimated half-life for this reaction ($k = 2x10^6M^{-2}s^{-1}$) is over thirty minutes.[79] In contrast the reaction of NO with haemoglobin to form nitrate is orders of magnitude greater ($k = 3.4x10^7M^{-1}s^{-1}$)[80] and would result in any auto-oxidation of NO to be completely overwhelmed leading to NO being converted almost exclusively to nitrate with little or no nitrite generation. For this reason mechanisms underlying the generation of nitrite were investigated. It was found that whereas plasma displays the ability to generate nitrite upon the addition of the NO donor Diethylamine nitric oxide (DEANO), phosphate buffered saline (PBS) did not, implying that an intrinsic component of plasma facilitates NO oxidation to nitrite.[81] Furthermore it was found that this process was inhibited by reductants such as glutathione and ascorbate but not by oxidants or by the thiol alkylating agent N-ethylmaleimide (NEM). Ultrafiltration of the plasma to remove high molecular weight proteins similarly decreased nitrite production focussing attention on caeruloplasmin. Removal of caeruloplasmin from plasma by immunoprecipitation decreased nitrite production to levels observed in PBS strongly suggesting a critical role for this enzyme in nitrite production. This was given further weight by the observation that caeruloplasmin knockout mice exhibit significantly lower plasma nitrite levels than their wild type equivalents, 0.51± 0.05µM vs and 0.79± 0.08µM respectively and the response to the addition of an NO donor was significantly blunted. These observations were mirrored in patients with acaerulaplaminaemia. The important role played by caeruloplasmin was re-emphasised by the finding that hepatic injury sustained during ischaemia-reperfusion was greater in knockout mice and that this was ameliorated by the exogenous administration of intraperitoneal nitrite[81].

1.12 Diet and endogenous Plasma Nitrite levels

Approximately 70% of plasma nitrite is formed intravascularly as a metabolite of NO generated from eNOS, the remainder being dietary in origin, [82] Spinach and Beets being particularly rich sources of nitrite (2.0 to 4.0mg/kg food) along with baked goods and cured meats. Reduction to NO takes place in the acidic conditions of the stomach leading to the NO generated being absorbed into the bloodstream where it is then oxidised to Nitrite.[83]

Plasma nitrite levels are also influenced by dietary nitrate intake. While small quantities of nitrate are present in fish and dairy produce, larger quantities are present in vegetables e.g lettuce and beetroot. The inorganic nitrates are absorbed into the bloodstream and concentrated in the salivary glands. Interestingly commensal bacteria in the oral cavity (posterior crypts of the tongue) and intestine appear to play an important role in the generation of nitrite due to the nitrate reductase properties of these organisms. Plasma nitrite levels were noted to increase after an oral nitrate load with attenuation of this effect following the administration of a mouthwash beforehand.[84]

Physical activity leads to increased arterio-venous gradients. This fact combined with variations in the nitrite pool secondary to dietary factors and social factors (e.g.smoking) leads to variance in measured plasma nitrite levels. However plasma nitrite levels tend to fall within the 150nM to 500nM range. [85] Levels have been found to be significantly increased in individuals suffering an intercurrent infective illness. Asymptomatic HIV patients were found to have normal levels of plasma nitrite (0.4µM) whereas those suffering an infective illness (Pneumonia/ CMV retinitis) had markedly raised levels (36.2µM). [86]

Similarly the physiological changes of pregnancy are associated with lower levels of circulating nitrite.[87] Levels of 0.48µM were observed in pregnant women in the third trimester as compared to 1.13µM in non pregnant women. No significant difference was seen between pre-eclamptic and non pre-eclamptic women.

Plasma nitrite concentrations are profoundly affected by physiological adaptations to altititude.[88]

The nitrite pool is composed of both a red blood cell (RBC) and a plasma component. RBCs contribute a larger fraction to this pool. The concentration in RBC's is approximately double that in plasma (121nM and 288nM in plasma and RBCs respectively). [89]

Both plasma and RBC Nitrite levels are significantly higher in some rodents as compared to man. Levels as high as 1.6μM have been reported in mouse plasma[90] and 10μM in Sprague-Dawley rats[91].

1.13 Nitrite and "The Mediterranean diet"

As mentioned above the dietary content of nitrite and nitrate contribute to plasma nitrite content. It has been hypothesised that the benefits of the "Mediterranean diet" may be explained by greater levels of nitrite and nitrate intake. The amount of vegetables consumed on average in higher is the Mediterranean diet as compared to most European and Northern American diets. In the UK the average daily vegetable consumption has been reported as 160mg per day [92] whereas Trichopoulos reported average vegetable consumption of 550g per day in the Mediterranean diet; approximately three times higher.[93] The greater nitrite/nitrate consumption that results from a greater vegetable intake (approximately 4-fold) has been suggested to contribute to the beneficial effects of the Mediterranean diet.

The Mediterranean diet has been associated with reduced morbidity and mortality. A recent meta-analysis suggested the Mediterranean diet was associated with a significant reduction of overall mortality [relative risk (RR) = 0.92], cardiovascular incidence or mortality (RR = 0.90), cancer incidence or mortality (RR = 0.94), and neurodegenerative diseases (RR = 0.87).[94, 95] A number of studies have reported an advantageous effect of increased inorganic nitrate intake upon human blood pressure profile. [95, 96] With the background of these observations Raat and colleagues examined the effect of a nitrate/ nitrite deplete diet versus a diet leading to normal plasma nitrite levels upon liver ischaemia reperfusion injury in mice.[97] Dietary nitrite depletion led to

significantly lower stomach content, plasma, liver and heart levels of nitrite. The severity of ischaemia reperfusion injury as assessed by liver infarct size was significantly greater in the animals receiving a nitrate/ nitrite deplete diet.

An interesting study by Webb and colleagues investigated the acute effects of an inorganic nitrate load (in the form of 500ml beetroot juice) upon blood pressure, endothelial function and platelet aggregation. The nitrate load equated to 2.79g/L; nitrite levels were undetectable. Ingestion of 500ml of water served as the control arm. Subjects receiving beetroot juice demonstrated a significant 16 fold increase in nitrate which remained elevated for almost 24 hours. Plasma nitrite levels in the study arm doubled and remained significantly elevated for 5 hours. There was no significant change in plasma nitrate or nitrite levels in the control group. Beetroot juice ingestion was associated with a significant 10.4mmHg drop in systolic blood pressure and 8.1mmHg drop in diastolic blood pressure, interestingly without a significant increase in heart rate. This drop in blood pressure was mirrored by an improvement in endothelial function (as measured by flow mediated dilatation) following I-R insult and an inhibition of platelet aggregation. Interruption of entero-salivary circulation by spitting out saliva over the 24 hour study period resulted in an abolition of the rise of nitrite levels but no change in the rise in nitrate levels. Spitting resulted in the eradication of the beneficial effects upon blood pressure and platelet function suggesting that the favourable effects of beetroot juice ingestion were nitrite rather than nitrate mediated.[98] A more recent study by the same group found the results mentioned above were reproduced when Beetroot juice was replaced by an equivalent quantity of inorganic nitrate in the form of potassium nitrate.[99] These results could potentially lead to improvements in public health through dietary interventions aimed at improving blood pressure control and hence cardiovascular fitness.

1.14 Mitochondrial Efficiency

A fascinating recent paper by Larsen and colleagues investigated the impact of oral sodium nitrate loading upon exercise capacity. [100] It has previously been reported that oral loading of

inorganic nitrate increases exercise capacity. A randomised double blind placebo controlled trial investigating oxygen consumption during exercise, with and without an oral nitrate load was performed. Healthy volunteers were randomised to receive oral sodium nitrate (0.1mmol/kg/day) or placebo (sodium chloride). All subjects performed cycle ergometer exercise tests before and after treatment at increasing exercise intensities. It was found that at equivalent exercise intensities, at the four lowest levels, the oxygen consumption (V02) was significantly lower in the nitrate treated group. This was despite a similar heart rate, lactate [Hla], ventilation (VE), VE/Vo2 and respiratory exchange ratio. These observations effectively re-wrote the received wisdom that oxygen consumption was fixed for a given workload and were the first to demonstrate an intervention that could modulate VO2 for a specified level of exercise.[101]

A more recent study by the same group investigated this phenomenon further.[100]

In a double blind cross-over trial Larsen and colleagues sought to ascertain the mechanisms underpinning this improved oxygen consumption efficiency. 14 healthy subjects were randomised to receive either oral sodium nitrate (0.1mmol/kg/day) or placebo (sodium chloride) for a total of three days prior to testing with a 6 day 'washout' prior to crossover. At the end to each stage a muscle biopsy and submaximal cycle ergometer test was performed. The mechanical work to oxygen uptake was significantly improved by 3% in the nitrate treated group, as previously described. Analysis of the muscle biopsies revealed a higher respiratory control ratio in the mitochondria of the nitrate treated subjects, indicating better coupling between respiration and oxidative phosphorylation. This increased oxidative phosphorylation efficiency suggests that a greater proportion of the membrane potential was dedicated to ATP synthesis and less was wasted as a result of uncoupling. This phenomenon was appeared to be due a reduced expression of ATP/ADP translocase. The results of this study not only inform our understanding of exercise physiology but may also prove relevant to the protective effect of

nitrite in the ischaemia-reperfusion setting because this channel appears to be a constituent of the mitochondrial permeability transition pore, the opening of which is central to IRI [102]. Further studies are clearly indicated.

1.15 Dietary Nitrate/ Nitrite – Health concerns

Public health concerns were voiced regarding dietary nitrate ingestion in 1945.[103] Nitrates have long been used as plant fertilisers and anxieties have been expressed regarding the contamination of drinking water by such agents. Children in Iowa, USA drinking reconstituted milk that had been prepared using water from rural wells were found be suffering from vomiting, cyanosis and failure to thrive. Laboratory examination revealed evidence of methaemoglobinaemia which corrected when alternative water supplies were used. This observation was attributed to a high nitrate content in the well water. Further studies have suggested that it was in fact the combination of high nitrate content and bacterial contamination that was causing harm. It was argued that the bacterial contamination lead to reduction of nitrate to nitrite which then oxidised oxyhaemoglobin (ferric) to methaemoglobin (ferrous) *in vivo*. [104]

$$NO_2^- + \text{oxyHb}(Fe^{2+}) \rightarrow \text{metHb}(Fe^{3+}) + NO_3^-$$

However since the initial concerns legislation across the industrial world is in place to limit the nitrate content of drinking water.

While fatal (sometimes deliberate) poisoning due to nitrite ingestion in adults has been reported [105] no cases of methaemogloinaemia have been reported in (non infant) children and adults secondary to elevated nitrate levels in drinking water. There are a number of reasons for this. Firstly, the pH of gastric contents is higher in infants (>4.0) resulting in less degrading of nitrite and hence more

absorption. Secondly, the methaemoglobin reductase enzyme system does not mature until after weaning. Thirdly, the intakes of food and water in infants is roughly three times that of adults relative to body weight. Fourthly, the levels of reducing agents such as, ascorbic acid, secreted in the stomach of infants of much lower than that of adults.

Magee and Barnes reported that preformed nitrosamines and nitrosamides are carcinogenic in animals. Anxieties were expressed regarding the potential for ingested nitrates and salivary nitrites to nitrosate ingested amines to form potentially carcinogenic nitrosamines.

Epidemiological studies do not correlate with these theoretical concerns since it has been reported that populations which consume a high vegetable intake (and therefore high inorganic nitrate) enjoy lower rates of malignancy.[106]

Studies investigating the potential link between dietary nitrate and gastrointestinal malignancy have produced conflicting results. Hill and colleagues found higher than average rates of death from gastric cancer in Worksop in 1973 prior to restrictions on the nitrate content of drinking water [107]. The average weekly nitrate consumption was twice that of the national average (980mg vs 440mg). Interestingly although death rates from gastric cancer were significantly greater in this population when compared to towns with a similar socio-economic make up, rates of gastric cancer per se were not significantly elevated. Moreover the authors report significantly increased rates of death from gastric cancer in Sutton-in-Ashfield where the dietary nitrate content was no higher than other 'control towns'. This observation casts doubt on the assumption of a causative link between the increased death rates and dietary nitrate intake in Worksop. More recent retrospective epidemiological studies have found no positive association between malignancy rates and nitrate intake. Some even report and negative correlation.[108] The case against a causative role is supported by the temporal separation between ingested amines and the formation of nitrite via the entero-salivary circulation. Moreover gastric pH rises during a meal leading to an environment that is hostile to the formation of nitrosamines.[109] Gastric secretions contain ascorbic acid which promotes reduction of nitrite to NO and helps prevent any potential nitrosamine formation.[110] The World

Health Organisation [111] and the American Cancer Society [112] have each concluded that there is no evidence for a link between inorganic nitrate consumption and increased risk of cancer.

1.16 Non NO mediated Nitrite Effects

Sodium Nitrite has an established use in the treatment of hydrogen sulphide and cyanide poisoning. The pharmacological doses of inorganic nitrite leads to the formation of abundant levels of methaemoglobin which acts as a scavenger, deactivating both hydrogen sulphide and cyanide. [113]

Nitrite infusion over 24 hrs has been shown to lead to alterations in the expression of heat shock proteins in a range of organs. These effects were not inhibited by NO scavengers. Oxy-Hb had no effect *in vitro* and C-PTIO had no effect *in vivo*. As such it has been suggested that nitrite may act as a signalling molecule in its own right at normal physiological oxygen levels.

1.17 Therapeutic Avenues

There is currently great interest in the potential role of the nitrite anion in pathological states, particularly in the context of ischaemia-reperfusion in many organ systems[114]. The role of nitrite in hypoxic signalling includes vasodilatation, cytoprotection following ischaemic attack and alteration of mitochondrial respiration, potentially through both NO and non-NO mediated pathways.

Gonzalez and colleagues (2008) investigated the effect of administering intravenous nitrite systemically in a canine model of myocardial infarction. An impressive 70% reduction in myocardial infarct size (as a percentage of the area at risk assessed by first pass myocardial perfusion magnetic resonance imaging) was observed in these animals.[115] Furthermore the infusion of nitrite to achieve

plasma concentrations of between 5 - 10µmol/L tended to result in subendocardial infarcts as opposed to transmural infarcts in the control group.

The cytoprotective properties of nitrite may be mediated via hypoxic NO release mediated by myoglobin resulting in inhibition of cytochrome oxidase C.[116]

In vitro studies utilising horse heart homogenate observed that deoxymyoglobin acted as a potent nitrite reductase 36 times faster than deoxyhaemoglobin. The NO generated from nitrite was shown to inhibit mitochondrial respiration in rat heart and liver isolates.[76] NO has previously been shown to inhibit mitochondrial respiration by binding to cytochrome *c* oxidase particularly at lower oxygen concentrations. [117]

Infusion of nitrite containing perfusate into isolated pig lungs has been shown to lead to a decrease in pulmonary vascular resistance and an increase in exhaled NO. [118]

In support of an enhanced role for nitrite in hypoxia, *in vitro* nitrite administration has been shown to confer protection against ischemia/reperfusion injury in the kidney, brain and liver.[119-122] Duranski and co-workers (2005) found that intraperitoneal injections of nitrite (48nmol) in a murine model of hepatic ischaemia reperfusion injury led to significantly lower rises in serum tranaminase levels despite a very modest rise in serum nitrite levels (594 to 727nM). Similarly intraventricular administration of nitrite conferred protection after coronary artery occlusion and reperfusion. 48nmol of nitrite injected into the left ventricle resulted in an impressive 67% decrease in infarct size. In these studies neither nitrate nor buffer conferred any protection to ischaemia reperfusion injury. [123]

In addition, nitrite treatment has also proved beneficial in models of hypertension [124] and pulmonary hypertension [125]

The potential benefits of nitrite are not however limited to the setting of acute ischaemia reperfusion injury. Kumar and colleagues (2008) studied the effects of chronic nitrite injection (given and twice daily intraperitoneally) in chronic hind limb ischaemia induced through common femoral artery ligation in mice. Nitrite administration was found to result in a greater vascular density through angiogenesis and the formation of collaterals as well as endothelial cell proliferation. Over a seven day period hind limb blood flow returned to close to normal with nitrite treatment but not without.

The NO scavenger carboxy PTIO abolished these effects suggesting that they are mediated through the generation of NO.[126]

Nitrite may have a positive role to play in the treatment cerebrovascular accidents. 'Thread occlusion' of the middle cerebral artery was employed to induce cerebral infarction in adult male rats [127]. Intravenous infusion of sodium nitrite at the time of reperfusion resulted in a significant reduction in cerebral infarct size measured using triphenol tetrazolium chloride (TTC) staining 24 hours after ischaemia reperfusion. Infusion of 48nmol and 480nmol of nitrite produced a 33% and 77% reduction in infarct respectively. Interestingly higher doses of nitrite (4800nmol) failed to achieve the same outcome. Infusion of sodium nitrate (480nmol) failed to demonstrate any reduction in infarct magnitude. The protective effects of nitrite infusion were abolished by pre treatment with Carboxy-PTIO. These results may translate into clinical benefit. Neurological outcomes were assessed using Modified Limb Placing and Rotorod behavioural tests. Nitrite treated animals demonstrated a 25% improvement as compared to the saline treated group.

Subarachnoid haemorrhage (SAH) often presents with catastrophic consequences in young patients. While the initial bleeding leads to significant cerebral damage, late vasospasm has been recognised as a serious life threatening c omplication. This has traditionally been treated prophylactically with nimodipine. Pluta and workers (2005) investigated the part that nitrite infusion may play in treating this complication [128]. SAH was induced in cynomolgus monkeys and angiographic evidence of delayed vasospasm was assessed. Continuous intravenous infusion of sodium nitrite, with additional daily boluses, resulted in no observable angiographic spasm. In contrast, animals treated with intravenous saline all displayed angiographic vasospasm with one of this control group dying as a consequence.[128]

Chronic Heart Failure (CHF) is often complicated by Diastolic Ventricular Interaction (DVI) leading to left Ventricular (LV) filling being compromised despite high left ventricular diastolic pressure[129]. A volume overloaded Right Ventricle forces the septum to be pushed towards the LV and also leads

to a rise in pericardial pressure resulting in LV constraint. In this situation, relief of this DVI leads to an improvement in cardiac output. An 'un-balanced' vasodilator that preferentially dilates the venous capacitance bed more than the resistance bed could potentially achieve relief of DVI. Organic nitrates remain an important and widespread form of treatment for patients with both acute and chronic heart failure. However, organic nitrates suffer from a number of major drawbacks in the treatment of decompensated heart failure. They markedly dilate resistance as well as capacitance vessels at higher concentrations, with the resulting hypotension often limiting dose escalation. Furthermore, resistance and the development of tolerance are significant clinical issues[130]. Intra-arterial nitrite infusion leads to a relatively selective venodilatation[131] and therefore has the potential of being invaluable in the treatment of patients suffering with heart failure particularly during an acute decompensation. We have recently studied the vascular effects of nitrite in patients with chronic heart failure and found the 'venoselectivity' to be preserved at higher doses although at lower doses the arterial response to nitrite in the patients group appears to be amplified possibly as a result of greater resting oxidative stress in these subjects (chapter 3). Furthermore, recent published data suggests that continuous treatment with nitrite may not induce tolerance[46] seen with organic nitrate treatment.

1.18 Future Directions

A wealth of *in vitro* and *in vivo* animal data presented herein has demonstrated the potential role of nitrite as a potential reservoir of bioactive NO. This alongside the paucity of human data available has paved the way for human studies particularly in disease states, such as heart failure and ischaemic conditions. The impressive reduction in myocardial infarct size demonstrated raises the possibility of nitrite being used as adjunctive therapy in conditions affected by ischaemia-reperfusion injury. The resultant salvaging of critically ischaemic but viable tissue could lead to profound improvements in patient recovery. Patients with heart failure may benefit from a vasodilator with selective venodilatory properties and lack of tolerance. Further work needs to be undertaken in this area to fully elucidate the benefit of nitrite in human pathology.

1.19 The current studies presented in this thesis

With this context in mind we designed a series of studies to investigate the role of inorganic nitrite in health and heart failure.

Our aims were:

1) To investigate the role of nitrite in hypoxic vasodilatation at physiological and pharmacological doses.
2) To investigate the responses of the vasculature in both health and heart failure subjects.
3) To examine the mechanisms that may subserve the conversion of nitrite to NO
4) To study the effects of systemic infusion of sodium nitrite and to determine its safety prior to larger investigations.
5) To investigate the role of myoglobin in mediating NO release and the persistent vasodilatation observed. (This aim developed during the study).

Our first objective was to establish the effect, in man, of intra-arterial sodium nitrite infusion upon both the resistance and capacitance beds as models of high oxygen tension environments and low oxygen tension environments respectively.

Secondly, in order to determine whether any difference between the two beds was due to hypoxia *per se* and not due to an intrinsic difference between arteries and veins, we proposed to render the arterial circulation hypoxaemic (by volunteers breathing 12% oxygen) and to compare the resistance bed response in hypoxia to that in normoxia.

Thirdly, to establish the effects to intra brachial nitrite infusion upon both the resistance and capacitance beds of patients with stable chronic heart failure, comparing the responses with those of healthy controls.

Fourthly, to determine the role of Nitric Oxide Synthase and Xanthine Oxidase mediating the vasodilatation and venodilatation induced by an intrabrachial infusion of nitrite at rest, in man breathing room air.

Fifthly, to establish the effects to intravenous nitrite infusion upon peripheral blood pressure, central aortic pressures, cardiac output, total peripheral resistance, cardiac index, heart rate variability and baroreceptor sensitivity in healthy volunteers at rest.

Finally to determine, using murine vascular rings, to the role of vascular myoglobin in mediating nitrite induced vasodilatation and the persistent relaxation observed in these studies. We proposed to inhibit myoglobin using carbon monoxide and employ the use of myoglobin knockout mice (with and without myoglobin transfection) to establish its role.

In the following chapter I will describe the first set of experiments that provided the basis for all subsequent studies. We aimed to establish the effect of inorganic nitrite upon the vasculature of healthy volunteers at rest in normoxia and hypoxia.

Chapter 2

Hypoxic Modulation of exogenous nitrite induced vasodilation in man

The data presented in this chapter has been published in the journal "Circulation". The reference is as follows:

- Maher AR, Milsom AB, Gunaruwan P, Abozguia K, Ahmed I, Weaver RA, Thomas P, Ashrafian H, Born GV, James PE, Frenneaux MP. *Hypoxic modulation of exogenous nitrite-induced vasodilation in humans. **Circulation***. 2008 Feb 5;117 (5):670-7

Data has also been presented at international meetings as follows:

- Maher AR, Gunaruwan P, Abozguia K, Palin TP, James PE, and Frenneaux MP. *Sodium Nitrite is a Potent Venodilator*. The 56th Annual American College of Cardiology (ACC) scientific sessions, March 2007, New Orleans, USA

- Maher AR, Gunaruwan P, Abozguia K, Palin , James P, Frenneaux MP. Venoselectivity of Nitrite: Evidence favouring Hypoxic release of NO. The Annual Scientific Conference of The British Cardiovascular Society (BCS) June 2007, Glasgow, UK

Execution

I performed all of the recruitment and organisation of the appointments involved in the study. I performed assessment of forearm blood flow, radionuclide plethymography and radionuclide ventriculography studies in the University of Birmingham. Biochemical analysis was performed by Dr Phil James and his team at the University of Cardiff, Wales. The *in vitro* work described in this chapter was performed by Alexandra Milsom, University of Cardiff, Wales.

Abstract

Background: It had been proposed that under hypoxic conditions nitrite may release Nitric Oxide (NO) causing potent vasodilatation. We hypothesized that nitrite would have a greater dilator effect in capacitance than in resistance vessels due to lower oxygen tension, and that resistance vessel dilation should become more pronounced during hypoxemia. The effect of intra-arterial infusion of nitrite on Forearm Blood Flow (FBF) and on Forearm Venous Volumes (FVV) was assessed during normoxia and hypoxia.

Methods and Results: 40 healthy volunteers were studied. Following baseline infusion of 0.9% saline, sodium-nitrite was infused at incremental doses from 40nmol/min to 7.84µmol/min. At each stage, FBF was measured using strain gauge plethysmography. FVV was assessed by radionuclide plethysmography. Changes in FBF and FVV in the infused arm were corrected for those in the control arm. The peak percentage venodilation during normoxia was 35.8%±3.4% (Mean±SEM) at 7.84µmol/min (P<0.001) and similar during hypoxia. In normoxia, arterial blood flow, assessed by the FBF-ratio (FBFR), increased from 1.04±0.09 (baseline) to 1.62±0.18 (nitrite) (P<0.05) vs 1.07 ± 0.09 (baseline) to 2.37 ± 0.15 (nitrite) (P<0.005) during hypoxia. This was recapitulated *in vitro*, in vascular rings.

Conclusions: Nitrite is a potent venodilator in normoxia and hypoxia. Arteries are modestly affected in normoxia but potently dilated in hypoxia, implying the important phenomenon of hypoxic augmentation of nitrite mediated vasodilation *in vivo*. There are therapeutic implications for the use of nitrite as a selective arterial-vasodilator in ischemic territories, and as a potent venodilator in heart failure.

Key words: Nitric oxide, nitrite, veins, hypoxia

2.1 Introduction

Debate surrounds the relative importance of different endogenous NO species in mediating physiological vasodilatation. These species include nitrite, S-nitrosothiols, N-nitrosamines, iron-nitrosyls, and nitrated lipid.[132] Nitrite has traditionally been considered a weak vasodilator. In aortic specimens concentrations of nitrite as high as 100-1000μM are typically required under normoxic conditions to induce relaxation.[132, 133] The discrepancy between plasma nitrite levels *in vivo* and the levels required to elicit a biological effect *in vitro* coupled with a study demonstrating lack of vasodilator activity of 200 μM nitrite in the forearms of healthy volunteers, diminished interest in nitrite.[134] Nitrite thus ceded to hemoglobin acting as an NO store, oxygen sensor and condition-sensitive NO donor, operating through release of the NO group from SNO-Hb. [33]

More recently nitrite has been recognized as a powerful signaling molecule and regulator of gene expression.[135, 136] Whilst in normoxia nitrite is a relatively inefficacious vasodilator when acting directly, during hypoxia this may be enhanced. NO released from nitrite as a result of an hypoxic environment may play an important physiological role.[132] Arteriovenous gradients of nitrite in the human forearm have been observed; these gradients are enhanced during exercise and during regional NO synthase inhibition.[5, 44] Thus it has been suggested that nitrite is consumed to liberate bioactive NO.[5, 44, 132] A vasoactive role for nitrite at normal physiological concentrations is still disputed as the arteriovenous gradient in nitrite may result from decreased venous nitrite production through decreased NO synthase (NOS) activity coupled with increased venous nitrite consumption.[75]

It has recently been proposed that deoxyhemoglobin is capable of acting as a nitrite reductase; hence hemoglobin would act as ‘oxygen sensor’ and would liberate NO from

nitrite along the physiological oxygen gradient. The work of Gladwin and others is at least hypothesis generating with respect to a role for nitrite in the modulation of vascular tone *in vivo*.[132]

In support of an enhanced role for nitrite in hypoxia, *in vitro* nitrite administration has been shown to confer protection against ischemia/reperfusion injury in the heart, kidney, brain and liver.[119-122] In addition, nitrite treatment has also proved beneficial in models of hypertension [124], pulmonary hypertension [125] and cerebral vasospasm .[137]

Accordingly, it was hypothesized: (1) While breathing room air, nitrite would be a potent dilator of capacitance vessels *in vivo* because of their relatively low ambient oxygen tension; (2) On breathing 12% oxygen and rendering the resistance vessels relatively hypoxic, nitrite would become a potent arteriolar-dilator.

This chapter focuses on the forearm vascular bed specifically assessing the resistance vasculature and the capacitance vasculature (composed principally of the small veins and venules which behave quite differently from conduit veins such as dorsal hand veins.) [138] To test the direct effect of nitrite on vascular tissue (haemoglobin-independent), isolated rabbit vessels (aorta and vena cava) were exposed to a range of nitrite concentrations in both normoxia and hypoxia.

2.2 Methods

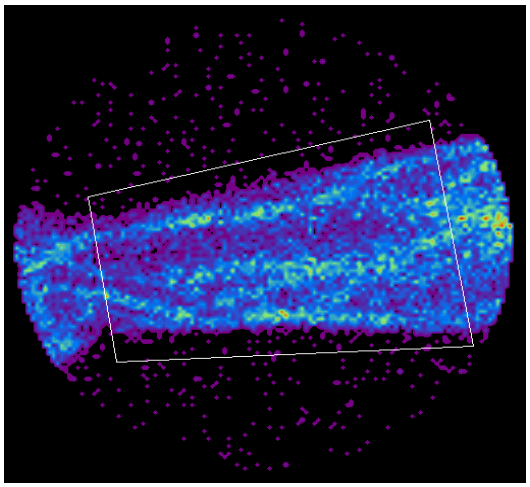
2.2.1 Subjects

Twenty six healthy volunteers were recruited to Protocol A and fourteen to Protocol B. Subjects had no history of active smoking, hypertension, diabetes, hypercholesterolemia or family history of ischemic heart disease. None took cardioactive medication or vitamin supplements. All had a normal cardiovascular examination, electrocardiogram and gave written informed consent. The study was approved by the Local Research Ethics Committee. Investigations were performed at the University of Birmingham Clinical Research Block in a dedicated vascular laboratory (22 to 24°C) with subjects having had a light breakfast and abstained from caffeine-containing drinks for at least 6hrs.

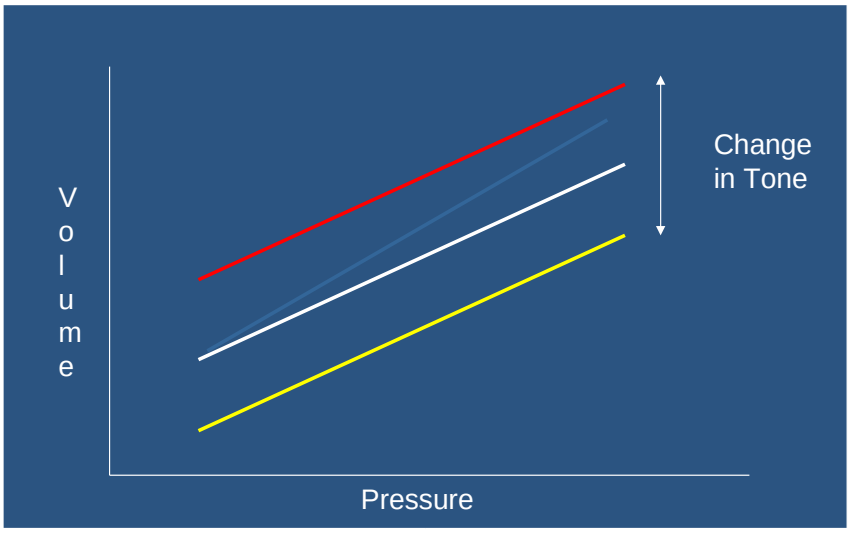
2.2.2 Measurement of Venous Volume

FVV was assessed using radionuclide plethysmography. The details and advantages of the technique have been described.[139] Briefly, after the administration of Stannous Fluoride venous blood was withdrawn, radio-labeled with Technetium99m and then re-injected into the subject after ten minutes incubation. After fifteen minutes for stabilization images were obtained. A region of interest was defined and images were obtained during 1-minute acquisition periods. At least 90% of the injected isotope is confined to the intravascular space; therefore forearm radioactive counts are proportional to forearm blood volume. Since the vast majority of blood in the peripheral circulation is contained within the veins, changes in counts reflect changes in FVV.[139] During each cycle of acquisition cuffs were inflated over both upper-arms at pressures of 0, 10, 20 and 30mmHg. From this data scintigraphic vascular volumes were plotted against cuff pressure to form venous Pressure Volume Relationship

(PVR). A parallel shift of the relationship indicated a change in venous tone.[139] Data were acquired from both the study and control arm and any changes observed were corrected for those occurring in the control arm. This technique has been validated by a number of groups.[139-141] Importantly, it has been shown that the position of the PVR is not altered by large changes in arterial inflow.[138] To allow presentation of grouped data, results were presented as percentages. The data were corrected for physical decay of technetium.



