Inorganic Nitrite and Conduit Artery Function

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Inorganic Nitrite and Conduit Artery Function

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Abstract

Background Inorganic nitrite, a metabolite of endogenously produced nitric oxide (NO) from NO synthases, provides the largest endocrine source of directly bioavailable NO. The conversion of nitrite to NO occurs mainly through enzymatic reduction, which is particularly favoured under hypoxia. Thus, current evidence shows that nitrite dilates small resistance arterioles where conditions of hypoxia predominate. Although organic nitrates/nitrites also mediate their principal effects via NO, they are not hypoxia dependent; hence, they selectively dilate muscular conduit arteries, lowering central blood pressures. Inorganic nitrite would be expected to lack such effects.

Methods and Results The effects of local and systemic administration of sodium nitrite on the radial artery (RA) a muscular conduit artery, forearm resistance vessels (forearm blood flow) and systemic haemodynamics in healthy male volunteers (n=43) were examined. Intra-brachial sodium nitrite (8.7 μmol/min) increased RA diameter by 28.3% (95% CI 20.3 to 36.2). Nitrite (0.087-87 μmol/min) displayed similar selectivity as glyceryl trinitrate (0.003-1 μg/min) for conduit arteries, compared to resistance arterioles. Nitrite dose-dependently increased local cGMP production from the dose of 2.6 μmol/min, by 1.1 pmol/min/100ml tissue (95% CI 0.5 to 1.8). Vasodilatation of the RA by nitrite was enhanced by administration of acetazolamide (oral or i.a.) and oral raloxifene ($P=0.0248$, $P<0.0001$ and $P=0.0006$, respectively) but was inhibited under hypoxia ($P<0.0001$) and hyperoxia ($P=0.0006$) compared to normoxia. Systemic intravenous administration of sodium nitrite (8.7 μmol/ min) dilated the RA by 10.7% (95% CI 6.8 to 14.7) and reduced central systolic BP by 11.6

mmHg (95% CI of difference 2.4 to 20.7), augmentation index and pulse wave velocity, without changing peripheral BP.

Conclusions Nitrite is a normoxia-dependent selective conduit artery dilator. The mechanism is via cGMP, and the effect is enhanced by acetazolamide and raloxifene. The selective central blood pressure-lowering effects of nitrite have therapeutic potential to reduce cardiovascular events.

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Table of Abbreviations

2D –	2-dimensional
2K1C –	Two-kidney one-clip
3D –	3-dimensional
ABG –	Arterial blood gas
ADP –	Adenosine diposphate
AIx –	Augmentation index
ALDH –	Aldehyde dehydrogenase
AMPK –	Adenosine monophosphate activated protein kinase
ANOVA –	Analysis of variance
AO –	Aldehyde oxidase
aPWV –	Aortic pulse wave velocity
ATP –	Adenosine triphosphate
AV –	Arterial-to-venous
bfPWV –	Brachial-femoral pulse wave velocity
BP –	Blood pressure
C-PTIO –	Carboxy 2-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl-3-oxide
CA –	Carbonic anhydrase
CABG –	Coronary artery bypass graft
CAD –	Coronary artery disease
CAFE –	Conduit artery functional endpoint
cAMP –	Cyclic adenosine monophosphate
CcOx –	Cytochrome C oxidase
CFR –	Coronary flow rate
Cgb –	Cytoglobin

cGMP – Cyclic guanosine monophosphate

CLD – Chemiluminescence detection

COX – Cyclooxygenase

CPAP – Continuous positive airway pressure

cPP – Central pulse pressure

CPR – Cardiopulmonary resuscitation

CRP – C-reactive protein

cSBP – Central systolic blood pressure

CVD – Cardiovascular disease

CYP450 – Cytochrome P450

CysNO – Nitrocystine

CytC – Cytochrome C

DASH – Dietary Approach to Stop Hypertension

DBP – Diastolic blood pressure

DPI – Diphenylene iodonium

Drp1 – Dynamin-related protein 1

ECG – Electrocardiogram

ED – Endothelial dysfunction

EDRF – Endothelium derived relaxing factor

EDTA – Ethylene diamine triacetic acid

ELISA – Enzyme-linked immunosorbent assay

eNOS – Endothelial nitric oxide synthase

FAD – Flavin adenine dinucleotide

FAO – Food and agriculture organization of the United Nations

FBF – Forearm blood flow

FMD – Flow mediated dilatation

GSNO – S-nitrosoglutathione

GTN – glyceryl trinitrate

GTP – Guanosine triphosphate

Hb – Haemoglobin

Hb-NO – Nitrosylated Haemoglobin

HO-1 – Haem oxygenase-1

HPLC – High performance liquid chromatography

HR – Heart rate

IH – Intimal hyperplasia

IL-1b – Interleukin-1 beta

IL6 – Interleukin 6

INFγ – Interferon-γ

iNOS – Inducible nitric oxide synthase

IPC – Ischemic preconditioning

IRI – Ischemia reperfusion injury

ISDN – Isosorbide dinitrate

ISIS-4 – Fourth international study of infarct survival

ISMN – Isosorbide mononitrate

LVDP – Left ventricular developed pressure

LVEF – Left ventricular ejection fraction

MAP – Mean arterial pressure

Mb – Myoglobin

MI – Myocardial infarction

MPTP – Mitochondrial membrane permeability transition pore

NAD – Nicotinamide adenine dinucleotide

NADPH – Nicotinamide adenine dinucleotide phosphate

NFAT – Nuclear factor of activated T cells

Ngb – Neuroglobin

nNOS – Neuronal nitric oxide synthase

NO – Nitric oxide

NOA – Nitric oxide analyser

NOS – Nitric oxide synthase

NSB – Non-specific binding

PAD – Peripheral artery disease

PAP – Pulmonary artery pressure

PDE5 – Phosphodiestersae type 5

PEdiN – Pentaerithrityl dinitrate

PEEP – Positive end expiratory pressure

PEmonoN – Pentaerithrityl mononitrate

PETN – Pentaerithrityl tetranitrate

PEtriN _ Pentaerithrityl trinitrate

PGC1α – Proliferator-activated receptor-γ coactivator 1α

PKA – Protein kinase A

PMT – Photomultiplier tube

pSBP – Peripheral systolic blood pressure

PWV – Pulse wave velocity

RA – Radial artery

ROS – Reactive oxygen species

SBP – Systolic blood pressure

SD – Standard deviation

sGC – Soluble guanylate cyclase

SNO – S-nitrosothiol

SNO-Hb – S-nitrosylated Haemoglobin

SO – Sulphite oxidase

TMB – 3,3’,5’5’-tetramethylbenzidine

TNFα – Tumour necrosis factor-α

US – Ultrasound

V-HEFT – Vasodilator heart failure trial

VOP – Venous occlusion plethysmography

WHO – World health organization

XDH – Xanthine dehydrogenase

XO – Xanthine oxidase

XOR – Xanthine oxidoreductase

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“Count the waves of the sea. Events in your life to come are many more; and like the waves either you ride them out or they ride you down.”

Arabic proverb

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Publications

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Chapter 1

Introduction

1.1 Inorganic nitrite

1.1.1 Overview

In contrast to the structurally complex and chemically synthesised organic nitrates/nitrites, inorganic nitrite (NO_2^-) is a simple naturally occurring anion. Under normal physiological conditions, the majority (~70%) of circulating and stored nitrite is derived from the oxidation of nitric oxide (NO), endogenously produced via the L-arginine NO synthase (NOS) pathway [1-3]. The remainder (~30%) is acquired through dietary intake, via the recently described Nitrate-Nitrite-NO pathway [4]. While concentrations in the circulation are low under basal conditions (90-350 nM) [5-8], nitrite accumulates substantially in tissues (1-20 μM) [9-11], with the highest concentrations in the liver, kidney, heart and topmost in the aorta [11,12].

Although nitrite is freely available in the environment, it is only found in trace amounts compared to inorganic nitrate (NO_3^-) [13]. Both are formed by the fixation of atmospheric nitrogen and oxygen, either directly through lightning, or indirectly through the actions of specialized bacteria; the latter being the more significant of the two processes. These anions are normally bonded to metal cations (commonly Na^+ or K^+) and exist as hydrophilic salts that permeate into water and soil [14,15]. Plants, especially green leafy vegetables and beetroot, take up nitrate avidly [4,13]. In turn the ingestion of these nitrate rich plants by mammals provides a major source of nitrite through the entero-salivary circuit [4]. Once ingested, nitrate is readily absorbed via the upper gastrointestinal tract avoiding first pass metabolism [7,16,17]. Within a 24 hour period ~75% of the absorbed nitrate is excreted by the kidneys; of the remainder ~25% is taken up

by the salivary glands, with only trace amounts secreted via sweat glands [6,7]. The nitrate rich saliva is then excreted into the oral cavity where bacteria found on the dorsal part of the tongue convert it to nitrite via nitrate reductases. A number of bacterial species contribute to this process to varying extents, the most notable of these being the *Veillonella* species, *Actinomyces* species, and *Rothia* species [18]. Nitrite is swallowed, and absorbed via the upper gastrointestinal tract leading to a rise in circulating levels. However, a proportion of this nitrite is protonated under the acidic conditions normally found in the stomach, forming nitrous acid which in turn decomposes to NO and other derivatives (see Equations 1-4) [19,20]. Although the entero-salivary circuit is the major pathway for nitrate reduction to nitrite in mammals, another possible mechanism described in a murine model, and in rodent and human liver homogenates, suggests direct reduction of nitrate to nitrite via the actions of hepatic xanthine oxidoreductase [21].

1.1.2 Nitrite in tissue and circulation

As recently as 2001, nitrite was viewed as biologically inactive, and its only utility was as a marker of endogenous NO production, thus reflecting NOS activity [5]. Furchgott demonstrated in 1953 that high concentrations of nitrite (100 and 1000 μM) relaxed strips of rabbit thoracic aorta [22]. Although lower concentrations were not tested, it was generally inferred that at physiological concentrations nitrite was biologically inactive. In 1994, Benjamin et al, and Lundberg et al, independently demonstrated for the first time that nitrite can be reduced to NO in a biological system - under the acidic conditions present in the human stomach [19,20]. A year later, in 1995, Zweier et al, suggested that nitrite,

via a similar mechanism of direct reduction, may provide an alternative source of NO in the ischemic heart, where NOS plays a diminishing role [23]. By looking at both NO metabolites (endogenous nitrate and nitrite) Cicinelli et al, suggested in 1999 that an arterial-to-venous (AV) gradient may exist [24]. A year later Gladwin et al, specifically investigated endogenous nitrite, and convincingly demonstrated the existence of an AV gradient, suggesting that nitrite is reduced to NO across the vascular bed under normal physiology [25]. However, others maintained the gradient in fact reflected differences in NOS activity and NO production in the arterial vs. venous system [26]. In 2001, Modin et al, found that nitrite in physiological concentrations was an effective vasodilator of rat aortic rings under conditions of low pH (6.6) and normoxia (6.5% CO_2 and 93.5% O_2). With this finding, an active biological role for nitrite in vascular tone was hypothesised [27]. It was Cosby et al, who demonstrated in 2003, that intra-brachial infusion of minimally supra-physiological concentrations of nitrite caused a significant increase in forearm blood flow [28]. This discovery resulted in the acceptance of nitrite as a physiological source of biologically active NO, making nitrite the largest directly accessible storage pool for NO. Although nitrate is found in much higher concentrations in the circulation and tissues, nitrate requires a two-step reduction to NO, via nitrite. In 2008, Maher et al, demonstrated significant enhancement of nitrite-induced vasodilatation under hypoxic conditions in humans, suggesting greater rates of reduction of nitrite to NO [29].

1.1.3 Mechanisms of nitrite reduction to nitric oxide

1.1.3.1 Acidic disproportionation

It has long been established that eNOS plays an important role in myocardial NO production [30-32], with an estimated ~1000 pM/s of NO being produced under normal physiological conditions [33]. Ischemia however markedly reduces eNOS activity and NO production [33,34]. These same conditions of low oxygen tension and low pH significantly enhance nitrite reduction, releasing quantities of NO which far exceed those produced by eNOS under such conditions [35]. It has been estimated that under ischemic conditions, NO production from nitrite (10 μM) in the heart is ~560 pM/s [33].

Under acidic conditions, nitrite exists in equilibrium with nitrous acid (Equation 1). Both exist in equilibrium with other intermediates (Equations 2 & 3), including dinitrogen trioxide (N_2O_3), which breaks down to form nitrogen dioxide (NO_2) and NO (Equation 4) [28,35,36].

$$H^+ + NO_2^- \leftrightarrow HNO_2 \quad (1)$$

$$HNO_2 \leftrightarrow NOOH \quad (2)$$

$$NOOH + NO_2^- \leftrightarrow N_2O_3 + OH^- \quad (3)$$

$$N_2O_3 \leftrightarrow NO_2 + NO \quad (4)$$

Thus simple nitrite disproportionation, which increases under acidic conditions, with abundant availability of H^+ ions, favours the release of free NO.

1.1.3.2 Enzyme facilitated reduction

However, in tissues, the process of disproportionation accounts for only ~15-20% of the total NO produced from nitrite [35]; the remainder is derived from enzymatic nitrite reduction [33]. A host of proteins have now been shown to possess nitrite reductase activity; these include haem-associated globins, molybdenum metallo-enzymes, mitochondrial proteins, cytochrome P450 and NOS enzymes (See Table 1.1).

	Protein/ enzyme	Expression	Ref
Haem-containing globins	Haemoglobin	Predominantly contained within red blood cells	28,37
	Myoglobin	Predominantly in striated skeletal, cardiac and smooth muscle; but also in brain, kidney, and liver of some animals	38-40
	Neuroglobin	Brain, peripheral nervous system, CSF, retina, pituitary, adrenal and testis	41-44
	Cytoglobin	All tissues with greater expression in brain, eyes, liver, heart, skeletal muscle	43-46
Mitochondrial proteins	Complex III	Spans the mitochondrial membrane as part of the electron transfer chain	47
	Cytochrome C	In the mitochondrial membrane, located within the inter-membrane space	48
	Cytochrome C Oxidase	Spans the mitochondrial membrane as part of the electron transfer chain	49,50
Molybdenum metallo-enzymes	Xanthine oxidoreductase	In humans predominantly expressed in the GI tract and liver. Also expressed in most organs especially blood and hearts of other mammalian species Commonly expressed as XDH; however, readily converted to XO (reversibly and irreversibly)	51-53
	Aldehyde oxidase	Found in the CNS, CVS, and respiratory and GI tracts with highest expression in the liver.	33,54,55
	Sulphite oxidase	Skeletal muscle, spleen and brain but higher expression in kidney, liver and myocardium	56,57
Others	Cytochrome P450	Ubiquitous throughout the animal and plant kingdom. In humans highest expression in hepatocytes	58-61
	Carbonic anhydrase	Ubiquitous throughout all cell compartments and all cell types. Commonest human isoenzyme is CAII	62,63
	Nitric Oxide Synthase	eNOS – endothelial cells, epithelial cells of respiratory and GI tract, hepatocytes and skeletal muscles nNOS – Neuronal cells, cardiomyocytes, vascular and smooth muscle cells, skeletal muscle iNOS – inducible in a host of cells which express eNOS and nNOS	64,65

Table 1.1: List of enzymes implicated in the production of NO from nitrite. CSF – Cerebrospinal fluid, GI– gastrointestinal tract, XDH – Xanthine dehydrogenase, XO – Xanthine oxidase, CNS – central nervous system, CVS – cardiovascular system, CA – Carbonic anhydrase, eNOS – endothelial nitric oxide Synthase, nNOS – neuronal nitric oxide Synthase, iNOS – inducible nitric oxide synthase.

1.1.3.2.1 Haem-associated globins

Haemoglobin (Hb), myoglobin (Mb), neuroglobin (Ngb) and cytoglobin (Cgb), belong to an extended family of haem-associated globin proteins, found in almost all living organisms. They have evolved from a single ancestral protein, and are mainly concerned with handling oxygen storage and delivery [37,42,66]. While the haem portion, composed of an iron atom within a tetrapyrrole ring [42], provides the redox-capacity of the protein, it is the globin subunit that guards against rapid oxidation. This directs the redox potential which allows for the reversible binding of oxygen [67]. In addition to this oxygen binding capacity, haem-associated globins manifest enzymatic activity, reacting with a range of compounds such as sulphides, peroxides, NO and its metabolites [42]. However, this enzymatic activity is limited under normal physiological conditions [42].

All the mammalian haem-associated globins have been shown to possess nitrite reductase activity in their deoxygenated state, in a well-characterized reaction (Equation 5) [68].

$$\text{Deoxy Fe(II)} + NO_2^- + H^+ \rightarrow \text{Fe(III)} + NO + OH^- \qquad (5)$$

However, the somewhat intriguing, parallel paradox is that haems, particularly Hb and Mb, are long-established avid scavengers of NO in their oxygenated state (see Equation 6). Thus there exists a delicate balance between nitrite reduction to NO and NO oxidation to nitrate in the presence of haem. This led to the proposal of a responsive oxygen-sensing role of Hb through nitrite and NO, which serves as an important modulator of vasodilatation [28].

1.1.3.2.1.1 Haemoglobin

Haemoglobin is a complex protein composed of four globin protein subunits, each linked to a haem molecule. The four subunits are composed of two α– and two β– globin proteins, which share only 50% of their polypeptide sequence [37]. Normal blood Hb concentrations in humans are 12-15 mmol/L (~150 g/L), located mostly in erythrocytes [37,69] where levels of nitrite exceed those of plasma (~290 nM and ~120 nM respectively) [70].

Oxygenated Hb (O_2-$Hb^{Fe(II)}$) acts as a nitrite oxidase producing nitrate and metHb ($Hb^{Fe(III)}$) (Equation 6) [26], a reaction described almost 140 years ago by Arthur Gamgee [68,71].

$$4\ O_2\text{-}Hb^{Fe(II)} + 4NO_2^- + 4H^+ \rightarrow 4\ Hb^{Fe(III)} + O_2 + 4NO_3^- + 2H_2O \quad (6)$$

An interaction between deoxygenated Hb (deoxy$Hb^{Fe(II)}$) and nitrite was recorded by John Haldane in 1901 [72]. It was Doyle et al, who in 1981, further elucidated the role of deoxyHb as a nitrite reductase leading to the production of NO and metHb via a nitrosylhemoglobin intermediate (Equation 7) [73].

$$deoxyHb^{Fe(II)} + NO_2^- + H^+ \rightarrow HONO + Hb \rightarrow Hb\text{-}NO + OH^- \rightarrow Hb^{Fe(III)} + NO + OH^- \quad (7)$$

The potential biological significance of nitrite as a source of NO under hypoxic conditions was then highlighted by Reutov et al, in 1993 [74]. The oxygen-dependence of this reaction, was demonstrated by Cosby et al, in 2003, where the use of deoxygenated erythrocytes resulted in significantly more NO being released [28]. However, rather than occurring at complete deoxygenation, peak NO production was found to occur at ~50% oxygen saturation of both free-, and erythrocyte-bound Hb [75,76]. This may be explained by a differential rate of

reaction between Hb in the “R” (relaxed, oxygenated) state, which reacts with nitrite ~10-fold faster than Hb in the “T” (tense, deoxygenated) state [77,78]. The nitrite reductase capacity of Hb occurs most rapidly when it is partially oxygenated and adopts the R3 state, with three of the four haem moieties bound to oxygen [77].

In support of nitrite’s biological significance as a source of NO during hypoxia, the addition of nitrite in physiologically relevant doses (10 nM to 10 μM) to rabbit or rat aortic rings in the presence of erythrocytes at high oxygen tensions resulted in only a small vasodilatory effect when compared to controls [76]. However, nitrite’s dilatory effect became considerably more potent as the oxygen tension was reduced, so that concentrations of nitrite as low as 200 nM resulted in a significant dilatation [76]. And so it has been suggested that as erythrocytes traverse the vasculature from the arterial to the venous side, the fraction of deoxyHb increases and on reaching the arteriolar bed, where the partial pressure of oxygen approaches the p50 of Hb, production of NO from nitrite is optimally supported [28,79].

A limitation of this elegant mechanism of nitrite reduction is the explanation of how NO escapes scavenging by oxyHb, within the confines of the erythrocyte, to exert its effects on the vasculature. Haemoglobin is such an avid scavenger of free NO, that free Hb in low concentrations (1000-fold less than in erythrocytes) leads to marked depletion of circulating NO, elevating basal vascular tone, resulting in hypertension in humans and animal models alike [69,80,81]. The affinity of free Hb for free NO, is thought to be a property of both oxyHb and deoxyHb alike. OxyHb reacts with free NO, yielding metHb and

nitrate (Equation 8). Whereas deoxyHb reacts with free NO producing nitrosylated-Hb (Hb-NO) (Equation 9) [79,82].

$$O_2\text{-}Hb^{Fe(II)} + NO \rightarrow Hb^{Fe(III)} + NO_3^- \quad (8)$$

$$\text{deoxy-}Hb^{Fe(II)} + NO \rightarrow \text{Hb-NO} \quad (9)$$

Several hypotheses attempt to tackle the conundrum of how NO escapes scavenging by cell bound Hb. The first of these suggests that NO is produced in specific compartments of the erythrocyte then actively released into the circulation [28]. Another hypothesis, proposes the formation of an s-nitrosothiol-Hb (SNO-Hb) intermediate, which releases NO during transit from the oxygen-rich arterial system, to the oxygen-deplete venous system [26,83]. However, although levels of SNO-Hb have been detected in animal models, their presence in humans at physiologically relevant concentrations has not been confirmed [84,85]. A further hypothesis, suggests that the reaction of nitrite and deoxyHb leads to the formation of N_2O_3, or other non-specified intermediates, which can more readily escape the erythrocyte, and once outside, dissociate releasing NO [28,86,87]. Thus, although the reductase capacity of Hb has been demonstrated, its relative biological contribution remains to be established.

All the reactions of nitrite and nitrate with deoxy-, oxy- and met- haems are summarised in Table 1.2.

1.1.3.2.1.2 Myoglobin

Originally described as “muscle haemoglobin” [88], Mb comprises a single unit which shares a near-identical 3D structure with Hb’s α- and β-subunits [37]. In mammalian models, Mb predominates in skeletal, cardiac and smooth muscle [38]. However, Mb has also been shown to be present in the brain, liver and kidneys of hypoxia tolerant carp [39]. Concentrations of Mb are ~0.33 mmol/L in human myocardium, ~0.5 mmol/L in the skeletal muscles of terrestrial mammals, but are ~10-fold greater (~3.8 mmol/L) in marine mammals [89]. Given the abundance of this haem-associated globin in the skeletal, and cardiac muscle of diving marine mammals and animals living in high altitudes [90], Mb’s sole role was considered to be oxygen storage and handling in mitochondria-rich muscle cells. By enhancing oxygen diffusion, especially under acidic conditions [42], Mb aids endurance muscle activity during periods of hypoxia and anaerobic exercise [91]. And so it came as a surprise when Mb-knockout mice displayed no adverse effects, and no reduction in their endurance capacity or response to hypoxia [90].

In view of these findings, one may well have questioned Mb’s actual function. However, there is now evidence of an important reaction between deoxyMb and nitrite [92]. For example, in an *ex-vivo* model, deoxyMb was shown to be an effective nitrite reductase [40,75,77], catalysing the production of NO ~36-fold faster than deoxyHb [40]. The dependence of nitrite reduction on Mb, was further confirmed *in-vivo*, in a study utilising a mouse model deficient in the protein [88]. Fully deoxygenated Mb produces NO at a rate of ~1.5 pmol/L/s from nitrite (10 μmol/L) [88], resulting in the formation of metMb. That NO is readily detectable suggests that it evades scavenging by metMb, which has a low affinity for NO

[88]. Thus under hypoxic conditions, deoxyMb acts as an NO generator [40,88], with an important role in hypoxia-mediated vasodilatation [12,93]. However, under aerobic conditions, oxyMb rapidly scavenges NO, producing metMb and nitrate [42,82,88,94], displaying an oxygen-sensing role similar to that of Hb (See table 1.2.).

1.1.3.2.1.3 Neuroglobin and Cytoglobin

Neuroglobin and cytoglobin are hexa-coordinated haem-associated globins, which share less than 25% of their structure with Mb and Hb. They are found in much lower concentrations (~0.2 μM) in vertebrates; but their expression is significantly up-regulated in hypoxia [45,95,96]. With Cgb this up-regulation is by a factor of ~1.8 to 2.5 depending on the tissue and the duration of hypoxia [96]. Neuroglobin was originally isolated from neuronal cells in the brain in 2000 [41], but is also expressed in endocrine cells [41-43]. Cytoglobin is found in the cytoplasm of all tissues and organs of vertebrates, with greatest expression in the brain, eyes, liver, heart and skeletal muscle [43,46].

As with Hb and Mb, the deoxygenated forms of Ngb and Cgb reduce nitrite to NO [44,97]. In addition, Cgb's action is potentiated under conditions of low pH: rates of nitrite reduction double when the pH falls from 7 to 6, increasing 15-fold at pH 5.5 [97]. Transformation to a penta-coordinated structure is required for nitrite reductase activity. In Ngb, this is achieved by the oxidation of two of the globin's surface cysteine residues (cysteine-46 and -55) forming a disulfide bridge [44,97]. This diminishes the globins' affinity for the haem-iron, resulting in the transformation from a hexa- to a penta-coordinated structure, with greater affinity for nitrite [98]. The resulting mechanism of nitrite reduction is similar to that of Hb and Mb (see Table 1.2).

Neuroglobin has been shown to have a protective effect limiting neuronal cell death [44], suggesting a role similar to that of Mb in muscle cells. However, the low abundance of Ngb and Cgb questions their significance as nitrite reductases under physiological conditions [99,100].

Table 1.3 compares the nitrite reductase activity of all the haem-containing globins discussed above.

	NO_2^-	NO
DeoxyFe(II)	$MetFe^{(III)}$ + NO	$Fe^{(II)}$-NO (iron-nitrosylated Hb)
OxyFe(II)	$MetFe^{(III)}$ + NO_3^-	$MetFe^{(III)}$ + NO_3^-
MetFe(III)	$MetFe^{(III)}$-NO_2^- (reversible) (+ $NO \rightarrow N_2O_3$)	$MetFe^{(III)}$-NO (reversible)

Table 1.2: Summary of nitrite's and NO's reactions with deoxy-, oxy- and met- haem-containing globins.

Protein		**Haemoglobin**	**Myoglobin**	**Neuroglobin**	**Cytoglobin**
Transition required for nitrite reduction		Allosteric transition between the R and T state	Transition between oxy- and deoxy-genated state	Transition between hexa- and penta-coordinated state	Transition between hexa- and penta-coordinated state
Optimum O_2 tension – p50		20 -26 mmHg	2.2-2.4 mmHg	2.2 mmHg	Unknown
Reaction with nitrite	*pH*	7.4	7.4	7.4	7.0
	Protein	50 µM	25 µM	10 µM	10 µM
	Nitrite	2.5 mM	20 µM	10 mM	2 mM
	k ($M^{-1}s^{-1}$)	~4.4	~12.4	~0.26	~0.14
Refs		42,75,101	40,42,101	42,44,101	96,97

Table 1.3: Comparison of the nitrite reductase activities of haem-containing globins

1.1.3.2.2 Mitochondrial enzymes

The capacity of mitochondria to reduce nitrite under anoxia was recognised by Taylor as early as 1965 [102]. Although the observation pertained to nitrite's action in preserving meats, this has also been demonstrated in a physiological setting, in a process which involves the terminal components of the oxidative chain: complex III, Cytochrome C (CytC) and complex IV [47,50,101,103]. A role for complex III was suggested in an early paper through the demonstration of inhibition of mitochondrial nitrite reduction, under anaerobic conditions, by myxothiazol (a competitive inhibitor of ubiquinol and the activity of complex III) [47].

Complex IV, also known as Cytochrome C oxidase (CcOx), the terminal part of the mitochondrial chain, reduces 95% of inspired oxygen [49]. Nitrite, is also reduced by CcOx; however, extremely low concentrations of oxygen (~2%) are required [50], as oxygen inhibits nitrite reduction in a competitive process. Furthermore, different CcOx isoforms exist, which possess a range of nitrite reductase activities, with the relative expression of each isoform being modulated by oxygen [104]. However, the physiological implications of these finding are uncertain given the requirement for such low oxygen concentrations.

Cytochrome C is an inter-mitochondrial membrane haemprotein, which transfers electrons from complex III to CcOx [48]. Thus, CcOx is dependent on CytC to reduce nitrite [50]. However, CytC also reduces nitrite independently of CcOx [48]. This activity is dependent on its adoption of a penta-coordinated geometry in a manner similar to that required by Ngb and Cygb [48]. However, CytC is unable to shuttle electrons in its penta-coordinated form, bringing into question the physiological relevance of this reaction.

1.1.3.2.3 Molybdenum metallo-enzymes

The family of molybdenum-containing enzymes includes xanthine oxidoreductase, aldehyde oxidase, sulphite oxidase and bacterial nitrate/nitrite reductases [105,106]. These enzymes support the catalysis of a wide range of redox/ hydroxylation reactions [105,106] dependent on molybdenum (Mo) which can exist in a range of oxidation states: Mo(IV), Mo(V) and Mo(VI) [106].

1.1.3.2.3.1 Xanthine oxidoreductase

Xanthine oxidoreductase (XOR) refers to two inter-convertible enzymes found in mammalian cells: xanthine oxidase (XO) and xanthine dehydrogenase (XDH) [51]. Xanthine oxidoreductase exists as homodimer and plays a key role in purine metabolism by catalysing the oxidation of hypoxanthine to xanthine, which in turn is further oxidised to uric acid [51,52]. Thus purine oxidation donates electrons to the Mo site, with reduction of Mo(VI) to Mo(IV) [106]. The Mo site may be similarly reduced following oxidation of NADH at the flavin adenine dinucleotide (FAD) site, which results in the reduction of FAD to $FADH_2$, from where electrons may be transferred to the Mo site [106]. The reduced FAD site ($FADH_2$) of XOR also supports the reduction of oxygen to reactive oxygen species (ROS) including superoxide and H_2O_2 (see Table 1.4). Xanthine dehydrogenase is the predominant intracellular isoform, whereas XO is mainly located on the extracellular membrane [107,108]. Indeed, XOR is present almost exclusively as XO in plasma, due to the action of serine proteases on XDH. Hypoxia induces the up-regulation of XOR expression, activity, and its release into the circulation, from endothelial cells [109]. Fasting and ischemia irreversibly convert XDH to XO, through the oxidation of a critical cysteine, and proteolysis [107,110].

Xanthine oxidoreductase has been shown to reduce nitrite to NO [33,51,106,111]. In rat and human heart tissue, the addition of the molybdenum-site XO-inhibitor allopurinol attenuates NO production from nitrite by ~50%, in a pH-dependent manner [112]. The nitrite reductase activity of XOR is attributed to the reduced Mo(IV) site (resulting from purine or NADH oxidation as described above) which donates electrons to nitrite [51,111] with concomitant oxidation to Mo(V) [106]. However, reduction of oxygen at the FAD site creates competition with nitrite for electrons across XOR, inhibiting nitrite reduction with the molybdenum substrate, xanthine [113]. Therefore, conditions of low oxygen tension favour XOR-mediated nitrite reduction, which is abolished at normal to high oxygen tension [51,111]. However, in the presence of NADH, which competes with oxygen at the FAD site, nitrite reduction is maintained at ~70% of anaerobic levels [113], thus providing a mechanism of XOR-mediated normoxic nitrite reduction. Also, blockade of the FAD site with diphenylene iodonium (DPI) in the presence of oxygen enhances nitrite reduction to NO in rat aorta [114]. In addition to the heart, functional XOR has been demonstrated on the surface of erythrocytes [115]. Erythrocytic XOR may thus provide an additional pathway for nitrite reduction, which avoids scavenging by oxyHb. Indeed, this appears to have a role in hypertension, where erythrocytic XOR is up-regulated with greater nitrite reductase activity [116].

Relative Activities	XO	XDH	Ref
Reduction of O_2 to O_2^-	100%	25% for Rate But amount >100%	117
Reduction of NAD^+ to NADH	0%	100%	118
Oxidation of NADH to NAD^+	50%	100%	119

Table 1.4: The relative activities of XO compared to XDH. The relative activities relate to the redox functions as stated for each substrate.

1.1.3.2.3.2 Aldehyde oxidase

Aldehyde oxidase (AO), another molybdenum-containing enzyme, is involved in the oxidation of aldehydes, and hydroxylation of heterocyclic compounds [54]. Aldehyde oxidase is an intracellular cytosolic enzyme, found in multiple organ systems, including the cardiovascular system, the central nervous system, the respiratory tract and the GI tract, with the highest expression and activity in the liver [54,55]. While mammals possess multiple isoforms of the enzyme, humans possess only one [54]. Sharing up to 86% of its structure with XOR, AO also reduces oxygen, generating ROS [55]. However, it is unable to function as a dehydrogenase [33,110], and is not to be confused with the aldehyde dehydrogenases. Similar to XOR, AO functions as a nitrite reductase, a process enhanced under acidosis and hypoxia [33]. This was demonstrated by inhibition of NO production from nitrite by ~40%, in the liver with raloxifene [33].

1.1.3.2.3.3 Sulphite oxidase

Sulphite oxidase (SO) is also a molybdenum-containing enzyme, involved in sulphur amino acid degradation [120], catalysing the oxidation of sulphite (SO_3^{2-}) to sulphate (SO_4^{2-}) [121]. Expressed in various tissues [57],the structure of human SO is different from XOR and AO, resembling that of plant nitrate reductases [111], but possesses nitrite reductase activity [56].

1.1.3.2.4 Carbonic anhydrase

Carbonic anhydrases are a class of metallo-enzymes, where the zinc ion plays a fundamental role, catalysing the hydration of carbon dioxide, yielding bicarbonate and a proton (Equation 10) [62,122,123].

$$H_2O + CO_2 \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+ \quad (10)$$

This hydration reaction takes place in all living organisms and is essential in many physiological processes, including respiration, CO_2 transport, pH regulation, excretion, secretion and metabolic synthesis [62,124]. The key role of CA is the removal of CO_2 from respiring tissues and buffering pH transients occurring with metabolic activity [125].

Sixteen isoforms have been identified in vertebrates, fifteen of which have been isolated in man [122,126], located in the cell membrane, the cytosol and the mitochondria [62]. The commonest and most active isoenzyme in humans is CAII [62,122,127], which is also the most abundant isoform in erythrocytes. Indeed, CAII possesses one of the greatest rates of enzyme catalysis known [62,122,127]. This supports a rapid local decrease in the capillary pH of active tissues, which in turn facilitates the release and delivery of oxygen from Hb (via the Bohr effect) [128]. Given the similarities between NO_2^- and HCO_3^-, it has been hypothesized that CA may play a key role in nitrite metabolism [63]. This hypothesis is supported by the observations that nitrite can inhibit CA by binding to the HCO_3^- active site [129], and that CA acts on nitrite to release NO under conditions of low pH and normal oxygen tension [63]. Surprisingly, inhibition of CA with dorzolamide or acetazolamide potentiates total NO release ~6-fold at pH 7.2, and ~2 fold at pH 5.9; however, the rate of production was unaffected. The zinc active site

cannot participate in redox reactions to support nitrite reduction, but instead behaves as a nitrous anhydrase [63], catalysing the following reaction (Equation11):

$$2NO_2^- + 2H^+ \leftrightarrow 2HNO_2 \leftrightarrow H_2O + N_2O_3^- \quad (11)$$

N_2O_3 then rapidly breaks down to form NO_2 and NO (see Equation 4). Alternatively, the reaction may be supported by stepwise nitrosation in the presence of thiols (e.g. glutathione), with the formation of zinc thiolate and nitrous acid (HNO_2); the latter further reacting with thiols to give a nitrosothiol [130].

