

**EFFECTS OF NITRITE AND
NITROXYL ON HUMAN
VASCULAR AND PLATELET
FUNCTION**

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*Dedicated to my parents,
my love Inara
and children
Zilya, Latípha and Temír*

Table of Contents

Abstract	x
Declaration	xii
Publications, presentations and awards related to the work conducted towards this thesis	xiii
Acknowledgements	xv
List of abbreviations	xvii
Chapter 1: Introduction	1
1.1 Nitric oxide physiology	2
1.1.1 Introduction	2
1.1.2 Nitric oxide effects on vascular tone	2
1.1.3 Nitric oxide production	4
1.1.4 Nitric oxide diffusion	7
1.1.5 Nitric oxide consumption	7
1.1.6 Molecular bases of NO action, including alternatives to direct activation of soluble guanylate cyclase	9
1.1.7 Nitric oxide bioreactivity.....	9
1.2 Nitric oxide pathophysiology: “endothelial dysfunction”	11
1.2.1 Normal endothelial function.....	11
1.2.2 Endothelial dysfunction.....	12
1.2.3 Clinical measurements of endothelial function	13
1.2.3.1 Invasive intra-coronary studies	14
1.2.3.2 Assessment of endothelial function in peripheral vascular beds.....	15
1.2.3.2.1 Venous occlusion plethysmography	15
1.2.3.2.2 Flow-mediated vasodilatation	16
1.2.3.2.3 Pulse wave analysis	16
1.2.3.2.4 Peripheral arterial tonometry	17
1.2.4 Biochemical assessment of endothelial function	17
1.2.5 Does arginine supplementation reverse endothelial dysfunction?	19
1.2.6 Mechanism of endothelial dysfunction.....	19

1.2.6.1 NO half-life and oxidative stress	21
1.2.6.2 Impaired nitric oxide synthesis: L-arginine and BH ₄ availability	22
1.2.6.3 Enhanced activity and expression of arginase.....	22
1.2.6.4 Dysregulation of dimethylarginine dimethylaminohydrolase	23
1.2.6.5 Reduced sensitivity to NO	23
1.3 Nitric oxide resistance	23
1.3.1 Introduction	23
1.3.2 Relationship between NO resistance and endothelial function.....	25
1.3.3 Platelet function and NO resistance.....	26
1.3.4 Epidemiology of NO resistance.....	27
1.3.5 Mechanisms of NO resistance	29
1.3.5.1 Nitric oxide bioavailability	30
1.3.5.2 Integrity of the NO/cGMP signaling pathway	31
1.3.5.3 cGMP-independent effects of NO.....	33
1.3.6 Prognostic value of NO resistance.....	34
1.3.7 Amelioration of NO resistance	37
1.3.7.1 Ascorbic acid	37
1.3.7.2 ACE inhibitors	37
1.3.7.3 Perhexiline	38
1.3.7.4 Glycaemic control.....	39
1.3.7.5 Statins	40
1.3.8 Influence of thioredoxin-interacting protein (TXNIP) on NO signaling: implications regarding NO resistance and its amelioration	41
1.4 Platelets: anatomy, physiology and pathophysiology	43
1.4.1 Normal platelet function and anatomy	43
1.4.2 Platelet adhesion.....	45
1.4.2.1 Von Willebrand Factor (vWf).....	45
1.4.2.2 GP Ib-IX-V receptor complex.....	45
1.4.3 Platelet activation	46
1.4.3.1 Platelet activation via ADP receptors	48
1.4.3.2 Platelet activation via thrombin receptors.....	48
1.4.3.3 Platelet activation via thromboxane A ₂ receptors	48
1.4.4 Platelet aggregation	49
1.4.5 Characterization of platelet aggregation.....	49
1.4.5.1 Platelet aggregometry	50

1.4.5.1.1 Optical platelet aggregometry	50
1.4.5.1.2 Impedance platelet aggregometry	51
1.4.5.1.3 The rapid platelet function analyzer	51
1.4.5.1.4 Shear-dependent assay: PFA-100	52
1.4.5.2 Measures of platelet activation	52
1.4.5.2.1 Soluble platelet activation markers	52
1.4.5.2.2 Flow cytometry	53
1.5 Nitrate-Nitrite-Nitric oxide pathway	53
1.5.1 Nitrogen living cycle	53
1.5.2 Sources of nitric oxide	55
1.5.3 Sources of nitrate and nitrite	56
1.5.4 Bioconversion of nitrate in the digestive tract	57
1.5.5 Nitrite bioactivation and hypoxic potentiation in humans	58
1.5.5.1 Role of haemoglobin	59
1.5.5.2 Role of myoglobin	60
1.5.5.3 Role of xanthine oxidase and xanthine oxidoreductase (XOR)	61
1.5.5.4 Role of eNOS	62
1.5.5.5 Role of cytochrome P450	63
1.5.5.6 Role of mitochondria	63
1.5.6 Dietary nitrate	65
1.5.6.1 Effects of nitrate-containing vegetables on blood pressure and cardiovascular diseases	65
1.5.6.2 Dietary sources of nitrate and nitrite	65
1.5.6.3 Acceptable daily intake (ADI) of nitrate	67
1.5.6.4 Dietary nitrate/ nitrite and cancer risk	68
1.5.6.5 Potential anti-cancer effects of beetroot	69
1.6 Nitroxyl	69
1.6.1 Introduction	69
1.6.2 Biochemical properties of HNO	71
1.6.3 Biological/endogenous generation of HNO	71
1.6.4 Vasodilator action of HNO	72
1.6.4.1 sGC/cGMP-dependent signaling	73
1.6.4.2 cGMP-independent signaling	73
1.6.4.3 Potassium channel activation	74

1.6.5 HNO and NO donors: distinct vasodilator properties.....	74
1.6.6 Anti-aggregatory actions of HNO	75
1.6.7 HNO donors and oxidative stress	77
1.6.8 HNO donors modulate vascular cells growth and proliferation	78
1.6.9 HNO and myocardial function	78
1.6.10 Therapeutic potential of novel HNO donors including CXL-1020	79
1.6.11 Scope of present studies	80
Chapter 2: Materials and Methods	81
2.1 Materials	82
2.1.1 Subject selection.....	82
2.1.1.1 Vascular <i>in vitro</i> experiments	82
2.1.1.2 Platelet aggregation studies.....	82
2.1.2 Blood sampling	83
2.1.3 Preparation of platelet-rich plasma.....	83
2.1.4 Preparation of washed red blood cells.....	83
2.1.5 Chemicals	83
2.2 Methods.....	84
2.2.1 Techniques for evaluation of platelet function	84
2.2.1.1 Whole blood impedance aggregometry	84
2.2.1.2 Instrumentation	85
2.2.1.3 PRP impedance aggregometry	85
2.2.1.4 Hypoxic chamber aggregometry experiments.....	86
2.2.2 Techniques for evaluation of vasodilator function	86
2.2.2.1 <i>In vitro</i> vascular human techniques	86
2.2.2.1.1 Organ bath experiments	86
2.2.2.1.2 Organ bath experiments in hypoxia.....	88
2.2.2.3 Other assays.....	88
2.2.2.3.1 Intra-platelet cyclic guanosine monophosphate (cGMP) assay.....	88
2.2.2.3.2 Electronparamagnetic resonance (EPR) spectroscopy for ROS measurements	89
2.2.2.3.3 DeoxyHb and pO ₂ measurements	89
2.3 Tables and figures for chapter 2.....	90
Chapter 3: Nitrite vasodilator effects <i>in vitro</i>: impact of hypoxia	95
3.1 Summary of the study	96
3.1.1 Objectives.....	96

3.1.2 Methods.....	96
3.1.3 Results.....	96
3.1.4 Conclusions.....	97
3.2 Introduction	97
3.3 Objectives of the study	98
<i>Primary</i>	98
<i>Secondary</i>	98
3.4 Methods.....	98
3.4.1 Study population	98
3.4.2 Vessel organ bath experiments.....	99
3.4.3 Subjecting vessel to hypoxia <i>in vitro</i>	100
3.4.4 Statistical analysis	101
3.5 Results	101
3.5.1 Patient characteristics	101
3.5.2 <i>In vitro</i> tolerance/ cross-tolerance study.....	102
3.5.3 Hypoxic potentiation of nitrite vasodilator effect in comparison to GTN.....	102
3.5.4 Evaluation of mechanisms of nitrite effect.....	103
3.5.5 Nitrite reductases: RBC, Hb and Mb.....	103
3.5.6 Does hypoxia affect nitrite responses equally in veins and arteries?.....	104
3.6 Discussion	104
3.7 Study limitations	107
3.8 Conclusion	107
3.9 Tables and figures for chapter 3.....	108
Chapter 4: Anti-aggregatory effects of nitrite in normoxia and hypoxia....	120
4.1 Summary of the study	121
4.1.1 Objectives.....	121
4.1.2 Methods.....	121
4.1.3 Results.....	121
4.1.4 Conclusions	122
4.2 Introduction	122
4.3 Objectives of the study	123
<i>Primary</i>	123

<i>Secondary</i>	124
4.4 Methods	124
4.4.1 Study population	124
4.4.2 Platelet aggregation studies	124
4.4.3 Induction of controlled hypoxia <i>in vitro</i>	125
4.4.4 ROS measurements	126
4.4.5 <i>In vivo</i> induction of mild hypoxia: effects on deoxyHb	126
4.4.6 Statistical analysis	126
4.5 Results	127
4.5.1 Patients/normal subjects characteristics	127
4.5.2 Nitrite anti-aggregatory effects in venous whole blood.....	127
4.5.3 Anti-aggregatory effects of NO ₂ ⁻ are mediated via NO	128
4.5.4 Nitrite effects in the presence of NO resistance	128
4.5.5 Arterio-venous differences of nitrite anti-aggregatory effects.....	129
4.5.6 Hypoxic potentiation of nitrite anti-aggregatory effects: role of deoxyHb.....	130
4.5.7 DeoxyHb and deoxyMb effects	131
4.6 Discussion	132
4.7 Study limitations	135
4.8 Conclusion	135
4.9 Figures for chapter 4	136
Chapter 5: Nitroxyl effects in vasculature and platelets: circumvention of nitric oxide resistance	155
5.1 Summary of the study	156
5.1.1 Objectives.....	156
5.1.2 Methods.....	156
5.1.3 Results	156
5.1.4 Conclusions	157
5.2 Introduction	157
5.3 Objectives of the study	159
<i>Primary</i>	159
<i>Secondary</i>	159
5.4 Methods	160
5.4.1 Study population	160

5.4.2 Study protocol of anti-aggregatory effects of HNO in human platelets	160
5.4.3 Vascular <i>in vitro</i> studies protocol.....	161
5.4.4 Platelet aggregation studies	161
5.4.5 Vascular studies.....	162
5.4.6 ROS measurements	163
5.4.7 cGMP studies	163
5.5 Data analysis	164
5.6 Results	164
5.6.1 Patients/normal subjects characteristics	164
5.6.2 Circumvention of NO resistance	165
5.6.3 Mechanisms underlying responses to IPA/NO.....	166
5.6.4 Anti-aggregatory effects of SNP and IPA/NO in the presence of diabetes mellitus.....	167
5.6.5 Impact of blood oxygenation on anti-aggregatory response of IPA/NO	168
5.6.6 Vasodilator effect of IPA/NO in saphenous veins.....	168
5.7 Discussion	169
5.8 Study limitations	174
5.9 Conclusion	175
5.10 Tables and figures for chapter 5.....	176
Chapter 6: Summary and future directions	192
Bibliography.....	205
Appendix: Published works related and conducted towards this thesis	255

Abstract

The identification of Nitric oxide (NO) as an endothelium-derived relaxing factor stimulated research into the physiology of this most important biological messenger, which maintains a healthy vascular endothelium and an anti-thrombotic intravascular environment.

Healthy endothelial cells constantly produce NO to create 'basal' vasorelaxation via the classical L-arginine/sGC/cGMP activation cascade. Under physiological conditions this NO pathway is the fundamental to maintenance of normal cardiovascular health, and conversely it is the substrate for development of many cardiovascular disease states, when the balance in this system becomes impaired.

Endothelial dysfunction, with the closely associated phenomenon of "NO resistance", can affect any NO-sensitive tissues including blood vessels and platelets, and is now believed to trigger atherogenesis and thrombogenesis.

Treatment of cardiovascular diseases associated with this phenomenon utilizing NO donors often has proved to be ineffective. Furthermore, treatment with organic nitrates is subject to development of nitrate tolerance, limiting efficacy of this class of agents. Several agents can ameliorate NO resistance over days or weeks, but there remains a problem in circumventing NO resistance in cardiac emergencies. In this thesis we demonstrate for the first time in humans partial circumvention of NO resistance with nitroxyl, a structural analogue of NO.

Additionally, another NO sibling nitrite (NO_2^-) has been attracting substantial interest in the last decade. Evidence has been accumulating that effects of nitrite are increased during hypoxia: - nitrite becomes a potent vasodilator and anti-aggregant when compared to normoxic environment. This is especially important in the situation of chronic tissue hypoxia or in acute vascular emergencies.

Key findings from the experiments in this thesis are:

1. Nitrite is a potent vasodilator compared to GTN: in general nitrite vasodilator effects are significantly potentiated in hypoxia in human saphenous veins. However, in human internal mammary arteries, nitrite-induced vasodilation is not potentiated under hypoxia. Prolonged exposure of human saphenous vein to nitrite does not cause tolerance or cross-tolerance to GTN. Nitrite effects in saphenous veins are substantially inhibited by ODQ, suggesting that they are largely mediated by soluble guanylate cyclase. Haemoglobin, myoglobin and red blood cells significantly increase hypoxic potentiation of nitrite vasodilator effects in human saphenous veins. Hypoxic potentiation of nitrite is diminished when saphenous vein intrinsic myoglobin is blocked by ferricyanide.
2. In platelets, the anti-aggregatory effects of nitrite are markedly and selectively potentiated under hypoxia. However, nitrite is subject to “NO resistance”. Anti-aggregatory actions of nitrite are more potent in venous relative to arterial blood and correlate with (greater) deoxyhaemoglobin levels. Deoxyhaemoglobin is the primary nitrite reductase in blood. We have also presented evidence that continuous generation of NO from endogenous nitrite is important in homeostasis of platelet aggregability.
3. Nitroxyl is a more potent anti-aggregant than SNP. Anti-aggregatory effects of nitroxyl are partially sGC mediated. Nitroxyl partially circumvents the phenomenon of “NO resistance” in platelets. Nitroxyl is also a potent dilator of human saphenous veins. Its effects are not NO-mediated but partially sGC-mediated.

Declaration

I, Rustem Dautov, certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Rustem Dautov

May 2014

Publications, presentations and awards related to the work conducted towards this thesis

Publications related to the work conducted in this thesis:

1. **Dautov, RF**, Stafford, I, Liu, S, Cullen, H, Chirkov, YY & Horowitz, JD 2014, 'Hypoxic potentiation of nitrite effects in human vessels and platelets', *Nitric Oxide*, May 22. In press. Doi: 10.1016/j.niox.2014.05.005
2. **Dautov, RF**, Ngo, DT, Licari, G, Liu, S, Sverdlov, AL, Ritchie, RH, Kemp-Harper, BK, Horowitz, JD & Chirkov, YY 2013, 'The nitric oxide redox sibling nitroxyl partially circumvents impairment of platelet nitric oxide responsiveness', *Nitric Oxide*, Sep 4.
3. Maher, AR, Arif, S, Madhani, M, Abozguia, K, Ahmed, I, Fernandez, BO, Feelisch, M, O'Sullivan, A, Christopoulos, A, Sverdlov, AL, Ngo, D, **Dautov, R**, James, PE, Horowitz, JD & Frenneaux, MP 2013, 'Impact of chronic congestive heart failure on pharmacokinetics and vasomotor effects of infused nitrite', *Br J Pharmacol*, vol. 169, no. 3, Jun, pp. 659-670.

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1. **Dautov, RF**, Chirkov, YY, Ngo, Sverdlov, AL, Kemp-Harper, BK, Ritchie, RH, Horowitz, JD. The Nitric Oxide Redox Sibling Nitroxyl Partially Circumvents Platelet Nitric Oxide Resistance in Patients With Ischaemic Heart Disease. Presented at AHA Scientific Sessions 2012, Los Angeles, USA. *Circulation 2012; 126: A12677*.
2. **Dautov, RF**, Liu, S, BK, Chirkov, YY, Horowitz, JD. Anti-aggregatory effects of nitrite are augmented in venous, relative to arterial blood. Presented at World Congress of Cardiology 2014, Melbourne, Australia. *Global Heart Journal 2014; 9:1S:PT366*.

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List of abbreviations

ADP	Adenosine diphosphate
ACEi	Angiotensin converting enzyme inhibitor
ADMA	Asymmetric dimethylarginine
ARB	Angiotensin receptor blocker
ATP	Adenosine triphosphate
BH ₄	Tetrahydrobiopterin
CABG	Coronary artery bypass grafting
cAMP	Cyclic adenosine monophosphate
CCB	Calcium channel blocker
cGMP	Cyclic guanosine monophosphate
CO ₂	Carbon dioxide
CPTIO	Carboxy-PTIO
DDAH	Dimethylarginine dimethylaminohydrolase
DeoxyHb	Deoxygenated haemoglobin
DeoxyMb	Deoxygenated myoglobin
EDRF	Endothelium-derived relaxing factor
eNOS	Endothelial nitric oxide synthase
FMD	Flow mediated dilatation
GP	glycoprotein
GTN	Glyceryl trinitrate
Hb	Haemoglobin
HNO	Nitroxyl
IHD	Ischaemic heart disease
IMA	Internal mammary artery
iNOS	Inducible nitric oxide synthase
IPA/NO	Isopropylamine NONOate
KPSS	Kreb's solution with KCl substituted for NaCl on an equimolar basis
Mb	Myoglobin

mcg	microgram
mg	milligram
N ₂	Nitrogen
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NaNO ₂	Sodium nitrite
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NO ₂ ⁻	Nitrite
NOS	Nitric oxide synthase
O ₂	Oxygen
O ₂ ⁻	Superoxide
PDE	phosphodiesterase
PKC	Protein kinase C
PKG	Protein kinase G
PLC	Phospholipase C
pO ₂	Partial pressure of oxygen
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
RBC	Red blood cells
ROS	Reactive oxygen species
SD	Standard deviation
SEM	Standard error of the mean
sGC	Soluble guanylate cyclase
SNP	Sodium nitroprusside
SV	Saphenous vein
TXNIP	Thioredoxin-interacting protein
VASP	Vasodilator-stimulated phosphoprotein
vWf	Von Willebrand factor
XOR	Xanthine oxidoreductase

Chapter 1: Introduction

1.1 Nitric oxide physiology

1.1.1 Introduction

Nitric oxide (NO) is a simple molecule as regards its chemical structure but on the other hand it is the most important biological messenger in the cardiovascular system to maintain a normal endothelium and an anti-thrombotic intravascular environment. Its existence was foreshadowed in 1980 by Furchgott and Zawadzki (Furchgott & Zawadzki 1980), who originally recognized it as endothelium-derived relaxing factor (EDRF). Subsequent evidence revealed that NO is the main component of EDRF (Ignarro, Byrns, et al. 1987; Moncada 1992) and that it is a key regulator of processes of vascular homeostasis including vasorelaxation, antithrombotic actions and vessel permeability (Palmer, Ferrige & Moncada 1987). For their pioneering work in identifying NO and its physiological properties, Drs. Furchgott, Ignarro and Murad received The Nobel Prize in medicine in 1998.

1.1.2 Nitric oxide effects on vascular tone

NO is a potent vasodilator that is principally involved in the regulation of vascular tone and in supporting the antithrombotic actions of the endothelium. NO acts in different ways to achieve the regulation of vascular tone. Healthy endothelial cells constantly produce NO at minimal level to create a 'basal' vasorelaxation. NO diffuses from endothelial cells directly across the membrane of the underlying vascular smooth muscle cells (VSMC). This increases production of 3,5-cyclic guanosine monophosphate (cGMP) via activation of soluble guanylyl cyclase (sGC) (Napoli & Ignarro 2001). Soluble guanylate cyclase is the biological target of NO. NO binds to the haem moiety of sGC and activates it. Further, cGMP is produced and this leads to NO-dependent vasodilation through VSMC relaxation. Increased cGMP production activates cGMP-dependent protein kinase that phosphorylates myosin light chain kinase and regulates actomyosin ATPase activity with resulting vasorelaxation ensues (see Fig. 1.1) (Hathaway, Konicki & Coolican 1985; Napoli et al. 2006; Nishikawa et al. 1984). Activation of cGMP-dependent protein kinase also leads to inhibition of voltage-gated Ca²⁺ channels and activation

of protein kinases that phosphorylate proteins in the sarcoplasmic reticulum, as well as Ca^{2+} -dependent potassium channels (Bolotina et al. 1994). Decrease in intracellular Ca^{2+} concentration (Ignarro & Kadowitz 1985) leads to reduction of calcium-calmodulin myosin light chain kinase complex formation in VSMC, resulting in vasorelaxation (Horowitz, A et al. 1996).

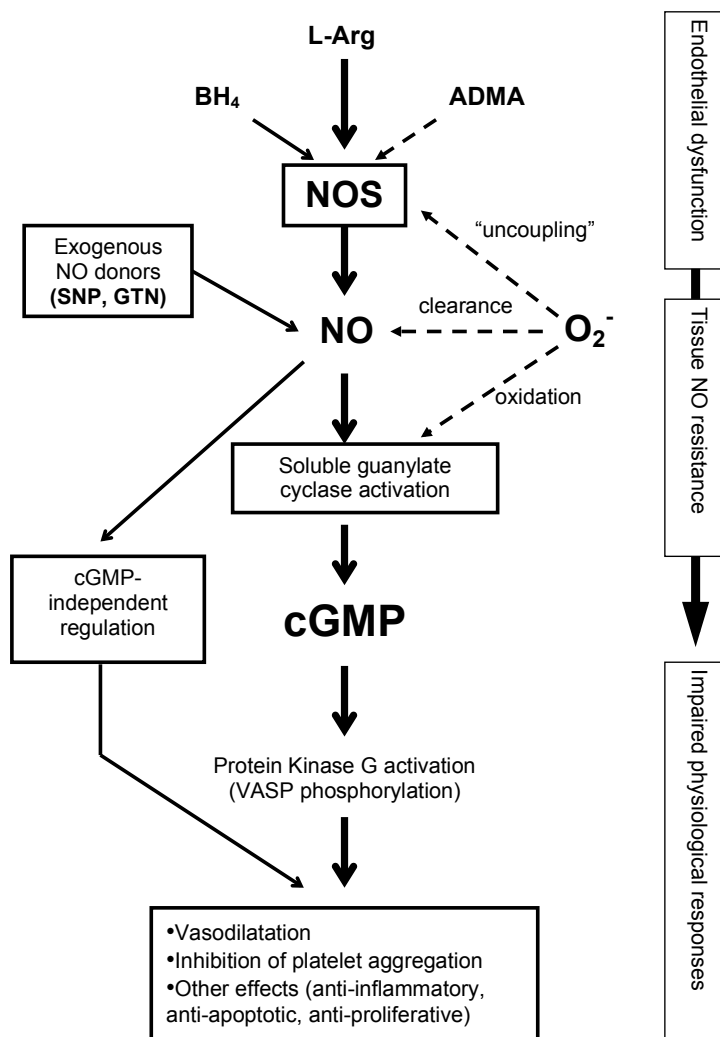


Figure 1.1 Nitric oxide (NO) signaling pathway: schematic view (reproduced with permission from (Rajendran & Chirkov 2008)). Nitric oxide synthase (NOS) requires L-Arginine (L-Arg) as a substrate and tetrahydrobiopterin (BH_4) as a cofactor. Asymmetric dimethylarginine (ADMA) is endogenous inhibitor of NOS. A constitutive, endothelial, form of NOS (eNOS) has an important role in regulation of the vascular tone. Impairment of eNOS, due to “uncoupling”, lack of BH_4 , or inhibition by ADMA leads to “endothelial dysfunction” eventuating in a diminished release of NO from endothelium. A constitutive isoform of NOS is also

present in platelets, where it provides a negative feed-back on platelet activation and aggregation. Phenomenon of “NO resistance”, occurring in both tissues (vasculature and platelets), is a result of decreased NO bioavailability, due to clearance by superoxide radicals (O_2^-), and impaired responsiveness of soluble guanylate cyclase to NO stimulation, because of the oxidation of the enzyme by O_2^- . NO resistance also accounts for reduced pharmaco-activity of exogenous NO donors, e.g. organic nitrates (GTN). Both phenomena, endothelial dysfunction and NO resistance, eventuate in impaired physiological responses and contribute to vasoconstriction, hypertension and thrombosis.(other abbreviations used: cGMP, cyclic guanosine-3,'5'-monophosphate; SNP, sodium nitroprusside; VASP, vasodilator-stimulated phosphoprotein).

Basal vascular tone is maintained by low NO levels produced by endothelium, although it is also important to mention the unloading of erythrocyte-bound NO in hypoxic conditions (Diesen, Hess & Stamler 2008). NO concentrations may increase under influence of shear stress or biochemical stimuli: ADP, thrombin, acetylcholine, serotonin, etc. Inhibition of NO production can predispose to hypertension (Stamler et al. 1994) or a stroke (Howard et al. 2005), reflecting the effects of NO on thrombotic regulation (Loscalzo 2001). Furthermore, NO can increase endothelial production of tissue plasminogen activator (t-PA), which converts plasminogen to plasmin and activates endogenous fibrinolysis (Giannarelli et al. 2007). Another targets for NO are sulfhydryl groups on proteins, to form nitrosothiol (SNOs) compounds.

1.1.3 Nitric oxide production

There are several pathways of NO production in the human body, for example nitrite acidification in the stomach or reduction in different tissues (see section 1.5), though the majority of NO is generated via classical L-arginine cascade. NO is synthesized in large variety of cells, including vascular endothelium, platelets, macrophages and neuronal cells (Giustarini et al. 2003). The main pathway for NO formation in humans is enzymatic via oxidation of the terminal guanidino nitrogen atom of L-arginine to L-citrulline and NO in NADPH- and O_2 -dependent manner (Palmer, Ashton & Moncada 1988). The enzymes catalyzing this reaction belong to

a group of nitric oxide synthases (NOS). Each NOS isoform has an N-terminal oxygenase domain and a C-terminal reductase domain with a calmodulin recognition sequence between the two (Masters et al. 1996). NOS activity is controlled via changes in intracellular Ca^{2+} content that influence the binding of calmodulin for efficient electron transfer from NADPH to the haem iron, which then catalyzes NO production. The predominant isoform is endothelial (eNOS) with partial contribution to total NO formation from nNOS and iNOS isoforms or neuronal and inducible forms respectively (Stuehr et al. 2004). Even though all three isoforms are similar in structure (Lamas et al. 1992), they differ biochemically and also in their transcription, translation, and post-translation mechanisms as well as in the duration of NO synthesis and the NO concentration achieved in the microenvironment locally (Bredt 1999; Fulton, Gratton & Sessa 2001; Stuehr 1997).

For nitric oxide synthases to produce NO, the following are required: - (1) substrate (L-arginine); (2) co-substrates: oxygen and nicotinamide adenine dinucleotide phosphate (NADPH); (3) cofactors: tetrahydrobiopterin (BH_4), flavin mononucleotide (FMN), flavin mononucleotide (FAD), calmodulin and haem (Marletta 1994). NOS activity depends immensely on the availability of substrate, cofactors and on the electron transfer rate (Ghosh, DK & Salerno 2003; Stuehr et al. 2004).

In the presence of cofactors (NADPH, flavin mononucleotide, BH_4 , flavin mononucleotide), activity of NOS also depends on the availability of substrate (L-arginine) and co-substrate (oxygen). L-arginine can also take part in three different pathways that indirectly influence NOS activity: - (1) breakdown by arginases; (2) L-arginine uptake and methylation; (3) formation of asymmetric dimethylarginine (ADMA) (Morris 2007). Arginase competes for cellular L-arginine stores and hence may have a significant impact on NOS activity (Morris 2005).

It is suggested that L-arginine bioavailability regulates NOS activity. Reduced bioavailability of L-arginine changes NOS to become a generator of superoxide rather than NO (Pou et al. 1999; Vasquez-Vivar et al. 1999), a process called eNOS

“uncoupling” which is discussed further in sections 1.2.6.2, 1.3.5.1.

Production of ADMA is also a component of L-arginine metabolism. ADMA is a known NOS inhibitor and is derived through L-arginine methylation of proteins and proteolysis (Tran, Leiper & Vallance 2003). ADMA not only inhibits NOS, but also transforms it from producer of NO to superoxide generator (Cardounel, Xia & Zweier 2005); this can have important consequences in many cardiovascular disease states. Overall, NO generation and its physiological actions are affected by L-arginine uptake, competitive metabolism and the production of ADMA. Furthermore, changes in L-arginine metabolism can potentially affect both NO and superoxide production.

Oxygen is a critically vital substrate in NOS enzymatic activity. NOS enzyme activity depends on tissue oxygen concentration resulting in a direct correlation between pO_2 and NO generation (Rengasamy & Johns 1996; Stuehr et al. 2004). As the norms for oxygen concentration are tissue specific, NOS activity varies between different tissues (Buerk 2007). Furthermore, NO inhibits mitochondrial respiration, which is the main tissue oxygen consumer (Buerk 2007; Mason et al. 2006). This maintains oxygen concentration and hence elevates NO generation by boosting NOS activity. On the other hand, when tissue oxygen concentration is low, NOS produces superoxide instead of NO (Abu-Soud, Rousseau & Stuehr 1996). Potentially these, the above metabolic mechanisms could mean continuous deterioration of NO generation, but there are some protective means that exist to limit this pathophysiological scenario. When generated NO concentrations attain certain levels, NOS enzymatic activity slows down to limit NO generation (Stuehr et al. 2004). Furthermore, NO utilization in cells depends on the oxygen concentration in the tissue (Abu-Soud, Rousseau & Stuehr 1996). When oxygen levels increase, NO utilization in the tissues also increases (Thomas, DD et al. 2001). Overall, the mechanisms of relationship between nitric oxide and oxygen are very important in the control of tissue oxygenation and perfusion.

1.1.4 Nitric oxide diffusion

Another important characteristic of NO that determines its local concentration is its high diffusion capacity. Given that the NO molecule is an uncharged and it is more soluble in hydrophobic milieu than in water, it diffuses easily across cell membranes. At the same time, due to its short half-life, NO is active mainly in the local tissue in which it is generated. Upon its production, NO may diffuse across membranes into target cells, where it activates soluble guanylyl cyclase to generate cGMP from guanosine triphosphate (GTP) (Murad et al. 1978). However this is not the sole biochemical action of NO.

In the majority of tissues, cells are within several cell lengths of circulating blood, where NO undergoes rapid uptake. NO diffuses at rate of 5 cell lengths per second on average (Chen, YY et al. 2007). Tissue diffusion of NO falls according to the square of the distance involved: - for example, when NO spreads to a distance of a cell length, its concentration decreases approximately 8-fold. Hence, within one second of diffusion the concentration of NO can decrease 200-fold. NO generation occurs in specific sites in the cell, but diffusion through a small distance can decrease NO concentration significantly. In the endothelium, NOS is situated between the endothelial cells and vascular smooth muscle cells. This close distance allows NO to diffuse to soluble guanylyl cyclase in the vascular smooth muscle cell without being markedly scavenged by intraluminal blood. Computer modeling of the vessel wall has revealed that around 100nM of NO diffuses from each endothelial cell to corresponding vascular smooth muscle cell to initiate vasorelaxation (Chen, X et al. 2007). Furthermore, microelectrode study in the perivascular space showed that about 200nM of NO is generated in this space (Vukosavljevic et al. 2006).

1.1.5 Nitric oxide consumption

Apart from NO production and diffusion, another mechanism that regulates the amount of NO in the tissue is its consumption. This consists of many reactions,

including those between NO with erythrocytes and ROS, as well as cell metabolism. Overall, consumption of NO, like NO generation, is oxygen dependent. This means that the availability of oxygen determines the rate of NO consumption and extent of NO reactions with the metabolic targets, suggesting a direct correlation between NO signaling and local tissue oxygen content (Thomas, DD et al. 2001). Elevated oxygen content augments NO consumption, while at the same time NO controls oxygen consumption by suppressing mitochondrial respiration. This dual association between NO and oxygen provides direct feedback, affecting control of tissue content of both gaseous molecules (Shiva et al. 2005). The respiratory suppression by NO relies on several factors, for example mitochondrial respiration rate (Mason et al. 2006). In cells with low respiratory rates suppression of mitochondrial respiration requires NO levels close to those attained during nitrosative stress. At low respiratory rates it has been shown in tissue models that 300nM of NO is needed to halve mitochondrial oxygen consumption (Mason et al. 2006). This suggests that the correlation between NO and oxygen depends on the cellular metabolic state.

The principal mechanism for cellular NO consumption remains uncertain. However, NO scavenging by ROS is probably the most important one (Thomas, DD et al. 2006). There are two main pathways by which NO can react with ROS: scavenging by superoxide and peroxidase (Fenton chemistry). NO interacts with superoxide at diffusion rates, resulting in formation of peroxynitrite (ONOO⁻) which then is converted in the presence of NO to nitrogen dioxide (NO₂) and dinitrogen trioxide (N₂O₃). These reactive nitrogen species (RNS) are thiophilic and influence thiol-mediated signaling pathways (Wink & Mitchell 1998). The second pathway of NO consumption by ROS is via reaction with peroxidases and Fenton-type reactions (Abu-Soud & Hazen 2000; Thomas, DD et al. 2006). The reaction of hydrogen peroxide (H₂O₂) with haem proteins can also accelerate NO clearance (Galijasevic et al. 2003).

NADPH oxidase (NOX) and xanthine oxidase (XO) are the major sources of non-mitochondrial ROS (Jacobson et al. 2007; Tabima, Frizzell & Gladwin 2012).

Above all, NO has very short life duration *in vivo*: this has been estimated at

between 1 ms and 1 second (Hakim et al. 1996).

1.1.6 Molecular bases of NO action, including alternatives to direct activation of soluble guanylate cyclase

Given that nitric oxide has an unpaired electron, it easily reacts with other radical intermediates (van der Vliet et al. 1999). The molecular mechanisms regulating the biological actions of NO can be separated into three groups. The most recognized mechanism is NO reaction with transition metals (iron, copper, and zinc). These metals are present in enzymes and other proteins, and by this mechanism, NO mediates the activity of many enzymes. The classical, and indeed central, example of this is the interaction with the haem-protein soluble guanylate cyclase. In a second important mechanism, NO combines with cysteine residues in various proteins. This is termed *S*-nitrosylation (Stamler, Lamas & Fang 2001) and is associated with changes in activity of the proteins concerned. Finally, NO rapidly reacts with superoxide, forming peroxynitrite. Peroxynitrite is powerful oxidant capable of modifying proteins, lipids, and nucleic acids. The second and third mechanisms are referred to as indirect effects of NO and are largely or entirely cGMP-independent (Davis et al. 2001).

1.1.7 Nitric oxide bioreactivity

NO can exist in three related redox forms: the free radical ($\text{NO}\cdot$), nitrosonium (NO^+) resulting from a 1-electron oxidation of $\text{NO}\cdot$, and nitroxyl anion (NO^-) resulting from one electron reduction of NO (Stamler, Singel & Loscalzo 1992). Each redox form has its own reactivity and chemical properties. Nitrite is the main metabolic product of NO in aqueous solutions. These NO species (NO_x) (reactive nitrogen species) and, in some cases, NO itself, can react with oxygen, thiols, reduced haem-containing proteins, and redox metals that determine its metabolism and half-life and in biological systems. Among these reactions is the reaction of NO with haem iron leading to sGC activation with cGMP production; and the reaction

of nitrosonium with sulfhydryl (SH) group-containing molecules in the presence of oxygen to produce S-nitrosothiols (RSNO). Relatively stable S-nitrosothiols, when compared with NO, may represent a storage pool for bioavailable NO (Feelisch et al. 1994; Stamler et al. 1992). S-nitrosoalbumin is responsible for the majority of plasma RSNO, and like other S-nitrosothiols, protects NO from inactivation with a half-life in circulating blood of around 30 minutes. Cysteine is the only thiol source in proteins and also represents the reactive thiol residue of glutathione. S-nitrosoglutathione (GSNO) has shorter half-life than RSNO. The significantly slower reaction between superoxide and RSNO when compared with superoxide and NO suggests that this RSNO stabilizes NO, protecting it from oxidative inactivation and thus increasing its bio-availability (Aleryani et al. 1998). Furthermore, as many S-nitrosoproteins (Yang & Loscalzo 2005) are formed via trans-S-nitrosation from GSNO, GSNO reductase (Hogg 2002) is an important regulator of the bioavailability of (RS)NO.

NO is also capable of forming a complex with haemoglobin and this is physiologically important in the setting of hypoxic vasodilation. Hb becomes S-nitrosylated, resulting in SNO-Hb formation when erythrocytes become oxygenated in the lungs. This SNO-Hb complex can be in the R-state (relaxed and unreactive state, high oxygen affinity Hb conformation) or the T-state (tense reactive state, low oxygen affinity Hb conformation) in which SNO-Hb quickly interacts with thiols and produces a vasodilatation (Jia et al. 1996; Pawloski, Hess & Stamler 2001). Impairment of this pathway has been associated with heart failure (Datta et al. 2004), diabetes mellitus (James et al. 2004), and pulmonary hypertension (McMahon et al. 2005). This SNO-Hb model can have therapeutic implications for conditions with abnormal microcirculatory perfusion (Allen, Stamler & Piantadosi 2009).

NO can react with oxygen and several reactive oxygen species, including superoxide, hydrogen peroxide, and hydroxyl radical, to limit its bioactivity. Both endothelium and macrophages are sources of these ROS. NO reacts with superoxide to form peroxynitrite (OONO^-) (Radi et al. 1991). This rate of formation of peroxynitrite is higher than the rate of superoxide reduction to hydrogen

peroxide by superoxide dismutase, the major antioxidant responsible for superoxide metabolism (Miles et al. 1996). The deleterious actions of high concentrations of peroxynitrite include NO loss and direct cytotoxic actions that result in apoptosis or necrosis (Virag et al. 2003).

Through the reduction of hydrogen peroxide via Fenton chemistry, hydroxyl radical (OH[•]), can react with NO, decreasing its bioavailability. ROS in the vessel wall can react with polyunsaturated fatty acid species in the low density lipoprotein (LDL) particle, initiating lipid peroxidation, resulting in oxidized LDL formation (Steinberg 1997). Oxidized LDL has also been demonstrated to decrease NO bioavailability via a number of mechanisms. For example oxidized LDL inhibited L-arginine uptake, eNOS expression, cGMP production, and subsequent NO and nitrite generation (Chen, LY, Mehta & Mehta 1996).

1.2 Nitric oxide pathophysiology: “endothelial dysfunction”

1.2.1 Normal endothelial function

A monolayer of endothelial cells forming vascular endothelium is found both in arterial and venous macro- and microvascular beds. This serves as a barrier between moving blood components and the vessel wall. In a healthy state the endothelium has powerful autocrine and paracrine capacities releasing a multitude of substances, the best known being nitric oxide. The endothelium maintains vascular homeostasis by providing a balance between release of vasodilator (for example nitric oxide, prostacyclin) and vasoconstrictor substances (endothelin, angiotensin II) (Anderson, TJ 1999). This balance retards development of atherosclerosis, and also associated adhesion of platelets and inflammatory activation. Distension of endothelium by laminar flow stimulates nitric oxide production, thereby suppressing expression of the inflammatory activator thioredoxin-interacting protein (TXNIP) (Forrester et al. 2009; Yamawaki & Berk

2005).

After an injury to the endothelium, new endothelial cells can be formed via recruitment of circulating endothelial progenitor cells derived from bone marrow or they can come from adjacent endothelium. A deficiency of endothelial progenitor cells (Hill et al. 2003) and/or reduced nitric oxide activity (Aicher et al. 2003) can prevent efficient vascular injury repair and cause endothelial dysfunction. There is evidence that nitric oxide plays an important role in the mediation of production of endothelial progenitor cells (Thum et al. 2005).

1.2.2 Endothelial dysfunction

Over the last century prevalence of infectious diseases has declined significantly. The incidence of cardiovascular diseases has been on the rise over past several decades culminating in a global epidemic, and representing the main cause of morbidity and mortality in the western world (Murray & Lopez 1997). The same trend is now seen in the developing Asian and African countries (Bonow et al. 2002). Atherosclerosis with its consequences in the form of coronary artery disease and cerebrovascular disease has replaced rheumatic heart disease as the primary cause of cardiovascular morbidity (Murray & Lopez 1997). Furthermore, the situation has been worsened by the pandemic of diabetes mellitus via increased incidence and accelerated progression of atherosclerosis and its complications (Bonow & Gheorghide 2004; Malmberg et al. 2000). Increasing incidence of coronary artery disease and diabetes mellitus has also been engendered in part by increased longevity (Fleg et al. 1990). Population aging with increased life expectancy is a risk factor for heart disease and diabetes (Harris, MI et al. 1998; King, Aubert & Herman 1998). The triad of heart disease, diabetes and aging are likely to represent the major predisposing factor towards mortality in developing and Western countries in the immediate future (Murray & Lopez 1997).

Sixty years ago, the largest epidemiological study in the history of cardio-vascular research, The Framingham Heart Study, convincingly revealed a strong association between diabetes mellitus and aging with development of heart disease, stroke and

peripheral vessel disease (Kannel et al. 1961). This provided a stimulus to the area of vascular biology research (Gibbons & Dzau 1996). For long time vascular endothelium was believed to be just a barrier between the blood and interstitial compartment. This view has dramatically changed since discovery of role of endothelium in production of nitric oxide in 1980 (Furchgott & Zawadzki 1980). This discovery augmented and changed vascular biology research forever (Ignarro, Byrns, et al. 1987; Moncada 1992).

The important physiological roles of endothelium and nitric oxide have been outlined above (see section 1.1). On the other hand, endothelial “dysfunction” has been shown to have direct association with the development of atherosclerosis (Bonetti, Lerman & Lerman 2003; Ludmer et al. 1986; Quyyumi et al. 1995). In many pathophysiological conditions the endothelial cells experience a reduced availability of nitric oxide but are surrounded by many vasoconstricting substances. Reduced availability of nitric oxide also means increased inflammation and a pro-atherosclerogenic state, via increased expression of TXNIP (Dunn et al. 2010; Forrester et al. 2009) (see more on TXNIP in section 1.3.8). The events that follow consist of impaired endothelium-dependent vasodilation, vasoconstriction and vascular inflammation. This leads to chronic inflammation and a pro-inflammatory state of the endothelium (Packard & Libby 2008). Therefore, endothelial dysfunction plays a key role in the atherosclerotic process from its start until vascular thrombotic complications (Hansson 2005; Libby 2002).

1.2.3 Clinical measurements of endothelial function

Evaluation of endothelial function is an important tool for diagnostic and prognostic purposes (Kasprzak, Klosinska & Drozd 2006) and to carry research into the pathophysiology of endothelial dysfunction, which comprises of an abnormal vasomotor function, inflammation and thrombosis. A large array of laboratory markers have been utilized, including TNF- α , t-PA, E-selectin, VCAM-1, von Willebrand factor, Il-1, CD40L, CRP, microalbuminuria and tests of apoptosis (Ochodnický et al. 2006; Szmítko et al. 2003; Widlansky et al. 2003).

These tests are complementary to imaging studies for evaluation of endothelial function and, even though being non-specific, carry additional information on endothelium physiology. Although endothelial-related vasomotor activity results from a release of numerous constrictor and dilator materials, nitric oxide is the most frequently utilized marker of endothelial function: - its effects are assessed by studying the vasomotor response to a number physiological or pharmacological stimuli, such as acetylcholine.

1.2.3.1 Invasive intra-coronary studies

Quantitative coronary angiography (QCA) post intracoronary injection of acetylcholine has been widely accepted as a test of endothelial function. Potential areas of coronary artery at risk typically develop vasoconstriction after acetylcholine injection or do not change coronary artery diameter (Schachinger, Britten & Zeiher 2000).

Angiographically “normal” coronary arteries may exhibit endothelial dysfunction, which is a marker of a long-term morbidity in otherwise low-risk population (Halcox et al. 2002; Schindler et al. 2003). A prospective study of 308 patients, who underwent intracoronary acetylcholine testing, revealed that endothelial dysfunction was independently associated with future cardiovascular events, even in the absence of a prior history of ischaemic heart disease (Halcox et al. 2002). Another study looked into 281 patients with known coronary artery disease: those with poor vasodilator response to acetylcholine had more subsequent cardiovascular events (Heitzer et al. 2001).

Further, it was demonstrated that diminished vasodilator response to GTN (consistent with the phenomenon of “NO resistance”, described below) was also associated with cardiovascular events in the future (Schachinger, Britten & Zeiher 2000): - the prognostic impact of NO resistance was similar to that of endothelial dysfunction, but relationships between the two phenomena were not sought.

It is theoretically possible for endothelial dysfunction to be extremely regionally heterogeneous: a common example is pulmonary hypertension (Klinger, Abman &

Gladwin 2013). The demonstration of endothelial dysfunction within the coronary artery requires invasive assessment and also carries a small risk of complications. The question therefore arises: - to what extent does detection of endothelial dysfunction in peripheral blood vessels (potentially using non-invasive techniques) recapitulate the situation in the coronary vascular bed?

1.2.3.2 Assessment of endothelial function in peripheral vascular beds

1.2.3.2.1 Venous occlusion plethysmography

Venous occlusion plethysmography is a semi-invasive tool for assessment of endothelial function by evaluating forearm blood flow. This method and its reproducibility have been published previously (Benjamin et al. 1995). Briefly, a 23-gauge cannula in the brachial artery of the non-dominant arm is utilized for infusion of endothelium-dependent (acetylcholine) or endothelium-independent (GTN, sodium nitroprusside) pharmacological stimuli and for the recording of blood pressure via a pressure transducer. A strain gauge is positioned around the forearm and connected to a plethysmography device and supported above the level of the right atrium. A forearm cuff is inflated to 50mmHg above the systolic blood pressure to eliminate the hand circulation during measurements of forearm blood flow. The upper arm-congesting cuff is inflated to 40mmHg for 7 seconds in each 15-second cycle to occlude venous outflow from the arm with a period cuff inflator. Rates of increase in calculated forearm volume are utilized to measure blood flow rates.

