TOWARDS NITRITE AS A THERAPY IN CARDIOVASCULAR DISEASE

by

Julian Oliver Macbeth Ormerod

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ATTRIBUTIONS

All experiments presented in Chapter 3 were designed and performed by the candidate. Dr Houman Ashrafian assisted with the design of these experiments. Dr Paul Robinson and Violetta Steeples created the myoglobin-insert adenovirus in the University of Oxford. Professor Jürgen Schrader of Heinrich-Heine-Universität in Düsseldorf kindly provided the myoglobin-knockout mice. These were re-derived, held and bred in the University of Birmingham under the authority of the candidate's personal license. The candidate gained approval for the use of genetically modified organisms from the University Committee.

All studies in Chapter 4 were designed and performed by the candidate. The candidate wrote the protocol and applied for ethical approval. Mr Majid Mukadam placed the right heart catheter for each participant, as part of a clinically indicated assessment for transplantation. Dr Sayqa Arif provided hands-on assistance with the majority of the studies in Chapters 4 and 5, and applied for ethical approval for the studies in healthy volunteers presented in Chapter 5. The candidate performed the majority of studies presented in Chapter 5, with assistance from Dr Sayqa Arif, Jonathan Evans and Dr Roger Beadle. The candidate designed the studies in Chapter 5, and gained ethical approval for the heart failure studies.

The studies in Chapter 6 were performed by the candidate, with the assistance of Jonathan Evans. The candidate wrote the protocol (with Dr Hussain Contractor) and applied for ethical approval. Dr Matteo Beretta, under the supervision of Professor Bernd Mayer in Vienna, performed the ALDH2 biochemistry. Dr Bernadette Fernandez, of Warwick University, performed the NO_x and RXNO measurements in the same chapter.

ABSTRACT

a) The nitrite anion has traditionally been considered an inert by-product of nitric oxide (NO) metabolism, but is now thought to be an important source of bioactive NO. Work from our department and others have implicated intrinsic vascular wall heme-proteins in nitrite-induced vasodilatation. These experiments were designed to test the hypothesis that vascular wall myoglobin is the protein responsible. Aortic rings from wild-type and myoglobin knockout mice were challenged with nitrite, before and after exposure to the heme-protein inhibitor carbon monoxide (CO). CO inhibited vasodilatation in wild-type rings but not in myoglobin-deleted rings. Restitution of myoglobin using a genetically modified adenovirus both increased vasodilatation to nitrite and reinstated the wild-type pattern of response to CO. cGMP accumulated in tissue exposed to nitrite. This was lower in myoglobin-deficient aorta and was abolished by an NO-scavenger. These results imply that myoglobin contributes significantly to nitrite-induced vasodilatation in the *in vitro* model, and acts through release of free NO. The prolonged vasorelaxation previously seen in the human forearm model was confirmed *in vitro*, and found to be partially myoglobin-dependent.

b) Reduction by heme proteins such as myoglobin contributes to a potentially clinically useful feature of nitrite – hypoxia-targeted release of NO. We hypothesised that sodium nitrite, which preferentially dilates veins rather than arteries, may have advantages over organic nitrates in the treatment of acute decompensated heart failure. The use of nitrates is often limited by development of hypotension and tolerance; limitations that the specific attributes of nitrite may overcome. We investigated the haemodynamic effects of short systemic sodium nitrite infusions in patients with advanced but stable heart failure. 18 consecutive patients undergoing right heart catheterisation as part of a clinical heart transplant assessment protocol were recruited to the study. Sodium nitrite was infused into the right internal jugular vein at three escalating doses (1, 10 and 50 µg/kg/min), and pressure and cardiac output measurements were taken using a Swan-Ganz catheter. Patients were divided by baseline pulmonary capillary wedge pressure (PCWP) into predefined groups (≥ or < 15mmHg, n = 13 and 5 respectively). Nitrite reduced pulmonary vascular resistance (by 30%, n = 13, p = 0.03), and increased stroke volume (by 7.1ml, p = 0.008) in the high PCWP group, with a minimal effect on systemic

vascular resistance or blood pressure (p = NS for both). Change in estimated trans-septal gradient (PCWP - right atrial pressure), a measure of effective left ventricular filling pressure) positively correlated with change in cardiac output (R = 0.79, p < 0.001) and stroke volume (R = 0.67, p = 0.004). This is consistent with relief of diastolic ventricular interaction through systemic venodilatation and reduced pulmonary artery pressure. Over this short time period, the increase in methaemoglobinaemia was small (maximum of 2.4%), but we went on to test this potential problem in longer infusions.

c) Longer infusions of nitrite were tested in both health and heart failure. Patients with decompensated heart failure often need treatment for hours or days, so we sought to establish the safety and haemodynamic profile of sodium nitrite over a more clinically relevant time frame. Initially, long infusions were given to healthy volunteers, principally to assess the development of methaemoglobinaemia. A safe dose for longer-term infusion was established at 10 μg/kg/min. The maximum metHb measured in this group was 2.2%, which appeared to reach a plateau at approximately three hours. This dose was then tested in participants with advanced heart failure, as before, to assess the haemodynamic effects of this dose over a period of three hours. Early results were favourable, though recruitment was extremely challenging. The haemodynamic effects of longer nitrite infusions in advanced heart failure (*n* = 3) appeared to increase throughout the three-hour infusion. This regime also appeared to be safe. Further work will establish the full clinical effect of this protocol.

d) Nitric oxide plays a central role in preconditioning – the protection of tissues from ischaemic injury by a preceding "warning shot", consisting of short periods of ischaemia. This effect can be mimicked pharmacologically and is an exciting avenue of current research in cardiovascular disease. Nitrite has been shown to protect against ischaemic injury in a variety of animal models. Human studies are underway but are yet to report. Mitochondrial aldehyde dehydrogenase (ALDH2) has been implicated as a central player in preconditioning with a variety of stimuli. We investigated the effects of systemic nitrite in the ischaemic human forearm model, with genetic and pharmacological inhibition of ALDH2, in order to establish the efficacy of nitrite and the role this enzyme may play in conferring protection. 35 volunteers underwent 51 studies divided in three protocols. Venous occlusion plethysmography was used to measure changes in forearm blood flow in response to intra-arterial ACh (25, 50, 100

nmoles/min), before and after 20 minutes of forearm ischaemia. This established model of transient forearm endothelial dysfunction was used to assess the protective effects of intravenous nitrite (1 μg/kg/min for 10 minutes) in three different protocols. In protocol 1, 1 μg/kg/min sodium nitrite for 10 minutes, given 24 hours preceding ischaemia prevented ischaemic endothelial dysfunction. Participants of East Asian origin were recruited to protocol 2, utilising a common genetic polymorphism in order to assess the role of ALDH2 in protection. 1 μg/kg/min sodium nitrite for the latter 10 minutes of ischaemia protected those with the variant ALDH2*1/*2 enzyme. Participants with the wild-type enzyme (ALDH2*1/*1) were not protected by peri-ischaemia nitrite. In protocol 3, pharmacological inhibition of the wild-type enzyme with disulfiram did not recapitulate the variant phenotype. In vitro studies revealed differences between the two enzymes in nitrite reduction and superoxide formation. This study provides evidence that systemic nitrite may be clinically useful, either in the pre-operative setting or during ischaemic events, in certain individuals. The role of ALDH2 in protection by systemic nitrite is complex and invites further study.

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LIST OF ABBREVIATIONS

Acute decompensated heart failure, ADHF

Cardiac output, CO

Diastolic ventricular interaction, DVI

Endothelial nitric oxide synthase, eNOS

Glyceryl trinitrate, GTN

Haemoglobin, Hb

Heart failure, HF

Ischaemia-reperfusion, IR

Left atrium, LA

Left ventricle, LV

Methaemoglobin, metHb

Mitochondrial aldehyde dehydrogenase, ALDH2

Mitochondrial permeability transition pore, mPTP

Myoglobin, Mb

Pulmonary capillary wedge pressure, PCWP

Pulmonary hypertension, PH

Reactive oxygen species, ROS

Right atrium, RA

Right ventricle, RV

Reperfusion Injury Salvage Kinase pathway, RISK pathway

Sodium nitroprusside, SNP

Soluble guanylate cyclase, sGC

Survivor Activating Factor Enhancement pathway, SAFE pathway

Xanthine oxidase, XO

1. INTRODUCTION

1.1. Biology of Nitric Oxide

The discovery of nitric oxide (NO) as the endothelium-derived relaxation factor was one of the most important scientific advances of the 20th century. NO is generated by a group of nitric oxide synthases (NOSs) via the oxidation of the amino acid L-arginine to citrulline. Oxygen and a variety of co-factors also participate in this reaction. NO gas crosses the cell membrane to bind and activate the heme group of soluble guanylate cyclase (sGC). This leads to increased conversion of GTP to cGMP, which exerts multiple effects to produce smooth muscle relaxation. NO is inactivated by superoxide or scavenged by heme-containing proteins, with a half-life of only a few seconds.

While the earliest work concentrated on the vasodilating effects of NO, over the last 15 years myriad other effects have been elucidated. Experiments investigating the effects of NO in human biology are complicated by the precise temporal-spatial production of NO. NO-donors are a crude tool to investigate such subtle effects (Seddon *et al.*, 2007). Expression of the various NOSs is not limited to their classical cell-types, for example "neuronal" NOS (nNOS) will have an effect in whole organ preparations which may previously have been assumed to contain only "endothelial" NOS (eNOS), and high-output "inducible" NOS (iNOS) can be expressed in many different tissues in various pathological states.

Nitrite has in the past been considered a relatively inert end-product of NO metabolism. However, it can be reduced (back) to NO by several mechanisms and in recent years

excitement has grown that it may in fact be a circulating store of physiologically active NO. This is discussed in detail in **section 1.3.** below.

1.2. Control of Vasodilatation

1.2.1. Hypoxic vasodilatation

Hypoxic vasodilatation is a fundamental physiological process: decreased vascular resistance in response to local reduction in oxygen saturation of Hb. It refers to local means of regulation of blood flow to specific groups of cells, as opposed to centrally mediated effects through circulating hormones or the autonomic nervous system, which subserve globally increased flow. Guyton and colleagues first established hypoxic vasodilatation in a series of studies in the late 1950s and early 1960s (Ross et al., 1962).

Hypoxic vasodilatation matches oxygen delivery to metabolic demand, possibly through signalling to pre-capillary resistance vessels (Segal and Duling, 1986). Nitric oxide is a critical determinant of vasodilatation and there is broad support for the involvement of NO or related species (S-nitrosothiols, N-nitrosamines, iron nitrosyls, nitrated lipid (Freeman *et al.*, 2008), and nitrite (Gladwin *et al.*, 2006)) in this process. Other work has implicated adenosine (Edmunds *et al.*, 2003), ATP, vasoactive peptides, K^+ and H^+. The relative contribution of the thus far established pathways remains open to debate, and in some cases controversial. The current consensus position is that there are multiple overlapping pathways, of which some may remain undiscovered at present (Segal, 2005).

1.2.2. Functional and exercise hyperaemia

Exercise hyperaemia is the much-studied physiological phenomenon of increased blood flow to working muscles. Functional hyperaemia is a term for demand-induced increase in blood flow to any organ, including non-muscular tissues such as brain, liver and kidneys. Exercising muscles promote increased blood flow through a variety of mechanisms: especially adenosine, potassium, and acidosis. These mechanisms are shown in **figure 1.1**.

The high-energy phosphate bond of ATP is hydrolysed to generate contractile force in muscle, leaving ADP and AMP. AMP is metabolised to adenosine by 5'-nucleotidase. Adenosine is particularly important in the regulation of coronary blood flow. K^+ leaves the cell with every action potential in contracting cardiac and skeletal muscle, and with sustained exercise begins to accumulate in the extracellular space. Extracellular K^+ produces hyperpolarization of the membrane potential of vascular smooth muscle, stimulates the Na^+/K^+-ATPase pump and increases membrane conductance to K^+ through K^+ activated K^+ channels. Hyperpolarisation of the membrane closes voltage-dependent calcium channels, leading to smooth muscle relaxation and vasodilatation. Carbon dioxide is a by-product of oxidative metabolism. CO_2 diffuses from muscle cells to vascular smooth muscle, causing vasodilatation directly or through decreased pH. CO_2 is also particularly important in regulation of cerebral blood flow. Lactic acid is produced during anaerobic metabolism, and also vasodilates through decreased pH. Inorganic phosphate, released by the hydrolysis of adenine nucleotides may also have a role in exercise hyperaemia.

Around 50% of the total resistance to blood flow across muscles resides in small muscular arteries outside the muscle itself. These arteries are not directly exposed to vasodilating

signals from exercising muscle units, but vasodilatation spreads proximally through cell-to-cell signalling with increasing recruitment of motor units (Segal and Jacobs, 2001).

1.3. The nitrite anion

Nitrite (NO_2^-) is present in human plasma at a concentration of approximately 150 – 500 nM (Dejam *et al.*, 2005; van Faassen *et al.,* 2009). The exact concentration varies from day-to-day, depending on endogenous production and diet, and is higher in the plasma of arterial blood than venous blood (**Table 1.1.**). Circulating nitrite is lower in pregnancy (Hata *et al.*, 1999), and higher with adaptation to altitude (Erzurum *et al.*, 2007). Nitrite is concentrated in red blood cells compared to plasma. Erythrocytes represent about half of total blood volume, making them the largest nitrite pool in whole blood (Dejam *et al.,* 2005). Tissue concentrations of nitrite vary widely: brain and aortic tissue contain significantly higher concentrations than heart, liver, kidney or lung (**Table 1.2.**).

Physiological nitrite has three sources: ingestion of nitrite in the diet, reduction of dietary nitrate (NO_3^-) following absorption into the boodstream and concentration in the salivary glands, and metabolism of NO released by the endothelium. Nitrite-rich foods include cereals, corn, and green leafy vegetables such as pak choi or spinach, which contain 2.0 - 4.0 mg of nitrite per kilogram of food. Cured meats have artificially increased levels of nitrite, which is used in the curing process. Nitrate is ingested in a wide variety of foods, especially vegetables such as spinach, lettuce or beetroots. Plasma nitrate is secreted and concentrated by the salivary glands (Spiegelhalder *et al.*, 1976). Bacteria in the oral cavity efficiently reduce salivary nitrate to nitrite, producing a 1,000-fold increased concentration of nitrite in saliva compared to plasma (Lundberg *et al.*, 2004). Humans produce around 1.5 litres of saliva per

day, which is generally swallowed. Nitrite is absorbed across the gastric mucosa, though some is reduced directly to NO in the strongly acidic milieu of the stomach lumen. Production is maximal at the gastro-oesophageal junction where salivary nitrite first meets gastric acid. The junction is also the most common site for the development of squamous metaplasia and gastric carcinoma. Restriction of nitrite- and nitrate-rich foods can reduce plasma levels of these species by about 50% (Gladwin *et al.*, 2000b).

The remainder of plasma nitrite is derived from endothelially-released NO. Mice with disrupted function of eNOS show lower levels of both plasma and cardiac nitrite (Bryan *et al.*, 2008). Conversely, mice with enhanced eNOS function (over-expressing human eNOS), exhibit higher levels (Elrod *et al.*, 2008). Oxidation of NO radicals is slow *in vitro*, but is significantly faster in blood (Rassaf *et al.*, 2002). Firstly, NO is apolar, and naturally partitions into low polarity lipid or protein fractions, thereby increasing its relative concentration by a factor of 10. This is particularly important, as the oxidation reaction is second order in NO concentration (Liu *et al.*, 1998). Secondly, the copper-storage plasma enzyme ceruloplasmin catalyses the first step of nitrite synthesis, oxidation of NO to NO^+. NO^+ is then rapidly hydrolysed to NO_2^- (Shiva *et al.*, 2006).

Infused sodium nitrite diminishes with a half-life of several minutes (Lundberg and Weitzberg, 2005). Nitrite reacts with haemoglobin to form metHb and nitrate, which is then excreted in the urine and, to a lesser extent, the faeces as an inert salt. A small amount of nitrite is excreted in the urine. The lethal dose of nitrite is considered by the US Food and Drug Administration to be 22 mg sodium nitrite per kilogram, in the adult human (corresponding to an average concentration of 320 μM). Death results from

methaemoglobinaemia, which is discussed in **section 1.3.4.** (van Faassen *et al*., 2009). Nitrite in food has previously been restricted due to concerns over carcinogenicity, though such concerns have now been largely refuted (van Faassen *et al*., 2009).

1.3.1. NO release

NO can be generated from nitrite directly: by acid disproportionation, or reduction by oxidoreductase enzymes (e.g. xanthine oxidase (Baker *et al.,* 2007; Godber *et al.*, 2000; Li *et al*., 2001), aldehyde dehydrogenase (Li *et al.*, 2008) or by deoxy- forms of heme globins (Li *et al.,* 2008), and recently proposed was carbonic anhydrase (Aamand *et al.*, 2009). The relative contribution of each of these methods in physiology is controversial.

1.3.1.1. Acid disproportionation

Benjamin and Lundberg independently demonstrated that nitrite derived from dietary nitrate was a substrate for NOS-independent production of NO in the acidic conditions of the human stomach (Benjamin *et al*., 1994; Lundberg and Govoni, 2004). In normoxic conditions elsewhere in the body, this mechanism of NO production probably does not occur. However, pH in the ischaemic myocardium may fall as low as 5.5 (Zweier *et al.*, 1995), making NO release from nitrite possible in these conditions.

1.3.1.2. Nitrite reductase: haemoglobin

The most-studied (and most controversial) proposed nitrite reductase is haemoglobin. In 2003, Gladwin and colleagues sought to definitively prove that nitrite was a vasodilator *in vivo* (Cosby *et al.*, 2003). This study is discussed in more detail later, but briefly they showed that nitrite could vasodilate the forearm of healthy volunteers at pharmacological and near-

physiological doses, with a greater increase on exercise. The observation that vasodilatation was associated with the formation of iron-nitrosyl-haemoglobin and SNO-Hb, led the authors to investigate the *in vitro* nitrite reductase activity of haemoglobin. They found that both 100 and 200 μM nitrite plus deoxygenated erythrocytes (5 ml RBCs at 1000 μM of heme), released NO into the gas phase. The level of oxygen is not given, but can be assumed to be at or about anoxia as the whole volume was purged with helium. The level of nitrite is also much higher than physiology (**Table 1.1.**) so this experiment pertains more to pharmacological nitrite. Further studies in aortic rings suggested that added red blood cells potentiate the vasodilating effect of nitrite. Remarkably, 2 μM nitrite had no vasodilating effect, even in hypoxia.

Nagababu and colleagues also used chemiluminescence to follow the production of NO from nitrite directly (Nagababu *et al.*, 2003). They extended the observations above by showing that the majority of iron-nitrosyl-Hb was in the ferric (3^+) state. The authors claim that manipulation of samples led to experimental error when using previous photolysis-chemiliuminescence techniques.

Huang and colleagues showed that the greatest rate of NO generation occurs at the R-to-T transition point of Hb oxygen saturation (Huang *et al.*, 2005b). This was shown to be due to the concerted action of two opposing forces. First, reduction of nitrite to NO proceeds at a faster rate at a more negative heme reduction potential. This is highest at 100% oxygen saturation. On the other hand, nitrite is reduced primarily by deoxyHb, with oxyHb acting solely as a potent scavenger of NO, and therefore NO production is also favoured by 0% oxygen saturation. The balance of the two forces at the R-to-T transition point explains the

maximal generation of NO at this point. Isbell and colleagues, in further studies using aortic rings, contend further that deoxyhaemoglobin has a unique dual-function, with both NO-production and scavenging abilities (Isbell *et al.*, 2007). This, they say, allows deoxyHb to play a central role in intravascular NO homeostasis, though they also admit a role for vessel wall enzymes.

The major problem with theories of NO release from nitrite by Hb or red cell enzymes is that Hb is a very efficient scavenger of free NO. Proposed explanations include the formation of *S*-nitrosothiols (SNOs) or N_2O_3 or as intermediates, both of which possibilities are discussed below. A minority of mathematical models also purport to explain how this could occur (Tsoukias, 2008).

1.3.1.3. Nitrite reductase: myoglobin

Myoglobin is a 16,700 KDa heme-containing protein, consisting of a densely packed 153 amino acid polypeptide chain. It is a molecular relative of haemoglobin and is abundantly expressed in skeletal and cardiac muscle. *In vitro*, Mb can act either as an NO scavenger (in normoxia) or as a nitrite reductase (in hypoxia) (Shiva *et al*., 2007a; Rassaf *et al*., 2007). The redox potential of Mb is lower than that of R-state Hb, meaning a greater ability of the heme to donate an electron to nitrite, and deoxyMb may in fact reduce nitrite and generate NO at a faster rate than deoxyHb (Huang *et al.*, 2005a). The bimolecular rate constant for the reaction of deoxyMb in isolated cardiomyocytes was measured at 12.4 mol/L/s, compared to 0.35 mol/L/s for deoxyHb (Shiva *et al*., 2007a). In this preparation, deoxyMb released NO in proximity to the mitochondria, and inhibited respiration through cytochrome C oxidase (Shiva *et al*., 2007a), confirmed in Hendgen-Cotta *et al.* (Hendgen-Cotta *et al.*, 2008). Myoglobin

has other important effects in the cell, which may be particularly relevant to ischaemia-reperfusion injury. For example, the dioxygenase function of oxygenated Mb ($NO + O_2 + NAD(P)H \leftrightarrow NO_3^- + NAD(P)^+ + H^+$) can prevent damage from the overproduction of NO radicals in the heart (Ishibashi *et al.,* 1993; Flogel *et al.,* 2001) by protecting the respiratory chain against nitrosative stress (Brown, 2000).

In studies in our department we observed a prolonged vasodilating response to nitrite in the forearms of healthy volunteers, which persisted for around 45 minutes after the nitrite infusion was stopped (unpublished data, Maher *et al.,* 2007). Intriguingly, this prolonged vasodilatation was only observed in the infused arm, even though levels of nitrite were raised in both arms during the infusion. Vasodilatation persisting after 30 minutes cessation of nitrite infusion was also reported in Cosby *et al.* (Cosby *et al*., 2003), but is not discussed further. This led us to hypothesise that an intrinsic vascular wall protein was involved. We had found only minor effects of xanthine oxidase inhibition with allopurinol in this model (unpublished data, Maher *et al*., 2007) and so we resolved to test the role of myoglobin in nitrite-induced vasodilatation. This forms the rational for the work presented in **Chapter 3**.

1.3.1.4. Nitrite reductase: eNOS, XO and ALDH2

Endothelial NOS (eNOS) has been proposed as a nitrite reductase in ischaemia or hypoxia, when its NO synthase activity is inhibited (Webb *et al.*, 2008a; Gautier *et al.*, 2006; Vanin *et al.*, 2007; Mikula *et al.*, 2009). Milsom and colleagues used renal-targeted knockout of eNOS to investigate this role in their model of bilateral kidney IR (Milsom *et al.*, 2010). Topical nitrite prior to reperfusion was protective in the wild-type animal ($n = 8$ vs. 4, $p < 0.001$). Interestingly, in this study nitrite appeared to worsen renal dysfunction post-IR in the eNOS

knockout (n = 7 vs. 4, $p < 0.001$). Also, eNOS knockout in itself appeared to be protective (n = 8 vs. 7, $p < 0.001$). This may reflect the dual nature – protective or harmful - of NO release in ischaemia-reperfusion, discussed in greater detail in **section 1.8.3**.

Xanthine oxidase (XO) catalyses the oxidation of hypoxanthine to xanthine and xanthine to uric acid. It is also capable of reducing nitrite to NO *in vitro* (Li *et al.*, 2001). Erythrocyte XO has been proposed as an important intravascular nitrite reductase (Webb *et al.*, 2008a), though this assertion suffers from the same drawbacks as that proposing haemoglobin – namely, how can the NO generated escape from the cell? Inhibition of xanthine oxidase prevented cytoprotection by nitrite in a number of studies (Webb *et al.*, 2004; Baker *et al.*, 2007) but this result was not universally seen (Shiva *et al.*, 2007a; Rassaf *et al.*, 2007).

Work from the Zweier group implicated xanthine oxidase and aldehyde dehydrogenase as the critical vascular wall nitrite reductases and discounted the role of red blood cells (Li *et al.*, 2008). Overall, NO production from red blood cells was very limited, except when nitrite was in large excess (500 μM). Inhibition of haemoglobin with carbon monoxide (CO) increased NO production from red blood cells, implying that scavenging activity of Hb was dominant. Importantly, this study relates to production of free NO, and therefore does not rule out the possibility that Hb, through intermediates, might have a role in nitrite-derived NO-like effects (this is discussed in more detail below). They went on to investigate liver and heart samples with rotenone, oxypurinol and raloxifene to inhibit mitochondrial complex I, XO and ALDH2 respectively. They do not report results of inhibition of tissue nitrite reductase activity with CO, and in any case the effects of this non-specific heme-inhibitor would be difficult to interpret in this model. We adapted this method, but (critically) combined with genetic

knockout to investigate the role of myoglobin in vasodilatation in the work reported in **Chapter 3**.

1.3.2. Protein S-nitros(yl)ation

NO has important cGMP-independent cellular effects, most of which involving post-translational protein modification. *S*-nitros(yl)ation of protein cysteine residues by NO generates *S*-nitrosothiols (SNOs), which may also regulate cell function. (Foster *et al.*, 2003; Hess *et al.*, 2005). Other SNOs, for example those of albumin (Stamler *et al.*, 1992) or haemoglobin (Singel and Stamler, 2005; Allen *et al.*, 2009) are believed to act as a reservoir of NO bioactivity, ready to be released in areas of hypoxia or low pH. Terminology in this area is variable: both S-nitrosation and S-nitrosylation refer to the covalent binding of a nitroso group (NO) to a thiol. However, S-nitrosylation is frequently used specifically to mean post-translational modification of proteins, as found in both cell-to-cell and intracellular signalling (Allen *et al.*, 2009). The composite term, S-nitros(yl)ation is often used to signify the broad effects of nitroso group binding.

Consistent with an important role in cellular signalling, S-nitros(yl)ation is not merely an indicator of NO production. Individual components of the signalling network are in a constant balance between nitros(yl)ation and de-nitros(yl)ation (Lima *et al.*, 2010). High molecular weight SNOs are present in the plasma at a concentration of approximately 50 nM (van Faassen *et al.*, 2009) and *in vivo* levels of SNOs depend linearly on nitrite concentration (Bryan *et al.*, 2005).

Stamler and colleagues initially proposed a theory of hypoxic vasodilatation in which SNO-Hb releases NO by R-to-T transition of Hb to produce vasodilatation. Free NO is bound by

heme to produce iron(II)-nitrosyl-Hb, and then finally re-nitrosylates the β-93 cysteine of Hb when the heme-bound NO is replaced by O_2 in the pulmonary circulation (Gow *et al.*, 1999). Nitrite was not included in the early formulations of this theory. The theory was challenged on several levels. Firstly, measured arterial-venous gradients of iron-nitrosyl-haemoglobin (Stamler *et al.*, 1997; McMahon *et al.*, 2002) could be confirmed in rodents but not in humans (Rassaf *et al.*, 2003). There was no detectable iron-nitrosyl-Hb found in humans in the latter study. Gladwin and colleagues reported much lower levels of both iron-nitrosyl-Hb, and of SNO-Hb (Gladwin *et al.*, 2000a). There were also no AV gradients of either product. Secondly, there were several problems with the proposed chemical processes: the majority of endothelium-derived NO is produced in the arterial circulation, where deoxyHb is low (Huang *et al.*, 2001; Joshi *et al.*, 2002); NO bound by ferrous (Fe^{2+}) may be too tightly bound to transfer to the β-93 cysteine to replenish SNO-Hb; and finally the R-to-T transition of Hb was not shown to facilitate release of NO from SNO-Hb in subsequent studies (Patel *et al.*, 1999; Wolzt *et al.*, 1999)

The theory was modified to include a direct SNO synthase activity of haemoglobin itself, using nitrite as substrate, to overcome the objections raised above (Angelo *et al.*, 2006). The observation that scavenging by haemoglobin should prevent haemoglobin release from red blood cells, despite the undoubted ability of deoxyhaemoglobin (and erythrocytic xanthine oxidase) to act as a nitrite reductase under certain conditions, has led to the hypothesis that SNOs, rather than free NO, produce NO-like bioactivity from nitrite. In this model, nitrite nitrosylates Hb, which shifts NO to protein intermediates by S-transnitrosation, with sGC (or Ca2+-dependent K+ channels (Bolotina *et al.*, 1994) and vasodilatation as a final target (Jia *et*

al., 1996). It has been shown SNOs can have NO-like effects which do not require the release of free NO (McMahon *et al.*, 2000)

Against this paradigm, the effects of nitrite-derived NO – particularly vasodilatation (Crawford *et al.*, 2006; Dalsgaard *et al.*, 2007) and cardioprotection (Duranski *et al.*, 2005; Webb *et al.*, 2004) - have been consistently shown to be inhibitable by NO scavengers, often using the cell-impermeable scavenger carboxy-PTIO. Substitution of the β-93 cysteine on haemoglobin, the critical cysteine for S-nitrosylation, actually increase the rate of nitrite reduction, by decreasing the heme redox potential (Crawford *et al.*, 2006). The authors in the latter study argue that the short burst of vasodilatation seen when red cells are added to a hypoxic preparation is in fact due to ATP release, rather than SNOs.

1.3.3. N_2O_3 as an intermediate

Dinitrogen trioxide (N_2O_3) is the anhydrous form of nitrous acid (HNO_2). N_2O_3 has been suggested to be both an NO donor and an effective nitrosylating agent. It can be generated by the reaction of nitrite with ferric heme-proteins, such as metHb. The metHb binds nitrite to form a nitrite radical, which generates N_2O_3 through a radical-radical reaction with NO (Gladwin and Kim-Shapiro, 2008; Perissinotti *et al.*, 2008):

$$\text{metMyoFe}^{3+}\text{-NO}_2^- + \text{NO} \rightarrow \text{MyoFe}^{2+} + \text{N}_2\text{O}_3$$

N_2O_3 is relatively more stable than NO, and is lipophilic and diffusible (Gladwin and Kim-Shapiro, 2008). While the involvement of such or similar intermediates is theoretically possible, practical demonstration is challenging and has not been achieved as yet.

1.3.4 Generation of methaemoglobin

Methaemoglobin (metHb) is an oxidised form of the heme-protein haemoglobin, where the iron of the heme group is in the ferric (Fe^{3+}) rather than ferrous (Fe^{2+}) state. It is chocolate brown in colour. This change removes the capacity of haemoglobin to carry oxygen and, consequently, high levels of methaemoglobinaemia cause morbidity and death (Atkinson, 1888; Oppe, 1951). The normal level of metHb is below 1%. Higher levels can be caused by genetic disorders or environmental factors such as drugs (anti-malarials, some older analgesics, and sulphonamide antibacterials) or industrial chemicals (chromates, chlorobenzene, arsine and aromatic amines). Importantly, nitrite can oxidise Hb to form metHb:

$$NO_2^- + HbFe^{2+} + H^+ \rightarrow NO + HbFe^{3+} + OH^-$$

The reaction is complex and depends upon the oxygenation level of Hb (Kim-Shapiro *et al.*, 2005). *In vivo*, metHb is reduced back to Hb by methaemoglobin reductases, also known as cytochrome b5 reductases, a widely expressed group of NADH-dependent enzymes.

The induction of metHb by nitrite has proved useful in the treatment of cyanide poisoning (Vale, 2001). Untreated, cyanide binds and inhibits cytochrome c oxidase, a crucial component in cellular respiration. In this multi-step process, cyanide preferentially binds sacrificial metHb, produced by nitrite, giving cyanmethaemoglobin. Thiosulfate, given intravenously as the sodium salt, reacts with cyan-metHb to form thiocyanate, and sulfite.

Thiocyanate can be excreted in the urine and remaining methaemoglobinaemia treated with methylene blue.

The reaction of metHb with very high levels of nitrite in conditions of low pH gives a green product known as nitri-Hb. A similar product, nitri-myoglobin, is responsible for the "greening" seen in cured meats (Kim-Shapiro *et al.*, 2005).

1.4. A short history of research into nitrite

Sodium nitrite was observed to be a dilator of smooth (then known as "non-striped") muscle as early as 1888 (Atkinson, 1888). The reaction of nitrite with haemoglobin, to form methaemoglobin, was also noted. When studied in aortic ring preparations, this vasodilating effect was weak (Furchgott and Bhadrakom, 1953) with supra-physiological amounts of nitrite required. This supposition limited the interpretation of experiments that later showed that nitrite can activate soluble guanylate cyclase (sGC) (Mittal *et al.*, 1978; Ignarro and Gruetter, 1980).

The limiting factor in these early experiments appears to be the high level of oxygenation. Nitrite reduction to NO is strongly favoured by hypoxia, such that physiological levels may produce significant vasodilatation *in vivo* when tissue oxygenation is low (Dezfulian *et al.*, 2007; Lundberg and Weitzberg, 2005). Additionally, the discovery that nitrite is consumed across the vascular bed, with significant arterial-to-venous differences, implied that nitrite might have a signalling role in physiology (Gladwin *et al.*, 2000b; Gladwin *et al.*, 2005). This realization kick-started a rapidly growing field of research into several areas of potential therapeutic interest:

1. Therapeutic vasodilatation, including in pulmonary and systemic hypertension

2. Therapy in diseases of haemolysis and NO-scavenging such as sickle cell anaemia

3. Cytoprotection from ischaemia-reperfusion (IR) injury

1.5. Nitrite in physiological and therapeutic vasodilatation

As mentioned above, the consumption of nitrite across the vascular bed suggests that nitrite has a physiological role in the circulation (Gladwin *et al.*, 2000b). It was long thought, largely on the basis of *ex vivo* studies, that nitrite could not vasodilate in micromolar concentrations (at the upper end of the physiological range) (Lauer *et al.*, 2001). However, recent evidence from both *in vitro* and *in vivo* studies has called this assumption into question.

1.5.1. *In vitro* studies

Early *in vitro* studies established that nitrite could activate sGC and relax arterial and venous tissue at supra-physiological levels (0.1 - 10 mM) (Moulds *et al.*, 1981), and focused on the role of S-nitrosothiols as intermediates in this reaction (Ignarro and Gruetter, 1980; Ignarro *et al.*, 1981). Nitrite was not considered a physiological vasodilator due to the large amounts required in these studies.