Example of image from which scintigraphic vascular volumes were potted.



Parallel shifts of the volume – pressure relationships represent changes in tone.

2.2.3 Biochemistry

Chemicals and reagents

Chemicals were purchased from Sigma other than glacial acetic acid, HPLC grade nitrite free water and hydrochloric acid which were purchased from Fisher Scientific.

Sodium nitrite in the human studies was purchased from Martindale Pharmaceuticals, UK.

Ozone-based chemiluminescence

Plasma nitrite and protein-bound NO were measured using a tri-iodide reagent linked to ozone based chemiluminescence as described previously.[142] The tri-iodide reagent is probably the most widely used in the NO metabolite field and has been validated against standards in several laboratories.[5, 143-146] The reader is referred to a complete discussion of the assay.[33, 45, 142, 147-151]

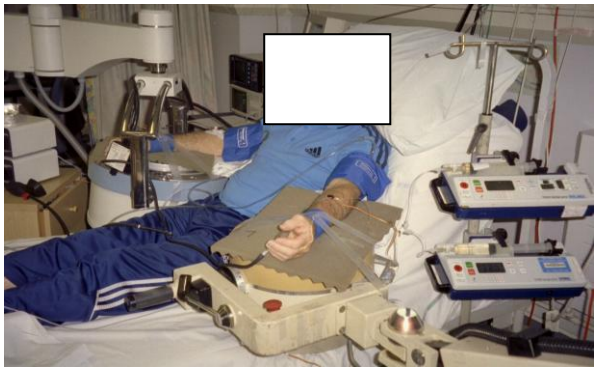
Stock solution of tri-iodide reagent was prepared fresh each day.[142, 146] Immediately prior to analysis frozen plasma samples were thawed in a water bath at 37°C for 3 minutes. We have previously shown sample freezing has no effect on plasma NO metabolite stability.[152] Area under curve was used in analysis and concentrations were calculated from a standard curve of sodium nitrite.

2.2.4 Study Protocols

Protocol A (Normoxia)

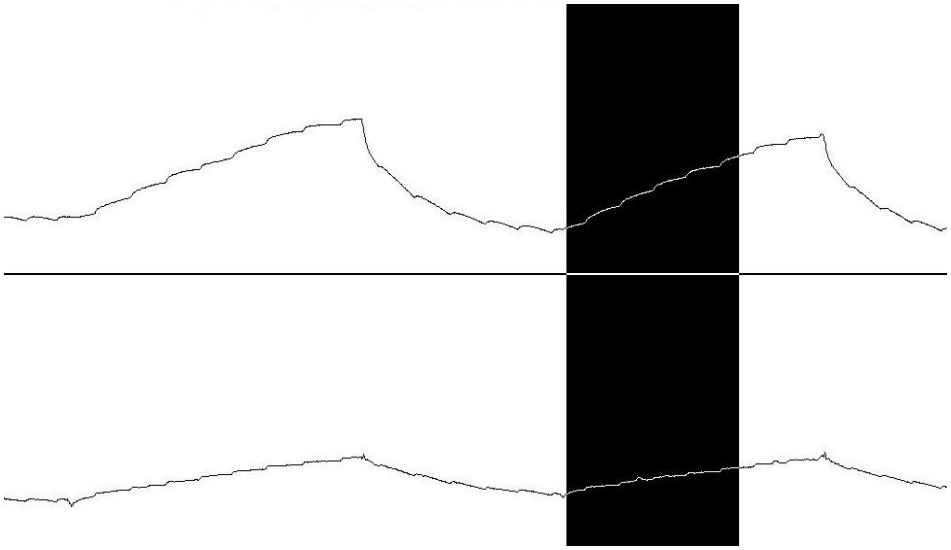
Subjects were rested supine. Both forearms were positioned on the face of a gamma camera (Scintron, MIA and ADAC-Transcam). A 20-gauge venous cannula was inserted into an ante-cubital vein in each arm. Blood was drawn and sent for a Full Blood Count and Biochemical Profile. A 27-gauge arterial needle (Coopers Engineering, United Kingdom)

mounted onto a 16-gauge epidural catheter and sealed with dental wax was then inserted into the non-dominant brachial artery under sterile conditions and kept patent by the continuous infusion of normal-saline.


Example of subject during a study.

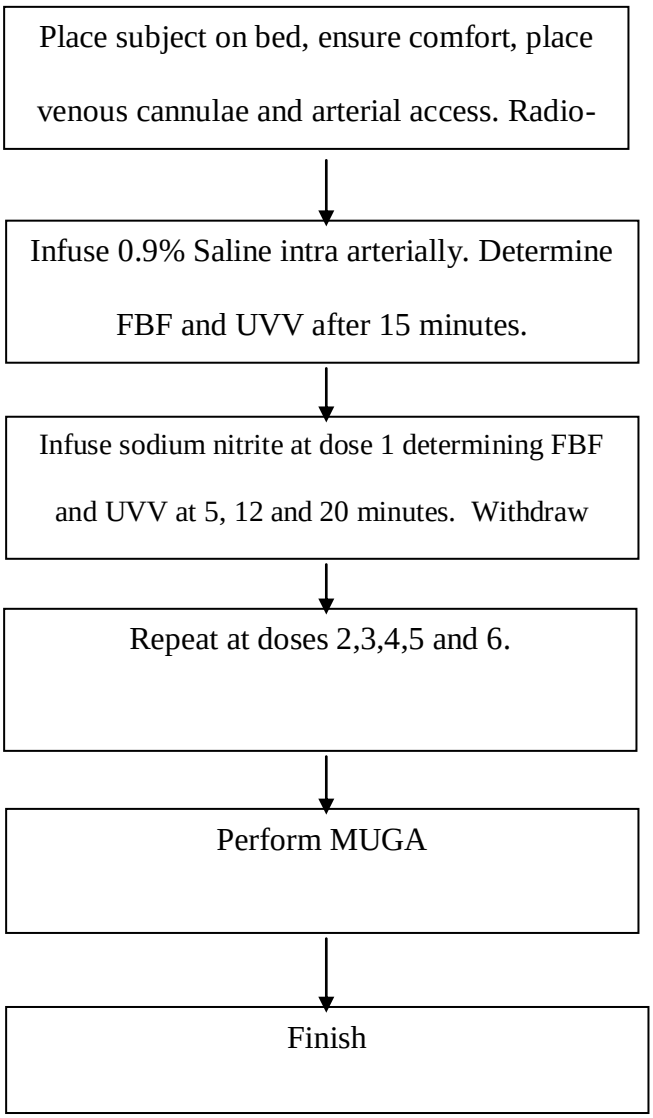
Heart rate and rhythm were monitored continuously (Dash 3000, Marquette, GE). Blood pressure was monitored continuously with finger plethysmography (TNO TPD Biomedical Instrumentation, Delft, Netherlands). Mercury in silastic strain gauges were used for the measurement of FBF (D.E. Hokanson, Inc., Bellevue, Washington), as described in the published literature.[153] Changes in FBF observed in the study arm were corrected for those occurring in the control arm and expressed as a ratio (FBFR) (infused:control). Two venous PVR were recorded and FBF was assessed during the infusion of normal-saline. Blood was drawn from both venous cannulae (infusion and control arm) at baseline and at each stage of the study. Blood samples were transferred after blood gas analysis (Bayer Rapidlab 865) into 4ml EDTA collection tubes and were centrifuged at 2000rpm for 10 minutes at 4^0C. Plasma was snap frozen in liquid nitrogen and stored at -80ºC for subsequent analysis. Plasma nitrite and protein-bound NO were measured at each stage in both protocols.

Intra-brachial nitrite was then commenced at a dose of 40nmol/min for thirty minutes. FVV was assessed at 5, 12 and 20 minutes followed by the assessment of FBF. The above was then repeated with doses of nitrite of 100nmol/min, 314 nmol/min , 784 nmol/min, 3.14µmol/min and 7.84µmol/min. Left ventricular ejection fraction was calculated by Multiple Gated Acquisition. In a subset of subjects (N=3) radial arterial samples were taken at baseline and at peak nitrite infusion in which levels of nitrite and protein-bound NO were measured.



Example of FBF determination using strain gauge plethysmography.

Protocol A


Place subject on bed, ensure comfort, place venous cannulae and arterial access. Radio-
Infuse 0.9% Saline intra arterially. Determine FBF and UVV after 15 minutes.
Infuse sodium nitrite at dose 1 determining FBF and UVV at 5, 12 and 20 minutes. Withdraw
Repeat at doses 2,3,4,5 and 6.
Perform MUGA
Finish

Protocol B (Hypoxia)

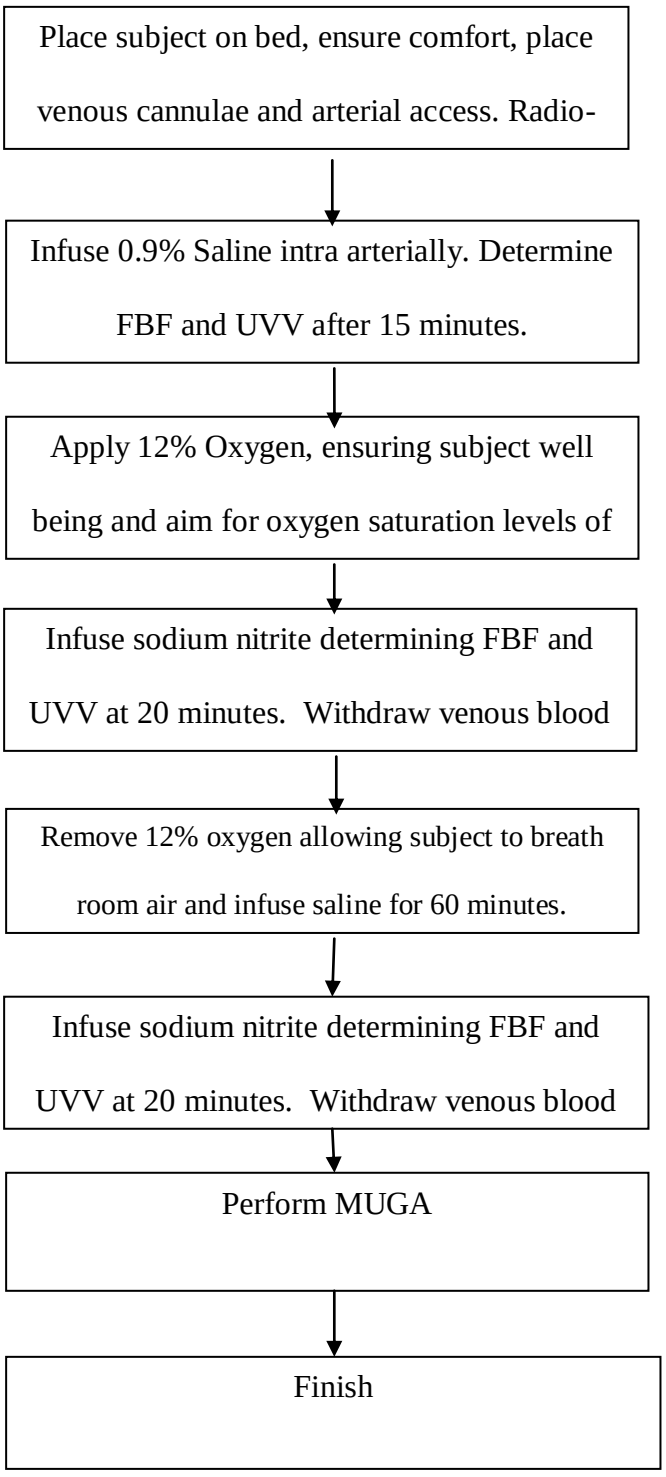
Subjects were prepared as for protocol A. Oxygen saturation levels were monitored continuously using pulse oximetry (Nellcor N-180). Baseline measurements of all parameters were taken during the initial saline infusion.

Hypoxia was induced with the subject breathing 12% oxygen via a facemask connected to a two way valve. When arterial oxygen saturation levels (as measured by pulse oximetry) were stable in the range 83-88% [estimated p02 = 47.5-55mmHg, based on Severinghaus' equation], FVV and FBF were assessed during saline infusion ('hypoxia alone') prior to the infusion of nitrite.

In seven subjects intra-brachial nitrite was infused at a dose of 7.84μmol/min for 20 minutes in either normoxia or hypoxia, in random order. Saline was infused between the two infusions of nitrite for 60 minutes. FVV was assessed at ten minutes into each nitrite infusion followed by the assessment of FBF.

In the remaining seven subjects protocol B was performed using nitrite at a dose of 314nmol/min in order to investigate the effects of hypoxia in the context of a plasma concentration of nitrite close to that found physiologically.

Protocol B


Place subject on bed, ensure comfort, place venous cannulae and arterial access. Radio-
Infuse 0.9% Saline intra arterially. Determine FBF and UVV after 15 minutes.
Apply 12% Oxygen, ensuring subject well being and aim for oxygen saturation levels of
Infuse sodium nitrite determining FBF and UVV at 20 minutes. Withdraw venous blood
Remove 12% oxygen allowing subject to breath room air and infuse saline for 60 minutes.
Infuse sodium nitrite determining FBF and UVV at 20 minutes. Withdraw venous blood
Perform MUGA
Finish

2.2.5 Vascular Tissue Assay

These studies were perfomed by Dr Alex Milsom, School of Medicine, Cardiff University, Cardiff, United Kingdom

Male New Zealand white rabbits (2 to 2.5Kg) were terminally anaesthetized by intravenous injection of sodium pentobarbitone (0.75ml/Kg). Abdominal aorta and vena cava were excised and placed in fresh Krebs buffer (NaCl 109.0mM, KCl 2.7mM, KH_2PO_4 1.2mM, $MgSO_4.7H_2O$ 1.2mM, $NaHCO_3$ 25.0mM, $C_6H_{12}O_6$ 11.0mM, $CaCl_2.2H_2O$ 1.5mM). Vessels were cleaned and cut into 2mm rings. Rings were mounted on matched stainless steel hooks for isometric tension recording in 8ml baths containing 5ml of Krebs's buffer.

Tissue was allowed to equilibrate for 60 min under a resting tension of 2g at 37^0C, the buffer being exchanged every 15 min. After final adjustment of the passive tension to 2g, vascular segments were constricted with phenylephrine (PE, 1µM). Once tension had reached a plateau, acetylcholine (10 µM) was added to demonstrate endothelium viability.

Following extensive washout, under normoxic conditions (95% O_2/5% CO_2) vessels were again constricted with 1µM PE, and sodium nitrite in Krebs's buffer (10µM, 100µM, and 1000µM final concentration) was administered. Under hypoxic conditions (95% N_2/5% CO_2 resulting in ~1% tissue bath O_2) the tissue was first incubated for 10 minutes before exposure to 3µM PE (to achieve a similar gram tension observed at 95% O_2) before administration of sodium nitrite. Tension was recorded for a further 20 minutes using 'Chart for Windows' (AD Instruments). Minimum tension achieved was used in all calculations. All relaxations are presented as a % of the maximum tension induced by PE under the relevant conditions.

2.2.6 Data and Statistical Analysis

All data are expressed as mean ± SEM, and *P* values of <0.05 were considered statistically significant. Repeated measures analyses were carried out for changes in FBFR and FVV. *In vitro* data was analyzed using two-way ANOVA.

2.3 **Results**

Subject characteristics

Table 2.1: Grouped baseline characteristics for all subjects. Continuous data are presented as mean±SEM.

Demographic and Clinical Features	
Age (y)	56.5±1.4
Sex, M/F	27/6
Body mass index (kg/m^2)	27.0±0.6
Smoking history, (n)	
Nonsmoker	32
Previous smoker	1
Current smoker	0
Serum cholesterol (mmol/L)	5.6±0.2
Plasma glucose (mmol/L)	4.8±0.1
Serum Creatinine (µmol/l)	99.6±3.1
Heart rate	64.0±2.0
Blood pressure (mmHg)	
Systolic	129±2.2
Diastolic	73.1±1.7
Left Ventricular Ejection Fraction	58.3±2.7

2.3.1 Protocol A: Normoxia

Venous Tone

At doses of nitrite 784nmol/min, 3.14µmol/min and 7.84µmol/min very large decreases in forearm venous tone were observed (Figure 1). The time course over which the dilatation occurred is presented in Table 2 and Figure 2. The peak venodilation(%) at 3.14µmol/min

and 7.84μmol/min was (mean ± SEM) 20.6± 4.2 (P<0.05) and 35.8± 7.5 (P<0.005) respectively.

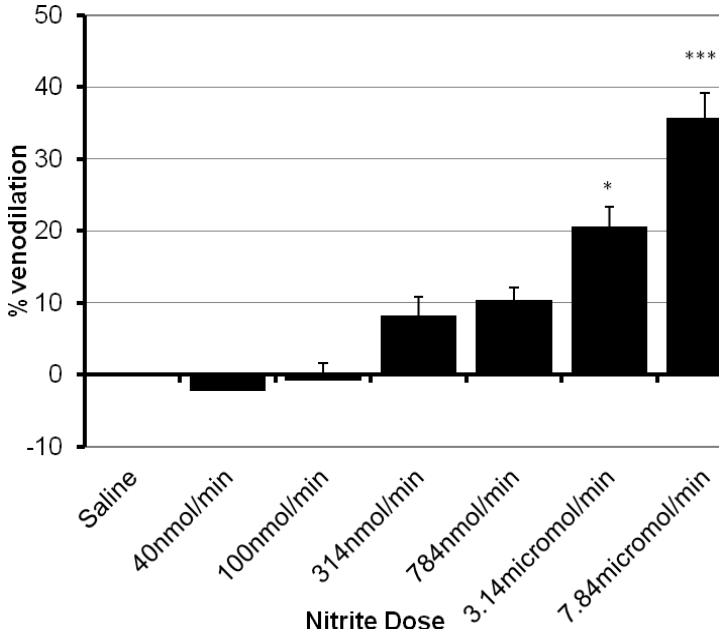


Figure 2.1: Change in peak forearm vascular volume under normoxic conditions. Data expressed as percentage change from baseline (saline infusion). All values are presented as mean±SEM, where * = P<0.05, and *** = P<0.001 compared with saline infusion.

Dose of Nitrite	% venodilation 5 minutes	% venodilation 12 minutes	% venodilation 20 minutes
40nmol/min	-2.2 ± 1.7	-2.8 ± 2.7	-4.2± 1.5
100nmol/min	-5.1 ± 2.6	-3.1 ± 2.3	-0.8± 4.6
314nmol/min	1.6 ± 3.1	8.2 ± 4.0	8.1± 4.3
784nmol/min	4.0 ± 2.5	7.3 ± 2.7	10.3± 2.8
3.14μmol/min	17.6 ± 4.1	19.1 ± 3.3*	20.6± 4.2*
7.84μmol/min	29.1 ± 10.1**	30.7 ± 4.3***	35.8± 7.5***

Table 2.2. Venodilation at doses of sodium nitrite at 5, 12 and 20 minutes of infusion. All values are presented as mean±SEM, where * = P<0.05, ** = P<0.005 and *** = P<0.001 compared with baseline.

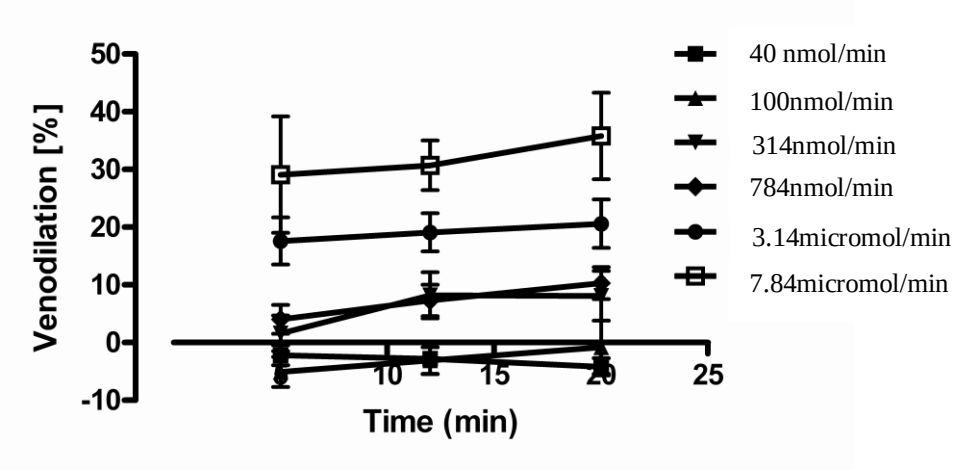


Figure 2. 2: Venodilation measured at 5, 12 and 20 minutes during infusion of nitrite at each of the infusion doses during normoxia.

Arterial Blood Flow

Only at 3.14µmol/min and at 7.84µmol/min were there noticeable increases in FBFR (Figure 2.3). At baseline the FBFR was 1.0 ± 0.1 (mean ± SEM). At 3.14µmol/min and 7.84µmol/min the FBFR (mean ± SEM) was 1.8 ± 0.3 and 1.6 ± 0.2 respectively (P<0.05).

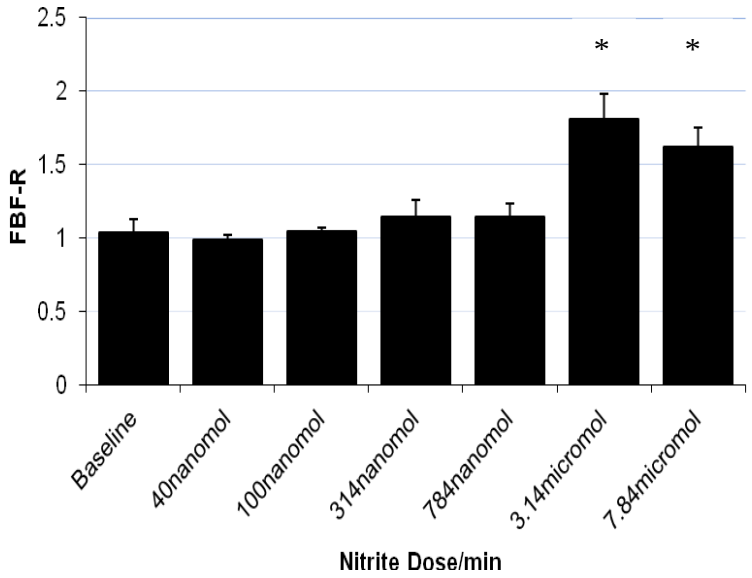


Figure 2.3: Forearm blood flow at incremental doses of sodium nitrite under normoxic conditions. Data expressed as a ratio of infused:control arm (FBF-R) to correct for any systemic changes, values are mean±SEM, where * = $P<0.05$,

Plasma Nitrite and Protein-bound NO levels

Venous plasma nitrite levels were significantly greater in the infused versus control arm at doses of 314nmol/ml upwards ($P<0.05$) (Figure 2.4)

There was no statistically significant difference the venous levels of plasma protein-bound NO between the two arms at any dose (Figure 2.4).

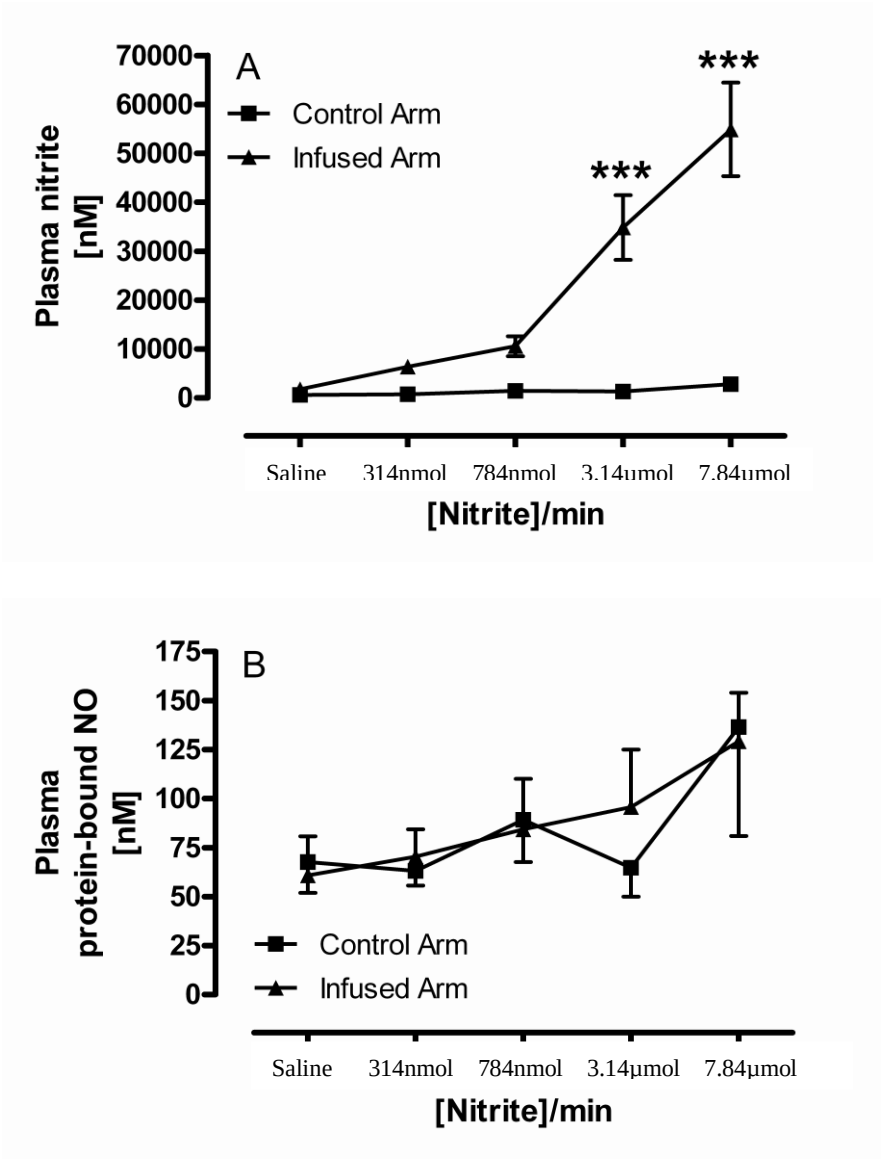


Figure 2.4: Plasma nitrite (A) and protein-bound NO (B) levels under normoxic conditions. All values are presented as mean±SEM, where *** = $P<0.001$ compared with control arm.

Arterial levels of plasma nitrite in the infused arm increased from 233nM ± 27.0nM at baseline to 34.1µM ± 12.3 µM (Figure 5A) ($P<0.05$). Arterial levels of plasma protein-bound NO in the infused arm increased from 64.0nM ± 5.8nM at baseline to 83.8nM ± 23.8nM (Figure 5B) (P=NS).

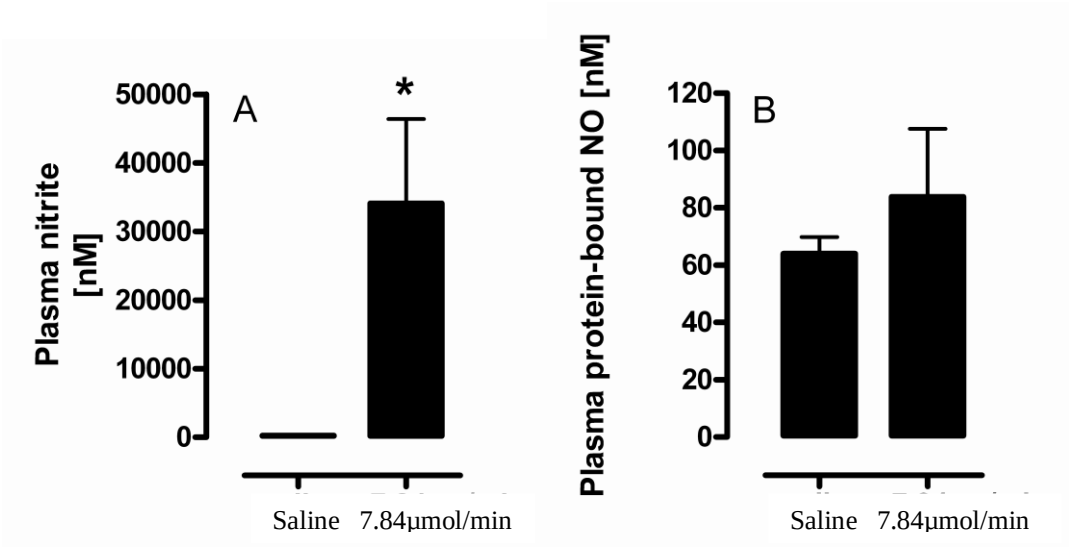


Figure 2.5. Arterial levels of plasma nitrite (A) and protein-bound NO (B) in the infused arm under normoxic conditions. Values presented as mean±SEM, where * = $P<0.05$,

2.3.2 Protocol B: Hypoxia

Venous Tone and Forearm Blood Flow

The degree of venodilation in response to nitrite infusion was similar in patients breathing room air to those breathing 12% oxygen. However hypoxia significantly enhanced the increase in FBFR during nitrite infusion. The mean increase in FBFR during high dose nitrite infusion (7.84micromol/min) in hypoxia was significantly greater than that in normoxia ($P<0.05$) (Figure 2.6). Low dose nitrite (314nmol/min) had little effect during normoxia but increased FBFR during hypoxia ($P<0.05$) (Figure 2.7).

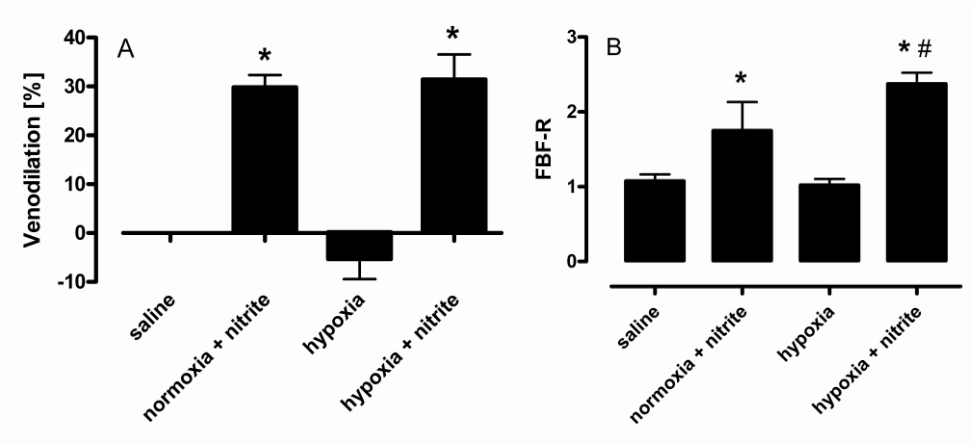
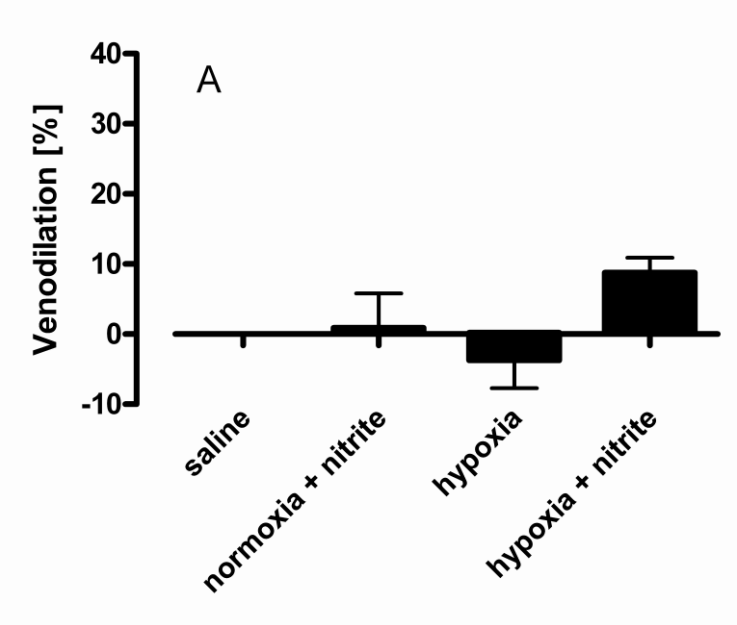


Figure 2.6: The effect of hypoxia on the venodilatation (A) and FBF-R (B) induced by intrabrachial nitrite (7.84μM/min). Values presented as mean±SEM, where * = $P<0.05$ compared with baseline and # = $P<0.05$ compared with normoxia and nitrite.



