The Significance of Nitric Oxide Metabolites in the Human Circulation in Health and Disease

by

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This thesis is dedicated to the memory of my father

Hadi Khalatbari

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Overview of the thesis

In this thesis I have investigated the profile and significance of nitric oxide metabolites in two human models: 1) Across healthy human coronary and pulmonary vascular beds 2) In the peripheral venous blood from patients with type 1 diabetes mellitus.

Chapters 1-3 offer a comprehensive background detailing issues of relevance to the rest of this work. Chapter 4 outlines precise methodological protocols and materials used. The results of the above clinical and laboratory studies are presented and discussed in Chapters 5 and 6. Chapter 7 attempts to summarise the results obtained, drawing together conclusions and highlighting perspectives for future research.

I found that nitric oxide was dynamically metabolised across the heart and that the compartmentalisation of its metabolites between plasma and erythrocytes was driven primarily by the oxygen saturation of the blood. Study of the changes in coronary arterial diameter and flow in response to exercise and inhibition of nitric oxide generation suggested the presence of an endothelium derived hyperpolarising factor-like activity in the epicardial coronary arteries.

Among patients with type 1 diabetes, blood levels of nitric oxide metabolites were generally lower compared to controls; and lower in those with microvascular complications comparing to those without. Vessel relaxation experiments suggested the existence of a red blood cell-related vasodilating factor (RRVF) which was present in

both diabetics and controls but exerted stronger vasodilator activity when erythrocytes from the former group were added to aortic ring preparations in a hypoxic tissue bath system *ex vivo*. Another novel finding was a positive correlation between this RBC-related vasodilator activity and HbA_{1C}; which was stronger in that group of patients who were generally younger with shorter duration of disease.

Abbreviations

6-PG	6-phosphoglucolactone
8-PT	8-phenyltheophylline
ACE	Angiotensin converting enzyme
ADP	Adenosine diphosphate
AGE	Advanced glycation end products
ALS	Amyotrophic lateral sclerosis
AMP	Adenosine monophosphate
AS	Angeli's salt
ATP	Adenosine triphosphate
AUC	Area under curve
BH4	Tetrahydrobiopterine
bpm	Beats per minute
CBF	Coronary blood flow
CcO	Cytochrome c oxidase
CFV	Coronary flow velocity
cGMP	Cyclic guanosine monophosphate
CO_2	Carbon dioxide
CS	Coronary sinus
CSA	Cross sectional area
CVD	Cardiovascular disease
CVR	Coronary vascular resistance
DAN	2, 3-diaminonaphthalene
DNA	Deoxyribonucleic acid
EBC	Exhaled breath condensate
EC50	Median effective concentration (required to induce a 50% effect)
EDHF	Endothelium derived hyperpolarising factor
EDRF	Endothelium derived relaxing factor
EDTA	Ethylenedinitrilotetraacetic acid
EPR	Electron paramagnetic resonance
ESR	Electron spin resonance spectroscopy
FAD	Flavin adenine dinucleotide
FiO_2	Fraction of inspired oxygen
FMN	Flavin mononucleotide
G6P	Glucose-6-phosphate
G6PD	Glucose-6-phosphate dehydrogenase
GAPDH	Glyceraldehyde-3 phosphate dehydrogenase
GFAT	glutamine: fructose-6-phosphate amidotransferase

GLUT	Glucose transporter
GSNO	*S*-nitrosoglutathione
GTP	Guanosine triphosphate
HbNO	Nitrosylhaemoglobin
HbO_2Sat	Haemoglobin oxygen saturation
HBP	Hexosamine biosynthesis pathway
HbSNO	S-nitrosohaemoglobin
HCL	Hydrochloric acid
HPLC	High performance liquid chromatography
IHD	Ischaemic heart disease
km/h	kilometre/hour
LAD	Left anterior descending artery
LADA	Latent autoimmune diabetes in adults
LCx	Left circumflex artery
LDL	Low density lipoprotein
LMS	Left main stem
L-NAME	Nitro-L-arginine methyl ester
L-NMMA	N^G-monomethyl-L-arginine
LNNA	N^G-nitro-L-arginine
$LOO^{\bullet}$	Lipid peroxyl radical
LV	Left ventricle
MAP	Mean arterial pressure
metHb	Methaemoglobin
MHR	Maximum heart rate
mM	Millimolar
MnSOD	Manganese superoxide dismutase
MVO_2	Myocardial oxygen consumption
MWCO	Molecular weight cut off
NADPH	Nicotinamide adenine dinucleotide phosphate
NAT	2, 3-naphthotriazole
NICE	National institute of health and clinical excellence
NLA	Nitro-L-arginine
nM	Nanomolar
NO	Nitric oxide
NOA	Nitric Oxide Analyzer
NOS	Nitric oxide synthase
NR	Nitrate reductase
O_2ct	Oxygen content
$ONOO^-$	Peroxynitrite
PAI	Plasminogen activator inhibitor

PARP	Poly (ADP-ribose) polymerase
PE	Phenylephrine
PGI_2	Prostacyclin
PKC	Protein kinase C
PMN	Polymorphonuclear
PMT	Photon multiplier tube
PO_2	O2 partial pressure
ppm	Part per million
PVC	Polyvinyl chloride
QCA	Quantitative coronary analysis
RAGE	Receptor for AGE
RAO	Right anterior oblique
RBC	Red blood cells
RCA	Right coronary artery
RNNO	N-nitrosamines
ROS	Reactive oxygen species
RRVF	RBC-related vasodilating factor
RSNO	S-nitrosothiols
SD	Standard deviation
SEM	Standard error of mean
sGC	Soluble guanylate cyclase
SOD	Superoxide dismutase
TGF	Transforming growth factor
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
VIP	Vasoactive intestinal peptide

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CHAPTER ONE

Nitric oxide: history, chemistry, metabolism

History

Nitric Oxide[2-4]

Nitric oxide (NO) was first prepared by the Belgian physician and scientist Jan Baptist van Helmont (1577-1644) in 1620. British scientists Robert Boyle (1627-1691) and Robert Hooke (1635-1703) independently generated NO in the 1660s. In 1772, Joseph Priestly (1733-1804), also the discoverer of oxygen, found that NO (nitrous air) was incompatible with plant life. J.A. Murray gave "nitrous air" its modern name- nitric oxide- in 1806.

Thereafter, NO was mainly known as an important air pollutant generated by fuel combustion, especially in motor vehicles and power plants, with a major role in the formation of photochemical smog. It was not until mid 1970s when scientists started to recognise its role in human physiology.

In 1977, Dr Ferid Murad from the University of Virginia found that organic nitrates like sodium nitroprusside and glyceryl trinitrate (GTN), which had been used as antianginal agents for nearly 100 years, released NO, which relaxed smooth muscle cells and resulted in vasodilation.

In 1980, Robert F. Furchgott from the State University of New York hypothesised that endothelial cells produce an unknown signal molecule that makes vascular smooth muscle cells relax. He called the signal molecule EDRF, or endothelium-derived relaxing factor. Later he suggested that EDRF may be NO.

Finally in 1987, Salvador Moncada from University College London and Louis J. Ignarro from University of California, through a series of experiments confirmed that EDRF was identical to NO.

In 1992, 372 years after its discovery; NO was voted "Molecule of the Year" by Science magazine.

In 1998, the Noble prize in Medicine and Physiology was awarded jointly to Furchgott, Ignarro, and Murad for their discoveries concerning NO as a signalling molecule in the cardiovascular system.

The new nomenclature of inorganic chemistry designates NO as "nitrogen monoxide". However, in biomedical and biochemical circles, it is still mainly known as "nitric oxide".

Glyceryl trinitrate (GTN)[2-4]

No history of nitric oxide is complete without mention of GTN, the most widely used antianginal agent in the world.

In 1847, GTN, also known as nitroglycerine, was first synthesised by the Italian chemist Ascanio Sobrero (1812-1888) when he was an assistant to Professor J.T. Pelouze in Paris. GTN, which was produced by mixing glycerine with sulphuric and nitric acid, turned out to be a highly explosive liquid. Sobrero considered GTN to be too dangerous to be of any practical use. He also noticed putting a small amount of GTN on the tongue can cause severe headache.

In 1851, Alfred Nobel (1833-1896) who had gone to Paris to work for Professor Pelouze, met with Sobrero and became very interested in GTN and its potential use in excavations and construction work. He soon found that mixing GTN with silica would turn the liquid into a more stable paste which could be shaped into rods of a size and form suitable for insertion into drilling holes. He called his new invention dynamite.

In 1867, Thomas Lauder Brunton, the father of modern pharmacology, used amyl nitrite to relieve angina.

In 1876, William Murrel, a London doctor, was the first to prescribe GTN as a treatment for the relief of anginal pain. GTN was soon established as the mainstay of angina pectoris relief.

Ironically, neither Nobel nor Murrel agreed to take GTN when they suffered from angina pectoris and heart failure.

In the early 1900s and following the flourish of GTN industry, two new medical conditions- "Monday disease" and "Sunday heart attacks" were described in workers exposed to high levels of nitrates during the week. Nitrate tolerance and nitrate withdrawal phenomena were recognised and taken responsible for the above conditions respectively.

In 1977, Dr Ferid Murad suggested that the activation of the enzyme guanylate cyclase by sodium nitroprusside and GTN might be due to the formation of nitric oxide.

While GTN remains the treatment of choice for angina pectoris, even today the actual mechanism of NO release from it is unclear. Several nonenzymatic and enzymatic systems have been found capable of metabolising GTN[5]. Recently mitochondrial aldehyde dehydrogenase has been suggested to have an important role in GTN bioactivation[6].

Chemistry[7-10]

General

NO is a colourless, hydrophobic gas with a low water solubility. It is a free radical with a single unpaired electron and can react rapidly with other molecules that contain unpaired electrons. NO can also interact with transition metals to produce an enormous range of complexes that have both theoretical significance and practical importance[11].

Figure 1.1: Molecular structure of nitric oxide.

The half life of NO in the blood is less than 5 seconds[11-13]. *In vitro*, in aqueous, haemoglobin-free solutions, NO has been reported to have a half life of at least 8 minutes[14;15]. The half life of NO in plasma *in vitro* is 68±12 seconds[16].

The maximum solubility of NO (at 1atm partial pressure) in water at room temperature (25°C) and pressure is approximately 2mM which is slightly higher than the solubility of O_2 in water. Due to its lipophilic properties, NO is 6-8 times more soluble in nonpolar solvents and lipid membranes compared to water. This property

makes NO highly diffusible across cell membranes and allows it to elicit its effects relatively far from its site of production.

Air

In air NO is almost immediately oxidised to NO_2 which then dimerises to N_2O_4:

$$2NO + O_2 \rightarrow \quad 2NO_2 \rightarrow N_2O_4$$

Aqueous Solutions

N_2O_4 (from air) in water forms nitrite and nitrate in roughly equal amounts:

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

However, when NO is directly introduced into water, the result is almost exclusively comprised of nitrite ions and protons:

$$NO + H_2O \rightarrow \quad NO_2^- + 2H^+$$

This suggests the presence of an intermediate N_2O_3 in aqueous solutions as below:

$$2NO + O_2 \rightarrow \quad N_2O_4$$

$$N_2O_4 + 2NO \rightarrow 2N_2O_3$$

$$N_2O_3 + H_2O \rightarrow 2NO_2^- + 2H^+$$

Oxidative Stress

Oxidative stress (or oxidant-derived tissue injury) occurs when production of oxidants or reactive oxygen species (ROS) exceeds local antioxidant capacity[17].

In the presence of oxidative stress, reactive oxygen species (ROS) like the superoxide anion can combine with NO to yield peroxynitrite which can degrade into nitrate. This happens even in the absence of haemoglobin. The more the oxidative stress in the fluid the higher the production of nitrate.

$$NO + .O_2^- \rightarrow ONOO^-$$

$$ONOO^- + H^+ \rightarrow ONOOH \rightarrow NO_3^- + H^+$$

Because peroxynitrite is physiologically less active than NO, this reaction essentially represents an inactivation mechanism for NO and has been utilised as a biological marker or evidence of oxidative stress.

NO^+ and NO^- [9;18]

As stated above, NO has an unpaired electron in its highest molecular orbit. Loss of this electron through oxidation forms the nitrosonium cation (NO^+). Gaining an additional electron through reduction forms the nitroxyl anion (NO^-).

NO^+ reacts readily with water to form nitrous acid (HNO_2) which is a strong oxidising agent. Nitroso- compounds act as NO^+ equivalents and can be regarded as NO^+ carriers in physiological conditions. The most important form of nitroso- compounds in biological systems is S-nitrosothiols. In addition, NO^+ can form N-nitroso compounds with amines. However, ordinary amines are usually protonated at

physiological pH to prohibit the nitrosation. S-Nitrosothiol can release NO spontaneously but in true chemical terms is an NO^+ donor:

$2RS—N=O \rightarrow R—SS—R + 2NO$

Little is known about the potential nitrosative and nitrative chemistry of the nitroxyl anion (NO^-/HNO). Angeli's salt (AS; $Na_2N_2O_3$), a well-known nitroxyl donor, has a large variety of biological effects including positive cardiac inotropy[19], selective venodilation in vivo, relaxation of vascular smooth muscle in vitro, and reducing blood pressure in vivo[9]. The latter two properties of AS have been associated with the formation of iron-nitrosyl complexes.

While NO^-/HNO is an integral component of the redox biology of NO, its physiological chemistry is not well-understood.

NO production *in vivo*[8;9]

NO is produced in various tissues in the body. It is synthesised from the semi-essential amino acid L-arginine by the enzyme nitric oxide synthase (NOS). The nitrogen atom of NO is derived from the guanidino group of the arginine side chain, and the oxygen atom of NO is derived from molecular oxygen (Figure 1.2)

L-arginine + $O_2 \rightarrow$ L-citrulline + NO

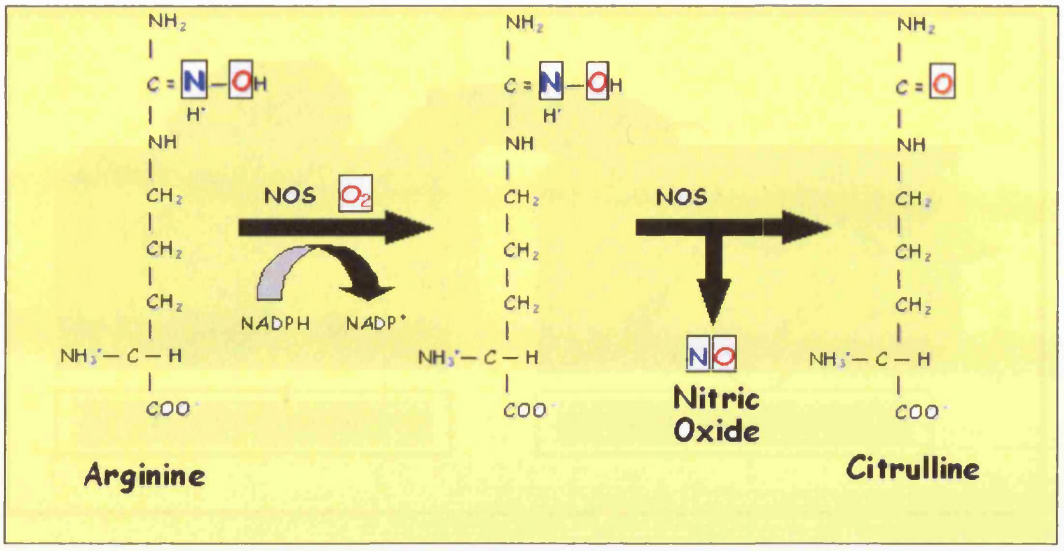


Figure 1.2: Nitric oxide synthesis from L-arginine.

As it can be seen in figure 1.2, the main substrates for NOS are L-arginine, oxygen, and NADPH (nicotinamide adenine dinucleotide phosphate). The main known cofactors are BH_4 (tetrahydrobiopterine), FAD (flavin adenine dinucleotide), FMN (flavin mononucleotide), haem, and zinc.

Endothelial cells constantly produce NO at a rate of 1-4nM/s[20].

Nitric Oxide Synthases (NOSs)[9]

Nitric oxide synthase (NOS) is the general term for a group of haem- and flavin-containing enzymes that catalyse the synthesis of NO in the body. They all convert L-arginine to L-citrulline and NO is produced in the process. The NOSs are functional dimers and consist of two major domains; a haem containing oxygenase domain and a flavin containing reductase domain. The domains are connected by a calmodulin-binding site which upon the binding of calmodulin acts as a bridge for electron transfer from the reductase to the oxygenase domain (Figure 1.3).

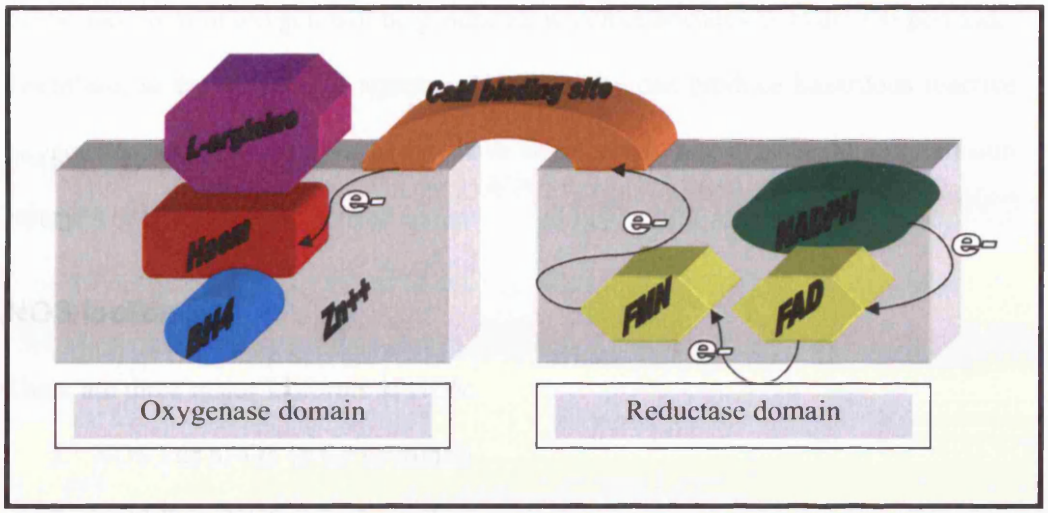


Figure 1.3: Schematic structure of nitric oxide synthase (NOS).

The electron donor is NADPH which donates two electrons to FAD, which are then passed to FMN. The reaction starts when calmodulin binds to the enzyme, allowing the electrons to reach the oxygenase domain and reduce the haem iron to its ferrous form, to which oxygen can then bind.

The oxidation of L-arginine to L-citrulline is a 2 step process. Arginine is first oxidised to N-hydroxy-L-arginine which is then oxidised to L-citrulline and NO.

For calmodulin to bind the enzyme, an influx of calcium is required to increase intracellular calcium and hence the binding of calcium to calmodulin. Calmodulin does not bind NOS at basal intracellular calcium levels. The exception is iNOS (NOSII) (see below).

If arginine is not present, the electron transferred to the haem will reduce oxygen and form superoxide. If the second electron is transferred before superoxide is released,

the peroxy form of oxygen will be generated, which dissociates as hydrogen peroxide. Therefore, in the absence of arginine, NOS activity can produce hazardous reactive oxygen species. This mechanism may have an important role in ischaemia/reperfusion injury.

NOS Isoforms

There are three major Isoforms of NOS:

1. NOS I or nNOS (n for neuronal)
2. NOS II or iNOS (i for inducible)
3. NOS III or eNOS (e for endothelial)

Table 1.1 summarises the properties of NOS isoforms. The net effect of the NO produced by NOSs is dependant on where it is being produced, regulation of its production, how much is being produced, and which other reactive species are present.

	nNOS	**iNOS**	**eNOS**
Molecular mass	160kD	130kD	135kD
Expression	Constitutive	Inducible	Constitutive
Cell fraction	Cytoplasmic	Cytoplasmic	Membrane-bound
Dependence on calcium influx	Dependent	Independent	Dependent
Physiological action	Neurotransmission	Cytotoxicity	Vasodilation

Table 1.1: Properties of NOS isoforms.

nNOS- is found primarily in neurons and skeletal muscle. The NO produced by nNOS serves as a neurotransmitter in the peripheral and central nervous system. In the central nervous system, NO has been implicated in neural signalling, neurotoxicity, synaptic plasticity, learning and memory, and perception of pain. In the peripheral nervous system, NO is involved in gut motility and control of the pyloric sphincter, bronchodilation pathway in the human pulmonary system, and neural control of the cerebral blood flow. It is also involved in urethra and bladder control and in penile erection[9].

In the skeletal muscle, NO serves as a mediator of contractile force. Activation of nNOS requires an influx of calcium to raise the intracellular calcium concentration, allowing calmodulin to bind to the enzyme[9].

iNOS- is found primarily in activated neutrophils and macrophages, astrocytes, and hepatocytes. iNOS is induced at the transcriptional level by cytokines, such as interferon-γ, interleukin 1, and tumour necrosis factor-α, or endotoxins such as lipopolysaccharides. iNOS is involved in the early immune response and its induction occurs over several hours via the inflammatory NFκB pathway. Calmodulin is bound to iNOS under basal physiological conditions (i.e. independent of calcium influx), so the enzyme is always activated once it is synthesised. The amount of NO produced by iNOS is approximately 1000 times greater than that of either nNOS or eNOS.

The NO produced by iNOS is a potent cytotoxic agent and its principal role is to destroy intracellular pathogens engulfed by neutrophils and macrophages (e.g. plasmodia, leishmania, mycobacteria, fungi, and even tumour cells). NO reacts with

various proteins and metabolic enzymes in these pathogens to alter their function. It also forms highly reactive nitrogen and oxygen species- such as peroxynitrite- which can damage the pathogen's DNA.

Overproduction of NO by iNOS has been implicated in septic/cytokine-induced circulatory shock. Excess NO leads to massive systemic vasodilation- via activating soluble guanylate cyclase and relaxing smooth muscle cells- which results in severe hypotension and may lead to the potentially fatal condition of "multiple organ failure".

It is of note that exogenous NO exerts regulatory effects on neutrophils function and may thus act as a local modulator in the inflammatory process. High concentrations of exogenous NO increase endothelial-neutrophil adhesion[21]. Exposure to NO donors inhibits human neutrophil functions[22].

iNOS is also present and active in a number of chronic inflammatory conditions such as rheumatoid arthritis and Crohn's disease.

eNOS- is found primarily in vascular endothelial cells and cardiac myocytes. It is bound to the cell membrane and like nNOS is constitutively expressed and is activated by the influx of calcium. The influx of calcium is a response to either receptor-dependent agents such as acetylcholine, histamine, bradykinin, ATP, insulin...or shear stress. Shear stress is the mechanical force of blood flow on the luminal surface of the vascular endothelium. Increased blood velocity stimulates calcium influx which activates eNOS to generate NO. The mechanisms by which flow-imposed shear stress elevates intracellular Ca^{2+} in endothelial cells are not fully understood. A recent study

by Yamamoto et al. on human pulmonary artery endothelial cells suggested that shear stress stimulates endothelial cells to release ATP, which activates Ca^{2+} influx via a subtype of ATP-gated cation channels also known as $P2X_4$ receptors[23].

NO diffuses out of the endothelial cells and into the adjacent smooth muscle cells where it activates the cytosolic enzyme soluble guanylate cyclase (sGC) by binding to its haem group. sGC transforms GTP (guanosine triphosphate) to cGMP (cyclic guanosine monophosphate). cGMP acts as a second messenger to activate protein kinase G. Protein kinase G phosphorylates a variety of channels and receptors, all leading to inhibition of calcium influx into the smooth muscle cells. Decreased intracellular calcium concentrations leads to smooth muscle cell relaxation and vasodilation (Figure 1.4).

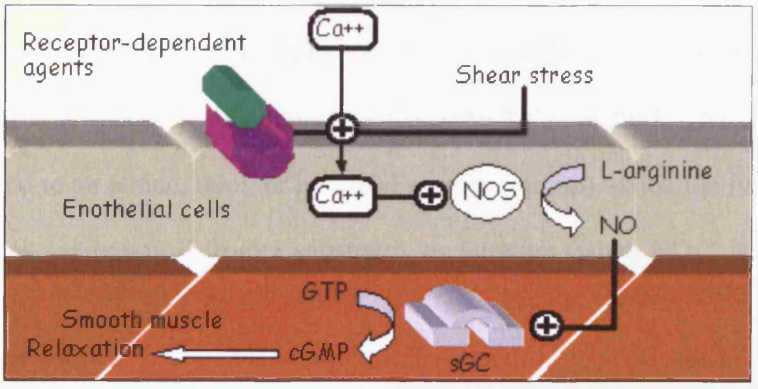


Figure 1.4: Endothelial NO production and vasorelaxation.

The NO produced by eNOS has a crucial role in maintaining normal vascular tone. Although endothelial-derived NO serves mainly as a vasodilator, it also exerts anti-platelet and anti-inflammatory functions by inhibiting platelet and leukocyte adhesion

and aggregation, respectively. EC50 for NO-mediated relaxation of intact and endothelium-denuded aortic rings is 10.5nM and 13nM respectively[24].

Biological effects and fate of NO[9;11;25;26]

Direct and indirect effects

The effects of NO in biological systems can be divided into two major categories: direct and indirect effects (figure 1.5).

Direct effects-are those reactions that are fast enough to occur between NO itself and specific biological molecules. The most important direct effects of NO are the reactions between NO and haem containing proteins.

Indirect effects-do not involve NO, but are rather mediated by reactive nitrogen oxide species (RNOS) formed from the reaction of NO either with oxygen or superoxide (O_2^-). The range of reactions mediated by RNOS include nitrosation (when NO^+ is added to an amine, thiol, or hydroxyl aromatic group), oxidation (when one or two electrons are removed from a substrate), or nitration (when NO_2^-) is added to a molecule.

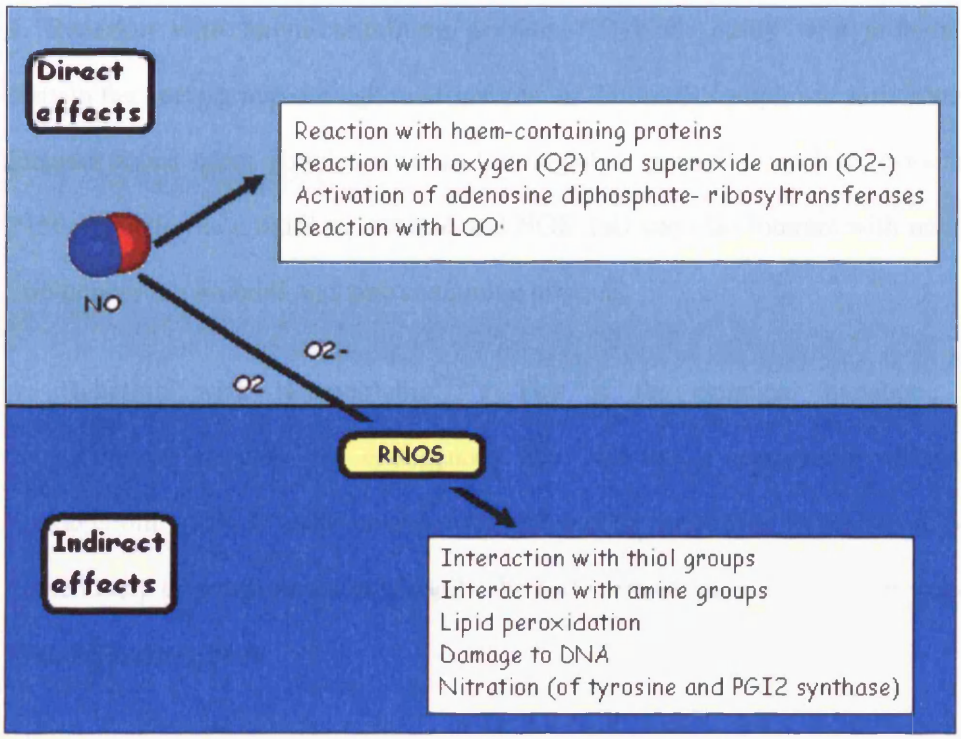


Figure 1.5: Direct and indirect effects of NO (LOO•: lipid peroxyl radical, PGI_2: prostacyclin, RNOS: reactive nitrogen oxide species).

At low concentrations (<1uM), the direct effects of NO predominate. At higher concentrations (>1uM), the indirect effects mediated by RNOS prevail.

Fate of NO in biological systems

The biological fate of NO is determined by two main factors: 1. NO concentration and source of production. 2. The surrounding milieu in which NO is released.

In cell types that contain eNOS and nNOS, NO is produced in relatively low concentrations and is therefore mainly engaged in its direct effects. Where iNOS is induced, NO is produced in high concentrations and the indirect and often pathologic effects of NO predominate.

The biological fate of NO can be summarized in 7 basic pathways:

1. Reaction with haem-containing proteins-NO binds easily with proteins that contain the haem group through the formation of dinitrosyl complexes with iron. This includes those haem proteins such as haemoglobin, guanylate cyclase, cytochrome P450, cytochrome c oxidase, catalase and NOS. NO can also interact with nonhaem iron-containing proteins and zinc containing proteins.

A. Reaction with haemoglobin[27;28]: This is the principal metabolic (and detoxification) pathway for endogenous NO. NO has a remarkable affinity for haemoglobin which is 1500 times greater than carbon monoxide. In the blood, NO is immediately taken up in the red blood cells and is transformed to nitrate by reaction with oxyhaemoglobin:

Oxyhaemoglobin + NO → Nitrate + Methaemoglobin

(HbO_2 + NO → NO_3^- + metHb)

MetHb is then reduced back to normal Hb by the house-keeping enzyme methaemoglobin reductase- also known as NADH-cytochrome b5 reductase.

Overall, 75-90% of all NO formed in the body converts to nitrate which is then eliminated via the kidneys. The half life of nitrate in plasma is about 5 hours[29].

Some NO will meet non-oxygenated haemoglobin in the blood and nitrosylate the Fe^{2+} to a fairly stable HbNO (nitrosylhaemoglobin) adduct (half life ~ 30-40 minutes)[29;30].

Hb + NO → HbNO

The above reaction mainly happens when NO is liberated into partly deoxygenated blood. As a result, the concentration of HbNO depends on the degree of oxygen saturation of haemoglobin [31].

NO reaction with cell-free haemoglobin is nearly 1000-fold faster than the reaction with RBCs.

S-nitrosohaemoglobin (Hb-S-NO) – NO can interact directly with the thiol groups on haemoglobin (S-nitrosation of Hb ß- chain cysteine 93) to produce SNO-Hb. However, this happens relatively slowly compared to the quicker oxidation rate of NO alluded to above. Oxides of NO (including nitrite, N_2O_3) can transfer quickly the NO^+ to form SNO-Hb by a favourable process termed transnitrosation.

SNO-Hb may play an important role in the control of blood perfusion and oxygen delivery to tissues, as discussed later in this chapter.

B. Soluble guanylate cyclase (sGC)[32;33]: Most of the biological effects of NO- including its muscle relaxant properties- are mediated through soluble guanylate cyclase. NO activates sGC by binding to its haem group. Binding of NO to sGC increases its activity by up to 500 fold. sGC transforms guanosine 5'-triphoshphate (GTP) to cGMP which acts as a second messenger and facilitates vasorelaxation and phosphorylation of various proteins by activating cGMP-dependent protein kinases.

sGC is activated by fairly low concentrations of NO, i.e. 10-100nM[34] (EC_{50} of ≈10nM[24]) which reflects the high affinity of NO for the sGC haem moiety.

C. Cytochrome P450[35]: NO inhibits cytochrome P450 enzymes in two ways, reversibly (by direct binding of NO to haem) and irreversibly (as an action of reactive

nitrogen species). The inhibition of cytochrome P450 isoforms alters the hepatic metabolism of many drugs and can have deleterious effects in high NO-production conditions such as septic shock.

D. Cytochrome c oxidase[25;36]: NO inhibits mitochondrial respiration by binding and inhibiting cytochrome c oxidase (CcO) in a reversible fashion. CcO is one of the enzymes in the mitochondrial respiratory chain with both haem and copper groups in its structure. Which metal group binds NO and causes the inhibition is still not clear.

Inhibition of mitochondrial respiration by NO is regarded as one mechanism of macrophage-derived cytotoxicity.

E. Catalase[37]: Catalase is a haem protein which is critical in protecting cells against hydrogen peroxide damage. NO can reversibly bind to the haem moiety of catalase molecule and inhibit its activity. This will result in increased hydrogen peroxide concentrations and enhance the cytotoxic effect of NO.

F. NOS[38]: NO can also attenuate NOS activity, serving as a negative feedback to control NO production. NO binds the haem group associated with NOS and prevents the binding of oxygen to the active site and thus inhibits the oxidation of arginine. nNOS and eNOS are more sensitive than iNOS to the inhibitory action of NO

2. Reaction with oxygen (O_2) and superoxide anion (O_2^-)[9]: NO reacts with O_2 and O_2^- to form reactive nitrogen oxide species (RNOS).

Oxygen- In aqueous solutions NO can undergo autoxidation (i.e. reaction with oxygen) to produce N_2O_3. N_2O_3 is the predominant RNOS formed from the autoxidation of NO in biological systems. N_2O_3 is rapidly hydrolysed to nitrite with a half-life of 1ms. The resultant nitrite is taken up by RBCs where it is further oxidised to nitrate and released back to plasma[39].

Superoxide anion (O_2^-)[40] -In physiological conditions O_2^- concentrations are kept low by its neutralisation by superoxide dismutase (SOD), antioxidants, and by its extremely rapid reaction with NO (almost diffusion limited). However, under pathological conditions (e.g. atherosclerosis, myocardial ischaemia, sepsis, distressed lung, inflammatory bowel disease, and amyotrophic lateral sclerosis) when the levels of O_2^- may be very high, NO combines rapidly with O_2^- to form peroxynitrite. Peroxynitrite ($ONOO^-$) is itself toxic and acts as a selective oxidant and nitrating agent to modify proteins (e.g. tyrosine→ nitrotyrosine), lipids, and nucleic acids.

Two major sources of $ONOO^-$ formation in our body are mitochondria and immune cells. In mitochondria, $ONOO^-$ is produced as a result of aerobic respiration. The generation of $ONOO^-$ in the mitochondria is intensely controlled by several regulatory mechanisms including manganese superoxide dismutase (MnSOD). The primary source for large amounts of $ONOO^-$ is immune cells through either NADPH oxidase or xanthine oxidase. Neither enzyme is directly inhibited by NO. Therefore as NO migrates near the source of O_2^-, it reacts to form peroxynitrite. However, as peroxynitrite moves from its source, it is converted by excess NO to N_2O_3. Thus, the primary chemistry of $ONOO^-$ would be within close proximity of the superoxide source.

Nitrotyrosine[40]- Nitrotyrosine is frequently used as a marker of in vivo production of $ONOO^-$. It is formed by the nitration of tyrosine by $ONOO^-$. Nitrotyrosine levels are increased in conditions associated with increased oxidative stress. Nitrotyrosine has been found in atherosclerotic plaques, motor neurons of patients with ALS, rejected renal allografts, inflammatory bowel disease, the synovial fluid of arthritis patients and the placental tissues from pre-eclamptic pregnancies. Animal studies have demonstrated nitrotyrosine formation in ischaemia-reperfusion injury in the heart.

Nitration of prostacyclin synthase[41]-Nitration of endothelial prostacyclin synthase by $ONOO^-$ inhibits its activity and impairs vasorelaxation.

3. Interaction with thiol (-SH) groups[9;25]**:** Nitrosation of thiols in proteins such as albumin results in long-lived S-nitrosothiols (RSNO) with a variety of different effects in biological systems. Peptides with thiol groups have strong affinity for N_2O_3. This makes thiols the primary target for reactive nitrogen oxide species (RNOS) in biological aqueous solutions.

The amino acid cysteine, which is found in most proteins, contains a thiol group. S-nitrosylation of cysteine residues resulting from the addition of a NO^+ group has been shown to modify the activity of several proteins. Although it is unlikely that NO acts directly on the cysteine residue, NO interacts with O_2 or O_2^- to produce RNOS capable of nitrosylating cysteine residues. Nitrosylation is a non-enzymatic chemical reaction.

In recent years RSNOs have attracted increasing attention as possible preservers of NO bioactivity in the circulation. They prevent loss of NO from oxidative degradation and also create bioactive low molecular weight nitrosothiols such as *S*-

nitrosoglutathione (GSNO) which demonstrate vasodilator properties that are equal to native NO. RSNOs provide a reservoir of NO bioactivity that might be utilised in states of NO deficiency.

96% of plasma RSNOs are S-nitrosoproteins of which 82% is S-nitroso-albumin[11].

4. Interaction with amine groups[9]: RNOS can also nitrosate the amine group of proteins to form nitrosamines (R_2NNO). Nitrosamines have a stable structure and are well known for their carcinogenic and mutagenic properties.

5. NO modifications of lipids: NO derivatives may react with unsaturated lipids to oxidise or nitrate them. One example is the low density lipoprotein (LDL) which is converted to its atherogenic form by oxidation and nitration.

The reaction between nitric oxide (NO) and lipid peroxyl radicals ($LOO^{\bullet}$) has been proposed to account for the potent inhibitory properties of NO toward lipid peroxidation processes[42].

6. NO damage to DNA[9]: NO is not reactive enough to damage DNA directly, but its derivatives (i.e. RNOS) can reach the nucleus to oxidise, nitrate, or deaminate genomic DNA, resulting in strand breaking and mutations. One apparent suspect is $ONOO^-$. $ONOO^-$ can travel up to 9μm and easily pass through biological membranes to reach the nucleus and modify DNA- preferentially reacting with guanine. Another powerful reactive nitrogen species is the nitrosating agent N_2O_3 which can damage DNA through reactions with its amines.

Chemical modification of DNA by RNOS may be an important contributor to the age- and inflammatory-related development of cancer or other diseases.

7. Activation of adenosine diphosphate- ribosyltransferases[25]: This leads to ribosylation of ADP which may have a role in the control of vascular tone via a mechanism independent of cGMP.

It has been suggested that reduced NO bioavailability increases vascular tone by two mechanisms. An acute decrease in NO levels leads to vasoconstriction due to a decrease in cGMP production. If NO levels continue to decrease for a longer time, ADP-ribosylation by NO is altered which leads to an increased sensitivity to vasoconstrictor agents such as endothelin.

NO transport in the bloodstream, role of NO reservoirs

Given the short life of NO in the blood, the presence of NO reservoirs which can preserve NO bioactivity and provide an "NO back up" in the circulation has been both the subject of much interest and controversy in the past 15 years. This mechanism of NO transport in the blood stream may be of particular importance when NO production or bioavailability is reduced and during increased tissue oxygen demand.

NO bioavailability, i.e. the availability of NO to exert its physiological activities, is determined by the balance between NO production and its degradation into other molecules which are either physiologically inert or have different physiological properties to NO.

The role of intravascular NO metabolites in the preservation of NO bioactivity is supported by NO inhalation studies. With the extremely short half life of NO in the

blood, one would expect a merely local pulmonary vasodilator effect for inhaled NO. However, animal studies have shown that inhaled NO can induce several extra-pulmonary cardiovascular effects[43-47]. Of interest, is a human study by Cannon et al. which showed NO inhalation results in peripheral vasodilation targeted to regions with pharmacologically reduced NO production[48].

While there is broad unanimity among scientists regarding the existence of NO reservoirs in the blood, the actual nature of these compounds has been a subject of ongoing debate; with *S*-nitros(yl)ated proteins (particularly haemoglobin) and the anion nitrite being the main candidates. Two alternative theories have formed around these two metabolites:

1. S-nitrosohaemoglobin theory: S-nitrosohaemoglobin serves as a stable storage form of intravascular NO.
2. Nitrite theory: NO is formed from the anion nitrite by the nitrite reductase activity of deoxygenated haemoglobin.

These theories will be discussed in detail below.

Both theories also try to explain the underlying mechanism of hypoxic vasodilation, i.e. local vasodilation in response to tissue hypoxia. Tissue hypoxia occurs when a region of the body is deprived of adequate oxygen supply. Hypoxic vasodilation is discussed in additional detail in CHAPTER TWO.

The main NO metabolites of interest and their significance in vascular physiology are discussed below. Methods of quantitative analysis of nitric oxide metabolites are discussed in CHAPTER FOUR.

Can NO transport as NO?-Nitric oxide produced by the endothelium diffuses both into the lumen and to the smooth muscle cells according to the concentration gradient in each direction. The rapid reaction between NO and oxyhaemoglobin (k_{Hb}= 3-5 x 10^7 M^{-1} s^{-1}), raises the question of how NO can escape from this large trap in the blood vessel lumen to reach and relax vascular smooth muscle cells. If specific mechanisms were not in place to reduce NO consumption by RBCs, NO would not have been able to exert any direct physiological effects locally. Moreover, a longer-than-expected survival of NO in the blood may mean that NO can travel as NO from its site of synthesis to many other tissues.

Several mechanisms have been proposed to explain the preservation of authentic NO bioactivity in the circulation. The most important mechanisms are:

1. A RBC-free zone near the vessel wall which reduces the consumption of NO by RBCs[49].

2. Limited extracellular diffusion by an unstirred layer surrounding each RBC[50].

3. A submembrane consisting of the RBC cytoskeleton and other relatively NO inert proteins such as methaemoglobin which can reduce NO uptake rate by RBCs[51].

Plasma NOx (NO_2^- + NO_3^-)

Definition- NOx accounts for the most abundant NO end products in the plasma, nitrate (NO_3^-) and nitrite (NO_2^-). To measure NOx, nitrate is typically first reduced to

nitrite. Because nitrate levels in plasma are about 100 times greater than nitrite, NOx mainly reflects plasma nitrate levels.

Source- Nitrate is an inert by-product of NO metabolism in the circulation. Its plasma level not only depends on NO production from various endogenous sources (eNOS, iNOS, etc.) but also on exogenous sources such as food and water.[52]

Nitrate is a normal component of the human diet and is principally derived from green leafy vegetables. It is absorbed rapidly and almost completely from the stomach and proximal small intestine into the plasma[53;54]. Approximately 25% of absorbed dietary nitrate undergoes enterosalivary circulation, recirculates in the blood, and is concentrated in saliva subsequently[55].

Approximately 20% of salivary nitrate (5-8% of nitrate intake) is reduced to nitrite by oral bacteria. In the acidic stomach, nitrite is further reduced to bioactive nitrogen oxides, including nitric oxide (NO), which increase with oral nitrate intake[55].

Increased dietary intake of nitrates therefore may have beneficial (e.g. antimicrobial activity[56]) as well as possibly deleterious (e.g. carcinogenesis by forming nitrosamines in the stomach[57]) effects on human health[58]. Classen et al. suggested that a nitrate-derived NO formation pathway is a possible mechanism for the hypotensive effect of vegetable- and fruit-rich diets[59].

Excretion- Most of the nitrate is eventually excreted in the urine. Wagner et al.[29] studied the metabolic fate of an oral dose of ^{15}N-labeled nitrate in humans over a period of 48 hours. An average of 60% of the $^{15}NO_3^-$ dose appeared in the urine as nitrate within 48 hours. Less than 0.1% appeared in the faeces. The ^{15}N label of nitrate

was also found in the urine (3%) and faeces (0.2%) in the form of ammonia or urea. The fate of the remaining 35% of the $^{15}NO_3^-$ dose administered remained unknown. The half-life of nitrate in the body was found to be approximately 5 hours, and its volume of distribution was about 30% of body weight. Daily endogenous biosynthesis of nitrate was estimated to be about 1 mmol/day.

Nitrate as an index of NO formation- Considering its diverse sources, nitrate is not a sensitive nor specific index of endothelial NO production.

Plasma nitrate can be used as an index of *in vivo* formation of NO provided that the oral intake of nitrate is restricted for at least 48 hours. Due to the large distribution volume and the low clearance of the ion, wide-spread, marked, and chronic changes in NO formation are required to significantly affect the levels of nitrate in blood[60]. For the same reason as well as the inert nature of nitrate, no arterial-venous gradient exists for circulating nitrate[61;62].

In disease states, such as heart failure, in which renal blood flow and extracellular volume are altered, caution should be exercised when plasma nitrate is evaluated as an index of NO formation[63].

Plasma nitrite

History- It has been known for many years that nitrites are active in biological systems and can relax smooth muscle cells. Brunton wrote in 1870[64]:

"It has been known for many years that the nitrites produce vasodilation . . . due to a direct action of the drug upon the vessel walls".

Vasodilator properties of nitrites were investigated further in early 20th century and high doses of sodium nitrite and amyl nitrite were found to dilate the coronary arteries[65;66]. Before the advent of modern and more effective antihypertensives, sodium nitrite was used in the treatment of hypertension. Today, sodium nitrite is used medically to treat cyanide poisoning.

Nitrite-mediated vasodilation is believed to occur through NO formation[67].

Physiology versus pharmacology- It is important to differentiate between physiological and pharmacological effects of nitrite. Physiological concentrations of plasma nitrite (<500nM[68]) do not induce vasodilation in bioassays[69] or in vivo[61;70]. Although Cosby et al. reported that infusion of physiological concentrations of sodium nitrite into the brachial artery in 10 human subjects increased forearm blood flow, the actual nitrite levels achieved in their study were 2564± 462nM; several folds higher than physiological concentration.

Source- Two major sources of nitrite in our body are oxidation of NO by oxygen; and reduction of nitrate by bacteria in the digestive system (see above). In addition, small amounts of body nitrite come from food, such as cured meat. Nitrite is primarily absorbed in the small intestine[53].

Biochemistry- The half-life of nitrite in human blood is about 110 seconds[71]. Plasma nitrite constantly enters the RBCs[72] where it reacts with oxyhaemoglobin to form nitrate and methaemoglobin (equation 1)[27]. The average elimination half life of nitrite following an oral dose of sodium nitrite was reported to be 30 minutes[73]. When nitrite

is added to human blood *ex vivo*, it is completely converted to nitrate within 10-12 minutes[74;75].

$$Hb\ (Fe\ II^{2+})\ O_2 + 2NO_2^- \rightarrow MetHb\ (Fe\ III^{3+}) + 2NO_3^- \qquad \textbf{(Equation 1)}$$

Excessive intake of nitrite may result in methaemoglobinaemia, especially in young infants whose methaemoglobin reductase system is not yet mature[58].

In low oxygen conditions- such as venous blood- some of the nitrite will react with deoxyhaemoglobin to form HbNO[28]. Under fully deoxygenated conditions, the product stoichiometry is 1:1 (methaemoglobin: HbNO)[76]. In vivo formation of HbNO following intravenous infusion of nitrite is inversely proportional to haemoglobin oxygen saturation[77]. Adding nitrite to blood, either *in vivo* or *ex-vivo*, also produces SNO-Hb in concentrations lower than HbNO[77;78].

Physiology- There has been an increasing interest in the physiological effects of the nitrite anion in recent years. Recent studies have shown that plasma nitrite reflects constitutive NOS activity[68] and may possibly be an index of regional endothelium-derived NO production[61;79]. In addition, nitrite has been proposed to be a potential circulatory pool of NO bioactivity with a possible role in regulating NO-dependent hypoxic vasodilation[67]. Consistent with this theory is the finding that hypoxia facilitates NO-dependent vasodilation by nitrite in vitro[80]. This theory tries to link reduced tissue O_2 with release of NO from NO stores.