1.1.3.2.5 Nitric oxide synthase

The NOS family of enzymes comprises three isoforms: neuronal NOS (nNOS / NOS1), inducible NOS (iNOS / NOS2) and endothelial NOS (eNOS / NOS3), with ~50% variation in protein structure between them [64,131]. Through an oxygen-dependent reaction, NOS oxidises the guanidino nitrogen atoms of L-arginine, forming NO and L-citrulline [131]. In anoxia, when this reaction is impaired, it is now appreciated that eNOS can use nitrite as a substrate, restoring NO production to baseline levels [65,132]. In elegant experiments, the nitrite reductase activity was located to the oxygenase domain of eNOS [65]. The biological relevance of nitrite's reduction by eNOS has been shown with human erythrocytes, whereby inhibition with L-NAME attenuated the production of NO from nitrite [115].

1.1.3.2.6 Cytochrome P450

Cytochrome P450 (CYP450) is a ubiquitous family of enzymes, belonging to the monooxygenase superfamily [58,59]. They are haem-containing proteins, mainly functioning as catalysts in the oxidation of organic compounds (RH) (Equation 12) [60,133].

$$O_2 + RH + NAPDH + H^+ \rightarrow ROH + H_2O + NADP^+ \quad (12)$$

Feelisch et al, found that the inhibition of microsomal CYP450 1A1, with ethoxyresorufin, attenuated the nitrite reductase activity of rat liver homogenates by ~40%, under hypoxic conditions [61]. Curtis et al, also demonstrated a role for hepatic CYP450 in nitrite reduction, however no effect in heart, lung or brain was found [134].

1.1.4. Nitrite and cardiovascular protection

The decline in circulating NO has been implicated in a number of cardiovascular disease processes [135,136]. In humans, the number of cardiovascular risk factors is inversely correlated with plasma nitrite concentrations [8]. The restoration of NO levels, through exogenous nitrite, has a positive impact on a number of mammalian models of cardiovascular disease [137-143]. A summary of a selection of nitrite studies in cardiovascular protection is presented in Tables 1.5 and 1.6, and detailed descriptions in sections 1.1.4.1-1.1.4.6.

Organ	Model	Nitrite administration			Outcome Targets	Ref
		Route	Time	Dose		
Heart	MI	i.v. Infusion	5min prior to reperfusion	70 µmol over 5 min	Infarct size LVEF	144
	Dobutamine induced ischemia		20min during stress echo	1.5 µmol/min	Peak systolic velocity Strain rate	145
Forearm	IRI-induced endothelial dysfunction		20min prior to or during ischemia	1.5 µmol/min	FMD	
		p.o.	Prior to ischemia	*Nitrate*	FMD	146

Table 1.5: Summary of studies into nitrite's effects on cardiovascular disease models in humans: MI – Myocardial infarction, LVEF – Left ventricular ejection fraction. FMD – Flow mediated dilatation.

Organ	Species	Model	Nitrite administration			Outcome target	Ref
			Route	Time	Dose		
Hind limb	Mouse	Ligation of femoral artery	i.p.	During ligation	165 µg/kg or 3.3 mg/kg	Angiogenesis Limb perfusion	138
	Mouse		i.p.	5 days Post ligation	165 µg/kg	Angiogenesis Limb perfusion	147
	Mouse	Ligation of femoral artery in hypertension	p.o.	Post ligation	50 mg/L	Angiogenesis Limb perfusion	148
	Mouse	Ligation of femoral artery in diabetes	i.p.	5 days Post ligation	165 µg/kg	Angiogenesis Limb perfusion	149
Cardiac arrest	Mouse	Induced cardiac arrest and resuscitation	i.v. bolus	At initiation of CPR	50 nmol	Cardiac and neurological function Survival	140
Heart	Mouse	MI by occlusion of coronary vessel	i.v. bolus	20 min before reperfusion	2.4 to 1920 nmol	Infarct size	137
	Mouse		p.o.	For a week prior to ischemia	50 mg/L	Infarct size	150
	Mouse		p.o.	24 hrs prior to ischemia	9.6 µmol/Kg	Infarct size	151
	Mouse		i.p.	24 hrs or 5min before reperfusion	48 nmol	Infarct size	
	Mouse		Intra-ventricle	5 min before reperfusion	48 nmol	Infarct size	152
	Rat		i.v. bolus	15 min before or after onset of ischemia	0.4 – 1.0 mg/kg	Infarct size	153
	Rat	Isolated perfused heart	i.v. infusion	15 min before ischemia	1 – 300 µg	CFR LVDP	
	Rat			15 min before reperfusion	10 µM and 100 µM	Infarct size LVDP	112
	Dog	MI by occlusion of coronary vessel	i.v. infusion	During ischemia – 60 or 5 min before reperfusion	5 min – 0.2 µmol/kg/min 60 min – 0.2 then 0.17 µmol/kg/min	Infarct size LVEF Microvasc-perfusion	139

Table 1.6: Summary of studies into nitrite's effects on cardiovascular disease in animal models: MI – Myocardial infarction, ED – Endothelial dysfunction, CPR – Cardiopulmonary resuscitation, CFR – coronary flow rate, LVDP – Left ventricular developed pressure, LVEF – Left ventricular ejection fraction, FMD – Flow mediated dilatation.

1.1.4.1 Endothelial dysfunction

Hypercholesterolemia [154] and advancing age [155] have a clear association with the development of cardiovascular disease (CVD). This augmented risk is thought to be related to their induction of endothelial dysfunction, through a reduction in NO bioavailability, either as a result of reduced NO production, or increased NO consumption - mainly by ROS.

Endothelial dysfunction, caused by hypercholesterolemia, leads to a proinflammatory phenotype in the microcirculation [156,157]. Stokes et al, demonstrated that the supplementation of dietary nitrite in hypercholesterolemic mice prevented elevated C-reactive protein (CRP), micro-vascular inflammation, and endothelial dysfunction [158]. Notably, nitrite had no effect on serum cholesterol levels (although triglyceride levels were reduced) suggesting a direct anti-inflammatory effect.

Endothelial dysfunction, associated with old age, affects the arterial system leading to stiffening of the large elastic arteries [155]. Sindler et al, found that older mice had ~45% lower levels of circulating and tissue nitrite [141]. Supplementation with nitrite restored nitrite levels to those found in younger mice, and significantly reduced age related oxidative stress, endothelial dysfunction and arterial stiffening [141]. This mechanism was proposed to be via nitrite's inhibition of elevated expression of NADPH oxidase (an important source of ROS) and cytokines (IL-1b, IL6, INFγ and TNFα).

1.1.4.2 Hypertension

Nitrite, via reduction to NO (which has been proposed to be mediated by deoxyHb) vasodilates resistance arterioles, reducing mean blood pressure in healthy volunteers [28]. Therefore nitrite has the potential to control hypertension, an important risk factor for CVD [154]. Indeed, nitrite administration to hypertensive rats reduces blood pressure [116,159-161], via reduction to NO by erythrocytic XOR [116], and down-regulation of vascular NADPH oxidase [161].

Several studies show that supplementation with nitrate, as a source of nitrite (via the entero-salivary circulation), reduces BP in healthy volunteers [146,162-167] and hypertensives [116]. In hypertensives, erythrocytic XOR expression is double that of normotensives, and this is associated with XOR-dependent nitrite reductase activity at pH 7.4 [116], which was not seen in normotensives [115]. It is therefore likely that this contributed to the substantial BP lowering effect when nitrate (~3.5 mmol) was given to grade 1 drug naive hypertensive patients [116]. In a rat model of hypertension, the BP lowering effects of nitrite were abolished by the XOR inhibitor allopurinol further suggesting a key role for the enzyme in nitrite reduction to NO in vascular tissue [116]. In addition, recent evidence suggests that nitrate derived from NOS under 'basal' (i.e. non-nitrate-supplemented) conditions, also reduces blood pressure, as demonstrated by a consistent ~3 mmHg increase in systolic BP during a 7-day period of sustained antibacterial mouthwash use [168]. This was associated with a ~25% decrease in circulating nitrite levels. Both effects were rapidly reversed on cessation of the mouthwash.

1.1.4.3 Intimal hyperplasia

Vascular damage usually leads to an appropriate healing response. However, dysfunctional endothelial cells can cause an exaggerated healing response, leading to inappropriate remodelling and intimal hyperplasia (IH). This in turn leads to the development of plaques and flow-limiting disease [169,170]. Furthermore, there is evidence that this inappropriate healing response is precipitated by attenuated NO availability [8,171,172].

In a murine model of vascular injury, restricting nitrate/nitrite intake intensified the damage. By contrast, supplementation with nitrite protected injured vasculature from an inappropriate healing response, and resulted in the regression of IH [142]. Although vascular injury was associated with amplified expression of XOR, the chronic inhibition of XOR worsened the vascular injury [142]. Furthermore, the protective effects of nitrite supplementation were lost, when nitrite was given in conjunction with the Mo-site XOR inhibitors, allopurinol and tungsten [142].

1.1.4.4 Peripheral artery disease

Peripheral artery disease (PAD) results from atheroma in the arterial system, leading to interruption of blood flow to the extremities. Using a murine model, Kevil's group demonstrated that intra-peritoneal (i.p.) nitrite administration during acute femoral artery ligation, had multiple beneficial effects, promoting endothelial cell proliferation, encouraging angiogenesis and arteriogenesis, enhancing vascular density, and re-establishing vascular flow into the ischemic limb [138]. Many of the effects were evident within 3 days following ligation. These effects were abrogated by the NO scavenger carboxy 2-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl-3-oxide (C-PTIO). No nitrite-induced changes occurred in the non-ischemic limb [138]. Using the same model in hypertensive mice, the group demonstrated that oral supplementation with nitrite in the drinking water for 2 weeks following ligation, also resulted in total restoration of blood flow in the ischemic limb, associated with a rise in cGMP levels [148]. These effects were prevented by XOR inhibition with allopurinol, suggesting a key role for nitrite-derived NO. In addition to producing similar beneficial effects in diabetic mice, post-ligation nitrite supplementation was also found to prevent tissue necrosis [149]. In humans with PAD, dietary nitrate supplementation (as a source of circulating nitrite) improved exercise time and time to exhaustion by ~18% and 17% respectively [173].

1.1.4.5 Myocardial infarction

Myocardial infarction is a result of coronary occlusion interrupting blood flow, causing myocardial cell necrosis through ischemia. Ischemia leads to acidosis and ion imbalance within the cell. While reestablishment of flow is necessary to reverse this process and salvage tissue, the reintroduction of oxygen consequent to reperfusion causes increased production and release of ROS, which leads to inflammation and accelerated cell death [174,175]. The majority of experimental studies have demonstrated an important cardio-protective role for NO in ischemia reperfusion injury (IRI) [32,176]. Despite inorganic nitrite acting as a source of NO under ischemia and acidosis, nitrite was originally thought to be detrimental in IRI [23]. However, in 2004 a protective effect in IRI was demonstrated in the heart by Webb et al, whereby nitrite (10 μM and 100 μM) attenuated infarct size by ~63% in an *ex-vivo* model of IRI, in the Langendorff rat heart [112]. These protective effects were abolished by the NO scavenger C-PTIO, demonstrating the requirement for nitrite reduction to NO. Since then, nitrite has been shown to have protective effects in the setting of myocardial infarction [137,139,152] as well as other organs subjected to an ischemic insult and reperfusion injury [137,177,178]. For example, Duranski et al, showed that the *in-vivo* administration of intra-ventricular nitrite in mice, prior to reperfusion, resulted in a ~67% reduction in infarct size [137]. This protective effect was also abolished with the NO scavenger C-PTIO, but was unaffected in eNOS knockout mice. This supports a role for nitrite-derived NO, as opposed to eNOS-derived NO.

Shiva et al, showed that the administration of nitrite to mice, 24 hours before an ischemic insult, either via the i.p. or oral route (via needle gavage), attenuated

infarct size ~33.9% and ~52.7% respectively. In addition, i.p. administration immediately before reperfusion reduced infarct size ~66% [151]. Also in a murine model, Bryan et al, demonstrated a protective effect of 7 days oral supplementation with nitrite (50 mg/L in the drinking water), reducing infarct size ~48% [150]. Nitrite supplementation was associated with increased tissue stores of nitrite and SNOs – suggesting a protective mechanism via s-nitrosylation, in addition to that conferred by nitrite-derived NO [150]. Importantly, dietary depletion of nitrate and nitrite had the opposite effects, increasing infarct size ~59% when compared to controls. Gonzalez et al, simulated a myocardial infarction with revascularization therapy in a canine model, whereby nitrite was given intravenously either during the last 60 min or last 5 min of a 2 hour ischemic insult, reducing infarct size by ~67% and ~48.5% respectively [139].

A role for XOR-mediated nitrite reduction was demonstrated by Webb et al, via inhibition of NO production using allopurinol and (-)BOF-4272, in homogenised rat and human myocardium, the latter collected from patients undergoing mitral valve replacement surgery [112]. Baker et al, confirmed the functional significance of XOR in the rat Langendorff IRI model, whereby oxypurinol blocked the protective effect of nitrite, and also showed important roles for NADPH oxidase, and sarcolemmal and mitochondrial ATP-sensitive potassium (K_{ATP}) channels in nitrite-mediated cardioprotection [153]. Several studies provide evidence for Mb. For example, Rassaf et al, highlighted a functional role for nitrite reduction by deoxyMb, diminishing myocardial oxygen consumption and cardiac contractility in the Langendorff rat heart [88]. Moreover, Hendgen-Cotta et al, demonstrated that the administration of intra-ventricular nitrite prior to reperfusion attenuated infarct size by ~61%, an effect that was abolished in Mb-deficient mice [152].

Nicholson et al, recently reported surprising findings, whereby 4-weeks voluntary exercise in mice down-regulated myocardial Mb (via inhibition of calcineurin and NFAT signalling) attenuating myocardial nitrite reductase activity, and the beneficial effect of nitrite supplementation on myocardial IRI [179]. Overall, it is likely that myocardial nitrite reduction proceeds with variable relative contributions from XOR and Mb, depending on the conditions and the species.

While these studies were conducted in animal models, there is some evidence that this may translate to humans. For example, in an IRI model in the forearm of healthy volunteers, supplementation with dietary nitrate, as a source of nitrite, significantly attenuated IRI-induced endothelial dysfunction [146,165]. Using a similar IRI model in healthy volunteers, Ingram et al, demonstrated comparable protection when intravenous nitrite was administered prior to, but not during the ischemic event [145]. In patients with stable, but inducible myocardial ischemia, the intravenous administration of nitrite (1.5 μmol/min for 20 min) during a dobutamine stress echocardiographic study, resulted in a significant improvement in myocardial function in the ischemic territories, with no effect in the normally perfused areas [145]. Though, in a recently reported trial, which attempted to replicate the design of the Gonzalez et al study, administering 70 μmol of sodium nitrite to patients presenting with an acute ST elevation MI (as an i.v. infusion over 5 min prior to revascularization therapy) was found to be ineffective in mitigating the deleterious effects of IRI [144]. This may in part be related to the dose of nitrite used which resulted in only a small rise in circulating nitrite concentration to 1.4 μmol/L (in the Gonzalez et al study the rise in circulating nitrite was ~5 μmol/L). The results of a further trial in which

nitrite was administered as an intracoronary bolus prior to reperfusion are still awaited [180].

1.1.4.6 Cardiac arrest

In a murine model of cardiac arrest, plasma heart and brain nitrite levels were found to be significantly depleted post-arrest [140]. Treatment with nitrite peri-arrest, partially restored these levels, improved cardiac function, reduced the incidence of arrhythmias and hypotension, and significantly enhanced left ventricular and right ventricular ejection fractions post-arrest [140]. This in turn translated into a significantly higher survival rate in the nitrite-treated group compared to the placebo arm. A study looking at the same model in humans is underway [181].

1.1.5 Nitrite, Mitochondria and cardiovascular protection

It is now clear that nitrite mitigates the deleterious effects of several cardiovascular pathological processes. The most widely investigated of which is IRI, where nitrite has been shown to be beneficial in a number of mammalian models, over a wide dose range and a broad temporal window. Furthermore, the conversion of nitrite to NO is required to achieve most, but not all of its cytoprotective effects [112,137,143,151].

While mitochondrial proteins act as nitrite reductases (as discussed in section 1.1.3.2.2), nitrite also modulates mitochondrial function [40,151] structure [143] and density [182]. Also, tissues with higher mitochondrial oxygen consumption demonstrate greater nitrite reductase activity, suggesting a further link [61]. Besides ATP generation, normal mitochondrial function includes redox signalling [183] and regulation of cell apoptosis [184]. However, these functions are also implicated in the progression of injury during ischemia and reperfusion [185]. During ischemia, mitochondria shift from fatty acid to glucose metabolism, a less efficient source of energy and ATP generation [186]. This anaerobic glycolysis results in decreased intracellular pH, mediated through the accumulation of H^+, NADH and lactic acid [187-189]. The continuation of these disturbances through persistent hypoxia leads to the cessation of ATP production, depletion of high-energy phosphate stores and cell death. With oxygen influx on reperfusion, mitochondria revert to oxidative metabolism of fatty acids, reinitiating electron transfer and ATP synthesis [187]. However, this leads to excessive ROS production at complexes I and III [190,191], with deleterious effects on mitochondrial and cellular proteins [192,193]. In particular

opening the mitochondrial membrane permeability transition pore (MPTP), which releases membrane-bound CytC into the cytoplasm, instigating cellular apoptosis [184,194,195].

During ischemia, nitrite appears to confer cytoprotection via its reduction (e.g. by Mb) to NO, modulating mitochondrial function. For instance, NO binds reversibly to the $copper_B/heme_{a3}$ binuclear centre of CcOx, inhibiting mitochondrial respiration by competing with oxygen [196-198]. This inhibition becomes more effective in hypoxia [199], conserving oxygen (short-term hibernation) during ischemia. Furthermore, nitrite has also been shown to increase the efficiency of oxygen utilization by mitochondria. Larsen et al, found that, following supplementation with dietary nitrate (as a source of circulating nitrite) in humans, mitochondria displayed an improvement in oxidative phosphorylation efficiency (P/O ratio) through a reduction in the expression of ATP/ADP translocase limiting proton leak [200]. In essence ATP production became more efficient requiring less oxygen [200]. Although the mitochondria were harvested from the musculoskeletal system of healthy volunteers, it is likely that a similar process occurs in myocardial mitochondria. Therefore, such a reduction in oxygen requirements would confer partial protection of the myocardium during ischemia.

On reperfusion, nitrite has direct effects on complex I and CytC. S-nitrosation of complex I is known to confer cytoprotection in myocardial IRI [201,202]. Nitrite has been shown to reversibly s-nitrosate and inhibit complex I, attenuating ROS production, stabilising MPTP and inhibiting CytC release [140,151]. In addition, NO also prevents CytC's release via s-nitrosylation of its haeme moiety, maintaining it in a hexa-coordinated membrane-stable form [203-208].

During normoxia, nitrite directly affects mitochondrial function, promoting mitochondrial fusion via protein kinase A (PKA) activation. This in turn inhibits dynamin-related protein 1 (Drp1) mediated mitochondrial fission [143]. Promotion of mitochondrial fusion and inhibition of fission is known to be cardioprotective during IRI [209,210]. Nitrite-mediated mitochondrial fusion resulted in an increase in the mitochondrial permeability potential, enhancing ROS production which activates AMP-activated protein kinase (AMPK) through oxidation and phosphorylation [143], a process which has an established protective role during IRI [211], and may form the basis for nitrite-mediated preconditioning. In addition, nitrite mediated AMPK phosphorylation has also been shown to mitigate smooth cell hyperproliferation in a murine model of carotid injury. This protective effect is caused by AMPK's activation of sirtuin-1, which in turn leads to the deacetylation of proliferator-activated receptor-γ coactivator 1α (PGC1α), a key receptor in the regulation of mitochondrial biogenesis [182]. The promotion of mitochondrial biogenesis increases cellular mitochondria content contributing to cytoprotection. Moreover, AMPK activation has been implicated in protection in diabetes mellitus [212] and drug-induced cardiomyopathy [213] and may explain part of the mechanism of (nitrate-)-nitrite-mediated protection against doxorubicin-induced cardiomyopathy, in addition to preservation of complex I activity, as demonstrated in the study by Zhu et al, [214].

The above proposed mechanisms of exogenous nitrite-mediated pre-conditioning resemble those observed during ischemic pre-conditioning (IPC). This suggests that IPC may confer its protective effects through endogenous nitrite-mediated production. Indeed, IPC has been shown to elevate endogenous myocardial nitrite levels via up-regulation of iNOS [215], and to

reversibly s-nitrosate and inhibit complex I [201]. Furthermore, the protection conferred through nitrite administration and IPC, have a dual temporal effect (termed acute and late) [151], with a similar magnitude at peak protection [143].

1.1.6 Alternative mechanisms of nitrite signalling

Nitrite may also indirectly mediate its effects through the production of SNOs [216], nitro-fatty acids [217] or directly, without the need for an intermediate or for conversion to NO [11,218].

1.1.6.1 S-nitrosothiols

S-nitrosothiols are formed through a process of s-nitrosation of cysteine, resulting in the addition of a nitroso group (RNO) to the sulphur atom [216,219]. S-nitrosothiols can be formed chemically through the reaction of a thiol and nitrous acid [219], the latter produced from nitrite as shown in Equation 1. Alternatively, generation of N_2O_3, e.g. according to Equation 3, also nitrosates thiols (see Equation 13) [219].

$$N_2O_3 + RS \rightarrow RSNO + NO_2^- \quad (13)$$

In addition, NO (formed from nitrite reduction or disproportionation, as per Equation 4) may react directly with a thiol radical [220]. The rate of this reaction is of a magnitude similar to that between NO and other free radicals or haems [216,220]. S-nitrosothiols may also be formed via enzymatic catalysis leading to a direct reaction between NO and a thiol where the metallic constituent of the enzyme allows for the oxidation of the intermediate thionitroxyl radical ($RSNOH^{\bullet}$) [216]. However, many of the proposed enzymatic reactions are thought to be unlikely either due to unfavourable kinetics or due to the immediate production of nitrite in preference to the production of nitrosothiols in the presence of water [221]. A kinetically favourable reaction involves mitochondrial CytC and its production of GSNO. Although this enzyme is confined to the inter-membrane space of the organelle, and GSNO is too large a molecule to

cross the membrane, it has been shown that a reduction in the expression of CytC in cells attenuates the production of SNOs [222]. S-nitrosothiols are present in human plasma primarily as s-nitrosoproteins. Albumin's abundance and the availability of its free cysteine group to nitrogen oxides makes s-nitrosoalbumin the most plentiful form of s-nitrosoproteins in the circulation [223]. However the ingestion of nitrate acutely increases plasma levels of nitrate and nitrite, but not necessarily SNOs [224]. By contrast, supplementation with nitrite (via the i.p. or oral route in mice) results in acute SNO-formation under normoxic conditions, suggesting nitrite-mediated nitrosation of thiols, independent of NO formation [11]. S-nitrosothiols mediate their cardioprotective effects via the transnitrosation of many proteins, including mitochondrial complex I, as described in section 1.1.5.

1.1.6.2 Nitro-fatty acids

Other nitrite-derived products, such as peroxynitrite ($ONOO^-$) lead to the nitration of unsaturated fatty acids. The endogenous production of these nitro-fatty acids is enhanced under conditions of hypoxia [225]. Nitro-fatty acids are found in the circulation in nanomolar to low micromolar concentrations [226]. In an *in vitro* model using an isolated Langendorff rat heart, IPC was found to promote the endogenous generation of nitro-fatty acids [227]. In an *in vivo* murine model, myocardial IRI was found to enhance fatty acid nitration in myocardial cells and mitochondria [228]. Furthermore, the administration of exogenous nitro-fatty acids 15 min prior to, or at reperfusion, had a significant protective effect leading to a reduction in infarct size by ~46% compared to control. This enhanced protection is probably mediated through increased expression of the antioxidant enzyme haem oxygenase-1 (HO-1), elevation of MicroRNA-499 (miRNA-499) levels, and abolition of the expression of p53 and Drp-1 [229].

1.1.6.3 Direct nitrite signalling

In addition, nitrite at physiologically relevant levels has been shown to directly induce haem-nitrosylation (NO-haem) and s-nitrosation in mammalian tissues (with the exception of the brain and the aorta) without the requirement for nitrite reduction [11]. The increase in RSNO and NO-haem was dose-dependent and occurred despite NO-scavenging with C-PTIO. Furthermore, under the prevailing conditions of normal oxygen and pH, nitrite increased cGMP levels, attenuated CYP450 activity, up-regulated expression of heat shock 70 protein (Hsp70) and down-regulated expression of HO-1 [11]. Moreover, in a different study that also demonstrated nitrite-mediated dilatation of endothelium-intact vessels without the need for reduction to NO, the dilatory effects were constrained by the inhibition of cyclooxygenase (COX) [218] suggesting a role for the enzyme in direct nitrite signalling.

1.2 Comparison between inorganic and organic nitrate/nitrite

1.2.1 History

In current clinical practice, the term ‘nitrate’ refers mainly to organic nitrates, such as glyceryl trinitrate (GTN), with an emerging, but generally still rather limited, understanding of the potential relevance of inorganic/dietary nitrate. Other than as a useful sign of a urinary tract infection on urinalysis, ‘nitrite’ holds little relevance therapeutically: sodium nitrite (inorganic) is a seldom-used treatment for cyanide and hydrogen sulphide poisoning; whilst the organic nitrite amyl nitrite is a drug of abuse (poppers).

However, to view amyl nitrite solely in this light would be to do it a great disservice. Amyl nitrite was first used by Sir Thomas Lauder Brunton in 1867 in the treatment of angina, making organic nitrates the first class of synthetic medical drugs [230,231]. Indeed, this explains the predominance of organic nitrates, a position further consolidated in 1876 when glyceryl trinitrate (GTN), renamed from Nitro-glycerine to distance it from its origins in the manufacturing of explosives, was introduced by William Murrell into medical application for the treatment of angina [231]. It superseded amyl nitrite due to its easier delivery and longer duration of action. Since then organic nitrates have been used extensively in the treatment of numerous conditions ranging from heart failure to anal fissures [232]; and have continued to be used by physicians for over 150 years, despite major limitations such as tolerance and headaches.

Despite this predominance of organic nitrates, inorganic nitrate has a much longer history in the treatment of coronary artery disease (CAD), being used as

early as 700 AD by the Chinese [233], although it was not until the period between the 14th-17th centuries until the therapeutic properties of inorganic nitrate and nitrite were recognised in the West [13], and not until 1880 that the cardiovascular uses of inorganic nitrite were recognised [233,234].

The first known use of inorganic nitrate was as a preservative to cure foods as a result of its antibacterial properties. Calcium nitrate, also known as wall saltpetre, was used in this capacity over 5000 years ago. Potassium nitrate (saltpetre) and sodium or potassium nitrite continue to be used to this day especially in the process of curing meats (now predominantly nitrite) and remain the most effective method to reduce bacterial growth and kill *botulinum* spores [235]. However, inorganic nitrate, added in the form of celery powder, continues to be used in the curing of organically produced meats, where the action of endogenous or artificially added bacteria eventually reduces it to nitrite [236]. The use of inorganic nitrate and nitrite continued until the early part of the 20th century being prescribed indiscriminately by physicians and in copious amounts, to treat not only CVD, but also other conditions such as epilepsy, lung disease and as diuretics to treat oedema [13]. This practice was discontinued when reports of nitrite toxicosis appeared. Concerns regarding the possible formation of potentially carcinogenic dimethylnitrosamine from sodium nitrite [237,238] and nitrate [239] led them to fall out of favour and their medicinal use had been limited to the treatment of cyanide and hydrogen sulphide poisoning, until very recently.

Richter and Mitchell, in 1880, acknowledged that amyl nitrite and inorganic nitrite may exert similar effects on the vasculature, an observation further extended by Atkinson in 1888 [240]. In the remainder of this chapter comparisons

will be made between organic and inorganic nitrate/nitrite with regards to the similarities and differences in their chemical structures, bio-activation pathways, pharmacological activity and effects, and current/possible future therapeutic applications.

1.2.2 Chemistry

As one would expect, the differences and similarities in the biological effects of organic and inorganic nitrates/nitrites are closely related to their underlying chemical structure.

Inorganic nitrate (NO_3^-) and nitrite (NO_2^-) are salts of nitric acid and nitrous acid respectively. The nitrate and nitrite anions are bonded to a metal cation, commonly Na^+ or K^+ (Figure 1.1). They are water-soluble and occur naturally in the environment through the fixation of atmospheric nitrogen and oxygen [14]. Inorganic nitrate (and nitrite in trace amounts) thus produced then find their way into water and soil with inorganic nitrate taken up by plants, especially green leafy vegetables and beetroot [13]. In addition, nitrate and/or nitrite is added to cured meat as a preservative [233,241]. Ingestion of nitrate by humans, results in its reduction to nitrite largely via the entero-salivary circulation [7,233]. However, the bulk of circulating and tissue nitrate/nitrite in mammals is formed endogenously via the oxidation of the NO derived from the enzymatic degradation of L-arginine by the NOS enzymes [2,3,242]. Endogenously produced NO is predominantly oxidised to nitrate via the actions of oxyhaemoglobin; however, about 20% is oxidised to nitrite via a reaction catalysed by ceruloplasmin [243]. Furthermore, nitrite can also be formed in mammalian tissue by the reduction of nitrate with xanthine oxidoreductase [21].

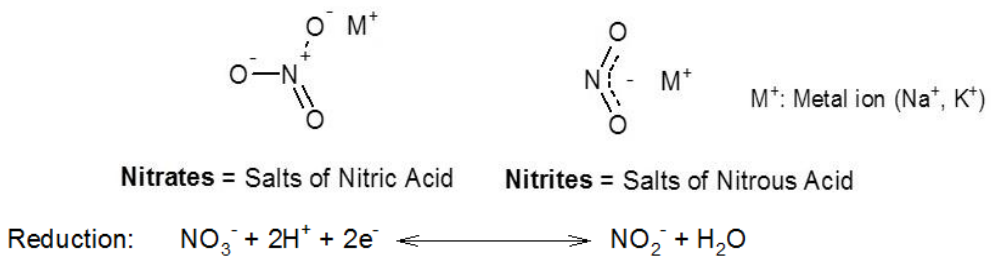

Figure 1.1: The chemical structure, activity and relationship of inorganic nitrate/nitrite

Organic nitrates are synthetic compounds (see Table 1.6) produced by nitrooxylation, a reaction between nitric acid (HNO_3) and an alcohol group (R-OH) where R- represents any organic residue (Equation 14).

$$R\text{-}OH + HNO_3 \rightarrow RONO_2 \quad (14)$$

This results in the formation of esters of nitric acid ($RONO_2$); hence, organic nitrates are small non-polar hydrocarbon chains attached to a nitrooxy- radical ($-ONO_2$), and it is this radical which imparts its biological effects. The only exceptions are amyl nitrite and ethyl nitrite which bear the nitro- radical (–ONO) functional group instead. Furthermore, the addition of aliphatic or aromatic groups of variable length and volume will further affect the lipophilic properties of the molecule [244]. The most commonly used medicinal organic nitrate is glyceryl trinitrate (GTN), however others include pentaerithrityl tetranitrate (PETN), isosorbide dinitrate (ISDN) and isosorbide mononitrate (ISMN) [245], the structures are displayed in Table 1.7.

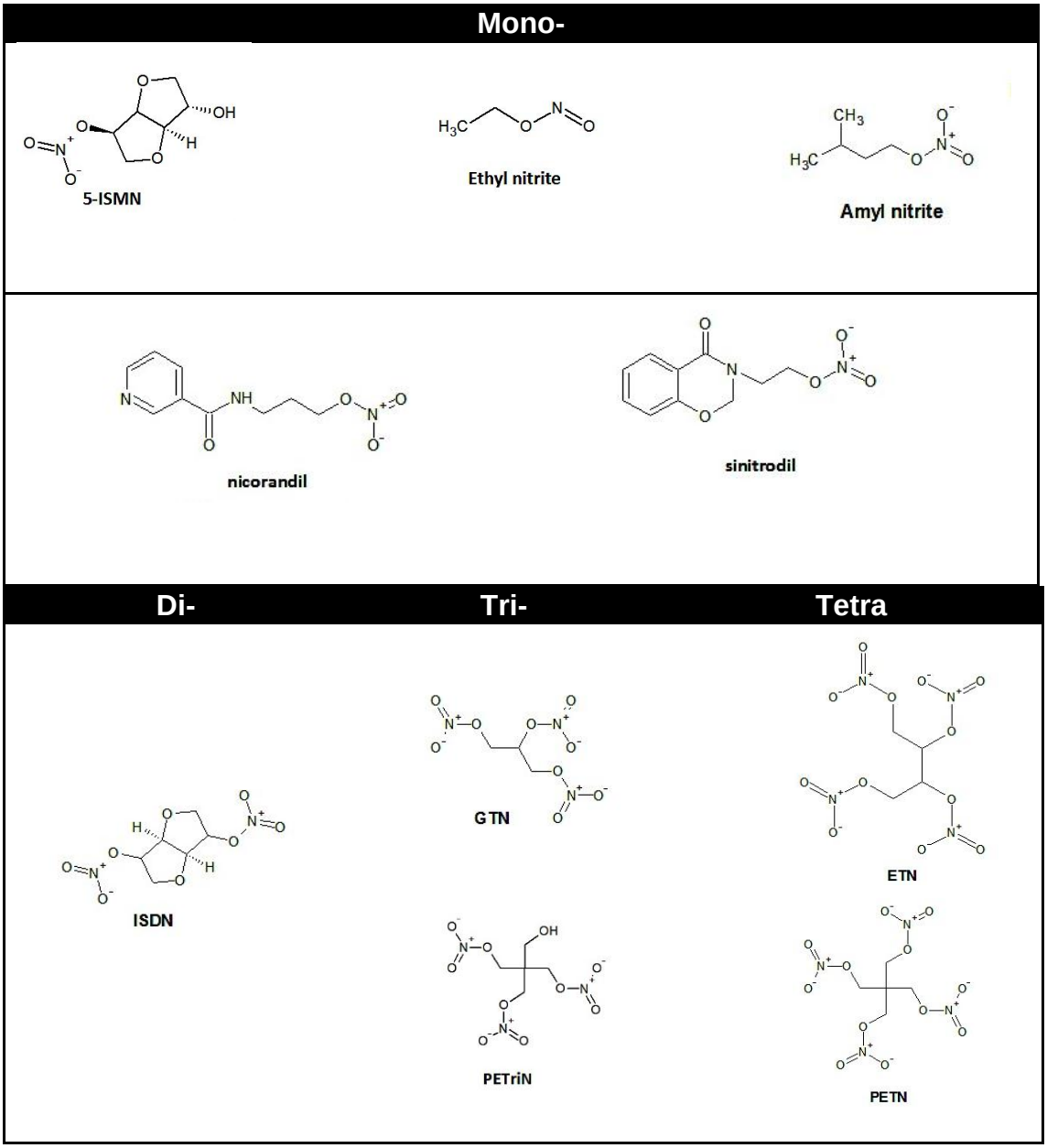

Table 1.7: Chemical structures of organic mono-, di-, tri- and tetra- nitrates/nitrites. (ISMN = isosorbide mononitrate, ISDN = isosorbide dinitrate, GTN = glyceryl trinitrate, PETN = pentaerithrityl tetranitrate, PETrin= pentaerythritol-trinitrate, ETN = erithrityl tetranitrate). *Drawn using ChemSketch software and adapted from* [246]

1.2.3 Pharmacokinetics

The pharmacokinetic profiles of organic and inorganic nitrates/nitrites differ significantly. This difference was alluded to as early as 1928 by Bodo who noted that although both classes of compounds resulted in vasodilatation, the magnitude and duration of the effects were different for sodium nitrite and amyl nitrite, where the latter had a more pronounced but shorter lived effect [247].