A reduction in blood flow response to acetylcholine in the forearm has been shown to be closely related to both the extent of coronary endothelial dysfunction and the presence of coronary artery disease (Anderson, TJ et al. 1995). This technique is also suitable for evaluation of the role of endothelial (dys)function during exercise or reactive hyperaemia (Bruning et al. 1996).

1.2.3.2.2 Flow-mediated vasodilatation

Flow-mediated dilation (FMD) is non-invasive and more clinically applicable method for evaluation of endothelial function by ultrasound imaging of a brachial artery (Uehata et al. 1997). FMD is used to examine the shear stress mechanism of NO production following transient ischemia (inflation and deflation of a blood pressure cuff): normally this leads to endothelial NO-dependent flow-mediated vasodilation. A number of investigators have shown that there is reasonably good correlation between the results of FMD and coronary responsiveness to acetylcholine (Anderson, TJ et al. 1995; Takase et al. 1998). For example Takase and colleagues demonstrated a strong correlation between endothelial function assessed by acetylcholine in the coronary arteries and FMD in the brachial arteries ($r=0.78$).

FMD results will vary according to time of day (Otto et al. 2004), but are also influenced by duration of vascular occlusion and cuff position (Berry, Skyrme-Jones & Meredith 2000). Therefore demonstration of “impaired FMD” requires considerable standardization of technique in any particular laboratory.

Impaired FMD is a characteristic of patients with ischaemic heart disease (Anderson, TJ et al. 1995) and is also manifest in most traditional cardiovascular risk factors (Celermajer et al. 1992).

FMD has been also useful to demonstrate the benefit of exercise training by improved FMD and a subsequent risk reduction of cardiovascular diseases in different populations (Camsarl et al. 2003; Gill et al. 2004; Gokce et al. 2002). Interestingly, even in overweight male population, acute exercise leads to increased FMD response in active men but those men who have sedentary lifestyle demonstrate decreased response (Harris, RA et al. 2008).

1.2.3.2.3 Pulse wave analysis

Pulse wave analysis is a relatively simple bedside non-invasive test to measure

arterial stiffness (O'Rourke, Pauca & Jiang 2001). Arterial stiffness, which is measured via evaluation of pulse wave reflection, increases with age and with many cardiovascular risk factors and has been shown to be an independent risk indicator of premature ischaemic heart disease (Weber et al. 2004). Endothelial function is assessed by measuring changes in arterial stiffness in response to the endothelium-dependent vasodilator salbutamol (Wilkinson et al. 2002).

1.2.3.2.4 Peripheral arterial tonometry

Another non-invasive test utilized to evaluate endothelial function is reactive hyperemia peripheral arterial tonometry (RH-PAT). It is a somewhat operator-independent technique and involves measurement of changes in digital pulse volume after cuff release in the arm. Normally response is attenuated in those with confirmed coronary endothelial dysfunction. Hence, RH-PAT could be utilized as a bedside test to screen patients for endothelial dysfunction (Bonetti et al. 2004). Results of RH-PAT correlate moderately with those of FMD (Woo et al. 2014), and the technique is comparatively simple.

1.2.4 Biochemical assessment of endothelial function

When nitric oxide bioavailability is decreased and the NOS-dependent pathways are impaired, the processes leading to formation of atherosclerotic plaques with accumulation of thrombocytes on the abnormal endothelium ensue (de Nigris et al. 2003; Napoli et al. 2006). Asymmetric dimethylarginine (ADMA), which functions at least in part as an endogenous competitive inhibitor of NOS, was shown to antagonize endothelium-dependent vasodilatation thirty years ago (Vallance et al. 1992). ADMA is a product of catabolism of arginine residues (see schematic Fig. 1.2). ADMA is partially cleared by renal excretion and accumulates in patients with chronic renal failure. Severe endothelial dysfunction is demonstrated in this group of patients and haemodialysis temporarily improves endothelium-dependent vasodilatation (Hand, Haynes & Webb 1998). ADMA is also partially metabolized,

by dimethylarginine dimethylaminohydrolases (DDAH). These enzymes are redox-sensitive: - hence redox stress could theoretically cause endothelial dysfunction via ADMA accumulation.

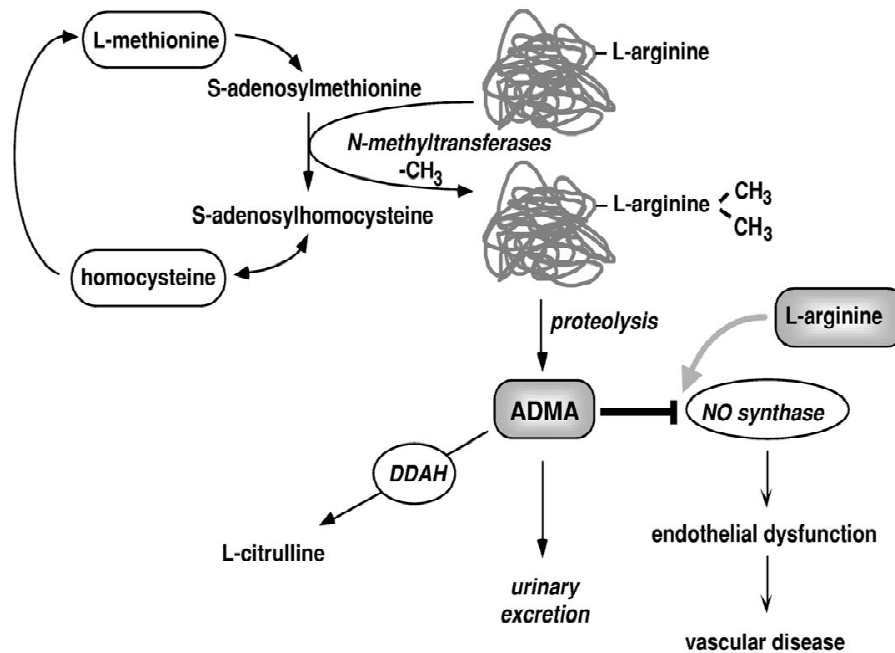


Figure 1.2 Schematic overview of the biochemical pathways related to ADMA. Methylation of arginine residues within proteins or polypeptides occurs through *N*-methyltransferases, which utilize *S*-adenosylmethionine as a methyl group donor. After proteolytic breakdown of proteins, free ADMA is present in the cytoplasm. It can also be detected circulating in human blood plasma. ADMA acts as an inhibitor of NO synthase by competing with the substrate of this enzyme, L-arginine, and causes endothelial dysfunction. ADMA is eliminated from the body via urinary excretion and more importantly, via metabolism by the enzyme dimethylarginine dimethylaminohydrolase (DDAH) to citrulline and dimethylamine. Reproduced with permission from (Boger 2003).

Plasma ADMA concentrations were shown to be elevated in individuals with normal renal function but vascular disease (Boger et al. 1998; Boger et al. 1997; Surdacki et al. 1999). Our group has recently shown that ADMA is elevated in patients with aortic stenosis compared with healthy controls, suggesting impaired nitric oxide generation in aortic stenosis (Ngo et al. 2007). Patients with high

cholesterol and atherosclerosis demonstrate raised ADMA levels correlating with cardiovascular risk factors and the severity of endothelial dysfunction (Juonala et al. 2007; Maas et al. 2008; Selcuk et al. 2007). An infusion of ADMA to healthy volunteers resulted in a significant reduction in plasma concentration of cGMP and in cardiac output thus demonstrating clinically significant NOS inhibition (Kielstein et al. 2004). A small change of ADMA plasma level in patients with ischaemic heart disease is enough to decrease NO production and further accelerate progression of coronary artery disease (Cardounel et al. 2007).

1.2.5 Does arginine supplementation reverse endothelial dysfunction?

A number of studies have documented amelioration of endothelial dysfunction with acute, subacute or chronic administration of arginine (Facchinetti et al. 2007; Lerman et al. 1998; Lucotti et al. 2006). However, these results are inconsistent, with many investigators failing to document any improvement from arginine treatment (Blum et al. 2000; Chin-Dusting et al. 1996).

These conflicting data suggest that arginine extracellular supplementation may fail, either because the arginine transport to sites of NOS activity is a limiting factor, or because in many circumstances deficiency of cofactors (eg. BH₄) rather than lack of arginine, impairs NO production. Furthermore, as explained in section 1.2, “endothelial dysfunction” also includes NO resistance, where the biochemical effects of NO, rather than its generation, represent the main limitation.

1.2.6 Mechanism of endothelial dysfunction

Endothelial dysfunction represents an impairment of vasomotor tone regulation, vessel wall inflammation and also an imbalance of coagulation factors at the vessel

wall luminal surface (Anderson, TJ 1999; Raitakari & Celermajer 2000). The imbalance of coagulation factors produced by endothelium (tissue factor, plasminogen activator inhibitor) may cause vascular thrombosis.

There are many known mechanisms of endothelial dysfunction. A result of the endothelial dysfunction pathophysiology is a decreased vasodilator and increased vasoconstrictor response. The former is most commonly a result of a dysfunctional NO pathway and could be considered as reductions in: (1) NO half-life, (2) synthesis of NO, (3) sensitivity to NO, or (4) NOS expression/activity, as schematically illustrated in Figure 1.3 (Naseem 2005).

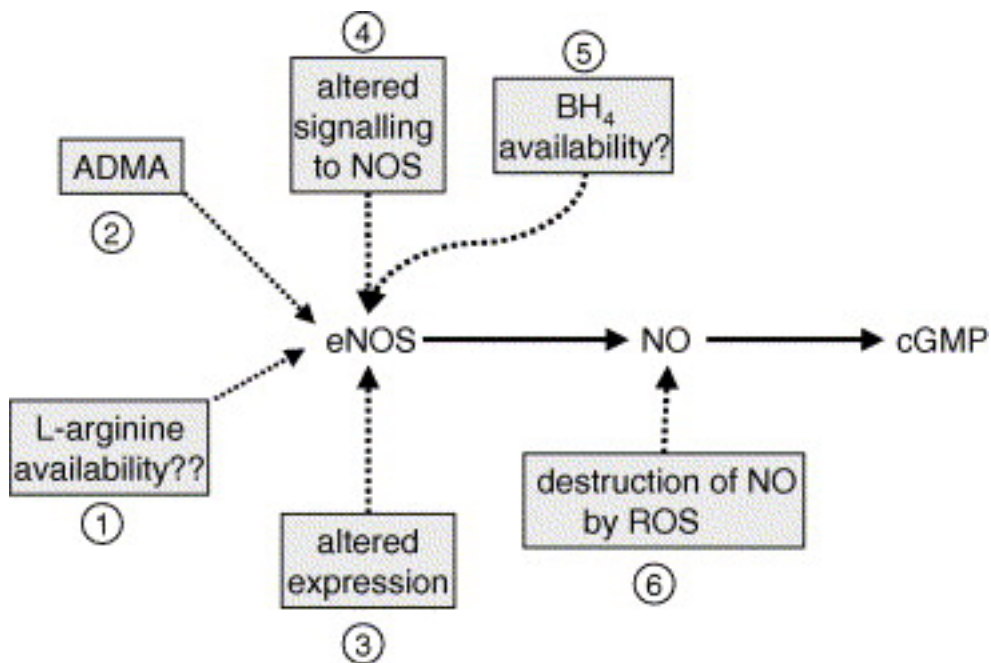


Figure 1.3 Schematic view of factors that may contribute to a reduction in nitric oxide bioavailability. There are many factors which could reduce NO availability including (1) a reaction in the availability of the substrate L-arginine, (2) increased concentration of circulating inhibitors such as ADMA, (3) altered levels of eNOS expression, (4) perturbed signal transduction reducing agonist-induced eNOS activation, (5) reduced availability of tetrahydrobiopterin (BH₄) an essential co-factor, or (6) the destruction of NO by other free radical species. Reproduced with permission from Naseem, KM (2005).

1.2.6.1 NO half-life and oxidative stress

Oxidative stress is caused by imbalance between the reactive oxygen species (ROS) production and their scavenging by antioxidant systems. ROS possess unpaired electrons and are free radicals: superoxide (O_2^-), hydrogen peroxide (H_2O_2), lipid peroxides (Halliwell & Gutteridge 1984). ROS are produced by xanthine oxidase, cytochrome P450 monooxygenase, uncoupled NOS and NADPH oxidases. ROS cause lipid oxidation, promote smooth muscle proliferation and lead to expression of proinflammatory genes (Ginnan et al. 2008; Griendling & FitzGerald 2003). ROS also stimulate endothelial cell apoptosis (Dimmeler & Zeiher 2000), a prothrombotic milieu in the blood vessel and activation of matrix metalloproteinases. This can result in plaque instability and rupture (Rajagopalan et al. 1996). Among endogenous antioxidants that scavenge ROS are glutathione (GSH), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px). Atherosclerotic disease has been associated with a reduction in the antioxidant protective mechanism (Belch et al. 1989; Loeper et al. 1991).

There is large body of evidence that oxidative stress plays an important role in cardiovascular disease. ROS have a significant role in myocardial ischaemia (Hammond & Hess 1985; McCord 1985) and in atherogenesis (Gesquiere et al. 1999). ROS release is associated with the classical cardiovascular risk factors: smoking (Morrow et al. 1995) hypertension (Minuz et al. 2002) and diabetes mellitus (Davi et al. 1999). ROS produced by acute and chronic oxidative stress represents the main substrate for endothelial dysfunction (Harrison, DG 1997). Superoxide can “scavenge” nitric oxide, while superoxide dismutase can stabilize it. In diabetes mellitus, hypertension, hypercholesterolemia, impaired endothelium-dependent vasorelaxation strongly correlates with nitric oxide scavenging by ROS (Lockette, Otsuka & Carretero 1986).

1.2.6.2 Impaired nitric oxide synthesis: L-arginine and BH₄ availability

Decreased nitric oxide bioavailability may theoretically be due to a lack of substrate (L-arginine) or cofactors (e.g. BH₄) for eNOS. Results of studies examining L-arginine supplementation on endothelial function have been both positive and negative (for review see (Herbaczynska-Cedro 1999)). Hence, the possible role of L-arginine deficiency in endothelial dysfunction still needs to be determined: it is possible that disordered intracellular distribution of arginine might also be relevant.

Tetrahydrobiopterin (BH₄) is an essential cofactor for nitric oxide synthesis (Schmidt, TS & Alp 2007). Normally, the dimeric structure of nitric oxide synthase is stabilized by BH₄. It couples oxidation of L-arginine to NADPH consumption and prevents degradation of the ferrous-O₂ complex. Decreased levels of this cofactor directly decrease nitric oxide production and also via a process of ‘uncoupling’, in which eNOS instead of catalyzing electron transfer to L-arginine reduces molecular oxygen to O₂⁻. Superoxide generated during ‘uncoupling’ can subsequently inactivate NO (Xia et al. 1998). The synthetic BH₄ analogue sapropterin has been shown recently to alternate endothelial dysfunction (for review see (Moens et al. 2011)).

1.2.6.3 Enhanced activity and expression of arginase

L-arginine deficiency resulting from increased arginase activity is another possible mechanism of eNOS uncoupling (Ryoo et al. 2008). Both isoforms of arginase metabolize L-arginine to urea and L-ornithine. Activity and expression of arginases increase with aging and this leads to reduced NO bioavailability that facilitates endothelial dysfunction with aging (Berkowitz et al. 2003; White, AR et al. 2006). Furthermore arginase activity may impose a limitation to access of arginine to the intracellular caveolae, where endothelial NOS is localized (Ryoo et al. 2008; Ryoo et al. 2006).

1.2.6.4 Dysregulation of dimethylarginine dimethylaminohydrolase

Asymmetric dimethylarginine (ADMA) inhibits endogenous NOS (see above) and represents another biochemical mechanism of endothelial dysfunction. ADMA is metabolized by dimethylarginine dimethylaminohydrolase (DDAH), an enzyme which is inactivated during oxidative stress (Ito et al. 1999). This mechanism may result in endothelial dysfunction. Indeed, it was demonstrated that over-expression of DDAH 1 *in vivo* leads to 2-fold reduction in plasma ADMA and to 2-fold increase in NOS activity (Dayoub et al. 2003).

1.2.6.5 Reduced sensitivity to NO

Reduced sensitivity to endogenous nitric oxide (also termed as ‘NO resistance’) at the vascular level may contribute to the mechanism of endothelial dysfunction and will be discussed below in more detail.

1.3 Nitric oxide resistance

1.3.1 Introduction

Nitric oxide (NO) is an important regulator of an array of physiological effects in humans. Soluble guanylate cyclase (sGC) is the principal intracellular receptor for NO, catalyzing the formation of cGMP. This further activates protein kinase G (PKG) and leads to phosphorylation of vasodilator-stimulated phosphoprotein (VASP) (for review see (Lohmann & Walter 2005)). Only recently it has been appreciated that dysfunction of this signaling pathway occurs frequently in the environment of oxidative stress, and contributes to the development of many cardiovascular disease states. This problem, termed “NO resistance” (Chirkov et al.

2001), is potentially present in all NO-responsive tissues, especially in blood vessels (Schachinger, Britten & Zeiher 2000) and platelets (Chirkov & Horowitz 2007). Resistance to NO in humans is most easily studied in platelets utilizing *in vitro* evaluation, but also occurs in leukocytes and other NO-responsive tissues, including blood vessels, and myocardium [for reviews see: (Chirkov & Horowitz 2007), (Ritchie et al. 2009)]. Scavenging of NO by superoxide anion radical (O_2^-) and impairment of sGC were identified as mechanisms for NO resistance in human platelets (Chirkov, Chirkova & Horowitz 1996; Chirkov et al. 1999; Chirkov et al. 2004; Lohmann & Walter 2005).

The phenomenon of NO resistance has extensive pathophysiological consequences and therapeutic implications. First, NO resistance may be a cause of overall platelet hyperaggregability and resultant thrombosis (Chirkov & Horowitz 2007). Second, NO resistance cannot be corrected by administration of NO donors commonly utilized in clinical practice (e.g. organic nitrates) (Chirkov & Horowitz 2007), even on a *de novo* basis (Armstrong, PW, Armstrong & Marks 1980). It has also been demonstrated that NO resistance represents an independent risk factor for cardiovascular morbidity and mortality (Schachinger, Britten & Zeiher 2000; Willoughby, S.R. et al. 2005). Up until this date, all therapeutic intervention studies have sought to restore the normal functioning of the NO/sGC pathway, a strategy termed amelioration of NO resistance. Several classes of therapeutic agents have been shown to ameliorate NO resistance to some extent, including angiotensin-converting enzyme inhibitors (Chirkov et al. 2004; Willoughby, S. R. et al. 2012), the cardiac metabolic agent perhexiline (Willoughby, S. R. et al. 2002), statins (Stepien et al. 2003) and utilization of intravenous insulin in diabetic patients presenting with hyperglycaemia and acute coronary syndrome (Worthley et al. 2007). However, the implications of such strategies relate to chronic disease management and even though the above therapeutics have been shown to ameliorate NO resistance over a period of days or weeks, they do not circumvent it. A residual clinical problem is posed by the occurrence of NO resistance in association with cardiovascular emergencies including acute coronary syndromes and heart failure (Chirkov & Horowitz 2007). Therefore, there is an urgent need for therapeutic agents, which may circumvent NO resistance, facilitating the

emergency treatment of cardiac crises.

1.3.2 Relationship between NO resistance and endothelial function

The vascular tone is determined by the combined effect of vasoconstrictor and vasodilator stimuli from the autonomic nervous system, humoral factors, and vessel endothelium. An imbalance of these mechanisms within the coronary arterial circulation can lead to myocardial ischemia especially when vasoconstrictor stimuli are more potent. The vessel endothelium is a complex endocrine and paracrine organ with a multitude of functions directed not only to vascular tone, but also proliferation of smooth muscle cells, aggregation of platelets, adhesion of monocytes and leukocytes, and vascular thrombosis. All these functions play significant roles in the pathophysiology of atherosclerosis (Schachinger, Britten & Zeiher 2000; Vane, Anggard & Botting 1990). Additionally, functional integrity of the endothelium provides potent protective effects against atherosclerosis and thrombosis (Benzuly et al. 1994; Radomski & Moncada 1993).

Although it is clear that the endothelium has multiple functions, the integrity of “endothelial function” is classically evaluated through changes of vasomotor tone in response to stimuli for NO release, either of pharmacological or mechanical nature. Acetylcholine is the conventional stimulus for endothelium-mediated vasorelaxation (Ludmer et al. 1986), acting via endothelial muscarinic membrane receptors, with signal transduction through G proteins to mediate the release of NO (Cohen & Vanhoutte 1995; Flavahan 1992), preventing vasoconstriction as a direct effect of acetylcholine on vascular smooth muscle cells. This theoretically means that endothelial function is “preserved” if there is any vasodilatation in response to acetylcholine.

The key mechanistic issue is: what precisely is detected by tests for “endothelial function”, given that the response in such tests (irrespective of whether the test is response to acetylcholine, flow-mediated dilatation, response to salbutamol, etc.) theoretically is determined by both kinetics/extent of NO generation and by the

integrity of the NO signal transduction cascade? To date, only limited vascular comparisons of responsiveness to stimuli for “endothelial function” and to NO donors have been reported in any vascular beds in humans. Schachinger et al. reported that what they termed “vasodilator dysfunction” (i.e. poor response to glyceryl trinitrate) carried similar prognostic implications to those of impaired acetylcholine responses in human coronaries (Schachinger, Britten & Zeiher 2000). Adams and colleagues (Adams et al. 1998) reported some relationship between FMD responses in human arm and responses to glyceryl trinitrate in the same vascular bed. However, in theory, the determinants of endothelial dysfunction will be more extensive than those of NO resistance, including all the determinants of NO generation (e.g. arginases, BH₄, ADMA: see section 1.2 for details) as well as NO signaling mechanism.

1.3.3 Platelet function and NO resistance

In blood vessels physiological evaluation of endothelial function in practice utilizes the responsiveness of vascular smooth muscle to NO released from adjacent endothelium. In the case of circulating platelets, the potential influence of NO release from adjacent tissues (endothelium and circulating blood cells, both erythrocytes and leukocytes) is considerable, and the modulating influence of leukocyte-derived reactive oxygen species is also relevant (for review see (Ferroni et al. 2012)). Indeed, the integrity of NOS within platelets has recently been questioned (Gambaryan et al. 2008). Hence the evaluation of integrity of platelet NO signaling revolves around determination of responsiveness to exogenous NO. While it is impossible in vitro to duplicate fully the circumstances of normal platelet physiology, there are substantial advantages in the whole blood aggregation technique described in section 1.4.5.1.

It has been demonstrated utilizing such aggregometry that decreased responsiveness of tissues to NO effects extends beyond vascular smooth muscle cells and also includes platelets (Chirkov et al. 1993; Terres et al. 1991). There is evidence that in patients with coronary risk factors, platelets in the blood circulation have functional abnormalities (Heeschen et al. 2003; Woods, Edwards & Ritter 1993). The same

applies to patients with ischemic heart disease (Chirkov et al. 2001; Diodati, Cannon & Quyyumi 1994; Furman et al. 1998). Our group have shown that even in chronic ischaemic heart disease patients platelet responsiveness to NO depends on the extent of the disease: in patients with acute coronary syndromes platelet responsiveness to NO is more impaired than in those with stable angina (Chirkov et al. 2001; Willoughby, S. R. et al. 2002).

It is important to distinguish poor platelet response to NO from platelet hyperaggregability although both may coexist (Chirkov et al. 1999). The biochemical correlation of NO resistance with impaired formation of cGMP in platelets in response to NO donors (assessed in the absence of ADP) with impairment of the antiaggregatory effects of NO donor was established at the outset of such studies (Chirkov, Chirkova & Horowitz 1996). Therefore, NO resistance in platelets is independent of increased platelet aggregation even though this phenomenon occurs in the presence of hyperaggregability, it can be regarded as a separate, additional feature of platelet (dys)function.

1.3.4 Epidemiology of NO resistance

The NO resistance phenomenon at the platelet level has been extensively documented in patients with ischemic heart disease. In patients with stable angina, when compared to subjects with no known ischaemic heart disease, a rightward shift was noted in the concentration-response curve for the anti-aggregatory actions of NO donors nitroglycerin (Chirkov et al. 1993) and sodium nitroprusside (Chirkov, Chirkova & Horowitz 1996). These results were obtained in platelet-rich plasma with optical aggregometry and ADP as inductor of aggregation. Furthermore, SNP-induced elevation of intraplatelet cGMP concentration was also significantly lower in platelets from patients with stable angina than in normal subjects (Chirkov, Chirkova & Horowitz 1996). In studies with whole blood aggregometry, similar findings of decreased platelet responsiveness to the anti-aggregatory and cGMP-stimulating effects of GTN and SNP were noted in acute coronary syndrome patients (Chirkov et al. 2001) and also in patients with stable

angina (Chirkov et al. 1999). Reduction in platelet sensitivity to NO donors was attributed to decrease in platelet sGC generation in response to NO and/or to inactivation of the released NO by superoxide (Chirkov et al. 1999).

Chronic heart failure, a condition characterized by increased oxidative stress and prothrombotic state (de Meirelles et al. 2011), is another example of platelet resistance to NO (Anderson, RA et al. 2004; Chirkov et al. 2004).

Platelets in patients with chronic heart failure, who were not treated with ACE inhibitors, demonstrated increased aggregability and impaired responsiveness to the anti-aggregatory and cGMP-stimulatory effects of SNP, when compared to normal subjects (Anderson, RA et al. 2004). Patients with chronic heart failure had also increased blood levels of superoxide. Platelet responses to SNP normalized after treatment with ACE inhibitor (Chirkov et al. 2004).

Diabetes mellitus is associated with a high incidence of cardiovascular disease states when compared to those with no diabetes (Funk, Yurdagul & Orr 2012). At the platelet level, it has been demonstrated that anti-aggregatory effects of SNP and GTN on ADP-induced platelet aggregation in diabetic patients were significantly weaker than in control subjects (Anderson, RA et al. 2005). Impaired platelet responsiveness in patients with diabetes appears to be partly due to increased blood glucose level (Rauch et al. 2000). Thrombus formation is increased in diabetics with long-term poor glycaemic control (for recent review, see (Vazzana et al. 2012)). On the other hand, normalization of platelet aggregability has been documented in association with improved glycaemic control (Demirtunc et al. 2009; Hara et al. 2012). Furthermore, there is a direct correlation between platelet hyperaggregability and plasma glucose in patients with acute myocardial infarction (Oswald et al. 1988). In a study of diabetic patients presenting with acute coronary syndrome, an inverse correlation between admission blood glucose and platelet responsiveness to SNP, and a positive correlation with blood superoxide levels was documented (Worthley et al. 2007).

NO resistance in platelets has also been described in aortic stenosis. Platelets from patients with aortic stenosis demonstrate hyperaggregability in response to ADP

(Chirkov et al. 2002). Furthermore, the anti-aggregatory actions of SNP were significantly diminished, representing NO resistance at the platelet level. In this study the majority of patients had at least moderate aortic stenosis. Although, there was no correlation between the severity of aortic stenosis and severity of platelet resistance to NO, suggesting that the observed platelet NO resistance develop early in the clinical course of aortic stenosis. The extent of NO resistance was similar in aortic stenosis patients with and without significant coronary artery disease. These results show that aortic stenosis represents an additional prognostic marker of platelet NO resistance.

1.3.5 Mechanisms of NO resistance

Nitric oxide is crucially important in the maintenance of vascular tone and in the inhibition of platelet aggregation. Impaired platelet sensitivity to exogenous NO *in vitro* implies decreased platelet responsiveness to endogenous NO *in vivo*, with resultant platelet hyperaggregability. Despite the extrapolation in this hypothesis, there is no technically good way for this to be tested experimentally. Nevertheless, NO resistance can contribute to the platelet hyperaggregability documented previously in a range of cardiovascular disease states (Bashour et al. 1988; Diiodati, Cannon & Quyyumi 1994). The antiaggregatory actions of NO donors (GTN and SNP) are mediated by NO release, which activates platelet sGC, leading to generation of cGMP (for review, see (Tziros & Freedman 2006)). A reduction in platelet sensitivity to NO donors documented in studies by our group (Chirkov et al. 2001; Chirkov et al. 1993) may be attributed to a problem in the NO/cGMP signaling cascade. The intracellular cGMP system incorporates the enzymes responsible for cGMP generation (soluble guanylate cyclase), decomposition (cyclic nucleotide phosphodiesterases), and signal transduction (cGMP-stimulated protein kinases).

1.3.5.1 Nitric oxide bioavailability

NO synthases are enzymes responsible for endogenous generation of NO (see figure 1.1). For NOS to be physiologically active, it requires the intracellular availability of L-arginine (potentially limited by activity of arginases) as the main substrate and tetrahydrobiopterin (BH₄) as the essential cofactor of endogenous production of NO (see section 1.1.3). Therefore, decreased NO generation could be the result of a lack of either or both of these necessary components. Reduced content of available BH₄ directly compromises NO generation. Additionally, this also leads to enzymatic “uncoupling” of NOS: instead of modulating transfer of electron to L-arginine and generating nitric oxide, NO synthase starts to reduce molecular oxygen to superoxide. NO is then “scavenged” by superoxide, forming peroxynitrite which oxidizes BH₄ to its enzymatically inactive form dihydrobiopterin (BH₂), this in turn augments NOS uncoupling. Enzymatic NOS impairment, as a result of “uncoupling” or suppression by its endogenous inhibitor asymmetric dimethylarginine (ADMA), causes “endothelial dysfunction”. These events lead to decreased NO production by endothelial cells. Although there is some controversy about the matter (Gambaryan et al. 2008), most investigators believe that platelets also have NO synthase (Freedman et al. 1998; Sase & Michel 1995), which provides a negative regulatory mechanism on activation and aggregation of platelets. Impaired NO generation by platelets is associated with vascular thrombosis and acute coronary syndromes (Freedman et al. 1998).

The interaction of NO with soluble guanylate cyclase and availability of NO for activation of sGC are fundamental to the NO/cGMP cascade. A reduction in platelet sensitivity to NO may be secondary to accelerated scavenging of NO by superoxide, concentration of which is increased in cardiovascular disease states (Riesenberg et al. 1997; Siminiak et al. 1993; Vaddi et al. 1994). In an elegant study, Chirkov and colleagues evaluated the relationship between superoxide and platelet responses to NO donors, utilizing a direct quantitation of superoxide concentration in blood samples (by chemiluminescence technique) and by assessing the effects of a superoxide scavenger superoxide dismutase on the antiaggregatory actions of NO donors (Chirkov et al. 1999). Blood samples from patients with stable angina revealed a 4-fold higher superoxide content, as compared with blood

from normal subjects. When superoxide dismutase was added to blood samples from normal subjects in order to reduce superoxide content, this did not alter platelet aggregation. However, in blood samples from patients with stable angina, superoxide dismutase suppressed platelet aggregation and augmented antiaggregatory efficacy of sodium nitroprusside. Results of this study imply that increased superoxide levels abate platelet responses to NO donors by reducing NO bioavailability and directly contribute to the extent of NO resistance (see figure 1.1).

Soluble guanylate cyclase is a haem-protein with its haem moiety serving as a “receptor” for NO and a regulator of NO-dependent enzyme activation. Given the high affinity of NO to any haem molecule, it also readily binds to haemoglobin. While this is one of mechanisms of NO stabilization and transportation within the circulation (Pawloski, Hess & Stamler 2001), on the other hand NO is consumed by reacting with oxyhaemoglobin under physiological conditions (Joshi et al. 2002) and in situations associated with intravascular haemolysis, for example during transfusion of red blood cells from a blood bank (Donadee et al. 2011). In vitro, hemoglobin neutralizes NO effectively (Joshi et al. 2002) in a haemoglobin oxygen saturation-dependent fashion (Diesen, Hess & Stamler 2008). Therefore, a scavenging effect of haemoglobin on NO availability can contribute to NO resistance and explain the relationship between raised haematocrit and endothelial dysfunction in cardiovascular diseases (Maio et al. 2011).

1.3.5.2 Integrity of the NO/cGMP signaling pathway

Activation of soluble guanylate cyclase by NO or its donors leads to generation of cGMP (see figure 1.1). Studies performed by our group have demonstrated a relationship between anti-aggregatory effects of SNP and GTN and cGMP activation. In fact, impaired platelet responsiveness to anti-aggregatory actions of SNP and GTN was associated with decreased generation of intraplatelet cGMP in response to these NO donors. Given that intraplatelet cGMP concentration represents net effects of generation of cGMP by soluble guanylate cyclase and

hydrolysis of cGMP by cyclic nucleotide phosphodiesterases, input of both enzymes needs to be taken into account when evaluating determinants of platelet responsiveness to NO.

The interaction of sGC with NO donors was evaluated in human intact platelets utilizing a selective sGC inhibitor ODQ (Chirkov et al. 1999). ODQ inhibited anti-aggregatory effects of both SNP and GTN. Interestingly the inhibition of those effects was more prominent in normal subject than in patients with ischaemic heart disease. These results implicate loss of platelet sGC sensitivity to nitric oxide in patients with ischaemic heart disease. It is possible that impairment of sGC function is caused by superoxide (see figure 1.1). It has been demonstrated that superoxide inhibits activity of human platelet sGC (Brune, Schmidt & Ullrich 1990) and augments platelet aggregation *in vitro* (Leo, R et al. 1997) and in animal models *in vivo* (Yao et al. 1993). Furthermore, an elevated superoxide production by neutrophils was demonstrated in patients with stable ischaemic heart disease (Vaddi et al. 1994) and myocardial infarction (Riesenberg et al. 1997; Siminiak et al. 1993).

To investigate if the decreased concentration of intraplatelet cGMP in patients with ischaemic heart disease was secondary to augmented activity of cGMP-specific phosphodiesterases, platelet-rich plasma from these patients was incubated with the cyclic nucleotide phosphodiesterase inhibitor 3-isobutyl-1-methyl-xanthine (IBMX) (Chirkov et al. 1999). As a result of this, the intraplatelet cGMP concentration doubled. At the same time when IBMX was added to platelet-rich plasma together with nitric oxide donor SNP, the resultant cGMP increase was not significantly different from the effect of SNP alone. Therefore, suppression of phosphodiesterase did not restore to normal intraplatelet cGMP responsiveness to NO donors in patients with ischaemic heart disease, suggesting that increased phosphodiesterase activity is not the main cause of the problem.

Intracellular cGMP activates cGMP-specific protein kinases (see figure 1.1). Once activated, these enzymes initiate a variety of effects (sequestration of cytosolic Ca^{2+} , suppression of extracellular Ca^{2+} influx, inhibition of phospholipases C and A_2), leading to platelet activation and aggregation (for review see (Moncada &

Higgs 2006)). To evaluate the mechanism of the cyclic nucleotide-mediated pathway distal to cGMP formation, the effects of lipophilic derivative of cGMP (db-cGMP) on platelet aggregability were studied (Chirkov, Chirkova & Horowitz 1996). Even though the patients with ischaemic heart disease manifested impaired responsiveness of platelets to SNP, there was no reduction in anti-aggregatory actions of db-cGMP. This findings therefore imply that the NO/cGMP pathway is intact distal to cGMP formation in this circumstance and thus the quantity of cGMP generated by sGC in response to stimulation by NO donor essentially predetermines the extent of anti-aggregatory effect.

1.3.5.3 cGMP-independent effects of NO

The intracellular cGMP mediates vasorelaxation and anti-aggregatory effects of endogenous NO and exogenous NO donors. The effect of NO and NO donors in inhibiting platelet aggregation also has a cGMP-independent component (see figure 1.1), which is only apparent in vitro at high concentrations (micromolar range) of NO donors.

The usual criterion for a response to endogenous NO or exogenous NO donor to be termed as cGMP-independent is lack of inhibition by the selective inhibitor of soluble guanylate cyclase, ODQ (Feelisch et al. 1999). Interestingly, in blood samples from patients with known platelet NO resistance, ODQ did not further diminish effects of NO donors: - residual platelet aggregation, after preincubation with ODQ, was similar to that seen in blood of normal subjects (Chirkov et al. 1999). While these results represent impaired NO-sensitivity of platelet soluble guanylate cyclase in these patients, they also indicate preservation of the cGMP-independent effects of NO donors. Since these effects are mediated largely by protein S-nitrosylation (Murphy et al. 2014), and given that this process is stabilized by thioredoxin-interacting protein (TXNIP) (Forrester et al. 2009), it is not entirely surprising that the sGC-independent effects are preserved under conditions of redox stress (see section 1.3.8 on TXNIP).

1.3.6 Prognostic value of NO resistance

Extensive data have demonstrated that there is a relationship between coronary and peripheral arterial atherogenesis and impairment of endothelium-mediated vasomotor regulation (Gokce et al. 2003; Lerman & Zeiher 2005; Ludmer et al. 1986; Raitakari, Seale & Celermajer 2001; Schachinger, Britten & Zeiher 2000). Additionally, endothelial dysfunction has been documented in patients with classical risk factors for coronary artery disease, despite the absence of frank atherosclerotic lesions. It was proposed that the endothelium is both a target and a regulator of atherosclerosis (Nishimura et al. 1995; Vita et al. 1990). Endothelial dysfunction of coronary arteries is a contributor to cardiovascular morbidity and mortality and it is potentially modifiable. A number of studies have shown that impaired coronary vasoreactivity is a prognostic marker of adverse cardiovascular outcomes. Furthermore, the evaluation of endothelial vasodilator function may be regarded as a diagnostic and prognostic tool in cardiovascular disease.

Schachinger and colleagues prospectively evaluated, in 147 patients with coronary artery disease, whether coronary endothelial and NO-mediated vasodilator dysfunction is a predictor of disease progression and cardiovascular event rates over a median follow-up period of 7.7 years (Schachinger, Britten & Zeiher 2000). Increased vasoconstrictor responses to acetylcholine infusion, as well as reduced vasodilatation to the intracoronary GTN, were indeed predictive of increased event rates. Therefore, in addition to endothelial vasodilator dysfunction, decreased vasodilator sensitivity to exogenous NO also represents a prognostic marker in patients with coronary artery disease (Table 1.1): this is not completely surprising, given the overlapping biochemical bases for these conditions.

Table 1.1 Prognostic impact of NO resistance (reproduced with permission from (Chirkov & Horowitz 2007)).

Authors	Disease	Tissue	NO donor	Follow up (yr)	Outcome measures	<i>P</i>
Schächinger et al., 2000	CAD	Coronary arteries	NTG	7.7	Cardiovascular morbidity/mortality	<0.01
von Mering et al., 2004	CAD	Coronary arteries	NTG	3.0	Cardiovascular morbidity/mortality	0.04
Heitzer et al., 2005	CHF	Brachial artery	SNP	4.8	Deterioration/heart transplantation/death	0.03
Willoughby et al., 2005	ACS	Platelets	SNP	7.0	Cardiovascular morbidity/mortality	0.04

This was the first study demonstrating that both endothelial dysfunction and vascular NO resistance have prognostic value. Other group confirmed these findings in a female cohort (von Mering et al. 2004) (Table 1.1). In this study, 163 women underwent coronary reactivity assessment with quantitative coronary angiography and intracoronary Doppler flow wire before and after intracoronary administration of acetylcholine and GTN. Over a study follow-up 36% had cardiac events: death or hospitalization for cardiovascular cause. On bivariate analysis, patients with an event had significantly less change in coronary cross-sectional area in response to acetylcholine ($p = 0.0006$) and to GTN ($p = 0.04$) compared to those without an event.

Another study demonstrated that both systemic endothelial dysfunction and impaired vasodilator responsiveness are both predictors of adverse outcomes in heart failure patients (Heitzer et al. 2005). In that study, endothelium-dependent and endothelium-independent vasodilatation was evaluated in 289 patients with mild left ventricular systolic impairment via measurement of forearm blood flow responses to acetylcholine and SNP utilizing venous occlusion plethysmography.

Patients who suffered adverse events had lower vasodilator responses to acetylcholine ($p < 0.001$) and to SNP ($p = 0.03$) than those without events (Table 1.1).

These studies demonstrate that both endothelial dysfunction and impaired vascular responsiveness to NO are prognostic markers of adverse outcome in patients with ischaemic heart disease and heart failure. Neither the precise mechanisms for this nexus, nor their potential therapeutic interactions have been explored to date. Additionally, it is not clear if there is any association between the extent of NO resistance identified in these studies, and that of inflammatory change/redox stress, as other factors, which potentially can contribute to adverse cardiovascular outcomes.

While the above studies evaluated the predictive value of NO resistance at a vascular level, a study by Willoughby and colleagues (Table 1.1) was the first to demonstrate that platelet NO resistance is also associated with adverse outcomes (Willoughby, S.R. et al. 2005). In a cohort of 52 patients with high-risk ACS, during a median follow-up of 7 years, impaired platelet NO sensitivity was associated with an elevated risk of cardiovascular readmission and/or death (relative risk, 2.7; $p < 0.04$) and all-cause mortality (relative risk, 6.3; $p < 0.03$).

Other studies have demonstrated relationships between impaired platelet NO responsiveness and different cardiovascular pathologies: aortic stenosis (Chirkov et al. 2002), chronic heart failure (Anderson, RA et al. 2004) and polycystic ovarian syndrome (Rajendran et al. 2009).

These data thus raise the question of whether identification of patients with marked NO resistance at the platelet or vascular level might constitute an effective means for improved therapeutic decision-making. The development of such strategies is to some extent contingent on demonstration of a relationship between amelioration of NO resistance and improved outcomes.

1.3.7 Amelioration of NO resistance

NO resistance is a potential target for pharmacological interventions. To date, a number of investigations have been undertaken to determine whether agents which potentially reduce redox stress might also improve NO signaling pathways and thus partially reverse NO resistance.

1.3.7.1 Ascorbic acid

Given that many of the determinants of NO resistance relate to ROS production, a study (Ellis, GR et al. 2001) evaluated effect of intravenous ascorbic acid on NO resistance in chronic heart failure patients utilizing *ex vivo* platelet aggregometry, measuring flow-mediated dilatation and ROS levels, finding that in patients with heart failure acute intravenous administration of ascorbic acid augmented platelet responsiveness to the anti-aggregatory effects of NO donors and improved endothelial function. This investigation can therefore be regarded as a “proof-of-principle” exercise: it demonstrates that NO resistance is potentially reversible, but does not identify a form of effective long-term treatment.

1.3.7.2 ACE inhibitors

The effectiveness of some ACE inhibitors in preventing ischemic events in high risk and particularly diabetic patients has been shown in large clinical trials (HOPE for ramipril and EUROPA for perindopril), but knowledge of the underlying mechanisms remains incomplete. ACE inhibitors have demonstrated pharmacodynamic effects beyond hypertension treatment. Among these effects are improvement of endothelial function and the normalization of vascular and cardiac structure and function (for review, see (Curran, McCormack & Simpson 2006)). For example, short-term therapy with ACE inhibitors improved flow-dependent vasodilation in the femoral artery of normotensive diabetic patients (Arcaro et al. 1999).

Commencement of therapy with ACE inhibitors in patients with heart failure was associated with an increase in platelet responsiveness to anti-aggregatory and cGMP-stimulating actions of SNP and a reduction in whole blood superoxide levels (Anderson, RA et al. 2004; Chirkov et al. 2004). More recently, it has been shown that ramipril ameliorates platelet NO resistance in HOPE study-type patients, with associated increases in soluble guanylate cyclase responsiveness to NO (Willoughby, S. R. et al. 2012). Thus, it is possible that reversal of platelet NO resistance contributes to the beneficial effects of ACE inhibitor therapy.

1.3.7.3 Perhexiline

Perhexiline is an effective cardiac metabolic agent (Lee, L, Horowitz & Frenneaux 2004), it is utilized in the management of patients with refractory angina or in those in whom coronary revascularization is impossible (Cole et al. 1990; Horowitz, JD et al. 1986). Clinical data suggest that perhexiline may be effective in suppressing symptoms in patients with acute coronary syndrome (Stewart et al. 1996) or with advanced and inoperable aortic valvular stenosis (Unger, Robinson & Horowitz 1997). Perhexiline has been also demonstrated to improve hemodynamic and functional status in chronic heart failure patients (Lee, L et al. 2005) and ameliorate symptoms in advanced cases of hypertrophic obstructive cardiomyopathy (Abozguia et al. 2010). Although perhexiline has at least several biochemical effects, it has been shown to inhibit the mitochondrial enzyme carnitine-palmitoyltransferase-1, potentially thus inhibiting long-chain fatty acid oxidation (Kennedy, Unger & Horowitz 1996) and also to inhibit Nox-2, thus reducing superoxide production (Gatto et al. 2013).

Multivariate analysis of potential correlates of the presence/ absence of platelet NO resistance in a cohort of patients with broad spectrum of ischaemic heart disease demonstrated that perhexiline therapy led to improved responsiveness to NO donors (Chirkov et al. 2001). In one prospective study, perhexiline was evaluated in regards to possible potentiation of platelet responsiveness to NO in patients with stable angina and acute coronary syndromes (Willoughby, S. R. et al. 2002). Treatment with perhexiline *per se* did not exhibit any anti-aggregatory effect when

evaluated *ex vivo* in these patients. Nevertheless, both in whole blood and in platelet-rich plasma from patients with acute coronary syndromes and stable angina, treatment with perhexiline for only 3 days significantly augmented SNP-induced inhibition of platelet aggregation and improved clinical symptoms. Furthermore, perhexiline treatment also potentiated SNP-induced intraplatelet cGMP content elevation and also suppressed release of superoxide from neutrophils *in vitro*. Similar observations in regards to amelioration of NO platelet resistance were documented in patients with aortic stenosis (Chirkov et al. 2002).

1.3.7.4 Glycaemic control

Hyperglycaemia is a hallmark of diabetes mellitus and metabolic syndrome. The key finding in patients with hyperglycaemia is the presence of endothelial dysfunction which is the major culprit of the associated vascular pathologies. *In vivo* and *in vitro* studies have shown that hyperglycaemia leads to the development of endothelial dysfunction and inhibits endothelium-dependent vasorelaxation (Cosentino & Luscher 1998; Kim et al. 2003). In type II diabetic patients, treatment aimed at intensive glucose-lowering improves endothelial function (Yasuda et al. 2006). Insulin therapy also helps to improve deleterious pro-aggregatory effects of hyperglycaemia, as it has been shown that hyperglycaemia inhibits platelet NO generation (De La Cruz et al. 2004). Anti-aggregatory effects of insulin *in vitro* are partially mediated by incremental NO release from platelets (Anfossi et al. 1996), perhaps due to the activation of platelet NOS (Trovati & Anfossi 2002). Additionally, intravenous insulin infusion in humans over 3 hours inhibited *ex vivo* platelet aggregation induced by ADP, epinephrine, collagen and thrombin receptor-activating peptide (Westerbacka et al. 2002).