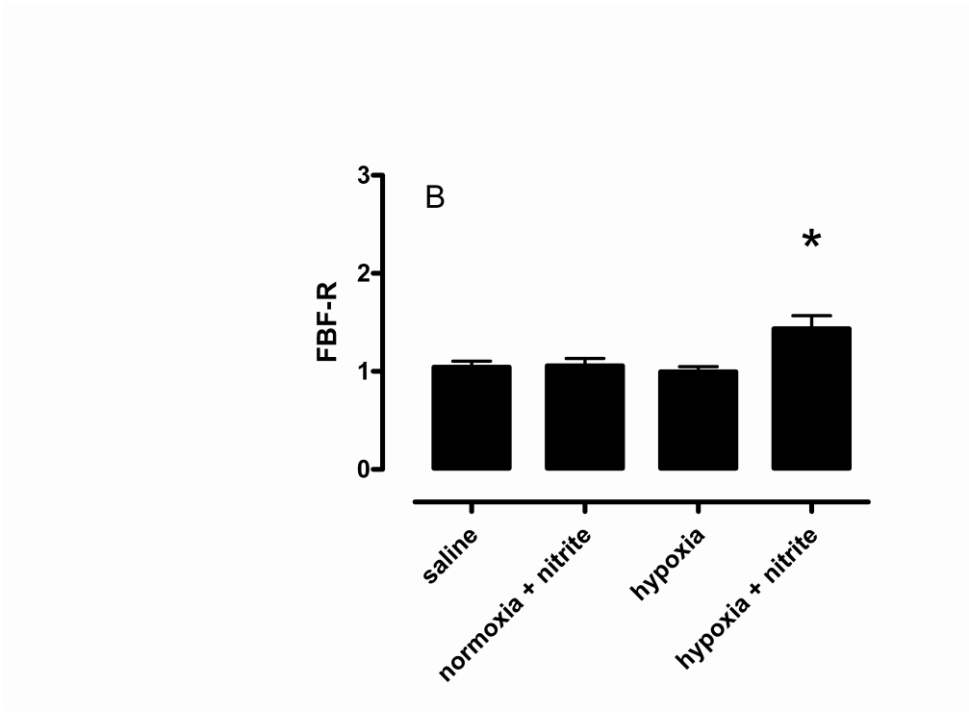


Figure 2.7: The effect of hypoxia on the venodilatation (A) and FBF-R (B) induced by intrabrachial nitrite (314nM/min). Values presented as mean±SEM, where * = $P<0.05$ compared with normoxia and nitrite.

2.3.3 *In vitro* Work

Nitrite-induced vessel relaxation increased proportionally with increasing nitrite concentrations in both aorta and vena cava ($P<0.05$) (Figure 2.8). No statistical difference was observed between the degree of nitrite-induced relaxation in the aorta versus vena cava under normoxia.

Hypoxia enhanced nitrite-induced relaxation in both vessel types when compared with relaxation under normoxia, however veins had a proportionally greater relaxation when compared with arteries at 100 and 1000µM nitrite (P<0.01).

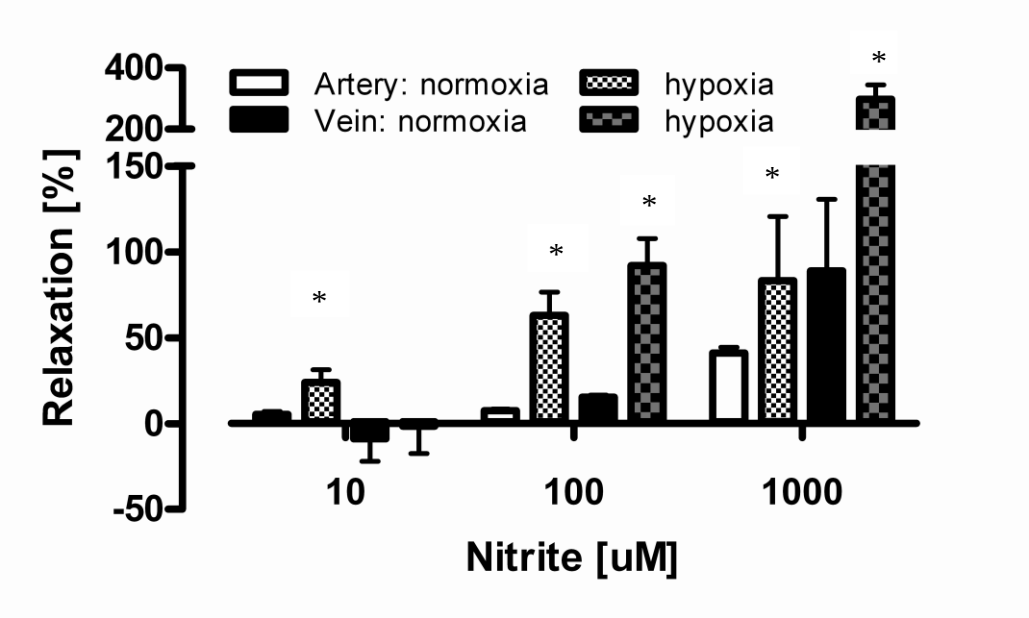


Figure 2.8: Relaxation of aorta and inferior vena cava following 20 minutes incubation with varying nitrite concentrations, under either normoxic or hypoxic conditions. Data presented as mean±SEM, each data set is n = 4-5. * = P<0.05 hypoxia vs normoxia.

2.4 Discussion

This study showed that under normoxic conditions exogenous nitrite-induced vasodilation in humans occurred predominantly in capacitance vessels. Under these conditions nitrite only modestly increased FBF (x1.6) even at maximal arterial nitrite concentrations (~30 μM). Although hypoxia had no incremental influence on the effects of nitrite on capacitance vessels, at similar concentrations (~55 μM) it had a profound relaxing effect on resistance vessels. The *in vitro* studies confirmed this influence of hypoxia on the nitrite-induced relaxation in both arteries and veins suggesting that oxygen tension is a major determinant of the degree of response to nitrite.

Until recently the nitrite-anion was regarded as a relatively inert by product of NO metabolism and even as a potentially carcinogenic source of N-nitroso compounds.[154] Recently, research has shown nitrite to be a distinct and important signalling molecule[136] Normoxic studies with animal aortic strips appeared to preclude a physiological role,[133] but in contradistinction a physiological function for nitrite has been supported by the capacity of physiological nitrite concentrations (~1-2 μM) to dilate human resistance vessels.[44]

We have now shown that exogenous nitrite is a potent dilator of the venous capacitance bed in humans, under both normoxic and hypoxic conditions. At peak dose we observed a ~40% venodilation; this is a large effect. Since up to 70% of the circulating blood volume resides within the capacitance bed, even subtle modifications of venous tone can result in large changes in central blood volume and cardiac 'preload'. Accordingly, a strong selective venodilator may confer distinct benefits in the treatment of heart failure. We have previously used carbachol extensively in studies assessing venous capacitance.[155] At peak dose a

maximal 3-4 fold increase in forearm blood flow and a 40% venodilation was observed [138]. Thus nitrite exerts similar venodilatory effects to carbachol with comparatively modest effects on resistance vessels during normoxia.

The enhanced resistance vessel response to nitrite during hypoxia is consistent with the findings of Cosby et al who observed that the vasodilatory effects of intra-arterial nitrite were enhanced by the tissue hypoxia induced by exercise of the forearm.[44] Moreover our study answers many of the questions that were raised following this publication and in particular demonstrates that hypoxia per se is a primary determinant in the action of nitrite. We found that in normoxia low dose nitrite (314nmol/min) had no discernable effect on FBF, whereas Cosby et al observed a 29% increase in FBF in response to nitrite infused at 400nmol/min in normoxia, but Lauer et al reported that exogenous nitrite had no effect at all on FBF even at doses as high as 36 micromol/min.[134] The reasons for this discrepancy may pertain to the differences in the subjects and the experimental protocols although such differences are not obvious as the preparation of nitrite and its infusion were similar. It is unlikely that our subjects differed significantly in sympathetic stimulation[156] It is however possible that there is heterogeneity in the handling of nitrite. In our own study we found a significant degree of variation in the responses observed. While in the majority of individuals nitrite doses as low as 314nmol/min had little measurable effect on FBFR, in a few subjects the effect was more pronounced. In one individual the FBFR increased by 27%. Such heterogeneity may explain some of the apparently contradictory findings in the literature and provides an interesting avenue for future work.

While in normoxia nitrite was a weak vasodilator of resistance vessels when compared with agents such as acetylcholine[157] and bradykinin[158] that can induce a 3-4 fold increase in FBF,

in hypoxia we show that it becomes an arterial vasodilator of comparable potency to these agents. Importantly, a significant augmentation in FBFR during hypoxia was also seen with low dose (314nmol/min) nitrite implying a possible physiological role under hypoxic conditions.

Our vascular ring studies confirmed that, (in the absence of haemoglobin), nitrite-induced dilatation of veins and arteries is more profound in the context of hypoxia than in normoxia confirming the findings *in vivo*. *In vitro* during hypoxia relaxation of veins was greater than that of arteries. However it is not valid to directly compare potency between arteries and veins and extrapolate to the *in vivo* situation because of the unphysiological nature of the preparation which measures relaxation from a preconstricted state rather than changes in basal tone.

Although this study confirms a role for hypoxia in facilitating exogenous nitrite-induced vasodilation, no conclusion can be drawn about the mechanisms subserving this effect. Both non-enzymatic chemical reactions[159] and nitrite reductase species [160] as diverse as hemoglobin,[44, 45, 161] xanthine oxidase, [122] endothelial NOS [162] and the mitochondrial enzymes cytochrome *c* oxidase and the bc1 complex [163] reduce nitrite hypoxically *in vitro*. However their relative contribution to the hypoxic vasodilatatory response *in vivo* remains unresolved.[75, 132, 160] In subjects infused with nitrite, NO adducts of hemoglobin are increased and it is suggested that hemoglobin is a key nitrite reductase.[44] Hemoglobin can subserve a number of roles, including nitrite reductase in hypoxia; as an acceptor of the resulting NO (e.g. through HbNO); and as donor of NO precisely where it is required i.e. at the site of hypoxia (through HbSNO). These multiple personalities make hemoglobin an attractive nitrite modulator. A recent *in vitro* study has observed that nitrite-dependant vasodilation

conforms to first-order reaction kinetics with respect to nitrite concentration. Using selective inhibitors during hypoxia, this study indicated that nitrite-induced vasorelaxation was independent of nitrite reductases listed above and importantly was inhibited by oxygenated haemoglobin but not by deoxygenated hemoglobin.[164] This study did not exclude a role for haemoglobin as a nitrite modulator *in vivo*. If nitrite is subject to hemoglobin-mediated NO production, how the resulting products or NO itself escape from the red blood cell is not yet clear as such an escape is difficult to reconcile with the diffusional limitations and the time constraints of an extremely quick A to V transit time.[75]

To reconcile nitrite's site and mechanism of action *in vivo*, vascular preparations will need to be further investigated. Specific agents and inhibitors are required to identify putative nitrite reductases, e.g. haemoglobin, myoglobin, neuroglobin and other heme proteins such as hypoxia-dependent nitrite reductases.[165] The species to which nitrite is converted and which activates guanylate cyclase e.g. NO, iron-nitrosyl, N-nitroso and S-nitroso complexes will also need to be identified. Parallel *in vivo* studies will supplement the *in vitro* studies. In the present study the venous plasma nitrosothiol levels rose similarly in both arms suggesting that protein-bound NO, in the plasma compartment, did not directly mediate the venous dilatation. This however does not preclude protein-bound NO within the red blood cells or within the vascular compartment contributing to the bioactive effects of nitrite.

2.5 Limitations

First, our study was performed in healthy volunteers and the response to nitrite may differ in disease. Second, organ chamber bioassays are limited by the complicating factor of varying baseline tension in pre-contracted vessels.[75] As different vessels (capacitance vs resistance)

show different and non-linear responses, such bioassay results should be assessed judiciously and any interpretation of apparent differences between vessel types should be guarded. Nonetheless, our *in vivo* and *in vitro* studies are internally consistent. Third, our results increase the understanding of the synergy between nitrite and hypoxia but provide limited insights into the role of endogenous nitrites in physiology. We speculate that this study may also implicate a role for endogenous nitrites in hypoxic vessels, but we have limited any discussion of the role of nitrite in physiological hypoxic vasodilatation.[75] Even in *in vitro* assays where unphysiologically high concentrations of nitrite (100μM) have previously been required, careful attention to pre-contraction and incipient conditions reveals that physiological concentrations of nitrite (0.1-10μM) can modulate tone.[164]

2.6 Conclusion

This study confirms that hypoxia *per se* is a prime determinant of the *in vivo* response to exogenous nitrite. Both capacitance and resistance vessels respond profoundly to relatively modest pharmacological concentrations of exogenous nitrite in hypoxia. While for some years, based on studies in normoxic and exercising patients, it has been speculated that "therapeutic application of nitrite should result in selective vasodilation to hypoxemic tissue, and could be used to treat diseases associated with ischemia,"[44] this study confirms the role for nitrite in resistance vessel dilatation in human hypoxic tissues. There are corresponding therapeutic implications; in conditions such as heart failure where selective venodilation would reduce intra-cardiac pressure in the absence of resistance vessel dilatation avoiding hypotension. Perhaps more importantly, pharmacological doses of nitrite may constitute a specific hypoxically bioactive agent that targets only hypoxic vessels.[119, 166, 167] A preliminary indication of the importance of nitrite has been afforded by data presented by Arai *et al*, who demonstrated that a brief nitrite infusion (plasma concentration of ~5 μM) decreased myocardial infarction size from 70% to 20%.[132] As well as confirming our findings, future studies will have to assess the therapeutic role of nitrite in disease.

In the following chapter we built on the findings in chapter 2 and went on to investigate the vascular responses of chronic heart failure patients to intra-arterial nitrite infusion.

A similar experimental set up was performed in order to compare the responses of these patients to those of healthy controls.

Chapter 3

Impact of chronic congestive heart failure on pharmacokinetics and vasomotor effects of infused nitrite

The data presented in this chapter is currently being considered for publication in the journal "British Journal of Pharmacology".

Execution

I performed all of the recruitment and organisation of the appointments involved in the study. I performed assessment of forearm blood flow, radionuclide plethymography and radionuclide ventriculography studies in the University of Birmingham. Biochemical analysis was performed by Dr Phil James and his team at the University of Cardiff, Wales.

Abstract:

Aims: Nitrite (NO_2^-) has recently been shown to represent a potential source of nitric-oxide (NO), with bio-activation of NO_2^- to generate NO being potentiated in the presence of hypoxia. We now compared the acute hemodynamic effects of infused nitrite (0.31 to 7.8μmoles/min) in normal subjects (n=20) and patients with stable congestive heart failure (CHF: n=21).

Methods and Results: NO_2^- infusion was well tolerated in all subjects. Forearm blood flow(FBF) increased markedly in CHF-patients at NO_2^- infusion rates which induced no changes in normal subjects (ANOVA: F=5.5; *P*=0.02).Unstressed venous volume (UVV) increased even with the lowest NO_2^- infusion rate in all subjects, with CHF patients being relatively hyporesponsive compared with normal subjects (ANOVA: F=6.2; *P*=0.01).There were no differences in venous blood pH or O2 concentrations between groups or during NO_2^- infusion. Plasma NO_2^- concentrations were lower in CHF-patients at baseline, and rose substantially less with NO_2^- infusion, without incremental oxidative generation of nitrate, consistent with accelerated clearance in these patients. Plasma protein-bound NO concentrations were lower in CHF-patients than normal subjects at baseline. This difference was attenuated during NO_2^- infusion.

Conclusions: The findings of arterial hyper-responsiveness to infused NO_2^- in CHF-patients, with evidence of accelerated transvascular NO_2^- clearance (presumably with concomitant NO release) suggest that NO_2^- effects may be accentuated in such patients, despite the similar venous oxygen saturations and pH in patients and controls. Despite the finding of apparent tissue resistance to NO, as previously documented in CHF, these findings provide a stimulus for the clinical exploration of NO_2^- as a therapeutic modality in CHF.

3.1 **Introduction**

Although the mechanism(s) of bioactivation remain incompletely understood, a number of reductases have been shown to "reactivate" NO from NO_2^-, independent of endothelial nitric oxide synthase (eNOS) activity[168, 169] . In the majority of studies, this process appears to be markedly potentiated in the presence of hypoxia, although once again it is not clear what mechanism(s) underlie this potentiation[131]. The implication of hypoxic potentiation of nitrite induced vascular relaxation is that exogenous administration of NO_2^- represents a means of selective release of NO to hypoxic (and presumably ischaemic) tissues and with selective vasodilatation in those regions, with minimal risk of inducing the "steal" phenomenon, and no dependence of effect on intact endothelial function. Thus NO_2^- represents a potential treatment for conditions such as myocardial ischaemia, congestive heart failure and pulmonary hypertension.

A desirable characteristic of a vasodilator agent for potential use in the management of acutely decompensated heart failure is that it exerts marked venodilator, rather than arteriolar, dilating effects. Salutary consequences of selective venodilator actions include relief of congestive symptoms without significant risk of precipitating symptomatic hypotension. Specifically, venodilator agents ameliorate the phenomenon of diastolic ventricular interaction, thus improving cardiac output and organ perfusion [129, 170, 171].

In this regard, organic nitrates such as glyceryl trinitrate (GTN) are frequently utilized as a component of the management of acute heart failure with pulmonary congestion. Although nitrate-based therapy exerts prominent venodilator effects[172, 173] and appears to have a number of advantages over a diuretic-based treatment regimen in such patients, there is no evidence that NO release from GTN is hypoxia-selective. Furthermore, therapy with GTN and other organic nitrates not only suffers the problem of attenuation of effect during prolonged therapy (nitrate tolerance and pseudo-tolerance) [174, 175], but also exhibits the

phenomenon of NO resistance (de novo hyporesponsiveness to all sources of NO) in the presence of congestive heart failure , so that some patients respond poorly to GTN, despite infusion at very high rates .[176-178] Moreover, the arteriolar vasodilatation induced by organic nitrates such as GTN frequently induces headache and can lead to deleterious reductions in systemic blood pressure.

We have described in chapter 2 the hemodynamic responses to infused NO_2^- in normal subjects, documenting a marked venodilatation and modest arteriolar vasodilatation under normoxic conditions. However, during hypoxia, there was selective potentiation of arterial vasodilation.[131] In the current study, we have compared vasomotor responsiveness to NO_2^- in stable congestive heart failure (CHF) and in normal subjects, relating response to concomitant plasma NO_2^- concentrations. We theorised that CHF patients might largely circumvent the problem of NO resistance at the arterial level by virtue of the potentiating effect of tissue hypoxia on NO_2^- bioactivation. As such, the primary null hypothesis was that the vasomotor effects of nitrite would not vary between patients with CHF and normal subjects. The secondary hypothesis was that the pharmacokinetics of nitrite would be identical between the two groups.

3.2 **METHODS**

Subject selection:

The study involved a comparison between patients with stable NYHA Class II-III CHF (n=21) and healthy volunteers (n=20). CHF patients were recruited from an 'advanced heart failure and cardiomyopathy' outpatient clinic.

Among CHF patients, contraindication to study entry were therapy including long-acting nitrates, symptomatic hypotension and clinically significant hepatic or renal dysfunction. None of the normal subjects had any known coronary risk factors, and none was taking cardioactive medications or vitamin supplements.

The study was approved by the Local Research Ethics Committee (Birmingham, UK), and all patients gave written informed consent. The study conformed to the principles of the Declaration of Helsinki.

Investigations were performed in a dedicated clinical research laboratory. Subjects had consumed a light breakfast and abstained from caffeine drink intake for at least 6 hours. Pre-study dietary nitrate/nitrite ingestion was not modified.

Protocol:

(a) Instrumentation:

Subjects rested supine in a dedicated vascular laboratory with ambient temperature maintained between 22-24°C. Twenty-gauge venous catheters were inserted into antecubital veins in each arm. A 27-gauge arterial needle (Coopers Engineering, UK) mounted onto a 16-gauge epidural catheter and sealed with dental wax was inserted into the non-dominant brachial artery under sterile conditions for infusion of NO_2^-, and kept patent by continuous saline infusion.

Heart rate was monitored continuously (Dash3000, GE-Marquette). Arterial blood pressure was monitored continuously with finger plethysmography (TNO TPD Biomedical Instrumentation, Delft, Netherlands).

(b) Hemodynamic assessment

The principal hemodynamic investigations performed were serial determination of unstressed venous volume (UVV) as an index of venodilator response, and forearm blood flow (FBF) as an index of arteriolar response to infused NO_2^-.

Both forearms were positioned on gamma-cameras (Scinton,MIA and ADAC-Transcam). Forearm venous volume(FVV) was assessed utilizing radionuclide plethysmography as previously described [179]. In brief, venous blood was withdrawn and radio-labelled with Tc99m, and then re-injected into subjects after 10 minutes incubation. Images were obtained after an equilibration period of 15 minutes. During each cycle of data acquisition, scintigraphic venous vascular volumes were obtained. The venous pressure-volume relationship was derived from serial estimates of FVV in order to calculate UVV. All data were corrected for decay of Tc99m. Results were expressed relative to baseline values of UVV, and those in the infused arm corrected for changes in the non-infused arm.

FBF was measured utilizing plethysmography with mercury-silastic strain gauges (DE Hokanson Inc., Bellevue, Washington) as previously described[180]. Again changes in FBF were expressed relative to baseline values, and changes in the infused arm corrected for those in the non-infused arm[131].

Left ventricular ejection fraction was calculated by multiple gated acquisition.

(c) NO_2^- infusion:

After determination of baseline data, nitrite was infused into the non-dominant brachial artery. Infusion rates were 0.31µmoles/min for 30 minutes, thereafter increasing to 0.78µmoles/min, 3.1µmoles/min and 7.8 µmoles/min, each for further 30-minute infusion periods.
Changes in hemodynamic parameters were measured 5, 12 and 20 minutes after initiation of each infusion rate.

(d) Blood sampling/analysis:

Blood was withdrawn via venous cannulae in both arms at baseline and after the conclusion of infusion of the three higher infusion rates of sodium nitrite.
Blood gas analysis was performed for pH, oxygen and methaemoglobin concentrations. (Bayer Rapidlab 865).
Blood for determination of venous plasma NO_2^-, nitrate and protein-bound NO (RXNO) concentrations (from both infused and non-infused arms) was taken into EDTA tubes and immediately centrifuged (200rpm for 10 minutes at 4C). Samples were stored at -80C prior to assay. Plasma NO_2^- ,nitrate and protein-bound NO content were determined via ozone-based chemiluminescence or HPLC[181] as previously described.[182]

In order to ensure that nitrite clearance *in vitro* did not vary between normal subjects and patients, additional experiments were performed in which blood samples from normal and CHF subjects were spiked with sodium nitrite *in vitro* to final concentrations of 2 and 20µM, after which blood samples were incubated under gentle agitation at 37ºC with aliquots being removed after 1, 2, 5, 10, 20 and 60 minutes prior to centrifugation and assay. Rates of in-vitro clearance of nitrite did not vary between groups (data not shown).

(e) Reagents:

Sodium nitrite was purchased from Martindale Pharmaceuticals, UK. HPLC-grade nitrite-free water (Fisher Scientific) was utilized for extractions and dilutions.

(f) Analysis of results:

Clinical characteristics of normal subjects and CHF patients were compared utilizing non-paired t-tests for normally distributed parameters, and Wilcoxon tests for skewed data.

Serial changes in FBF and UVV, pH and venous O_2 saturation, and concentrations of NO_2^- and protein-bound NO in both the infused and non-infused forearms were compared in normal and CHF subjects by 2-way ANOVA with repeated measures, utilizing Dunnett's t-test to assess for significance of changes at individual time points. Logit transformation of data was utilized to detect possible disparity of concentration-response relationships between groups of subjects.

All results are expressed as mean+/- SEM unless otherwise stated.

3.3 **RESULTS**

Subject/Patient Characteristics

These are summarised in Table 3.1 and 3.2. The normal and CHF groups differed significantly as regards body mass index, fasting plasma glucose and serum creatinine levels. CHF patients had both lower blood pressure and higher heart rates than normal subjects.

Table 3.1

Demographic and Clinical Features	CHF Patients (mean±SEM)	Healthy Controls (mean±SEM)	*P* Value
Age (y)	62.9±2.7	57.6±1.2	0.08
Sex, M/F (%)	18/3 (86/14)	15/5 (75/25)	0.70
Body mass index (kg/m^2)	30.0±1.2	26.8±0.6	0.05
Serum cholesterol (mmol/L)	5.3±0.4	5.4±0.2	0.69
Plasma glucose (mmol/L)	5.8±0.4	4.7±0.1	0.05
Serum Creatinine (µmol/l)	125±6.7	103.4±5.2	0.03
Heart rate	74±2	61.5±1.9	0.01
Blood pressure (mmHg)			
Systolic	117±3.7	128±2.0	0.03
Diastolic	74.8±1.9	73.4±1.4	0.73
Left Ventricular Ejection Fraction	34.8±2.5	56.6±1.7	0.001
Aetiology (Ischaemic/DCM)	10/11	N/A	

Amongst CHF subjects, the number of patients with an ischemic basis for CHF and those due to a dilated cardiomyopathy were similar. Most (95%) were in Class II-III NYHA functional class. Treatment for CHF included ACE inhibitors/ angiotensin receptor blockers in 95%, ß-adrenoceptor antagonists in 76%, digoxin in 19%, and aldosterone antagonists in 57%. As planned, no patient was taking long-acting nitrates and none had frequent episodes of angina pectoris necessitating administration of sublingual GTN.

Table 3.2

Medication	% of patients receiving
Beta blockers	76%
ACE Inhibitors / ARB	95%
Aldosterone Antagonists	57%
Loop diuretics	100%
Perhexiline	29%
Warfarin	57%
Antiplatelets	48%
'Statins'	52%
Digoxin	19%

Hemodynamic effects of sodium nitrite infusion

Sodium nitrite infusion was well-tolerated in all subjects. Mean arterial blood pressure and heart rate did not vary significantly during the course of the experiment. Maximal venous methemoglobin concentrations in the infused arm were less than 2% of total haemoglobin at all nitrite infusion rates.

Forearm blood flow (FBF) changes are shown in Figure 3.1. In the non-infused forearm, both in normal subjects and CHF patients, there was significant vasoconstriction during the course of the study (ANOVA: F=2.6;*P*=0.04) which was attenuated at the highest nitrite infusion rate, raising the

possibility of the onset of NO_2^- induced vasodilatation due to recirculation (Figure 3.1A) . In the infused arm (Figure 3.1B), the relationship between infusion rate and effect varied markedly between groups. For normal subjects, there was a progressive increase in FBF with infusion rates of 3.14μmoles/min or greater. On the other hand, in CHF patients, vasodilator responses were induced by the lowest infusion rates of nitrite, but with similar responses at > 3.14 μmoles/min. Logit transformation of concentration-response data confirmed that the gradients of the linearised relationships were significantly steeper (P=0.017) in normal subjects than in CHF patients; partitioned ANOVA methodology was therefore utilized for analysis of these data. At lower, but not higher, infusion rates there were considerably greater FBF responses (F=5.5;P=0.02) than those in normal subjects, in whom the threshold for increases in FBF was NO_2^- infusion rate of 3.1μmoles/min.

Unstressed venous volume (UVV) changes are summarised in Figure 3.2. In the non-infused arm (3.2A) both in normal subjects and patients, there was venoconstriction (F=9.6;P<0.0001) which was more marked in normal subjects (F= 15.4;P<0.001)). In the infused arm (Figure 3.2B), both groups exhibited evidence of increases in UVV commencing with the lowest infusion rate of sodium nitrite infusion, with a monophasic and progressive dose-related increase in UVV. However, overall responses in CHF patients were substantially lower than those in normal subjects (F=6.3;P=0.01) .