The study of vasodilatation by nitrite intensified with the nitrite revival at the turn of this century. In acidic conditions, physiological concentrations of nitrite were found to relax isolated aortic rings (Modin *et al.*, 2001), and the importance of oxygen tension in these studies was definitively shown by Dalsgaard and colleagues using the wire myograph (Dalsgaard *et al.*, 2007). This latter work used two concentrations of O_2 in the perfusing gas

(1% and 95%), and also high (1 μM) and low (0.02 μM) concentrations of norepinephrine (NE) for preconstriction. Hypoxia potentiated vasorelaxation to nitrite by two log orders. Removing endothelium, adding allopurinol (to inhibit xanthine oxidase), or adding myxothiazol (to inhibit mitochondrial bc_1 – complex III or cytochrome c oxidase) did not appear to affect the response to nitrite, though ODQ (a G-protein inhibitor) abolished response. Together, they interpreted these results to mean that nitrite vasodilates by conversion to NO, and that the bioconvertor is not xanthine oxidase, nor cytochrome c oxidase, nor is resident in the endothelium. They postulated that the bioconvertor is instead resident in the vessel wall, and that the reaction is first order with respect to concentration of nitrite, and is enhanced by hypoxia. They discuss nitrite reductase activity and possible candidates, focusing on haemoglobin (though they accept that their data do not support such a conclusion), and do not discuss myoglobin despite it being inherently oxygen-sensitive thanks to its heme group.

ODQ prevented nitrite-induced vasodilatation, which was interpreted as proving the requirement for free NO in this process. In fact, ODQ is not as specific an inhibitor of sGC as claimed, and may block nitrite reductase activity by other heme-proteins as well as sGC activation (Alzawahra *et al.*, 2008). The other inhibitors had no effect on nitrite-induced vasodilatation, suggesting that neither XO nor cytochrome c oxidase are important players in this process. Finally, haemoglobin was a net scavenger in all conditions studied.

1.5.2. *In vivo* studies

In an important study, Cosby and colleagues definitively showed that sodium nitrite vasodilates the forearm when infused into the brachial artery (Cosby *et al.*, 2003). Initially, a

high dose of 36 μmol/min (2.4 mg/min) was tested and found to significantly increase forearm blood flow (FBF) both with and without NOS inhibition by L-NMMA (n = 10, p <0.01). FBF increased further on forearm exercise, despite relative reduction in nitrite concentration due to increased blood flow. They went on to test a near physiological dose of 400 nmoles/min (27.6 μg/min), which significantly increased FBF from 3.5 ± 0.2 to 4.5 ± 0.3 ml/min/100ml tissue (n = 10, p < 0.006) at rest and to a greater extent on exercise. The authors concluded that basal levels of nitrite are capable of influencing resting vascular tone and subserving hypoxic vasodilatation.

In 2007, the same group extended their previous observations using systemic doses of sodium nitrite. A low dose of just 350 nM was found to cause a significant drop in blood pressure in healthy volunteers (Dejam *et al.*, 2007). In a clinically relevant aside, experiments in monkeys reported in the same paper suggest that nitrite might not induce tolerance in the same way as inorganic nitrates. Studies in our department revealed that sodium nitrite, when infused into the brachial artery of healthy volunteers, modestly dilates arterioles in normoxia, but profoundly vasodilates in conditions of hypoxia (Maher *et al.*, 2008).

Hypoxic vasodilatation requires a sensor, a signal and an effector. Many proposed nitrite reductases (discussed in detail in section 1.3.1.) contain heme and therefore have oxygen-sensing capacity. The reaction of haemoglobin with nitrite, in particular, requires both a proton and oxygen, making it a hypoxia or acidosis sensor (Huang *et al.*, 2005b), with direct release of NO or NO-like bioactivity and vasodilatation the consequence of activation. This theory is self-contained, which may explain part of its popularity, but it suffers from the drawbacks discussed above. The role of nitrite in physiological hypoxic vasodilatation is

therefore unresolved, but the ability of exogenous nitrite to act as a vasodilator – potently in certain conditions - is established beyond doubt.

A consistent feature of nitrite-induced vasodilatation is its targeting of hypoxia. Vasodilators are used in acute decompensated heart failure (ADHF), more for their effects on preload than afterload. There are no specific venodilators in clinical use, and so arterial hypotension is often the limiting factor in treatment of ADHF, requiring admission to intensive care and inotropic therapy. We hypothesized that nitrite, as a hypoxia-targeted vasodilator, would principally cause venodilation and preload reduction in normoxic patients with heart failure, without the drop in arterial blood pressure that comes with nitrate-therapy. This hypothesis was investigated in the experiments presented in **Chapters 4 and 5**.

1.5.3. Pulmonary hypertension

Pulmonary hypertension (PH) is defined as a mean pulmonary artery pressure of >25 mmHg at rest, or >30mmHg on exercise. It is conventionally divided into primary pulmonary hypertension, a disorder of unknown aetiology, and pulmonary hypertension from secondary causes. These secondary causes include cardiac causes (heart failure or primary valve disease), pulmonary disorders such as chronic obstructive pulmonary disease and chronic pulmonary embolic disease, and connective tissue disease (most commonly scleroderma).

Utilising the established hypoxia-potentiated vasodilatory properties of nitrite, several studies have investigated the possibility of treating pulmonary hypertension with nebulised or intravenous nitrite. Mark Gladwin and his associates did the majority of this work (references below).

Inhaled sodium nitrite decreased pulmonary hypertension to a similar degree as 20 ppm NO gas, in hypoxic and normoxic lamb models of PH (Hunter *et al.*, 2004). The pulmonary vasodilatation depended upon deoxyHb and was potentiated by low pH. The effect of nitrite was confirmed in a later study using both hypoxic and inflammatory (monocrotaline-induced) models in mice and rats (Zuckerbraun *et al.*, 2010). 3 – 300 mg sodium nitrite was nebulised in 5 ml of PBS, and given intermittently in an exposure chamber. Development of hypoxic PH was prevented by low-dose nebulised nitrite (3 times per week for 20 minutes per exposure) throughout the 28 days of hypoxia ($n = 9$, $p < 0.001$). It also reversed changes already seen when started two weeks into the hypoxic period ($n = 14$, $p < 0.001$). The effect of nitrite was not increased at the higher dose. Inflammatory PH was reversed by low-dose nebulised nitrite given 3 times per week during weeks 4 - 6 following monocrotaline exposure ($n = 8$, $p < 0.001$). Nebulised nitrite, 3 times per week, would be a cheap and safe intervention, especially when compared to inhaled NO gas. This study did show marked increase in superoxide generation in the lung, as part of experiments intended to show the necessity of XO in nitrite-dependent generation of NO, and long-term effects were not investigated. Research in Cardiff University has begun to extend these observations to human subjects. 18 healthy volunteers underwent nitrite infusions and showed a decrease in pulmonary arterial pressure, as estimated by echocardiography, in normoxia, as well as prevention of hypoxic vasoconstriction (Ingram *et al.,* 2010).

Pulmonary hypertension can occur acutely (as well as chronically) as a result of pulmonary embolism. Dias-Junior *et al.* induced PH by infusing clots of venous blood into the right atrium of mongrel dogs (Dias-Junior *et al.*, 2006), which produced the expected rise in

pulmonary vascular resistance (PVR) and pulmonary arterial (PA) pressure. One group of embolised animals received intravenous sodium nitrite at a dose of 6.75 μmol/kg nitrite over 15 minutes, followed by a maintenance infusion of 0.28 μmol/kg/min for the remainder (31.1 μg/kg/min followed by 19.3μg/kg/min), which increased the mean plasma concentration of nitrite by approximately 2 μM (from ~1 μM to ~3 μM). Cardiac index in embolised animals was significantly increased by nitrite (+28%, $n = 10$, $p < 0.05$ compared to placebo). Interestingly, this moderate dose of nitrite produced significant vasodilatation in control animals, which contrasted with the effect in embolised dogs and was associated with a drop in cardiac index. The authors suggest that this may be due to consumption of nitrite in the pulmonary vasculature. In a later small study, sildenafil was found to potentiate the effect of nitrite (Dias-Junior *et al.*, 2008). There was a transient but significant drop in mean arterial pressure (24 mmHg), which suggests that extreme caution is warranted in the co-administration of nitrite and sildenafil, as with organic nitrates.

1.5.4. Cerebral vasospasm

Stroke is the clinical syndrome of sudden loss of brain function due to cessation of blood supply. It is a common cause of death and disability in people of all ages. Strokes are pathophysiologically divided into ischaemic (caused by embolisation of material, commonly blood clot, into the cerebral circulation) and haemorrhagic (bleeding into the brain parenchyma). Ischaemic stroke causes damage through necrosis of brain tissue and ischaemia-reperfusion (IR) injury. The role of nitrite in prevention of IR injury is discussed in **section 1.8.4**.

Sub-arachnoid haemorrhage (SAH) caused by a burst aneurysm may also result in stroke. Damage is frequently exacerbated by cerebral vasospasm, caused by the irritating effect of blood surrounding small arteries. Nitrite prevented cerebral vasospasm after subarachnoid haemorrhage in a primate model (Pluta *et al.*, 2005). One group (n = 3) received 90 mg sodium nitrite intravenously over 24 hours plus a 45 mg bolus daily for 14 days. A second group (n = 3) received a 180 mg infusion of sodium nitrite every 24 hours continuously for 14 days. 90 mg over 24 hours equates to approximately 70 - 200 μg/kg/min assuming an average weight of 3 - 9 kg; weights and sexes are not given and the cynomolgus monkey is variable between subspecies as well as sexually dimorphic in this regard. There was a control group that received no nitrite (n = 8). This model uses a band of autologous clot around the middle cerebral artery. Interestingly, this intervention produced vasospasm in all control animals, peaking at 7 days. This contrasts with the clinical syndrome of human SAH, in whom vasospasm only develops in 50%. No animal in either nitrite group developed significant vasospasm, and the degree of clinically insignificant vasospasm correlated inversely with the level of nitrite measured in the cerebrospinal fluid.

Asymmetric dimethyl-L-arginine (ADMA), a naturally-occurring NOS inhibitor was increased in patients with SAH and cerebral vasospasm (n = 12), compared to a control group without vasospasm (n = 5), which underscores the potentially important role of NO in this pathology. With these results in mind, a human trial of nitrite in sub-arachnoid haemorrhage began recruiting in 2009 (reported by Calvert and Lefer (Calvert and Lefer, 2010)). Systemic infusions of nitrite will be given for 14 days following confirmation of a ruptured cerebral aneurysm with the intention of providing both safety and efficacy data for a future therapeutic role.

1.6. Nitrite in the treatment of systemic hypertension

It is estimated that 11% of global disease is directly related to chronic hypertension (WHO 2002). As discussed in the preceding section, nitrite causes vasodilatation in animal models (Kozlov *et al.*, 2005; Modin *et al.*, 2001) and human volunteers (Dejam *et al.*, 2007; Maher *et al.*, 2008). It is also cheap and orally bioavailable, both important attributes in a potential therapy.

Prehypertension is defined as elevation of blood pressure above "normal" levels but below those that trigger treatment. As such, this condition is a product of national and international treatment guidelines, and the recognition that blood pressure is normally distributed across a population. Currently, prehypertension is diagnosed with a systolic blood pressure of 120-139 mmHg or a diastolic pressure of 80-89 mmHg (Chobanian *et al.*, 2003). It is felt that people at increased risk of developing hypertension may benefit from therapy, albeit in a less aggressive manner (Julius *et al.*, 2006). Thus the risk-benefit balance is different; therapies must be safe and cheap. Dietary supplementation with nitrite has been suggested as a candidate that would meet these goals (Webb *et al.*, 2008b).

Supplementation of dietary nitrate by administration of sodium nitrate (0.1 mmol/kg/d) to healthy volunteers over 3 days reduced diastolic (but not systolic) BP by 3.7 mm Hg compared to sodium chloride (Larsen *et al.*, 2006). In a later randomised open-label study of 500ml beetroot juice or water, nitrate-rich (34.0 ± 0.1 mmol/L) beetroot juice caused a fall in systolic blood pressure of 10.4 ± 3.0 mm Hg at 2.5 hours (Webb *et al.*, 2008b). The test substance contained undetectable amounts of nitrite, but spitting saliva, rather than

swallowing, during the period after ingestion abolished the effects. This intervention interrupts the enterosalivary circulation and prevented the rise in plasma nitrite seen in the swallowing group. Thus, the effects of an oral nitrate load were attributed to bacterial reduction of nitrate to nitrite in the mouth.

Chronic kidney disease caused by long-standing hypertension is a major public health problem worldwide. Dietary nitrite at a very low dose prevents chronic renal injury caused by NOS inhibitor (Kanematsu *et al.*, 2008)

1.7. Nitrite in sickle cell disease

Sickle cell disease (SCD) is a single gene recessive disorder with high prevalence in the black African population. It is caused by a single nucleotide substitution in the short arm of chromosome 11 (A to T), which leads to the replacement of glutamate with hydrophobic valine at position 6 of the β-subunit of haemoglobin. Haemoglobin S (HbS) thus formed is relatively normal at high oxygen concentrations but conformational change in low oxygen concentration exposes a novel hydrophobic patch. HbS then aggregates to form long chains, which distort red cells into the characteristic sickle shape. Heterozygotes show mild sickling of red cells and possess relative protection against malaria (known as heterozygote advantage), while homozygotes experience the full clinical spectrum of SCD.

Polymerisation of HbS causes red blood cell rigidity, microvascular obstruction, inflammation and infarction. Red cell membrane damage leads to severe intravascular haemolysis and anaemia. The consequence of intravascular free haemoglobin is NO-scavenging, which causes a global deficiency of NO, leading to chronic vasoconstriction and activation of cell adhesion

molecules in both endothelium and platelets (Reiter *et al.*, 2002). Deficiency of NO is exacerbated by the release of red cell arginase, which converts arginine to ornithine, thereby reducing the availability of substrate for NO synthase (Schnog *et al.*, 2004; Morris *et al.*, 2005), and (as in subarachnoid haemorrhage) increased levels of the naturally occurring eNOS inhibitor, ADMA (Schnog *et al.*, 2005). As a whole, this process causes a diffuse vasculopathy in sickle cell patients, with complications including: pulmonary hypertension, acute chest syndrome, marrow and fat infarction, multiple stroke, priapism, dactylitis, and cutaneous ulceration (Gladwin *et al.*, 2004; Stuart and Nagel, 2004). Even with the best available therapy, life expectancy in SCD is severely reduced (Platt *et al.*, 1994).

1.7.1 Vaso-occlusive crisis in SCD

Most patients with SCD experience intermittent painful episodes known as vaso-occlusive crises. Damage can occur in any organ but pain commonly results from bone or fat infarction. Abdominal organs may be affected, leading to generalised pain and jaundice, and irreversible organ damage may result. Acute chest crisis is a form of vaso-occlusive crisis, which can be fatal.

The pathology of vaso-occlusive crisis is multi-factorial. Increased platelet activation in steady state SCD was noted over 25 years ago (Westwick *et al.*, 1983), and it has since been discovered that activation increases during vaso-occlusive crisis (Wun *et al.*, 1998). Again, the proportion of circulating platelets that are activated correlates well with intravascular haemolysis, and indeed pulmonary hypertension (Villagra *et al.*, 2007). Increased sickling leads to mechanical blockage of flow, worsened by NO-scavenging and inflammation. Intravenous nitrite targets NO-release to areas of hypoxia and acidity, in ischaemia and

infarction. It can be theorised that NO-dependent vasodilatation would increase blood flow in vaso-occlusive crisis, but modulation of inflammation and cell adhesion may be equally important in this pathology. This two-pronged action makes nitrite particularly worthy of investigation in SCD.

1.7.2 Nitrite as a therapy in sickle cell disease

The best-characterised consequence of NO-scavenging in SCD is pulmonary hypertension. Pulmonary hypertension is an independent risk factor for death in SCD and is found in around 30% of SCD patients. Even relatively mild elevations of pulmonary arterial pressure are associated with a 10-fold rise in early mortality (Gladwin *et al.*, 2004). This risk is further increased when combined with left ventricular diastolic dysfunction (Sachdev *et al.*, 2007). Rates of pulmonary hypertension are strongly correlated with lactate dehydrogenase levels (a red cell enzyme released in haemolysis), which supports a causative role of intravascular haemolysis in the development of this complication (Kato *et al.*, 2006; Hsu *et al.*, 2007).

Inhaled NO reduced pulmonary and systemic vasoconstriction in a canine model of intravascular haemolysis (Minneci *et al.*, 2005). Inhalation of the gas restored vascular reactivity to sodium nitroprusside (SNP) in this study, though it should be cautioned that SNP is not a pure NO-donor as claimed. A randomised double-blind placebo-controlled trial in 20 children with SCD and veno-occlusive pain crisis showed significantly lower pain scores and patient-controlled morphine use in those given NO compared to placebo gas (Weiner *et al.*, 2003). However, inhaled NO is expensive and inconvenient to administer, and is therefore not an option outside acute hospital care. The effects of inhaled NO, which occur beyond the

physiological diffusion distance of this ephemeral gas, imply that the therapeutic benefit may rely upon nitrite as an intravascular intermediate (Cannon, III *et al.*, 2001).

The relative resistance to nitrite seen in these studies is consistent with the diminished response to other NO donors seen in mouse models and in humans with SCD (Reiter *et al.*, 2002; Eberhardt *et al.*, 2003; Gladwin *et al.*, 2003; Kaul *et al.*, 2004). The mechanism of resistance is closely related to NO consumption by plasma cell-free haemoglobin (Hsu *et al.*, 2007)

The ability to release NO in a hypoxia-targeted manner, coupled with the central role of NO-deficiency in SCD, makes intravenous sodium nitrite an attractive candidate therapy in SCD. Preliminary data from the canine model of intravascular haemolysis provide reason for optimism with regard to potential benefits of nitrite therapy in PH and vaso-occlusive crisis. Nitrite appeared superior to SNP in both pulmonary and systemic vasodilatation in this model (Minneci *et al.*, 2008). However, in one study of 14 SCD patients, brachial artery nitrite infusion produced a substantially smaller increase in forearm blood flow when compared to that in healthy volunteers (Mack *et al.*, 2008). This blunting of vascular responsiveness was acknowledged to be typical of the response to NO-dependent therapies in SCD and should temper enthusiasm until trial evidence is available.

Beyond SCD, haemolysis and NO-scavenging may underlie some of the adverse outcomes associated with transfusion of stored blood (Gladwin and Kim-Shapiro, 2009). It may be hypothesized that addition of nitrite to banked blood might be beneficial in this regard.

1.8. Nitrite in protection from ischaemia-reperfusion (IR) injury

The phenomenon of reperfusion injury (i.e. injury distinct from that caused directly by ischaemia) was first described fifty years ago (Jennings et al., 1960). The hypothesis that such injury may be preventable led directly to the discovery of ischaemic preconditioning by Murry and colleagues (Murry *et al.*, 1986), an experiment wherein four five-minute canine coronary artery occlusions, before a final ligation, reduced absolute infarct size by 23%. Ischaemic preconditioning proved to be the archetype for a host of protective stimuli, initially given before ischaemia, and subsequently after the onset of reperfusion. This latter is now known as postconditioning, and again an ischaemic stimulus is the gold standard (Zhao *et al.*, 2003). Ischaemic postconditioning has definitively entered the clinical arena, with a real-life reduction in peak creatine kinase (a marker of infarction), and improved ejection fraction (Hansen *et al.*, 2010). Myocardium is the best-studied tissue, especially in terms of elucidation of the signalling pathways, but endothelial, renal, hepatic and brain tissues have all been shown to respond to similar stimuli and also represent legitimate therapeutic targets.

1.8.1. Ischaemia-reperfusion injury

Several factors contribute to ischaemia-reperfusion injury; including neutrophils cell adhesion, ATP depletion, generation of reactive oxygen species (ROS), and mitochondrial calcium overload (Buja, 2005). Seminal work by Crompton and colleagues proposed opening of the mitochondrial permeability transition pore (mPTP), a non-selective pore located on the inner mitochondrial membrane, as the critical determinant of cell death (Crompton *et al.*, 1987). Opening of this massive multi-potent channel results in loss of membrane selectivity; oxidative phosphorylation is uncoupled leading to ROS generation and energy loss; and

finally the mitochondrion swells and releases pro-apoptotic factors as the membrane breaks down, resulting in apoptosis or necrosis of the cell.

Prevention of mPTP opening appears to be the final common pathway of ischaemic preconditioning and postconditioning. The pore is "primed" by pro-cell death factors, but remains closed in the acidic conditions of ischaemia (Griffiths and Halestrap, 1995). Opening of the mPTP therefore occurs in the first few minutes of reperfusion, affording a window of opportunity for therapeutic intervention that extends into the post-ischaemic period (Halestrap, 2009). Illustrating this phenomenon, a direct inhibitor of mPTP opening, sanglifehrin-A, reduces infarct size by 50% when given during the first 15 minutes of reperfusion, but fails to do so when administered later in this period (Hausenloy *et al.*, 2003).

1.8.2. Mechanisms of cytoprotection

The evident risks of exposing tissue to repeated periods of ischaemia makes the identification of signalling pathways in cytoprotection, and therefore identification of downstream pharmacological stimuli, a priority. The pathways are detailed below and summarized in **figure 1.2**.

1.8.2.1. RISK pathway

The Reperfusion Injury Salvage Kinase (RISK) pathway, thus termed by Hausenloy and Yellon, was the first such signalling pathway described and remains the best studied (Hausenloy and Yellon, 2004). This pathway is activated in both ischaemic preconditioning and postconditioning, and involves binding of G-protein coupled receptors by their ligands, and downstream activation of PI3K and Akt, or activation of MEK then ERK (extracellular

regulated kinase). These kinases, perhaps combined in a "signalosome" (Garlid *et al.*, 2009), travel to mitochondria and activate final common pathway targets, leading ultimately to prevention of opening of the mPTP. This is shown in more detail in **figure 2**.

Cardio-protective signalling pathways within the mitochondrion have recently begun to be elucidated. Mitochondrial Protein Kinase Cε1 and 2 (PKCε1 and 2) have been identified as part of the final common pathway of cardio-protective signalling (Budas and Mochly-Rosen, 2007). Activation of PKCε1 results in opening of mitochondrial ATP gated potassium channels (K_{ATP}), causing a small burst of reactive oxygen species (ROS) production by complex 1 of the respiratory chain. This small increase in ROS activates PKCε2, which inhibits mPTP opening and prevents cell death (Garlid *et al.*, 2009).

The difference between a small burst of ROS being essential and a large one being detrimental - the same being true of NO and also TNFα - mirrors the fundamental paradox of cardio-protection: short bursts of ischaemia are beneficial, while ischaemic damage results from longer exposure.

1.8.2.2. SAFE pathway

TNFα plays a well-defined role in inflammation and cell death, and has been hypothesised to exacerbate IR injury (Mann, 2003). Recent evidence suggests it is essential in both pre- and post-conditioning (Deuchar *et al.*, 2007; Lacerda *et al.*, 2009; Smith *et al.*, 2002a) and activates a separate adaptive pathway in myocardial injury, termed the Survivor Activating Factor Enhancement (SAFE) pathway (Lecour *et al.*, 2005). This pathway is shown in more detail in **figure 1.2**.

1.8.2.3. Non-RISK non-SAFE signalling

Adenosine protects independently of activation of Akt/Erk, and (at least the early part of) the RISK pathway (Schulz *et al.*, 2001). However, TNFα knockout mice can still be preconditioned with adenosine, suggesting that neither is activation of the SAFE pathway required (Smith *et al.*, 2002b). The presence of multiple pathways is suspected (Heusch *et al.*, 2008) but there may also be significant cross-talk between the classical RISK and SAFE pathways (e.g. Erk phosphorylates STAT-3 (Nakagami *et al.*, 2001), at least in endothelium, and a SAFE pathway inhibitor also prevents MEK phosphorylation (Matsumiya *et al.*, 2002). Definitive proof of separate non-RISK non-SAFE pathways would require studies in multiple knockout animals, with knockout, silencing or specific inhibition of critical steps in both pathways (Lecour, 2009).

1.8.3. NO in cytoprotection

Endogenous NO from eNOS is essential for ischaemic postconditioning (Penna *et al.*, 2006) but not preconditioning (Schulz *et al.*, 2004). However, exogenous NO does trigger preconditioning (Hataishi *et al.*, 2006; Schulz *et al.*, 2004). Even though ischaemic preconditioning appears not to require endogenous NO production, this result should be interpreted with caution as NO may be released by acid disproportionation or reduction of endogenous nitrite, independent of eNOS. Endogenous NO also has an important role in delayed preconditioning, through expression of iNOS (Bolli *et al.*, 2007). The multiple levels of interaction of NO in cytoprotection are shown in **figure 1.3**.

1.8.4 Nitrite in cytoprotection

As discussed in the previous section, nitric oxide plays a variety of roles in cytoprotection. Any of these entry points may potentially be accessed by nitrite, as a hypoxia-targeted store of NO. Mitochondrial exposure to nitrite causes nitrosylation of critical thiols on mitochondrial complex I, inhibiting it (Raat *et al.*, 2009). Flux through the electron chain is reduced, which protects downstream complexes II-V from the increase in ROS associated with reperfusion. A wide variety of other mitochondrial proteins are also vulnerable to oxidative damage. Protection from damage prevents release of cytochrome c and induction of apoptosis (Shiva *et al.*, 2007b).

Nitrite-mediated protection is a recognized phenomenon in many different animal models, oral nitrate has been tested in humans, and several human studies of nitrite in IR injury are planned or underway but are some way from reporting (*clinicaltrials.org*). This rapidly growing field is summarized in the following sections. Where possible, doses are additionally given in the form μg/kg/min, for easier comparison. Even so, multiple routes of administration, among a variety of tissue models, make direct comparison challenging.

1.8.4.1. Nitrite in the protection of myocardium

NOS-independent generation of NO occurs in the ischaemic myocardium; this is attributed to reduction of the nitrite store (Zweier *et al.*, 1995). Low pH, hypoxia and reduced substrate availability results in uncoupling of eNOS and markedly reduced production of NO, while also strongly favouring nitrite reduction.

The first demonstration of preconditioning by nitrite was in the Langendorff isolated rat heart model in 2004 (Webb *et al.*, 2004). Nitrite administered immediately before reperfusion (48

nmol, ~110 μg/kg, intraperitoneally) produced a 67% reduction in infarct size in the whole mouse (Duranski *et al.*, 2005). Preconditioning with nitrite depends upon on the opening of K_{ATP} channels (Baker *et al.*, 2007) and was abolished in both animal models by addition of an NO scavenger (carboxy-PTIO), implying a free NO intermediate. Interestingly, in the second study (Duranski *et al.*, 2005) there was a biphasic dose-response, with the top dose of 1920 nmol (~ 4 mg/kg) providing no protection.

Nitrite confers delayed preconditioning (i.e. when the preconditioning stimulus is delivered 24-72 hours before ischaemia, sometimes known as "second-window") (Shiva *et al.*, 2007b), which may underlie the protective effects of high-NO_x diets, such as the "Mediterranean Diet" (Raat *et al*., 2009). The short half-life of nitrite *in vivo* suggests a mechanism involving post-translational modification (Shiva and Gladwin, 2009). Nitrite also protects myocardium when administered after the onset of ischaemia. This is termed peri- or per-conditioning, and is generally thought to be more similar to postconditioning than preconditioning, given that signalling molecules are unlikely to reach the ischaemic tissue in any quantity until reperfusion. Recently, Gonzalez and colleagues demonstrated a 34% absolute reduction in infarct size (from 70 ± 10% to 36 ± 8%) in a canine model, where nitrite was given during the last five minutes of a two hour ischaemic period (Gonzalez *et al.*, 2008). A significant increase in the levels of S-nitrosylated proteins was detected following the 60-minute infusion. However, after the 5-minute infusion, protection occurred in the absence of any significant increase in nitrosylated species. This suggests that nitrite itself is the primary mediator of protection (Gonzalez *et al.*, 2008).

1.8.4.2. Nitrite in the protection of endothelium

Endothelium is uniquely sensitive to ischaemic injury (Mankad *et al.*, 1997), and endothelial dysfunction is a ubiquitous finding in cardiovascular disease in general. In contrast to other tissue models, endothelial IR injury is more difficult to study in animals than in humans, which may explain the lack of data on endothelial protection by nitrite.

Webb and colleagues, in the second part of the study on blood pressure reduction discussed in section 1.6., found that ingestion of beetroot juice (500 ml, 2 hours before ischaemia, containing 45.0 ± 2.6 mmol/L nitrate and undetectable nitrite) prevented ischaemia-induced endothelial dysfunction as measured by flow-mediated dilatation (FMD) (n = 10, $p < 0.05$) (Webb *et al.*, 2008b). FMD is more convenient than venous-occlusion plethysmography (VOP), as it is quicker to perform and does not require arterial cannulation. However, it has the disadvantage that only one level of flow stimulus can be assessed before and after ischaemia, and the error associated with measuring arterial diameter by ultrasound is high. In this context, the group numbers are quite low. Unfortunately, the elegant spit-swallow differentiation of nitrite vs. nitrate effects was not performed in this part of the study. Even so, these data provide indirect evidence that nitrite might have an effect in first window preconditioning (stimulus given in the 1-2 hours before ischaemia). We decided to investigate the more clinically relevant windows – delayed (or second-window) preconditioning (stimulus given 24-72 hours before ischaemia), and periconditioning (stimulus given during ischaemia and before reperfusion). These experiments are reported in **Chapter 6**.

1.8.4.3. Nitrite in the protection of kidney, liver and brain

Nitrite has been investigated in the prevention of renal IR injury (Tripatara *et al.*, 2007). 103 male Wistar rats were subjected to 60 minutes of bilateral renal ischaemia and 6 hours of

reperfusion. Rats were randomly allocated to 3 groups: saline placebo, 30 nmol sodium nitrite administered topically 1 minute before reperfusion, and 30 nmol (~0.4 μg/kg/min) nitrite intravenously 1 minute before reperfusion. Renal injury was estimated by measurement of serum AST, and dysfunction by serum creatinine. The authors showed a significant attenuation in both renal injury and glomerular dysfunction in the topical nitrite group (n = 12 vs. 6 for each, $p < 0.05$ compared to IR alone) but the effect sizes were modest (~20% absolute reduction of creatinine rise, ~30% absolute reduction in AST rise). Surprisingly, systemic administration of nitrite had no effect on any parameter. These two results challenge interpretation of the finding that allopurinol abolished protection by nitrite, and the authors claim of a central role for xanthine oxidase (Tripatara *et al.*, 2007). It should be noted that larger reductions in renal dysfunction were shown in a later study in mice from the same group, previously discussed in **section 1.3.1**. (Milsom *et al.*, 2010).

Duranski and colleagues, in the study discussed above, additionally showed that intraperitoneal injection of 48 nmol (~110 μg/kg) sodium nitrite entirely prevented the rise in AST after hepatic IR (Duranski *et al.*, 2005). The NO-scavenger carboxy-PTIO prevented this protective effect, and only the most effective dose of nitrite was assessed in the liver. A study by Lu and colleagues of a similar vintage reported protection of the liver from IR injury in rats (Lu *et al.*, 2005) with a central role proposed for xanthine oxidase. Unusually, all animals were given sodium nitrite (2 mg/kg intraperitoneally, 45 minutes before ischaemia). This is a large dose, especially since IP nitrite is well absorbed and quickly distributed (Bryan *et al.*, 2005), and since there was no group without nitrite, it is difficult to evaluate the extent of the proposed inhibitory effect of allopurinol. In the previous study, 4 mg/kg was found to provide no protection to the myocardium, as it was at the top of the biphasic dose-response. It is

certainly possible that the dose chosen in the latter study is too high to provide meaningful support for the role of xanthine oxidase in this model.

In a more recent study of hepatic IR injury, dietary supplementation of nitrate or nitrite was given to mice in order to replicate the cardioprotective "Mediterranean Diet". Mice given either natural nitrate or a low NO_x diet supplemented with nitrite were protected from hepatic IR injury, as measured by release of ALT and AST, compared to those on the low NO_x diet alone (Raat *et al.*, 2009). Low NO_x mice were given de-ionised water to remove that source of dietary NO_x. As expected, plasma nitrite was much lower in the low NO_x group (29 ± 2 μM, $n = 12$) compared to the group given 300 mg/L sodium nitrite in the drinking water (47 ± 5 μM, $n = 12$), which were similar to those in mice on a normal chow/normal water diet (54 ± 7 μM, $n = 12$). An immediate concern is that all these levels are rather high compared with other published literature (van Faassen *et al.*, 2008) – in fact the low NO_x mice had plasma nitrite levels well above what is considered normal elsewhere. It is unlikely that this simply relates to the mouse strain, C57bl6 mice were used, and perhaps merely underscores the variability of NO_x levels seen across the literature.

Jung and colleagues investigated the protective effects of sodium nitrite and nitrate in a rat model of brain IR injury (Jung *et al.*, 2006). 48, 480 or 4800 nmoles sodium nitrite (in a volume of 500mcL PBS) was infused into the tail vein during the first minute of reperfusion. This is equivalent to 16.5 - 1656 μg/kg/min for 1 minute. 48 nmoles and 480 nmoles nitrite protected (with a 33% and 77% reduction in infarct size respectively, $n = 12$, $p < 0.05$ for both). The top dose of nitrite (4800 nmoles) showed no reduction in infarct size, and nor did 480 nmoles of sodium nitrate. Carboxy-PTIO given 30 minutes before ischaemia prevented

the protective effect of nitrite. Functional recovery was better in the nitrite-treated groups after 1 day ($p < 0.05$), but the clinical significance of functional tests using groups of only 3 animals is questionable. Later, the same group demonstrated that the protective effect of nitrite was still present when given at the same dose up to 3 hours into reperfusion (Jung *et al.*, 2009). Biochemical study of neuroglobin using absorption spectroscopy suggests that this newly characterised heme-globin should be at least as capable as myoglobin of acting as a nitrite reductase in hypoxic conditions, and may in fact be a less avid scavenger (Petersen *et al.*, 2008). Interestingly, in this study cytoglobin was unreactive with nitrite. The presence of neuroglobin may make brain tissue particularly susceptible to NO-overload when treated with nitrite, potentially shedding some light on the result above.

In contrast to the results above, Schatlo and colleagues found that nitrite in combination with thrombolysis (with rtPA) did not provide any additional protection over thrombolysis alone, in a model of middle cerebral artery stroke (Schatlo *et al.*, 2008). The investigators in this study administered a bolus then 7.5 or 0.5 μmol/L sodium nitrite for 50 minutes, which even at the low dose amounted to ~9mg/kg in total. This total dose is very high, even though the authors claim that the infusion rate is comparable to previous work, and this may account for the lack of effect.

1.8.4.4. Nitrite in organ transplantation

A major cause of graft failure in liver and lung transplantation is IR injury (Calvert and Lefer, 2010), making studies of nitrite in this area a priority. So far there are no published studies of nitrite in human transplantation, but inhaled NO has had success in both transplantation of the lung (Yerebakan *et al.*, 2009) and of the liver (Lang, Jr. *et al.*, 2007). However, the objections

to inhaled NO on the grounds of cost and invasiveness still apply. Dietary nitrite prevented rejection of cardiac allografts in one study in the rat (Zhan *et al.*, 2009). Unfortunately, the authors give only (undistinguished) plasma NO_x levels making interpretation of the dose the rats actually received even more challenging than usual. On the basis of an approximate doubling of plasma NO_x, it would seem that the dose is at least similar to previous dietary supplementation studies (Raat *et al.*, 2009; Shiva *et al.*, 2007b). All rats received immunosuppression with tacrolimus, a calcineurin-inhibitor, at a dose of 0.5 mg/kg/day for 7 days to prevent acute rejection. Clearly, the subsequent high rate of graft failure is due to the lack of immunosuppression, and so direct comparison to the human (who continues immunosuppressive therapy beyond the acute rejection stage) is not possible. Still, this study provides proof-of-concept for future investigation. Overall, a range of windows and doses have been tested *in vitro* and in animal models, but there has so far been very little clinical translation of the protective effects of NO and nitrite.