Given poor outcomes in acute coronary syndrome patients who present with hyperglycaemia, detailed physiological evaluation was performed to delineate this deleterious combination (Worthley et al. 2007). In diabetic patients admitted with acute coronary syndromes, increased blood levels of superoxide directly correlated with blood sugar levels on admission. Platelet responsiveness to the anti-

aggregatory effects of the NO donor SNP was poor, and varied inversely with superoxide levels. Finally, 12 hours of intravenous insulin infusion resulted in a significant reduction of blood sugar levels, improved platelet responsiveness to SNP and reduced superoxide blood levels. These findings provide a further rationale for utilization of insulin therapy in myocardial infarction (Malmberg et al. 1995; Ritsinger et al. 2014) and give a strong indication for extension of insulin therapy to patients with all acute coronary syndromes.

1.3.7.5 Statins

Hypercholesterolemia is a well documented risk factor associated with endothelial dysfunction on the vascular level (Landmesser, Hornig & Drexler 2000). While statins, in general, ameliorate endothelial dysfunction (Margaritis, Channon & Antoniades 2014; Tiefenbacher et al. 2004), impact on vascular NO responsiveness escaped initial evaluation. It has been suggested that statin-induced beneficial effects on endothelial function may be mediated primarily via activation of NO synthase (Sun et al. 2006), associated with reductions in plasma ADMA concentrations (Boger et al. 1998).

Minimal data are available in regards to the impact of statins on platelet responsiveness to NO. One study in patients with acute coronary syndromes demonstrated on multivariate analysis that NO resistance was less common in patients receiving statin therapy (Chirkov et al. 2001). Another study, in a group of asymptomatic adults with and without mild hypercholesterolaemia, evaluated the effects of 3 months treatment with pravastatin (Stepien et al. 2003). The study revealed that in platelet-rich plasma the aggregation response to ADP was significantly greater in subjects with hypercholesterolaemia. There was also more superoxide generated during aggregation in those with hypercholesterolaemia. Although statin therapy did not affect platelet aggregability *in vitro*, inhibition of aggregation with SNP was significantly potentiated. These observations can be interpreted as an improvement in platelet NO sensitivity and increase in NO bioavailability.

Overall, there are therefore several strategies nowadays which have been demonstrated to ameliorate NO resistance with its deleterious effects associated with many cardiovascular diseases.

However, in situations of cardiovascular emergencies, when NO resistance is generally at its worst, immediate circumvention of this problem would achieve significant clinical benefits. One of the studies in this thesis with the nitroxyl donor isopropylamine-NONOate addresses this therapeutic deficiency.

1.3.8 Influence of thioredoxin-interacting protein (TXNIP) on NO signaling: implications regarding NO resistance and its amelioration

The thioredoxin system, which consists of thioredoxin (Trx), NADPH and thioredoxin reductase (TrxR), has emerged as a major anti-oxidant maintaining the cellular physiology and survival. Dysregulation in this system has been associated with metabolic, cardiovascular, and malignant disorders (Lee, S, Kim & Lee 2013).

TXNIP is a critically important amplifier of intracellular redox stress, with marked effects in exacerbating ROS production, stress and inflammatory activation within mitochondria and endoplasmic reticulum. The question has recently arisen that TXNIP might modulate impairment of NO signaling various conditions, for example diabetes/hyperglycaemia and myocardial ischaemia (Mahmood et al. 2013; Yoshihara et al. 2014).

There are multiple bilateral interactions between TXNIP and NO signaling which result in suppression of TXNIP expression by NO and suppression of NO generation with its biological effects by TXNIP (Forrester et al. 2009). TXNIP-induced increases in superoxide generation lead to inactivation of dimethylarginine dimethylaminohydrolase, partial inactivation of sGC, and "scavenging" of NO to produce peroxynitrite. As a result, TXNIP reduces protein kinase G activation and

inhibits NO signaling. Therefore it would be expected that increased TXNIP expression, for example in diabetes, would be associated with endothelial dysfunction (Shah et al. 2013).

Conversely, it has been demonstrated that NO suppresses TXNIP expression: in rat pulmonary artery smooth muscle cells the NO donor S-nitrosoglutathione suppressed TXNIP expression (Schulze et al. 2006).

A recent study evaluated the relationship between TXNIP expression and efficacy of NO signaling in human platelets (Sverdlov et al. 2013). It was found that platelet TXNIP content tended to vary inversely with platelet NO responsiveness, with a highly significant negative correlation between these parameters in otherwise normal aging subjects.

Another recent study further shed new light on the understanding of TXNIP pathophysiology within the cardiovascular system (Yoshioka et al. 2012): authors evaluated the effect of TXNIP knockout on recovery from myocardial ischaemia in mice. Following a period of ischaemia/reperfusion, TXNIP knockout mice exhibited better residual mitochondrial activity, increased ATP generation together with decreased ROS formation within mitochondria, and consequently the potential for more rapid recovery of left ventricular function. To date, no studies reported the changes in TXNIP expression during human ischaemia-reperfusion, nor the determinants of myocardial TXNIP expression in these patients. However, it can be assumed that non-laminar flow observed in heart failure and atrial fibrillation and the presence of diabetes mellitus/insulin resistance would provide particular bases for vulnerability to TXNIP-mediated deleterious effects. Overall, the effects of TXNIP might arise largely through modulation of mitochondria metabolism (Yoshioka & Lee 2014), as well as via increased ROS production and antagonism of Trx effect.

Platelet TXNIP content increases with aging, varies inversely with responsiveness to NO, and decreases rapidly after treatment with ramipril (Sverdlov et al. 2013). These data suggest that TXNIP-induced oxidative stress may be a critical modulator of tissue resistance to NO, a fundamental basis for cardiovascular

disease. In practice, therefore, the effects of suppression of TXNIP expression may be beneficial to improve NO responsiveness and ameliorate NO resistance in many cardiovascular disease states for restoration of cardiovascular homeostasis. Importantly, both ACE inhibitors and perhexiline, agents which ameliorate NO resistance, also suppress TXNIP expression (Sverdlov et al. 2013), further suggesting that a cause-and-effect relationship may operate.

1.4 Platelets: anatomy, physiology and pathophysiology

1.4.1 Normal platelet function and anatomy

Platelets are circulating disc-like cells and are 2 to 5 μ m in diameter and 0.5 μ m in thickness, which are derived from megakaryocytes in the bone marrow. The release of platelets into the circulation is regulated by thrombopoietin, interleukins and probably by nitric oxide (Battinelli et al. 2001). Normal platelet count is 150,000 to 300,000 per microliter.

Platelets are the cornerstone of the normal clotting mechanism. Within seconds of vascular injury, platelets adhere to the damaged vessel wall. This leads to platelet aggregation (platelet-to-platelet adherence), which further results in the formation of a 'platelet plug' that temporarily seals off the damaged vessel wall. This platelet function is important to host preservation but can also contribute to host destruction: in cardiovascular disease states, by forming arterial thrombi, platelets may limit blood supply to distal tissues and cause local ischaemia (Holmsen 1989). Thus, platelet structure, biochemistry, physiology, and pathology are relevant both to normal function and to pathophysiology of occlusive vascular disease.

Platelets do not have nuclei but they have a complex infrastructure that allows them to function over a limited life-span of 7-10 days. This includes multiple active factors and organelles situated in the cytoplasm: contractile proteins actin, myosin and thrombosthenin; mitochondria, lysosomes, peroxisomes, glycogen particles; Golgi apparatus and endoplasmic reticulum. Furthermore, their cytoplasm contains two types of granules: α -granules and dense bodies, which play an important role during platelet activation (reviewed by (Michelson, A.D. 2007)). α -granules contain platelet factor-4, β -thromboglobulin, platelet-derived growth factor, fibrinogen, fibronectin, thrombospondins, plasminogen activator inhibitor-1 (PAI-1), P-selectin and von Willebrand factor (vWf) (Reed 2004). Dense granules are rich in calcium, serotonin and adenosine diphosphate (ADP).

All these structures within the cytoplasm are encompassed by a trilaminar membrane. The outer layer consists of glycoproteins serving as receptors for agonists/antagonists (e.g. glycoprotein IIb/IIIa complex interacts with fibrinogen and thus plays an important role in platelet aggregation and adhesion to endothelial surfaces (White, JG 1987)). The middle layer is a lipid bilayer to which glycoproteins are attached. The inner layer of platelet membrane consists of a system of thin contractile filaments resembling actin filaments (White, JG 1969). These filaments have an important role in the shape change and translocation of receptors on the exterior surface of the cell. Also in the submembrane compartment there is a network of tubules connecting the cytoplasm to the external surface of the platelet. This network is known as the open canalicular system and represents a large system of channels to provide two-way transport into and out of the platelet (e.g. excretion content of α -granules and dense bodies out of platelet during its activation).

Thrombus formation results from a combination of events that follow platelet adhesion to the damaged endothelium and includes activation of platelets and changes in their shapes, aggregation and granule secretion. These steps are described in more detail in the following sections.

1.4.2 Platelet adhesion

Platelet adhesion leading to thrombus formation is a key mechanism both in normal haemostasis and in the pathogenesis of acute coronary syndromes and thrombotic disorders. Through specific adhesion receptors circulating platelets adhere to sites of vascular injury. This is despite the hemodynamic forces in moving blood opposing this process. Adhesion is initiated by the reversible interaction between subendothelial bridging molecule of vWF and the GP Ib-IX-V complex, once the endothelial cell lining of a vessel is disrupted (Savage, Almus-Jacobs & Ruggeri 1998; Savage, Saldivar & Ruggeri 1996).

1.4.2.1 Von Willebrand Factor (vWf)

vWf is a polymeric adhesive protein in subendothelium, secretory granules in endothelial cells, plasma and platelet α -granules. vWf serves a key role in mediating platelet adhesion to the subendothelium via GP Ib-IX-V complex under conditions of high shear, and platelet aggregation by binding to the major platelet integrin α -IIb3 (GP IIb-IIIa) via arginine-glycine-aspartic acid sequence following platelet activation (Savage, Shattil & Ruggeri 1992).

1.4.2.2 GP Ib-IX-V receptor complex

Initially known as a vWf platelet receptor, the GP Ib-IX-V complex is now recognized to have a multitude of functions in physiological haemostasis and pathological thrombosis. For example, the GP Ib-IX-V complex initiates the interaction of non-activated platelets with vessel endothelium by binding to surface endothelial P-selectin (Romo et al. 1999) and with activated leukocytes through binding to integrin Mac-1 (Simon et al. 2000). Further to its adhesive role, the GP Ib-IX-V complex interacts with thrombin (Mazzucato et al. 1998), high-molecular-weight kininogen (Bradford et al. 1997), and coagulation factors XI (Baglia et al. 2002) and XII (Bradford, Pixley & Colman 2000). It also plays a key role in

regulating the cytoskeleton of platelets (Jackson, Mistry & Yuan 2000).

Platelets can also adhere to other proteins in the subendothelium including collagens I, III, IV and fibronectin. Further, platelets become irreversibly attached to the vessel due to interactions between their receptors and subendothelial proteins with plasma proteins immobilized at the site of injury (Andrews, RK et al. 1999). In addition to mediating platelet adhesion, platelet receptors trigger intracellular signaling events that lead to platelet activation.

1.4.3 Platelet activation

Platelet adhesion at sites of vascular endothelial damage generates a platelet monolayer. Following adhesion, platelets become activated by several physiological agonists. This process leads to the local accumulation of molecules secreted from platelets: including thromboxane A₂, ADP, serotonin, epinephrine, and arachidonic acid. These molecules act on their respective receptors on the platelet surface membrane that can respond rapidly to activate phospholipase C (PLC), increase cytosolic Ca²⁺ levels, and suppress production of cAMP (see figure 1.4). Further, thrombin generated at the platelet surface initiates platelet activation via its receptors and leads to strengthening of platelet thrombus by fibrin deposits.

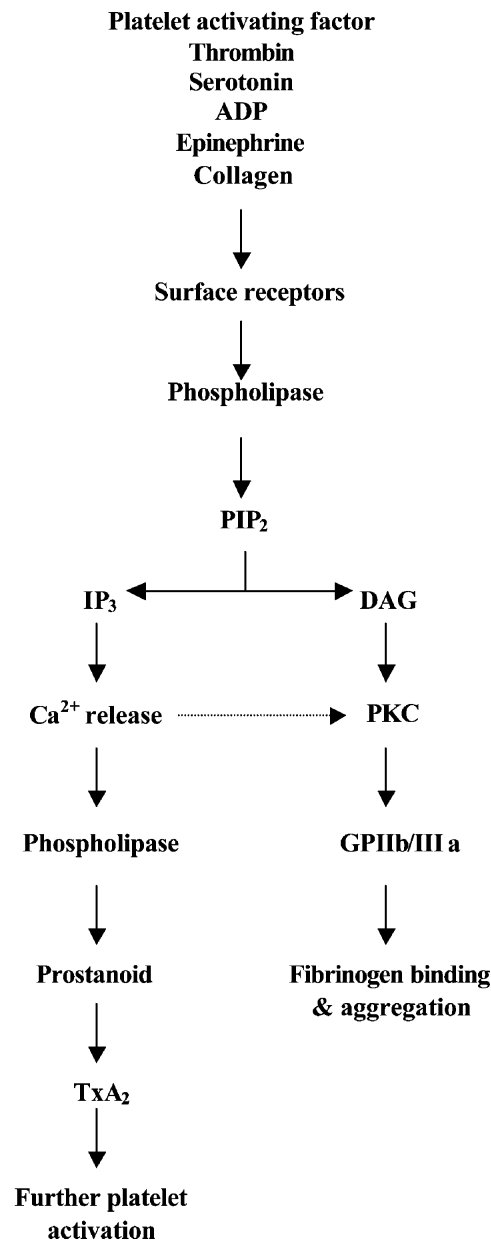


Figure 1.4 Mechanism(s) of platelet activation

PIP₂, phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol; IP₃, inositol 1,4,5-trisphosphate; PKC, protein kinase C; TxA₂, thromboxane A₂. This figure is reproduced with permission from Willoughby et al. 2002 (Willoughby, S, Holmes & Loscalzo 2002).

1.4.3.1 Platelet activation via ADP receptors

ADP is released from dense granules upon platelet activation. Despite being a weak activator, ADP has a key role in platelet function via potentiating the effect of other agonists by activating its both receptors P2Y₁ and P2Y₁₂ and hence amplify the response of platelets (Cattaneo & Gachet 1999; Jin, J & Kunapuli 1998). Activation of P2Y₁ and P2Y₁₂ receptors leads to activation of PLC with rise in cytoplasmic Ca²⁺ levels, inhibition of adenylyl cyclase and lowering cAMP levels, platelet shape change and aggregation (Gachet 2001; Jin, J & Kunapuli 1998; Savi et al. 1998).

1.4.3.2 Platelet activation via thrombin receptors

Thrombin is a serine protease that mediates formation of fibrin by cleaving fibrinogen and is a potent platelet activator.

Thrombin-induced platelet activation is initiated via protease-activated receptors (PARs) (Kahn et al. 1999). This leads to activation of PLC, with further production of the second messengers IP₃ (mobilizes intracellular Ca²⁺) and DAG (protein kinase C activator) (Berridge 1993) (see figure 1.4). These processes initiate cascade reactions that lead to platelet aggregation, production of arachidonic acid and thromboxane synthesis (Kramer et al. 1996). Also thrombin binding to GPIIb-IX-V (see above) triggers platelet adhesion, secretion of dense granules, and aggregation (Adam, Guillin & Jandrot-Perrus 2003).

1.4.3.3 Platelet activation via thromboxane A₂ receptors

Thromboxane A₂ (TXA₂) is a platelet agonist and vasoconstrictor. It is produced from arachidonic acid by cyclooxygenase with prostaglandin G₂ and prostaglandin

H₂ as intermediate products and hence depends on rate of release of arachidonic acid from the platelet membrane. TXA₂ potentiates activation of platelets after their stimulation by primary agonists (ADP and thrombin): - these primary agonists do not cause significant aggregation without TXA₂.

There are at least two forms of TXA₂ receptor on the platelet membrane (Hirata et al. 1996) that are coupled to several G proteins to activate phospholipase A₂ and phospholipase C. Activation of the TXA₂ receptor leads to activation of several signaling proteins leading to platelet aggregation.

1.4.4 Platelet aggregation

The process of platelet aggregation (crosslinking) (Parise 1999) centres on binding of soluble adhesive proteins such as plasma vWF and fibrinogen to the platelet IIb/IIIa receptor, while further facilitating platelet activation to form a stable and strong thrombus. The formation of a platelet plug strengthened by insoluble fibrin fibers helps to establish haemostasis in a damaged vessel.

Platelet adhesion and aggregation were previously considered as distinct processes in thrombus formation. It is now obvious that the key mechanism is similar (Ruggeri 2000), involving the interaction between platelet receptors, GP Ib-IX-V complex with fibrinogen and vWf (Ruggeri, Dent & Saldivar 1999; Wu et al. 2000). It is like a 'chain reaction': once platelets adhere, they bind fibrinogen and vWf from the blood; this creates an ideal surface for further recruitment of more platelets.

1.4.5 Characterization of platelet aggregation

There are several methods available to assess platelet function. These methods usually involve evaluation of platelet aggregation and/or platelet granule release studies.

Platelet function tests

All platelet function tests are based on the detection of platelet aggregate formation after stimulation with an agonist. Different techniques used to detect this aggregate formation.

1.4.5.1 Platelet aggregometry

1.4.5.1.1 Optical platelet aggregometry

Optical platelet aggregometry was invented in 1962 (Born 1962) and utilizes the principle that the absorbance of light through a suspension depends on the number of particles rather than on their size. During aggregation, the number of particles decreases, and light transmission increases. Briefly, blood is collected by venipuncture into plastic tubes containing 1:10 volume of acid citrate anticoagulant (2 parts of 0.1 mol/L citric oxide to 2 parts of 0.1 mol/L trisodium citrate). Platelet-rich plasma (PRP) is prepared by centrifugation of whole blood at a low speed (150g for 10 min). This PRP is added to a cuvette with a stirrer bar and stirred (900 rpm) at 37°C between a light source and a photocell within the aggregometer. Upon the addition of an agonist (thrombin, collagen, ADP or arachidonic acid), the aggregated platelets clump together and fall to the bottom of the cuvette, thus increasing light transmission. The level of aggregation is assessed by a maximum change in light transmission. Platelet-poor plasma (PPP) is used as a blank for the aggregometer. Variation between samples has been estimated to have a standard deviation in a range of 3.6 to 7.7%, with day-to-day variation accounting for the majority of the variation, followed by operator variability (Nicholson et al. 1998). The sources of this variation include sample preparation and its aging, diet with high levels of lipids in the sample, smoking. Modifications of optical platelet aggregometry have also been developed including an impedance-based aggregometry and bedside assays.

1.4.5.1.2 Impedance platelet aggregometry

An alternative method to optical aggregometry is impedance aggregometry in whole blood, a technique developed in 1980 (Cardinal & Flower 1980), which measures changes in impedance across two platinum wires as platelet aggregates form on them increasing the electrical impedance. This technique has the advantage of being fast, as no sample preparation is required; it is more relevant physiologically as all the components of the blood are present including leukocytes and erythrocytes (Harrison, P 2000; Mascelli et al. 1997). Aggregation in PRP can also be assessed with impedance aggregometry method. Several studies have compared platelet aggregation in whole blood to that in PRP and have reported similar results (Ingerman-Wojenski, Smith & Silver 1983).

Impedance platelet aggregometry was the only method utilized to evaluate platelet aggregation in the present study.

1.4.5.1.3 The rapid platelet function analyzer

Several analyzers have been invented for a rapid assessment of inhibition of platelet aggregation by anti-platelet agents at bedside. These analyzers are utilized also to measure platelet function. An example is the Ultegra™ Rapid Platelet Function Analyzer to monitor platelet inhibition by GPIIb/IIIa antagonists (Wheeler et al. 2002). This assay is based on thrombin receptor-activating peptide (TRAP)-induced platelet aggregation. It has a cartridge with fibrinogen-coated beads, a magnetic stirrer, and TRAP. Whole blood is added to the cartridge: as activated platelets bind and agglutinate the fibrinogen-coated beads, there is an increase in light transmittance which is analyzed digitally (Ostrowsky et al. 2004). The advantage of this assay is that it is fast bedside device, uses only a small amount of whole blood

and does not require a sample preparation.

1.4.5.1.4 Shear-dependent assay: PFA-100

In vivo platelets continuously experience shear conditions as they circulate through the vasculature: low shear in veins to high shear in stenosed arteries. Some of the platelet functions operate only under conditions of shear stress. In particular, the interaction of GPIb on platelets with immobilized von Willebrand factor only occurs under shear. As a result shear-based systems have been developed to assess platelet function. The original technique was described in 1985 (Kratzer & Born 1985). The Platelet Function Analyzer PFA-100 is an automated system for monitoring platelet responsiveness. It has a membrane coated with collagen-adrenaline or collagen-ADP. Whole blood is aspirated through a hole in the membrane under high shear stress. The endpoint is expressed as the time to closure - cessation of blood flow due to occlusion of aperture by platelet aggregates and is a measure of platelet function (Marshall et al. 1997). This assay has the advantage of using small amount of whole blood applied to a cartridge.

1.4.5.2 Measures of platelet activation

Many tests have been invented to assess activity of circulating platelets or to show that platelets have been activated *in vivo* by prothrombotic stimuli (Harrison, P 2000), for example: -

1.4.5.2.1 Soluble platelet activation markers

Platelet activation may be quantitated by measuring released platelet substances in platelet-poor plasma. Examples of this type are radioimmunoassays (RIAs) and enzyme-linked immunosorbent assays (ELISAs) for platelet-specific proteins, e.g. β -thrombomodulin and platelet factor 4, whose utility as platelet activation markers have been investigated in human thrombotic conditions (Kaplan 1978).

1.4.5.2.2 Flow cytometry

Flow cytometric analysis is a powerful approach for studying many properties of platelets and is widely used for assessment of platelet activation and function. The standard method is to use a marker of platelet degranulation. The two markers usually used are P-selectin (CD62p), a marker of α -granule release, or CD63 antigen, a marker of dense granule release (Rendu & Brohard-Bohn 2001). P-selectin is expressed on the platelet surface after α -granule secretion and initiates adhesion of activated platelet to leukocytes. Activated platelets *in vivo* rapidly lose their surface P-selectin but continue to function in the circulation (Berger, Hartwell & Wagner 1998). It has been demonstrated that circulating monocyte-platelet aggregates are a more sensitive marker of *in vivo* platelet activation than is platelet surface P-selectin in the clinical settings of stable coronary artery disease (Furman et al. 1998), human percutaneous coronary intervention and acute MI (Michelson, A. D. et al. 2001).

1.5 Nitrate-Nitrite-Nitric oxide pathway

1.5.1 Nitrogen living cycle

Before the Nitrate-Nitrite-Nitric oxide pathway, with its relevance to the current study is described here, it is important to discuss briefly nitrogen (N), which was identified as a chemical element (atomic weight 14), included in 1869 to the

Periodic Table published by the Russian chemist Dmitrii Mendeleev. The biggest source of nitrogen on our planet is atmospheric air.

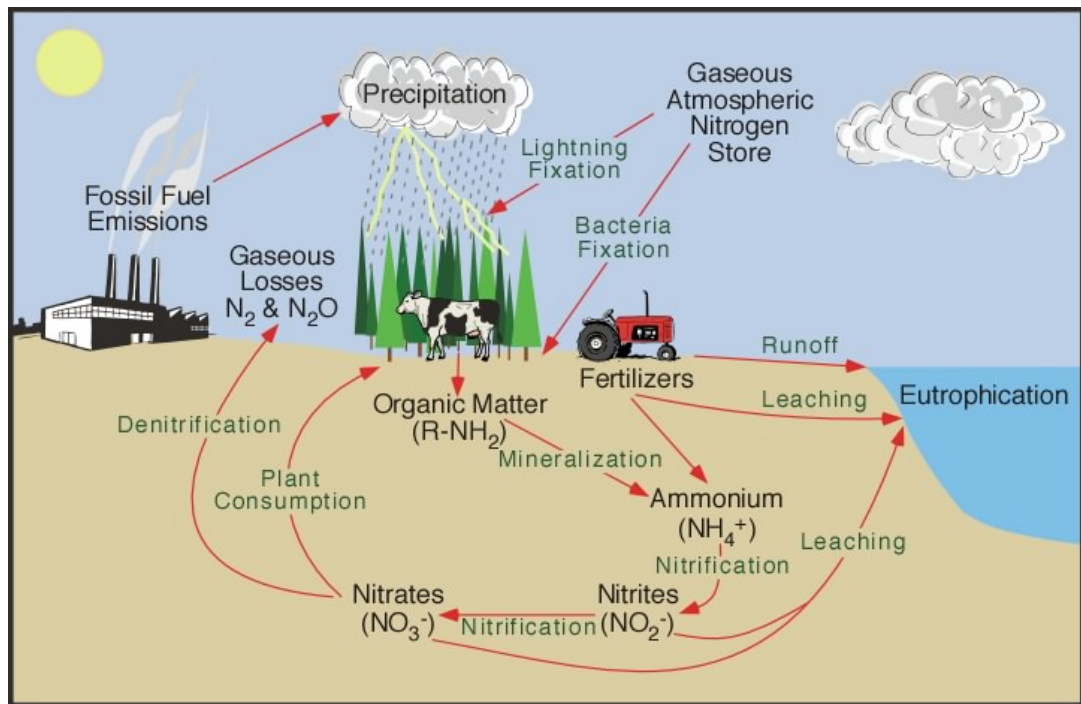


Figure 1.5 Schematic of Nitrogen cycle reproduced with permission from Pidwirny, M. (2006). "The Nitrogen Cycle". Fundamentals of Physical Geography, 2nd Edition. Date Viewed – 30.05.2014 at <http://www.physicalgeography.net/fundamentals/9s.html>

Nitrogen gas (N_2) is the largest gas component of atmospheric air we breathe (78% of air by volume) and represents a critical component of the nitrogen cycle. Once nitrogen undergoes the process of ‘fixation’, during which nitrogen gas is converted to ammonium mainly by bacteria in the soil, nitrogen enters a potential “biological” component of this cycle. Ammonium (NH_4^+) is oxidized to different nitrogen oxides including nitrite (NO_2^-) and nitrate (NO_3^-) in a process termed nitrification (see figure 1.5). Nitrate is eventually reduced by denitrifying bacteria to nitrite, nitric oxides and finally nitrogen gas that goes back to the atmosphere. These denitrifying bacteria, having bacterial reductases, use nitrogen oxides as terminal electron acceptors for mitochondrial respiration. This principle, where bacteria play a crucial role in the nitrogen living cycle, will become an important example, given the similar role of bacterial reductases in nitrite physiology in humans.

Almost a century ago an observation was made that humans excrete more nitrate in the urine than they ingest (Mitchell, Shonle & Grindley 1916). Sixty-five years later in bacteria-free rats it was shown that net nitrate production does not depend on presence of bacteria (Green, Tannenbaum & Goldman 1981). In 1980, Furchgott and Zawadzki described a substance derived from vascular endothelium that relaxed vessels and this was later found to be nitric oxide (Furchgott & Zawadzki 1980; Ignarro, Buga, et al. 1987). Since this discovery, substantial research has been conducted in the area of nitric oxide – now encompassing almost in every field of medicine.

1.5.2 Sources of nitric oxide

The majority of nitric oxide (NO) in human body is produced via the classical L-arginine pathway catalysed by nitric oxide synthases (NOS). There are three types of NOS: endothelial, neuronal and inducible. The NO production via this pathway is a complex oxygen-dependent mechanism involving five electron transfers and presence of L-arginine as a substrate and tetrahydrobiopterin and NADPH as cofactors (Moncada & Higgs 1993). The NO molecule has one unpaired electron and this property makes this substance very reactive, expediting its function as a signaling messenger in human physiology.

Other sources of NO in humans are nitrate and nitrite, which may be physiologically “recycled” in the blood and tissue to form NO (Lundberg et al. 1994; Zweier et al. 1995). This process does not require oxygen. In other words, nitrate and nitrite, initially regarded as products of NO catabolism only can also be regarded as a ‘storage pool’ of NO bioactivity functioning in addition to the classical L-arginine pathway of NO production. The production of nitrite/nitrate in the body represents not only the catabolism of NO, but also the establishment of a relatively stable potential source of re-generation of NO (exogenous nitrate/nitrite add to this “storage pool”: see section 1.5.3). Further, these substances can be transported in the body and stored in the tissues until there is a physiological need for NO. Hence, nitrate and nitrite could be also regarded as a “back-up mechanism”

for situations when there is not enough NO, for example when the L-arginine pathway is dysfunctional, especially in tissues subjected to hypoxia.

1.5.3 Sources of nitrate and nitrite

Nitrate and nitrite in humans come from two main sources. The major source remains the L-arginine pathway of NO generation. In the blood NO undergoes rapid oxidation to form nitrite and nitrate.

The second major source of nitrate and nitrite is diet. The highest content of nitrate is in vegetables, in particular, the green leafy vegetables (Hord, Tang & Bryan 2009; Santamaria 2006). This is discussed in detail in section 1.5.6.2. The potential impact of vegetable sources of nitrite can be seen from the fact that one plate of salad made of green leafy vegetables contains more nitrate than that formed by NOS isoforms in one day (Lundberg et al. 2009). Another dietary source of nitrate is drinking tap water. This occurs due to contamination of ground waters by fertilizers. The government always strictly regulates standards of drinking water (Ward et al. 2005) due to possibility of methaemoglobinaemia in babies up to six months of age (Butler & Feelisch 2008) and historical belief that nitrate can be a cause of gastric cancer (Tannenbaum & Correa 1985). The latter has not been confirmed to date (Powlson et al. 2008).

1.5.4 Bioconversion of nitrate in the digestive tract

In 1976, it has become clear that a substantial portion of nitrate circulating in the body is concentrated in the salivary glands leading to nitrate concentrations in saliva of 20-fold higher than of that in the serum (Spiegelhalder, Eisenbrand & Preussmann 1976). Once dietary nitrate is ingested and absorbed in the stomach, it then mixes in the blood with nitrate that was generated by oxidation of NO produced by NOS systems. The majority of nitrate is then excreted in the urine but up to a quarter of it ends up in the salivary glands with concentrations 100-1000-fold higher than in the blood (Govoni et al. 2008). Excreted in saliva, it is further reduced in the oral cavity to nitrite by commensal anaerobic bacteria living on the back of the tongue (Duncan et al. 1995; Li, H et al. 1997). Humans do not have endogenous nitrate reductases, hence the facultative bacteria with nitrate reductase enzymes serve this vital purpose. Furthermore, it is important to avoid using bacterial mouthwash solutions from this perspective (Govoni et al. 2008), as well as to avoid unnecessary antibiotic use (Park et al. 2013). Similarly, those who constantly spit out saliva or lack it due to Sjögren's syndrome are also at increased risk of lower nitrite levels which potentially can be deleterious in cardiovascular disease states (Webb, Patel, et al. 2008). As mentioned earlier, these bacteria utilize nitrate in their respiration process as an alternative to oxygen terminal electron acceptors to generate ATP in the anaerobic environment.

After generation of nitrite in the course of this functional symbiosis with tongue bacteria (Lundberg et al. 2004), nitrite enters the stomach. Due to the acidic environment in the stomach, part of nitrite is rapidly protonated to nitrous acid (HNO_2), and this further decomposes to nitrogen oxides including NO (Benjamin et al. 1994; Lundberg et al. 1994). This facilitates effective elimination of some common bacterial pathogens that could otherwise survive in the stomach acid for long time (Dykhuisen et al. 1996). Acidified nitrite in the stomach is also effective against *Helicobacter pylori* – bacteria that causes gastric and duodenal ulcers (Dykhuisen et al. 1998). Nitric oxides that are generated in the acidic gastric milieu act on many bacterial targets including cell wall, proteins and DNA. Despite this acidic barrier on the nitrate-nitrite-NO pathway and the large amount of NO

generated in the acidic milieu of the stomach, only a small portion of nitrite entering the stomach is lost during this stage:- the majority of salivary nitrite is absorbed into the bloodstream (Lundberg & Govoni 2004). This potent 'storage pool' of NO generation capacity can be reduced to NO by a multitude of nitrite reductases, the precise identities of which remain subject to debate (Feelisch et al. 2008). In addition to the gastrointestinal bioconversion of nitrate to NO via nitrite, it has been shown that similar processes occur on the skin surface (Weller et al. 1996), lower gastrointestinal tract (Sobko et al. 2005) and in urine (Lundberg et al. 1997), where higher local concentrations of NO are achieved, playing important antibacterial and wound healing roles.

1.5.5 Nitrite bioactivation and hypoxic potentiation in humans

In addition to the protonation of nitrite in the gastric acidic environment, research over the past decade revealed that humans are equipped with multiple enzymes and proteins, containing haem or molybdenum, that are capable of reducing nitrite to NO.

The concept of nitrite reduction in humans has been developing rapidly, in contrary to the long belief that nitrite anion was intermediate in the oxidation of nitric oxide to the more stable nitrate. Under normal physiological conditions, the NO oxidation pathway was thought to be irreversible. Conversely, data have been accumulating to prove that endogenous nitrite mediates a number of signaling events along an oxygen gradient with augmentation of nitrite effects at low tissue oxygenations. This phenomenon has been termed "hypoxic potentiation of nitrite" (for recent reviews see (Umbrello et al. 2013; van Faassen et al. 2009)). Hypoxic nitrite reduction leads to vasodilation, inhibition of platelet aggregation, modulation of mitochondrial respiration, and cytoprotection after ischaemic events. Currently, more than twenty enzymatic and non-enzymatic pathways involved in the endogenous reduction of nitrite have been identified and the number is growing.

Among those nitrite reductases are haemoglobin (Kim-Shapiro et al. 2005), myoglobin (Shiva et al. 2007), xanthine oxidoreductase (Tripatara et al. 2007),

mitochondrial enzymes (Heusch, Boengler & Schulz 2010; Shiva 2013), neuroglobin (Petersen, Dewilde & Fago 2008), ALDH2 (Li, H et al. 2008), even eNOS (Vanin et al. 2007) and many more. The predominant reductase is relatively tissue/organ specific and in some situations interplay of several nitrite reductases produces NO (van Faassen et al. 2009).

1.5.5.1 Role of haemoglobin

Vasodilator effects of nitrite have been shown to be potent in acidotic (pH=6.6) environment of organ bath without any enzymatic involvement (Modin et al. 2001). Cosby et al. made observation that infusion of low dose nitrite in human forearm produced significant vasodilatation at physiological pH (Cosby et al. 2003) without acidosis. Another observation was made by Cannon et al. of a significant gradient of nitrite between arterial and venous blood at baseline and after inhalation of NO gas with increased concentration of NO in microcirculation (Cannon et al. 2001). These and other studies, carried out around the same time, led to the establishment of a key concept that nitrite bioconversion is regulated by oxygen tensions in the tissue and is potentiated as oxygen tension falls, i.e. in hypoxic environment. Depending on oxygenation level haemoglobin exists in two forms: deoxyhaemoglobin and oxyhaemoglobin. Interaction of nitrite in the blood with deoxyhaemoglobin leads to its reduction to NO (Cosby et al. 2003; Crawford et al. 2006; Nagababu et al. 2003). On the other hand, nitrite is oxidized to nitrate when it reacts with oxyhaemoglobin (Isbell, Gladwin & Patel 2007). Deoxyhaemoglobin nitrite reductase activity is regulated allosterically, exhibiting the maximal reductase activity with a deoxyhaemoglobin fraction of 50% (Doyle et al. 1981; Huang et al. 2005). NO generation from the interaction between nitrite and deoxyhaemoglobin was evidenced from increased vasodilatation and vascular cGMP levels, which were reversed by the NO scavenger CPTIO (Crawford et al. 2006). Interestingly, both deoxyhaemoglobin and oxyhaemoglobin are scavengers of NO, hence the final result on how much NO is generated from nitrite reduction will depend on the deoxyhaemoglobin fraction's reducing capacity. Isbell et al.

studied interactions between oxygen, haemoglobin and nitrite bioactivity utilizing rat aortas (Isbell, Gladwin & Patel 2007). They demonstrated significant potentiation of nitrite-induced dilatation of rat aorta at low oxygen tensions as well as significant nitrite reductase activity of deoxyhaemoglobin. In their platelet experiments, Srihirun et al. showed no anti-aggregatory effect of nitrite in platelet-rich plasma (Srihirun et al. 2012), but when erythrocytes were added to platelet-rich plasma, nitrite showed anti-aggregatory effects. These effects were potentiated if erythrocyte mass was deoxygenated before its addition to platelet-rich plasma. Hence, they concluded that nitrite may have role in modulating platelet activity in circulation, especially during hypoxia.

1.5.5.2 Role of myoglobin

Myoglobin is another haem-containing protein capable of nitrite reduction to NO. It is abundant in the myocardium and is believed to be the main nitrite reductase in cardiac muscle. As it was demonstrated by Hendgen-Cotta et al., myoglobin knockout mice are not protected by nitrite against ischaemia-reperfusion injury compared to wild-type mice (Hendgen-Cotta et al. 2008). Myoglobin easily binds oxygen and has a low haem redox potential. In fact, myoglobin needs less than 50% oxygenation for nitrite reduction (Rassaf et al. 2007). This leads to fast nitrite reduction to NO when myoglobin is deoxygenated: deoxymyoglobin reduces nitrite 30-times faster when compared to haemoglobin (Huang et al. 2005; Shiva et al. 2007). In “real life” this means that when myoglobin becomes deoxygenated, for example in the subendocardium or in the exercising skeletal muscle, it will immediately reduce nitrite to NO. This has recently been demonstrated in isolated cardiomyocytes and in the working heart preparation (Rassaf et al. 2007; Shiva et al. 2007). NO generated by myoglobin can bind to cytochrome *c* oxidase of the mitochondrial electron transport chain, reducing electron flow and oxygen utilization (Shiva et al. 2007). These data suggests that nitrite reduction by myoglobin in the presence of hypoxia may influence energetics and myocardial oxygen consumption. This was recently supported by a study demonstrating significant decreases in oxygen utilization by exercising healthy subjects when they were given diet rich in nitrates (Larsen et al. 2007).

In addition to being the main nitrite reductase in the cardiac muscle, myoglobin has been recently shown to contribute significantly to nitrite-induced vasodilatation in murine aorta despite its very low concentrations in vascular smooth muscle (Ormerod et al. 2011). The efficacy of myoglobin as a nitrite reductase (relative to haemoglobin) can be deduced from the fact that it represents the predominant means of nitrite clearance despite low haem protein : nitrite concentrations ratios in blood vessel wall relative to red cells (Bryan et al. 2004; Gladwin & Kim-Shapiro 2008; Rodriguez et al. 2003). Myoglobin is also a more potent nitrite reductase in hypoxic and in acidic environments. A byproduct of nitrite reduction by myoglobin is metmyoglobin, which being a weak NO scavenger, allows NO to be released (Gladwin & Kim-Shapiro 2008). In erythrocytes, more methaemoglobin is produced during nitrite reduction and it is a more potent NO scavenger than metmyoglobin. This may result in smaller amounts of NO being reduced from nitrite by haemoglobin.

1.5.5.3 Role of xanthine oxidase and xanthine oxidoreductase (XOR)

Xanthine oxidoreductase is a known source of superoxide and hydrogen peroxide, generating it via reduction of oxygen, and is one of the key enzymes capable of superoxide-mediated cellular injury. This can have deleterious consequences in human physiology. At the same time at low oxygen tension and/or low pH xanthine oxidoreductase is capable of reducing nitrite to NO (Godber et al. 2000; Millar et al. 1998; Zhang et al. 1997). Normoxic superoxide generation from xanthine oxidoreductase is pH-dependent (Godber et al. 2000), and is increased at alkaline pH 8-9. On the other hand, anaerobic XOR-mediated NO generation increases 10-fold when pH becomes more acidic (8.0 to 6.0). With a lower pH, a more rapid NO generation rate was observed under aerobic than under anaerobic conditions. This would be expected, because under aerobic conditions, the acidification would significantly increase XO-mediated nitrite reduction and simultaneously slow down the competitive reaction of oxygen reduction (Godber et al. 2000), thus facilitating NO generation under aerobic conditions. This reaction is catalyzed at the molybdenum site of this enzyme. Xanthine oxidoreductase activity is up-regulated

under ischaemia and in inflammatory conditions (Harrison, R 2004). Xanthine oxidoreductase has always been considered to have little role as nitrite reductase in blood and vessels. However, recently Ghosh and colleagues demonstrated improved efficacy of inorganic nitrate and nitrite as a dietary supplementation in hypertension as a consequence of increased erythrocytic XOR nitrite reductase activity (Ghosh, SM et al. 2013). Xanthine oxidoreductase is predominantly a nitrite reductase in lung tissue (Casey et al. 2009) and plays significant role in the amelioration of pulmonary hypertension and inflammatory lung conditions (Zuckerbraun et al. 2010).

1.5.5.4 Role of eNOS

The endothelial isoform of NOS releases NO via the classical L-arginine pathway with the consumption of 1.5 NADPH equivalents and two oxygen molecules for each NO molecule generated. This reaction requires oxygen and presence of the cofactors Ca^{2+} -calmodulin and tetrahydrobiopterin (BH_4), and is tightly regulated via a combination of mechanisms (cytosolic Ca^{2+} - calmodulin, (de)phosphorylation, (de)palmitoylation, and intracellular relocation between the Golgi apparatus and membranes of the endothelial cells (Govers & Rabelink 2001)). Deficiency of L-arginine or BH_4 causes “uncoupling” (Katusic 2001) where the oxygenase domain of eNOS releases superoxide radicals instead of NO (Stroes et al. 1998). Oxygen deficiency is known to halt the L-arginine pathway if the oxygen levels fall below a threshold level (Wang et al. 1995). However, eNOS is not wholly inactivated by the absence of oxygen. Instead, in the presence of nitrite, it switches to a novel nitrite reductase activity which releases NO (Gautier et al. 2006; Vanin et al. 2007).

It was recently discovered that eNOS is the only NOS isoform capable of reducing nitrite to NO (Gautier et al. 2006). This gives eNOS a special significance when considering the effects of ischemia on various tissues. Anoxic NO release from reduction of nitrite by eNOS may be a possible explanation for several animal and clinical studies revealing a protective role of eNOS in the early stages of ischemia (Endres et al. 2004; Pozo-Navas et al. 2006). For example, eNOS located on

membranes of red blood cells could theoretically bioactivate nitrite in the presence hypoxia. Reduction of nitrite to NO was not detectable in normoxia (Milsom et al. 2010; Webb, Milsom, et al. 2008).

1.5.5.5 Role of cytochrome P450

Cytochrome P450 is a very large superfamily of haem proteins with almost 8,000 different members currently known. They are found in all human cells. These enzymes are responsible for metabolism of many drugs and have recently been demonstrated to play a role in reduction of nitrite to NO (Kozlov, Dietrich & Nohl 2003). The precise subtype(s) of cytochrome P450 involved in nitrite reduction have not yet been delineated.

1.5.5.6 Role of mitochondria

More than a decade ago, mitochondria were found to generate NO from nitrite (Kozlov, Staniek & Nohl 1999; Reutov & Sorokina 1998). Two different components of the mitochondrial respiratory chain, namely complex III (Kozlov, Staniek & Nohl 1999) and cytochrome c oxidase (complex IV) (Reutov & Sorokina 1998) were found to be responsible. The main question is how nitrite enters cells and gains access to mitochondria since the NO_2^- anion is negatively charged and should not diffuse easily through membranes. It was demonstrated recently that nitrite anions are partially protonated even at physiological pH, and may therefore cross model lipid membranes as neutral molecule HNO_2 (Samouilov et al. 2007). But in the real life the situation is less obvious: - Castello and colleagues have shown that only around 10% of nitrite added to isolated yeast mitochondria enters the mitochondria (Castello et al. 2006). Thus, nitrite concentrations available for mitochondrial nitrite reductases may be lower than nitrite concentrations in the cytoplasm. This explains why nitrite transport to mitochondrial nitrite reductase seems augmented by low pH. Nitric oxide can inhibit mitochondrial respiration by binding to the respiratory chain complexes (Bolanos et al. 1994; Brown & Cooper

1994; Cleeter et al. 1994). It has been proposed that this function of nitric oxide can spare the tissue from oxidative stress during reperfusion injury. On the other hand, a pathologic role of NO reaction with cytochrome c oxidase leading to increased ROS generation has been postulated (Taylor & Moncada 2010).

Once generated by the mitochondrial respiratory chain, NO can act in several different ways: NO and its metabolites like peroxynitrite (Pacher, Beckman & Liaudet 2007) are known to inhibit mitochondrial respiration by binding to the enzymes of the respiratory chain (Brown & Borutaite 2004; Cassina & Radi 1996). Prolonged inhibition of the respiratory chain by NO has been shown to cause apoptosis and cell death in rat thymocytes (Bustamante et al. 2000). As mitochondria reduce nitrite to NO one can expect that nitrite inhibits mitochondrial respiration. It has long been known that oxygenated mitochondrial enzymes are inhibited by very high nitrite concentrations exceeding 0.3 mM (Walters et al. 1975). In hypoxia, on the other hand, mitochondrial respiration is suppressed by much lower concentrations of nitrite (Nohl, Staniek & Kozlov 2005). This observation is consistent with the fact that hypoxia potentiates the release of NO from nitrite in biological systems.

Many other mitochondrial enzymes and proteins have been shown to be able to reduce nitrite to nitric oxide. Among them is aldehyde dehydrogenase which demonstrated vasodepressor effect in rats by reducing nitrite to nitric oxide (Golwala et al. 2009; Li, H et al. 2008). The same enzyme is also involved (variably) in bioactivation of GTN (Chen, Z, Zhang & Stamler 2002).