Figure 3.1

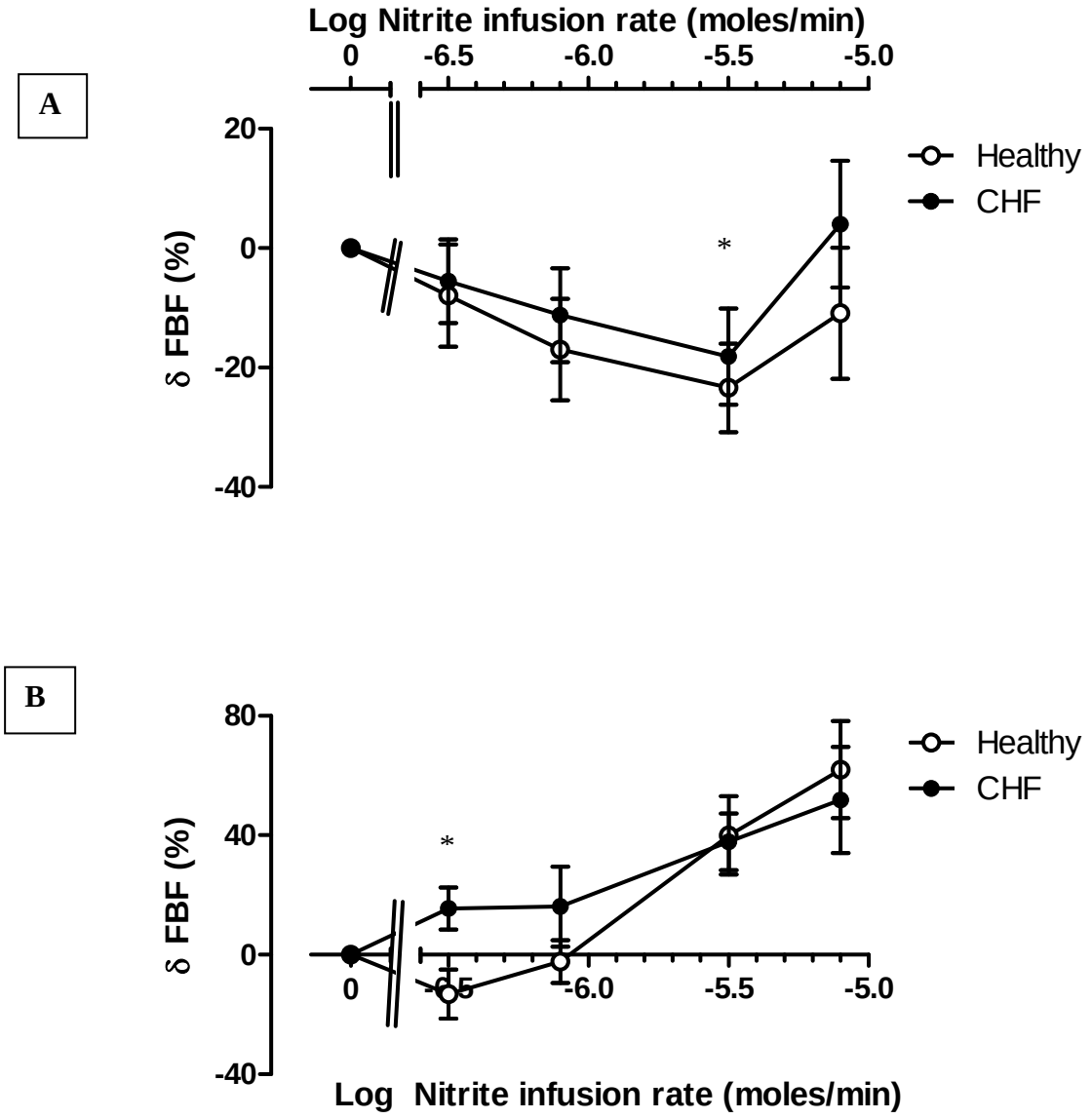


Figure 3.1: Changes in Forearm Blood Flow (FBF) in the non-infused arm (A) and Infused arm (B) in normal subjects (open symbols) and CHF patients (closed symbols). In the non-infused arm, FBF decreased significantly (F=2.6;*P*=0.04); in the infused arm there was a selective increase in FBF associated with lower NO_2^- infusion rates (F=5.5;*P*=0.0.2). *=*P*<0.05

Figure 3.2

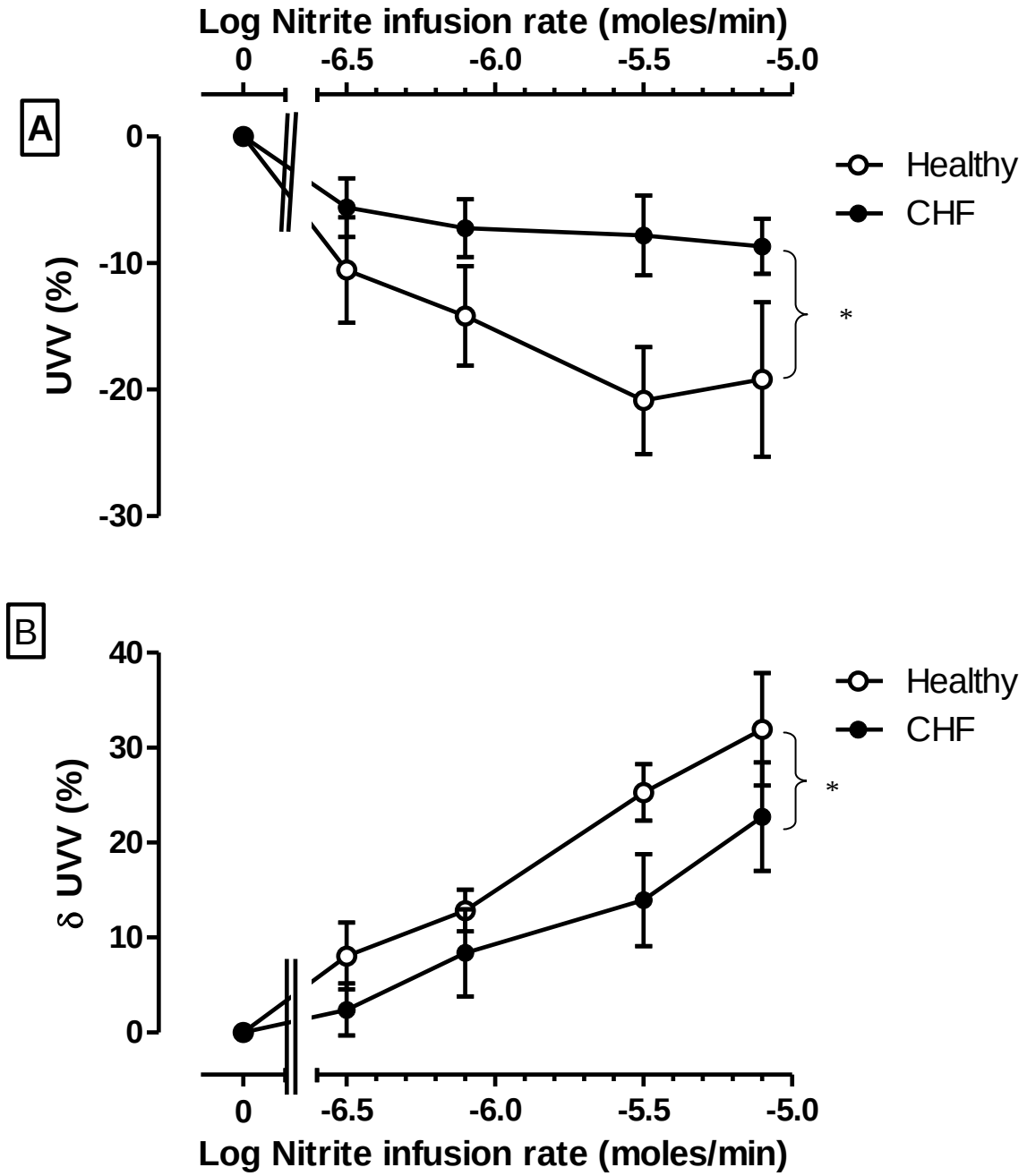


Figure 3.2: Changes in unstressed venous volume (UVV) in the non-infused arm (A) and infused arm (B) in normal subjects (open symbols) and CHF patients (closed symbols). In the non-infused arm, there was a decrease in UVV which was more marked in normal subjects

(F=15.4;*P*<0.001). In the infused arm the increase in UVV with NO_2^- infusion was attenuated in CHF patients (F=6.2;*P*=0.01). *=*P*<0.05

Changes in pH and venous O_2 saturations

pH in venous blood did not vary significantly between normal subjects and CHF patients , nor did it fluctuate significantly (Figure 3.3) during nitrite infusion in either arm.

Venous oxygen saturation also did not vary significantly between patient groups, *P*= 0.27 and *P*=0.35 for changes in the CHF group versus healthy controls in the non infused and infused arms respectively.

With nitrite infusion there was a trend to lower venous oxygen saturation with time in the non infused arm (*P*=0.07) which may be explained by immobility and decreases in blood flow with time. In comparison in the infused arm there was no significant change in venous oxygen saturation with time (*P*= 0.53). At peak dose venous oxygen saturation were 79.5% ± 7.2 and 83.4 ± 1.8 in the CHF and healthy volunteers respectively

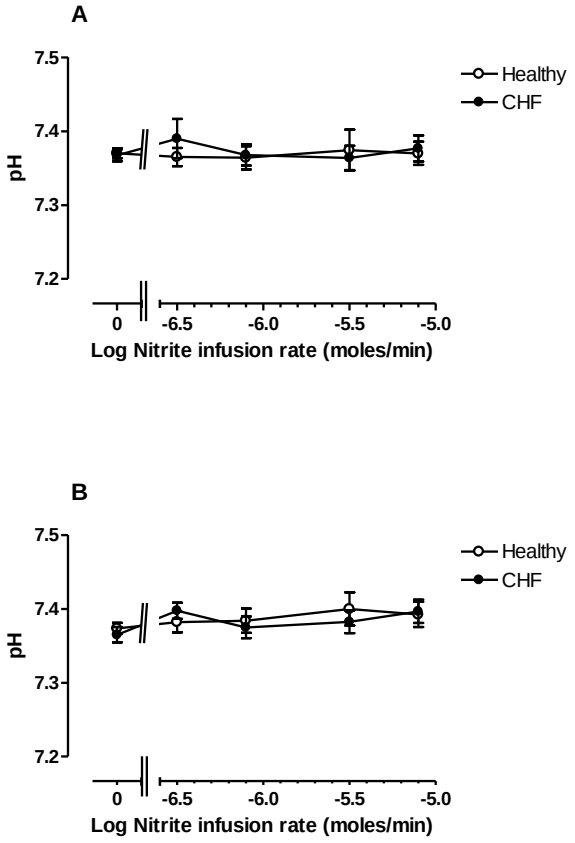


Figure 3.3: pH fluctuations in venous blood from (A) non-infused arm and (B) infused arm in normal subjects (open symbols) and CHF patients (closed symbols). pH did not vary significantly either with NO_2^- infusion rate or between groups.

Plasma NO_2^- and nitrate concentrations

Changes in NO_2^- concentrations in venous blood during nitrite infusion are summarised in Figure 3.4A and 3.4B. Resting plasma NO_2^- concentrations were non significantly (*P*=0.08) lower in CHF patients. NO_2^- concentrations increased marginally (F=2.3;*P*=0.09) in venous blood in the non-infused arm in both normal subjects and CHF patients (Figure 5A), with greater levels appearing (F=4.3;*P*=0.04) in the normal subject group.

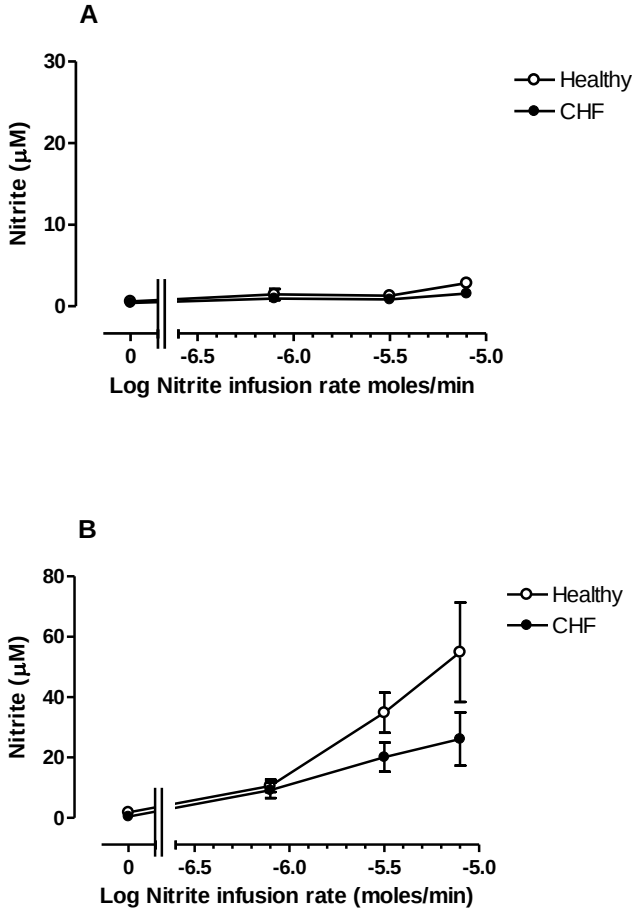


Figure 3.4: Venous plasma nitrite concentration (µM) in (A) non-infused arm and (B) infused arm in normal subjects (open symbols) and CHF patients (closed symbols). Baseline nitrite concentrations were marginally lower in CHF patients than in normal subjects (P=0.08). Nitrite concentrations rose significantly (F=32.7;P<0.0001) with NO_2^- infusion in the infused arm: this increase was attenuated significantly (F=10.6;P=0.002) in CHF patients.

In the infused arm (Figure 3.4B), NO_2^- concentrations increased approximately 70-fold during nitrite infusion (F=32.7;P<0.0001;Figure 3.3B). The increase in venous NO_2^- levels in the infused arm was

greater in the normal subjects than in CHF patients (F=10.6;*P*=0.002). The increase in venous NO_2^- concentrations for a 10-fold increase nitrite infusion rate increase was disproportionately small: approximately 7-fold for normal subjects and 3-4 fold for CHF patients.

Consistent with previous observations [183] basal nitrate levels in the venous effluent were found to be higher in the CHF patients compared to healthy volunteers (*P*=0.0034). Venous nitrate levels rose similarly (*P*= 0.96 and *P*=0.67 for the non infused and infused arms respectively) in the two groups of subjects (Figure3. 5A and 3.5B) suggesting that the degree of oxidation of nitrite to nitrate did not differ between groups.

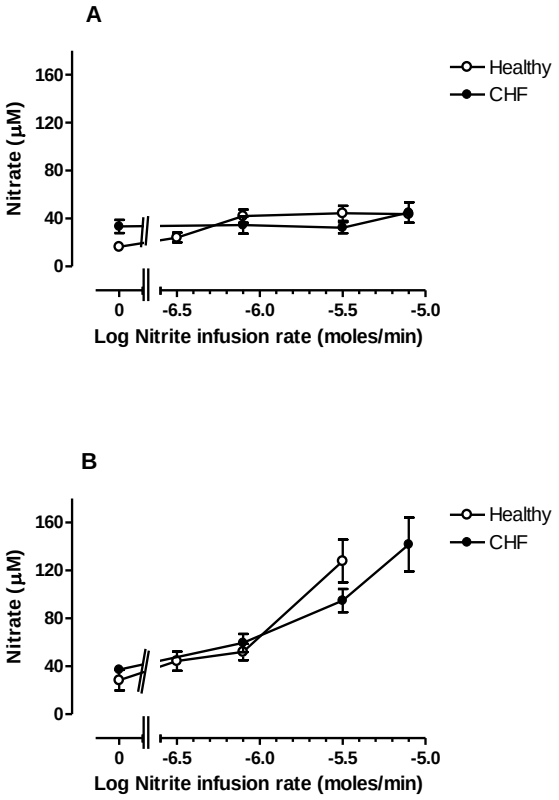


Figure 3.5: Venous plasma nitrate concentrations in (A) non infused arm and (B) infused arm in normal subjects (open symbols) and CHF patients (closed symbols). Baseline nitrate concentrations were greater (*P*=0.003) in CHF subjects. During nitrite infusion, nitrate

concentrations increased (F=16.2 P<0.0001)) in the infused arm, but without significant difference between normal subjects and CHF patients.

Protein-bound NO concentrations

Changes in protein-bound NO concentrations in venous blood from non-infused and infused arms throughout the study are summarised in Figure 3.6A and 3.6B. In the non-infused arm (Figure 3.6A), protein-bound NO concentrations were significantly greater in normal subjects than in patients with CHF (F=8.6; P=0.04). In the infused arm, this difference was attenuated during nitrite infusion, becoming non-significant. Furthermore, protein-bound NO tended to increase with the highest nitrite infusion rate.

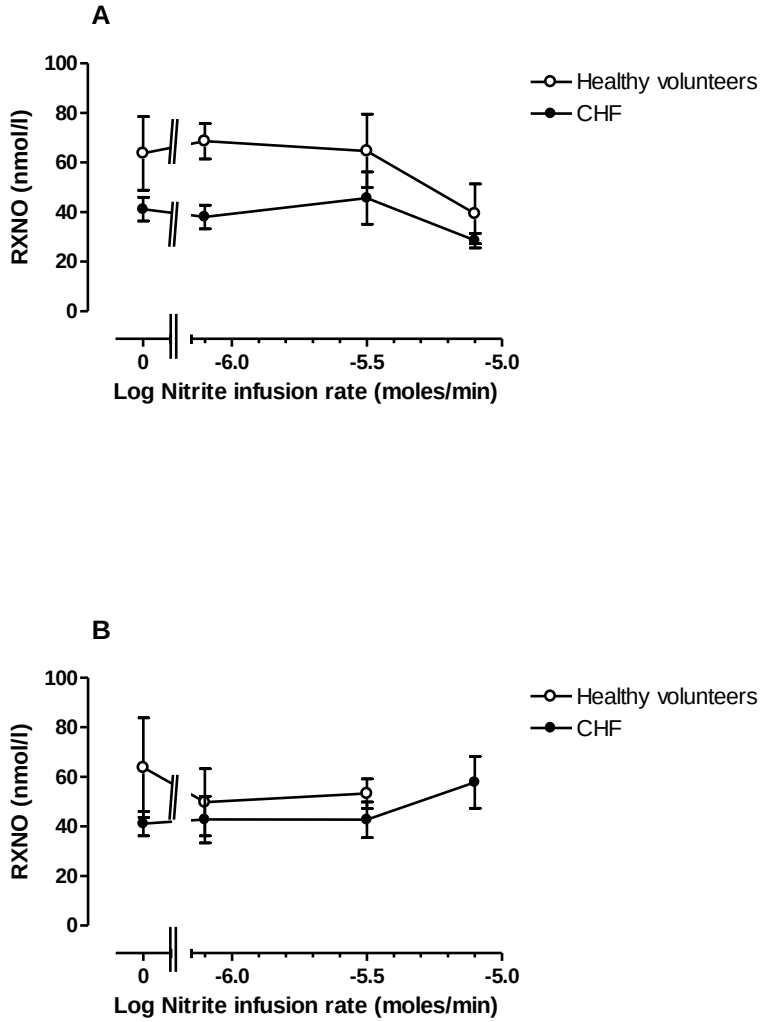


Figure 3.6: Venous plasma protein-bound NO (RXNO:nmol/L) in (A) non-infused arm and (B) infused arm in normal subjects (open symbols)and CHF patients (closed symbols). RXNO concentrations did not vary significantly with NO2- infusion in either arm (F= 0.44 *P*= 0.99), but were significantly lower in CHF patients than in normal subjects in the non-infused arm (*P*=0.04), while this difference was attenuated in the infused arm.

3.4 DISCUSSION

A number of previous studies, both in humans and animal models, have suggested that infused nitrite exerts vasomotor responses which are in many ways similar to those of the organic nitrates, with marked venodilatation, and moderate arteriolar dilatation which becomes more marked at high infusion rates or when augmented by hypoxia or exercise.[131, 184] These characteristics suggest that, like organic nitrates, NO_2^- is selectively bio-activated to NO in veins and large arteries. On the other hand, infused NO_2^- represents in many ways a particularly attractive agent for treatment of CHF complicated by fluid overload. Not only is it a potent venodilator, but also its effects appear to be (somewhat surprisingly) independent of the phenomenon of tolerance induction [46], at least in primate models. Furthermore, NO_2^- provides a "needs-based" vasodilator effect, with effects accentuated by hypoxia in many systems [76, 185, 186]. The multiple/precise mechanism(s) underlying this accentuated effect remain incompletely elucidated.

In this study, effects of nitrite infusion were compared in cohorts of normal subjects and patients with stable CHF without fluid overload. The previously documented[131] selective venodilator effects of NO_2^- at low infusion rates was again documented in normal subjects: the lowest two infusion rates of nitrite induced significant increases in UVV, without any change in FBF. On the other hand, CHF-patients exhibited marked and selective hyper-responsiveness to lower rates of NO_2^- infusion at the level of the forearm resistance vessels, whereas there was also evidence of substantially reduced venodilator responses in CHF patients. The latter finding may represent NO resistance. Alternatively it may be a consequence of increased clearance of NO_2^- across the vascular bed, resulting in lower concentrations in the capacitance vasculature at any given infusion rate (see below).

The finding that CHF-patients had lower protein-bound NO and marginally lower nitrite concentrations than normal subjects is consistent with previous findings in subjects with endothelial dysfunction[42, 187, 188]. Steady-state NO_2^- concentrations (in the absence of dietary or intravenous NO_2^- supplementation) may be regarded as being indicative of NO generation and therefore indicative of

diminished eNOS activity in CHF[42], whereas protein-bound NO concentrations reflect both the generation of S-nitrosoproteins from released NO, as well as a measure of prevalent redox stress[189]. With sodium nitrite infusion, venous NO_2^- concentrations increased, but not in proportion to the increases in infusion rates, suggesting some selective loss of NO_2^- during sampling at higher infusion rates. These changes were noted both in the infused and non-infused arms, indicating that the non-infused arm cannot be regarded as a simple "control" site: indeed the FBF changes (Figure 3.1, Inset) suggest the onset of some dilator effect with the highest nitrite infusion rate. Nevertheless, NO_2^- levels increased far less markedly with increasing sodium nitrite infusion rates in CHF patients than in normals (Figure 3.5B) indicating greater rates of NO_2^- clearance in these patients. Although NO_2^- may be reduced to NO (bio-activation), a major clearance mechanism for NO_2^- is oxidation to nitrate. Therefore, venous nitrate concentrations were measured in both infused and non-infused arms, revealing a similar increase in plasma nitrate levels in the two groups upon infusion of nitrite and thus making clearance through oxidation to nitrate less likely. Similarly our *in vitro* results suggest that there is no systematic difference in the rate of degradation of nitrite in the two groups, thus excluding sampling artefacts as a possible explanation for the differences in nitrite concentrations between normal subjects and CHF-patients.

As regards the additional potential formation of S-nitrosoproteins via NO_2^- metabolism, venous protein-bound NO concentrations tended to increase in the infused arm in the CHF patients (coupled with a relative decrease in protein-bound NO concentrations in the controls), attenuating the difference between baseline levels in the two groups. These findings are also consistent with selective bio-activation of NO_2^- in the CHF patients, and might have been accounted for by the presence of tissue hypoxia and/or acidosis in these patients, given the previously described incremental bio-activation of NO_2^- in the presence of hypoxia[131]. However, neither venous blood pH nor oxygen concentrations differed significantly between groups. There was a trivially (non significantly) lower venous oxygen saturation in the infused arm of the heart failure patients but this seems unlikely to provide an explanation for

the difference in clearance. It therefore appears either that a component of subcellular/microvascular hypoxia was present in the CHF patients without detection in venous samples (a possible but somewhat unlikely event) or that these results indicate that, apart from hypoxia, NO_2^- bioactivation to NO can be induced by factors other than hypoxia, such as changes in redox state. This issue is worthy of further investigation.

Taken together, these results show that CHF patients are hyper-responsive to the arteriolar dilating effects of infused sodium nitrite, presumably due to increased release of NO (despite the absence of arterial hypoxaemia). This finding may imply the need for some caution in the use of infused nitrite preparations in patients with decompensated CHF, for fear of precipitating falls in systolic blood pressure. On the other hand, symptomatic hypotension was not observed in the currently studied patient cohort.

However CHF patients were hyporesponsive both to the effects of infused sodium nitrite on venous capacitance and to a lesser extent, to the effects on FBF at high infusion rates. These findings may imply the existence of NO resistance, as previously documented in both arteries and platelets of such patients[176]. Although platelet NO resistance exhibits partial responsiveness to ACE inhibitor therapy[190], which was utilized in the majority of the CHF group, its interaction with vascular NO resistance is less certain. Alternatively the apparent resistance of the capacitance vessels may in part be due to a lower concentration of nitrite in venous blood due to the increased transvascular clearance. As net responsiveness to NO_2^- reflects both accelerated bioactivation and diminished tissue responses to NO, it is likely that individual responses might well vary markedly, according to each of these component factors.

A number of potential limitations and caveats apply to the current findings. Most importantly, the biochemical basis of the observed arterial hyper-responsiveness to NO_2^- was not determined, and indeed the possible relevance of tissue hypoxia to this phenomenon cannot be completely excluded with the methodology utilized in this study. Furthermore, changes in tissue redox stress were not

documented: this remains a major priority for future investigations. While the observation of attenuation of the increase in plasma NO_2^- in association with increasing rates of sodium nitrite infusion implies accelerated transvascular clearance of NO_2^- in such patients, the release of NO was not measured, and therefore it cannot be absolutely certain that there was a close relationship between such accelerated clearance and incremental rates of NO generation. Indeed, there is recent evidence both that nitrite may exhibit vasoactivity independent of bioconversion and that nitrite may exert a component of its effects independent of the formation of NO or NO adduct[191]. Furthermore, the proportion of nitrite clearance via oxidation to nitrate cannot be assessed completely from the current results, although it was possible to exclude the possibility that transvascular nitrate generation occurred selectively in CHF patients given the similar rise in nitrate levels. Finally, the findings of the current investigation, while relevant to the potential therapeutic administration of nitrites, may not be indicative of physiological modulation of NO_2^- effect at far lower concentrations.

While NO_2^- infusion has the potential to lead to the generation of methemoglobin, (a possible concern) we did not observe a significant increase in venous methemoglobin levels in this study.

We noted a significant vasoconstriction in the non-infused arm during the study. In theory this could have been secondary to hypotension however no significant drop in blood pressure was observed, in fact there appeared to be a trend towards an increase. It has been our experience in prolonged intrabrachial infusion studies with other agents that forearm blood flow falls in the contralateral arm over time despite no significant changes in blood pressure. We suspect this results from the effects of slight discomfort and often a full bladder during these long studies. It is because of such changes in flow in the non infused arm during prolonged studies that bilateral plethysmography with correction for the non infused arm is recommended for intrabrachial infusion studies.[192]

With regards to the medication received by the CHF patients, while there are data to suggest that angiotensin converting enzyme inhibitors and angiotensin receptor blockers improve NO production/ endothelial function we could not find any evidence to suggest any impact upon nitrite conversion. The possibility that the medication taken by the CHF patients may account for some of the differences observed remains a limitation of this study.

The findings of the current investigation extend the question of the potential clinical utility of NO_2^- as a component of the management of patients with both acute and chronic heart failure. The major residual issue to be explored is the extent of change in vascular responsiveness to be seen in such patients, especially in decompensated CHF, where significant tissue hypoxia is more likely to be present, with resultant potentiation of NO_2^-- bio-activation, perhaps counter-balanced in part by the phenomenon of tissue resistance to NO [193]. Despite the occurrence of NO resistance, treatment with organic nitrates is at least as effective as diuretic/morphine-based acute pharmacotherapy for decompensated heart failure with acute pulmonary edema [194, 195]. However, nitrate therapy is often associated with the unpleasant side effect of headache. In this study none of the subjects who received intravenous nitrite suffered with this symptom and potentially provides a distinct advantage over traditional nitrate therapies. A comparison with NO_2^- based therapy now seems indicated.

Having established the vascular response to nitrite in both health and heart failure subjects we now turned to investigating the mechanisms involved in nitrite reduction. We focussed specifically on two enzyme systems that had been proposed as being critically involved on nitrite conversion to NO.

Chapter 4

Xanthine Oxidoreductase and Endothelial Nitric Oxide Synthase Do Not Contribute Significantly To Vascular Relaxation by Nitrite in Man

The data presented in this chapter is currently being considered for publication in the journal "Clinical Science".

Execution

I performed all of the recruitment and organisation of the appointments involved in the study. I performed assessment of forearm blood flow, radionuclide plethymography and radionuclide ventriculography studies in the University of Birmingham. Biochemical analysis was performed by Dr Phil James and his team at the University of Cardiff, Wales.

Abstract

Aims: The nitrite anion is increasingly considered an important source of bioactive nitric oxide. Recent evidence suggests that xanthine oxidoreductase (XOR) and endothelial nitric oxide synthase (eNOS), in both vasculature and erythrocytes, may reduce nitrite to NO *ex vivo* under conditions of severe hypoxia/acidosis. To ascertain the importance of this mechanism in man, the effect of nitrite infusion with and without blockade of NOS and XOR, was studied in 25 healthy volunteers.

Methods : Uniquely effects of intra-brachial nitrite infusion on both the high and low ambient oxygen tension environments of resistance vessels and the capacitance bed, respectively, were studied.

Results: Peak percentage venodilation with nitrite and L-NMMA was 15.1% compared to 26.8% with nitrite alone, the difference being consistent with inhibition of pre-existing basal eNOS activity alone. Peak FBF-ratio(FBFr) was also similar, 40.4% and 42.7%, with and without eNOS inhibition respectively. Peak percentage venodilation in the allopurinol group (26.9%) was not different to controls(26.8%). Peak FBFr was also similar in the two groups, 51.8% and 42.7%, with and without XOR inhibition respectively.

Conclusion: In healthy volunteers at rest, neither intravascular XOR nor eNOS appear to play a significant role in nitrite-induced vasodilatation, in either high or lower ambient oxygen tension environments.

Key words: Nitric oxide, nitrite, vasodilation, xanthine oxidoreductase and nitric oxide synthase

4.1 Introduction

A number of important questions include: (i) what are the nitrite reductase(s) *in vivo*; (ii) how do these reductase(s) interact with endogenous/exogenous nitrite to elicit biological effects *in vivo* and (iii) what are the corresponding therapeutic implications?

A variety of different nitrite reductases have been identified. These include pH-dependent enzyme-independent nitrite conversion to NO,[47] deoxyhemoglobin,[48-54] myoglobin,[55-59] neuroglobin,[60] aldehyde oxidase,[61] microsomal cytochrome P-450[62] and cytochrome C.[63, 64], however the relative importance of these different mechanisms *in vivo* remains unclear. Since the different proposed reductases exhibit tissue specificity, synergy, redundancy and sensitivity to incipient conditions[66]; and since nitrite is reduced via a number of mechanisms *ex vivo*, there are significant challenges to translating *in vitro* nitrite reducing capacity to *in vivo* physiology. For example, there has been controversy regarding the role of red blood cells (RBCs), hemoglobin (Hb) and deoxyhemoglobin in nitrite metabolism.[48-54, 67-69] Although there is a clear chemical rationale for the Fe^{II} centre of Hb to reduce nitrite, the NO scavenging capacity of the plentiful heme groups in RBCs has led to ongoing discussion regarding their likely net NO yield.[61] To reconcile this discordance, the membrane compartment hypothesis contends that RBC membrane eNOS and XOR generate NO at a distance from the RBC heme, hence the NO is free to diffuse to the vasculature.

Similarly, building on existing evidence that the vasculature has intrinsic nitrite reductase activity,[70] and that XOR and eNOS have been implicated as nitrite reductases for several years;[58, 59, 61, 68, 70-72], it has recently been reported that nitrite conversion to NO by the vasculature and/or by RBCs is attenuated by inhibition of xanthine oxidoreductase (XOR) at very low oxygen tensions at pH 6.8. Furthermore, it has been shown that RBC endothelial

nitric oxide synthase (eNOS) inhibition with L-NMMA diminishes nitrite bioconversion.[73] These proposals have merit as they reconcile the long held belief that RBCs yield NO with the concern that high RBC Hb concentrations would attenuate NO release through scavenging. However, while these findings may be applicable to nitrite bioconversion in the context of pronounced pathological hypoxia, the implication of these observations to the vasodilation observed with pharmacological concentrations of nitrite under normoxic and mildly hypoxic conditions is unclear.

Accordingly, although the role of XOR[68] and NOS[54] have been studied to some extent, hitherto such studies have been limited to investigating the resistance bed, yet the mechanisms responsible for nitrite induced vasorelaxation may differ according to the prevailing oxygen tension.

In order to test the role of XOR and eNOS in nitrite reduction *in vivo* in normal resting non-pathological conditions in man, we used well-established pharmacological inhibitors of these enzymes, at doses shown to provide sufficient blockade in healthy volunteers. More specifically we study the role of XOR and eNOS in mediating nitrite-induced vascular effects in both the high oxygen tension environment of the forearm resistance bed but also, and more importantly the role in the relatively hypoxic environment of the capacitance vessels. We employ a similar experimental set up to that used in earlier chapters. We infused sodium nitrite intra-brachially with and without enzyme inhibition in order to ascertain the extent to which XOR and eNOS are contributing to NO generation from nitrite in both the resistance and capacitance beds.

4.2 Materials and Methods

Subject Recruitment

25 healthy volunteers were recruited; 15 to the NOS group and 10 to the XOR group. They were non-smokers with no history of hypertension, diabetes or hypercholesterolemia, and no family history of ischemic heart disease. None was taking cardiac medications or vitamin supplements and all had a normal cardiovascular examination and electrocardiogram.

Ethical Statement

The study was approved by the Local Research Ethics Committee and all subjects gave written informed consent. The study complied with the Declaration of Helsinki.

Subject preparation and Measurement of Forearm Blood Flow

Subjects rested supine in a dedicated vascular laboratory (22-24°C). A 27-gauge arterial needle (Coopers Engineering, United Kingdom) mounted onto a 16-gauge epidural catheter and sealed with dental wax was inserted under sterile conditions into the non-dominant brachial artery. Heart rate and rhythm (Dash 3000, Marquette, GE) and blood pressure (finger plethysmography: TNO TPD Biomedical Instrumentation, Delft, Netherlands) were monitored continuously. Forearm blood flow (FBF) was measured using mercury-in-silastic strain gauges (D.E. Hokanson, Inc., Bellevue, Washington).[196] In order to control for systemic and local passive changes with time changes in FBF observed were corrected for the control arm and thereby expressed as a FBF ratio.