1.9. Concluding remarks and hypothesis

The study of the physiology and clinical potential of the nitrite anion is a rapidly advancing, and occasionally controversial, field. Much has been discovered in the last 10 years, but many important questions remain; these include:

1. What is the predominant nitrite reductase *in vivo* in physiological conditions?
2. Does hypoxia-dependent vasodilatation give nitrite an advantage over current vasodilators?
3. Does nitrite protect against ischaemia-reperfusion injury in humans?

In this thesis I will present several groups of experiments, each intended to provide answers to these important questions and, ultimately, to assess the future of the nitrite anion as a therapeutic agent in cardiovascular disease. First, I present experiments comparing vasorelaxation in mouse aortas, using transgenic mice and virus vectors, in order to investigate the role of this vascular wall heme-protein in nitrite metabolism. I will show that myoglobin is an important bioconverter of nitrite, at least in hypoxia, and may play an important role in vasorelaxation by nitrite in vivo. Myoglobin contributes to a prolonged vasorelaxation by nitrite, as well as the phenomenon of hypoxia-potentiated vasorelaxation, both of which were initially observed in human forearm studies, and may be useful in the treatment of heart failure.

The second group of experiments investigates the safety and feasibility of nitrite infusions in severe heart failure, making use of the phenomenon of hypoxia-augmented vasorelaxation investigated in the preceding chapter. Five-minute infusions of nitrite produced varied and generally beneficial haemodynamic effects in severe heart failure, though this was at the expense of the development of methaemoglobinaemia. I went on to investigate the development of methaemoglobinaemia during longer infusions in healthy volunteers, in order to establish a safe dose of nitrite. Having established a safe dose, I investigated whether the beneficial haemodynamic effects would persist over the course of longer infusions in patients with heart failure.

The third group of studies assesses the ability of nitrite-infusion to act as a cytoprotective agent, using the human forearm model in healthy volunteers, with Mendelian randomisation to elucidate the role of ALDH2 in this phenomenon. Previous work has suggested that

cytoprotection by a variety of stimuli proceeds through activation of ALDH2. I will show that a ten-minute systemic infusion of nitrite, given 24 hours before the onset of an ischaemic insult, prevents endothelial ischaemia-reperfusion injury. The same infusion, given during the last ten minutes of ischaemia, protected those with the variant ALDH2*2 enzyme, but not those with the wild-type *1 enzyme. This result was contrary to our initial hypothesis, and I present preliminary data to begin to explain this unexpected result.

Hypothesis:

We aimed to test the hypothesis that the nitrite anion, at least in part through reduction to nitric oxide by heme proteins (including myoglobin), causes beneficial vasodilatation in heart failure and protects against ischaemic injury.

1.10. Tables

Table 1.1.

	Arterial	Venous
Nitrite (NO_2^-)	540 ± 74 nM	466 ± 79 nM
Nitrate (NO_3^-)	40.7 ± 4.5 μM	41.3 ± 4.5 μM
SNO-Hb	161 ± 42 nM	142 ± 29 nM

Table 1.1: Plasma levels of NO_x and nitrosylated Hb in humans on a normal diet
Data from van Faassen *et al.*, 2009; Dejam *et al.*, 2005. Note the arterial to venous plasma nitrite gradient.

Table 1.2.

	[Nitrite] (μM)
Mouse	
Plasma	1.6 - 20
$eNOS^{-/-}$ Plasma	0.7 ± 0.1
Rat	
Plasma	0.3 - 10
Heart	0.8 ± 0.1
Liver	0.5 ± 0.1
Kidney	0.6 ± 0.1
Lung	0.5 ± 0.1
Brain	1.7 ± 0.3
Aorta	22.5 ± 9.2

Table 1.2: **Plasma and tissue nitrite levels in mice and rats**

Note the wide range of values reported for plasma nitrite. Data from Bryan *et al.*, 2004; Tracey *et al.*, 1995; Godecke *et al.*, 1998.

1.11. Figures

Figure 1.1.

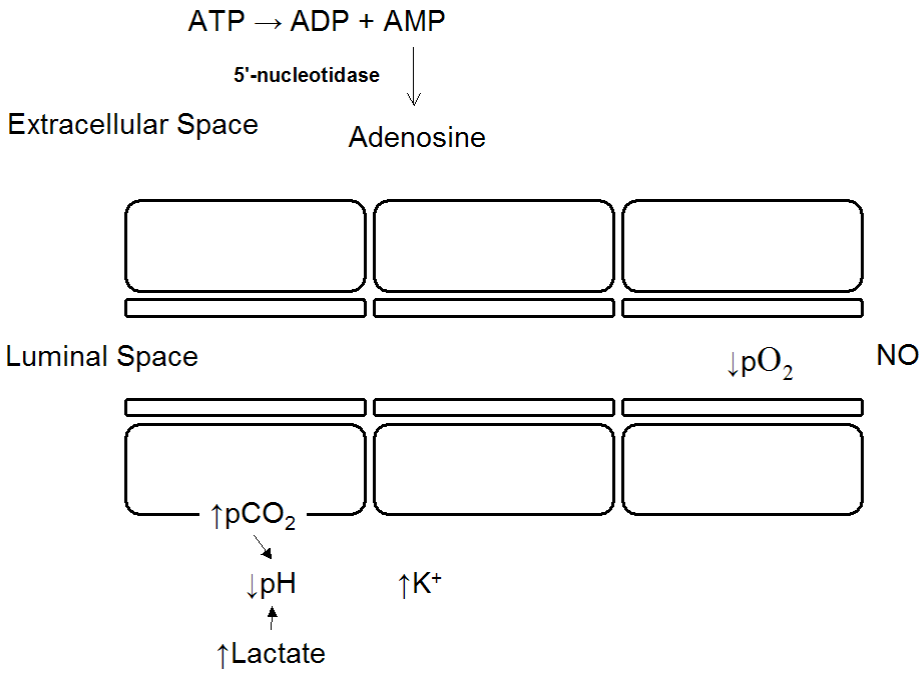


Figure 1.1: Exercise hyperaemia proceeds through several distinct and overlapping pathways

Figure 1.2.

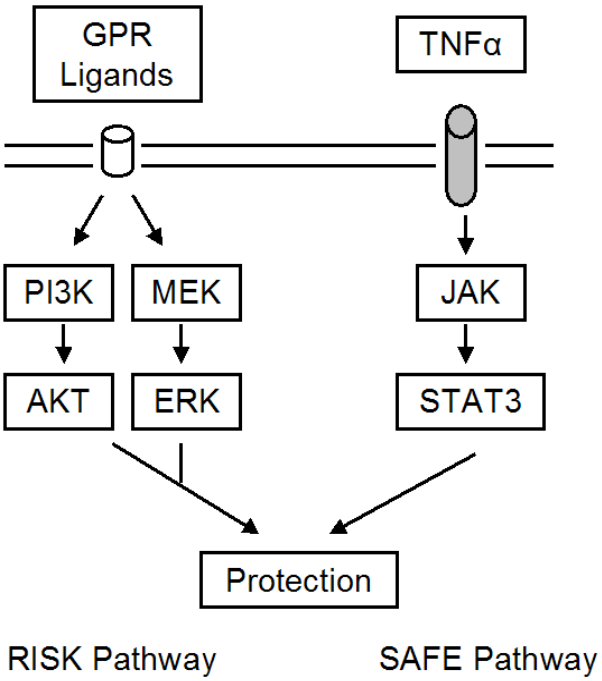


Figure 1.2: The RISK and SAFE pathways in cytoprotection

These pathways are stimulated by ischaemic preconditioning and postconditioning, and exert downstream mitochondrial effects leading to protection from IR injury.

Figure 1.3.

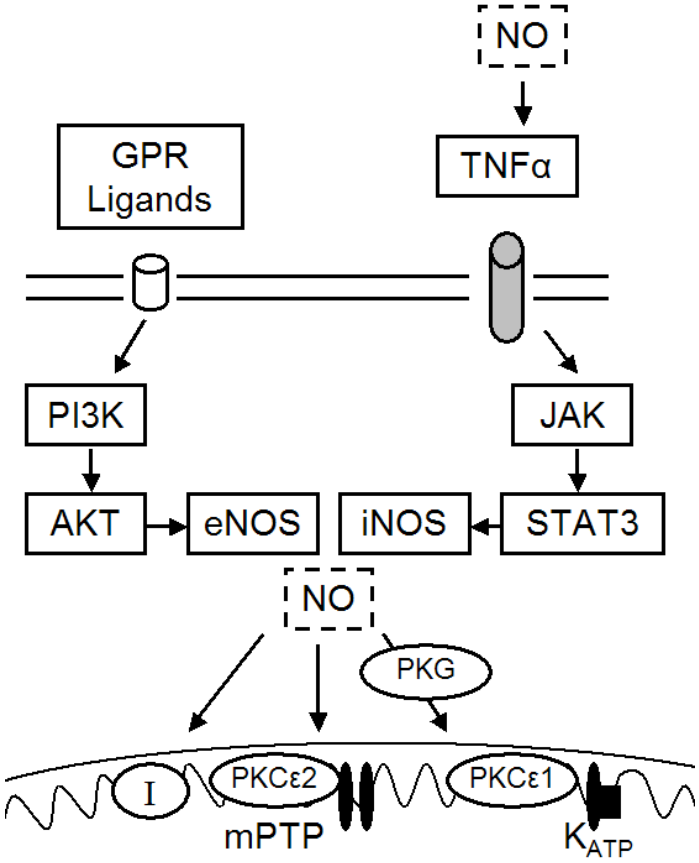


Figure 1.3: Nitric oxide interacts with established pathways of protection at multiple levels

NO can activate the SAFE pathway. NO is released by Akt (RISK pathway) and STAT3 (SAFE pathway) through activation of eNOS and induction of iNOS respectively. Activated PKCε1 phosphorylates and opens KATP channels, causing increased K+ uptake, increased pH and increased ROS. ROS stimulate both PKCε1 and PKCε2, the latter inhibiting the mPTP, leading to protection. NO can also directly stimulate PKCε2 and can inhibit complex I in a protective manner.

2. METHODS

This chapter contains generic methods for techniques used in **chapters 3 - 6**.

2.1. Mouse aortic myography

2.1.1 Generation of myoglobin-deleted mice

The myoglobin-deleted mice used in the experiments presented in **chapter 3** were developed and kindly provided by Professor J. Schrader of Heinrich-Heine-Universität, Düsseldorf (Godecke *et al.*, 1999). In brief, the vital heme-binding site encoded in myoglobin exon 2 was replaced by the neomycin-resistance gene in embryonic stem cells. Correctly targeted clones (3 of 98 screened) were identified using PCR (Primers: Myo, 59-GAGGGAGCTGGTGTCAACAG-39; neo, 59- TTGCAAAACCACACTGCTC-39) and were subsequently aggregated with 2.5-day old mouse embryos to generate chimeric mice (NMRI background). Heterozygous mice were rederived by the Biomedical Services Unit in Birmingham University and genotyped to identify homozygous animals. Where possible, littermate wild-type mice were used as controls in these experiments, but unrelated NMRI mice were also used.

2.1.2. Preparation of tissue

Mice were housed in an environment that conforms to the requirements set down in the Scientific Procedures (Animals) Act 1986. They were kept three to a cage, with bedding and environmental enrichment. Photoperiod was maintained in a 12:12 reversed light: dark cycle. Experiments were performed on male C57/BL6 mice (in-house), myo (colony), or NMRI mice (from colony or Charles Rivers) as indicated. All animals were provided with food

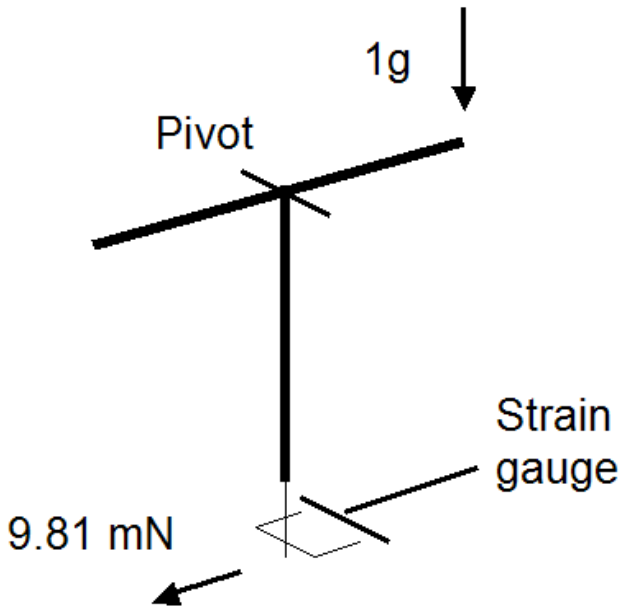
pellets (SDS RM3 diet, Lillico) and water *ad libitum*. Cage temperature was maintained at 21 ± 2ºC. Animal weights were recorded twice weekly during which cage cleaning took place. The colony was held under the authority of the Candidate's personal licence **(#23502).** All experiments were done on tissue harvested after schedule 1 killing, and so were exempt from the requirement for a project license. 6-7 week old male mice were killed humanely by cervical dislocation. Female mice were not used so as to avoid any effects of the oestrus cycle on vascular function.

The thoracic aorta from the arch to the diaphragm was removed *en bloc* and immediately immersed in ice-cold oxygenated buffer. Krebs-Hensleit buffer was used throughout, with the following composition: NaCl 120.0 mM, KCl 4.7 mM, $MgSO_4$ 1.2 mM, K_2PO_4 1.2 mM, Glucose 11.0 mM, $CaCl_2$ 2.5 mM, $NaHCO_3$ 25 mM. All chemicals were obtained from Sigma-Aldrich Co (St Louis, Missouri, USA), apart from sodium nitrite (Martindale Pharmaceuticals, Romford, UK). All adherent fat and adventitia was stripped under direct vision using a variable-magnification binocular microscope (Carl Zeiss AG, Jena, Germany), taking care not to over-stretch the aortic tissue. The tissue was held by pieces of adherent fat using two microsurgery forceps, which were then distracted. The effect is to remove fat from the aorta with as little stress on the muscular wall as possible. The endothelium was removed by bubbling vigorously with air; a 1ml syringe and 23G needle were used for this purpose.

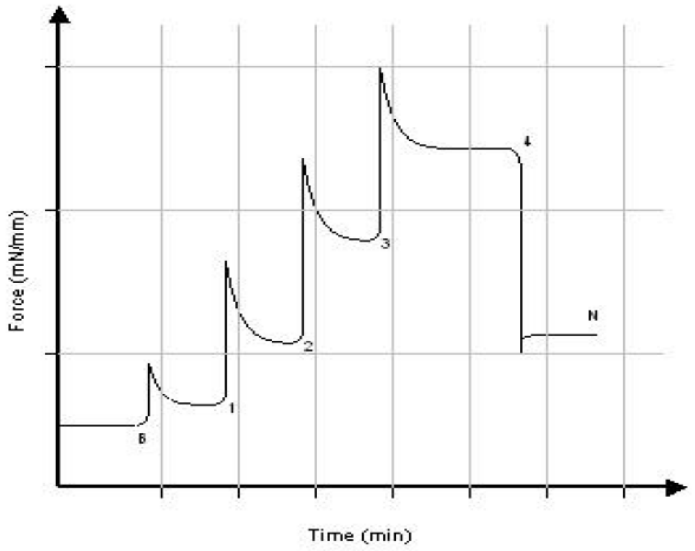
2.1.3. Myography

The aorta was cut into four 2mm rings and hung in a four-well wire myograph (Danish Myo Technology model 610M, A/S, Denmark) in Krebs-Hensleit buffer. The experimental setup is

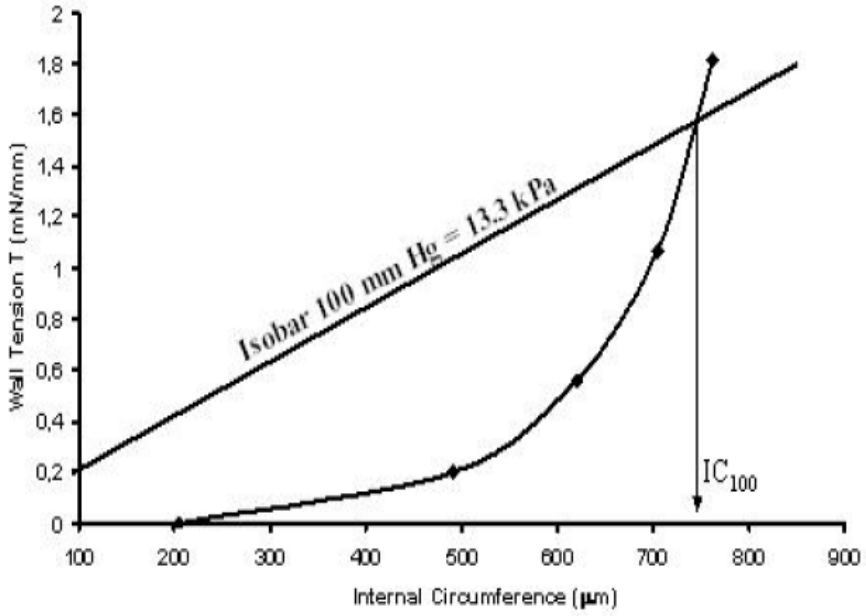
shown in **figure 2.1**. The strain gauge was calibrated using the moment force of a known weight (1g = 9.81 mN), as below:



For each experiment, the rings were attached to an isometric tension transducer and resting tension was increased stepwise (5 mN increase every 15 minutes) to a maximum of 20 mN. This resting tension is estimated to exert 90% of the wall tension, T, that would be experienced by the artery at a blood pressure of 100 mmHg, where T = 100(internal circumference/2π). This optimal resting tension was determined in a preliminary experiment: rings were mounted and the internal circumference was increased stepwise, using the built-in micrometer. The developed increase in force was measured by the force transducer and recorded as below:



When plotted, this produces an exponential curve, which intersects the straight line T = 100(internal circumference/2π), as below, giving the wall tension value for 100 mmHg:



The myograph wells were cleaned after each experiment by rinsing several times with double distilled water, incubating each chamber with 1 M HCl for 15 minutes, and again rinsing several times with water.

In each run, the segments were preconstricted with 50 mM KCl or 1 μM phenylephrine. This response was allowed to plateau at a higher tension force. Rings that failed to develop an additional 5% of the resting tension on preconstriction, were discarded as the response to vasodilators would be too small to measure accurately. The response to vasodilators was measured as the percentage reversal of this preconstriction, averaged over at least two segments. An example trace is shown in **figure 2.2**.

2.2. Polymerase chain reaction and immunoblotting

These experiments were performed by the candidate under supervision in Prof Watkins' group in Oxford University.

2.2.1 RNA extraction and PCR from aortic rings

Fresh tissue was harvested from *myo*$^{-/-}$ and NMRI mice to perform analysis of RNA and protein content. Several aortas were pooled in each group on order to gain sufficient quantities of RNA and protein. Tissue RNA was extracted from aorta and heart (as a positive control) using the Tri-reagent system (Applied Biosystems, Foster City, California, USA). The reagent contains a high-salt solution of guanidine thiocyanate and phenol. Homogenised tissue is then separated into an aqueous and organic phase by adding bromochloropropane (BCP). RNA partitions to the aqueous phase (ready to be precipitated, washed, quantified and used), DNA to the interphase (which was discarded in this experiment) and protein to the organic phase. Tri-reagent has the advantage over spin-column based methods of RNA extraction as the yield is often higher, with less contamination. Each aorta weighs approximately 2 mg, and myoglobin is a relatively rare transcript, so maximum yield was considered essential. With

this system, yield is approximately 1-1.5 µg RNA per milligram of tissue. The protocol was as follows:

- Tissue was homogenized with a blade homogeniser, in 10-20 volumes of Tri-reagent and incubated for five minutes at room temperature
- 100 µl BCP was added, incubate at room temperature for 15 minutes and centrifuge (12,000*g* for 15 minutes) to separate into aqueous and organic phases
- The aqueous phase (RNA) was separated to a new tube, the interphase (DNA) was discarded and the organic phase (Protein) was frozen for later use
- RNA was precipitated using isopropanol (500 µl per ml of initial Tri-reagent)
- Centrifuge at 4°C (12,000*g* for 8 minutes) and discard supernatant
- The pellet was washed with 75% ethanol (excess removed) and briefly air dried
- The pellet was then suspended in ultrapure water

RNA was quantified using a commercial analyser (Nanodrop spectrophotometer, Thermo Scientific, Waltham, Massachusetts, USA). The Nanodrop system requires only a very small volume (0.5 µl) to measure RNA content very accurately, and can also quantify contamination with DNA or RNA. It removes the need for a glass or plastic cuvette by creating a tube of solution between light source and detector. Effectively, the air-solution interface created by the surface tension of the drop of solution acts as the walls of the cuvette. cDNA was prepared using a reverse transcriptase kit (Promega, Fitchburg, Wisconsin, USA). 20 µl reaction volumes contained the following: 25 mM $MgCl_2$, 10X Buffer, 10 mM dNTP mixture, 25 u ribonuclease inhibitor, 15 u reverse transcriptase, 0.5 µg random primers, 1 µg total RNA, and nuclease-free water to make up volume. The mixture was incubated at room

temperature for 10 minutes, then at 42°C for 60 minutes, then at 95°C for 5 minutes and at 5°C for 5 minutes. These last two steps inactivate the reverse transcriptase enzyme and prevent it binding the cDNA product.

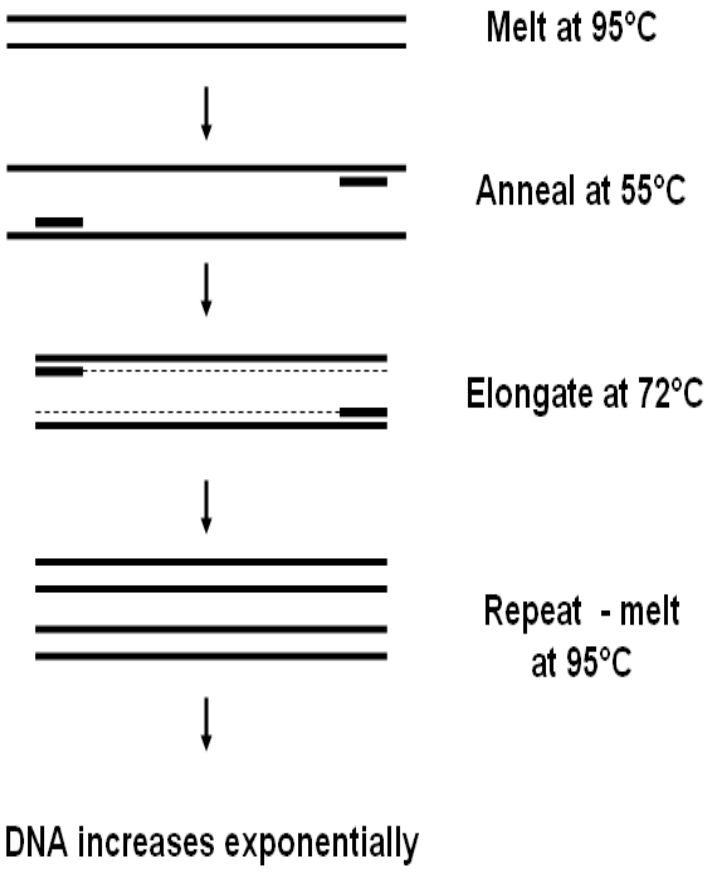
40 cycles of PCR were performed using cDNA to the following protocol: Melt at 95°C for 1 minute, anneal at 55°C for 1 minute, elongate at 72°C for 1 minute, repeat x 39. Each reaction mixture contained 500 ng template DNA, 200 nM forward and reverse primers, 10X reaction buffer with 15 mM $MgCl_2$, 200 μM dNTPs, Taq polymerase (all Promega, as before), and sterile water to make up reaction volume of 20 μL. Primer sequences and protocol are given below.

Murine primer sequences:

Myoglobin f: ATGGGGCTCAGTGATGGG r: TCAGCCCTGGAAGCCTAGCT

sGC f: CCCCTGGTCAGGTTCCTAAG r: GGAGACTCCCTTCTGCATTCT ;

GAPDH f: TCCCACTCTTCCACCTTC r: CTGTAGCCGTATTCATTGTC



2.2.2. Protein extraction and western blotting of aortic rings

The Tri-reagent system was less successful for protein extraction in our hands. We therefore used fresh tissue samples, which were snap frozen for storage and subsequently blade-homogenized in lysis buffer (25 mM Tris/HCl, pH 7.4, 1 mM EDTA, 1% SDS, 1 mM dithiothreitol and complete-mini protease inhibitor cocktail tablet (Roche Applied Science, Basel, Switzerland) and then boiled with Lamelli buffer. The general principle of western blotting is as follows: proteins are separated by mass, each sample using a separate vertical lane, and then transferred to a porous membrane. The membrane is incubated with antibodies specific to the target proteins, along with random protein (milk powder) to block non-specific interaction. The membrane with bound antibody is then incubated a second time with antibody specific to the Fc (constant) domain of the species of antibody used (in this case goat

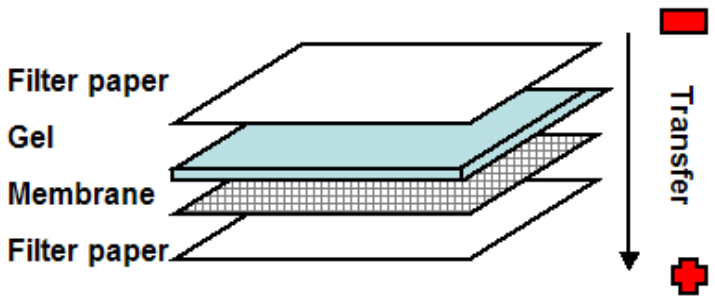
IgG specific to rabbit Fc). This antibody construction is fluorescent and can be quantified using a digital CCD in darkness.

SDS-polyacrylamide gels are used to separate proteins of different sizes. The pore size, and hence speed at which protein moves through the gel, depends upon the acrylamide concentration chosen (and so the number of cross-links formed). The size of the pore decreases as the polyacrylamide: bis ratio increases, making correct gel choice for a given polypeptide length absolutely critical. The gel consists of stacking and resolving components. Gels were mixed and poured into 1 mm plastic cassettes (NC2010, Invitrogen, Carlsbad, California, USA) with the following composition (15% gel; Tris HCL 375 μM (Stock 1 M, pH 8.8), Tetramethylethylenediamine (TEMED) 5.6 μM, Acrylamide/Bis-Acrylamide (ACR/BIS) 15 % (w/v), Ammonium persulfate (APS) 0.1 % (w/v), Sodium dodecyl sulfate 0.1 % (w/v)). Gels were topped with isopropanol and left to polymerise for 45 minutes. The addition of isopropanol produces a smooth surface at the interface between the stacking and resolving gels. Once the resolving gel was fully polymerised the isopropanol was poured off and replaced with a 5% stacking gel (Tris HCL 130 μM, TEMED 8.6 μM, ACR/BIS 5%, APS 0.1% (w/v), SDS 0.1% (w/v)), and combs for the loading lanes were inserted before the acrylamide had polymerised.

The combs were carefully removed, leaving loading lanes for protein samples. The tank is filled with electropheresis running buffer and loaded with two cassettes. 10 μL of sample was added to 5 μL of loading buffer (TRIS 60mM, Glycerol 25% (v/v), SDS 2% (v/v), β1-mercaptoethanol 14.4mM, bromophenol blue) samples were heated to 95ºC for 3 minutes to denature secondary structure and each sample was then loaded into a lane. SDS is an anionic detergent that denatures the secondary and non–disulfide–linked tertiary protein structures,

and applies a negative charge to each protein in proportion to its mass (allowing for a linear relationship between migration and mass during electrophoresis). β-mercaptoethanol is included to disrupt disulphide bonds within the sample. Glycerol increases the density of the sample, to ensure it sinks to the bottom of the well. One well in each gel was loaded with 10 µL of a pre-prepared standard that contains proteins of a known molecular weight. 100V is applied across the stacking gel, this is increased to 150V in the resolving gel, and continues until the bromophenol dye reaches the bottom of the gel.

Gel-separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (pore size 0.4 µm, Millipore, Fisher), pre-soaked in 100% methanol for 15 seconds. PVDF membrane, scotch brite pads and filter paper were soaked in transfer buffer (TRIS 25 mM, Glycine 192 mM, methanol 20% (v/v)). The stacking gel was removed from the resolving gel and discarded. The gel was then added to a sandwich constructed as below. From the bottom up this consisted of: negative cathode, fibre pad, filter paper, resolving gel, and PVDF membrane. The sandwich is completed with a second layer of filter paper, another fibre pad (or more if required) and the positive anode. Each layer was rolled to remove air bubbles, which would otherwise prevent efficient protein transfer. The tank was placed in a refrigerated room and run at a voltage of 25V for 12 hours.

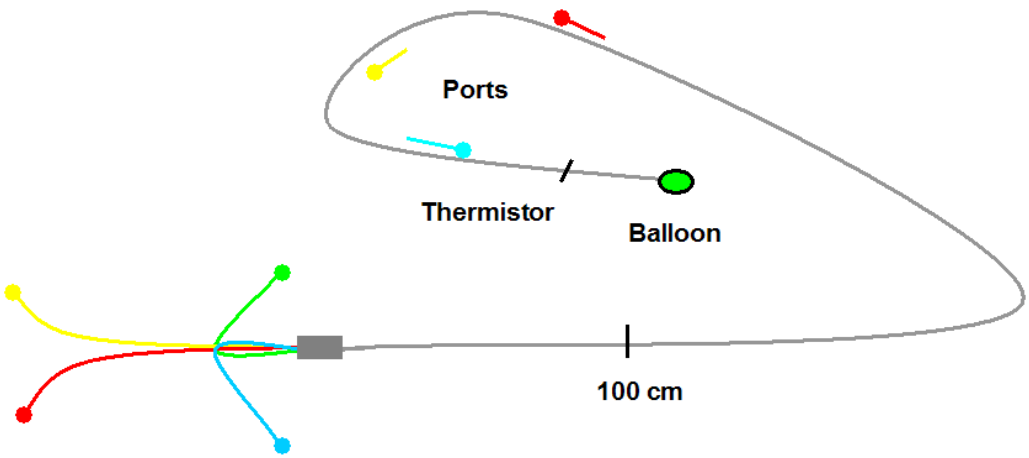


The PVDF membrane saturated with 5% fat-free milk in 0.1% Tween-20 (Sigma-Aldrich, St Louis, Missouri, USA) in phosphate buffered saline (PBS) (blocking solution) and hybridised with α-myoglobin rabbit polyclonal antibody (Santa Cruz, (FL-154) sc-25607) at 1:1000 dilution overnight at 4°C. The membranes were washed four times (10 minutes each at RT) with 0.1% Tween-20 in PBS and then incubated with goat anti-rabbit IgG (HRP) (Abcam ab2721-1) at 1:4000 dilution for 1 hour at room temperature, washed four times and finally visualized with the ECL advance immunoblotting detection system (GE Healthcare, Little Chalfont, Bucks, UK).

2.3. Measurement of intra-cardiac pressures

The pulmonary artery catheter (PAC) has been in clinical practice since its invention in 1970 by Swan and Ganz of the Cedars-Sinai Medical Centre (Swan *et al.*, 1970). It can be used to measure the pulmonary capillary wedge pressure (a surrogate of left ventricular end diastolic pressure or left atrial "filling pressure"), pressures in the right ventricle and atrium, and cardiac output by the thermodilution method. The same basic design has been widely used for over thirty years. Its popularity has waned somewhat in recent times due to large-scale studies failing to show an improvement in mortality or length of ITU stay (Sandham *et al.*, 2003); importantly, in these studies there was no increase in mortality associated with the use of a PAC (Harvey *et al.*, 2005). They are still commonly used in investigation, intensive care and high dependency contexts: over 1 million PACs per year are inserted in clinical practice in the USA alone. The risks of pulmonary artery catheterisation are markedly reduced by operator experience and good patient selection. The most severe complication is pulmonary artery rupture, which is uncommon (<1%) but more likely in high-risk patients (those with severe pulmonary hypertension or receiving anticoagulants).

The distal lumen is at the tip of the PA catheter, with a 1.5 cubic cm balloon positioned just proximal to this. Proximal to the balloon is a thermistor, which may be used to measure temperature changes over time for the thermodilution method of calculation of cardiac output. The catheter has two additional lumens at 19 cm and 30 cm proximal to the tip, residing approximately within the right ventricle and right atrium (or superior vena cava). The basic design is shown below:



A sheath is inserted into the right internal jugular vein under sterile conditions with lignocaine local anaesthetic (1%, 10mg/ml, maximum 3mg/kg), using the Seldinger technique. The Seldinger technique describes the use of a guide-wire, which is initially inserted into the jugular vein down the lumen of a wide-bore seeker needle. “Flashback” of venous blood confirms correct positioning of the seeker needle though care must be taken to ensure the carotid artery is not cannulated. The sheath is then threaded over the guide-wire, which is then withdrawn, and position is confirmed with fluoroscopy. The PA catheter is inserted into the sheath, with the natural curve pointing to the patient’s left shoulder, after being attached to monitoring equipment to measure pressure waveforms. A non-compressible tube with a

column of heparinised saline leads from the PA catheter tip to a fluid-filled pressure transducer. This transmits pressure measured at the tip and must be calibrated to ambient air pressure by opening the distal end to the air and zeroing the measuring equipment. The catheter is advanced to the right atrium using the pressure trace as a guide, or fluoroscopy depending upon operator preference. Pressure waveform is recorded in the right atrium (**figure 2.3a**), and the catheter is advanced through the tricuspid valve. The pressure waveform changes to reflect the new position.

The catheter is turned one-quarter clockwise to aim cranio-postero-medially, and the balloon is inflated to "float" the catheter tip into the right ventricular outflow tract, where it is followed with fluoroscopy into the pulmonary artery at about 40 cm of insertion. The pressure waveform can be measured in the PA (**figure 2.3b**) and, by inflating the balloon, the pulmonary capillary wedge pressure (PCWP) is achieved (**figure 2.3c**). The balloon prevents anterograde flow from proximal to the tip. The pressure at the tip therefore equalises across a continuous column of blood to the left atrium, thereby approximating to left atrial pressure. Example pressure waveform traces are given in **figure 2.3**. Each value used was an average of at least three cardiac cycles and was measured by an expert technician.