It has been suggested that in low oxygen conditions deoxyhaemoglobin acts as a nitrite reductase to produce NO (equation 2)[28;81-83] through an allosterically controlled reaction between its haem moiety and nitrite[84] which in turn stimulates vasodilation[62;67].

$$Hb\ (Fe\ II^{2+}) + NO_2^- + H^+ \rightarrow MetHb\ (Fe\ III^{3+}) + NO + OH^- \qquad \textbf{(Equation 2)}$$

Since NO is immediately scavenged by haemoglobin (equation 3) to form nitrosylhaemoglobin (HbNO) ($k_3 \approx 4 \times 10^7\ M^{-1}/s^{-1}$)[76] it has been difficult to conceive a mechanism whereby nitrite-derived NO inside RBC can stay away from rapid inactivation reactions with haemoglobin and translocate to the extracellular domain subsequently.

$$Hb\ (FeII^{2+}) + NO \rightarrow Hb\ (FeII^{2+})\ NO \qquad \textbf{(Equation 3)}$$

Moreover, as recently discussed[69], given the rapid arterial to venous transit time of RBC across the physiological O_2 gradient, it is unlikely that plasma nitrite enters this cycle of events in arteries and proceeds via the above system in time to deliver and recycle plasma NO to arterioles in the same vascular bed.

It is entirely possible that nitrite (or the HbNO produced inside the RBC) produces SNO-Hb, for which a mechanism already exists to translocate the "SNO" across RBC membrane via an anion exchange protein.

Another possibility is that the vasoactive effect of nitrite is intrinsic to the smooth muscle cells, i.e. nitrite first enters the smooth muscle cells and then causes vasodilation. Dalsgaard et al.[80] who recently proposed the latter, whilst confirmed the NO-dependant nature of nitrite vasoactivity, showed that nitrite-dependent vasodilation is independent of known NO-generating nitrite reductase nominees in the field, namely haemoglobin, xanthine oxidase, eNOS and the bc1 complex of the mitochondria. This theory is further supported by earlier studies which showed endothelium (which may act as a barrier to nitrite) removal augments vasodilation to sodium nitrite in a rat aortic ring bioassay[85].

One possible explanation for the above findings is the intracellular reduction of nitrite to NO in acidic conditions. It is a well-known phenomenon that nitrite, when acidified, dilates vessels through the release of NO[86]. Intracellular pH of smooth muscle cells decreases significantly (≈pH 6.6[87]) during hypoxia/ischaemia and increased metabolic activity. In these acidic conditions, NO can be generated from non-enzymatic reduction of inorganic nitrite[86] to activate sGC and relax the smooth muscle cells. Of note is that nitrite levels in tissues (e.g. 10μM in rat aorta[88]) are generally much higher than in plasma and comparable to the levels which induce vasodilation in bioassays.

The same mechanism may apply to the ischaemic tissue, e.g. during myocardial ischaemia. NO derived from nitrite in the acidic environment of the ischaemic myocardium can diffuse out and into the coronary vascular smooth muscle cells to induce vasodilation. It is likely that the major bioactive pool of NO in the body is intracellular rather than circulatory nitrite.

Arterial-venous gradients- Gladwin et al.[62] reported significantly higher levels of nitrite in arterial blood compared to venous blood across the forearm circuit of 3 human subjects. They took this as an evidence for nitrite bioactivity (delivery or metabolism) in the peripheral circulation. Similar results were reported by Kelm et al.[79]. Nonetheless, higher arterial nitrite levels may simply reflect higher eNOS activity and NO production on the arterial side, or a greater consumption of nitrite in veins.

Plasma N-nitrosamines (RNNO)

It has been known for many years that RNNOs are generated endogenously at various sites of the body such as the stomach (as a result of the reaction of nitrite in an acidic environment with amino groups of food constituents) and chronically infected or inflamed organs[89].

Most low-molecular-weight RNNOs are potentially mutagenic and have traditionally been associated with an increased risk for cancer[90;91] , however, epidemiological evidence of the carcinogenic potential of nitrosamines in humans remains inconclusive[92].

Recently, Feelisch *et al* found that N-nitroso proteins are also present in the plasma of healthy subjects, suggesting that they may serve a physiological role such as a novel NO storage and/or delivery system.[92].

Further studies are required to elucidate the potential role of RNNOs in cardiovascular physiology.

Plasma S-nitrosothiols (RSNO)

Biochemistry- Nitrosation of thiols in proteins such as albumin and peptides such as glutathione results in long-lived S-nitrosothiols (RSNO) with a variety of different effects in biological systems. Incubation of plasma with increasing concentrations of NO results in a significant and concentration-dependent formation of nitrite and RSNOs[93]. Similarly, intravenous infusion of NO increases plasma levels of nitrite and RSNO[94]. As the reaction between NO and free thiols is very slow, it is believed that the production of RSNOs in above conditions is mainly due to oxidation of NO to N_2O_3 which is a strong nitrosating agent and reacts with thiols to yield RSNO and nitrite[95].

Once formed, circulating RSNOs can release NO[96] in a reaction catalysed by reducing agents such as thiols, transition metal ions, or direct light[97]. They can also transfer NO^+ to another thiol via so-called transnitrosation reactions[98]. In the absence of reducing agents, spontaneous decomposition of RSNO is rather slow[92].

Physiology- Biological activities of RSNOs are comparable to NO. Similar to NO, RSNOs possess strong vasodilator and antiplatelet properties. Intravenous administration of the low molecular weight nitrosothiol GSNO, increases brachial artery diameter and forearm blood flow and decreases systemic blood pressure in a dose-dependent manner[99]. RSNOs inhibit platelet aggregation by stimulating platelet-sGC[100-102]

The role of *S*-nitrosothiols- and mainly *S*- nitrosoalbumin (SNO-albumin) and *S*-nitrosohaemoglobin (SNO-Hb)- as stable long distance transporters of NO bioactivity in the circulation was first proposed by Stamler in 1992[103] and has been promoted by

him and his team ever since[104;105]. They first discovered the presence of a circulatory pool of SNO-albumin in plasma whose levels were coupled to NO synthase (NOS) activity and hypothesised that it may serve as a reservoir of NO bioactivity[103]. Next, they found *S*-nitrosoglutathione (GSNO) - a low molecular weight SNO- to be a more potent relaxant than SNO-albumin. GSNO –unlike NO-can retain its vasodilatory activity in the presence of haemoglobin[105;106]. In due course, the *S-nitrosohaemoglobin (SNO-Hb) Hypothesis* was developed (see below).

While the *S-nitrosohaemoglobin hypothesis* and the contribution of SNO-Hb to the "hypoxic vasodilation" phenomenon remains controversial (see below), there is substantial evidence for the role of RSNOs in conserving and transporting NO bioactivity[39;97;107;108].

S-nitrosohaemoglobin (SNO-Hb)

Biochemistry- SNO-Hb is formed by the S-nitrosation of Hb ß- chain cysteine 93[109;110].

Physiology- SNO-Hb, similar to other RSNOs, possesses antiplatelet[111] as well as vasodilator properties both in vitro and in vivo at lower oxygen saturations[112].

Structure of haemoglobin; R and T states- Haemoglobin is a tetrameric protein. Each haemoglobin molecule consists of 2 pairs of polypeptide chains (globin subunits), i.e. 4 chains in total. The major form of human adult haemoglobin, HbA_1, consists of two α and two β chains. Each chain contains a haem prosthetic group with an iron atom in its centre that can bind an oxygen molecule (O_2). Therefore, there are four O_2-binding sites in each haemoglobin molecule. Binding oxygen can only occur when the iron atom is in its ferrous (Fe^{2+}) state. Haemoglobin has a high affinity for

NO. Ferric (Fe^{3+}) haemoglobin, also known as methaemoglobin (metHb) is not functional and does not bind oxygen or NO. The deoxy conformation of haemoglobin is the "tense" or **T** conformational state. The oxy form with higher oxygen affinity is the "relaxed" or **R** conformational state. The equilibrium between these two states controls the delivery of O_2 and CO_2 to their appropriate sites.

S-nitrosohaemoglobin hypothesis- It has been postulated that haemoglobin may serve as a carrier and preserver of NO bioactivity in the circulation. It has been shown that NO can react with Hb in two rather different ways: binding to haem-Fe^{2+} in a process comparable to the binding of O_2; and forming an adduct with surface-exposed Cys^{93} β side chains.

According to the model described by Stamler[105;110;113]- also known as *S-nitrosohaemoglobin hypothesis*- NO is first captured by the Fe^{2+} at the haem and then transferred to the sulfhydryl (-SH)group of the β-chain cysteine-93 (βCys^{93}) residues to form SNO-Hb. The haem iron preferentially binds NO when in the T (deoxy-) conformation. The NO is transferred to βCys^{93} when Hb is in the R (oxy-) conformation. Then when R changes again to the T conformation to deliver O_2 to the tissues, NO is also released in an allosterically dependant manner[114] and transferred (via trans-nitrosation reactions) to the sulfhydryl groups of small sulfhydryl molecules (X-SH) such as glutathione to form X-S-NO. X-S-NOs, while having the same vasodilator properties as free NO, are resistant to scavenging by haemoglobin. Inside the RBCs, there is equilibrium between NO bound to the thiol of glutathione and reactive thiols (cysß93) of haemoglobin on the one hand, and NO bound to the thiols of haemoglobin and membrane-associated band3 protein (AE1), on the other hand. In

low oxygen tensions, NO is transferred from SNO-Hb in the cytosol to the membrane to form membrane-SNO which can induce vasodilation[105].

The net effect is the conversion of unstable free NO to relatively stable X-S-NO which is oxygen-sensitive and can release NO in low oxygen conditions to relax the vascular smooth muscle cells and dilate the arteries (hypoxic vasodilation).

Stamler and colleagues have reported several observations that support the *S-nitrosohaemoglobin hypothesis* that have yet to be countered convincingly by other groups. First and foremost, they demonstrated the very existence of S-nitrosohaemoglobin in the circulation[113] and that SNO-Hb can be produced *in vitro* when Fe(II)NO species are subjected to mild oxidation[115]. The same group reported a significant arterio-venous gradient of SNO-Hb suggesting its cyclic metabolism in the circulation[116;117].

Challenges to the S-nitrosohaemoglobin hypothesis-Many aspects of the *S-nitrosohaemoglobin hypothesis* have been questioned by other groups. Other laboratories have not been able to measure the micromolar concentrations and artery-to-vein gradient of SNO-Hb reported by the Stamler group[62;118;119]. (A previous paper from our laboratory reported increased levels of SNO-Hb with oxygenation across the pulmonary circulation in patients with congestive heart failure but not in healthy controls[120].) Nor have they been able to reproduce the observation of preferential binding of NO on the deoxyhaems of R-state (oxygenated) haemoglobin (i.e., allosterically controlled *association* kinetics) or even the oxygenation dependent transfer of the NO from the ß-chain haem to the cysteine 93 and the deoxygenation-dependent transfer of NO from the cysteine 93 back to the haem (i.e., cycling)[84;121].

Nitrosylhaemoglobin (HbNO)

Most of the NO which enters the erythrocytes will react with oxyhaemoglobin to form nitrate and methaemoglobin. Nonetheless, some NO will meet non-oxygenated haemoglobin and nitrosylate the Fe^{2+} to a fairly stable HbNO adduct [77;122](in vivo half life ~ 40 minutes[123;124]). HbNO is also produced from the reaction between the continuous flux of plasma nitrite into the erythrocytes and non-oxygenated haemoglobin[28]. Therefore, HbNO is a co-index of NO and nitrite uptake by erythrocytes at any given haemoglobin oxygen saturation.

HbNO is eventually oxidised by O_2 to methaemoglobin and nitrate in three steps as shown by Herold and Rock[125]:

Hb ($FeII^{2+}$) NO + O_2 ↔ Hb (Fe^{2+}) + NO + O_2 ↔ Hb ($FeII^{2+}$) O2 + NO → MetHb + NO_3^- **(Equation 4)**

Figure 1.6: Three-step degradation of HbNO to nitrate and metHb. (From Herold and Rock 2005[125])

HbNO is one of the many molecules proposed to serve as potential preservers of NO bioactivity in the circulation. By binding to haemoglobin, NO avoids degradation to nitrate. However, HbNO does not show vasodilator properties in vivo[122;126] nor is there any evidence that it can dissociate efficiently to deliver NO to tissues directly.

HbNO has a characteristic EPR (electron paramagnetic resonance) spectrum. EPR has been widely used to measure changes in HbNO levels following addition of NO or its metabolites to blood, in both *in vitro* and *in vivo* studies. However, because the level

of HbNO present in both arterial and venous samples at base line are below the detectable level by EPR (<0.5 μM), EPR studies have not been able to show any measurable A-V gradient in HbNO levels[121;123]. Our triiodide studies show higher HbNO levels in venous blood (see CHAPTER FIVE).

RBC nitrite

Plasma nitrite constantly enters the RBCs[72]. Inside the erythrocytes, nitrite can either react with oxyhaemoglobin to form methaemoglobin and nitrate (Equation 3); or with deoxygenated haemoglobin to form methaemoglobin and nitrosylhaemoglobin (HbNO)[76;78] (Equation 4):

$$HbO_2 + 2NO_2^- \rightarrow MetHb + 2NO_3^- \qquad \textbf{(Equation 5)}$$

$$4Hb + 2NO_2^- + 2H^+ \rightarrow 2MetHb + 2HbNO + 2H_2O \qquad \textbf{(Equation 6)}$$

Nitrite concentrations in RBCs were long thought to be negligible because of the rapid kinetics of the above reactions. However, recent studies by Kelm/Gladwin/Feelisch teams have shown substantial levels of intraerythrocytic nitrite (200–500nM)[68;127]. Virtually all nitrite was located in the cytosol as bound to proteins. They also found an artery-to-vein gradient of RBC nitrite across the human forearm circulation, consistent with their previous finding of artery-to vein-gradient in plasma.

Nitrated lipids (Nitrolipids)

Unsaturated fatty acids are nitrated endogenously to produce nitrated lipids[128]. Recent studies have shown that these nitrated lipids may directly or through transnitrosation reactions act as NO-releasing agents[129]. Nitrolipids relax rat aortic rings in a concentration-dependent manner while releasing nitric oxide[129]. Nitrolinoleate, a

synthetic nitrated lipid, has been shown to inhibit platelet aggregation, probably through a non-NO dependant mechanism[130].

Further studies are needed to elucidate the biological importance of nitrated lipids.

CHAPTER TWO

Coronary physiology, role of nitric oxide

The heart as a pump: anatomy and physiology

The main task of the heart is to force blood throughout the circulation, supplying oxygenated blood to tissues and draining deoxygenated blood from tissues for reoxygenation in the lungs[1].

Deoxygenated blood from all over the body is carried to the right atrium by the venous system. Three main veins enter the right atrium. The two larger veins are called superior and inferior vena cava and drain the upper and lower limbs, respectively. The third vein, coronary sinus, drains the heart muscle itself[131;132].

From the right atrium and through the tricuspid valve, blood enters the right ventricle where it is pumped up to the lungs during right ventricular systole. Pulmonary artery carries the deoxygenated blood from the right ventricle to the lungs[131;132].

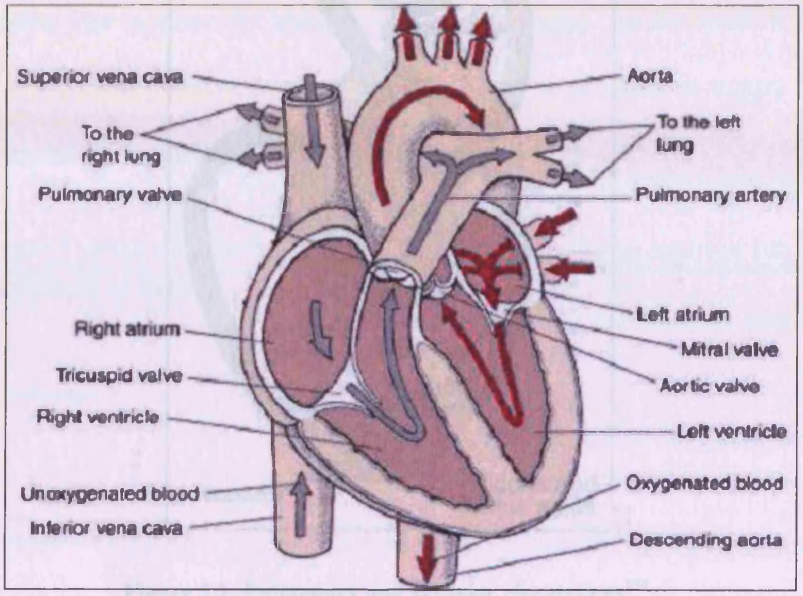


Figure 2.1: Anatomy of the heart and direction of normal blood flow[133].

After oxygenation in the lungs, blood flows into the left atrium of the heart via pulmonary veins. Oxygenated blood then enters the left ventricle (LV) through the mitral valve. During ventricular systole, blood is pumped into the aorta from where it travels throughout the circulation to reach all parts of the body[131;132] (Figure 2.1).

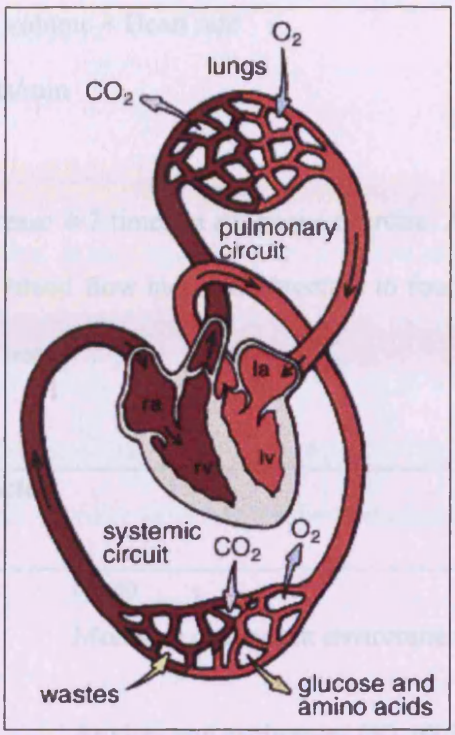


Figure 2.2: Pulmonary and systemic circulations[134].

As evident from Figure 2.2, normal circulation consists of two anatomically separate vascular beds or circuits: *pulmonary circulation* and *systemic circulation.* The right ventricle drives blood through the pulmonary circulation and the left ventricle drives it through the systemic circulation[131;132].

In a resting man of average size lying in the supine position, about 70ml of blood is pumped out of each ventricle at each heart beat. This is called the *stroke volume*. The output of the heart in one minute is called *cardiac output* and averages about 5L/min in a resting supine man[131;132]. Effects of various conditions on cardiac output are shown in table 2.1. Variations in cardiac output can be made by changes in heart rate or stroke volume:

Cardiac output= Stroke volume × Heart rate

5L/min= 70ml × 72beats/min

Cardiac output can increase 4-7 times in strenuous exercise. As the work of the heart increases, the coronary blood flow increases threefold to fourfold to supply the extra nutrients needed by the heart[135].

Condition or factor	
No change	Sleep
	Moderate changes in environmental temperature
Increase	Anxiety and excitement (50-100%)
	Eating (30%)
	Exercise (up to 700%)
	High environmental temperature
	Pregnancy
	Adrenaline
Decrease	Sitting or standing from lying position (20-30%)
	Rapid arrhythmias
	Heart disease
Approximate percent changes are shown in brackets.	

Table 2.1: Effects of various conditions on cardiac output [131].

Coronary circulation

Anatomy

The heart, unlike any other organ, not only provides flow to the entire organism but also has to generate its own perfusion pressure. This occurs through the coronary circulation. The main task of the coronary circulation is to match oxygen delivery to oxygen demand of the myocardium so that for any given oxygen need the heart will be supplied with a sufficient quantity to prevent underperfusion leading to ischaemia or infarction[1].

The left and right coronary arteries originate at the base of the aorta and supply blood to the myocardium. The left main coronary artery divides into left anterior descending (LAD) and circumflex arteries which supply blood to the left ventricular muscle. The right coronary artery supplies blood to the right ventricular muscle as well as- in 80-90% of people- the posterior wall of the left ventricle. Most of the cardiac venous blood from the left ventricle is drained to the right atrium via the coronary sinus (CS). Of coronary sinus outflow, 90-95% is derived from the left coronary artery[136]. Venous blood from the right ventricle is mostly drained directly into the right atrium via small anterior cardiac veins (figure 2.3). A small amount of venous drainage of the heart drains via Thebesian veins directly into the left atrium, and the right and left ventricles, so contributing to the physiological arteriovenous shunt.

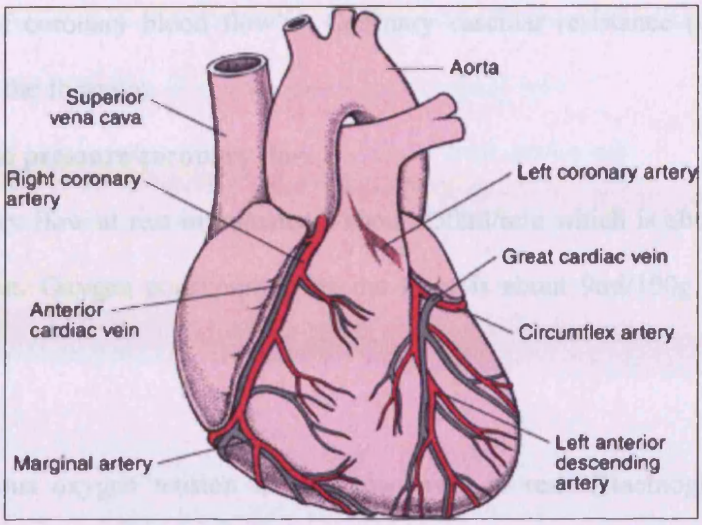


Figure 2.3: Blood supply of the heart. (Great cardiac vein=coronary sinus)[133]

Most of the coronary flow to the left ventricle occurs in diastole. This has two reasons. First and like any other muscle, contraction of the left ventricle during systole compresses the intramyocardial vessels and squeezes the blood out of the coronaries. Second, increased intraventricular pressure during systole stops subendocardial blood supply. Compressive forces are much smaller in the right ventricle; therefore right ventricular perfusion is reduced but not interrupted during systole[131;132].

Conductance versus resistance vessels

From a physiological point of view, there are two major types of arterial vessels in the coronary circulation: *conductance arteries* and *resistance arterioles*. *Conductance arteries* are the larger arteries that govern the quantity of blood arriving at the resistance vessels. Resistance arterioles are narrower (<150μm in diameter) and constitute the major resistance to flow. Their resistance increases by a power of four as their radius decreases (Poiseuille law). Resistance arterioles are the principal

controllers of coronary blood flow[137]. Coronary vascular resistance (CVR) can be estimated by the formula:

CVR= Aortic pressure/coronary flow

Total coronary flow at rest in humans is about 250ml/min which is about 5% of the cardiac output. Oxygen consumption by the heart is about 9ml/100g tissue/min at rest[1;135].

Cardiac venous oxygen tension is very low, even at rest. (Haemoglobin oxygen saturation in the coronary sinus is 25-30% at rest.) This is because the myocardium extracts 70-80% of the oxygen from each unit of blood delivered to it (oxygen extraction). Little additional oxygen can be extracted from the blood in the coronaries and therefore increases in oxygen demand/consumption can be satisfied only by increases in coronary blood flow. Indeed, an increase in myocardial metabolism is normally accompanied by increases in coronary blood flow. Increase in coronary blood flow is achieved by coronary vasodilation[1;135].

Determinants of myocardial oxygen demand/consumption

Myocardial oxygen demand/consumption is determined by three main factors[1;135]:

1. Myocardial contractility (inotropic state): Factors that increase myocardial contractility include adrenergic stimulation, digitalis, and other inotropic agents. At a molecular level, increased contractility is associated with either increased intracellular calcium or sensitisation of the contractile proteins to a given level of cytosolic calcium.
2. Myocardial wall tension: myocardial wall tension increases with increases in afterload (e.g. increased blood pressure, aortic stenosis), preload (i.e. increased

LV end-diastolic dimensions), and myocardial wall thickness (e.g. LV hypertrophy). This is in accordance with Laplace law:

Wall stress= Pressure× Radius/2(Wall thickness)

3. Heart rate: Heart rate is the most important determinant of myocardial oxygen demand/consumption. When heart rate doubles, myocardial oxygen demand approximately doubles (table 2.2).

Effects on MVO_2 of 50% Increase In
Wall stress, 25%
Contractility, 45%
Pressure work, 50%
Heart rate, 50%
Volume work, 4%

Table 2.2: The table demonstrates the dominant contribution to myocardial O_2 consumption (MVO_2) made by pressure work and prominent effects of increasing pressure work and heart rate on MVO_2. From Gould KL: Coronary Artery Stenosis. New York, Elsevier, 1991, p 8.[135]

Determinants of myocardial oxygen supply

Oxygen delivery to tissues involves a series of convective and diffusive processes. Convective oxygen transport refers to the bulk movement of oxygen in air or blood. Diffusive transport refers to the passive movement of oxygen down its concentration gradient across tissue barriers[138]. The two major factors influencing myocardial oxygen supply are oxygen carrying capacity and coronary blood flow[1;135]:

1. Oxygen carrying capacity: Satisfactory oxygen transport and delivery depends on:
    a. FiO_2: fraction of inspired oxygen, the percent concentration of oxygen in the gas entering the lungs.
    b. Lungs

c. RBCs
d. Haemoglobin

Hypoxia from pneumonia or carbon monoxide poisoning, anaemia, and haemoglobinopathies can alter oxygen delivery and cause ischaemia despite normal coronary flow.

2. Coronary blood flow: Human coronary arteries dilate in response to exercise or cardiac pacing to increase the blood flow and meet the elevated myocardial oxygen requirements[139;140]. Regulation of coronary circulation is discussed below.

Hypoxic vasodilation, active and reactive hyperaemia

Hypoxic vasodilation, active and reactive hyperaemia are all manifestations of the local "metabolic" blood flow regulation mechanism.

Hypoxic vasodilation- Hypoxic vasodilation is defined as local vasodilation in response to hypoxia. Local vasodilation leads to increase in blood flow. This is an acute physiological control mechanism that occurs within seconds to minutes to ensure adequate oxygen delivery and blood buffering capacity (including CO_2 elimination) to tissues under metabolic stress[141]. It is seen in myocardium, skeletal muscle, and many other tissues[132].

Hypoxic vasodilation is caused primarily by chemical factors acting directly on vascular smooth muscle cells to cause relaxation. The precise identity and mechanism of the oxygen sensor and mediators of vasodilation remain unknown. One of the most

important chemical factors is the tissue hypoxia itself. Hypoxia causes local arteriolar vasodilation both because the arteriolar walls cannot maintain contraction in the absence of oxygen and because oxygen deficiency causes release of vasodilator substances[132].

Other important vasodilator mechanisms are discussed below. Recently, circulatory NO metabolites, namely nitrite and S-nitrosohaemoglobin (SNO-Hb), have been proposed to play an important role in the hypoxic vasodilation phenomenon (see CHAPTER ONE).

Active hyperaemia- When any tissue becomes highly active, such as an exercising muscle, or the brain during rapid mental activity, the rate of blood flow through the tissue increases. This phenomenon is called active hyperaemia. Here, the increase in local metabolism, depletes the cells of oxygen and nutrients rapidly and causes them to release large quantities of vasodilator substances. The subsequent increase in local blood flow supplies the tissue with additional nutrients required to sustain its new level of function[132;142].

Reactive hyperaemia- When the blood supply to a tissue is blocked for a period of time and then is unblocked, blood flow through the tissue usually increases immediately. This phenomenon is called *reactive hyperaemia* and represents the local vasodilation which occurs in response to oxygen debt and accumulation of metabolic products due to interruption of blood flow[132;142].

Regulation of coronary blood flow

Coronary blood flow is regulated mostly by local vasodilation of resistance arterioles in response to cardiac muscle need for oxygen. Whenever myocardial oxygen demand is increased, regardless of cause, coronary blood flow also increases. Moreover, coronary blood flow increases almost in direct proportion to any additional metabolic consumption of oxygen by the heart. The exact means by which increased oxygen consumption causes coronary dilation has not been determined.

Four major interrelated mechanisms are thought to contribute to the regulation of coronary vascular tone:

1. Myogenic autoregulation
2. Neurohumoral control
3. Local metabolism
4. Endothelium-dependent factors

Impairment of these control mechanisms, e.g. in coronary atherosclerosis, may lead to myocardial ischaemia.

Myogenic autoregulation

In humans (and experimental animals) the heart has the ability to maintain coronary perfusion at relatively constant levels over a wide range of mean aortic blood pressure from 130mmHg to 40mmHg. This is due in part to the intrinsic vasoconstrictor response of smooth muscle to stretch. Vascular smooth muscle fibres contract when the blood vessels distend following a rise in intraluminal pressure. In contrast, a drop

in intraluminal pressure will relax the vascular smooth muscle fibres. This autoregulatory mechanism is called *myogenic control* and is present in other organs, most notably the kidneys. Its contribution to coronary circulation is however, small[143].

Neurohumoral control

The role of the autonomic system in neurogenic control of the coronary tone is summarised in table 2.3. The coronary arterioles contain both α- and β-adrenergic receptors, muscarinic receptors, and nonadrenergic noncholinergic receptors. Sympathetic innervation of coronary arteries is much more extensive than parasympathetic.

Innervation	Messenger	Receptor	Site and function
Adrenergic sympathetic	NE	α	Vasoconstrictive
		α_1	Larger conductance vessels
		α_2	Resistance vessels
	NE, E	β	Vasodilatory
		β_1	Larger vessels
		β_2	Resistance vessels
Cholinergic parasympathetic	Ach	Muscarinic	Vasodilation via NO (vasoconstriction when endothelium damaged)
Nonadrenergic noncholinergic nerves	CGRP	CGRP receptors	Modest vasodilation by opening K_{ATP} channel

NE: norepinephrine; E: epinephrine; Ach: acetylcholine; CGRP: calcitonin gene related peptide

Table 2.3: Summary of coronary neurogenic control (from Opie LH, Heart Physiology from Cell to Circulation, page 284, 4th edition, Lippincott Williams and Wilkins 2004.)

Physiologically, the net effect of sympathetic stimulation of the heart is coronary vasodilation. The cathecolamines norepinephrine and epinephrine mediate coronary vasodilation by β-adrenergic effects. In addition, their positive inotropic effect will

indirectly promote vasodilation by increasing the production of vasodilator metabolites in the myocardium. When the endothelium is dysfunctional and cannot generate nitric oxide, then overall vasoconstriction is more likely.

The direct effect of acetylcholine on healthy coronary arteries is vasodilation. However, stimulation of the parasympathetic (vagal) system decreases myocardial oxygen demand (by slowing down the heart and reducing contractility), and therefore indirectly constricts the coronary arteries.

Neural control of the coronary circulation is only complementary to other more important mechanisms. Denervation of the heart does not alter the increase in coronary blood flow in response to hypoxia[144;145].

Local metabolism

The close relationship between coronary blood flow and myocardial oxygen demand/consumption suggests that one or more products of metabolism contribute to coronary vasodilation. These include decreased oxygen and increased local concentrations of adenosine, adenine nucleotides, CO_2, H^+, lactate, and K^+ (table 2.4).

Factor	Mechanism of action
Adenosine	A_2 receptors, ↑cAMP
K_{ATP}	Hyperpolarisation, ↓intracellular calcium
$\downarrow O_2$	Opens K_{ATP}
$\uparrow H^+$	Direct effect on smooth muscle, sensitises the coronary arteries to adenosine
$\uparrow CO_2$	↑intracellular H^+
Lactate	?
K^+	Neurotransmitters, adrenergic receptors, ↑NO
ATP	Controversial. May not have a direct vasodilatory role[1].

Table 2.4: Metabolic vasodilators and their mechanism of action.

Two major mechanisms shown in experimental models to mediate ischaemic/hypoxic vasodilation in coronary arteries are[1]:

1. Breakdown of ATP to adenosine.
2. Activation of ATP sensitive potassium channels (K_{ATP})

Distal precapillary resistance arterioles are the main site of *metabolic* regulation of coronary blood flow[135]. Large conductance arteries are not affected directly by myocardial metabolites because of their extramural location.

Adenosine

Adenosine- a potent vasodilator- is believed to be the principal local mediator of metabolic vasodilation in coronary arteries in hypoxic/ischaemic conditions. Intracoronary infusion of adenosine is associated with a significant increase in

coronary blood flow and decrease in coronary vascular resistance[146]. In conditions of hypoxia or ischaemia, a large proportion of the myocyte's ATP degrades to AMP; then small proportions of this are converted to adenosine by the enzyme 5'-nucleotidase. Adenosine diffuses out of the myocyte into the interstitial space where it interacts with its vascular A_2 receptors to cause vasodilation and hence increase local coronary blood flow. Much of it is then reabsorbed into myocytes to be reused.

Three types of adenosine receptors have been identified:

1. A_1 or myocardial receptors: inhibit the formation of cyclic AMP and hyperpolarise nodal cells. This is how high doses of adenosine can arrest the heart.
2. A_2 or vascular receptors: are present on the vascular smooth muscle cells and exert vasodilation by stimulating the formation of cyclic AMP.
3. A_3 receptors: are present in the central nervous system. Their activation induces hypotension without affecting the heart rate[147].

Methylxanthines, including the bronchodilators aminophylline and theophylline, are nonselective adenosine receptor antagonists and can inhibit vasodilation caused by adenosine. This property of methylxanthines has been widely used in coronary flow studies (including my pacing study, see CHAPTER FIVE) to investigate the role and contribution of adenosine on coronary diameter and blood flow at rest and following increased myocardial oxygen demand.

Although the important role of adenosine in hypoxic or ischaemic conditions is widely accepted[148], it does not seem to play a dominant role in normal physiological conditions, at rest or during exercise, and other vasodilator mechanisms must be considered. Blocking adenosine receptors does not prevent augmentation of CBF velocity caused by increased myocardial activity during exercise or rapid atrial pacing, despite eliminating adenosine's contribution to the process[149;150].

ATP sensitive potassium channels (K_{ATP})[147;151]

K_{ATP} channels are widely distributed in many tissues and cell types including pancreatic islet cells and the coronary vascular smooth muscle cells. Their role is to couple the cell metabolic state to its membrane potential. K_{ATP} channels are normally inhibited (i.e. closed) by intracellular ATP and activated (i.e. opened) by MgADP[152].

In the coronary arteries, K_{ATP} channels contribute to the basal coronary tone by setting the membrane potential[153]. They are also believed to play an important role in the vasodilation response during myocardial ischaemia in human. When intracellular ATP/ADP ratio falls, for example during increased myocardial activity, hypoxia or local ischaemia; K_{ATP} channels open. This generates an outward current which hyperpolarises the cell membrane and leads to vasodilation. Inhibition of K_{ATP} channels by sulfonylureas such as tolbutamide and glibenclamide diminishes the vasodilation response induced by ischaemia and hypoxia[154;155].

In a recent study[153], Farouque et. al compared the effects of inhibition of K_{ATP} channels by intracoronary glibenclamide at rest and following rapid ventricular pacing (150 beats per minute) in human subjects with atherosclerotic coronary disease. At rest, glibenclamide reduced conduit coronary artery diameter by 4% but did not

significantly alter coronary blood flow velocity. Calculated coronary blood flow (CBF) showed a 9% decline; reflecting the changes in diameter. Following pacing, in addition to vasoconstriction, glibenclamide infusion also resulted in a trend to reduction of peak CBF velocity compared with 0.9% saline (42.8±4.4 versus 40.6±4.2 cm/second; P=0.12). Peak CBF was calculated to be 17% less during glibenclamide infusion compared with saline infusion.

Animal studies, however, suggest that K_{ATP} channels are not essential elements of the coronary vascular response to exercise in the normal porcine or canine heart [153;156;157].

Endothelium dependent factors

The endothelium is the largest organ in the body. It is strategically located between the circulating blood and the media and adventitia of the blood vessels. Endothelium dependent vasodilator factors include nitric oxide, prostacyclin, and EDHF (endothelium derived hyperpolarising factor). Endothelium also generates a powerful vasoconstrictor, the peptide endothelin-1, whose effects are more significant in diseased atherosclerotic arteries where endothelial damage is extensive[153;158].

Endothelial dysfunction is defined as an imbalance between relaxing and contracting factors, between procoagulant and anticoagulant mediators or between growth-inhibiting and growth-promoting substances[131;159].

Nitric oxide (NO)

The biochemistry of nitric oxide production and its role in vascular physiology has been discussed in CHAPTER ONE. NO is released from endothelial cells in response to shear stress[160].

In addition, there are a number of pharmacologic agents including acetylcholine, histamine (via H_1 receptors), bradykinin, VIP (vasoactive intestinal peptide), substance P, and some other polypeptides that exert their vasodilator properties via stimulation of endothelial NO release[131]. Released NO diffuses into vascular smooth muscle cells where it induces relaxation by activating the enzyme soluble guanylate cyclase, increasing intracellular cGMP.

NO formation can be pharmacologically inhibited by the administration of L-arginine analogues such as N^G-nitro-L-arginine (LNNA), nitro-L-arginine (NLA), nitro-L-arginine methyl ester (L-NAME), and N^G-monomethyl-L-arginine (L-NMMA). These compounds compete with the natural precursor L-arginine at the catalytic site of NOS[136].

Contribution of NO to coronary flow regulation has been studied in human, porcine, and canine models, in vivo. These studies have been reviewed at the end of this chapter. The potential role of circulatory metabolites of NO in the regulation of CBF had not been studied before and was the focus of this thesis.

When endothelial dysfunction occurs, the normal vasodilation-inducing response of endothelium to shear stress and other factors diminishes. This reduced response is attributed to reduced nitric oxide generation, increased activity of endothelin-1, oxidative excess and reduced production of hyperpolarising factor. Additionally, endothelial dysfunction initiates a proinflammatory prothrombotic state. Most forms of cardiovascular risk factors and diseases- e.g. hypertension, coronary artery disease,

chronic heart failure, peripheral artery disease, diabetes, and chronic renal failure- are associated with endothelial dysfunction.

Prostacyclin (PGI_2)

Prostacyclin (PGI_2) is produced by endothelial cells from its precursor arachidonic acid via the cyclo-oxygenase pathway[131]. It is released from the endothelium in response to shear stress, pulsatile flow, hypoxia, and several other substances that also release NO such as ADP, ATP, serotonin, and thrombin[136]. PGI_2 inhibits platelet aggregation and promotes vasodilation. In most blood vessels, however, its platelet inhibitory effects are probably more important than its vasodilatory ones[136].

In healthy men with no coronary disease, oral ibuprofen (to inhibit PGI_2 synthesis) had no effect on basal coronary tone or the coronary response to exercise[149]. In a slightly different group of patients with either coronary disease or risk factors for coronary disease, intracoronary aspirin (another inhibitor of cyclo-oxygenase) reduced resting epicardial coronary artery diameter and CBF and attenuated ventricular pacing-induced hyperaemia[161].

Endothelium derived hyperpolarising factor (EDHF)

EDHF is another distinct endothelial pathway involved in vasorelaxation and is likely to play an important role in cardiovascular physiology[162]. Similar to NO and PGI_2, EDHF is released from the endothelial cells in response to agonists (e.g. acetylcholine and bradykinin) and fluid shear stress[163]. The endothelial hyperpolarisation that initiates relaxation results from the opening of two populations of endothelial potassium channels, the small conductance and intermediate conductance calcium-activated potassium channels (SK(Ca) and IK(Ca), respectively)[162;164].

Hyperpolarisation of the smooth muscle membrane closes the voltage dependent calcium channels. The subsequent reduction in the influx of extracellular calcium ions (that normally sustains contraction) leads to vasorelaxation[163].

The chemical nature of EDHF is still a matter of debate and different substances have been identified to act as an EDHF in different vascular beds, e.g. epoxyeicosanoids, potassium ions, anandamide, hydrogen peroxide or C-type natriuretic peptide[165]. Despite this heterogeneity of proposed factors it is unclear if such a factor indeed exists in all vessels. An alternative explanation for the EDHF phenomenon is that direct intercellular communication via gap junctions allows passive spread of agonist-induced endothelial hyperpolarisation through the vessel wall[163].

Unlike nitric oxide which is dominant in larger conduit arteries, EDHF appears to be far more important in small arterioles[166].

NO inhibits the production of EDHF[167]. Therefore a decrease in NO bioavailability may result in the upregulation of EDHF.

Segmental distribution of regulatory mechanisms

Metabolic stimuli that increase CBF by relaxing resistance arterioles, increase shear in the upstream conduit vessels, and produce "flow mediated" vasodilation. It has been shown that neurogenic, metabolic, myogenic, and shear stress–induced mechanisms dominate resistance at specific microvascular sites[137;168]. For example in pigs, metabolic vasodilation occurs predominantly in the smallest arterioles (<30μm), whereas intermediate arterioles (30 to 60μm) are the principal site of myogenic

regulation. The large arterioles (100 to 150μm) appear to be the sites of flow-mediated dilation.

During metabolic stress and in response to local metabolic factors such as adenosine, the smallest arterioles dilate first, resulting in reduced microvascular resistance and increased CBF. As the upstream arteriolar pressure decreases, myogenic dilation of slightly larger arterioles upstream occurs and causes an additional decrease in resistance. Increased flow in the largest arterioles augments shear stress and triggers flow-mediated dilation, further reducing the resistance of this network[135].

Role of NO in the coronary circulation; in vivo studies

In this section, the main in vivo studies which investigated the role of NO in the regulation of coronary flow are reviewed. These studies were conducted in dog, pig, or human subjects. When comparing results of different studies, it is important to realise that significant species difference exists in regard to the coronary circulation. For example porcine hearts-like human hearts- have a dominant right coronary system, whereas the canine heart has a dominant left coronary system; or the dog has a relatively well developed collateral circulation whereas pigs (similar to humans without coronary artery disease) have practically no anatomically demonstrable collaterals[136].

While there are considerable differences between human and canine coronary structure, porcine heart shows a close similarity to human heart and the pig has been well characterised as an appropriate model for the study of coronary physiology, the coronary collateral circulation and exercise physiology[136;169-171].

Canine studies

In a series of experiments on chronically instrumented dogs, Bache and colleagues[172-175] (1993-1998) studied the significance of K^+_{ATP} channels, adenosine, and nitric oxide in regulating CBF under resting conditions and during increments in myocardial metabolic demand produced by treadmill exercise. Infusions of glibenclamide, 8-phenyltheophylline (8-PT), and N^G-nitro-L-arginine (LNNA) were used to inhibit K^+_{ATP} channels, adenosine receptors, and NO synthase respectively. During control conditions, CBF was 49±3 ml/min at rest and increased to 92±8 ml/min at peak exercise. The blockade of K^+_{ATP} channels decreased CBF at rest (from 51 +/- 4 to 42 +/- 6 ml/min) but did not impair the increases in CBF that occurred during exercise (although CBF was lower at each level of exercise per se). Combined K^+_{ATP} channel and adenosine blockade decreased resting coronary flow to 27±3 ml/min and attenuated the increase in coronary flow produced by exercise. However, exercise still increased flow to 45±5 ml/min. Consistent with findings by Bernstein et al. (see below), the NO synthase inhibitor LNNA, alone or in combination with 8-PT did not alter resting coronary flow and did not impair the normal increase in flow during exercise. Simultaneous blockade of all three mechanisms further decreased resting coronary flow to 20±2 ml/min and markedly blunted exercise-induced coronary vasodilation so that coronary flow both at rest and during exercise was below the control resting level.

These studies showed that in dogs:

1. K^+_{ATP} channels are critical for maintaining coronary vasodilation at rest and during exercise. When K^+_{ATP} channels are intact, neither NO nor adenosine-dependent mechanisms are obligatory for maintaining coronary blood flow.
2. When K^+_{ATP} channels are blocked, both adenosine and NO act to increase coronary blood flow during exercise.
3. The residual increase in coronary flow in response to exercise after adenosine receptor and K^+_{ATP} channel blockade is dependent on endogenous NO.
4. In the presence of combined K^+_{ATP} channel blockade and adenosine receptor blockade, NO is able to produce approximately one quarter of the coronary vasodilation that occurs in response to exercise when all vasodilator systems are intact.

In a complementary experiment[176], they measured plasma NOx in aortic and coronary sinus blood samples to compare coronary NO production in response to treadmill exercise with intracoronary administration of the endothelium-dependent agonists acetylcholine and bradykinin. A three-stage progressive exercise protocol was employed. No coronary NOx production could be detected at rest or during the first 2 stages of exercise; only at the highest level of exercise was a small increase in coronary NO_x production measured. In contrast, coronary production of NO_x was significantly increased in response to endothelium-dependent agonists. This study confirmed that coronary NO production in response to endothelium-dependent agonists is greater than in response to the increase in shear stress associated with exercise. It also supported previous evidence suggesting that NO is not essential to exercise-induced coronary vasodilation.

Bernstein et al.[177] (1996) studied the changes in NO production from the coronary circulation in conscious dogs during exercise. Plasma NOx from aortic and CS blood was measured with the dog standing at rest on the treadmill and at three successively increasing exercise speeds. After acute exercise was performed and blood samples were taken, nitro-L-arginine (NLA) was given to block NO synthesis and the exercise was repeated. Acute exercise caused significant elevations in NOx production by the coronary circulation. After NLA, there was no measurable NOx production at rest or during exercise. There was no significant change in CBF response to exercise after the blockade of NO synthesis. Blockade of NO synthesis resulted in elevations in myocardial oxygen consumption.

In a similar study to Bache et al., Tune et al.[156] (2001) examined the effect of combined inhibition of K^+_{ATP} channels (glibenclamide), nitric oxide synthesis (LNNA), and adenosine receptors (8-PT) in coronary exercise hyperaemia of 10 chronically instrumented dogs. During control exercise, myocardial oxygen consumption increased ~2.9-fold, CBF increased ~2.6-fold, and coronary venous oxygen tension decreased from 19.9 ± 0.4 to 13.7 ± 0.6 mmHg. In contrast to Bache's studies, triple blockade did not significantly change the myocardial oxygen consumption or CBF response during exercise. Triple blockade lowered the resting coronary venous oxygen tension to 10.0 ± 0.4 mmHg and during exercise to 6.2 ± 0.5 mmHg. Triple blockade increased coronary venous adenosine concentrations during exercise, but the adenosine levels did not increase sufficiently to overcome the adenosine receptor blockade.

Matsunaga et al.[178] (1996) studied the effects of NO synthesis inhibition on myocardial metabolism in pentobarbital sodium-anaesthetised dogs at baseline and during atrial pacing. NG-nitro-L-arginine methyl ester (L-NAME) infusion was used to inhibit NO synthase. CBF and PO_2 in the anterior interventricular vein at baseline were both significantly decreased by L-NAME. CBF was increased during pacing, which was not affected by L-NAME. Myocardial adenosine release remained unchanged during pacing before L-NAME, but it was significantly increased after L-NAME infusion. The experiment was repeated in dogs pretreated with the adenosine receptor blocker 8-PT. Combined blockade of NO and adenosine suppressed pacing-induced increase in CBF.

Unlike Bernstein et al., Sherman et al.[179] (1997) showed that blockade of NO synthesis with either systemic or intracoronary L-NAME decreased myocardial oxygen consumption in dogs in vivo.

Minamino et al.[180] (1997) studied the effect of NO synthesis inhibition on adenosine production in canine coronary arteries. The intracoronary administration of L-NAME for 30 minutes increased adenosine levels in coronary venous blood.

Summary- in dogs:

1. NO is not essential to CBF increase in response to exercise.
2. Exercise increases coronary NO production.
3. Loss of NO can be compensated for by increased participation of other vasodilator mechanisms.