Measurement of Forearm Venous Volume

Forearm venous volume (FVV) was assessed using radionuclide plethysmography, as described previously.[197] The advantage over strain gauge plethysmography is that results are independent of changes in interstitial fluid. 1 minute dynamic images were obtained in both arms using two gamma cameras (Scintron, MIA and ADAC-Transcam). Since >90% of the injected isotope remains intravascular, and the vast majority of blood in the peripheral circulation is venous, changes in counts reflect changes in FVV.[197] Vascular volumes were plotted against upper arm cuff pressures (of 0, 10, 20 and 30mmHg) to form a venous volume-pressure relationship (VPR). Linear regression was applied to each VPR, with R^2>0.9 taken to indicate linearity. Non-linear VPRs were discarded. Volumes were expressed as a percentage of the unstressed venous volume during normal saline infusion (the volume-axis intercept). A parallel shift of the relationship indicates a change in venous tone.[197] Our previous studies have shown that changes in arterial inflow do not alter venous VPR.[197] As with FBF, data were adjusted for changes in the control arm and corrected for physical decay of technetium. We, and others, have previously validated this technique.[197-199]

Inhibition of Nitric Oxide Synthase (n=15)

(i) Intra-brachial sodium nitrite was given at the dose of 7.84µmol/min for 30 minutes. FVV and FBF were measured a 20 minutes. 60 minutes was allowed to elapse for a return to baseline. L-NMMA alone was then infused at a dose of 12mg/min for a total of 20 minutes. L-NMMA and nitrite (7.84µmol/min) were infused concurrently for 20 minutes. This dose of L-NMMA has previously shown to provide local NOS blockade.[200] Moreover, this achieved a calculated arterial blood concentration of LNMMA of 4mmol/l, slightly higher than that previously shown to substantially inhibit red blood cell nitric oxide synthase activity (3mmol/l). [201]

Inhibition of Xanthine Oxidase (n=10)

Each subject received 600mg oral allopurinol at 6am on the morning of the study, a dose demonstrated to provide >95% inhibition of XO.[202-204] Subjects were otherwise prepared as in the eNOS inhibition group. Intra-brachial sodium nitrite was given at a dose 7.84μmol/min; FVV then FBF were measured at 5, 12 and 20 minutes.

Data and Statistical Analysis

Data were analyzed using SPSS version 14 for Windows. All data are expressed as mean ± SEM, and $P<0.05$ was considered statistically significant. One-way analysis of variance (repeated measures) was carried out for changes in FBF and FVV. Data was found to be normally distributed.

4.3 **Results**

Subject characteristics

Grouped baseline characteristics for all subjects are shown in Table 4.1.

Table 4.1 – Subject Characteristics

Demographic and Clinical Features		**(mean ± s.e.m.)**	
Age (y)		57.6 ± 1.2	
Sex, M/F		21/4	
Body mass index (kg/m^2)		26.8 ± 0.6	
Serum cholesterol (mmol/L)		5.4 ± 0.2	
Plasma glucose (mmol/L)		4.7 ± 0.1	
Serum Creatinine (µmol/l)		103.4 ± 5.2	
Heart rate		61.5 ± 1.9	
Blood pressure (mmHg)			
Systolic		128 ± 2.0	
Diastolic		73.4 ± 1.4	
Left Ventricular Ejection Fraction		56.6 ± 1.7	

NOS inhibition reduced baseline venodilation and peak nitrite-induced venodilation to a similar extent

L-NMMA alone caused a downward displacement of the basal VPR (venoconstriction) of 9.9 ± 2.8% (*P*=0.07) (Fig.4.1). This is consistent with inhibition of basal NO production and with previous observations. Nitrite (7.84µmol/min) alone caused an upward displacement of the VPR (venodilation) of 26.8 ± 4.6% (*P*=0.004). Nitrite (7.84µmol/min) and L-NMMA caused an upward displacement of the VPR of 15.1 ± 2.5% (*P*=0.011). The difference was 11.7 ± 2.1%, *P*=0.16 between groups.

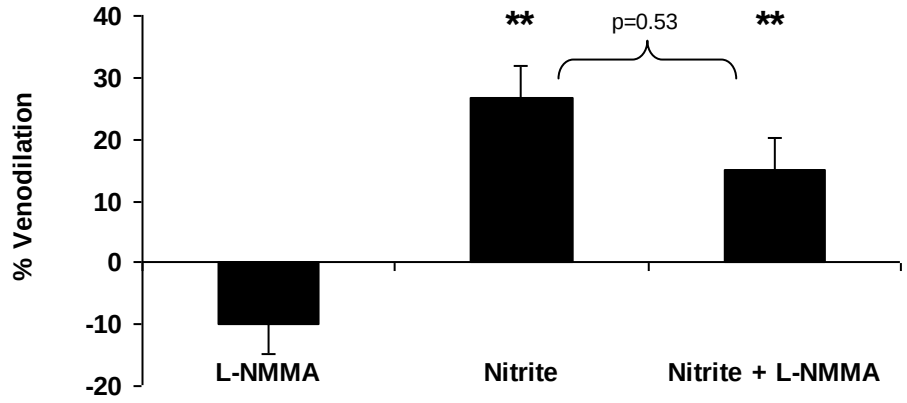


Figure 4.1 – NOS inhibition and FVV

eNOS inhibition with L-NMMA reduced baseline FVV and peak nitrite-induced venodilation by a similar amount. ***P*<0.01 compared to baseline, *n*=9.

NOS inhibition did not affect resistance bed response to nitrite

L-NMMA alone caused a 20% reduction in the basal FBF ratio (vasoconstriction), from 1.0 ± 0.02 to 0.8 ± 0.02 (*P*=0.001 by one-way ANOVA). Nitrite (7.84µmol/min) alone increased the FBF ratio (i.e. vasodilation) by 42.7% (from 1.00 ± 0.02 to 1.39 ± 0.08, *P*=0.007). Nitrite (7.84µmol/min) and L-NMMA increased the FBF ratio by 40.4% (from 1.0 ± 0.02 to 1.37 ± 0.11, *P*=0.032) (Fig.4.2). *P*=1.00 between groups.

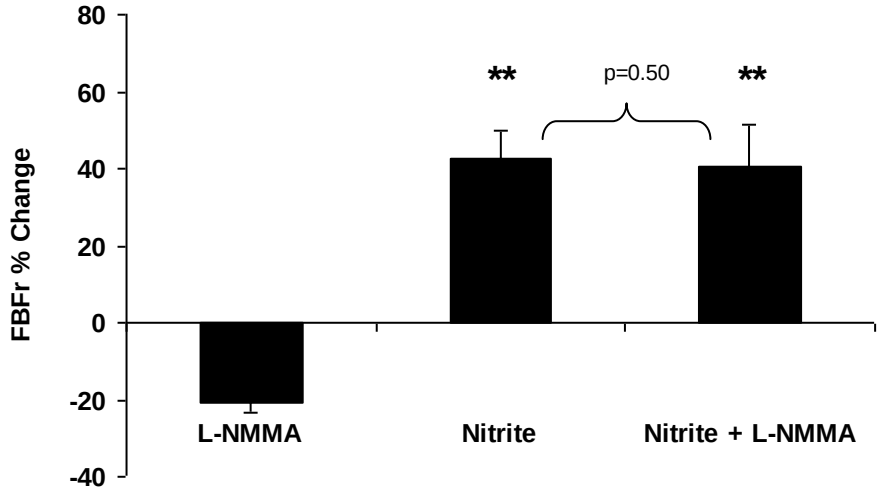


Figure 4.2 – FBFr and NOS inhibition

eNOS inhibition with L-NMMA did not alter the resistance vessel response to nitrite. *P<0.05 compared to baseline; **P<0.01 compared to baseline, n=9.

Xanthine oxidase inhibition did not affect nitrite-induced venodilation

In subjects pre-treated with the xanthine oxidase inhibitor allopurinol, nitrite (7.84μmol/min) caused an upward displacement of the VPR of 26.9 ± 3.8% compared to baseline (P=0.011) (Fig.4.3) as compared to an upward displacement of the VPR of 26.8 ± 4.6% in controls (P=0.004) (Fig.4.3). P=0.30 between groups.

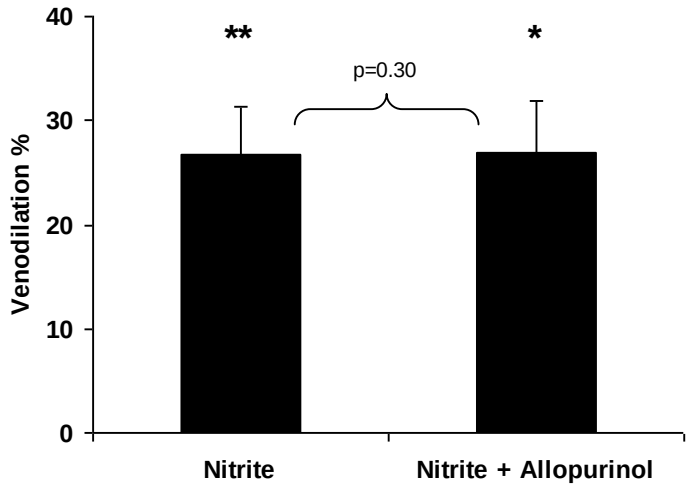


Figure 4.3 - XOR inhibition and FVV

XOR inhibition with allopurinol did not affect nitrite induced venodilation. *P<0.05 compared to baseline, n=10; **P<0.01 compared to baseline, n=9.

Xanthine Oxidase inhibition did not affect resistance bed response to nitrite

In subjects pre-treated with allopurinol, 7.84µmol/min nitrite increased the FBF ratio by 51.8% (to 1.5 ± 0.1, P=0.021) (Fig.4), as compared to an increase in the FBF ratio of 42.7% (from 1.00 ± 0.02 to 1.39 ± 0.08, P=0.007) with 7.84µmol/min nitrite alone. P=0.50 between groups.

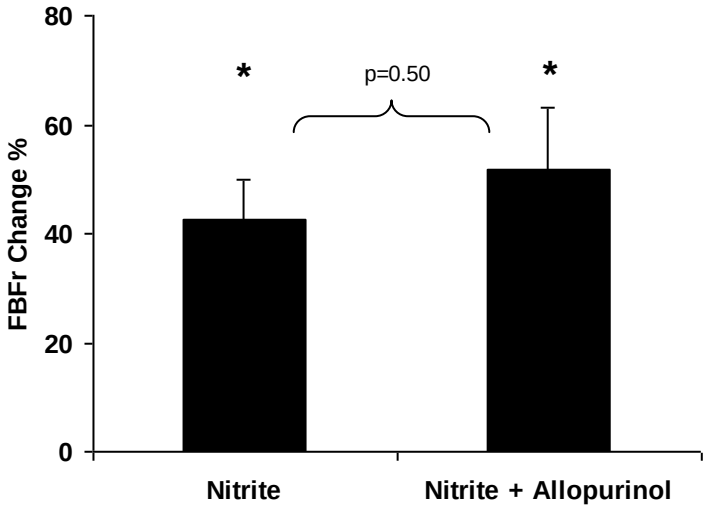


Figure 4.4 - FBFr and XOR inhibition

XOR inhibition with allopurinol did not affect resistance vessel response to nitrite, compared to controls. *P<0.05 compared to baseline, n=10 for allopurinol, n=8 for controls.

4.4 Discussion

The principal finding of the present study is that XOR and eNOS, irrespective of their cellular location, do not significantly contribute to nitrite-induced vasodilatation *in vivo* in healthy human volunteers under the conditions of this study. Our observations pertain to both resistance and capacitance vessels, and are based on the use of allopurinol and L-NMMA, which are well-established inhibitors of XOR and eNOS.

Role of NOS as a nitrite reductase

NO has a central role in maintaining basal vascular tone as evidenced by the observations that intra-arterial NOS inhibitors reduce resting blood flow[205] and that mice lacking eNOS exhibit increased vascular tone (as manifested by their hypertension).[206] This crucial role of vascular eNOS and NO in vasoregulation[207], coupled with the capacity of the vasculature to bioconvert nitrite,[61, 70] converged to proposals that nitrite may be reduced by eNOS *ex* and *in vivo*. Moreover, there is increasing evidence that RBCs express biochemically active eNOS on the cytoplasmic surface of their membranes. Accordingly, it has been reported *ex vivo* that RBC eNOS, at concentrations of nitrite closer to the physiological range represents a source of nitrite derived NO free from scavenging by intracellular oxyHb. Thus while RBCs may in the context of severe acidosis (pH = 6.8) liberate NO from nitrite, the results of the present study do not support such a role for eNOS under less extreme conditions (i.e. L-NMMA did not inhibit nitrite-induced vasodilatation *in vivo)*. In arteries and in the mildly hypoxic environment of veins the reduction in the venodilator effect of nitrite by co-infusion of L-NMMA was equivalent in magnitude to the effect of L-NMMA on basal venous tone[138]. At most eNOS therefore makes only a modest contribution to nitrite-induced venodilation (20% or less, and probably none) under physiological conditions.

In order to reconcile the observations made in previous studies with our observations, we note that: (i) The concentrations of nitrite often used ex vivo (e.g.100µM) are 1-2 orders of magnitude greater than both those occurring *in vivo* (0.2-5µM) or the vasodilating nitrite levels identified in previous studies (<10µM).[208] In the present study, while the initial nitrite dose was similarly high(~50µM)[208], nitrite-induced vasodilatation persisted as levels fell to near physiological concentrations. (ii) *Ex vivo* hypoxia is established by purging of experimental chambers with N2, yielding very low PO_2 levels (2-4mmHg). By contrast, in health (at rest), venous oxygen tension is typically around 70 -80mmHg. At physiological oxygen tensions in *vivo*, oxygen concentrations are not limiting for eNOS (Km 6-9 µMol). Thus while eNOS-related nitrite conversion may be of pertinence to severe hypoxia (anaeorobiosis and acidosis), biochemical considerations would not readily explain such a role *in vivo* under relatively normal circumstances. (iii) Simlarly, the pH yielding maximal NO elaboration *ex vivo* studies (~6.8) is extreme**.** (iv) Finally,RBC NO production, while potentially of importance in very severe hypoxia and acidosis, is approximately 10-fold less than that of blood vessels. Mitigating this lesser RBC NO production, it is recognized that in the context of hypoxia, NO donors appear to have a greater gain in activating cGMP (i.e. hypoxia increases NO bioavailability).

Role of XOR as a nitrite reductase

Xanthine oxidoreductase is a potential nitrite reductase.[59, 61] Whilst the present study does not exclude a role for XOR as a vascular reductase in pathological states, it does not support the proposal that XOR is a major *in vivo* reductase in healthy human volunteers under normoxic/mildly hypoxic conditions. Indeed, the demonstrated contribution by XOR to (rat vascular) NO production is less than 25%. This is consistent with recent biochemical data showing that heme proteins are the principal nitrite reductases.[209] Methodological differences

may again have contributed to the discrepancy between our findings and those of ex vivo studies. These include the high concentration of nitrite, low pH (~5.5), and low oxygen tension used in these studies.

4.5 Limitations

The present study has a number of limitations.

The dose of nitrite used is an order of magnitude greater than that present in normal physiology. We did however note that vasodilatation persisted into a period where nitrite concentrations were close to physiological concentrations. Moreover, the focus of the present study was the effect of exogenous nitrite.

Our interpretation of the effect of L-NMMA depends upon the subtraction of the effect of this non-specific NOS inhibitor on baseline flow. We have previously shown that infusion of L-NMMA alone leads to a 10% reduction in FVV, which has been interpreted as being due to an inhibition of basal NO production. It is however possible that a component of this may be due to the production of NO by NOS using nitrite as a substrate. Although this is a limitation of the study we feel that this is unlikely given that the magnitude of the venodilatation is not blunted once the 10% adjustment is made.

4.6 Conclusion

Many proteins, especially those with an established capacity for electron transfer (e.g. hemes, cytochromes, molybdenum dependant oxidoreductases etc), *can* reduce nitrite. Although the chemistry of these reduction reactions will vary and are interesting in their own right, there is an increasing focus on what *does* reduce nitrite *in vivo*. Our results are best seen as being

complementary to those of Webb et al.[168] While the nitrite reductase activity of eNOS and XOR may be relevant and important in pathological states, our *in vivo* studies in man would suggest that in resting healthy volunteers, eNOS and XOR do not play a significant role. Focus will therefore return to other contenders in the field including deoxyhaemoglobin and more recently haem containing proteins in the vascular wall.

Whilst conducting these studies we observed a phenomenon which initially caused some confusion and which had not been hitherto described. We observed that both the arterial and venous responses to nitrite endured despite plasma levels having dropped close to those at baseline. These observations are described in more detail in the following chapter.

Chapter 5

The Persisting Vascular Effects of Intra arterial Nitrite

This data has been presented at The 57th Annual American College of Cardiology (ACC) scientific sessions, March 2008:

- Maher AR, Thomas P, Anderson S, Abozguia, Ahmed I, Weaver R, James P, Frenneaux MP. Intra-arterial causes an enduring Vasodilation in Man. The 57th Annual American College of Cardiology (ACC) scientific sessions, March 2008, Chicago, USA

Execution

I performed all of the recruitment and organisation of the appointments involved in the study. I performed assessment of forearm blood flow, radionuclide plethymography and radionuclide ventriculography studies in the University of Birmingham. Biochemical analysis was performed by Dr Phil James and his team at the University of Cardiff, Wales.

Abstract

Background: We present the important observation that the vascular effects of nitrite following an intra-arterial infusion persist long after the intravascular concentration of nitrite has returned to levels close to baseline.

Methods: 17 Healthy volunteers were studied while either breathing room air or 12% oxygen. Following saline, sodium-nitrite was infused intra-arterially at 7.84μmol/min followed by an hour-long saline infusion. At each stage Forearm Blood Flow (FBF), Forearm Venous Volume/Pressure relation (an index of venous tone) and plasma levels of NO metabolites were assessed.

Results: Following cessation of nitrite infusion venodilation persisted for up to 30 minutes whereas venous nitrite levels had decreased from a peak of 48.9 microM to 4.4microM by 15minutes. Arterial blood flow expressed as FBF-ratio (FBFR), increased from baseline to 1.50±0.16 (P<0.05) and 1.99±0.17 (P<0.01) in normoxia and hypoxia respectively and returned to baseline only at 45 minutes and 15 minutes respectively.

Conclusions: We demonstrate a persisting effect of nitrite long after the intravascular levels have decreased suggesting a significant degree of protein binding. We propose that protein binding of NO in vessels (possibly by myoglobin) may be responsible. Our data may provide new insights into the role of nitrite in vascular physiology.

Key words: Nitric oxide, nitrite, veins, hypoxia, myoglobin

5.1 Introduction

Herein I focus on a fascinating aspect, namely the time-course of vasodilation post the termination of the nitrite infusion. We show that this vasodilation persists for an unexpectedly long period of time. This observation appears to be in distinct contrast to the phenomenon of rebound vasoconstriction that has been observed with nitrates and implicated in the ischaemia seen in patients taking nitrates during their 'nitrate-free period'.[210] Rabbits treated with glycerin trinitrate exhibit an increased coronary resistance after abrupt withdrawal.[211]

We have previously reported the acute effects of local nitrite infusion. We showed that in normoxia nitrite was a potent venodilator but had little effect on resistance vessels. However, in hypoxia nitrite became a potent arteriolar-dilator. We now present data on the persistence of this effect following cessation of nitrite infusion despite normalisation of nitrite levels.

5.2 Methods

Subjects

Seventeen healthy volunteers were studied. All subjects had no history of active smoking, hypertension, diabetes or hypercholesterolemia and no family history of ischemic heart disease. None of the healthy volunteers was taking cardioactive medication or vitamin supplements and all had a normal cardiovascular examination and electrocardiogram. All subjects gave written informed consent. The study was approved by the Local Research Ethics Committee. Studies were performed at the University of Birmingham Clinical Research Block in a dedicated vascular laboratory (22 to 24°C) and with the subject having had a light breakfast and abstained from caffeine for at least 6 h previously.

Biochemistry

Chemicals and reagents

All chemicals were purchased from Sigma other than glacial acetic acid, HPLC grade nitrite free water and hydrochloric acid (Fisher Scientific). Sodium nitrite was purchased from Martindale Pharmaceuticals, UK.

Ozone-based chemiluminescence

Plasma nitrite and protein-bound NO were measured using a tri-iodide reagent linked to ozone based chemiluminescence as described previously.[142] The tri-iodide reagent is probably the most widely used in the NO metabolite field and has been validated against standards in several laboratories.[5, 143-146] The reader is referred to a complete discussion of the assay.[33, 45, 142, 147-151]

A stock solution of tri-iodide reagent was prepared fresh each day.[142, 146] Immediately prior to analysis frozen plasma samples were thawed in a water bath at 37°C for 3 minutes. We have previously shown sample freezing has no effect on plasma NO metabolite stability.[152] Area under curve was used in analysis and concentrations were calculated from a standard curve of sodium nitrite.

Study Protocols

In vivo studies

FVV was assessed using radionuclide plethysmography. The details of the technique and its relative merits for assessing venous capacitance are detailed elsewhere.[139]

Subjects rested supine. Both forearms were positioned on the face of a gamma camera (Scintron, MIA and ADAC-Transcam). A 20-gauge cannula was inserted into an ante-cubital vein in each arm. Venous blood was drawn and sent for a Full Blood Count and Biochemical Profile. A 27-gauge arterial needle (Coopers Engineering, United Kingdom) mounted onto a 16-gauge epidural catheter, sealed with dental wax, was inserted into the non-dominant brachial artery under sterile conditions and kept patent by the continuous infusion of normal-saline.

Heart rate and rhythm were monitored continuously (Dash 3000, Marquette, GE) as was blood pressure with finger plethysmography (TNO TPD Biomedical Instrumentation, Delft, Netherlands). Mercury-in-silastic strain gauges were applied to measure FBF (D.E. Hokanson, Inc., Bellevue, Washington), as described previously.[153] Fifteen minutes after the administration of the radio-labeled red blood cells two venous Volume Presure Relationships (VPR) were recorded and FBF were assessed. Blood was drawn from both venous cannulae

at each stage of the study. Samples were collected in 5mL syringes and transferred into 4ml EDTA collection tubes. These were centrifuged at 2000rpm for 10 minutes at 4^0C. The plasma was snap frozen in liquid nitrogen and stored at -80ºC until analysis for plasma nitrite and protein-bound NO.

The study proceeded with the subject either breathing room air (9 subjects) or 12% oxygen (8 subjects).

In the subjects breathing 12% oxygen the study continued when arterial oxygen saturation levels were stable in the range 83-88%.

Oxygen saturation levels were monitored continuously using pulse-oximetry (Nellcor N-180).

In the subjects breathing 12% oxygen baseline measurements of all parameters were taken again during the initial saline infusion ('hypoxia alone' stage).

Intra-brachial sodium nitrite was then infused at a dose of 7.84µmol/min for 10 minutes following which FVV and FBF were assessed (hence nitrite was infused for 20 minutes in total). In the subjects breathing 12% oxygen the face mask was then removed. Saline was then infused for 60 minutes. Every 15 minutes FVV and FBF were assessed.

Data and Statistical Analysis

All data are expressed as mean±SEM; *P* values of <0.05 were considered statistically significant. Changes in FBF and FVV observed in the study arm were corrected for those in the control arm. One-way analysis of variance was carried out for changes in FBFR and FVV.

5.3 Results

Subject characteristics

Grouped baseline characteristics for all patients are shown in Table 5.1.

Table 5.1

Demographic and Clinical Features	
Age (y)	56.2±1.9
Sex, M/F	15/2
Body mass index (kg/m^2)	26.8±1.1
Smoking history, (n)	
Nonsmoker	17
Previous smoker	0
Current smoker	0
Serum cholesterol (mmol/L)	5.8±0.3
Plasma glucose (mmol/L)	4.5±0.1
Serum Creatinine (µmol/l)	98.4±2.7
Heart rate	66.0±3.0
Blood pressure (mmHg)	
Systolic	125±3.0
Diastolic	72.8±1.9

Venous Tone

Nitrite produced a 34% venodilatation (P=0.01) which was similar in hypoxia and normoxia. The venodilation remained significantly greater than baseline even at 15 minutes of saline infusion post nitrite in normoxia (P=0.016) and even at 30 minutes there was a trend towards significant venodilatation (P=0.057). In hypoxia the venodilatation persisted in a similar

pattern and remained significant until 30 minutes (P=0.009). (Figure 5.1 – Normoxia, Figure 5.2- Hypoxia)

Figure 5.1 - Venous Tone

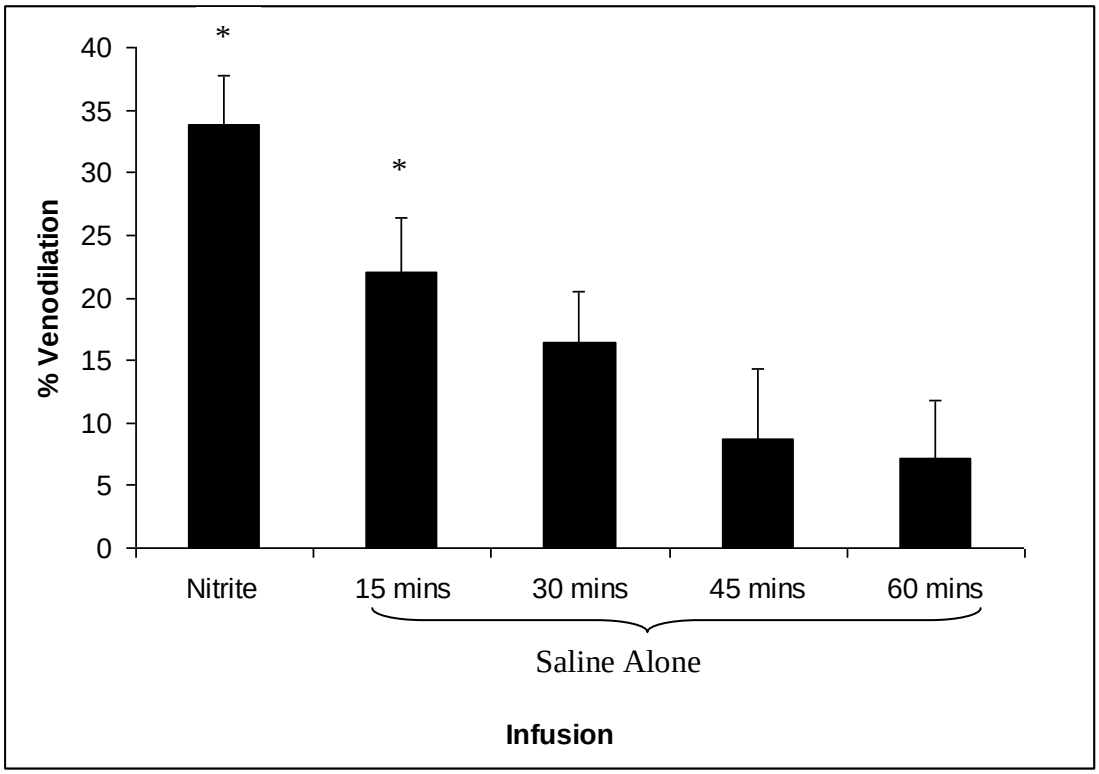


Figure 5.1 – Venodilation in 'normoxia'

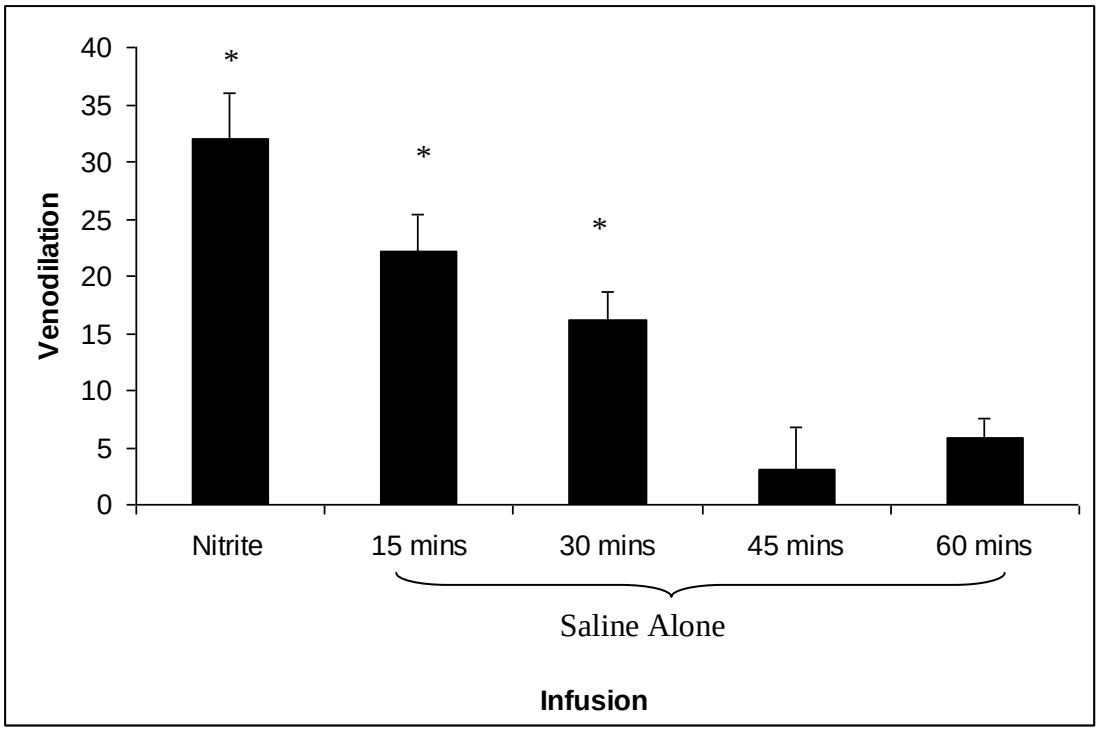


Figure 5.2 – Venodilation in 'hypoxia'

FBF

Nitrite caused a significant increase in FBFR (P<0.05). (Figure 5.3 – Normoxia, Figure 5.4 - Hypoxia) This increase was still significantly greater than baseline at 30 minutes (P=0.05) in normoxia but was no longer significant at 45 minutes. In hypoxia the pattern of response was similar although by 15 minutes of washout FBFR was no longer statistically greater than that at baseline.

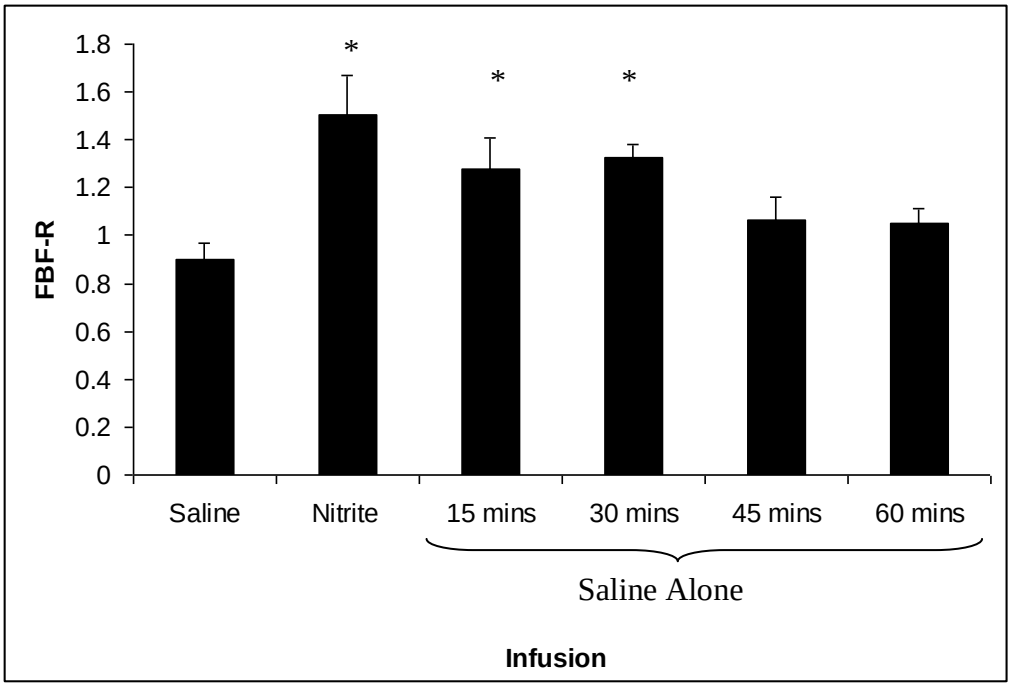


Figure 5.3 – FBF in 'normoxia'

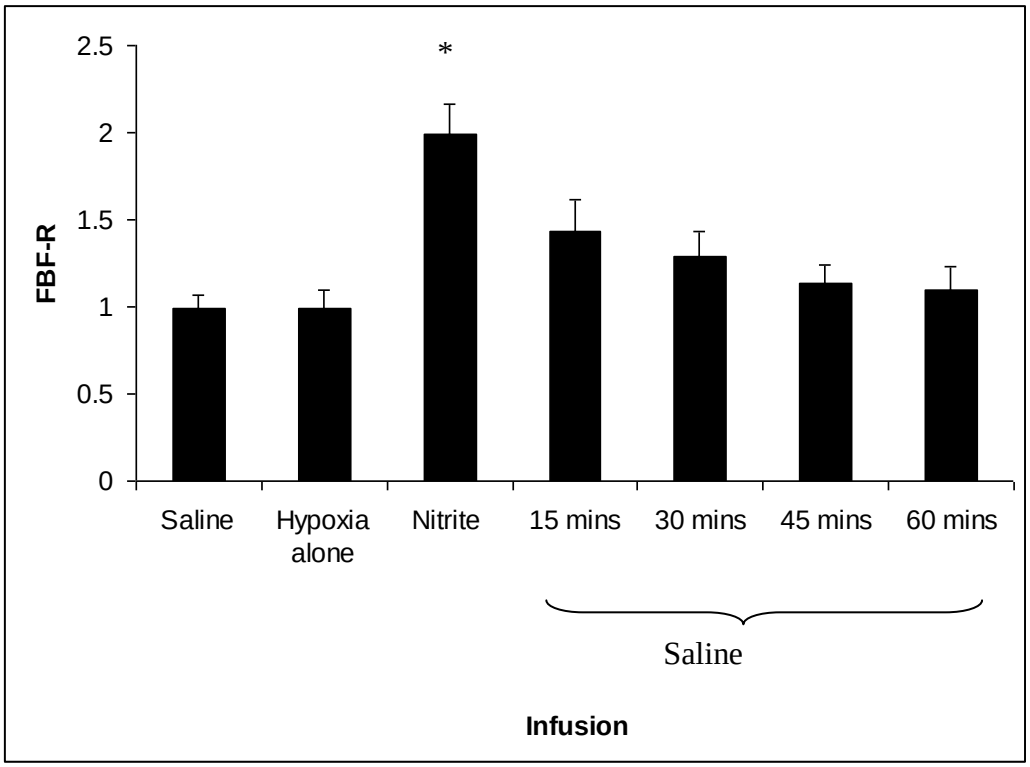


Figure 5.4 – FBF in 'hypoxia'

NO metabolite levels

The levels of nitrite rose in the infused arm during the administration of sodium nitrite.(P<0.05) However, by 15 minutes these levels had dropped 10 fold and were no longer significantly different to those in the control arm (Figure 5.5). There was no significant change in the levels of protein bound NO in either arm at any time point (Figure 5.6).