Resistance to flow is given by the change in pressure across a vascular bed, divided by the rate of flow. The rate of flow through both the pulmonary and systemic circulations must be equal and equivalent to the cardiac output. Pulmonary vascular resistance is given by the calculation:

$$\text{PVR (wood units)} = \frac{\text{PA (mmHg)} - \text{PCWP (mmHg)}}{\text{Cardiac Output (L/min)}}$$

The normal range is 1.25 – 2.5 wood units ($mmHgminL^{-1}$). Systemic vascular resistance is given by the calculation:

$$\text{SVR (wood units)} = \frac{\text{MABP (mmHg)} - \text{RA (mmHg)}}{\text{Cardiac Output (L/min)}}$$

The normal range is 11 - 15 wood units. The trans-pulmonary gradient (PA - PCWP) is an important prognostic factor in transplantation. If this value is > 12 mmHg, and not reversed by vasodilators, the patient is generally considered ineligible for transplantation. This is because the recipient's right heart will be accustomed and have adapted to high pressures, for example with a degree of muscular hypertrophy, but the donor heart will not. Exposing a naïve heart to such pulmonary pressures is likely to result in catastrophic failure of the right ventricle and death of the patient.

2.4. Measurement of forearm blood flow

Venous occlusion plethysmography (VOP) is a long-established technique and is considered the gold-standard against which other techniques are assessed (Roddie and Wallace, 1979). VOP has been used to study forearm blood flow responses for more than 90 years, though the method has been much simplified with the advent of mecury-in-silastic strain gauges, which are used in place of the original, unwieldy water jackets (Benjamin *et al.*, 1995)). The same technique can be used to measure baseline endothelial function between different groups of patients, or, as in the present work, the endothelial response to ischaemia-reperfusion injury. It has become a commonly used technique to assess preconditioning agents in man.

The alternative is flow-mediated dilatation (FMD). The technique relies on the physiological phenomenon whereby shear stress in the brachial artery (induced by a sudden increase in flow) leads to dilatation of the vessel. The dilatation relies on intact endothelium. Briefly, a cuff is inflated on the forearm (or upper arm in an alternative technique) and released while the brachial artery is visualised with ultrasound. The diameter before and after the flow stimulus can be measured later and a ratio determined. This ratio is proportional to endothelial function and so can be used to measure the effect of forearm ischaemia. FMD provides a single ratio whereas VOP measures endothelial function at three levels of ACh infusion. The spatial resolution of ultrasound also reduces the sensitivity of the technique. Due to these limitations, despite the clear advantage of not requiring cannulation of the brachial artery, we chose to use VOP in these experiments.

The patient sits upright in bed, with both arms outstretched at the level of the heart (**figure 2.4**). The hands are excluded from the circulation during measurement periods by inflation of a wrist cuff to 200 mmHg or 20 mmHg above systolic blood pressure (whichever is the greater). Blood flow in the hands is predominantly to the skin, with a great number of arterio-venous shunts as part of temperature regulation. These features contribute to a different pharmacology overall in the hands compared to the forearm (Robinson, 1984; Collier and Robinson, 1974). Forearm blood flow (FBF) is measured by occluding venous outflow (with a rapid-inflation cuff at 40 mmHg), and recording the rate of increase of forearm circumference. Mercury-in-silastic strain gauges measure forearm circumference dynamically and are attached to a recording computer (**figure 2.5**). This rate is constant initially, and is proportional to FBF, but will change as the forearm deep veins fill. Consequently, only the linear initial slope is used (**figure 2.6**). Rate of change of circumference is measured in both

arms, and recorded as a ratio of infused to control arm (FBF ratio). The values used in each experiment were an average of 3-5 cuff inflations.

Absolute forearm blood flow can be estimated using the forearm volume, but, as this is in itself an estimated value, FBF ratio may be considered more reliable. Some groups express measures as a calculated forearm resistance, though as the mathematics of flow assume a uniform ("Newtonian") fluid flowing at a constant rate through a non-distensible conduit, this is, again, an approximation when applied to pulsatile blood in arteries (Benjamin *et al.*, 1995). Vascular resistance is estimated using the formula:

$$\text{Resistance} = \frac{\text{Perfusion Pressure}}{\text{Blood Flow}}$$

Hence, it can be seen that, while vasodilating drugs will affect the tone of resistance vessels, flow may also be affected by arterial blood pressure. Every effort is made to keep blood pressure and sympathetic arousal constant during each study. The participant is made comfortable at the beginning of every study, and the experiment proceeds in a quiet, dedicated vascular laboratory at a constant temperature of 21-24ºC. A large change in perfusion pressure would affect the measurement of FBF ratio in an unpredictable way, but the effect of the inevitable small changes are compensated for by normalising measurements in the infused arm to those in the control arm (Greenfield and Patterson, 1954). Absolute responses to vasodilating drugs vary through the day, with a circadian rhythm (Panza *et al.*, 1991), but the ratio between arms should be unaffected.

2.5. Figures

Figure 2.1.

Figure 2.1: Photograph of the experimental setup used in Chapter 3

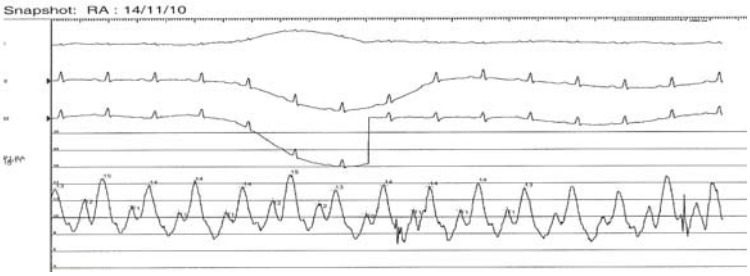
Two myographs are connected to a computer, which records real-time tension traces from each aortic ring.

Figure 2.2.

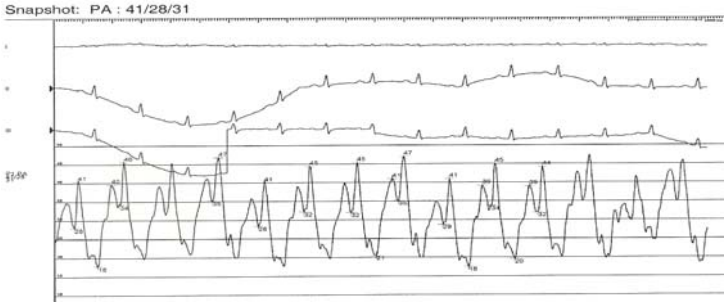
Figure 2.2: Example aortic myography trace

Recorded using aortic myography, as utilized in the work in **Chapter 3**. Shown above is the effect of preconstriction, then that of progressively increasing levels of nitrite.

Figure 2.3. a)



b)



c)

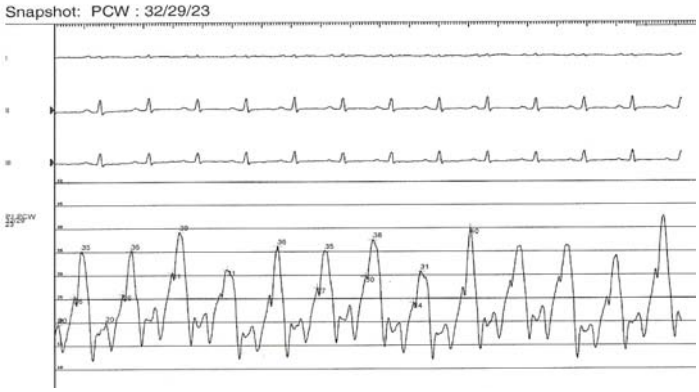


Figure 2.3: Example waveform recordings from the pressure probe of a Swann-Ganz catheter

a) Mean right atrial pressure of 10 mmHg **b)** mean pulmonary arterial pressure of 31 mmHg **c)** mean pulmonary capillary wedge pressure of 23 mmHg; from a high PCWP patient in the study of intravenous nitrite in advanced heart failure (**Chapter 4**). Three leads of electrocardiogram are shown above.

Figure 2.4.

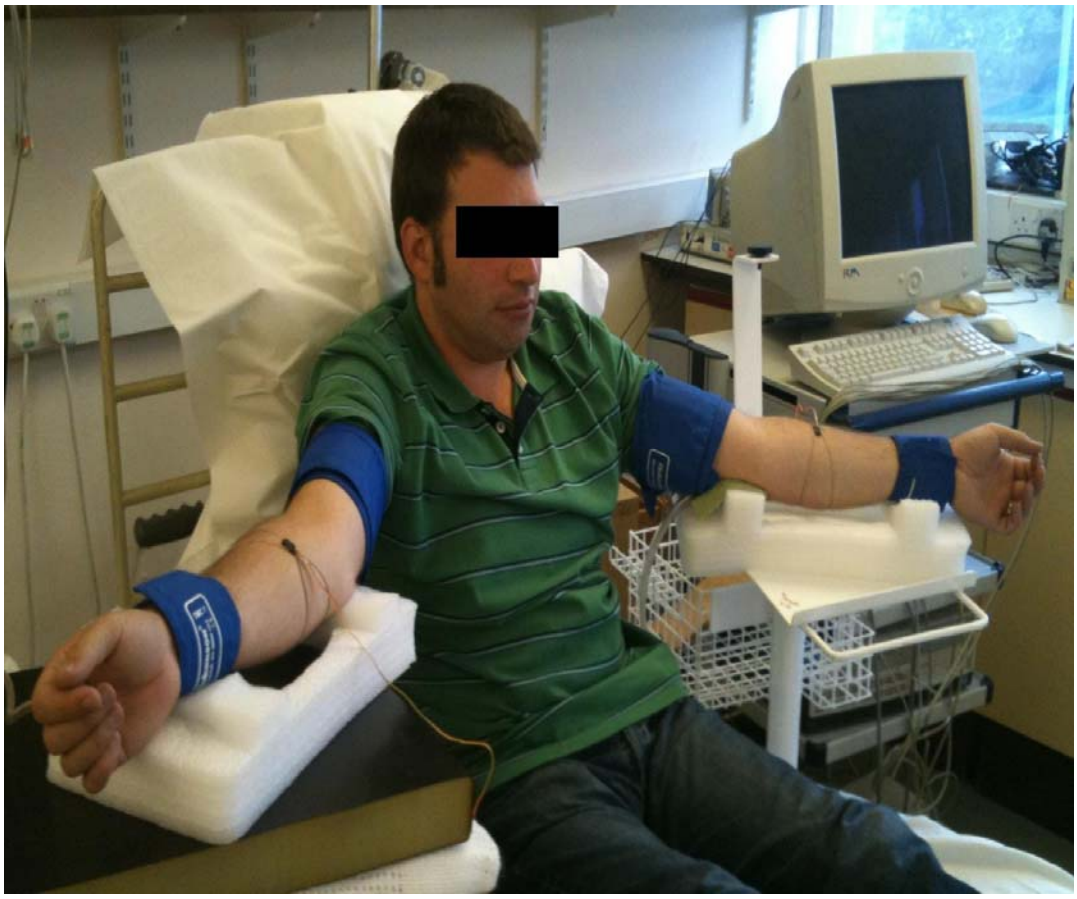


Figure 2.4: Photograph of the experimental setup used in chapter 6

The participant sits upright in bed with the arms outstretched at the level of the heart. Rapid-inflation blood pressure cuffs are around both wrists and both upper arms, with mercury-in-silastic strain gauges around both forearms.

Figure 2.5.

a)

b)

c)

d)

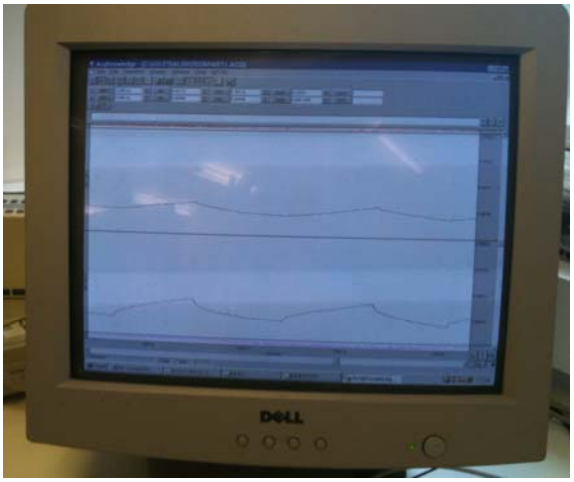
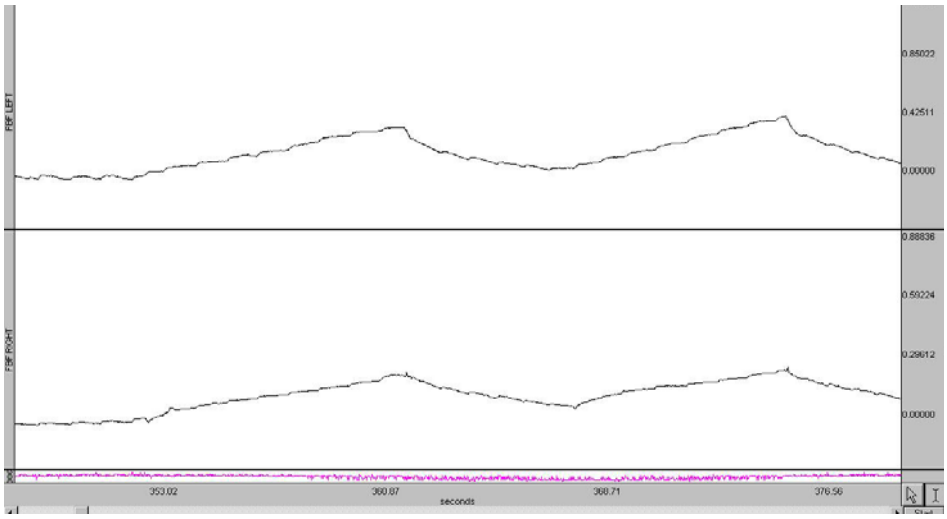


Figure 2.5: Further items of equipment from chapter 6

a) Vasodilating drugs were infused intra-arterially **b)** a rapid-inflation pump was used to occlude venous blood flow for each measurement of FBF **c)** strain gauges were attached to plethysmographs with computer recording **(d).**

Figure 2.6.

a)



b)

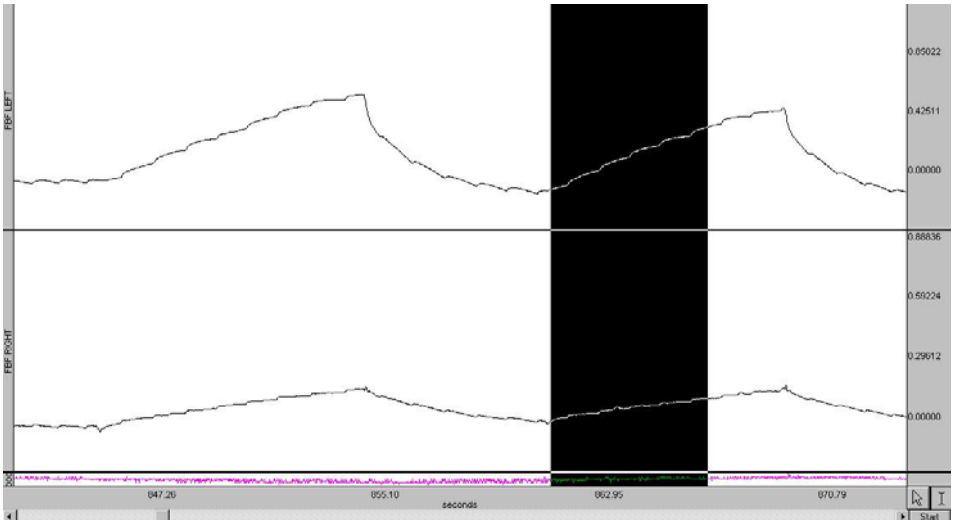


Figure 2.6: Example forearm plethysmography traces

Taken from control and infused arms during measurement of forearm blood flow (FBF); from a participant in the cytoprotection study (**Chapter 6**). The y-axis represents time, with voltage in mV up the x-axis **a)** FBF-ratio is measured at baseline and **b)** after infusion of vasodilators (acetylcholine or GTN). The initial slope in each arm was measured using the highlighted gradient tool (black box in **2.6b**). Each pair of slopes represents one measurement of FBF-ratio, which is given by the automatic measurement software; an average of at least 3 measurements was used for each data point.

3. MYOGLOBIN IS INVOLVED IN VASODILATATION BY NITRITE

3.1. 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 (Wittenberg and Wittenberg, 1989). The tertiary structure of the globular protein was derived using x-ray crystallography in the 1950s (Kendrew, 1961). 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 (Schenkman *et al.*, 1997) 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 (Garry *et al.*, 1998; Godecke *et al.*, 1999).

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 (Wittenberg, 1970). Experimental evidence for this phenomenon is far from unambiguous, with model calculations both supporting and refuting myoglobin's contribution to cellular O_2 flux (Gardner and Schubert, 1997).

The chemistry of nitrite activation *in vivo* remains uncertain (Li *et al.* 2008). Several (non-exclusive) bioconvertors have been identified including: pH-dependent enzyme-independent nitrite reduction (Benjamin *et al.* 1994); microsomal cytochrome P-450 (Kozlov, Staniek, & Nohl 1999; Li *et al.* 2006); xanthine oxidase (Li *et al.*, 2008); aldehyde oxidase (ALDH2) (Li *et al.*, 2008); neuroglobin (Petersen *et al.*, 2008); hemoglobin (Basu *et al.*, 2007; Crawford *et al.*, 2006; Grubina *et al.*, 2007; Huang *et al.*, 2005a; Isbell *et al.*, 2007); and myoglobin (Shiva *et al.*, 2007a). 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 (Alzawahra *et al.*, 2008). We devised experiments to test the hypothesis that vascular myoglobin, like cardiac myoglobin (Rassaf *et al.*, 2007), can act as a bioconvertor for exogenous nitrite.

In our department we have observed a prolonged vasodilating action of nitrite, which persisted for 45 minutes after the infusion of nitrite was stopped (unpublished data, Maher *et al.*, 2007). 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 (Alzawahra *et al.*, 2008; Li *et al.*, 2008). 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.

3.1.1 Hypothesis

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

3.2. Methods

3.2.1 Animals

Male C57bl6, 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. The 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).

3.2.2. Mouse aortic ring myography

Aortic myography was done according to standard procedures (detailed description in **Methods 2.1.**) Pretension force was 20 mN. Preconstriction was produced using 50 mM KCl except where stated. Myography wells were open to the air and bubbled with 95% N_2 - 5% CO_2, 95% O_2 - 5% CO_2, or 20% CO - 75% N_2 - 5% CO_2. Buffer was allowed to reach steady state, which using this equipment produced an oxygen tension of 82 ± 2 and 83 ± 2 mmHg

(~11 kPa) for the low oxygen gases (with and without CO respectively), and 158 ± 2 mmHg (~22 kPa) for the high oxygen mix as measured by blood gas analyser (Instrumentation Laboratory, Bedford, MA). 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) (van Faassen *et al.*, 2009). This level was stable over time. The same equipment was used to monitor pH, which was stable at 7.4 with both gas mixtures.

3.2.3. Generation of replication-defective adenoviruses

This virus was made on behalf of the candidate by Prof Watkins' group in the University of Oxford. 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 linearised 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 linearised 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.

3.2.4. Transfection and incubation of *myo*$^{-/-}$ aortic tissue

Thoracic aortas from myo2/2 mice were obtained as before. These rings were then exposed to either adenovirus expressing myoglobin (AdMYO) or control virus (AdCtl) (at a concentration of 1028 PFU/mL) diluted in Opti-MEM (Sigma), supplemented with 11 mM glucose, for 120 min under continuous bubbling with carbogen gas. Rings were washed with Opti-MEM to remove adherent virus particles, and then incubated at 37°C in Krebs-Henseleit buffer for 270 min. This incubation time was determined in preliminary experiments to be sufficient for recapitulation of the low levels of myoglobin seen in wild-type aorta. Response to nitrite both in the presence and absence of CO was assessed as before. Nitrite was used at a final concentration of 9 mM (~EC90) in order to focus solely on the effect of myoglobin (i.e. after saturating other pathways).

3.2.5. 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 (described in detail in **Methods 2.2**.). The RNA analysis and western blotting were done by the candidate in Prof Watkins's laboratory in the University of Oxford. Presented are the best examples of four blots and two gels.

3.2.6. 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 (Bryan *et al.*, 2005). 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).

3.3. Results

3.3.1. Protein and RNA analysis in the wild-type mouse aorta

Myoglobin is present at the mRNA (**figure 3.1a**) and protein (**figure 3.1b**) level.

3.3.2. The response of C57bl6 aortic rings to 9mM nitrite at high and low oxygen levels

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 3.2**).

3.3.3. The response of wild-type and knockout aortic rings to nitrite, in the presence and absence of carbon monoxide

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) (**figure 3.4a**). 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 were compared to wild-type controls. Overall response to nitrite was markedly reduced in *myo*$^{-/-}$ rings (**figure 3.4b**) ($n = 5$, $p < 0.001$ by two-way ANOVA). In contrast to wild-type aortae, CO did not inhibit vasorelaxation in *myo*$^{-/-}$ rings (**figure 3.4b**).

3.3.4. The response of transfected knockout rings to nitrite, in the presence and absence of 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 3.5**). 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%). 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 3.1b**).

3.3.5. The response of wild-type and knockout aortic rings to sodium nitroprusside, in the presence and absence of carbon monoxide

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 not significantly changed by the addition of CO (37.7 ± 3.5% to 43.7 ± 4.2%, p = NS 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 3.6**).

3.3.6. The effect of xanthine oxidase and aldehyde dehydrogenase inhibition in knockout aortic rings

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 repeated measures 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 repeated measures ANOVA, n = 4 for each group) (**figure 3.7**). 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.

3.3.7. The effect of the NO-scavenger carboxy-PTIO and the sGC inhibitor ODQ on cGMP accumulation and vasorelaxation

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 3.8a**). Moreover, relaxation of aortic rings by nitrite was completely abolished by preincubation with 20 μM oxygenated haemoglobin (oxyHb) or by the addition of 10 μM ODQ to the organ bath (**figure 3.8b**).

3.3.8. The effect of prolonged exposure to higher levels of nitrite in wild-type and knockout aortic rings

Aortic ring studies were also performed to investigate the initially recorded phenomenon of prolonged vasodilatation, first seen in human studies (Maher *et al*, unpublished data, as above). Ten-minute exposure to 18 mM nitrite induced a characteristic persistent vasorelaxation in aortic rings a) Typical traces for nitrite-induced relaxation in NMRI (broken lines) and *myo*$^{-/-}$ (solid lines) 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 3.9**).

3.4. Discussion

Myoglobin was present in the normal mouse aorta at the RNA and protein level, a result that has been previously disputed. This was therefore essential to confirm before embarking on an investigation of myoglobin's possible effects in the vasculature. Expression of myoglobin is however extremely low. The semi-quantitative tests performed cannot estimate the level of expression with any greater accuracy, but this may explain why the protein has been reported to be absent in the past. Experiments in low and high oxygen conditions show that nitrite-induced vascular relaxation is potentiated by hypoxia. This confirms previous observations (Dalsgaard et al., 2007), and again was important to confirm in these particular experimental

conditions. This result was fundamental in driving our hypothesis that myoglobin is an important biocionvertor as, containing a heme-group, it is exquisitely placed to sense oxygen-levels in the cell.

Deficiency (by knockout) or inhibition of myoglobin (by CO) in mouse aorta appeared to diminish vasorelaxation to nitrite. Confirming this observation with both chronic and acute inhibiton of myoglobin is important. In the first instance, it would appear likely that the absence of myoglobin from conception would induce compensatory changes elsewhere. In the second instance, CO will additionally inhibit any molecule containing a heme-group, and so it is only by comparing the response to CO in the presence and absence of myoglobin that the true role of myoglobin can be isolated. Finally, restitution of myoglobin to knockout aorta recapitulates the wild-type response to carbon monoxide. This supports the supposition that it truly is myoglobin, rather than a compensatory change in knockout animals, that underlies the difference between them and the wild-type mouse. Expression of virally-inserted myoglobin in the smooth muscle was very low, though it was similar to that seen in wild-type aorta, and functionally the tissue reacted in a similar manner.

Relaxation to sodium nitroprusside was increased by carbon monoxide in both wild-type and $myo^{-/-}$ rings, implying that the net effect of heme-molecules as a group is scavenging of NO. The difference in magnitude of the effect of CO is consistent with greater scavenging of NO by myoglobin in the wild-type mouse. This is an interesting result, and implies that myoglobin may be acting as both reductase and scavenger at all times, with the relative contribution of each activity determined by the level of oxygen.

Xanthine oxidase and aldehyde dehydrogenase also exhibit nitrite reductase activity, as shown in the myoglobin-knockout mouse, as inhibitors of these enzymes reduced residual nitrite effects. As with ODQ, compounds believed to be specific inhibitors may later be found to be relatively prolific in their effects, and further testing (perhaps demonstrating correlation with directly measured enzyme activity) would be needed to determine this result with certainty. Additionally, the knockout mouse may have undergone changes in other nitrite reductase pathways that are as yet unknown, so this result should be applied to the wild-type animal with caution.

The effects of nitrite were abolished by a G-protein inhibitor (with the caveats described above) and by NO-scavengers. This implies that the effects of nitrite are mediated by the stimulation of soluble guanylyl cyclase by free NO. This was not confirmed with direct measurement of NO

High levels of nitrite induced prolonged relaxation of mouse aorta, which was also seen in human studies form our department (Maher *et al.,* unpublished data, as before). Prolonged vasodilatation to nitrite appears to be partially myoglobin-dependent, in that the time required to revert to resting tension was significantly longer in wild-type aortas. This may be due to increased nitrite reductase activity in the initial nitrite exposure, but myoglobin could also act as a store of NO-activity in the form of a nitrosothiol, releasing small amounts of NO throughout this period. Other molecules can also become nitrosylated, which would explain the (shorter) latent period in *myo*$^{-/-}$ aortae.

In summary therefore, 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.

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 (Erzurum et al., 2007). Levels of nitrite recorded in aortic tissue are strikingly higher than in plasma (>20 μM) (**Table 1.1**). Oxygen tension in resting muscle is approximately 24 mmHg, and working

muscle exhibits even greater hypoxia (8 mmHg) (Bylund-Fellenius *et al.*, 1981), 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 (**section 1.5**).

3.4.1. 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 sub cellular 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*.

3.5. Conclusions

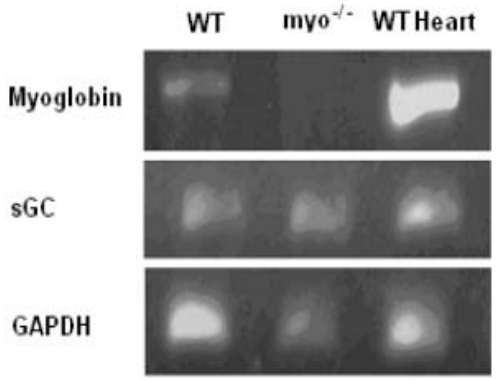
Low pharmacological concentrations of nitrite relax aortic rings at near physiological levels of oxygen. Nitrite may be present in much higher quantities in certain tissues. Vasorelaxation

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 3.10**. Prolonged latency is a hallmark of nitrite-induced vascular relaxation; it is also partially myoglobin-dependent. Overall, these properties of nitrite make it an attractive pharmacological candidate, as investigated in later chapters. Nitrite can have acute and chronic effects on blood flow, responsive to oxygen levels and pH (though this was not investigated in the present study). It is tempting to speculate that the nitrite store, reduced by myoglobin in the vessel wall, would be sufficient to underlie a primitive system of hypoxic or exercise-induced vasodilatation.

3.6. Figures

Figure 3.1.

a)



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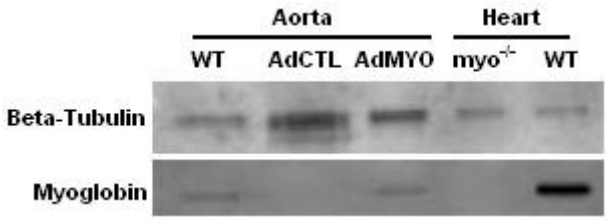


Figure 3.1: RNA and protein analysis in mouse aorta and heart

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 3.2.

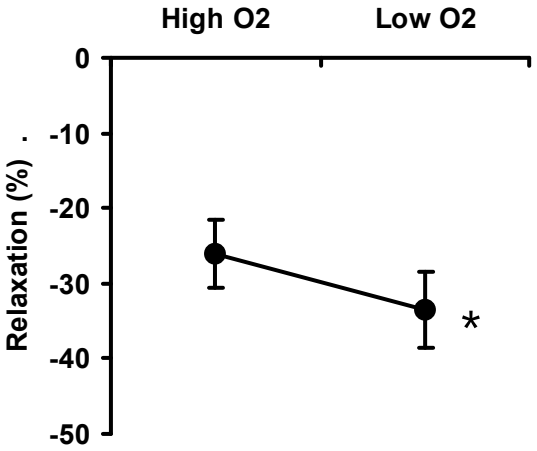
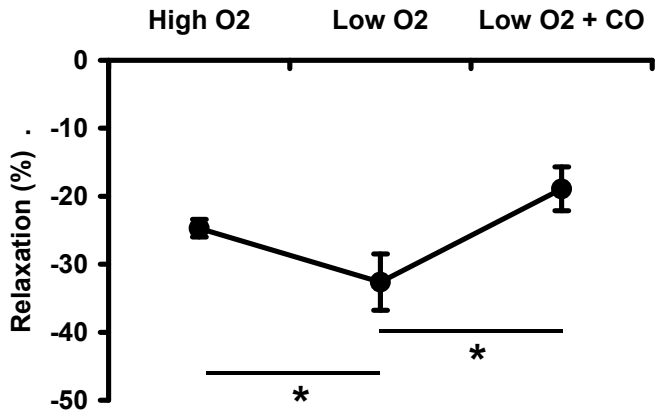


Figure 3.2: The effect of oxygen level in wild-type (C57bl6) mouse aorta

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 3.3.

a)



b)

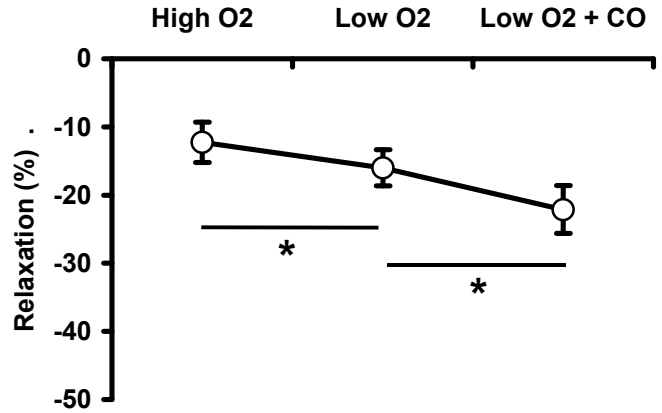


Figure 3.3: The effect of oxygen and carbon monoxide in wild-type (NMRI) and myoglobin knockout aortas

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; *$p < 0.01$.

Figure 3.4.

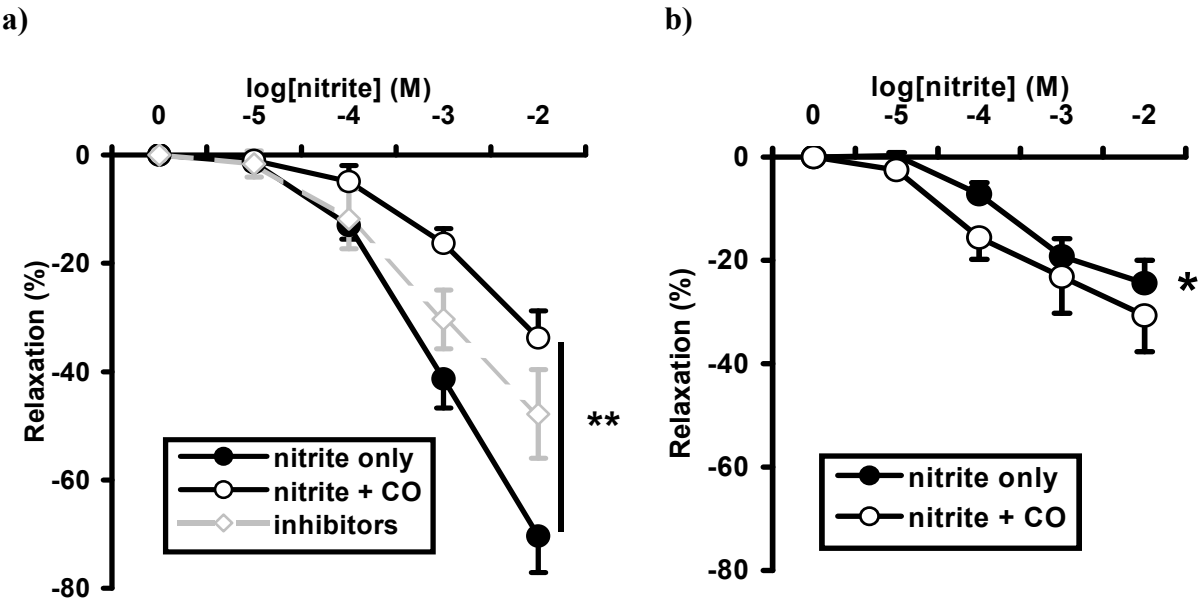


Figure 3.4: The effect of carbon monoxide on the concentration response curve in wild-type (NMRI) and myoglobin knockout aortas

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). *$p < 0.01$ compared to wild-type, **$p < 0.001$ compared to baseline

Figure 3.5.

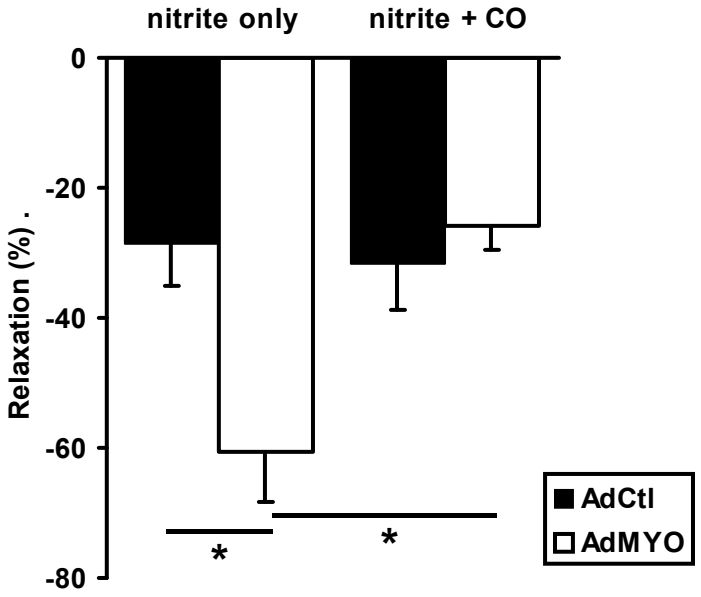


Figure 3.5: The effect of myoglobin-containing and blank virus exposure on myoglobin-knockout aortas

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 3.6.