Porcine studies

Mercus et al.[181](2004) investigated the integrated contribution of K_{ATP} channels, adenosine, and NO to the regulation of CBF in chronically instrumented swine at rest and during exercise. Swine exercised on a treadmill (0–5 km/h), during control and after blockade of K_{ATP} channels (with glibenclamide), adenosine receptors (with 8-PT), and/or NOS (with L-NNA). L-NNA, 8-PT, and glibenclamide each caused coronary vasoconstriction and reduced myocardial O_2 delivery and coronary venous O_2 tension at rest and during exercise. These effects were not modified by simultaneous blockade of the other vasodilators. Thus in swine, loss of K_{ATP} channels, adenosine, or NO was not compensated for by increased participation of the other two vasodilator mechanisms. These findings suggest a parallel (additive) rather than a redundancy (backup) design of CBF regulation in the porcine circulation. Combined blockade of the above 3 vasodilator mechanisms increased myocardial oxygen extraction to >90%. Although CBF was significantly impaired by triple blockade, it still doubled in response to exercise. This indicates that other mechanisms are also in place to mediate metabolic vasodilation.

Human studies

Lefroy et al.[182](1993) studied the effect of inhibition of NO synthesis (by L-NMMA) on epicardial coronary artery calibre and CBF in humans. L-NMMA caused a significant reduction in basal distal (but not proximal) LAD diameter and basal CBF. Coronary venous oxygen saturation dropped from 37.5 +/- 2.8% to 34.3 +/- 2.8% (P = 0.019).

Quyyumi et al.[183] (1995) studied the effect of inhibiting nitric oxide synthesis with N^G-monomethyl-L-arginine (L-NMMA) on the coronary vasodilation during rapid atrial pacing (mean heart rate 141 beats per minute) in patients with angiographically normal coronary arteries with and without multiple risk factors for coronary atherosclerosis (hypertension, hypercholestrolaemia, diabetes). Endothelium-dependent vasodilation was estimated with intracoronary acetylcholine and endothelium-independent dilation with intracoronary sodium nitroprusside and adenosine. All the measurements were repeated after intracoronary infusion of L-NMMA. During the control study, cardiac pacing at 141±11 bpm produced a mean 50% increase in blood flow, 21% reduction in coronary vascular resistance, and a 9% increase in proximal and distal coronary artery diameters. At rest, L-NMMA produced a 16±25% (mean±SD) increase in coronary vascular resistance and an 11% reduction in distal epicardial coronary artery diameter, indicating tonic basal release of nitric oxide from human coronary epicardial vessels and microvessels. L-NMMA depressed pacing-induced decrease in coronary vascular resistance and eliminated pacing-induced vasodilation in the epicardial coronary arteries. Epicardial coronary dilation during control pacing (9±13%) was converted to constriction after L-NMMA and pacing (-6±9%). L-NMMA specifically inhibited endothelium-dependent vasodilation with acetylcholine but did not alter endothelium-independent dilation with sodium nitroprusside and adenosine. Patients with one or more cardiac risk factors had depressed microvascular vasodilation during cardiac pacing. Moreover, the inhibitory effect of L-NMMA on pacing-induced coronary epicardial and microvascular vasodilation was observed only in patients without risk factors, whereas those with risk factors had an insignificant change, indicating that nitric oxide contributes significantly to pacing-induced coronary vasodilation in patients free of risk factors

and without endothelial dysfunction. Patients with risk factors also had reduced vasodilation with acetylcholine but the responses to sodium nitroprusside were similar in both groups.

The authors concluded that:

1. Persistent (although lower) coronary microvascular dilation with cardiac pacing after administration of L-NMMA, as evident by the decrease in coronary vascular resistance, confirms the presence of non-NO related mechanisms that contribute to metabolic vasodilation of the coronary microvasculature in humans.
2. Failure of the epicardial coronary arteries to dilate in response to pacing after L-NMMA suggests that coronary epicardial vasodilation during metabolic stimulation of the human heart is likely to be mediated entirely by the endothelium-derived release of nitric oxide.

Egashira et al.[184] (1996) studied the role of NO in coronary vasodilation induced by rapid atrial pacing in patients without significant coronary artery disease who were undergoing coronary angiography for evaluation of chest pain. 80% of patients had at least one cardiovascular risk factor. An increase in the heart rate (120-130bpm) increased CBF and the coronary artery diameter. L-NMMA reduced basal CBF but did not significantly affect basal coronary artery diameter, arterial pressure, or heart rate. L-NMMA markedly attenuated the pacing-induced dilatation of the large epicardial coronary artery, whereas it did not affect pacing-induced increase in CBF. These findings suggest that:

1. Dilatation of the large epicardial coronary arteries in response to pacing is mediated by NO.
2. NO may not significantly contribute to the dilatation of resistance arterioles and consequently the overall increase in CBF in response to rapid atrial pacing. Alternatively, there may be other vasodilatory systems in place in resistance arterioles which can compensate for the effects of NO inhibition by increasing their metabolic signals.

Duffy et al.[161] (1999) assessed the contribution of endothelium-derived nitric oxide and vasodilator prostanoids to resting blood flow, metabolic vasodilation, and flow reserve in the human coronary circulation. 25 patients scheduled for percutaneous intervention or diagnostic studies for investigation of chest pain were recruited. Angiographically smooth or mildly irregular coronary arteries were studied. In single-vessel disease, the study was performed in an adjacent vessel after the percutaneous intervention. Metabolic vasodilation was induced by 2 minutes of ventricular pacing (150 beats per minute). Coronary haemodynamics were assessed before and after inhibition of vasodilator prostanoids and NO with intracoronary aspirin and L-NMMA, respectively. Aspirin reduced resting conduit vessel diameter by 11% and CBF by 27% and increased coronary vascular resistance (CVR) by 24%. Pacing increased the coronary artery diameter from 2.36±0.1 to 2.54±0.2 mm, but this increase was abolished by aspirin. Pacing increased CBF by 78% during vehicle infusion, but aspirin attenuated the pacing-induced hyperaemia to a 42% increase compared with before aspirin. Pacing reduced CVR by 40% during vehicle infusion. With ASA, CVR decreased with pacing by 27%. L-NMMA reduced resting conduit vessel diameter by 9% and CBF by 20% and increased CVR by 19%. Pacing increased coronary artery diameter (from 2.32±0.1 to 2.60±0.1 mm), but this increase

was abolished by L-NMMA. Pacing increased CBF by 60% during vehicle infusion. With L-NMMA, pacing increased CBF to a similar extent (by 60%) although maximum pacing-induced hyperaemia was 20% less with L-NMMA. Thus, although the percent increase in CBF was similar after L-NMMA, the maximum CBF achieved was less. Pacing reduced CVR by 39% before and 32% after L-NMMA infusion. Thus, minimum CVR after pacing was greater with L-NMMA.

They concluded that tonic release of vasodilator prostanoids and NO contributes to resting conduit and resistance vessel tone and to peak functional hyperaemia and flow-mediated dilation after metabolic stimulation.

Summary- in humans:

1. There is a tonic basal release of NO from the coronary epicardial vessels.
2. Pacing-induced vasodilation in epicardial coronary arteries is nitric oxide dependent.
3. Microvascular vasodilation in response to pacing may not be exclusively dependent on nitric oxide.
4. Contribution of nitric oxide to coronary vasodilation is reduced in patients with coronary artery disease or cardiovascular risk factors and leads to a net reduction in vasodilation during stress.

CHAPTER THREE

Diabetes mellitus type 1, microvascular complications, and nitric oxide metabolism

General

Definition

Diabetes mellitus is a group of metabolic diseases characterised by chronic hyperglycaemia due to defects in insulin secretion, insulin action or both[185]. In 2000, there were an estimated 171 million cases in the world, and this number is projected to increase to 366 million by 2030[186]. Symptoms include excessive excretion of urine (polyuria), thirst (polydipsia), constant hunger, weight loss, vision changes and fatigue.

Classification

Diabetes mellitus is commonly classified as type 1 and type 2. There are also other less common types of diabetes, e.g. gestational diabetes.

Type 1 diabetes represents about 10% of cases of diabetes mellitus around the world[187] and is due to insulin deficiency caused by T-cell mediated autoimmune destruction of the B cells in the pancreatic islets of Langerhans[131]. Type 1 diabetes usually develops before the age of 40, with a peak incidence between 10 and 14 years of age[188].

Type 1 diabetes is further subclassified based on the presence (type 1a, 90% of cases in Europe) or absence (type 1b) of serological evidence of autoimmunity. Both subtypes have similar clinical presentations[188].

Latent (or late-onset) autoimmune diabetes in adults (LADA) is a form of type1a which is commonly misdiagnosed as type 2. Patients are generally older at presentation (>40) and may pass several years before becoming insulin-dependant. This form of diabetes should particularly be suspected in patients with no signs of metabolic syndrome[188].

In contrast to type 1, type 2 diabetes has more complex and less well-understood aetiology. It is characterised by insulin resistance and impaired B cell insulin secretion[131]; which are believed to be the result of an interaction between various environmental factors and multiple diabetogenic genes[189]. Moreover, type 2 diabetes is frequently part of a wider collection of metabolic disorders (dyslipidaemia, truncal

obesity, hypertension), i.e. the "metabolic syndrome" or "syndrome X"[189;190]. These high risk conditions alter endothelial function and NO bioavailability independently and can complicate the interpretation of NO studies in diabetes.

To avoid the confounding factors frequently associated with type 2 diabetes, the focus of this thesis has been on patients with type 1 diabetes with no other cardiovascular risk factors.

Tissue damage in diabetes

Both types of diabetes are associated with long term tissue damage which can be categorised into macro- and microvascular complications (Table 3.1). The focus of this thesis is on the micro- rather than macrovascular complications of type 1 diabetes.

Hyperglycaemia (glucose toxicity) plays a pivotal role in the pathogenesis of macro- and microvascular complications of diabetes and there is good evidence that intensive glycaemic control reduces the risk of developing microvascular complications significantly[191].

Certain types of cells are more vulnerable to the toxic effects of hyperglycaemia. These include capillary endothelial cells in the retina, mesangial cells in the renal glomerulus, and neurons and Schwann cells in peripheral nerves[192]. In these cells, glucose uptake is regulated by GLUT proteins other than GLUT-4[193] and is therefore independent of insulin. Unlike most cells in our body, these cells are unable to down-regulate the transport of glucose inside the cell when they are exposed to hyperglycaemia[194;195]. This leads to highly "toxic" glucose levels inside the cell which can trigger several detrimental biochemical pathways.

Microvascular complications
Retinopathy, cataract
Nephropathy
Neuropathy
Macrovascular complications
Coronary heart disease
Cerebrovascular disease
Peripheral vascular disease

Table 3.1Vascular complications of diabetes mellitus.

Miscellaneous factors

There are other modifying factors which can either accelerate or decelerate the process of hyperglycaemia-induced tissue damage in diabetics. These include genetic factors, duration of disease, smoking, blood pressure, lipid profile, and von Willebrand factor levels.

Genetic factors play a role in determining individual susceptibility to tissue damage[192].

The frequency of microvascular complications of diabetes increases as the duration of disease increases. In the EURODIAB[196] study, the prevalence of microvascular disease was 25% in subjects with short duration of disease (≤5 years) and 82% in the long duration (≥14 years) group.

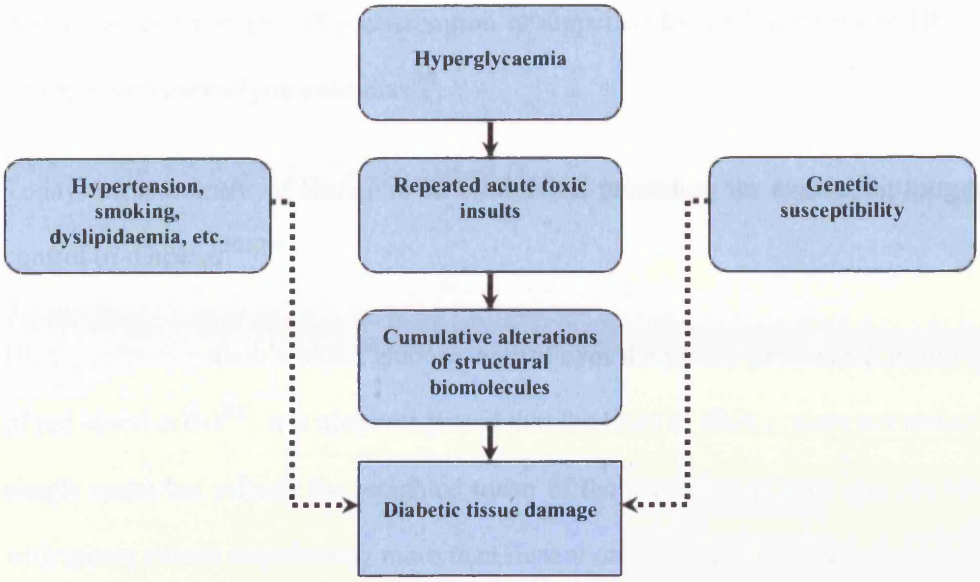


Figure 3.1: General features of hyperglycaemia-induced tissue damage in diabetes

Several factors can expedite or hinder the development of microvascular complications. In the same study and in the short duration group, factors associated with early development of complications were cigarette smoking and a family history of hypertension. Subjects free of microvascular complications in spite of long duration of diabetes had better glycaemic control, lower blood pressure, better lipid profile and lower von Willebrand factor levels.

HbA_{1C}: A measure of long-term glycaemic control

HbA_{1C} is a minor component of adult haemoglobin (Hb A) which forms from the non-enzymatic attachment of glucose to the amino-terminal valine of the β chain[197]. It was first identified by Rahbar in late 1960s as a minor "abnormal fast-moving haemoglobin band" in diabetic patients[198]. The conversion of Hb A to HbA_{1C} occurs continuously throughout the 120-day life-span of normal erythrocytes and is a slow

post-translational event. This observation is supported by the low levels of HbA_{1C} in patients with haemolytic anaemias[199].

Today, measurement of HbA_{1C} is an established procedure for evaluating long-term control of diabetes[200;201].

HbA_{1C} reflects patient's blood glucose control over the past 8-12 weeks (i.e. life-span of red blood cells)[202]. It is also recognised that the level of HbA_{1C}, does not reflect the simple mean but reflects the weighted mean of the preceding plasma glucose levels, with recent events contributing more than distant ones[203]. The blood glucose levels in the one month before sampling contribute to about 50% of the HbA_{1C} value, whereas 3-4 months previously contributes only about 10%[202].

The reference range for HbA_{1C} depends on the method and laboratory. In 2002, the National Institute for Health and Clinical Excellence recommended that all HbA_{1C} assays should be aligned with the assay used in the Diabetes Complications and Control Trial (DCCT). Using a DCCT-aligned HPLC-based fully automated glycohaemoglobin analyser (Tosoh Bioscience), normal HbA_{1C} levels in our affiliated hospital (University Hospital of Wales) ranges between 4%-6% of total haemoglobin. In diabetics it is usually above 7%.

Research value

Since its discovery, the magnitude of HbA_{1C} percentage has been widely studied in epidemiologic studies and found to be an independent risk factor for the development of microangiopathy[191] and cardiovascular mortality and morbidity[204;205], even in non-diabetics[204;206;207]. Some of these studies are mentioned below.

The Diabetes Control and Complications Trial (DCCT) in 1441 patients with insulin dependent diabetes mellitus showed that reduction of HbA_{1C} from 9% to about 7% significantly reduced the development and/or progression of microvascular complications of diabetes[191].

The Norfolk cohort of European Prospective Investigation of Cancer and Nutrition (EPIC-Norfolk)[204] reported on 4662 men aged 45-79, followed for 6 years. HbA_{1c} was continuously related to subsequent all cause, cardiovascular, and ischaemic heart disease mortality through the whole population distribution, with lowest rates in those with HbA_{1c} concentrations below 5%. An increase of 1% in HbA_{1c} was associated with a 28% ($P<0.002$) increase in risk of death independent of age, blood pressure, serum cholesterol, body mass index, and cigarette smoking habit.

The Rancho-Bernardo study[206], a community-based study of 1,239 nondiabetic older adults followed for an average of 8 years, showed that glycated haemoglobin was significantly related to cardiovascular disease (CVD) and ischaemic heart disease (IHD) mortality in women but not men. The age-adjusted relative hazard for those in the highest quintile of glycated haemoglobin ($\geq$ 6.7%) compared with women with lower levels was 2.37 for fatal CVD and 2.43 for IHD.

The EURODIAB study in type 1 diabetics across Europe recognised HbA_{1C} as an independent risk factor for retinopathy[208] and nephropathy[209].

Postprandial glucose excursions- Increasing evidence suggests that excessive excursions of postprandial glucose might be important for the development of micro- and macroangiopathic complications of diabetes. Postprandial hyperglycaemia has been reported to be at least as important as fasting hyperglycaemia in relation to death

from cardiovascular and coronary heart disease[210] and the development of retinopathy and nephropathy[211]. One study showed that postchallenge glucose was more strongly associated with carotid intima-media thickness (as an index of atherosclerosis) than fasting glucose or HbA_{1C} level in non-diabetic subjects who were at risk of type 2 diabetes[212]. There is ongoing research on how to best control postprandial hyperglycaemia.

Nonetheless, postprandial glucose is a single measurement and unlike HbA_{1C} does not reflect glycaemic control over a substantial period of time.

It is also worth mentioning that the evidence for the significance of postprandial glucose comes from studies on patients with type 2 diabetes. It is not known if the conclusions can be extended to type 1 diabetes.

Pathophysiology of microvascular complications in type 1 diabetes

Despite the established link between chronic hyperglycaemia and long term microvascular complications of diabetes, the biochemical mechanism(s) of this association is not well understood.

Hyperglycaemia can affect many cellular pathways. The main challenge is to identify those pathways that are involved in causing vascular dysfunction. The importance of the elucidation of these mechanisms is supported by the fact that maintaining normoglycaemia at all times is almost impossible throughout the life of diabetic patients[213;214]. Moreover, restoration of normoglycaemia does not always stop the progression of established disease[215]. It is therefore vital to develop therapeutic agents

which can inhibit the intermediate biochemical pathways between hyperglycaemia and microvascular complications. Four such biochemical pathways have been discovered and studied so far:

1. Increased polyol (sorbitol) pathway flux
2. Increased formation of advanced glycation end products (AGEs)
3. Increased protein kinase C (PKC) activation
4. Increased hexosamine (glucosamine) pathway flux

Hyperglycaemia-induced overproduction of superoxide by the mitochondrial electron transport chain has been proposed to be a unifying mechanism linking all the above elements to each other[192].

Increased polyol (sorbitol) pathway flux

The polyol pathway of glucose metabolism is normally relatively inactive[193] and most of the intracellular glucose is phosphorylated to glucose 6-phosphate by hexokinase. It is estimated that under normoglycaemic conditions, polyol pathway accounts for approximately 3% of glucose utilisation in erythrocytes[216]. This ratio can increase to about one third of the total glucose turnover under hyperglycaemic conditions.

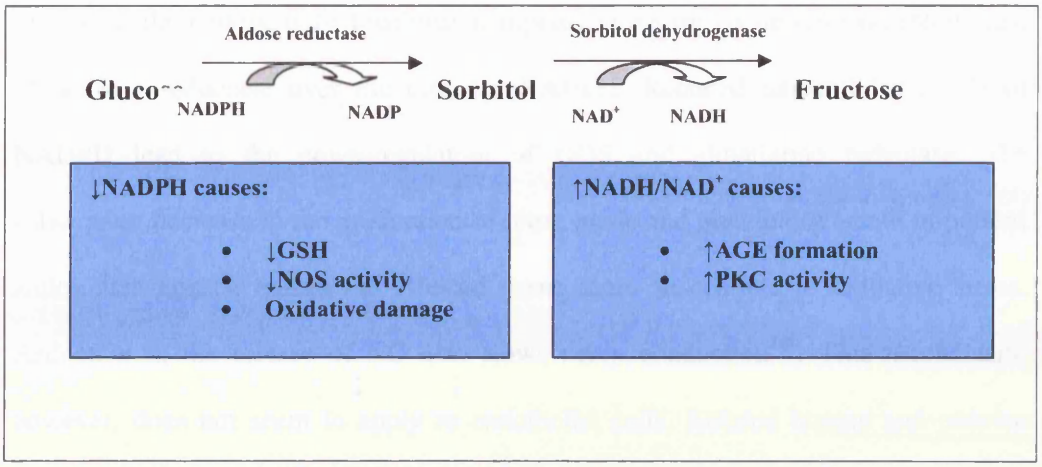


Figure 3.2: The polyol pathway and consequences of increased glucose flux through it. (GSH: Glutathione, NOS: NO synthase, AGE: advanced glycation end products, PKC: protein kinase C)

The polyol pathway becomes active when intracellular glucose levels are elevated (figure 3.2). Aldose reductase, the first and rate-limiting enzyme in the pathway, reduces glucose to sorbitol using NADPH as a cofactor. Sorbitol is then oxidised to fructose by sorbitol dehydrogenase, which uses NAD^+ as a cofactor. This is a normal reaction sequence in testis but not other tissues which often lack sorbitol dehydrogenase. Therefore, chronic hyperglycaemia and increased activity of aldose reductase lead to accumulation of sorbitol in these tissues[217].

Several mechanisms have been proposed to explain the role of polyol pathway in the pathogenesis of diabetic tissue damage.

In the ocular lens, excess sorbitol induces hyperosmotic oedema which by disturbing the cell membrane leads to the leakage of aminoacids, glutathione, and myoinositol which in turn provoke cataract. However, sorbitol levels in diabetic vessels and nerves are too low to suggest this mechanism is important in these tissues[192;218].

The pathophysiology behind neuronal dysfunction in diabetes mellitus is more likely to be aldose reductase-induced oxidative stress[219]. It is believed that the excess flow

of glucose through the polyol pathway competes with nitric oxide synthase (NOS) and glutathione reductase over the cofactor NADPH. Reduced intracellular levels of NADPH lead to the down-regulation of NOS and glutathione reductase. The subsequent decrease in the production of nitric oxide and glutathione –both important antioxidant agents- makes the affected tissue more susceptible to oxidative stress. Reduction in the release of NO also slows nerve conduction[220]. This mechanism, however, does not seem to apply to endothelial cells. Isolated human and porcine coronary endothelial cells show an increase in basal NO production in response to acute hyperglycaemia (see below)[221].

The leading role of aldose reductase in glucose toxicity has made its inhibition a potential strategy in preventing long term complications of diabetes, especially cataract, retinopathy, and peripheral neuropathy[222-225]. Unfortunately, most of the clinical trials performed so far have either failed to show a definite therapeutic/prophylactic role for aldose reductase inhibitors or caused various adverse effects.

Increased formation of advanced glycation end products (AGEs)

Glycation is the result of non-enzymatic reaction between the carbonyl group of a simple sugar (i.e. glucose, fructose and galactose) and free amino group of proteins. Reactive sugars can also bind to other macromolecules such as lipids and nucleic acids. The rate of the reaction is proportional to the concentration of sugar (mainly glucose) in the blood[193]. HbA_{1C} is an example of glycation end products in the circulation.

Glycation is initially reversible but through a series of slow reactions known as Amadori reactions, Schiff base reactions, and Maillard reactions; leads to the formation of irreversibly modified molecules collectively known as advanced glycation end-products (AGEs). There is an increased accumulation of AGEs in diabetes mellitus, renal failure, and aging[226].

Glycation interferes with the function of many important proteins throughout the body. In the renal glomeruli, advanced glycation of proteoglycans alters the selective filtration properties of basement membrane by inducing a decrease in the electronegative charge[227]. AGEs also increase the production of transforming growth factor-beta (TGF-β) which in turn induces vascular hypertrophy and mesangial extracellular matrix expansion both of which play an important role in the pathogenesis of microvascular complications of diabetes[228].

AGE-modified proteins in the circulation can affect a range of cells and tissues including polymorphonuclear (PMN) leukocytes, monocytes and macrophages, glomerular mesangial cells, and vascular endothelial cells. This effect is conveyed through specific receptors for AGEs (so-called "RAGE"). In endothelial cells, AGE binding to its receptor generates oxygen free radicals that may induce oxidative damage and favour coagulation. In addition, endothelial AGE receptor binding appears to increase vascular permeability through the induction of vascular endothelial growth factor (VEGF)[193]. The latter may play an important role in the pathophysiology of diabetic retinopathy. Indeed, it has been shown that inhibition of AGE production by aminoguanidine prevents late structural changes of experimental diabetic retinopathy[229].

AGEs depress superoxide production in stimulated PMN leukocytes. As superoxide plays an essential role in bactericidal activity, this inhibitory effect may be a contributory factor to the increased prevalence and severity of bacterial infection seen in diabetic patients. In contrast, baseline superoxide production of PMN leukocytes is increased by AGEs[230].

AGEs can also bond together and, consequently, increase protein crosslinking. In the circulatory system, increased collagen crosslinking caused by AGEs increases cardiovascular stiffness as well as the risk for cardiovascular morbidity and mortality[231].

Increased protein kinase C (PKC) activation

PKC is a ubiquitous family of protein kinases with at least 11 isoforms[232]. PKC phosphorylates various target proteins and its persistent and excessive activation in hyperglycaemia is associated with diabetic vascular disease and tissue damage.

Raised intracellular glucose levels increase PKC activity by increasing the synthesis of diacylglycerol (DAG) from glucose. DAG is a powerful enhancer of PKC activity[193] (figure 3.3).

Increased activity of PKC has a variety of effects on gene expression. Endothelial nitric oxide synthase (eNOS) is decreased and the vasoconstrictor endothelin-1 is increased, leading to alteration of normal tissue blood flow. Activation of PKC-α[233] as well as increased expression of VEGF[234] enhance endothelial permeability. Increased expression of TGF-β_1, type IV collagen[235] and fibronectin[236] increase extracellular matrix accumulation in glomerular mesangial cells. PKC increases the production of reactive oxygen species (ROS) via activation of NADPH oxidase[237].

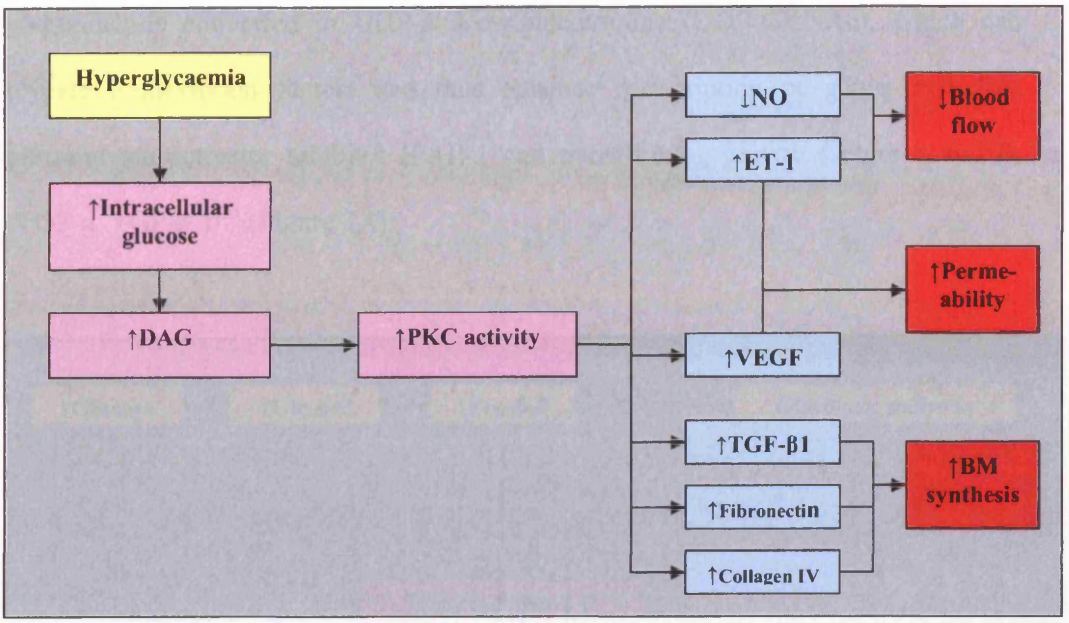


Figure 3.3: Consequences of hyperglycaemia-induced activation of PKC. (PKC: protein kinase C, BM: basement membrane, DAG: diacylglycerol, ET: endothelin, VEGF: vascular endothelial growth factor, TGF: transforming growth factor)

The importance of PKC in the development of complications of DM is evident from studies in which inhibitors of PKC have been shown to prevent early changes in the diabetic retina[238] and kidney[239]. Most pharmacological studies are restricted to in vitro models because common inhibitors of PKC are non-specific and are associated with unacceptable toxicity.

Increased hexosamine (glucosamine) pathway flux

Shunting glucose into the hexosamine pathway is the most recent proposed mechanism by which hyperglycaemia can cause diabetic complications[192]. It has been shown that increased hexosamine pathway flux induces oxidative stress, apoptosis, and increased extracellular matrix expression in mesangial cells[240].

The hexosamine biosynthesis pathway (HBP) is a relatively minor branch of glycolysis[241]. Glucosamine-6-phosphate, generated from fructose-6-phosphate and

glutamine, is converted to UDP-*N*-acetylglucosamine (UDP-GlcNAc), which can glycate transcription factors and thus enhance transcription of genes including plasminogen activator inhibitor (PAI)-1 and transforming growth factors α and β_1 (TGF-α, TGF-β_1)[193] (Figure 3.4).

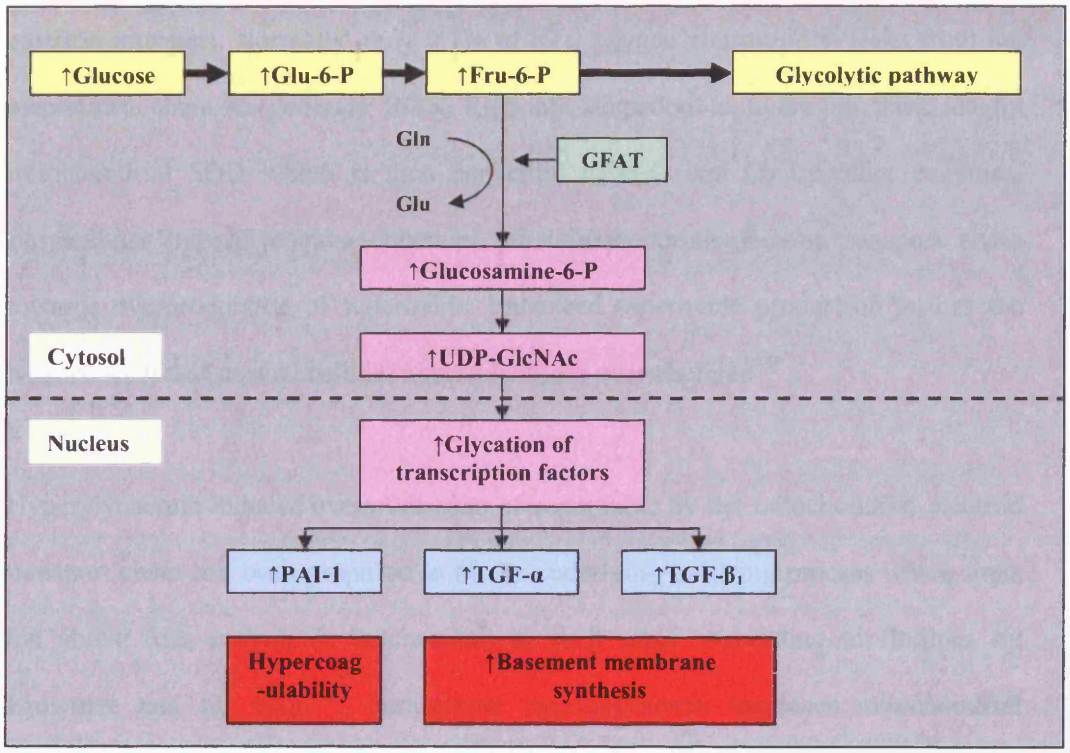


Figure 3.4: The glucosamine pathway. (Gln: glutamine, Glu: glucose, Fru: Fructose, GFAT: glutamine: fructose-6-phosphate amidotransferase, UDP-GlcNAc: Uridine diphosphate glucose - *N*-acetylglucosamine, PAI: plasminogen activator inhibitor, TGF: transforming growth factor)

The conversion of glucose to glucosamine is catalysed by the rate-limiting enzyme glutamine: fructose-6-phosphate amidotransferase (GFAT). It has been reported that GFAT overexpression in muscle and fat[242] or liver[243] results in insulin resistance.

A unified mechanism: increased oxidative stress

The occurrence of oxidative stress in diabetes has been extensively documented[244-249]. A consistent feature common to all cell types that are damaged by hyperglycaemia is an increased production of reactive oxygen species (ROS); primarily superoxide[250;251]. Mitochondria are the principal source of ROS in cells as the result of uncoupled electron transport. Normally, only 0.1% of total oxygen consumption leaks from the respiratory chain to generate ROS. ROS are degraded to hydrogen peroxide by mitochondrial SOD which is then converted to H_2O and O_2 by other enzymes. Intracellular hyperglycaemia enhances the mitochondrial electron transport chain towards overproduction of superoxide. Enhanced superoxide production pushes the balance such that normal cellular antioxidants are overwhelmed[249].

Hyperglycaemia-induced overproduction of superoxide by the mitochondrial electron transport chain has been proposed to be the underlying initiating process which links the above four pathogenic mechanisms to each other. According to findings by Brownlee and his team[192], intracellular hyperglycaemia increases mitochondrial production of superoxide. Superoxide damages nuclear DNA strands. Damage to DNA activates the intranuclear enzyme poly (ADP-ribose) polymerase (PARP). PARP is a DNA repair enzyme which is activated by damage to DNA. PARP then inhibits the key glycolytic enzyme glyceraldehyde-3 phosphate dehydrogenase (GAPDH) by modifying its molecular structure with polymers of ADP-ribose (figure 3.5).

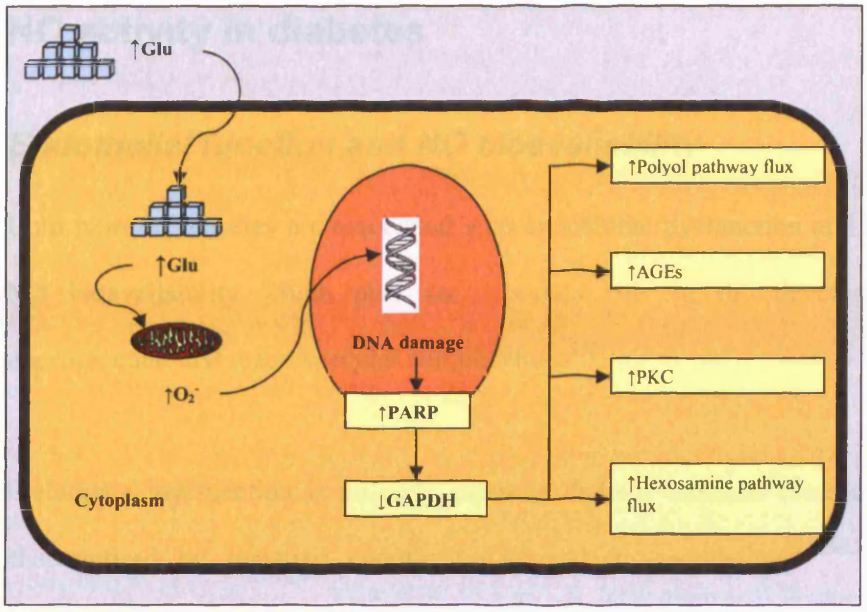


Figure 3.5: The unified mechanism of hyperglycaemia-induced cellular damage (Glu: glucose, PARP: poly (ADP-ribose) polymerase, GAPDH: glyceraldehyde-3 phosphate dehydrogenase, AGE: advanced glycation end products, PKC: protein kinase C).

Inhibition of GAPDH activity leads to accumulation of all the glycolytic metabolites that are upstream of GAPDH. Increased levels of these metabolites activate the four pathogenic pathways described above. As seen in figure 3.5, increased levels of glyceraldehyde-3-phosphate activate AGE and PKC pathways. Increased levels of fructose-6-phosphate increases flux through the hexosamine pathway. A further increase in intracellular glucose levels increases flux through the polyol pathway.

Other mechanisms have also been proposed to explain increased oxidative stress in diabetes. It has been shown that xanthine oxidase (a superoxide-generating enzyme) is increased in plasma and liver of diabetic rats. Xanthine oxidase leaks from the liver into plasma (only in diabetic rats and not in controls) and binds to vascular endothelial cells where it can produce superoxide in the presence of xanthine. This process can be inhibited by heparin (which releases xanthine oxidase from the endothelial surface) and allopurinol, an inhibitor of xanthine oxidase[248].

NO activity in diabetes

Endothelial function and NO bioavailability

Both types of diabetes are associated with endothelial dysfunction and alterations in NO bioavailability which play an important role in the development of its microvascular and macrovascular complications[252].

Endothelial dysfunction is an early event in diabetic vascular disease[253-255] and is characterised by impaired endothelium-dependent vasodilation[253;256;257], increased platelet aggregation, increased expression of leukocyte adhesion molecules, increased vascular smooth muscle proliferation, and increased endothelial permeability for macromolecules and lipoproteins[258]. These abnormalities accelerate atherosclerosis and promote the development of diabetic macroangiopathy. Clinically, the "syndrome" of endothelial dysfunction can be described as generalised or localised vasospasm, thrombosis, atherosclerosis, and restenosis[259].

Several mechanisms can be considered to explain impaired NO bioavailability in diabetes:

1. Increased oxidative destruction of NO.
2. Decreased NO production by:
    a. Reduced eNOS activity due to reduced available substrates L-arginine, BH_4, intracellular NADPH.
    b. Insulin deficiency: Insulin is known to stimulate vasodilation via increased NO production[260;261]. Reduced insulin levels in type 1 diabetes may decrease NO production.

3. NO quenching by AGEs[262].

Most of the current evidence support number 1; the oxidative stress theory[253;256-258;263-265]. There is ample evidence for increased oxidative stress in diabetes as discussed before. Acute hyperglycaemia, has been shown to increase eNOS gene expression and NO production in isolated endothelial cells[266-268]. Paradoxically, upregulation of eNOS in a hyperglycaemic milieu is also associated with a marked concomitant increase of O_2^- production; which is several times larger than the increase in NO release[266]. This evidence provides the molecular basis for the so-called "eNOS uncoupling" concept[266;269] suggesting that eNOS itself in pathological states may be an important source of O_2^-.

In summary, hyperglycaemia-induced imbalance between NO and O_2^- and the subsequent destruction of NO by reacting with O_2^- is thought to be the main mechanism underlying reduced bioavailability of NO in diabetes.

NO metabolites

NO studies in diabetes have mainly focused on its vasodilatory effects. Very few studies have ever looked into the biochemical fate of individual NO metabolic species in diabetes (table 3.2) and none has been inclusive of all the metabolites of interest. This is at least partly due to the lack of a general consensus over standard methods for the analysis of NO metabolites. The controversy extends to the very nature, *in vivo* importance, and biological levels of these metabolites.

Reference	Method	Study subjects	Metabolite measured	Levels compared to controls
Milsom AB *et al*[270] (2002)	EPR	Human	HbNO	Higher
Thule PM *et al*[271] (2006)	ESR	Rat	HbNO	Lower
Padron J *et al*[272] (2000)	Saville, Spec	Rat	SNO-Hb	Higher
Milsom AB *et al*[270] (2002)	Saville, Spec	Human	RSNO	Same

Table 3.2: Levels of blood NO metabolites other than NOx (nitrate + nitrite) in diabetics compared to controls. (EPR: Electron paramagnetic resonance; ESR: Electron spin resonance spectroscopy, Spec: spectrophotometry)

Where NO metabolites have been studied, the less controversial plasma NOx (nitrate+ nitrite) has been used as a rough and indirect estimate of overall NO turnover in the blood (table 3.3)[273-281]. The majority of these studies reported higher plasma levels of NOx in diabetics. One study[255] showed lower NOx in diabetics compared to healthy controls. Another study[282] showed similar fasting but decreased postprandial levels in diabetics. A previous paper from our laboratory in 2002 reported similar concentrations of NOx in 23 diabetics and 17 controls, however, the tendency was toward higher levels in diabetics (7.64±0.75 uM in diabetics versus 5.93±0.75 uM in controls)[270].

Reference	Method	Metabolite measured	Levels compared to controls
Kobylianskii AG[277] (2003)	HPLC	Nitrite, nitrate	Similar nitrite, higher nitrate
Chiarelli F[281] (2000)	HPLC	NOx	Higher
Hoeldtke RD[278] (2002)	ELISA	NOx	Higher
Hoeldtke RD[279] (2003)	Colorimetric Griess reaction	NOx	Higher
Hoeldtke RD[280] (2003)	Colorimetric Griess reaction	NOx	Higher
Mylona-Karayanni C[275] (2006)	Colorimetric Griess reaction	NOx	Higher
Savino A[274] (2006)	Colorimetric Griess reaction	NOx	Higher
Wierusz-Wysocka B[273] (1998)	Colorimetric Griess reaction	NOx	Higher
Farkas K[282] (2004)	Fluorometric	NOx	Similar fasting, lower postprandial
Milsom AB[270] (2002)	Fluorescent Spectrophotometer	NOx	Similar
Correa RC[255] (2003)	Colorimetric Griess reaction	NOx	Lower

Table 3.3: List of human studies comparing plasma NOx in diabetics and controls. (NOx: nitrite + nitrate).

This thesis, for the first time, presents a comprehensive investigation into the profile of individual NO metabolites (i.e. nitrate, nitrite, RSNO, HbNO, SNO-Hb, RBC nitrite) in plasma and RBCs from type 1 diabetics and controls. One should bear in mind that changes in these metabolites do not provide concrete information regarding the location and source of NO production. Nonetheless, it provides an in-depth insight into the metabolic fate of NO in diabetics. Any correlation found between alterations in NO metabolism and long term microvascular complications may have application for the prevention and/or treatment of these complications in diabetes and other

diseases characterised by endothelial dysfunction. In addition, such information can potentially be utilised to develop a screening test for the early detection of microvascular complications at a pre-clinical stage.

CHAPTER FOUR

Methods and Measurement of nitric oxide

Introduction

Interest in NO measurement increased exponentially with the discovery that NO is the endothelium-derived relaxing factor (EDRF).

NO is very unstable in biological specimens. It rapidly reacts with O_2, O_2 radicals and oxidising agents such as oxyhaemoproteins. The instability of NO makes its direct measurement in blood and other body fluids difficult.

Measurement of NO metabolites (e.g. nitrite, nitrate) and biological adducts (e.g. S-nitrosothiols) is another way to assess NO production without the measurement problems associated with the unstable nature of NO. These molecules are generally more stable than NO. Further interest in the measurement of NO biological by-products emerged when it was proposed in 1992 that one of these adducts, S-nitrosothiols, may preserve and transport NO bioactivity in the circulation[103]. Since then, other NO metabolites such as nitrite and SNO-Hb have also been considered as potential reservoirs of NO bioactivity (see CHAPTER ONE). This has led to a universal appeal by different groups, including our laboratory at the Wales Heart Research Institute, to develop and advance reliable methods to analyse NO metabolites in blood.

Table 4.1 shows the methods currently employed in our laboratory to measure NO metabolites. The chemiluminescence assay is the most sensitive and specific of them all.

Method	**Yield**	**Reference**
Fluorometry (DAN assay)	NOx (nitrate + nitrite)	Misko et al.[283]
Tri-iodide based chemiluminescence	Plasma nitrite, RSNO	Yang et al.[284]
Modified tri-iodide based chemiluminescence	RBC nitrite, HbNO, SNO-Hb	Rogers et al.[285]
Electron paramagnetic resonance (EPR)	HbNO	Bemski et al.[286]

Table 4.1: Methods of NO measurement at the Department of Cardiology, Wales Heart Research Institute, 2007.

Electron paramagnetic resonance (EPR), the primary method for measuring HbNO[286], is not sensitive enough to detect HbNO at physiological concentration (detection limit: 200[123]-500[121]nM). For this reason, I modified and improved the tri-iodide based

chemiluminescence assay to distinguish HbNO from other haemoglobin-bound species and quantify its concentration in blood. The precise detail of the systematic testing and derivation of the assay systems for the individual NO metabolites and the optimised methods are described later in this chapter.

Real time assessment of NO production at a cellular level is possible using electrochemical (amperometric) NO-specific microelectrodes. Microelectrodes are placed close to the cellular source of NO. However, the practical use of microelectrodes is extremely limited by their time-consuming methodology as well as susceptibility to temperature, flow, and pH artefacts [287;288].

Fluorometry (DAN assay)

Introduction

This is our method of choice for the measurement of plasma NOx (nitrate + nitrite). As mentioned in CHAPTER ONE, NO_3^- is abundant in certain foods, beverages and in most water. Therefore if one systematically studies plasma NOx in humans, a diet with defined NO_3^- content should be considered.

DAN assay was originally developed for the measurement of nitrite in biological samples[283]. To measure plasma NOx, nitrate is first reduced to nitrite using a nitrate reductase. Subsequently, under acidic conditions, nitrite releases NO^+ which reacts rapidly with 2, 3-diaminonaphthalene (DAN) to form the highly fluorescent product 2, 3-naphthotriazole (NAT) (figure 4.1). Because nitrate levels in plasma are about 100 times greater than nitrite, NOx mainly reflects plasma nitrate levels.

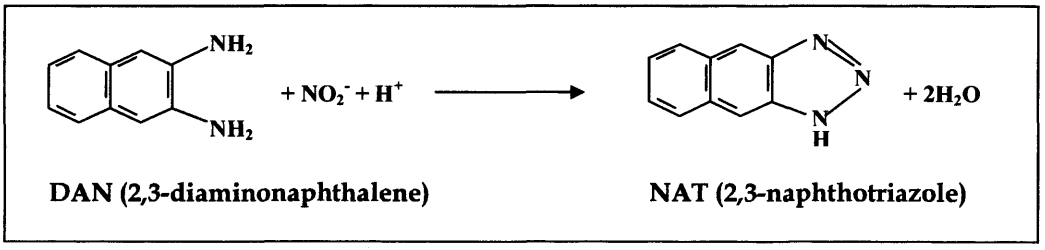


Figure 4.1: Reaction of nitrite with 2, 3-diaminonaphthalene (DAN) to form 2, 3-naphthotriazole (NAT) under acidic conditions[289].

An alternative method to measure plasma nitrate is vanadium-based chemiluminescence. In acidic conditions at high temperature (80-95°C), vanadium (III) exerts very strong reducing properties and reduces both NO_2^- and NO_3^- to NO. The strong reducing environment can also create problems by reducing arginine-based NOS inhibitors to NO. Thus, one should not use the assay in samples containing L-

NMMA, L-NA, etc. In my cross-heart study, half of the blood samples contained L-NMMA. To avoid the problems just mentioned, DAN assay was used to measure plasma nitrate throughout.

Protocol

Blood samples were collected into EDTA tubes. Care was taken not to use heparin as an anticoagulant as it inhibits nitrate reductase and could interfere with this assay[290].

All chemicals were purchased from Sigma-Aldrich (UK) with the exception of DAN, which was available from Janssen Chimica. For composition of reagents and buffer see table 4.2.

Sodium phosphate buffer	Stock solution 1.4M (8.06 grams Na_2HPO_4, 2.08 grams $NaH_2PO_42H_2O$ in 50ml water). Working solution 14mM. Dilution of stock solution in water
NADPH	Stock solution 30μM (0.3 grams in 1ml buffer). Diluted with buffer to 10μM working solution.
Glucose-6-phosphate	Sock solution 50mM (in buffer). Diluted in buffer to a 5mM working solution
Glucose-6-phosphate dehydrogenase	Stock solution 100 units/ml (in buffer). Diluted in buffer to 1.6 units/ml working solution
Nitrate reductase	Stock solution 5 units/ml (in buffer). Diluted in buffer to 0.8 units/ml working solution
Enzyme mixture (Master mix)	Equal quantities of sodium phosphate buffer, glucose-6-phosphate, glucose-6-phospate dehydrogenase and nitrate reductase.
HCl	Diluted in water to a 0.62 M working solution.
DAN	Stock solution 0.5mg ml^{-1} (in 0.62 M HCl). Diluted with 0.62 M HCl to 0.05mg ml^{-1} working solution and stirred for 30 min before use. Both solutions protected from light.
NaOH	Diluted in water to a 2.8 N working solution.