Many organic nitrates/nitrites, such as GTN, amyl nitrite and PETN have poor oral bioavailability as they undergo first pass metabolism. In the case of GTN up to 80 – 90% of the drug is metabolised by the liver. Hepatic glutathione reductase and glutathione-S-transferase progressively denature the drug yielding nitrate, nitrite and the other by-products, such as glyceryl-1,3-dinitrate which is inactive; this is in contrast to it's metabolism in the vasculature which yields the active by-product glyceryl-1,2-dinitrate [248]. However, the high lipophilicity of organic nitrates allows for other routes of administration such as buccal, sublingual or trans-dermal [249]. Other organic nitrates such as ISMN, ISDN and nicorandil have better oral bioavailability profiles with 0%, 50% and 25% of the drug being metabolised in the liver respectively [250-252]. Once introduced into the blood stream, levels quickly rise which account for the rapid onset of action (1 – 3 min); however, they are also rapidly cleared from the plasma, which in turn gives them a rapid offset (15 - 30 min) [253].

Inorganic nitrate and nitrite are hydrophilic salts which do not undergo first pass metabolism by the liver and so can be readily administered orally. Ingested inorganic nitrate is readily absorbed across the upper gastrointestinal tract; ~75% is excreted by the kidneys, with trace amounts being secreted by sweat gland and ~25% concentrated in the salivary glands [6,7,14,254]. Upon secretion

into the oral cavity, the nitrate in the saliva is reduced to nitrite by the action of commensal bacteria found on the back of the tongue [18]. The swallowed nitrite, under the influence of the acidic conditions in the stomach, becomes protonated to nitrous acid (see Equation 1). In turn, a proportion of the nitrous acid decomposes to produce NO and other derivatives (see Equations 2-4) [19,20,255]. This reaction is further enhanced by low pH, ascorbate and polyphenols [256,257]. After the ingestion of nitrate, either in its dietary or medicinal form, there is a sharp rise in the salivary, plasma and urinary levels of nitrate and nitrite [7]. In the plasma the levels of nitrate rise within 30 min and peak at 3 hours and are sustained for up to 24 hours; in contrast the levels of nitrite rise more gradually to a significant level by 1 - 1.5 hours and plateau at ~2.5 hours and remain significantly elevated for up to 6 hours [165]. If inorganic nitrite is ingested orally, its bioavailability is around 95-98% [258].

In addition, the interaction of inorganic nitrite with alcohol under acidic conditions leads to the production of the organic nitrite ethyl nitrite. These conditions can be replicated *in vivo* when tissue nitrite interacts with ingested alcohol (red wine or distilled spirits) in the stomach [259]. The quantities of ethyl nitrite produced are governed by the amount of nitrite in the saliva available for this reaction in the gut, the concentration of alcohol and the stomach acidity, the latter being more important. Interestingly the vasoactive effects of ethyl nitrite (which are mediated through NO generation) were found to be more potent than those of nitrite itself [259].

1.2.4 Metabolism and bioactivation

The biological effects of organic and inorganic nitrates are predominantly a result of their NO donor capacity. Both organic nitrates/nitrites and inorganic nitrate are pro-drugs requiring conversion to their active metabolites (although nitrite as described in section 1.1.6.3 has been shown to posses direct signalling capabilities)[11,218]. The mechanisms by which organic nitrates are activated are not fully understood. However they are divided into two subsets those with high potency and 3 or more nitrate groups (GTN, PETriN and PETN) and those with low potency and 1 or 2 nitrate groups (ISDN and ISMN). Both subsets are thought to be activated via the CYP450 system leading to direct NO release [260,261]. However, high potency nitrates at low concentrations are also activated by mitochondrial aldehyde dehydrogenase (ALDH-2) enzyme [262]. Other mechanisms of activation have been suggested, these include xanthine oxidase, haemoglobin, glutathione-S-transferase and glutathione dependent reductases [263,264]. Recently, it has been demonstrated that GTN mediates some of its effects directly through the oxidation of protein kinase G1α (PKG1α) [265].

In contrast, inorganic nitrate is metabolised via the Nitrate-Nitrite-NO activation pathway. Nitrite present in the blood stream is reduced directly to NO, a process which is enhanced in hypoxic and acidic conditions, by a number of possible mechanisms, which include haem-associated globins [28,44,68,97], molybdenum metallo-enzymes [33,112,115,266], mitochondrial proteins [47,48,50], cytochrome P450 [61,134], NOS enzymes[65,132], carbonic anhydrase [63], ALDH-2 [267], protons [19], ascorbate [256] and polyphenols [257]. Therefore, differences in the modes and rates of activation of organic nitrates/nitrites and inorganic nitrate/nitrite are the predominant determinants of their potency as vasodilators.

1.2.5 Structure – Activity relationship

The pharmacophore (the component of the molecule which elicits the pharmacological effects) in organic nitrates is the -ONO_2 group [244]. The prefix mono-, di-, tri-, or tetra- describes the number of ONO_2 groups present in the molecule; recently it has been suggested that the greater the number of ONO_2 groups, the greater the potency of the drug [268]. The only exception is PETN, which despite possessing four -ONO_2 groups and demonstrating the highest potency in an in vitro model, displays poor clinical potency [269]. This is thought to be related to the poor systemic bioavailability profile of its active metabolites PEmonoN, PEdiN and PEtriN [269,270]. In addition, it is suggested that the potency of an organic nitrate is also dependant on its lipophilicity, which is in turn governed by the structure of the organic residue (R-); this is usually an alkyl- or an aryl- group; however, amino alkyl- groups have also been described (NH_2-$(CH_2)_n$-ONO_2) [245,246].

Furthermore, the stereochemistry of the organic nitrate molecule influences its potency, as nitrates with nitrooxy-groups in the endo position are less potent than those with the nitrooxy-group in the exo position (see Figure 1.2 A & B), as the latter position makes the -ONO_2 group more available for bioactivation (Figure 1.2) [245]. However, the duration of action of nitrates with nitrooxy-groups in the exo position is shorter due to greater systemic and metabolic clearance [271]. Thus of the dinitrates, ISDN is used clinically and has intermediate potency (one exo and one endo nitrooxy-group). ISDN is metabolised to two active mononitrates: 5-ISMN (endo nitrooxy-group) and 2-ISMN (exo nitrooxy-group) (Figure 1.2 C). Whilst 5-ISMN is used therapeutically, given the endo nitrooxy-group it would appear to be less potent than 2-ISMN; however, other factors

such as the position of the OH- group aid denitration; in addition, 5-ISMN has a lower plasma clearance and a longer half-life of ~5 hours, compared to ~2.5 hours with 2-ISMN, and thus has a longer duration of action.

The presence of other functional groups in the molecule will further modify their effects, for example nicorandil's K^+ channel opening action is thought to enhance vasodilatation, on the other hand, sinitrodil has improved antianginal action with diminished vasorelaxant activity [244,272].

Considerations of structure-activity relationships are not relevant to inorganic nitrate/nitrite, given their existence as simple anions in the circulation (as seen in Figure 1.1).

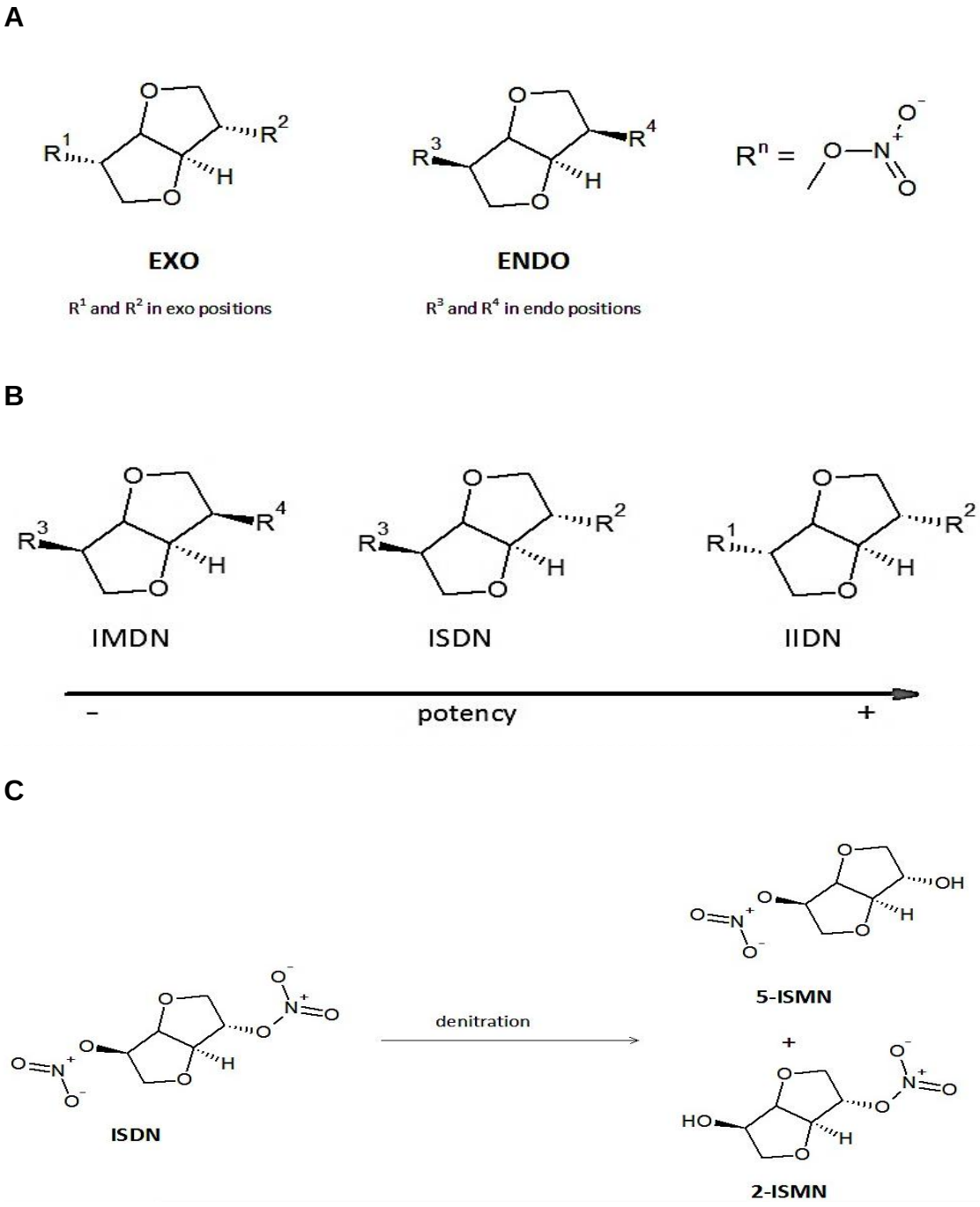

Figure 1.2: ENDO and EXO nitrooxy-group positions. (A) An example of the ENDO and EXO positions using dinitrates; (B) Effect of ENDO and EXO nitrooxy-group positions on relative potencies of dinitrates (isoiodide dinitrate (IIDN), isosorbide dinitrate (ISDN) and isomannide dinitrate (IMDN)); (C) Metabolism of ISDN to its two active metabolites: 5-ISMN (ENDO) which the form administered therapeutically, and 2-ISMN (EXO). (*Drawn using ChemSketch software and adapted from* [271])

1.2.6 Pharmacodynamics

The most important and well established effects of organic nitrates/nitrites are venodilatation and vasodilatation. At low dose, organic nitrates induce peripheral venodilatation, redistributing blood flow from the cardio-pulmonary circulation to the mesenteric and splanchnic vasculature [261]. This leads to a fall in pulmonary artery and left ventricular end diastolic pressures and thus a reduction in the preload. At high dose they also induce arterial and arteriolar dilatation, reducing after-load [261]. This effect on preload and after-load, which leads to a reduction in the heart's oxygen demand, coupled with the coronary dilatation, leading to enhanced oxygen supply, accounts for therapeutic actions of organic nitrates in CAD, angina and heart failure. In addition, organic nitrates possess anti-thrombotic and ant-platelet effects which may enhance their therapeutic effects in CAD [273].

In marked contrast to the long-established recognition of the profound vascular effects of organic nitrates, as recently as a decade ago inorganic nitrite was thought to be inert in the vasculature at physiological or near-physiological concentrations, being nothing more than a metabolite and biomarker of endogenous NO production by NOS enzymes [5]. Whilst it had been demonstrated in 1995 that nitrite could be converted to NO in tissues such as the heart under hypoxic/ischemic conditions [23], with what appeared to be damaging effects, this was thought to have limited relevance under more normal physiological conditions. It was believed that the effects of inorganic nitrite were only mediated at markedly elevated/toxic concentrations, which were well recognised to result in vasodilatation/hypotension and vascular collapse.

However, following the demonstration of an AV gradient of nitrite by Gladwin et al, [84] suggesting consumption of nitrite across the microcirculation, and relaxation of rat aorta under acidic conditions by Modin et al, [27] Cosby et al, demonstrated that the intra-brachial infusion of near-physiological concentrations of nitrite resulted in vasodilatation of forearm resistance vessels [28]. Since then it has become evident that nitrite has a role as an important reservoir of NO in the vasculature. The associated formation of iron-nitrosylated haemoglobin suggested that the effects were due to the reduction of nitrite to NO and that deoxyhaemoglobin was involved in this process supporting the reduction of nitrite to NO in the small resistance arterioles, particularly of muscular vascular beds, where oxygen extraction from the circulation to the tissues is most marked. Here, the oxygen saturation of haemoglobin approaches the P_{50}, the oxygen concentration at which half the haem is saturated with oxygen. This represents an optimum balance point between the greater reductive potential of haem in the R (oxy) state tetramer and the number of unligated deoxyhaem sites necessary for nitrite binding, which are more plentiful in the T state tetramer. Thus, this results in near-maximal conversion rates of nitrite to NO and hence vasodilatation [61,68,75,76].

Thus in addition to vasodilatation, the enhanced reduction of nitrite to NO in conditions of hypoxia and low pH supports its important role in hypoxic signalling, and cytoprotection in IRI. This role has been supported further by the elegant demonstration that nitrite has a more marked dilatory effect in veins than resistance vessels, where the oxygen content is lower, highlighting nitrite's role in oxygen sensing [29]. Several 'enzymes' that catalyse reduction of nitrite to

NO under hypoxic and or acidotic conditions have been described (see table 1.1).

The NO produced by the reduction of nitrite activates soluble guanylate cyclase (sGC), an intracellular receptor and transducer that potentiates the conversion of GTP to cGMP [274,275]. For many years it was assumed that the activity of nitrite on the vasculature was solely mediated through its reduction to NO and that nitrite has no activity of its own. Recently, however, it has been suggested that nitrite possesses vasodilator effects independent of those mediated by NO, or of known nitrite reductases [11,218,276]. Besides reduction to NO, nitrite undergoes other reactions leading to nitrosylation of proteins [9,11], and fatty acids [225,227,228] leading to modification their activities. (See section 1.1.6)

Table 1.8. Summarises and compares the pharmacokinetic properties of organic and inorganic nitrates / nitrites.

	Organic Nitrates / Nitrites	Inorganic Nitrate / Nitrite
Absorption	• Readily absorbed via buccal, sublingual, trans-dermal, rectal, routes [249]; also via inhalation [277] • Variable oral bioavailability due to variable rate of hepatic first pass metabolism [248,253] • Organic nitrates with poor bioavailability tend to be more potent [248]	• Readily absorbed across the upper GI tract [254]; also via inhalation [278,279] • Do not undergo first pass metabolism [14]
Distribution	• Widely distributed in the body via the circulation • Blood levels rise rapidly with onset of action within 1 – 3 min [253]	• Widely distributed in the body via the circulation • 25% of absorbed nitrate is concentrated in the salivary glands [254] • Following ingestion there is a sharp rise in salivary, plasma and urinary levels of nitrate and subsequently nitrite • Serum nitrite levels start to rise at about 1 hour and peak at about 3 hours [165,255]
Metabolism	• Rapidly metabolised by various enzymes leading to rapid offset within 15 – 30 min [253] • Cytochrome P450 system: low potency nitrate & high doses of high potency nitrates [260,261] • ALDH-2: low doses of high potency nitrates [262] • Also: haemoglobin, xanthine oxidase, glutathione -S-transferase, and glutathione dependant reductase [248,260,261]	• Via the Nitrate – Nitrite – NO pathway: • Enterosalivary circulation: nitrate reductase activity of bacteria on tongue generates nitrite: • Nitrite is metabolised to NO in the stomach and circulation • Circulation: haemoglobin, myoglobin, xanthine oxidase, eNOS, Aldehyde oxidase, ALDH2, carbonic anhydrase, ascorbate, polyphenols [28,68,115]
Excretion	• Renal failure does not markedly influence kinetics of ISDN [280]	• 75% of absorbed nitrate is excreted by the kidney [254]

Table 1.8: A comparison of the pharmacokinetic properties of organic and inorganic nitrates / nitrites.

1.2.7 Adverse effects

Although organic nitrates are potent vasorelaxant drugs, their untoward effects continue to limit their clinical application. Adverse reactions linked to organic nitrates fall into two categories: acute adverse effects associated with their vasodilator effect such hypotension, dizziness, nausea and headache; and adverse effects resulting from chronic administration such as nitrate tolerance, increased oxidative stress and endothelial dysfunction [248,281]. The mechanism accounting for nitrate tolerance is a complex phenomenon that is not fully understood. Its hallmark is the attenuated pharmacological effects of the drug with chronic administration. Chronic use may also lead to endothelial dysfunction and the development of vascular tolerance to other endothelium-dependant vasodilators. Furthermore endothelial dysfunction triggers the production of ROS, which in turn further aggravates the tolerance [248]. It has been demonstrated that the phenomenon of tolerance is consistent throughout most organic nitrates; the exceptions are PETN, amino-alkyl nitrates and nicorandil. Bioactivation of the latter two drugs is not dependant on the ALDH-2 enzyme, which may explain this observation [246,260].

Recent evidence has emerged suggesting the important role mitochondrial ALDH-2 plays in the development of tolerance. It is thought that ROS formation following GTN bioactivation by ALDH-2 cause oxidisation of the thiol groups, which form the active sites of the enzyme, thus leading to irreversible inhibition of ALDH-2 which in turn decreases the bioactivation of GTN. However, it is not fully clear if oxidation is the only process which can be implicated or if other metabolites may also contribute to the inactivation of ALDH-2 [248,262].

Although inorganic nitrate/nitrite does not appear to exhibit the phenomenon of tolerance [282], other adverse effects, such as methaemoglobinemia (with high doses; also an adverse effect of organic nitrates), and a possible carcinogenenic effect have limited their use [13]. Cases of methaemoglobinemia in infants after exposure to high levels of nitrate in drinking water were first reported in the 1940's [283]. As a consequence, concerns about the potential adverse effects of nitrate in water provoked the need for further regulatory measures aimed at limiting nitrate levels to 45 mg/L as the maximum allowed content [14]. Methaemoglobinemia, also known as "Blue Baby Syndrome", usually occurs in infants under six months of age as the result of nitrate intoxication from vegetables and drinking water. After nitrate reduction to nitrite, the ferrous ion in oxyhaemoglobin is oxidized to the ferric ion; this deprives haemoglobin of its oxygen binding ability. The increase of methaemoglobin in blood leads to a reduction in the oxygen supply to the tissues. This becomes physiologically significant when methaemoglobin exceeds 5-10%, and symptoms such as cyanosis, tachypnoea, irritability, cardiovascular problems and altered mental status may occur [284]. The susceptibility of infants to this phenomenon is thought to be caused by their higher gastric pH allowing for bacterial colonisation and thus enhancing nitrate reduction to nitrite; the higher circulating levels of foetal haemoglobin which is more readily oxidized to methaemoglobin; and the reduced levels of the enzyme methaemoglobin reductase hindering the reduction of methaemoglobin back to oxyhaemoglobin [283,285,286].

Concerns regarding the carcinogenicity of inorganic nitrate/nitrite stem from observations and studies performed in animals. The unexpected formation of the carcinogenic nitrosamine, dimethylnitrosamine in ruminants from their feed

of herring preserved with sodium nitrite was discovered during an outbreak of severe liver disease in 1961 and 1962 in Norway [237]. Dimethylnitrosamine had previously been demonstrated to disrupt nucleic acids in the rat and cause liver tumours [287] and as it could be formed *in vitro* from secondary amines and nitrite mixing together in the acidic conditions of human gastric juices [288] concerns that ingestion of high levels of nitrite could have similar effects in humans seemed well founded.

In addition to acidification of nitrite in the human stomach results in the formation of two potent nitrosating agents: nitrous acid, HNO_2 and dinitrogen trioxide, N_2O_3 (Equations 1-4), which react with secondary amines in the diet producing N-nitrosamines. However whilst N-nitroso compounds were shown to be carcinogenic in animals [289-291], chronic feeding of nitrite to rats, even when diethylamine was given at the same time, did not induce tumours [292]. Subsequently, dietary nitrate was also proposed as a potential source of N-nitrosoamines due to its conversion to nitrite in the entero-salivary circulation [239,293]. Although numerous studies have been conducted implicating nitrate/nitrite in the development of cancer in humans [294-296] many other studies have failed to confirm this and the evidence remains equivocal [297-299]. In view of this contradictory data, the Joint FAO/WHO Expert Committee on Food reviewed all the available evidence, but failed to establish a definite link between nitrate intake and risk of developing cancer [299,300]. Furthermore, The World Cancer Research Fund/American Institute of Cancer Research found no evidence linking ingestion of vegetables which are known to be high in nitrate with the development of cancer [301].

However, there may be exceptions to this in some patient subgroups. Patients with Barrett's oesophagus have been shown to have increased nitrosation at the gastro-oesophageal junction, an area highly associated with the development of adenocarcinoma [302]. Furthermore there is an observed association between high nitrate (but not nitrite) intake with an increased risk of thyroid cancer [303,304]. This in turn is thought to be related to the fact that the ion channel responsible for the transport of nitrate in the salivary gland is also responsible for the transport of iodide [305]. This may also explain why the ingestion of high levels of inorganic nitrate competes with the uptake of iodine and may lead to thyroid hypertrophy and dysfunction [306]. However, whether nitrate is causally associated with the development of thyroid cancer, or any other type of cancer, remains to be established. This is an area that has recently been comprehensively reviewed by Milkowski et al, and highlights the problems of interpreting associations between dietary factors and disease from epidemiological studies (such as weak associations, residual confounding etc.) and advises extreme caution [307]. Given the potential large benefits on cardiovascular health, these concerns should not stall progress, but need to be studied in large long-term prospective studies.

1.2.8 Therapeutic applications in cardiovascular disease

While inorganic nitrite/nitrate has numerous beneficial effects, special consideration will be given to its therapeutic application CVD as this allows for a direct comparison with organic nitrates (see tables 1.9 & 1.10).

Organic nitrates have been used in the treatment of CVD since their introduction in the mid 1800's and continue to be used to this day. In current clinical practice, short-acting organic nitrates are primarily indicated in the symptomatic treatment of MI and angina pectoris due to their rapid onset of action when administered sublingually. When used in long-term prevention of angina pectoris a washout interval of 10 hours a day is recommended in order to avoid tolerance. Furthermore, the development of tolerance and endothelial dysfunction due to the continued administration of GTN, usually for more than 24 hours, may lead to an increased risk of re-infarction [249]. In addition organic nitrates are also indicated for the treatment of acute cardiac heart failure and in hypertensive urgencies and emergencies *per se* and when associated with an aortic dissection.

Despite a decline in the incidence of mortality from CVD it remains the leading cause of death worldwide. Furthermore it accounts for 48% of the total mortality rates in Europe [308-310]. Although the development of CVD is governed by many factors, the most influential modifiable life style factors include smoking, obesity, lack of exercise and poor diet [311]. In the Dietary Approach to Stop Hypertension (DASH) study the supplementation of dietary intake with fruit and vegetables for 3 weeks resulted in a reduction in both systolic and diastolic BP [312].

Furthermore it has been suggested by retrospective studies that a diet rich in fruits and vegetables can improve the outcomes of CVDs [313-315]. Originally these effects were attributed to the vitamin and antioxidant content of fruits and vegetables [316], however the strongest associations were found with diets rich in green leafy vegetables and therefore inorganic nitrate (found in high concentrations in these vegetables) may account for these effects [317]. Indeed, several effects of inorganic nitrate/nitrite that would likely be beneficial in terms of CVD outcomes have been reported. These include vasodilatation [28], cytoprotection against tissue IRI [112,139], inhibition of platelet aggregation [146], regression of intimal hyperplasia [142], improvement in peripheral artery disease [138,173], blood pressure reduction [146], and reduction in oxygen cost during exercise [318]. Furthermore, the role of NO in vascular homeostasis in general is well established and an attenuation in its production or bioavailability is implicated in many of the cardiovascular disorders [135,136,319] and thus the provision of an alternative source of NO from nitrate/nitrite is likely to be of long term benefit.

	Organic Nitrates / Nitrites	Inorganic Nitrates / Nitrites
Endothelial Dysfunction	• Short duration treatment has positive impact on endothelial function [320] • Long duration treatment leads to tolerance and ED [320] • Enhances ATP release from RBC [321,322]	• Has a positive impact on endothelial function regardless of duration of supplementation [141,158] • More effective at enhancing ATP release from RBC especially during hypoxia [321-323]
Pulmonary Hypertension	• Leads to a reduction in PAP [324]	• Leads to a reduction in PAP [325] • Effects more pronounced when inhaled [326]
Myocardial Infarction	• Has a myocardial protective effect if given and discontinued at a specific window of time prior to ischemic injury [327] • Has a myocardial destructive effect if tolerance develops and is given throughout the ischemic event [328]	• Has a myocardial protective effect in I/R injury model [112] • Improves angiogenesis [138] • May improve outcomes in cardiac arrest [140]
Platelet Function	• Inhibits platelet aggregation [329] • Anti-platelet effect varies between different nitrates [329] • Is dependent on xanthine oxidoreductase [330]	• Inhibits platelet aggregation [146,331] • Effects are likely to be mediated by nitrite-NO [146]

Table 1.9: Comparing and contrasting the therapeutic effects of inorganic and organic nitrates/ nitrites in animal and preclinical models.

	Organic Nitrates / Nitrites	Inorganic Nitrates / Nitrites
Endothelial Dysfunction	• Have a negative impact on endothelial function through the production of ROS [332]	• Has a positive impact on endothelial function regardless of duration of supplementation [146,165]
Blood Pressure	• Leads to a fall in systemic BP • Rapid in onset (around 15 – 30) [247,253] • Limited by the development of tolerance which ameliorates its BP lowering effects [248,281]	• Leads to a fall in systemic BP [146,162] • Nitrate - Slow in onset (around 3 hours) • Effects more sustained with no evidence of tolerance [162,165] • Dose dependent [165]
Pulmonary Hypertension	• Leads to a reduction in PAP when inhaled [277]	• Leads to a reduction in PAP when inhaled [279]
Platelet Function	• Inhibits platelet aggregation with no evidence of attenuation of this effect with chronic administration [333]	• Inhibits platelet aggregation [146,331]
Other therapeutics	• Treatment of acute pulmonary oedema • Treatment of Angina • Treatment of anal fissures	• Antimicrobial effects in different parts of the GI tract [334,335] • Enhancement of gastric blood flow and mucosal healing[336,337] • Improve exercise capacity[318]

Table 1.10: Comparing and contrasting the therapeutic effects of inorganic and organic nitrates/ nitrites in humans.

1.2.8.1 Endothelial function and vasodilatation

Endothelial function is related to the activity of endothelial NOS to generate NO (endothelium derived relaxing factor (EDRF)) [338,339], NO bioavailability and sGC signalling [319,340]. An impairment in any of these pathways leads to endothelial dysfunction, which has been recognized as a major precursor of CVD [341,342].

Although a short duration of treatment with GTN may have a positive impact on endothelial function, prolonged treatment has a negative impact by attenuating eNOS activity [320] and inducing the production of ROS [343]. It has also been suggested that tolerance to GTN is caused by down regulation of mitochondrial ALDH-2 providing another source of ROS [344].

In contrast, studies in mice with endothelial dysfunction, induced through a high cholesterol diet, demonstrated that inorganic nitrite supplementation had a positive impact on arteriolar endothelial function [158]; In addition, nitrite treatment lead to the reversal of age-related endothelial dysfunction in another mouse model [141]. In humans, similar effects were suggested when the ingestion of beetroot juice, or potassium nitrate lead to the prevention of IRI induced endothelial dysfunction as measured by flow mediated dilatation (FMD)[145,146,165]. In addition to enhancing endothelium dependent dilatation, it is worth considering that inorganic nitrite might enhance endothelium-independent dilatation by enhancing NO bioavailability, as has been demonstrated for other substances with anti-inflammatory effects [345].

An alternative mechanism for the activation of eNOS is via the release of ATP from erythrocytes [321]. The release of erythrocyte derived ATP is enhanced by both GTN and inorganic nitrite [322]. However, nitrite has a greater effect which becomes more pronounced during hypoxia [323].

1.2.8.2 Blood Pressure

Studies undertaken in healthy volunteers demonstrated a significant reduction in BP following the ingestion of inorganic nitrate [146,162,165]. Ingestion of nitrate-rich beetroot juice leads to blood pressure reduction that was maximal at around 2.5 – 3 hours; this is consistent with the peak nitrite plasma levels. In addition, when the entero-salivary circuit was interrupted, by spitting or by antibacterial mouthwash, there was no significant rise in plasma nitrite levels and the blood pressure lowering effects were abolished, providing further evidence that these effects were a result of the increase in circulating nitrite levels [146,346]. Once in the circulation inorganic nitrate is estimated to have a half-life between 5 - 8 hours, a result of its active reabsorption in the proximal renal tubule [347]. The half-life of an oral dose of nitrite on the other hand has been demonstrated to be around 110 seconds [348]. In addition nitrates BP lowering effects of nitrate were dose-dependent [165]. Although these studies were conducted in healthy volunteers these effects were also replicated in patients with hypertension [116].

In a rat model of hypertension, induced through a unilateral nephrectomy and a chronic high salt diet, intervention with high dose nitrate resulted in a reduction in BP which was greater in magnitude than that observed in normotensive rats. In addition this effect was maintained for the 8 week duration of the study indicating the lack of any tolerance [349]. Furthermore, the rats treated with nitrate supplementation demonstrated a reduction in renal hypertrophy, fibrosis and injury leading to the mitigation of protein urea [349]. These effects of reduced hypertrophy and fibrosis were not exclusive to renal tissue but were also observed in the myocardium, albeit to a lesser extent. The protective effects on

cardiac and renal remodelling evoked by nitrate supplementation are likely to be due to the reduction in oxidative stress rather that the reduction in BP *per se* [349]. Also, sodium nitrite has been shown to have antioxidant effects: supplementing the drinking water for 4 weeks in two-kidney one-clip (2K1C) hypertensive rats with sodium nitrite resulted in a dose dependent decrease in BP associated with an inhibition of NADPH oxidase activity [161]. While organic nitrates may be effective in lowering BP, and are used, for example, in the management of acute hypertensive emergencies, the effect is often variable, and longer term use is limited by the rapid development of tolerance.

1.2.8.3 Pulmonary hypertension

Both organic nitrates and inorganic nitrite have been shown to affect pulmonary arterial pressure (PAP). In studies on anesthetised dogs, intravenous GTN was found to have no effect on PAP, though inhaled GTN lead to a reduction in PAP [350]. However, a more recent study in the same animal model using higher doses of intravenous GTN found that it decreased PAP [324]. The use of inhaled GTN in humans also resulted in a reduction in PAP [277,351]. It should be noted that in all these studies the GTN was administered for long enough for the effects of tolerance to become evident.

Dietary nitrate supplementation in a mouse model improved PAP in a mouse model of the disease [352]. A study in sheep has demonstrated that inhaled inorganic nitrite also leads to dilatation in pulmonary vascular beds ameliorating pulmonary hypertension [326]. And in a canine model of pulmonary embolus the administration of intravenous nitrite had similar effects on PAP [325]. The use of inorganic nitrite in human studies resulted in vasodilatation in the pulmonary vasculature leading to reduction in PAP [278,279].

1.2.8.4 Myocardial Infarction

A key determinant of post MI mortality and morbidity is the degree of left ventricular systolic dysfunction, which is in turn directly determined by the infarct size [353]. Besides reperfusion therapies, there are two strategies that could potentially reduce infarct size. The first is preconditioning, either ischemia-induced or pharmacological; first described in 1986, IPC describes the protection acquired by tissue from the effects of a prolonged ischemic event, when the same tissue is subjected to non sustained periods of ischemia [354]. In addition, protection can be afforded if the repeated ischemic insult occurs in a different organ, the phenomenon of remote IPC [355]. The second strategy involves the attenuation of further cell death and apoptosis incurred following the reestablishment of coronary perfusion (IRI) a phenomenon known as post conditioning [356].

Nitric oxide has been demonstrated to play an important role in IPC [357-359]. GTN was used as a NO donor in this disease model in animals and was found to impart a significant degree of myocardial protection [327]. In this rabbit model, a 60 min intravenous infusion of GTN was discontinued in different groups at different time points (1, 24, 72 and 96 hours) prior to the onset of the ischemic insult. There was no group in which GTN treatment was continued throughout the ischemic insult as it was felt that this would represent direct cardio-protection. A myocardial protective effect in the 1 hour and 96 hour groups was comparable to that in the group treated with conventional IPC. The protective effect in the 24 and 72 hour groups was greater than the control. [327].