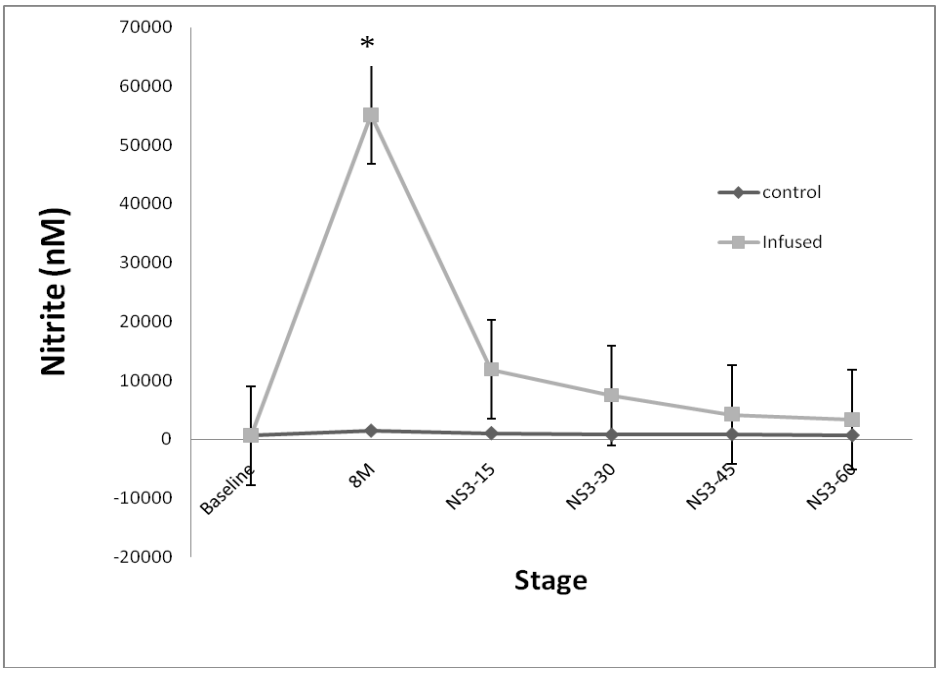


Figure 5.5

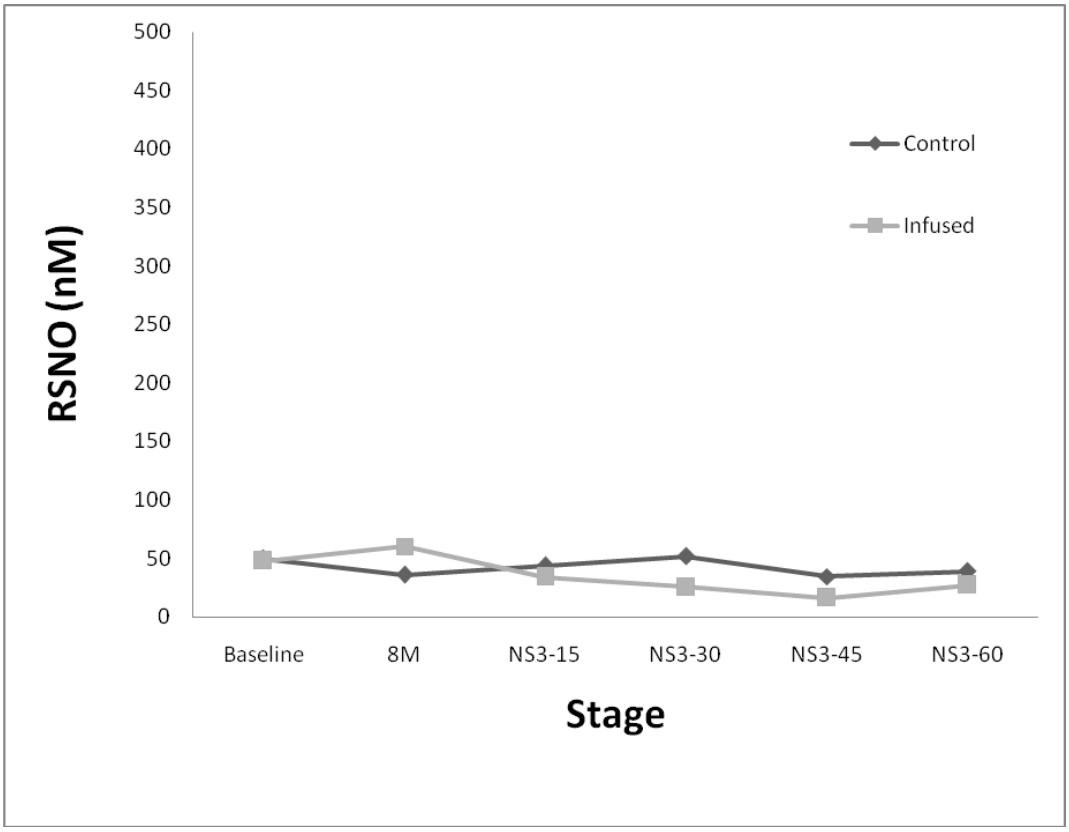

500
450
400
350
300
250
200
150
100
50
0
RSNO (nM)
Control
Infused
Baseline
8M
NS3-15
NS3-30
NS3-45
NS3-60
Stage

Figure 5.6

5.4 Discussion

We report an enduring effect of nitrite after the levels had dropped 10-fold. In fact in some individuals we actually observed a further increase in vasodilation after termination of the nitrite infusion before a gradual return to baseline (data not shown). In contrast, in previous studies in which we have examined the responses to Bradykinin and Carbachol[155, 212] venodilation disappeared within five minutes compared to the 30-45 minutes observed with nitrite. Our data would suggest that a potential reservoir may have been created from which NO was slowly released leading to the ongoing vasodilation.

While this study does not investigate the mechanisms underlying this phenomenon it does point towards some important possibilities. Our observations were not explained by an increase in circulating protein bound NO metabolites.

We hypothesise that a proportion of the NO released from nitrite was bound to a component of the vessel wall and then released over a period of time. It is possible that the myoglobin in the vascular smooth muscle may serve this function. In fact, there has been considerable recent interest in the role of myoglobin in NO homeostasis.[213, 214]

In a paradigm similar to that of haemoglobin, we propose that myoglobin may act as a store of NO. NO released from nitrite by deoxymyoglobin could bind to the sulphydryl group of the cysteine residue[215] (position 110) of human oxyhaemoglobin forming S-NO oxyMb. This S-NO oxyMb could act as a stable store of NO with the bound NO being slowly released. Rayner et al have demonstrated *in vitro* that mixtures containing human ferrous oxymyoglobin with NO donors such as S-nitrosoglutathione and S-nitrosocysteine could lead to the generation of S-NO oxyMb. Moreover S-NO oxyMb relaxed preconstricted aortic rings in a cyclic GMP dependent manner.[213, 216, 217] It has been demonstrated that binding of

NO to the sulphydryl group of myoglobin is dependent, at least in part, on the oxygen tension of the environment.[218]

We did observe, as in Chapter 2, a greater initial increase in FBFR in hypoxia and a slightly quicker return to baseline, however the overall pattern of response was similar in the two oxygen environments when comparing the 'areas under the curve'. Although we expected the response to be modulated by hypoxia, due to an increased level of deoxymyoglobin, this was not found in our study. However an important limitation was the fact although the nitrite was infused with the subject breathing 12% oxygen (20 minutes) it was not possible to expose the subjects to this degree of hypoxia through the hour long period of saline washout as this was beyond the remit of our ethical approval.

In summary, we have found intra-arterial nitrite to cause a vasodilation that endures for a significant length of time after the infusion has ceased, probably due to vascular protein binding. This is both a novel and exciting observation. While myoglobin could possibly mediate this phenomenon further mechanistic studies are needed to elucidate this.

This possibility and the role of vascular myoglobin in nitrite reduction was investigated further. The studies and findings are presented in chapter 7.

In order to investigate the effects of intravenous infusion of inorganic nitrite and to inform future studies by providing data regarding the safety of its use in this context, a series of studies were performed in both normoxia and hypoxia. These studies are described in the next chapter.

CHAPTER 6

Systemic Effects of Intravenous Sodium Nitrite

Execution

The studies described in this chapter were designed and organised by myself with the normoxic studies being executed by myself and the hypoxic studies by Dr Sayqa Arif. Biochemical analysis was performed by Dr Phil James and his team at the University of Cardiff, Wales.

Abstract

Aims

The discovery of nitrite as a source of nitric oxide, particularly in hypoxic and ischemic conditions (at least at pharmacological concentrations) has generated great interest amongst researchers. When administered intra-arterially under normoxic conditions, nitrite has a selective vasodilatory effect on the capacitance bed but during hypoxia marked dilation of forearm resistance vessels was also observed. Accordingly, we hypothesised that an intravenous infusion of sodium nitrite would cause a significantly greater reduction in blood pressure during hypoxia compared to normoxia, in humans.

Methods

33 healthy subjects were recruited. An intravenous infusion of sodium nitrite was administered at escalating doses (3, 8, 30 and 60μmol/min) during either normoxia or hypoxia in random order. Haemodynamic measurements were determined non-invasively through the use of a hemodynamic monitoring system (Taskforce®). Aplanation tonometry (Sphygmocor®) was used to delineate central aortic blood pressure.

Results

To allow for the time-dependent effect of hypoxia all measurements in this group were placebo-corrected. During hypoxia systolic blood pressure fell by a mean of 11mmHg ($P \leq 0.05$) from baseline at the highest dose of sodium nitrite. There was a non-significant reduction in systolic blood pressure during normoxia of 6mmHg. Heart rate increased in association with blood pressure reductions. No significant changes were observed in stroke volume, cardiac output or cardiac index with nitrite during either hypoxia or normoxia. In the hypoxia group total peripheral resistance mirrored changes in systolic blood pressure. Central aortic blood pressure fell in both groups (9 v 13 mmHg in normoxia [$P>0.05$] v hypoxia [$P>0.05$]) from baseline in both groups.

Conclusion

An acute intravenous infusion of nitrite was safe and well tolerated in healthy volunteers. A significant decrease in blood pressure was observed during hypoxia but not during normoxia, thus supporting the role of nitrite as a hypoxia-specific vasodilator.

6.1 Introduction

Several potential mechanisms by which nitrite may be reduced to NO have been demonstrated in animal models, including deoxyhemoglobin, deoxymyoglobin, eNOS, xanthine oxidoreductase, acidic disproportionation, and mitochondrial complex IV [76, 114, 168, 219-224]. However, one major commonality to all of these mechanisms is that more NO is released from nitrite during hypoxic conditions [225]. Furthermore we were the first to demonstrate in man that intra-arterial sodium nitrite infusion only modestly dilated forearm resistance vessels but potently dilated capacitance vessels under normoxic conditions *in vivo*. However, under hypoxic conditions it markedly dilated forearm resistance vessels[131].

In this chapter, we sought to investigate the effect of intravenous sodium nitrite on acute haemodynamic parameters in healthy volunteers, during both normoxia and hypoxia. We hypothesized the following: 1) While subjects are breathing room air, there would be no significant vasodilatation of the resistance vessels as verified by an unchanged arterial blood pressure and systemic vascular resistance; 2) When subjects inspire 12% oxygen, rendering arterial blood relatively hypoxic a significant reduction in arterial blood pressure and systemic vascular resistance would be observed.

6.2 Methods

Subjects

33 healthy volunteers (20 male and 13 female) were recruited into the study. 18 were allocated to protocol A (normoxia) and 15 into protocol B (hypoxia). Participants had no history of smoking, were free of known cardiovascular disorders and were not on any regular

medication or vitamin supplements. All had a normal cardiovascular examination and electrocardiogram. The investigation conforms to the principles outlined in the Declaration of Helsinki. The study was approved by the local research ethics committee (ref: 07/Q2707/10 and 08/H1207/69). All subjects gave written, informed consent. The study was performed at the University of Birmingham Clinical Research Block in a quiet vascular laboratory which was temperature-controlled at 22 - 24ºC. All subjects had a light breakfast and were requested to abstain from alcohol for 24 hours and caffeine-containing drinks for 12 hours before the study.

Subjects were allocated randomly between protocols and A and B of the study.

Measurements

Cardiac haemodynamic variables were measured noninvasively by transthoracic electrical bioimpedance (Taskforce® Monitor, CN Systems, Graz, Austria), as described previously [226]. Blood pressure and heart rate were measured continuously. Impedance cardiography was used to determine changes in stroke volume, cardiac output and total peripheral resistance. Although absolute values of these parameters measured by the bioimpedance technique differ significantly from those measured using the invasive thermodilution technique it has been shown to be a valid way of assessing changes in these parameters [227]. Furthermore, as the subject group consisted of healthy volunteers it would have been unjustified to use invasive haemodynamic monitoring for the sole purpose of this study.

The haemodynamic data were used for calculation of BRS using the sequence method [228]. The algorithm searches for episodes of spontaneous activation of the baroreceptor reflex. Episodes of baroreceptor reflex activation/inactivation are defined when blood pressure rises/falls for at least 1 mm Hg for at least four consecutive heartbeats and when,

simultaneously, decrements/increments of RR-interval of at least 4 ms/beat, respectively, occur. Linear regressions of increments/decrements in systolic blood pressure and increments/decrements in RR-interval were computed. Only episodes with correlation coefficients of greater than 0.8 were selected, and from all regressions, a mean slope of baroreceptor sensitivity was calculated for each steady state period. Spectral analysis of RR-interval (RRI) to determine HRV was performed as described elsewhere [229]. Parameters of low frequency (LF) and high frequency (HF) components were expressed as normalised units (nu).

The technique of aplanation tonometry using SphygmoCor® (AtCor Medical, Gloucestershire, UK) was used to estimate central aortic pressure non-invasively, and has been described elsewhere [230]. Pulse wave contour analysis was performed by taking a recording at the radial artery. Arterial waveforms were recorded non-invasively over 10 seconds using a high-fidelity hand-held tonometer. The radial pressure wave was calibrated to brachial blood pressure (Taskforce®). The central aortic pressure waveform was calculated using a validated generalized transfer function.

Study medication

Sodium Nitrite was obtained from Martindale Pharmaceuticals UK (Brentwood, United Kingdom). It was diluted to the required concentration using 0.9% sodium chloride solution. An intravenous infusion of sodium nitrite at a rate of 1ml/min was used as placebo.

Study Protocols

Protocol A - normoxia

Subjects were placed in a semi recumbent position on a hospital bed. This position was used as it allowed 12% oxygen to be administered to participants entered into protocol B. An intravenous cannula (Venflon, 20 Gauge) was inserted into a vein in each antecubital fossae. Blood was drawn and sent for a full blood count and biochemical profile (plasma urea, electrolytes, creatinine, glucose and cholesterol levels). Each subject underwent placement of a finger cuff for continuous blood pressure recording, an upper arm cuff for the oscillating blood pressure measurement and ECG and band electrodes on the trunk for cardiography.

Following 10 minutes of rest, an intravenous infusion of 0.9% sodium chloride ('normal saline') was started for 20 minutes. 10 minutes into the infusion, haemodynamic variables were recorded using the Taskforce® over a 10-minute period. During this recording period three 10-second measurements of pulse wave analysis at the radial artery were performed, using the Sphygmocor technique. A mean of the three recordings was calculated and used for subsequent data analysis. The above measurements were repeated during intravenous infusion of sodium nitrite at 3 escalating doses of 8µmol/min, 30µmol/min and 60µmol/min. The starting dose of 8µmol/min was used during normoxia in light of our findings in chapter 2, where a dose of 7.84µmol/min was well-tolerated and produced significant changes in the forearm vasculature [131].

Protocol B - hypoxia

Subjects were prepared as for protocol A. Oxygen saturation levels were monitored continuously with pulse oximetry (Nellcor N-180, Nellcor, Pleasanton, California). Hypoxia was induced by subjects breathing in 12% oxygen via a facemask connected to a two-way valve. Target oxygen saturation as measured by pulse oximetry was 83% - 88% (estimated pO2 of 47.5 – 55 mmHg, based on Severinghaus' equation). The hypoxia protocol was

executed in two stages, at least 24 hours apart. Once patients reached target oxygen saturations the saline infusion was commenced. Initially saline was infused for 1 hour 15 minutes in total. On the second visit at a similar time in the day, three concentrations of sodium nitrite (3µmol/min, 30µmol/min and 60µmol/min) were then infused. As a precaution, a lower starting dose of sodium nitrite was used in this Group due to the anticipated effect on systemic blood pressure during hypoxia. Each infusion lasted 25 minutes each resulting in a total infusion time of 1 hour 15 minutes. As described in protocol A, cardiac haemodynamic measurements were recorded 10 minutes into each infusion. Autonomic parameters were recorded during the last 5 minutes of the 25-minute infusion. Hence, both the saline and nitrite arms of this protocol lasted for the same duration to allow for the time-dependent effect of hypoxia on our measurements.

Data and Statistical Analysis

Data was analyzed using SPSS version 15.0 software (SPSS Inc., Chicago, USA). Study results that are normally distributed are expressed as mean ± standard deviation (mean±SD) and non-normally distributed data are presented as median plus interquartile range (IQR). Probability values of <0.05 were considered statistically significant. For normally distributed data repeated measures ANOVA was used to analyze the data within groups and between group data was analyzed using ANCOVA. The non-parametric equivalents used were Friedman's tests with Wilcoxon-Rank Sign Test for *post hoc* analysis and the Mann-Whitney U test for between-groups analysis. The Bonferroni correction was applied where necessary. The authors had full access to the data and took responsibility for its integrity.

6.3 Results

Baseline Characteristics

There were no significant differences in the general characteristics of the study subjects in the two protocols (Table 6.1).

	Hypoxia n=15	Normoxia n=18	P value
Age (years)	31±11	32±9	0.72
Gender (n)	♂ 10 / ♀ 5	♂ 10 / ♀ 8	
Mean weight (kg)	72±18	69±11	0.35
Body mass index (kg/m^2)	24±5	24±3	0.98
Haemoglobin (g/dL)	13.8±0.8	13.8±1.1	0.65
Creatinine (µmol/l)	85±15	90±17	0.94
Cholesterol (mmol/l)	4.6±1.1	4.5±0.8	0.25
Glucose (mmol/l)	4.6±0.6	4.5±0.7	0.64

Table 6.1

Baseline characteristics of the study subjects

(Apart from gender all values are expressed as mean±standard deviation)

Cardiac haemodynamic data

Blood pressure

There was no significant reduction in systolic blood pressure with nitrite infusion at any concentration during normoxia with a mean BP reduction from baseline of 120±13mmHg to 114±22mmHg at the 60µmol/min dose of sodium nitrite (P>0.05). During hypoxia, however there was a significant mean reduction in blood pressure of 11mmHg from 123±19mmHg to 112±15mmHg at the 60µmol/min dose from baseline (P<0.05). Sodium nitrite was infused at the 60µmol/min dose in 7 and 6 subjects in the hypoxia and normoxia groups respectively. The mean reduction in blood pressure between the two groups was not significantly different (P>0.05).

Fig. 6.1a)

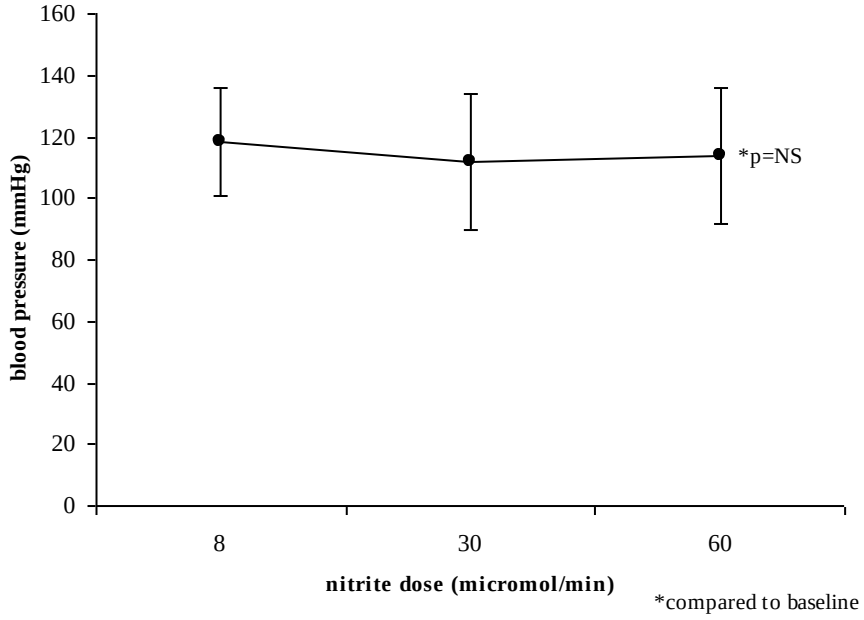


Fig. 6.1b)

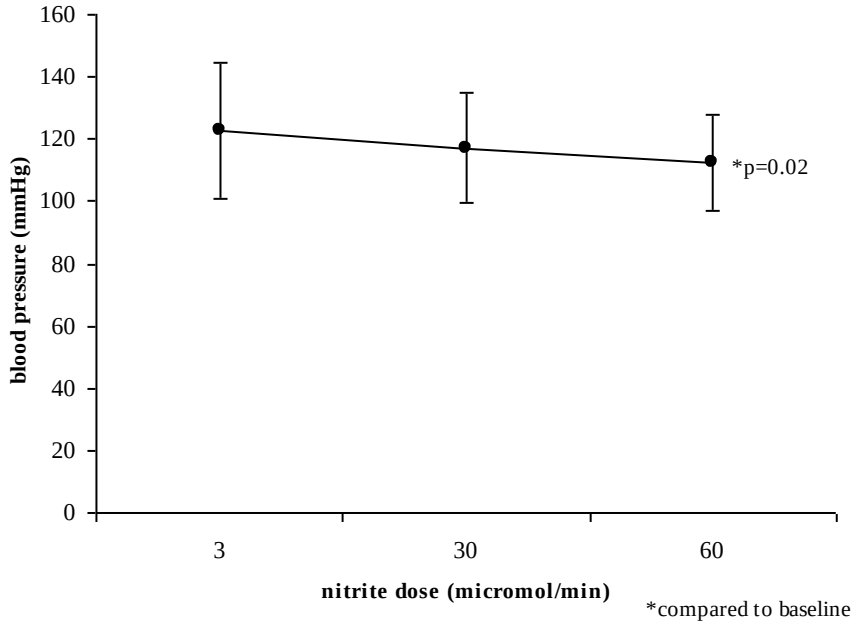
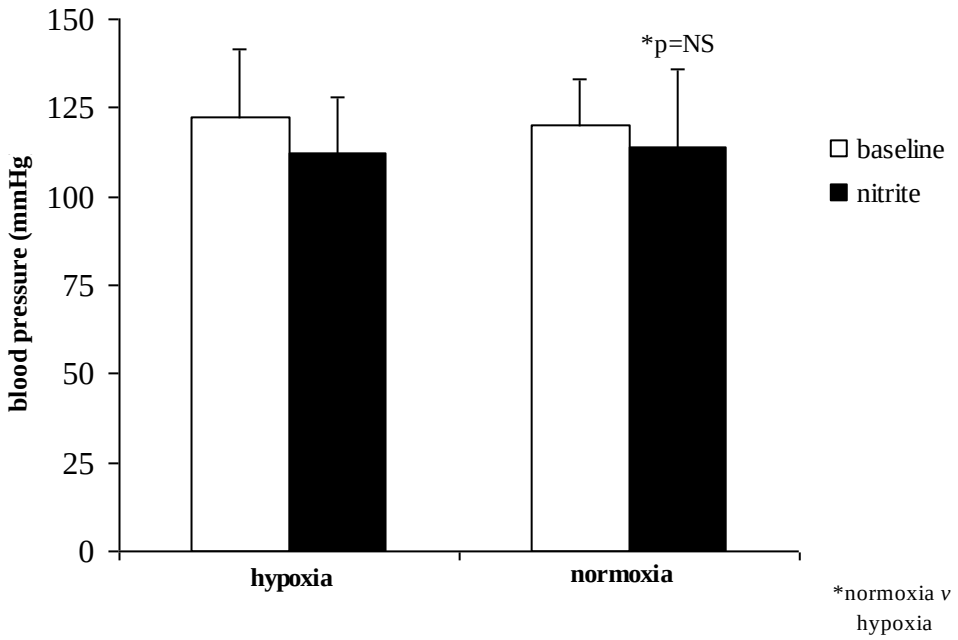


Fig. 6.1c)



Systolic blood pressure during a)normoxia ,b)hypoxia and c)both at 60 micromol/min dose. N=6 and 7 at the 60 micromol/min dose during normoxia and hypoxia respectively.

Heart rate

An increase in heart rate was observed in both groups. In normoxia, a reduction in systolic blood pressure of 6mmHg (at the 60µmol/min dose) was associated with an increase in heart rate of 8bpm (61±4 to 69±9bpm, $P<0.05$). During hypoxia, the heart rate at this dose increased by 13bpm (73±15 to 86±17bpm, $P<0.05$) in association with a mean reduction in blood pressure of 11mmHg (fig. 6.2).

Fig. 6.2a)

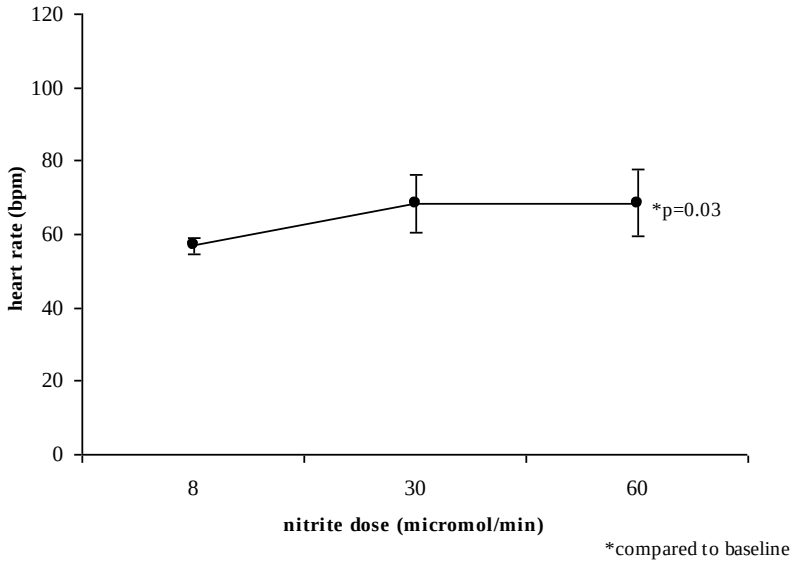


Fig. 6.2b)

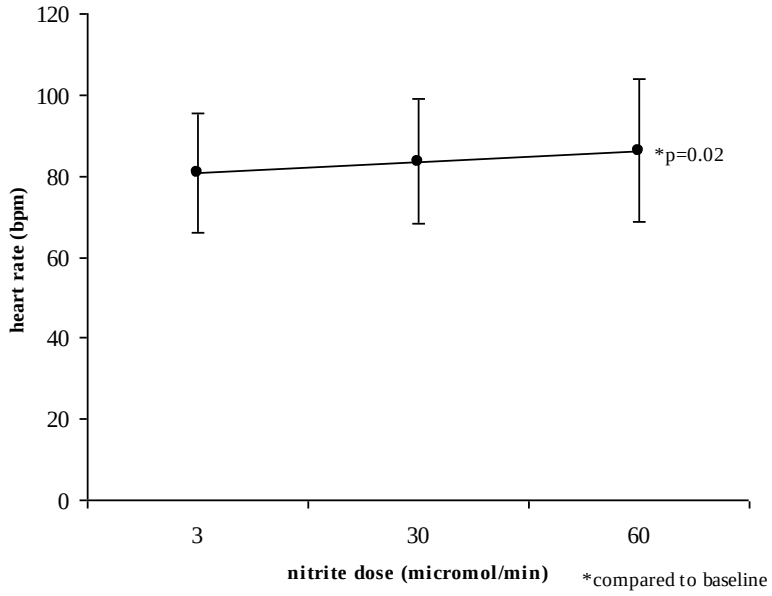


Fig. 6.2 Heart rate in response to an infusion of sodium nitrite during a)normoxia and b)hypoxia. N=6 and 7 at the 60 micromol/min dose during normoxia and hypoxia respectively.

Stroke volume, cardiac output, cardiac index, total peripheral resistance, total peripheral resistance index

There was no significant change in SV, CO or CI in either group during nitrite infusion compared to placebo (Table 6.2). TPR and TPR index mirrored changes in systolic blood pressure (fig. 6.3). The reduction in TPR and TPRI was significant in the hypoxia group at the 60µmol/min dose ($P<0.05$) but not in the normoxia group ($P>0.05$). However the change in TPR and TPRI was not significantly different between the normoxia and hypoxia groups.

	normoxia n=6	*P*-value*	hypoxia n=7	*P*-value*
SV (ml/min)	82.6±16.4	0.14	75.5±22.3	0.1
CO (L/min)	5.7±1.7	0.93	6.3±1.1	0.16
CI (l/[min*m²])	3.2±0.9	0.79	3.4±0.6	0.14
TPR (dyne*s/cm^5)	1154±265	0.25	1069±239	0.05
TPRI (dyne*s*m²/cm^5)	2064±587	0.29	1993±564	0.05
* compared to baseline				

Table 6.2.

Cardiac haemodynamic parameters following an infusion of sodium nitrite at the 60µmol/min dose.