Table 4.2: Composition of reagents for the fluorometric analysis of NOx. All solutions were prepared fresh on the day of the experiment.

All reagents were made at room temperature in HPLC grade water to reduce nitrite contamination. Fluorescence excitation and emission spectra of NAT were obtained in a white opaque Dynatech fluorescence 96-well microtitre plate.

1. Frozen plasma samples were thawed in a water bath at 37°C for 3 minutes.
2. Plasma was filtered through a 10,000 *MWCO* cut-off ultrafilter (Vivaspin filters from Sartorius VivaScience) at 10,000 *g* for 30 minutes to remove haemoglobin (resulting from cell lysis) and other high-molecular weight proteins. High protein levels exert a negative effect on fluorescence. Haemoglobin, in levels as low as 10μM, can completely abolish the signal[283].
3. Aliquots of filtrate (50μl) were placed into 96-well plate in triplicate.
4. Standards were added in triplicate 50μl volumes into 96-well plates.
5. 10μl of10μM NADPH was added (to initiate the reaction)[283].
6. 40μl of the following enzyme mixture (master mix) containing the following reagents (in 14mM sodium phosphate buffer) was added to each well:
    a. 0.8U/ml nitrate reductase (NR) (isolated from *Aspergillus niger*)
    b. 5mM glucose-6-phosphate (G6P)
    c. 1.6U/ml glucose-6-phosphate dehydrogenase (G6PD)

NADPH/$NADP^+$ can interfere in the assay by quenching the fluorescence produced[291]. To minimise this interference, we used a lower concentration of NADPH than the one originally described by Misko et al. (i.e. 40μM). Instead, we included an NADPH recycling system in our mixture to maintain a constant NADPH concentration and allow the reaction to proceed for longer

periods of time. $NADP^+$ is recycled to NADPH by the dehydrogenation of G6P to 6-phosphoglucolactone (6-PG). This reaction is catalysed by G6PD[292]:

$$NO_3^- + NADPH + H^+ \xrightarrow{NR} NO_2^- + NADP^+ + H_2O$$

$$H_2O + NADP^+ + G_6P \xrightarrow{G6PD} 6\text{-}PG + NADPH + H^+$$

7. Samples were incubated for 1.5 hours at 37°C. Plates were covered with cling film. During this time, nitrate was converted to nitrite by the action of nitrate reductase[283]. The rate of conversion of nitrate to nitrite is 98%[289].
8. After incubation, 10µl of freshly prepared DAN (0.05mg/ml in 0.62M HCl) was added to each well. Samples were further incubated for 10 minutes at room temperature, protected from light.
9. The reaction was terminated with 5µl of 2.8N NaOH. NaOH also enhances the fluorescent signal produced by NAT[283]. NAT is stable in alkaline solutions[289].
10. Formation of NAT was measured after 10 minutes, using a Perkin Elmer luminescence spectrophotometer. Fluorescence excitation and emission spectra of NAT was measured using wavelengths of 365nm and 450nm, respectively.

Standard curves were made daily with sodium nitrate ranging from zero to 100µM (figure 4.2).

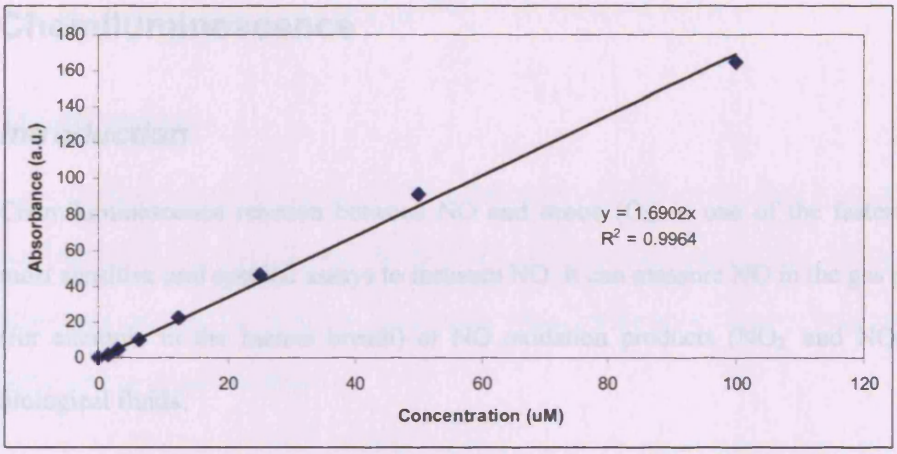


Figure 4.2: Standard curve of sodium nitrate.

Chemiluminescence

Introduction

Chemiluminescence reaction between NO and ozone (O_3) is one of the fastest and most sensitive and specific assays to measure NO. It can measure NO in the gas phase (for example in the human breath) or NO oxidation products (NO_2^- and NO_3^-) in biological fluids.

The NO chemiluminescence assay is based on the gas phase interaction between NO and O_3 in an NO Chemiluminescence Analyzer. Some of the NO_2 produced in this reaction is in the excited state and as the unstable electrons of NO_2* return to their original ground state, they dissipate energy which is released as a light photon and this signal is amplified in a photon multiplier tube (PMT). This light emission is linearly related to the NO content of the sample.

$$NO + O_3 \rightarrow NO_2^* + O_2$$

$$NO_2^* \rightarrow NO_2 + \text{energy}$$

The amount of NO in biological fluids is very small, if any, and stripping alone would not reflect at all the amount of NO present since most of it would already be oxidised to NO_2^- or NO_3^- . In an acidic environment and with strong reducing agents (such as KI, NaI, or vanadium III chloride) these oxides can be reduced back to NO. In body fluids under these reducing conditions, other bioactive NO adducts, like nitrosothiols and nitrosamines are reduced to NO as well.

Nitric Oxide Analyzer (NOA)[293]

Characteristics

Our chemiluminescence experiments are undertaken using a model 280i Nitric Oxide Analyzer (NOA®) from Sievers Instruments. This is a high-sensitivity detector for measuring nitric oxide based on a gas-phase chemiluminescent reaction between NO and ozone as described above.

Emission from electronically excited nitrogen dioxide is in the red and infrared region of the spectrum, and is detected by a thermoelectrically cooled, red-sensitive photomultiplier tube (PMT).

An electrostatic ozone generator and high voltage transformer are used to generate ozone at a concentration of ~2% by volume from oxygen. This large excess of ozone is sufficient for measurement of NO up to 500ppm.

The detection limit for measurement of NO and its reaction products in liquid samples is ~1 picomole (table 4.3).

Sensitivity	~1 picomole
Range	nanomolar to millimolar
Repeatability	± 5-10%
Sample size	0.001-5ml

Table 4.3: Some specifications of Sievers 280i Nitric Oxide Analyzer for liquid measurements.

Set up of Purge Vessel

Measurement of NO and its reaction products in liquid samples is performed using a glass purge vessel with a rubber septum-covered injection inlet. An oxygen-free inert

gas (nitrogen in our studies) bubbles through chemical reducing agents (e.g. tri-iodide) at a constant flow rate of 200ml/min. Samples are injected through the rubber septum into the purge vessel and the inert gas carries the NO over to the NOA for detection. The carrier gas is passed through a NaOH gas bubbler trap containing 25ml of 1 N NaOH before entry to the NOA. The NaOH trap filters the gas from any corrosives. The purge vessel is placed in a water bath heated at 50°C to speed up the chemical reactions (figure 4.3).

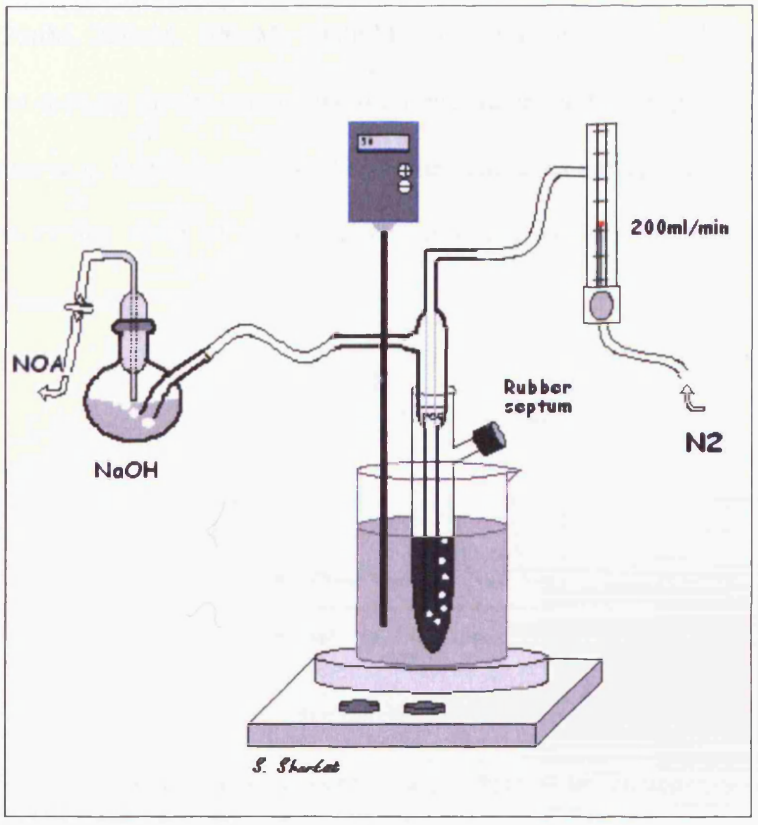


Figure 4.3: Overall schematic of the purge vessel system.

Injection is performed using a 500µl gas tight Hamilton syringe. Calibration is performed everyday by injection of standard solutions of sodium nitrite.

Preparation of Nitrite Standard Solutions

To prepare 100ml of 10mM NO_2^-, 69mg of $NaNO_2$ is dissolved in 100ml nitrite-free HPLC water. This stock solution of 10mM NO_2^- can be kept in an air tight glass bottle at room temperature for 1 week. It is stable for several weeks if stored at 4°C or lower and not exposed to light.

The above standard stock is used to prepare dilutions; typically 200µl injections are made at the following concentrations to construct a standard curve for the NOA: 62.5nM, 125nM, 250nM, 500nM, 1000nM. An initial injection of HPLC water is performed to account for the contamination in the water used to prepare the standards and containers (e.g. Eppendorf tubes). Thereafter, standards are injected into the purge vessel in ascending order of concentration starting from the most dilute standard (62.5nM) (figure 4.4).

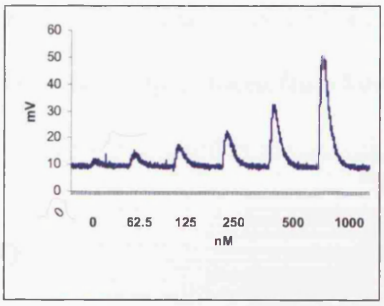


Figure 4.4: NOA signals from injections of standard sodium nitrite solutions into tri-iodide.

To prepare a concentration-based calibration curve (figure 4.5), the same volume should be injected for all of the standards and samples.

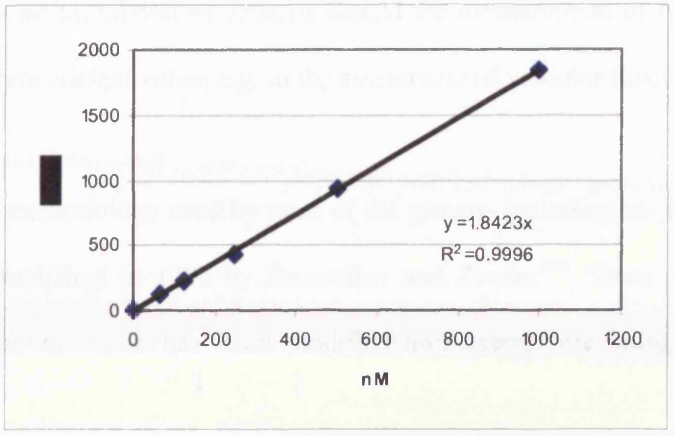


Figure 4.5: Standard curve of sodium nitrite.

Data are imported into Origin and smoothed using the Adjacent Averaging method (number of data points: 150). Peak Analysis is used to calculate the area under curve (AUC).

Sample Analysis

The procedure for sample analysis is the same as described above. In our studies, we measured NO metabolites in blood samples taken from human subjects. Particulars of plasma and RBC measurements are discussed in the next section.

Tri-iodide (I^{3-}) assay

General

The tri-iodide assay is the most widely used ozone based chemiluminescence technique to detect NO metabolites in the blood. It has the benefit of measuring several metabolites in the same plasma or red blood cell (RBC) sample in one session therefore reducing the variations related to analysing the samples with different methods on different occasions. This also saves a lot of time which would be an

advantage in hospital laboratory settings should the measurement of NO metabolites ever gain definite clinical value, e.g. in the assessment of vascular function.

The tri-iodide methodology used by most of the groups- including us- is based on the method first published in 1998 by Samouilov and Zweier[294]. Since then, tri-iodide based chemiluminescence has been modified and extensively validated by many groups.

Chemistry

The classic tri-iodide reagent contains glacial acetic acid, water, KI, and I_2. This reagent can release NO from nitrite, S-nitrosothiols (including SNO-Hb), N-nitrosamines (RNNO), and haem-nitrosyls (including HbNO). We use the classic triiodide reagent for the analysis of plasma samples.

When analysing RBC lysate, the sensitivity of tri-iodide assay is greatly affected by the high concentrations of haem in the injectate into the reaction chamber. NO is immediately quenched by haem iron[285].

To overcome this problem, our group developed a modified tri-iodide reagent by adding potassium hexacyanoferrate $K_3Fe^{III}(CN)_6$ to triiodide[285]. $K_3Fe^{III}(CN)_6$ oxidises haem from its ferrous (Fe^{2+}) to its ferric (Fe^{3+}) form which has a much lower affinity for NO and is thus a less potent scavenger of NO. Oxidising the haemoglobin to methaemoglobin also helps to cleave NO from HbNO and improves the yield of NO from these species.

Pre-treatment with acidified sulfanilamide and mercuric chloride ($HgCl_2$) is used to distinguish between several NO species present in the samples (table 4.4).

	Pre-treatment	Yield
Plasma	nil	Nitrite+ RSNO+ RNNO+ haem-nitrosyls
RBC lysate		***Nitrite+ SNO-Hb + HbNO***
Plasma	Acidified Sulfanilamide	RSNO+ RNNO+ haem-nitrosyls
RBC lysate		***SNO-Hb+ HbNO***
Plasma	Acidified Sulfanilamide + $HgCl_2$	RNNO+ haem-nitrosyls
RBC lysate		***HbNO***

Table 4.4: Pre-treatment of blood samples and their associated yields of NO metabolites.

Nitrite (NO_2^-) assay- In the presence of acid, NO_2^- is converted to the nitrosonium ion (NO^+):

$$NO_2^- + 2H^+ \rightarrow NO^+ + H_2O$$

NO^+ reacts rapidly with nucleophiles like iodide (from KI), to form NO:

$$NO^+ + I^- \rightarrow ONI$$

$$2ONI \rightarrow 2NO + I_2$$

I_2 is highly soluble in the acidified KI solution and further forms I_3^- in the reagent:

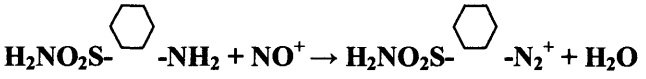
$$I_2 + I^- \rightarrow I_3^-$$

During the experiment when the reagent is bubbled with N_2, some of the I_2 escapes the purge vessel as a purple-brown vapour which can stain PVC tubes. Iodine vapour is trapped and decolourised in the NaOH trap.

As evident from the above equations, I_2 is not necessary in the reaction mixture for the reduction of nitrite. However, when working with biological samples where both nitrite and nitrosation products (RNO) are present, uncontrolled amounts of I_2/I_3^- produced during the process can also reduce unknown amounts of RNOs and therefore make the results unpredictably inaccurate. For this reason, crystal I_2 is added to the reagent to stabilise the yield of nitrite and RNO[284] (see below).

In order to distinguish the RNO fraction from the total nitrite + RNO signal obtained above, samples are pre-treated with and without 5% acidified sulfanilamide. Acidified sulfanilamide forms a diazonium complex with nitrite which is not reducible to NO in the tri-iodide reagent and therefore does not produce a signal:

$$NO_2^- + 2H^+ \rightarrow NO^+ + H_2O$$



(Sulfanilamide)

Nitrite is calculated as the difference between the un-treated sample and the "sulfanilamided" sample.

It is important to remember that the tri-iodide reagent is not strong enough to reduce NO_3^-.

Nitrosation products (RNO) - The main nitrosation products of NO in the blood are S-nitrosothiols (RSNO), N-nitrosamines (RNNO) and haem-nitros(yl)ated compounds.

RSNO includes S-nitrosoalbumin (78% of total plasma RSNO), low molecular weight RSNOs like S-nitrosoglutathione (GSNO), and intra-erythrocytic SNO-Hb. I_3^- releases NO from nitrosothiols as originally described by Samouilov and Zweier[294]:

$$\mathbf{I_3^- + 2RSNO \rightarrow 3I^- + 2RS\cdot + 2NO^+}$$

$$\mathbf{2RS\cdot \rightarrow RS\text{-}SR}$$

$$\mathbf{2NO^+ + 2I^- \rightarrow 2NOI \rightarrow 2NO + I_2}$$

The tri-iodide reagent also releases NO from RNNO and haem-nitros(yl)ated compounds in the plasma or RBC lysate (e.g. HbNO); the mechanism of which is still not confirmed. In order to distinguish the signal coming from RNNO/haem-nitrosyls in the samples from RSNO, samples can be pre-treated with and without mercuric chloride ($HgCl_2$). The –SNO bond is mercury-labile whereas RNNO and haem-nitros(yl)ated compounds are stable in the presence of mercury. This forms the basis of Saville-Griess reaction for the detection of RSNOs in body fluids:

$$RSNO + Hg^{2+} \rightarrow RS\cdot + Hg^{+} + NO^{+}$$

Mercury can displace NO^{+} from the thiol[284]. NO^{+} is then taken up by sulfanilamide as above. RSNO is calculated as the concentration difference between the sample treated with sulfanilamide alone and the sample treated with $HgCl_2$ and sulfanilamide.

The tri-iodide method detects nitrosothiols in biological buffers or blood plasma down to 10nM concentration with high accuracy and reproducibility[127;284].

A similar methodology can be used to release NO from SNO-Hb.

Feelisch et al.[92] have reported that at physiological pH, complete SNO cleavage by $HgCl_2$ would not occur if the samples are incubated for less than 20 minutes. They did not find any difference between sequential pre-treatment with $HgCl_2$, followed by sulfanilamide/H^{+} and coincubation with both agents under acidic conditions.

Materials

Chemicals

Iodine, potassium iodide, potassium hexacyanoferrate ($K_3Fe^{III}(CN)_6$), sulfanilamide, sodium hydroxide, sodium nitrite, and mercuric chloride were purchased from Sigma. Acetic acid glacial, HPLC water, and hydrochloric acid were purchased from Fisher Scientific. Spray coated EDTA vacutainer tubes (VACUETTE, 1.8mg EDTA per 1 ml blood) were purchased from Greiner Bio-One.

Stock solutions and reaction mixtures

Stock solutions for measuring NO metabolites in plasma and RBCs are shown in table 4.5. Tri-iodide, potassium hexacyanoferrate, and pre-sulfanilamided water solutions were made fresh daily. Potassium hexacyanoferrate solution was kept on ice in dark. 5% acidified sulfanilamide was made weekly and kept in dark at room temperature.

For plasma measurements, 5ml of the tri-iodide reagent was put into the purge vessel.

For RBC measurements, 7.2ml of the tri-iodide reagent was mixed with 0.8ml of the hexacyanoferrate solution in the purge vessel immediately before analysis. N_2 gas was bubbled through the reagent from the beginning.

Tri-iodide
- 70ml glacial acetic acid
- 650mg I_2
- 20ml HPLC water
- 1g KI

Potassium hexacyanoferrate
- 823mg $K_3Fe^{III}(CN)_6$
- 10ml HPLC water

$HgCl_2$ (50mM)
- 67.9mg $HgCl_2$
- 5ml HPLC water

5% Acidified sulfanilamide (290mM)
- 500mg sulfanilamide
- 10ml of 1N HCl

Pre-sulfanilamided water
- 1ml 5% acidified sulfanilamide
- 10ml HPLC water

Table 4.5: Stock solutions for measuring NO metabolites in plasma and RBCs. Reaction mixture for plasma analysis: 5ml of Tri-iodide. Reaction mixture for RBC analysis: 7.2ml Tri-iodide+ 0.8ml potassium hexacyanoferrate.

Blood sample collection and preparation

Blood was collected into 4ml EDTA vacutainer tubes. Blood samples were centrifuged at 670g, 4°C for 5 minutes. Plasma was rapidly separated from RBCs and snap-frozen in liquid nitrogen. Buffy coat was discarded and the RBCs were snap-frozen in liquid nitrogen. Plasma and RBC samples were stored at -80°C; and left to defrost in dark at 37°C for 3 minutes just before analysis.

Potential concerns

1. **Separation of plasma and RBCs-** Care was taken to separate plasma and RBC compartments as soon as possible after collection. Previous studies have shown that plasma nitrite rapidly enters the RBCs[72;75]. Therefore, the more plasma and RBCs are left together the lower the plasma nitrite levels and the higher the RBC-contained NO metabolites would become.

2. **Contaminating pre-treatments-** Filtering, columning or adding chemicals/stabilisers were avoided as they are all potential sources for nitrite contamination. Nitrite contamination not only increases the nitrite content of the samples but is also metabolised by the biological samples and may lead to artificial production of other NO species (e.g. RSNO, HbNO and SNO-Hb) in vitro. Therefore, every effort was made to protect the samples from nitrite contamination. If nitrite reaches the samples, its thorough elimination would be almost impossible.

   Erythrolytic solutions are another potential source of contamination. It is not necessary to lyse RBCs in EDTA or similar erythrolytic solutions. Simple

shock-freezing and thawing the RBCs will lyse them without subjecting the samples to potential contamination associated with hypotonic lysis.

3. **Freezing**- Freezing the biological samples has been suggested to be a potential confounding factor in nitric oxide measurements[295]. What makes the problem even more challenging is the fact that in most of the clinical studies due to the large number of samples and/or unavailability of NO measurement facilities locally, blood samples cannot be analysed immediately and have to be frozen and stored for future analysis.

   It has been reported that freezing nitrite containing biological samples may facilitate the conversion of nitrite to nitrate via intermediates which may also nitrate tyrosine to form 3-nitrotyrosine; a biomarker for peroxynitrite formation in vivo or nitros(yl)ate cysteines in proteins to form S-nitrosothiols: RSNOs. In their recent paper, Wang et al.[127] reported a 66±19% increase in SNO-Hb-derived chemiluminescence signal when RBCs were rapidly thawed at 37°C from a storage temperature of -80°C, compared to RBC lysate that was immediately subjected to analysis.

   The underlying mechanism is believed to be based on a decrease in pH in sodium phosphate buffered solutions during the freezing process before the temperature drops below 4°C. In our methodology, we did not use any buffers at any stage. Moreover, snap-freezing the samples in liquid nitrogen, minimizes the time during which the above chemical reaction may occur.

Sample freezing was found to have no effect on RBC or plasma NO metabolite stability in our studies[296].

4. **Exposure to air and light-** Blood samples should not be left exposed to room air or light. Nitrogen oxides pollution in the atmosphere may react with the water compartment of blood/plasma to form nitrite (NO_2^-) and nitrate (NO_3^-).

NO in the atmosphere is immediately oxidised to form NO_2 which then dimerises to N_2O_4. N_2O_4 dissolves in the water compartment of plasma to form NO_2^- and NO_3^-[7]:

$$\mathbf{2NO + O_2 \rightarrow 2NO_2 \rightarrow N_2O_4}$$

$$\mathbf{N_2O_4 + H_2O \rightarrow NO_2^- + NO_3^- + 2H^+}$$

In our laboratory conditions, leaving the plasma samples at room temperature for more than 15-20 minutes increases the plasma nitrite levels by 13-17%. The same phenomenon is seen when a water sample is left exposed to air for a few hours. Air pollution would be less a problem in areas where the air is generally cleaner and if the laboratory is well-ventilated.

RSNOs (including SNO-Hb) are photosensitive and the samples should be kept in dark pre-analysis. Not only UV but even visible light can break down RSNOs to release NO[297].

5. **Collection tubes and choice of anticoagulant-** We generally prefer to collect our blood samples into EDTA vacutainer tubes. Heparin can be used alternatively but at the price of extensive foaming in the reaction chamber which can potentially alter the chemiluminescence signal. As explained earlier, heparin cannot be used in the DAN assay because it inhibits nitrate reductase. Vacutainer tubes protect the blood from exposure to air and keep the oxygen content of blood constant.

Protocol for the measurement of plasma nitrite and nitrosation products

Except for the initial preparations already mentioned, plasma samples were analysed for nitrite and S-nitrosothiols using a methodology relatively similar to the one described by Yang et al.[284]

In summary, 200µl of plasma was injected into 5ml tri-iodide reagent in the reaction chamber. The associated chemiluminescence signal represented total plasma nitrite + protein-bound NO (RNO). 30µl of 5% acidified sulfanilamide was mixed with 300µl of plasma and incubated at room temperature in dark for 15minutes. Our experiments show that incubating plasma samples with acidified sulfanilamide for less than 15 minutes may not eliminate nitrite from the sample completely. 200µl of this sample was then injected into the reaction chamber. The produced signal represented protein-bound NO species (figures 4.6a and b). Plasma nitrite was calculated as the difference between the two signals. Before any calculations were made, areas under curves were computed by Origin/Peak Analysis software, read against a standard curve, and the concentrations corrected for dilutional adjustments.

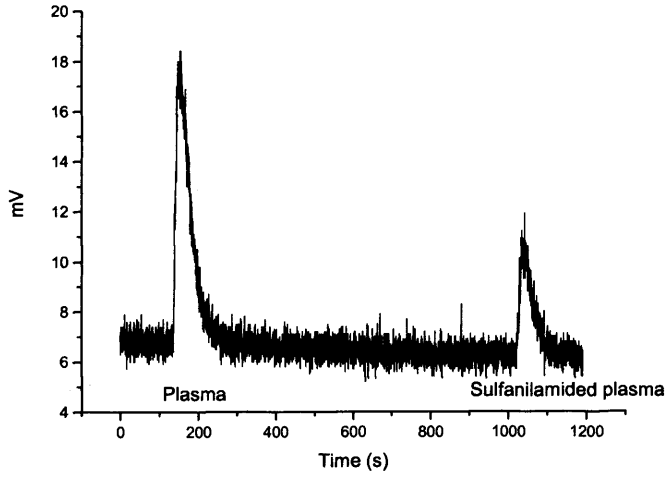


Figure 4.6a: Signals represent 200µl injections of plasma and sulfanilamided plasma, respectively.

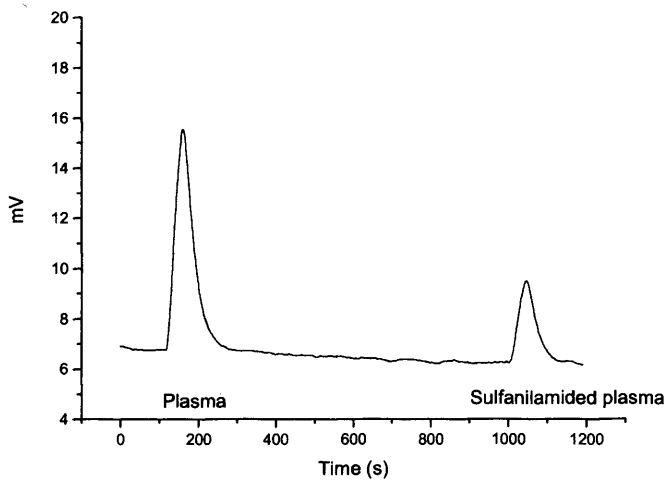


Figure 4.6b: Signals from 4.6a after being smoothed in Origin software.

Two major components of RNO, S-nitrosothiols and iron-nitrosyl complexes, can be further distinguished by pretreating the sulfanilamided sample with $HgCl_2$ in advance as described by Yang et al.[284]

Protocol for the measurement of RBC nitrite, HbNO, and SNO-Hb

1. *Frozen RBCs lyse when they are thawed at 37°C for 3 minutes. 100µl of the undiluted lysate is directly injected into the reaction chamber containing 8ml of modified tri-iodide reagent. The resulting signal represents RBC nitrite+ HbNO+ SNO-Hb (figures 4.7a and b). The reagent is changed after the signal returns to baseline.

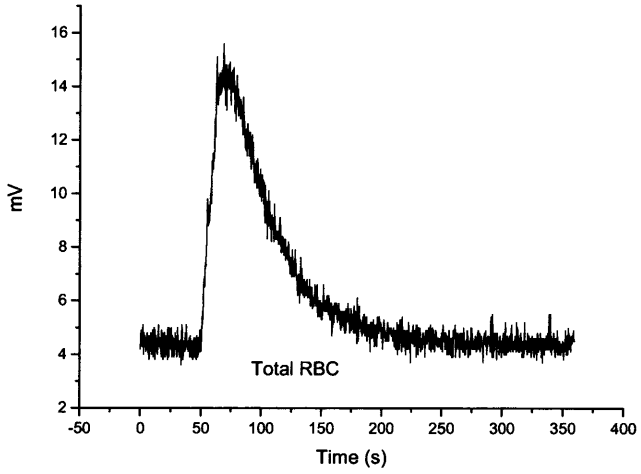


Figure 4.7a: Signal represents 100µl injection of undiluted haemolysate.

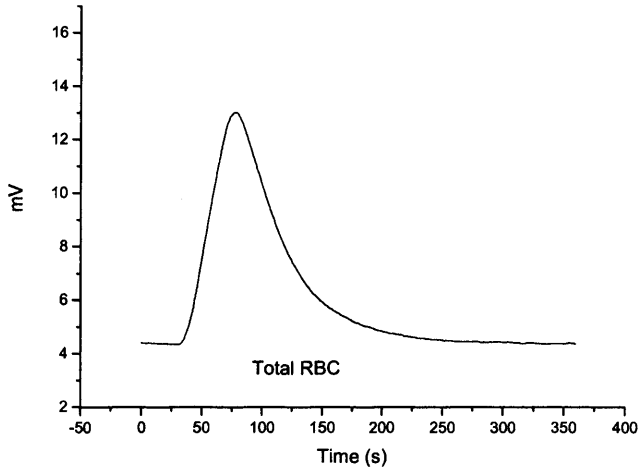


Figure 4.7b: Signal from 4.7a after being smoothed in Origin software.

2. 1ml of 5% acidified sulfanilamide is added to 10ml of HPLC water.
3. 200µl RBC lysate is diluted in 800µl pre-sulfanilamided water (mixture S).
4. 30µl of $HgCl_2$ solution is added to 270µl pre-sulfanilamided water (mixture H).
5. Mixtures S and H are incubated at room temperature in dark for 15 and 25 minutes respectively.
6. 200µl of each of the mixtures S and H are injected into the reaction chamber separately. The signal from mixture S represents HbNO+ SNO-Hb. The signal from mixture H represents HbNO (figures 4.8a and b).

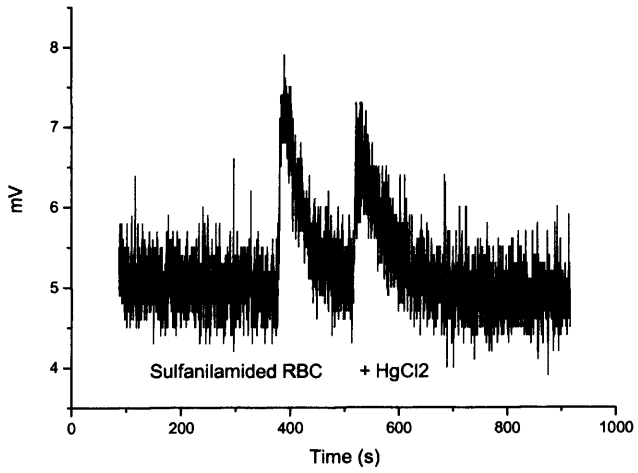


Figure 4.8a: Signals represent 200µl injections of sulfanilamided and mercuried haemolysate respectively.

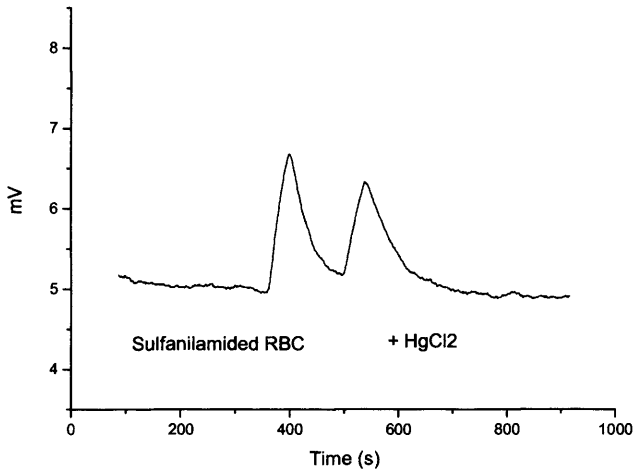


Figure 4.8b: Signals from 4.8b after being smoothed in Origin software.

7. Data are transferred from Liquid to Origin for smoothing. Areas under curves are measured by Peak Analysis and converted to concentrations by reading

them off a standard curve. Concentrations are then corrected for dilutional adjustments. The standard curve is plotted daily by injecting ascending concentrations of standard nitrite solutions into the system.

8. RBC nitrite is calculated as the difference between the undiluted RBC lysate and mixture S. SNO-Hb is calculated as the difference between mixtures S and H.

Notes

♠ Direct injection of undiluted RBC lysate

To avoid adding nitrite contamination while diluting the RBC lysate, I avoided the initial dilution of RBC lysate (with water or buffer) altogether.

For the total RBC NO (nitrite+ SNO-Hb+ HbNO), I developed a technique to inject 100μL of undiluted RBC lysate directly into the reaction chamber. Undiluted RBC lysate is thick and viscous. Care was taken to introduce it directly into the reagent; otherwise it might stick on the glassware.

♥ "Sulfanilamided" water

For the rest of the assay where RBC lysate needs to be pre-treated with sulfanilamide, I used a "sulfanilamided water" solution (10ml water + 1ml 5% acidified sulfanilamide) to both dilute the samples and eliminate contaminating nitrite. Diluting the RBC lysate makes it easier to aspirate and inject with the syringe. Nitrite contamination was not a concern anymore as the water was already treated with sulfanilamide.

Sulfanilamided water prepared as above is a milder acid (pH=1.6) compared to the stock solution of 5% acidified sulfanilamide which contains 1 N HCl (pH≈ 0), but is still acidic enough to react with nitrite efficiently.

My experiments showed that sulfanilamided water can eliminate nitrite from standard solutions as strong as 100μM nitrite.

The above were 2 key methodological developments that allowed the robust quantification and identification of specific NO metabolites with a considerable degree of certainty, not shown to date by other laboratories.

CHAPTER FIVE

Results I
Coronary utilisation of a stable nitric oxide reservoir: importance during increased metabolic demand

Introduction

The main task of the coronary circulation is to match oxygen delivery to myocardial oxygen demand. Coronary blood flow (CBF) normally increases in response to increased myocardial oxygen demand; e.g. during rapid atrial pacing. Multiple anatomical, physiological, and biochemical factors work together to allow this precise matching to occur (CHAPTER TWO).

Endothelium-derived nitric oxide (NO) is one of the key factors for the maintenance of normal vascular physiology. NO is released in response to agonists and shear stress; and mediates vessel dilatation. Recent evidence suggests that microvascular NO activity may also depend upon its more stable circulating metabolites which may act as biological "reservoirs" to preserve NO bioactivity (CHAPTER ONE). These potential reservoirs could be particularly useful in conditions where vascular endothelium is dysfunctional, bioavailability of NO is reduced, or the metabolic demand for oxygen is increased.

The potential role of circulatory metabolites of NO in the regulation of CBF has not been studied previously. The main aims of this clinical study were to:

1. **Establish the profile of NO metabolites across the healthy human heart and lungs, at rest and with increased oxygen demand.**
2. **Study the physiological importance of NO in the regulation of coronary blood flow.**
3. **Study the association of NO metabolites and their apportionment between RBC and plasma compartments, with blood oxygen.**

NO metabolism and the vasodilator response of the coronary vasculature were studied across the coronary and pulmonary beds at rest and during rapid atrial pacing, before and after L-NMMA infusion, in patients with normal coronary arteries. N^G-monomethyl-L-arginine (L-NMMA) is an analogue of L-arginine that specifically inhibits nitric oxide synthase[298]. In order to address the role of NO and NO metabolites as distinctively as possible, the potential effect of adenosine, another

important coronary vasodilator, was removed by administering aminophylline, a nonselective adenosine receptor antagonist, throughout the study.

Both blocking agents, aminophylline and L-NMMA, were administered as continuous systemic intravenous infusions. Intracoronary infusion would not be suitable because recirculation of the blocking agents would result in ever increasing coronary concentrations[299], which might confound the measurement of changes in coronary diameter, coronary flow, myocardial oxygen consumption, and NO metabolites in response to pacing .

Methods

Study population

Eight otherwise healthy subjects (3 men and 5 women aged 51±15 years) undergoing electrophysiology (EP) testing for paroxysmal supraventricular arrhythmias were studied. All subjects were in sinus rhythm with no past history of ischaemic heart disease, smoking, diabetes mellitus, hypertension (blood pressure>140/90mmHg) or hypercholestrolaemia (total cholesterol>5.2mmol/L). All gave fully informed written consent approved by the North West Surrey Local Research Ethics Committee and the Bro Taf Local Research Ethics Committee. The study was also reviewed and approved by the Research and Development Department at St. Peter's Hospital, Chertsey. The investigation conformed to the principles outlined in the Declaration of Helsinki[300].

Clinical study

Catheterisation laboratory study protocol

The catheterisation laboratory study protocol is summarised in figure 5.1. Prior to catheterisation subjects underwent an 8 hour fast during which time they were allowed water. Diagnostic coronary angiography was performed by the Judkins technique with 6 French catheters through a femoral sheath (contrast agent: Visipaque, GE Healthcare Limited, UK). The left heart catheter was maintained in the left main stem (LMS) and 2 catheters were introduced via the right femoral vein and positioned in the coronary sinus (CS) and pulmonary artery (PA) for further blood

sampling. A bipolar pacing wire was placed into the right atrium via the right femoral vein. The position of the sampling catheters was confirmed with contrast injection.

Intravenous infusion of aminophylline (Phoenix Pharma Ltd) was commenced via a peripheral line at the beginning of the study and continued throughout the pacing protocol. Aminophylline was administered at a loading dose of 5 mg/kg body weight over 20 minutes followed by 500 micrograms/kg/hour maintenance dose until the end of the study (British National Formulary protocol).

After baseline haemodynamic parameters were maintained for 5 minutes, brachial blood pressure was measured and blood samples were obtained from the LMS, CS, and PA (baseline samples) for blood gas and NO metabolite analysis. LMS-CS difference was taken to reflect "transcardiac" whereas PA-LMS was taken to reflect "transpulmonary" exchange. Strictly speaking, "transpulmonary" should be accurately measured as PA to pulmonary vein; however, it is technically difficult to obtain such samples from healthy human subjects.

Following baseline sampling rapid atrial pacing at 65% and then 85% of maximum heart rate (MHR) was commenced. MHR was calculated for each subject individually as 220 minus age in years. Subjects were paced for 5 minutes at each heart rate. At the end of each 5-minute pacing interval, blood was sampled for blood gas and NO metabolite analysis and the left coronary angiogram was repeated.

After discontinuation of pacing and confirmation that blood pressure had returned to levels observed before pacing, an infusion of L-NMMA (Clinalfa, Merck Biosciences

AG) was introduced intravenously. L-NMMA was administered as a loading dose of 5mg/kg for 7 minutes, followed by a maintenance dose of 50 micrograms/kg/min throughout the rest of the study, as described by Zhang *et al*[301]., to achieve stable blood concentrations of L- NMMA and sustained inhibition of NOS. Blood samples were then taken from the same anatomical sites (LMS, CS, PA). Blood pressure was measured and the whole protocol was repeated (65% and 85% MHR) with the continued infusion of L-NMMA.

Overall, each study took about 50 minutes and therefore heparin (5000 units IV) was administered following insertion of left coronary catheter to prevent clot formation around the catheter.

At the end of the study, all the infusions were discontinued and the operator carried out the electrophysiology study as planned.

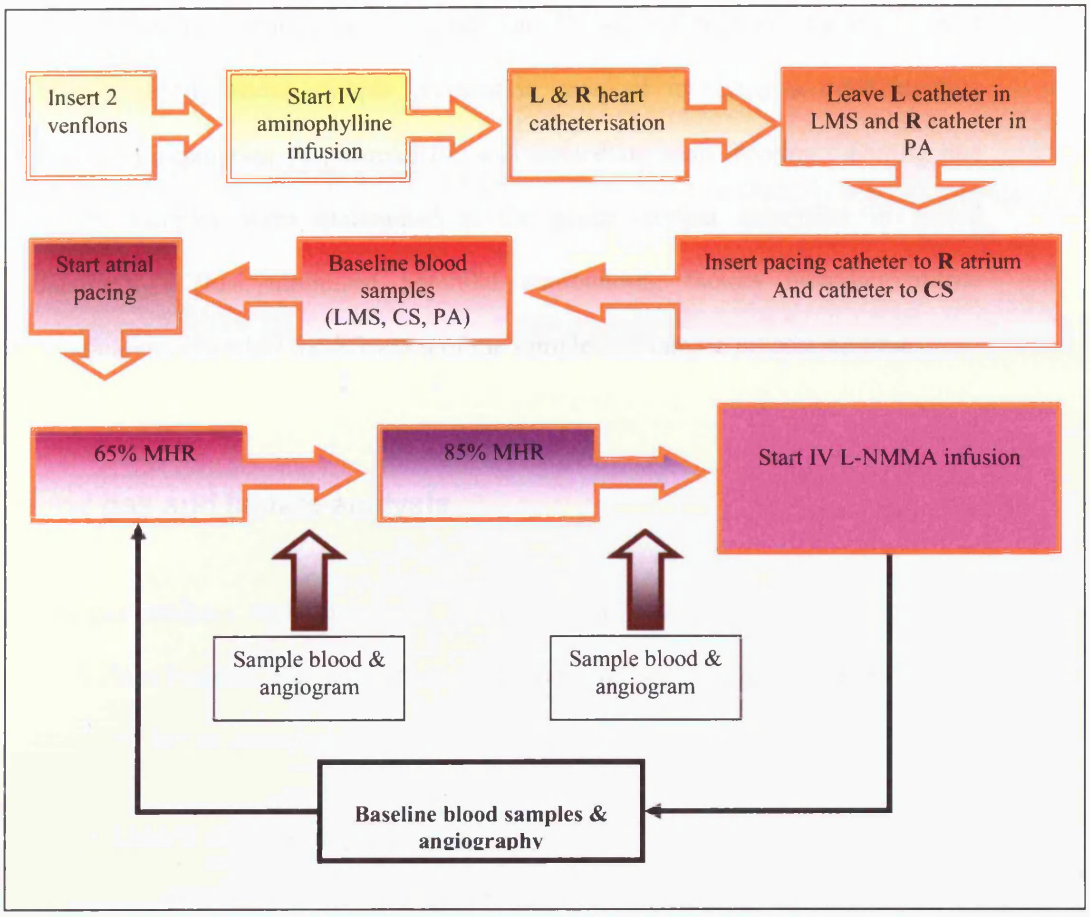


Figure 5.1: Clinical protocol. CS: coronary sinus, LMS: left main coronary artery, PA: pulmonary artery, L: left, R: Right, MHR: maximum heart rate.

Blood collection and storage

Blood samples were collected in 10ml syringes and transferred after blood gas analysis into 6ml gas tight EDTA vacutainers. These were centrifuged at 600g for 10 minutes at room temperature. The red cell fraction and plasma were immediately separated, snap frozen in liquid nitrogen and stored at -80°C for subsequent analysis. Although other authors have developed techniques to potentially inhibit in vitro

chemistry occurring during sample processing[284], we preferred to adhere to a well validated, strictly timed sample preparation protocol involving a centrifugation followed by separation of plasma/RBC and immediate snap freezing. During this time the samples were maintained at the given oxygen saturation in sealed vacutainers. This minimised potential confounding factors including nitrite contamination, chemical modification of the sample and sample processing time.

Blood gas and lactate analysis

Blood gas analysis was carried out immediately in the catheterisation laboratory (OSM3 Hemoximeter, Radiometer). In addition samples from the LMS and CS were assessed for lactate concentration.

Quantitative angiography

Angiography studies

GE's Centricity Cardiology CA1000 workstation was employed retrospectively for image review and analysis.

To detect changes in vessel diameter at each stage, quantitative coronary analysis (QCA) was performed in proximal, mid, and distal LAD and left circumflex (LCx) in right anterior oblique (RAO) view. Side branches of the relevant coronary artery were used as a reference guide to perform serial measurements at the same sites. The size of the intracoronary catheter was used for calibrating the arterial diameter. Cross sectional area (CSA) was calculated for each measurement point ($CSA = \pi r^2$, r =

vessel radius in centimetres) and an average of the three measurements was taken as the mean CSA of the vessel. Each of the above measurements was repeated three times and the average was taken into account. Measurements were standardised by dividing the calculated values to the baseline diameter of the coronary artery at rest at the beginning of the study.

Estimation of coronary flow

The principals of TIMI frame count method[302] were employed to estimate coronary blood flow (CBF). The number of cineframes needed for dye to reach standardised distal landmarks was counted for LAD and LCx separately. Frame count was converted to time (*t* in seconds) by being divided by 30, i.e. the number of frames in each second. The distance (*d* in cm) between proximal and distal landmarks was measured by QCA. Coronary flow velocity (CFV) was calculated as *d/t* (cm/s). CBF was taken as the product of CFV times average CSA of each LAD and LCx. Blood flow in LMS (LMS_{BF}) was calculated as the sum of blood flow in LAD (LAD_{BF}) and LCx (LCx_{BF}).

In summary, the following formulas were used:

CBF (ml/s) = CFV (cm/s) X mean CSA (cm^2)

CBF (ml/s) = [*d* (cm)/*t*(s)] [π r $(cm)^2$]

$LMS_{BF} = LAD_{BF} + LCx_{BF}$

Calculations

Formulas for calculating myocardial oxygen extraction, oxygen consumption, transpulmonary oxygenation, and transcardiac consumption/production of any metabolite are given in table 5.1.

Variable	**Formula**
Myocardial oxygen extraction (%)	$100(LMS_{O2} - CS_{O2})/ LMS_{O2}$
Myocardial Oxygen consumption (ml/min)	LMS_{BF} X $(LMS_{O2} - CS_{O2})$
Transpulmonary oxygenation (%)	$100(LMS_{O2} - PA_{O2})/ PA_{O2}$
Transcardiac "metabolite" consumption	LMS_{BF} X $(LMS_{[metabolite]} - CS_{[metabolite]})$
O_2 delivery (ml/min)	LMS_{O2} X BF

Table 5.1: LMS_{O2}: oxygen content in LMS (ml O_2/ml blood), CS_{O2}: oxygen content in CS (ml O_2/ml blood), PA_{O2}: oxygen content in PA (ml O_2/ml blood), LMS_{BF}: blood flow in LMS, $LMS_{[metabolite]}$: metabolite level in blood from LMS, $CS_{[metabolite]}$: metabolite level in blood from CS, BF: blood flow.