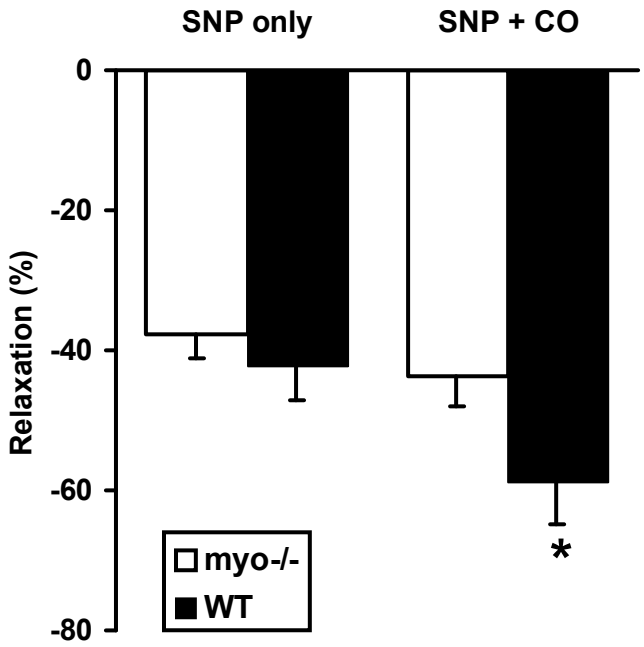


Figure 3.6: The effect of the NO-donor SNP on wild-type and myoglobin knockout aortas

Response to the NO-donor SNP (0.1 μM) 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 3.7.

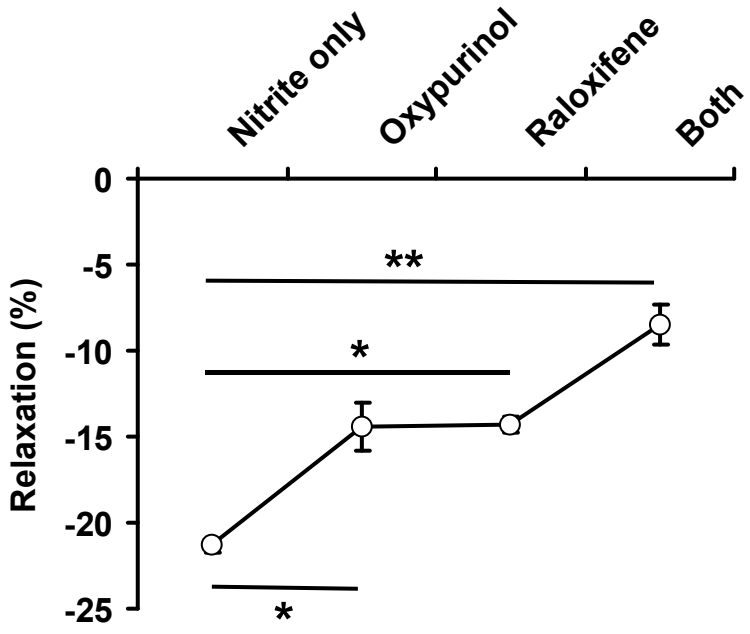
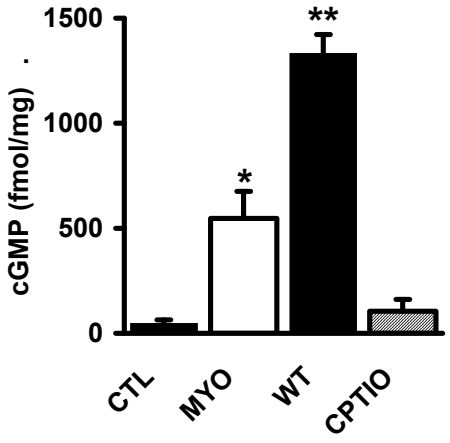


Figure 3.7: The effect of other candidate nitrite reductase inhibitors on myoglobin-knockout aortas

The majority of the remainder of (9 mM) nitrite-dependent vasodilatation 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 3.8.

a)



b)

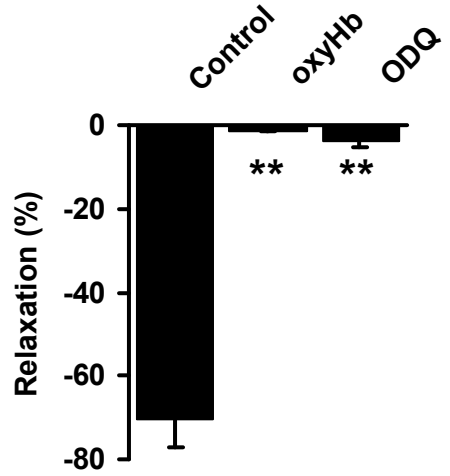
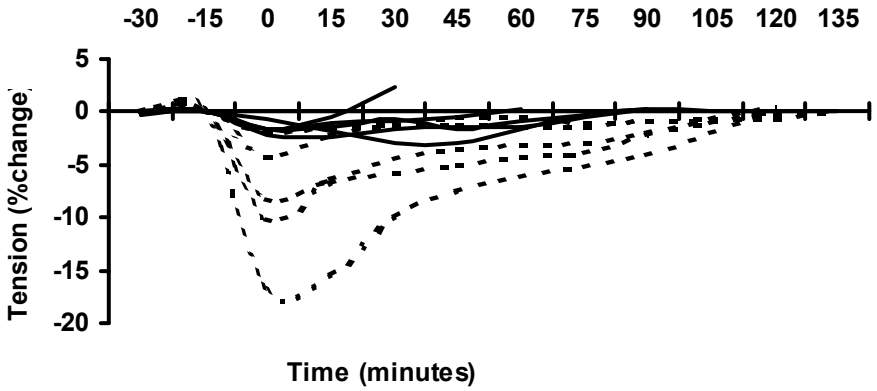


Figure 3.8: The effect of ODQ and NO-scavengers on wild-type and myoglobin-knockout aortas

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 3.9.

a)



b)

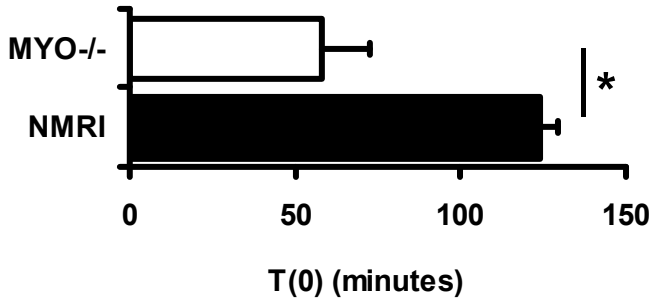


Figure 3.9: The effect of 10 minute exposure to 20 mM nitrite on wild-type and myoglobin-knockout aortas

a) Incubation of aortic tissue with 20 mM sodium nitrite for 10 minutes produces a prolonged vasorelaxation, which persists after removal of sodium nitrite (broken lines: WT, solid lines: *myo*$^{-/-}$, %change from baseline measured every 15 minutes, smoothed curve of intermittent data points (Excel)) **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 3.10.

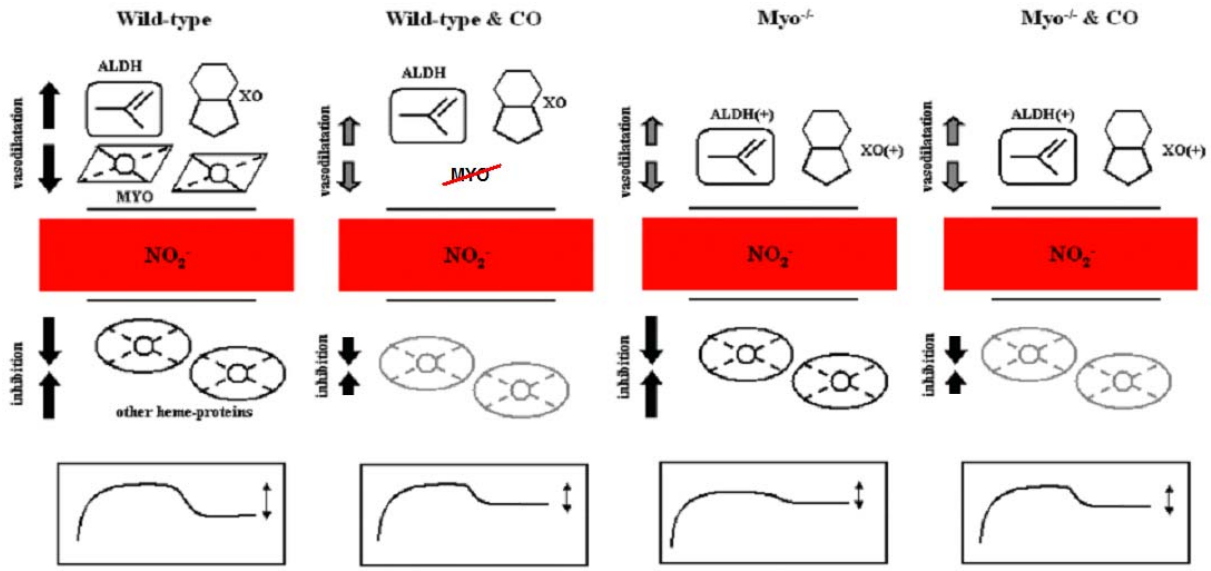


Figure 3.10: A model of myoglobin's role in vasorelaxation induced by nitrite

NO is liberated by various species, including myoglobin, xanthine oxidase and ALDH (above). Other heme-containing proteins have a net scavenger role (below). A typical myography response to nitrite is shown (bottom). Exposure to CO inhibits myoglobin (second left), reducing magnitude of vasorelaxation in wild-type aortas. Myoglobin-deficient aortas (second right) may have partially compensatory upregulated nitrite reductases. When exposed to CO gas (far right), relief of scavenging (and hence increased magnitude of vasorelaxation) is the only effect.

4. INVASIVE MEASUREMENTS OF THE ACUTE HAEMODYNAMIC EFFECTS OF INTRAVENOUS NITRITE IN HEART FAILURE

4.1. Introduction

Heart failure is a syndrome in which there is insufficient cardiac output to meet the metabolic needs of the body, initially on exercise but in severe cases at rest. It is a common cause of admission to hospital, intensive care, and a common cause of death. Intensive therapy often includes inotropic agents which improve cardiac function at the expense of increased myocardial lactic acidosis, and which do not improve mortality. The only established options available to patients with end-stage heart failure are ventricular assist devices and transplantation, both of which are resource intensive and have limited availability in the NHS. This is due to expense but also to scarce organ supply for transplantation. Both interventions are far from universally available worldwide and new therapies are desperately needed. Overall, around 1.8% of Europeans and 2% of Americans meet a diagnosis of chronic heart failure (Davies *et al.*, 2001; Lloyd-Jones, 2001). Heart failure is a dynamic disease, with most patients suffering episodes of acute decompensation, interspersed with periods of a variable duration where their symptoms are stable.

Therapy for acute decompensated heart failure (ADHF), which often manifests as pulmonary oedema, includes morphine for anxiolytic and pulmonary vasodilating effects, and intravenous diuretics and organic nitrates which both act as vasodilators (Davies *et al.*, 2001;

Lloyd-Jones, 2001; Cohn, 1985; Cohn *et al.*, 1986). Organic nitrates, such as glyceryl trinitrate (GTN), dilate both veins and arteries, and therefore relieve both preload (i.e. ventricular filling) and afterload (i.e. resistance to flow). Though a small reduction in afterload is often beneficial in ADHF, too great a drop in arterial blood pressure will reduce perfusion of vital organs, especially the kidneys and the brain. Thus, development of severe hypotension is often a reason to reduce or discontinue nitrate therapy.

Venodilation decreases right atrial pressure, and hence right ventricular filling. Patients with dilated heart failure often exhibit a phenomenon known as diastolic ventricular interaction (DVI), wherein increased right ventricular diastolic volume and pressure, contained within the relatively non-distensible pericardium, impedes left ventricular filling, and thereby reduces stroke volume (Atherton *et al.*, 1997b). When right ventricular filling is reduced, the septum moves across towards the right side, reducing the impediment to left ventricular filling and thereby increasing cardiac output. This can be measured directly by nuclear cardiology techniques, or estimated by a change in the trans-septal gradient, which gives the net left ventricular filling pressure (left ventricular end-diastolic pressure – right ventricular end-diastolic pressure) (Atherton *et al.*, 1997a).

As discussed in detail in **section 1.5.**, nitrite has recently been shown to be an active vasodilator in humans, with the unique property of hypoxia-targeted vasodilatation (Maher *et al.*, 2008). The balance of evidence seems to suggest that nitrite, through O_2-sensing nitrite reductases such as myoglobin (as discussed in **chapter 3**), releases more NO in areas of low oxygen tension, such as the relatively hypoxic venous circulation. Recently, a study in rats showed that intravenous sodium nitrite significantly decreased pulmonary vascular resistance

and increased cardiac output (Casey *et al.*, 2009). Harnessing this unique property, we hypothesised that nitrite would provide beneficial venodilation in ADHF, without the drop in systemic vascular resistance and arterial blood pressure that characterises therapy with organic nitrates.

ADHF is a medical emergency, which poses significant challenges to the successful, ethical conduct of research. As Birmingham is a transplant centre, we took advantage of the opportunity to study nitrite infusions in those with advanced but stable heart failure. We prospectively split these patients into two groups; those predicted to exhibit significant DVI (PCWP ≥ 15 mmHg), and those predicted not to exhibit DVI (PCWP < 15 mmHg). Measurements of intra-cardiac pressures were taken using a right heart catheter that was inserted for clinical reasons as part of the transplant assessment protocol, after clinical studies had been completed. Measurement of intra-cardiac pressures using a right heart (Swan-Ganz) catheter is discussed in more detail in **Methods 2.3.**

4.1.1. Hypothesis

Short systemic infusions of sodium nitrite will have beneficial haemodynamic effects in advanced heart failure with significant DVI (pulmonary capillary wedge pressure ≥ 15 mmHg), but not in heart failure without DVI (PCWP < 15 mmHg).

4.2. Methods

18 consecutive patients who were undergoing right heart catheterisation as part of the heart transplant assessment protocol and who gave informed consent were recruited to the study (baseline characteristics are summarised below). The Local Research Ethics Committee

approved the study protocol (**REC #08/H1207/67**). Methaemoglobinaemia may trigger haemolysis in patients with G6PD deficiency. This is a very common condition worldwide but rare in the Caucasian population (Cappellini and Fiorelli, 2008), in which redox stress causes haemolysis, which may be severe. It is an X-linked condition, so is extremely rare in females. For safety, we excluded males with a known history of G6PD deficiency and those with a high risk of having this condition.

4.2.1. Inclusion Criteria

1. Aged 18 years or over
2. Admission to hospital for pulmonary artery catheterisation, under the transplant assessment protocol

4.2.2. Exclusion Criteria

1. Pre-existing inotrope therapy
2. Recent ST-elevation myocardial infarction or thrombolysis
3. G6PD Deficiency, or high risk of G6PD deficiency
4. Women of child-bearing potential or nursing mothers

4.2.3. Right heart catheterisation and protocol

A Swan-Ganz catheter was inserted via a sheath into the right internal jugular vein under local anaesthesia with positioning confirmed by fluoroscopy and pressure waveform (a detailed description is given in **Methods 2.4.**). Right atrial and ventricular pressures were measured at the beginning of the study and again at the end of the protocol only, to avoid multiple insertions of the catheter into the pulmonary artery. Consequently, RA pressure was only

available at baseline and peak nitrite dose, which also limited measurements derived from RA pressure. After baseline measurements, subjects received three five-minute infusions of sodium nitrite at escalating doses (1, 10 and 50 μg/kg/min) into the sheath (right internal jugular vein). At the end of each infusion, pulmonary artery (PA) pressure, pulmonary capillary wedge pressure (PCWP) and CO were measured. Blood pressure, electrocardiogram and oxygen saturation were monitored continuously. The protocol is summarised in **figure 4.1.**

4.2.4. Measurement of cardiac output by the Fick principle

Cardiac output as measured by the Fick principle was used in preference to thermodilution. Indicator dilution techniques become unreliable in the presence of significant tricuspid regurgitation. This is because ventricular blood freely mixes with blood in the right atrium, diluting the indicator substance (in the case of thermodilution, cold dextrose solution), and giving an inappropriately low reading of cardiac output. In indicator dilution techniques all blood is assumed to move forwards through the circulation. In dilated cardiomyopathy, the fibro-cartilage skeleton of the heart expands, forcing the atrio-ventricular valve leaflets apart. This is known as functional mitral/tricuspid regurgitation.

The Fick principle states that the amount of a substance taken up by an organ (or the whole body) per unit of time is equal to the blood flow multiplied by the difference in concentration of that substance between arterial and mixed venous blood, so for O_2:

$$\dot{V}O_2 = \text{Cardiac Output (L/min)} \times (artO_2 - mvO_2)$$

$$\therefore \text{Cardiac Output (L/min)} = \frac{\dot{V}O_2}{(artO_2 - mvO_2)}$$

Whole body uptake of O_2 is estimated from body surface area:

$$\dot{V}O_2 = \sqrt{\frac{\text{height(cm) x weight (kg)}}{3600}}$$

Though there are several factors that are estimated in this equation, they remain constant within a single study, therefore this technique is an exceptionally reliable way to measure change in cardiac output. In one case thermodilution was used due to mechanical failure of the blood gas analyser. Additionally, pressure data was discarded from one patient due to failure of the catheter after baseline measures. Mixed venous blood gas was analysed using a clinical analyser (Radiometer ABL800, Denmark).

4.2.5. Power calculation

This was planned as a 16 patients per group pilot study, with participants acting as their own control (baseline measures compared to three different doses of nitrite infusion), which increases the statistical power. This number was chosen to give a representative sample, based on our experience of physiological studies with nitrite and the typical patient groups undergoing transplant assessment. We had no metric to estimate the effect size of nitrite in this specific group (i.e. PCWP $\geq$ 15mmHg) but were guided by the calculation that 10 patients would give 80% power to detect a 20% rise in cardiac output with a standard deviation of 0.9 L/min, at a p-value of < 0.05.

4.2.6. Statistical Analysis

Changes in trans–septal pressure gradient (i.e. LVEDP – RVEDP) during nitrite infusion were estimated as change in (PCWP – RA pressure) (Morris-Thurgood & Frenneaux 2000). We prospectively chose to divide patients into those with PCWP ≥ 15mmHg (n = 13) vs those with PCWP < 15 mmHg (n = 5). This was based on our observation in a previous study that PCWP ≥ 15mmHg was reasonably predictive of a 'paradoxical' increase in LV end-diastolic volume during application of lower body negative pressure (implying marked DVI) (Atherton *et al.* 1997a). Blood pressure and heart rate changes were assessed by linear mixed models analysis (SPSS v14), which is a variant of ANOVA that can correct for single missing data points. All other comparisons were by paired two-tailed t-test unless otherwise specified. Data are presented as mean ± SEM; a p-value of less than 0.05 was taken to indicate statistical significance.

4.3. Results

4.3.1 Patient Characteristics

Baseline patient characteristics are given in **Table 4.1.** Two patients complained of orthopnoea on presentation.

4.3.2. The effect of systemic nitrite infusion on pulmonary vascular resistance and systemic vascular resistance

Overall, PVR fell from 2.55 ± 0.50 to 1.83 ± 0.30 wood units (p = 0.03). In the subgroup with PCWP ≥ 15 mmHg (n = 13), PVR fell from 2.96 ± 0.61 to 2.04 ± 0.41 wood units (p = 0.03)

at the highest dose of nitrite (50 μg/kg/min). In the subgroup with PCWP < 15mmHg (n = 5), PVR was unchanged (from 1.19 ± 0.27 to 1.15 ± 0.12 wood units, p = NS) (**figure 4.2a**). By comparison, SVR fell from 17.4 ± 1.3 to 16.0 ± 1.1 wood units (p = 0.04) at the highest dose of nitrite. In the high PCWP subgroup, SVR fell from 18.3 ± 1.6 to 16.4 ± 1.3 wood units (p = 0.02). In the low PCWP subgroup, SVR was unchanged (from 14.3 ± 0.7 to 14.8 ± 0.8 wood units, p = NS) **(figure 4.2b**).

4.3.3. The effect of systemic nitrite infusion right atrial pressure and pulmonary capillary wedge pressure

Overall, RA pressure fell from 11.2 ± 2.0 to 6.6 ± 1.7 mmHg compared to baseline (p = 0.003). In the high PCWP subgroup, RA pressure fell from 13.7 ± 2.1 to 7.9 ± 1.8 mmHg (p = 0.002). In the low PCWP subgroup, RA pressure was unchanged (from 3.0 ± 1.7 to 2.0 ± 2.7 mmHg, p = NS) (**figure 4.3a**). Overall, PCWP fell from 20.7 ± 2.3 to 18.4 ± 2.5 mmHg compared to baseline (p = 0.06). In the high PCWP group, PCWP fell from 25.2 ± 1.6 to 22.4 ± 2.1 mmHg (p = 0.07). In the low PCWP subgroup PVR was unchanged (from 6.3 ± 0.3 to 5.3 ± 1.3 mmHg, n = 5, p = NS) (**figure 4.3b**).

4.3.4. The effect of systemic nitrite infusion on blood pressure and heart rate

Heart rate was unchanged at any dose of nitrite (**figure 4.3c**). There was a 3 mmHg fall in MAP, p = 0.005 (**figure 4.3d**). This was predominantly in the high PCWP group (4.0 mmHg vs. 0.4 mmHg in the low PCWP group).

4.3.5. The effect of systemic nitrite infusion on cardiac output and stroke volume

There was a significantly higher resting stroke volume (SV) in the low PCWP group ($p = 0.03$ by two tail unpaired *t-test*). SV was unchanged in the entire group (from 51.2 ± 5.1 to 54.4 ± 4.2 ml, p = NS). SV increased from 44.9 ± 5.0 ml to 51.9 ± 4.9 ml in the high PCWP group ($p = 0.008$) and was unchanged in the low PCWP group (from 67.8 ± 8.7 ml to 60.8 ± 6.6 ml, p = NS) (**figure 4.4a**)). In all patients, CO rose from 3.79 ± 0.25 L/min to 4.11 ± 0.23 L/min ($p = 0.02$). In the high PCWP subgroup, CO rose from 3.49 ± 0.25 L/min to 3.98 ± 0.25 L/min ($p = 0.009$), but was unchanged in the low PCWP group (4.58 ± 0.46 L/min to 4.60 ± 0.38 L/min, p = NS) (**figure 4.4b**).

4.3.6. The effect of systemic nitrite infusion on estimated trans-septal gradient

Trans-septal gradient increased (11.5 ± 1.9 to 14.5 ± 1.4 mmHg, $n = 18$, $p = 0.01$) in the high PCWP group overall (**figure 4.4c**), and was unchanged in the low PCWP group.

4.3.7. The effect of systemic nitrite infusion on methaemoglobinaemia

Methaemoglobinaemia rose from 0.3 ± 0.1% to 1.0 ± 0.5% at the highest dose of nitrite ($n = 18$, $p = 0.0001$) (**figure 4.5**).

4.4. Discussion

Nitrite infusion decreased pulmonary vascular resistance to a greater degree than systemic vascular resistance. This fits with the observation that hypoxia potentiates vasorelaxation to nitrite in the mouse aorta, as the pulmonary artery is relatively hypoxic compared to the systemic circulation. Clearly, this is not the only difference between the two circulations, as

evidenced by the phenomenon of hypoxic vasoconstriction in the pulmonary circulation as opposed to vasodilatation in the systemic. This result may appear to discount a significant role of nitrite in physiological flow-regulation in the pulmonary circulation, but it is important that the doses of nitrite used in this study are far higher than physiological levels. Overall, this observation confirms our hypothesis, and may support future uses of nitrite in pathology such as pulmonary hypertension.

The change in cardiac output correlated well with change in estimated trans-septal gradient. This cannot, of course, prove causation, but it lends support to the hypothesis that relief of DVI is involved. Estimated TS gradient rose overall and fell in only two patients (highlighted). CO also only fell in these two (**figure 4.4c**), which suggests that initial PCWP ≥ 15mmHg is predictive of significant DVI. One of these two patients had a previous opration for congenital aortic stenosis, and may have had a pericardiectomy. It proved impossible to access the original operation notes to confirm this. If so, they would therefore not be subject to diastolic-ventricular interaction, as the phenomenon requires the constriction of an intact and relatively non-distendable pericardium. The trans-septal gradient was unchanged in the low PCWP subgroup. Overall, change in trans-septal gradient positively correlated with change in both SV (n = 18, R = 0.67, p = 0.004, figure 4.3d), and CO (n = 18, R = 0.79, p < 0.001 figure 4.3e), suggesting that relief of DVI plays a major role in the increase in SV and CO.

Cardiac output rose by approximately 8%. The change was largest in two patients that spontaneously reported orthopnoea: 60% and 40%. Symptoms were not formally assessed in the group as a whole during this study. In the 13 patients with a baseline PCWP ≥ 15 mmHg

the rise in CO was greater, at 15%, which implies the mechanism may be relief of diastolic ventricular interaction, and this hypothesis is supported by the correlation of cardiac output with the trans-mural gradient. Intravenous nitrite had a limited effect on pulmonary capillary wedge pressure itself which, combined with the profound drop in right atrial pressure, means that the trans-mural pressure is reduced, and consequently true left ventricular filling pressure is increased. This dramatic change with nitrite therapy underlies the increase in stroke volume and hence cardiac output.

Overall, intravenous nitrite infusion in humans with advanced heart failure is safe and efficacious. Methaemoglobinaemia rose within safe levels during these infusions. Nitrite infusion appears to increase venous capacitance and to be a potent pulmonary vasodilator, while the effect on the systemic vascular resistance is much less profound. This may be due to consumption of nitrite and nitrosylated species across the pulmonary vascular bed, or reduced liberation of NO in conditions of higher oxygen tension (Maher *et al.*, 2008).

Glyceryl trinitrate is an established therapy for acute decompensated heart failure. There is evidence that patients with heart failure are resistant to organic nitrates, with at least an order of magnitude lower potency (Katz *et al.*, 1992). Tolerance develops quickly, probably within 12 hours (Elkayam, 1996). A drop in systolic blood pressure commonly prompts reduction in dose or cessation of GTN therapy. The average drop was 19% in one study of GTN in ADHF (Young *et al.*, 2002), compared to 6% in the present study; such a drop may be accompanied by reflex tachycardia. Side effects are common with GTN therapy: 60-68% of patients experience one or more adverse effect; this is most commonly headache (in 20-29%) or

nausea (in 6%) (Loh *et al.*, 2001; Young *et al.*, 2002). None of the patients in the present study complained of any adverse effects.

The low PCWP group had a significantly higher baseline stroke volume (67.8 ml vs. 44.9 ml, $p = 0.03$) and were also significantly older (57 vs. 42 years old). It may well be that a high PCWP predicts a "sicker" group of patients, with greater systolic dysfunction. Diastolic dysfunction may be a greater problem in the older low PCWP group, and indeed in the general population with heart failure, who tend to be elderly. Clearly, it is also not yet possible to extrapolate these results to patients with acute decompensated heart failure, though the favourable haemodynamic result along with high tolerability of nitrite infusion in the short term provides good pilot data for the study of such a therapeutic intervention.

Sodium nitrite is known to cause methaemoglobinaemia, an effect that has been long utilised in the treatment of cyanide poisoning. The maximum level measured in any participant was 2.9% (average 1.02 ± 0.13%) in this study, which is similar to the previous findings of Gladwin and colleagues (Dejam *et al.*, 2007). This would need to be monitored over a longer time period to confirm the safety of systemic nitrite at vasodilating doses. These infusions were very short, at only five minutes per dose. Tolerance may occur within the time-frame of hours to days needed for successful treatment of ADHF, though the persistent vasodilatation seen in **Chapter 3** suggests that the haemodynamic effects may in fact increase over a longer infusion.

4.4.1. Limitations

Ideally, we would have compared the haemodynamic effects of nitrite with both a placebo group and a group treated with GTN. It proved challenging to recruit sufficient numbers of patients with a low (< 15 mmHg) PCWP. It appears that such patients represent a very small group on the transplant waiting list, which in view of their self-selection for severe disease should not, in retrospect, be surprising. We considered attempting to recruit patients from the general heart failure clinic, but it was felt that inserting a right heart catheter for non-clinical reasons in these patients would substantially change the risk-benefit ratio. We felt that this step may be unethical as the pilot data in 5 patients removes equipoise on the question of haemodynamic effects of nitrite in this group.

4.5. Conclusions

By invasively measuring haemodynamic effects, we have shown that the selective venodilator and pulmonary vasodilator actions of nitrite extend to patients with severe heart failure. Nitrite infusion is safe, and cardiac output can be markedly increased in this group, seemingly through relief of diastolic ventricular interaction. There is a negligible effect on blood pressure and therefore an absence of reflex tachycardia, in contrast to GTN therapy. These characteristics make nitrite an attractive candidate therapy for acute decompensated heart failure, though longer studies are needed to measure the stability of the haemodynamic response, and the development of metHb represents a potential barrier to its use. These will be investigated in the next chapter.

4.6. Tables

Table 4.1.

	Low PCWP Mean ± SEM	High PCWP Mean ± SEM	Sig.
M/F	4/1	8/5	
Age (years)	57 ± 3	42 ± 4	*
Heart Rate (bpm)	71 ± 8	82 ± 5	
MAP (mmHg)	78 ± 6	74 ± 2	
PCWP	6 ± 0	25 ± 2	**
Diagnosis:			
Dilated Cardiomyopathy	2 (40%)	9 (69%)	
Ischaemic Heart Disease	2 (40%)	1 (8%)	
Primary Valve Disease	0 (0%)	2 (16%)	
Cardiac Amyloid	1 (20%)	1 (8%)	
Medications:			
ACE Inhibitor/AgIIRB	4/1 (100%)	11/2 (100%)	
B-Blocker	3 (60%)	9 (69%)	
Digoxin	0 (0%)	3 (23%)	
Loop Diuretic	4 (80%)	13 (100%)	
Spironolactone/Eplerenone	4/0 (80%)	7/1 (62%)	
Warfarin/Enoxaparin	1/0 (20%)	4/2 (46%)	

Table 4.1: Clinical characteristics of patients with advanced heart failure

Baseline patient characteristics ($n = 18$). *$p < 0.05$, **$p < 0.001$

4.7. Figures

Figure 4.1.

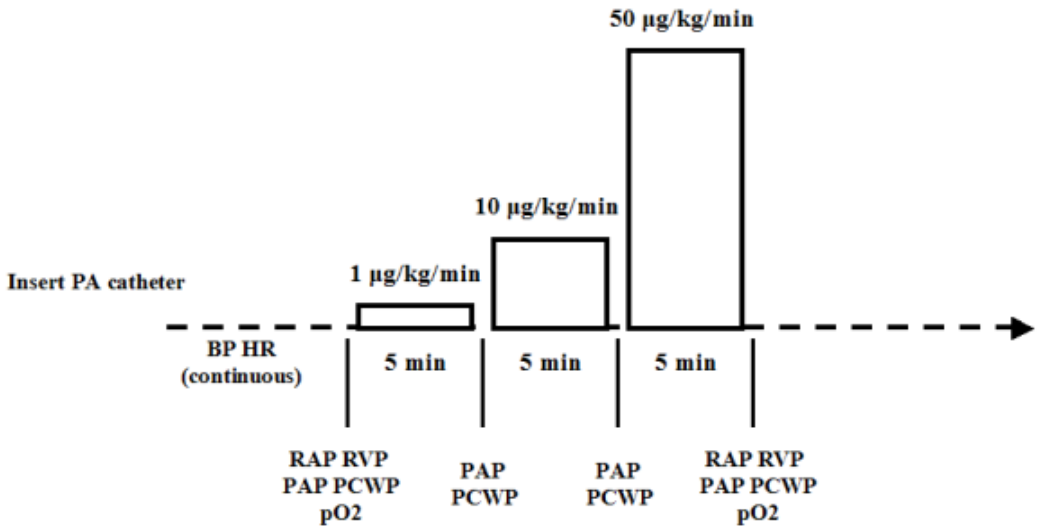


Figure 4.1: Protocol for acute nitrite infusions

A diagram summarising the protocol for the acute nitrite infusions performed in **chapter 4**. Measurements performed at each time point are listed below the horizontal axis.

Figure 4.2.

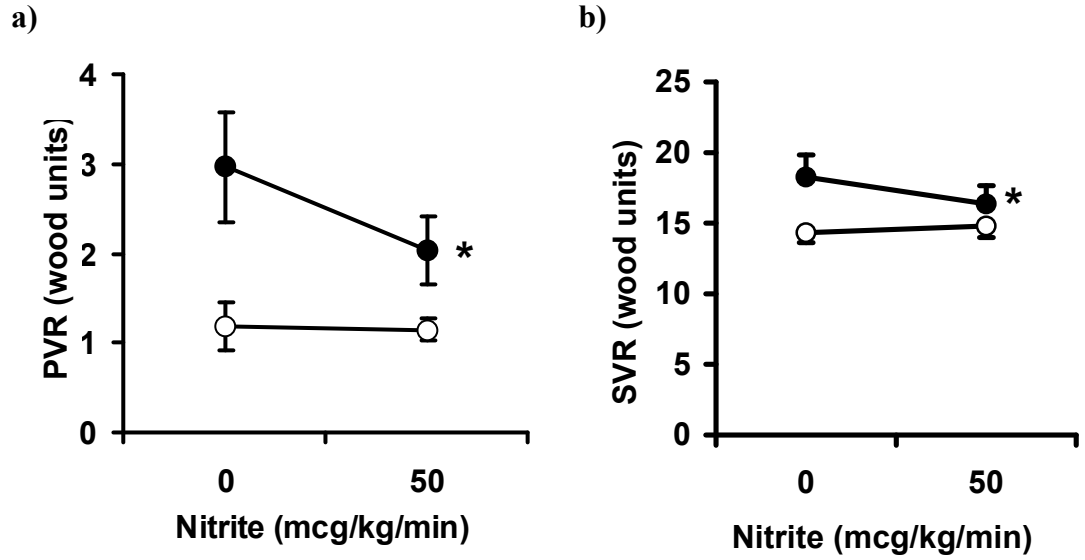


Figure 4.2: The effect of systemic nitrite on pulmonary and systemic vascular resistance
a) Pulmonary vascular resistance (PVR) fell from 2.96 ± 0.61 to 2.04 ± 0.38 wood units in the high PCWP subgroup, (filled circles, n = 13, p = 0.03). PVR fell from 1.19 ± 0.27 to 1.15 ± 0.12 wood units in the low PCWP group, (hollow circles n = 5, p = NS). **b)** Systemic vascular resistance dropped from 18.3 ± 1.5 to 16.4 ± 1.3 wood units in the high PCWP group, (filled circles, n = 13, p = 0.02). SVR increased from 14.3 ± 0.7 to 14.8 ± 0.8 wood units in the low PCWP group, (hollow circles, n = 5, p = NS; * $p < 0.05$ compared to baseline

Figure 4.3.