Fig.6.3a)

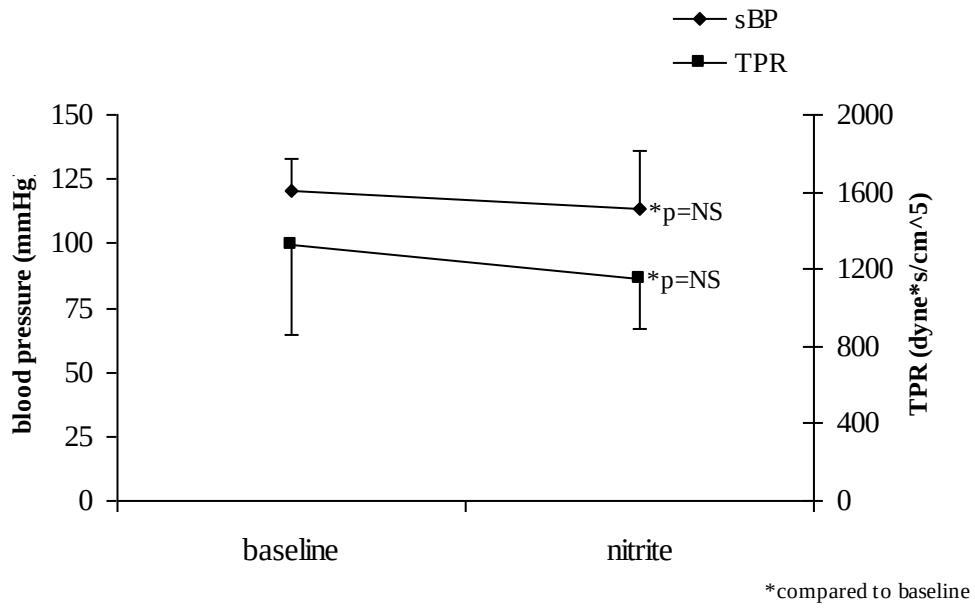


Fig. 6.3b)

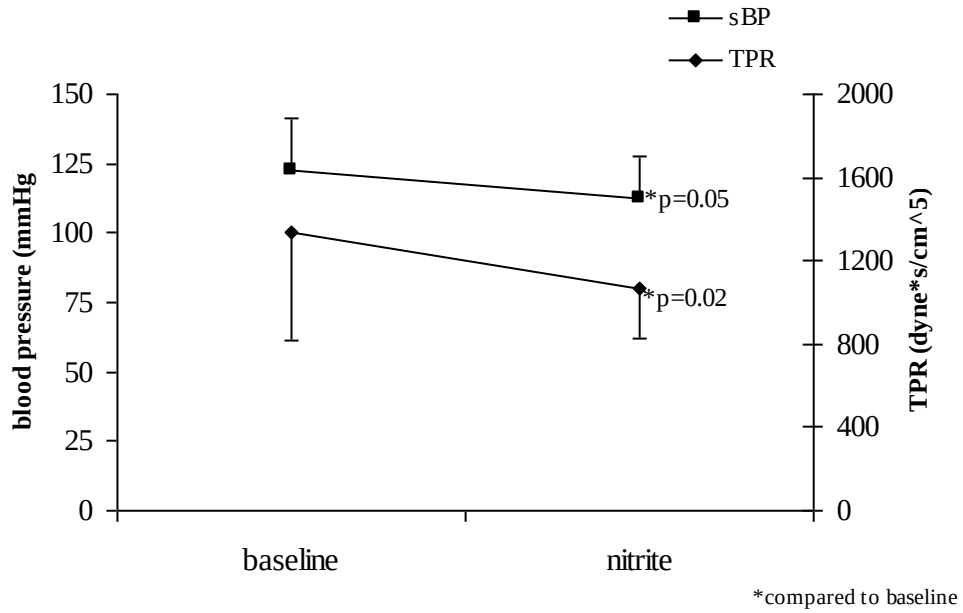


Fig. 6.3

Changes in TPR mirrored changes in systolic blood pressure during a)normoxia (n=6) and b)hypoxia (n=7). Data shown is for baseline and sodium nitrite at the 60µmol/min dose.

Autonomic data

In a sub-set of subjects in the hypoxia group (n=5) baroreceptor sensitivity and heart rate variability were studied (fig. 6.4). Data are presented as median (IQR). During hypoxia alone both baroreceptor sensitivity and heart rate variability remained unchanged (data not shown). Following intravenous infusion of 60µmol/min nitrite under hypoxic conditions, BRS was unchanged (median of 14.7 [11.5 – 34.8]) ms/mmHg baseline to a median of 14.1 (5.6 –

25.1) ms/mmHg during 60µmol/min nitrite infusion (*P*>0.05). However, HFnuRRI decreased significantly from 63.3% (50.8 – 71.7) to 39.8% (19.3 – 47.2, *P*<0.05). During nitrite infusion LFnuRRI increased by 36.7% (28.3 – 49.1) to 60.2% (52.8 – 80.6, *P*>0.05).

Fig. 6.4a)

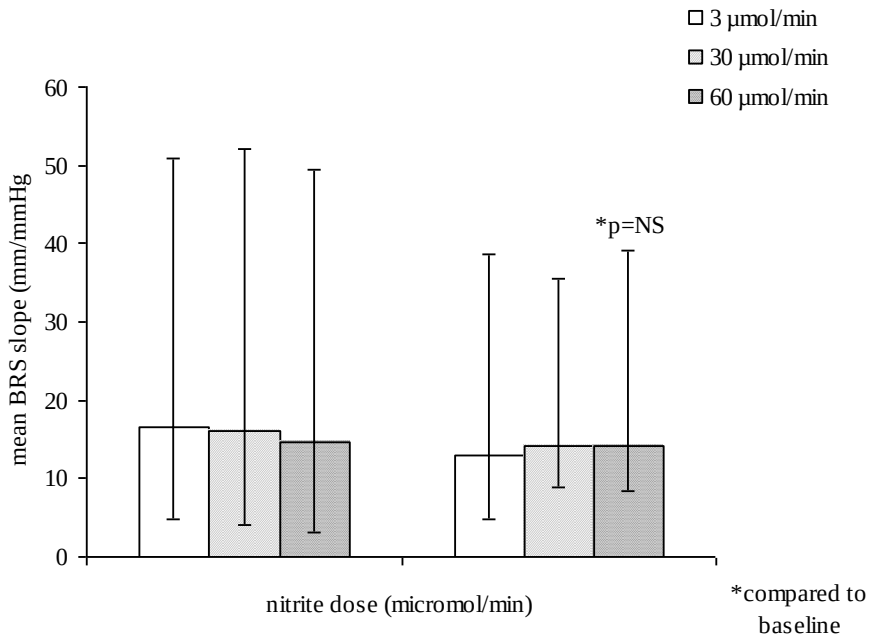


Fig. 6.4b)

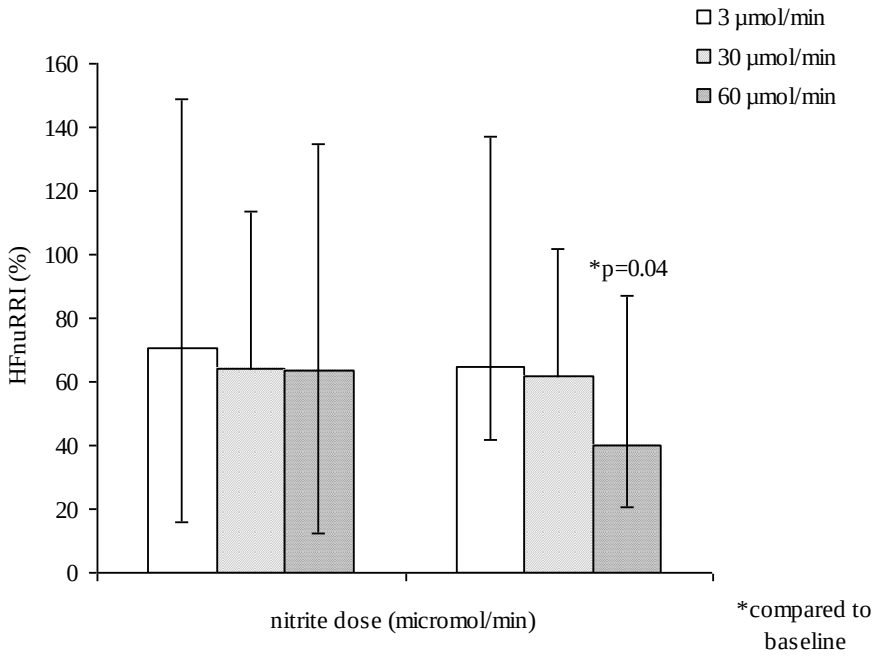


Fig. 6.4c)

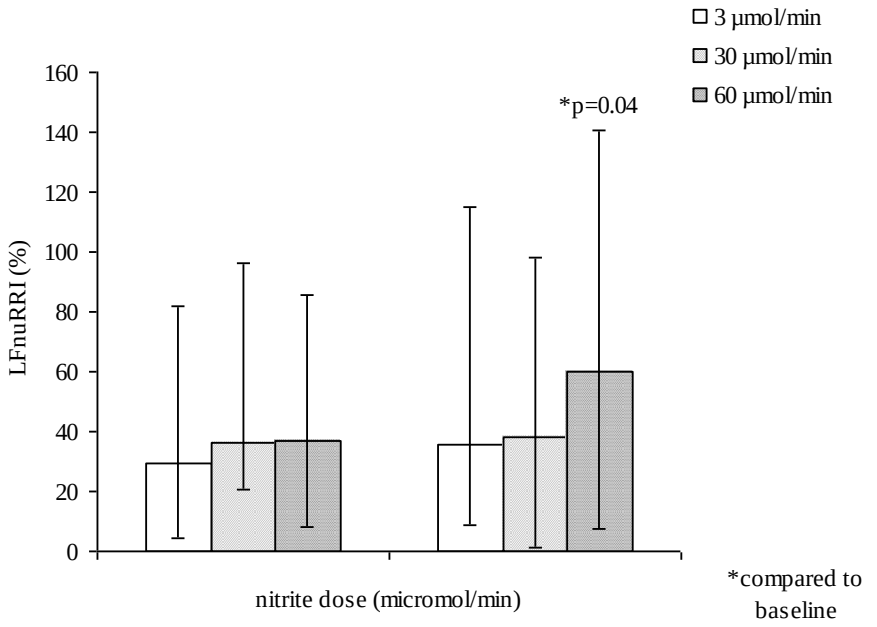


Fig. 6.4 Changes in autonomic parameters during hypoxia following an infusion of sodium nitrite

a)baroreceptor sensitivity, b)HRV - HFnuRRI and c) HRV – LFnuRRI. Data shown is expressed as median (IQR).

Central aortic pressures and aortic augmentation index

There was a trend towards reduction in central aortic pressure during hypoxia and normoxia at the 60µmol/min dose (Table 6.3). Data is presented as median (IQR). The central (aortic) systolic blood pressure decreased by 9mmHg from 104(95-112)mmHg to 95(90-98)mmHg at the 60µmol/min during normoxia, compared to baseline (P>0.05). During hypoxia there was a reduction of 13mmHg from 102(97-122)mmHg to 89(86-104)mmHg, compared to baseline (P>0.05).

	baseline	nitrite[#]	*P*-value*
Normoxia (n=8)			
pSBP (mmHg)	120(113 – 128)	114 (108 – 116)	0.12
cSBP (mmHg)	104 (95 – 112)	95 (90 – 98)	0.06
Hypoxia (n=5)			
pSBP (mmHg)	117 (113 – 138)	105 (100 – 128)	0.08
cSBP (mmHg)	102 (97 – 122)	89 (104 – 86)	0.08

*compared to baseline; Abbreviations used: peripheral systolic blood pressure (pSBP); central systolic blood pressure (cSBP).
Data shown is for nitrite at the 60µmol/min dose.

Table 6.3. Changes in cardiac haemodynamics as measured by applanation tonometry. Data shown is expressed as median (IQR).

6.4 Discussion

This study demonstrated that under normoxic conditions, an intravenous infusion of sodium nitrite up to a dose of 60μmol/min did not significantly alter cardiac haemodynamic parameters, with the exception of heart rate which increased by 8bpm equating to a 13% increase from baseline (P<0.05). There was a 5% reduction in systolic blood pressure but this did not reach statistical significance. In contrast, during hypoxic conditions, 60μmol/min of intravenous sodium nitrite significantly reduced systolic blood pressure by 9% (P<0.05). Furthermore, both TPR and TPRI decreased significantly in the hypoxia group thus in keeping with a reduction in systemic blood pressure.

Central aortic pressure as measured by applanation tonometry decreased non-significantly from baseline in both normoxic and hypoxic groups. However, there was a greater reduction associated with hypoxia of 13mmHg than during normoxia (9mmHg). The findings in hypoxia did not reach significance but this could be explained by missing data due to a measurement error resulting in values for only 5 subjects in the hypoxia group and hence under-powering of data in this group.

Autonomic measurements appropriately mirrored the changes in blood pressure with a resultant tachycardia. Vagal tone decreased as evidenced by a decreased in HFnuRRI and sympathetic tone increased reflecting the increase in LFnuRRI during hypoxia. It should however be noted as a limitation that, although changes in HFnuRRI and LFnuRRI have generally been interpreted as reflecting changes in vagal and sympathetic tone respectively, neither of these measures has a high degree of specificity.

During hypoxia, the measurements were corrected for the time dependent effect of hypoxia. Furthermore, an acute intravenous infusion of sodium nitrite was well tolerated and safe in both the hypoxia and normoxia group. No adverse events were seen. To our knowledge this is the first study in humans investigating the acute effect of an intravenous infusion of nitrite on cardiac haemodynamic parameters.

Following renewed interest in nitrite, numerous mechanisms for nitrite reduction to bioactive NO have been investigated. The potential of nitrite as a source of NO during hypoxia was first demonstrated in 1937, and then later in 1981 when Doyle et al. reported that nitrite reacts with deoxyhemoglobin (in the presence of protons) to produce methemoglobin and NO [231]. Hemoglobin deoxygenation is closely associated with the physiological oxygen gradient, advocating the role of nitrite as an effector of hypoxic vasodilation. This was demonstrated in humans by Cosby et al who demonstrated that nitrite at near physiological and supra physiological concentrations was associated with vasodilation with deoxyhemoglobin as an important nitrite reductase [184]. It has been suggested that nitrite reduction by non-enzymatic acid disproportionation and enzyme reduction by XOR both require low oxygen tensions further lending support to nitrite as an effector of hypoxic vasodilation [114]. More recently deoxymyoglobin has been reported to be an oxygen-independent nitrite reductase [76, 232]. We have demonstrated that in isolated mouse aortic rings from wild-type and myoglobin knockout mice, there is a markedly reduced (>50%) reduction in vascular reactivity in the knockout mice compared to the wild types during hypoxia (unpublished data). This has been confirmed *in vivo* in mice and in isolated mice heart preparations where myoglobin not only serves as a functional nitrite reductase, but regulates responses to cellular hypoxia and reoxygenation [116]. It has been proposed that these numerous mechanism may allow for generation of bioactive NO *in vivo* across the physiological oxygen gradient [85].

Although, the present study has not addressed the mechanisms related to nitrite reduction it adds important new information into the role of nitrite during hypoxia in humans. Significant blood pressure reductions were seen in the hypoxia group at pharmacological doses of nitrite. These results corroborate our earlier findings that under hypoxic conditions, nitrite is an arteriolar as well as venous dilator strengthening the case for nitrite as an effector of hypoxic vasodilation. [131]

6.5 Limitations

The limitations of this study include the small numbers of subjects used. For greater accuracy and reliability invasive methods of measuring cardiac haemodynamics would ideally have been utilised. Sodium nitrite as a vasodilator, and more importantly a selective venodilator, has the potential to reduce cardiac filling pressures with an increase in stroke volume without hypotension. Recently, a study in rats showed that intravenous sodium nitrite significantly decreased pulmonary and systemic arterial pressure and increased cardiac output [233]. This information can only be reliably obtained by invasive measurements. However, the use of such investigative tools in healthy volunteers would be deemed unjustified for the purpose of research alone.

We recognize that the main potential adverse effect of sodium nitrite is the development of methaemoglobinaemia [233]. In this study we did not measure methaemoglobin levels. However we investigated the cardiac hemodynamic effects of intravenous sodium nitrite in patients (n=16) with advanced congestive heart failure (Chapter 3). An infusion of sodium nitrite of 50µg/kg/min was associated with an increase in methemoglobin levels, which is well within safe levels, of 0.3±0.1% to 1.1±0.5%. Future studies will need to measure methaemoglobin

levels especially after administration of longer infusions of sodium nitrite to ensure these results are repeated.

We have demonstrated that an intravenous infusion of sodium nitrite is safe and well tolerated in humans. When administered systemically, nitrite retains venoselectivity during normoxia with arterial hypotension observed during hypoxia. Furthermore, intravenous sodium nitrite has been associated with a favourable cardiac hemodynamic response in animal models [233, 234]. This coupled with its lack of development of tolerance [46] makes nitrite an attractive potential therapeutic option for patients with acutely decompensated heart failure to ease cardiac congestion and regain symptomatic control. However, its role in such a group of patients needs to be investigated further.

6.6 Conclusion

A short intravenous infusion of sodium nitrite was safe and well tolerated by healthy subjects with a significant reduction in blood pressure during hypoxia thus, supporting the role of nitrite as an effector of hypoxic vasodilation. Further studies need to be performed to explore the role of sodium nitrite as a potential therapeutic option for heart failure.

Finally, in order to investigate the role of vascular myoglobin in nitrite reduction and building on the results presented in chapter 5 we conducted a series of experiments investigating the effects of nitrite upon vascular ring relaxation of vessels taken from wild type and myoglobin knock-put mice. These studies and their findings are presented in chapter 7.

Chapter 7

The Role Of Myoglobin In Vasodilatation By Nitrite

The data in this chapter has been published in the journal "Cardiovascular Research":

- Julian O.M. Ormerod[#], Houman Ashrafian[#], Abdul Maher[#], Sayqa Arif, Violetta Steeples, Gustav V.R. Born, Stuart Egginton, Martin Feelisch, Hugh Watkins, Michael P. Frenneaux. **The Role of Vascular Myoglobin in Nitrite mediated blood vessel relaxation.** Cardiovasc Res. **2011 Feb 15;89(3):560-5. Epub 2010 Oct 1.**

Execution:

The studies in this chapter were performed in collaboration with Dr Jules Ormerod – The initial washout studies were designed and performed by myself. Myoglobin knockout mice were obtained by me. The transfection and restitution studies in this chapter were performed by Dr Ormerod.

7.1. Introduction

Myoglobin (Mb) is widely expressed in the cytoplasm of vertebrate muscle cells, both skeletal and cardiac, with a much lower or absent expression in smooth muscle [235]. The tertiary structure of the globular protein was derived using x-ray crystallography in the 1950s [236]. Myoglobin consists of a densely packed, 153 amino acid polypeptide chain, with an iron-porphyrin heme group that is identical to that of Hb. This feature allows Mb to reversibly bind and release O_2, though the saturation kinetics are different (due to differences in the globular protein as well as the lack of quaternary structure) and act as a short-term store of oxygen in exercising muscle. The heme of myoglobin achieves 50% saturation at intracellular levels of O_2 as low as 2.4 mmHg [237] and so is fully saturated under normal conditions. Exercising muscle can, however, reach near anoxia at very high workloads. Intriguingly, despite this important physiological role, the myoglobin knockout mouse is remarkably healthy, with tissue oxygenation maintained primarily by an increased capillary density [238, 239].

It is believed that myoglobin subserves an intracellular process known as "facilitated diffusion" of oxygen. Deoxygenated Mb binds O_2 at the cell membrane and diffuses through the cytoplasm to the mitochondria, where it releases O_2 to the respiratory chain. The deoxygenated Mb then diffuses back down its concentration gradient to the membrane to repeat the cycle [240]. Experimental evidence for this phenomenon is far from unambiguous, with model calculations both supporting and refuting myoglobin's contribution to cellular O_2 flux[241].

The chemistry of nitrite activation *in vivo* remains uncertain[61] . Several (non-exclusive) bioconvertors have been identified including: pH-dependent enzyme-independent nitrite

reduction[242] ; microsomal cytochrome P-450 (Kozlov, Staniek, & Nohl 1999; Li *et al.* 2006); xanthine oxidase [61]; aldehyde oxidase (ALDH2) [61]; neuroglobin [243]; hemoglobin [49, 50, 52, 53, 244]; and myoglobin [57]. It is uncertain which of these, alone or in combination, contributes to the *in vivo* conversion of endogenous and exogenous nitrite. In particular, the role of heme moieties, e.g. hemoglobin, in nitrite metabolism, is still uncertain [245]. We devised experiments to test the hypothesis that vascular myoglobin, like cardiac myoglobin [246], can act as a bioconvertor for exogenous nitrite.

We have observed a prolonged vasodilating action of nitrite, which persisted for 45 minutes after the infusion of nitrite was stopped. Intriguingly, this vasodilatation was only observed in the infused arm, even though plasma nitrite levels rapidly equilibrated between the two arms. We hypothesized that an intrinsic vascular wall protein was therefore responsible. This hypothesis was supported by work from the Zweier lab indicating the same [61, 245]. Their experiments also implicated an intrinsic vascular wall protein, and narrowed the search to heme-proteins by inhibition of such using carbon monoxide gas. Accordingly, we undertook experiments to investigate directly the role of myoglobin in nitrite-induced vasorelaxation.

7.1.1 Hypothesis

Ablation or inhibition of myoglobin, genetically or with carbon monoxide gas, will reduce the vasodilating response of aortic tissue to sodium nitrite

7.2. Methods

7.2.1 Animals

Male NMRI and *myo*$^{-/-}$ mice were used at 6-7 weeks of age (26-32g in weight). We housed all animals under standard lighting parameters and provided food and water *ad libitum*. Animals were killed by cervical dislocation as approved under Schedule 1 of the Animals (Scientific Procedures) Act 1986. Aorta from arch to diaphragm was quickly removed *en bloc*, and immediately plunged into ice-cold oxygenated Krebs solution. Aorta was cleaned of all adherent fat and adventitia, and cut into four 2 mm rings. These were mounted in organ baths on a wire myograph (Danish Myo Technology).

7.2.2. Mouse aortic ring myography

Aortic myography was done according to standard procedures. Pretension force was 20 mN. Preconstriction was produced using 50 mM KCl or x mM phenylephrine. Myography wells were open to the air and bubbled with 95% nitrogen- 5% CO_2 (with and without carbon monoxide, CO), or with 95% O_2 - 5% CO_2. Buffer was allowed to reach steady state, which using this equipment produced an oxygen tension of 83 ± 2.5 mmHg (11 kPa) for the low oxygen gases, and 158 ± 2.4 mmHg (22 kPa) for the high oxygen mix. The lower level, which was used for the majority of experiments, was chosen in order to approximate to *in vivo* intravascular conditions, as it is between the average oxygen tension of arteries (90 mmHg) and veins (40 mmHg) [247]. This level was stable over time.

7.2.3. Generation of replication-defective adenoviruses

Both Ad5-Myo-GFP and Ad5-GFP (control virus) were generated using the AdEasy XL Adenoviral vector system (Stratagene). Human wild-type myoglobin cDNA was cloned into

the pShuttle-IRES-hrGFP-1 vector (Stratagene), allowing co-expression of GFP with myoglobin (a stop codon was introduced to prevent fusion of the FLAG tag). The vector was linearized by PmeI digestion and recombined with the pAdEasy-1 (viral backbone vector) in BJ5183 Escherichia coli. The recombined adenoviral constructs were transfected into DH10β E. coli to allow higher plasmid yields. After confirmation of recombination by BstXI and Pac-1 restriction digestion and sequencing, Pac1 linearized recombinant Ad plasmids were then transfected into AD293 cells (Stratagene) using oligofectamine (Invitrogen). AD293 cells were scraped from cell culture vessels and lysed by 4 freeze-thaw vortex cycles. Lysates containing recombinant adenoviruses used to infect further AD293 cells for amplification. The viruses were purified by caesium chloride gradient ultracentrifugation. The viral titre was estimated by infecting HEK293 cells (not expressing capsid proteins) and counting GFP expressing cells.

7.2.4. End-point PCR and western blotting

Aortas from *myo*$^{-/-}$ and wild-type control mice were harvested as before and flash frozen in liquid nitrogen. RNA and protein were extracted using TriReagent (Sigma). cDNA was transcribed from purified RNA using a standard kit (Applied Biosystems) and 40 cycles of PCR were performed. The protein fraction was purified and used for western blotting.

7.2.5. Measurement of cGMP accumulation

Fresh aortas were harvested as before. Tissue was exposed to 10 mM nitrite in Krebs-Hensleit buffer at 37 ºC for 15 minutes, with and without the NO scavenger carboxy-PTIO. A phosphodiesterase inhibitor cocktail was used in the buffer, as in previous work in the Feelisch group [248]. Samples were flash frozen in liquid nitrogen and homogenised by

crushing in a frozen mortar and pestle, as the blade homogeniser was ineffective for aortic tissue. cGMP was measured using a commercial ELISA kit (Amersham Biotech, UK).

7.3. Results

7.3.1. Myoglobin is present in the normal mouse aorta

Myoglobin is present at the mRNA (**figure 7.1a**) and protein (**figure 7.1b**) level.

7.3.2. Nitrite-induced vascular relaxation is oxygen-dependent

In preliminary experiments in C57bl6 mice (a commonly-used laboratory mouse), reducing the buffer oxygen tension increased vasorelaxation to 9 mM sodium nitrite (n = 6, $P < 0.05$ by two-tailed t-test) (**figure 7.2**). This confirms previous observations.

7.3.3. Deficiency or inhibition of myoglobin in mouse aorta diminishes vasorelaxation to nitrite

Acute vasorelaxation induced by sodium nitrite was assessed during exposure to 95% N_2/5% CO_2 and then to 20% CO/ 75% N_2/ 5% CO_2. The level of CO was chosen to inhibit only heme-proteins, as previously described[23], and did not in itself cause vasorelaxation. Preliminary experiments with a fixed dose of sodium nitrite (9 mM) showed a differential response to carbon monoxide. Bubbling with the 20% CO mix inhibited relaxation in the wild-type (NMRI) group (n = 5, $P < 0.05$ by ANOVA), but potentiated relaxation in the *myo*$^{-/-}$ mouse vessels (n = 4, $P < 0.01$ by ANOVA). Further experiments assessed the contribution of myoglobin at several increasing doses of sodium nitrite. CO inhibited nitrite-induced vasorelaxation in the murine wild-type aortae (n = 5, $P < 0.001$ by two-way ANOVA) (f**igure 7.4a**) suggesting that an intrinsic vascular heme moiety significantly contributes to vascular nitrite bioconversion. The effect of 50 μM oxypurinol (an inhibitor of xanthine oxidase) and 50 nM raloxifene (an inhibitor of aldehyde dehydrogenase) is shown for comparison. To assess whether myoglobin was this heme moiety, *myo*$^{-/-}$ mice[24] were compared to wild-type

controls. Overall response to nitrite was markedly reduced in *myo*$^{-/-}$ rings (**figure 7.4b**) (n = 5, $P < 0.001$ by two-way ANOVA), implying that myoglobin significantly contributes to vascular nitrite bioconversion. In contrast to wild-type aortae, CO did not inhibit vasorelaxation in *myo*$^{-/-}$ rings (**figure 7.4b**).

7.3.4. Restitution of myoglobin recapitulates wild-type response to carbon monoxide

To confirm the role of myoglobin as a nitrite bioconvertor and to exclude the possibility that the changes noted in the *myo*$^{-/-}$ mice were artefactual adaptations to gene manipulation, we investigated the effect of restoring myoglobin in *myo*$^{-/-}$ mouse vessels. Transduction of myoglobin augmented the baseline response to nitrite from 28.6 ± 6.5% to 60.7 ± 7.6% (P = 0.024 by ANOVA), compared to control virus-treated rings (**figure 7.5**). The pattern of response to CO was similar. Thus, treatment with control virus did not change the effect of CO (from 28.6 ± 6.5% to 31.6 ± 7.0%). Crucially, viral transduction of myoglobin reinstated the inhibition of nitrite relaxation by CO seen in wild-type mice (from 60.7 ± 7.6% to 25.8 ± 3.7%, P = 0.014 by ANOVA). The concentration of nitrite (~EC_{90} at 9 mM) used in this study was chosen to facilitate accurate determination of the vasorelaxing effect of nitrite with other pathways saturated. Western blotting confirmed the presence of myoglobin in aortic tissue treated with adMYO (**figure 7.1b**).

7.3.5. Relaxation to sodium nitroprusside is increased by carbon monoxide in both wild-type and myo$^{-/-}$ rings

Sodium nitroprusside (SNP) was used to assess the effect of CO on vasorelaxation by a nitrite-independent NO donor. Importantly, *myo*$^{-/-}$ aortic rings retained their response to NO (i.e., the extent of vasorelaxation to a fixed concentration of SNP (0.1 μM) was comparable in tissues from wt and *myo*$^{-/-}$ mice) and relaxation was increased from 37.7 ± 3.5% to 43.7 ±

4.2% by the addition of CO (P = 0.032 by ANOVA). In wild-type mice, relaxation was increased from 42.3 ± 4.7% to 58.7 ± 6.0% (P = 0.029 by ANOVA) (**figure 7.6**). The difference in magnitude of effect of CO is consistent with greater scavenging of NO by myoglobin in the wild-type mouse.

7.3.6. Xanthine oxidase and aldehyde dehydrogenase also exhibit nitrite reductase activity

50 μM oxypurinol caused a 32% inhibition of vasorelaxation (from 21.3 ± 0.4% to 14.4 ± 1.4%, $P < 0.05$ by ANOVA) whereas 50 nM raloxifene produced a 32% inhibition (from 21.3 ± 0.4% to 14.3 ± 0.5%, $P < 0.05$ by ANOVA). The two agents in combination inhibited nitrite-induced vasodilation by 60% (from 21.3 ± 0.4% to 8.5 ± 1.1%, $P < 0.001$ by ANOVA, n = 4 for each group) (**figure 7.7**). Preincubation time of vascular rings with inhibitors was five minutes with each agent separately or together; optimal concentrations of inhibitors used were determined in preliminary experiments.

7.3.7. Nitrite vasodilates through release of nitric oxide

In the presence of a phosphodiesterase inhibitor cocktail, exposure of vascular tissue to 9mM nitrite for 15 minutes caused accumulation of cGMP, which was lower in *myo*$^{-/-}$ tissue than in wild-type and abolished by preincubation with the NO-scavenger, carboxy-PTIO (**figure 7.8a**). Moreover, relaxation of aortic rings by nitrite was completely abolished by preincubation with 20 μM oxygenated hemoglobin (oxyHb) or by the addition of 10 μM ODQ to the organ bath, suggesting the effects of nitrite are mediated by the stimulation of soluble guanylyl cyclase by free NO (**figure 7.8b**).

7.3.8. Prolonged vasodilatation to nitrite is partially myoglobin-dependent

Aortic ring studies were also performed to investigate the initially-recorded phenomenon of prolonged vasodilatation, first seen in human studies. Ten-minute exposure to 18 mM nitrite induced a characteristic persistent vasorelaxation in aortic rings a) Typical traces for nitrite-induced relaxation in NMRI (grey) and *myo*$^{-/-}$ (black) aortic rings b) Latency time, T(0), is shorter in *myo*$^{-/-}$ compared to wild-type controls, (n = 4 for each, $P < 0.05$ by two-tailed t-test) (**figure 7.9**). This implies that the phenomenon relies in part upon the presence of myoglobin.

7.4. Discussion

The main findings of the *in vitro* study are that: a) vascular bioconversion of exogenous nitrite is sensitive to CO, and therefore is likely to depend upon a heme-protein; b) in contrast, when CO is used in rings from myoglobin-deleted mice vasorelaxation is unchanged or increased; this implies that the contribution of remaining heme-proteins (other than myoglobin) is net NO scavenging; c) deletion of myoglobin substantially diminishes exogenous nitrite-mediated vasorelaxation; indicating that myoglobin is a major bioconvertor of exogenous nitrite in aortic rings; d) restitution of myoglobin to *myo*$^{-/-}$ mouse aortas results in increased vasodilation; and the wild-type pattern of response to CO (i.e. inhibition of vasodilation) is restored; e) further studies using inhibitors imply that the other major bioconvertors in this model are non-heme enzymes, including xanthine oxidase and aldehyde dehydrogenase.