Biochemistry

Chemicals

Glacial acetic acid, HPLC grade nitrite free water and hydrochloric acid (HCl) were purchased from Fisher Scientific. The rest of the chemicals were purchased from Sigma.

NO measurements

Plasma NOx was measured using the DAN assay[283]. Tri-iodide based chemiluminescence was used to measure NO metabolites in plasma and RBC samples as described in CHAPTER FOUR. RBC NO analysis for this study was undertaken before our current method which enables us to distinguish between HbNO and SNO-Hb was validated. Therefore RBC-related NO metabolites are reported as RBC nitrite and total haemoglobin-bound NO (table 5.2).

Method	Yield	Reference
Fluorometry (DAN assay)	NOx (nitrate + nitrite)	Misko et al.[283]
Tri-iodide based chemiluminescence	Plasma nitrite, RSNO	Yang et al.[284]
Modified tri-iodide based chemiluminescence	RBC nitrite, Hb-bound NO	Rogers et al.[285]

Table 5.2: Methods of NO measurement and their yield in this study.

Data presentation and statistical analysis

All chemiluminescence signals were smoothed using Origin 7.0 and the area under the curve was analysed using Origin peak analysis. Data are presented as means ± SEM. Data were tested for normality using the Shapiro-Wilks test. To compare differences in means between groups a Student's paired *t* test was used for normally distributed data and a Wilcoxon matched pairs test for non-normally distributed data. For multiple comparisons (i.e. LMS, CS, PA) a repeated measures one way ANOVA was performed. A bivariate correlation (Pearson's correlation coefficient) assessed the relationship between Hb-bound NO, plasma nitrite, RBC nitrite, and HbO_2Sat (%). A two tailed P value < 0.05 was considered significant throughout.

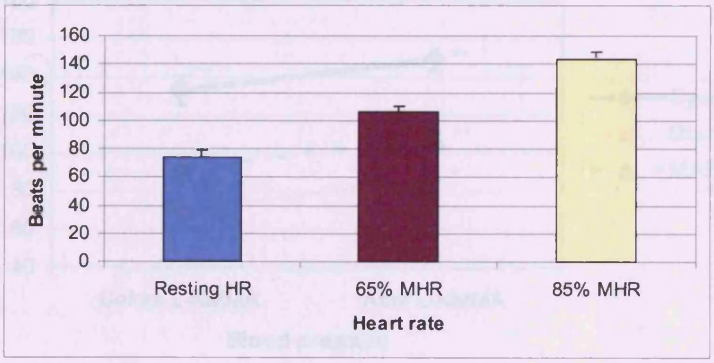
Results

The procedure was well tolerated by all the patients without any complications or side effects. None of the patients developed angina or 2nd degree heart block in response to rapid atrial pacing. None of the patients showed evidence of significant coronary artery disease on angiography.

Haemodynamic variables

Changes in heart rate (HR)

Maximum heart rate (MHR) was calculated as 220-age. Average HR at rest and following atrial pacing is shown in figure 5.2. Pacing increased heart rate from74±4.7 at rest to 106±4.6 and 143±5.5 at 65% and 85% MHR, respectively (P<0.0000001). This equals 43% and 93% increase in HR from baseline, respectively. L-NMMA infusion did not affect heart rate.



	Resting HR	65% MHR	85% MHR
Beats per minute	74±4.7	106±4.6	143±5.5

Figure 5.2: Changes in heart rate with pacing at 65% and 85% maximum heart rate (MHR).

Values represent mean ± SEM.

Changes in blood pressure

Right arm blood pressure was measured using a standard automatic inflation sphygmomanometer. Mean arterial pressure (MAP) was calculated as:

MAP = [(2 x diastolic) +systolic] / 3

L-NMMA infusion increased systolic, diastolic and mean arterial blood pressure as summarised in table 5.3 and illustrated in figure 5.3.

	Baseline	+L-NMMA
Systolic BP (mmHg)	133±8	149±6** (P<0.01)
Diastolic BP (mmHg)	70±6	87±2* (P<0.05)
MAP (mmHg)	91±6	108±3** (P<0.01)

Table 5.3: Blood pressure (BP) data for subjects comparing baseline to post L-NMMA infusion. MAP: mean arterial pressure. * denotes significant difference.

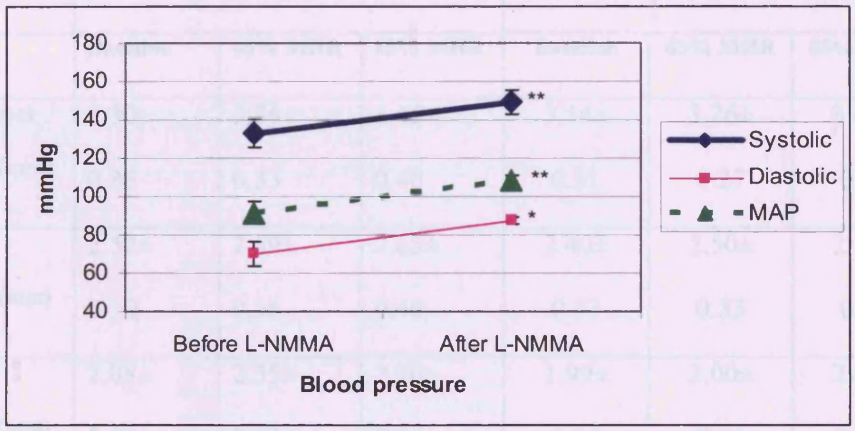


Figure 5.3: Systolic, diastolic and mean arterial blood pressure increased after L-NMMA infusion (P<0.01, *P<0.05).**

Pacing did not have a significant effect on blood pressure.

Coronary artery diameter

Summary: Inhibition of NO synthesis decreased LAD diameter. Pacing increased LAD diameter but reached statistical significance only after L-NMMA infusion. The extent of this effect was dependent on the anatomical location with smaller and more distal segments showing higher relative (percent) increase in diameter.

Coronary vasodilatation in response to pacing was studied before and after L-NMMA infusion in LAD. Overall, a similar trend was observed along the LAD: LAD constricted with L-NMMA and dilated with pacing. The extent of this effect was dependent on the anatomical location (see below). Vessel diameter measurements from proximal, middle, and distal LAD are given in table 5.4.

	Before L-NMMA			After L-NMMA		
	Baseline	**65% MHR**	**85% MHR**	**Baseline**	**65% MHR**	**85% MHR**
Proximal LAD (mm)	3.30± 0.35	3.36± 0.33	3.45± 0.40	3.14± 0.31	3.26± 0.27	3.44± 0.25
Mid LAD (mm)	2.52± 0.38	2.59± 0.36	2.65± 0.40	2.40± 0.37	2.50± 0.33	2.71± 0.41
Distal LAD (mm)	2.08± 0.40	2.35± 0.43	2.20± 0.58	1.99± 0.35	2.00± 0.36	2.42± 0.47

Table 5.4: Vessel diameter measurements from proximal, middle, and distal LAD (mm).

Effect of L-NMMA infusion at rest- L-NMMA infusion decreased vessel diameter in proximal LAD by 5.6±1.3 % (P=0.06) and in mid LAD by 3.4± 1.2 % (P<0.05). Distal LAD did not constrict significantly in response to L-NMMA (figure 5.4).

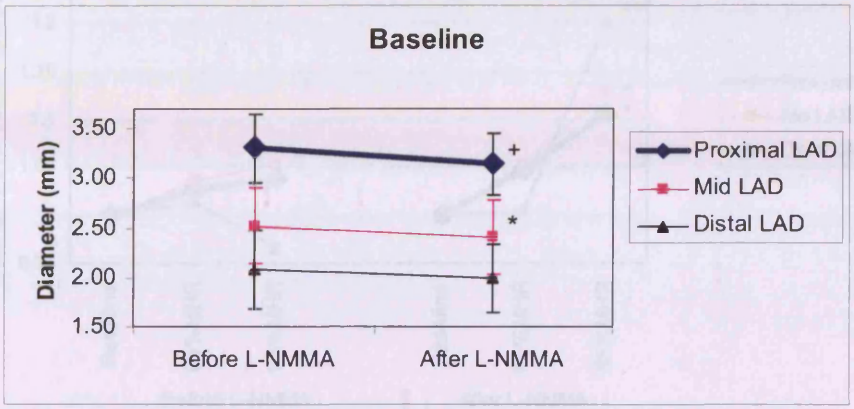


Figure 5.4: Changes in LAD diameter in response to L-NMMA at rest. (+ P=0.06, * P<0.05).

Effect of pacing- (Figure 5.5) LAD diameter increased in response to pacing in all three segments but reached statistical significance only after L-NMMA infusion.

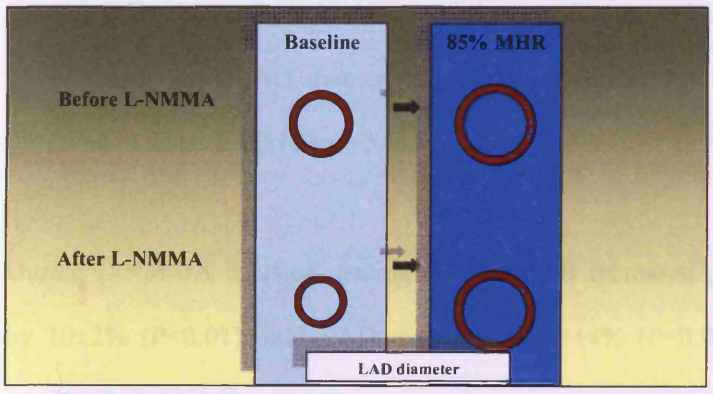


Figure 5.5: Changes in vessel diameter in response to pacing before and after L-NMMA infusion.

When vessel diameters were standardised for baseline (i.e. measured vessel diameter/baseline vessel diameter), this increase was more significant after L-NMMA

infusion (P value for proximal and mid LAD<0.01, P value for distal LAD<0.001) (figure 5.6).

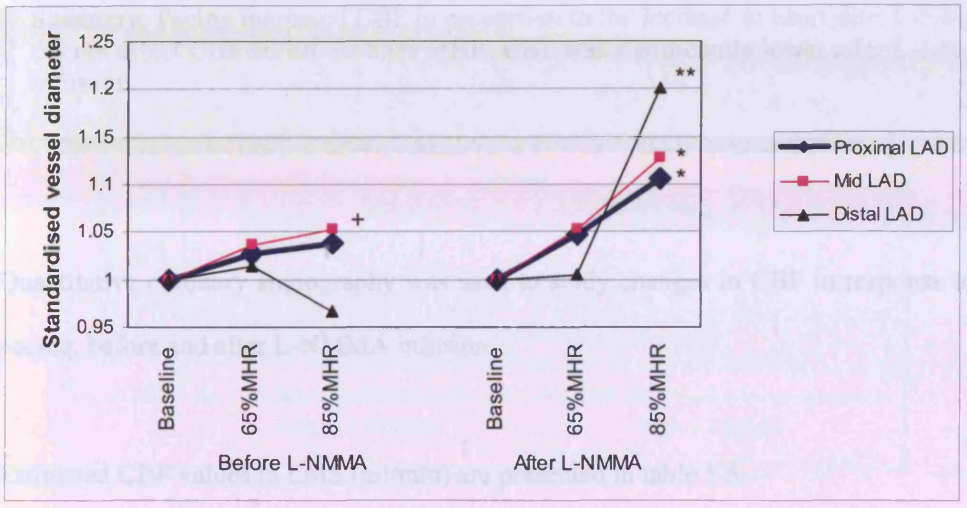


Figure 5.6: Standardised changes in vessel diameter in response to pacing before and after L-NMMA infusion. (+ P<0.05, * P<0.01, **P<0.001).

Before L-NMMA infusion, pacing at 85% MHR, i.e. approximately twice the resting heart rate for the subjects in this study, increased proximal LAD diameter by 4±2% (P=0.16) and mid LAD diameter by 5±1% (P<0.05). No significant change was observed in distal LAD (figure 5.6).

During L-NMMA infusion, pacing at 85% MHR increased proximal LAD diameter by 10±2% (P<0.01), mid LAD diameter by 13±4% (P<0.01), and distal LAD by 20±4% (P<0.001) (figure 5.6).

Coronary blood flow (CBF)

Summary: Pacing increased CBF in proportion to the increase in heart rate. L-NMMA did not affect CBF at rest. At 85% MHR, CBF was significantly lower after L-NMMA infusion.

Quantitative coronary angiography was used to study changes in CBF in response to pacing, before and after L-NMMA infusion.

Estimated CBF values in LMS (ml/min) are presented in table 5.5.

	Before L-NMMA			After L-NMMA		
	Baseline	**65% MHR**	**85% MHR**	**Baseline**	**65% MHR**	**85% MHR**
LMS flow (ml/min)	85 ±10	134 ±17	181± 25	77± 9	122± 18	143 ±19

Table 5.5: Estimated blood flow in LMS (ml/min) throughout the study.

Effect of L-NMMA infusion- Coronary flow was not reduced significantly by L-NMMA at rest (P=0.15) or 65% MHR (P=0.35). At 85% MHR, coronary flow was 20±5% lower following L-NMMA infusion (P<0.05) (figure 5.7).

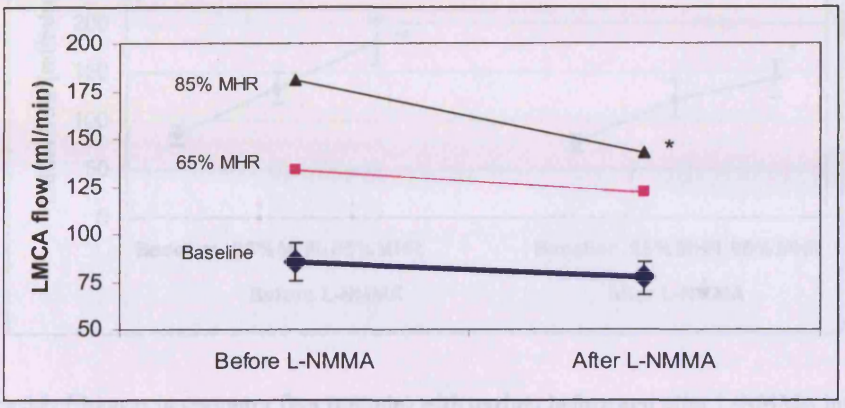


Figure 5.7: LMS flow before and after L-NMMA infusion. CBF was not significantly altered by L-NMMA at rest and 65% MHR. When the heart was paced at 85%MHR, L-NMMA reduced coronary flow by 20±5% (* P<0.05).

Effect of pacing- Pacing increased coronary flow both before (P<0.01) and after (P<0.05) L-NMMA infusion (figure 5.8).

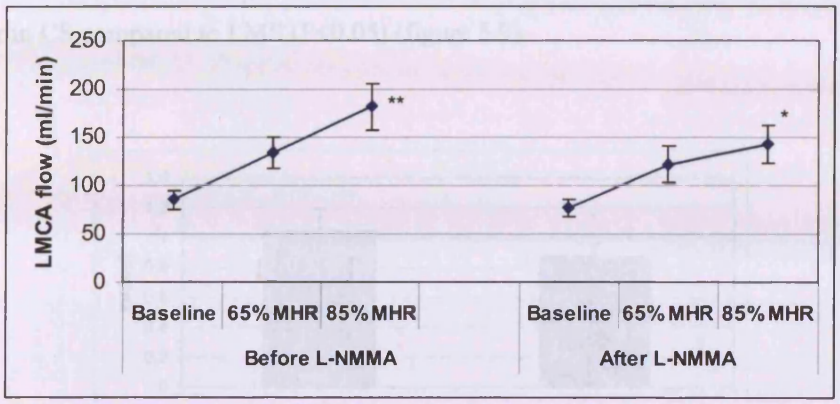


Figure 5.8: Changes in coronary flow (ml/min) with pacing; before and after L-NMMA infusion (ANOVA * P<0.05, ** P<0.01).

Pacing the heart at 85% MHR, i.e. approximately twice the resting heart rate for the subjects in this study, increased the coronary flow by 113±15 % and 86±17% before(P<0.01) and after(P<0.01) L-NMMA infusion, respectively. The magnitude of increase (i.e. comparing 113±15 % to 86±17%) was not statistically different before and after L-NMMA (P=0.3).

Lactate

Lactate was measured in blood from LMS and CS at rest and only in CS after atrial pacing (normal levels in peripheral venous blood <2mmol/l). Lactate levels were lower in CS compared to LMS (P<0.05) (figure 5.9).

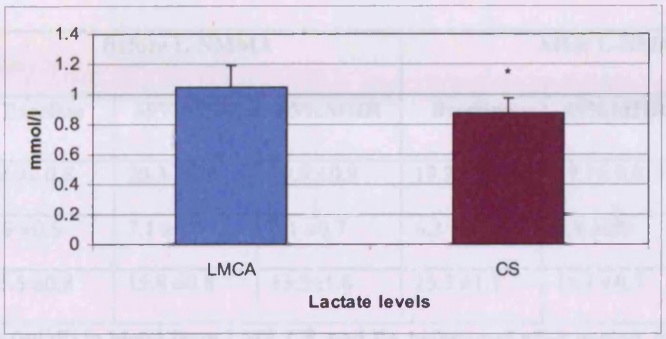


Figure 5.9: Lactate levels in blood from LMS and CS. *P<0.05.

Pacing did not affect CS lactate levels (figure 5.10).

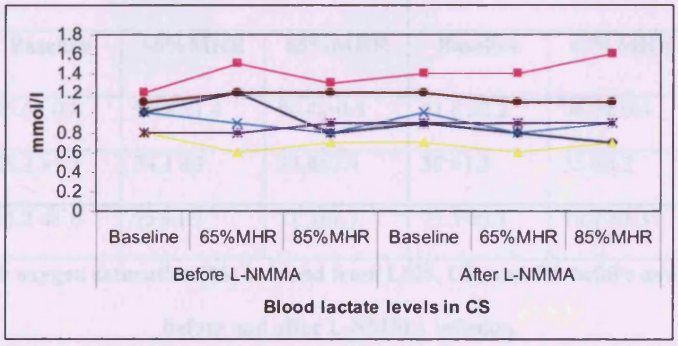


Figure 5.10: Lactate levels in blood from CS throughout the study protocol. CS lactate levels were not affected by pacing.

Oxygen

Levels

O_2ct and HbO_2Sat of the blood samples taken during the study are given in tables 5.6 and 5.7, respectively.

	Before L-NMMA			After L-NMMA		
	Baseline	**65%MHR**	**85%MHR**	**Baseline**	**65%MHR**	**85%MHR**
LMS	19.9± 0.8	20.3 ±0.8	19.9 ±0.9	19.2 ±0.9	19.7± 0.8	20 ±0.7
CS	5.9 ±0.5	7.1 ±0.7	7.1 ±0.7	6.2 ±0.5	6.8 ±0.9	7.8 ±1.0
PA	15.5 ±0.8	15.8 ±0.8	13.5±1.8	15.3 ±1.1	15.1 ±0.7	14.1 ±0.8

Table 5.6: O_2ct (ml/dl) in blood from LMS, CS, and PA before and after pacing, before and after L-NMMA infusion.

	Before L-NMMA			After L-NMMA		
	Baseline	**65%MHR**	**85%MHR**	**Baseline**	**65%MHR**	**85%MHR**
LMS	95.6± 0.8	94.8 ±1.4	96.4 ±0.4	93.8 ±2.2	96.7± 0.4	97.1 ±0.4
CS	28.2 ±1.3	34.1 ±3	34.4±2.4	30 ±1.5	35 ±3.2	39 ±3.4
PA	74.2 ±1.6	75 ±1.5	71.3±1.7	73.3 ±3.1	73.7 ±1.3	68.8 ±1.9

Table 5.7: Hb oxygen saturation (%) in blood from LMS, CS, and PA before and after pacing, before and after L-NMMA infusion.

Physiological differences at baseline

At baseline heart rate, haemoglobin oxygen saturation (HbO_2Sat) (%) and blood oxygen content (O_2ct) (ml/dl) decreased significantly across the heart ($P<0.0001$), increased significantly when CS effluent mixed with systemic venous return in the PA ($P<0.0001$) and increased further across the lungs ($P<0.0001$) (figures 5.11 and 5.12).

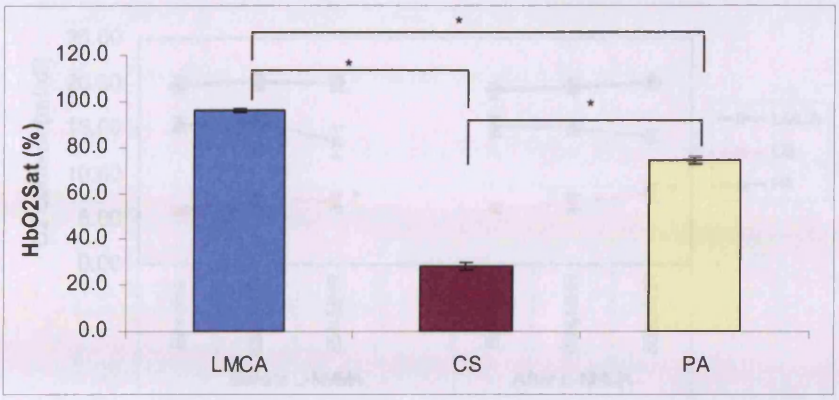


Figure 5.11: Baseline HbO_2Sat in the LMS, CS, and PA (* P<0.0001).

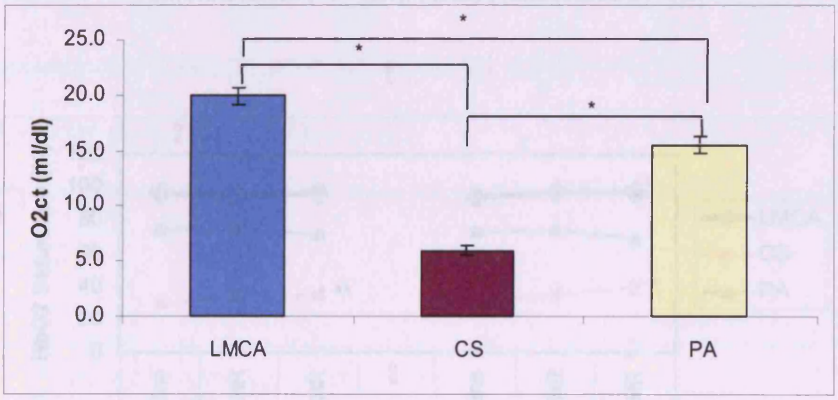


Figure 5.12: Baseline O_2ct in the LMS, CS, and PA (* P<0.0001).

Effect of L-NMMA infusion- L-NMMA infusion did not change the oxygen content (O_2ct) or haemoglobin oxygen saturation (HbO_2Sat) of blood taken from any of the anatomical sites.

Pacing

Changes in O_2ct and HbO_2Sat with pacing before and after L-NMMA infusion are illustrated in figures 5.13 and 5.14.

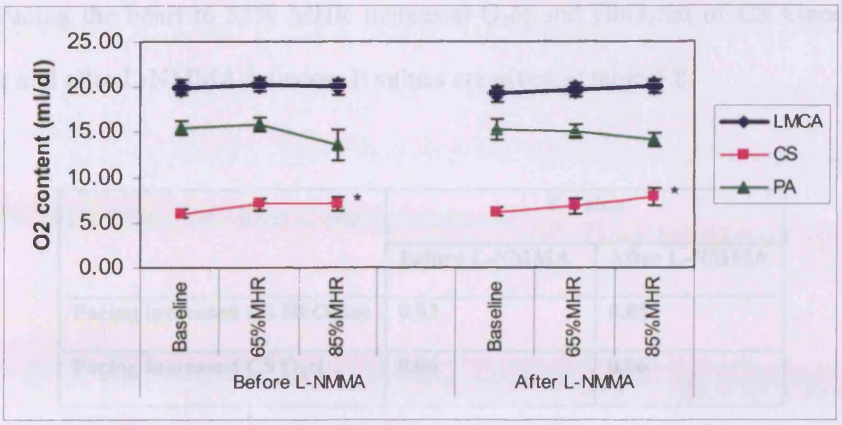


Figure 5.13: Changes in O_2ct of blood from LMS, CS, and PA with pacing, before and after L-NMMA infusion (* P=0.06). MHR: Maximum heart rate.

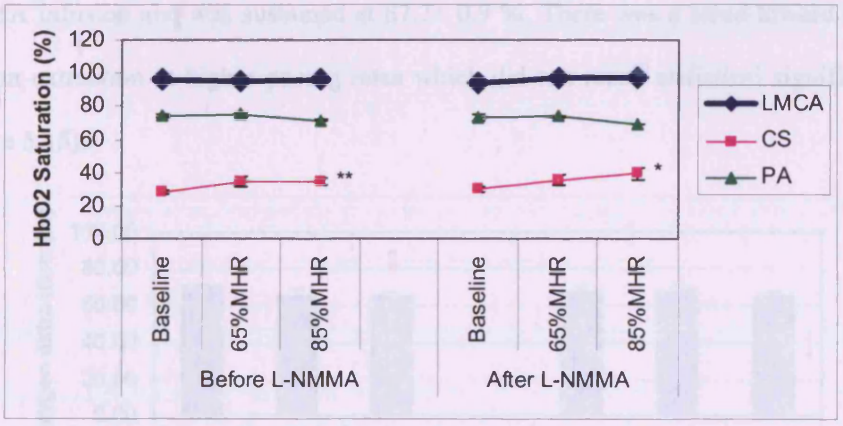


Figure 5.14: Changes in Hb oxygen saturation of blood from LMS, CS, and PA with pacing, before and after L-NMMA infusion (* P=0.05, ** P<0.05). MHR: Maximum heart rate.

LMS- Atrial pacing did not affect oxygen measurements in blood from LMS.

PA- There was a trend towards a decrease in O_2ct and HbO_2Sat in PA blood with pacing which did not reach statistical significance.

CS- Pacing the heart to 85% MHR increased O_2ct and HbO_2Sat of CS blood both before and after L-NMMA infusion. P values are given in table 5.8:

	P Value	
	Before L-NMMA	**After L-NMMA**
Pacing increased CS HbO_2Sat	**0.03**	**0.05**
Pacing increased CS O_2ct	**0.06**	**0.06**

Table 5.8: P values for changes in CS HbO_2Sat and CS O_2ct before and after L-NMMA infusion.

Myocardial oxygen extraction

Oxygen extraction across the heart did not change significantly with pacing or after L-NMMA infusion and was sustained at 67.2± 0.9 %. There was a trend toward lower oxygen extraction at higher pacing rates which did not reach statistical significance (figure 5.15).

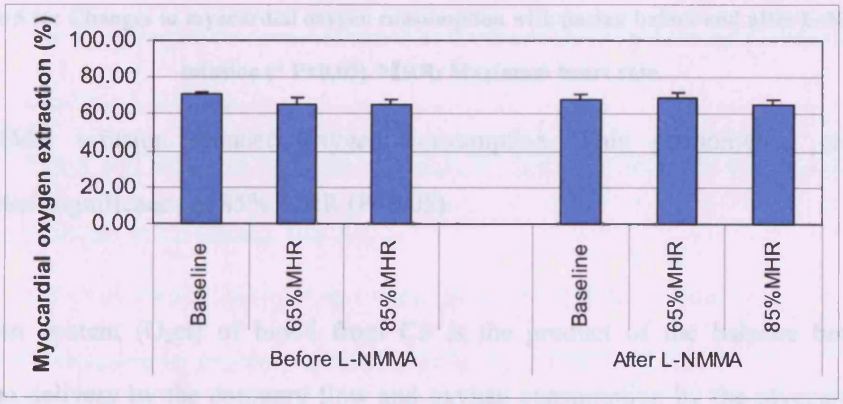


Figure 5.15: Changes in myocardial oxygen extraction with pacing before and after L-NMMA infusion. MHR: Maximum heart rate.

Myocardial oxygen consumption

Oxygen consumption (ml O_2/min) increased in proportion to increasing HR both before ($P<0.05$) and after ($P<0.05$) L-NMMA infusion (figure 5.16). On average, 91% increase in HR was associated with 100% increase in oxygen consumption before L-NMMA and 88% increase after L-NMMA infusion.

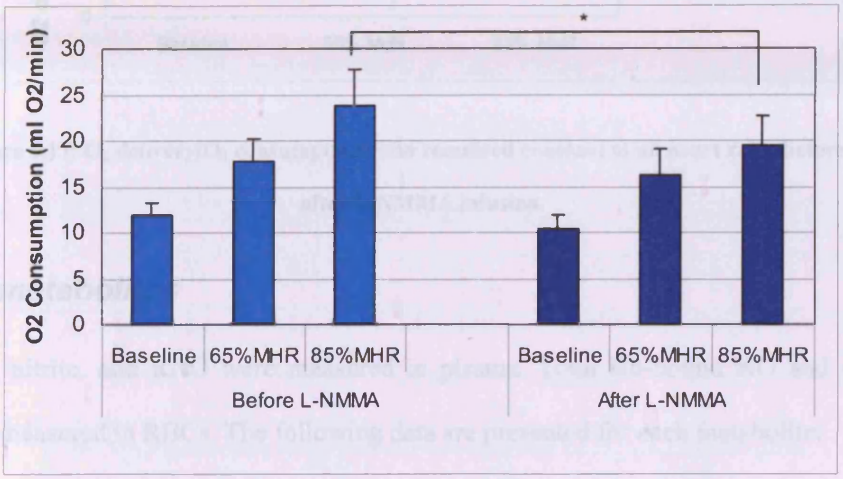


Figure 5.16: Changes in myocardial oxygen consumption with pacing before and after L-NMMA infusion (* $P<0.05$). MHR: Maximum heart rate.

L-NMMA infusion reduced oxygen consumption. This phenomenon reached statistical significance at 85% MHR ($P<0.05$).

Oxygen content (O_2ct) of blood from CS is the product of the balance between oxygen delivery by the coronary flow and oxygen consumption by the myocardium. This balance was maintained at an almost constant ratio at higher oxygen consumption rates regardless of NO production. Figure 5.17 demonstrates the ratio between oxygen delivery and oxygen consumption in different HR stages of the study protocol before and after L-NMMA infusion.

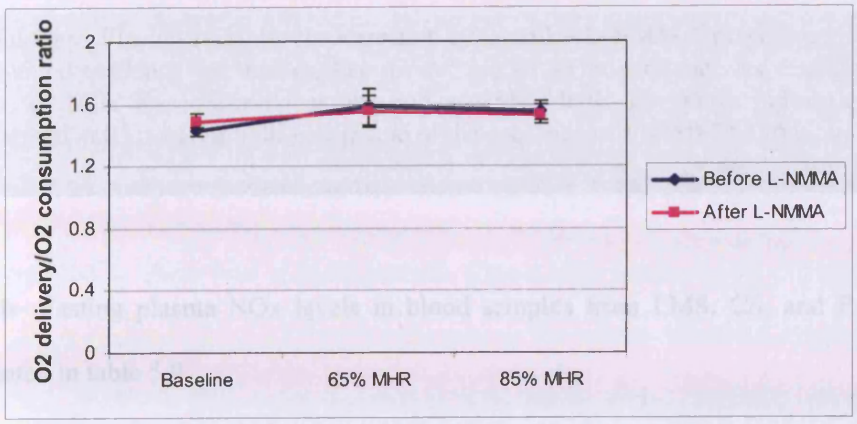


Figure 5.17: O_2 delivery/O_2 consumption ratio remained constant at all heart rates before and after L-NMMA infusion.

NO metabolites

NOx, nitrite, and RNO were measured in plasma. Total Hb-bound NO and nitrite were measured in RBCs. The following data are presented for each metabolite:

- **Levels**: Metabolite levels at rest (baseline) and after pacing; before and after L-NMMA infusion.
- **Effect of L-NMMA infusion:** comparing metabolite levels before and in the presence of L-NMMA infusion
- **Effect of Pacing:** comparing metabolite levels at different heart rates.
- **Transcardiac:** comparing metabolite levels in LMS and CS.
- **Transpulmonary:** comparing metabolite levels in PA and LMS.

Plasma NOx

Summary: Plasma NOx levels were not affected by L-NMMA or pacing. The study provided evidence for transcardiac production of NOx. Evidence for transpulmonary loss of NOx was observed at rest and at 65% MHR. The latter phenomenon was reversed at 85% MHR both before and in the presence of L-NMMA infusion.

Levels- Fasting plasma NOx levels in blood samples from LMS, CS, and PA are presented in table 5.9.

	Before L-NMMA			After L-NMMA		
	Baseline	**65% MHR**	**85% MHR**	**Baseline**	**65% MHR**	**85% MHR**
LMS	11.5±2.8	14.4±3.5	14.1±4.2	12.9±3.8	16.3±3.7	15.9±3.8
CS	14.0±3.6	15.5±3	15.5±4.2	15.5±3.7	16.9±3.8	12.7±3.7
PA	15.5±3.7	18.8±4	11.8±3.3	16.9±3.5	18.1±3.7	13.4±3.6

Table 5.9: Plasma NOx levels in blood samples from LMS, CS, and PA (µM).

Changes in plasma NOx levels with pacing before and after L-NMMA infusion are illustrated in figure 5.18

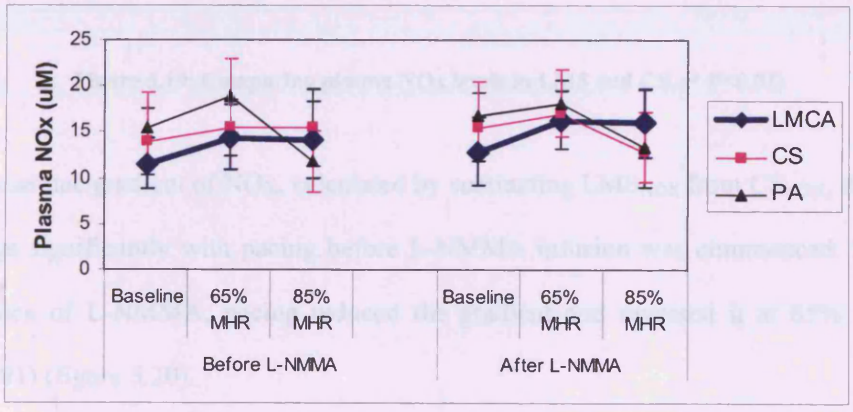


Figure 5.18: Changes in plasma NOx levels (µM) with pacing, before and after L-NMMA infusion. Neither pacing nor L-NMMA affected NOx levels.

Effect of L-NMMA infusion- Plasma NOx levels did not change after L-NMMA infusion in any of the sample sites at any pacing rate.

Effect of pacing- Pacing did not affect plasma NOx levels.

Transcardiac - There was a trend towards higher NOx levels in CS compared to LMS which reached statistical significance only in the resting (baseline) sample after L-NMMA ($P<0.01$). This trend reversed at 85% MHR after L-NMMA; where CS NOx was consistently lower than LMS ($P<0.01$) (figure 5.19).

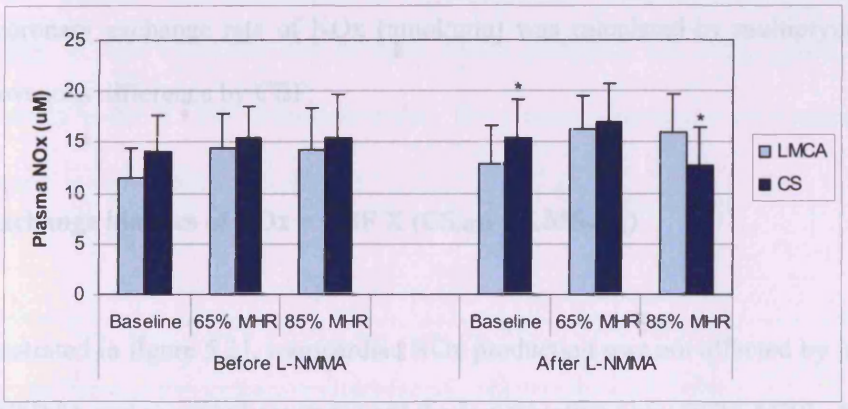


Figure 5.19: Comparing plasma NOx levels in LMS and CS. (* P<0.01)

Transcardiac gradient of NOx, calculated by subtracting LMS_{NOX} from CS_{NOX}, did not change significantly with pacing before L-NMMA infusion was commenced. In the presence of L-NMMA, pacing reduced the gradient and reversed it at 85% MHR ($P<0.01$) (figure 5.20).

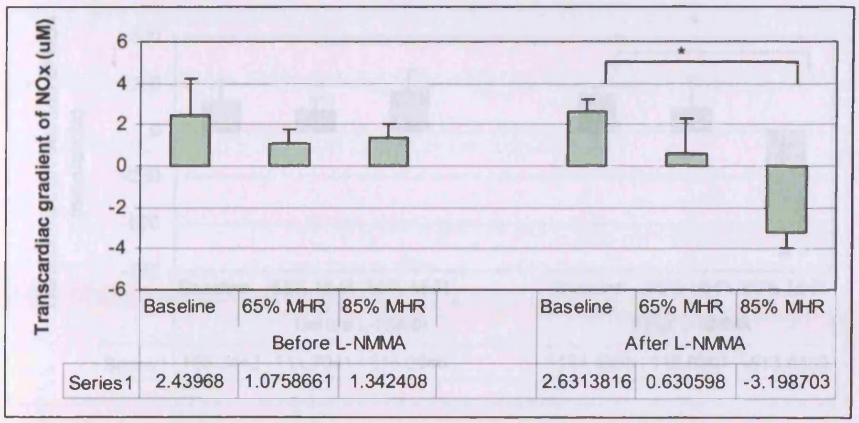


Figure 5.20: Transcardiac gradient of NOx at rest and during pacing, before and after L-NMMA infusion (* P<0.01).

The coronary exchange rate of NOx (nmol/min) was calculated by multiplying the arteriovenous difference by CBF:

Net exchange kinetics of NOx = CBF X (CS_{NOX} – LMS_{NOX})

As illustrated in figure 5.21, transcardiac NOx production was not affected by pacing or L-NMMA and remained constant until the last sampling point (85% MHR after L-NMMA) where a net loss of NOx was observed (P<0.01).

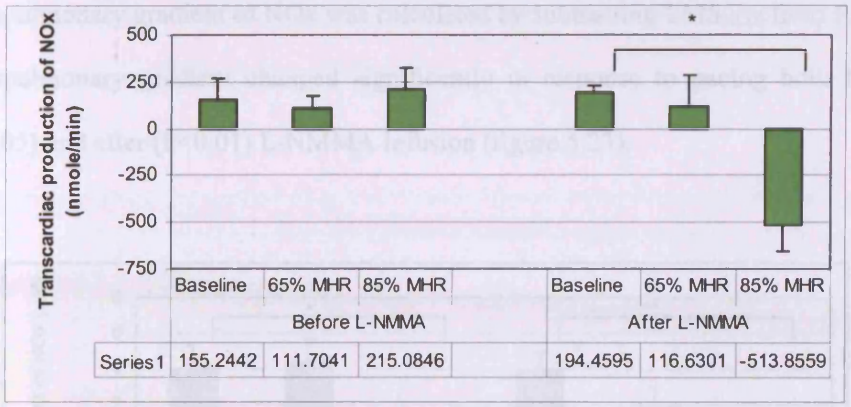


Figure 5.21: Transcardiac exchange of NOx at rest and during pacing, before and after L-NMMA infusion (* P<0.01).

Transpulmonary - A negative gradient was observed in plasma NOx levels across the lungs at rest (P<0.05) and at 65% MHR (P<0.01), i.e. NOx levels were consistently lower in LMS compared to PA. The gradient reversed at 85% MHR both before and after L-NMMA infusion (P=0.06) (figure 5.22).

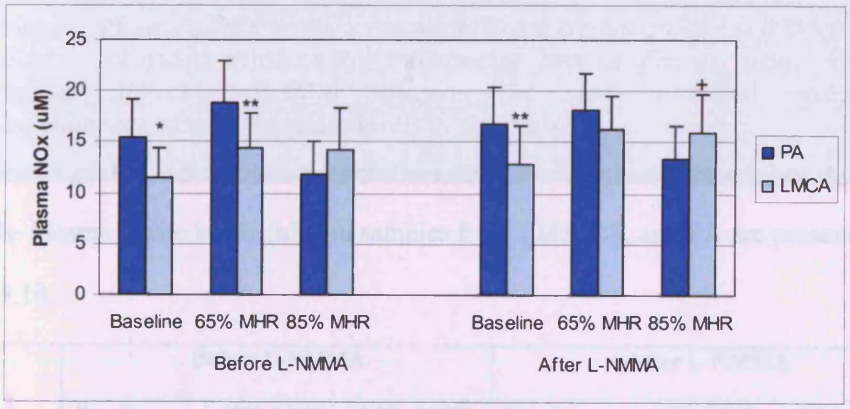


Figure 5.22: Comparing plasma NOx levels in PA and LMS (* P<0.05, ** P<0.01, + P=0.06).

Transpulmonary gradient of NOx was calculated by subtracting LMS_{NOX} from PA_{NOX}. Transpulmonary gradient changed significantly in response to pacing both before ($P<0.05$) and after ($P<0.01$) L-NMMA infusion (figure 5.23).

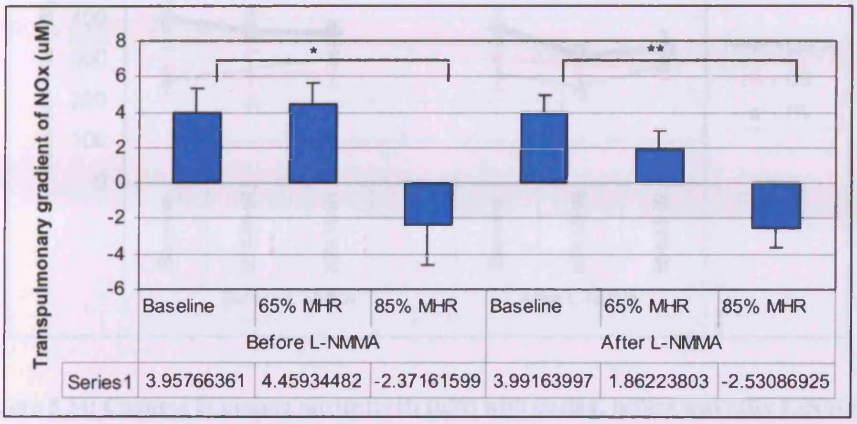


Figure 5.23: Changes in transpulmonary gradient of NOx with pacing, before and after L-NMMA infusion (* P<0.05, ** P<0.01)

Plasma nitrite

Summary: Plasma nitrite levels were not affected by pacing or L-NMMA infusion. The study provided evidence for transcardiac loss of plasma nitrite which was significant before L-NMMA infusion. The study provided evidence for transpulmonary increase in nitrite levels in plasma.

Levels- Plasma nitrite levels (nM) in samples from LMS, CS, and PA are presented in table 5.10.

	Before L-NMMA			After L-NMMA		
	Baseline	**65% MHR**	**85% MHR**	**Baseline**	**65% MHR**	**85% MHR**
LMS	396.3±50	368.3±46	363.4±26	376.3±41	311.4±23	327.5±42
CS	271.5±56	256.5±65	276.1±43	302.3±47	282.1±41	284.7±36
PA	247.1±54	281±68	300.3±35	269±43	234.2±29	285.1±37

Table 5.10: Plasma nitrite levels (nM).

Changes in plasma nitrite levels with pacing before and after L-NMMA infusion are illustrated in figure 5.24.

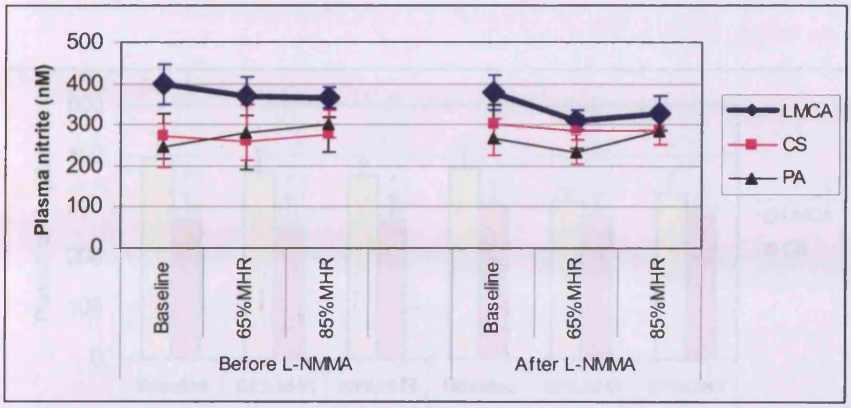


Figure 5.24: Changes in plasma nitrite levels (nM) with pacing, before and after L-NMMA infusion. Neither pacing nor L-NMMA infusion affected nitrite levels significantly.

Effect of L-NMMA infusion- L-NMMA infusion did not affect plasma nitrite levels in any of the sample sites at any pacing rate.

Effect of pacing- Pacing did not affect plasma nitrite levels.

Transcardiac- Plasma nitrite levels were generally lower in CS than in LMS. The difference was significant before L-NMMA infusion at 65% MHR and 85% MHR ($P<0.05$) (figure 5.25).

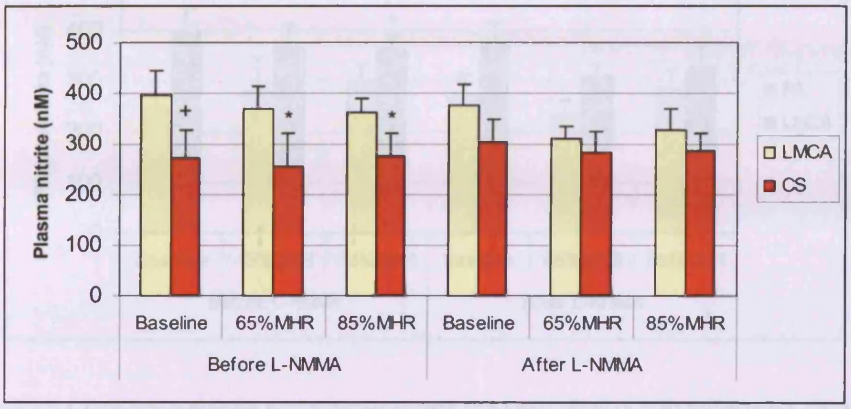


Figure 5.25: Comparing plasma nitrite levels in LMS and CS (+ P=0.15, * P<0.05).

When transcardiac nitrite gradient was corrected for flow, a general "loss" of nitrite was observed across the coronary bed which was not significantly affected by pacing. The "loss" was a smaller amount in the presence of L-NMMA ($P<0.04$) (figure 5.26).

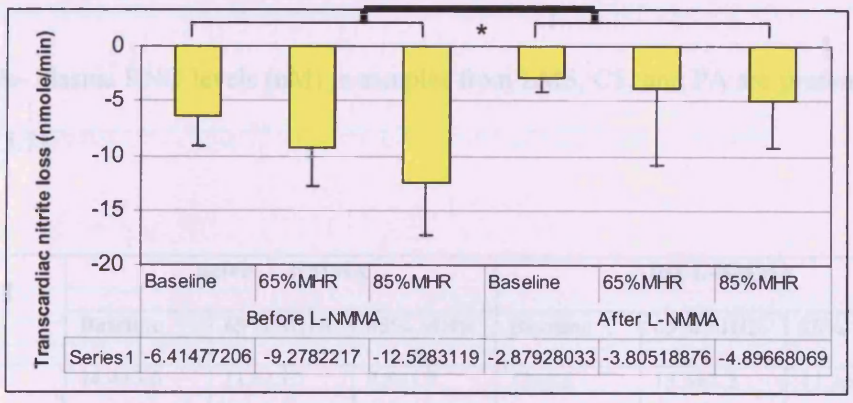


Figure 5.26: Transcardiac loss of plasma nitrite (nmol/min) (* P<0.05).