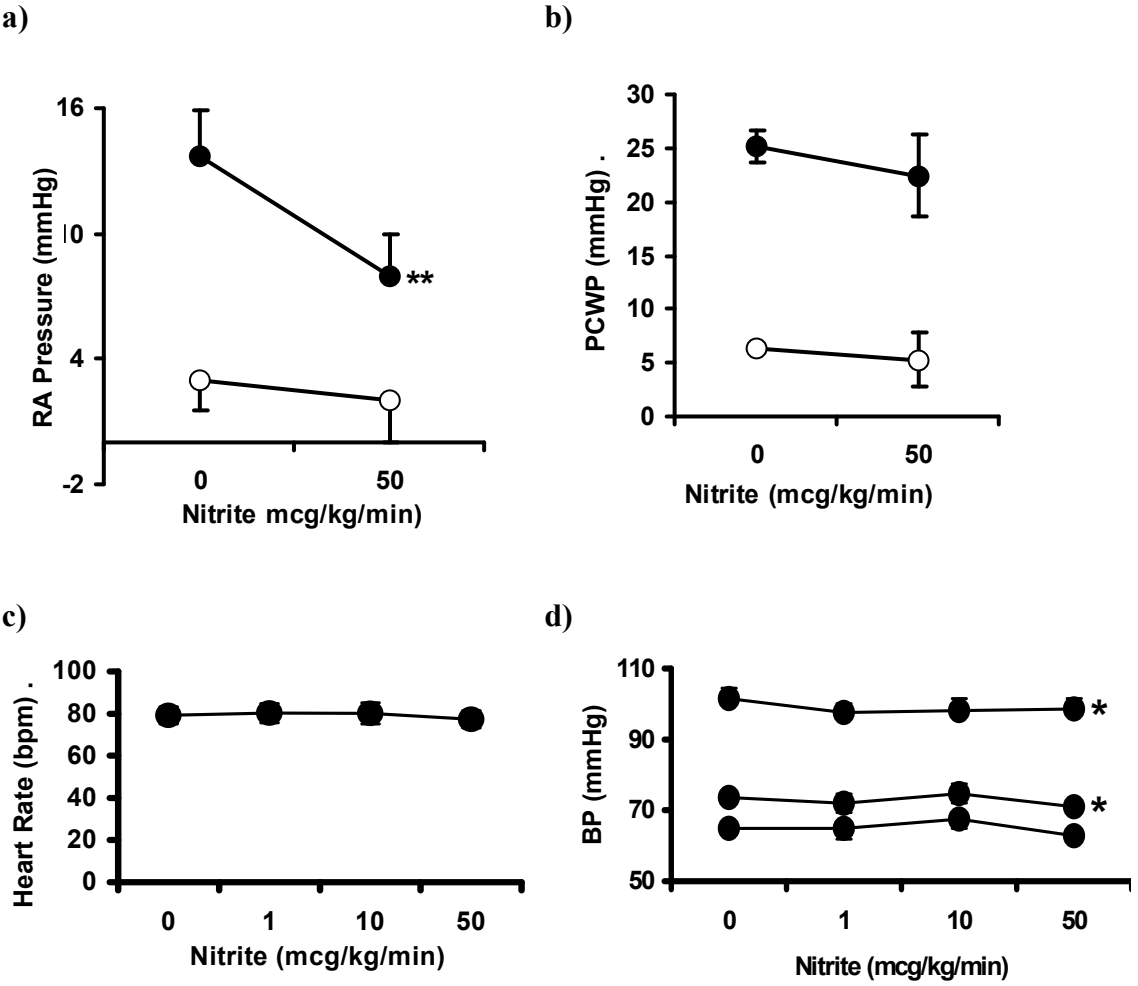


Figure 4.3: The effect of systemic nitrite on right atrial pressure, pulmonary capillary wedge pressure, heart rate and blood pressure

a) In the high PCWP subgroup, RA pressure fell from 12.7 ± 2.1 to 6.8 ± 1.8 mmHg, (filled circles, $n = 13$, $p = 0.003$). In the low PCWP subgroup, RA pressure fell from 3.0 ± 1.5 to 2.0 ± 2.0 mmHg, (hollow circles, $n = 5$, p = NS) **b)** In the high PCWP group, PCWP fell from 24.8 ± 1.7 to 22.0 ± 2.2 mmHg, (filled circles, $n = 13$, $p = 0.09$). In the low PCWP subgroup, PCWP fell from 6.3 ± 0.3 to 5.3 ± 1.3 mmHg, (hollow circles, $n = 5$, p = NS) **c)** Heart rate was unchanged at any dose of nitrite ($n = 18$, p = NS) **d)** Systolic and mean arterial pressures were reduced by an average of 3 mmHg, ($n = 18$, $p < 0.05$ for each); * $p < 0.05$ compared to baseline, ** $p < 0.01$ compared to baseline

Figure 4.4.

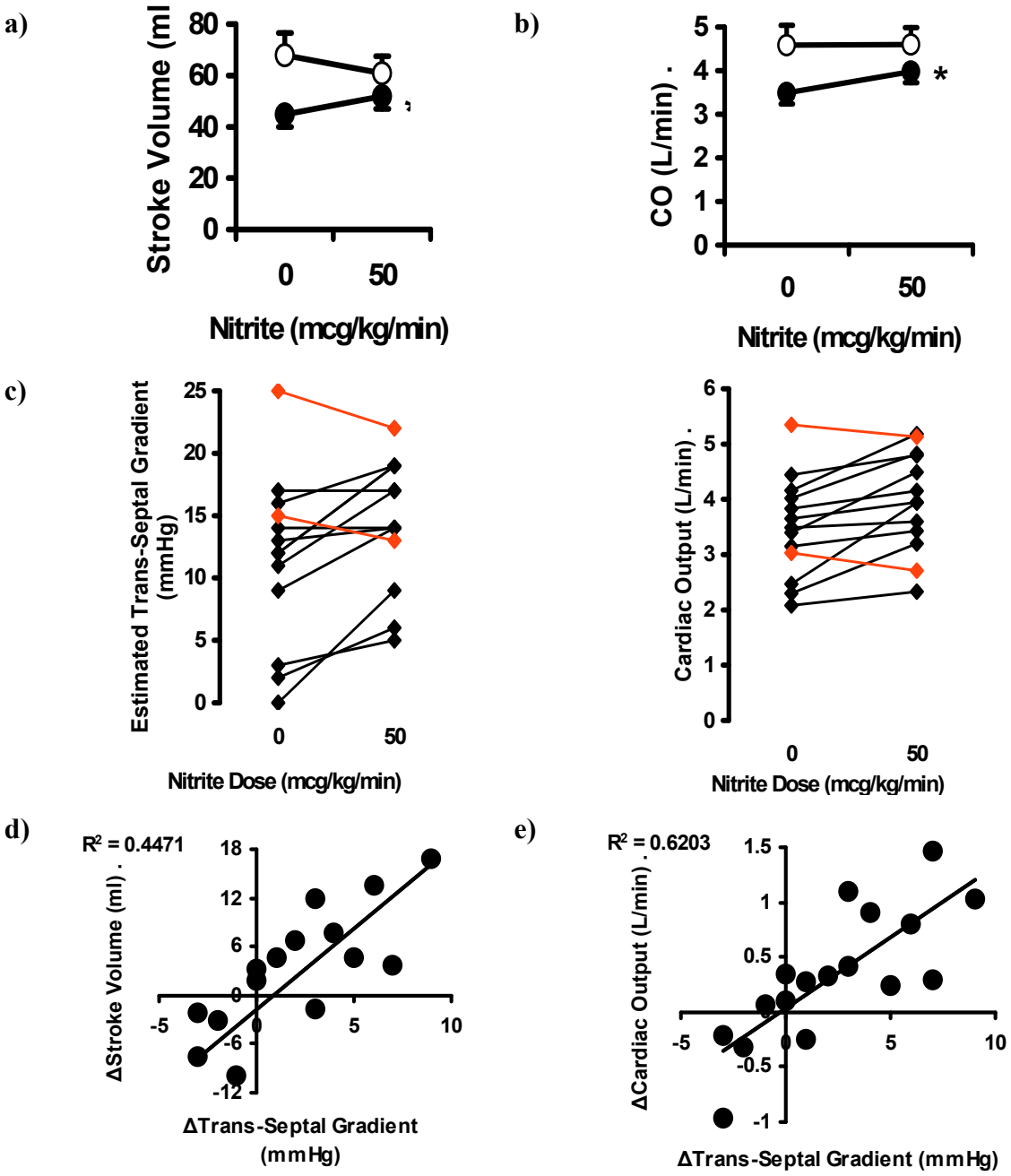


Figure 4.4: The effect of systemic nitrite on cardiac output, stroke volume and estimated trans-septal pressure

a) Stroke volume increased in the high PCWP group from 44.9 ± 5.0 ml to 51.9 ± 4.9 ml, (filled circles, n = 13, p = 0.008) and fell in the low PCWP group by from 67.9 ± 8.7 ml to 60.8 ± 6.6 ml (hollow circles, n = 5, p = NS). SV at baseline was significantly higher in the low PCWP subgroup, (n = 18, p = 0.03) **b)** Cardiac output increased in the high PCWP group

from 3.5 ± 0.3 L/min to 4.0 ± 0.3 L/min, (filled circles, $n = 13$, $p = 0.009$) and unchanged in the low PCWP group (from 4.58 ± 0.5 L/min to 4.60 ± 0.4 L/min, hollow circles, $n = 5$, $p =$ NS) **c)** Change in estimated trans-septal gradient and cardiac output in high PCWP group - trans-septal gradient and cardiac output only fell in 2/13 patients (highlighted in red) **d)** Change in estimated trans-septal gradient positively correlated with change in SV in all patients, ($n = 18$, $R = 0.67$, $p = 0.004$) **e)** and with change in cardiac output, ($n = 18$, $R = 0.79$, $p < 0.001$); $^{*}p < 0.05$ compared to baseline

Figure 4.5.

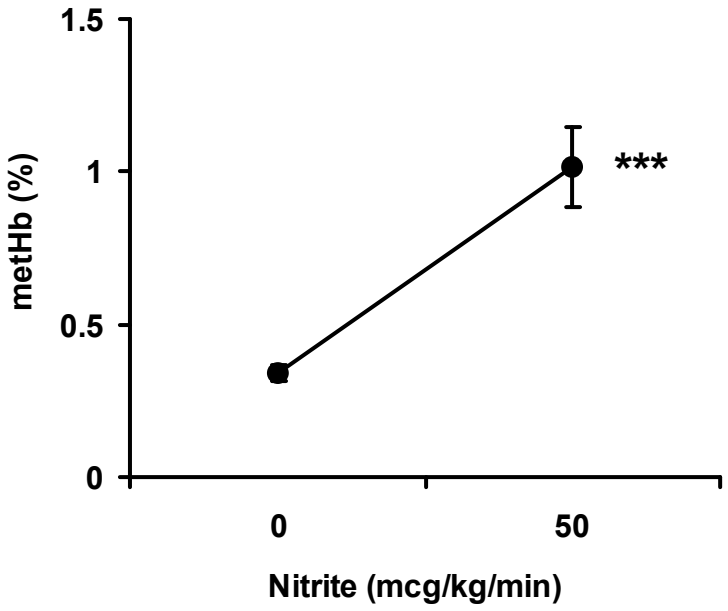


Figure 4.5: The effect of systemic nitrite on methaemoglobinaemia

Methaemoglobinaemia rose from 0.3 ± 0.1% to 1.0 ± 0.5% at the highest dose of nitrite (n = 18, p = 0.0001)

5. CHRONIC INFUSION OF SODIUM NITRITE IN HEALTH AND HEART FAILURE

5.1. Introduction

It has been proposed that haemodynamic profiling of patients presenting with acute decompensated heart failure may provide useful information on prognosis and response to therapy. This was investigated in a landmark study by Nohria and colleagues (Nohria *et al.*, 2003).

In one study, 452 heart failure patients were divided into four profiles on the basis of the presence or absence of congestion (pulmonary or systemic) and hypoperfusion. These were: patients with no evidence of congestion or hypoperfusion (dry-warm, n = 123); congestion with adequate perfusion (wet-warm, n = 222); congestion and hypoperfusion (wet-cold, n = 91); and hypoperfusion without congestion (dry-cold, n = 16). Wet-cold patients had more severe disease than wet-warm patients, on the basis of lower LV ejection fraction (p = 0.0004), serum sodium (p = 0.01), and systolic blood pressure ($p < 0.0001$), and higher NYHA functional class ($p < 0.0001$), and resting heart rate (p = 0.0004). Dry-warm patients had least severe disease, and the authors were guarded about interpreting the dry-cold group with only 16 patients. Wet-cold status was the strongest univariate predictor of one-year mortality tested, with a hazard ratio of 3.66 (2.16-6.21, $p < 0.001$) compared to dry-warm patients. It remained strongest in the multivariate analysis. By comparison, the next strongest were a diagnosis of ischaemic cardiomyopathy at 1.90 (1.35-2.68, $p < 0.001$), and NYHA functional class at 1.51 (1.23-1.85, $p < 0.001$).

Wet-cold ADHF patients are often, but not universally, given therapy with intravenous nitrates (Forrester *et al.* 1976). Reduction in preload by venodilatation causes a paradoxical rise in cardiac output, through relief of DVI. Even so, the usefulness of organic nitrate vasodilators is limited by hypotension and the development of tolerance.

As discussed in **Chapter 3**, NO-release from nitrite will preferentially occur in the venous system, unless substantial arterial hypoxaemia is present (pO_2 < 85mmHg, 11.31kPa). We have seen in **Chapter 4** that systemic nitrite in advanced heart failure induces less systemic hypotension for a given effect on preload, increasing cardiac output through relief of DVI. Inorganic nitrite (Dejam *et al.*, 2007) and nitrate (Petersson et al., 2009) do not seem to induce tolerance, which would be a further advantage over therapy with organic nitrates. However, nitrite also has a unique disadvantage: the development of methaemoglobinaemia. The maximum level of metHb achieved in the previous study was low, at 2.9%, but may continue to rise during longer infusions.

Nitrite has been known to oxidise Hb to metHb for over 100 years. Oxygen cannot bind to metHb, and therefore high levels in the blood ultimately inhibit cellular respiration and can be fatal. Production of metHb depends upon the relative concentration of nitrite and Hb, as well as the ability of nitrite to enter the cell. The reduction of metHb to Hb depends upon the metHb reductase system, which includes NADH- and NADPH-dependent enzymes (reviewed in (Umbreit, 2007). The former, known as NADH-cytochrome b5 reductase or diaphorase I, is responsible for 95% of physiological metHb reduction and exists in two main forms. The first is soluble and restricted in its expression to the erythrocyte. The second is membrane bound,

either in the endoplasmic reticulum or the mitochondrial inner membrane, and is more involved in lipid metabolism. Both enzymes are expressed as different transcripts of the same gene. Absence or lack of function of these enzymes leads to congenital methaemoglobinaemia, which is generally inherited as an autosomal recessive condition but may be sporadic, with the type depending on the particular enzyme subtype that is lacking. Type 1 is due to lack of the erythrocytic enzyme only and leads to visible cyanosis and relatively mild symptoms such as headaches and nausea on exercise. These patients have around 25% metHb at rest. Type 2 is due to lack of both types, including membrane-bound diaphorase I and is characterised by microcephaly, unremitting cognitive decline, athetosis and generalised hypertonia, caused by the loss of essential lipid synthesis. Type 3 is a historical entity, now considered identical to type 1, and type 4 has only been described in a single patient (Umbreit, 2007).

The second enzyme system, NADPH methaemoglobin reductase or diaphorase II, is responsible for only 5% of metHb reduction in physiology, but has a central role in the treatment of methaemoglobinaemia with methylene blue. Methylene blue is a blue-coloured dye, which is stable in its oxidised form. Diaphorase II reduces methylene blue to leukomethylene blue using NADPH. This reduced form can chemically reduce metHb to Hb(Fe^{2+}). Acquired methaemoglobinaemia results when the reductase system is overwhelmed by production. Until the oxidising agent is removed, metHb inexorably accumulates in the blood stream.

As evidenced by the work presented in the preceding **Chapter 4**, short infusions of sodium nitrite have beneficial haemodynamic effects in heart failure. Taken with observations from

Chapter 3 that the vasorelaxing effects of nitrite persist even after its removal, we hypothesised that the beneficial haemodynamics we have shown would also persist, and may in fact increase, during a longer time frame of hours. Prior to testing this theory in our invasive heart failure model, we first performed a dose-ranging safety study in healthy volunteers, as patients with advanced heart failure may be particularly sensitive to the effects of methaemoglobinaemia.

5.1.1. Hypothesis

A dose of systemic sodium nitrite can be found that balances production of metHb with its elimination. This dose will have haemodynamic effects in heart failure, which persist, and may increase, over time.

5.2. Methods

5.2.1. Power calculation

As a preliminary safety study we planned to recruit groups of 3-4 healthy volunteers to each dose of nitrite. As the safety threshold for heart failure patients (5% methaemoglobinaemia) was reached rather quickly at both upper doses of nitrite, no further participants were recruited to these groups.

The intended group size in the heart failure study was guided by the calculation that 8 participants would give a statistical power of 80% to detect a 0.9 L/min difference in cardiac output at a standard deviation of 0.9 L/min (Bonferroni post-hoc comparison of baseline and 3 hour level, (Lenth, 2010)).

5.2.2. Healthy volunteer protocol

7 non-smoking healthy volunteers who gave written informed consent were recruited to the study. A cannula was placed in an antecubital vein in each arm. Continuous infusions at three different doses of nitrite (1, 10 and 60 mcg/kg/min) were given over a period of up to six hours into the dominant arm, while the second cannula was used to withdraw blood for analysis of metHb level at 30 minute intervals. Blood pressure and heart rate were monitored continuously and recorded at 30 minute intervals. Studies were discontinued once the safety cut-off point (metHb of 10%) was reached, or continued for up to six hours. Symptoms are reported to occur at levels of methaemoglobinaemia of 20-30% in healthy volunteers. One study was discontinued prematurely due to failure of the intravenous cannula (the participant refused a replacement). Approval for the study was given by the local Research Ethics Committee **(REC #08/H1207/69).**

5.2.3. Heart failure patient protocol

3 volunteers who were attending hospital for right heart catheterisation as part of the transplant assessment programme were recruited after giving informed consent. Infusions of nitrite at a dose of 10 μg/kg/min, which was established as a safe dose in the earlier protocol, were given to patients with advanced heart failure over a period of three hours. Blood pressure and heart rate were monitored continuously. Haemodynamic measurements were taken at baseline, and at 5, 15 and 30 minutes, and then at every subsequent 30 minutes. As in **chapter 4**, cardiac output was determined using the Fick method, for the reasons given in the preceding chapter, but also because thermodilution requires several 10 ml boluses of cold 5% dextrose solution for each measurement. Over the multiple measurements in this study, we

felt that this could affect haemodynamics in our heart failure patients. For this reason, it was additionally ensured that blood samples taken were replaced by an equal volume of normal saline. Approval for the study was given by the local Research Ethics Committee (**REC #08/H1207/67**). Inclusion and exclusion criteria for the study in heart failure are given below. The protocol is detailed in **figure 5.1.**

5.2.4. Inclusion Criteria

1. Aged 18 years or over
2. Admission to hospital for pulmonary artery catheterisation, under the transplant assessment protocol
3. High wedge pressure or significant DVI identified in clinical procedure

5.2.5. Exclusion Criteria

1. Pre-existing inotrope therapy
2. Recent ST-elevation myocardial infarction or thrombolysis
3. G6PD Deficiency, or high risk of G6PD deficiency
4. Women of child-bearing potential or nursing mothers

5.3. Results

5.3.1. Participant characteristics

Baseline characteristics are given for healthy volunteers (**Table 5.1**) and heart failure patients (**Table 5.2**). All participants in the healthy volunteer studies at higher doses of nitrite were withdrawn early from the study due to participant request or unsafe levels of

methaemoglobinaemia. One participant was withdrawn early from the 10 μg/kg/min group as one venous cannula tissued and the participant refused a second. One participant in the heart failure group was withdrawn early due to discomfort from the Swan-Ganz sheath. Studies were performed in the afternoon to minimise the effect of diurnal variation in vascular response.

5.3.2. The effect of systemic nitrite infusion on methaemoglobinaemia in healthy volunteers

At the highest dose of 60 μg/kg/min, metHb levels continued to increase, up to our safety cut-off level of 10%. Infusions were discontinued at this point. At 30 μg/kg/min metHb also continued to climb, albeit at a slower rate, necessitating cessation of both studies. At a dose of 10 μg/kg/min, metHb stabilised at a safe level of 1.75 ± 0.35% (maximum 2.4%) (**figure 5.2)**. The increase in variability in the 30 μg/kg/min group results from withdrawal of participants as they reach the safety cut-off of 10% metHb; one was withdrawn at 150 minutes, then a second at 270 minutes.

5.3.3. The effect of systemic nitrite infusion on heart rate and blood pressure in healthy volunteers

Heart rate and blood pressure were stable in the healthy volunteers at the 10 μg/kg/min dose of nitrite (**figure 5.3**). At a dose of 60 μg/kg/min, both participants felt unwell towards the end of the study, and the study was ceased due to participant request. During this time an average BP fall of 8 mmHg was recorded.

5.3.4. The effect of systemic nitrite infusion on cardiac output and trans-septal gradient in heart failure patients

Cardiac output rose from 3.3 ± 0.5 L/min to 4.4 ± 0.7 L/min at 3 hours, an increase of 33% (**figure 5.4a**). This change did not reach statistical significance but appears comparable to the 15% rise recorded in the acute studies at the highest dose of nitrite (from 3.5 ± 0.3 L/min to 4.0 ± 0.3 L/min, $p = 0.009$). This change appeared to correlate with change in trans-septal gradient at both 30 minutes (**figure 5.4b**) and 3 hours (**figure 5.4c**).

5.3.5. The effect of systemic nitrite infusion on pulmonary and systemic vascular resistance, heart rate and blood pressure in heart failure patients

The pulmonary vascular resistance appeared to trend towards a decrease throughout the 3-hour infusion (from 3.5 ± 1.4 to 1.1 ± 0.5 wood units, **figure 5.5a**), though this did not reach statistical significance. Systemic vascular resistance was measured at 19.4 ± 1.8 and 16.1 ± 1.4 wood units, at the beginning and end of the study repectively (**figure 5.5b**). As in the healthy volunteers at dose of 10 μg/kg/min nitrite, heart rate (**figure 5.5c**) and mean arterial BP (**figure 5.5d**) were steady throughout the 3-hour infusion.

5.3.6. The effect of systemic nitrite infusion on methaemoglobinaemia in heart failure patients

Methaemoglobinaemia rose throughout the study and had not reached plateau at 3 hours (2.0 ± 0.2%, maximum 2.2%, **figure 5.6**).

5.4. Discussion

Blood pressure dropped by 8 mmHg towards the end of the infusions at 60 μg/kg/min. This may have been due to the vasodilating effect of nitrite, but it might be expected that this drop would occur cumulatively. As this drop correlated with symptoms of dizziness and nausea, it may in fact have been due to central vasovagal activity rather than vasodilatation by nitrite *per se*. Methaemoglobinaemia is known to cause sysmptoms such as nausea and shortness of breath, though these tend to occur at higher levels of metHb (Umbreit, 2007).

Our preliminary dose-ranging study in healthy volunteers established that 10 μg/kg/min nitrite produced a safe level of methaemoglobinaemia (< 2%) that was stable over the six-hour time frame of the study. Both higher doses led to an inexorable rise in metHb, and the two participants who received the highest dose felt symptomatically unwell as they reached the biochemical safety threshold of 10% metHb. Clearly, higher doses are unlikely to be useful in heart failure and were not tested for safety reasons.

We went on to investigate the physiological effects of nitrite at 10 μg/kg/min in patients with advanced heart failure and a PCWP of ≥ 15 mmHg. The kinetics of metHb production and reduction appear to have a "threshold" dose of nitrite, between 10 and 30 μg/kg/min, above which metHb will continue to accumulate until the nitrite infusion is ceased. This is consistent with a physiological model of constant metHb production with a saturable metHb reductase system of enzymes.

The resting cardiac output in the heart failure group was 3.3 ± 0.5 L/min, which is around half that expected in healthy individuals. They represent a group at the severe end of the heart failure continuum. Such patients are admitted regularly to hospital with decompensation of

their fluid status, requiring intravenous diuretics and perhaps inotropic therapy to restore compensation. These therapies increase myocardial ischaemia and can compromise renal function, and so managing these patients is a significant challenge. A rise in cardiac ouput from 3.3 ± 0.5 L/min to 4.4 ± 0.7 L/min, should this prove to be sustained in a larger group in trial conditions, would be clinically significant. This rise was achieved with a tolerable level of metHb, and no significant fall in blood pressure or rise in heart rate. Tentatively, and extrapolating from data in the acute studies presented in the preceding chapter, the mechanism appears to be relief of diastolic interaction, which would limit the benefit to carefully selected patients.

It proved extremely challenging to recruit heart failure patients to the long infusions study. Each study required the availability of a bed in the Coronary Care Unit at the Queen Elizabeth Hospital, which necessarily must prioritise high-dependency patients ahead of research subjects. Participants were most generous with their time, but recruitment was significantly lower than in the acute study. Patients with advanced heart failure are very prone to fatigue, and while we took this into account with the reduced length of the study (3 hours vs. 6 hours in the healthy volunteers), one patient could not complete the protocol. The exacting nature of the study was off-putting to other potential participants. With the limited datasets available, it is not surprising that no change in any parameter met statistical significance and these must therefore be regarded as very preliminary data.

We considered attempting to recruit patients with less severe heart failure, but rejected this for two reasons. Firstly, such patients do not in general receive right heart catheters on clinical grounds, which changes the risk-benefit ratio of the study and may be a significant barrier to

recruitment (as we have found in other invasive research studies). Right heart catheterisation carries a small risk of death or serious injury, which is greater in patients with heart failure. Secondly, as shown in the preceding chapter, patients with a lower PCWP (who are in general less unwell) do not show dramatic haemodynamic responses to nitrite so may not be an adequate group in which to investigate a possible therapy for ADHF.

5.4.1. Limitations

We were unable to follow the clearance of metHb after cessation of the study, when the 10% safety level was reached, due to limitations in our ethical approval. Studies in animals suggest that metHb would decline to normal levels over the following 4-6 hours (Emerick *et al.*, 1965). Both participants given 60 μg/kg/min nitrite felt dizzy and nauseated when they approached a metHb level of 10%, and consequently we limited studies to 2 participants in this and the 30 μg/kg/min group. We felt this was reasonable as even levels of metHb over 5% would be unacceptable in the group with advanced heart failure, and even more so in the eventual clinical target group with ADHF. The study in heart failure patients is ongoing, and we aim to recruit at least 8 participants to this group.

5.5. Conclusions

Systemic administration of 10 μg/kg/min sodium nitrite is safe in both healthy volunteers and patients with advanced heart failure. Although the data are very preliminary, these data are consistent with an increase in haemodynamic effects over time, to approximately the levels seen in the acute studies at the highest dose (50 μg/kg/min). This should encourage further investigation into sodium nitrite as a treatment for decompensated heart failure.

5.6. Tables

Table 5.1.

	Mean ± SEM
M/F	5/2
Age (years)	27 ± 2
Weight (kg)	72 ± 5
Height (m)	1.77 ± 0.04
BMI (kg/m^2)	22.7 ± 0.5
Hb (g/dL)	14.4 ± 0.6
Na^+ (mmol/L)	141.1 ± 0.8
K^+ (mmol/L)	3.90 ± 0.05
HCO_3^{2-} (mmol/L)	23.9 ± 0.6

Table 5.1: Baseline characteristics (healthy volunteers)

Table summarising the baseline characteristics of healthy volunteers in the long infusion study

Table 5.2.

	Mean ± SEM
M/F	2/2
Age (years)	54 ± 5
Height (m)	1.66 ± 0.0
Weight (kg)	70.8 ± 0.8
BMI (kg/m^2)	25.7 ± 0.8
Heart Rate (bpm)	63 ± 6
Mean Arterial Pressure (mmHg)	71 ± 7
PCWP (mmHg)	15 ± 4
Diagnosis:	
Dilated Cardiomyopathy	4
Ischaemic Heart Disease	0
Primary Valve Disease	0
Cardiac Amyloid	0
Medications:	
ACE Inhibitor/AgIIRB	4
B-Blocker	4
Digoxin	1
Loop Diuretic	4
Spironolactone/Eplerenone	3
Warfarin/Enoxaparin	0

Table 5.2: Baseline characteristics (heart failure patients)

Table summarising the baseline characteristics of participants in the long infusion (chronic heart failure) study

5.7. Figures

Figure 5.1.

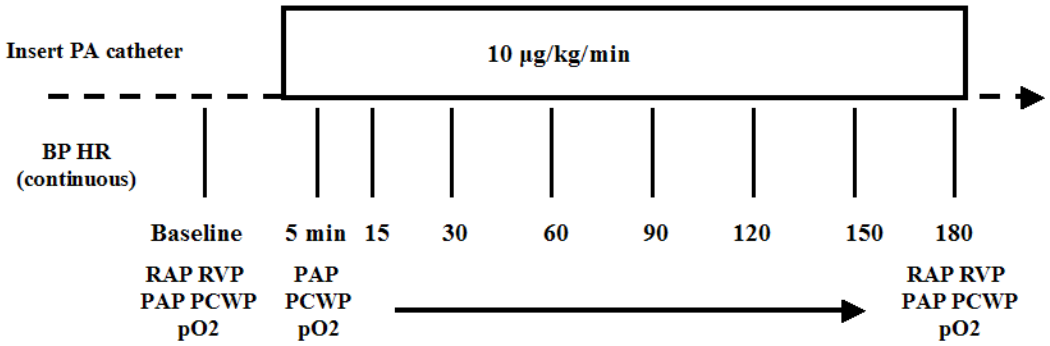


Figure 5.1: Protocol for long infusions of nitrite in heart failure patients

Details of measurements taken at each time point are listed below the horizontal axis

Figure 5.2.

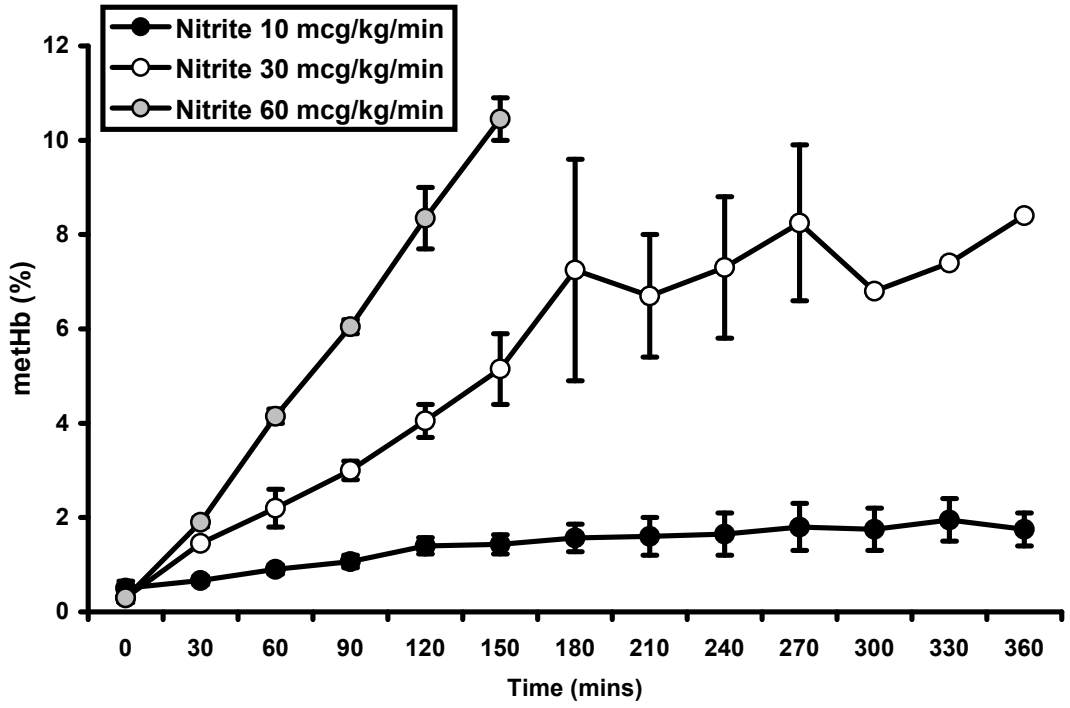
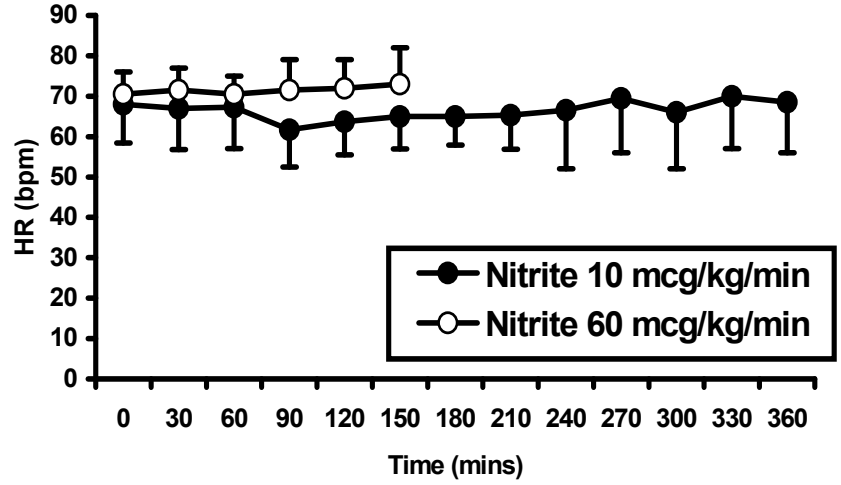


Figure 5.2: The effect of longer systemic infusions of nitrite on methaemoglobinaemia in healthy volunteers

Intravenous sodium nitrite given systemically at 10, 30 and 60 μg/kg/min caused a dose-dependent rise in metHb (% of total Hb) over time (*n* = 3, 2, 2 respectively)

Figure 5.3.

a)



b)

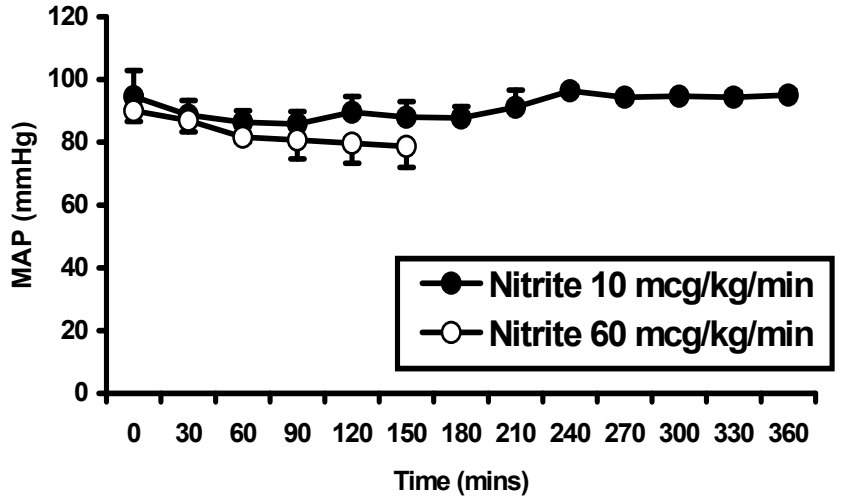


Figure 5.3: The effect of longer systemic infusions of nitrite on heart rate and blood pressure in healthy volunteers

a) Heart rate was steady over the course of the study **b)** Mean arterial blood pressure fell by an average of 8 mmHg in both volunteers at the 60 μg/kg/min dose of sodium nitrite. The studies were terminated for safety reasons. Data from the experiments with the 30 μg/kg/min dose are omitted for clarity.