In summary, there is a multitude of nitrite reductases that can bioactivate nitrite to nitric oxide. In contrast to classical the L-arginine pathway for NO production, the above-mentioned pathways do not require oxygen and are activated in hypoxic environments. They can be regarded as a “backup” for NO generation especially in the situations when NOS systems could be dysfunctional.

1.5.6 Dietary nitrate

1.5.6.1 Effects of nitrate-containing vegetables on blood pressure and cardiovascular diseases

Traditional Mediterranean and Japanese diets are known as healthy and potentially contributing to extended longevity of those populations and decreased prevalence of cardiovascular diseases (Keys 1995; Mente et al. 2009; Sobko et al. 2010; Yamori, Miura & Taira 2001). Even though these regions are far away from each other, they are similar in their dietary content. Both are high in fruit and vegetables, sea fish, and low in red meat and saturated fats. These two regionally distant diets also both include a high dietary nitrate content, particularly of green leafy vegetables such as spinach, lettuce and rocket in the Mediterranean diet and ta cai, garland chrysanthemum, laver, spinach in the Japanese traditional diet (Tsuji et al. 1993). It is well known that vegetarian diets are associated with lower blood pressure (Armstrong, B et al. 1979; Armstrong, B, van Merwyk & Coates 1977; Donaldson 1926; Sacks, Rosner & Kass 1974). Furthermore, intake of green leafy vegetables may reduce the prevalence of heart attack and stroke (Joshiyura et al. 1999; Joshiyura et al. 2001). However, the conclusions from these studies need to be interpreted with caution, as these studies were observational only (Kapil, Webb & Ahluwalia 2010). More definitive evidence of an effect (even though short term) on the blood pressure reduction needs to come from prospective controlled intervention trials, such as the DASH study (Appel et al. 1997).

1.5.6.2 Dietary sources of nitrate and nitrite

Around 80 % of dietary nitrate comes from vegetables (Gangolli et al. 1994; White, JW, Jr. 1975), with the rest from drinking tap water, though the concentration of this may vary (Knight et al. 1987). Dietary nitrite is mainly derived from cured meats, where it is added to prevent the growth and accumulation of botulinum toxin

(Binkerd & Kolari 1975). Inorganic nitrate has also been used for meat curing for centuries (Lundberg, Weitzberg & Gladwin 2008), and has several similarities, but also marked differences from the organic nitrates, such as GTN which also has an important history as an explosive and medicinal product.

Vegetables can be categorized according to their nitrate content (see Table 1.4): high nitrate content vegetables ($>1000 \text{ mg kg}^{-1}$) belong to the following families: Brassicaceae (rocket, the highest nitrate accumulating vegetable), Chenopodiaceae (beetroot, spinach), Asteraceae (lettuce) and Apiaceae (celery) (Santamaria 2006; Tamme et al. 2006). Nitrate content also varies across the plant: the highest concentration is in the leaf and the lowest is in the root (Meah, Harrison & Davies 1994).

Table 1.4 Nitrate content in vegetables. Reproduced with permission from (Lidder & Webb 2013).

Vegetables	Nitrate content Mean [range] (mg kg^{-1})	Nitrate content mean [range] [$\text{mmol per UK portion (80 g)}$]
High	1890 [1213–2650]	2.44 [1.57–3.42]
Rocket	2597 [2597]	3.35 [3.35–3.35]
Spinach	2137 [965–4259]	2.76 [1.24–5.50]
Lettuce	1893 [970–2782]	2.44 [1.26–3.60]
Radish	1868 [1060–2600]	2.41 [1.37–3.35]
Beetroot	1459 [644–1800]	1.88 [0.84–2.32]
Chinese cabbage	1388 [1040–1859]	1.79 [1.34–2.40]
Medium	316 [168–518]	0.41 [0.22–0.67]
Turnip	624 [307–908]	0.80 [0.40–1.18]
Cabbage	513 [333–725]	0.66 [0.44–0.94]
Green beans	496 [449–585]	0.64 [0.58–0.76]
Leek	398 [56–841]	0.51 [0.06–1.08]
Spring onion	353 [145–477]	0.46 [0.19–0.61]
Cucumber	240 [151–384]	0.31 [0.19–0.50]
Carrot	222 [121–316]	0.29 [0.16–0.40]
Potato	220 [81–713]	0.28 [0.10–0.92]
Garlic	183 [34–455]	0.24 [0.05–0.58]
Sweet pepper	117 [93–140]	0.15 [0.11–0.18]
Green pepper	111 [76–159]	0.14 [0.10–0.21]
Low	78 [25–203]	0.10 [0.03–0.27]
Onion	87 [23–235]	0.11 [0.03–0.31]
Tomato	69 [27–170]	0.09 [0.03–0.23]
Water	(mg l^{-1})	(250 ml glass)
Tap	26 [22.8–30.3]	0.10 [0.09–0.12]
Mineral	2.6 [<0.1 –6.3]	0.01 [<0.0004 –0.025]

In the same sort of vegetables nitrate content could be influenced by environmental, agricultural and genetic factors (Knight et al. 1987). Important environmental factors are humidity, temperature, watering and sun exposure. For example, lettuce grown in winter contains more nitrate than that grown in summer (Ysart, Clifford & Harrison 1999). Utilization of nitrogen fertilizers significantly influences the nitrate content of vegetables (Maynard et al. 1976) and therefore also potential risk of nitrate toxicity (see below).

1.5.6.3 Acceptable daily intake (ADI) of nitrate

The Acceptable Daily Intake (ADI) set by the European Food Safety Authority for nitrate is 3.7 mg kg^{-1} ($0.06 \text{ mmol kg}^{-1}$). This equates to $\sim 260 \text{ mg day}^{-1}$ for a 70 kg adult ($\sim 4.2 \text{ mmol}$) (Katan 2009). The average diet of a vegetarian contains $\sim 4.3 \text{ mmol nitrate day}^{-1}$, close to the ADI, and 3-4 times greater than a 'normal' diet, containing $\sim 1.2 \text{ mmol nitrate}$.

The norms for drinking water originate from 1940s when an association was recognized between methaemoglobinaemia in infants ('blue baby syndrome') and high nitrate concentrations in well water (Comly 1987). This resulted in the creation of the limits for drinking water of 45 and 50 mg l^{-1} in the USA and Europe respectively. Importantly, methaemoglobinaemia is unlikely unless the wells are contaminated with bacteria, which are required to generate high concentrations of nitrite causing methaemoglobinaemia, via reduction of nitrate (Avery 1999).

Well-known large outbreaks of watermelon 'toxicity' cases in children in former USSR in early 1990's related to use of large quantities of fertilizers. Agricultural factors include nitrogen fertilization, degree of atmospheric nitrogen fixation by bacteria in non-legumes in addition to legumes (Bhattacharjee, Singh & Mukhopadhyay 2008) and the nitrate reductase activity in the root, deficient in the lettuce, but active in peas which generally have low concentrations of nitrate (Wallace 1986). This is why farmers grow peas or beans every 3-4 years on the same soil to enrich it with nitrogen so other plants or cereals in the following years

could utilize it.

1.5.6.4 Dietary nitrate/ nitrite and cancer risk

Nitrate and nitrite have been used for curing meat for centuries, and remain the most effective method to reduce bacterial growth and kill botulinum spores. Major concern emerged in the 1960s ('Nitrites, nitrosamines, and cancer' 1968), with the demonstration of carcinogenic dimethylnitrosamine formation (known to disrupt nucleic acids in the rat and cause liver tumours (Magee & Barnes 1956)) from sodium nitrite (Sander 1967). However, chronic feeding of nitrite to rats, even when diethylamine was given at the same time, did not induce tumours (Druckrey et al. 1963). Concern for dietary nitrate arose in 1976, when Spiegelhalder *et al.* (Spiegelhalder, Eisenbrand & Preussmann 1976) and Tannenbaum *et al.* (Tannenbaum, Weisman & Fett 1976), both independently suggested that conversion to nitrite in the enterosalivary circulation could result in the formation of N-nitrosoamines. Whilst N-nitroso compounds have repeatedly been shown to be carcinogenic in animals (Bartsch, Ohshima & Pignatelli 1988; Magee & Barnes 1956), reviews such as the 2003 Joint FAO/WHO Expert Committee on Food Additives (JEFCA) of the epidemiological and toxicological studies in humans have not established a definite link between nitrate intake and risk of developing cancer ('Evaluation of certain food additives. Fifty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives' 2002). However, as with most substances, some groups of individuals are likely to be more susceptible than others. For example, work from McColl's group has demonstrated that patients with Barrett's oesophagus exhibit increased tissue nitrosation at the gastro-oesophageal junction, the major site of adenocarcinoma of the human upper gastrointestinal tract (Winter et al. 2007).

The World Cancer Research Fund/American Institute of Cancer Research, Second Report 2007, did not show any increased risk of cancers, such as stomach or ovarian, with green leafy vegetables, and even showed trends towards beneficial effects. Indeed, the recommendation from the report was to eat at least five portions/servings (at least 400 g) of a variety of non-starchy vegetables and fruits

every day, specifically including green, leafy vegetables. Some epidemiological studies have shown that fruit and vegetables have protective effects against certain cancers (Steinmetz & Potter 1996).

1.5.6.5 Potential anti-cancer effects of beetroot

Quite independently of the nitrate/nitrite field, several *in vitro* and *in vivo* studies have demonstrated that red beet/ beetroot extract has protective effects in various cancer cell lines, such as prostate and breast, liver, lung, oesophagus and skin (Kapadia et al. 2011; Kapadia et al. 2003; Kapadia et al. 1996; Lechner et al. 2010). These effects of beetroot (juice) have generally been ascribed to betanin, the major beta-cyanin constituent, which has strong antioxidant activity, and is particularly high in betalain extracts obtained from hairy root cultures of the red beetroot *B. vulgaris* (Georgiev et al. 2010). Beetroot may represent a particularly safe source of dietary nitrate, with the potential to reduce, rather than increase cancer risk. Indeed, beetroot juice has even achieved a considerable degree of acceptance as an alternative medicine for cancer patients (Morant et al. 1991). However, the mechanisms require further clarification.

1.6 Nitroxyl

1.6.1 Introduction

The various redox forms of nitrogen oxides have been discussed in section 1.1.7. Of these, the one electron reduced and protonated form of nitric oxide, nitroxyl (HNO), while initially neglected, has recently started to attract immense interest (Kemp-Harper 2011). It has been documented that nitroxyl might be generated

endogenously (Fukuto, Dutton & Houk 2005) and possesses unique biological properties different from those of nitric oxide (Miranda, Paolucci, et al. 2003; Paolucci et al. 2007; Wink et al. 2003). These different actions include direct targeting of thiols by nitroxyl, positive inotropic effect on myocardium, and elevation of plasma level of calcitonin gene-related peptide (CGRP) which itself induces positive cardiac inotropy and vasodilation (Paolucci et al. 2007).

The potential therapeutic benefits of the NO signaling pathway have been widely evaluated for decades. However, the clinical efficacy of NO donors is limited to some extent by the phenomena of NO tolerance (Henry, Horowitz & Louis 1990; Sage et al. 2000) and NO resistance (Chirkov et al. 1999). With these clinical issues in mind, HNO donors may offer new opportunities in the treatment of cardiovascular disease states. Superficially, the cardiovascular effects of nitroxyl and nitric oxide seem to resemble each other with both nitrogen oxides being potent vasorelaxants (Irvine, Favalaro & Kemp-Harper 2003; Irvine et al. 2007; Paolucci et al. 2007) and platelet anti-aggregants (Bermejo et al. 2005). However, nitroxyl is different to nitric oxide, demonstrating unique reactive properties with many biological substrates. Nitroxyl reacts directly with thiols (Bartberger, Fukuto & Houk 2001) and activates vascular voltage-dependent potassium channels (Irvine, Favalaro & Kemp-Harper 2003). Additionally, nitroxyl elevates plasma CGRP and is a potent cardiac inotrope (Paolucci et al. 2003; Paolucci et al. 2001). Nitroxyl also exerts positive inotropic effects (see section 1.6.9) which might be useful in the context of acute or chronic heart failure. In contrast to the above-mentioned limitations of therapy with NO donors, nitroxyl could preserve its effects under pathophysiological conditions as it is resistant to scavenging by superoxide (Miranda et al. 2002), is not prone to develop vascular tolerance (Irvine et al. 2007) and reacts with ferric (Fe^{3+}) rather than ferrous (Fe^{2+}) haem proteins (Miranda, Nims, et al. 2003).

1.6.2 Biochemical properties of HNO

HNO is a weak acid (Bartberger et al. 2002) and is a highly reactive molecule that rapidly decomposes to nitrous oxide (N₂O) (Fukuto, Switzer, et al. 2005). Angeli's salt (Na₂N₂O₃) discovered over a century ago, is the main "HNO compound" that had been studied as an HNO donor until recently. It has a relatively short half-life of 2.5 minutes and decomposes to nitroxyl and nitrite in pH-dependent manner (Hughes & Cammack 1999). Short half-life and release of nitrite makes this agent less than ideal for study of nitroxyl effects. Another HNO donor isopropylamine NONOate (IPA/NO), has been increasingly used to evaluate HNO actions in the cardiovascular system (Miranda et al. 2005). At physiological pH, IPA/NO predominantly decomposes to HNO but also has a similar short half-life to that of Angeli's salt (Miranda et al. 2005). It is obvious that new donors of HNO with longer half-lives need to be synthesized in order to expedite their potential therapeutic utility.

Physiological properties of HNO are regulated via its high reactivity with proteins containing metals and thiols (Fukuto, Bartberger, et al. 2005; Paolucci et al. 2007). Soluble guanylyl cyclase represents the main haem-containing target for HNO in blood vessels (Miranda, Nims, et al. 2003). Furthermore, interactions of HNO with vascular receptors, enzymes and ion channels containing thiols, distinguish HNO actions from those of NO and underlie its main physiological effects. Presence of thiols (e.g. L-cysteine, N-acetylcysteine) leads to diminution in effects of HNO donors, but not those of NO (Paolucci et al. 2001; Pino & Feelisch 1994). On the other hand, presence of NO scavengers CPTIO or vitamin B12, selectively diminishes effects of NO (Ellis, A, Li & Rand 2000; Wanstall et al. 2001).

1.6.3 Biological/endogenous generation of HNO

It can be contemplated that given the unique chemistry and biological activity of HNO, it might serve as an endogenous signaling molecule, similar to NO, although

at present the endogenous production of HNO in humans remains to be conclusively demonstrated. This stems partially from the lack of direct detection methods for HNO. However, there is some in vitro evidence for the potential endogenous production of HNO. Biochemical studies demonstrate that HNO can be formed directly from NOS itself, especially in the absence of BH₄ (Fukuto, Dutton & Houk 2005), or after oxidation of the NOS intermediates N-hydroxy-L-arginine (NOHA) (Donzelli et al. 2006; Fukuto, Dutton & Houk 2005) and hydroxylamine (Donzelli et al. 2008). Some therapeutically utilized agents, such as the alcohol deterrent cyanamide, are also HNO donors (Fukuto, Gulati & Nagasawa 1994).

HNO can also be generated via non-NOS sources: after reduction of NO by xanthine oxidase, haemoglobin, mitochondrial cytochrome c, ubiquinol and superoxide dismutase (Fukuto, Dutton & Houk 2005). Furthermore, interaction of S-nitrosothiols and other thiols yields HNO (Donzelli et al. 2006; Spencer et al. 2003). Given that HNO is potentially generated from the above sources, it could explain physiological responses such as endothelium-dependent relaxation. There is in vitro evidence that endogenously produced HNO contributes to endothelium-dependent relaxation in both conduit (Ellis, A, Li & Rand 2000; Wanstall et al. 2001) and resistance vessels (Andrews, KL et al. 2009). HNO also acts as a neurotransmitter (Costa et al. 2001; Li, CG, Karagiannis & Rand 1999) and participates in septic vasorelaxation (Crawford et al. 2004).

Up until now, it has been suggested that HNO production increases under oxidative stress and in cardiovascular disease states accompanied by NOS uncoupling (Fukuto, Dutton & Houk 2005). At the same time HNO should have relatively low clearance, secondary to its resistance to scavenging by superoxide (Miranda et al. 2002). With these mechanisms in mind, HNO theoretically might at least partially compensate for impaired NO-signaling.

1.6.4 Vasodilator action of HNO

It has been known for some time that HNO is a potent vasodilator (Irvine et al. 2008). Since the pioneering work by Fukuto and colleagues, demonstrating

vasodilatory effects of Angeli's salt in rabbit aorta and bovine pulmonary artery (Fukuto et al. 1992), more studies demonstrated similar effects of HNO donor Angeli's salt in animal blood vessels of different anatomy and caliber. Vasodilator actions of HNO donors are mediated via several signaling pathways, including sGC/cGMP-dependent, cGMP-independent effects, via potassium channel activation.

1.6.4.1 sGC/cGMP-dependent signaling

It was believed initially that only NO was capable of directly activating sGC (Dierks & Burstyn 1996) and that HNO required first to be oxidized to NO before it could activate sGC. Recent studies have revealed that HNO activates sGC directly (Miller, TW et al. 2009; Zeller et al. 2009). Furthermore, it has been demonstrated that activation of sGC/cGMP is the main signaling pathway for HNO-induced vasodilatation. Vasodilator effects of HNO are markedly suppressed by the sGC inhibitor ODQ (Favaloro & Kemp-Harper 2007; Irvine et al. 2007), while nitric oxide scavengers such as CPTIO and vitamin B12 do not significantly affect responses (Andrews, KL et al. 2009; Favaloro & Kemp-Harper 2009; Wanstall et al. 2001). Similarly, HNO increases vascular cGMP concentrations (Fukuto et al. 1992; Irvine et al. 2007). These findings confirm the importance of sGC/cGMP signaling pathway in the HNO-induced vasorelaxation.

1.6.4.2 cGMP-independent signaling

Studies have demonstrated that the HNO donor Angeli's salt increases vascular cGMP content (Fukuto et al. 1992; Ritchie et al. 2009). However intravenously administered HNO donors including Angeli's salt did not increase plasma cGMP content (Miranda et al. 2005; Paolucci et al. 2003). This may have several explanations (Bullen, M. L., Miller, Andrews, et al. 2011): - (1) plasma cGMP content is not purely representative of changes at the intracellular levels, (2) differential sensitivity in the detection of plasma vs intracellular cGMP, and (3)

HNO targeting sGC/cGMP-independent pathways *in vivo*. The latter statement has been supported by studies demonstrating elevation of sensory neuropeptide, CGRP, when conscious animals were given HNO donors, while this was not observed with NO donors (Miranda et al. 2005; Paolucci et al. 2003; Paolucci et al. 2001). Calcitonin gene-related peptide, by activating CGRP1 receptors on endothelial and vascular smooth muscle cells, stimulates NOS and adenylate cyclase, respectively (Brain & Grant 2004). While, the role of CGRP in HNO-mediated vasorelaxation *in vivo* is still uncertain (Paolucci et al. 2001), CGRP is regarded as an important biomarker for detecting HNO involvement in vasomotor responses.

1.6.4.3 Potassium channel activation

The third pathway that is utilized in HNO-mediated vasodilatation is activation of both voltage-gated (K_V) and ATP-sensitive (K_{ATP}) potassium channels (Favaloro & Kemp-Harper 2007). Several animal studies utilizing the K_V channel inhibitor, 4-aminopyridine (Andrews, KL et al. 2009; Favaloro & Kemp-Harper 2009; Irvine, Favaloro & Kemp-Harper 2003) and the K_{ATP} channel inhibitor, glibenclamide (Favaloro & Kemp-Harper 2007), have demonstrated significant reduction of HNO-mediated vasodilatation in rat mesenteric and coronary arteries, consistent with partial modulation of HNO-mediated vasodilatation by activation of these K^+ channels.

1.6.5 HNO and NO donors: distinct vasodilator properties

Different pharmacological characteristics of HNO, combined with its vasodilator potential, can signify therapeutic advantages compared to NO donors. One of the major limitations to therapy with “traditional” NO donors – organic nitrates (e.g. GTN) is development of nitrate tolerance after their prolonged use, making beneficial utilization of these agents somewhat limited. The phenomenon of nitrate tolerance is multifactorial in origin: it comprises of impaired biotransformation of GTN, dysfunction of sGC, increased activity of cGMP-degrading

phosphodiesterases, and/or reduced bioavailability of NO (Klemenska & Beresewicz 2009; Sage et al. 2000). It has been demonstrated that HNO donors do not induce tolerance when assessed acutely on rat aorta in vitro (Irvine et al. 2007) and chronically over 3 days subcutaneous infusion in rats (Irvine, Kemp-Harper & Widdop 2011). Furthermore, there are now data showing no cross-tolerance to HNO when given in animals with induced GTN tolerance (Irvine et al. 2007; Irvine, Kemp-Harper & Widdop 2011). These important characteristics of HNO donors will translate to significant clinical interest once HNO donors safe for human use become available. The wide applications of such use are expected to be in daily clinical practice in the treatment of acute and chronic cardiovascular disease states including acute coronary syndromes and heart failure.

Another valuable characteristic of HNO donors is their ability to preserve their biological activity in the situations of cardiovascular pathologies associated with increased redox stress (Chirkov & Horowitz 2007; Forstermann 2008) when traditional NO donors lose their efficacy (Chirkov & Horowitz 2007) due to the phenomenon of “NO resistance” (see section 1.3). Reactive oxygen species including superoxide react with NO resulting in generation of peroxynitrite (ONOO^-), which can oxidize sGC haem to its ferric form (Fe^{3+}) (Stasch et al. 2006). Oxidized sGC cannot be activated by NO, thus worsening NO resistance. Compared to NO, HNO is resistant to the scavenging effects of superoxide (Miranda et al. 2002) and furthermore, HNO is capable of activating oxidized sGC. Additionally, bioavailability of HNO may be increased in cardiovascular disease states normally associated with depletion of thiols (Irvine et al. 2008), given that HNO is normally cleared by combination with tissue thiols.

1.6.6 Anti-aggregatory actions of HNO

Similarly to NO, HNO also shares anti-aggregatory properties. Bermejo and colleagues evaluated anti-aggregatory effects of Angeli's salt in human platelets

(Bermejo et al. 2005) and demonstrated significant inhibition of platelet aggregation and ATP release induced by different agonists. Angeli's salt also significantly increased intraplatelet sGMP content in an ODQ-sensitive manner. A scavenger of HNO, L-cysteine, decreased anti-aggregatory effects of Angeli's salt but increased those of the NO donor SNP. In summary, this study demonstrated nitroxyl to be a potent human platelet anti-aggregant.

HNO donors potentially may be useful in the treatment of atherothrombotic syndromes and promise to offer benefits over traditional NO donors. Patients with cardiovascular diseases states, for example ischemic heart disease, heart failure and diabetes mellitus commonly exhibit platelet NO resistance (Chirkov & Horowitz 2007; Willoughby, S.R. et al. 2005). The phenomenon of NO resistance is independent of prior exposure to nitrates and occurs as a result of decreased NO efficacy due to scavenging by superoxide and sGC dysfunction (Chirkov et al. 1999). As HNO is resistant to scavenging effect of superoxide, it is expected that HNO will sustain its anti-aggregatory effects during oxidative stress.

Mondoro and colleagues studied the anti-aggregatory effects of Angeli's salt in patients with sickle cell disease, a condition accompanied by oxidative stress and NO resistance (George et al. 2010; Wood, Hsu & Gladwin 2008), and showed that these effects are preserved (Mondoro et al. 2001).

Bullen and colleagues studied anti-aggregatory effects of SNP and isopropylamineNONOate on washed platelets from 12-week old male wild type and ApoE^{-/-} mice, all maintained on a high fat diet for 7 weeks (Bullen, M.L. et al. 2012). ApoE^{-/-} mice had a 4-fold increase in cholesterol and 2-fold increase in superoxide production. In hypercholesterolaemic mice, the anti-aggregatory effects of SNP were significantly decreased when compared with wild type mice. By contrast, platelet responsiveness to IPA/NO was not significantly reduced.

Whilst the mechanisms underlying platelet NO resistance in hypercholesterolaemia need to be properly evaluated, the anti-aggregatory actions of IPA/NO appeared to be preserved. Therefore, as NO-independent sGC stimulators, HNO donors need to be evaluated in the context of NO resistance in humans, in order to be further considered as alternative therapy to NO donors in cardiovascular disorders

associated with NO resistance.

1.6.7 HNO donors and oxidative stress

Increased ROS production, including that of superoxide, hydrogen peroxide, hydroxyl and peroxynitrite, leads to oxidative stress with associated endothelial dysfunction, inflammation, vascular remodeling and atherogenesis, resulting in the development of many cardiovascular pathologies (Forstermann 2008; Miller, AA, Budzyn & Sobey 2010; Thomas, SR, Witting & Drummond 2008). Even though there are several sources of reactive oxygen species in the body, there is evidence that HNO inhibits oxidative stress through several pathways. Importantly, HNO may donate one of its hydrogen atoms and hence act as a one-electron reductant. HNO has been demonstrated to suppress membrane lipid peroxidation in a yeast model system (Lopez et al. 2007) and potentiate the activity of the antioxidant protein haem oxygenase-1 (Mondoro et al. 2001).

A recent study by Miller and colleagues demonstrated that both Angeli's salt and IPA/NO rapidly decrease superoxide production by suppressing vascular Nox2 oxidase activity via a sGC/cGMP-independent pathway, in mouse cerebral arteries (Miller, AA et al. 2013). In this study, the ability of IPA/NO to attenuate superoxide content was reversible and abolished by the nitroxyl scavenger L-cysteine, but was unaffected by the NO scavenger vitamin B12, the sGC inhibitor ODQ, or by cGMP-dependent protein kinase inhibition. Furthermore, angiotensin II-stimulated superoxide release was significantly diminished in arteries from Nox2-deficient (Nox2(-/y)) compared to wild type mice. In contrast to wild type, IPA/NO had no effect on superoxide content in arteries from Nox2(-/y) mice. Finally, angiotensin II-induced constriction of wild type cerebral artery was almost disappeared by IPA/NO, whereas vasoreactivity to other constrictors thromboxane A2 mimetic U46619 or high potassium was unchanged. While suppressing superoxide generation, HNO donors help to sustain the bioavailability of endogenous NO and limit oxidation of sGC, therefore preserving vascular NO- and HNO-mediated signaling. Given that NADPH oxidase has been identified as a

major contributor to oxidative stress in the vasculature (Miller, AA et al. 2013; Selemidis et al. 2008), ability of HNO to regulate its activity is of significant potential therapeutic benefit and therefore the mechanisms underlying such actions need to be further elucidated.

1.6.8 HNO donors modulate vascular cells growth and proliferation

A large body of data is available regarding the beneficial vasoprotective effects of NO, including regulation of vascular smooth muscle cell proliferation and migration (Rudijanto 2007). Furthermore, NO has been shown to accelerate re-endothelization of blood vessels after vascular damage (Ahanchi, Tsihlis & Kibbe 2007). Only in the last few years have data started to emerge regarding influence of HNO on proliferation and migration of vascular smooth muscle and endothelial cells (Tsihlis et al. 2010).

The current evidence suggests that HNO has potential anti-angiogenic properties: the HNO donor IPA/NO demonstrated inhibition of vascular smooth muscle cell proliferation in culture; reduction of neointimal hyperplasia, macrophage infiltration and vascular smooth muscle proliferation two weeks post rat carotid artery balloon injury (Tsihlis et al. 2010). Another recent study revealed that Angeli's salt decreased vascular endothelial growth factor plasma levels and density of mouse tumour blood vessels (Norris et al. 2008). This may potentially mean that HNO donors could have clinical importance in suppressing tumour-related angiogenesis.

1.6.9 HNO and myocardial function

Excitingly, recent studies documented positive inotropic and lusitropic effects of HNO donors on the myocardium (Gao et al. 2012; Miranda et al. 2005; Paolocci et al. 2003; Paolocci et al. 2001; Tocchetti et al. 2011). Conversely, neither traditional NO donors or classical nitrovasodilators utilized in the clinical practice (e.g. GTN)

have marked influence on cardiac contractile function (Paolucci et al. 2003; Tocchetti et al. 2007). Interestingly, HNO effects of positive inotropy are preserved (and in the case of lusitropy even increased) when used concomitantly with beta-blocker therapy (Paolucci et al. 2003). This latter finding combined with positive inotropy and lusitropy makes utilization HNO donors very promising in the management of acute heart failure. As mentioned previously, the only limiting factor before this goes into large scale clinical research is absence of synthesized pure and safe HNO donors for use in humans.

1.6.10 Therapeutic potential of novel HNO donors including CXL-1020

As outlined above, HNO donors are capable of inducing vasodilatation, suppressing platelet aggregation, and inhibiting superoxide production. When these effects are combined with ability of HNO to resist scavenging by superoxide and HNO's lack of tolerance development indicate that HNO donors potentially may be utilized in the treatment of cardiovascular disease states (Bullen, M. L., Miller, Andrews, et al. 2011) where traditional nitrosoagents including GTN maybe subject to nitrate tolerance, nitrate/NO resistance.

Therapies with HNO donors are promising and are discussed in the recent position paper from the Working Group on Myocardial Function of the European Society of Cardiology (Tarone et al. 2014).

Recently Sabbah and colleagues studied a new stable and pure HNO donor (CXL-1020) in isolated murine myocytes and intact hearts in experimental canine heart failure models and in patients with heart failure (Sabbah et al. 2013). CXL-1020 decomposes solely to HNO. In adult mouse ventricular myocytes: - it increased sarcomere shortening by up to 210% in a dose-dependent fashion. Suppression of protein kinase A or sGC activity did not change this contractile response. CXL-1020 was equally effective in myocytes from normal or failing hearts. In anaesthetized dogs with heart failure induced by coronary embolization, CXL-1020

decreased left ventricular end-diastolic pressure and myocardial oxygen consumption while improving ejection fraction from 27% to 40%. In conscious dogs with tachypacing-induced heart failure, CXL-1020 augmented left ventricular contractility and caused venoarterial dilatation. Heart rate was minimally altered. In patients with systolic heart failure, CXL-1020 reduced both left and right heart filling pressures and systemic vascular resistance, while increasing cardiac and stroke volume index. Heart rate was unchanged, and arterial pressure was lowered by around 10%. These data demonstrate the clinical efficacy of a novel pure HNO donor to augment myocardial function and represent first-in-human evidence for its potential application in heart failure patients (Sabbah et al. 2013).

1.6.11 Scope of present studies

The critical issues raised in this Introduction which interface with the material in the current thesis are:

- (a) The influence of hypoxia on responsiveness of blood vessels and platelets to nitrite, and
- (b) The possibility that platelet reactivity to HNO might be independent of soluble guanylate cyclase dysfunction (with the associated phenomenon of nitric oxide resistance).

To date, there has been substantial work on hypoxic potentiation of vascular NO responsiveness, but only limited evaluation of platelet reactivity changes and uncertainty as to the mediation of these changes.

Similarly, the possibility that HNO might circumvent platelet NO resistance has not previously been examined.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Subject selection

2.1.1.1 Vascular *in vitro* experiments

In order to study vascular reactivity *in vitro*, we recruited patients undergoing coronary artery bypass grafting surgery (n=49). During the surgery, discarded segments of saphenous veins or left internal mammary artery were collected and immediately stored on ice-cold Krebs for transportation to the laboratory. The mean transportation time to the laboratory was 30 minutes. The protocol was approved by the Ethics of Research Committee of The Queen Elizabeth Hospital and the Ethics of Research Committee of Ashford hospital (Bellberry Limited). Informed consent was obtained prior to study entry.

2.1.1.2 Platelet aggregation studies

All subjects (n=159) in the platelet aggregation experiments of this thesis were recruited at the Queen Elizabeth Hospital, being stable patients with known ischaemic heart disease (n=98), including individuals undergoing non-emergent cardiac catheterization. A proportion of experiments was done on apparently healthy volunteers (n=61). In order to create a group of individuals with widely variable platelet responsiveness to NO, we studied both healthy subjects and patients with ischaemic heart disease, given that the latter have a high prevalence of NO resistance (Chirkov et al. 1999; Chirkov et al. 2001; Willoughby, S. R. et al. 2012). The only exclusion criterion was current therapy with ADP receptor antagonists, given that this would preclude assessment of reversal of ADP induced aggregation by NO donors and NO₂⁻. The protocols were approved by the Ethics of Research Committee of The Queen Elizabeth Hospital and informed consent was obtained prior to study entry.

2.1.2 Blood sampling

This was performed as described previously (Chirkov et al. 1999). Blood samples were collected from an antecubital or femoral vein or a femoral artery into plastic tubes containing 1:10 volume of acid citrate anticoagulant (two parts of 0.1 M citric acid to three parts of 0.1 M trisodium citrate); acidified citrate was utilized to minimize deterioration of platelet function during experiments.

2.1.3 Preparation of platelet-rich plasma

Venous whole blood was centrifuged twice at 250 g for 10 min at room temperature to obtain platelet-rich plasma (PRP). To assure complete removal of red blood cells (RBC), PRP was spun to 2500 g for 10 seconds and the supernatant was collected. Absence of Hb was proved with Radiometer Copenhagen NPT 7 Series Blood Gas Analyser.

2.1.4 Preparation of washed red blood cells

Washed RBC were utilized in vascular *in vitro* experiments to assess their impact on the nitrite concentration response curve (see section 3.5.5). In these experiments, whole blood from healthy volunteers was centrifuged twice at 250 g for 10 min at room temperature to obtain RBC and PRP. RBC were immediately washed 3 times in normal saline and centrifuged at 2500 g for 5 min.

2.1.5 Chemicals

Adenosine 5'-diphosphate (ADP) sodium salt, sodium nitroprusside (SNP), sodium nitrite (NaNO₂), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (CPTIO), 1H-(1,2,4-) oxadiazolo(4,3-a)quinoxalin-1-one (ODQ), L-cysteine, 3-isobutyl-1-methylxanthine (IBMX), human Hb, equine Mb were obtained from Sigma (St. Louis, Missouri). The HNO donor IPA/NO was kindly provided by Dr

Katrina Miranda, University of Arizona, USA. cGMP kit was purchased from Biomedical Technologies Inc. (Stoughton, MA).

ADP, SNP, NaNO₂, CPTIO, L-cysteine, human haemoglobin (Hb), equine myoglobin (Mb) were freshly reconstituted in physiological saline (0.9% NaCl) prior to each experiment. ODQ was prepared using DMSO. To prevent decomposition before use, IPA/NO stock solution was always made fresh in 0.01M NaOH just 10 minutes before each experiment, and kept on ice and away from light. Further dilutions were made with physiological saline just before it was added to the blood sample. Vehicle controls with 0.9% NaCl, diluted NaOH and DMSO, and controls for decomposed IPA/NO (24 hours in physiological saline at 37°C) were performed, with no effect on the parameters measured.

The Krebs solution (Krebs 1932) was of the following composition (in mmol/L): NaCl 118, KCl 3.89, KH₂PO₄ 1.18, NaHCO₃ 25, MgCl₂ 1.05, CaCl₂ 2.34, EDTA 0.01, and glucose 5.56 (pH 7.4). The Krebs solution was prepared on the morning of each organ bath experiment and refrigerated prior to use. During its preparation the solution was gassed with carbogen (95% O₂ and 5% CO₂) to provide adequate dissolution of all compounds. During the *in vitro* vascular protocol this carbogen gas mixture was utilized to create a hyperoxic environment around the suspended vascular rings. Alternatively, utilizing a hypoxic gas mixture (95% N₂ and 5% CO₂), hypoxic environments were ensued.

2.2 Methods

2.2.1 Techniques for evaluation of platelet function

In this study platelet aggregation was assessed in venous or arterial whole blood and venous platelet-rich plasma using impedance aggregometry.

2.2.1.1 Whole blood impedance aggregometry

Whole blood impedance aggregometry is designed to measure electrical impedance between two electrodes in whole blood samples (Cardinal & Flower 1980) with an electrical current circulating between them. When electrodes are immersed in the blood sample, they become coated with a thin layer of platelets. After platelet

aggregation is induced by an agonist (in this study by ADP 2.5 μM), the impedance increases due to the build up of platelets on the electrodes. The impedance is directly proportional to extent of platelet aggregation induced by ADP and is recorded over time.

2.2.1.2 Instrumentation

Platelet aggregation was examined utilizing impedance aggregometers (Models 560 and 700, Chrono-Log, Pennsylvania, USA). Aggregation was induced with ADP and was monitored continually for 7 min; responses were recorded for electrical impedance in Ohms. Data was collected automatically via a Chrono-log Aggro/Link computer interface connected to an IBM computer. Inhibition of aggregation was evaluated as a percentage comparing the extent of maximal aggregation in the presence and absence of the anti-aggregatory agent studied (figure 2.1).

Tests were performed at 37 °C and stirring speed of 900 rpm. Samples of whole blood or PRP were diluted twofold with normal saline (final volume 1 ml) and pre-warmed for 5 min at 37 °C. Aggregation was induced with ADP (final concentration of 2.5 μM). Aggregation was monitored continually for 7 min, and responses were recorded as electrical impedance (in Ohms). SNP, NaNO_2 , IPA/NO, NONOates (in appropriate final concentrations) were added prior to ADP (figure 2.1). Preincubation times, depending on the anti-aggregatory agent used ranged between 1 and 2 minutes and are specified below. Inhibition of aggregation was evaluated as a percentage comparing the extent of maximal aggregation in the presence and absence of the anti-aggregatory agent studied.

2.2.1.3 PRP impedance aggregometry

These experiments were performed similarly to whole blood aggregometry. The PRP was prepared from freshly collected venous whole blood sample as described above in the Materials section.

2.2.1.4 Hypoxic chamber aggregometry experiments

We utilized a custom-made hypoxic chamber from Plexiglas (see schematic figure 2.2) so that aggregation, both in whole blood and in PRP, could be evaluated under controlled hypoxia. Hypoxic gas mixture consisting of 95% nitrogen (N₂) and 5% carbon dioxide (CO₂) was supplied to the hypoxic chamber. Oxygen concentration in the hypoxic chamber was monitored with an oxygen analyzer (Teledyne Brown Engineering, Inc., USA) placed in the chamber. The flow-rate of hypoxic gas into the chamber was regulated depending on what oxygen concentration was desired to be in the chamber based on the specific protocol. Blood pH, deoxyHb concentration, pO₂ were recorded as described below.

2.2.2 Techniques for evaluation of vasodilator function

2.2.2.1 *In vitro* vascular human techniques

2.2.2.1.1 Organ bath experiments

These were performed as described previously (He, Angus & Rosenfeldt 1988; Sage et al. 2000) (figure 2.3). Vascular rings were immersed and suspended under tension in 15-mL water-jacketed organ bath chambers containing modified Krebs solution (Krebs 1932) bubbled with a special gas mixture “carbogen” (95% O₂ and 5% CO₂) at 37°C, unless otherwise stated (figure 2.4). Replacement of solution in the organ bath was accomplished by the over-flow principle with fresh solution from the reservoir flask when necessary. Resting tensions in human saphenous vein (SV) and internal mammary artery (IMA) segments were normalized for internal diameter. Mean resting tension was 1 g for SV and 2 g for IMA. These resting tensions were set at 1g and 2g because this gave optimal contractions to KCl solution (120 mmol/L) in preliminary experiments. The segments were equilibrated for 60 minutes before exposure to KCl solution; segments contracting <1 g were discarded. After a further 30 minutes of washout, the segments were contracted with increasing concentrations of phenylephrine (0.01 to 100µmol/L).

After a further 45 minutes of washout, the segments were precontracted with phenylephrine to produce 70% of maximum tension in the SV and in the IMA. Once the contractile response had reached a plateau, each segment was exposed to increasing concentrations of GTN (0.0001 to 10 $\mu\text{mol/L}$) or NaNO_2 (0.01 to 100 $\mu\text{mol/L}$) for SV and NaNO_2 (0.01 to 100 $\mu\text{mol/L}$) for IMA. All concentrations of phenylephrine, GTN and NaNO_2 were freshly prepared and kept in an ice-bath throughout the experiments. Time from harvest of vessels to assessment of relaxant responses was held constant at 3 hours. To study effects of hypoxia on nitrite concentration-response curves in SV and in IMA, we chose to utilize two environments with different O_2 concentrations: -

(1) high content of oxygen (O_2) with our laboratory standard carbogen gas mixture consisting of 95% O_2 and 5% CO_2 . This has been the most commonly used gas in vascular organ bath experiments for more than 50 years since Furchgott's studies of vasoreactivity of rabbit's aorta strips (Furchgott & Bhadrakom 1953). Given high content of oxygen in carbogen gas mixture we termed the resultant environment in the organ bath as 'hyperoxia' rather than 'normoxia'.

(2) the creation of 'hypoxia' is described in section 2.2.2.1.2 below.

Additionally, in some experiments, suspension of RBC (10% of organ bath volume) was added. RBC were obtained after separation from PRP, and were washed 3 times in normal saline and centrifugation at 2500 g for 5 min.

Force displacement of vascular rings throughout the experiments was recorded using a PowerLab (ADInstruments) eight-channel data recorder on an IBM compatible computer (for representative data sample see figure 2.5).

2.2.2.1.2 Organ bath experiments in hypoxia

Hypoxic *in vitro* vascular experiments were performed similarly to those in hyperoxia but the gas perfusing the organ bath consisted of 5% CO₂ balanced with 95% N₂. The presence of CO₂ in this gas mixture was necessary to keep pH in the organ in the physiological range. Oxygen content in the hypoxic organ bath was constant at 1% and regularly tested with an oxygen electrode (MI-730, Microelectrodes, Inc., USA). pH in both hyperoxic and hypoxic organ baths was monitored and was constant at 7.3-7.4.

The gas mixture containing 5% CO₂ balanced with 95% N₂ was found to be ideal for our *in vitro* vessel work. When 100% N₂ was tried in our pilot experiments the Krebs solution in organ bath became cloudy with precipitate within 10 minutes of bubbling with nitrogen and pH indicator showed pH of 9-9.5. When 1% O₂ with 5% CO₂ balanced with 95% N₂ gas mixture was bubbled through Krebs solution organ bath – pH remained constant at 7.5 but hypoxia could not be achieved. Hence, the gas mixture containing 5% CO₂ balanced with 95% N₂ was used for all hypoxic organ bath experiments.

2.2.3 Other assays

2.2.3.1 Intra-platelet cyclic guanosine monophosphate (cGMP) assay

Intraplatelet cGMP content was assayed as described previously by our laboratory (Chirkov et al. 1999). Briefly, platelet-rich plasma (0.5 mL) was incubated at 37°C with SNP (10 µM) and IPA/NO (10 µM) for 1 minute, as in aggregation studies. PDE inhibitor IBMX (0.5 mM) was added to plasma 5 minutes before SNP. After incubation, plasma was filtered through GF/C Glass Microfibre Filters (Whatman) for harvesting the platelets. Filters with absorbed platelets were rinsed with physiological saline and placed into 0.5 mL of 4 mM EDTA for further extraction of cGMP in a boiling water bath for 5 minutes. After centrifugation of samples at 3000g for 10 minutes, cGMP concentration in supernatant was estimated using cGMP [¹²⁵I] assay system (Biomedical Technologies Inc). Results were expressed

as increase (%) in intraplatelet cGMP accumulation in comparison with IBMX alone.

2.2.3.2 Electronparamagnetic resonance (EPR) spectroscopy for ROS measurements

Blood samples were collected by venipuncture into lithium-heparin tubes. Quantitation of total reactive oxygen species (ROS) in whole blood was performed utilizing EPR spectroscopy, as previously described (Mariappan et al. 2009). All EPR samples were prepared using 20 mM Krebs HEPES buffer (pH 7.4) in a final concentration 25 μ M of deferoxamine, 2 μ M of diethyldithiocarbamate (chelating agents), 200 μ M of the spin probe 1- hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) and placed in 50 μ L glass capillaries (Corning, NY). The EPR spectra were recorded using an e-scan M EPR spectrometer (Bruker Bio-Spin e-scan, Rheinstetten/Karlsruhe, Germany) and super-high Q microwave cavity with the following settings: field sweep, 10G; microwave frequency, 9.75 GHz; microwave power, 19 mW; modulation amplitude, 2G; conversion time, 10.24 ms; time constant, 40.96 ms; receiver gain, 3.2×10^2 . ROS content was expressed as EPR signal intensity in arbitrary units. The coefficient of variability for replicate estimates was 4%.

2.2.3.3 DeoxyHb and pO₂ measurements

To check pH, haemoglobin fractions and oxygen saturation of blood utilized in the platelet aggregation studies, the samples were collected into PICO 70 Radiometer blood gas syringes containing 60IU of heparin and processed on the Radiometer Copenhagen NPT 7 Series Blood Gas Analyser after being incubated in the aggregometer as per protocol. The same gas syringes and the blood gas analyser were utilized to measure those parameters in organ baths when vessels were incubated with RBC.

2.3 Tables and figures for chapter 2

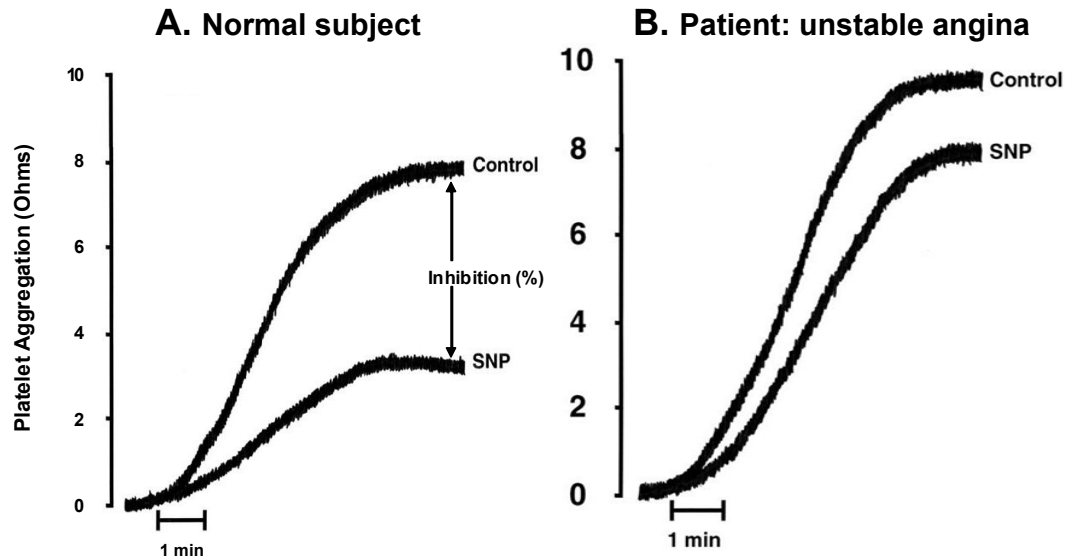


Figure 2.1 Schematic of SNP (10 μM) anti-aggregatory effect on ADP (2.5 μM) induced platelet aggregation (adapted from Chirkov 2007). Note (1) hyperaggregability to ADP in unstable angina, (2) NO resistance in unstable angina, demonstrated by impaired anti-aggregatory response to SNP.