Transpulmonary- Plasma nitrite levels were higher in LMS comparing to PA, indicating a positive gradient across the lungs (figure 5.27).

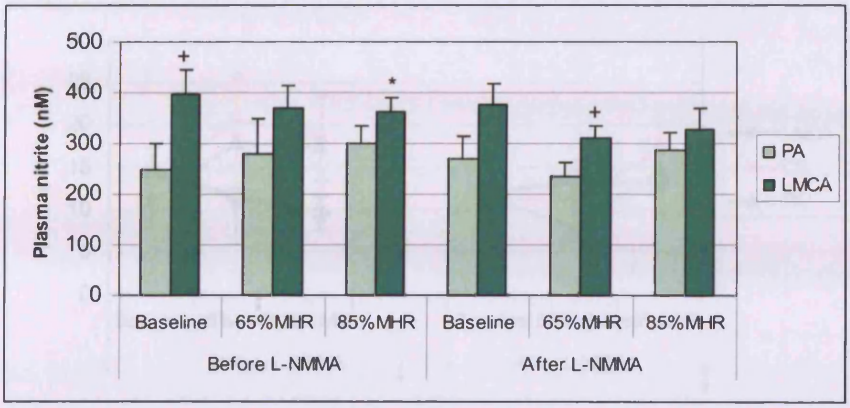


Figure 5.27: Comparing plasma nitrite levels before (PA) and after (LMS) pulmonary circulation (+ P<0.1, * P<0.05).

Plasma RNO

Summary: Plasma RNO levels were not altered by pacing or L-NMMA infusion. No significant transcardiac or transpulmonary gradient was found.

Levels- Plasma RNO levels (nM) in samples from LMS, CS, and PA are presented in table 5.11.

	Before L-NMMA			After L-NMMA		
	Baseline	**65% MHR**	**85% MHR**	**Baseline**	**65% MHR**	**85% MHR**
LMS	14.4±3.0	11.5±5.7	9.5±1.9	12±0.8	13.5±2.2	13.2±0.9
CS	13.2±1.8	20.7±5.3	18.5±5.0	11.2±1.0	11.2±3.2	17.1±3.0
PA	11±2.3	17.9±7.5	17.4±6.0	11.4±3.1	7.83±1.5	14.6±5.0

Table 5.11: Plasma RNO levels (nM).

Changes in plasma RNO levels with pacing before and after L-NMMA infusion are illustrated in figure 5.28.

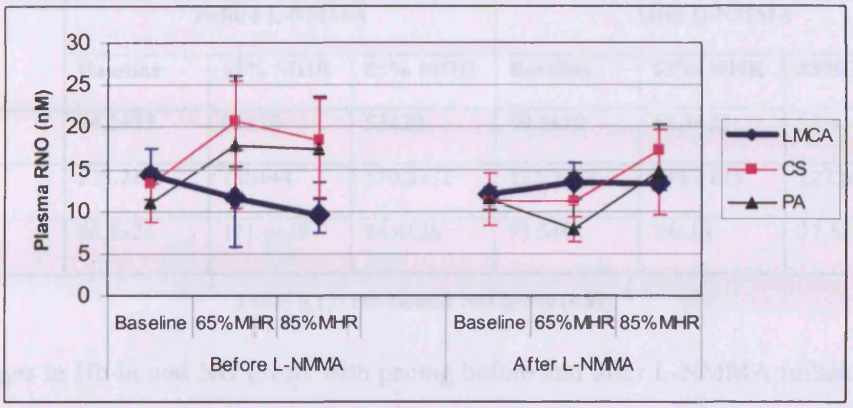


Figure 5.28: Changes in plasma RNO levels (nM) with pacing, before and after L-NMMA infusion. Neither pacing nor L-NMMA infusion affected RNO levels significantly.

Effect of L-NMMA infusion- L-NMMA infusion had no effect on RNO levels.

Effect of pacing- Pacing had no effect on RNO levels.

Transcardiac- No significant transcardiac gradient was observed.

Transpulmonary- No significant transpulmonary gradient was observed.

Total Hb-bound NO

Summary: Total Hb-bound NO levels were not affected by L-NMMA or pacing. The study provided evidence for transcardiac formation of Hb-bound NO. No significant transpulmonary gradient was detected.

Levels- Hb-bound NO levels (nM) in samples from LMS, CS, and PA are presented in table 5.12.

	Before L-NMMA			After L-NMMA		
	Baseline	**65% MHR**	**85% MHR**	**Baseline**	**65% MHR**	**85% MHR**
LMS	74.2±33	91±22	53±20	59.5±22	62.3±22	52.4±17
CS	228.2±77	188±44	170.2±71	132.2±47	149.1±25	127.6±27
PA	96.8±26	111.4±38	84.4±26	75.5±9	75±18	77.5±18

Table 5.12: Hb-bound NO levels (nM).

Changes in Hb-bound NO levels with pacing before and after L-NMMA infusion are illustrated in figure 5.29.

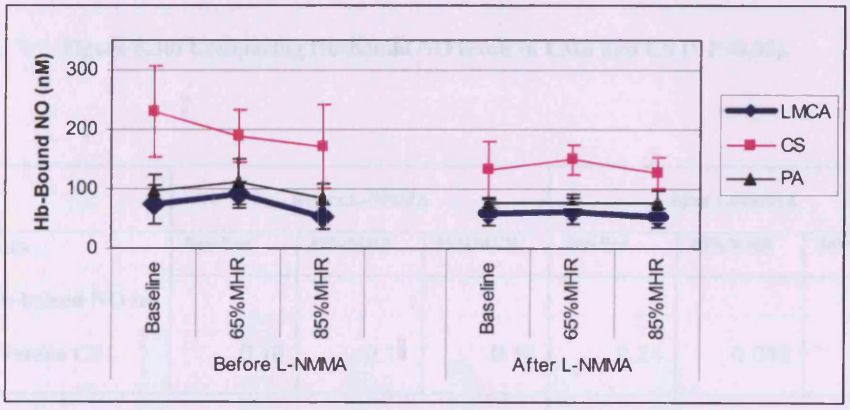


Figure 5.29: Changes in Hb-bound NO levels (nM) with pacing, before and after L-NMMA infusion. Neither pacing nor L-NMMA infusion affected Hb-bound NO levels significantly.

Effect of L-NMMA infusion- L-NMMA infusion did not alter Hb-bound NO levels at any heart rate.

Effect of pacing- pacing did not affect Hb-bound NO levels in blood samples from LMS, CS, and PA.

Transcardiac- Hb-bound NO levels were higher in CS than LMS at baseline and higher pacing rates indicating transcardiac formation of Hb-bound NO. This pattern was not affected by L-NMMA infusion (figure 5.30). P values are given in table 5.13

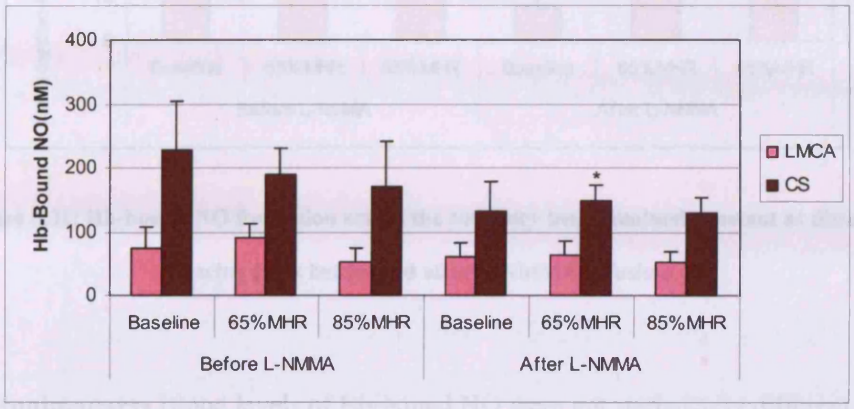


Figure 5.30: Comparing Hb-bound NO levels in LMS and CS (* P<0.05).

	Before L-NMMA			After L-NMMA		
P values	**Baseline**	**65%MHR**	**85%MHR**	**Baseline**	**65%MHR**	**85%MHR**
for Hb-bound NO in LMS versus CS	0.18	0.11	0.18	0.24	0.048	0.09

Table 5.13: P values for figure 5.30, comparing Hb-bound NO levels in LMS and CS.

Transcardiac formation of Hb-bound NO, calculated by multiplying transcardiac gradient by CBF, remained constant at different heart rates and was not affected by L-NMMA infusion (figure 5.31).

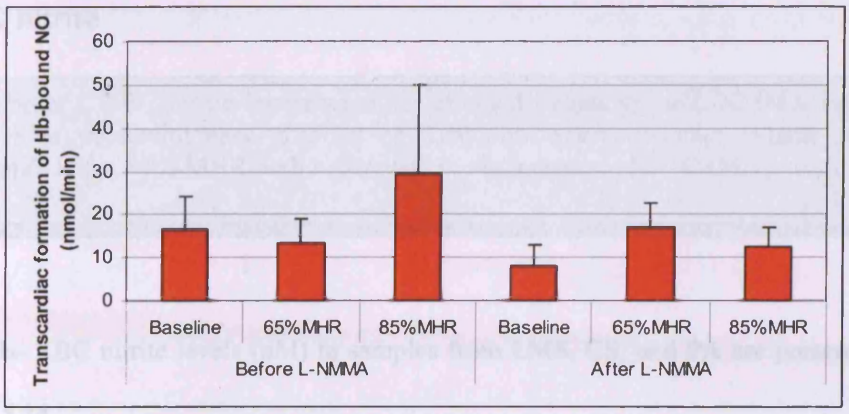


Figure 5.31: Hb-bound NO formation across the coronary bed remained constant at different pacing rates before and after L-NMMA infusion.

Transpulmonary- Blood levels of Hb-bound NO were not statistically different across the pulmonary bed (figure 5.32).

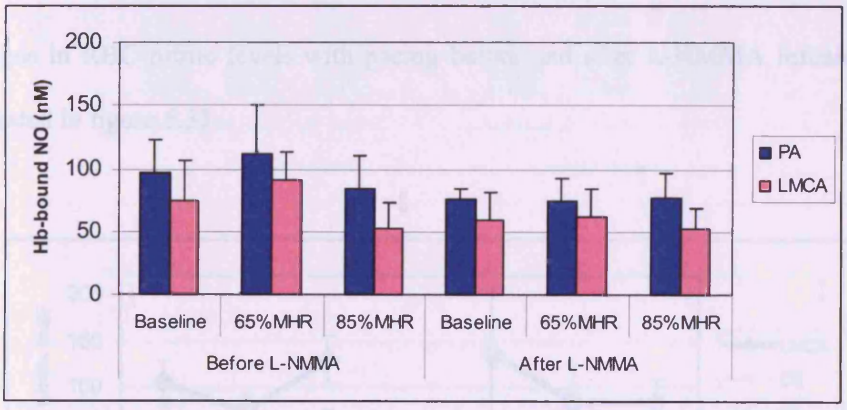


Figure 5.32: Comparing Hb-bound NO levels in PA and LMS. Hb-bound NO levels did not change across the lungs.

RBC nitrite

Summary: RBC nitrite levels were not affected by pacing or L-NMMA infusion. A positive transpulmonary gradient of RBC nitrite was detected which was more significant at 85% MHR both before and in the presence of L-NMMA infusion.

Levels- RBC nitrite levels (nM) in samples from LMS, CS, and PA are presented in table 5.14.

	Before L-NMMA			After L-NMMA		
	Baseline	**65% MHR**	**85% MHR**	**Baseline**	**65% MHR**	**85% MHR**
LMS	105.9±25	74.2±13	129.7±27	137.8±69	86.2±37	85.0±26
CS	66.4±23	70.1±23	65.2±11	32.0±9.3	90.1±34	43.6±27
PA	47.3±15	38.7±20	51.7±17	41.2±16	46.6±30	16.2±7

Table 5.14: RBC nitrite levels (nM).

Changes in RBC nitrite levels with pacing before and after L-NMMA infusion are illustrated in figure 5.33.

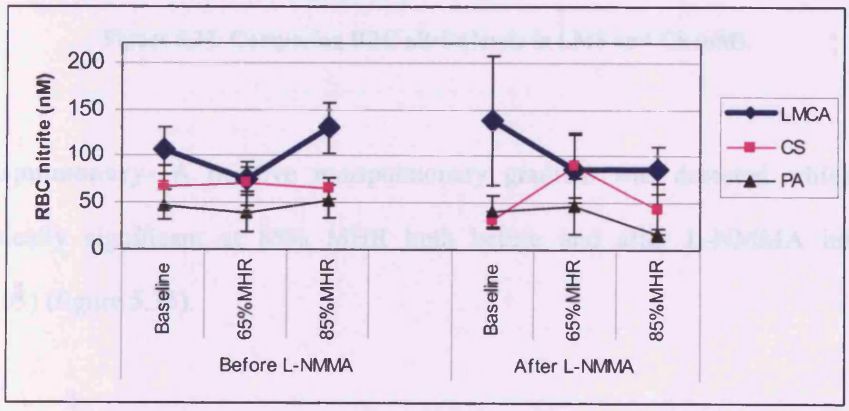


Figure 5.33: Changes in RBC nitrite levels (nM) with pacing before and after L-NMMA infusion. Neither pacing nor L-NMMA infusion affected RBC nitrite levels significantly.

Effect of L-NMMA infusion- L-NMMA infusion did not alter RBC nitrite levels at any heart rate.

Effect of pacing- pacing did not affect RBC nitrite levels in blood samples from LMS, CS, and PA.

Transcardiac- RBC nitrite levels tended to be lower in CS than LMS but the difference did not reach statistical significance (figure 5.34). Correction for flow did not change the pattern, nor did it alter the significance of the findings.

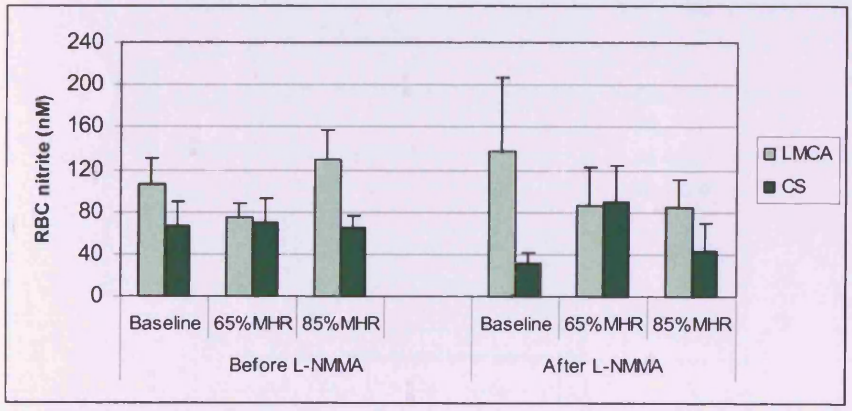


Figure 5.34: Comparing RBC nitrite levels in LMS and CS (nM).

Transpulmonary- A positive transpulmonary gradient was detected which was statistically significant at 85% MHR both before and after L-NMMA infusion ($P<0.05$) (figure 5.35).

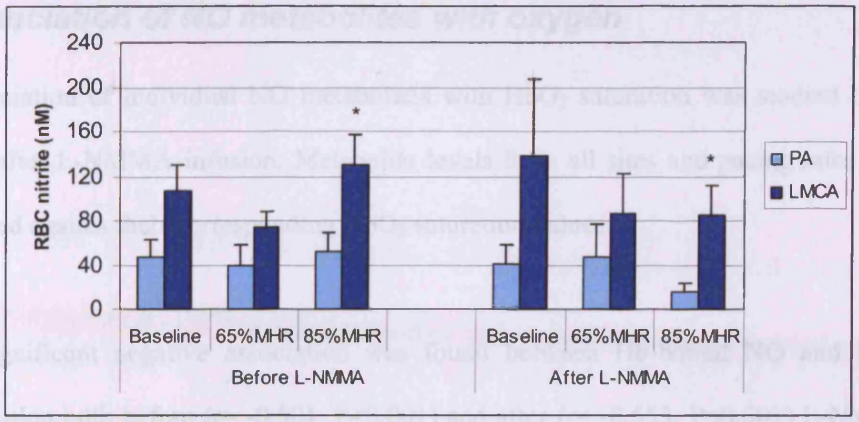


Figure 5.35: Comparing transpulmonary levels of RBC nitrite in PA and LMS (* P<0.05)

Association of NO metabolites with oxygen

Association of individual NO metabolites with HbO_2 saturation was studied before and after L-NMMA infusion. Metabolite levels from all sites and pacing rates were plotted against their corresponding HbO_2 saturation values.

A significant negative association was found between Hb-bound NO and HbO_2 saturation both before (r= -0.501, P<0.001) and after (r= -0.553, P<0.001) L-NMMA infusion (figures 5.36 and 5.37).

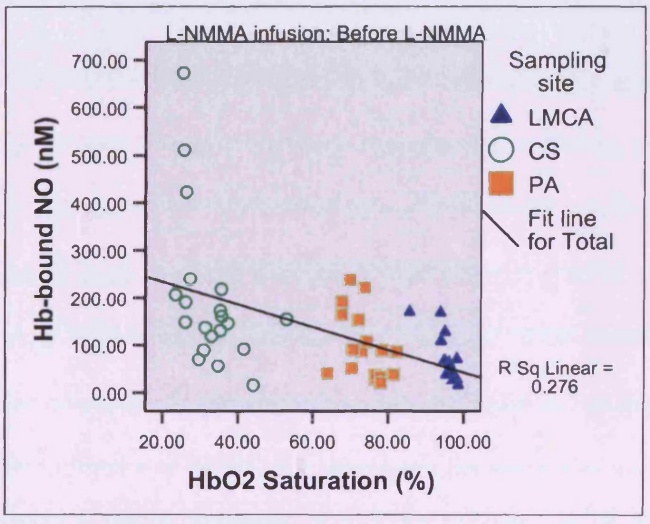


Figure 5.36: Association between Hb-bound NO and HbO_2 saturation before L-NMMA infusion (r= -0.501, P<0.001).

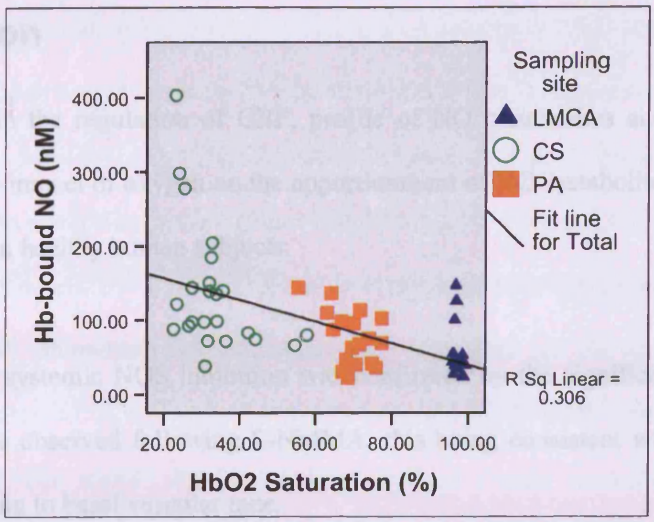


Figure 5.37: Association between Hb-bound NO and HbO_2 saturation following L-NMMA infusion (r= -0.553, P<0.001).

Discussion

Role of NO in the regulation of CBF, profile of NO metabolites across heart and lungs, and the impact of oxygen on the apportionment of NO metabolites in the blood were studied in healthy human subjects.

In this study, systemic NOS inhibition was confirmed by the significant increase in blood pressure observed following L-NMMA, this being consistent with endothelial NO contributing to basal vascular tone.

Role of NO in coronary vasodilation and the regulation of CBF

Vasodilation- Inhibition of NO synthesis decreased LAD diameter at rest confirming the contribution of NO to basal coronary vascular tone. Pacing increased LAD diameter but reached statistical significance only after L-NMMA infusion. The extent of this effect was dependent on the anatomical location with smaller and more distal segments showing higher percent increase in diameter. This particular mode of coronary arterial response to pacing when NO synthesis is inhibited raises the possibility of the existence of EDHF-like activity in the epicardial coronary arteries. Two typical characteristics of EDHF favour this postulation. Firstly, EDHF is normally inhibited by NO[167;303], and EDHF-mediated responses are most prominent after NO synthase inhibition. Similarly, the inhibition of NO synthesis by L-NMMA in this study, revealed the presence of a vasodilator mechanism which not only maintained but also enhanced pacing-induced vasodilation. Secondly, unlike NO, relaxation by EDHF is known to be more prominent in arteries with smaller diameters[304]. Similarly in this study, distal measurements in smaller segments of LAD showed more vasodilation in response to pacing.

The results show that coronary vasodilation in response to increased myocardial oxygen demand is not exclusively NO dependent and that there are other vasodilator mechanisms to take over when NO synthesis is impaired. Consistent with this result, previous canine studies have also shown that NO is not required for exercise induced coronary vasodilation[149;156;175;177;305;306]. In contrast, previous human studies showed that L-NMMA attenuates the pacing-induced dilatation of the large epicardial coronary arteries[161;182-184]. Two main methodological differences exist between the present study and earlier human studies. First, I chose systemic rather than intracoronary infusion of L-NMMA to avoid the potentially confounding effects of recirculation of L-NMMA (as explained above) as well as the direct effect of intracoronary infusion on the coronary haemodynamic parameters. Second, unlike the other studies, adenosine receptors were blocked throughout this study.

CBF- Previous studies in canine and human models have shown that in both species inhibition of NO synthesis with arginine analogues results in little[161;178;184]or no change[177;183;307-313] in CBF at rest. However, the importance of NO in mediating coronary vasodilation and matching CBF to increased myocardial oxygen demand in response to exercise or pacing appears to be different between the two species.

Canine studies show that inhibition of NO does not attenuate exercise-induced increases in CBF in response to exercise[175;177;305;306;314;315].

In contrast, human studies[161;182-184] show that inhibition of NO synthesis reduces CBF when myocardial oxygen demand is increased by atrial pacing, although the percent increase in CBF is preserved.

The results of this study are in line with previous human studies. Pacing increased CBF in proportion to the increase in heart rate. At rest, inhibition of NO synthesis did not affect CBF. At 85% MHR, CBF was significantly lower after L-NMMA infusion. These findings would suggest that in humans, NO is not necessary for the maintenance of CBF at rest, but it plays an important role in enhancing the CBF in response to increased myocardial oxygen demand. It is also noteworthy that a hyperaemic response exists even in the presence of L-NMMA, consistent with the accepted dogma that several metabolic factors combine in order to mediate the overall response.

Profile of NO metabolites across healthy human heart and lungs

This study, for the first time, looked into the apportionment of individual NO metabolites in blood across 1) the coronary and 2) the pulmonary vascular beds.

Plasma NOx levels-mainly reflecting plasma nitrate- were not affected by L-NMMA or pacing. The study provided evidence for transcardiac production of NOx. The study also provided evidence for transpulmonary loss of NOx. Transpulmonary loss of nitrate has not been reported before. Following the ingestion of an oral dose of ^{15}N-labeled nitrate in humans, approximately 65% of the $^{15}NO_3^-$ dose appears in the urine and faeces as nitrate, ammonia, or urea within 48 hours. So far, the fate of the remaining 35% of the $^{15}NO_3^-$ dose administered remains unknown [29]. Transpulmonary loss of nitrate describes an alternative route for nitrate excretion from the body and suggests that nitrate is taken up directly by the lower respiratory tract. Further evidence comes from a recent study from the Karolinska Institute in Sweden which

showed that some of the ingested nitrate is excreted through the respiratory tract by aerosolisation of mucus and saliva. Sixty minutes after an oral dose of nitrate, nitrate concentrations in exhaled breath condensate (EBC) sampled from trachea increased by 2.5 fold [316].

Plasma nitrite levels were not affected by pacing or L-NMMA infusion. The study provided evidence for transcardiac loss of plasma nitrite which was significant before L-NMMA infusion was commenced. Whether this nitrite "loss" reflects transcardiac consumption–e.g. by the myocardium or vascular smooth muscle cells- or is simply the result of higher nitrite production on the arterial side (due to higher NOS activity) and/or higher nitrite consumption in the venous blood (due to a presumed nitrite reductase activity by deoxyhaemoglobin) remains to be clarified. The study also provided evidence for transpulmonary increase of nitrite in plasma.

Plasma RNO levels were not altered by pacing or L-NMMA infusion. No significant transcardiac or transpulmonary gradient was found.

Hb-bound NO levels were not affected by L-NMMA or pacing. The study provided evidence for transcardiac formation of Hb-bound NO. No significant transpulmonary gradient was detected.

RBC nitrite levels were not affected by pacing or L-NMMA infusion. Similar to plasma nitrite, RBC nitrite increased across the pulmonary vascular bed. This phenomenon was more significant at 85% MHR both before and in the presence of L-NMMA infusion.

The **total NO metabolite pool** excluding nitrate (i.e. plasma nitrite+ RNO+ Hb-bound NO+ RBC nitrite) remained unchanged across the coronary and pulmonary beds (P=0.91) with no significant net loss or gain of NO across the heart or lungs. However, significant re-apportionment of NO metabolites occurred between the plasma and RBC compartments as already described for individual metabolites. Total NO metabolite pool was not significantly affected by L-NMMA infusion. Findings are summarised in figure 5.38.

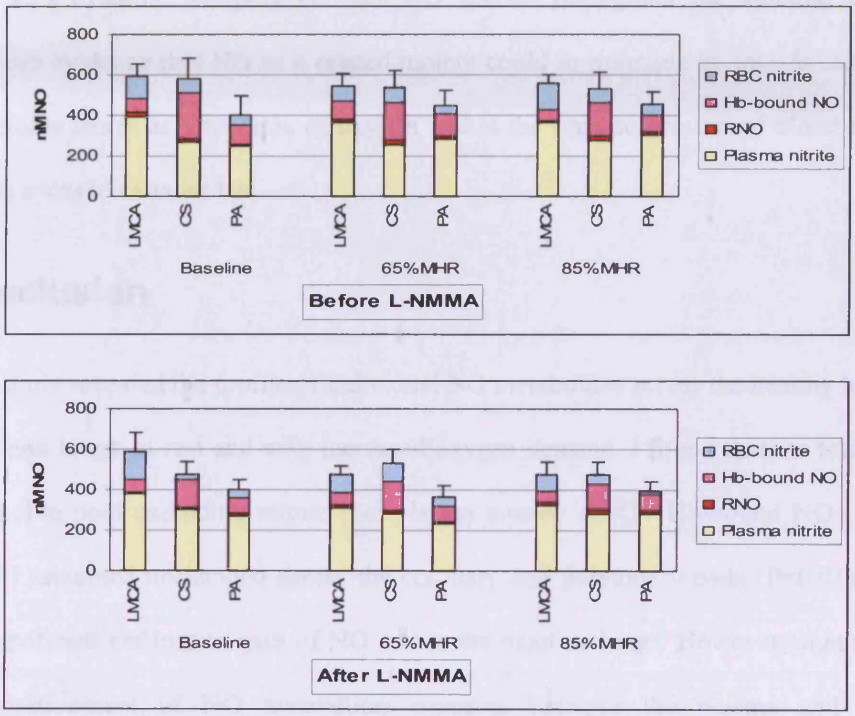


Figure 5.38: Total NO metabolite pool did not change significantly throughout the study and remained comparable among blood samples taken from LMS, CS, and PA. However, significant changes in the apportionment of NO metabolites occurred. Error bars represent SEM for the total sum of NO metabolites (excluding nitrate).

Effect of oxygen in the apportionment of NO metabolites

In this first study to measure NO metabolites across the coronary circulation, I observed significant re-apportionment of NO between metabolite species in plasma and RBC across the coronary circulation driven by the substantial transcoronary oxygen gradient. The strongly negative association observed between HbO_2Sat and Hb-bound NO was mirrored by a positive association between HbO_2Sat and plasma NO. This dynamic interplay between NO species throughout the vascular circuit provides evidence that NO or a related moiety could in principle be transferred from metabolite stores as a function of oxygen within the time constraints of blood transit across a single vascular bed.

Conclusion

This study revealed the profile of individual NO metabolites across the healthy human heart and lungs, at rest and with increased oxygen demand. I found that the total NO metabolite pool excluding nitrate (i.e. plasma nitrite+ RNO+ Hb-bound NO+ RBC nitrite) remained unchanged across the coronary and pulmonary beds ($P=0.91$) with no significant net loss or gain of NO across the heart or lungs. However, significant re-apportionment of NO metabolites occurred between the plasma and RBC compartments driven by the substantial transcoronary oxygen gradient.

The study also investigated the importance of NO in the regulation of coronary blood flow and found that in humans NO is not necessary for the maintenance of CBF at rest, but it plays an important role in enhancing the CBF in response to increased myocardial oxygen demand.

Nonetheless these data do not provide direct evidence to identify the particular NO moiety responsible for the transfer of NO bioactivity between the plasma and RBC compartments. Furthermore, the study does not address whether NO metabolites may accrue greater importance when myocardium is compromised or when there is tissue ischaemia. In other words, we have dealt with a healthy heart under normal and stressed conditions.

A less intuitive but equally important finding was that administration of L-NMMA had little bearing on NO metabolite levels in arterial or venous blood in the acute setting (i.e. within minutes). This finding suggests that blood levels of specific NO metabolites (e.g. NOx or nitrite) do not accurately reflect immediate endothelial NOS production. This finding has far reaching implications for a large number of studies utilising such measurements to draw conclusions on endothelial function. Although L-NMMA did not affect NO metabolite levels in the acute setting, it is likely that chronic endothelial dysfunction (or enhancement) will result in proportional changes.

CHAPTER SIX

Results II
The profile of nitric oxide metabolites in type 1 diabetes mellitus; correlation with microvascular complications

Introduction

Microvascular complications are major causes of morbidity and mortality in diabetes but their aetiology remains poorly understood.

Nitric oxide (NO) produced by vascular endothelium plays an important role in maintenance of normal vascular physiology by modulating haemostasis, inflammation, blood flow, and tissue perfusion. NO in blood rapidly reacts with proteins (including haemoglobin and albumin), oxygen, or reactive oxygen species (mainly O_2^-) to form its more stable metabolites. These metabolites can potentially serve as NO reservoirs and transport NO bioactivity in the circulation for a longer duration and distance than NO itself (see CHAPTER ONE).

Recent data suggest that abnormal NO metabolism may be pathogenic in diabetes[253-255;317-321]. Reduced bioavailability of NO has been demonstrated in both type 1 and type 2 diabetes mellitus and has been attributed either to reduced production by NO synthase (NOS) or increased inactivation, perhaps by increased levels of superoxide (O_2^-)[253;317;318].

There has been limited work on NO metabolism in type 1 diabetes. None of the few studies which have looked into the metabolic fate of NO in type 1 diabetes has been inclusive of all the metabolites of interest. (For a review of published work in this field please see CHAPTER THREE).

In this study, I established the profile of NO metabolites in patients with type 1 diabetes mellitus and investigated whether and how glycosylation affects 1) apportionment of NO between metabolic pathways 2) hypoxic release of bioactive NO from RBCs 3) relationship between NO metabolism and microvascular complications.

I studied blood/NO interaction in 2 subgroups of diabetic patients; with and without microvascular complications, and compared it to age- and gender-matched controls. RBC and plasma NO components were measured directly in venous blood samples and again following addition of increasing doses of NO *ex vivo* to test the capacity of blood to apportion NO to its various metabolites. An isolated aortic vessel system was used to assess NO release and vessel relaxation induced by RBCs from patients and controls under low oxygen conditions.

I found that NO metabolism and the apportionment of exogenous NO are altered in diabetics and that these abnormalities precede the clinical manifestation of microvascular complications. Vessel relaxation studies implied the existence of a RBC-related vasodilating factor (RRVF) which operates in close correlation with HbA_{1C} in diabetics.

New information about NO metabolism in diabetes improves our understanding of the pathophysiology of its microvascular complications and points to new prophylactic and therapeutic strategies.

Metabolites studied- The main NO metabolites of interest and their significance in vascular physiology are as follows.

Plasma NOx (NO_2^- + NO_3^-)

NOx accounts for the most abundant NO end products in the plasma, nitrate (NO_3^-) and nitrite (NO_2^-). Because nitrate levels in plasma are about 100 times greater than nitrite, NOx mainly reflects plasma nitrate levels.

Nitrate is an inert by-product of NO metabolism in the circulation. Its plasma levels not only depend on NO production from various endogenous sources (eNOS, iNOS, etc.) but also on exogenous sources such as food and water.[52]

Due to the above facts, nitrate is not a sensitive nor specific index of endothelial NO production.

Plasma nitrite

Plasma nitrite, generated from the reaction of NO and oxygen, reflects constitutive NOS activity[68]. More recently, nitrite has been suggested by some groups to be the major bioactive NO store in human blood[77].

Plasma S-nitrosothiols (RSNO)

Nitrosation of thiols in proteins such as albumin results in long-lived S-nitrosothiols (RSNO) with a variety of different effects in biological systems.

In recent years RSNOs have attracted increasing attention as possible preservers of NO bioactivity in the circulation. They prevent loss of NO from oxidative degradation and also create bioactive low molecular weight nitrosothiols such as *S*-nitrosoglutathione (GSNO) which demonstrate strong vasodilator properties[322]. RSNOs provide a reservoir of NO bioactivity that might be utilised in states of NO deficiency.

Nitrosylhaemoglobin (HbNO)

Most of the NO which enters the erythrocytes will react with oxyhaemoglobin to form nitrate and methaemoglobin. Nonetheless, some NO will meet non-oxygenated haemoglobin and nitrosylate the Fe^{2+} to a fairly stable HbNO adduct [77](in vivo half life ~ 40 minutes[123]). HbNO is also produced from the reaction between the continuous flux of nitrite into the erythrocytes and non-oxygenated haemoglobin. Therefore, HbNO is a co-index of NO and nitrite uptake by erythrocytes at any given haemoglobin oxygen saturation.

HbNO is one of the many molecules proposed to serve as potential preservers of NO bioactivity in the circulation. However, HbNO does not show vasodilator properties in vivo[122] nor is there any evidence that it can dissociate efficiently to deliver NO to tissues directly.

S-nitrosohaemoglobin (SNO-Hb)

SNO-Hb is formed by the S-nitrosation of Hb ß- chain cysteine 93[109;110]. SNO-Hb shows vasodilator properties both in vitro and in vivo at lower oxygen saturations and may play an important role in the control of blood flow and oxygen delivery to tissues[112].

RBC nitrite

Plasma nitrite constantly enters the RBCs[72]. Inside the erythrocytes, nitrite can either react with oxyhaemoglobin to form methaemoglobin and nitrate (Eq. 1); or with deoxygenated haemoglobin to form methaemoglobin and nitrosylhaemoglobin (HbNO)[76;78] (Eq. 2):

$$HbO_2 + 2NO_2^- \rightarrow MetHb + 2NO_3^- \qquad \text{Eq. 1}$$

$$4Hb + 2NO_2^- + 2H^+ \rightarrow 2MetHb + 2HbNO + 2H_2O \qquad \text{Eq. 2}$$

Methods

Study population

Recruitment

Patients were recruited from two specialist diabetes outpatient clinics at the University Hospital of Wales (Consultant Diabetologists, Dr. John Peters and Dr. Mark Evans). Controls were recruited from healthy volunteers working at Cardiff University and associated departments. 28 healthy volunteers and 46 patients with type 1 diabetes mellitus were recruited. The diabetic group was further divided into two subgroups: Group A: those without microvascular complications (n=20); and Group B: those with microvascular complications (n=26).

To avoid the risks of fasting diabetic patients in an outpatient setting, research subjects were non-fasting; with the intention of providing a mid-afternoon blood sample under "normal" conditions (insulin, diet) for each individual. Controls were sampled in exactly the same way and the same time of the day, i.e. 2-3 p.m.

Microvascular complications- Microvascular complications were defined as nephropathy, neuropathy, and diabetic eye disease. Patients with at least one of the following complications were recruited to Group B:

1. **Nephropathy:** Patients were screened for diabetic nephropathy with urine dipstix, urine albumin, and albumin: creatinine ratio. Microalbuminuria is the earliest indicator of diabetic nephropathy[323]. Consistent with NICE guidelines[323],

microalbuminuria was defined as albumin: creatinine ratio greater than or equal to 2.5mg/mmol (men) or 3.5mg/mmol (women), or urine albumin concentration greater than or equal to 20mg/l. Proteinuria was defined as albumin: creatinine ratio of ≥30mg/mmol or albumin concentration ≥200mg/l.

2. **Established neuropathy** – Patients were screened with history and physical examination for evidence of peripheral sensorimotor neuropathies (using a 10-g nylon monofilament), acute mononeuropathies, autonomic neuropathies (neuropathic bladder, erectile dysfunction), diabetic foot, and cranial nerve palsies.

3. **Diabetic eye disease** – Patients were screened for either of background retinopathy, maculopathy, or proliferative retinopathy. Screening was performed with direct ophthalmoscopy and retinal photography.

The most common microvascular complication among Group B was retinopathy (88%) followed by neuropathy (15%) and nephropathy (4%). Four patients (15%) showed evidence of microvascular complications in more than one system.

Exclusion criteria

Patients with other modifiable cardiovascular risk factors (i.e. smoking, cholesterol>5.2mmol/l, triglyceride>2mmol/l, hypertension>140/90mmHg), those with a history of ischaemic events (angina, myocardial infarction, stroke), any acute illness, and those taking medications which might alter/improve endothelial function (e.g. ACE inhibitors) or affect vascular tone were excluded from the study.

Demographics

Groups were matched for age and gender (table 6.1). Within the diabetic group, Group B were generally older than Group A ($P<0.001$) and had a longer duration of disease ($P<0.001$).

	Age (years)	Gender	Duration of disease (years)	*n*
Controls	33.36±11	14M; 14F		28
Diabetics	33.61±12	24M; 22F	15.33±11	46
Group A	27.15±8	10M, 10F	9.22± 7.36	20
Group B	38.58±12	14M; 12F	20.31±11	26

Table 6.1: Age and gender distribution and duration of disease. M: Male, F: Female. Values are in mean± SD.

Ethics

All subjects were provided with a detailed information sheet and asked to give fully informed written consent. Relevant staff at the Diabetes Clinic received an introductory leaflet which explained the study procedure (Appendix 2). The study was approved by the Local Research Ethics Committee.

Blood collection and storage

Venous blood samples were taken from an antecubital vein and stored in standard hospital EDTA vacutainers. HbA_{1C} was immediately measured by a fully automated glycohaemoglobin analyser (Tosoh's ion-exchange HPLC). This measurement is

quoted as the "last" HbA_{1C} in this chapter. An average of HbA_{1C}s over the last five years (HbA_{1C}-mean) was calculated using the hospital's computer data base and taken as an indicator of glycaemic control over the past 5 years. Plasma glucose was measured with a calibrated Optium Xceed Blood Glucose Meter (Medisense).

To test the capacity of blood to metabolise exogenously added NO, the NO donor, NOC-9 (MAHMA NONOate, half life at pH=7.4 and 37°C: 1 minute) was added to the EDTA samples to provide final added concentrations of 0, 1, 10 and 100 μM NO. Samples were incubated at 37°C for 5 minutes. Plasma and red blood cells (RBC) were separated after centrifugation at 670g for 5 minutes and stored at -80°C for future analysis.

Care was taken to ensure all samples were treated within comparable time intervals. There was a 30 minute lapse between the time samples were taken in the field and the time they were placed in the freezer in the laboratory.

Chemicals

Iodine, potassium iodide, potassium hexacyanoferrate, sulfanilamide, sodium hydroxide, and mercuric chloride were purchased from Sigma. NOC-9 (MAHMA NONOate) was purchased from Axxora. Acetic acid glacial, HPLC water, and hydrochloric acid were purchased from Fisher Scientific. NaCl 0.9% was purchased from Fresenius Kabi Limited.

Nitric oxide measurements

Plasma nitrate was measured using the DAN assay[283]. Tri-iodide based chemiluminescence was used to measure other NO metabolites in plasma and RBC samples as described in detail in CHAPTER FOUR.

Vessel relaxation experiments

In vitro, it has been demonstrated that RBCs release an NO-type species that dilates pre-constricted aortic vessels under low oxygen tensions[116;324]. Addition of RBCs to pre-constricted aortic vessel rings incubated under fully oxygenated conditions (95%) results in only a further constriction above that induced by phenylephrine (PE). Addition of similar RBC to aortic vessel rings incubated at low oxygen tensions results in vessel relaxation.

NO release and vessel relaxation induced by RBCs was studied under low oxygen conditions (O_2 1%) in an 8-channel tissue bath system. Endothelium denuded rings of thoracic aorta from male New Zealand White rabbits (2-2.5 Kg) were prepared for isometric tension recordings. The tissues were pre-constricted with phenylephrine (PE: 10^{-6} mol/l). As soon as the response reached a plateau, 20µl aliquots of fresh saline-washed RBCs were added to each channel.

Relaxation was calculated as a percent of the tension induced by 10^{-6}mol/l PE for each aortic ring. The average from 8 channels was calculated and was taken to reflect mean relaxation for each subject. The responsiveness of the tissues was assessed at the end of each experiment by constricting with PE followed by relaxation to a standard NO donor, S-nitroso-glutathione (GSNO; 10^{-7}mol/l).

Details of the Tissue Organ Bath System protocol can be found in Appendix 3.

Statistical analysis

Data were analysed and processed using SPSS 14.0 for Windows. Shapiro-Wilks test was used to assess the data for normality. Mann-Whitney or Kruskal-Wallis tests were used to compare the mean values of the metabolites between diabetics and controls when indicated. One way ANOVA was used to compare the mean values of parametric variables (e.g. HbA_{1C}). Significance was assumed when $p<0.05$. Stepwise multiple regression analysis was used to adjust the data for age and duration. Correlation between variables was examined using Pearson's correlation coefficient.

Results

Glucose, HbA_{1C}, cholesterol (table 6.2)

Plasma glucose and HbA_{1C} were higher in diabetics than controls ($p<0.001$). Plasma glucose, last HbA_{1C}, and 5-year average HbA_{1C} were higher in Group A than Group B ($p<0.05$).

	Glucose (mmol/l)	HbA1C (%)	5-year HbA1C (%)	Cholesterol (mmol/l)
Controls	6.40±0.32	5.21±0.07	N/A	5.12±0.23
Diabetics	12.22±0.88	8.23±0.25	8.57±0.25	4.52±0.11
Group A	14.41±1.18	8.92±0.46	9.36±0.43	4.51±0.19
Group B	10.47±1.16	7.72±0.22	7.96±0.25	4.53±0.13

Table 6.2: Plasma glucose, last and 5-year HbA1C, and cholesterol levels. "Diabetics" represent pooled data from groups A and B. Values are in mean +/- SEM.

In the diabetic group, mean and last HbA_{1C}s were very strongly correlated ($r= 0.852$, $P<0.001$) (figure 6.1).

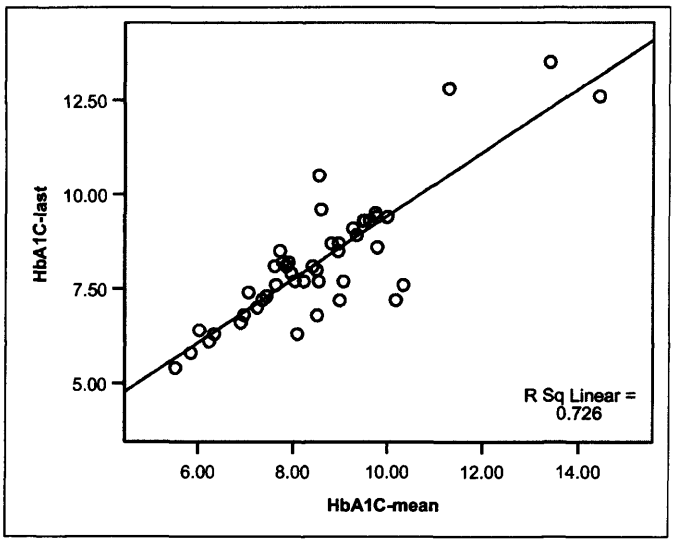


Figure 6.1: Correlation between HbA_{1C}-mean (%) and HbA_{1C}-last (%) in diabetics ($P<0.001$).

HbA_{1C} increases with age- There are discrepancies in the literature regarding the influence of age on HbA_{1C} in normal subjects. While some groups have demonstrated an increase in HbA_{1C} with age[325;326], other groups have not been able to verify such a correlation[327;328]. My study, although in a relatively small group (n=28) of young non-diabetic subjects (average age 33.36±11 years), confirmed a positive linear relationship between HbA_{1C} and age(r=0.527, P=0.004) (figure 6.2). A similar correlation existed between non-fasting plasma glucose and age (P<0.05).

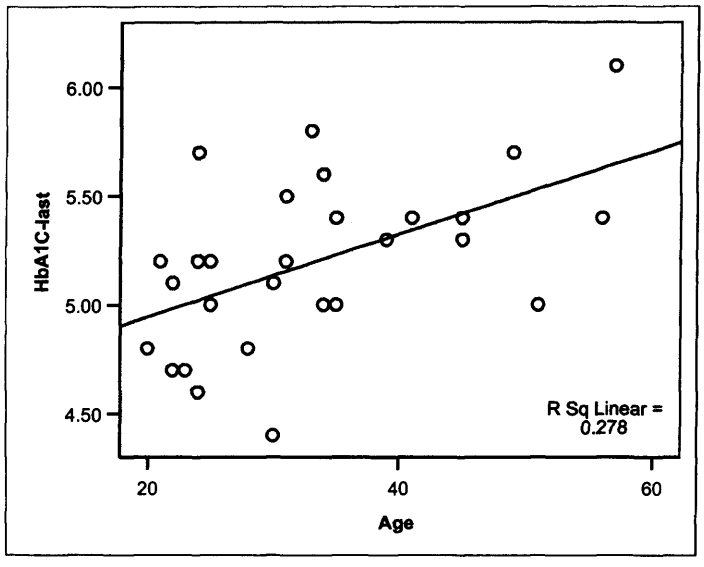


Figure 6.2: Correlation between HbA_{1C} (%) and age (years) in controls.

NO metabolites

Basal (un-doped) levels- Means and medians of basal levels of NO metabolites in plasma and RBC are given in table 6.3. No significant correlation was found between NO metabolites and age or duration of disease. Adjusting the data for age and duration did not affect the statistical significance of results.

	Plasma			**RBC**		
	Nitrate (μM)	**Nitrite (nM)**	**RNO (nM)**	**Nitrite (nM)**	**HbNO (nM)**	**SNO-Hb (nM)**
Controls	14.6±1.4	63.7±12.5	18.6±2.8	65.1±12	67.9±9	30.6±5.3
Diabetics	16.1±1.2	23.7±4.3	16.8±1.7	31.3±8.3	42.1±6.3	26.2±5.4
Group A	15.2±1.4	35.9±9	16.6±2.7	14.4±5.4	53.9±13.1	33.4±11.3
Group B	16.6±1.6	14.8±2.5	17.1±2.3	42.9±13	33.1±4.6	20.8±4.3

Table 6.3: NO metabolites in diabetics and controls. Values represent *mean +/- SEM.*

Diabetics versus controls- Plasma nitrate (figure 6.3) was slightly higher in diabetics (group A + group B) than controls but did not reach statistical significance. Plasma nitrite was lower in the diabetic group taken as a whole ($p<0.001$) (figure 6.4) as were RBC nitrite ($p<0.01$) and HbNO ($p<0.01$) (figure 6.5). Plasma RNO (figure 6.4) and SNO-Hb (figure 6.5) levels were similar between the two groups.