However, in a different study, conducted in a rat model, the continuous delivery of GTN throughout the ischemic insult lead to an increase in the infracted area

from 45% to 59% [328]. On the other hand the discontinuation of the GTN treatment 3 hours prior to the ischemic event caused a decrease in the infarct size from 45% to 33% [328]. This unusual finding may be explained by the results of more recent studies which demonstrated that ALDH2 is involved in protecting the myocardium from IRI damage when it is up-regulated, but the reverse is true when it is down-regulated [360,361]. One must note that the above studies have been conducted in animal models and the findings are yet to be validated in humans. However, clinical trials have not demonstrated any clear benefit of organic nitrates. Although the Vasodilator Heart Failure Trial (V-HEFT) suggested a small improvement in outcomes between the ISMN-hydralazine and placebo, this did not reach statistical significance [362]. A possible mechanism relates to observation from *in vitro* experiments which suggest that hydralazine may inhibit the development of tolerance [363]. Whilst in the fourth International study of Infarct survival (ISIS-4), 30 days administration of ISMN post infarct appeared to be safe, but had no mortality benefit vs. placebo [364]. However, two more recently published studies demonstrated increased cardiac events and worse outcomes in some patient populations treated with ISMN [365,366]. Therefore, the current place of organic nitrates as a symptomatic treatment of patients with CAD may need to be investigated further.

Following an initial indication that inorganic nitrite was deleterious in a model of IRI [23], nitrite was found to have profound cytoprotective effects, significantly decreasing infarct size and restoring cardiac function in an *in vitro* rat myocardial IRI model [112], which was substantiated in an *in vivo* model [137]. Many subsequent studies have confirmed these cytoprotective effects in other animal models and other organs such as the brain, liver and kidneys in addition to the

heart [139,367,368]. Whilst these effects are dose dependent, it is striking that low micromolar concentrations are effective, indeed dietary nitrate and nitrite depletion results in increased IRI in the liver [367] and the heart [150]. Furthermore long-term administration of sodium nitrite has been demonstrated to enhance angiogenesis in ischemic tissue [138]. In addition, treatments with inorganic nitrite lead to improved outcomes in a mouse model of cardiac arrest [140]. Although such findings in animal models provided considerable promise of inorganic nitrite as a treatment of MI in humans, a recently published trial showed no increased benefit from intra-venous nitrite administration 5min prior to reperfusion therapy [144] (as described in section 1.1.4.5). However, the results of another trial where nitrite was administered intracoronary immediately prior to revascularization are still awaited [180].

1.2.8.5 Platelet function

In addition to playing an important role in normal coagulation and vascular healing, platelet activation and aggregation also plays a key role in the pathophysiology of atherosclerosis, atherothrombosis and acute coronary events [369-371].

An early study conducted by Schafer et al, investigated the effects of GTN and ISDN on platelet function [329]. The investigators found that GTN inhibited platelet aggregation and that this inhibition was both dose and time dependent. Furthermore these effects were reversed when the exposure of the platelets to the GTN was abolished. These effects seemed to be mediated by disrupting the arachidonic acid – thromboxane A2 pathway and were not related to an increase in intra-cellular cyclic adenosine monophosphate (cAMP); ISDN had similar effects, however those of GTN were more pronounced [329]. The antiplatelet effect of GTN also appears to be partly dependent on activation via XOR [330]. Furthermore, the effects of GTN on platelet aggregation do not appear to be affected by chronic administration and the development of vascular tolerance [333]. However, continuous administration of GTN for 3 days via trans-dermal patch lead to increased production of superoxide by platelets [372]. The clinical applications of this are yet not fully clear as clinical studies have demonstrated conflicting results; for example a further study which evaluated the effects of organic nitrates (GTN / ISDN) on platelet function in patients undergoing coronary artery bypass grafting (CABG) failed to demonstrate any measurable effects on platelet function when using bedside coagulation parameters [373].

Whilst inorganic nitrite was originally thought to lack any effect on platelet aggregation, as supported by studies in the rat [11], inorganic nitrate was found to inhibit platelet aggregation in humans [146,331]. Originally it was proposed that SNO formation might play a role [331]; however SNOs were not detected in the systemic or portal circulations. In humans, it became clear that the effect of nitrate on platelets was dependent on its conversion to nitrite, as spitting of all saliva for 3 hours abolished both the rise in serum nitrite levels and the antiplatelet effect [146].

1.3 The vascular bed, nitrite and the role of deoxyhaemaglobin

The arterial tree functions as a conduit, transferring blood from the heart to the organs and the extremities, and a cushion, dampening the pulsatile nature of blood ejected from the heart [374]. Thus, it delivers oxygen and nutrients to the peripheral organs at a steady rate and pressure [374]. The branching design coupled with a constant wall shear rate allows the arterial vessels to operate as the optimal conduit system with a minimum of work requirement [375]. The larger conduit vessels have intrinsic elastic properties, making them more compliant and allowing them to expand in systole and recoil in diastole propelling blood onwards and dampening the oscillatory flow in the process [376]. The smaller muscular arteries have a higher prevalence of smooth muscle cells allowing them to dilate providing a low resistance path to the organs and the extremities [376]. Arterioles are responsible for further smoothing of the pulsatile flow to a more laminar flow in addition to the regulation of blood distribution to organs

and within an individual organ. Furthermore, by controlling the peripheral vascular resistance, arterioles determine the mean blood pressure.

This level of control on the part of resistance arterioles requires a sophisticated level of regulation in which NO plays an important part, acting as an endogenous dilator produced by the vascular endothelium offering background dilatation of the arteriolar bed, moreover, the production of NO can be changed in response to variations in blood flow which alters the shear stress[377].

Nitric oxide donors cause a dilatory effect in muscular conduit arteries that is more pronounced than in resistance arterioles . It has been proposed that this heightened sensitivity to NO donors is consequent to the minimal basal levels of endogenous NO found in conduit vessels [378]. It is suggested that endogenously produced NO down regulates the expression of sGC and diminishes its sensitivity to NO . In addition, NO reduces G kinase expression and enhances the activity of phosphodiesterase V, further desensitising the vascular smooth muscle cells. Hence, the dilatory selectivity of NO donors for a particular vessel type may in part be dependent on the basal levels of NO found in each vascular bed.

Indeed, the organic nitrate GTN is a selective dilator of muscular conduit arteries . However. It has been suggested that Inorganic nitrite is dependent on hypoxic conditions to facilitate its reduction to NO, via the actions of deoxyhaemoglobin amongst other enzymes. However, nitrite's observed vasodilatation of resistance arterioles does suggest that nitrite may act as an NO donor under conditions of near normal oxygen tension. Thus it is probable that nitrite may continue to act as an NO donor under conditions of normal oxygen tension and in the absence of high levels of deoxyhaemoglobin.

The proposal that nitrite is mainly reduced by deoxyhaemoglobin to NO may well hold true for the prominent vasodilation observed in veins, where deoxyhaemoglobin is prevalent, and for the enhanced dilatation of resistance arterioles when comparing hypoxia to normoxia . However, the role deoxyhaemoglobin plays in physiological systems remains controversial as it does not adequately explain nitrite's actions on the resistance arterioles under conditions of normoxia, where modest dilatation occurred , as these vessels still display oxygen tensions (and levels of deoxyhaemoglobin) comparable to those found in other segments of the arterial tree.

In fact, when doses of 10 μM of sodium nitrite (designed to mimic near physiological levels) were add to rabbit aorta (Ao), inferior vena cava (IVC) and pulmonary artery (PA) dilatation to all vessel types was evident [379]. Under conditions of normoxia the PA dilated more than the Ao (the IVC response was variable). However, under conditions of hypoxia, the Ao dilatation was significantly greater than that observed in the PA and the IVC. During hypoxia the nitrite-induced dilatory response was enhanced in all vessels, however, this increase was more marked in the Ao (700%) than in the PA (60%); furthermore, these effects were independent of the presence of haemoglobin [378]. This suggests that deoxyhaemoglobin plays a relatively minor role. Instead, these differences may be related to the significantly greater smooth muscle mass in the Ao when compared to the two other vessels. Indeed, this would be consistent with the has been suggestion that nitrite-induced dilatation under aerobic conditions is mediated via sGC and/or other haem enzymes .

1.4 Summary

Nitrite is both a metabolite of endogenously produced NO and the largest directly accessible mammalian source of the messenger. Biologically active NO can be liberated from this pool when nitrite is enzymatically reduced via the actions of a number of candidate proteins. The relative contribution of these proteins to nitrite's reduction is dictated by its tissue distribution, level of expression, and modifiable activity dependent on the prevailing conditions. Indeed, they all exhibit increased activity under conditions of hypoxia and/or acidosis, making nitrite a selective donor of NO in hypoxic/ischemic tissue, compensating for the diminished NOS activity. Nitrite, either through reduction to NO, or via direct signalling, modifies mitochondrial structure and function improving a host of cardiovascular disease processes.

Although organic nitrates/nitrites also mediate their effects principally via NO and share many similarities with inorganic nitrite (and nitrate) they also have striking differences in terms of their pharmacokinetics, pharmacodynamics, chemical structure and mode of activation with associated differential effects on their modification of cardiovascular function and disease.

1.5 Hypothesis

The cardiovascular actions of inorganic and organic nitrates / nitrites on conduit and resistance vessels are believed to be different. The organic nitrate GTN (which is able to act as an NO donor under conditions of normoxia) is a selective dilator of muscular conduit arteries , the accepted mechanism by which GTN selectively lowers central blood pressure [378]. It has been proposed that inorganic nitrite, however, is dependent on hypoxic conditions to facilitate its reduction to NO . Moreover, the suggested requirement for hypoxia to facilitate nitrite's action, in addition to oxygenated haemoglobin and myoglobin being avid scavengers of nitrite-derived NO, suggests that nitrite will lack any activity in conduit vessels. However, under conditions of normoxia, nitrite has been shown to effectively dilate resistance arterioles . And although a degree of oxygen extraction does occur at the level of resistance arterioles the levels of deoxyhaemoglobin in these vessels is not too dissimilar from that in conduit vessels. Thus although the current literature suggests that inorganic nitrite is a selective hypoxia-dependent resistance arteriolar dilator, with minimal effect on conduit arteries. We propose that inorganic nitrite may have a dilatory effect in conduit vessels that would become more pronounced under conditions of induced hypoxia. To test this hypothesis a number of studies were conducted where an intra-arterial or an intra-venous infusion of sodium nitrite was administered; with the concomitant measurement and assessment of vascular / hemodynamic parameters; selectivity in normoxia, hypoxia and hyperoxia; and effect of inhibition of AO with raloxifene or inhibition of CA with acetazolamide.

Chapter 2

Methods

2.1 Volunteers

The studies were approved by the St Thomas' Research Ethics Committee (South East London REC2) and NRES Committee London – Westminster. Subjects were healthy male volunteers recruited from the community. Informed written consent was obtained from all the participants.

2.2 Reputability and reproducibility

Once recruited, volunteers were allocated to one or more groups of studies. Pre set dates were agreed upon in which the volunteers were to present to the research facility. If more than one visit was required, in order to compare the effects of different interventions, then visit dates were set to be at least one week apart. The order of the intervention being administered during each visit was randomised in an equal fashion so that the number of interventions administered first were the same within a complete study set (with 3 visits a randomised balanced Latin square design was implemented). The participants were blinded to the intervention being used in each visit.

Data acquired from each visit was saved under the allocated participant number and the visit order (i.e. 1^{st}, 2^{nd} or 3^{rd}) without any indication of the intervention applied. Analysis of the data set was conducted only at the conclusion of the entire study (i.e. when all the participants completed all of their visits). Analysis was first conducted by the investigator, and then repeated by a second experienced party, which was blinded, with no access to the randomisation table containing the order of interventions applied in each visit.

2.3 Standard study protocol

Participants were asked to avoid ingesting nitrate-rich foods (including green leafy vegetables, beetroot and processed meats), caffeine, smoking and undertaking heavy exercise for 24 hours prior to the study. They were also instructed to fast overnight (though encouraged to take clear fluids to avoid dehydration). The studies were conducted at a similar time of the day, for repeated visits in the same subject, under controlled temperatures (23 - 27 °C) and with minimal sensory stimulation to minimise confounding. For intra-arterial (i.a.) studies, the brachial artery was cannulated using a 27 gauge needle (Cooper's Needle Works, Birmingham, UK®). This part of the procedure was performed with the use of local anaesthesia and under strict aseptic techniques. For intravenous (i.v.) studies, an 18 gauge cannula in an antecubital fossa vein was used. On successful cannulation the needle was secured in position and a saline infusion was commenced at a rate of 1 ml/min (Injectomat Agilia syringe driver, Fresenius Kabi, Homburg, GR®). Following an initial equilibration period of 15 min and recording of baseline measurements with 0.9% saline, the effects of i.a./ i.v. Sodium nitrite (Martindale Pharmaceuticals, UK and Ipswich Hospital Pharmacy Manufacturing Unit, UK®)/ GTN (Hospira Ltd, UK®) on radial artery (RA) diameter, forearm blood flow (FBF) and systemic haemodynamic parameters were assessed as described in detail in the following sections. Blood samples for the assessment of local concentrations were taken using an 18 gauge cannula placed in the radial vein of the infused arm. A similar sized cannula placed in the antecubital vein of the control arm was used for the aspiration of blood samples intended for the assessment of systemic spill over.

2.4 Measurement of conduit and resistance vessel function

2.4.1 Measurement of local radial artery diameter (conduit vessel)

An Acuson-Aspen advanced 2-dimensional ultrasound (2D US) machine with a 10 MHz Linear probe (Siemens GR®) was used for direct imaging of the radial artery (Figure 2.1). The probe was fixed in position using an adjustable magnetic flexible stand (Mitutoyo JP®). Facilitated by ECG triggering, images were acquired at the end-diastolic phase for the duration of 120 seconds at a rate of 1 frame every 3 seconds, giving a total of 40 frames per sequence of acquisition. The Radial artery diameter for each individual frame was measured using computer aid edge-to-edge detecting software (Brachial analyzer, Medical Imaging Applications®) and the mean of the measurements of the 40 frames was used to give the vessel diameter at that time point (Figure 2.2).

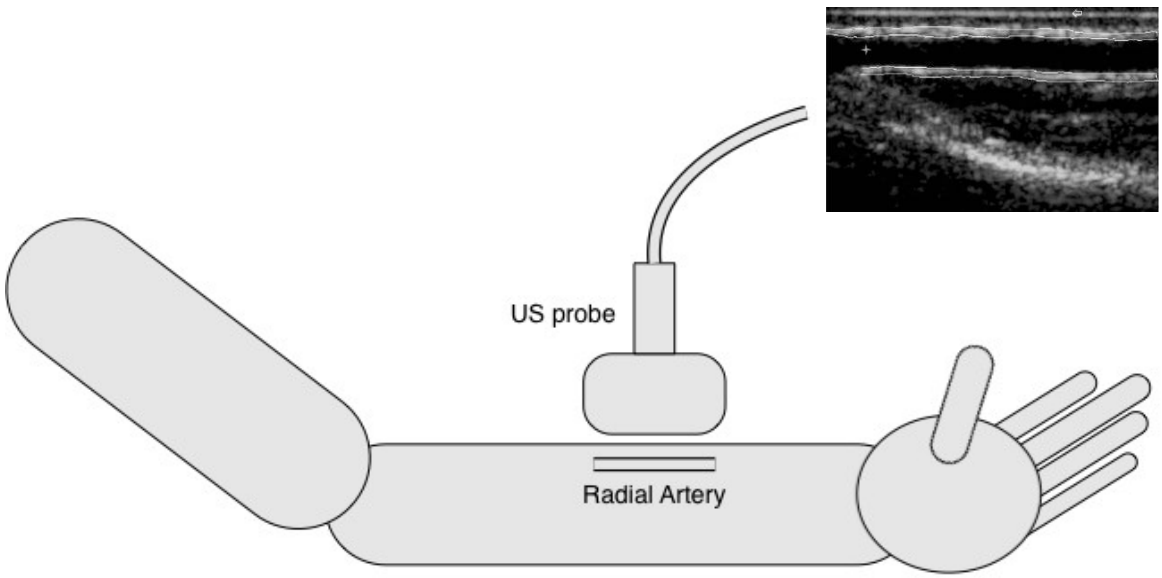

Figure 2.1: Diagrammatic representation of 2-D US imaging used for the assessment of RA diameter. 2-D US – 2-dimensional ultrasound, RA – Radial Artery.

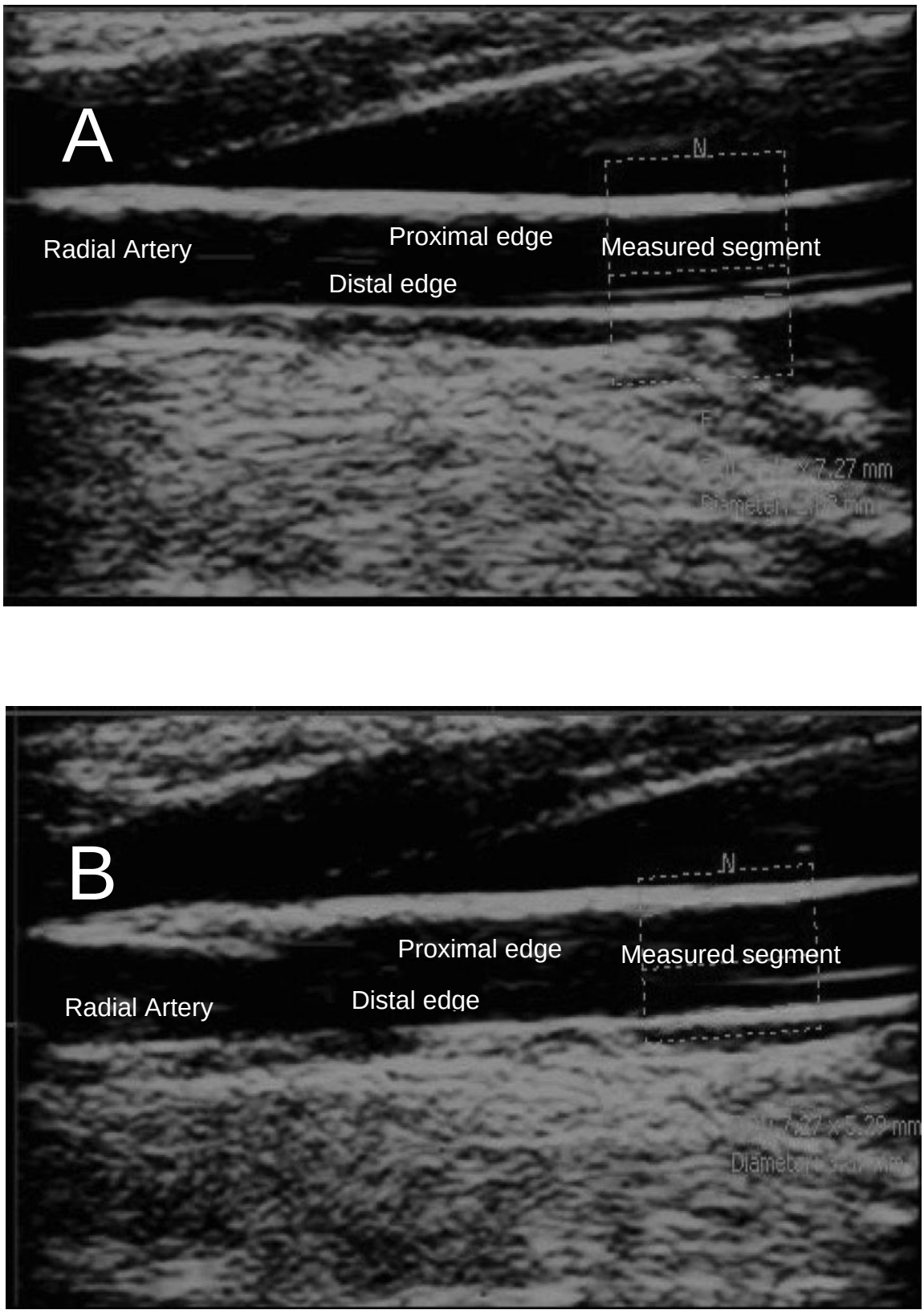

Figure 2.2: 2-D US images depicting a single acquisition frame. The RA lumen and RA proximal and distal and vessel wall edges can be clearly seen. The dashed box delineates the segment being measured by the software. A) A single acquisition frame of the RA at baseline during saline infusion (measures 2.83mm). B) A single acquisition frame of the RA following infusion with inorganic nitrite 2.6 µmol (measures 3.37mm). This is equivalent to dilatation of ~16%. 2D US – 2-dimensional ultrasound, RA – radial artery.

2.4.2 Measurement of local forearm blood flow (resistance arterioles)

Although arterial flow can be calculated from the measurements of arterial diameter (acquired via 2D US as described in 2.3.1) and blood velocity (measured using Doppler US) [378] the technique suffers from a number of limitations. It is heavily operator dependant, requiring extreme precision during repeat studies, as small errors in the measurement of arterial diameter will lead to magnification of the error when calculating flow; this is in addition to the inability to generate a dose response curve for the effects of drugs infused locally on the resistance vessels [378].

Venous occlusion plethysmography (VOP) however, provides a simple, accurate and reproducible assessment of FBF. And when combined with the minimally invasive procedure of intra-arterial drug administration, allows for detailed analysis of the vasoactive pharmacological effects of a drug [378].

2.4.2.1 Venous occlusion plethysmography

First described by Hewlett & van Zwaluwenburg in 1909 [380], combining venous occlusion with occlusion plethysmography is now an established method for the measurement of FBF. The key principal is that the occlusion of venous return from the arm without affecting arterial inflow will lead to tissue swelling, as blood continues to enter the limb but is unable to leave. Moreover, the tissue swelling (or increase in forearm volume) over time, remains linear for as long as the veins are not fully distended, and the rising venous pressure remains less than that of the occluding pressure [378]. Measuring this linear rate of increase in forearm volume gives an indication of the rate of arterial inflow.

Originally the measurement of tissue swelling was awkward and challenging relying on air and then fluid displacement [378]. However, in 1953 Whitney refined the technique by demonstrating that changes in limb volume could be measured using a mercury-in-rubber elastic strain gauge [381], making the technique far less cumbersome and gaining it wide appeal. Whitney surmised that excluding the circulation of the hand approximated the shape of the forearm to that of a cylinder allowing its volume to be calculated by the following equation: Volume of a cylinder (V) = circumference x the height (or length). Therefore V = πd xl (where (d) is the diameter and (l) the length) Figure 2.3. As the length of the arm remains constant, the rate of change in circumference is proportional to the rate of change in volume (i.e. Δ Volume = Δ circumference).

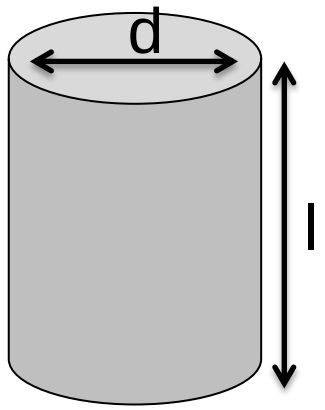

Figure 2.3: Volume of a cylinder is equal to its circumference (πd) multiplied by its length (l).

To measure the change in circumference Whitney employed the elastic mercury-in-rubber strain gauge, such that alterations in the circumference of the forearm at the level of the strain gauge would lead to alterations in its length; this in turn would cause a (recordable) proportional variance in the strain gauge's resistance [378]. This technique has changed little over the years being only recently refined by the introduction of computerisation giving rise to traces as seen in Figures 2.4 and 2.5.

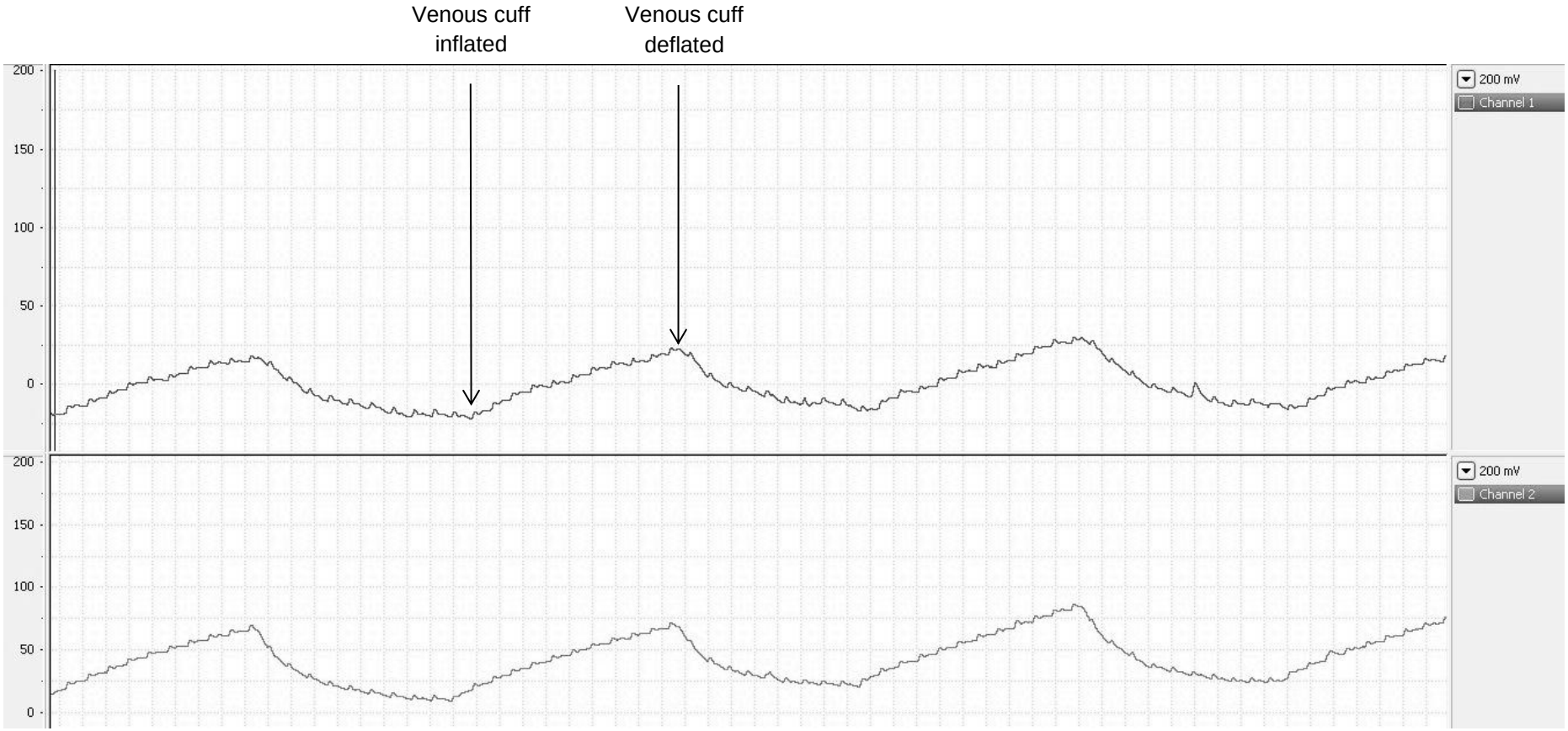

Figure 2.4: A venous occlusion plethysmography computer enhanced tracing taken at baseline (i.e. during saline infusion). The above tracing (Channel 1) is of the intervention (infused arm) and the bottom tracing (Channel 2) is of the control (non-infused) arm. The rate of increase in forearm volume in both arms appears to be equal. The points at which the venous cuffs are inflated and deflated are clearly marked.

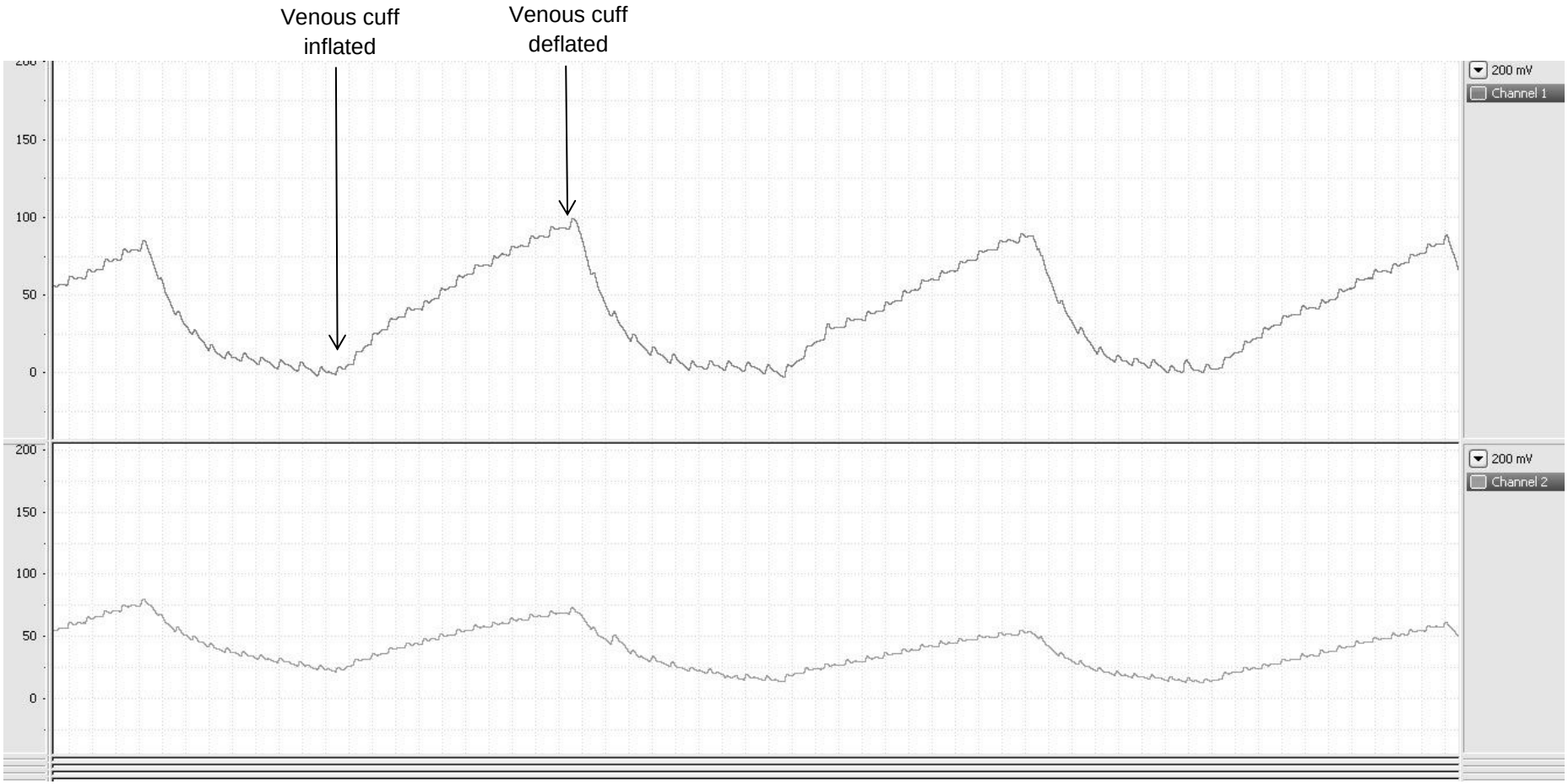

Figure 2.5: A venous occlusion plethysmography computer enhanced tracing taken during inorganic nitrite infusion. The above tracing (Channel 1) is of the intervention (infused arm) and the bottom tracing (Channel 2) is of the control (non-infused) arm. The rate of change in the volume of the intervention arm has increased from that seen on baseline (Figure 2.4) and is clearly greater than that of the control arm. The points at which the venous cuffs are inflated and deflated are clearly marked.

In addition to approximating the volume of the forearm to that of a cylinder, exclusion of the hand's circulation is required in view of the different pharmacological and physiological responses displayed by its vasculature when compared to that of the forearm [378,382]. The difference is consequent to the high number of arterio-venous shunts contained in the hand and the larger proportion of blood flowing through the skin; whereas in the forearm the bulk of blood flow (~50 - 70%) is through the muscle [382,383].

Excluding the hand's circulation is achieved by rapidly inflating a cuff placed around the wrist to a supra-systolic pressure (~170 - 180 mmHg). The wrist cuffs are inflated for around 30 - 60 sec prior to taking measurements to allow FBF to reach a stable and steady state [384]. Occlusion of venous return is accomplished via inflating a cuff placed around the upper arm to a pressure that is supra-venous but well below the diastolic pressure (~40mmHg) [385] (Figure 2.6). Although the wrist cuffs remain inflated throughout the time period in which the measurements are taken (this should be minimised as the hands remain ischemic for the duration) the venous cuffs are inflated for 10 seconds then deflated for ~5 seconds to allow the veins to empty and the recording to return to base line [386] (Figures 2.4 & 2.5). To facilitate rapid emptying once the cuffs are deflated, the arm is supported using foam pads and the forearm positioned slightly above the level of the right atrium. The measure of blood flow in the roughly cylindrical area delineated by the two cuffs is expressed in ml of flow per min per 100 ml of tissue (ml/min/100 ml tissue).

Combining intra-arterial drug administration with VOP, indirectly assess the vasoactive effects of drugs on the tone of resistance vessels, by measuring the changes in flow [382,387] (Figure 2.6). However, the relationship between changes

in flow and vascular tone will only hold true as long as there are no significant changes in arterial BP, as a vessel's resistance results from the interplay of the underlying tone and the distending pressures [388].

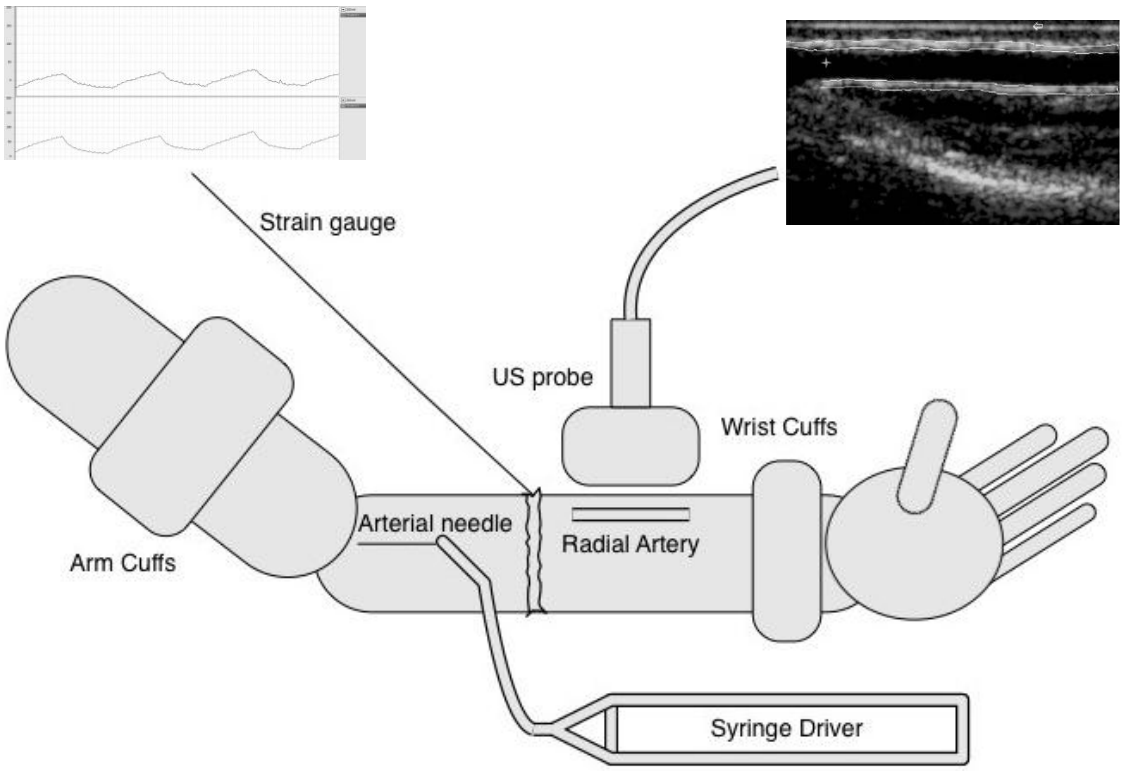

Figure 2.6: Diagrammatic representation of the 2D US, venous occlusion plethysmography and intra-brachial infusion set up to measure changes in conduit (RA) and resistance (FBF) vessel changes. 2D US – 2-dimensional ultrasound, RA – Radial Artery, FBF – fore arm blood flow.