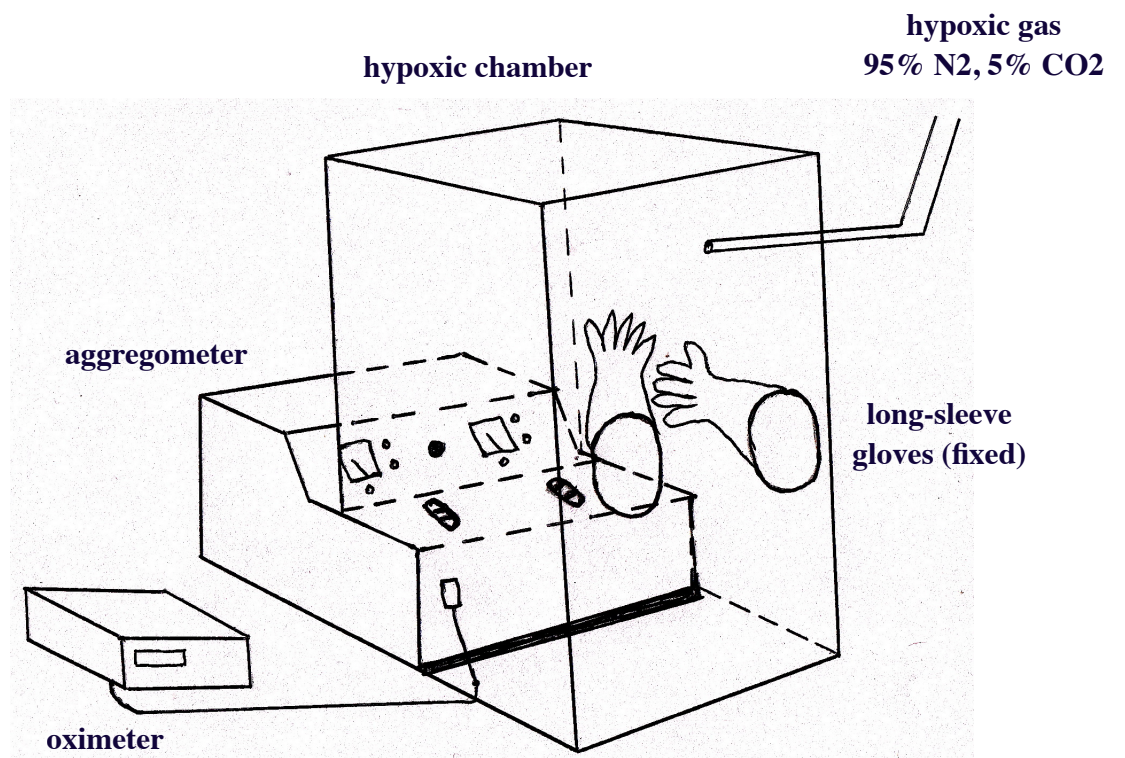


Figure 2.2 Schematic: hypoxic chamber

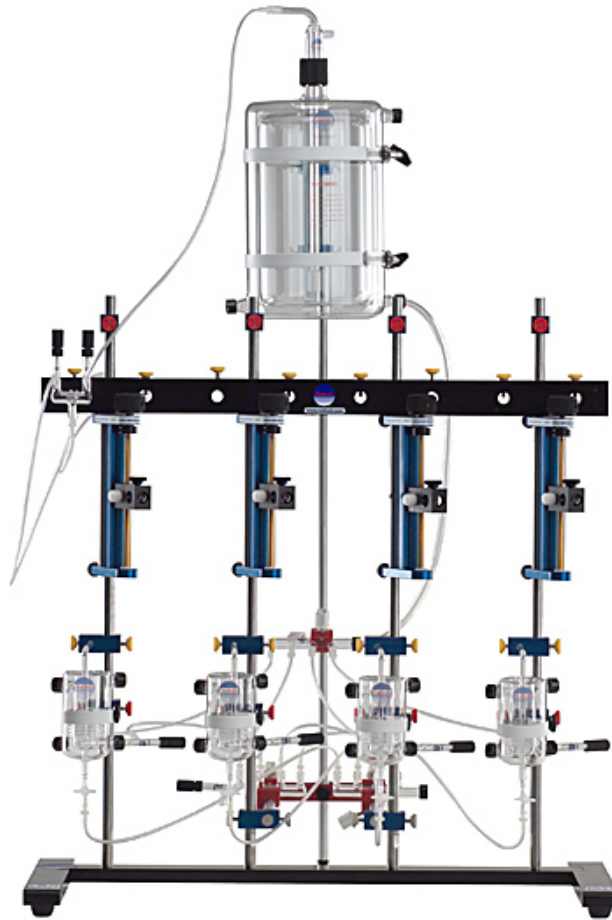


Figure 2.3 Organ bath set up (4 channels system is shown for representation purposes only: our *in vitro* vascular experiments were performed utilizing 8 organ baths).

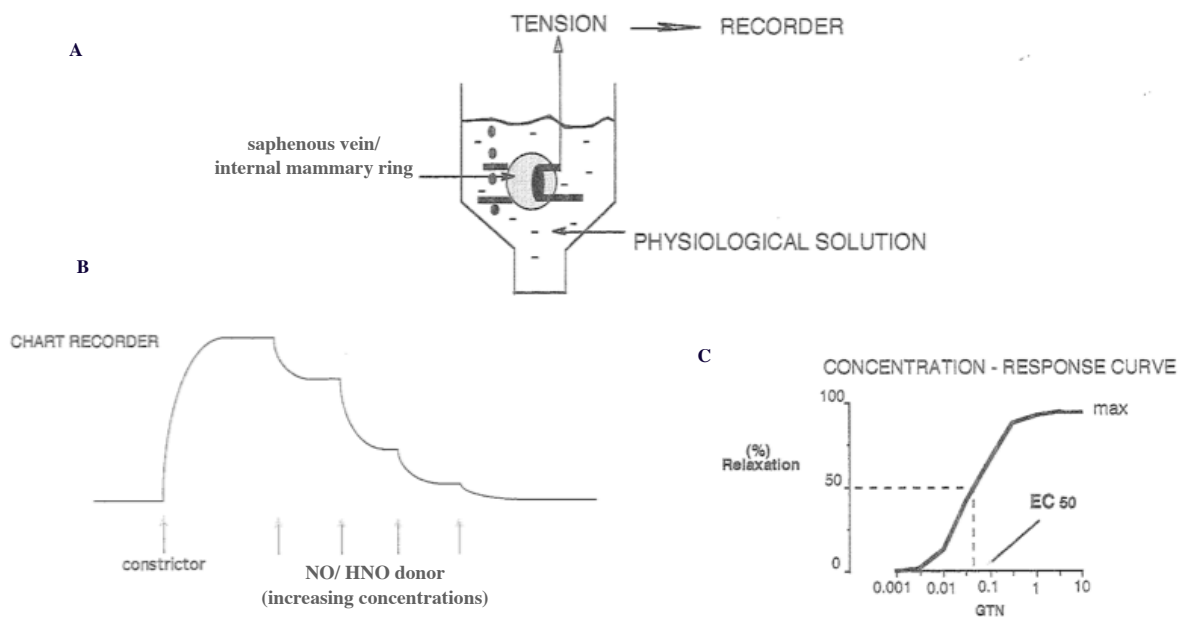


Figure 2.4 Schematic: methods for determination of vascular responsiveness.

A. Organ bath set-up: The vascular segment is suspended and connected to a force-displacement transducer, while immersed in physiological solution \pm vasoactive material.

B. Determination of vasomotor responses: Vessel segments are constricted (in these experiments, with phenylephrine throughout) and cumulative concentration-response curves constructed to the NO or HNO donor under investigation.

C. EC₅₀ values are derived from concentration-response curves via curve-fitting (Prism 6, GraphPad Software, Inc.).

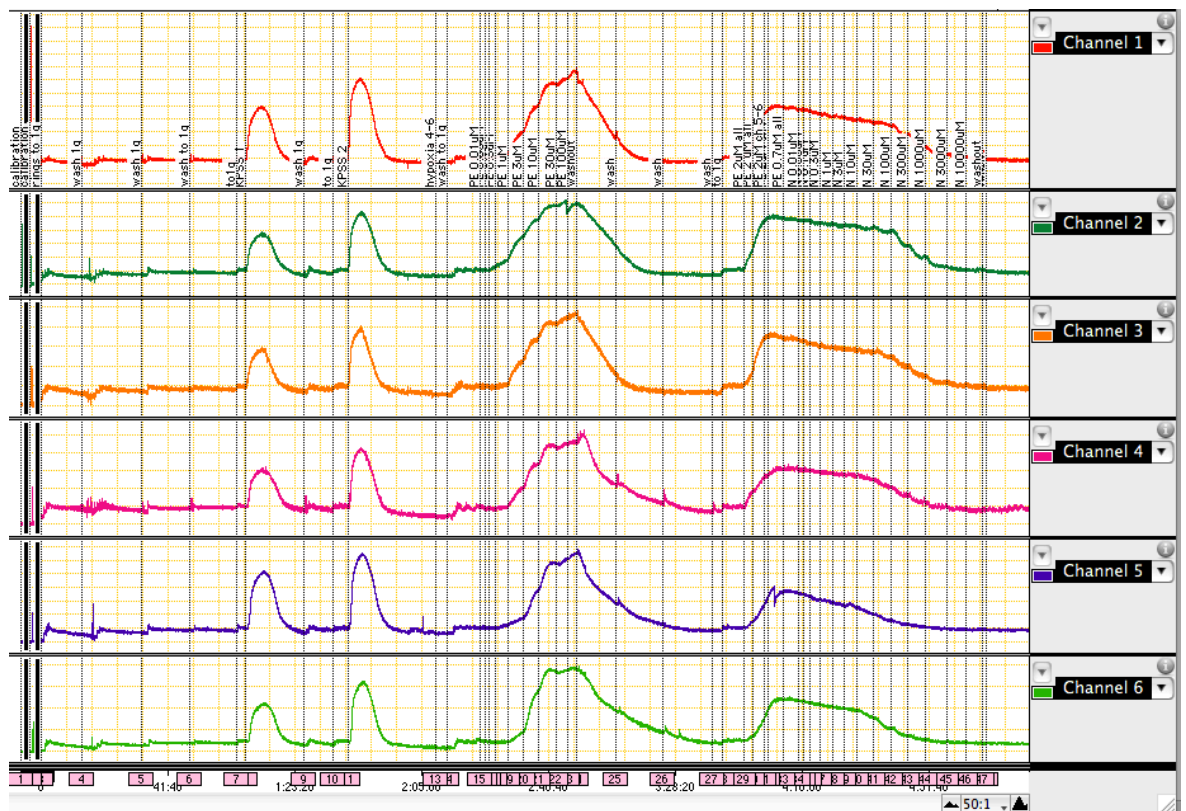


Figure 2.5 Sample tracings from one organ bath experiment with saphenous vein: here only 6 good out of 8 rings were used. Initial 2 spikes are vasoconstriction to KPSS. The third one is phenylephrine concentration-response curve and the fourth is nitrite concentration-response curve after constriction of saphenous vein with phenylephrine to 70% of maximum contraction. The data shown were collected over 5 hours (screenshot from LabChart Reader).

KPSS = Krebs's solution with KCl substituted for NaCl on an equimolar basis.

Chapter 3: Nitrite vasodilator effects
***in vitro*: impact of hypoxia**

3.1 Summary of the study

3.1.1 Objectives

Hypoxic potentiation of NO generation from nitrite (NO_2^-) might facilitate NO_2^- -mediated vasodilatation at sites of myocardial ischaemia and in acute heart failure. The current study was designed to investigate hypoxic potentiation of NO_2^- effects in human vasculature *in vitro*, addressing underlying mechanisms.

3.1.2 Methods

The vasodilator efficacy of NO_2^- , in comparison with glyceryl trinitrate (GTN), was evaluated *in vitro*, using segments of human saphenous vein. First, the potential development of NO_2^- tolerance and of cross-tolerance to GTN was evaluated in isolated saphenous veins. Next, the impact of hypoxia on vasodilator responses to NO_2^- and GTN was compared. Hypoxic effect on NO_2^- vasodilator properties in saphenous veins was also assessed in the presence of red blood cells (RBC), haemoglobin (Hb) and myoglobin (Mb). The effects of preincubation of saphenous vein with the xanthine oxidase inhibitor allopurinol, the soluble guanylate cyclase inhibitor ODQ, and the myoglobin blocker ferricyanide were also examined. Pilot experiments were performed to evaluate changes in vasodilator effects of NO_2^- in segments of human internal mammary artery (IMA) under hypoxia. Vasodilator response to NO_2^- in human internal mammary artery was compared to that in saphenous veins.

3.1.3 Results

Prolonged incubation with NO_2^- did not result in attenuation of either NO_2^- or GTN effects. Conversely, prolonged incubation with GTN induced nitrate tolerance without attenuation of NO_2^- responses.

Under hypoxic conditions, there was a leftward shift of the NO_2^- concentration-response curve (EC_{50} : 22 μM in hyperoxia vs. 3.5 μM in hypoxia; $p < 0.01$), but no significant potentiation of GTN effect. In the presence of red blood cells, hypoxic potentiation of NO_2^- vasodilator effect was accentuated. SV vasodilator response to

NO_2^- increased ($p < 0.05$) in hypoxia when preincubated with Hb or Mb. Ferricyanide inhibited nitrite vasodilator effects. However, experiments with IMA revealed no significant impact of hypoxia on reactivity to NO_2^- .

3.1.4 Conclusions

In human saphenous vein, NO_2^- does not cause tolerance or cross-tolerance with GTN. For the first time we show significant potentiation of vasodilator properties of NO_2^- by hypoxia in human saphenous veins *in vitro*. The results of this work provide further mechanistic insights on hypoxic potentiation of vasodilator actions of NO_2^- . The balance of evidence suggests differential rates of NO release from NO_2^- , largely modulated by deoxyMb.

3.2 Introduction

NO_2^- exhibits two potential advantages as a treatment for acute cardiovascular disorders. First, vasodilator responses of NO_2^- are potentiated in the presence of hypoxia (Cosby et al. 2003; van Faassen et al. 2009). In theory, this might facilitate selective vasomotor effect at sites of myocardial ischaemia, and might also potentiate NO_2^- -mediated dilatation in the presence of acute heart failure (Maher et al. 2013). Furthermore, NO_2^- vasomotor effects are not subject to tolerance induction and also do not exhibit cross-tolerance with organic nitrates (Dejam et al. 2007; Maher et al. 2013). Potentiation of NO generation from NO_2^- under hypoxia might also substantially augment the anti-aggregatory effects of NO_2^- (Srihirun et al. 2012; Totzeck et al. 2012), and this might be of particular relevance in myocardial ischaemia/heart failure.

We have therefore undertaken a study in human subjects, in order to evaluate the potential efficacy of NO_2^- in hypoxia and to study the phenomenon of ‘hypoxic potentiation’ of NO_2^- in human vasculature as regards its underlying mechanisms. We assessed vasodilator effects of NO_2^- in comparison to those of the NO donor

glyceryl trinitrate (GTN) on human saphenous veins *in vitro* under hypoxia. We further studied effects of hypoxia and NO_2^- on vasoreactivity in segments of human internal mammary artery. In series of additional experiments with preincubation of vessels with different inhibitors and blockers, we investigated the mechanistic bases of NO_2^- vasodilator effect. The results provide incremental insights into the therapeutic potential of NO_2^- as a treatment for acute cardiac illnesses.

3.3 Objectives of the study

The following hypotheses were tested in this study:

Primary

In human saphenous vein, vasodilator effect of NO_2^- is subject to the phenomenon of hypoxic potentiation, which is specific to NO_2^- .

Secondary

Vasodilator effects of NO_2^- are equally potentiated by hypoxia in veins and arteries.

Prolonged administration of NO_2^- does not result in induction of tolerance, and there is no cross-tolerance between NO_2^- and GTN.

3.4 Methods

3.4.1 Study population

Patients of only one cardiac surgeon were screened for this study to minimize bias due to different surgical technique, treatment of discarded blood vessels, intraoperative medications used by anaesthetist assigned to a particular surgeon, etc. Potentially eligible study patients (n=53) were screened the night before coronary artery bypass surgery on cardio-thoracic ward before any pre-medication was given. In the current study, subjects were consented for once only participation with no further follow up. Routine demographic information, medical history,

medications list were obtained at the time of enrollment to the study for each patient.

One patient did not sign the consent form and hence did not participate in the study. In a further 4 patients who originally signed the consent form – there was no discarded vein or artery segments available at the time of surgery due to complexity of the case or poor quality of the grafts. A total of 48 patients (mean age 67 ± 12 (SD) years) undergoing coronary artery bypass grafting surgery (CABG) were recruited to study effects of NO_2^- on vascular reactivity *in vitro*. During the surgery, discarded segments of saphenous vein or internal mammary artery were collected and immediately stored on ice-cold Krebs solution for transportation to the laboratory. The protocols were approved by the Ethics of Research Committee of The Queen Elizabeth Hospital and informed consent was obtained prior to study entry. Of the remaining 48 patients, vessel segments of a further 6 patients were not used as they could not pass minimal ‘vasoreactivity’ threshold at the start of organ bath experiments assessed by response to potassium solution (KPSS). Thus, vessel segments of 42 patients were used in this study. Ideally, each patient’s discarded vessel segment would have enough rings to mount in eight organ baths in our laboratory on the day of the experiment. There was an opportunity to use discarded human vessels from varicose vein stripping surgery but this was not done due to the likelihood of abnormal physiology of such vascular segments.

3.4.2 Vessel organ bath experiments

The discarded segments of proximal saphenous vein (SV) or distal left internal mammary artery (IMA) were cleaned and cut into 2 mm wide rings.

Vascular rings were suspended under tension in 15-mL organ baths containing Krebs solution at 37°C. SV and IMA segment resting tension was normalized for internal diameter, as previously described (He, Angus & Rosenfeldt 1988; Sage et al. 2000). Mean resting tension was 1 g for SV and 2 g for IMA. SV segment resting tension was set at 1 g and IMA resting tension at 2 g because this tensions

gave optimal contractions to KCl solution (120 mmol/L) in preliminary experiments. The segments were equilibrated for 60 minutes before exposure to KCl solution; segments contracting <1 g were discarded. After a further 30 minutes of washout, the segments were contracted with increasing concentrations of phenylephrine (0.01 to 100 μ mol/L).

After a further 45 minutes of washout, the segments were precontracted with phenylephrine to produce 70% of maximum tension in the SV and IMA. Once the contractile response had reached a plateau, each segment was exposed to increasing concentrations of GTN (0.0001 to 10 μ mol/L) or NaNO₂ (0.01 to 100 μ mol/L) for SV and NaNO₂ (0.01 to 100 μ mol/L) for IMA. Time from vessel harvest to the assessment of relaxant responses was held constant at 3 hours.

In some experiments, a suspension of washed red blood cells (RBC) (10% of organ bath volume) was added to SV. Blood samples were obtained by venipuncture from normal subjects for this purpose. After the separation from platelet-rich plasma (PRP), RBC were washed 3 times in normal saline with centrifugation at 2500 g for 5 min before their addition to the organ bath.

3.4.3 Subjecting vessel to hypoxia *in vitro*

To assess the effects of hypoxia on vasodilator properties of NO₂⁻ and to study the phenomenon of hypoxic potentiation of NO₂⁻, ideally it would be interesting to use a range of gas mixtures with different concentrations of O₂ in order to create a concentration-response curve for O₂. Given the limited availability of vascular tissue for the experiments, we elected to utilize only two concentrations of O₂ in the gas mixtures: 95 and 0%.

Standard carbogen gas mixture consisting of 95% O₂ and 5% CO₂ was utilized to gas through Krebs solution in the organ bath to create 'hyperoxia'. Hypoxia in organ baths was induced by bubbling a gas mixture consisting of 5% CO₂ balanced with 95% N₂ (instead of O₂). Oxygen content in the hypoxic organ bath was constant at 1% and regularly confirmed with Oxygen electrode (MI-730, Microelectrodes Inc., NH, USA). pH in both hyperoxia and hypoxia remained

stable at 7.3-7.4 and was confirmed with pH indicator paper strips.

3.4.4 Statistical analysis

All data are means \pm SEM. Data processing and statistical analysis were performed utilizing GraphPad Prism version 6 (GraphPad software Inc., San Diego, CA). Vascular relaxant responses were compared using the EC₅₀ parameters (concentration eliciting half the maximum response) with an unpaired *t* test. The limit of statistical significance was set 0.05 (two-tailed) throughout. Post-hoc comparisons using Bonferroni adjustment was applied for EC₅₀ ratios (hyperoxia vs hypoxia) between control, RBC, Hb and Mb groups in vascular experiments.

3.5 Results

3.5.1 Patient characteristics

Seven patients participated in the NO₂⁻ tolerance and cross-tolerance *in vitro* study. A further 29 patients undergoing CABG surgery participated in the study of vasodilatory effects of nitrite *in vitro*. Six patients were subsequently excluded from the study because their vessels showed poor vasoreactivity on initial test with potassium solution. The remaining 23 patients participated either in saphenous vein studies (n=20) or in internal mammary artery studies (n=3). Tables 3.1 and 3.2 show demographics and pharmacotherapy of both groups of patients. All of the subjects were males. This was purely coincidental. Pharmacotherapy was similar for all subjects.

Each patient's discarded blood vessel provided up to 8 vascular rings to study; individual vascular rings were discarded if they showed poor contractile responses to potassium. In saphenous veins the following studies were performed: -

- NO₂⁻ tolerance and cross-tolerance studies,
- nitrite concentration-response curves in hyperoxia and under hypoxia,

- comparative studies with GTN,
- mechanistic studies, including influence of preincubation of SV with soluble guanylate cyclase inhibitor ODQ, xanthine oxidase inhibitor allopurinol, red blood cells, purified haemoglobin and myoglobin, myoglobin blocker ferricyanide

In arterial vascular rings we evaluated mainly the influence of hypoxia on vasodilator responses to nitrite, compared to those in saphenous veins.

3.5.2 *In vitro* tolerance/ cross-tolerance study

In pre-constricted saphenous vein rings *in vitro*, NO₂⁻ induced concentration-dependent vasorelaxation (figure 3.1). After exposure to very high concentrations of NO₂⁻ (10 mM) for 45 min followed by 30 min washout, there was no significant shift in the NO₂⁻ concentration-response curve (log EC₅₀ for the low-affinity components -3.7 ± 0.1 vs. -4.0 ± 0.1 M (n = 7; p = NS, for before and after attempted tolerance induction respectively). Furthermore, there was no cross-tolerance to GTN (log EC₅₀ -7.9 ± 0.1 vs. -7.9 ± 0.1 M; n = 7, p = NS for before and after NO₂⁻ tolerance induction respectively).

3.5.3 Hypoxic potentiation of nitrite vasodilator effect in comparison to GTN

Under hypoxia, vasoconstrictor responsiveness to phenylephrine (PE) was marginally diminished without any change in EC₅₀ values (figure 3.2).

Hypoxic potentiation of vasodilator effects of NO₂⁻ was assessed *in vitro* (in organ bath) using segments of human saphenous vein obtained from 14 patients. Effects of NO₂⁻ (figure 3.3A) were significantly potentiated under hypoxia: - there was a leftward shift of the NO₂⁻ concentration-response curve under hypoxic conditions (EC₅₀ = 22 μM in hyperoxia, EC₅₀ = 3.5 μM in hypoxia; p<0.01). However, under the same conditions there was no significant potentiation of GTN vasodilator effect in saphenous veins (figure 3.3B).

Of note, hypoxia *per se* caused significant relaxation of saphenous vein segments within 2 minutes of hypoxia induction in organ bath (by $24.2 \pm 2.9\%$) when compared to segments remaining in hyperoxic conditions ($7.5 \pm 1.2\%$; $p=0.003$) (figure 3.4).

3.5.4 Evaluation of mechanisms of nitrite effect

In order to study the mechanisms underlying nitrite vasodilator effects, several series of organ bath experiments were performed. Firstly, the soluble guanylate cyclase (sGC) inhibitor ODQ ($10 \mu\text{M}$) (Garthwaite et al. 1995) was added to organ baths before NO_2^- ($n=3$). This significantly attenuated NO_2^- responses, and shifted the concentration-response curve to the right, precluding extensive assessment of NO_2^- vasodilator response (figure 3.5). These data therefore suggest that NO_2^- vasodilator effects are sGC-mediated. Secondly, some saphenous vein rings ($n=3$) were preincubated with allopurinol (1 mM) an inhibitor of xanthine oxidoreductase (XOR) (O'Byrne et al. 2000) (figure 3.6). XOR has been shown to act as a NO_2^- reductase in some tissues, e.g. in lung tissue (Zuckerbraun et al. 2010). In the current scenario, however, allopurinol did not affect NO_2^- vasodilator effects (figure 3.6). This implies that XOR does not exert a significant NO_2^- reductase capacity in saphenous veins.

3.5.5 Nitrite reductases: RBC, Hb and Mb

We sought to identify the mechanism(s) of the hypoxic potentiation of NO_2^- effect. Considering that NO_2^- exerts its vasodilator and anti-aggregatory actions via reduction to NO by reductases including Hb and Mb, we evaluated the impact of washed red blood cells (as carriers of Hb, 10% of organ bath volume), Hb ($50 \mu\text{M}$), and Mb ($50 \mu\text{M}$), both in hyperoxia and hypoxia (figures 3.7 and 3.8).

In the presence of added RBC, there was a selective impairment of NO_2^- -induced vasodilatation under hyperoxia, resulting in a marked increase in hypoxic potentiation, which we calculated as EC_{50} ratio of the NO_2^- vasodilator effects in

hyperoxia vs hypoxia (EC₅₀ ratio 62). Incubation with Hb (EC₅₀ ratio 41) or Mb (EC₅₀ ratio 13) also caused significant increases in hypoxic potentiation when compared to NO₂⁻ alone (EC₅₀ ratio 6; p<0.01, ANOVA).

In order to assess NO₂⁻ reductase potency of intrinsic vessel Mb, a series of experiments with ferricyanide were performed. This protein when incubated with murine myocardial cell culture has been shown to block NO₂⁻ reductase effects of Mb (Shiva et al. 2007). In the current experiments we observed significant rightward shift in the NO₂⁻ concentration-response curve after saphenous vein rings were preincubated with ferricyanide (1 mM) (figure 3.9). This shift was more pronounced in hypoxia. This strongly supports deoxyMb being the key NO₂⁻ reductase in blood vessels.

3.5.6 Does hypoxia affect nitrite responses equally in veins and arteries?

Although vasodilator effects of NO₂⁻ in human internal mammary arteries *in vitro* have been studied in the past (Buikema et al. 1992), effects of hypoxia on vasodilator actions of NO₂⁻ in human internal mammary arteries have not been assessed.

A study evaluating NO₂⁻ effect on segments of internal mammary artery was also conducted in the hyperoxia and under hypoxic conditions. There was no significant difference in vasoreactivity of internal mammary artery between hyperoxia and hypoxia (figure 3.10); indeed responsiveness was virtually unchanged. This was in contrast to the hypoxic potentiation of vasodilator properties of NO₂⁻ observed in saphenous veins (see figure 3.3 for comparison).

3.6 Discussion

The results of this work provide further mechanistic insights on hypoxic potentiation of vasodilatory actions of NO₂⁻. For the first time we show significant potentiation of vasodilator properties of NO₂⁻ by hypoxia in human saphenous veins

in vitro. At the same time we show no hypoxic potentiation of NO_2^- vasodilatory effects in human internal mammary artery. These data, along with the results of platelet work presented in chapter 4 of this thesis, provide a better understanding the phenomenon of hypoxic potentiation of NO_2^- effects in different tissues and settings.

The current results emphasize some differences between NO_2^- and the organic nitrate GTN. Unlike GTN, NO_2^- has previously been shown not to be prone to tolerance induction with prolonged exposure (Crandall et al. 1931; Dejam et al. 2007; Maher et al. 2013); furthermore there is no cross-tolerance to GTN (Maher et al. 2013). As GTN tolerance is engendered largely by acquired impairment of enzymatic bioactivation (Sage et al. 2000), this implies that different bioactivation pathways are operable for NO_2^- . Furthermore, current results demonstrate that the vasodilator effect of NO_2^- is selectively potentiated by exposure to hypoxia in saphenous veins. GTN, on other side, did not significantly show this phenomenon. Hence the mechanism(s) of hypoxic potentiation in blood vessels also are specific for NO_2^- , and do not apply to GTN. In particular, these findings imply that the mechanisms of hypoxic potentiation relate not to the biochemical pathways of NO signaling but specifically to the processes underlying NO release from NO_2^- in saphenous veins.

The next issue was identification of the mechanism(s) underlying hypoxic potentiation of the venodilator effects of NO_2^- . In view of previous findings by other groups (Dalsgaard, Simonsen & Fago 2007; Isbell, Gladwin & Patel 2007; Nagababu et al. 2003), we considered the possibility that deoxygenated Mb might be largely responsible, and also that *in vivo* Hb within blood vessels might also modulate hypoxic potentiation *in vivo* (Crawford et al. 2006).

The experiments to test these hypotheses were complicated by apparent NO “scavenging” by Hb (Donadee et al. 2011; Olson et al. 2004) and Mb (Flogel et al. 2001). For example, under hyperoxia, addition of RBC to perfusate markedly impaired responses to NO_2^- ; smaller changes occurred with free Hb and Mb.

With hypoxia, however, given these above effects of NO “scavenging”, it also emerged that potentiation of NO_2^- effect was greatly enhanced in the presence of RBC (as shown in Fig 3.7). These data suggested that because of the combined impact of NO “scavenging” and extensive hypoxic potentiation, the vasodilator effects of NO_2^- are far more likely to be physiologically relevant under hypoxia.

The vessel’s intrinsic Mb remains the main NO_2^- reductase. A series of experiments with ferricyanide supported this statement (Fig. 3.7) and confirmed previous findings by another group (Shiva et al. 2007).

The phenomenon of hypoxic potentiation of NO_2^- effects is of considerable clinical interest: the concept of being able to deliver a vasodilator (and also anti-aggregatory) stimulus selectively to hypoxic regions has a potential clinical application in all conditions in which hypoxia occurs in the presence of intact mechanisms for NO/sGC signaling. Conversely, it is possible that the presence of arterio-venous shunting (for example in haemodialysis patients) may attenuate venodilator/anti-aggregatory effects of NO_2^- by virtue of induction of increased oxygen content in venous blood. These areas remain to be explored with these specific mechanisms in mind. Although the data on asymmetry of hypoxic potentiation of NO_2^- effect are currently limited to a comparison between saphenous vein (extensive potentiation) and internal mammary artery (apparently no potentiation), this finding is of potential physiological interest. For example, the “diving reflex” includes sympathetic and parasympathetic discharges triggered by cold stimulation of trigeminal nerve afferents and maintained by hypoxia, inducing intensive arterial constriction in all but cerebral, coronary and adrenal circulations (for review see (Campbell, Gooden & Horowitz 1969)). NO_2^- -induced hypoxic potentiation would alternate this vasoconstriction. Therefore it would be of interest to see whether NO_2^- hypoxic potentiation in arteries is most intense in the cerebral, coronary and adrenal circulations. Furthermore, the molecular mechanisms responsible for hypoxic potentiation remain to be elucidated.

3.7 Study limitations

The current study has a number of limitations. The vascular work is limited to saphenous vein and internal mammary artery preparations, and is therefore incompletely representative of the spectrum of physiological and pharmacological effects of NO_2^- . In a variety of vascular beds, previous *in vivo* studies have shown that NO_2^- is a selective venodilator (Maher et al. 2008) but its effects on arterial and arteriolar function are also of clinical interest, as are effects within the pulmonary circulation (Ingram et al. 2010). Furthermore, there is uncertainty regarding the clinical relevance of effects of the high concentrations of NO_2^- utilized in these experiments.

3.8 Conclusion

This *in vitro* study has shown significant venodilator effect of NO_2^- under hypoxia. Furthermore, this was specific for NO release from NO_2^- - there was no significant potentiation of vasodilator properties of GTN under hypoxia. On the other hand, NO_2^- vasodilator effect on internal mammary artery is not subject to hypoxic potentiation in *in vitro* setting.

3.9 Tables and figures for chapter 3

Table 3.1: Patient demographics and pharmacotherapy in SV group

Characteristic	Total
Men/women, n	17/3
Age (mean \pm SD), years	72 \pm 8
Diabetes, n (%)	7 (35)
Hypertension, n (%)	17 (85)
Hypercholesterolaemia, n (%)	19 (95)
Smoking, n (%)	5 (25)
Pharmacotherapy	
Aspirin, n (%)	19 (95)
Nitrates, n (%)	5 (25)
ACE inhibitors, n (%)	7 (35)
ARB, n (%)	8 (40)
CCB dihydropyridine, n (%)	5 (25)
CCB non-dihydropyridine, n (%)	2 (10)
Beta-blockers, n (%)	6 (30)
Statins, n (%)	17 (85)
Perhexiline, n (%)	1 (5)

Characteristics of patients (n= 20) undergoing CABG whose saphenous vein (SV) segments were utilized to study vasodilator effects of nitrite (n=14) and effects of GTN (n=6) in organ bath experiments under hyperoxia and under hypoxia. Effects of GTN were studied on the same cohort of patients as the nitrite group. Further, in some of these experiments effects of incubation with red blood cells (n=4), myoglobin (n=5) and haemoglobin (n=4) on saphenous vein reactivity under hyperoxia and under hypoxia were evaluated.

Table 3.2 Patient demographics and pharmacotherapy in IMA group

Characteristic	Total
Men/women, n	3/0
Age (mean \pm SD), years	57 \pm 11
Diabetes, n (%)	0 (0)
Hypertension, n (%)	2 (67)
Hypercholesterolaemia, n (%)	2 (67)
Smoking, n (%)	0 (0)
Pharmacotherapy	
Aspirin, n (%)	3 (100)
Nitrates, n (%)	1 (33)
ACE inhibitors, n (%)	1 (33)
ARB, n (%)	1 (33)
CCB dihydropyridine, n (%)	0 (0)
CCB non-dihydropyridine, n (%)	2 (67)
Beta-blockers, n (%)	1 (33)
Statins, n (%)	2 (67)
Perhexiline, n (%)	0 (0)

Characteristics of patients (n = 3) undergoing CABG whose internal mammary artery segments were utilized to study vasodilator effects of nitrite in hyperoxia and under hypoxia.

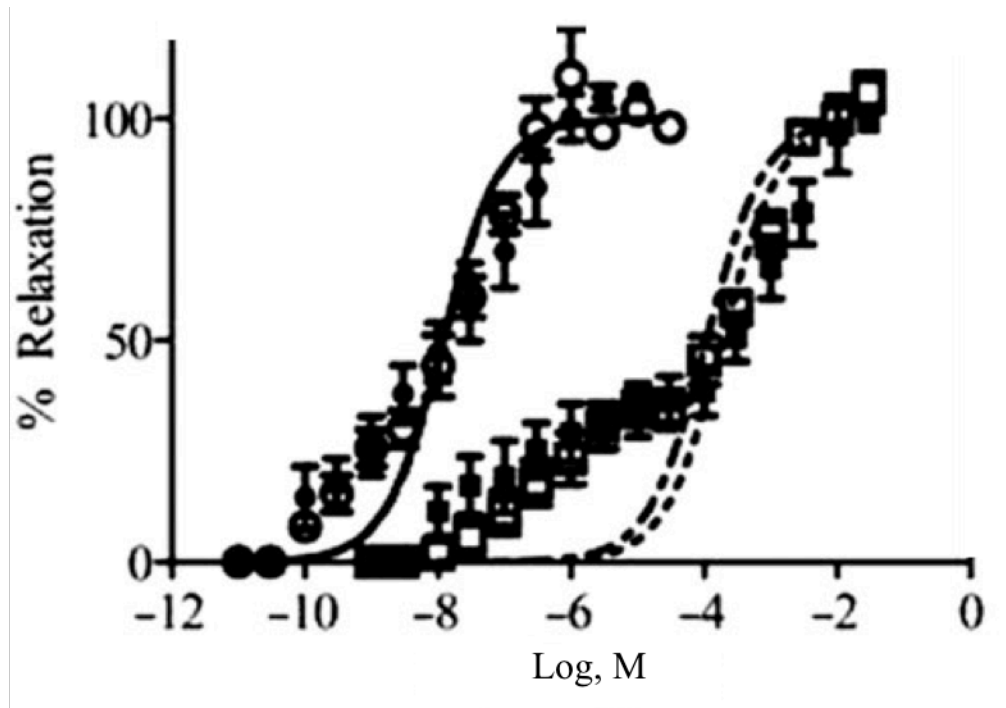


Figure 3.1 Concentration-response curves to GTN and NO_2^- in saphenous vein rings (n=7).

- GTN before prolonged NO_2^- exposure
- GTN after prolonged NO_2^- exposure
- NO_2^- before prolonged NO_2^- exposure
- NO_2^- after prolonged NO_2^- exposure

These data therefore show:-

- (1) GTN response is unaffected by prolonged NO_2^- exposure
- (2) NO_2^- response is unaffected by prolonged NO_2^- exposure

(These data were published in Br. J. Pharmacol: (Maher et al. 2013)). Reproduced with permission from the publisher (Br. J. Pharmacol.).

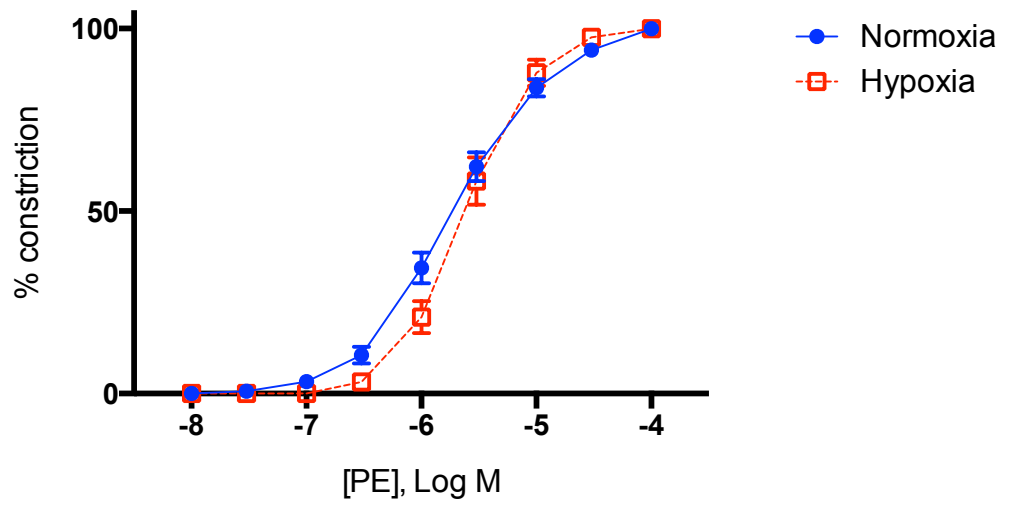
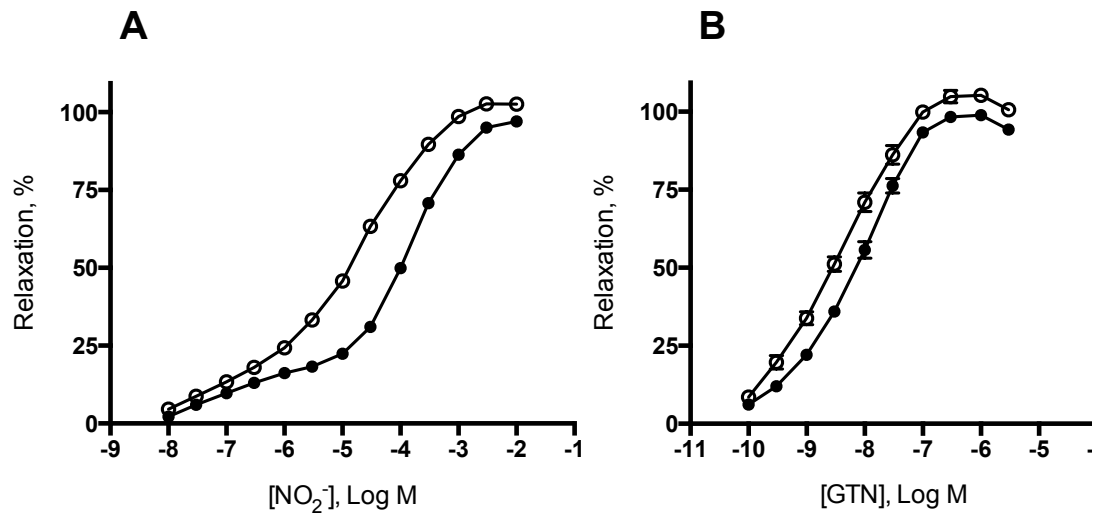


Figure 3.2 Phenylephrine concentration-response curve: differences in vasoconstrictive effect of phenylephrine in hypoxia (open symbols) and hyperoxia (closed symbols) in saphenous vein rings (n=8). EC₅₀ values did not significantly differ.



	NO ₂ ⁻ hyperoxia	NO ₂ ⁻ hypoxia	GTN hyperoxia	GTN hypoxia
logEC ₅₀ ±SEM	-3.87±0.03	-4.70±0.02	-8.09±0.04	-8.45±0.07

Figure 3.3 Vasodilator effects of (A) sodium nitrite (NaNO₂, n=14, error bars fall within the size of the symbols) and (B) glyceryl trinitrate (GTN, n=6) in human saphenous vein in hyperoxia (closed symbols) and in hypoxia (open symbols). EC₅₀ values were calculated from concentration-response curves and compared via paired t test for hyperoxia vs hypoxia: p<0.01 for NO₂⁻ and p=0.1 for GTN.

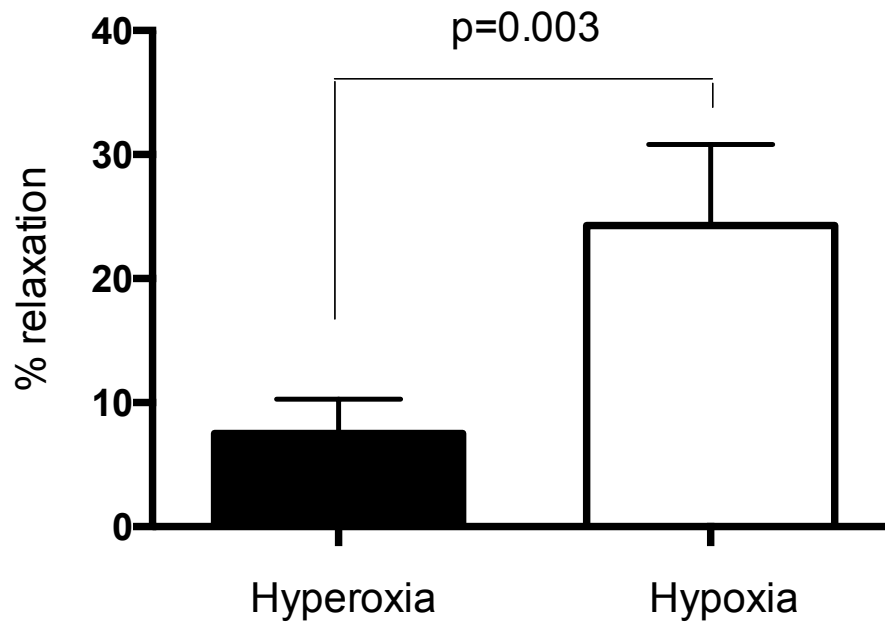


Figure 3.4 Effect of hypoxia on baseline tension of saphenous vein rings (SV; n=5): SV rings relaxed by 24.2 ± 2.9 % within 2 minutes of hypoxia induction in organ baths vs 7.5 ± 1.2 % relaxation in vessels continuing to bubble with hyperoxic gas mixture ($p=0.003$).