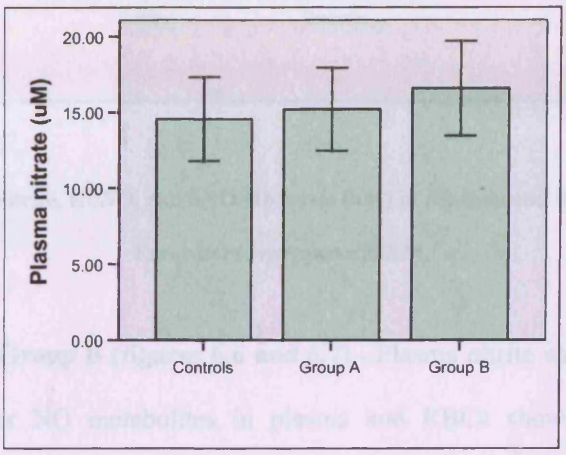


Figure 6.3: Plasma nitrate levels (uM) in controls, group A, and Group B. Error bars represent ±2SEM.

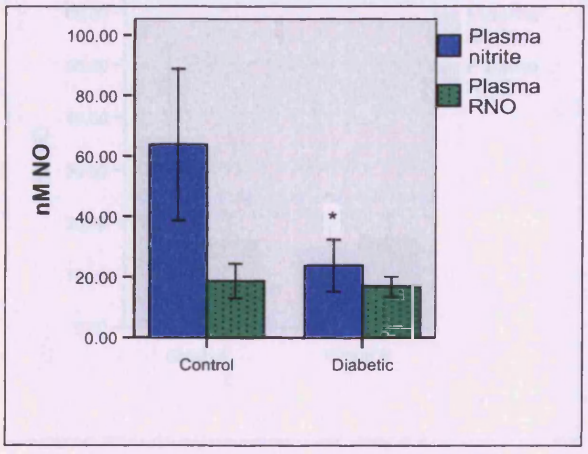


Figure 6.4: Plasma nitrite and RNO (nM) levels in controls and diabetics. *$P<0.001$. Error bars represent ±2SEM.

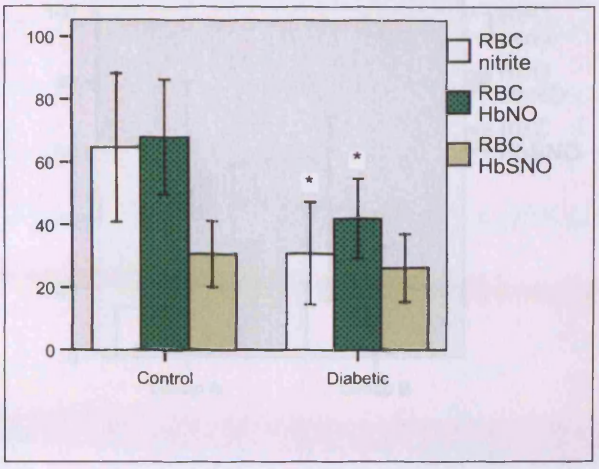


Figure 6.5: RBC nitrite, HbNO, and SNO-Hb levels (nM) in controls and diabetics. *$P<0.01$.

Error bars represent ±2SEM.

Group A versus Group B (figures 6.6 and 6.7) - Plasma nitrite was lower in Group B ($p<0.05$). Other NO metabolites in plasma and RBCs showed similar levels between groups except for RBC nitrite which was lower in Group A (P=0.054).

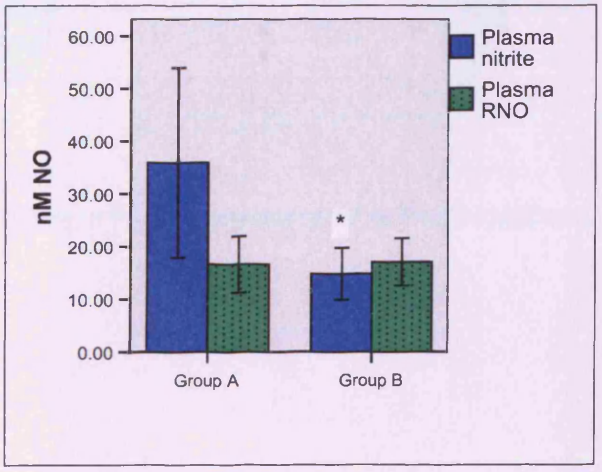


Figure 6.6: Plasma nitrite and RNO (nM) in Groups A and B. *$P<0.05$.

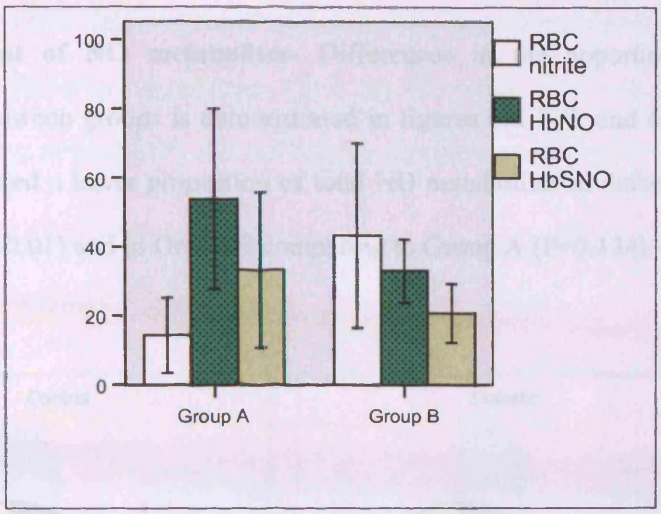


Figure 6.7: RBC nitrite, HbNO, and SNO-Hb (nM) in Groups A and B. Error bars represent ±2SEM.

Apportionment of NO metabolites- Differences in the apportionment of NO metabolites between groups is demonstrated in figures 6.8, 6.9, and 6.10. In plasma, nitrite constituted a lower proportion of total NO metabolites in diabetics comparing to controls ($P<0.01$) and in Group B comparing to Group A ($P=0.134$).

Figure 6.8: Apportionment of NO metabolites in plasma.

A similar trend was observed for RBC nitrite between controls and diabetics which did not reach statistical significance (P=0.16). However, RBC nitrite constituted a lower proportion of total RBC NO in Group A comparing to Group B (P=0.082).

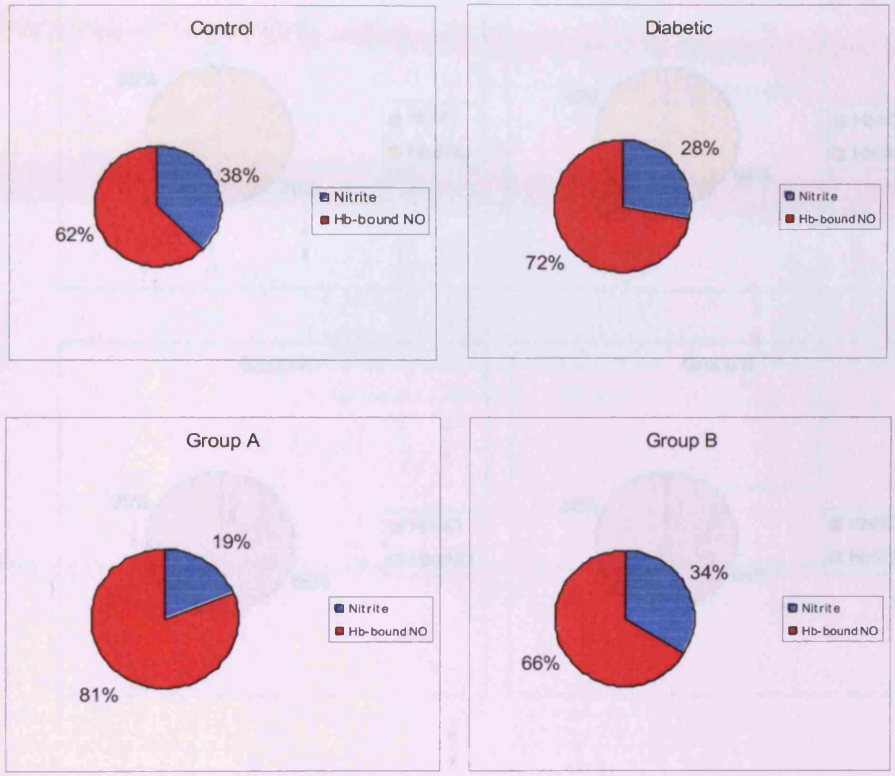


Figure 6.9: Apportionment of nitrite and Hb-bound NO (HbNO+ SNO-Hb).

HbNO constituted the majority of Hb-bound NO. HbNO: SNO-Hb ratio was identical among all groups and subgroups.

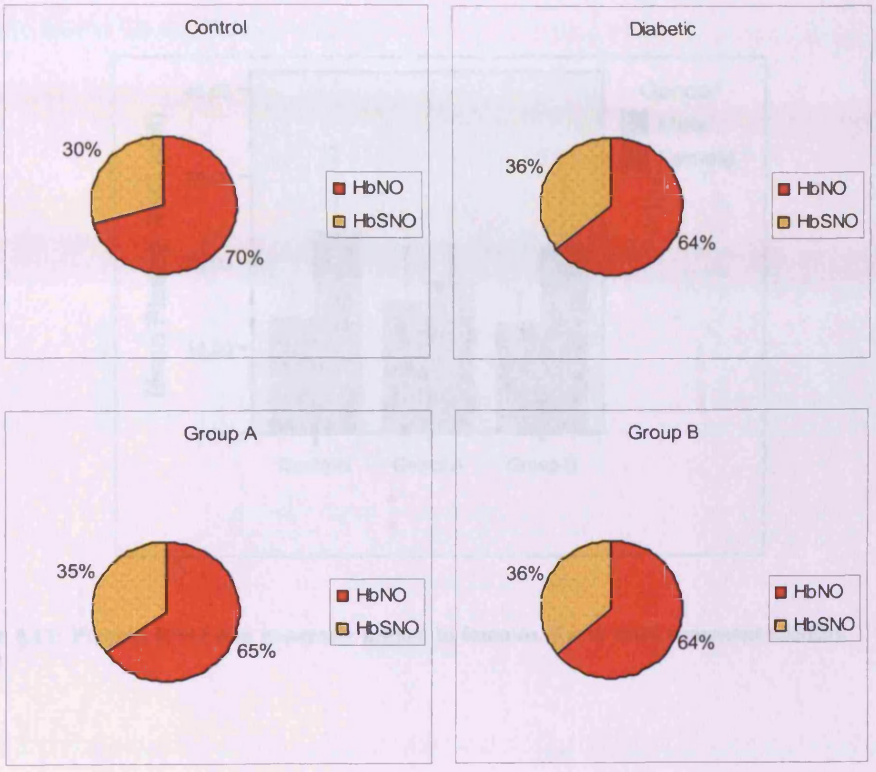


Figure 6.10: Apportionment of Hb-bound NO.

Gender difference- There was no difference in NO metabolites between genders except for plasma RNO which was generally higher in females compared to males ($P<0.05$ for all males vs. all females) (figure 6.11).

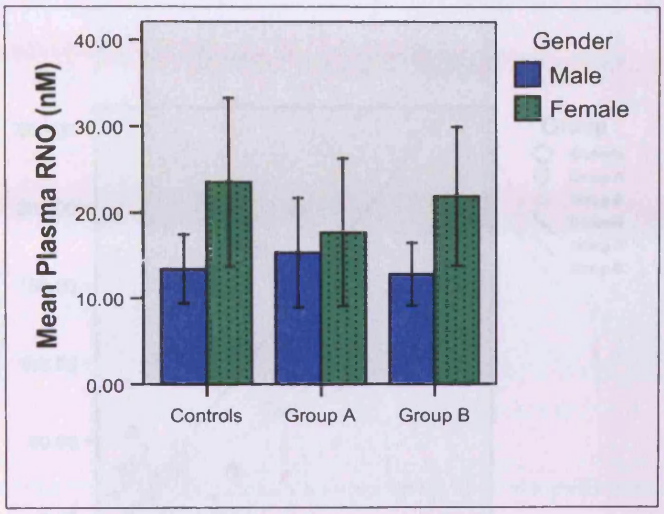


Figure 6.11: Plasma RNO was generally higher in females. Error bars represent ±2SEM.

Correlation between NO metabolites

Strong correlation was found between plasma nitrite and HbNO in controls (r=0.762, P<0.001). The correlation became weaker in Group A (r=0.509, P<0.05) and was lost in Group B (figure 6.12)

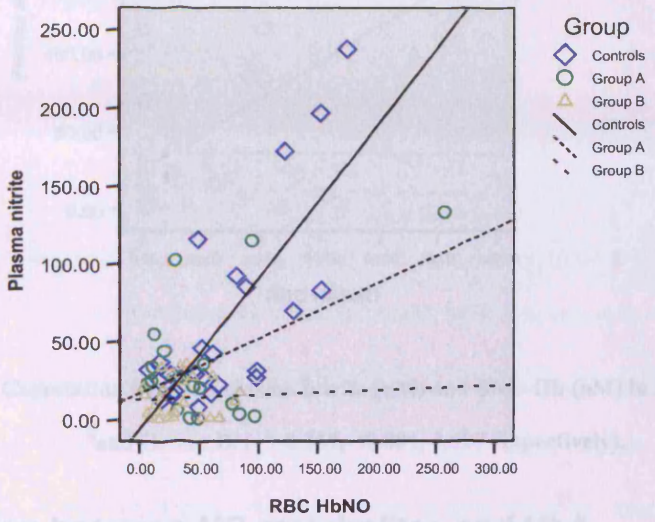


Figure 6.12: Correlation between plasma nitrite (nM) and HbNO (nM) in controls, Group A, and Group B (r^2= 0.581, 0.259, 0.003 respectively).

Plasma nitrite showed a similar correlation with SNO-Hb in controls (r= 0.507, P=0.01) but not in the diabetic groups (figure 6.13).

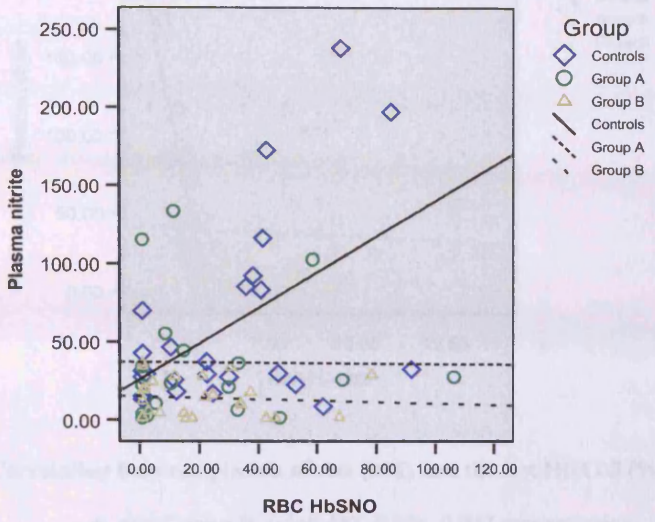


Figure 6.13: Correlation between plasma nitrite (nM) and SNO-Hb (nM) in controls, Group A, and Group B. (r^2=0.257, <0.001, 0.007 respectively).

Correlation between NO metabolites and HbA_{1C}

Among NO metabolites, plasma nitrite and HbNO correlated with HbA_{1C}. Plasma nitrite showed a strong negative correlation with HbA_{1C} in controls (r=-0.655, P<0.001) but not in diabetics (figure 6.14).

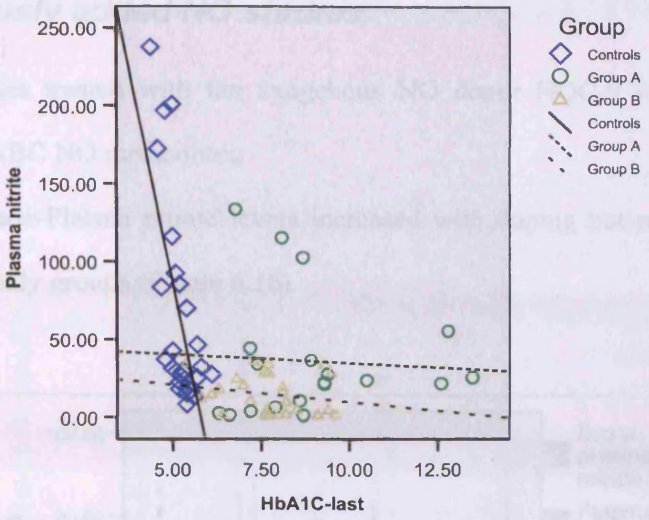


Figure 6.14: Correlation between plasma nitrite (nM) and the last HbA1C (%) in controls, Group A, and Group B. (r^2=0.442, 0.004, 0.042 respectively).

HbNO was negatively correlated with HbA_{1C} in all three groups but the correlation and statistical significance was stronger in controls than Group A than Group B (controls: r= -0.591, P<0.001; Group A: r= -0.456, P=0.05; Group B: r= -0.186, P=0.3) (figure 6.15).

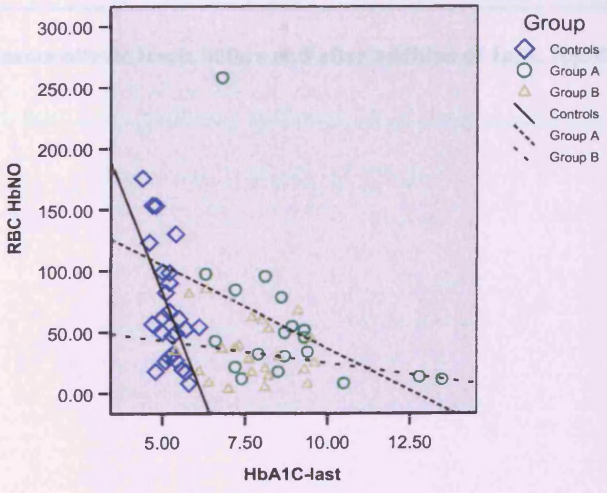


Figure 6.15: Correlation between HbNO (nM) and the last HbA1C (%) in controls, Group A, and Group B. (r^2=0.349, 0.208, 0.035 respectively).

Exogenously added NO studies

Blood samples treated with the exogenous NO donor NOC-9 were analysed for plasma and RBC NO metabolites.

Plasma nitrate-Plasma nitrate levels increased with doping but remained identical among the study groups (Figure 6.16).

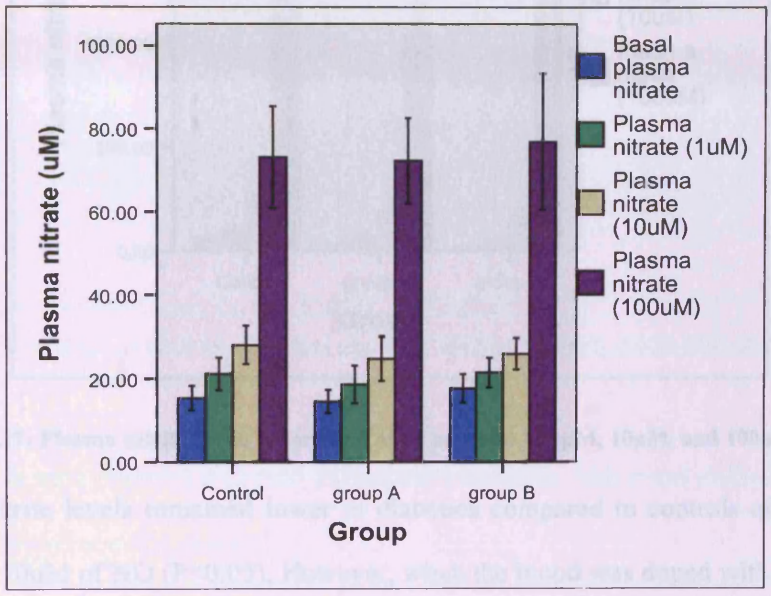


Figure 6.16: Plasma nitrate levels before and after addition of 1µM, 10µM, and 100µM of NO.

Plasma nitrite- Addition of NO to blood increased plasma nitrite levels in all groups (Figure 6.17).

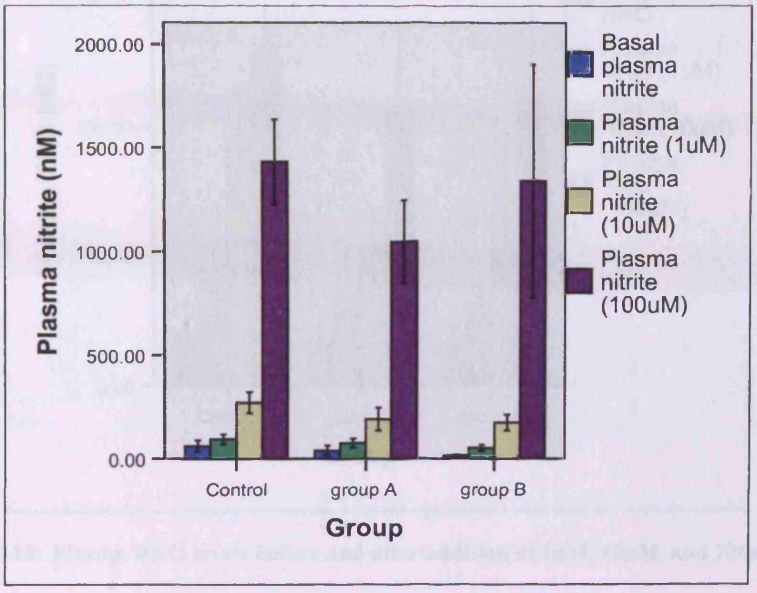


Figure 6.17: Plasma nitrite levels before and after addition of 1μM, 10μM, and 100μM of NO.

Plasma nitrite levels remained lower in diabetics compared to controls after adding 1uM and 10uM of NO ($P<0.05$). However, when the blood was doped with 100uM of NO, plasma nitrite levels reached statistically comparable levels in diabetics and controls. There was no significant difference between plasma nitrite levels in groups A and B after doping with 1, 10, and 100uM of NO.

Plasma RNO- Plasma RNO levels increased after doping (Figure 6.18).

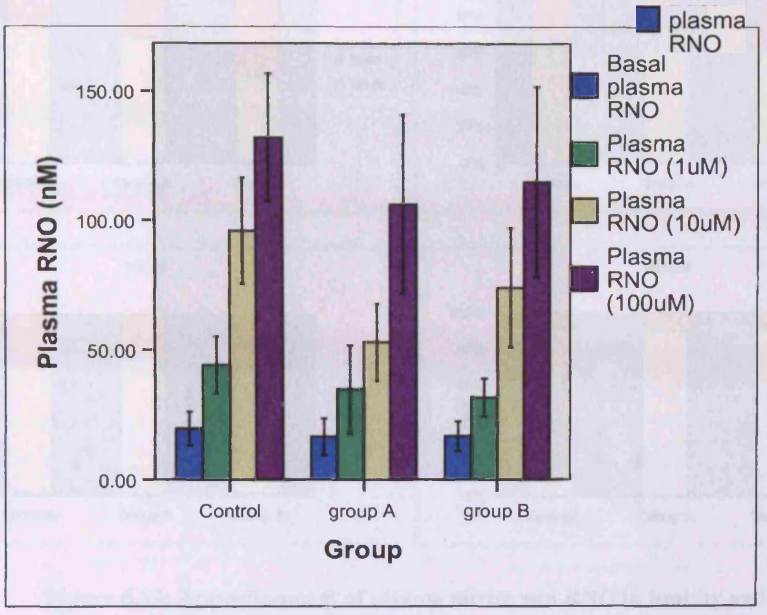


Figure 6.18: Plasma RNO levels before and after addition of 1µM, 10µM, and 100µM NO.

RNO levels were lower in diabetics compared to controls. This trend reached statistical significance at 10uM NO.

Apportionment of NO in plasma

The apportionment of NO between plasma nitrite and RNO after addition of exogenous NO was identical among controls, group A, and group B at each concentration of added NO. Adding NO led to relatively higher proportions of nitrite than RNO (figure 6.19 and table 6.4).

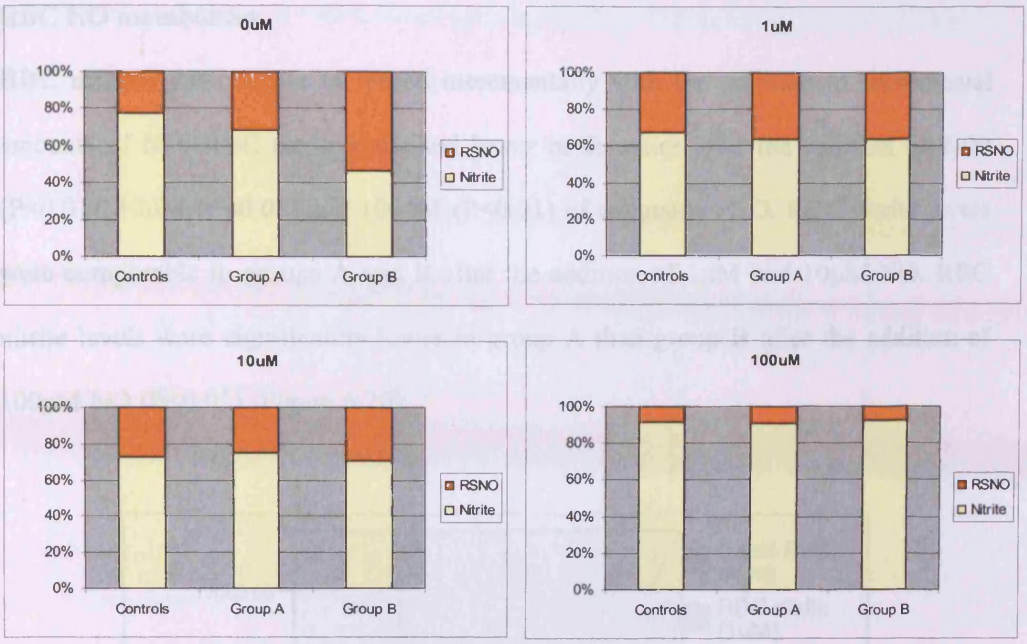


Figure 6.19: Apportionment of plasma nitrite and RNO in healthy and diabetic samples before and after being doped with increasing concentrations of exogenous NO.

		nM				%		
		Controls	Group A	Group B		Controls	Group A	Group B
Controls	Nitrite	63.69	35.87	14.79		77.36	68.26	46.40
	RNO	18.64	16.68	17.08		22.64	31.74	53.60
1uM NO	Nitrite	96.11	68.57	53.29		67.39	63.98	63.96
	RNO	46.50	38.60	30.02		32.61	36.02	36.04
10uM NO	Nitrite	253.11	178.12	186.17		72.43	74.87	70.84
	RNO	96.33	59.77	76.64		27.57	25.13	29.16
100uM NO	Nitrite	1369.66	967.58	1302.49		91.71	90.70	91.95
	RNO	123.85	99.26	113.97		8.29	9.30	8.05

Table6.4: Levels (nM) and proportions (%) of nitrite and RNO in control and diabetic plasma before and after doping with NOC-9 at 1μM, 10μM, and 100μM of NO.

RBC NO metabolites

RBC nitrite- RBC nitrite increased incrementally with the addition of incremental amounts of NO. RBC nitrite remained lower in diabetics after the addition of 1μM ($P<0.01$), 10μM ($P<0.05$) and 100μM ($P<0.01$) of exogenous NO. RBC nitrite levels were comparable in groups A and B after the addition of 1μM and 10μM NO. RBC nitrite levels were significantly lower in group A than group B after the addition of 100μM NO ($P<0.01$) (Figure 6.20).

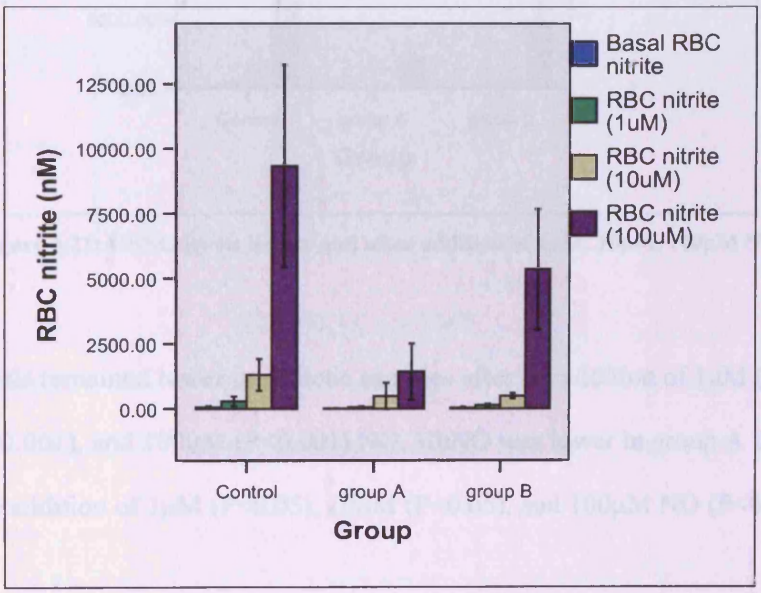


Figure 6.20: RBC nitrite levels before and after addition of 1μM, 10μM, and 100μM NO.

RBC HbNO-Addition of NO to blood increased HbNO levels in all groups (Figure 6.21).

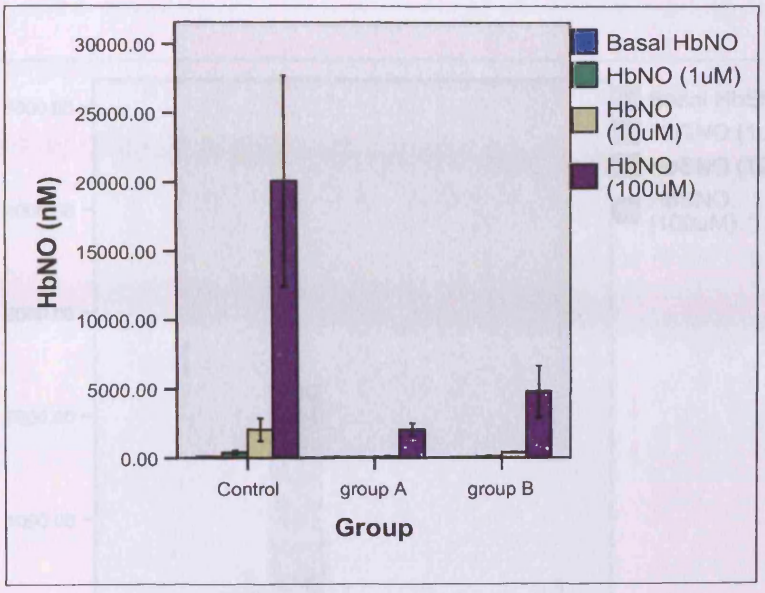


Figure 6.21: HbNO levels before and after addition of 1μM, 10μM, 100μM NO.

HbNO levels remained lower in diabetic samples after the addition of 1μM ($P<0.01$), 10μM ($P<0.001$), and 100μM ($P<0.001$) NO. HbNO was lower in group A than group B after the addition of 1μM ($P<0.05$), 10μM ($P<0.05$), and 100μM NO ($P<0.05$).

RBC SNO-Hb- Addition of NO to blood increased SNO-Hb levels in all groups (Figures 6.22 and 6.23).

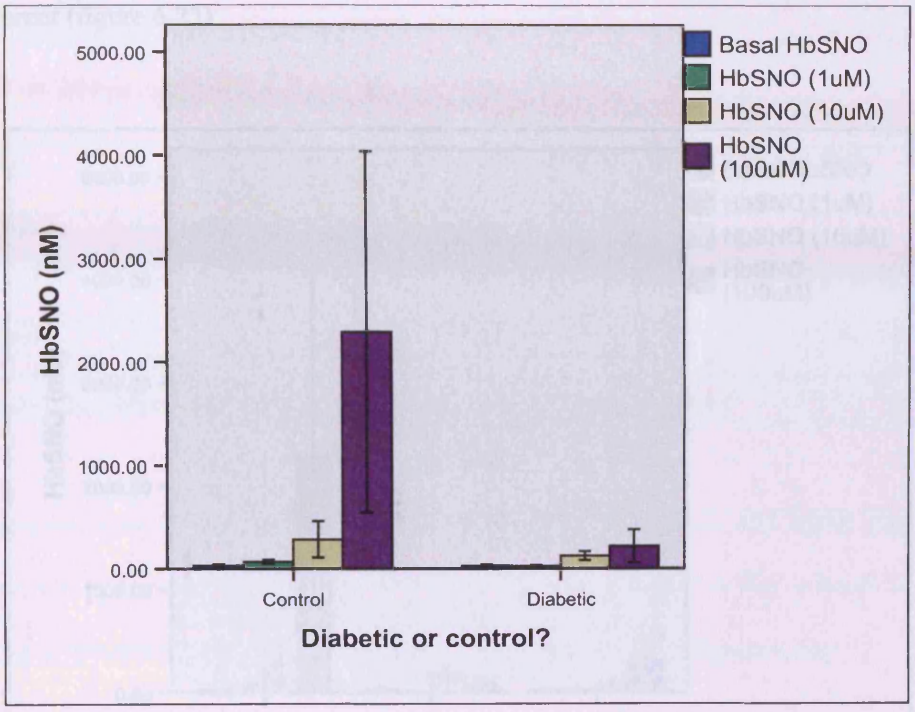


Figure 6.22: SNO-Hb levels after addition of 1µM, 10µM, 100µM NO.

SNO-Hb levels were lower in doped diabetic samples than controls. The difference reached statistical significance at 1μM ($P<0.001$) and 100μM NO ($P<0.01$).
SNO-Hb levels in doped samples from group A and group B were not statistically different (figure 6.23).

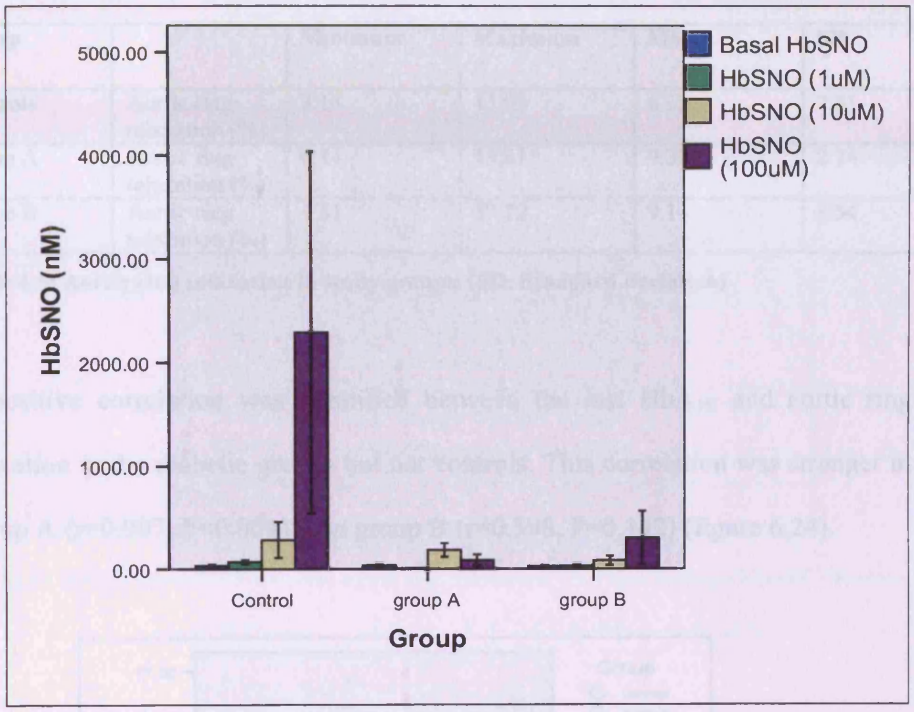


Figure 6.23: SNO-Hb levels after addition of 1μM, 10μM, 100μM NO.

Vessel relaxation experiments

Consistent with previous findings from our laboratory[324], in a 1% oxygen environment, diabetic RBCs showed higher vasodilator properties compared to RBCs from controls (P=0.005) (table 6.5).

Group		Minimum	Maximum	Mean	SD
Controls	Aortic ring relaxation (%)	3.14	13.02	6.65	2.51
Group A	Aortic ring relaxation (%)	6.14	15.81	9.32	2.74
Group B	Aortic ring relaxation (%)	1.51	17.12	9.14	3.54

Table 6.5: Aortic ring relaxation in study groups. (SD: Standard deviation)

A positive correlation was identified between the last HbA_{1C} and aortic ring relaxation in the diabetic groups but not controls. This correlation was stronger in Group A (r=0.907, P<0.001) than group B (r=0.398, P=0.142) (figure 6.24).

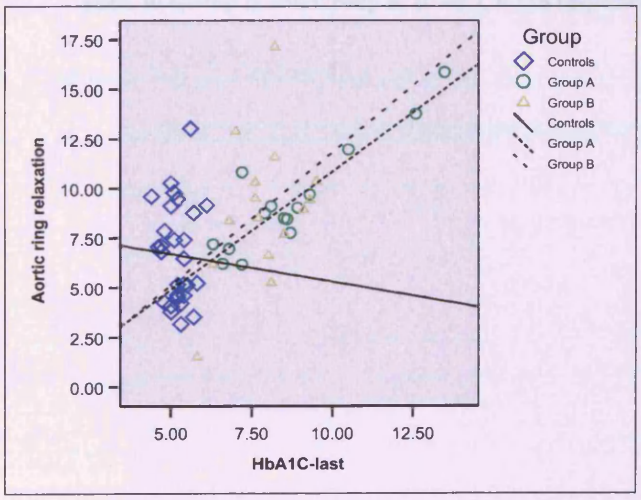


Figure 6.24: Correlation between aortic ring relaxation (%) and the last HbA1C (%) in controls, Group A, and Group B. (r^2=0.002, 0.823, 0.158 respectively).

A similar correlation was found in the diabetic groups between aortic ring relaxation and the calculated average 5-year HbA_{1C} (Group A: r=0.836, P<0.001; Group B: r=0.358, P=0.19) (figure 6.25).

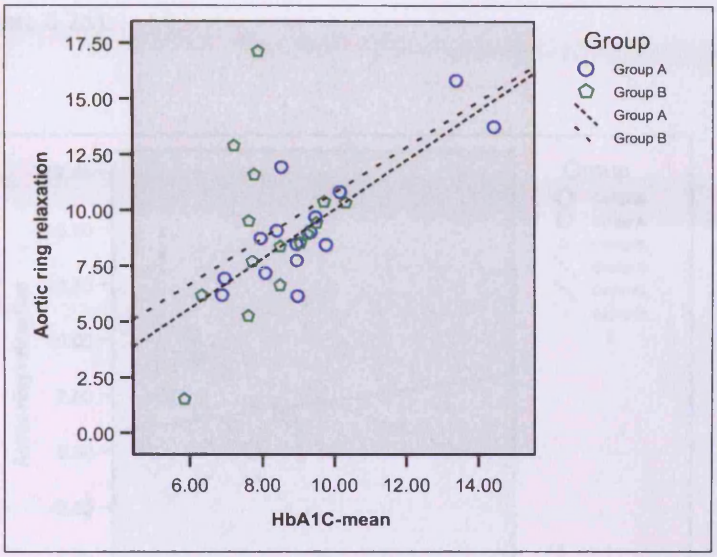


Figure 6.25: Correlation between aortic ring relaxation (%) and average HbA1C (%) over 5 years in Group A and Group B. (r^2=0.7, 0.128 respectively).

A much weaker correlation was observed between plasma glucose and aortic ring relaxation, ($r=0.057$, $P<0.05$). Aortic ring relaxation did not correlate with any of the measured NO metabolites except for HbNO. HbNO was negatively correlated with relaxation in Group A ($r= -0.472$, $P=0.089$) and more so in Group B ($r= -0.532$, $P<0.05$) (figure 6.26).

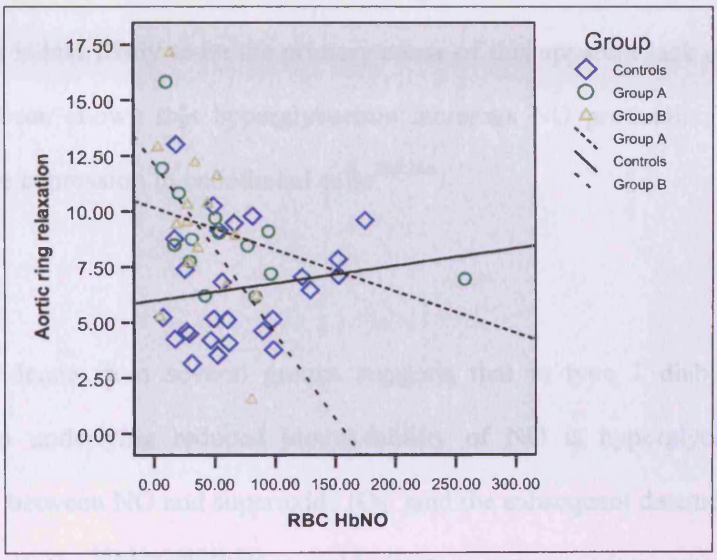


Figure 6.26: Correlation between HbNO (nM) and aortic ring relaxation (%) in controls, Group A, and Group B. (r^2=0.021, 0.222, 0.283 respectively).

Discussion

Effect of diabetes on NO metabolite levels

Endothelial dysfunction associated with reduced NO bioavailability is an established feature of diabetes mellitus. Impaired NO bioavailability can be attributed to decreased production of NO, increased degradation of NO, or both. Decreased production is less likely to be the primary cause of this apparent lack of vascular NO as it has been shown that hyperglycaemia increases NO production by increasing eNOS gene expression in endothelial cells[266-268].

Ample evidence from several groups suggests that in type 1 diabetes, the main mechanism underlying reduced bioavailability of NO is hyperglycaemia-induced imbalance between NO and superoxide (O_2^-)and the subsequent destruction of NO by reacting with O_2^- [253;256-258;263-265] to yield nitrate or peroxynitrite; some of which will eventually rearrange to form nitrate[329].

Lower levels of plasma nitrite, RBC nitrite and HbNO in diabetics can be explained by decreased formation, increased degradation, or both (figure 6.27). **Decreased formation-** It is possible that in the presence of factors such as hyperglycaemia, advanced glycation end products (AGEs) and reactive oxygen and nitrogen species, NO metabolism is shifted away from the formation of these metabolites towards the formation of other by-products such as nitrate. **Increased degradation-** it is entirely possible that diabetes-related pathometabolic factors either directly react with plasma

nitrite, RBC nitrite and HbNO to eliminate them; or affect their metabolism in a way to make them degrade faster. The nature and individual pathometabolic role of these factors remains to be clarified.

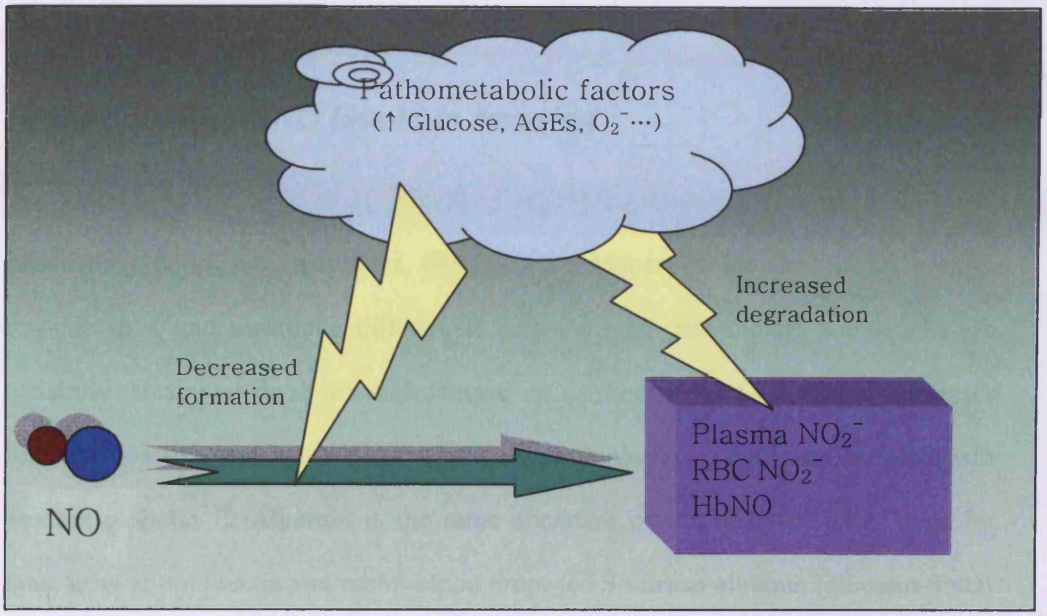


Figure 6.27: Pathometabolic factors may affect both the formation and degradation of plasma nitrite, RBC nitrite, and HbNO in diabetics and lower their blood levels.

It is interesting to note that the levels of nitrosoproteins in both plasma (i.e. RNO) and RBCs (i.e. SNO-Hb) remained stable across the groups. Nitrosoproteins display NO-like activity in vitro and have been proposed to be an important circulatory pool of NO bioactivity (CHAPTER TWO). Whether there are mechanisms in place to maintain their blood levels and the potential impacts of alterations in their levels and release kinetics on the cardiovascular system deserves further investigation.

Not only the levels but also the distribution of NO metabolites in plasma and RBC compartments was altered in diabetes; as illustrated in figures 6.8, 6.9, and 6.10. Since NO metabolites are thought to have a regulatory role in microvascular physiology, alterations in their apportionment may potentially disturb tissue perfusion, especially under low oxygen conditions.

Higher plasma RNO levels in females

Plasma levels of nitrosoproteins (RNO) were higher in females in all groups. Physiological and metabolic differences between male and female populations are generally attributed to hormonal factors. It is known that oestrogen increases important carrier proteins in plasma such as ceruloplasmin, transferrin, and thyroxin binding globulin[330]. Albumin is the most abundant plasma protein. Early work by Stamler et al. on human and rabbit blood proposed S-nitroso-albumin (albumin-SNO) as the principal S-nitrosoprotein in mammalian plasma[103]. Nonetheless, albumin-SNO cannot be taken accountable for the increased levels of RNO in females in this study. Firstly, oestrogen does not affect albumin levels[330]. Secondly, in this study females showed lower levels of serum albumin compared to males (Female: 41.94± 0.75 (g/l) versus Male: 44.26±0.56 (g/l); $P<0.05$). Total serum protein was also lower in females but did not reach statistical significance ($P=0.144$). It is likely that female milieu promotes the formation of RNO species which are independent of plasma albumin and protein levels.

Correlation between NO metabolites

It is not surprising to find a strong correlation between plasma nitrite and both HbNO and SNO-Hb in healthy individuals. HbNO is the direct product of reaction between

nitrite (or NO) with deoxyhaemoglobin[11;28;30]. Adding nitrite to blood, either *in vivo* or *ex-vivo*, also produces SNO-Hb in concentrations lower than HbNO[77;78]. The more nitrite in the plasma, the more nitrite will enter the RBCs to form HbNO and SNO-Hb (CHAPTER ONE). Therefore it is expected that in healthy individuals, levels of HbNO and SNO-Hb reflect plasma nitrite closely as well as direct formation of these metabolites from NO (figure 6.28).

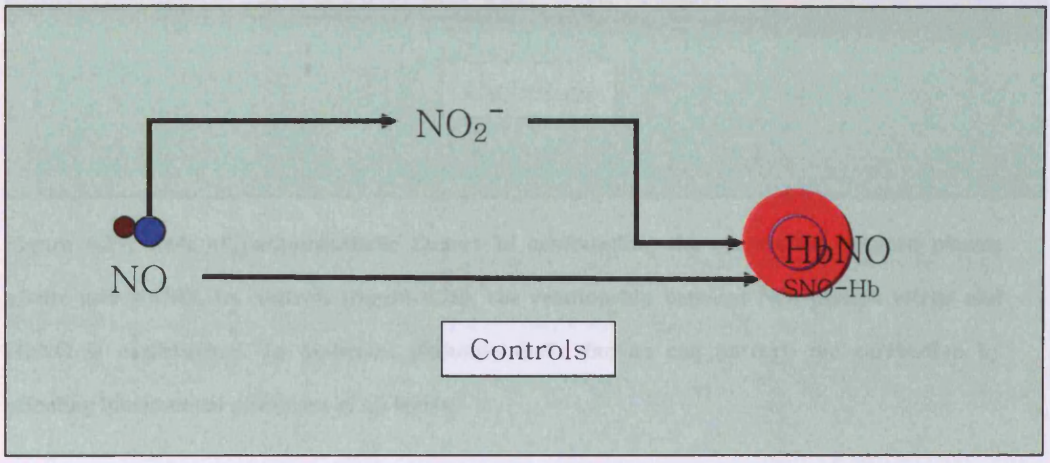


Figure 6.28: Both NO and nitrite can react with deoxyhaemoglobin to form HbNO inside the erythrocytes.