It is important to note that by altering FBF, a vasoactive drug will also cause changes in its own concentrations found in the plasma; thus a vasodilator which enhances FBF will lead to a fall in plasma concentrations, whereas a vasoconstrictor will reduce FBF and increase plasma concentrations [389]. Hence, the vasoactive effect of a drug is expressed in the form of a cumulative dose response curve not the concentration of the drug in the plasma. Although the vessels can reach maximal dilatation (or constriction) meaning that there is a sigmoid relationship, at the lower doses used the association between the

response and the log of the infused dose is a linear one [387]. Results can be expressed as an absolute change from baseline FBF or as a change in ratio between the infused arm and control arm. The latter allows for the negation of any external factors that may influence general basal tone. However, although it may seem that using the non-infused arm as a control when expressing and analysing the data is advantageous, allowing for better reproducibility, this only seems to hold true when evaluating vasoconstrictors. When assessing vasodilators the data is improved when expressed in absolute terms using the infused arm only, as small changes in the FBF in the control arm will have a magnified effect on the FBF ratio in the high flows induced by the drug [390]. Indeed percentage change in ratio of FBF was found to be more reproducible when assessing the effects of vasoconstrictors [391]. However, when assessing the effects of vasodilators it was found that reproducibility was enhanced when the changes were quoted as absolute values of FBF rather than as a percentage change in the FBF ratio [392].

2.4.3 Measurement of radial artery and forearm blood flow protocol

The volunteer remained supine for the duration of the study. Pressure cuffs were placed around the upper arms and the wrists. The cuffs themselves were connected to E20 Rapid Cuff Inflators, which in turn were supplied by an AG101 Cuff Inflator Air Source (Hokanson Inc. WA US®). The circumference of the forearms were measured, using a measuring tape, and an appropriately sized mercury-in-silastic strain gauge was placed around each forearm and connected to an EC6 Plethysmograph (Hokanson Inc. WA US®). The wrist cuffs were inflated to supra-systolic pressure (180±2 mmHg) to exclude the circulation in the hand. After a 30-60 sec pause, to allow the changes in the forearm circulation to reach equilibrium, the cuffs around the upper arm were inflated to supra-venous pressures (40±2 mmHg) and deflated a number of times in cycles consisting of ~10 sec inflation and ~5 sec deflation. The degree of change in the circumferential size of the forearm measured by the mercury-in-silastic strain gauge was acquired by channelling the signal through the EC-6 Plethysmograph, and recorded and analysed using Chart 5 software® (see figures 2.4 & 2.5).

2.5 Measurement of systemic haemodynamics

Following a minimum period of 5 min laying supine, baseline readings of non-invasive measurements of the following systemic haemodynamic parameters were performed: pulse wave velocity (PWV), brachial-femoral (bfPWV) was measured over the brachial to radial path from simultaneous pressure cuff recordings at each site using the Vicorder system (Skidmore Medical Ltd, Bristol UK®). The path distance was taken from the proximal edge of the upper arm cuff to that of the wrist cuff. Peripheral BP and heart rate were also measured (IntelliVue MP30, Phillips, NL®): during forearm blood flow studies, a thigh cuff was placed over the arm cuff, however, measurements were also taken at baseline and at the end of the infusion with the BP cuff placed directly on the arm; these latter measurements were used to calibrate the Finometer (Finapres Medical systems, Amsterdam NL®) used to determine central blood pressure, augmentation index and heart rate. An average of 2 readings (rather than 3) was taken for the final results, due to time limitations. Measurements were repeated during and/or at the end of the study. In calculating the central pulse pressure (cPP), the peripheral diastolic BP (DBP) measurement was used, as central DBP was not recorded. However, DBP changes by only 1-2 mmHg from the aorta to the periphery, and therefore our measurement of peripheral DBP is a close estimate of central DBP. In addition, SpO2, SpMet (Radical-7, Rainbow, CA, US) were measured continuously in studies using higher doses of nitrite or in those in which hypoxia or hyperoxia were induced.

2.6 Hypoxia/normoxia/hyperoxia studies Protocol

A continuous positive airway pressure (CPAP) mask, fixed with a one-way 5.0 cm H_2O positive end expiratory pressure (PEEP) valve system, was securely fitted around the participant's nose and mouth. The mask was connected via 1.5 meters of tubing to either: (i) a room air port (normoxia), (ii) a 100% oxygen port (hyperoxia) or (iii) a 12% oxygen/ nitrogen balanced gas mix cylinder (hypoxia). When the latter was used, the aim was to achieve stable arterial oxygen saturation levels (as measured by pulse oximetry) above 83%, as previously described by Maher et al. [393]. All three inhaled gases were delivered at a pressure of 1.5 to 2 bars and the flow of gas was adjusted to achieve the desired oxygen saturations at a level that was comfortable for the volunteer (15 - 20 litre/minute). At the completion of the study an arterial blood gas sample was aspirated, into a heparinised gas syringe, via the brachial needle and analysed through a blood gas machine.

It should be noted that a number of limitations of this study design do persist. The use of a facemask resulted in a sudden drop in inspired oxygen levels and hinders the maintenance of a steady state of hypoxia. Acute hypoxia (with oxygen concentrations of < 17%) affects haemodynamics, inducing tachycardia [394] and increasing cardiac output and BP [386]. In addition, it affects respiratory physiology causing pulmonary hypertension and hyperventilation [382].

Moreover, prolonged exposure to hypoxia (up to 2 hours) is required to allow for equilibration and the attainment of a steady physiological state , however, the need to immobilise the participants for the total duration of the study restricted it's length, limiting the interval of exposure to hypoxia.

2.7 Measurement of plasma nitrite and s-nitrosothiols

A number of techniques are available to measure nitrite and other NO-related species in biological samples, either directly (e.g. capillary zone electrophoresis, high-performance capillary electrophoresis and high-performance liquid chromatography) or indirectly (e.g. Griess reaction or chemiluminescence detection method) [388]. However, ozone based reductive chemiluminescence detection (CLD) has become the method of choice for the detection and quantification of nitrite and other NO metabolites in biological matrices due to its sensitivity and specificity when combined with different reductive methods [382,395].

2.7.1 Chemiluminescence detection

Originally developed by Cox and Frank in 1982, CLD measures nitrite levels in fluid samples indirectly by reducing it to NO and measuring the stoichiometric amount of NO that is released [382]. It has been reported to detect levels of nitrite as low as 5nM in biological fluids [388].

Cox and Frank noted that in the presence of an acid (they used glacial acetic acid) nitrite reacts to produce free nitrous acid (see Equation 1), the nitrous acid can then further be protonated to $H_2NO_2^+$ (Equation 15) which exists in equilibrium with a nitrosonium cation (NO^+) (Equation 16).

$$H^+ + HNO_2 \leftrightarrow H_2NO_2^+ \quad (15)$$

$$H_2NO_2^+ \leftrightarrow NO^+ + H_2O \quad (16)$$

However, they also noted that to release NO a reducing agent was required, and for that they used potassium iodide (KI), where the free iodide anion (Equation 17) reacts with NO^+ converting it to NO via a nitrosyle iodide (ONI) intermediate (Equations 18 & 19).

$$KI \rightarrow I^- + K^+ \quad (17)$$

$$NO^+ + I^- \rightarrow ONI \quad (18)$$

$$2ONI \rightarrow 2NO + I_2 \quad (19)$$

This method of quantification is not exclusive to nitrite alone as different redox agents can be used; the chemical nature of which depends on the specific NO-species being measured. Using the appropriate reducing agent allows chemiluminescence to be highly selective with a level of imprecision reported to be as low as 5% [382].

A glass purging-vessel (Figure 2.7) is used to hold the redox chemical solution. The sample which is to be tested is injected in 50 µL – 200 µL aliquots directly

into the purging vessel so that the redox agent reduces the nitrogen containing species releasing NO into the solution at the base of the vessel.

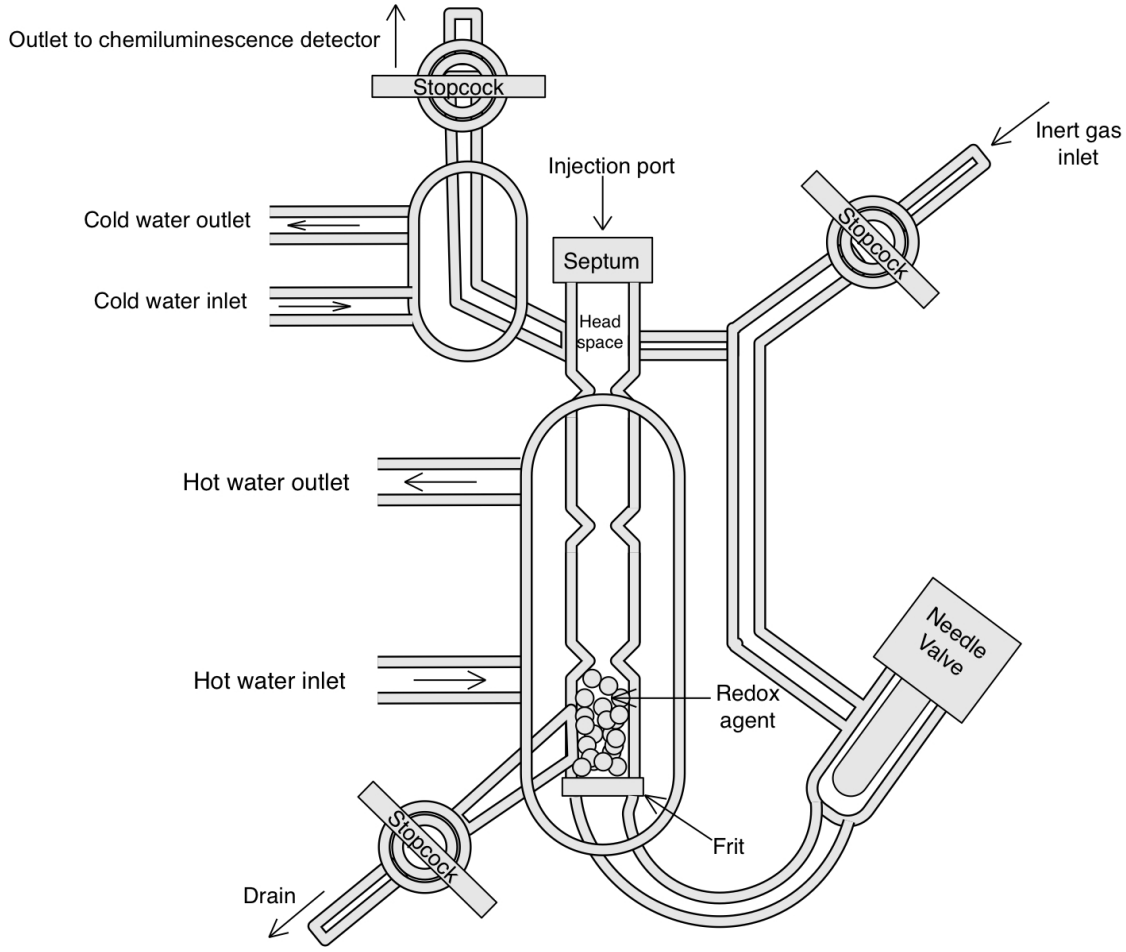

Figure 2.7: Diagrammatic representation of a purge vessel set up. The stopcocks in this diagram are all depicted in the closed position. When running an assay the inert gas inlet and NO outlet stopcocks should be open. The drain stopcock is opened when the redox chemical is due to be changed. The needle valve is used to adjust the cell pressure and control the rate of flow of the inert gas into the reaction chamber. NO – nitric oxide.

An inert gas is purged through the system forcing the NO out of the solution, and into a gaseous form that collects in the headspace. The inert gas continues to carry the NO from the headspace, through a glass chemical trap containing 10 - 15 ml of 1 M NaOH, then to the nitric oxide analyser (NOA) where it enters the reaction chamber and is eventually detected and quantified. The principle of

the NOA is dependent on the rapid reaction between NO and ozone (O_3) which produces nitrogen dioxide in an excited state (NO_2*) (Equation 20) [396].

$$NO + O_3 \rightarrow NO_2^* + O_2 \quad (20)$$

As the excited electron of the NO_2* returns to the ground state it releases a photon ($h\nu$) in the form of light in the red and infrared part of the spectrum (~640 - 3000 nm) [397] (Equation 21).

$$NO_2^* \rightarrow NO_2 + h\nu \quad (21)$$

A photomultiplier tube (PMT) then amplifies the sensed light generating an electrical signal that can be recorded and quantified by a computer and appropriate software. Examples of the traces obtained can be seen in Figure 2.8. The complete set up can be seen in Figure 2.9.

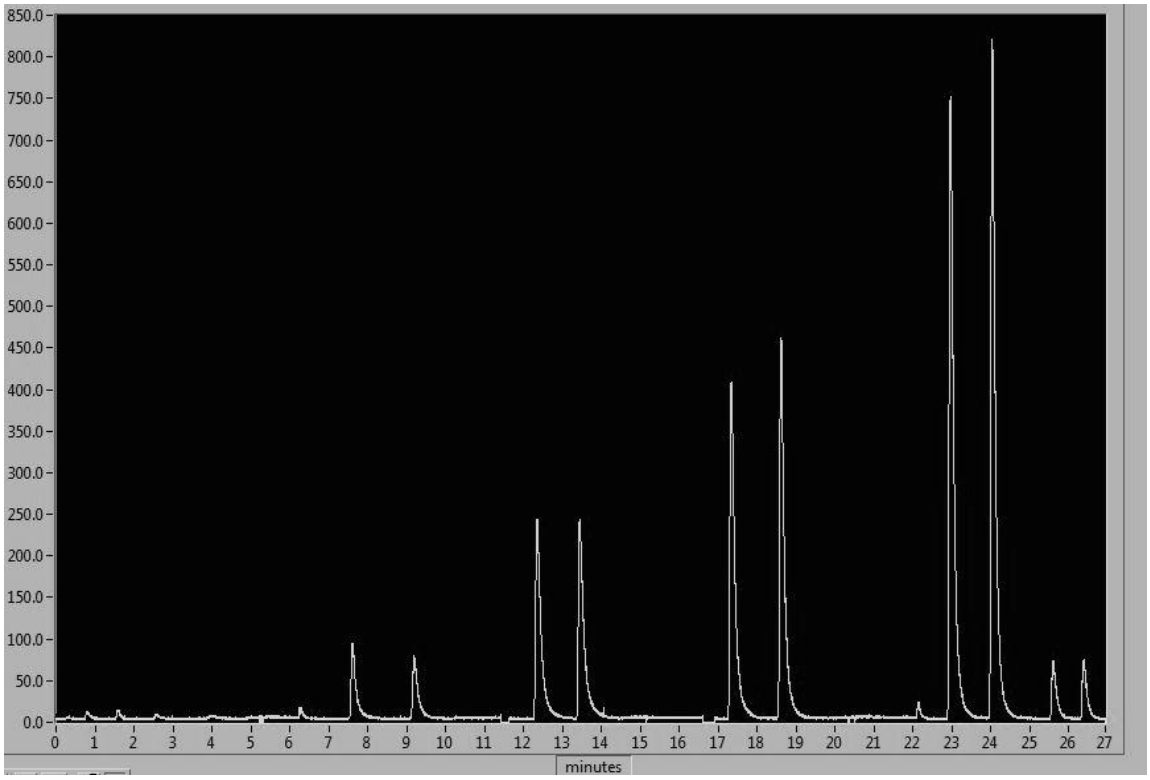

Figure 2.8: A signal tracing recorded by the data analysing software. Each peak corresponds to a sample injection into the purge vessel. The size of the peak (area under the curve) is proportional to the quantity of NO detected. NO – nitric oxide.

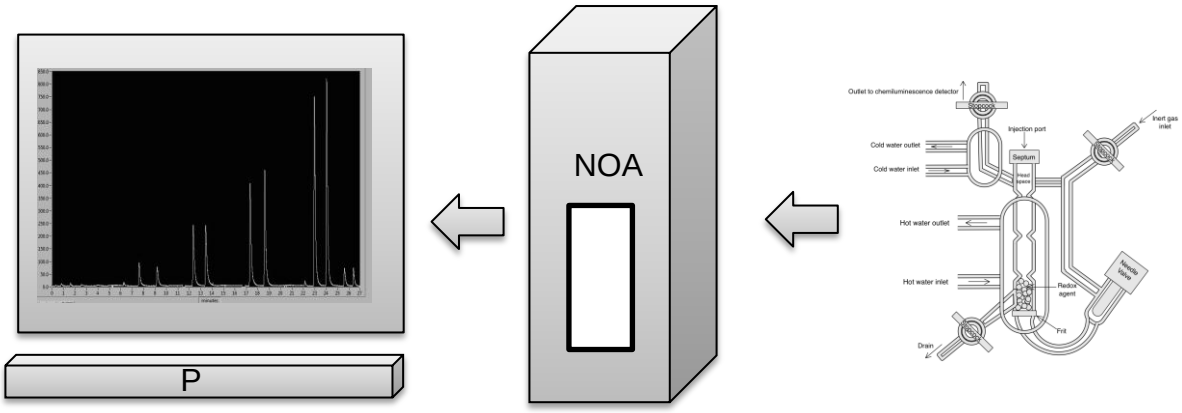

Figure 2.9: Chemiluminescence apparatus set up. The sample is injected into the purge vessel containing the redox agent. The NO-containing species is reduced and NO is released. The inert gas carries the NO from the purge vessel to the NOA. NO reacts with ozone releasing a photon that is detected by the analyser as a signal. The signal is translated and recorded by the data recording software. NO - nitric oxide, NOA - nitric oxide analyser, PC - personal computer.

When measuring SNOs the purge vessel is filled with acidified tri-iodide (I_3^-), a redox chemical that is able to reduce a host of NO-containing species [29,398]. Tri-iodide exists in the acidified solution as I_3^- but also as free iodine (I^-) and iodide (I_2) (Equation 22) and readily reduces SNOs, forming a disulphide (RSSR) intermediate and NO^+ (Equation 23), the later forming NO in a sequence of reactions as seen in Equations 18 and 19.

$$I_3^- \rightarrow I^- + I_2 \quad (22)$$

$$I_3^- + 2RS\text{-}NO \rightarrow 3I^- + RSSR + 2NO^+ \quad (23)$$

However, as the free iodine from the tri-iodide reacts with nitrite reducing it and releasing free NO, it is necessary to treat the sample in a way as to render nitrite undetectable via chemiluminescence. Depleting the sample of nitrite is accomplished by the addition of acidified sulfanilamide which reacts with the nitrite to form a diazonium cation that is stable in tri-iodide and does not generate a signal [399]. It has been proposed that treatment with acidified sulfanilamide also degrades SNOs [400]. However, it has been shown that SNOs have a high degree of stability:

SNO-Hb (87.6%), SNO-HAS (90.8%), GSNO (96.8%) and Cys-SNO (99.5%), when treated with acidified sulfanilamide [378].

However, using CLD to measure circulating levels of SNO's in this study does present a unique set of difficulties. For instance the standards used to establish standard curve were based on nitrite and not artificial SNO's. Furthermore, it has been suggested that chemicals used in preparing the samples for SNO analysis do not completely remove nitrite from the sample and may cause the decomposition of SNOs, thus, hindering accurate quantification [401]. Besides, if nitrite were to be successfully depleted from the sample, this alone would affect the levels of SNOs, as the two exist in equilibrium [401]. Moreover, SNOs posses variable half lives with some only lasting for seconds, which makes measuring them in samples stored for even a short period of time especially challenging [402]. Other factors affecting this assay are the nature and conformation of the SNO moiety being measured. Thus, although SNO's have a high degree of stability following acidification (as stated above), a change in their confirmation may affect the assay.

Although CLD is highly sensitive there is a wide disparity in baseline plasma nitrite levels reported in different studies [403-406]. These discrepancies are likely to be a result of variations in sample preparation, assay techniques and possible contamination.

Due to its rapid reaction with haemoglobin, nitrite has a short half-life (~10 min) in the circulation [401]. However, once the sample has been removed (without *in vivo* production) nitrite has a half-life of less than 2 min [405]. Hence, it is imperative that the sample be processed immediately, and the plasma separated from the erythrocytes as swiftly as possible, either physically (via

centrifugation) or chemically (via the use of ferricyanide or carbon monoxide) [407]. Furthermore, nitrite is scavenged more rapidly by free haemoglobin, therefore the blood sample should be aspirated through a large bore cannula to avoid haemolysis. Regardless, a degree of degradation during the processing of the sample is unavoidable; however, doing this rapidly, using pre-chilled vacutainers, in addition to centrifugation at a low temperature (4°C) limits the degradation.

Assay techniques can be improved by: (i) conducting the assay at temperatures of 40 °C by circulating warm water around the purge vessel reaction chamber, (ii) ensuring the vacuum in the purge vessel is intact and that there are no gas leaks in the system, (iii) maintaining the cell pressure at a constant level by adjusting the flow of the inert gas, (iv) minimising foaming by adding an antifoaming agent to the redox chemical when analysing proteinated samples, (v) changing the redox agent regularly and frequently after multiple injections, (vi) using a gas-tight Hamilton long (5 inch) bevelled needle syringe to inject the samples swiftly and directly into the centre of the bottom of the purge vessel, (vii) allowing sufficient time between consecutive injections.

The pervasive nature of nitrite makes it a ubiquitous lab contaminant, present in tap water, tainting glassware and almost all clinical vacutainers [407,408]. Although found only in trace amounts in tap water, nitrite's low concentrations in plasma makes it imperative to use nitrite free water in all aspects of the analysis from preparing standards to cleaning the equipment. Nitrite's levels are imperceptible in Millipore treated water [402] and so this should be used preferentially when conducting assays. Finally, heparin treated vacutainers should be used as they have been shown to have far less contamination than EDTA vacutainers [408].

2.7.2 Measuring plasma nitrite protocol

Blood was sampled serially through an 18 gauge Venflon® cannula placed in a forearm vein. In some studies blood was taken from the arm receiving the infusion (ipsilateral arm) in addition to the contralateral arm. Five ml samples of blood were aspirated and immediately transferred to pre-chilled Lithium Heparin tubes (Vacuette®) and immediately spun at 4 °C for 5 min at 4700 RPM (Mikro 220R centrifuge, Hettich GR®). The haemolysis-free supernatant plasma was removed and placed into two 1.5 ml pre-labelled Ependorff tubes. These samples were snap-frozen in liquid nitrogen and stored at -80 °C until the day of the analysis, when they were thawed and stored on ice. The levels of nitrite in whole plasma were analysed using the 280i Nitric Oxide Analyzer (Sievers Instruments, GE analytic instruments®). The Purge vessel was filled with sodium iodide dissolved in 99.8% glacial acetic acid to which a few drops of antifoaming agent were added. A stock solution of 100 mM sodium nitrite was used to prepare the standards which were used in generating the calibration curves. The series of standard dilutions was constructed by serial dilution of the stock solution giving concentrations of 100 nM, 0.5 µM, 1 µM, 5 µM, 10 µM, 50 µM and 100 µM. HPLC nitrite free water was used in the preparation of the stock and standard solutions. Using an analytical syringe (Hamilton Bonaduz, GR, SW®), 50 µL samples of selected dilutions were injected, in duplicate, into the purge vessel directly onto the reducing agent. The NO generated was detected by the NOA and analysed by the software to generate the calibration curve. To maintain consistency, close attention was paid to the volume of reducing agent, the maintenance of the cell pressure and the maintenance of the gas pressure. Once a satisfactory calibration curve had been generated, 50

µL of the plasma samples were injected, in duplicate, into the purge vessel. The reducing agent in the purge vessel was changed after each duplicate. The concentrations of nitrite were derived from the calibration curve. For most studies whole plasma was used, with antifoam added to the chamber to reduce frothing. In the intra-venous nitrite study, plasma was deproteinated using 3K Microcon filters (Merck Millipore®).

2.7.3 Measuring plasma s-nitrosothiols protocol

Using an 18 gauge needle, blood samples were collected from the contralateral arm at baseline and from the infused arm at the end of the study. Blood (~5 ml) samples were aspirated and treated in an identical manner as those described above for plasma nitrite. On the day of the analysis the samples were thawed and placed on ice. At least three minutes prior to analysis part of the sample was treated with 5% acidified sulfanilamide (prepared daily by dissolving 5 g of sulfanilamide in 100 ml of 1 M HCl) in a ratio of 9:1 of sample to acidified sulfanilamide. Samples were analysed using the 280i Nitric Oxide Analyzer (Sievers Instruments, GE analytic instruments®) as per nitrite. The Purge vessel was filled with acidified tri-iodide (prepared daily by dissolving 2.0g of potassium iodide (KI) with 1.3 g of Iodine (I_2) in 40 ml of distilled water (H_2O) and 140 ml of 99.8% glacial acetic acid) to which a few drops of antifoaming agent were added. The series of standard dilutions was constructed by serial dilution of the stock solution (100 mM sodium nitrite) giving concentrations of 5 nM, 10 nM, 25 nM, 50 nM, 100 nM, and 250 nM. Using an analytic syringe (Hamilton Bonaduz, GR, SW®), samples were injected into the purging vessel in aliquots of 100 µL. Otherwise all remaining aspects of the analysis were conducted in a manner identical to the one described for plasma nitrite (Section 2.6.2).

2.8 Measurement of plasma cGMP

2.8.1 Enzyme-immunoassay

The Enzyme-Linked ImmunoSorbent Assay (ELISA) is an analytical technique that employs antibodies, antigens and enzymes in detecting and quantifying an analyte of interest (in this case cGMP) in a liquid or liquefied sample [408]. The quantification of the analyte is achieved through a controlled sequence of biochemical reactions, culminating in the generation of a signal (usually a colour change) that can be measured then translated to express the concentration of analyte present in the sample. Maintaining the same basic principle but varying the sequential addition of the antibodies and antigens gives rise to slightly different assay methods; these are described as being either indirect, sandwich or competitive.

The Amersham cGMP Enzymeimmunoassay Biotrak system (GE Healthcare ®) that was used to conduct the assessment of plasma cGMP levels relied on the competitive sequence.

The first step of the competitive technique is known as the solid substrate or solid phase. In it a plate composed of multiple wells is pre-coated with an antigen that is then immobilised onto its surface (in this particular assay the solid substrate is a donkey anti-rabbit antigen). The following step involves the addition of an antibody (in this assay a rabbit anti-cGMP antibody) with a particular affinity for the solid substrate. However, although part of the antibody does bind irreversibly to the solid substrate, an epitope with specificity for the analyte (cGMP) remains free. When the analyte is added to the wells it binds to the available antibody epitope, forming a reversible complex. The next step of the sequence involves the addition of a pre-prepared enzyme linked form of the

analyte (in this assay it is a peroxidase conjugated cGMP). This construct (peroxidase conjugated cGMP) is in direct competition with the analyte (cGMP) giving the technique its name. In the penultimate step, the plate is washed so that only the ligands (cGMP or conjugated cGMP), which are bound by the antigen-antibody interactions to the solid phase (donkey anti-rabbit and rabbit anti-cGMP), remain fixed to the plate, while the unbound components are washed away. The proportion of cGMP vs. peroxidase-conjugated cGMP that remains bound depends on the concentration of the former. In the final step The plate is developed by adding the peroxidase enzymatic substrate 3,3',5'5'-tetramethylbenzidine (TMB), a chromogenic reporter which when cleaved leads to a detectable colour change that can be translated into a measurable signal. This is achieved via the transmission of a specific wavelength of light through the liquid and detecting the transmitted intensity by spectrophotometry. This signal is then used to indicate the quantity of the analyte (cGMP) in the sample (Fig 2.10).

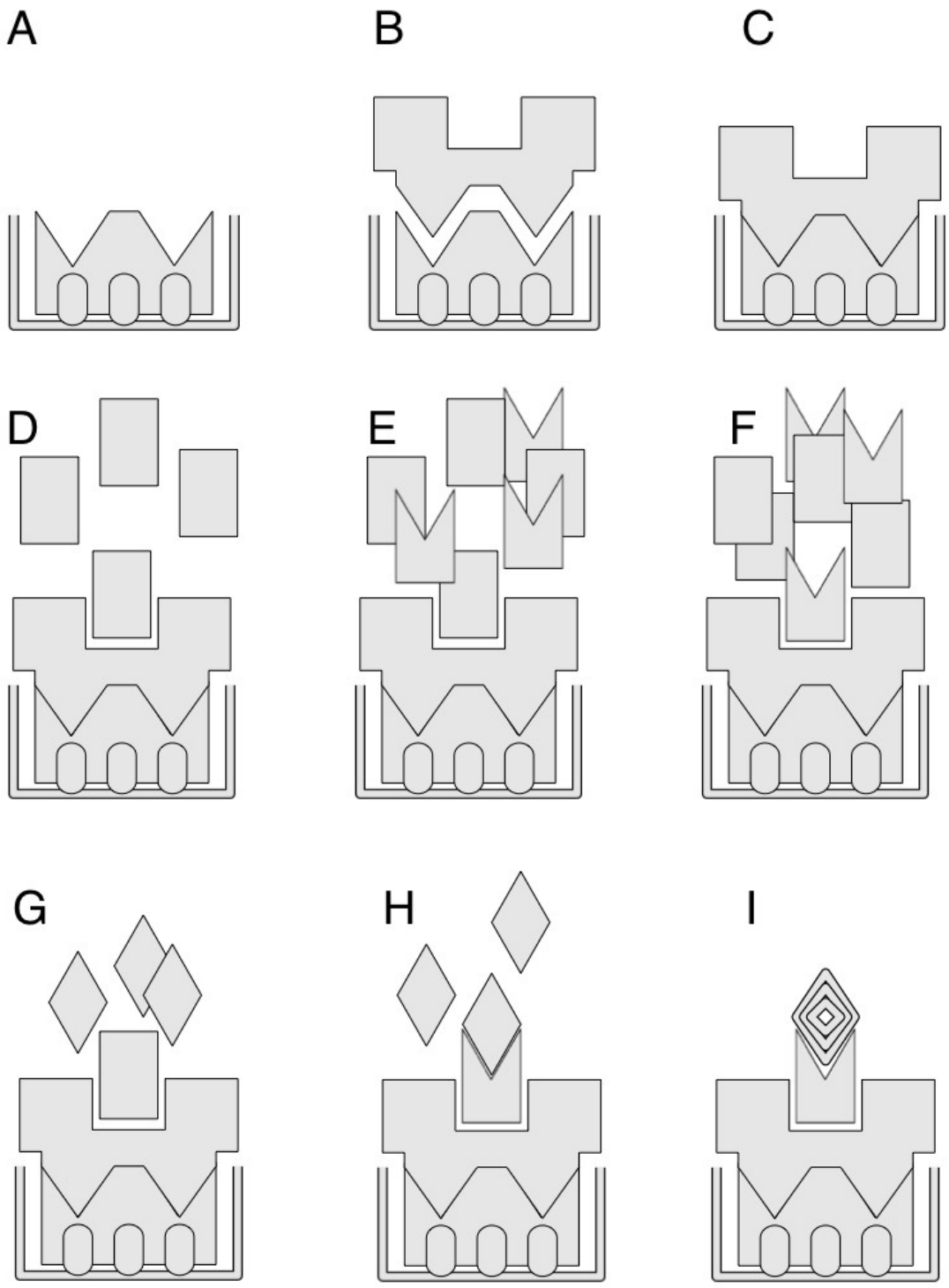

Figure 2.7: Competitive cGMP enzyme-immunoassay: A) The solid phase where the antigen is fixed to the well. B) The antibody is added to the well. C) The antibody binds to the solid phase antigen, however an epitope remains free. D) The analyte (cGMP) is added to the well and binds to the free epitope. E) The enzyme-linked analyte (peroxidase conjugated cGMP) is added to the well and competes with cGMP, however here cGMP remains un-displaced. F) Conjugated-cGMP displaces cGMP. G and H) after washing the wells TMB is added: In (G) cGMP remains bound thus TMB remains inactivated. In (H) conjugated-cGMP is bound thus TMB binds to it and is activated as seen in (I). TMB - 3,3',5'5'-tetramethylbenzidine.

2.8.2 Cyclic GMP assay protocol

Blood samples (~4 ml each) were collected at appropriate time points, via an 18 gauge cannula, placed in the anticubital fossa vein, of either the infused or contralateral arm and transferred to pre-chilled EDTA tubes (Vacuette®) and immediately spun at 4 °C for 5 min at 4700 RPM (Mikro 220R centrifuge, Hettich GR®). The haemolysis-free supernatant plasma was removed and placed into two 1.5 ml pre-labelled Ependorff tubes. These samples were snap-frozen in liquid nitrogen and stored at -80 °C until the day of the analysis.

The assessment was conducted using the Amersham cGMP Enzymeimmunoassay Biotrak system (GE Healthcare®). The reagents provided, in the assay kit, were allowed to equilibrate to room temperature prior to use. Diluted assay buffer was then reconstituted by adding, and thoroughly mixing, the contents of the provided concentrate to distilled water, bringing the total volume to 500 ml. The dilute wash buffer was reconstituted in an identical manner.

Both the lyophilised cGMP antibody and the lyophilised cGMP conjugate were reconstituted by adding 11 ml of the diluted assay buffer to each of the individual bottles and gently mixing until completely dissolved. Care was taken to avoid excessive agitation and foaming.

The stock standard of cGMP (10.24 pmol/ml) was reconstituted by adding 2.5 ml of the diluted assay buffer to the acetylation standard bottle; the contents were carefully mixed until completely dissolved. Eight further working standards were constructed by consecutive serial dilutions of the stock solution, with an equal volume of dilute assay buffer, giving concentrations of 5.12, 2.56, 1.28, 0.64, 0.32, 0.16, 0.08 and 0.04 pmol/ml.

The plasma samples for analysis (or unknowns) were prepared by diluting the plasma with the assay buffer in a ratio of 10:1. A volume of 1 ml of each of the standards and each of the unknowns was transferred to an appropriately pre-labelled polypropylene tube.