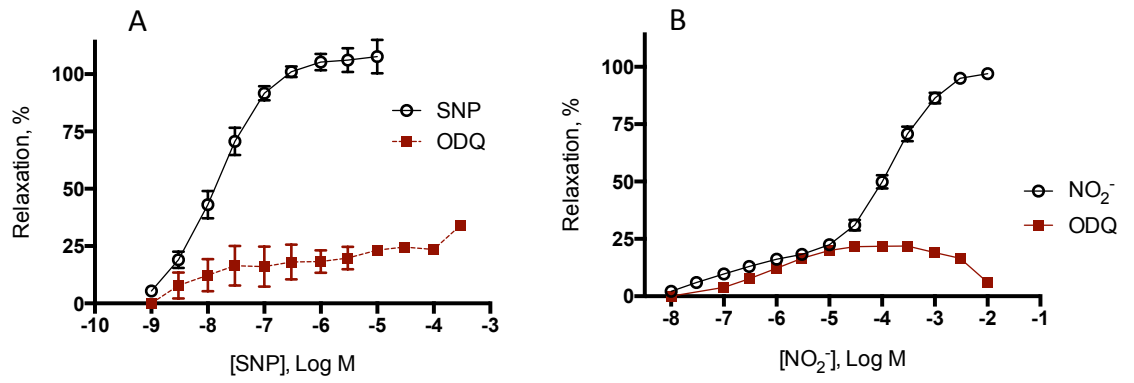


Figure 3.5 Effects of pre-incubation (5 minutes) with ODQ (10 μM; n=3) on SNP and NO₂⁻ concentration response curves:

(A) SNP vs SNP + ODQ (n=4)

(B) NO₂⁻ vs NO₂⁻ + ODQ (n=14)

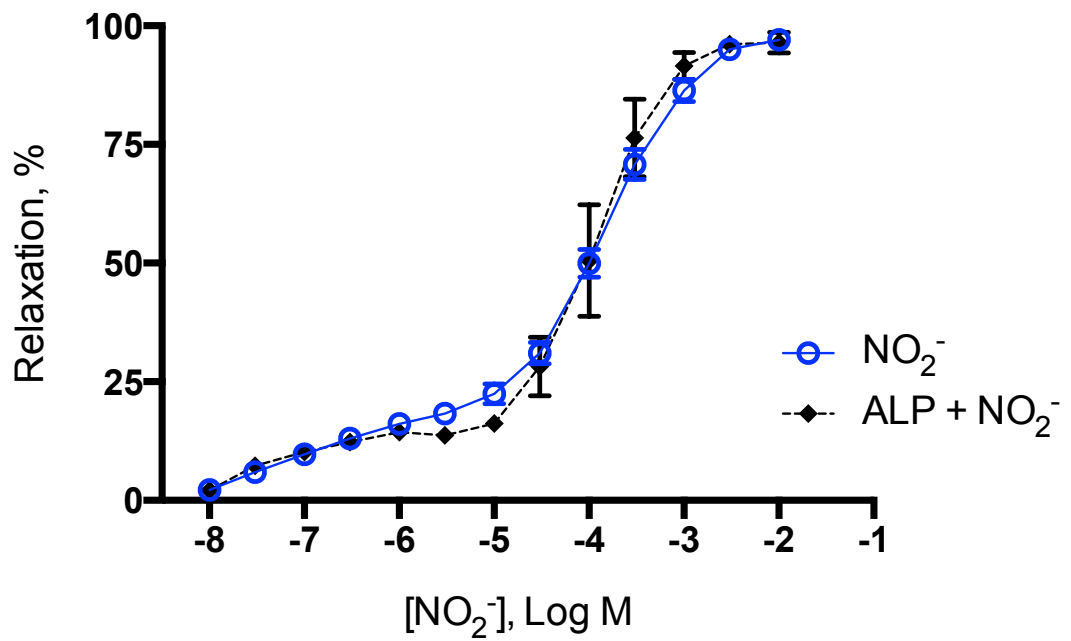
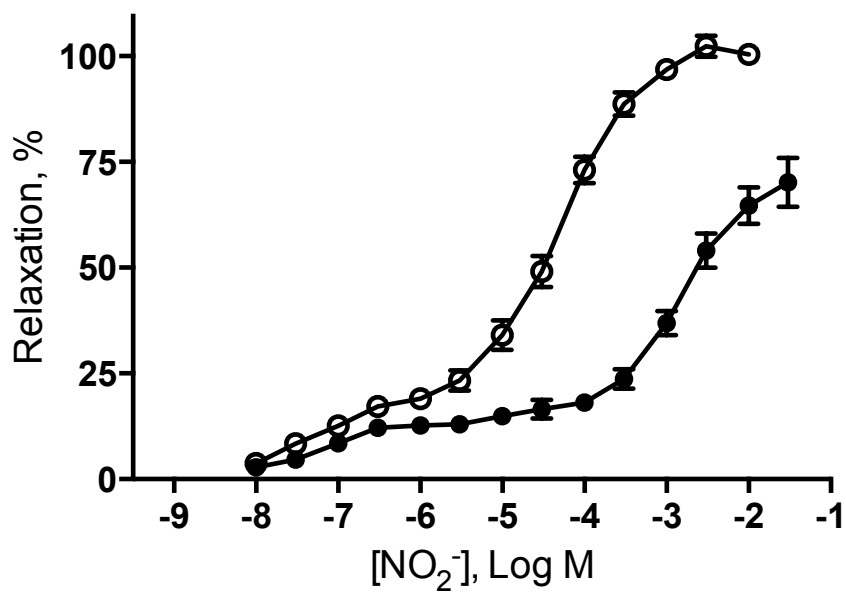


Figure 3.6 Effects of pre-incubation (5 minutes) with allopurinol (ALP; 1 mM; n=3) on NO₂⁻ (n=14) concentration response curve; p=NS



	RBC hyperoxia	RBC hypoxia
Log EC ₅₀	-2.70 ± 0.22	-4.38 ± 0.06

Figure 3.7 Impact of presence of red blood cells (RBC: 10% by volume) on NO₂⁻ vasodilator effect in hypoxia vs hyperoxia in saphenous vein rings (n=5 for all experiments), p=0.01 for EC₅₀; closed symbols = hyperoxia, open symbols = hypoxia.

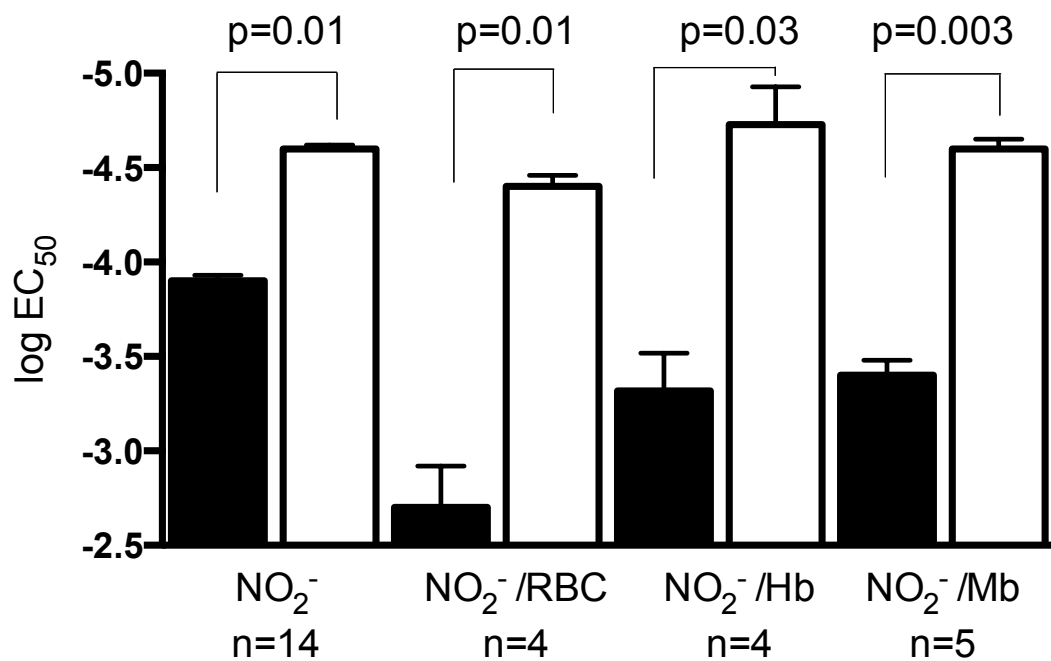
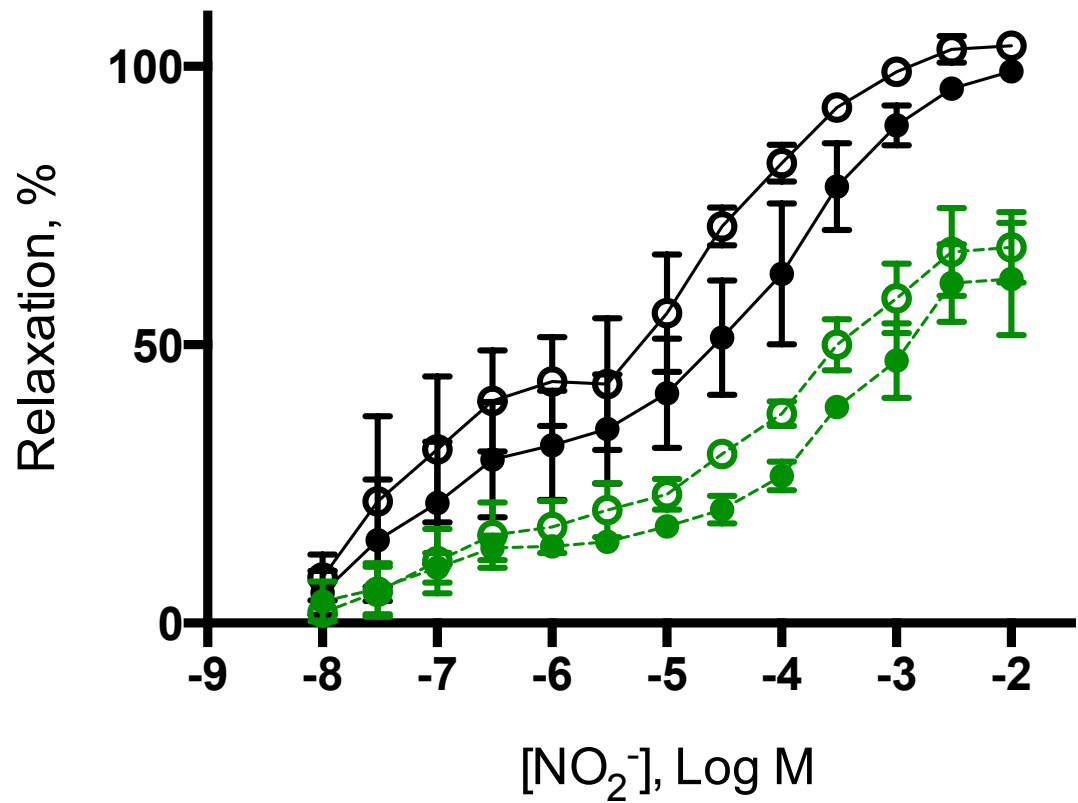
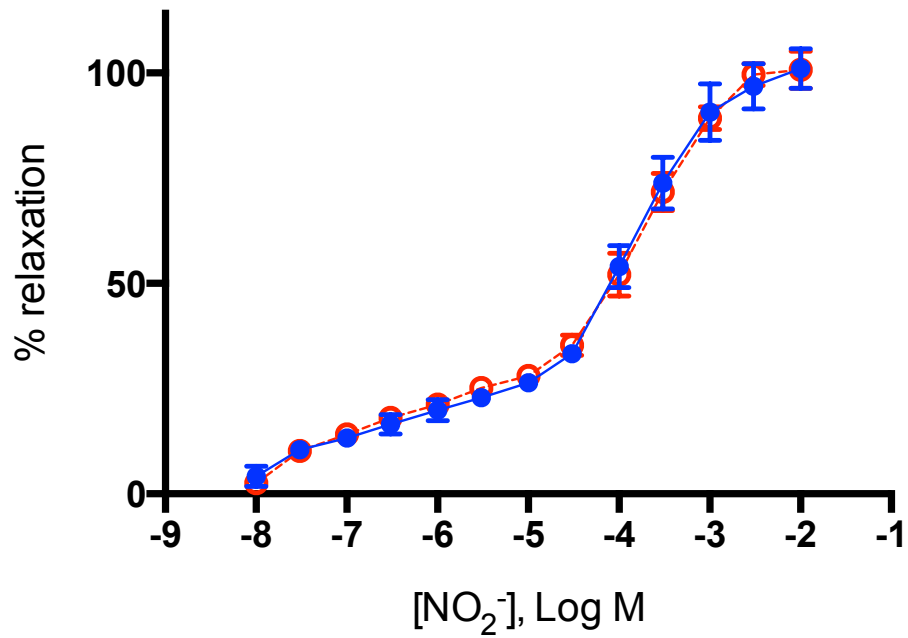


Figure 3.8 Impact of presence of red blood cells (RBC: 10% by volume), haemoglobin (Hb; 50 μ M) and myoglobin (Mb; 50 μ M) on hypoxic potentiation of NO₂⁻ vasodilator effect in saphenous vein rings, displayed as a difference in EC₅₀ values; closed bars = hyperoxia, open bars = hypoxia.



	NO ₂ ⁻ , hyperoxia	NO ₂ ⁻ , hypoxia	FC + NO ₂ ⁻ , hyperoxia	FC + NO ₂ ⁻ , hypoxia
LogEC ₅₀	-3.8 ± 1.1	-5.0 ± 0.5	-3.2 ± 0.5	-3.6 ± 0.8

Figure 3.9 Effect of pre-incubation with ferricyanide (FC; 1 mM; dotted lines) on NO₂⁻ vasodilator actions in hyperoxia (closed symbols) vs hypoxia (open symbols; n=3; F=4.7, p=0.006 for ferricyanide inhibition). There was no evidence of differential inhibition by FC according to hyperoxic or hypoxic conditions.



	Hyperoxia	Hypoxia
Log EC ₅₀	-3.9 ± 0.1	-3.8 ± 0.1

Figure 3.10 Vasodilator effect of NO₂⁻ (n=3) in human internal mammary artery in hyperoxia (closed symbols) and in hypoxia (open symbols), p=0.7 for EC₅₀ comparison

**Chapter 4: Anti-aggregatory effects
of nitrite in normoxia and hypoxia**

4.1 Summary of the study

4.1.1 Objectives

Hypoxic potentiation of NO generation from nitrite (NO_2^-) might facilitate NO_2^- -mediated suppression of platelet aggregation at sites of myocardial ischaemia and in acute heart failure. In the current study, we characterized hypoxic potentiation of NO_2^- effects in human platelets, addressing underlying mechanisms.

4.1.2 Methods

In whole blood (WB) samples and platelet-rich plasma (PRP) we assessed inhibition of platelet aggregation by NO_2^- (100 μM and 1 mM), in comparison with that of the more direct NO donor sodium nitroprusside (SNP, 10 μM). Further, pre-incubation of WB samples with the NO scavenger CPTIO (50 μM and 200 μM) before addition of SNP or NO_2^- was performed to delineate the proportion of the antiaggregatory effect of these compounds that is NO-mediated. Inhibition of platelet aggregation by NO_2^- was compared in venous and in arterial blood, and deoxygenated haemoglobin (deoxyHb) concentration was correlated with anti-aggregatory response. We also evaluated the influence of incremental hypoxia applied to venous blood samples (in hypoxic chamber) on deoxyHb level and anti-aggregatory effect of NO_2^- . In human experiments *in vivo* the effect of brief inhalation of hypoxic gas mixture on blood deoxyHb fraction was assessed.

4.1.3 Results

In individual subjects (n=37), there was a strong correlation ($r=0.75$, $p<0.0001$) between anti-aggregatory effects of NO_2^- and those of SNP in whole blood. In PRP, the effects of NO_2^- were less marked than in whole blood ($p=0.0001$), suggesting a role for Hb (within RBCs) in the bioconversion of NO_2^- to NO. Experiments with the NO scavenger CPTIO proved that anti-aggregatory effects of NO_2^- are NO-mediated ($p=0.006$ for inhibition by CPTIO). Inhibition of platelet aggregation by NO_2^- was almost 3-fold greater in venous than in arterial blood ($p<0.0001$), and deoxyHb concentration directly correlated ($r=0.69$, $p=0.013$) with anti-aggregatory response. Incremental hypoxia applied to venous blood samples caused a

progressive increase in both deoxyHb level and anti-aggregatory effect of NO_2^- . When subjects inhaled a gas mixture containing 12% O_2 balanced with nitrogen (N_2) for 20 minutes, there was a 3-fold rise in blood deoxyHb fraction ($p < 0.01$). In PRP, response to NO_2^- also increased under hypoxia, and was further enhanced ($p < 0.01$) by deoxyHb and deoxyMb. Furthermore, deoxyHb and deoxyMb exerted significant anti-aggregatory effects even in the absence of added NO_2^- , suggesting a role for endogenous NO_2^- .

4.1.4 Conclusions

The results of this work provide further mechanistic insights on hypoxic potentiation of anti-aggregatory actions of NO_2^- . Anti-aggregatory actions of NO_2^- are NO-mediated. The responses to NO_2^- are diminished in the presence of NO resistance, and resultant sGC activation underlies biological effect. Anti-aggregatory effects of NO_2^- in whole blood were increased under hypoxia, and directly correlated with deoxyHb level. The balance of evidence suggests differential rates of NO release from NO_2^- (largely modulated by deoxyHb) as the fundamental mechanism.

4.2 Introduction

The extensive physiological roles of nitric oxide (NO) in cardiovascular control and homeostasis of platelet function have been delineated over the past 30 years (for recent review see (Jin, RC & Loscalzo 2010)). However, the physiological bases for NO generation are now understood to include not only bioconversion of arginine by NO synthases (NOS) (Moncada & Higgs 1993) but also reduction of nitrite (NO_2^-) (Cosby et al. 2003; Gladwin et al. 2005; Lundberg, Weitzberg & Gladwin 2008; Lundberg et al. 1994), which was previously considered to be an inert product of NO metabolism. As NO can be generated from NO_2^- independently of NOS function, both the physiological and potential therapeutic roles of NO_2^- are of increasing interest (for review see (van Faassen et al. 2009)).

NO_2^- exhibits two potential advantages as a treatment for acute cardiovascular disorders. First, vasodilator responses of NO_2^- are potentiated in the presence of hypoxia (Cosby et al. 2003; van Faassen et al. 2009). In theory, this might facilitate selective vasomotor effect at sites of myocardial ischaemia, and might also potentiate NO_2^- -mediated dilatation in the presence of acute heart failure (Maher et al. 2013). Furthermore, NO_2^- vasomotor effects are not subject to tolerance induction and also do not exhibit cross-tolerance with organic nitrates (Dejam et al. 2007; Maher et al. 2013). Potentiation of NO generation from NO_2^- under hypoxia might also substantially augment the anti-aggregatory effects of NO_2^- (Srihirun et al. 2012; Totzeck et al. 2012), and this would be of particular relevance in myocardial ischaemia/heart failure. In fact, potentiation of NO generation from NO_2^- has been demonstrated to correlate with deoxyhaemoglobin concentrations in studies in vessels from rats (Isbell, Gladwin & Patel 2007) and also in platelet-rich plasma (Srihirun et al. 2012).

We have therefore undertaken the current study in human subjects, in order to evaluate the potential efficacy of NO_2^- in hypoxia and to study the phenomenon of ‘hypoxic potentiation’ of NO_2^- in human platelets as regards its underlying mechanisms. We examined anti-aggregatory effects of NO_2^- , in comparison with those of the more direct NO donor sodium nitroprusside (SNP), in venous and arterial whole blood and platelet-rich plasma samples obtained from healthy subjects and patients with ischaemic heart disease (IHD). The results provide incremental insights into the therapeutic potential of NO_2^- as a treatment for acute cardiac illnesses.

4.3 Objectives of the study

The following hypothesis were tested in this study:

Primary

The anti-aggregatory effect of NO_2^- is subject to the phenomenon of hypoxic potentiation in humans and is directly correlated to deoxyHb concentration.

Secondary

NO₂⁻ is a more potent inhibitor of platelet aggregation in venous vs arterial blood and its effects are NO-mediated.

4.4 Methods

4.4.1 Study population

In order to evaluate a group of individuals with variable platelet responsiveness to NO, we studied both healthy subjects and patients with ischaemic heart disease, including individuals undergoing non-emergent cardiac catheterization, given that the latter have a high prevalence of NO resistance (Chirkov et al. 1999; Chirkov et al. 2001; Willoughby, S. R. et al. 2012). The only exclusion criterion was current therapy with ADP receptor antagonists, given that this would preclude assessment of reversal of ADP induced aggregation by NO donors and NO₂⁻.

The protocols were approved by the Ethics of Research Committee of The Queen Elizabeth Hospital and informed consent was obtained prior to study entry.

4.4.2 Platelet aggregation studies

These studies were performed as described previously (Chirkov et al. 1999). After 6 to 12 hours of fast subjects/patients were consented and once agreed to participate, venous or arterial blood samples were collected from an antecubital / femoral vein or a femoral / radial artery into plastic tubes containing 1:10 volume of acid citrate anticoagulant (two parts of 0.1 M citric acid to three parts of 0.1 M trisodium citrate); acidified citrate was utilized to minimize deterioration of platelet function during experiments. Blood was centrifuged at 250 g for 10 min at room temperature to obtain PRP. To assure complete removal of RBCs, PRP was spun at 2500 g for 10 seconds and the supernatant was collected. Absence of Hb was proved with Radiometer Copenhagen NPT 7 Series Blood Gas Analyser.

Platelet aggregation was examined utilizing impedance aggregometers (Models 560

and 700, Chrono-Log, Pennsylvania, USA). In brief, tests were performed at 37 °C and stirring speed of 900 rpm. Samples of whole blood or PRP were diluted twofold with normal saline (final volume 1 ml) and pre-warmed for 5 min at 37 °C. Aggregation was induced with ADP (final concentration of 2.5 μM). Aggregation was monitored continually for 7 min, and responses were recorded for electrical impedance in Ohms. SNP and NaNO₂ (final concentration of 10 μM and 1 mM or 100 μM, respectively) were added to samples 1min before ADP. If CPTIO was utilized, then it was added to the whole blood sample 3 minutes before SNP or NaNO₂. Inhibition of aggregation was evaluated as a percentage comparing the extent of maximal aggregation in the presence and absence of the anti-aggregatory agent studied.

4.4.3 Induction of controlled hypoxia *in vitro*

We utilized a custom-made hypoxic chamber from Plexiglas (see schematic figure 2.2) so that aggregation could be evaluated under controlled hypoxia. Hypoxic gas mixture comprising of 95% N₂ and 5% CO₂ was connected to the hypoxic chamber. The gas flow rate was easily adjustable to the desired level of O₂ concentration within the chamber.

To check pH, Hb fractions and oxygen saturation of blood utilized in the platelet aggregation studies, the samples were collected into PICO 70 Radiometer blood gas syringes containing 60IU of heparin and processed on the Radiometer Copenhagen NPT 7 Series Blood Gas Analyzer after being incubated in the aggregometer as per protocol.

Oxygen concentration in the hypoxic chamber was monitored with an oxygen analyzer (Teledyne Brown Engineering, Inc., USA).

4.4.4 ROS measurements

Blood samples were collected by vascular puncture into lithium-heparin tubes. Quantitation of total ROS in whole blood was performed utilizing electronparamagnetic resonance (EPR) spectroscopy, as previously described (Mariappan et al. 2009). All EPR samples were prepared using 20 mM Krebs HEPES buffer (pH 7.4) and 25 μ M of deferoxamine, 2 μ M of diethyldithiocarbamate (chelating agents), 200 μ M of the spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) and placed in 50 μ L glass capillaries (Corning, NY). The EPR spectra were recorded using an e-scan M EPR spectrometer (Bruker Bio-Spin e-scan, Rheinstetten/Karlsruhe, Germany) and super-high Q microwave cavity with the following settings: field sweep, 10G; microwave frequency, 9.75 GHz; microwave power, 19 mW; modulation amplitude, 2G; conversion time, 10.24 ms; time constant, 40.96 ms; receiver gain, 3.2×10^2 . ROS content was expressed as EPR signal intensity in arbitrary units. The coefficient of variability for replicate estimates was 4%.

4.4.5 *In vivo* induction of mild hypoxia: effects on deoxyHb

In six stable Coronary Care Unit patients deoxyHb levels were determined via arterial blood gases sampling from the radial artery under local anaesthetic (1% lignocaine) at baseline and after 20 minutes of breathing 12% oxygen mixture (balanced with nitrogen) (Maher et al. 2008). All participants underwent ST segment monitoring and had blood pressure, heart rate and oxygen saturation regularly monitored.

4.4.6 Statistical analysis

All data are means \pm SEM. Data processing and statistical analysis were performed utilizing GraphPad Prism version 6 (GraphPad software Inc., San Diego, CA). Comparison of SNP and NO₂⁻, CPTIO effects on platelet aggregation in arterial vs venous blood and in hypoxic chamber involved paired analysis; in PRP vs WB – non-paired *t*-test. The relative effects of Mb, Hb on responses to NO₂⁻ in PRP were

compared utilizing non-paired t-test. Effects of various degrees of hypoxia on pO₂, deoxyHb fraction and NO₂⁻ anti-aggregatory response were evaluated utilizing ANOVA with repeated measures. The limit of statistical significance was set 0.05 (two-tailed) throughout.

4.5 Results

4.5.1 Patients/normal subjects characteristics

The population evaluated in this study was stratified regarding the presence of ischaemic heart disease (IHD), diabetic status, gender and age, in order to facilitate evaluation of the full spectrum of NO responsiveness, and comprised of:

(i) Patients with stable IHD (n=63):

(a) undergoing diagnostic cardiac catheterization (n=23) including routine femoral arterial and femoral venous cannulation (and thus facilitating paired arterial and venous blood sampling) or

(b) evaluated incidentally (n=40) with blood sampling from antecubital vein.

Mean age was 69±13 years, with 63% male.

(ii) Asymptomatic adults with no known cardiovascular disease (n=39) were included to facilitate evaluation of the full spectrum of NO responsiveness. Mean age of patients with IHD was 69±1.3 years, with 63% male, while for normal subjects mean age was 42±1 years, with 52 % of subjects male.

4.5.2 Nitrite anti-aggregatory effects in venous whole blood

To study anti-aggregatory properties of NO₂⁻, we first assessed the effects of NaNO₂ (100 μM and 1 mM) in venous WB and compared those effects with SNP (10 μM) in paired samples of 15 subjects (Fig. 4.1). There was a significant difference between anti-aggregatory effects of the two concentrations of NaNO₂ (100 μM and 1 mM, p=0.0001). The higher concentration of NaNO₂ had similar effects to those of 10 μM SNP (Fig. 4.1).

4.5.3 Anti-aggregatory effects of NO₂⁻ are mediated via NO

We examined whether the physiological effects of NO₂⁻ are mediated by release of NO. We utilized the widely accepted NO scavenger CPTIO (Goldstein, Russo & Samuni 2003) as a tool in these series of experiments. In these studies we used two concentrations of CPTIO (50 μM and 200 μM) to investigate if there is a concentration-dependent response. 50 μM CPTIO significantly suppressed the anti-aggregatory effects of SNP in WB (from 36 ± 6 % to 19 ± 5 %; p=0.004), but not those of NaNO₂ (1 mM) (30 ± 6 % to 22 ± 6 %; p=0.2) (Fig 4.2). Nevertheless, this concentration of CPTIO is lower than that utilized in most cases in the literature (Dautov et al. 2013; Srihirun et al. 2012). Hence, in further series of experiments we utilized 200 μM CPTIO. This concentration of CPTIO was sufficient to significantly suppress anti-aggregatory effect of NO₂⁻ (from 24 ± 2 % to 5 ± 3 %; p=0.006) (Fig. 4.2). These data therefore prove that anti-aggregatory effects of NO₂⁻ are at least largely NO-mediated.

4.5.4 Nitrite effects in the presence of NO resistance

The phenomenon of NO resistance (for review see (Chirkov & Horowitz 2007)) represents the main limitation to the anti-aggregatory effect of NO and NO donors. If NO₂⁻ acts primarily via NO release, it would be expected that the extent of its anti-aggregatory effects might be subject to this phenomenon. Given that NO responsiveness can be modulated by ROS (Chirkov et al. 1999; Worthley et al. 2007), we assessed ROS levels in arterial and venous whole blood samples of 10 patients (Fig. 4.3). These were similar (1,013,000 ± 72,309 AU for arterial vs 990,579 ± 70,262 AU for venous blood, p=NS). Further, anti-aggregatory effects of NO₂⁻ and SNP were compared in a heterogeneous patient/subject population comprising both normal subjects (n=14) and patients with ischaemic heart disease (n=23), in whom NO resistance occurs commonly (Chirkov et al. 1999; Chirkov et al. 2001; Willoughby, S. R. et al. 2012). We compared anti-aggregatory effects of SNP and NaNO₂ in WB samples. The results, shown in Figure 4.4, demonstrate a strong correlation (r=0.75, p<0.0001) between anti-aggregatory effects of the two

agents in individual patients, showing that responses to NO_2^- are diminished in the presence of NO resistance.

4.5.5 Arterio-venous differences of nitrite anti-aggregatory effects

In view of previous studies implicating Hb as a powerful NO_2^- reductase and thus activator of NO generation (for review see (Kim-Shapiro et al. 2005)), and in parallel with the current experiments in blood vessels showing marked increase in hypoxic potentiation of NO_2^- vasodilator effect in saphenous veins in the presence of RBC (Fig. 3.7 and 3.8 of this thesis), we conducted comparative experiments in WB and PRP (see figure 4.5). In PRP (n=13) anti aggregatory effects of NO_2^- were significantly less marked than in WB (n=38, p=0.0001), consistent with a contributory effect of Hb (within RBCs) into bioconversion of NO_2^- to NO.

We then assessed the anti-aggregatory effect of NO_2^- in comparison with those of SNP in paired PRP samples of 11 subjects/patients. The anti-aggregatory effect of NO_2^- (1 mM) was significantly weaker compared to that of 10 μM SNP ($4 \pm 2\%$ vs $38 \pm 12\%$, respectively; p=0.01) (Fig. 4.6). On the other hand, the anti-aggregatory effect of SNP in PRP was comparable to that of NO_2^- in whole blood (see figure 4.5). These data are therefore consistent with a central role of Hb (in RBC) in NO_2^- bioactivation.

Indeed deoxygenated haemoglobin (deoxyHb) has previously been demonstrated to play a major role in NO_2^- bioactivation (Isbell, Gladwin & Patel 2007). We now also evaluated its potential impact on platelet aggregation. As would be expected, deoxyHb concentrations were greater (p<0.0001) in venous (more hypoxic) than in arterial blood (Fig. 4.7). There was a direct correlation (r=0.69, p=0.013) between deoxyHb concentration (including both arterial and venous samples) and anti-aggregatory response to NO_2^- (1mM) as shown in figure 4.8.

In view of the possible involvement of Hb (within RBC) in NO_2^- reduction to NO, O_2 level might be an important modulator of NO_2^- activity. Therefore, we became interested in arterial blood with its higher oxygenation. To investigate whether the level of blood oxygenation affects the anti-aggregatory potency of NO_2^- , we

performed experiments in human arterial and venous blood samples. For comparison we ran parallel experiments with the more direct NO donor SNP (Fig. 4.9). In venous blood samples anti-aggregatory effect of NO_2^- was almost three-fold greater than in arterial blood ($p < 0.0001$, $n = 16$) (Fig. 4.9). In contrast, there was no difference in anti-aggregatory effects of SNP in arterial vs venous blood samples ($p = 0.83$, $n = 13$).

Interestingly, in the absence of additional NO_2^- , platelet aggregation was greater in arterial (6.9 ± 0.6 Ohms) than in venous blood samples (5.8 ± 0.5 Ohms, $p = 0.002$, $n = 19$) (Fig. 4.10). In the presence of the NO scavenger CPTIO ($200 \mu\text{M}$), there was a differential impact on ADP response. Application of CPTIO in venous blood significantly increased ADP-induced aggregation ($p = 0.04$), while there was no significant effect in arterial blood (Fig. 4.11). These results are consistent with a greater release of NO from endogenous NO_2^- in venous blood.

4.5.6 Hypoxic potentiation of nitrite anti-aggregatory effects: role of deoxyHb

We evaluated whether incremental hypoxia in venous blood samples might further potentiate the anti-aggregatory effect of NO_2^- in parallel with increases in deoxyHb level. We reduced oxygen (O_2) concentrations, utilizing a hypoxic chamber attached to the platelet aggregometer (Fig. 2.2 of this thesis). Oxygen concentration was monitored as described in the Methods (see 2.2.1.4). Corresponding pO_2 in the blood samples was determined using a blood gas analyzer (see Methods, 2.2.3.3). Within 15 min of incubation of blood samples in the hypoxic chamber, there was a decline in pO_2 levels down to 30 mmHg (Fig. 4.12A). Simultaneously, deoxyHb content rose from 42 ± 13 % to 69 ± 11 % (Fig. 4.12B). There was a progressive increase in anti-aggregatory effect of NO_2^- with reductions in ambient O_2 concentrations (Fig. 4.12C).

To validate the physiological relevance of this experimental approach, we investigated whether we could increase *in vivo* deoxyHb levels in blood ($n = 6$) by inhalation of a 12% O_2 (balanced with N_2) mixture for 20 minutes (Maher et al. 2008). In arterial blood, this induced a significant rise in deoxyHb fraction from 3.8 ± 0.5 % to 13 ± 2 %, $p = 0.006$ (Fig. 4.13).

We next determined whether the increase in hypoxic potentiation of anti-aggregatory effect is specific for NO_2^- , by comparison with SNP. While there was no significant change in SNP-induced inhibition of aggregation (Fig. 4.14), there was 3-fold potentiation of the anti-aggregatory effect of NO_2^- by hypoxia ($p=0.005$).

If venous whole blood samples were kept capped on the bench for 2 hours, there was a small rise in deoxyHb level. There was an associated trend towards potentiation of NO_2^- anti-aggregatory effect (both with 1 mM and 100 μM concentrations) (Fig. 4.15) with tendency towards greater potentiation of NO_2^- anti-aggregatory effects with 100 μM ($p=0.08$) than with 1mM. This finding potentially can be explained by the fact that for supraphysiological concentration of NO_2^- (1 mM), evaluating changes towards the upper limit of possible platelet responses, a small rise in deoxyHb does not make a substantial difference. However with a lower concentration of NO_2^- (100 μM), a small rise in deoxyHb content might lead to more NO release and potentiation of NO_2^- anti-aggregatory effects.

4.5.7 DeoxyHb and deoxyMb effects

We also utilized PRP to further investigate the physiological and therapeutic implications of hypoxic potentiation of the anti-aggregatory effects of NO_2^- . First, we found that responses to 1 mM NO_2^- were substantially potentiated (Fig. 4.16) as ambient oxygen concentration fell. Construction of the concentration-response curve of NO_2^- in PRP under hypoxic conditions, revealed that the threshold for inhibition of aggregation by NO_2^- had fallen to 10 μM (Fig. 4.17). We then evaluated, under peak hypoxic conditions (zero oxygen concentration in hypoxic chamber):

- (a) the roles of deoxyHb and deoxyMb and
- (b) the potential impact of endogenous NO_2^- on platelet aggregation.

As shown in Fig. 4.18, NO_2^- (1mM) inhibited ADP-induced aggregation by about 50 %, and this effect was markedly increased ($p<0.01$) by both deoxyHb and deoxyMb. Furthermore, both deoxyHb and deoxyMb exerted significant anti-

aggregatory effects in the absence of added NO_2^- , consistent with enhancement of the anti-aggregatory actions of endogenous NO_2^- .

4.6 Discussion

The results of this work provide further mechanistic insights on hypoxic potentiation of anti-aggregatory actions of NO_2^- . Indeed, it could be asserted that the anti-aggregatory effects of nitrite are trivial except under hypoxia. Utilizing SNP as a comparator NO donor, we demonstrated significantly more potent anti-aggregatory effects of NO_2^- in human venous vs arterial whole blood, and that this difference was directly correlated with deoxyHb concentration. This is supported by *in vitro* platelet work with artificially induced different hypoxia levels in a custom-made hypoxic chamber. Additionally we have demonstrated that tissue responsiveness to NO_2^- , like that of SNP, is constrained in the presence of NO resistance.

The next issue was identification of the mechanism(s) underlying hypoxic potentiation of the anti-aggregatory effects of NO_2^- . In view of previous findings by other groups (Dalsgaard, Simonsen & Fago 2007; Isbell, Gladwin & Patel 2007; Nagababu et al. 2003), we considered the possibility that deoxygenated Mb might be largely responsible, and also that *in vivo* Hb within blood vessels might modulate hypoxic potentiation (Crawford et al. 2006).

Since the initial demonstration of anti-aggregatory effects of NO_2^- in 1991 (Laustiola et al. 1991), consideration of the anti-aggregatory effects of NO_2^- in humans has been somewhat limited. We speculated that NO_2^- anti-aggregatory effects might be increased under hypoxia, and that this might result in greater effects even within venous blood. In the current experiments it was documented that in the absence of additional NO_2^- , ADP-induced aggregation was significantly greater in arterial than in venous blood. This raised the possibility that endogenous NO_2^- might be selectively limiting platelet aggregation in venous blood (Srihirun et

al. 2012), supporting this hypothesis and consistent with the previously reported findings of Park et al (Park et al. 2013) in murine platelets. When experiments were performed in platelet-rich plasma under hypoxia, additional evidence of the impact of endogenous NO_2^- was evident in that addition of deoxyHb or deoxyMb exerted anti-aggregatory effects in the absence of exogenous NO_2^- (Fig. 4.18). Results with exogenous NO_2^- effects were analogous: NO_2^- inhibition of platelet aggregation was significantly greater in venous than in arterial blood (Fig. 4.9). Indeed when venous blood was rendered hypoxic there was a further increase in response to NO_2^- (Fig. 4.14) and responses to NO_2^- were markedly potentiated in PRP under hypoxic conditions, with a leftward shift in the concentration-response curve (Fig. 4.16 and 4.17).

As regards mechanism(s) underlying both hypoxic potentiation of anti-aggregatory effects of NO_2^- and accentuation of NO_2^- effects in venous blood, we demonstrated that no similar changes occurred with SNP, either regarding arterio-venous differences or effects of *in vitro* hypoxia (figures 4.9 and 4.14). Experiments in platelet-rich plasma suggested that both deoxyHb and deoxyMb might be contributors to the potentiation of anti-aggregatory effects of NO_2^- in venous blood and under hypoxia. Correlation data suggested that increases in deoxyHb concentrations during progressive hypoxia might play a primary role (Fig. 4.12). The lack of impact of sampling site (arterial vs venous) and hypoxia on SNP responses effectively excludes changes in function of soluble guanylate cyclase as a basis for the potentiation of NO_2^- effect (figures 4.9 and 4.14). Furthermore, as total ROS content did not vary between arterial and venous blood it is unlikely that differential “scavenging” of NO by superoxide (O_2^-) played a role: the balance of evidence suggests differential rates of NO release from NO_2^- (largely modulated by deoxyHb) as the fundamental mechanism. However, the fact that hypoxic potentiation also occurred in platelet-rich plasma (Fig. 4.16) suggested that factors other than deoxyHb (e.g. xanthine oxidase, eNOS, ALDH, etc.) might play a role, consistent with previous findings by other investigators (Feelisch et al. 2008; Vanin et al. 2007).

Given the differences in mode of activation of NO_2^- compared with other sources of NO, an important clinical issue is whether the effects of NO_2^- might circumvent not only NOS dysfunction but also distal impairment in the NO/sGC signaling pathway, termed “NO resistance” (for review see (Chirkov & Horowitz 2007)). For example, we recently showed that the nitroxyl donor IPA/NO can partially circumvent this phenomenon (Dautov et al. 2013). Correlation data (see Fig. 4.4) established that inter-individual variability in anti-aggregatory effects of NO_2^- closely parallel those in SNP responses. Thus, NO_2^- does not circumvent NO resistance, and this is consistent with previous data in vascular tissue (Maher et al. 2013) suggesting that conversion to NO and resultant sGC activation represents the main biochemical mode underlying physiological effect of NO_2^- . Three patients (fig 4.4) exhibited moderate responses to SNP, but zero response to nitrite. The cause of these outlying data was not determined.

One of the critical issues regarding interpretation of the current data, especially those concerning inhibition of platelet aggregation, is the high (supraphysiological) concentration of nitrite utilized in the platelet experiments. In preliminary concentration-response studies under normoxia, we demonstrated that the threshold for definite inhibition of aggregation by NaNO_2 in whole blood was 10 μM , which induced 7.4 ± 4.1 % inhibition (Fig. 4.19). However, the steep part of the concentration-response curve was around 1 mM under normoxic conditions with a mean response of 41.5 ± 8.3 % inhibition. Effects of vehicle (saline) are unlikely to have contributed to this observation. Since we wished to quantitate the extent of potentiation of NO_2^- effects under hypoxia in a most accurate way that was referable to leftward shifts in the concentration-response curve, 1 mM was utilized. (Of note, we utilized a similar approach when selecting 10 μM SNP concentration). This should not be taken to imply lack of physiological significance of NO_2^- - induced vasodilation or inhibition of platelet aggregation. Indeed, numbers of other investigators have confirmed pharmacological activity of NO_2^- at far lower concentrations. For example, Modin et al utilized rat aortic rings at pH 6.6, and demonstrated that 10 μM NO_2^- induced significant vasodilation (Modin et al. 2001). In previous experiments regarding platelet aggregation, NO_2^- concentrations as low as 0.1 μM were shown to have some inhibitory effects on platelet aggregation

(Srihirun et al. 2012). Importantly, the key experiments in this chapter were performed at concentrations of perhaps 100-fold greater than physiological: yet the findings may well be physiologically relevant.

The whole phenomenon of hypoxic potentiation of NO_2^- effects is of considerable clinical interest: the concept of being able to deliver a vasodilator and anti-aggregatory stimulus selectively to hypoxic regions has a potential clinical application in conditions in which hypoxia occurs in the presence of intact mechanisms for NO/sGC signaling. Conversely, it is possible that the presence of arterio-venous shunting (for example in haemodialysis patients) may attenuate venodilator/anti-aggregatory effects of NO_2^- by virtue of induction of increased oxygen content in venous blood. These areas remain to be explored with these specific mechanisms in mind.

4.7 Study limitations

The current study has a number of limitations. There is uncertainty regarding the clinical relevance of effects of high concentrations of NO_2^- utilized in these *in vitro* experiments. Similarly, it was impossible to evaluate the effects of either prolonged NO_2^- exposure or of “true” anoxia ($\text{pO}_2 < 13$ mmHg) in platelet aggregation studies.

4.8 Conclusion

The overall implication of our findings is that anti-aggregatory effects of NO_2^- on human platelets are potentiated by hypoxia. This was evident both from differences in NO_2^- effects in arterial vs venous blood and from experiments with controlled hypoxia. It is also clear that deoxyHb plays a significant role in reducing NO_2^- to NO in human blood circulation.

4.9 Figures for chapter 4

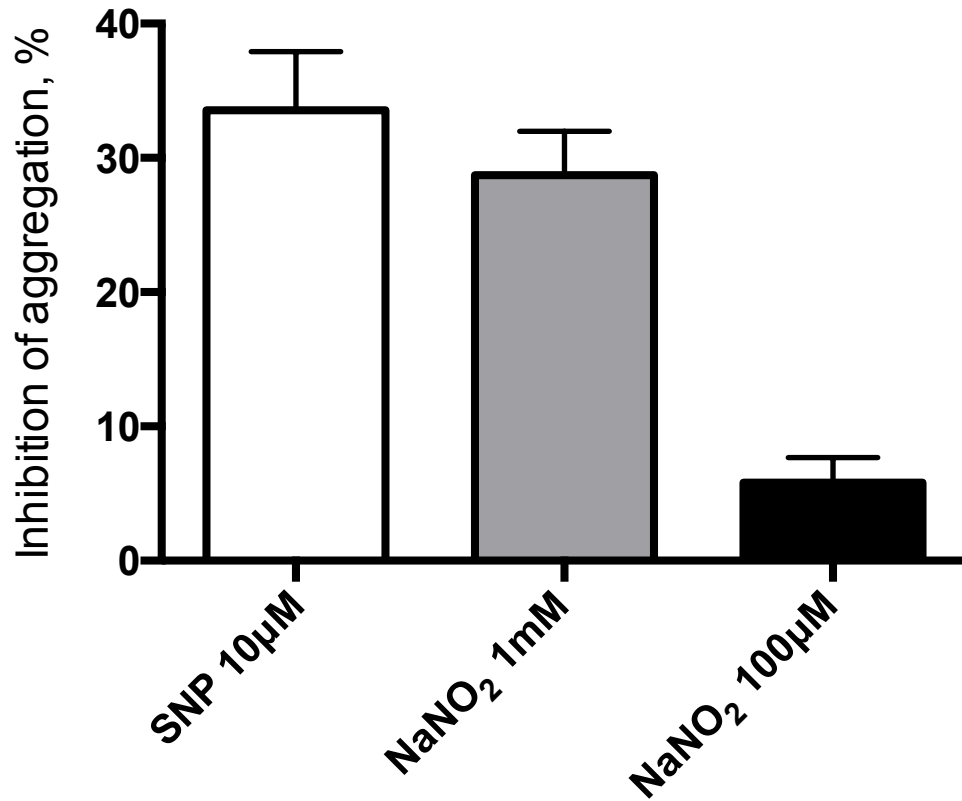


Figure 4.1 Comparison of anti-aggregatory effects of SNP (10µM; n=37) and NaNO₂ (1mM; n=37 and 100µM; n=15) in venous whole blood (repeated measures ANOVA: p=0.0003)

Post hoc comparisons (Bonferroni):

p=NS for SNP vs NaNO₂ 1mM

p=0.0002 for SNP vs NaNO₂ 100µM

p=0.003 for NaNO₂ 100µM vs 1mM

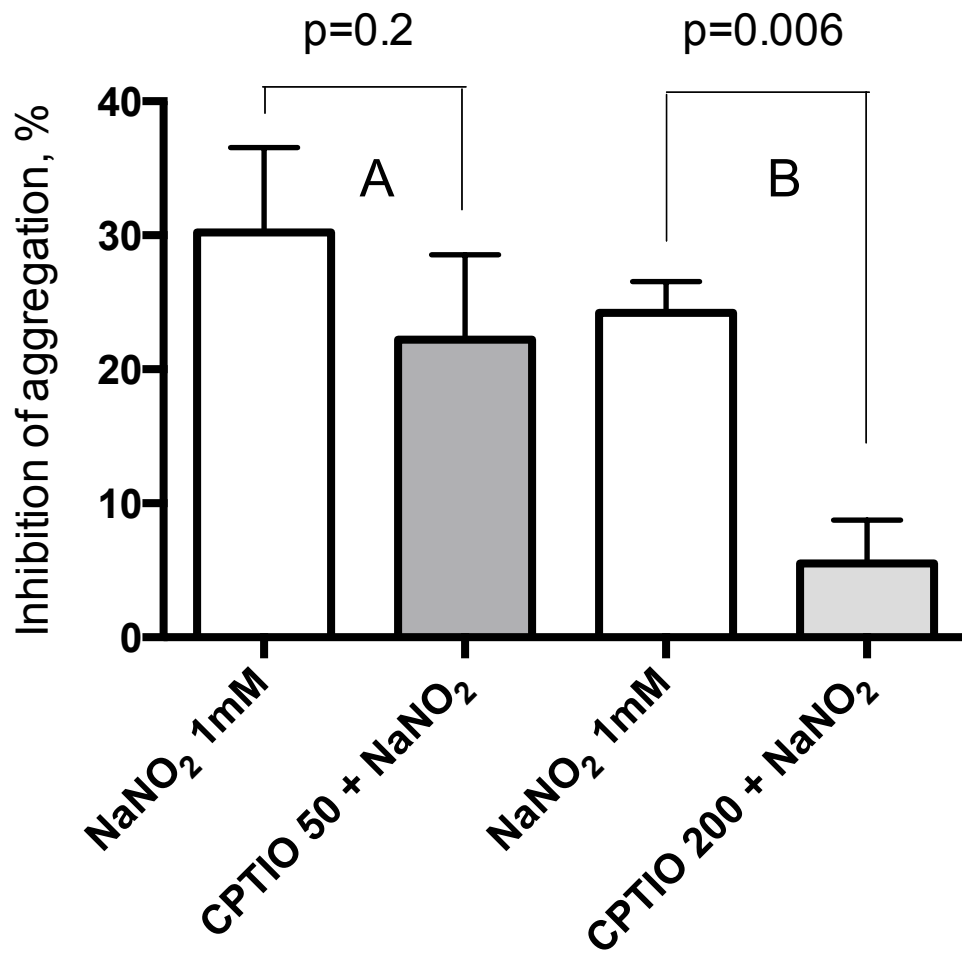


Figure 4.2 Concentration dependent effect of CPTIO (50 μ M; n=10 vs 200 μ M; n=4) on anti-aggregatory actions of NaNO₂ (1mM) in venous whole blood.