The correlation becomes weaker and less predictable in diabetics where pathometabolic factors perturb the availability of both NO and plasma nitrite (figure 6.29).

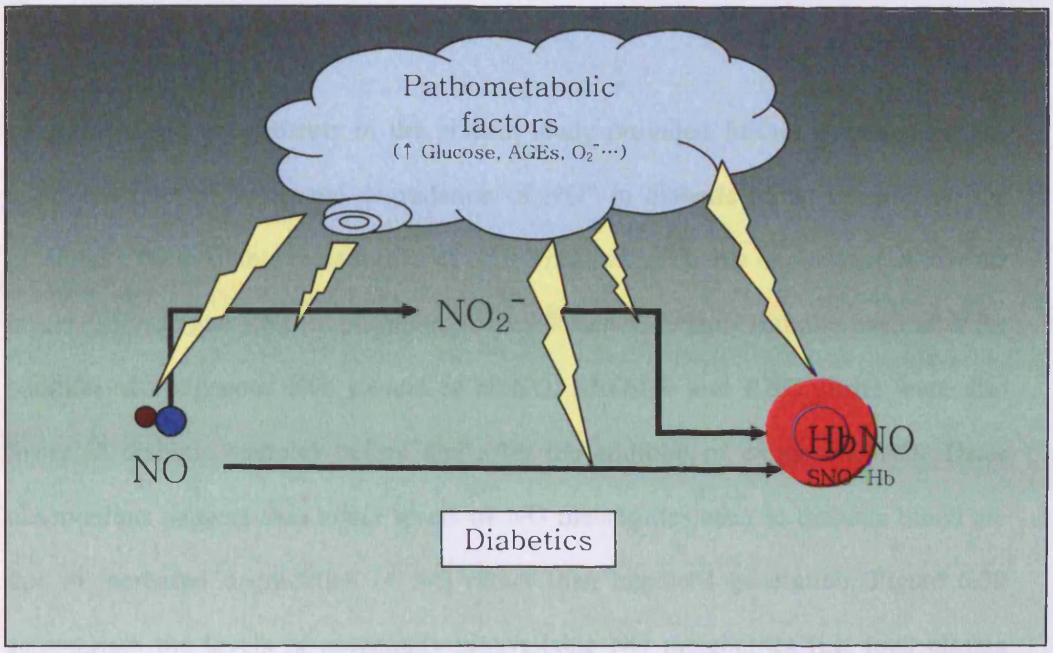


Figure 6.29: Role of pathometabolic factors in confounding the correlation between plasma nitrite and HbNO. In controls (figure 6.28), the relationship between NO, plasma nitrite and HbNO is undisturbed. In diabetics, pathometabolic factors can perturb the correlation by affecting biochemical pathways at all levels.

Correlation between NO metabolites and HbA_{1C}

Consistent with the above findings, I also found that diabetes perturbs both the correlation between plasma nitrite and HbNO, on one hand and nitrite with HbA_{1C} on the other hand. The negative linear relationship observed between plasma nitrite and HbA_{1C} in controls did not exist in diabetics. In the case of HbNO, the moderate negative correlation with HbA_{1C} in controls became gradually weaker in Group A and more so in Group B.

Exogenously added NO studies

Exogenous NO experiments in the present study provided further evidence for the important role of "enhanced degradation of NO" in diabetic blood in reducing the potentially bioavailable metabolites of NO. These experiments demonstrated that the levels of nitrite and RNO in plasma remained lower in diabetic samples even after the addition of exogenous NO. Levels of HbNO, SNO-Hb and RBC nitrite were also lower in diabetic samples before and after the addition of exogenous NO. These observations suggest that lower levels of NO metabolites seen in diabetic blood are due to increased degradation of NO rather than impaired generation. Figure 6.30 summarises the levels of potentially bioavailable NO metabolites (i.e. total plasma and RBC NO metabolites excluding nitrate) in control and diabetic groups at baseline and after the addition of 1μM, 10μM, and 100μM exogenous NO.

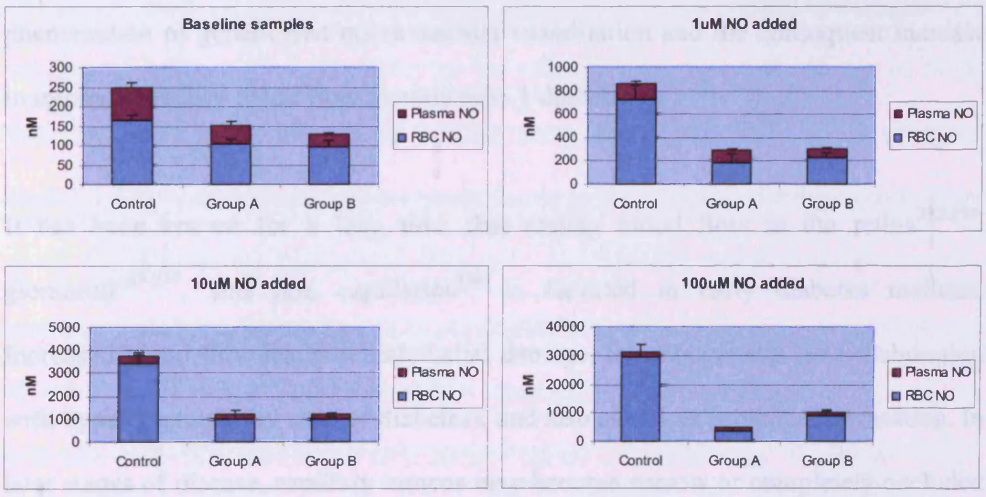


Figure 6.30: Total levels of potentially bioavailable NO metabolites (i.e. total plasma and RBC NO metabolites excluding nitrate) in control and diabetic groups at baseline and after the addition of 1μM, 10μM, and 100μM exogenous NO.

Interestingly, although the absolute levels of plasma nitrite and RNO were lower in diabetic samples; their apportionment in plasma was identical among all 3 study groups at each concentration of exogenous NO (figure 6.19).

Vasodilator properties of diabetic blood: RBC-related vasodilating factor (RRVF)

Diabetic patients are known to have accelerated endothelial dysfunction which has been linked to abnormal metabolism of NO. Given the fact that the bioavailability of NO is reduced in diabetic blood, one may expect it to have lower vasodilator properties. Nonetheless, my observations showed that RBCs from diabetic patients exert stronger vasodilator properties than RBCs from controls, *ex vivo*. This property of RBCs was more significant in Group A patients who were generally younger and had a shorter history of diabetes. These findings may help to explain the established phenomenon of generalised microvascular vasodilation and the consequent increase in microcirculatory blood flow in early type 1 diabetes[331].

It has been known for a long time that resting blood flow in the retina[332-334], glomeruli[335-337], and skin capillaries[338] is elevated in early diabetes mellitus. Increased blood flow leads to endothelial damage, thrombogenesis (in collaboration with hypercoagulability state of diabetes), and also enhances protein extravasation. In later stages of disease, capillary lumens may become narrow or completely occluded due to the thickening of basement membrane, proliferation of endothelial cells, and the formation of microthrombi[339]. Injury to the endothelium leads to adaptive microvascular sclerosis which deters vasodilatory reserve and autoregulatory

capacity of the microvasculature[338]. Blood flow may get further impaired by macrovascular disease in larger arteries upstream.

Microvascular vasodilation in diabetes has been attributed to several factors including altered levels of vasoactive substances; altered vasomotor responsiveness, chronic plasma volume expansion, and tissue hypoxia[331]. My observations suggest the existence of a RBC-related vasodilating factor (RRVF) which is present in both diabetics and controls but exerts stronger vasodilator activity when RBCs from the former group are added to aortic ring preparations in a hypoxic tissue bath system *ex vivo*. Moreover, this RBC-related vasodilator activity is in a strong correlation with HbA_{1C}; more so in my Group A patients who were generally younger diabetics with shorter duration of disease.

The exact nature of RRVF remains to be clarified. Previous work has suggested an NO-type species to be responsible for the relaxation instigated by RBCs in tissue bath experiments[324]. The results of this study neither support nor reject those findings. I did not find a positive correlation between aortic ring relaxation and any of the NO metabolites analysed in this study. Nonetheless, this does not necessarily exclude a role for NO metabolites in aortic ring relaxation. It is entirely possible that RRVF is an NO adduct which cannot be detected by the tri-iodide and DAN assays. It is also possible that RRVF is a distinct factor from NO (e.g. K^+ or adenosine) which acts in parallel to RBC-NO to exert vasorelaxation.

The role and position of HbA_{1C} in the RRVF system needs to be defined. Like other advanced glycation end products (AGE)[262;340], HbA_{1C} might be expected to inactivate

NO more efficiently than unglycated Hb, and to mediate relative vasoconstriction. Paradoxically in this study RBCs with higher Hb_{A1C} content showed stronger RRVF activity. A finding which may imply RRVF activity is not exclusively dependent on NO. An alternative explanation is that Hb_{A1C} promotes the formation of a special NO adduct which is inactive in normal conditions but becomes active to exert RRVF activity in a hypoxic tissue bath. If RRVF proves to be independent of NO, glycation of Hb may still have a role in promoting the formation of RRVF or enhancing its release from the diabetic RBC, the mechanism of which remains to be elicited.

Finally, HbA_{1C} may not have any cause-effect relationship with vasodilation and simply reflect the general diabetic hyperglycaemic state. The three possible links between HbA_{1C} and RRVF are summarised in figure 6.31.

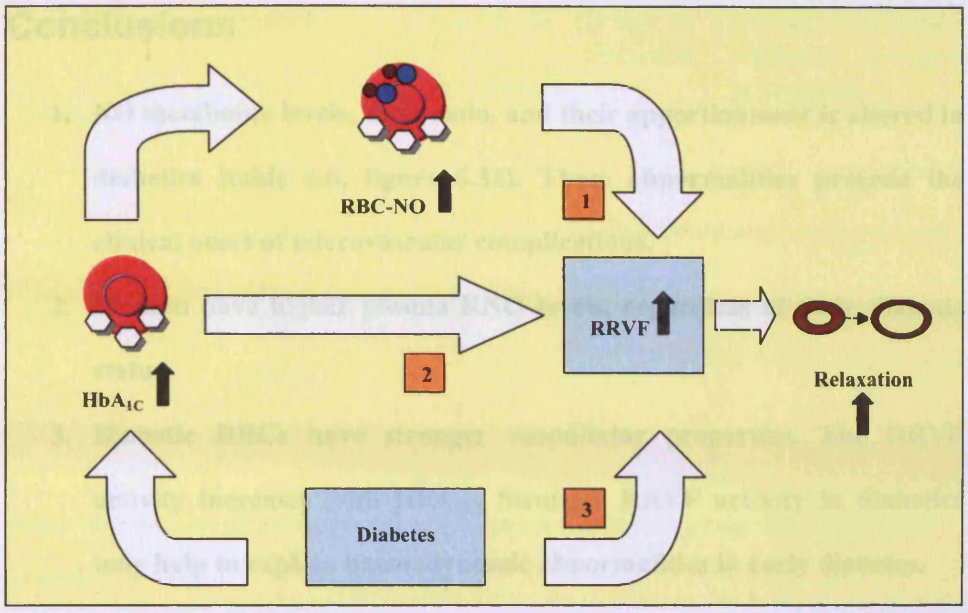


Figure 6.31: Potential explanations for the correlation between HbA_{1C} and RRVF activity. 1) RRVF is a NO adduct and HbA_{1C} increases its formation by attracting more NO into RBCs. 2) RRVF is not a NO adduct. Increased HbA_{1C} leads to increased RRVF activity directly or via non-NO dependant pathways. 3) Increased HbA_{1C} and RRVF activity are independent features of diabetes mellitus. There is no direct link between HbA_{1C} and RRVF.

The negative correlation observed between aortic ring relaxation and HbNO in diabetics can be explained by the co-operative nature of the binding of NO to haemoglobin as demonstrated by Gow et al.[341]. HbNO has a higher affinity for NO than free haemoglobin. This makes HbNO a stronger "NO quencher" which can eliminate free NO from the system more efficiently than Hb and therefore mitigate NO-related vasorelaxation.

Conclusions

1. **NO metabolite levels, their ratio, and their apportionment is altered in diabetics (table 6.6, figure 6.32). These abnormalities precede the clinical onset of microvascular complications.**
2. **Women have higher plasma RNO levels, regardless of their diabetic status.**
3. **Diabetic RBCs have stronger vasodilator properties. The RRVF activity increases with HbA_{1C}. Stronger RRVF activity in diabetics may help to explain haemodynamic abnormalities in early diabetes.**

Haemodynamic abnormalities can be detected in the glomerular microcirculation and the retina many years before nephropathy or retinopathy become clinically detectable[331].Correspondingly, this study demonstrated that alterations in NO metabolism precede microvascular complications in type 1 diabetes. Prospective studies are required in the future to investigate a cause-effect relationship between altered NO metabolism and microvascular complications of diabetes.

Compartment	Metabolite	Levels in Group A diabetics compared to controls	Levels in Group B diabetics compared to controls
Plasma	nitrate	↔	↔
	nitrite	↓	↓↓
	RNO	↔	↔
RBC	nitrite	↓↓	↓
	HbNO	↓	↓↓
	SNO-Hb	↔	↔

Table 6.6: Summary of changes in NO metabolites in the diabetic groups in comparison to controls.

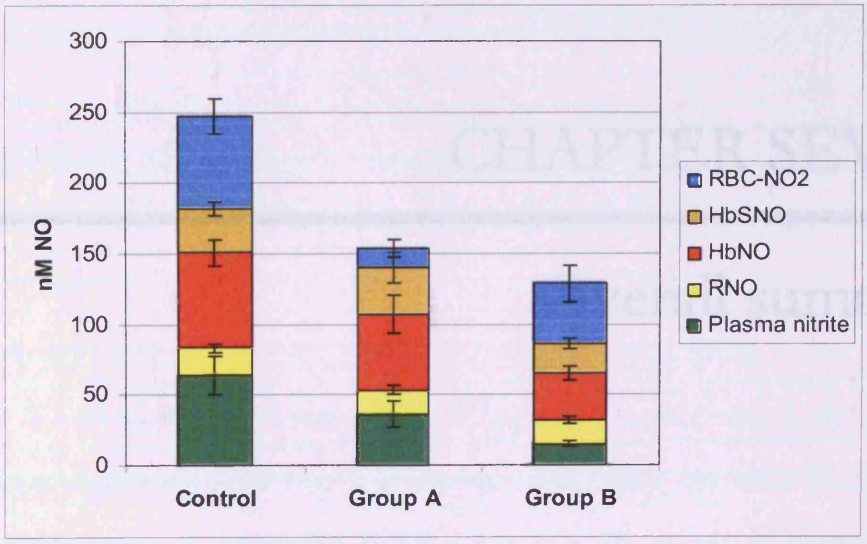


Figure 6.32: Summary of NO metabolites levels in controls, Group A, and Group B (plasma nitrate not included).

CHAPTER SEVEN

Overall summary

It is well accepted that endothelial NO plays an important role in the control of vascular tone and blood flow[25]. *In vivo* radical NO has a very short life span in the blood prior to its metabolism to other nitrogen oxide species. Consequently direct effects of NO are confined to a short distance from its site of production[51]. However, the biological metabolites of NO which circulate in the plasma and RBC are more stable compounds with considerably longer circulatory life spans. These species have been proposed to act as a potential reservoir of bioactive NO in the circulation. However, the identity of the metabolite(s) responsible for conserving bioactivity and

the mechanism(s) involved in the release of bioactivity and its transport to the site of action still remain to be fully elucidated. Several scientific groups have studied various NO metabolites in plasma and RBC over the past 15 years in a quest to identify which metabolite(s) and what mechanism(s) play primary roles. One particular issue which has hindered progress in this research field has been the lack of a standard methodology for NO metabolite measurements. As a result, wide differences can be found among laboratories in the reported values of circulatory NO metabolites.

The focus of this PhD project

The present PhD project focused on the significance of NO metabolites both in health and in the pathophysiology of cardiovascular disorders. Firstly as a result of the issues related to methodology I examined, modified, and improved the available methodologies. The development of assays to analyse various NO metabolites with the least risk of contamination allowed collection and accurate assessment of plasma and RBC samples within the context of two human model systems.

Secondly I went on to examine the significance of NO metabolites in health and investigated the profile and dynamics of NO metabolism across the heart and lungs in patients with normal coronary arteries.

Finally I looked at NO metabolites in a disease state; type 1 diabetes mellitus, one of the most important risk factors for vascular disease.

Cross-heart study

I studied NO metabolism across the cardiac and pulmonary beds in healthy subjects. In this work I explored variations in plasma and RBC NO metabolites in response to increased oxygen demand. To study the physiological importance of NO and its metabolites in the regulation of coronary blood flow, each study was repeated after inhibition of endothelial NO production by a systemic infusion of L-NMMA. I also looked at the association of NO metabolites and their apportionment between RBC and plasma compartments in relation to blood oxygen and the cross heart, cross pulmonary oxygen gradients.

I established the profile of individual nitric oxide metabolites and the dynamic changes in their concentration, production/consumption across human heart and lungs. These changes were related to coronary function and I used the TIMI-frame count method to estimate coronary flow. The study provided evidence for transcardiac gain and transpulmonary loss of NOx –mainly reflecting plasma nitrate. The novel finding of transpulmonary loss of plasma nitrate may account for a fraction of the 35% unknown fate of ingested nitrate. In addition, this study provided evidence for transcardiac loss and transpulmonary addition of nitrite from/to plasma. Plasma RNO levels remained stable throughout the study. Contrary to the changes in nitrite, the study provided further evidence for transcardiac formation of Hb-bound NO and transpulmonary formation of RBC-nitrite.

In terms of relevance to the two main theories which propose either S-nitrosohaemoglobin or plasma nitrite as the stable "long-distance" transporters of NO

bioactivity in the blood, my study did not support the S-nitrosohaemoglobin hypothesis as no dynamic changes nor any significant gradient were detected between SNO-Hb levels across the coronary vascular bed. Regarding the nitrite hypothesis, transcardiac "loss" of nitrite may be interpreted as transcardiac "consumption" of nitrite to favour a role for nitrite in coronary physiology. Together with the increase in intraerythrocytic Hb-bound NO, it is tempting to speculate the traffic of nitrite from plasma into RBC as a function of deoxygenation. However, as mentioned previously in CHAPTER FIVE, arteriovenous differences can be misleading and could be the result of higher production of nitrite on the arterial side or higher nitrite consumption in the low oxygen milieu of venous blood.

Consistent with previous studies, my study confirmed the important contribution of NO to the basal coronary tone in human subjects and demonstrated that coronary vasodilatation in response to increased myocardial oxygen demand is not exclusively NO dependent and occurs even when endothelial NO production is inhibited by L-NMMA. Moreover, I found that coronary vasodilator response to pacing is enhanced with the inhibition of NO production; particularly in more distal and hence smaller segments of the coronary arteries. Based on this observation, I postulate the presence of an EDHF-like activity in the epicardial coronary arteries. Further studies into the exact nature of this EDHF-like factor may have important clinical implications in the medical treatment of patients suffering from angina pectoris, particularly in those with more distal lesions in their coronary arteries which are anatomically less amenable to revascularisation.

Regarding coronary blood flow (CBF), this study showed that NO is not necessary for the maintenance of CBF at rest, but it plays an important role in enhancing the CBF in response to increased myocardial oxygen demand. When the heart was paced at 85% maximum heart rate, CBF was reduced in the presence of L-NMMA.

Studying the role of oxygen in the apportionment of NO metabolites, I observed that hypoxaemic conditions favour the formation of intraerythrocytic NO metabolites at the cost of decreased NO metabolites in plasma. One can postulate from this observation that NO or a related moiety could in principle "swap" between plasma and RBCs as a function of oxygen. More importantly, this study shows that this phenomenon may occur during a single arteriovenous transit; a finding that bears relevance to the question whether plasma or RBC sources can diffuse quickly enough between intra- and extravascular compartments in order to exert meaningful physiological effect.

In conclusion from studying the significance of NO metabolites in the coronary circulation, I conclude that NO is dynamically metabolised across the heart and that the compartmentalisation of its metabolites between plasma and RBC is driven primarily by the oxygen saturation of the blood.

Diabetic study

To investigate the potential alterations in nitric oxide metabolism in a disease state, I chose diabetes mellitus as a model. Diabetes mellitus is associated with endothelial dysfunction and is a major risk factor for cardiovascular disease[321]. Patients with type

1 diabetes with no other cardiac risk factors or a previous history of macrovascular disease were recruited to this study. The profile of NO metabolites was investigated in this group of patients and potential correlations were drawn between the formation of advanced glycation end products, alterations in nitric oxide metabolism, and the existence of microvascular complications of diabetes. I found blood levels of NO metabolites to be generally lower in diabetics compared to controls; and lower in those with microvascular complications compared to those without. I also discovered a physiological correlation between plasma nitrite and HbNO on one hand and HbA_{1C} on the other hand in normal subjects which is perturbed in diabetics. Studying NO metabolites in diabetic patients without and with microvascular complications showed that disturbance in NO metabolism occurs prior to clinically apparent sequelae.

My vessel relaxation experiments suggested the existence of a RBC-related vasodilating factor (RRVF) which is present in both diabetics and controls but exerts stronger vasodilator activity when RBCs from the former group are added to aortic ring preparations in a hypoxic tissue bath system *ex vivo*. Another novel but yet unexplained finding was a positive correlation between this RBC-related vasodilator activity and HbA_{1C}. This was stronger in that group of patients who were generally younger with shorter duration of disease. Given that no significant correlation was found between NO metabolites and vasorelaxation and that diabetics exhibited less total NO metabolites, it is unlikely that this phenomenon relates to different release kinetics from the RBC. A plausible explanation is that the recapture of released NO in the tissue bath system may be significantly reduced in type 1 diabetes and therefore more NO or NO moiety would be bioactively available to relax the aortic rings. Other

possibilities are that RRVF is either not a NO moiety at all or is a NO moiety but the current methodology is not sensitive or specific enough to detect it.

The above findings also provide a potential basis for the development of diagnostic tests which can clarify the diagnosis of diabetes in uncertain cases as well as screening tests to identify patients who are at higher risk of developing microvascular disease or those who are in the subclinical phase of microvascular complications. Further treatment strategies can then be implemented to delay the progress of the pathologic process in these patients.

Propositions for future studies

This study like any other PhD project has specifically addressed a select few questions and simultaneously created many more.

The particular NO moiety responsible for the transfer of NO bioactivity in blood remains unidentified. It is entirely possible that the preservation of NO bioactivity is not dependent on a single moiety but occurs through interplay among a group of NO metabolites. The biochemistry of such an interplay and the potential impact of physiological (e.g. oxygen) and pathological (e.g. free radicals) factors warrant further investigation. In terms of existing hypotheses (Stamler versus Gladwin) the truth is likely somewhere in between. My study supports exchange of NO-"moiety" between blood metabolites and blood compartments during a single arteriovenous transit.

It is entirely possible that the major bioactive pool of NO in the body is intracellular rather than circulatory nitrite. Intracellular pH of smooth muscle cells decreases significantly (≈pH 6.6[87]) during hypoxia/ischaemia and increased metabolic activity. In these acidic conditions, NO can be generated from non-enzymatic reduction of inorganic nitrite[86] to activate sGC and relax the smooth muscle cells.

This study investigated transcardiac NO metabolism in human subjects with normal coronary arteries. There is evidence from the diabetic work that NO metabolites may behave differently in disease. Transcardiac NO metabolism in the presence of coronary disease needs to be explored further. It is also important to recognise my studies involved sampling from sites under normal oxygenation status, even at higher heart rates. It is entirely possible that NO metabolites may accrue greater physiological importance when oxygenation is perturbed, such as during ischaemia.

Another area which deserves further investigations is the observed EDHF-like activity in coronary arteries. Pharmacological agents which enhance this effect could potentially become the new class of antianginals in the future.

From the diabetic study, the RBC-related vasodilating factor (RRVF) remains to be identified. Why is RRVF more potent in diabetics? What is the biochemical basis of the correlation between HbA_{1C}, NO metabolites, and vessel relaxation? How can these findings be pursued to the development of clinical diagnostic and screening tests? Would a tighter control of hyperglycaemia rectify alterations in NO metabolism?

Diabetes is only one of the risk factors for cardiovascular disease. The impact of other risk factors such as smoking and hyperlipidaemia on NO metabolism should also be studied.

• • •

Taken together, the role(s) of blood NO metabolites are interlinked and in constant flux. Largely based on the work presented in this thesis but also ongoing studies, it is our contention that most NO metabolites are in equilibrium with each other and that perturbation of any one particular metabolite or route has influence on the amounts and roles of the others. By definition, each NO metabolite can (depending on conditions) biochemically form any other. It has also become apparent to us that each NO metabolite may have distinct bioactivity that may only in part be mediated by classical NO pathways.

This work therefore provides new and exciting data from human subjects that are accurate and robust. They also provide the foundation for next generation research into possible ways to manipulate these processes to benefit human health.

APPENDICES

Appendix 1: Coronary utilisation of a stable nitric oxide reservoir: importance during increased metabolic demand.

Infusions

1. Aminophylline 250mg/10ml vials

- Loading dose: Add 5mg/kg to 100ml Normal Saline 0.9% and infuse over 20 minutes (100 drops/minute).
- Maintenance dose: Add 250mg to 250ml Normal Saline, thus, make a 1mg/ml solution. Infuse this solution at the rate of 500 µg/kg/hour using table 1:

Table 1:

Weight (kg)	Maintenance dose, Infusion rate (ml/hour)
50	25
55	27.5
60	30
65	32.5
70	35
75	37.5
80	40
85	42.5
90	45
95	47.5
100	50

2. L-NMMA 260mg/vial

- Loading dose: 5mg/kg over 7 minutes.
- Maintenance dose: 50 µg/kg/minute

- Loading dose: Add 500mg (2 vials) of L-NMMA to 50ml Normal Saline 0.9 % (10mg/ml). Infuse the calculated dose over 15 minutes.
- Maintenance dose: Add 250mg to 25ml Normal Saline (10mg/ml). Put into a 50ml syringe and infuse with a syringe driver. (Table 2)

Table 2

Weight (kg)	Loading dose mg/7 min	(drops/min)	Maintenance dose (ml/hour)
50	250	(33)	15
55	275	(36)	16.5
60	300	(40)	18
65	325	(43)	19.5
70	350	(47)	21
75	375	(50)	22.5
80	400	(54)	24
85	425	(57)	25.5
90	450	(60)	27
95	475	(67)	30

PATIENT INFORMATION SHEET

1. Study Title

Coronary utilisation of a stable nitric oxide reserve: Importance during increased metabolic demand.

(Measuring a messenger produced by the lining of healthy blood vessels to keep them open, at rest and as the heart rate is increased (pacing))

2. Invitation Paragraph

Your doctors will have told you that you need to undergo electrophysiological studies (EPS) to further investigate your rapid heart beat. Dr James, Professor Frenneaux, Dr Paul and their research groups, at the Wales Heart Research Institute and St Peter's Hospital, are conducting this study. Before you decide to help with it, it is important that you understand what the research is about. PLEASE READ THIS INFORMATION SHEET CAREFULLY and discuss it with friends and relatives. Please ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. You will be given a copy of this information and consent form if you decide to take part.
Thank you for taking the time to read this.

3. What is the purpose of the study?

The overall purpose of the study is to look at the effects of nitric oxide (NO), a messenger produced by the lining of healthy blood vessels to keep them open. You are being asked to enter this study as a healthy patient without diseased arteries to the heart. The necessary blood samples will be taken at the time of your electrophysiological studies.
The study will compare the use of the messenger (NO) in the healthy heart (yours), to those patients' with diseased arteries. Patients' in both groups will have blood samples taken with a resting heart rate and also when the heart rate is increased during the procedure.

4. Why have I been chosen?

You have been chosen because you are a healthy man undergoing electrophysiological studies as recommended by your doctors. Also you have had a normal exercise test, suggesting that you have normal arteries to the heart.

5. Do I have to take part?

Your participation in this study is entirely voluntary. Should you decide not to take part you will of course still have your planned electrophysiological studies. Your care will remain unchanged.

6. What will happen to me if I take part?

If you decide to take part in the study you will be asked not to take any of your heart medication for 48 hours beforehand. You will also be asked not to eat or drink for 12 hours before the study. We will then fully explain the procedure and ask you to sign a consent form.
Just before we start the study we will give you a small tablet to swallow in order to relax you. A slow intravenous infusion of a drug called aminophylline (which is commonly used in patients with asthma to help with their breathing) will be started and continued throughout the study. Next the skin at the top of your leg will be cleaned and some sterile towels placed over your legs. A small injection of local anaesthetic will be put into the top of the right leg to numb the area. A needle will be inserted into the artery and another into the vein. Plastic tubes will then be used, so that no further needles are required. Via these small tubes special catheters will be inserted around into the veins and arteries of the heart and also a wire fed around into one chamber. A small amount of special dye will be injected through the tubes as an X ray is taken. This will allow us to see the outline of your arteries clearly. After this we will take several small samples of blood out of the tubes.
By using the wire already in position we will the pace your heart and slowly increase your heart rate. During this time we will repeat the blood tests.
Finally, after a few minutes we will then, via the tubes, inject a small amount of a drug which stops the production of extra messenger, and repeat the slow increase in your heart rate.
After this Dr Paul will carry out the electrophysiological study as planned. All of the tubes, catheters and wire already inserted will be used for the standard procedure in your EPS.
The study part of the procedure will only add about 30 minutes to the procedure time.

7. What do I have to do?

Once you have read this form and had time to think about the study, you will be contacted by Dr Paul's research team. If you agree to participate then you will be contacted again before the date of your electrophysiological study.

8. What is the drug or procedure that is being tested?

No drugs are being tested, but the amount of messenger released into your blood from your healthy heart is measured.

9. What are the alternatives for diagnosis or treatment?

This study does not affect your future care in any way as it is being done in addition to your planned management.

10. What are the side effects of taking part?

The side effects of taking part are the same as for your planned EPS. This includes, bleeding from the artery or vein after the procedure, and also an awareness of the increase in your heart rate.

11. What are the possible disadvantages and risks of taking part?

You will feel some discomfort while the tubes are placed in your leg and possibly some palpitations while the catheters are placed in your heart (both should last no more than a few seconds)
Lying flat following the procedure can also be uncomfortable. The main risks relate to the bleeding from the leg (discussed above) and of rhythm disturbances (palpitations) caused by the catheters in your heart. If these occur for more than a few seconds the catheters will be withdrawn. You may notice some leg discomfort for a few days after the procedure and sometimes some bruising.

12. What are the possible benefits of taking part?

The benefits of taking part are that the procedure involves no further risk to you than having the EPS alone. The benefit is that by studying a healthy heart, we are able to better understand other patients' with diseased arteries.

13. What if new information becomes available?

If you agree to participate in this study you are being studied as a healthy patient, and therefore the information obtained will be of help to those patients' with coronary artery disease.

14. What happens when the research study stops?

Your care will continue as normal. You will not be asked to attend any additional follow up visits for the purpose of the study.

15. What if something goes wrong?

If taking part in this research project harms you, there is no special compensation arrangement. If you are harmed due to someone's negligence then you may have grounds for legal action, but you may have to pay for it. Regardless of this, if you wish to complain about any aspect of the way you have been approached or treated during the course of this study the normal National Health Service complaints mechanisms may be available to you.

16. Will my taking part in this study be kept confidential?

Dr James, Professor Frenneaux, Dr Paul and their study personnel will collect information about you. This will remain confidential.
This data will be kept in a secure office at the University of Wales College of Medicine.
Anonymity will be maintained throughout the trial.

17. What will happen to the results of the research study?

The data from this study may be used in publications. However, your name will not appear in the publications.

18. Who is organising and funding the research?

The study is organised and funded jointly by the Cardiology Research Department's at St Peter's Hospital and the University Of Wales College Of Medicine.

19. Who has reviewed the study?

This study has been reviewed by the North West Surrey Local Research Ethics Committee, the Bro Taf Local Research Ethics Committee. Each of the above hospitals' Research and Development Departments have also reviewed and approved the study on behalf of the Trusts'.

20. Contact for further information

If you or your relatives have any questions about the study, please call

Dr Sue Ellery or Dr V. Paul 01932 723534

YOU WILL BE GIVEN A COPY OF THIS INFORMATION SHEET AND A SIGNED CONSENT FORM TO KEEP.

Centre Number

Study Number:

Patient Identification Number for this Trial:

CONSENT FORM

Coronary utilisation of a stable nitric oxide reservoir: importance during increased metabolic demand.

(Measuring a messenger produced by the lining of healthy blood vessels to keep them open, at rest and as the heart rate is increased (pacing))

Name of Researcher: Dr Afshin Khalatbari, Dr Vince Paul, Dr Phillip James& Professor Michael Frenneaux

Please initial box

1. I confirm that I have read and understood the information sheet ☐ dated..... (Version.....) For the above study and have had the opportunity to ask questions.

2. I understand that my participation is voluntary and that I am ☐ free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.

3. I agree to take part in the above study ☐

Name of Patient	Date	Signature
Name of Person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes

Appendix 2: The profile of nitric oxide metabolites in type 1 diabetes mellitus; correlation with microvascular complications

Information sheet for medical and nursing staff at the

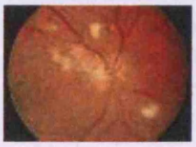
Diabetes Clinic, University Hospital of Wales, Cardiff

6 February 2005

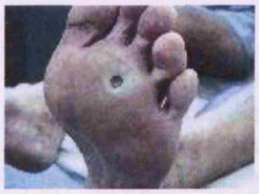
Impaired release of Vascular Nitric Oxide (NO) by Highly Glycated Haemoglobin: Correlation with Microvascular Disease.

Dr Afshin Khalatbari, Dr John Peters, Dr Philip James
Wales Heart Research Institute, University Hospital of Wales

To: Staff at Diabetes Clinic/ UHW



Microvascular complications remain major causes of morbidity and mortality in diabetes, but the aetiology is poorly understood. Our recent data suggest that abnormal nitric oxide (NO) metabolism may have a pathogenetic role in microvascular complications of diabetes.



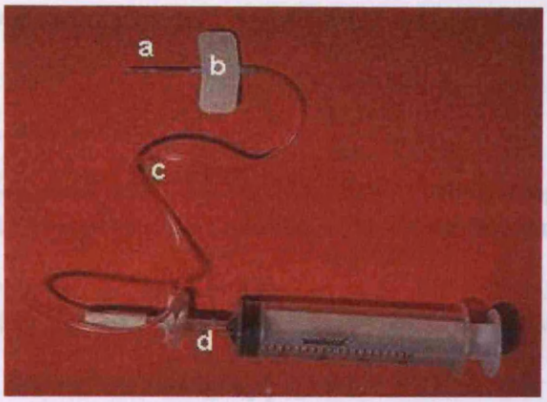
To study this further we need to take (up to) 40ml blood from the patients with type 1 diabetes mellitus attending the Diabetes Clinic, who do not have any other cardiovascular risk factors (non-smoker, normal blood pressure, total cholesterol<5.2, triglyceride<2, no history of ischaemic heart disease) and fall into one of the following categories:

A) 30 patients without any microvascular complications.
B) 30 patients with moderate to severe microvascular complications (neuropathy, nephropathy, retinopathy).
C) 30 newly diagnosed type 1 diabetics.

For groups A and B, this will be a one off blood sample. For group C, I shall see them in 6 months time when I take a second sample.

We also need 30 non-diabetic blood samples as our controls. This can be taken from healthy companions of the patients upon their consent.

Blood will be taken with a 50ml syringe via a butterfly catheter.



Due to technical limitations, we can only take and analyse blood from one patient (or control) each day. I shall attend the Clinics early and review the notes beforehand to choose the suitable patient and record the relevant past medical history. Then I will speak to the patient, explain the study, and ask for his/her consent. If none of the patients met the criteria, I will ask a healthy relative to give blood which will be analysed as control.

After taking the blood, HbA1C will be measured in the Clinic and I will store the rest of the blood in blue (clotting) and purple (FBC) topped blood tubes and take them to WHRI for further analysis.

Please do not hesitate to contact me if you have any questions regarding the above study.

Dr Afshin Khalatbari
Clinical Research Fellow
Wales Heart Research Institute
Extension: 2912, E-mail: khalatbaria@cf.ac.uk

PATIENT INFORMATION SHEET

1. Study Title

Impaired release of Vascular Nitric Oxide (NO) by Highly Glycated Haemoglobin: Correlation with Microvascular Disease.

(Measuring chemicals produced by the lining of blood vessels and heart tissue and studying their correlation with diabetes control and chronic complications.)

2. Invitation Paragraph

Dr James, Dr. Peters, Dr. Khalatbari and their research group at the University Hospital of Wales and the Wales Heart Research Institute are running this study. Before you decide to help with it, it is important that you understand what the research is about. PLEASE READ THIS INFORMATION SHEET CAREFULLY and discuss it with friends and relatives. Please ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. You will be given a copy of this information and consent form if you decide to take part. Thank you for taking the time to read this.

3. What is the purpose of the study?

The overall purpose of the study is to find any correlation between chronic complications of type 1 diabetes, level of blood sugar control, and nitric oxide metabolites in the blood.

4. Why have I been chosen?

You are chosen because you have type 1 diabetes with no other cardiovascular risk factors.

5. Do I have to take part?

Your participation in this study is entirely voluntary. Should you decide not to take part your care will remain unchanged.

6. What will happen to me if I take part?

We will fully explain the procedure and ask you to sign a consent form. Afterward, up to 40ml venous blood will be taken from a peripheral vein. The procedure will be similar to any routine venesection in the hospital. This is only a one-off blood sample.

7. What do I have to do?

Once you have read this form and had time to think about the study, you will be asked to sign the consent form.

8. What is the drug or procedure that is being tested?

No drugs or procedures are being tested.

9. What are the alternatives for diagnosis or treatment?

This study does not affect your future care in any way as it is being done in addition to your planned management.

10. What are the side effects of taking part?

The side effects of taking part are the same as any routine venesection.

11. What are the possible disadvantages and risks of taking part?

There are no major risks or disadvantages.

12. What are the possible benefits of taking part?

The benefit is that by studying your blood, we are able to better understand other patients' with diabetes.

13. What if new information becomes available?

If you agree to participate in this study, the new information obtained will be of help to those patients' with diabetes.

14. What happens when the research study stops?

Your care will continue as normal. You will not be asked to attend any additional follow up visits for the purpose of the study.

15. What if something goes wrong?

If taking part in this research project harms you, there is no special compensation arrangement. If you are harmed due to someone's negligence then you may have grounds for legal action, but you may have to pay for it. Regardless of this, if you wish to complain about any aspect of the way you have been approached or treated during the course of this study the normal National Health Service complaints mechanisms may be available to you.

16. Will my taking part in this study be kept confidential?

Dr James, Dr. Peters, Dr. Khalatbari and their study personnel will collect information about you. This will remain confidential. This data will be kept in a secure office at the Wales Heart Research Institute. Anonymity will be maintained throughout the trial.

17. What will happen to the results of the research study?

The data from this study may be used in publications. However, your name will not appear in the publications.

18. Who is organising and funding the research?

The study is organised and funded jointly by the Cardiology Department at the University Hospital of Wales and the Wales Heart Research Institute, Cardiff University.

19. Who has reviewed the study?

Research and Development Office at Cardiff & Vale NHS Trust, Local Research Ethics Committee

20. Contact for further information

If you or your relatives have any questions about the study, please call
Dr Afshin Khalatbari 029 2074 2912

YOU WILL BE GIVEN A COPY OF THIS INFORMATION SHEET AND A SIGNED CONSENT FORM TO KEEP.

Centre Number:
Study Number:
Patient Identification Number for this Trial:

CONSENT FORM

Impaired Release of Vascular Nitric Oxide (NO) by Highly Glycated Haemoglobin: Correlation with Microvascular Disease.
(Measuring chemicals produced by the lining of blood vessels and heart tissue at rest and after coronary angioplasty.)

Name of Researchers: Dr. John Peters, Dr Philip James& Dr. Afshin Khalatbari

Please initial box

1. I confirm that I have read and understood the information sheet dated.....(version.....) for the above study and have had the opportunity to ask questions. ( )

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ( )

3. I agree to take part in the above study. ( )

Name of Patient	Date	Signature
Name of Person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes
Version 1 05/03/2005 PRO/03/06

Questionnaire

Impaired release of Vascular Nitric Oxide (NO) by Highly Glycated Haemoglobin: Correlation with Microvascular Disease.

Date:

Patient number: Addressograph:

☐ Group A- Type 1 DM No complications
☐ Group B-Type 1 DM with complications

What complications:
☐ *Retinopathy* ☐ *Nephropathy* ☐ *Neuropathy*

☐ Group C- Type 1 newly diagnosed (Return date :.....)

HbA1C:

Cholesterol:

PMH: DH:

Appendix 3: Tissue Organ Bath System protocol

Tissue Organ Bath System protocol

Radnoti 8 Channel tissue bath System was used.

1. Tissue bath was turned on and set to 37°C.
2. New Zealand white rabbits weighing 2-3kg were anaesthetised with sodium pentobarbital and killed.
3. The thoracic aorta was removed from the rabbit and placed immediately in Krebs-Ringer buffer of the following composition (m*M*): NaCl 118.7, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24.8, *D*-glucose 10.1, pH 7.4 and aerated with 95% O_2 and 5% CO_2 gas mixture.
4. The artery was cleared from all adherent fat and connective tissue surrounding the adventitia. The aorta was then cut into 8 equal rings (each 3 mm). This was accomplished with a slicing device consisting of five razor blades arranged in parallel and separated by spacers clamped rigidly in place. When part of the aorta was laid at a right angle over the cutting edges of the blades and the barrel of a plastic pipette was rolled over it, four rings of equal length were cut.
5. Endothelium was removed in order to avoid any influence of basally released endothelium derived relaxing factor(s) on the response. In order to remove endothelium, the intimal surface of the ring, while kept moist with Krebs' solution, was rubbed gently with a shaved down wooden stick for about 45 seconds and the ring was then mounted in the organ chamber. To ascertain the effectiveness of the rubbing procedure, the ring was tested with acetylcholine (ACh) once it had undergone initial equilibration and pre-contraction. A lack of relaxation response to ACh at concentrations up to 1uM confirmed the loss of essentially all endothelial cells.

6. Each ring was placed on the hooks of the gut bath. 5 ml of oxygenised Krebs buffer was added to each well of the bath.
7. The computer system and the graphs were calibrated.
8. Relaxation studies were performed after a pre-contraction with phenylephrine (PE) (10^{-6} M). Relaxation was expressed as a percent of change from the pre-contracted tension with PE.
9. When the fluid in each organ chamber was to be replaced it was sucked out using a syringe and replaced from the top. Each chamber was normally washed out three times when required.
10. The tension on each ring was continuously recorded. For rabbit experiments the initial tension when the ring was first mounted was set at about 3 g and as the ring stretched during the first hour or so, the position of the force transducer was adjusted at intervals to keep basal resting tension close to 2-2.5 g, which represented the optimal resting tension for this vascular preparation.
11. Blood was collected from patients into 4ml EDTA vacutainer tubes. Blood samples were centrifuged at 670g, 4°C for 5 minutes. Plasma was rapidly separated from RBCs.
12. One millilitre of the above spun RBC sample was mixed with 0.5 ml normal saline and ultra-centrifuged at 6000rpm for a further 5 minutes to obtain a dense layer of RBCs at the bottom of the tube.
13. The concentration of O_2 in the Krebs buffer (KB) bathing the tissues was reduced by bubbling gas of 5%CO_2/95% N_2 mix directly into the bottom of the tissue baths to create a low oxygen tension medium to mimic the physiological O_2 gradient in arterioles.

14. 20µL of washed RBC was added to each one of the 8 aortic ring wells in the gut bath.
15. Additions were made with manual automatic pipettes with disposable plastic tips.
16. Relaxations were calculated as a percent of the tension induced by 10^{-6}mol/L PE for each aortic ring and the average was calculated and recorded for each subject at the end.
17. The responsiveness of the tissues was assessed at the end of each experiment by constricting with PE followed by relaxation to a standard NO donor, nitroso-glutathione (GSNO; 10^{-7}mol/L).
18. Average relaxation was calculated among 8 channels.

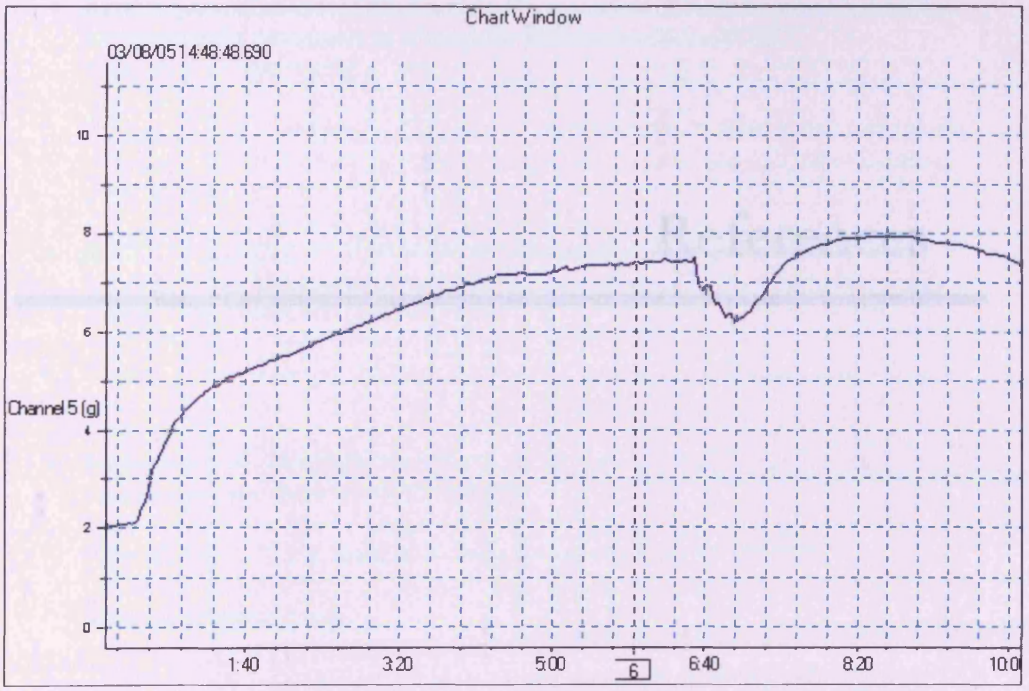


Figure 2: Sample chart window representing aortic ring relaxation in response to RBC in one of the chambers. Time (min) is shown on axis X. Tension (g) is shown on axis Y. Dotted line marks the time when the RBCs were added. The dip in the tension curve represents relaxation. In organ chamber bioassays, RBCs dilate blood vessels at low pO_2 (<1% O_2), which is characteristic of tissues. The hypoxic vasodilator response is followed by vasoconstriction in vitro (representing scavenging of endothelial NO), which starts at approximately 1 min following addition of RBCs.

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