At the completion of the above steps, the acetylation reagent was created by adding 2.5 ml of acetic anhydride to 5 ml of triethylamine in a glass vessel and mixing thoroughly. The resulting reagent was added in aliquots (100 µL) to each of the polypropylene tubes containing 1ml volumes of the standards or unknowns; each tube was then immediately agitated using a vortex mixer.

The pre-treated micro-plate was set up with sufficient wells to run non-specific binding (NSB) blanks, standards and unknowns. Excluding the wells designated as NSB, 100 µL of the diluted antiserum was added to all the wells. This was followed by adding 150 µL of the assay buffer to the NSB wells and 50 µL aliquots from all the acetylated standards and diluted plasma samples (unknowns) to the appropriate pre-designated wells. All samples were added in duplicate. On completion the micro-plate was covered, gently mixed and incubated at 3 - 5 ^{o}C for 2 hours.

At the end of that time period the micro-plate was uncovered and aliquots of the dilute conjugate (100 µL) were added to each of the wells. The micro-plate was re-covered, gently mixed and allowed to incubate at 3 - 5 ^{o}C for 1 hour.

At the completion of this time period the plate was carefully and thoroughly washed using the diluted wash buffer. The plate was blotted to ensure the removal of any residual wash buffer. Room temperature TMB hydrogen peroxide was immediately added to the wells in 200 µL aliquots. The micro-plate was covered and agitated at room temperature on a shaker for 30 min.

The reaction was halted by adding 100 µL of 1 M sulphuric acid to all the wells. The end point value was determined using a spectrophotometric plate reader capable of measuring optical density at 450 nm. SoftMax Pro® microplate acquisition and analysis software was used to construct a standard curve of absorbance vs. log10 of the antigen concentration which was then used to quantify the concentration of cGMP in the (unknown) test samples.

Chapter 3

Results – 1

Intra-arterial sodium nitrite and GTN

3.1 Subjects

A total of 43 healthy males were requited for the studies, of whom 16 took part in more than 1 study. The demographic data for each study are shown in Table 3.1.

Study	Intervention	Dose /min	Number	Age (years)	Height (cm)	Weight (kg)
1	i.a. Nitrite	8.7 µmol	8	34.9 ±7.8	NR	NR
2a	i.a. Nitrite	0.087-87 µmol	8	23.9 ±3.9	182.8 ±4.1	76.9 ±13.5
2aii	i.a. Nitrite (buffered)	0.087-26 µmol	4	32.75 ±4.4	180.8 ±2.1	90.75 ±19.9
2b	i.a. GTN	0.003 -1 µg	7	26.3 ±8.0	180.2 ±8.0	76.3 ±8.4
3	p.o. ACZ & RX	0.087-26 µmol	14	28.0 ±7.4	179.8 ±6.0	79.7 ±21.4
4	i.a ACZ	2.6 µmol	8	33.6 ±13.1	179.2 ±6.3	82.4 ±14.1
5	O_2	0.087-26 µmol	8	34.6 ±15.4	177.6 ±8.7	79.4 ±13.62
6	i.v. Nitrite	8.7 µmol	9	30.3 ±9.4	NR	NR

Table 3.1: Demographic data. Data shown as mean±SD. NR – not recorded, i.a. – intra-arterial, i.v. – intra-venous, ACZ – acetazolamide, RX – raloxefine, O_2 – Oxygen.
Study 1: Fixed dose i.a. sodium nitrite
Study 2: Dose responses to i.a: (a) sodium nitrite (b) GTN
Study 3: Effect of oral acetazolamide and raloxifene on the dose response to i.a. sodium nitrite
Study 4: Effect of a co-infusion of i.a. acetazolamide and sodium nitrite
Study 5: Effect of normoxia, hypoxia, and hyperoxia on the dose response to i.a. sodium nitrite
Study 6: Fixed dose i.v. sodium nitrite

3.2 Statistical Analysis

The data from the studies was analysed using the Graph Pad Prism Software. All data are expressed as mean (95% confidence intervals) unless otherwise stated. Data were compared by repeated-measures ANOVA (2-tailed) with Dunnett's post test for comparison with baseline and Bonferroni post test for comparison with the control group. P values <0.05 were considered statistically significant, unless Bonferroni corrections for multiple comparisons were required, as stated.

3.3 (Study 1) Intra-arterial sodium nitrite - fixed dose

Sodium nitrite at a fixed dose of 8.7 μmol/ min was infused into the brachial artery, of 8 volunteers, for the duration of 60 min. Radial artery response was assessed at baseline, 5 min, 15 min, 30 min, 45 min and 60 min; FBF was assessed immediately following the end of each 2 min RA diameter acquisition.

The intra-arterial infusion of sodium nitrite resulted in a marked dilatation of the RA at 5 min by 21.4 % (95% CI 15.3 to 27.6) compared to baseline saline, Figure 3.1. This was ~75% the value at 60 min, which was 28.3% (95% CI 22.1 to 34.4). The data from one volunteer was excluded from the analysis as their radial artery dilatation to nitrite exceeded the mean + 2 SDs (of the entire group) at all time points. Furthermore, of particular note, the mean oxygen saturation of haemoglobin was 99% (range 97-100%), i.e. this dilatation did not occur under conditions of reduced oxygen tension found in small arterioles, or hypoxia (or ischemia) which have been considered essential for the effects of nitrite via its reduction to NO, but rather occurred under fully oxygenated conditions that are generally considered to inhibit nitrite bioactivation.

As expected, sodium nitrite infusion increased FBF: this was by 2.1 ml/ min/ 100 ml tissue (95% CI 0.7 to 3.5) at 5 min, see Figure 3.2, with no further significant increase over 60 min in the infused arm. However, FBF remained unchanged in the control forearm throughout the 60 min (Figure 3.2). There was an eight-fold elevation in systemic plasma nitrite concentration from 64±19 nmol/L at baseline to 528±128 nmol/L at 5 min and a 45-fold elevation to 2853±314 nmol/L at 60 min (Figure 3.3). However, despite this rise in circulating nitrite there were no significant changes in heart rate nor in peripheral brachial blood

pressure (systolic, SBP, mean arterial, MAP, or diastolic, DBP) were detected during the 60 min infusion of sodium nitrite compared to baseline, i.e. time 0 min (Figure 3.4).

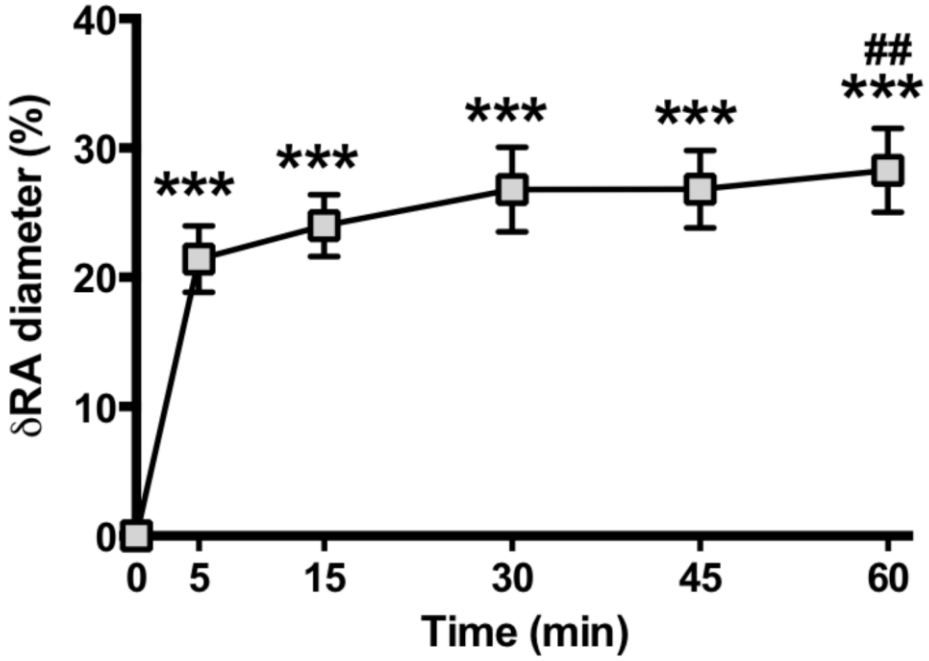

Figure 3.1: Effect of 60 min intra-brachial infusion of sodium nitrite (8.7 μmol/ min) on change in artery (RA) diameter (%); Data shown as mean±SEM, *n*=7, ***P<0.001 compared to baseline, ##P<0.01 compared to 5 min.

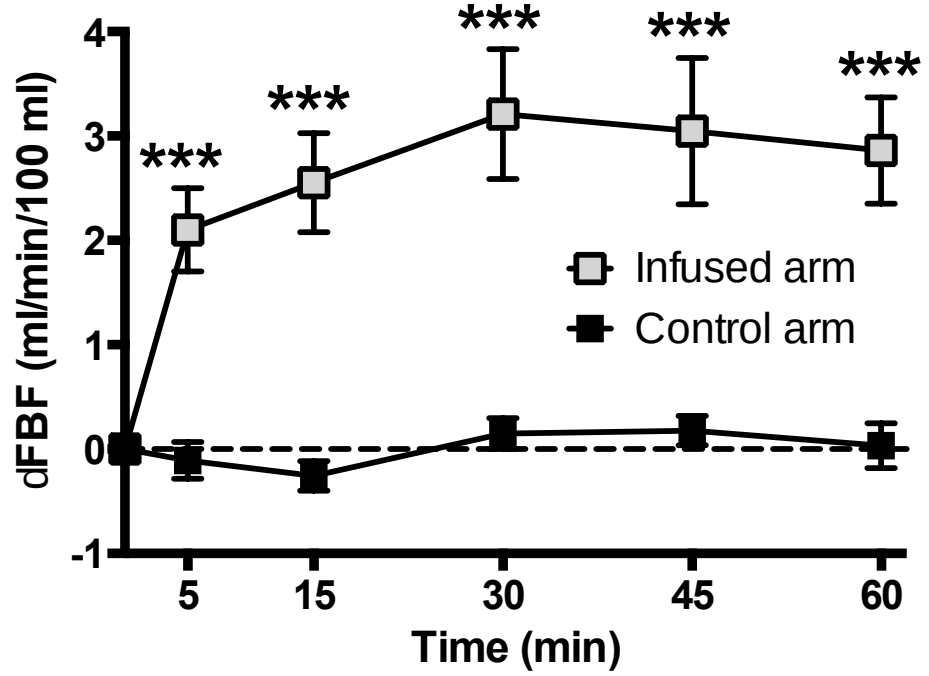

Figure 3.2: Effect of 60 min intra-brachial infusion of sodium nitrite (8.7 μmol/ min) on change in forearm blood flow (FBF). Data shown as mean±SEM, *n*=7,***P<0.001 compared to baseline.

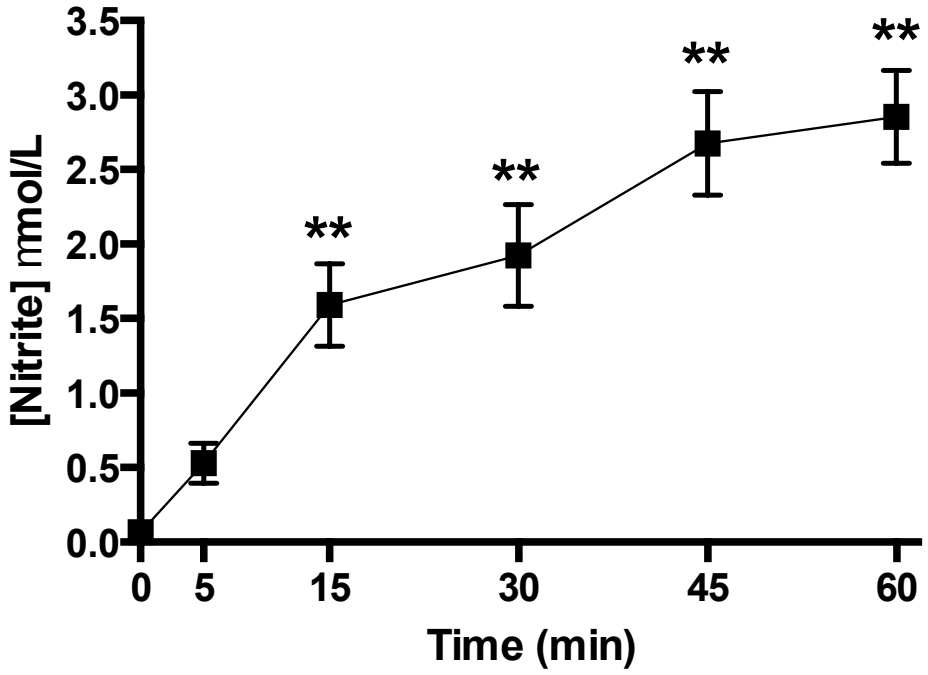

Figure 3.3: Effect of 60 min intra-brachial infusion of sodium nitrite (8.7 µmol/ min) on systemic nitrite concentrations (blood sampled from contralateral arm). Data shown as mean±SEM, *n*=7, **P<0.01 compared to baseline.

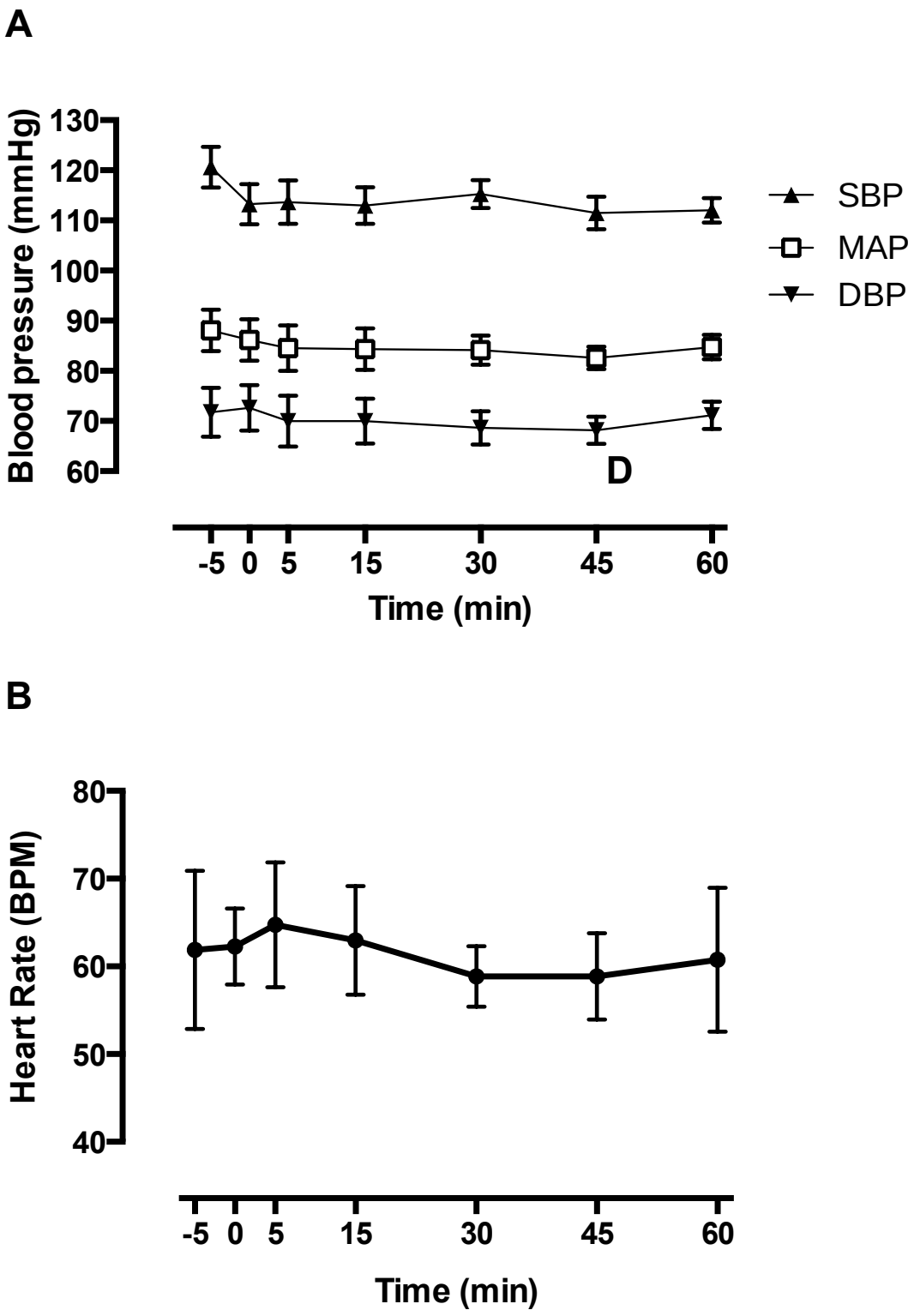

Figure 3.4: Effect of 60 min intra-brachial infusion of sodium nitrite (8.7 µmol/ min) on (A) peripheral brachial blood pressure (systolic, SBP, mean arterial, MAP, or diastolic, DBP). (B) Heart rate. Data shown as mean±SEM, *n*=7.

3.4 (Study 2) Intra-arterial sodium nitrite versus GTN - dose response

In this study an intra-brachial infusion of sodium nitrite, was administered in a low (0.087 - 2.6 μmol/ min) and a high (8.7 - 87 μmol/ min) dose series spanning a dose response curve to 8 healthy volunteers. A different group of 7 healthy volunteers were given an intra-brachial infusion of GTN in a single continuous series of dose steps (0.003 - 1 μg/ min). Radial artery responses were assessed at 5 - 7 min following each change in dose, while FBF changes were assessed at 20 - 22 min following each change with the change in the next dose occurring at 25 min.

3.4.1 Intra-arterial infusion of sodium nitrite - radial artery

With nitrite the study comprised a low dose series (0.0, 0.087, 0.26, 0.87 and 2.6 μmol/ min) with subjects returning to complete the higher dose series (8.7, 26 and 87 μmol/ min), with each dose being infused for 25 min. The mean baseline radial artery measurements between the first and second visit were similar: 2.32±0.53 mm and 2.50±0.28 mm respectively (mean±SD). Figure 3.5A demonstrates a clear dose-response to nitrite in terms of radial artery diameter, with significant dilatation (compared to baseline) seen from doses of 0.87 μmol/ min, resulting in a dilatation of 11.2% (95% CI 2.6 to 19.8). The highest dose of 87 μmol/ min resulted in a dilatation of 35.0% (95% CI 26.4 to 43.6).

Radial artery measurements were made at 5 min following the initiation of each dose, while FBF was measured at 20 min. However, with nitrite (26 μmol/ min) radial artery measurements were also made at 20 min, and no differences were seen between this time point and 5 min.

3.4.1.1 Intra-arterial sodium nitrite (in buffered saline) - radial artery

Given that 0.9% saline has a pH of ~5.5, a separate series experiments were performed, in 4 volunteers, with sodium nitrite (0.087-26 μmol/ min) in saline, pH-balanced with sodium bicarbonate to pH 7.5±0.13 (mean±SD). Similar degrees of radial artery dilatation were found. (Figure 3.5B).

3.4.2 Intra-arterial GTN - radial artery

The dose of GTN used (0.003 -1 µg/min = 1.3^{-5}-4.4^{-3} µmol/min) resulted in significant radial artery dilatation (compared to baseline), with the dose of 0.01 µg/ min, resulting in a dilatation of 13.6% (95% CI 2.9 to 22.4). The highest GTN dose (1.0 µg/ min) resulted in a dilatation of 33.3% (95% CI 22.5 to 44.1) see Figure 3.5C.

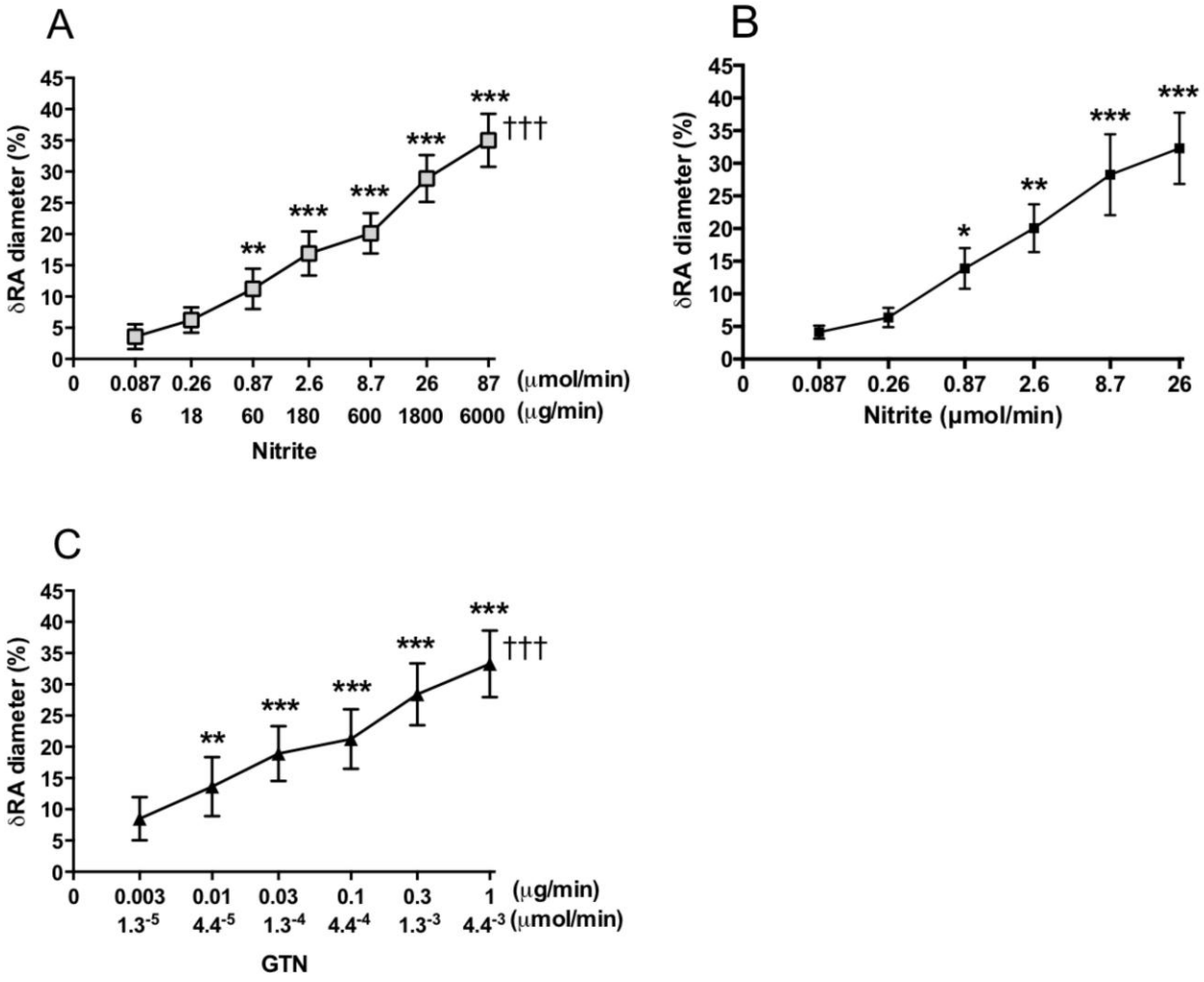

Figure 3.5: Changes in radial artery (RA) diameter (%) with intra-brachial infusions of (A) sodium nitrite in saline (0.087-87 µmol/ min), (B) sodium nitrite in pH balanced saline (0.087-26 µmol/ min) and (C) GTN (0.003-1 µg/min). Data shown as mean±SEM, *n*=8 (nitrite in saline), *n*=4 (nitrite in pH balanced saline), and *n*=7 (GTN), *P<0.05 **P<0.01, ***P<0.001, compared to baseline, †††P<0.0001 across the dose range.

3.4.3 Intra-arterial sodium nitrite vs. GTN – FBF

When assessing FBF both GTN and nitrite resulted in a significant increase. When compared to baseline the highest dose of nitrite (87 µmol/min) resulted in an increase of 6.21 ml/min/100ml (95% CI 4.82 to 7.59). The highest dose of GTN (1 µg/min) resulted in an increase of 4.31 ml/min/100ml (95% CI 2.58 to 6.04). See figure 3.6

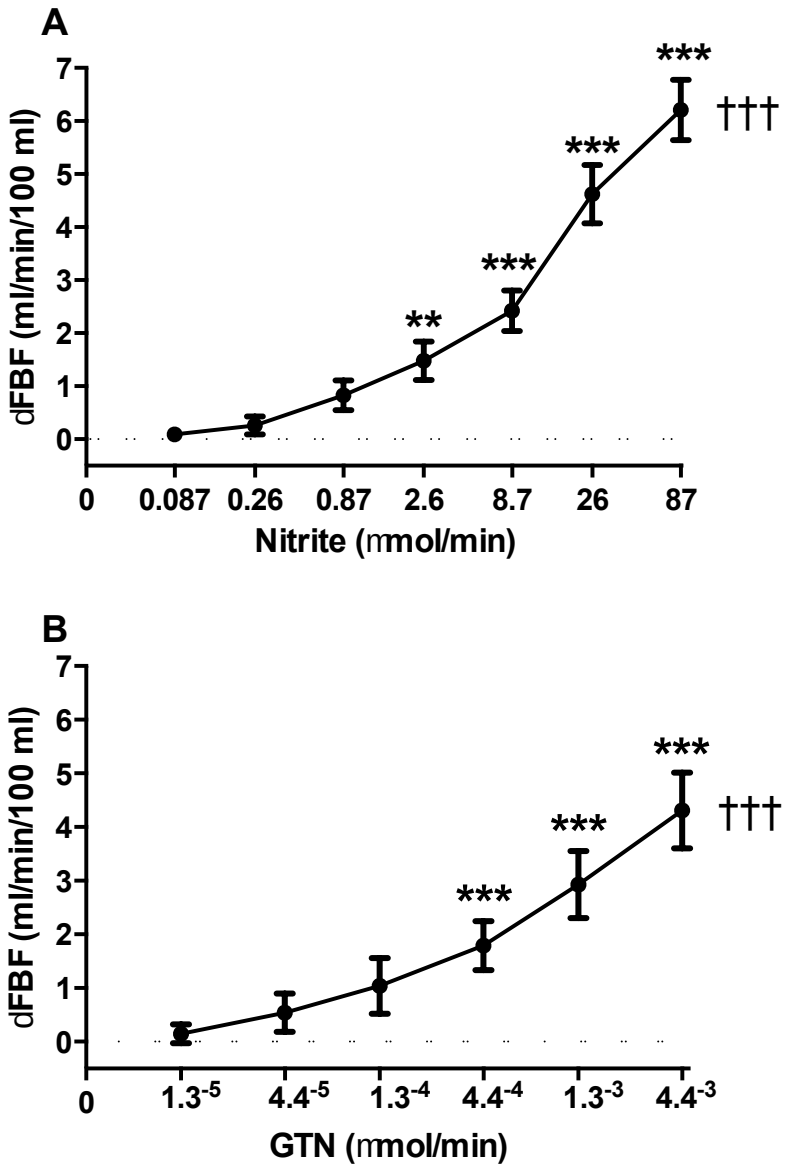

Figure 3.6: Changes in radial artery forearm blood flow (FBF) with intra-brachial infusions of (A) sodium nitrite in saline (0.087-87 µmol/ min), (B) GTN (0.003-1 µg/min). Data shown as mean±SEM, n=8 (nitrite in saline), n=4 (nitrite in pH balanced saline), and n=7 (GTN), **P<0.01, ***P<0.001, compared to baseline, †††P<0.0001 across the dose range

3.4.4 Intra-arterial sodium nitrite vs. GTN - selectivity

The selectivity of nitrite and GTN for vasodilating conduit arteries versus small resistance arterioles was compared by plotting the change in radial artery diameter, against the change in FBF at each dose step (see Figure 3.7). Nitrite resulted in a gradient of 4.9±0.6, with a strong correlation between conduit artery and resistance arteriole responses, r^2 = 0.58, P<0.0001. With GTN the gradient was 5.7±1.3, but with a weaker association than nitrite, r^2 = 0.33, P<0.0001. The Y-intercept was estimated at 6.4±1.8% for nitrite, compared to 10.5±2.9% for GTN, (i.e. change in radial artery dilatation with no change in FBF), although the relationship is not linear at the lower doses for GTN, and the actual intercept is likely to be closer to ~6%. Overall, the large/conduit artery selectivity of GTN and nitrite were similar.

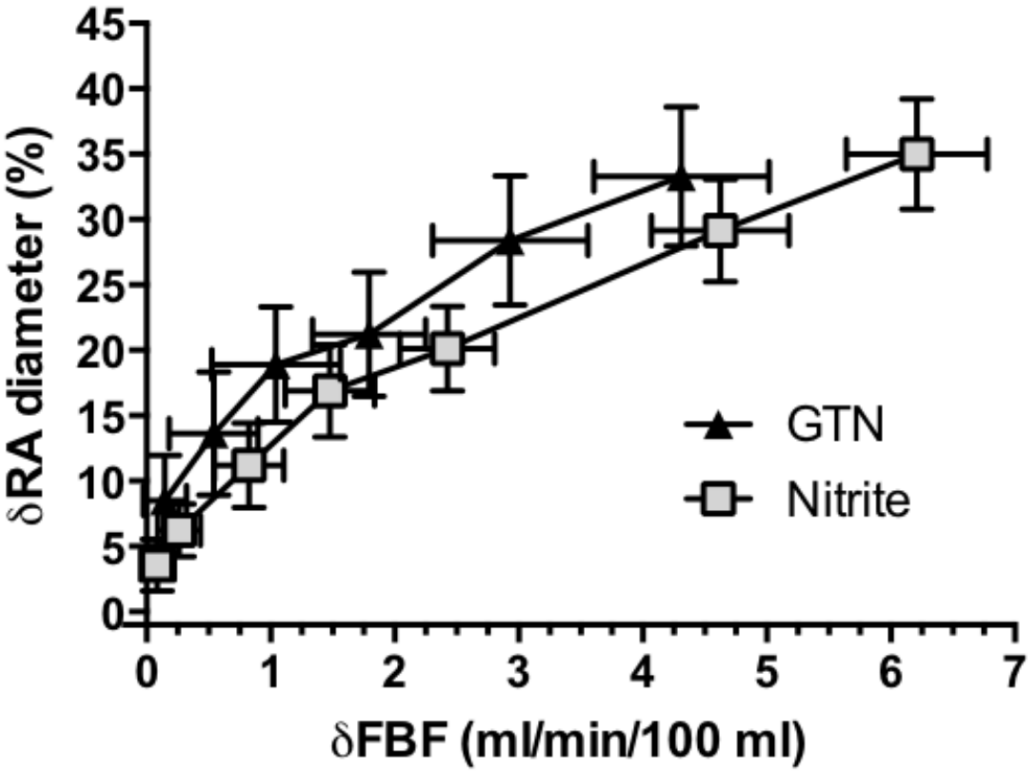

Figure 3.7: Changes in radial artery (RA) diameter (%) versus change in forearm blood flow (FBF) with intra-brachial infusion nitrite (0.087-87 μmol/ min) and GTN (0.003-1 μg/min) at each dose step. Data shown as mean±SEM, *n*=8 (nitrite) and *n*=7 (GTN).

3.4.5 Intra-arterial infusion of sodium nitrite - radial artery flow

To determine whether conduit artery dilation was flow-mediated, an analysis of the Doppler US flow images was undertaken. This demonstrated that there was no change in radial artery peak flow (see Figure 3.8) despite the increase in flow in resistance vessels (FBF).

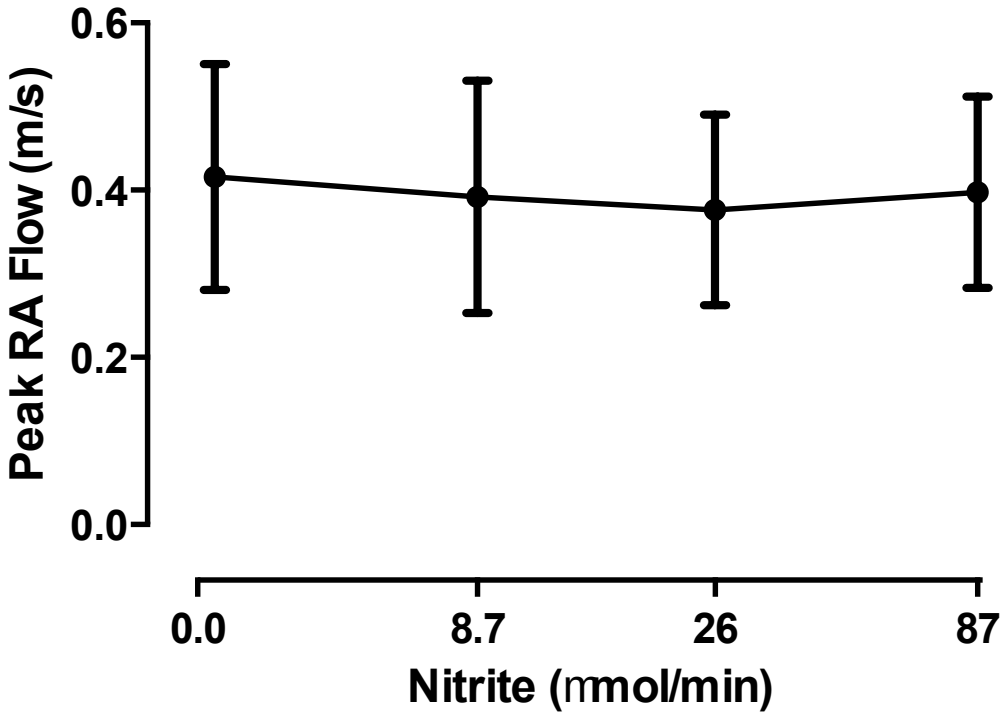

Figure 3.8: Effect of high dose sodium nitrite intrabrachial infusion of sodium nitrite (8.7, 26, 87 µmol / min) on RA flow. Data shown as mean±SEM, *n*=7. RA – Radial artery.

3.4.6 Intra-arterial sodium nitrite vs. GTN - haemodynamics

No changes in peripheral SBP, DBP or MAP or HR were found following the highest GTN dose, or with nitrite up to the penultimate dose of 26 μmol/min (see Figure 3.9). However, at the highest dose of nitrite (87 μmol/min) for 5 min, BP decreased by ~5.5/14 (11) mmHg (SBP/DBP (MAP) respectively) and HR increased by 19 bpm (P<0.001). The MAP transiently fell by more than the pre-specified threshold of 15 mmHg in only one subject, at the end of the infusion of the highest dose.