Note that NaNO₂ responses were evaluated in blood from different individuals for experiments A and B. Statistical comparisons were made by paired *t*-test.

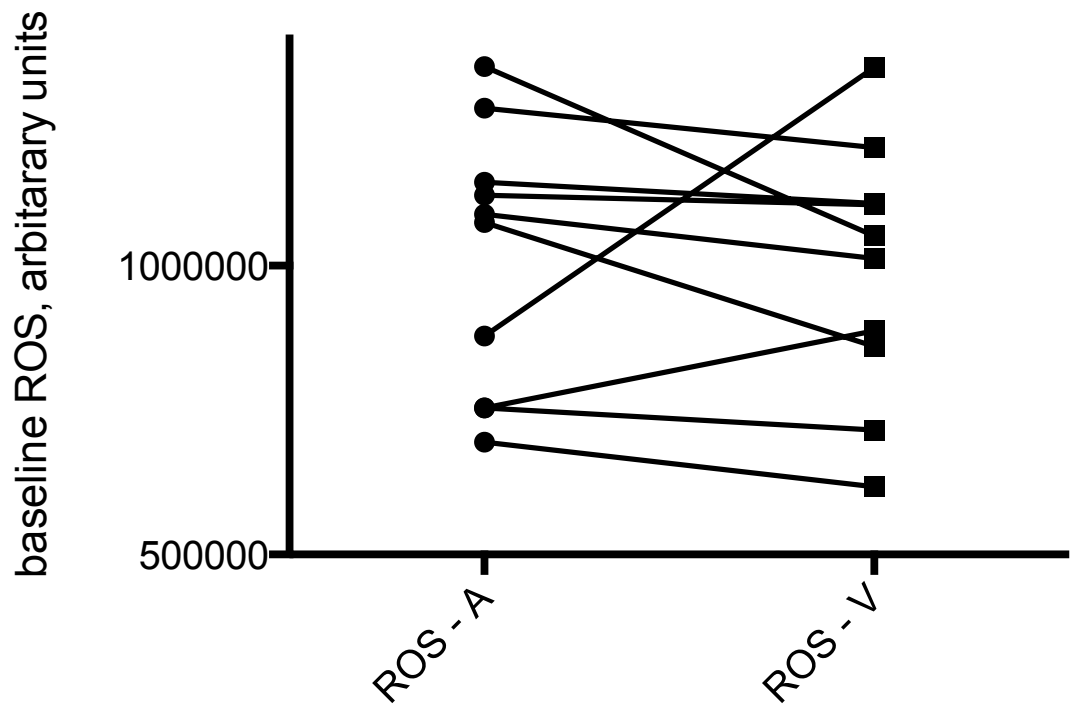


Figure 4.3 Baseline reactive oxygen species (ROS) in arterial vs venous whole blood (n=10): no significant differences between the blood samples (p=0.74).

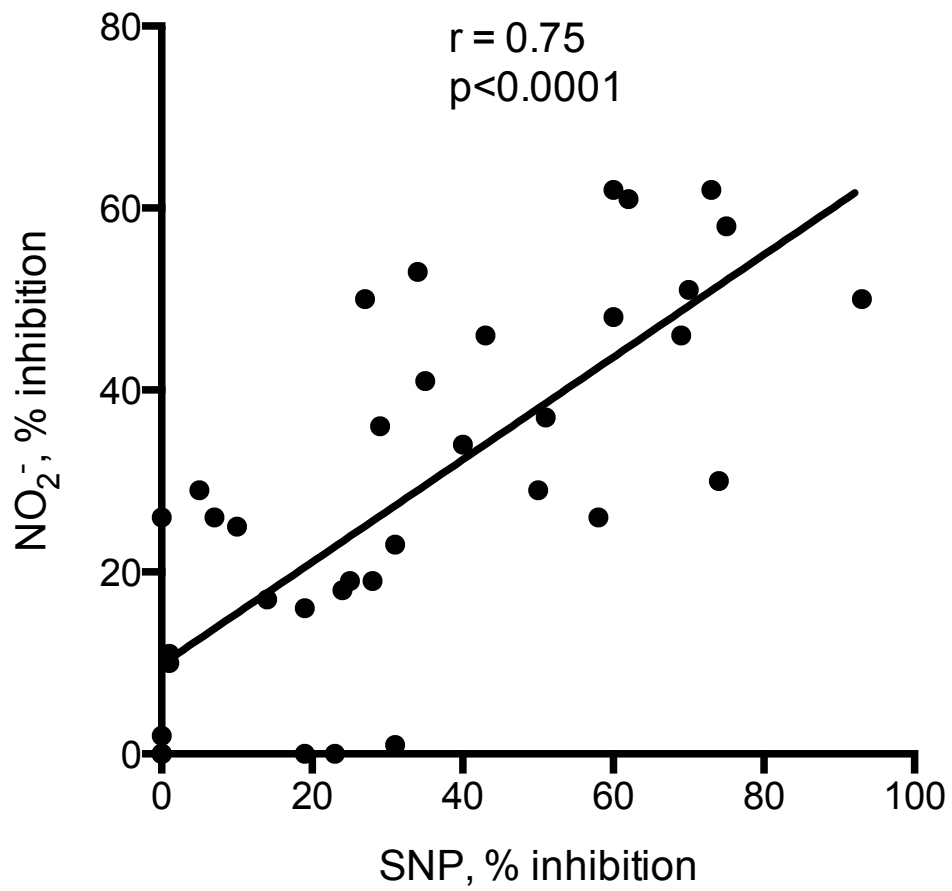


Figure 4.4 Correlation between inhibition of platelet aggregation by SNP (10 μM) and NO₂⁻ (1 mM) in venous blood (n=37).

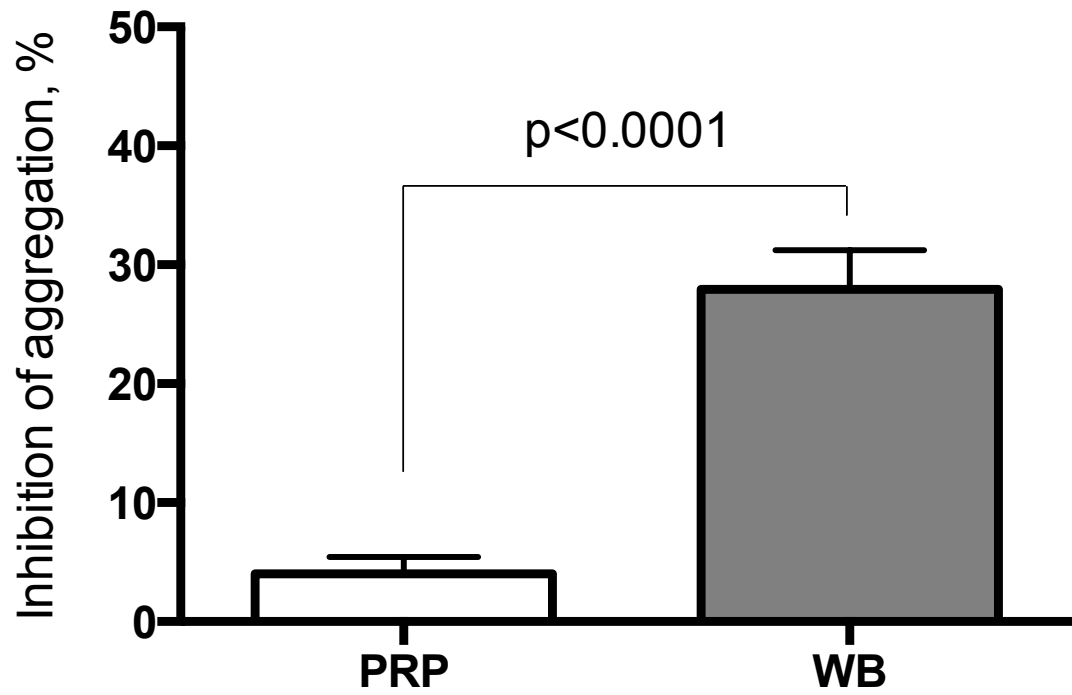


Figure 4.5 Comparison of anti-aggregatory effects of NO_2^- (1mM) in PRP (n=13) and whole blood (n=38) under normoxic conditions.

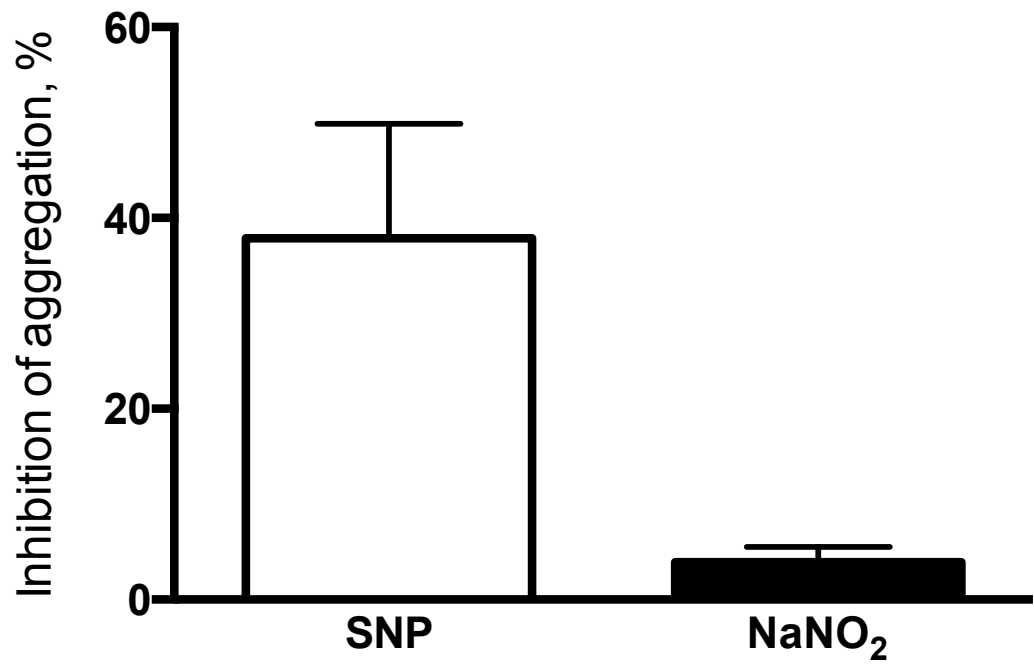


Figure 4.6 Comparison of anti-aggregatory effects of SNP (10 μ M) and NaNO₂ (1 mM) in platelet-rich plasma (n=11, paired samples; p=0.01).

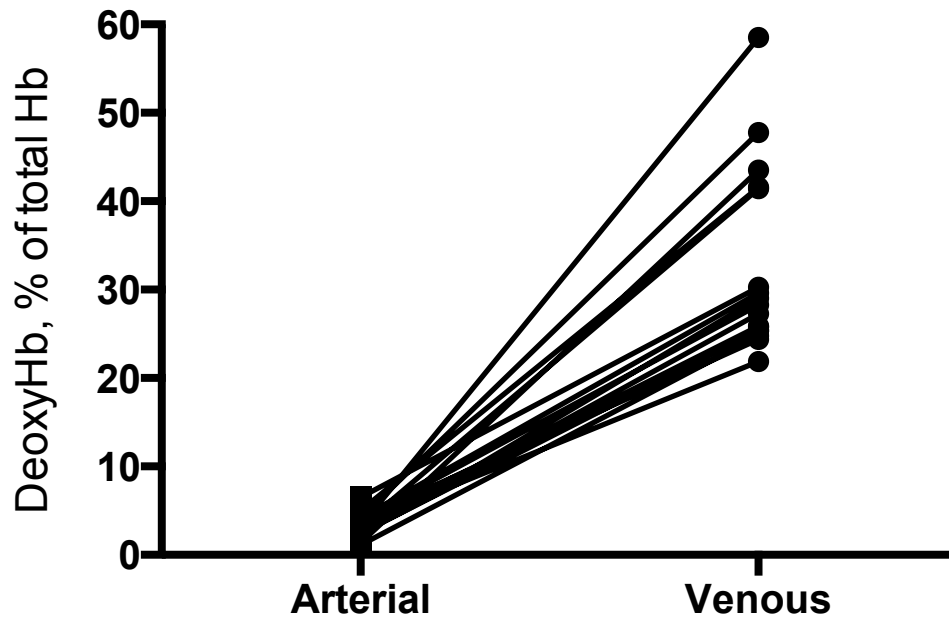


Figure 4.7 DeoxyHb content in venous vs arterial blood: paired analysis (n=17), $p < 0.0001$ for difference

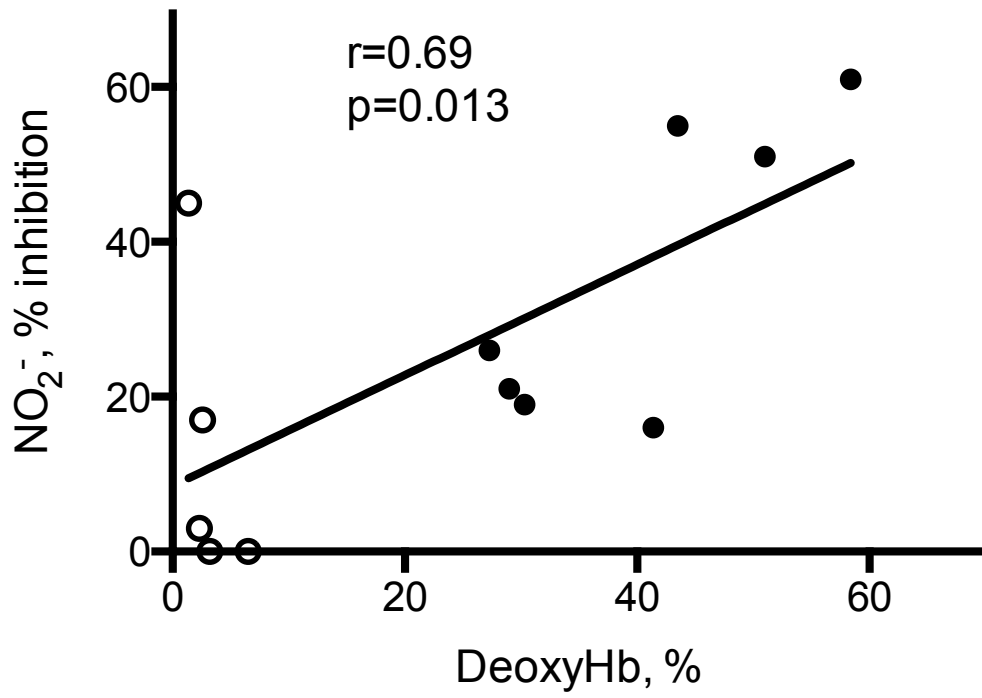


Figure 4.8 Correlation between anti-aggregatory effects of NO₂⁻ (1mM) and heterogeneity of deoxyHb content (combined data from venous (n=7) and arterial (n=5) blood). Open symbols refer to arterial samples, closed to venous samples

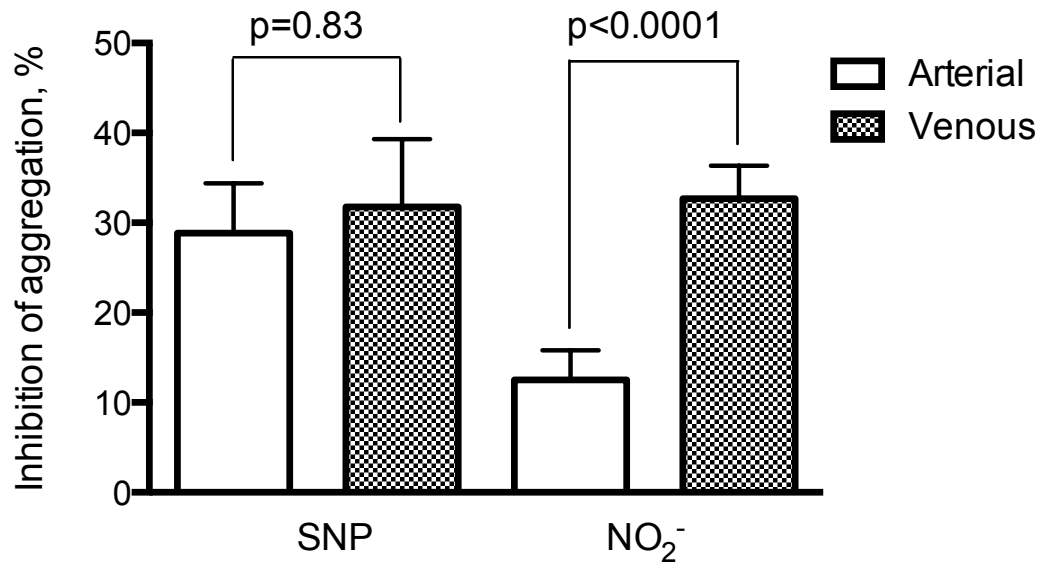
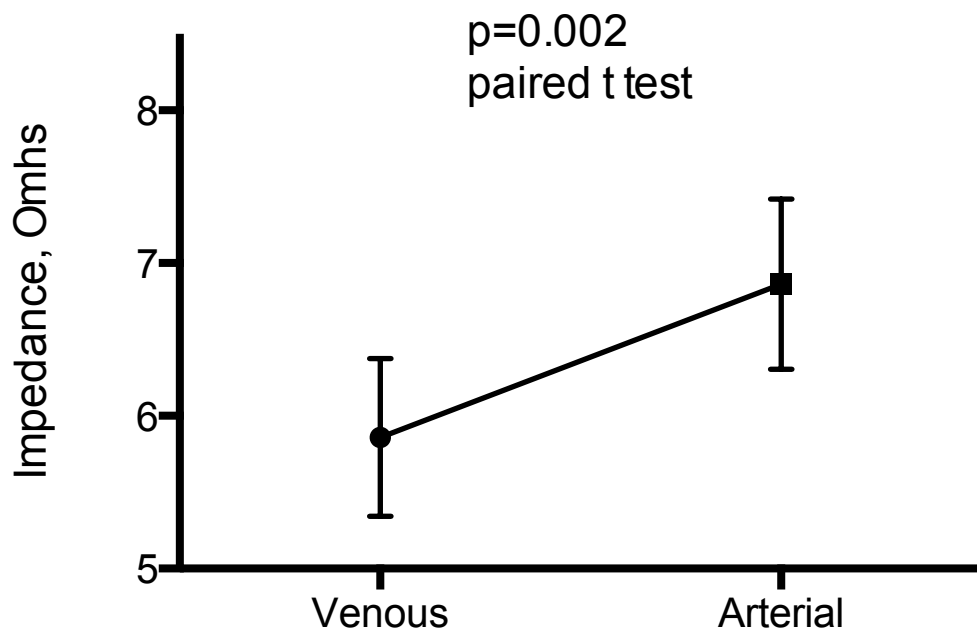


Figure 4.9 Anti-aggregatory effects of NO₂⁻ (1 mM; n=16) and SNP (10 μM; n=13) in arterial and venous whole blood samples



	Venous	Arterial
Mean ± SEM	5.8 ± 0.5	6.9 ± 0.6

Figure 4.10 Baseline aggregatory response to ADP (2.5 μM) in arterial vs venous whole blood (n=19).

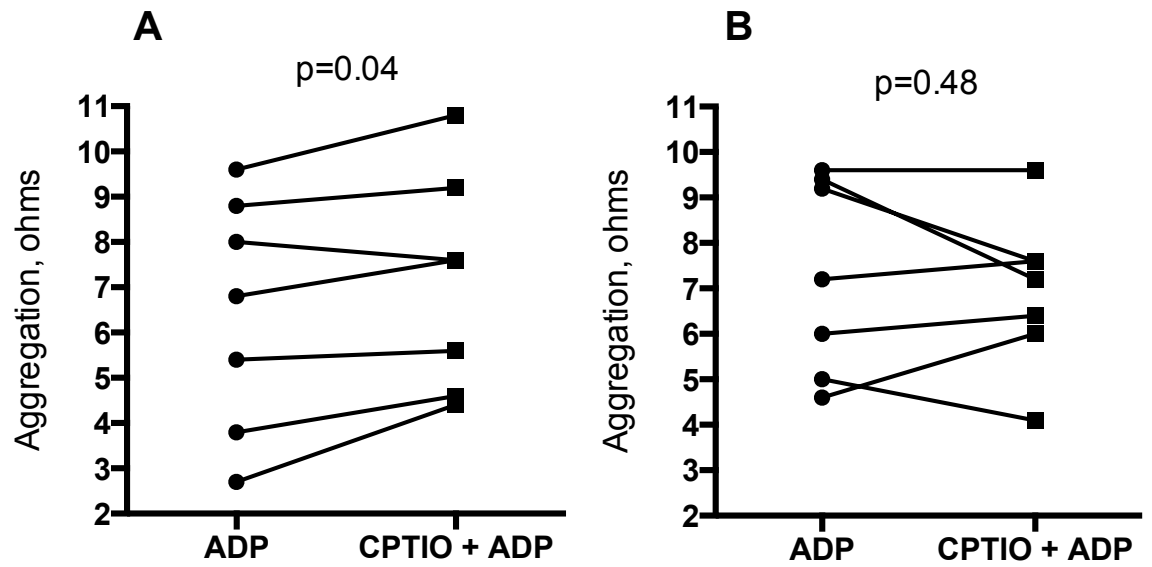


Figure 4.11 Effects of the NO scavenger CPTIO (200 μM) on ADP-induced (2.5 μM) aggregation in (A) venous and (B) arterial whole blood samples ($n=7$ in both groups, paired t -tests)

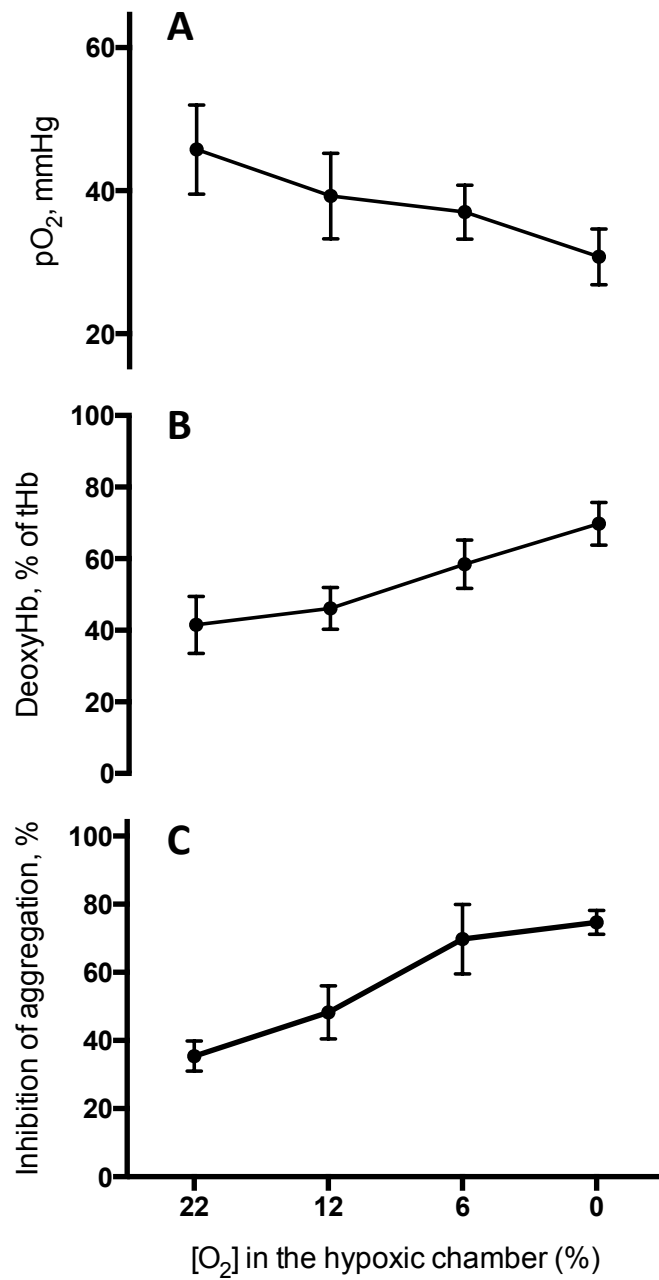


Figure 4.12 Effects of decreasing O₂ concentrations in hypoxic chamber on whole blood. Data analysis via repeated measures ANOVA: -
 (A) actual blood pO₂ (F=21; p=0.01);
 (B) deoxyHb (% of total Hb) (F=89; p<0.001);
 (C) NO₂⁻ -induced inhibition of platelet aggregation (F=6.3; p=0.002) in venous blood samples *in vitro* (n=4 in all cases).

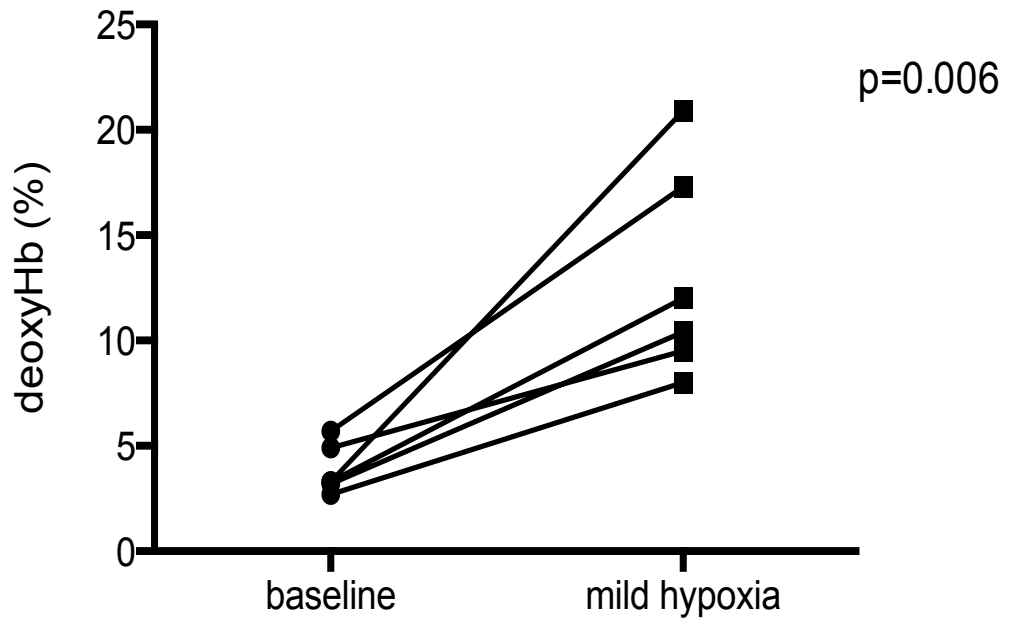


Figure 4.13 Increase in arterial deoxyHb level with mild hypoxia *in vivo* (n=6): patients breathing 12% oxygen gas mixture for 20 minutes.

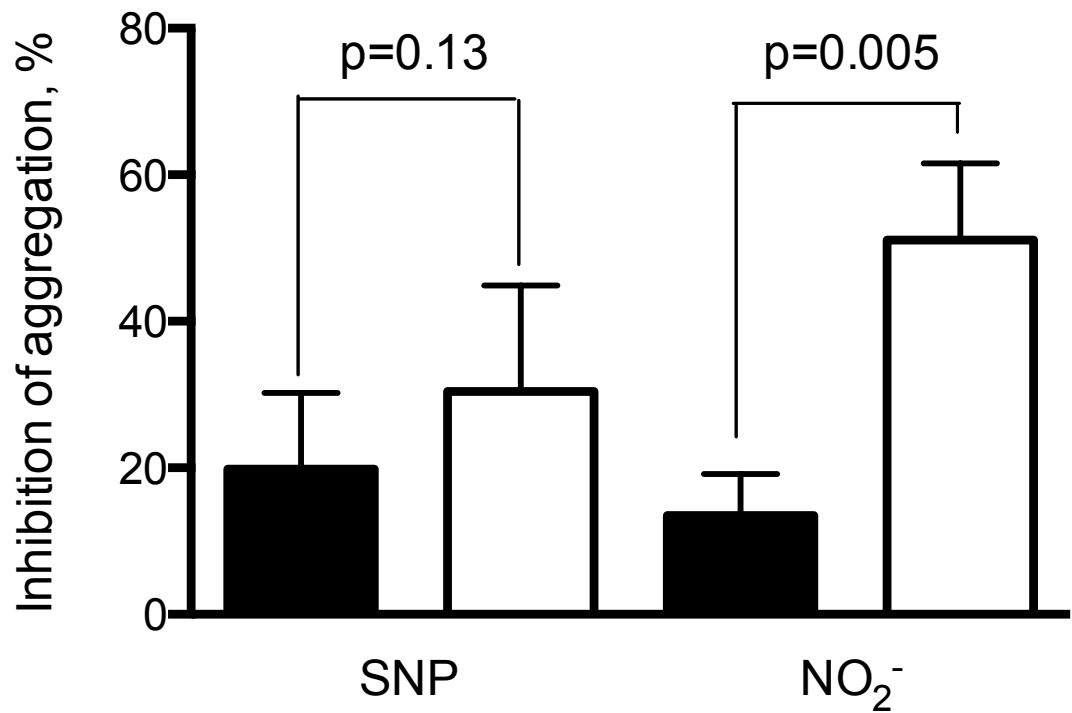


Figure 4.14 Selective potentiation of NO₂⁻ (1 mM) vs SNP (10 μM) anti-aggregatory responses under *in vitro* hypoxia (rendering venous blood more hypoxic in hypoxic chamber) in venous whole blood (n=6-8 for all groups); closed bars = normoxia, open bars = hypoxia

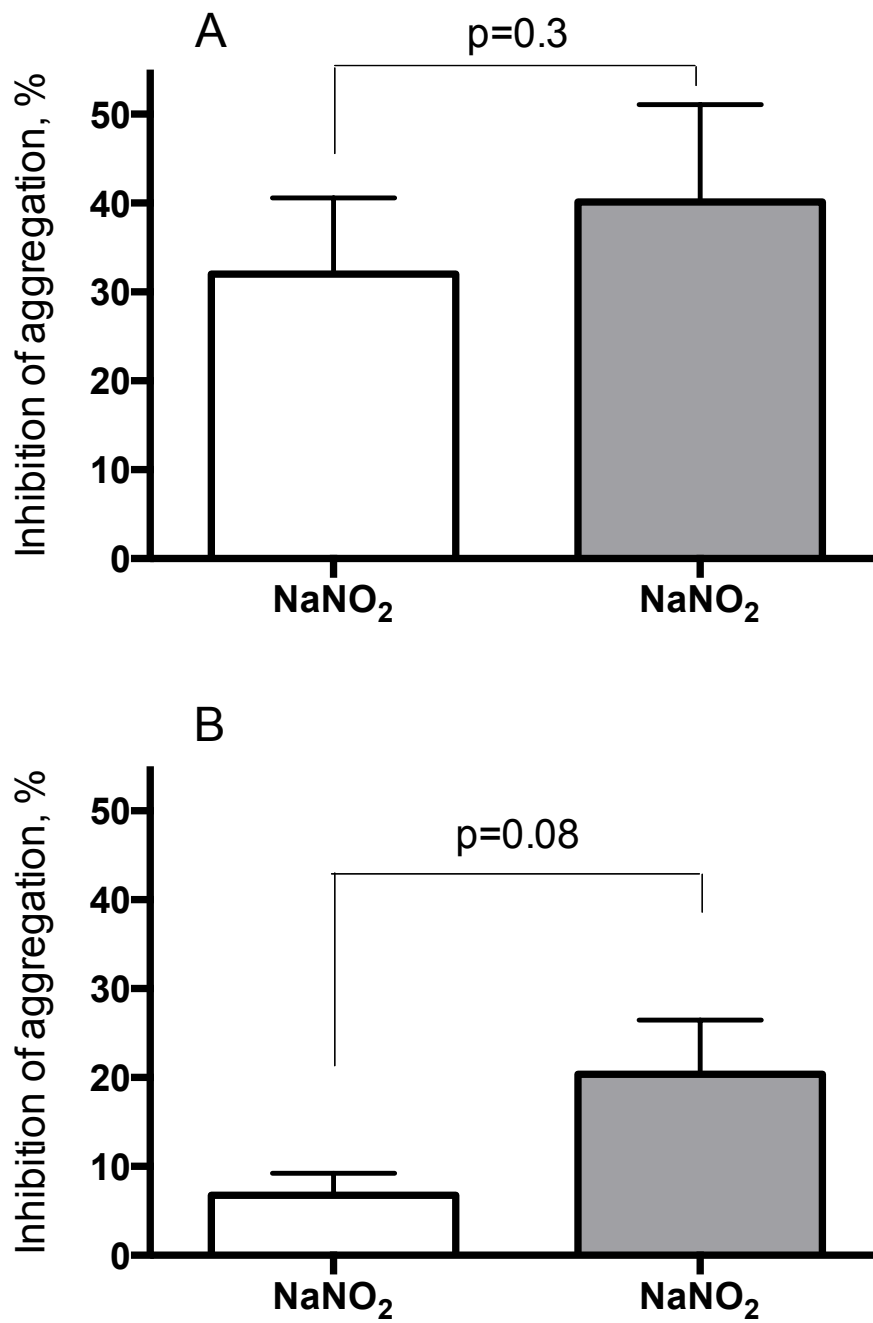


Figure 4.15 Effect of prolonged (2 hours) exposure to room temperature *in vitro* of venous whole blood samples on anti-aggregatory effects of (A) 1 mM, and (B) 100 μM of NaNO₂ (n=8 for both groups, clear bars – at baseline, shaded bars – after 2 hours on the bench).

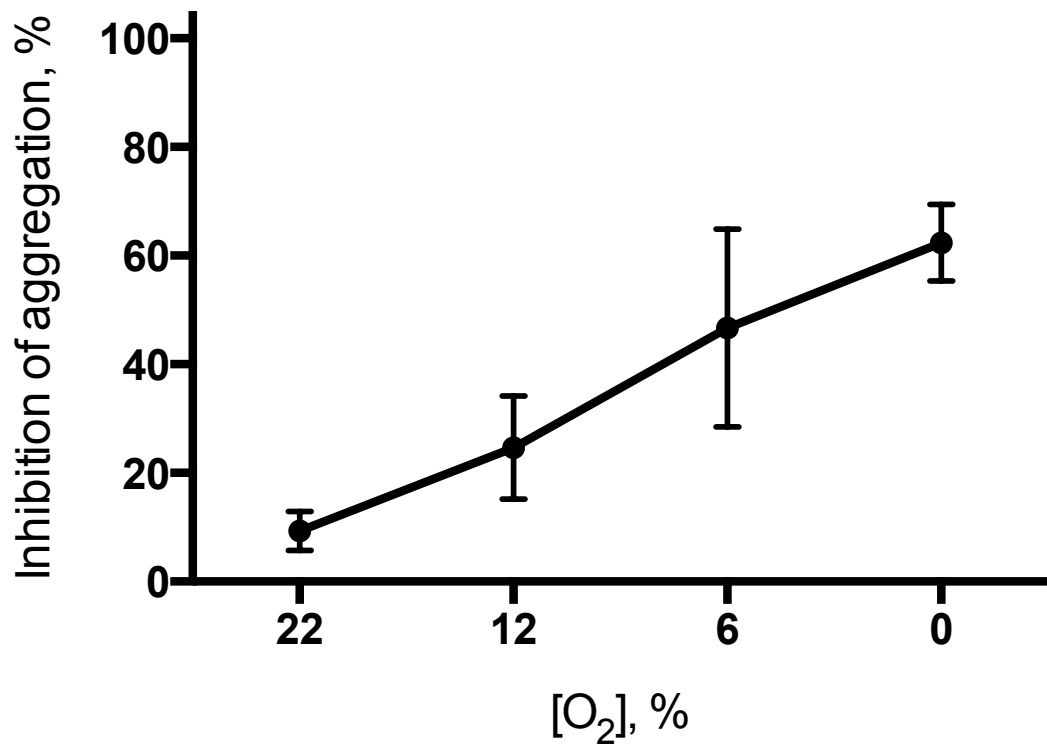


Figure 4.16 Effect of decreasing O₂ concentration in hypoxic chamber on NO₂⁻ (1 mM) anti-aggregatory effects in venous PRP (F=9.4; p<0.001; n=3 for all points)

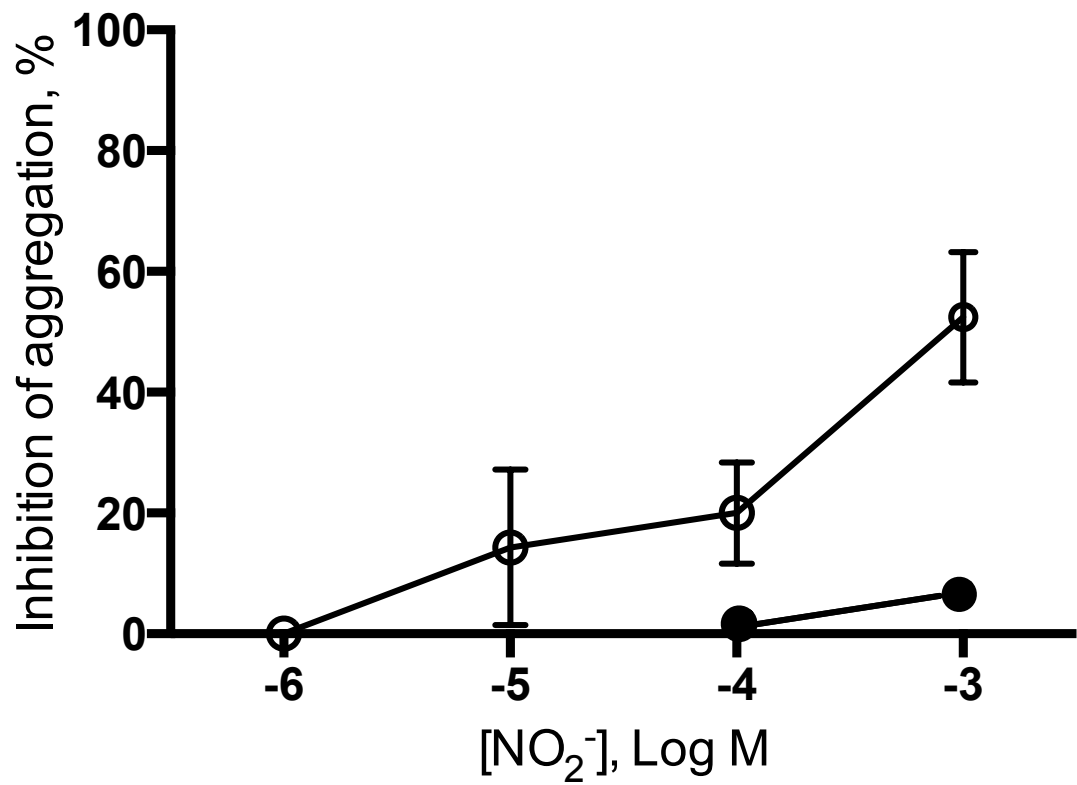


Figure 4.17 Concentration-response curve for NO_2^- in PRP at 0% oxygen concentration in hypoxic chamber (open symbols) and in normoxia (closed symbols) ($p=0.03$, ANOVA with repeated measures for difference between curves).

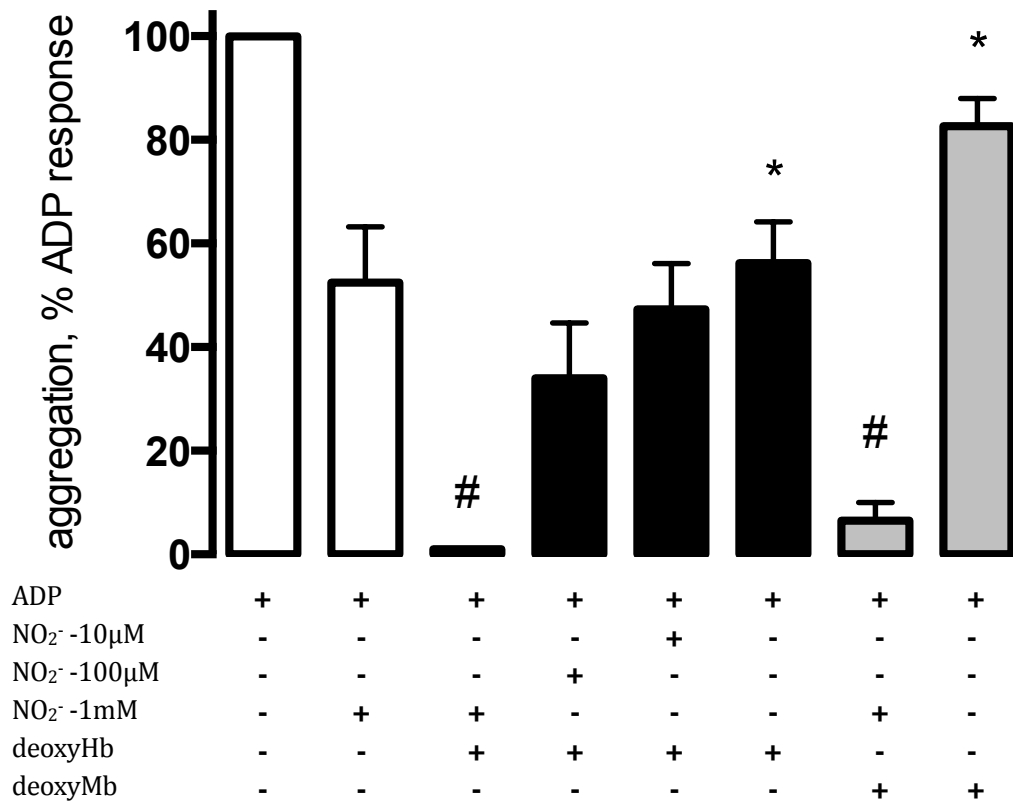


Figure 4.18 Effects of deoxyHb (50µM), deoxyMb (50µM) and NO₂⁻ (10µM, 100µM and 1mM) on ADP (2.5µM) induced aggregation in PRP in hypoxia (zero O₂ concentration in hypoxic chamber), n=3-5 (* p<0.05 vs ADP, # p<0.01 vs ADP/NO₂⁻ 1mM, p<0.0001, ANOVA with repeated measures).

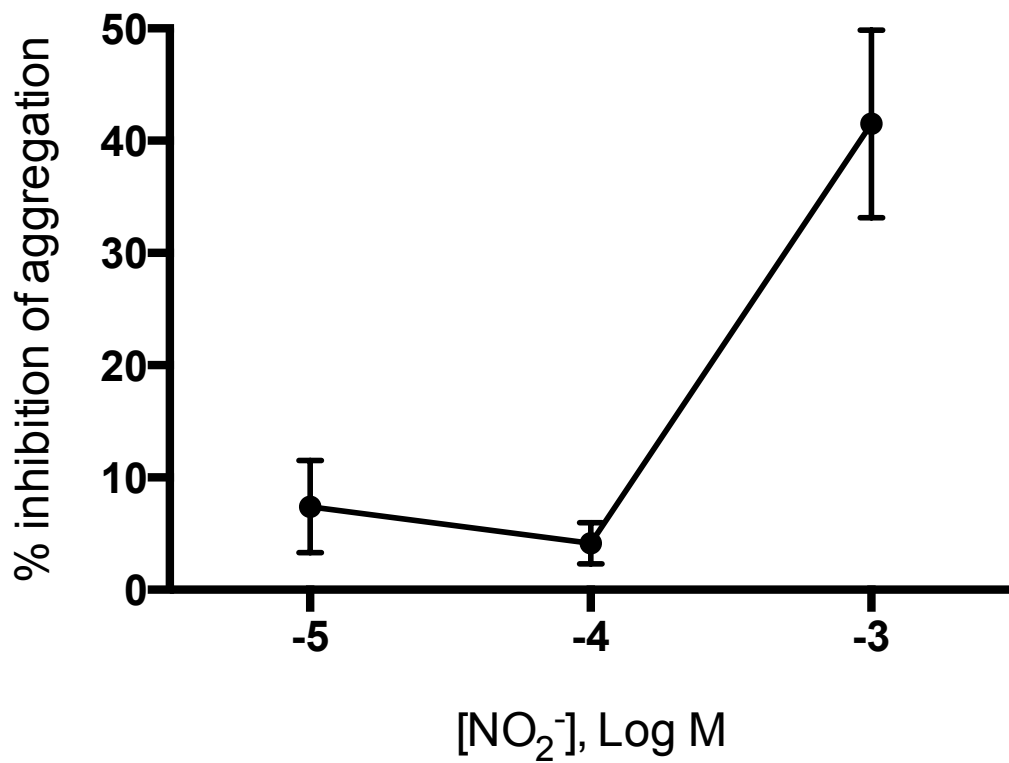


Figure 4.19 Inhibitory effects of NO₂⁻ on ADP-induced (2.5μM) platelet aggregation in whole blood from healthy subjects (n=5). NO₂⁻ in concentrations 0.1μM and 1μM produced no inhibition (not shown); p=0.0002, ANOVA.