Figure 5.4.

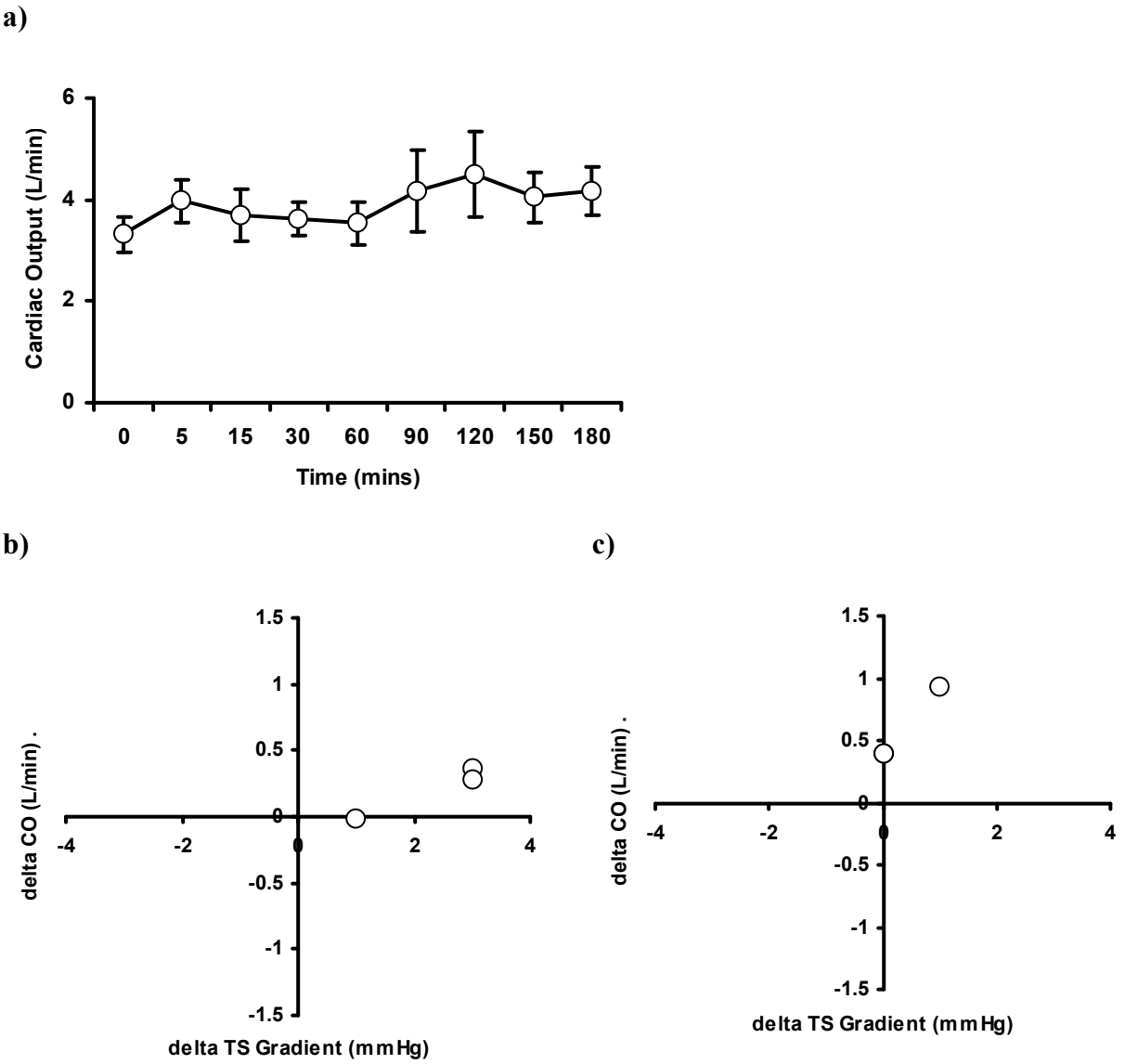


Figure 5.4: The effect of longer systemic infusions of nitrite on cardiac output in heart failure patients

a) Cardiac output appears to rise over the course of the three-hour infusion (from 3.3 ± 0.5 L/min at baseline to 4.4 ± 0.7 L/min, $n = 3$, $p = 0.08$ by single comparison one-way ANOVA) **b)** This increase appeared to correlate with the change in trans-septal gradient at 30 minutes ($n = 3$, $R^2 = 0.9594$, p = NS) **c)** and at 180 minutes ($n = 2$)

Figure 5.5.

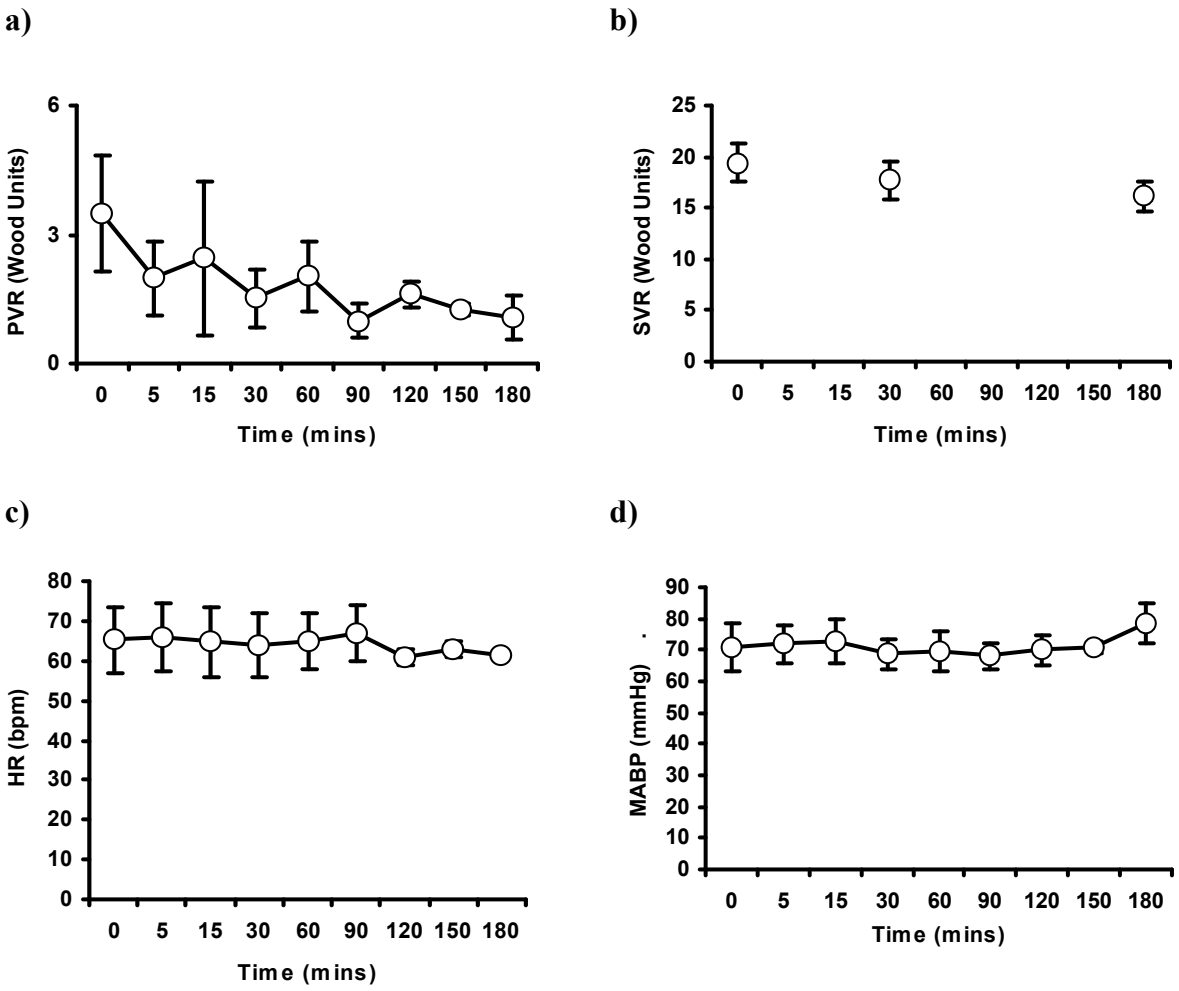


Figure 5.5: The effect of longer systemic infusions of nitrite on pulmonary and systemic vascular resistance, heart rate and blood pressure in heart failure patients

a) The pulmonary vascular resistance appeared to decrease throughout the 3 hour infusion (from 3.5 ± 1.4 to 1.1 ± 0.5 wood units, n = 3) **b)** There was little change in systemic vascular resistance (from 19.4 ± 1.8 to 16.1 ± 1.4 wood units) **c)** Heart rate was steady throughout the infusion **d)** Mean arterial BP was also steady throughout

Figure 5.6.

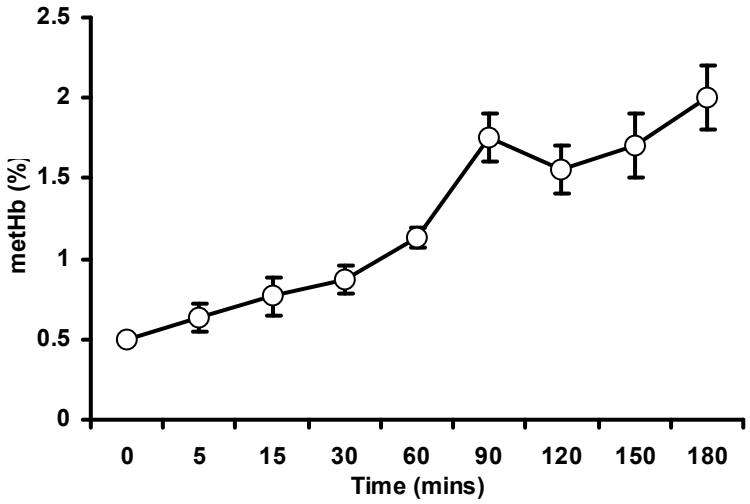


Figure 5.6: The effect of longer systemic infusions of nitrite on methaemoglobinaemia in heart failure patients

Maximum metHb levels were similar to those in the healthy volunteers at 3 hours (2.0 ± 0.2% vs. 1.6 ± 0.2%, maximum 2.2% vs. 2.1%, $p = 0.36$ for a difference by two-tailed *t-test*)

6. NITRITE AS A CYTOPROTECTIVE AGENT: THE ROLE OF ALDH2

6.1. Introduction

Several properties of NO make it a candidate cytoprotective agent: i) it reversibly inhibits mitochondrial respiration, thereby reducing the oxygen demand of ischaemic tissue, and extending the zone of adequate oxygenation to a greater distance from blood vessels (Loke *et al.*, 1999; Thomas *et al.*, 2001) ii) it causes vasodilatation of collateral vessels, promoting maximal oxygen delivery (Johnson, III *et al.*, 1991); iii) NO-dependent S-nitrosylation of L-type calcium channels prevents reperfusion injury by calcium overload (Hu *et al.*, 1997); iv) NO also has a variety of effects on inflammatory pathways – it prevents platelet aggregation (Walter and Gambaryan, 2004), neutrophil adherence (Ma *et al.*, 1993) and apoptosis (Li *et al.*, 1999).

In ischaemic conditions, NO synthesis by NOS may be diminished owing to scant availability of substrate, co-factors and oxygen (Becker *et al.*, 2000). As demonstrated by the work described in **Chapter 3**, nitrite generates NO by a NOS-independent mechanism that is enhanced by ischaemia, making it an intriguing potential therapy in preventing IR injury. Indeed, as detailed in **section 1.8.4**, nitrite has now been shown to protect renal tissue (Milsom *et al.*, 2010; Dezfulian *et al.*, 2007)) liver (Duranski *et al.*, 2005), brain (Jung *et al.*, 2006) and myocardium (Duranski *et al.*, 2005; Gonzalez *et al.*, 2008) from ischaemia-reperfusion (IR) injury in animal models. Nitrate (NO_3) given orally in beetroot juice, protects endothelium from IR injury in healthy human volunteers (Webb *et al.*, 2008b). The authors

attributed this protection to a two-fold increase in plasma nitrite. Studies are underway to investigate first window preconditioning with nitrite (clinicaltrials.gov) but are yet to report. Mitochondrial aldehyde dehydrogenase (ALDH2) has been implicated as a central player in preconditioning with a variety of stimuli (Chen 2008).

Mitochondrial aldehyde dehydrogenase (ALDH2) is a member of the 19-strong human aldehyde dehydrogenase family of NAD(P)+-dependent enzymes (Vasiliou and Nebert, 2005). A common polymorphism in exon 12 (Glu487Lys, or Glu504Lys in the unspliced protein), known as the ALDH2*2 allele, is present in up to 50% of individuals of East Asian descent (Goedde *et al.*, 1983). Heterozygosity at this allele results in a near inactive enzyme and produces the "Asian Flushing" phenotype; mutation of a single subunit destabilises the co-factor binding site and dimer interface such that heterozygotes are functionally similar to homozygotes with the variant allele (Larson *et al.*, 2005). Individuals possessing one or two copies of the ALDH2*2 allele may be at greater risk of coronary artery disease (Guo *et al.*, 2010) and myocardial infarction (Takagi *et al.*, 2002). Recently, ALDH2 has been postulated to be central to cardioprotection by a range of agents (Chen *et al.*, 2008). In this study, increased ALDH2 dehydrogenase activity faithfully correlated with reduction in myocardial infarct size in rats. Additionally, such individuals have a diminished response to GTN, consistent with diminished bioactivation, both systemically (Li *et al.*, 2006) and in the forearm (Mackenzie *et al.*, 2005). Nitrite-dependent phosphorylation of ALDH2 was found in a recent proteomic search: this phosphorylation occurred in a dose-dependent manner after ischaemia-reperfusion injury (Perlman *et al.*, 2009). Finally, ALDH2 itself exhibits intrinsic nitrite reductase activity (Li *et al.*, 2008). We hypothesised that an interaction between ALDH2 and nitrite might contribute to IR protection in humans.

Disulfiram is an irreversible inhibitor of ALDH2. Administration mimics the "Asian Flushing" phenotype, which is used to deter from relapse in abstinent former alcoholics (Chick *et al.*, 1992). Supporting this, disulfiram treatment reduces GTN-induced vasodilatation in wild-type individuals, to a level similar to that seen in people with the East Asian variant enzyme (ALDH2*2) (Mackenzie *et al.*, 2005).

We used two tools, genetic and pharmacological, in an established model of IR injury in the human forearm (Kharbanda *et al.*, 2001) to investigate protection by nitrite and the role of ALDH2. Given the likelihood that nitrite given before ischaemia (in the first preconditioning window) prevents IR injury in the forearm model, we chose initially to investigate the more clinically relevant second window. Having established a protocol for systemic nitrite that mitigates IR injury when given 24 hours before ischaemia, we went on to investigate whether such a protocol would prevent IR injury when used after the onset of ischaemia. This, potentially most useful method of protection, is known as periconditioning.

6.1.1. Hypothesis

Intravenous systemic sodium nitrite will protect from IR injury in wild-type individuals, but not in those with the Asian variant ALDH2*2 enzyme.

6.2. Methods

This study was approved by the local Research Ethics Committee (**REC #09/H1207/99).** All participants gave written informed consent. All studies were performed in a dedicated vascular laboratory, maintained at 22-24°C in quiet conditions. All participants were non-smokers and none were on medications of any kind. All participants avoided nitrite/nitrate-

rich foods (e.g. green leafy vegetables) or alcohol for 24 hours before the study, and abstained from caffeine on the day of the study (a standard measure in studies of vascular reactivity). Baseline characteristics are given in **Table 6.1.**

6.2.1. Power calculation

Based on previous studies we predicted a pre- vs. post-ischaemia difference in mean area under the curve of 1.5 with a standard deviation of 0.9. A group of 10 gives 90% power to detect a difference at a p-value of < 0.05.

6.2.2. Forearm plethysmography

Participants were seated upright on the bed with both arms exposed, fully extended and supported at heart level. Mercury-in-silastic strain gauges of an appropriate size were placed around the widest portion of each forearm. The non-dominant brachial artery was cannulated using a 27-gauge needle (Cooper's Needleworks) attached to an epidural catheter and sealed with dental wax under aseptic conditions, with lignocaine anaesthesia at the participant's discretion. Saline (0.9% NaCl) (or drugs) were infused at 0.5ml/min at all times to maintain needle patency. Venous access was gained in the antecubital fossa of the control arm. Blood pressure and heart rate were continuously monitored. Forearm blood flow (FBF) was measured simultaneously in both arms using venous occlusion plethysmography (VOP) to determine the FBF-ratio (detailed description of the technique is given in **Methods 2.3.**). Wrist cuffs were inflated to 200mmHg in order to exclude the hand circulation during measurements. FBF-R was measured at rest and again after 3-minute intra-arterial infusion of ACh into the study arm at each of 3 ascending doses (25, 50 and 100 nmol/min). Endothelial function was assessed using the change in FBF-ratio from baseline. Endothelium-independent

forearm blood flow was only measured at the end of each study in the main group (3 ascending doses: 4, 8 and 16 nmol/min), in order to prevent potential confounding effects of GTN infusion before ischaemia (Chen 2008).

6.2.3. Ischaemia-reperfusion protocol

Following 15 minutes of acclimatization, baseline endothelial function was measured. 5 minutes washout was allowed to elapse from the end of the last infusion, then the upper arm cuff on the study arm was inflated to >200 mmHg for a period of 20 minutes. Endothelial function was then re-measured after 15 minutes of reperfusion, to assess the level of IR injury. The study design is summarised in **figure 6.1a**, with progression through the study for each group of volunteers detailed in **figure 6.1b**.

6.2.4. Nitrite infusion

Sodium nitrite or saline placebo was infused intravenously into the control arm at a dose of 1μg/kg/min. This dose was chosen as it does not cause significant vaso- or venodilation (Maher *et al.*, 2008). Randomisation to nitrite or placebo was performed by coin toss before the first study day for each participant. They then received the other preparation on a separate research day at least 72 hours later.

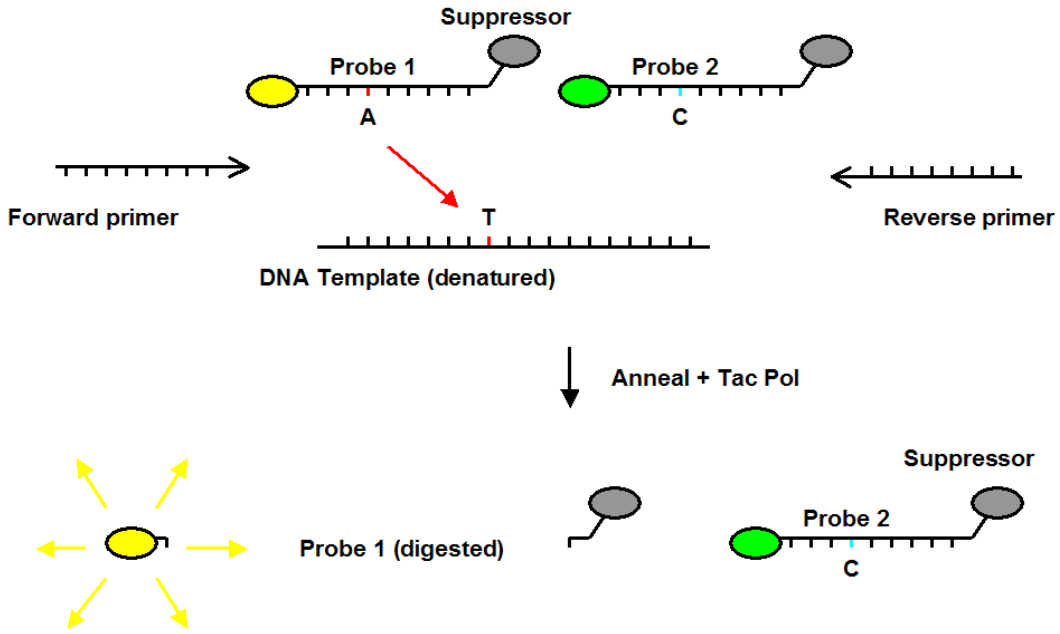
6.2.5. Blood sampling

Venous blood samples were taken prior to ischaemia and then after 1 minute of reperfusion for the measurement of plasma nitrite, nitrite and RXNOs. Samples were placed in tubes spiked with Ethylenediaminetetraacetic acid (EDTA) and *N*-ethylmaleimide (NEM),

transferred on ice to a chilled (4°C) centrifuge and spun at 4000 rpm for 15 minutes. Plasma and cell samples were then snap frozen in liquid nitrogen and stored at -80°C.

6.2.6. Genetic analysis

Genetic analysis for ALDH2 genotype was performed after the completion of all studies, in order to maintain blinding – participants were recruited and studied blind to their ALDH2 status, with the assumption that around 40% of East Asian participants would have the variant allele. DNA was extracted from venous blood samples (QIAamp, Qiagen) and analysed using commercial probes and primers (Taqman System, Applied Biosciences). The Taqman probe consists of a short complementary sequence (one for each allele), a dye (different colours for each allele) and a quenching molecule. Tightly bound probes are cleaved by the DNA polymerase, allowing the dye to fluoresce. This signal is read by the rt-PCR analyser and flags up the existence of one, other or both alleles, as below.



One participant was found to be homozygous at the *2 allele. This dataset was excluded from the analysis. Anonymised genotyping for each individual in the East Asian group is given in **Table 6.2**.

6.2.7. Protocols

Protocol 1: 12 Caucasian/West Asian participants were recruited to the delayed preconditioning group. Nitrite (1μg/kg/min for 10 minutes) was given intravenously 24 hours before the onset of ischaemia.

Protocol 2: Having established an effective dose of nitrite, 22 healthy volunteers of East Asian origin were recruited to the main randomised double-blind placebo-controlled crossover study. Nitrite (1μg/kg/min for 10 minutes) or placebo (Normal saline at the same rate) was given for the latter 10 of 20 minutes of ischaemia.

Protocol 3: 6 Caucasian/West Asian individuals were pre-treated with disulfuram 600mg daily for 2 days prior to the study. In previous studies this dose has been found to inhibit ALDH2 fully. Nitrite (1μg/kg/min for 10 minutes) was given for the latter 10 minutes of ischaemia as in protocol 2.

6.2.8. Statistical Analysis

Data from each study was analysed by an investigator who was blinded to the identity of the participant, their genetic status, and the protocol used. Statistical testing was performed using Graphpad Prism v5.03. Pre-and post-ischaemia dose-response curves were compared using

two-way ANOVA throughout, unless otherwise stated. A *p*-value of less than 0.05 was considered statistically significant.

6.2.9. ALDH2-dependent soluble guanylate cyclase (sGC) activation in the presence of nitrite

This work was done on behalf of the candidate by Prof Mayer's group in the University of Zurich, Switzerland. Expression and purification of ALDH2 was performed as before (Beretta *et al.*, 2010). Purified bovine lung sGC (50 ng) was incubated at 37 °C for 10 min in a final volume of 100 µL with the indicated concentrations of $NaNO_2$ in the presence of 100 µg purified ALDH2*1 or ALDH2*2. Assay mixtures contained 50 mmol/L triethanolamine HCl (pH 7.4), 5 mmol/L $MgCl_2$, 0.5 mmol/L [α-32P]GTP (~250,000 cpm) and 1 mmol/L cGMP. Reactions were terminated by the addition of 0.45 ml of zinc acetate (120 mmol/L) and 0.45 ml of sodium bicarbonate (120 mmol/L), followed by isolation and quantification of [32P]cGMP as described previously (Schultz and Böhme, 1984). Blank values were determined in the absence of sGC. A second set of experiments was performed with a constant $NaNO_2$ concentration (10 mmol/L). Where indicated, superoxide dismutase (SOD, 1,000 U/mL) was additionally present. Chloral-hydrate (CH) inhibitable sGC stimulation was derived subtracting the activity values obtained in the presence of 10 mmol/L CH measured under the corresponding experimental conditions.

6.2.10. ALDH2-dependent inactivation by nitrite

This work was done on behalf of the candidate by Prof Mayer's group in the University of Zurich, Switzerland. Dehydrogenase activity was measured by monitoring the formation of NADH as increase in light absorbance at 340 nm in 50 mmol/L sodium pyrophosphate buffer

(pH 7.5) containing 0.4 mmol/L acetaldehyde, 10 mmol/L $MgCl_2$ and 5 mmol/L NAD. After 2 min of equilibration, the reactions were started by the addition of ALDH2*1 or ALDH2*2 (19 and 111 μg/mL final concentrations, respectively) and monitored for ~3 min to obtain initial reaction rates. Activities were subsequently measured after the addition of 10 mmol/L $NaNO_2$ and of 1 mmol/L DTT.

6.2.11 Measurement of plasma nitrite, nitrate and RXNO

This work was done on behalf of the candidate by Prof Feelisch's group in the University of Warwick. Plasma NO_x (nitrite and nitrate) was measured from samples supplemented with 10 mM EDTA and 2 mM N-ethyl-maleimide, using gas-phase chemiluminescence. The addition of NEM and EDTA serves the purpose of blocking SH-groups and inhibiting metal-catalysed transnitrosation reactions. Quantification of NO, RSNOs and RXNOs was achieved by group-specific denitrosation (Bryan *et al.*, 2004). Briefly, samples were injected into either a triiodide-containing reaction mixture (for the release of RXNO species) or a solution containing potassium ferricyanide (NO-heme species), which was constantly purged with nitrogen. NO was measured with a gas phase chemiluminescence detector (CLD 77am sp, EcoPhysics) under reduced ambient light conditions (<15 lux), as NO and adducts are susceptible to photolytic decomposition.

6.3. Results

6.2.1. Participant characteristics

All subjects tolerated the procedures with no complications. Baseline characteristics of volunteers are given in **Table 6.1**.

6.3.2. Delayed preconditioning with nitrite in individuals with wild-type ALDH2

1μg/kg/min sodium nitrite given intravenously 24 hours before the study prevented post-ischaemia endothelial dysfunction in a group of homozygous wild-type (ALDH2*1/*1) participants ($p = 0.78$, $n = 7$) (**figure 6.2**).

6.3.3. Periconditioning in individuals with wild-type and variant ALDH2

The IR protocol did not affect heart rate or blood pressure in any group; resting heart rate and blood pressure was similar on each study day and endothelium-independent forearm blood flow was similar between groups. Twenty minutes of forearm ischaemia induced significant endothelial dysfunction in individuals with ($p < 0.0001$, $n = 9$) or without ($p = 0.0001$, $n = 9$) the ALDH2*2 allele (**figures 6.3a&b**). 1 μg/kg/min sodium nitrite given intravenously during the latter ten minutes of ischaemia prevented endothelial dysfunction in participants that were heterozygous for the ALDH2*2 allele ($p = 0.63$, $n = 8$)(**figure 6.3c**). Wild-type individuals did not display protection ($p = 0.006$, $n = 10$)(**figure 6.3d**) ($p < 0.001$ for a difference between groups).

6.3.4. Periconditioning in individuals with wild-type ALDH2, pre-treated with the ALDH2 inhibitor disulfiram

Homozygous wild-type participants pre-treated with disulfiram (600mg daily for two days) and given 1μg/kg/min sodium nitrite given intravenously during the latter ten minutes of ischaemia, displayed significant post-ischaemia endothelial dysfunction ($p < 0.0001$, $n = 6$)(**figure 6.4**).

6.3.5. *In vitro* measurements of the activity of wild-type and variant ALDH2 enzyme

The *1/*2 variant enzyme exhibits a rightward shift in the NO-generation curve in response to nitrite when compared to the wild-type enzyme ($p < 0.0001$, $n = 3$) (**figure 6.5a**). The majority of the difference in chloral hydrate inhibitable sGC stimulation is abolished by the addition of superoxide dismutase (SOD) (**figure 6.5b**). Sodium nitrite inhibits ALDH2*1 aldehyde dehydrogenase activity but not that of the variant *2 enzyme – though the resting level of activity is far lower (**figure 6.5c**).

6.3.6. Plasma NO_x and RXNO levels before and after nitrite infusion in individuals with wild-type and variant ALDH2

Plasma nitrite was identical in both groups at baseline and rose significantly in wild-type individuals after nitrite infusion ($n = 7$, $p < 0.05$). This rise did not reach statistical significance in variant individuals (**figure 6.6a**). Plasma nitrate did not increase significantly in either group and was not significantly different at baseline ($n = 9$ for each) (**figure 6.6b**). Plasma RXNO was not significantly different at baseline and did not rise significantly in either group ($n = 7$ for variant, $n = 9$ for wild-type) (**figure 6.6c**).

6.4. Discussion

There is growing interest in the potential of inorganic nitrite to prevent reperfusion-reperfusion (IR) injury (Lundberg *et al.*, 2009). Hitherto, the mechanism of protection by nitrite in animals remains incompletely understood, and protective effects have not been confirmed in humans. The present study addresses a number of these uncertainties. We have demonstrated for the first time that nitrite, given 24 hours before reperfusion, prevents IR injury in the human forearm model. Furthermore, we have shown that the same protocol of

nitrite administration, started after the onset of reperfusion, is efficacious in certain individuals, with protection depending upon ALDH2 genotype. This protection, in contrast to previous preconditioning work in myocardium (Chen *et al.*, 2008) and in refutation of our original hypothesis, did not depend upon activation of the ALDH2 enzyme.

In order to establish whether simple ALDH2 activity was responsible for the observed difference, a group of wild-type homozygous participants was pre-treated with disulfiram. Pharmacological inhibition of ALDH2 in healthy volunteers did not recapitulate the ALDH2*1/*2 phenotype with respect to nitrite efficacy. While we cannot exclude potential inhibitory effects of disulfiram elsewhere in the signalling cascade, this discrepancy suggests that downstream differences in individuals with the ALDH2*1/*1 and *1/*2 genotypes may in fact be responsible. Finally, further investigation of the variant and wild-type enzymes at the biochemical level confirmed significantly reduced aldehyde breakdown, as expected, but additionally revealed mildly reduced nitrite reductase activity as well as marked differences in ROS generation. It has been proposed that nitrite triggers a protective signalling cascade by reversible inhibition of mitochondrial complex I, with modulation of mitochondrial ROS generation at reperfusion (Shiva *et al.*, 2007b), however, the relevance of this mechanism in humans is unclear.

Toxic aldehydes, such as 4-HNE, are known to contribute to mPTP opening and subsequent cell death, and are among the toxic species which accumulate during ischaemia. ALDH2 metabolises these aldehydes and this has been postulated to underlie its protective effects (Budas and Mochly-Rosen, 2007). ALDH2 activation is sufficient to induce cardioprotection in PKCε2 knockout mice, suggesting a downstream position in the mitochondrial signalling

cascade (Budas *et al.*, 2010). Since PKCε2 has been suggested to be common to all cardioprotective signalling pathways, and ALDH2 is activated by a variety of successful stimuli, this places ALDH2 in a central position in cardioprotection (Garlid *et al.*, 2009). It is intriguing that activation of ALDH2 does not appear to be required in periconditioning by nitrite and it may be that nitrite activates other targets, or a downstream target of ALDH2. It appears that reactive oxygen species may be important in this process.

The failure of disulfiram to recapitulate the phenotype of the *2 variant individuals was unexpected. However, disulfiram is known to have effects on systems other than merely ALDH2, including NF-κB, which is known to have a role in cytoprotection (Lovborg *et al.*, 2006). Disulfiram may also, in itself, increase oxidative stress and thereby aggravate IR injury (Ningaraj and Rao, 1998). It may also be that chronic inactivity of ALDH2 induces compensatory changes that acute inhibition of ALDH2 with disulfiram does not mimic.

Plasma NO_x and RXNO levels are very variable between individuals and also vary from day-to-day in the same person. It is therefore impossible to exclude subtle differences in these species that may contribute to the observed differences. However, the level of plasma nitrite achieved in the wild-type group was similar to that reported to protect endothelium in humans when given prior to ischaemia (Webb *et al.*, 2008b), and is several orders of magnitude lower than doses which have been seen to prevent protection in other studies (Jung *et al.*, 2006) which, taken together, implies that the observed difference (and unexpected result) is not merely a result of incorrect dosing. Repeating the study at a higher dose would be instructive but may be impractical.

6.4.1. Limitations

There are several limitations to the interpretation of this study. The endothelial model of IR injury is well established but is not necessarily a surrogate for other organs. The present study is relatively small, though was powered as such. As the study involves puncture of the brachial artery, it was felt that it was not reasonable to require more than two runs from each participant. Ideally, the effect of disulfiram and delayed protection would have been studied in the East Asian population, but insufficient participants were recruited. Finally, our disulfiram group were all male, but there were no significant effects of gender found in any group. Our biochemical studies were performed with the homozygous variant enzyme since it is a dominant-negative polymorphism (Beretta *et al.*, 2010).

6.5. Conclusions

The possibility cannot be excluded that different protocols of nitrite periconditioning might be efficacious in wild-type individuals (Hausenloy and Yellon, 2010). The effective dose of nitrite in the present study is rather low when compared with those successfully used in animal models (cf. 13.8 μg/kg/min, 0.2 μmol /kg/min, aiming for a plasma concentration of 5-10 μmol/L in Gonzalez *et al.*, 2008), but the protective effect seen in those with the variant enzyme, which our biochemical data suggests releases less NO and more superoxide for a given level of nitrite, and the protection afforded in previous preconditioning studies by this level of plasma nitrite (Webb *et al.*, 2008b), argues against the lack of efficacy simply being due to inadequate dose. It has been seen that high levels of NO may prevent protection (Bolli, 2001), but again our low dose of nitrite is unlikely to produce such levels.

A major difference between the endothelial model used in the present study, and previously successful myocardial models, is that nitrite or related species could not access the ischaemic endothelium until the onset of reperfusion. In studies of myocardial infarction, small penetrating arteries or collaterals in the left ventricle may allow a small amount of nitrite to enter. The protective effect previously reported in the ischaemic canine myocardium (Gonzalez *et al.*, 2008) therefore leaves the door open to further study of nitrite during percutaneous coronary intervention in humans.