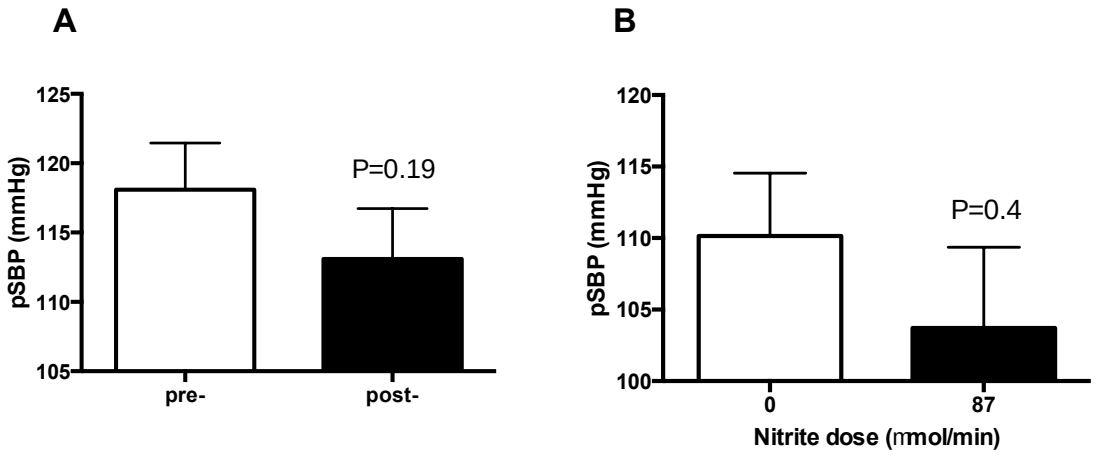

Figure 3.9: Effect of intra-brachial sodium nitrite across a high dose series (8.7, 26 and 87 μmol/ min, each dose infused for 25 min, 25 min and 5 min, respectively) on (A) peripheral Systolic Blood Pressure (pSBP) measured before and immediately after cessation of the infusion, and (B) peripheral Systolic Blood Pressure (pSBP) measured during the infusion of sodium nitrite at 0 and 87 μmol/ min, n=7.

3.4.7 Intra-arterial sodium nitrite vs. GTN - venoselectivity

During i.a infusion of the low dose series of nitrite (n=3) and i.a. infusion of GTN (n=3), venous capacitance was measured by keeping the arm cuffs inflated at 40 mmHg and assessing changes in forearm volume at 2.5 minutes [5]. Low dose nitrite increased forearm venous capacitance in the infused arm compared to the control arm at a dose of 2.6 µmol/min (see Figure 3.10). Nitrite resulted in conduit artery dilatation with lower doses (0.26 and 0.87 µmol/min). Forearm venous capacitance was also increased by GTN compared to baseline in the infused arm, but not on comparison with the control arm. Therefore venodilatory effects were observed for nitrite and GTN. Since these studies were performed in different subjects, it is not possible to determine directly which had greater venoselectivity.

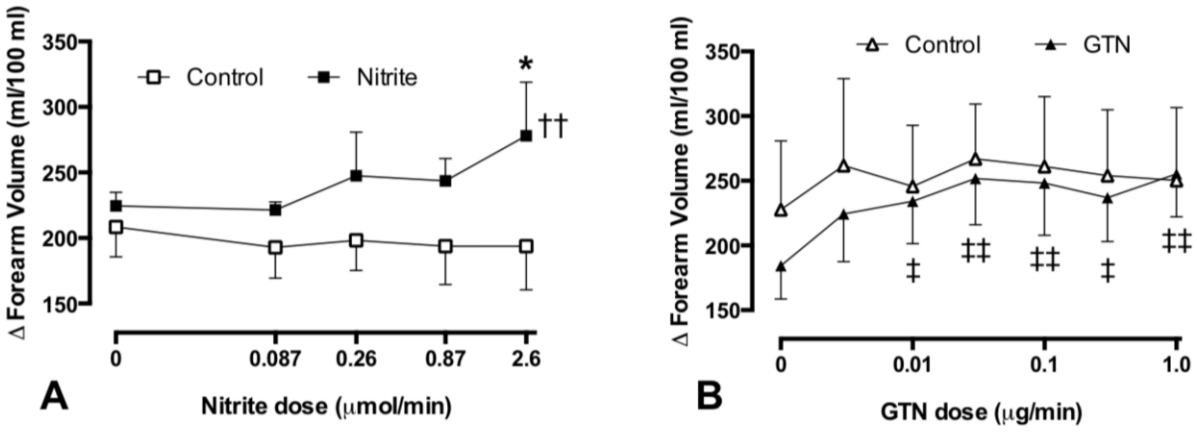

Figure 3.10: Venous Capacitance: change in forearm volume with intra-brachial infusions of (A) sodium nitrite (0.087-2.6 µmol/ min) and (B) GTN (0.003-1 µg/min). Data shown as mean±SEM, *n=3* in both groups; ††P<0.01 compared to control on 2-way ANOVA, *P<0.05 on Bonferroni comparison with control, ‡P<0.05, ‡‡P<0.01compared to baseline (1-way ANOVA).

3.4.8 Intra-arterial sodium nitrite and systemic methaemoglobin

Systemic methaemoglobin levels increased from 1.3±0.1% to 3.5±0.2% with the highest dose of nitrite, associated with a concomitant decrease in haemoglobin oxygen saturations from 98.5±0.1% to 97.0±0.4% (see Figure 3.11). Methaemoglobin levels in the infused arm increased to 6.0±1.3%.

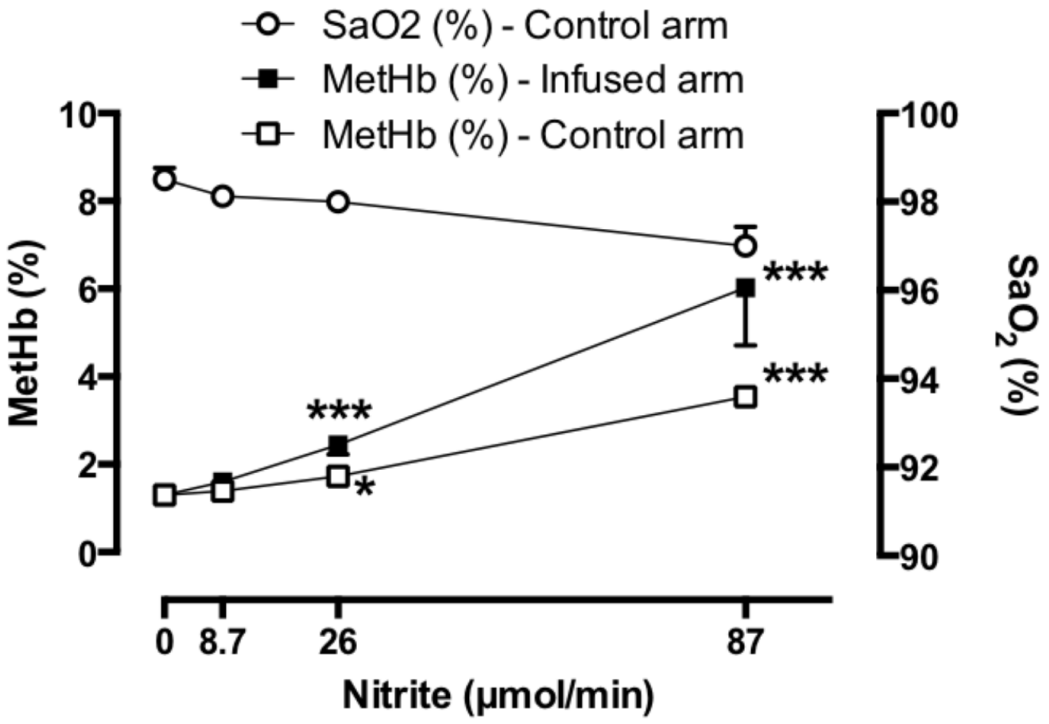

Figure 3.1: Effect of intra-brachial infusion of sodium nitrite (dose response 0.087-87 µmol/ min) on percentage methaemoglobin (MetHb (%)) in infused and control arm (left Y-axis) and percentage haemoglobin oxygen saturations (SaO_2 (%)) in control arm (right Y-axis). Data shown as mean±SEM, *n*=8, *P<0.05, ***P<0.001, compared to baseline.

3.4.9 Plasma nitrite, cyclic GMP and s-nitrosothiols levels

Plasma nitrite concentrations, following sodium nitrite infusion, in the infused and contralateral arms are shown in Figure 3.12A. Nitrite levels at baseline were comparable in the infused and control arm (0.046±0.015 µmol/L and 0.054±0.022 µmol/L respectively). It should be noted that the concentrations depicted in the graph are absolute concentrations that were not corrected for changes in flow.

To determine changes in local cGMP production, we multiplied cGMP plasma concentrations by FBF; units are expressed in picomoles produced per min per 100 ml of tissue.[6] As shown in Figure 3.12B, nitrite infusion (2.6, 26, 87 µmol/min) increased cGMP production in a dose-dependent manner, by 1.1 pmol/min/100 ml tissue (95% CI 0.5 to 1.8), 2.2 pmol/min/100 ml tissue (95% CI 1.2 to 3.2), and 4.7 pmol/min/100 ml tissue (95% CI 3.3 to 6.2), respectively. However, no systemic changes in cGMP production (in the contralateral arm) were detected. One set of cGMP data pertaining to nitrite (2.6 µmol/min) in the infused arm, was excluded from the analysis as the cGMP levels exceeded the mean + 2 SDs (of the entire set).

Plasma SNOs in the infused arm were below the level of detection at baseline, and at most of the doses of nitrite infused. However, with nitrite infusion at the highest dose (87 µmol/min), plasma SNOs production was 348±130 pmol/min/100 ml of tissue.

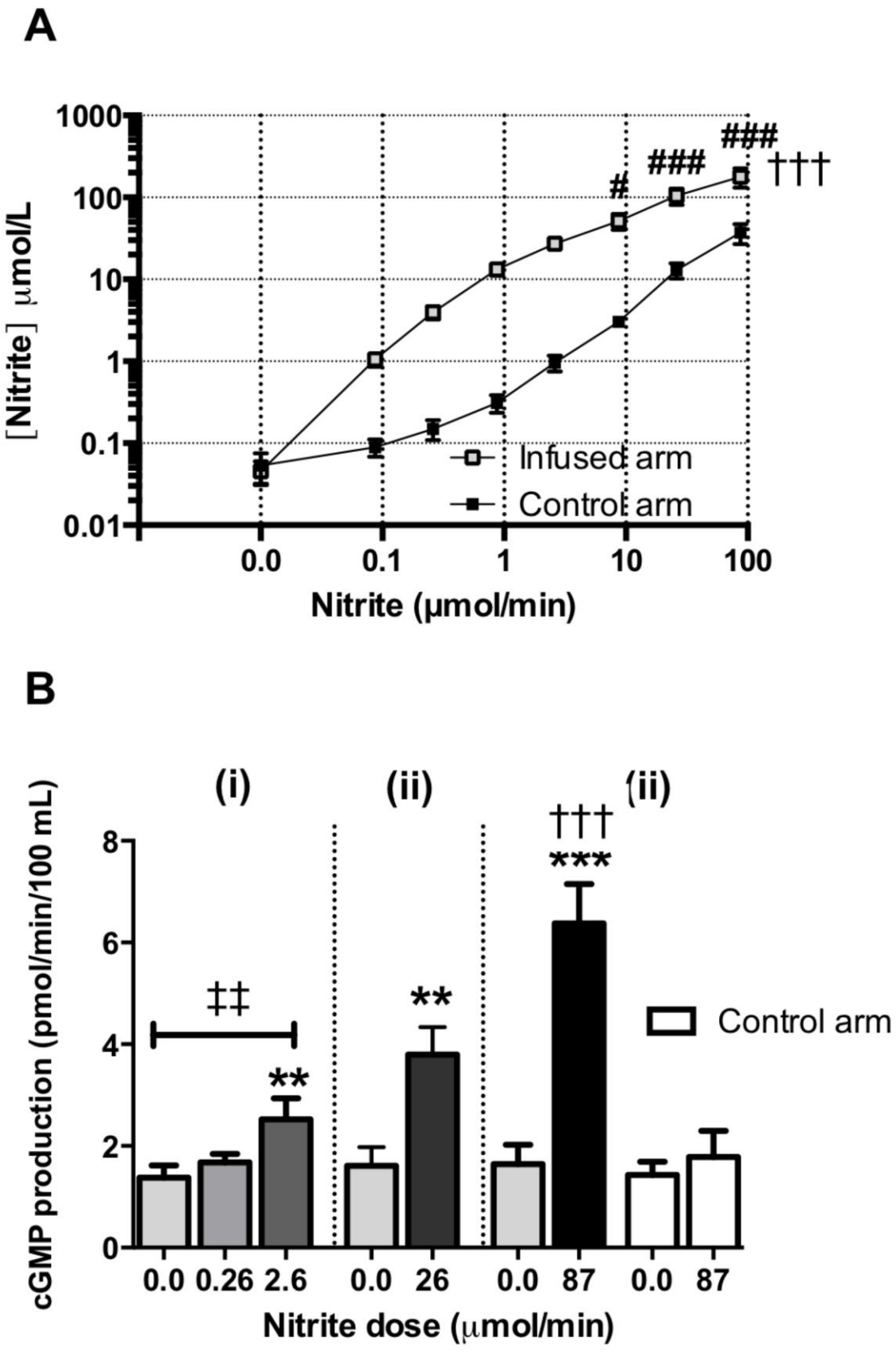

Figure 3.12: Effect of intra-brachial infusions of sodium nitrite on (A) plasma nitrite concentration in infused and contralateral arms (*n*=8), and (B) plasma cGMP production: (i) nitrite (0, 0.26 and 2.6 μmol/ min), cGMP in intervention arm (*n*=6), (ii) nitrite (0, 26 μmol/min), cGMP in intervention arm (*n*=7), (iii) nitrite (87 μmol/min), cGMP in intervention and control arms (*n*=5). Data shown as mean±SEM, $^{\#}$P<0.01 $^{\#\#\#}$P<0.001 compared to control arm, **P<0.01, ***P<0.001 compared to nitrite (0 μmol/ min), ‡‡P<0.01 for dose response, †††P<0.0001 compared to control arm on ANOVA.

Chapter 4

Results – 2

Intra-arterial sodium nitrite with acetazolamide or raloxifene

4.1 (Study 3) Intra-arterial sodium nitrite with oral acetazolamide and raloxifene

As described previously (in chapter 1) both carbonic anhydrase [7] (through nitrite anhydartion) and aldehyde oxidase [8] (through nitrite reduction) have been shown to potentiate the release of NO from nitrite. Carbonic anhydrase is found on erythrocytes [62] while AO is located in the vascular tree [70,224]. To investigate the role played by each enzyme, in nitrite's effects on human conduit vessels, the CA inhibitor acetazolamide and the AO inhibitor raloxifene were used in an attempt to modify the effects of intra-arterial nitrite on the RA. In addition, AO has also been implicated in the generation of ROS [402], thus the use of raloxifene would also allow for the determination of the net effect of inhibition of AO-dependent nitrite reduction versus inhibition of AO-derived ROS, with concomitant increase in NO bioavailability.

The sample size calculation conducted was based on quantitative data set estimating a standard deviation in the population of 7% in RA diameter dilatation and a 25% mean difference between the control group and the intervention groups to be relevant to be detected with a confidence interval of 5% and a confidence level of 95% the sample size required was calculated to be ~ 8.

The infusion of nitrite alone dilated the RA by ~ 25%. Using the infusion of nitrite alone as the control and comparing that with the intervention (nitrite + acetazolamide or nitrite + raloxifene) and aiming to detect a variation in RA dilatation of 50% between the two groups results in an actual target difference of 25% / 2 = 12.5% in actual RA dilatation. Allowing for a standard deviation of 7%, thus, the standardised difference would calculated as follows:

Standardised difference = target difference / standard deviation

Standardised difference = 12.5 / 7

Standardised difference = 1.78

Using the appropriate sample size formula: Sample size = (2 / standardised difference ^{2}) x $C_{p,power}$, n = (2 / 1.78^2) x 13.0, n = (2/3.2) x 13.0 , n = 8.12

Allowing for a Latin square design and the multiple comparisons between the control group and two intervention groups the total calculated sample size was 8 x 2 = 16. We attempted to recruit 18 volunteers however regretfully due to multiple dropouts only Fourteen healthy male volunteers attended on 3 occasions to receive an oral dose of either: (i) acetazolamide (500 mg), (ii) raloxifene (120 mg), or (iii) placebo (standard lactose tablet), according to a single-blind, randomized, balanced cross-over (Latin-square) design, 2 h before brachial artery cannulation and commencement of intra-arterial sodium nitrite infusion.

Oral administration of acetazolamide (500 mg) or raloxifene (120 mg) had no effect on baseline radial artery diameter: 2.689±0.255 mm with placebo, 2.711±0.265 mm with acetazolamide and 2.737±0.257 mm with raloxifene (mean±SD). However, acetazolamide enhanced radial artery dilatation to sodium nitrite: reaching the required significance level (P=0.0248), as did Raloxifene (P=0.0006), see Figure 4.1.

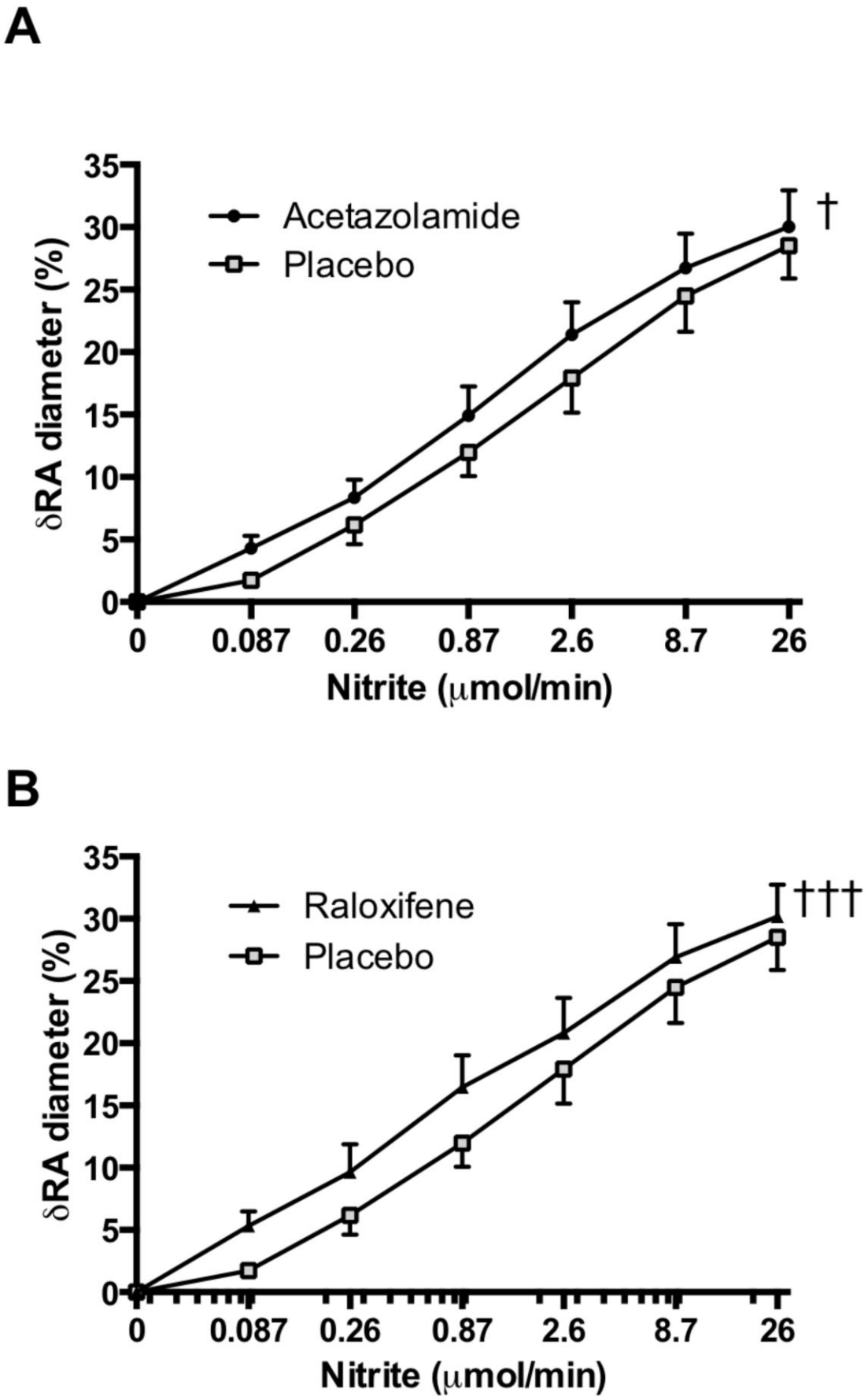

Figure 4.1: Effect of the administration of (A) oral acetazolamide and (B) oral raloxifene on the change in conduit artery (radial) diameter (%) during an intrabrachial infusion of sodium nitrite (dose response 0.087-26 μmol/ min). Data shown as mean±SEM, n=14, $^{\dagger}P<0.025$, $^{\dagger\dagger\dagger}P<0.001$, compared to placebo.

4.2 (Study 4) Intra-arterial sodium nitrite with intra-arterial acetazolamide

In an attempt to negate any systemic effects of acetazolamide (when taken orally) this study was conducted to isolate local effects by administering a co-infusion of i.a. acetazolamide and sodium nitrite and measuring their effects on RA diameter and FBF. Eight healthy volunteers attended on 3 occasions. On the first two visits nitrite was infused alone at a fixed dose (2.6 μmol/ min), or co-administered with acetazolamide (0.1 – 3.0 mg/ ml), each dose for 7 min with measurements taken at the end of this period, in a single-blind, randomized, balanced design. On the 3rd visit i.a acetazolamide was infused alone (0.1 – 3.0 mg/ ml).

Co-infusion of acetazolamide (0.1 - 3.0 mg/ min) with sodium nitrite (2.6 μmol/ min) enhanced nitrite-induced radial artery dilatation compared to sodium nitrite alone ($P<0.0001$), see Figure 4.2A. However, FBF was not increased, rather, there was a trend for acetazolamide to blunt the vasodilatory effect of nitrite in the resistance arterioles ($P=0.10$), see Figure 4.2B. The infusion of acetazolamide alone had no effect on FBF; however, it paradoxically diminished (rather than increasing) radial artery diameter ($P=0.03$).

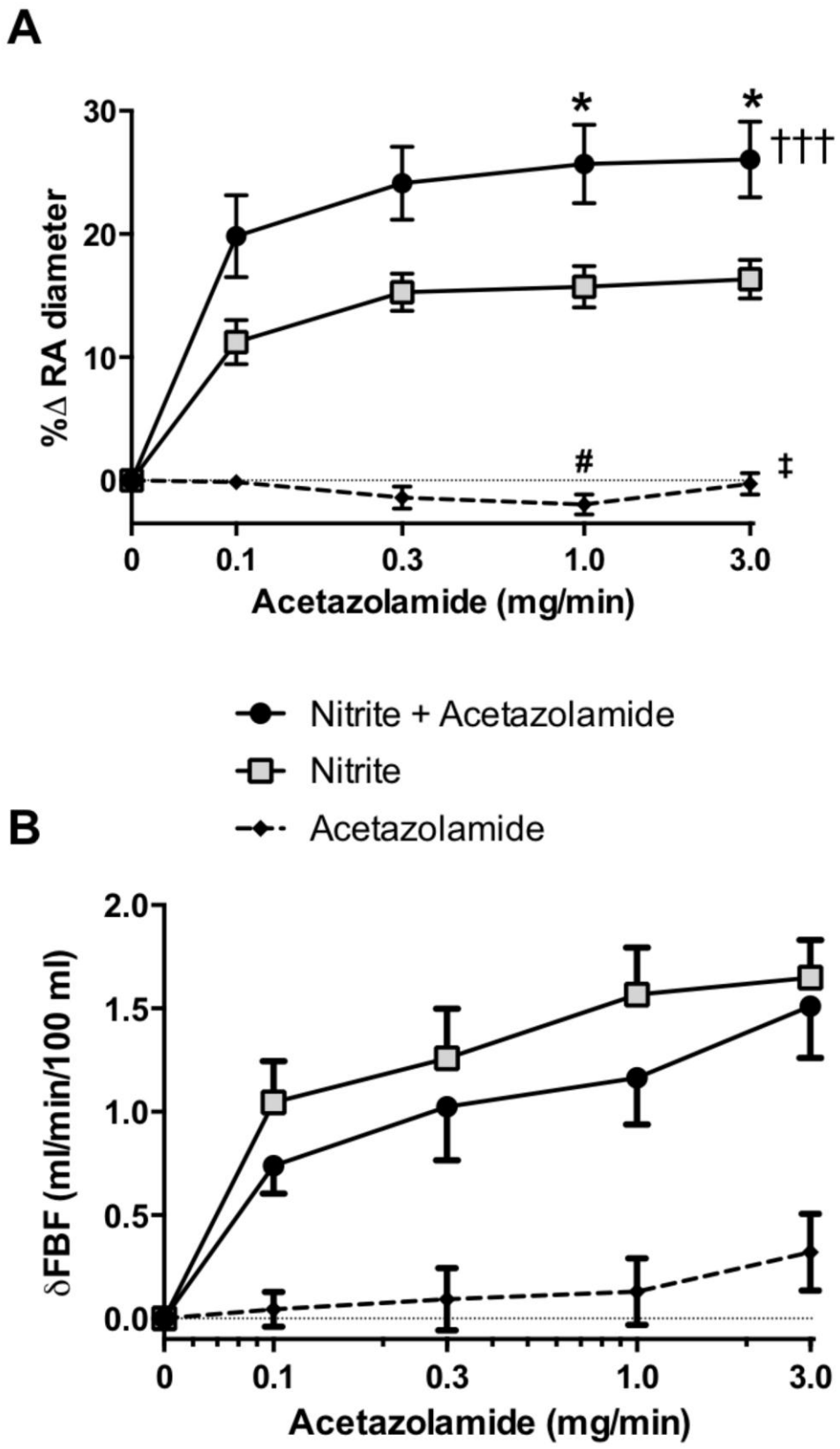

Figure 4.2: Effect of the intra-brachial administration of acetazolamide (0.1-3 mg/min), nitrite (2.6 µmol/ min) or both in combination on (A) the change in radial artery (RA) diameter and (B) the change in forearm blood flow (FBF). Data shown as mean±SEM, *n*=8, †††P<0.0001, *P<0.05 compared to nitrite alone (2-way ANOVA, with Bonferroni post-testing respectively); for acetazolamide alone: ‡P<0.05, $^{\#}$P<0.05 compared to baseline (1-way ANOVA, with Dunnett's post-testing respectively).

4.2.1 Intra-arterial sodium nitrite - onset of action

In this cohort of volunteers, acquisition of continuous US imaging of the radial artery, following the commencement of nitrite (2.6 µmol/ ml) was undertaken to asses nitrite's rate of action. This revealed a rapid onset of dilatation (half life 109 s (95% CI 61 to 489) which was near maximal by the end of 5 minutes (Figure 4.3).

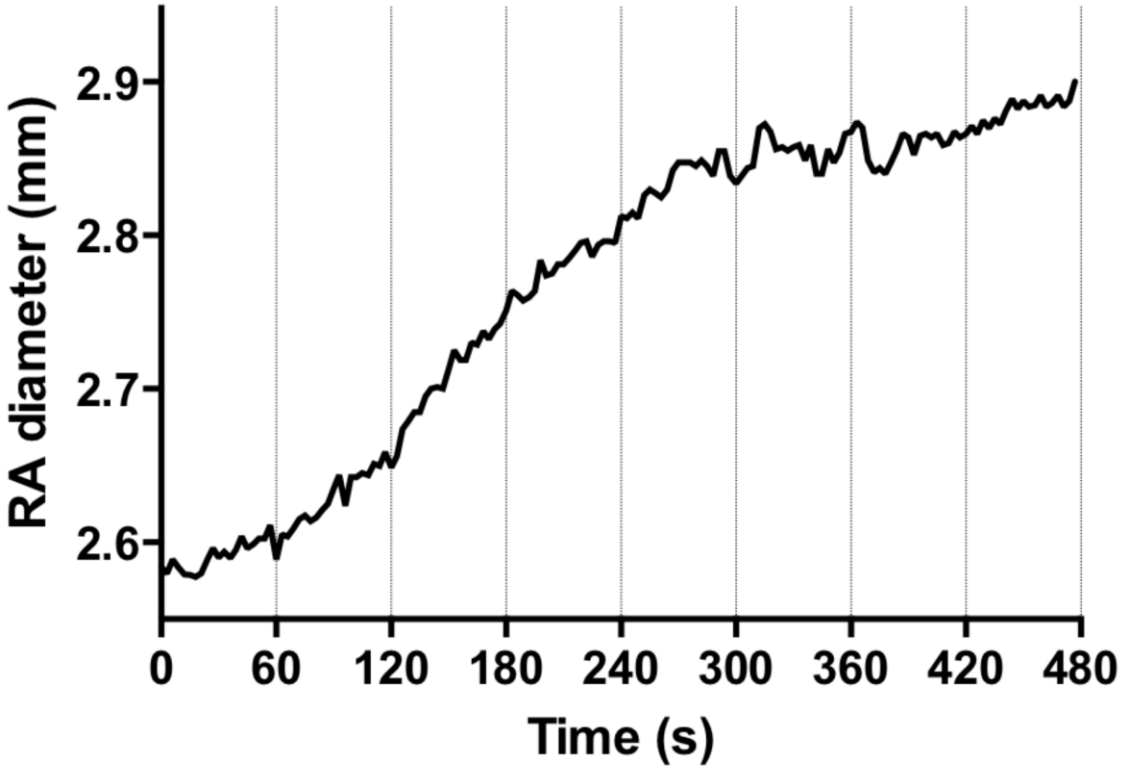

Figure 4.3: Change in RA diameter over the first 8 minutes of intrabrachial nitrite infusion (2.6 µmol/ min). Data represents mean, recordings every 3 seconds, *n*=8.

Chapter 5

Results – 3

Intra-arterial sodium nitrite under normoxia, hypoxia and hyperoxia

5.1 (Study 5) Intra-arterial sodium nitrite under normoxia, hypoxia and hyperoxia

The Effect of normoxia, hypoxia, and hyperoxia on the dose response to i.a. sodium nitrite (0.087 – 26 µmol/ ml) in the radial artery was investigated in 8 volunteers. Healthy male participants attended on 3 occasions where sodium nitrite was infused while inhaling (i) room air (21%O_2), (ii) 12% O_2, or (iii) 100% O_2 in a single-blind, randomized, Latin-square balanced design. Radial artery responses were assessed at 5-7 min following each change in dose.

As shown in Figure 5.1, hypoxia and hyperoxia both inhibited radial artery dilatation to nitrite (to a similar extent) compared to normoxia ($P<0.0001$ and $P=0.0006$, respectively). Sodium nitrite (8.7 µmol/ ml) dilated the radial artery by only 19.0±2.6% under hypoxia, compared to 29.1±4.1% under normoxia (absolute difference of 10.1%, 95% CI of difference 1.90 to 18.3). Importantly, baseline radial artery diameter was almost identical under hypoxia, normoxia and hyperoxia: 2.743±0.221 mm, 2.841±0.292 mm, 2.728±0.218 mm (mean ±SD) respectively. In addition, there were no differences in BP or HR between the conditions from baseline to the end of the study (see Figure 5.2). Therefore, there was no physiological evidence of increased sympathetic activity, or any effect on conduit artery tone as a result of hypoxia *per se*.

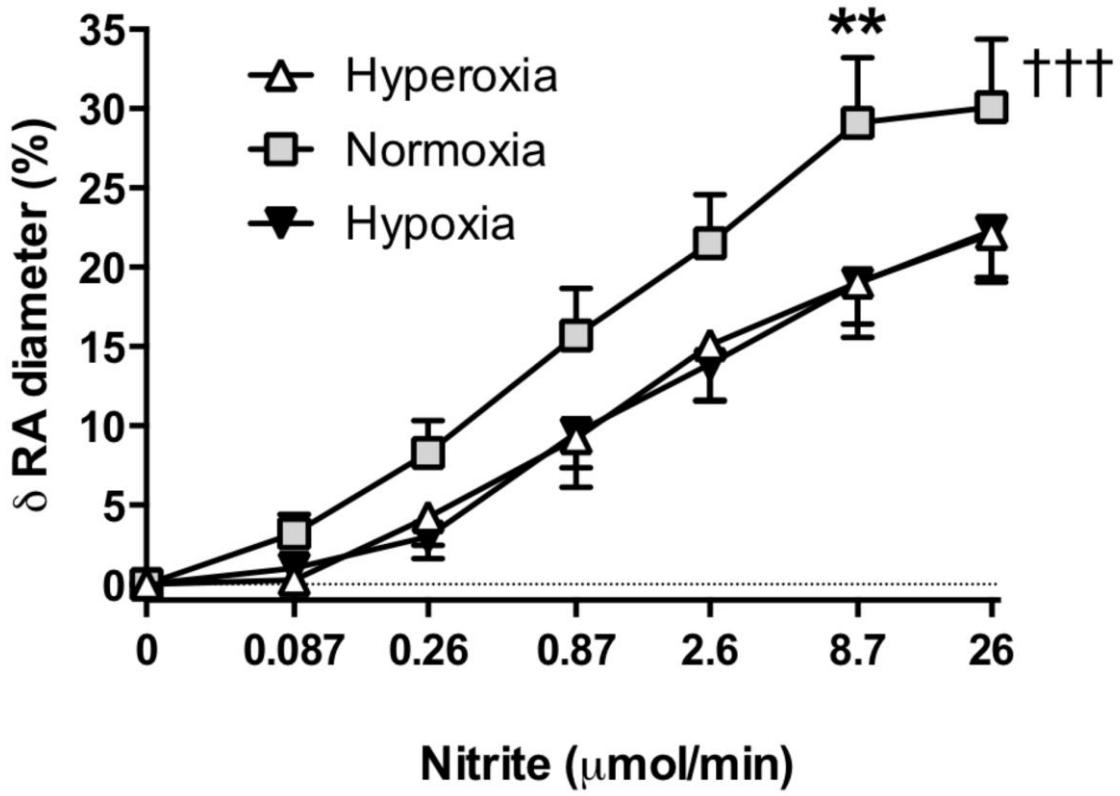

Figure 1.1: Effect of systemic hypoxia, normoxia and hyperoxia on the change in radial artery (RA) diameter during an intra-brachial infusion of sodium nitrite (0.087-26 μmol/ min); *n*=8, **P<0.01 compared to hypoxia, †††P<0.001 compared to hypoxia and hyperoxia.