Carbon monoxide, an inhibitor of heme-proteins, had differing effects on wild-type and myoglobin-deleted aortic rings; this is only explicable by the presence of myoglobin in murine vasculature. This is confirmed by the demonstration of myoglobin at the transcript and protein level. The results summarised in a) to d) above support the hypothesis that myoglobin is a significant bioconvertor of exogenous nitrite.

7.4.1. Limitations

A major controversy that cannot be resolved by this system is the relative contribution of haemoglobin to nitrite-induced vasodilatation, either in physiology or therapeutics. Stamler and others have proposed that SNO-Hb, created by nitrite, can provide a source of NO-like bioactivity, without need for free NO. Certainly, these results (particularly those using the

scavenger carboxy-PTIO) imply that free NO is important, but the lack of either haemoglobin or intact erythrocytes in the experimental setup means that this work can shed no light on the controversy. The oxygen tension in these studies was chosen to represent low arterial levels, and it was assumed that tissue levels would quickly equilibrate with the surrounding buffer. It cannot be excluded that certain areas of aortic tissue, or subcellular compartments, experience much lower oxygen levels. This would explain the apparent discrepancy between biochemical studies of myoglobin's nitrite reductase activity and the present organ bath study. It is of course similarly possible that such effects would be present *in vivo*.

The experiments were designed to focus on smooth muscle myoglobin, and so the majority of aortic rings had the endothelium removed. Also, in this model the relative contribution of myoglobin is only clear at higher levels of nitrite than are found in normal human physiology. However, nitrite levels recorded in humans vary over more than an order of magnitude; for example, micromolar levels a have been measured in altitude-adapted individuals (Himalayan study). Levels of nitrite recorded in aortic tissue are strikingly higher than in plasma (>20 μM). Oxygen tension in resting muscle is approximately 24 mmHg, and working muscle exhibits even greater hypoxia (8 mmHg) [249], which together mean that myoglobin is likely to participate in vasodilatation during physiological circumstances, and not just in pathology (anoxia, extreme acidosis) as previously hypothesised.

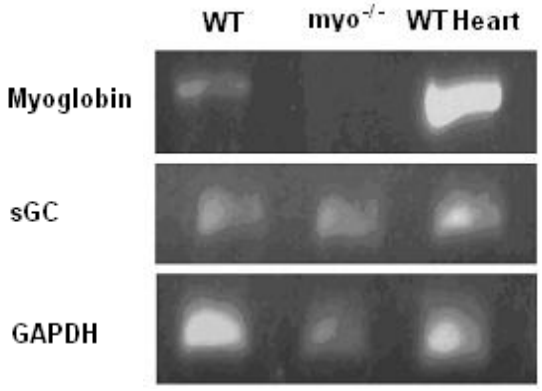
7.5. Conclusions

Nitrite relaxes aortic rings at near physiological levels of oxygen, but low pharmacological levels of nitrite. Nitrite may be present in much higher quantities in certain tissues. Vasorelaxation proceeds through the liberation of NO, as it may be blocked by G-protein inhibitors or NO-scavengers, and myoglobin is important in this process. Xanthine oxidase and ALDH2 contribute to the remainder of the vasorelaxing effect. A summary of this model is given in figure 7.10. Prolonged latency is a hallmark of nitrite-induced vascular relaxation; it is also partially myoglobin-dependent.

7.6. Figures

Figure 7.1.

a)



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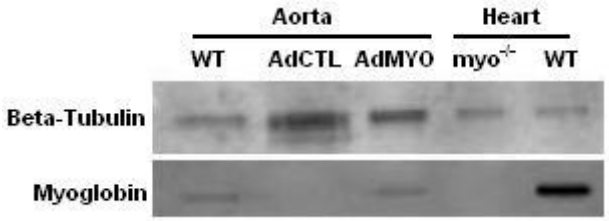


Figure 7.1: Myoglobin is present in the normal mouse vasculature

a) End-point PCR showing presence of myoglobin mRNA in aortic and cardiac tissue from a wild-type (NMRI) mouse, and absence in *myo*$^{-/-}$ tissue **b)** Western blot showing presence of myoglobin in aortic and cardiac tissue from a wild-type (NMRI) mouse, and absence in *myo*$^{-/-}$ tissue. Myoglobin is restored in *myo*$^{-/-}$ tissue using a recombinant adenovirus (AdMYO)

Figure 7.2.

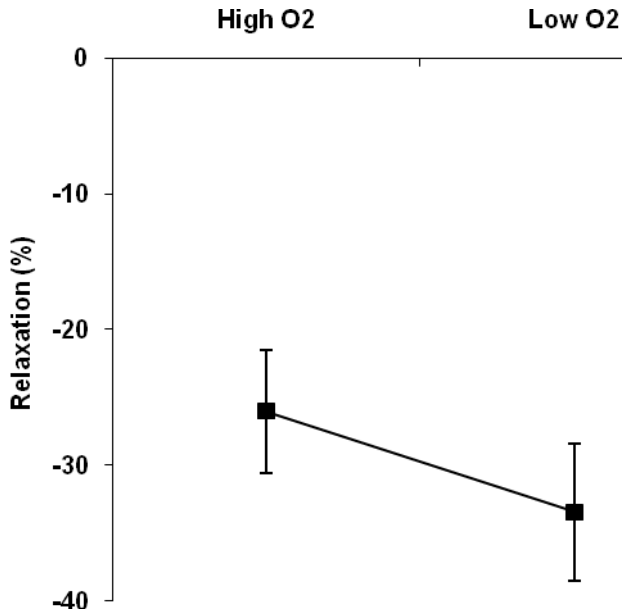
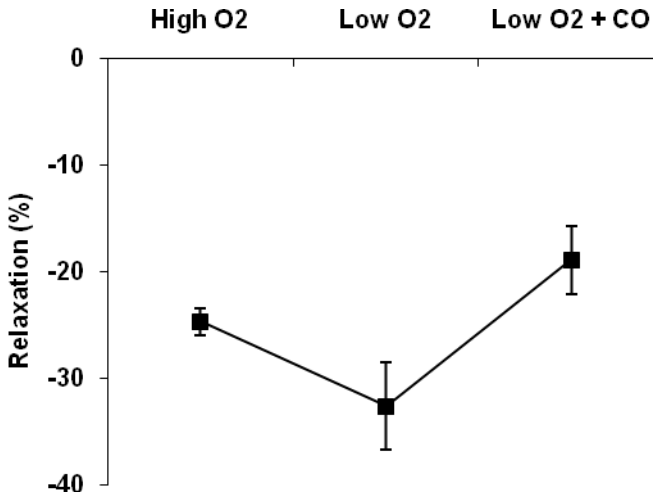


Figure 7.2: Nitrite relaxes mouse aorta in an oxygen-dependent manner

Confirming previous work, lowering the buffer oxygen tension from 158 mmHg to 84mmHg increased relaxation to 9 mM sodium nitrite from 26.0 ± 4.5 to 33.1 ± 5.1 in C57bl6 mouse aortas, *P< 0.05

Figure 7.3.

a)



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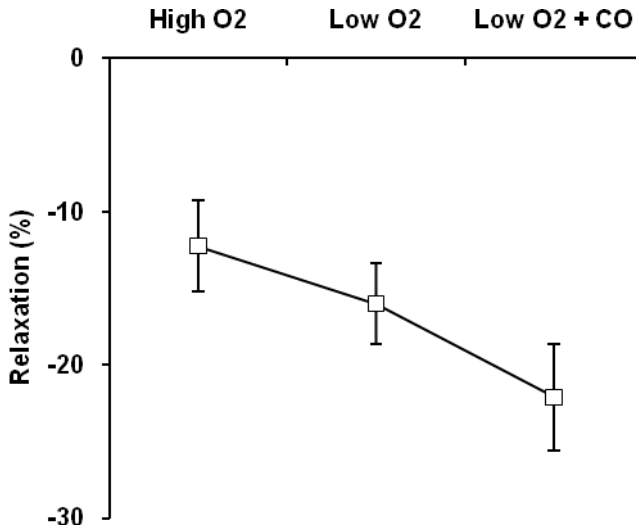
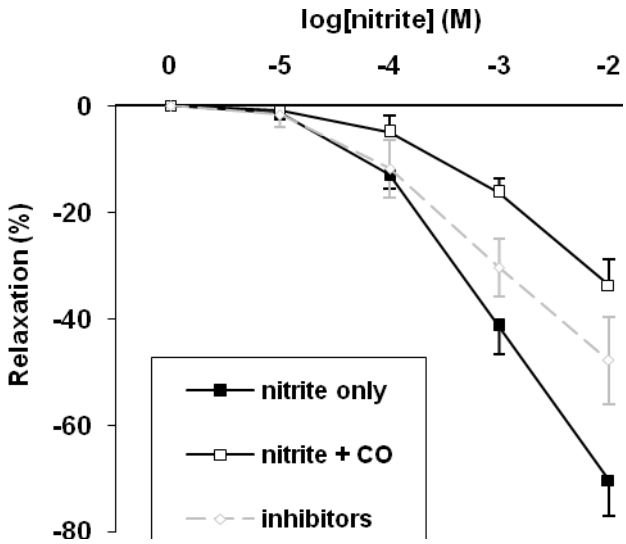


Figure 7.3: Exposure to carbon monoxide causes a differential response in wild-type aortas compared to myoglobin knockout

a) **Relaxation to 9 mM sodium nitrite is inhibited by exposure to 20% CO gas in NMRI mouse aorta, whereas** b) **relaxation to 9mM sodium nitrite is lower overall in *myo*$^{-/-}$ aorta compared to NMRI and is potentiated by CO gas; the effect of reducing oxygen tension is similar to that in C57bl6 aortas (**figure 3.2**) for both.**

Figure 7.4.

a)



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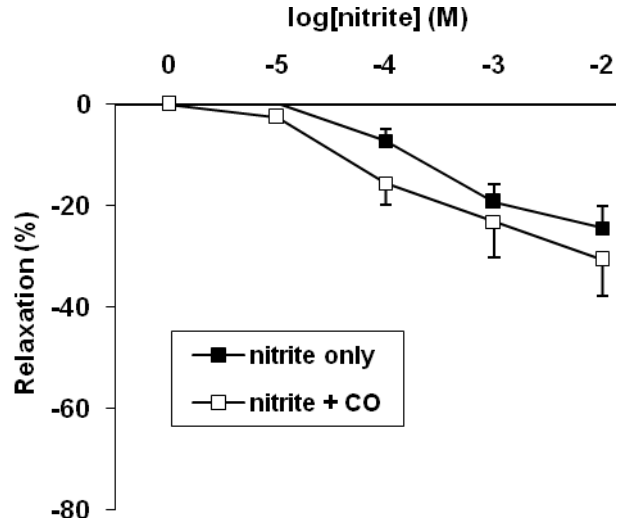


Figure 7.4: Vasorelaxation to nitrite is reduced by both inhibition of myoglobin with CO and ablation by genetic knockout

a) Concentration response curve showing the response of wild-type (NMRI) aorta to bubbling with CO gas. Wild-type aorta shows an inhibition of nitrite–dependent vasorelaxation in response to CO (n = 5, P < 0.001 by two-way ANOVA). The combined effect of oxypurinol and raloxifene is shown for comparison **b)** Inhibition with CO is not seen in *myo*$^{-/-}$ aortas, (n = 5, P = 0.44 by two-way ANOVA).

Figure 7.5.

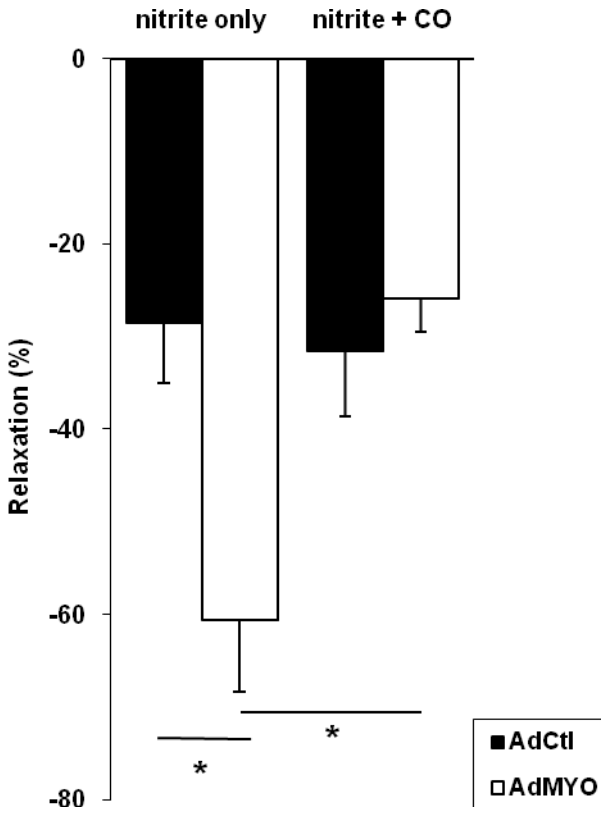


Figure 7.5: Replacement of myoglobin to the knockout mouse recapitulates the wild-type phenotype

Restitution of myoglobin to *myo*$^{-/-}$ aorta using a recombinant adenovirus (AdMYO) increases vasodilatation to 9 mM sodium nitrite compared to *myo*$^{-/-}$ aorta treated with control virus (AdCtl) (n = 4 for each, $P < 0.01$). The inhibitory effect of carbon monoxide is restored, *$P < 0.05$ by ANOVA

Figure 7.6.

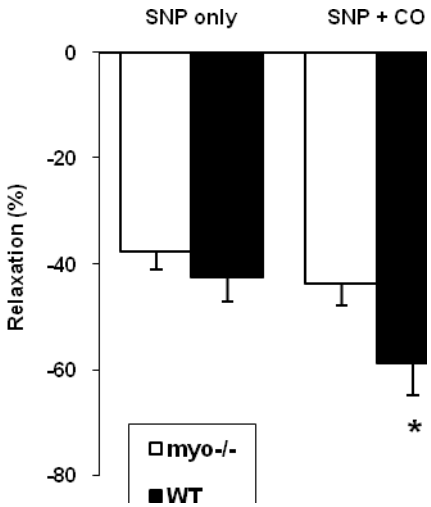


Figure 7.6: Vasorelaxation in the myo$^{-/-}$ mouse is otherwise unimpaired

Response to the NO-donor SNP is not diminished in *myo-/-* rings. CO has no effect in knockout aortas but increases response to nitrite in wild-type rings, consistent with increased scavenging of NO in the presence of myoglobin, *$P < 0.05$ by ANOVA compared to baseline

Figure 7.7.

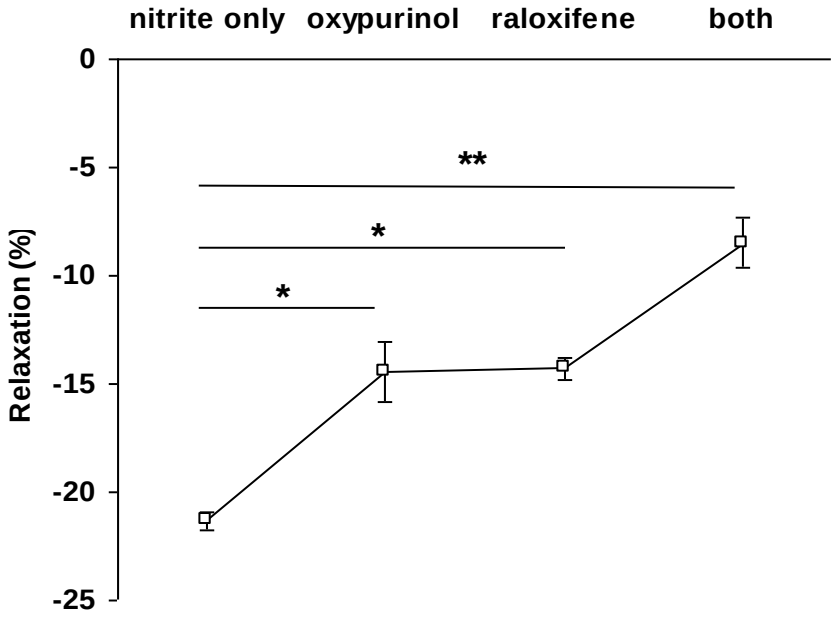
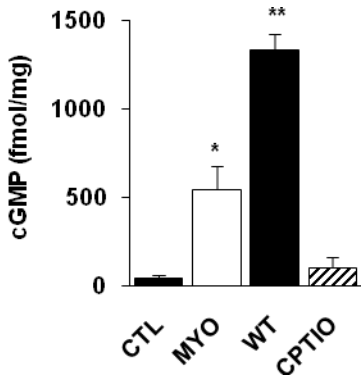


Figure 7.7: Other species contribute to vasorelaxation

The majority of the remainder of nitrite-dependent vasodilation in *myo*$^{-/-}$ aorta is due to xanthine oxidase and aldehyde dehydrogenase as shown by the addition of specific inhibitors (oxypurinol and raloxifene) (n = 4 for each), *P < 0.05 compared to nitrite only, **P < 0.05 compared to oxypurinol or raloxifene only, and P < 0.001 compared to nitrite only (repeated measures ANOVA with post-hoc tests)

Figure 7.8.

a)



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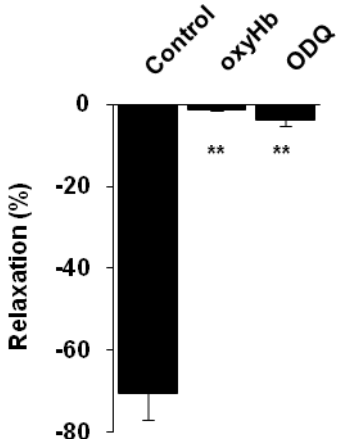
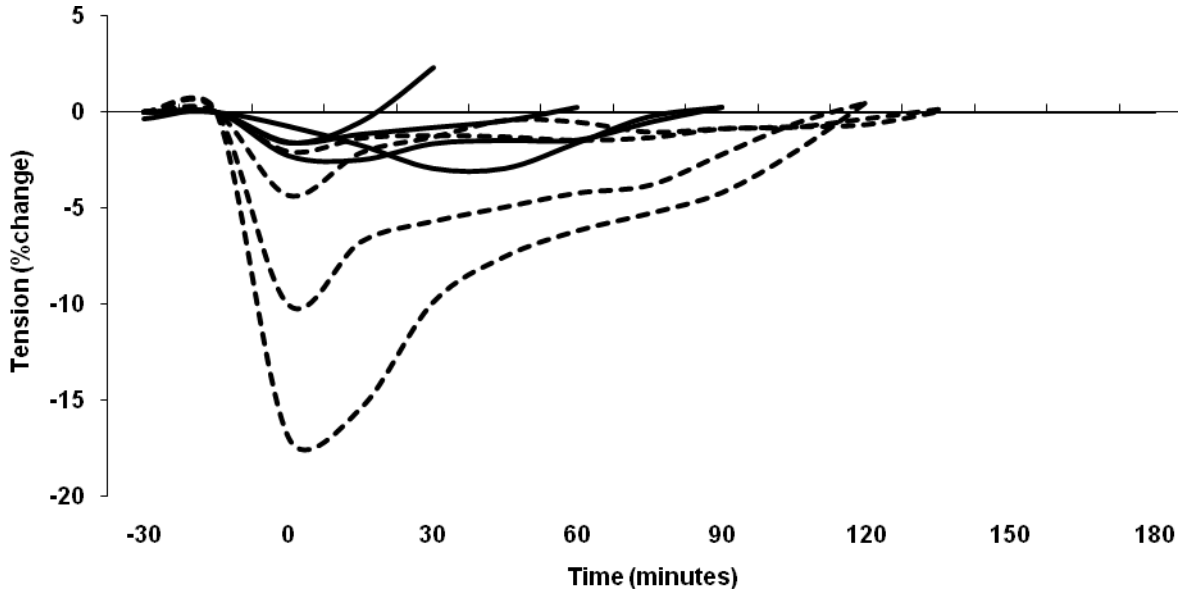


Figure 7.8: Vasorelaxation by nitrite proceeds through the liberation of NO

a) 15 minute exposure to 9 mM sodium nitrite causes accumulation of cGMP which was lower in *myo*$^{-/-}$ tissue than wild-type (n = 6 for each), and was prevented by co-administration of the NO-scavenger carboxy-PTIO (n = 5); **b)** Vasorelaxation to 9 mM sodium nitrite is prevented by the presence of the NO-scavenger oxyHb and the G-protein inhibitor ODQ (n = 4 for each). *P < 0.05 compared to control, **P < 0.001 compared to control and *myo*$^{-/-}$ by ANOVA.

Figure 7.9.

a)



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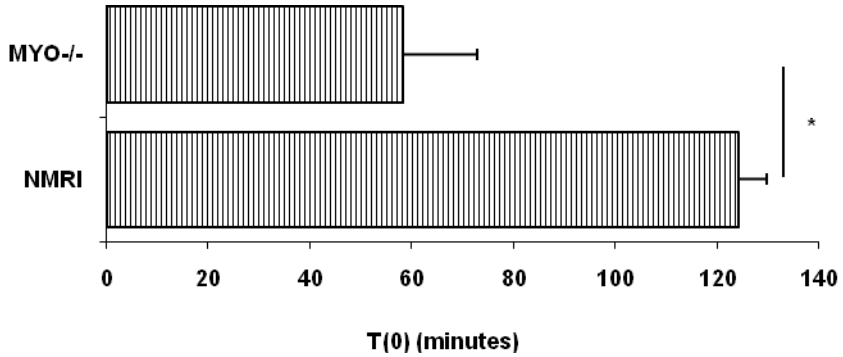


Figure 7.9: Myoglobin prolongs the duration of vasorelaxation

a) Incubation of aortic tissue with 20 mM sodium nitrite for 10 minutes produces a prolonged vasorelaxation, which persists after removal of sodium nitrite (solid lines: WT, broken lines: *myo*$^{-/-}$) **b)** The duration of prolonged vasorelaxation is markedly reduced in *myo*$^{-/-}$ aortas, T(0) taken as first time-point where tension reaches baseline (n = 4 for each), *P < 0.05 by two-tailed t-test

Figure 7.10.

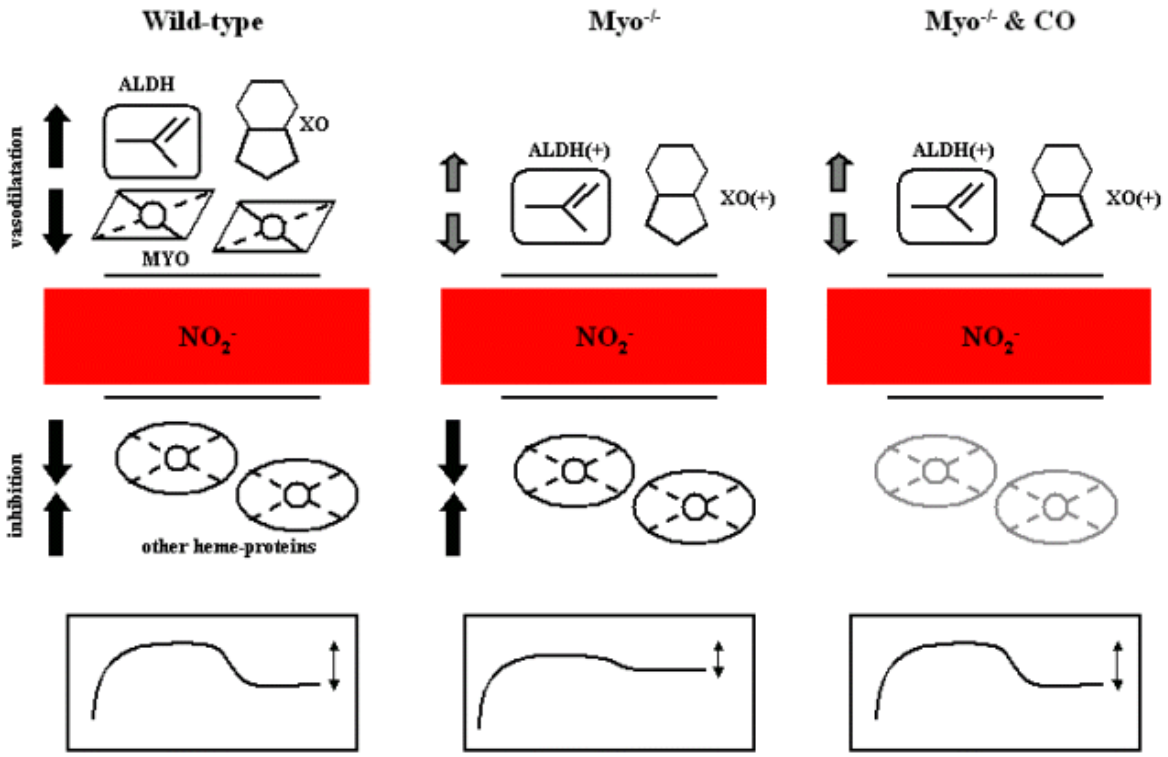


Figure 7.10: A model of myoglobin's role in vasorelaxation induced by nitrite

NO is liberated by various species, including myoglobin, xanthine oxidase and ALDH. Other heme-containing proteins have a net scavenger role. Exposure to CO inhibits myoglobin, reducing vasorelaxation in wild-type aortas, but not in myoglobin-deficient aortas, where relief of scavenging is the only effect.

Chapter 8

Summary

Our understanding of the control of vascular tone, regulation of blood flow and the body's response to injury in the context of ischaemia-reperfusion continues to grow. It is now understood that inorganic nitrite plays an important role in these functions (and potentially others). While its mechanism of action continues to be investigated the physiological importance of this anion is being increasingly established. Exciting therapeutic avenues are being explored and sodium nitrite may prove to play a very important pharmacological role in conditions including acute myocardial infarction, pulmonary hypertension, coronary artery bypass surgery and acute decompensated heart failure.

The studies presented in this thesis have added to our understanding in this area in the following ways:

1) We have ascertained that intrabrachial sodium nitrite infusion produces a profound venodilatation of the capacitance bed in this hypoxaemic environment.
2) Under normal physiological conditions, with subjects breathing room air, only a modest arteriolar vasodilatation occurs as manifested by a small increase in forearm blood flow. However breathing 12% oxygen, rendering the resistance bed hypoxaemic, resulted in an exaggerated forearm blood flow response to nitrite infusion even at physiological levels of nitrite.
3) These findings support very strongly that the NO metabolite, sodium nitrite, plays an important role in hypoxic vasodilatation.
4) We have investigated the mechanisms that may lead to nitrite reduction. *In vitro* studies have suggested that NOS and XOR may play a role in the bioconversion of nitrite to NO, particularly under severely hypoxic and anoxic conditions. In healthy volunteers, at rest, we did not find that inhibition of these enzymes affected the

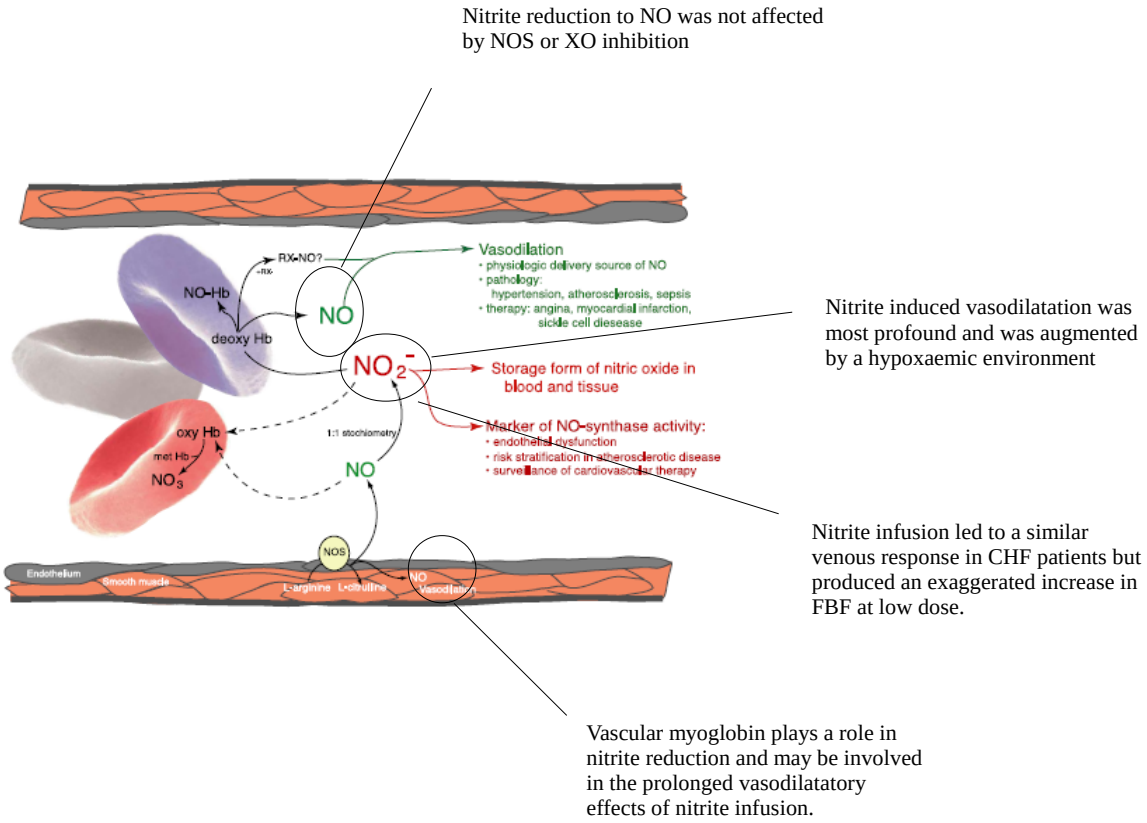
vascular response to sodium nitrite. This does not however preclude a role in pathological states.

5) During these studies we have a found the vascular response to nitrite to persist long after the plasma levels have returned to near-baseline levels. This observation may be explained by possible protein binding of nitrite or NO and led to studies investigating the role of vascular myoglobin. We demonstrated that myoglobin was present in murine aortic rings and that myoglobin knock-out mice manifested a diminished nitrite induced vasodilatation. This response was corrected in the knock-out mice by the restoration of myoglobin using a genetically modified adenovirus vector.
6) We investigated the "translational" implications of these findings. We have found that the pharmacokinetics of nitrite in such patients to be different to that observed in healthy volunteers. Interestingly, although parameters such as pO2 and pH did not differ between the groups, at low dose intra-arterial nitrite infusion CHF patients manifested an enhanced increase in FBF. This observation may be explained by differences in redox stress and is worthy of further investigation.
7) We have found systemic intravenous infusion of nitrite to be well tolerated and safe. During the breathing of 12% oxygen, nitrite infusion led to reductions in blood pressure and total peripheral resistance.

These studies are supportive of an important physiological role for inorganic nitrite and have investigated the mechanisms underlying its action. We were the first to demonstrate the critical role of hypoxaemia per se in facilitating nitrite induced vasodilatation in man and have investigated the handling of nitrite infusions in patients with heart failure, a group that could potentially benefit greatly from its use therapeutically.

Figure 8.1:

Findings of these studies that have advanced our knowledge:



Recommendations:

Given our knowledge now of the dietary effects of nitrate and nitrite intake future studies should control for this variable by defining a suitable and standardised diet prior to any experiments.

Further studies should be considered to address the following issues:

1) More detailed “cardiac catheter laboratory” based studies to define accurately the effects on cardiac haemodynamics and pulmonary artery pressures and resistance.

2) Given the preferential venodilatation that we observed, studies investigating the effect on intravenous nitrite infusion in both acute and chronic decompensated heart failure.

3) The evidence supporting a cytoprotective role for inorganic nitrite provides a strong basis for clinical studies investigating the effect of nitrite infusion upon myocardial infarction size in the context of patients being treated by primary percutaneous coronary intervention.

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