Epidemiologically speaking, this result is difficult to reconcile with the increased cardiovascular risk proposed to result from possession of the variant genotype. It seems unlikely that such a high prevalence of this allele should have persisted in the face of it causing serious harm to the reproductive ability of the individual, though it might be argued that coronary disease tends to affect people later in life. It does not exist simply though a founder effect (though that may explain the particularly high prevalence among Han Chinese people), and it is tempting to speculate that it might have a protective effect, similar to that proposed for sickle cell/thallasaemia against malaria, cystic fibrosis against cholera, or haemochromatosis against bacterial assault. It has been proposed that the variant enzyme protects against alcoholism (though it should be noted that data from Japan are contradictory to those from China in this respect) but it is hard to believe that this would have an effect over an evolutionary timescale. If people with variant ALDH2 are protected against certain types of ischaemia-reperfusion injury then this is an important result for millions of people worldwide.

6.6. Tables

Table 6.1.

	Delayed	ALDH2*1/*2	ALDH2*1/*1	Disulfiram
Number	12	11	10	6
Height (m)	1.72 ± 0.05	1.72 ± 0.03	1.68 ± 0.03*	1.83 ± 0.03*
Weight (kg)	65.9 ± 5.8	61.6 ± 4.4	63.7 ± 2.6	77.5 ± 4.5
BMI (kgm^{-2})	21.9 ± 0.7	20.6 ± 0.8	22.6 ± 0.6	23.1 ± 1.0
Forearm (cm)	23.6 ± 0.9	23.0 ± 0.8	24.2 ± 0.4	25.6 ± 0.9
Age (years)	25 ±1	23 ± 0.7	23 ± 0.7	24 ± 1.3
Gender (male)	4(57%)	6(60%)	7(64%)	6(100%)
HR Placebo (bpm)		75 ± 3	61 ± 1	
HR Nitrite (bpm)	66 ± 3	73 ± 3	64 ± 3	62 ± 5
mBP Placebo (mmHg)		84 ± 4	84 ± 5	
mBP Nitrite (mmHg)	87 ± 4	85 ± 3	81 ± 3	89 ± 3

Table 6.1: Baseline characteristics

Table summarising the baseline characteristics of participants in each group; *$p < 0.05$ for difference between marked columns by one-way ANOVA (Bonferroni post-hoc test)

Table 6.2.

Sample	*2 Allele Detected	*1 Allele Detected	Interpretation
1		1	*1/*1
2		1	*1/*1
3	1	1	*1/*2
4		1	*1/*1
5		1	*1/*1
6		1	*1/*1
7		1	*1/*1
8		1	*1/*1
9		1	*1/*1
10	1		*2/*2
11		1	*1/*1
12	1	1	*1/*2
13	1	1	*1/*2
14		1	*1/*1
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18	1	1	*1/*2
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23		1	*1/*1
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26		1	*1/*1
27		1	*1/*1
28		1	*1/*1
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31		1	*1/*1
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33		1	*1/*1
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35		1	*1/*1
36		1	*1/*1
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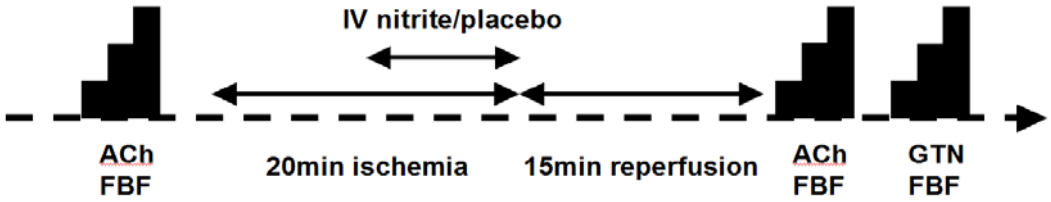
Table 6.2: Summary of genotyping results and interpretation

6.6. Figures

Figure 6.1.

a)



b)

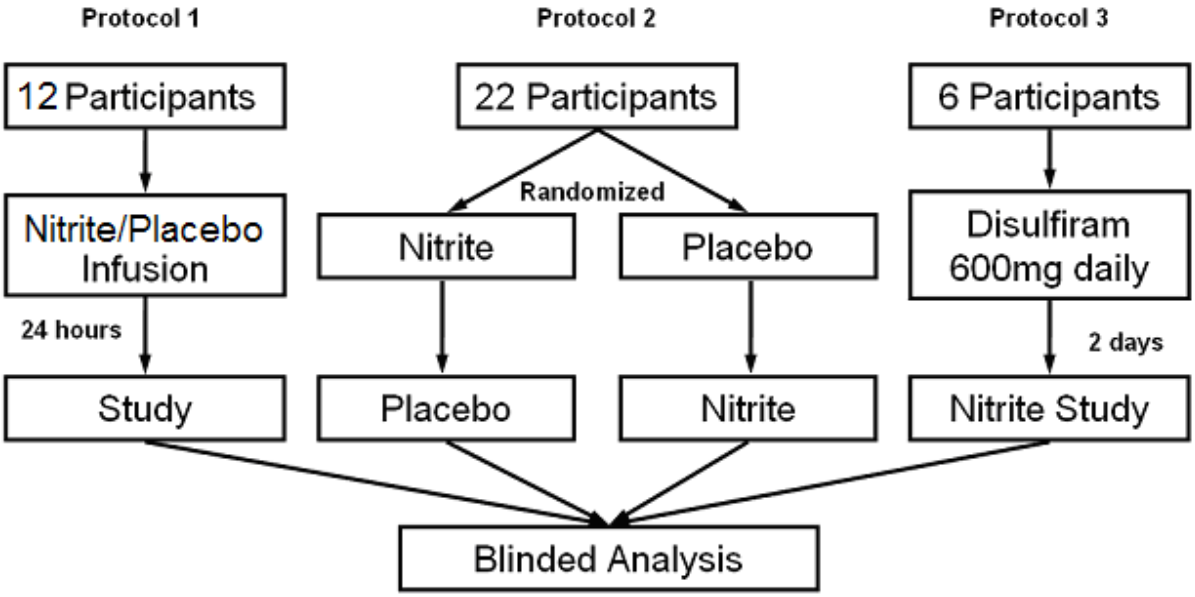


Figure 6.1: Recruitment and study design

a) Figure summarising the basic study design and plan of each study day, **ACh FBF:** measurement of endothelium-dependent forearm blood flow; **GTN FBF:** measurement of endothelium-independent forearm blood flow **b)** Figure showing progression through study for each group.

Figure 6.2.

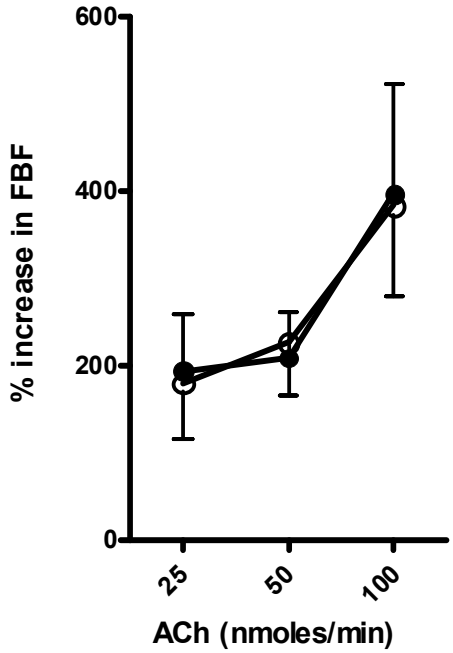


Figure 6.2: The effect of nitrite infusion 24 hours prior to ischaemia in ALDH2*1/*1 individuals

Intravenous sodium nitrite (1 μg/kg/min) given 24 hours prior to ischaemia prevented endothelial dysfunction in wild-type individuals (pre- vs. post-ischaemia FBF-R, $p = 0.78$, $n = 7$); filled circles: pre-ischaemia FBF-R, hollow circles: post-ischaemia FBF-R

Figure 6.3.

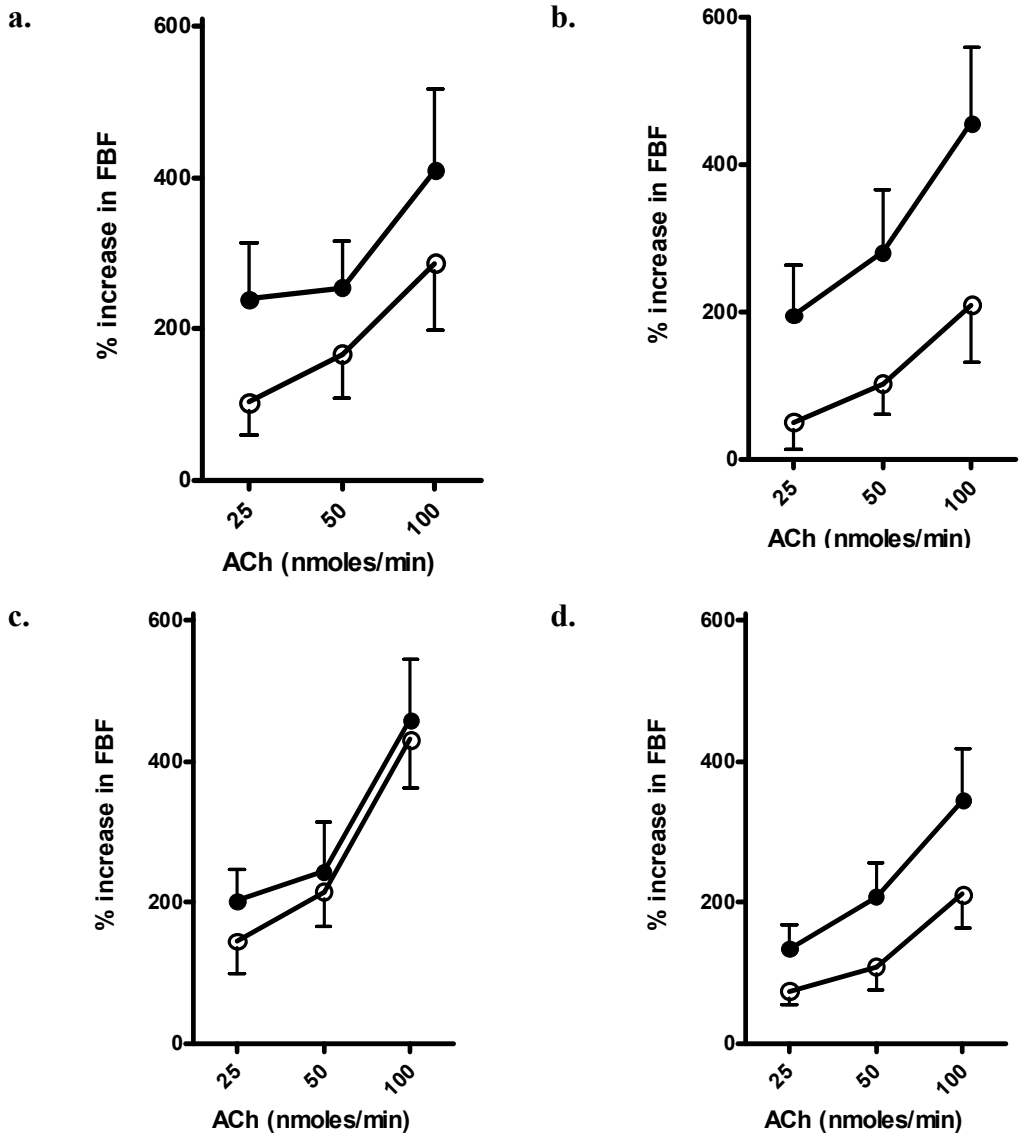


Figure 6.3: The effect of nitrite or placebo infusion during ischaemia in ALDH2*1/*1 and ALDH2*1/*2 individuals

a) IR produced significant endothelial dysfunction in ALDH2*1/*2 individuals, ($p < 0.0001$, $n = 9$) **b)** IR produced significant endothelial dysfunction in ALDH2*1/*1 individuals ($p = 0.0001$, $n = 9$) **c)** Intravenous sodium nitrite (1μg/kg/min) given during the final 10 minutes of ischaemia prevented endothelial dysfunction in ALDH2*1/*2 individuals ($p = 0.63$, $n = 8$) **d)** Intravenous sodium nitrite (1μg/kg/min) given during the final 10 minutes of ischaemia did not prevent endothelial dysfunction in ALDH2*1/*1 individuals ($p = 0.006$, $n = 10$); filled circles: pre-ischaemia FBF-R, hollow circles: post-ischaemia FBF-R

Figure 6.4.

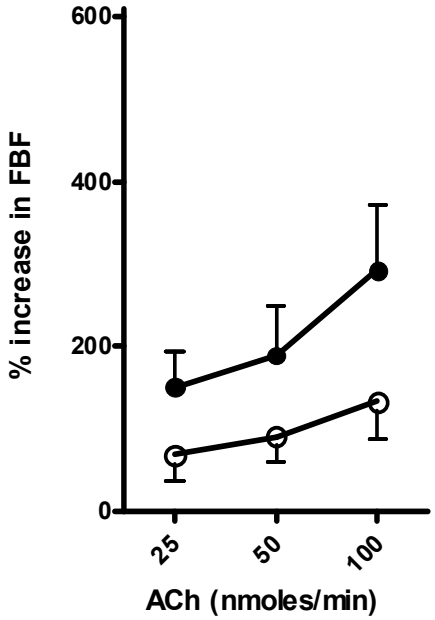


Figure 6.4. The effect of disulfiram pre-treatment and nitrite infusion during ischaemia in ALDH2*1/*1 individuals

Pre-treatment with disulfiram (600mg daily for 2 days) did not rescue the wild-type phenotype ($p < 0.0001$, $n = 6$); filled circles: pre-ischaemia FBF-R, hollow circles: post-ischaemia FBF-R

Figure 6.5.

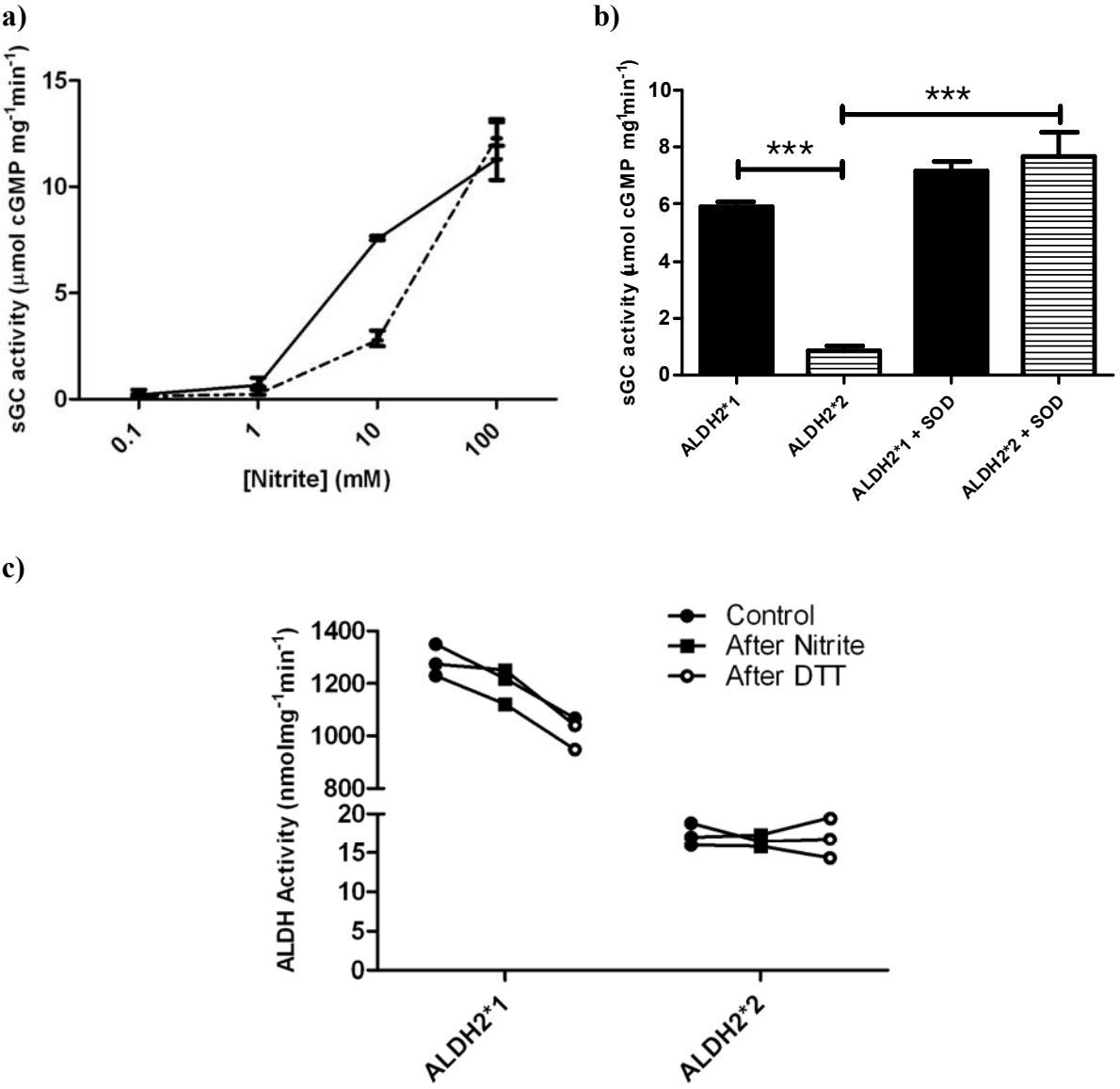


Figure 6.5: *In vitro* studies of wild-type and variant ALDH2 enzymes

a) The ALDH2*2 variant enzyme exhibits a rightward shift in the NO-generation curve in response to nitrite when compared to the wild-type enzyme ($p < 0.0001$, $n = 3$); solid line: ALDH2*1, dotted line: ALDH2*2 **b)** The majority of the difference in chloral hydrate inhibitable sGC stimulation is abolished by the addition of superoxide dismutase (SOD) (solid columns, ALDH2*1; hatched columns, ALDH2*2) ($n = 3$ for each) **c)** Sodium nitrite inhibits ALDH2*1 ($p < 0.01$ compared to baseline) but not *2 ($n = 3$ for each), **d)** blood results; $^{**}p < 0.01$, $^{***}p < 0.0001$

Figure 6.6.

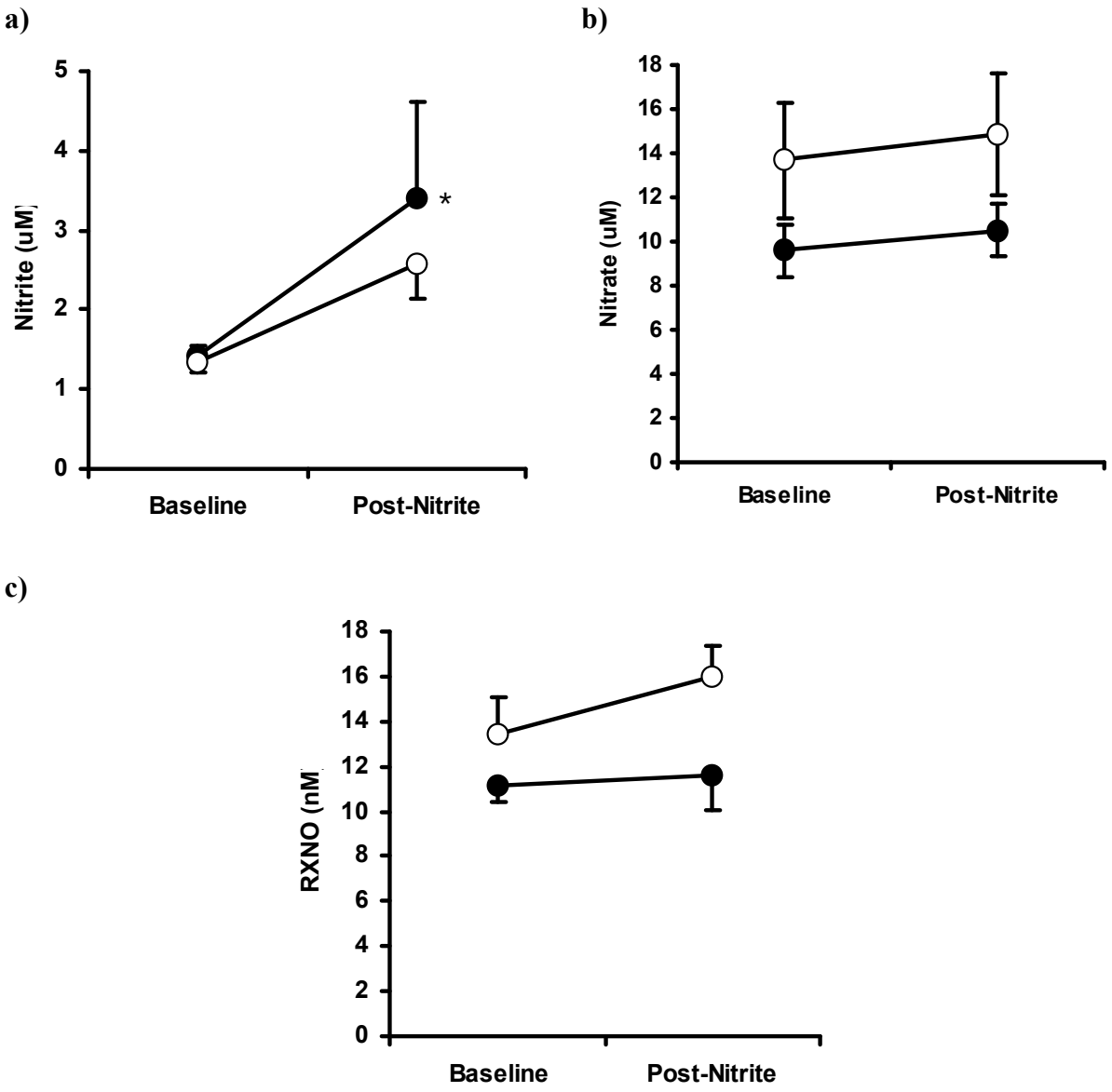


Figure 6.6: Plasma nitrite, nitrate and RXNO levels

a) Plasma nitrite increased significantly in wild-type but not variant individuals (n = 7 for each) **b)** Baseline and post-nitrite differences in plasma nitrate did not reach statistical significance (n = 9 for each) **c)** Baseline and post-nitrite differences in plasma RXNO did not reach statistical significance (n = 7 for variant, n = 9 for wild-type); filled circles, wild-type; hollow circles, *1/*2 variant; *$p < 0.05$ by two-way ANOVA.

7. GENERAL DISCUSSION AND CONCLUSIONS

- **Myoglobin is a nitrite reductase in vascular tissue, it affects the pharmacokinetic profile of exogenous nitrite, and may be important in vasodilatation *in vivo***
- **Nitrite shows acutely beneficial haemodynamic effects in patients with severe heart failure**
- **Above a threshold dose of nitrite, metHb accumulates to dangerous levels**
- **Below the threshold dose, metHb levels plateau, making it possible to select safe doses for chronic nitrite infusion**
- **Chronic nitrite infusion produces beneficial haemodynamic effects in patients with severe heart failure, that persist and increase beyond the acute time-frame investigated above**
- **Nitrite protects healthy endothelium from ischaemia-reperfusion injury when given 24 hours before injury**
- **Nitrite protects endothelium when given during ischaemia in people with variant ALDH2, but not in those with classical ALDH2**

7.1. Myoglobin participates in the reduction of nitrite, and affects its pharmacokinetic profile

The identity of the nitrite bioconverter is elusive. It appears likely that a number of species participate in this reaction, with different proteins taking the lead role under different physiological conditions. The work in **Chapter 3** implicates myoglobin in this role, even in conditions of mild hypoxia that are surprisingly close to normal oxygenation in the

vasculature. The mechanism of this vasodilatation certainly involves release of NO, and seems to proceed along the established path of sGC stimulation, though the mechanism may be more esoteric. The intracellular positioning of the relatively sparse smooth muscle myoglobin, with direct access to the mitochondria, may have particular relevance in this regard. Recent work has shown that reactive oxygen species (and particularly hydrogen peroxide) can themselves vasodilate (Prieto *et al.*, 2010; Feng *et al.*, 2010). Nitric oxide, released by the reaction of nitrite and myoglobin in the vicinity of mitochondria, could inhibit the respiratory chain (Complex I in Hendgen-Cotta *et al.*, 2008) to produce a burst of ROS, leading to vasodilatation. This is consistent with the relatively slow development of nitrite-induced vasodilatation, when compared to that produced by other NO-donors.

The duration of vasodilatation is increased by the presence of myoglobin. This feature of nitrite appears to have clinical relevance, as shown by the long infusions in **Chapter 5**, wherein the haemodynamic effects of sodium nitrite increased over a time period of minutes to hours. In the future, it would be most useful to investigate the mechanism of this prolonged effect. Primate myoglobin has an extra cysteine residue compared to that of other animals, which can also be nitrosylated, therefore this ability of myoglobin may be even more pronounced in primates. Living vessels from humans are difficult to source, and are technically challenging to use. I performed some preliminary studies with internal mammary artery rings, harvested at the time of cardiac surgery (and which are normally discarded), but these were very unreliable and it has not as yet been possible to produce reproducible results. The artery samples we were given initially had been sprayed with papaverine. This is standard practice among some surgeons, to prevent vasospasm, but it unfortunately makes the artery unusable for myography. When the surgeons harvested the tissue before papaverine, the rings

were still unresponsive, and it was only when I doubled the glucose content of the buffer that a proportion of the rings contracted. The failure of other samples may relate to differences between patients (i.e. level of atheroma) or surgical collection technique. The results gained from a small number of rings were very heterogeneous, and this technique will need further optimisation. Once perfected, future work could compare human and rodent rings, or perhaps make use of the gene transduction techniques used earlier in **Chapter 3**, to investigate a difference between knocked-in human and rodent myoglobin, with and without the unique cysteine 110, both on the *myo*$^{-/-}$ background.

The experiments in **Chapter 3** and studies discussed in the introduction together suggest that nitrite may have clinical utility in two distinct areas: selective vasodilatation in heart failure and cardioprotection in myocardial infarction. Both areas rely on the ability of nitrite to release NO in a NOS-independent manner, particularly in conditions of hypoxia or ischaemia.

7.2. Nitrite infusion in decompensated heart failure

The work in **Chapter 4** demonstrates that intravenous sodium nitrite has beneficial haemodynamic effects in advanced heart failure. However, as shown by the work in healthy volunteers and in patients with advanced heart failure (**Chapter 5**), the maximum dose is limited by the development of methaemoglobinaemia, which accumulates over time. At a safe dose of 10 μg/kg/min, effects were more modest but increased over the course of the three-hour infusion. As before, nitrite had more effect in the relatively hypoxic pulmonary circulation, while the reduction in systemic vascular resistance was minimal. Pulmonary vasodilatation, along with venodilatation (signified by a reduction in right atrial pressure), caused an increase in cardiac output through relief of diastolic ventricular interaction. It

remains to be investigated whether this statistically significant rise in cardiac output is clinically relevant; this can only be evaluated in a future trial.

A randomised controlled trial of 10 μg/kg/min nitrite vs. GTN in acute decompensated heart failure should be performed to assess the true clinical impact of nitrite in this condition. Sodium nitroprusside has the disadvantage of being metabolised to cyanide, which accumulates during longer infusions. Inclusion criteria must be especially rigorous, as only a subset of such patients ('cold and wet' in the previous authors' scheme (Forrester *et al.*, 1976)) would be expected to benefit from vasodilator therapy, and poor patient selection would dilute any difference in effect. A larger trial could stratify patients in the manner of Nohria et al. (2003). Nitrite would be predicted to produce better outcomes in the "cold and wet" group than in the remainder. Such a trial would examine the additive benefit of nitrite on standard therapy, as it would be unethical to replace standard therapies in the other groups. Endpoints should include clinically significant hypotension and reflex tachycardia, and tolerance must be assessed, as this is a potential advantage over nitrates. It may be that the narrow therapeutic window of nitrite with regard to methaemoglobinaemia means that it has no advantage over traditional inorganic nitrates. Another condition in which nitrite therapy may be beneficial would be pulmonary hypertension. Unfortunately, very few pulmonary hypertension patients are admitted to the transplant assessment programme in the Queen Elizabeth Hospital, Birmingham, and such patients are also very heterogeneous in clinical profile. In our centre the majority are secondary in aetiology to heart failure or lung disease of various types. Such a study could really only be performed in a regional specialist centre. With access to such a group, one could assess a variety of nitrite therapies, including ambulatory infusions or dietary nitrite supplementation in primary pulmonary hypertension

patients. Haemodynamic assessment of pulmonary artery pressures can be done non-invasively using echocardiography, which would be an advantage over the present work, and more global measures such as quality of life and exercise performance could be measured over a longer period. Dietary nitrite could also be assessed in heart failure, perhaps compared to a nitrate/hydralazine regimen.

7.3. Nitrite before surgery or at intervention in myocardial infarction

The work in **Chapter 6** demonstrates for the first time that nitrite infusion 24 hours prior to ischaemia protects the human endothelium. This provides a rationale for investigation of nitrite protection for planned events such as surgery or percutaneous coronary intervention. In most individuals, nitrite given during the latter part of ischaemia does not protect endothelium. It must be noted, however, that the endothelial model is imperfect to investigate the ischaemic myocardium, as no nitrite may enter the tissue until the beginning of reperfusion. The model therefore reflects postconditioning more than periconditioning and should not prevent investigation of nitrite periconditioning for the myocardium. The observation that nitrite post-conditioning protects the endothelium in ALDH2*2 variant individuals provides some encouragement that such a strategy may be successful. Periconditioning may have more clinical utility – most ischaemic events are unpredictable and a safe intervention given before reperfusion would be a valuable addition to the medical armoury.

A clinical trial of nitrite infusion before primary percutaneous coronary intervention (for acute coronary syndrome) would establish whether this might be a useful clinical therapy in the future. Certainly, it would be practically simple and cheap as an intervention. There is also

good evidence that nitrite, especially at the extremely low doses required, would be very safe. The endpoints however might be more complicated. Indices of myocardial damage such as troponin release are very variable after acute coronary syndromes and would necessitate a very large trial to prove an effect. A more powerful strategy might be to use cardiac magnetic resonance imaging (with T2 weighting to show oedema) to gain an assessment of the area at risk of infarction, and an extremely accurate measurement of infarct size. Longer-term follow-up could include the presence of heart failure (which is related to initial infarct size), and could be done using clinical assessment, diuretic use and echocardiography. Transgenic mouse models may be used to probe the relationship between nitrite and variant ALDH2.

Myoglobin may have a particularly important role in cardioprotection by nitrite. *In vivo* administration of nitrite reduced infarct size by 60% in a mouse model, whereas the same protocol of nitrite administration did not protect in *myo*$^{-/-}$ mice (Hendgen-Cotta *et al.*, 2008). This was attributed to the unique position of myoglobin within the cell, with NO released directly beside mitochondria where it nitrosylates mitochondrial complex I (Raat *et al.*, 2009), inhibiting it, and protecting the mitochondrion from excess oxidative stress (Shiva *et al.*, 2007b).

Nitrite is present in our food. The amount we ingest varies greatly depending on one's diet, with high-nitrite food sources such as cereals and leafy green vegetables containing 2.0-4.0 mg of nitrite per kilogram (van Faassen *et al.*, 2008). While a change in diet is unlikely to cause central haemodynamic changes comparable to those in **chapter 4**, the approximate doubling of plasma nitrite by the low doses given in **chapter 6** is achievable. This may explain some of the cardiovascular protection afforded by a diet rich in vegetables, though

admittedly it would be brave to commend a (similarly nitrite-rich) daily bacon sandwich to those seeking the same effect.

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9. PUBLICATIONS

Below are listed the publications achieved during the period of research leading to the submission of this thesis (August 2007 – December 2010).

Original research articles

Ormerod JO, Ashrafian H, Maher AR, Arif S, Steeples V, Born GV, Egginton S, Feelisch M, Watkins H, Frenneaux MP: The Role of Vascular Myoglobin in Nitrite-Mediated Blood Vessel Relaxation; *Cardiovascular Research* 2010 Oct 19. [Epub ahead of print]

Abstracts

International:

Ormerod JOM, Evans J, Contractor H, Kharbanda R, Frenneaux MP, Ashrafian H: Systemic nitrite induces second window preconditioning in healthy volunteers; *American Heart Association Scientific Sessions 2011*

Ormerod JOM, Evans J, Arif S, Contractor H, Kharbanda R, Frenneaux MP, Ashrafian H: ALDH2 Genotype Influences the Protection of Endothelium against Ischemic Injury by Systemic Nitrite; *American College of Cardiology Scientific Session 2011* **[Prize for highest-ranked research from UK]**

Arif S, Maher AR, **Ormerod JOM**, Mohan S, Madhani M, Frenneaux MP: The acute hemodynamic effects of intravenous sodium nitrite in healthy volunteers during normoxia and hypoxia; *European Society of Cardiology Congress 2010*

Ormerod JOM, Arif S, Mukadam ME, Scott G, Ashrafian H, Bonser RS, Frenneaux MP: Systemic nitrite dilates veins and pulmonary vasculature, relieving diastolic ventricular interaction in advanced heart failure; *European Society of Cardiology Congress 2009* **[oral presentation]**

Ormerod JOM, Ashrafian H, Steeples V, Egginton S, Watkins H, Frenneaux MP: Myoglobin is involved in vasodilatation by nitrite; *European Society of Cardiology Congress 2009*

National:

Ormerod JOM, Evans J, Arif S, Contractor H, Kharbanda R, Frenneaux MP, Ashrafian H: Nitrite Protects Endothelium From Ischaemic Injury, Dependent Upon ALDH2; *Medical Research Society Meeting for Clinician Scientists in Training 2011*

Ormerod JOM, Arif S, Mukadam ME, Scott G, Evans J, Ashrafian H, Bonser RS, Frenneaux MP: Systemic Sodium Nitrite Selectively Dilates Veins and Pulmonary Vasculature, Relieving Diastolic Ventricular Interaction in Advanced Heart Failure; *Medical Research Society Meeting for Clinician Scientists in Training 2010*

Ashrafian H, Howell NJ, **Ormerod J**, Hammer F, Drury N, Steeples V, Lygate C, Frenneaux MP, Pagano D, Watkins H: Aortic stenosis with impaired ventricular function manifests impaired cardiac metabolism: implications for prognosis and surgical intervention; *British Cardiovascular Society* 2009

Maher AR, Arif S, **Ormerod J**, Nasimizadeh M, Ahmed I, Frenneaux M: Intra-arterial inorganic nitrite preferentially dilates the capacitance bed in chronic heart failure; *British Cardiovascular Society* 2009

Ormerod JOM, Ashrafian H, Egginton S, Watkins H, Frenneaux MP: Vascular Smooth Muscle Reduces Nitrite to NO, and is Involved in Vasorelaxation; *103rd Meeting of The Association of Physicians of Great Britain and Ireland*

Review Articles

Gamble JH, Scott G, **Ormerod JO,** Frenneaux MP: Pathophysiology of coronary artery disease: the case for multi-parametric imaging; *Expert Reviews in Cardiovascular Therapy* 2009 Mar;**7(3):**299-310

Gamble JH, **Ormerod JO,** Frenneaux MP: Exercise can be effective therapy for depression; *The Practitioner* 2008 Sep;**252(1710):**19-20, 23-4.

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Other

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10. APPENDICES

Appendix I:

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