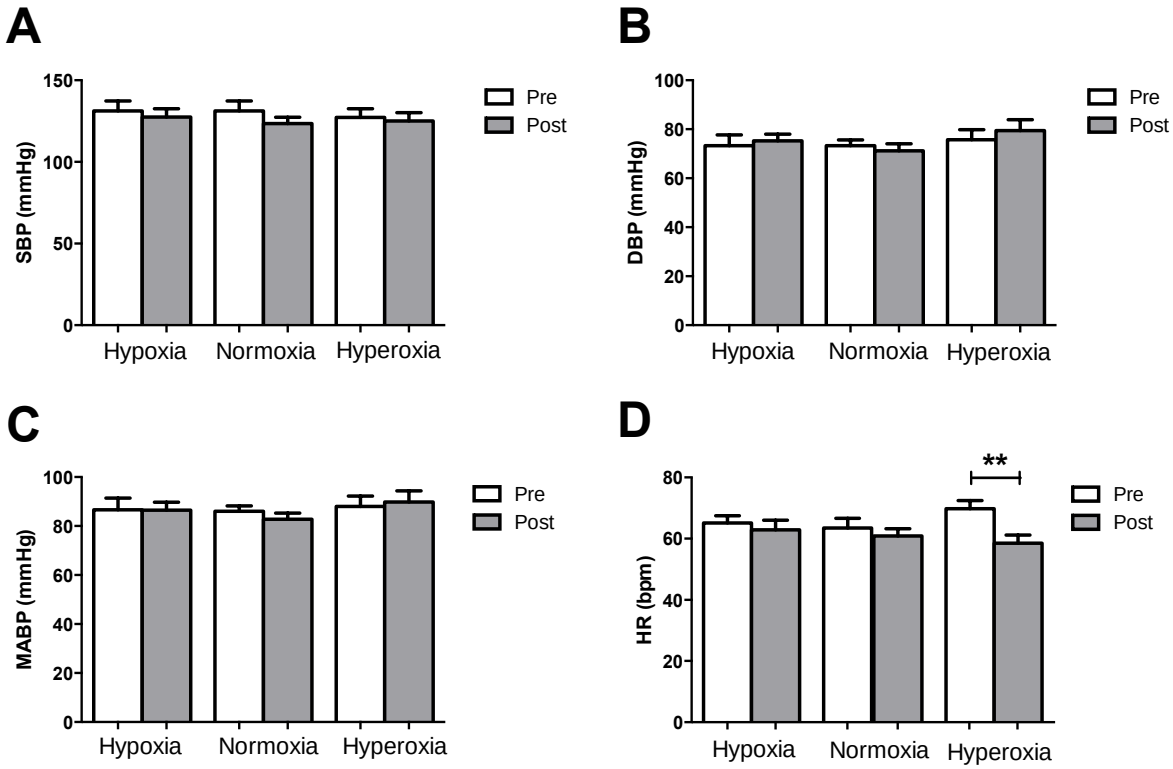

Figure 5.2: Effect of hypoxia v normoxia v hyperoxia during an intra-brachial infusion of sodium nitrite (0.087-26 μmol/ ml) on peripheral brachial blood pressure, BP, ((A) systolic, SBP, (B) diastolic, DBP, (C) mean arterial, MABP and (D) heart rate (HR). Data shown as mean±SEM, *n*=8, **P<0.01 compared to pre-nitrite (1-way ANOVA, with Bonferroni multiple post-testing).

In contrast to radial artery responses, but in keeping with previous studies,[402,409] the change in FBF in the intervention arm (assessed in the last 3 subjects) was 2.2 ml/ min/ 100 ml greater under hypoxia than normoxia (95% CI 0.6 to 3.8) at the highest dose of nitrite (26 µmol/ ml) as shown in Figure 5.3. There was no significant difference in baseline FBF between hypoxia and normoxia, again showing no physiological evidence of increased sympathetic activity. The overall haemoglobin O_2 saturation recorded during the hypoxia study was 91.6±3.4%, compared to 98.4±0.8% and 100±0.0% in normoxia and hyperoxia, respectively (mean ±SD). As shown in Figure 5.4, analysis of the ABGs taken at the end of the study, reveal that the pCO_2 was greater during normoxia (5.1±0.2 kPa) than during hypoxia (4.5±0.3 kPa), P=0.015 with no difference in bicarbonate; pH was lower during normoxia (pH 7.396±0.006) compared to pH 7.44±0.01 during hypoxia (P<0.05). Besides pO2, there were no differences in pH, pCO_2 or plasma bicarbonate under hyperoxia compared to normoxia and hypoxia.

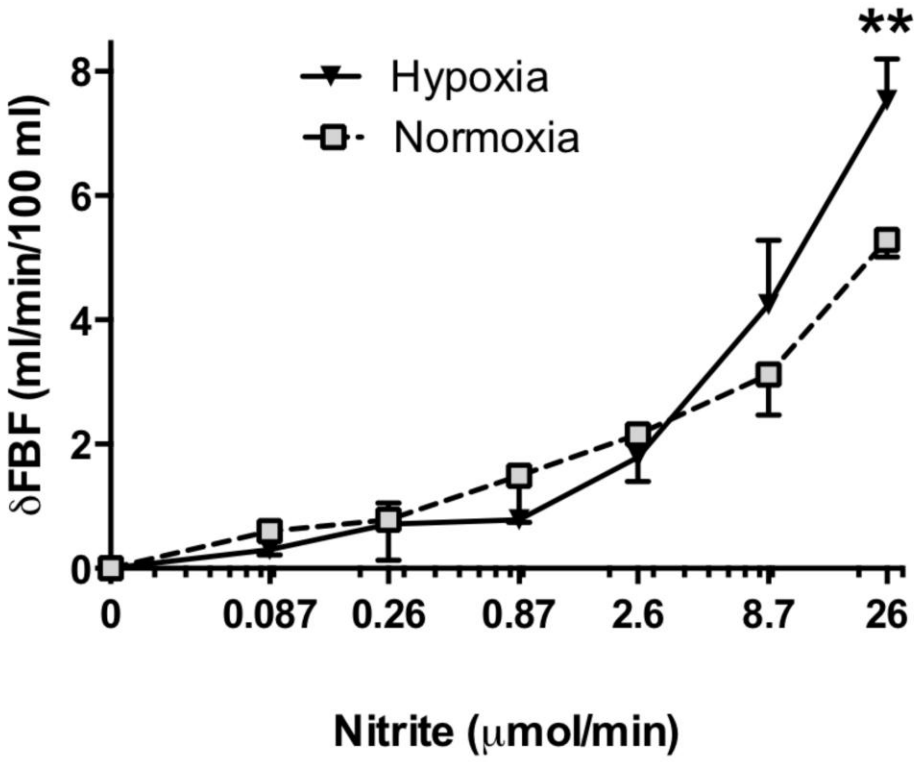

Figure 5.3: Effect of systemic hypoxia and normoxia on the change in forearm blood flow during an intra-brachial infusion of sodium nitrite (0.087-26 µmol/ ml). Data shown as mean±SEM, *n*=3, **P<0.01 compared to normoxia.

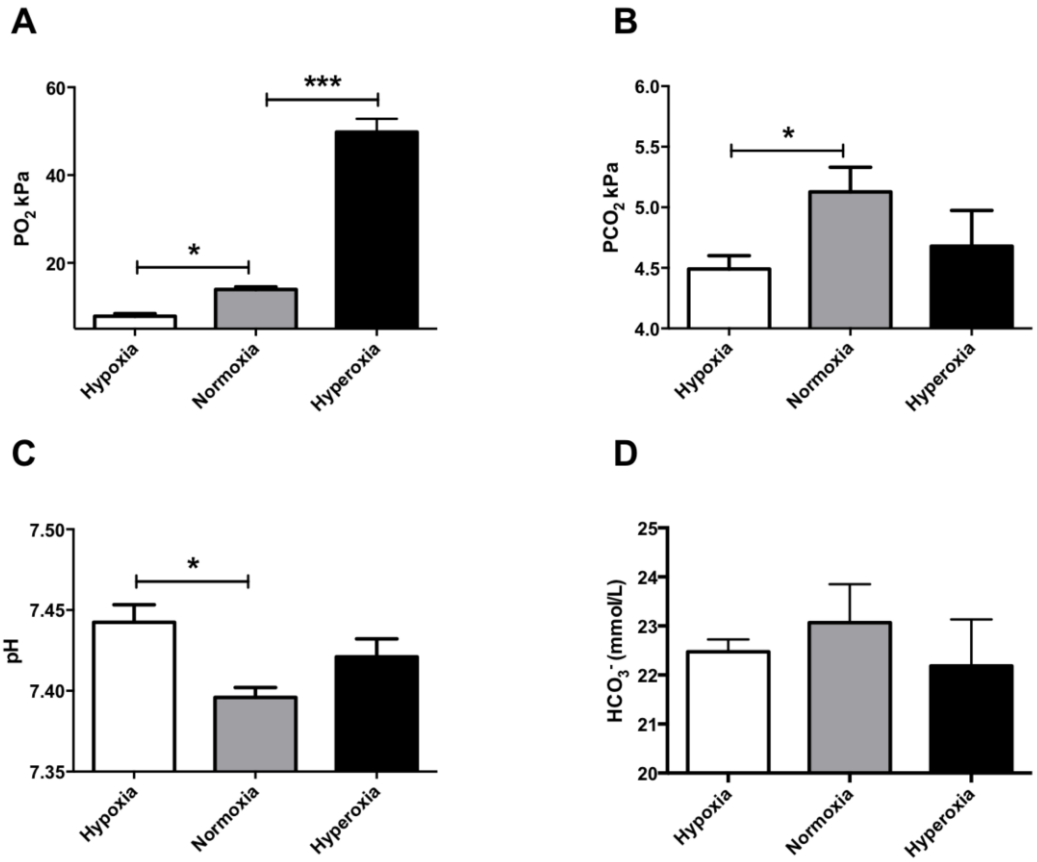

Figure 5.4: Effect of hypoxia v normoxia v hyperoxia on arterial blood gas parameters: (A) O_2 partial pressure, (B) CO_2 partial pressure, (C) pH, (D) HCO_3^-.

Chapter 6

Results – 4

Intra-venous sodium nitrite

6.1 (Study 6) Intra-venous sodium nitrite

To investigate the systemic effects of sodium nitrite, an intra-venous infusion was administered at a fix dose (8.7 μmol/min) for the duration of 60 min in 9 healthy male volunteers. Sodium nitrite was also effective systemically dilating the RA in the contralateral forearm by 10.7% (95% CI 6.8 to 14.7) at 45 min, see Figure 6.1A.

The profile of the increase in systemic plasma nitrite concentration with intravenous nitrite was similar to the intra-arterial study (see figure 6.1B), although the baseline concentration was higher (0.379±0.065 μmol/L, compared to 0.064±0.019 μmol/L previously) a consequence of the different method (deproteination of plasma using filters versus whole plasma, respectively) the former being typically associated with higher levels in this range, which may be due to contamination of the filters with nitrite.

As with the intra-arterial administration of sodium nitrite, intravenous sodium nitrite did not result in any significant changes in peripheral brachial BP (SBP, MAP, or DBP) during the 60 min infusion of sodium nitrite compared to baseline, time 0 min (see Figure 6.2A). Assessments of central haemodynamics were performed from the third subject onwards (n=7). Sodium nitrite produced large reductions in central SBP of 11.6 mmHg, (95% CI of difference 2.4 to 20.7, see Figure 6.2B and peripheral augmentation index (AIx) by 11.9% (95% CI of difference 0.5 to 23.2, see Figure 6.2C. Nitrite also reduced central pulse pressure (cPP) by 11 mmHg from 28 (19, 38) to 17 (15, 31) (median, IQR) (P=0.042) and pulse wave velocity (brachial-femoral) by 1.23 m/s (95% CI 0.28 to 2.19).

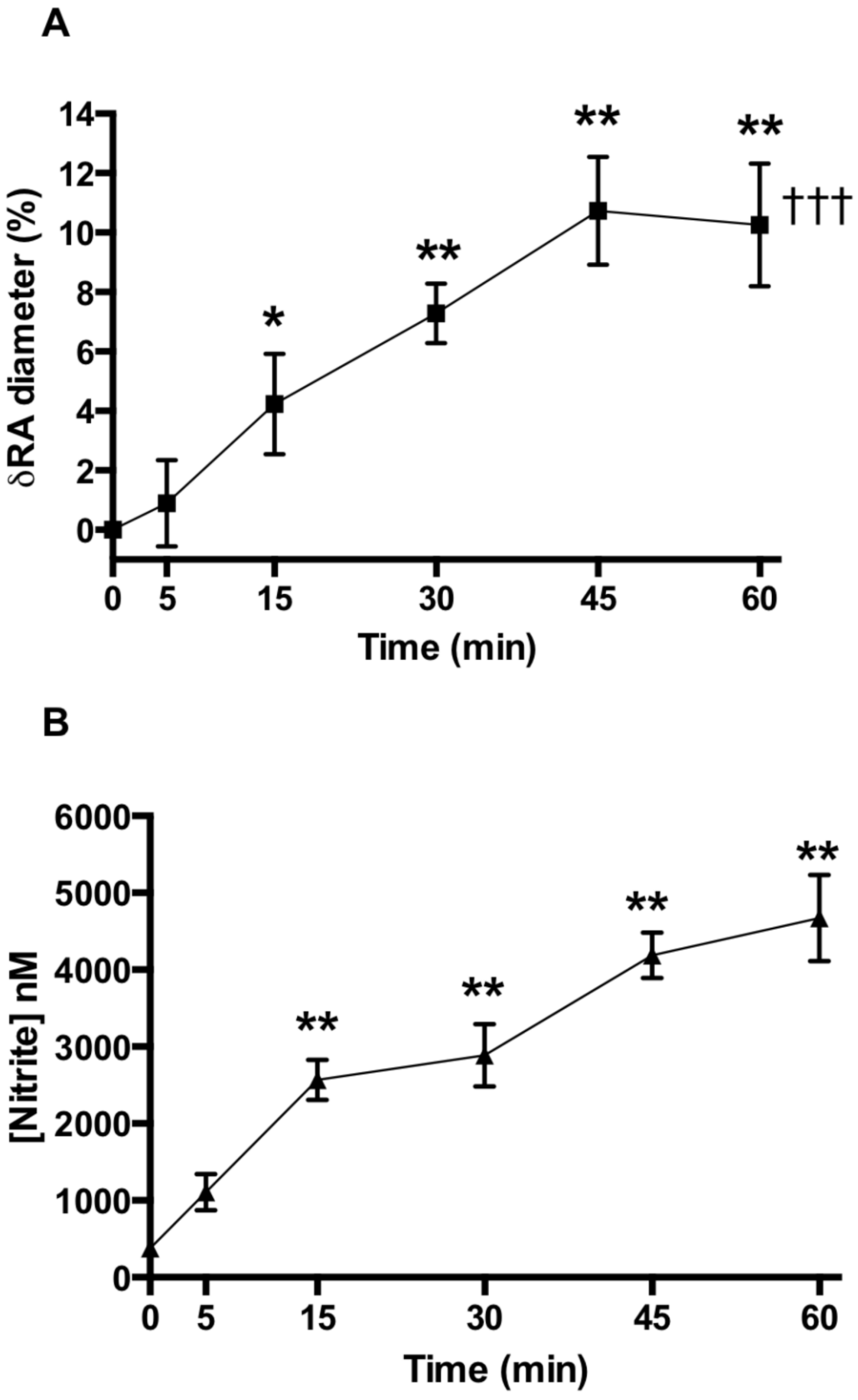

Figure 6.1: Effect of intravenous sodium nitrite (8.7 µmol/ ml over 60 min) on, (A) change in radial artery (RA) diameter (%) in the contralateral arm, (B) on systemic plasma nitrite concentrations. Data shown as mean±SEM, n=9, *P<0.05, **P<0.01 compared to baseline, †††P<0.001 overall.

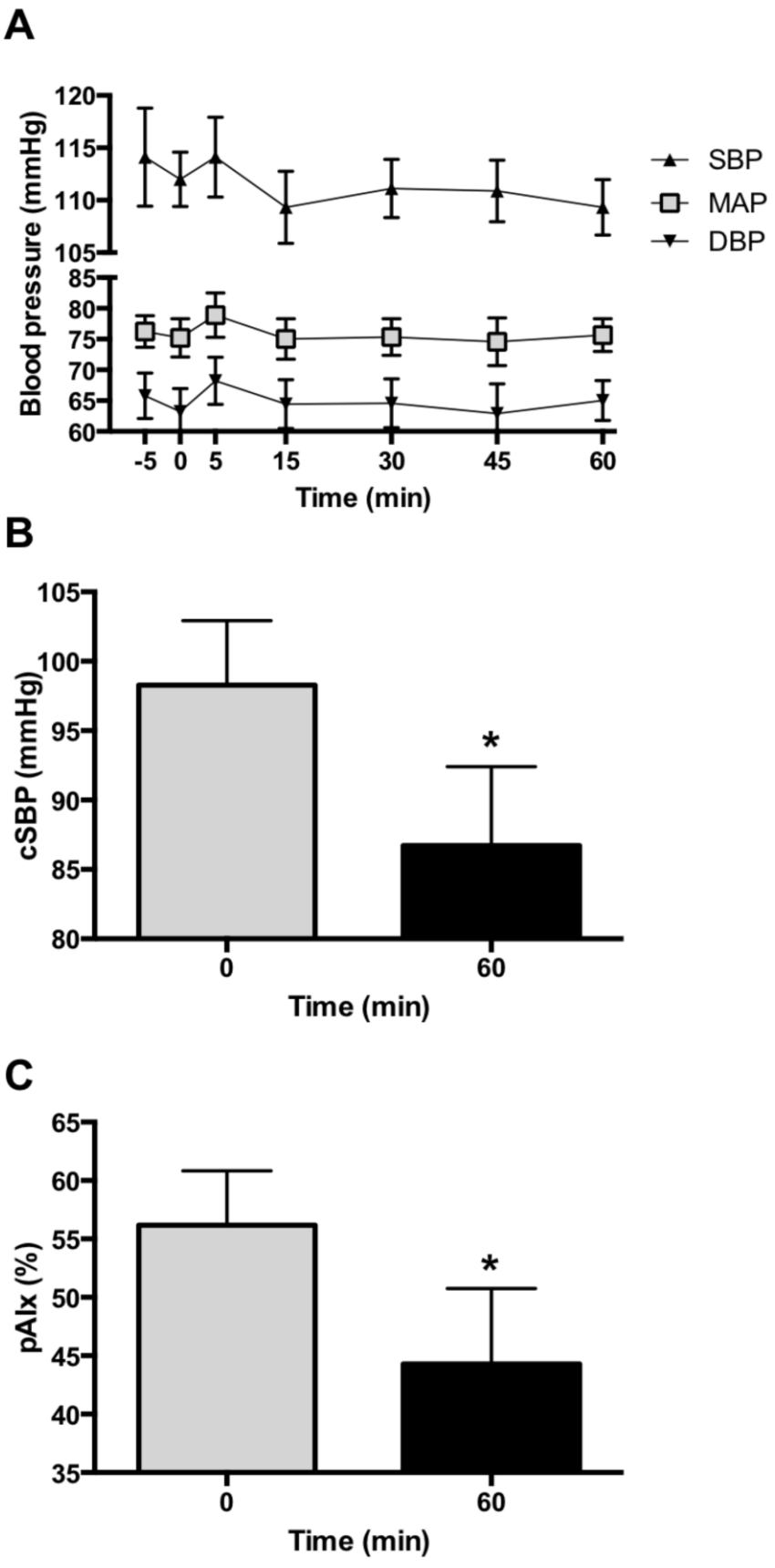

Figure 6.2: Effect of intravenous sodium nitrite (8.7 µmol/ ml over 60 min) on (A) peripheral brachial blood pressure (BP) measurements (systolic, SBP, mean arterial, MAP, or diastolic, DBP), (B) central Systolic Blood Pressure (cSBP), and (D) peripheral augmentation index (pAIx) performed before and after the 60 min infusion of sodium nitrite. Data shown as mean±SEM, *n*=9 for A, *n*=7 for B&C, *P<0.05.

Chapter 7

Discussion

7.1 Discoveries

These studies have led to several discoveries:

(i) Sodium nitrite at supra-physiological concentrations, administered via the intra-arterial route causes rapid and marked dilatation of the radial artery under normal oxygenated conditions.

(ii) Nitrite's actions were highly selective for conduit arteries, to a similar degree as GTN, one of the most selective large/conduit artery dilators identified to date.

(iii) Nitrite resulted in enhanced cGMP production in a dose-dependent manner, suggesting NO-mediated vasodilatory effects.

(iv) The effects of nitrite in the radial artery were enhanced by (a) acetazolamide, suggesting a role for carbonic anhydrase, and (b) raloxifene, possibly via inhibition of AO-mediated ROS generation.

(v) Nitrite-induced radial artery dilatation was maximal under conditions of normoxia, being inhibited by hypoxia and hyperoxia,

(vi) Intravenous sodium nitrite was also an effective systemic conduit artery dilator, causing significant dilatation of the contralateral radial artery (~11%), associated with a lowering of cSBP (by ~12 mmHg) and cPP (by ~11 mmHg), at a dose that was not associated with any change in peripheral BP.

7.2 Local activity of sodium nitrite

The rapid effect of nitrite in the radial artery – within minutes, suggests a direct local effect. This was not related to a flow-mediated dilatory mechanism, as no change in conduit artery flow was detected. In addition, the variable selectivity of different vasodilators for conduit versus resistance vessels indicates that conduit artery dilatation occurs independently of forearm blood flow [409]. For example, alpha blockers and calcium channel blockers are effective at increasing FBF but have minimal effect on conduit artery diameter [410]. Furthermore, intravenous nitrite (8.7 μmol/min) did not increase FBF systemically, but resulted in radial artery dilatation of ~11%. Also, intra-arterial co-infusion of acetazolamide enhanced nitrite-induced radial artery dilatation, despite causing a borderline reduction in FBF (P=0.10).

Rather than being a key difference between organic and inorganic nitrates/nitrites as previously thought [411], nitrite displayed similar selectivity as GTN, one of the most selective large artery dilators known [412]. Indeed, it appears that NO donors in general, such as organic nitrates and sodium nitroprusside show the greatest selectivity for muscular arteries over resistance arterioles [413]. The principle mechanism underlying the vascular effects of organic nitrates is via activation of soluble guanylyl cyclase (sGC), increasing cGMP levels and activating cGMP-dependent protein kinases, and/or cyclic nucleotide-gated ion channels [25,63]. These are the same targets for nitrite-derived NO activity. Indeed, intra-arterial nitrite infusion increased local plasma cGMP production (regarded as the most sensitive marker of NO availability)[33] in a dose-dependent manner, supporting nitrite-derived NO as the mechanism of dilatation. The precise mechanism for nitrite conversion to NO is not clear, as a

number of enzymes have been implicated in the catalysis of nitrite to NO in different tissue compartments. However, a key pathway considered to account for the effects of nitrite in small resistance vessels is nitrite reduction to NO via deoxHb [54]. In addition, it is possible that nitrite reduction to vasodilating NO is supported by fully oxygenated red cells which possess appreciable nitrite reductase activity in vitro; i.e., ~50% of the capacity of deoxygenated red cells to reduce nitrite (10 μM) to NO [55], reflecting the greater reductive potential of haem in the R (oxy) state tetramer [55], Thus, HbO_2 saturations ~99% during normoxia could support nitrite reduction, although this is difficult to confirm or refute in this design of *in vivo* experiments.

However, certain alternative enzymes that may enhance nitrite-derived NO *production*, or *activity*, are amenable to pharmacological manipulation *in vivo*. For example, carbonic anhydrase, an enzyme found in abundance in vessels and erythrocytes, has been demonstrated by Aamand et al, [29] to possess nitrite anhydrase activity (as the isolated enzyme and in tissue homogenates) increasing NO production, and enhancing nitrite-dependent dilatation in the rat aorta (at 1% O_2). The CA inhibitor acetazolamide, prevents the hydration of CO_2 but not the anhydration of nitrite. It has been suggested that the two substrates bind to different groups in the active site and that acetazolamide may increase the affinity for nitrite, by occupying non-productive binding sites on the enzyme, thus enhancing its activity as a nitrite anhydrase [279]. Notably, such activity appears to predominate around physiological pH, as the initial rates of NO production from nitrite via CA in the presence of acetazolamide were greater at pH 7.2 than pH 5.9, albeit under strict anaerobic conditions. Thus, administering acetazolamide (either systemically via the oral route, or locally via the intra-

arterial route) significantly enhanced radial artery dilatation to nitrite, supporting this mechanism of enhanced NO *production*.

Mechanisms which may enhance nitrite-derived NO *activity* include inhibition of ROS production. While XO is established as an important source of ROS, the activity of AO to generate ROS is ~25-fold greater than XO in human liver and rat hearts [414]. Raloxifene, a potent inhibitor of AO [414], enhanced nitrite-induced dilatation of the human radial artery, and the rat aorta suggesting that AO-mediated ROS production predominates over AO-mediated nitrite reduction in normoxia. On the other hand, conditions which increase ROS production such as hyperoxia [15], will diminish nitrite-derived NO activity. Indeed, hyperoxia inhibited nitrite-induced radial artery dilatation.

Hypoxia has also been shown to amplify ROS production [414]. It is therefore possible that this increased ROS production accounts for the attenuated dilatory effect of nitrite in the radial artery - to a similar degree as during hyperoxia, and this overrides the increased rate of nitrite reduction resulting from the greater proportion of deoxyHb encountered under hypoxic conditions in the conduit artery. By contrast, in the resistance vessels, hypoxia augmented nitrite-increased FBF, as reported previously [414]. This suggests less scavenging of NO in the arterioles, which may be, at least in part, a consequence of the erythrocyte-free zone [415] (see Figure 7.1). In smaller vessels, a narrower erythrocyte-free zone results in a closer proximity of the erythrocyte to the vessel wall, minimizing the diffusion distance for NO and the duration in which it may be scavenged by ROS. In the larger radial artery, the wider erythrocyte-free zone results in the opposite. Thus, in the resistance arteriole, the effects of enhanced reduction of nitrite via deoxyHb predominates, whereas in the conduit

vessel, scavenging by ROS prevails. A further mechanism for nitrite's selective vascular effects in normoxia versus hypoxia, may involve the recently described endothelial Hbα, which is absent from larger arteries, but present in the myoendothelial junctions of small arteries, where it dampens responses to (eNOS-derived) NO [416]. However, in hypoxia, it is possible that deoxyHbα supports further nitrite reduction to NO which is in the immediate vicinity of the vascular smooth muscle cells, enhancing vasodilatation of resistance arterioles.

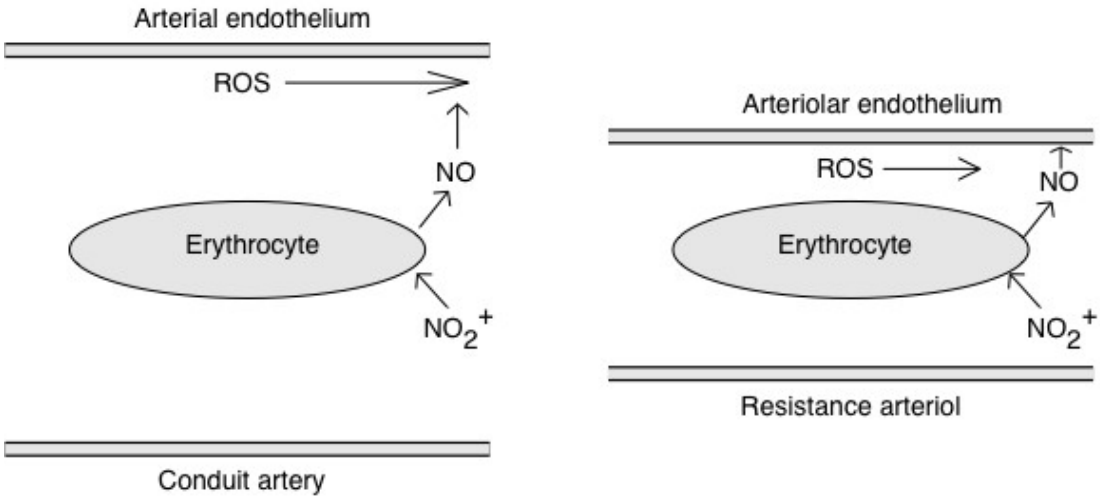

Figure 7.1: The larger gap between the erythrocyte and the vessel wall in the conduit artery (erythrocyte-free zone) increases the distance traversed by the NO messenger to reach the vessel wall which allows for greater scavenging by ROS. In the arteriole however, a smaller gap improves NO's chance of reaching the vessel wall and exerting its effects.

Furthermore, it is conceivable that nitrite exerts dual activity on the vasculature; where on one hand its effects are mediated through the classic NO-cGMP pathway and on the other, they are mediated via NO-independent signalling. The latter being effected either by nitrite directly [75,417] or through the formation of an intermediate that is not dependent on nitrite's reduction to NO. Thus, in conduit vessels nitrite's NO-independent signalling pathway dominates whereas in resistance arterioles its actions are contingent on its conversion to NO.

S-nitrosothiols make an attractive candidate for such an intermediate. They are formed without the need for nitrite's conversion to NO [115], being produced in both normoxic as well as hypoxic conditions (in various rodent tissues) [75] and have been shown to induce vasodilatation via cGMP-independent mechanisms [63].

In humans, nitrite-related SNO formation was observed following systemic intravenous infusions of sodium nitrite in healthy volunteers [63]. And although in another study the infusion of nitrite intravenously did not result in SNO formation this is probably a result of the low amount of nitrite (30 μmol) administered as no dilatation in the brachial artery was observed [55]. However in this same study the direct systemic infusion of GSNO resulted in dilatation of the brachial artery [25]. The inability to record SNO levels at baseline or at most of the doses of nitrite infused in the studies reported in the results section, makes supporting or refuting this hypothesis problematic. Although SNO levels were easily recordable at the highest dose of 87 μmol it is difficult to ascertain if the rise in SNO levels was due to increased local intravascular production or due to production at other sites such as the liver due to systemic overspill.

7.3 Systemic activity of sodium nitrite

Nitrite was effective systemically, with a modest dose (8.7 µmol/ ml, 0.6 mg/min) selectively dilating the radial artery by ~11% after 45 min with no effect on FBF. The delayed response represents the time for the systemic concentration of nitrite to accumulate, given an initial half-life of nitrite of ~20 min (with continuous infusion) [418]. Consistent with this selective dilatation of conduit arteries, nitrite selectively lowered cSBP/cPP by ~12 mmHg/ ~11 mmHg respectively with no effect on peripheral BP. Nitrite also reduced peripheral AIx by 11.9±4.6%, which equates to a change in central AIx of ~10% [419]. Selective dilatation of muscular conduit arteries is an accepted mechanism by which GTN selectively lowers central BP through a reduction in wave reflection [29]. It is therefore likely that the effects of nitrite on central haemodynamics are mediated by a similar mechanism. The lack of effect of nitrite on peripheral BP is consistent with the findings from a recent study performed in 55 patients with peripheral arterial disease, which found no reduction in BP with sustained release sodium nitrite up to doses of 160 mg twice daily [420].

7.4 Clinical relevance of nitrite's central effects

Central pressures are different to brachial pressures due to (variable) pulse pressure amplification when moving from the aorta to the periphery [421]. Central (aortic, carotid) pressures more closely reflect the load on the heart and brain and are at least as predictive of cardiovascular events and may be more predictive [11]. In the CAFE study, the amlodipine±perindopril combination resulted in a lower central pulse pressures than the atenolol±thiazide combination, and was significantly associated with a composite outcome of total cardiovascular events [218]. Central pulse pressures may therefore serve as a target in intervention strategies and the pharmacological use of a treatment, such as sodium nitrite, that selectively lowers cSBP/cPP/AIx, has the potential to reduce cardiovascular risk independently of any effect on peripheral blood pressure. A specific advantage of inorganic nitrate/nitrite (in contrast to organic nitrates) is that it does not appear to induce tolerance [11].

Normalization of elevated aortic pulse wave velocity (aPWV, a marker of large elastic artery stiffness) was recently demonstrated in old mice given oral dietary nitrite supplementation for 3-weeks [134]. While acutely, dietary nitrate has recently been demonstrated to reduce aPWV by 0.3 m/s in healthy volunteers and by ~0.5 m/s in grade 1 hypertensives, this was in parallel with reductions in brachial systolic pressures of ~5 mmHg and ~12 mmHg respectively [422,423]. Our current studies show an acute effect of nitrite, lowering bfPWV by ~1.2 m/s, independent of peripheral BP.

7.5 Limitations and Future studies

All the studies reported involved healthy male volunteers. It remains to be determined whether these mechanisms would be similar in females and patients with vascular disease.

Furthermore, other enzymatic pathways were not tested such as XOR, ALDH-2, CYP450, and interaction with PDE5. In addition, nitrite's prospective capability of direct signalling (which has been attenuated using a COX inhibitor) was also not investigated.

Conducting these studies would help further elucidate nitrite's mechanism of action on conduit vessels and resistance arterioles and the possibility of a dual activity and other possible complex interactions.

In a clinical setting these findings are exacting as they allow for the possibility of a multitude of new uses of inorganic nitrite. Glyceryl trinitrate is already extensively used to pre-dilate an occluded coronary vessel, prior to revascularization, in order to make a more accurate assessment of the vessel's calibre prior to the implantation of a suitably sized stent. If nitrite's dilatory effects in the RA can be demonstrated in the epicardial coronary arteries (which are similar in calibre to the RA but differ in flow dynamics) when combined with nitrite's mitigation of the deleterious effects of reperfusion, this would allow it to be used in preference to GTN prior to coronary intervention.

Furthermore, patients presenting with an acute coronary syndrome and on-going chest pain who require prolonged treatment with intravenous GTN prior to percutaneous coronary intervention (which is in essence does cause a short period of interruption of blood flow and reperfusion) may benefit more from pre-

treatment with intravenous nitrite. The argument here is that prolonged administration of GTN has been shown to exacerbate myocardial damage from reperfusion whereas inorganic nitrite has been found to be protective regardless of the duration of systemic delivery.

The selective central haemodynamic effects of nitrite may have potential utility in medical emergencies where such an effect would be desirable such as an aortic dissection. Although GTN is already used as a drug of choice in this clinical setting, nitrite would provide a more superior option as tolerance does not develop with prolonged administration.

These are few of the many new exciting potential clinical application of inorganic nitrite. One may argue that nitrite has fallen out of favour due to its unpredicted effects on the vascular system which on some occasions may lead to circulatory collapse. However, the studies that were conducted and came to this conclusion are out-dated and used preparations of nitrite that were far from ideal, described as “grains of nitrite” which could have contained widely varying quantities of the ion. It maybe that it is time for the circle to be complete and for inorganic nitrite / nitrate to transplant organic nitrite / nitrate in clinical.

7.6 Conclusion

Over the last decade, the status of the nitrite anion has been transformed from an inactive metabolite of NO to a key source of the messenger. Although direct activity and the formation of other intermediates without the need for reduction have been demonstrated, the bulk of its actions are mediated through its conversion to NO. This conversion was thought to be contingent on conditions of hypoxia and/or ischemia thus making nitrite activity in the oxygenated arterial tree unlikely. However, contrary to expectation, inorganic nitrite selectively dilates conduit arteries under normal oxygenated conditions, to a similar degree as GTN, and selectively lowers central systolic/pulse pressure, with important therapeutic potential. These effects appear to be mediated via a carbonic anhydrase catalysed – cGMP-dependent mechanism in normoxia. The inhibition of conduit artery dilatation in hypoxia and hyperoxia, and the enhancement of dilatation by aldehyde oxidase inhibition, suggest an important role for ROS production in modulating these responses. These findings are a further step in the continuing evolution of the understanding of nitrite's fundamental role in the cardiovascular system an understanding that is likely to advance further over the next decade.

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