**Chapter 5: Nitroxyl effects in
vasculature and platelets:
circumvention of nitric oxide resistance**

5.1 Summary of the study

5.1.1 Objectives

Impaired platelet responsiveness to nitric oxide (NO resistance) is a common characteristic of many cardiovascular disease states and represents an independent risk factor for cardiac events and mortality. NO resistance reflects both scavenging of NO by superoxide (O_2^-), and impairment of the NO receptor, soluble guanylate cyclase (sGC). There is thus an urgent need for circumvention of NO resistance in order to improve clinical outcomes. Nitroxyl (HNO), like NO, produces vasodilator and anti-aggregatory effects, largely via sGC activation, but is not inactivated by O_2^- . We tested the hypothesis that HNO circumvents NO resistance in human platelets. In addition, we assessed vasodilator properties of HNO in human saphenous veins *in vitro*.

5.1.2 Methods

In 57 subjects with or without ischaemic heart disease, platelet responses to the HNO donor isopropylamine NONOate (IPA/NO) and the NO donor sodium nitroprusside (SNP) were compared. Vasodilator properties of the HNO donor IPA/NO relative to SNP effects were assessed *in vitro* in saphenous veins of 5 patients undergoing CABG surgery.

5.1.3 Results

While SNP (10 μ M) induced 29 \pm 3% ($p<0.001$) inhibition of platelet aggregation, IPA/NO (10 μ M) caused 75 \pm 4% inhibition ($p<0.001$). In NO-resistant subjects ($n=28$), the IPA/NO:SNP response ratio was markedly increased ($p<0.01$), consistent with partial circumvention of NO resistance. Similarly, cGMP accumulation in platelets was greater ($p<0.001$) with IPA/NO than with SNP stimulation. The NO scavenger carboxy-PTIO (CPTIO, 200 μ M) inhibited SNP and IPA/NO responses by 92 \pm 7% and 17 \pm 4% respectively ($p<0.001$ for differential inhibition), suggesting that effects of IPA/NO are only partially NO-mediated. ODQ (10 μ M), an inhibitor of sGC suppressed IPA/NO responses by 36 \pm 8 % ($p<0.001$), consistent with a contribution of sGC/haem to IPA/NO inhibition of aggregation. There was no significant relationship between whole blood reactive

oxygen species (ROS) content and IPA/NO responses. IPA/NO produced significant vasodilator effects, on equimolecular basis, more powerful than SNP. These vasodilator effects were at least partially sGC-mediated and potentially NO-mediated.

5.1.4 Conclusions

The HNO donor IPA/NO substantially circumvents platelet NO resistance while acting, at least partially, as a haem-mediated sGC activator. IPA/NO is also a potent vasodilator with partial sGC-mediated vasodilatation.

5.2 Introduction

Nitric oxide (NO) is a pivotal modulator of numerous physiological effects. The intracellular receptor for NO is soluble guanylate cyclase (sGC), which catalyses the formation of cGMP. This in turn leads to activation of protein kinase G (PKG) and phosphorylation of vasodilator-stimulated phosphoprotein (VASP) (for review see (Lohmann & Walter 2005)). It has not been fully appreciated until recently that dysfunction of this signaling pathway occurs under conditions of oxidative stress, and is manifested in many cardio-vascular disease states. This problem, termed “NO resistance” (Chirkov et al. 2001), is manifested in NO-responsive tissues, notably in blood vessels (Worthley et al. 2007) and platelets (Chirkov & Horowitz 2007). Scavenging of NO by superoxide anion radical and impairment of sGC were identified as component mechanisms for NO resistance in human platelets [(Lohmann & Walter 2005), (Chirkov, Chirkova & Horowitz 1996), (Chirkov et al. 1999), (Chirkov et al. 2004)].

The pathophysiological and therapeutic implications of this phenomenon are extensive. First, NO resistance cannot be corrected by administration of NO donors (e.g. organic nitrates) (Chirkov & Horowitz 2007). Second, NO resistance may be a cause of net platelet hyperaggregability and, consequently, thrombosis (Chirkov & Horowitz 2007). Finally, NO resistance represents an independent risk factor for cardiovascular morbidity and mortality (Schachinger, Britten & Zeiher 2000),

(Willoughby, S.R. et al. 2005). A variety of drugs have been shown to be useful in ameliorating this problem, including ACE inhibitors (Chirkov et al. 2004), (Willoughby, S. R. et al. 2012), and the metabolic anti-ischaemic agent perhexiline (Willoughby, S. R. et al. 2002). While these therapies may ameliorate NO resistance, they do not circumvent it. Therefore these therapeutic strategies offer no benefit in circumstances requiring emergency treatment to activate NO signaling-related mechanisms. Examples might include acute heart failure and/or myocardial ischaemia. Thus, there is an urgent need for agents which may circumvent NO resistance.

Recent reports have implicated nitroxyl (NO^-) as another nitrogen oxide capable of activating sGC [(Ellis, A, Li & Rand 2000), (Wanstall et al. 2001), (Bermejo et al. 2005), (Irvine, Favalaro & Kemp-Harper 2003), (Miller, TW et al. 2009)]. *In vivo*, nitroxyl is thought to exist in its protonated form (HNO); it has no unpaired electron, and because of that is resistant to scavenging by O_2^- under conditions of increased O_2^- release. Therefore, HNO donors might be expected to induce continued sGC stimulation despite oxidative stress, commonly associated with cardio-vascular diseases.

A series of studies of the last decade [(Paolocci et al. 2003), (Paolocci et al. 2001), (Ritchie et al. 2009) (Tocchetti et al. 2007), (Lin et al. 2012)] have shown in animal models that HNO exerts a number of clinically relevant physiological effects: it suppresses cardiomyocyte hypertrophy, via stimulation of sGC, and elicits cGMP-dependent vasodilatation; HNO also suppressed O_2^- generation (Miller, AA et al. 2013). In murine platelets, it has been recently shown (Bullen, M. L., Miller, Dharmarajah, et al. 2011), that the HNO donor isopropylamine-NONOate (IPA/NO) induces concentration-dependent inhibition of platelet aggregation, which is predominantly sGC/cGMP dependent. Importantly, in hypercholesterolaemic mice, manifesting NO resistance, the anti-aggregatory effect of IPA/NO was preserved. Similar results obtained by others in vascular studies in animal models also suggest that either endogenous (Leo, CH et al. 2012), or exogenous (Wynne et al. 2012) HNO may circumvent NO resistance. The potential advantages of HNO donors for the emergency treatment of cardiovascular crises

associated with NO resistance also include their lack of cross-tolerance with organic nitrates (Irvine, Kemp-Harper & Widdop 2011).

The current human study was undertaken in order to evaluate the potential efficacy of the HNO donor IPA/NO as a possible means of circumventing NO resistance. We assessed anti-aggregatory effects of IPA/NO, in comparison with the NO donor sodium nitroprusside (SNP), in blood samples obtained from subjects exhibiting different extent of NO resistance. Furthermore, we assessed *in vitro* vasodilator properties of IPA/NO, with mechanistic aspects of this process in comparison with SNP.

5.3 Objectives of the study

The study was designed to assess effects of the HNO donor IPA/NO on ADP-induced human platelet aggregation and its vasodilator properties on human saphenous vein *in vitro*. For that purpose, a cohort comprising patients with ischaemic heart disease and healthy adults, with a full spectrum of NO responsiveness, was evaluated. The following hypotheses were tested in this study:

Primary

Nitroxyl circumvents NO resistance in human platelets when compared to the direct NO donor SNP.

Secondary

Nitroxyl exerts potent vasodilator properties in human saphenous veins.

5.4 Methods

5.4.1 Study population

In order to evaluate a group of individuals with variable platelet responsiveness to NO, we combined healthy subjects and patients with ischaemic heart disease, given that the latter have a high prevalence of NO resistance (Chirkov et al. 2001), (Chirkov et al. 1999), (Willoughby, S. R. et al. 2012). The only exclusion criterion was current therapy with ADP receptor antagonists, given that this would preclude assessment of reversal of ADP induced aggregation by NO and HNO donors.

For the vascular component of this study, patients undergoing coronary artery bypass grafting surgery were recruited. Discarded segments of saphenous veins were utilized for *in vitro* study of HNO vasodilator effect.

The protocols were approved by the Ethics of Research Committee of The Queen Elizabeth Hospital and informed consent was obtained prior to study entry.

5.4.2 Study protocol of anti-aggregatory effects of HNO in human platelets

The experimental studies consisted of the following components:

- 1) A comparison of the effects of the HNO donor IPA/NO and of the NO donor SNP in inhibiting platelet aggregation in whole blood and in platelet-rich plasma.
- 2) An evaluation of the putative mechanisms underlying differential platelet responsiveness to these NO and HNO donors. Specifically:
 - (a) In order to test the hypothesis that differences in response might result from differential scavenging (specifically NO, but not HNO) by reactive oxygen species (ROS), we quantitated whole blood total ROS and correlated responses with this parameter.
 - (b) In order to determine whether responses to SNP and IPA/NO might be modulated by NO and/or HNO release, we examined the impact of co-incubation with carboxy-PTIO (CPTIO, 200 μ M), as a widely accepted NO scavenger (Akaike et al. 1993; Irvine, Favalaro & Kemp-Harper 2003) and L-cysteine (300 μ M and 3mM), as a HNO scavenger (Ellis, A, Li & Rand 2000; Pino & Feelisch 1994).
 - (c) In order to determine to what extent responses were dependent on haem-mediated sGC stimulation, the effects of inhibition of sGC with ODQ (10 μ M)

(Garthwaite et al. 1995) were evaluated in platelet-rich plasma (PRP) as previously described (Chirkov et al. 1999).

(d) In order to evaluate the relationship between cGMP accumulation, and anti-aggregatory responses, we assayed intraplatelet cGMP content after incubation of PRP with SNP and IPA/NO in presence of 3-isobutyl-1-methylxanthine (IBMX), non-specific phosphodiesterase inhibitor.

In this study we used SNP as a reference NO releasing drug, because its effects on platelet aggregation are predominantly cGMP dependent, in contrast to other NO donors (e.g. DEA-NONOate and S-nitrosoglutathione) which are capable of initiating cGMP-independent pathways (Sogo et al. 2000).

5.4.3 Vascular *in vitro* studies protocol

The vascular studies comprised the following:

1) A comparison of the vasodilator effects of the HNO donor IPA/NO and of the NO donor SNP in segments of human saphenous veins *in vitro*.

2) An investigation of the mechanisms underlying vasodilator properties of IPA/NO. Specifically:

(a) In order to determine whether responses to SNP and IPA/NO might be modulated by NO and/or HNO release, we examined the impact of co-incubation with carboxy-PTIO (CPTIO, 200 μ M) and hydroxocobalamin (100 μ M), as a widely accepted NO scavengers and L-cysteine (3 mM), as a HNO scavenger.

(b) In order to assess to what extent vasodilator responses are sGC mediated, the vessels rings were pre-incubated with ODQ (10 μ M) before applying IPA/NO.

Similarly to platelet aggregation studies, the reference compound for IPA/NO was the NO donor SNP.

5.4.4 Platelet aggregation studies

Blood samples were collected by venesection from an antecubital vein into plastic tubes containing 1:10 volume of acid citrate anticoagulant (two parts of 0.1 M citric

acid to three parts of 0.1 M trisodium citrate); acidified citrate was utilized to minimize deterioration of platelet function during experiments (Kinlough-Rathbone, Packham & Mustard 1983). Blood was centrifuged at 250 g for 10 minutes at room temperature to obtain PRP. Platelet aggregation was examined utilizing a dual-channel impedance aggregometer (Model 560, Chrono-Log, Pennsylvania, USA) as described previously (Chirkov et al. 1999). In brief, tests were performed at 37°C and stirring speed of 900 rpm. Samples of whole blood or PRP were diluted twofold with normal saline (final volume 1 ml) and pre-warmed for 5 min at 37°C. Aggregation was induced with ADP (final concentration of 2.5 µM). Aggregation was monitored continually for 7 min, and responses were recorded for electrical impedance in ohms. Both SNP and IPA/NO (final concentration of 10 µM) were added to samples 1 min before ADP. Duration of pre-incubation with SNP was estimated as that which was optimal in our previous experiments ((Chirkov et al. 2001), (Chirkov, Chirkova & Horowitz 1996), (Chirkov et al. 1999)). IPA/NO has a half-life of ~2.5 minutes under physiological conditions (pH 7.4, 37°C) (Maragos et al. 1991), therefore 1 min of pre-incubation time was also applied. Inhibition of aggregation was evaluated as a percentage comparing the extent of maximal aggregation in the presence and absence of the anti-aggregatory agent studied.

5.4.5 Vascular studies

During the CABG surgery, the discarded segments of the proximal saphenous vein (SV) were collected. The vessels were placed in ice-cold Krebs solution for transportation to the laboratory. Once cleaned, vessels were cut into 2 mm-wide rings. These segments were suspended under tension in 15-mL organ baths containing Krebs solution at 37°C. Resting tension was normalized for internal diameter, as previously described (He, Angus & Rosenfeldt 1988; Sage et al. 2000). The vascular rings were equilibrated for 60 minutes before exposure to KCl solution; rings contracting <1 g were discarded. After a further 30 minutes of washout, the rings were contracted with increasing concentrations of phenylephrine (0.01 to 100µM). After a further 45 minutes of washout, the rings were precontracted with phenylephrine to produce 70% of maximum tension in the

vessel. Once the contractile response had reached a plateau, each ring was exposed to increasing concentrations of SNP (0.001 to 100 μ M) or IPA/NO (0.001 to 100 μ M). Time from vessel harvest to the assessment of relaxant responses was held constant at 3 hours. In some mechanistic experiments, the vascular rings were preincubated with CPTIO (200 μ M), hydroxocobalamin (100 μ M), or ODQ (10 μ M).

5.4.6 ROS measurements

Blood samples were collected by venipuncture into lithium heparin tubes. Quantitation of total reactive oxygen species (ROS) in whole blood was performed utilizing electronparamagnetic resonance (EPR) spectroscopy, as previously described (Mariappan et al. 2009). All EPR samples were prepared using 20mM Krebs HEPES buffer (pH 7.4) in a final concentration 25 μ M of deferoxamine, 2 μ M of diethyldithiocarbamate (chelating agents), 200 μ M of the spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) and placed in 50 μ L glass capillaries (Corning, NY). The EPR spectra were recorded using an e-scan M EPR spectrometer (Bruker Bio-Spin e-scan, Rheinstetten/Karlsruhe, Germany) and super-high Q microwave cavity with the following settings: field sweep, 10 G; microwave frequency, 9.75 GHz; microwave power, 19 mW; modulation amplitude, 2 G; conversion time, 10.24 ms; time constant, 40.96 ms; receiver gain, 3.2 x 10². ROS content was expressed as EPR signal intensity in arbitrary units. The coefficient of variability for replicate estimates was 4%.

5.4.7 cGMP studies

Intraplatelet cGMP content was assayed as described previously (Chirkov et al. 1999). Briefly, platelet-rich plasma (0.5 mL) was incubated at 37°C with SNP (10 μ M) and IPA/NO (10 μ M) for 1 minute, as in aggregation studies. IBMX (0.5 mM) was added to plasma 5 minutes before SNP. After incubation, plasma was filtered

through GF/C Glass Microfibre Filters (Whatman) for harvesting the platelets. Filters with absorbed platelets were rinsed with physiological saline and placed into 0.5 mL of 4 mM EDTA for further extraction of cGMP in a boiling water bath for 5 minutes. After centrifugation of samples at 3000 g for 10 minutes, cGMP concentration in supernatant was estimated using cGMP [¹²⁵I] assay system (Biomedical Technologies Inc., MA, USA). Results were expressed as increase (%) in intraplatelet cGMP accumulation in comparison with IBMX alone.

5.5 Data analysis

Comparisons of subject characteristics were performed using chi-square and Fisher's exact test as appropriate. Comparison of SNP and IPA/NO effects on platelet aggregation and cGMP generation involved paired analysis. The relative effects of CPTIO and ODQ on responses to SNP and IPA/NO were compared utilizing non-paired t-test. Evaluation of the ROS : IPA/NO and ROS : SNP response relationships utilized linear regression. The limit of statistical significance was set 0.05 (two-tailed) throughout. Data are expressed as mean ± SEM.

5.6 Results

5.6.1 Patients/normal subjects characteristics

Platelet studies

Individuals with or without ischaemic heart disease (IHD), aged 66±16 years (n = 57) were studied. Patients (n = 37) were assessed during hospital admission for elective cardiac catheterization, or admission due to unstable myocardial ischaemia. 20 subjects participated as healthy controls. It was anticipated on the basis of the previous studies (Chirkov et al. 2001), (Chirkov et al. 1999), (Willoughby, S. R. et al. 2012) that this strategy of patients/subject inclusion would result in a wide spectrum of platelet responsiveness to NO. The population was anticipated to include a substantial number of subjects among the IHD subgroup whose platelets were severely hypo-responsive to NO and NO donors (“NO resistance”) and indeed

the presence of NO resistance (response < 30% inhibition of ADP-induced aggregation) in some of the apparently healthy cohort was possible (Chirkov et al. 2001; Dautov et al. 2013). As illustrated in Table 5.1, the presence of NO resistance was not associated with any significant differences in demographics or treatment profile in the subject/patient cohort examined, except the history of diabetes mellitus (p=0.01). Consistent with previous findings, NO resistance occurred more commonly in elderly individuals, patients with IHD and hypertensives (Chirkov & Horowitz 2007) although none of these trends reached statistical significance. This might perhaps have reflected therapy with agents such as ACE inhibitors (Chirkov et al. 2004; Willoughby, S. R. et al. 2012).

Vascular work

Patients undergoing CABG surgery, aged 60 ± 7 years (n = 6, all males) were consented to participate in the study. One patient's vessel was utilized in the validation experiment comparing IPA/NO effect with "classical" HNO donor Angeli's salt. The other 5 patients were studied according to the general experimental protocol (as in 5.4.5). Patient characteristics are listed in Table 5.2.

5.6.2 Circumvention of NO resistance

As shown in Figure 5.1, mean responses to SNP (10 μ M) and IPA/NO (10 μ M) in cohorts of individuals including some normal subjects and others with known coronary disease, were $29 \pm 3\%$ and $75 \pm 4\%$ of inhibition of aggregation respectively (p<0.0001).

Importantly, 49% of patients had responses to SNP of <30%, representing the equivalent of NO resistance relative to norms (Chirkov et al. 2001; Willoughby, S.R. et al. 2005). Therefore platelet responsiveness to SNP was categorized relative to the mean response in the heterogeneous population evaluated, comparisons being made between individuals with <30% vs $\geq 30\%$ inhibition of platelet aggregation in response to SNP (Fig. 5.2). The relationship between responsiveness to SNP and to

IPA/NO was fitted to a hyperbolic function (Fig. 5.2) and comparisons made of responsiveness for NO-resistant and NO-responsive patients via IPA/NO:SNP ratios (Fig. 5.3). From both of these analyses it was apparent that many NO-resistant patients were fully responsive to IPA/NO, and that differences between SNP and IPA/NO responses reflected primarily the greater responses to IPA/NO in NO-resistant individuals. In these subjects, even with reduced IPA/NO responsiveness, the IPA/NO:SNP response ratio (Fig. 5.3) was markedly increased ($p < 0.01$), consistent with partial circumvention of NO resistance.

5.6.3 Mechanisms underlying responses to IPA/NO

Decomposition of IPA/NO is pH-dependent: at pH 8 and above IPA/NO is primarily a HNO donor, with transition to NO donor at lower pH. Importantly, at pH 5–8 IPA/NO functions as a dual donor of HNO and NO (Miranda et al. 2005), (Salmon et al. 2011). To delineate the possible role of the NO component in the currently described anti-aggregatory effects of IPA/NO, we used the NO scavenger CPTIO (Akaike et al. 1993). In the presence of CPTIO, SNP responses were inhibited by $92 \pm 7\%$ ($p < 0.01$). However, there was only minor ($17 \pm 4\%$, $p = 0.01$) reduction in the anti-aggregatory effects of IPA/NO (Fig. 5.4). Thus the inhibitory effects of CPTIO were selective ($p < 0.001$) for the NO donor SNP. These data therefore suggest that effects of IPA/NO are predominantly HNO-mediated.

The haem-dependent inhibitor of sGC ODQ (Garthwaite et al. 1995) in a concentration of $10\mu\text{M}$ attenuated responses to both SNP and IPA/NO (Fig. 5.5), although the extent of this inhibition was significantly greater for SNP ($p < 0.03$). These data therefore suggest that the anti-aggregatory effect of IPA/NO is partially mediated by haem-dependent sGC activation.

We attempted to delineate the role of HNO release as a component of IPA/NO effect using the HNO scavenger L-cysteine (Ellis, A, Li & Rand 2000; Pino & Feelisch 1994). However, when L-cysteine ($300\mu\text{M}$ and 3mM) was co-incubated with ADP ($2.5\mu\text{M}$), both in platelet-rich plasma and whole blood, it exerted an intrinsic anti-aggregatory effect, which precluded this assessment.

The relationship between baseline whole blood total ROS content and responsiveness to SNP and IPA/NO is shown in Figure 5.6. There was a non-significant trend, towards a negative relationship between whole blood ROS and SNP responses. Regarding IPA/NO responses, this relationship was in the form of horizontal line (Fig. 5.6), suggestive of no influence of ROS content on IPA/NO responses.

As a measure of sGC activity, we assessed intraplatelet cGMP content in the presence of the phosphodiesterase inhibitor IBMX at baseline and after incubation of platelet-rich plasma with SNP and IPA/NO. cGMP accumulation in platelets was greater ($p < 0.001$) with IPA/NO than with SNP stimulation. As shown in figures 5.7 and 5.8, maximal response to SNP was a nearly 4-fold increase in cGMP generation, while with IPA/NO this was nearly 20-fold (median/mean response was 10-fold). With IPA/NO (Fig. 5.8), in the majority of the experiments the anti-aggregatory effects were very strong. Data points clustered in the range of 80 to 100% inhibition of aggregation, thus precluding the assessment of the relationship between cGMP generation and inhibition of aggregation by IPA/NO. cGMP accumulation in response to SNP varied directly with extent of inhibition of aggregation (Fig. 5.7) but no similar relationship was seen with IPA/NO responses (Fig. 5.8; Table 5.3).

5.6.4 Anti-aggregatory effects of SNP and IPA/NO in the presence of diabetes mellitus

We analyzed the differences in anti-aggregatory effects of SNP and IPA/NO in the presence and absence of diabetes mellitus. In whole blood samples of patients with diabetes ($n=21$), SNP had a weak anti-aggregatory effect ($20 \pm 5\%$) (Fig. 5.9), as platelets of diabetic patients are prone to NO resistance (Worthley et al. 2007). Anti-aggregatory effects of SNP were significantly ($p=0.03$) stronger in subjects without diabetes ($34 \pm 4\%$). No stratification for the greater relationship between blood glucose levels and SNP response was undertaken.

Anti-aggregatory effects of IPA/NO were 2-3 fold greater than those of SNP and did not significantly differ between diabetics and non-diabetics ($70 \pm 5\%$ and $79 \pm 4\%$, respectively). Thus, the HNO donor (IPA/NO) demonstrated powerful anti-aggregatory properties regardless of NO resistance (Fig. 5.9).

5.6.5 Impact of blood oxygenation on anti-aggregatory response of IPA/NO

Similarly with the experiments described for nitrite in Chapter 4, the impact of site of blood sampling was evaluated for IPA/NO in 6 subjects. In each case, paired arterial and venous blood samples were obtained.

The results indicated that while ADP-induced aggregation did not vary according to sampling site (data not shown) there was significantly greater ($p < 0.01$) inhibition of aggregation with IPA/NO in venous than arterial blood, as shown in Fig. 5.10.

These data therefore imply that the bio-activation of IPA/NO, like that of NO_2^- , may be potentiated by hypoxia. The precise mechanism of this potentiation was not explored in the current studies.

5.6.6 Vasodilator effect of IPA/NO in saphenous veins

Experiments in saphenous veins ($n=5$; for patient characteristics see Table 5.2) revealed more potent ($\log EC_{50} -6.7 \pm 0.1$) vasodilator effects of the HNO donor IPA/NO compared to the NO donor SNP ($\log EC_{50} -7.9 \pm 0.1$) (Fig. 5.11). Preincubation of SV rings with the NO scavengers CPTIO and hydroxocobalamin shifted SNP concentration-response curves to the right (from $\log EC_{50} -7.9 \pm 0.1$ for SNP to -7.1 ± 0.1 for CPTIO; $p = 0.06$, and -5.7 ± 0.1 for hydroxocobalamin; $p = 0.01$) (Fig. 5.12A). This confirmed that the vasodilator effect of SNP is NO-mediated. Both NO scavengers had only minimal (non-significant) inhibitory effects on vasodilator effects of IPA/NO (from $\log EC_{50} -6.7 \pm 0.1$ for IPA/NO to -6.5 ± 0.2 for CPTIO; $p = 0.6$, and -5.6 ± 0.7 for hydroxocobalamin; $p = 0.1$) (Fig 5.12B). This suggests that NO release contributes minimally to the vasodilator effects of IPA/NO.

After inhibiting sGC with ODQ, vasodilator responses to SNP almost disappeared, signifying the fact that the vasodilator effects of SNP are NO/haem-mediated (Fig. 5.13, left). With IPA/NO, there was a two orders of magnitude right-ward parallel shift of the concentration-response curve ($p < 0.0001$), with vasodilator effect of IPA/NO still substantial (Fig. 5.13, right). This suggests that the physiological effect of IPA/NO is predominantly NO/haem-independent, although haem-dependent component also exists.

5.7 Discussion

The results of this work provide evidence that the nitroxyl donor IPA/NO can partially circumvent NO resistance in human platelets. While there are theoretical reasons on the basis of previous animal studies [(Bullen, M. L., Miller, Andrews, et al. 2011), (Bullen, M.L. et al. 2012)] to suggest that this would occur, this is the first demonstration of circumvention of NO resistance in humans.

Resistance to NO in humans is most easily studied in platelets utilizing *in vitro* evaluation, but also occurs in leukocytes and other NO-responsive tissues, including blood vessels, and myocardium [for reviews see: (Chirkov & Horowitz 2007), (Ritchie et al. 2009)]. NO resistance represents both an independent marker of increased cardiovascular risk ((Willoughby, S.R. et al. 2005), (Schachinger, Britten & Zeiher 2000)) and also a therapeutic target. NO resistance limits utility of organic nitrates, even on *de novo* basis (Armstrong, PW, Armstrong & Marks 1980), and potentially of nitrite (Maher et al. 2013). To date, all intervention studies have been directed at restoration of normal functioning of the NO/sGC cascade, a strategy which can be termed amelioration of NO resistance. This has been achieved via correction of hyperglycaemia (Worthley et al. 2007), use of ACE inhibitors (Chirkov et al. 2004), (Willoughby, S. R. et al. 2012), perhexiline (Willoughby, S. R. et al. 2002) and possibly statins (Stepien et al. 2003). However, the implications of such strategies relate to chronic disease management. A residual

clinical problem is posed by the occurrence of NO resistance in association with cardiovascular emergencies such as acute heart failure and acute myocardial ischaemia/infarction (Chirkov & Horowitz 2007). Under these circumstances a therapy which is effective despite NO resistance is desirable. The current data suggest that HNO donors might to some extent solve this problem.

In the current study, we evaluated a “mixed” cohort of individuals including both normal subjects and patients with known IHD. Therapy with ACE inhibitors, which is known to ameliorate NO resistance (Chirkov et al. 2004; Willoughby, S. R. et al. 2012) was not an exclusion criterion. The result was a wide spectrum of platelet responsiveness to NO, including approximately 50% subjects whose platelets were “NO resistant”, and thus offering a suitable background for evaluation of the potential acute benefits of HNO donor therapy.

In this study, we used SNP, which is a well-documented NO donor known to engage predominantly cGMP-dependent pathways, which therefore can be used as a reference drug when studying sGC-mediated physiological effects, in the current case – inhibition of platelet aggregation. We have demonstrated that subjects, in whom responsiveness to the SNP was intact, manifested uniformly good responses to IPA/NO (Figs. 5.2 and 5.3). In the presence of NO resistance (poor response to SNP), platelet responses to IPA/NO, although heterogeneous, were substantial, representing partial circumvention of NO resistance. We have previously demonstrated that SNP responses are modulated by rates of O_2^- generation in whole blood ((Chirkov et al. 1999), (Worthley et al. 2007)). However in the current study total ROS content, rather than O_2^- generation rate was measured. This less specific technique failed to show the expected differential relationships between SNP vs IPA/NO responses and total ROS content (Fig. 5.6).

As regards mechanisms of platelet responses, IPA/NO (10 μ M) stimulated platelet sGC (as assessed via intraplatelet cGMP accumulation in the presence of IBMX), substantially more than SNP in equimolecular concentrations (Table 5.3). The effect of SNP, but not IPA/NO, declined in the presence of NO resistance. These results are in accordance with the previous animal studies [(Bullen, M. L., Miller,

Andrews, et al. 2011), (Bullen, M.L. et al. 2012)], which documented sGC stimulation and increased intracellular cGMP with IPA/NO, and showed that these effects were preserved in conditions of experimental NO resistance. The classical HNO donor Angeli's salt also inhibits platelet aggregation and increases intraplatelet cGMP content (Bermejo et al. 2005). In that study, cGMP accumulation in response to 10 μ M Angeli's salt was about 4 times less than with 10 μ M SNP. Taking into account our results (IPA/NO is more potent than SNP, as shown in Figs. 5.5, 5.7 and 5.8), it could be inferred that IPA/NO is more potent than Angeli's salt. However, this has to be examined in a direct experiment. There are differences in the decomposition of these two compounds. While IPA/NO is generally regarded as a HNO donor, at neutral pH in buffer it releases some NO (Miranda et al. 2005; Miranda, Paolocci, et al. 2003; Salmon et al. 2011), which could contribute to cGMP-stimulating effect of HNO. However, our experiments with CPTIO (Fig. 5.4) suggest that the effects of IPA/NO in physiologically relevant circumstances (whole blood) are largely independent of NO release. In the current experimental setting (platelet-rich plasma and whole blood) L-cysteine inhibited platelet aggregation. Therefore we were unable to examine whether this HNO scavenger would cancel IPA/NO effects on platelets. Nevertheless, it has been shown previously in vascular settings that L-cysteine substantially inhibits IPA/NO vasodilatation, confirming that its physiological effects are primarily HNO-mediated (Bullen, M. L., Miller, Andrews, et al. 2011).

We sought to determine whether the effects of IPA/NO were mediated by sGC activation, via its haem moiety. For this purpose we employed ODQ, a sGC-specific inhibitor, which acts by oxidizing sGC haem to a ferric form, which exhibits only poor NO sensitivity (Garthwaite et al. 1995). ODQ dose dependently inhibits sGC activation by NO. Inhibition of sGC by ODQ is NO-competitive. Enzyme activity cannot be restored following extensive dilution, suggesting an irreversible inactivation (Schrammel et al. 1996). However, ODQ-oxidized sGC could be re-reduced by dithionite, and this re-reduced sGC has identical NO-stimulated activity as the original ferrous sGC (Zhao et al. 2000). In our experiments in platelets, ODQ partially inhibited IPA/NO effects. However, this

inhibition was less marked than that of SNP effects (Fig. 5.5). Thus we conclude that inhibition of platelet aggregation by IPA/NO is only partially mediated by sGC-associated ferrous haem.

Numerous investigations with purified sGC from animal tissues showed that ODQ induces a rightward-shift of the concentration–response curves for different NO donors, and a reduction in V_{\max} [for Review see: (Hobbs 1997)]. This inhibition of sGC causes a depression in physiological response to NO donor (Tseng, Tabrizi-Fard & Fung 2000). Sensitivity of sGC activators (SNP or IPA/NO) to ODQ inhibition is a determinant of the extent to which haem-independent mechanisms are involved. Therefore, the pattern of inhibition of haem-dependent functional response would be characterized by a non-parallel shift in the concentration-response curve with progressive depression of maximum response, as in our experiments with SNP in saphenous vein rings (Fig. 5.13, left). We documented an apparent difference in ODQ impact on the vasodilator effects of SNP (NO donor) and IPA/NO (HNO donor). A parallel shift of the curve in the experiments with IPA/NO (Fig. 5.13, right) is indicative of the existence of haem-dependent component for this predominantly HNO donor. Indeed, if there were no haem-mediated component, there would be no target for ODQ, and there will be no alteration of the concentration-response curve. Here, with IPA/NO, ODQ eliminated contribution from haem-mediated component, but most of the physiological effect of IPA/NO was not ferrous haem-mediated.

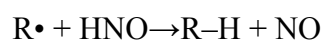
According to current views, the interaction of HNO with sGC may occur at least at two levels: haem and sGC surface cysteines [(Miller, TW et al. 2009), (Fukuto, Cisneros & Kinkade 2013)], analogously to the recently proposed double-site activation of sGC by NO [(Fernhoff, Derbyshire & Marletta 2009), (Fernhoff et al. 2012)]. Indeed, ferrous haem-mediated activation of purified sGC preparation by HNO donors was documented by two groups of researchers [(Miller, TW et al. 2009), (Zeller et al. 2009)]. However, it was not clear whether HNO directly activates this enzyme via coordination to the ferrous haem (Miller, TW et al. 2009), or has to be oxidized to NO first (Zeller et al. 2009). In our study, ODQ-independent component of the IPA/NO effect on platelet aggregation (Fig. 5.5) could be due to interaction with oxidized (ferric) form of sGC, given the general

preference of HNO for binding to ferric (Fe^{3+}) *versus* ferrous (Fe^{2+}) haem groups, with further transition to ferrous nitrosyl haem via reductive nitrosylation (Miranda, Nims, et al. 2003). However, it has been reported by two research groups that HNO donor Angeli's salt does not activate oxidized (ferric) sGC [(Miller, TW et al. 2009), (Zeller et al. 2009)]. It could be argued that experimental results of these studies, obtained with the purified enzyme preparations in buffer and forced enzyme oxidation with 100 μM ferricyanide (Miller, TW et al. 2009) or 100 μM ODQ (Zeller et al. 2009), cannot be directly projected on IPA/NO effects in biological tissues (i.e. platelets and blood vessels). However the current study, physiological by nature, was not equipped to delineate HNO interactions with different red/ox states of platelet sGC.

Effects of HNO on sGC also may involve interactions with the enzyme thiols (Fukuto & Carrington 2011). Experiments utilizing purified enzyme preparation (Miller, TW et al. 2009) revealed that while Angeli's salt in concentrations up to 10 μM activated sGC, high concentrations (100 μM) caused inhibition, which was counteracted by 10 mM dithiothreitol. This observation was attributed to interaction of HNO with the sGC thiols, on the premise that thiol modification inhibits the NO-activated sGC. While, in general, direct oxidation of sGC cysteines (with formation of disulfides) is detrimental for enzyme functionality [(Miller, TW et al. 2009), (Fernhoff et al. 2012)], non-oxidative binding of NO to surface cysteines is beneficial [(Fernhoff, Derbyshire & Marletta 2009), (Fernhoff et al. 2012)]. Thus, further studies are warranted to address thiol-mediated effects of HNO on sGC.

It appears (Figs. 5.2 and 5.3) that NO resistance does not impact on platelet responsiveness to HNO donors, unless NO resistance is extreme, which might result from extensive oxidation and haem depletion of sGC. This may occur in cardiovascular disease states associated with oxidative stress (i.e. ischaemia, heart failure and diabetes) and affects both platelets and vascular tissues (Schmidt, HH, Schmidt & Stasch 2009).

As asserted by Fukuto and co-workers [(Fukuto, Cisneros & Kinkade 2013), (Lopez et al. 2007)], HNO can be regarded as an anti-oxidant, capable of quenching reactive radical species via hydrogen atom donation with consequent release of NO:



Furthermore, HNO donors (Angeli's salt and IPA/NO) while having no direct effect on superoxide levels (generated in a xanthine/xanthineoxidase cell-free assay), suppress superoxide production by vascular Nox2 oxidase (Miller, AA et al. 2013). Both possibilities are worth examining as regards the mechanism of circumvention of NO resistance by IPA/NO, both in platelets and vasculature.

The current findings invite translation to the clinical setting and suggest that HNO donors may be particularly useful in acute cardiovascular disease states associated with NO resistance, such as acute heart failure. In this regard, the positive inotropic, but not chronotropic, effects of HNO donors demonstrated in the scenario of heart failure ((Paolocci et al. 2003), (Tocchetti et al. 2011)) are also attractive when combined with their vasodilatory and anti-aggregatory properties. Another clinically relevant finding is that HNO lacks cross-tolerance with organic nitrates (Irvine, Kemp-Harper & Widdop 2011). New HNO donors have been recently synthesized from acyl nitroso compounds and acyloxy nitroso compounds [(DuMond, Wright & King 2013), (Shoman et al. 2011)], and await appropriate translational investigations.

5.8 Study limitations

The current study has some possible limitations. First, IPA/NO was the only HNO donor examined; it remains to be determined whether the effects vary within this emerging class of drugs. Second, in comparison between platelets from diabetics and non-diabetic subjects, no consideration was taken of current blood glucose levels, although these are known to influence NO response (Worthley et al. 2007). In practice, it is likely that the combined scenario of acute ischaemia or heart failure with poorly controlled diabetes might represent an ideal setting for therapy with HNO donors. Last, comparing NO resistant and NO responsive vessels would be very interesting. For example this could be achieved by dividing patients into diabetic and non-diabetic groups.

5.9 Conclusion

The results of the current study shed new light on the phenomenon of NO resistance and open new horizons regarding its potential management. The study revealed, that:

- (1) The nitroxyl donor IPA/NO can partially circumvent NO resistance in human platelets.
- (2) Anti-aggregatory effects of IPA/NO are partially mediated by sGC-associated haem, and are largely NO-independent.
- (3) The nitroxyl donor IPA/NO is also a potent vasodilator of human saphenous veins *in vitro*.

Circumvention of NO resistance utilizing HNO donors is therefore a concept which is ripe for testing in the clinical arena in settings such as acute myocardial ischaemia and/or heart failure

5.10 Tables and figures for chapter 5

Table 5.1 Subject Characteristics: comparison of demographics according to platelet response to anti-aggregatory response to SNP.

Characteristic	NO resistant (n = 28) *	NO responsive (n = 29)
Men/women, n	12 vs 16	15 vs 14
Age (mean \pm SD), yrs	70 \pm 2	63 \pm 4
Normal subjects, n (%)	7 (25)	13 (45)
Known ischaemic heart disease, n (%)	18 (64)	11 (38)
Diabetes, n (%)	15 (54) #	6 (21)
Hypertension, n (%)	22 (79)	19 (66)
Hypercholesterolaemia, n (%)	23 (82)	18 (62)
Smoking, n (%)	6 (21)	3 (10)
Drugs used		
Aspirin, n (%)	18 (64)	16 (55)
Nitrates, n (%)	13 (46)	12 (41)
ACE inhibitors, n (%)	16 (57)	15 (52)
ARB, n (%)	6 (21)	5 (17)
ACE inhibitors + ARB, n (%)	22 (79)	20 (69)
Perhexiline, n (%)	5 (18)	5 (17)
Statins, n (%)	22 (79)	18 (62)
CCB, n (%)	11 (39)	10 (34)
Beta-blockers, n (%)	13 (46)	9 (31)

SD – standard deviation, *ACE* – angiotensin converting enzyme, *ARB* – angiotensin II receptor blocker, *CCB* – calcium channel blocker.

No differences were found between groups on chi-square/ Fisher's exact test, except with diabetes mellitus (p=0.01).

* NO resistance was defined as < 30% inhibition of aggregation with 10 μ M SNP.

Table 5.2 Characteristics of patients (n=5) undergoing CABG surgery whose saphenous vein segments were utilized to study vasodilator effects of IPA/NO in organ bath experiments

Characteristic	
Men/women, n	5/0
Age (mean \pm SD), yrs	60 \pm 7
Diabetes, n (%)	1 (20)
Hypertension, n (%)	3 (60)
Hypercholesterolaemia, n (%)	5 (100)
Smoking, n (%)	1 (20)
Drugs used	
Aspirin, n (%)	4 (80)
Nitrates, n (%)	1 (20)
ACE inhibitors, n (%)	3 (60)
ARB, n (%)	1 (20)
CCB dihydropyridine, n (%)	1 (20)
CCB non-dihydropyridine, n (%)	1 (20)
Beta-blockers, n (%)	2 (40)
Statins, n (%)	5 (100)
Perhexiline, n (%)	0 (0)

Table 5.3 Generation of cGMP induced by SNP and IPA/NO in the presence of IBMX in NO-resistant vs NO-responsive individuals. Data represent % of baseline.

	NO resistant	NO responsive	P value
SNP	166 ± 8	238 ± 26	0.011
IPA/NO	909 ± 198	1163 ± 191	0.4

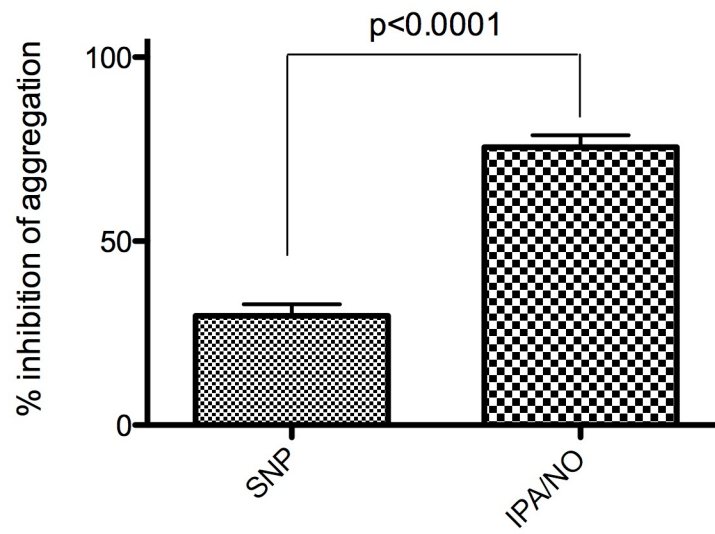


Figure 5.1 Comparison of anti-aggregatory effects of SNP (10 μ M) and IPA/NO (10 μ M) in whole blood (n=57).

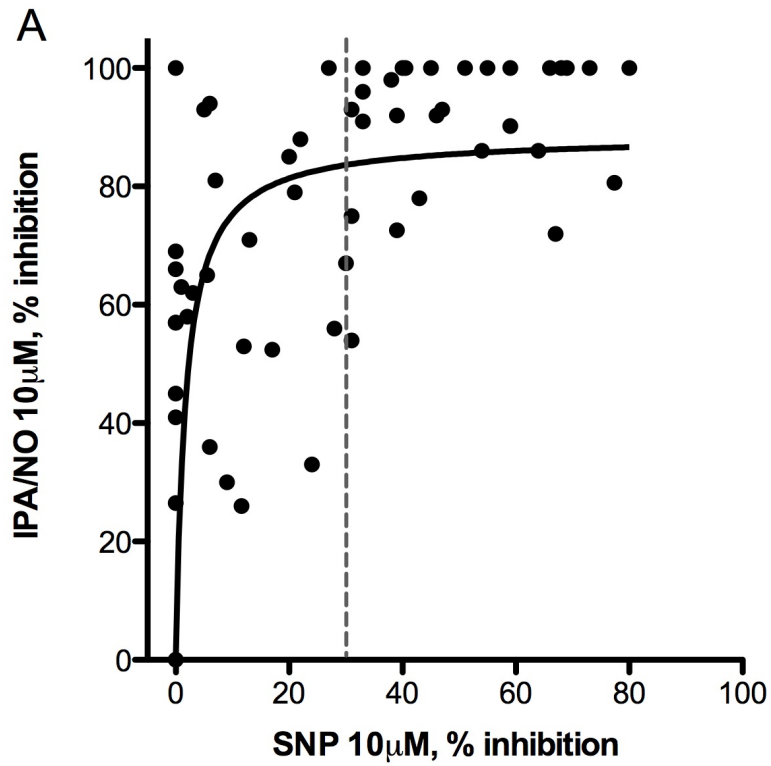


Figure 5.2 The relationship between platelet responsiveness to SNP (10 μ M) and to IPA/NO (10 μ M) in whole blood. The dotted line delineates the lower limit of normal SNP response in healthy individuals.

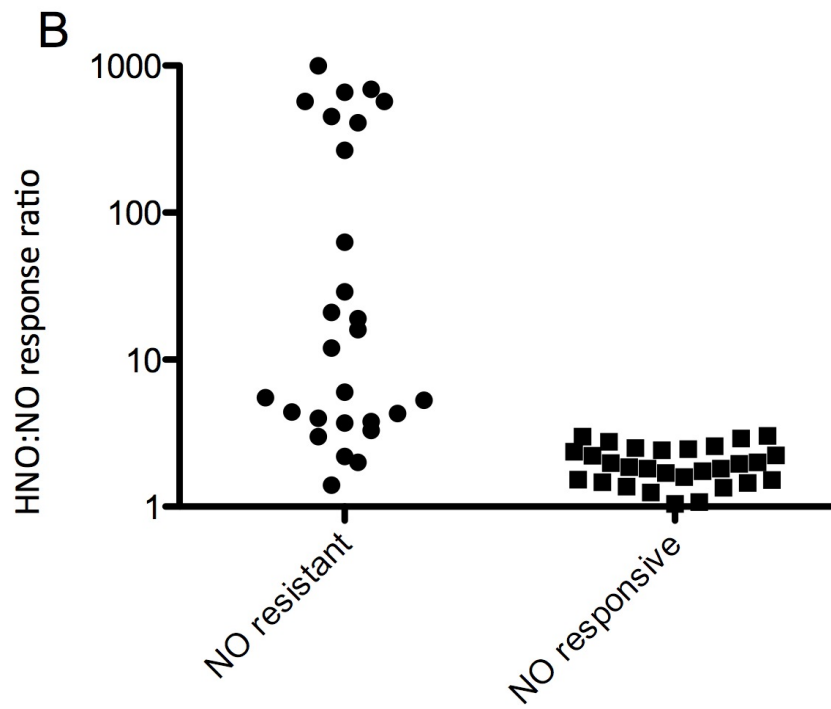


Figure 5.3 IPA/NO:SNP response ratios in NO-resistant and NO-responsive individuals: data are from evaluation *in vitro* in whole blood: comparison by non-paired *t*-test.

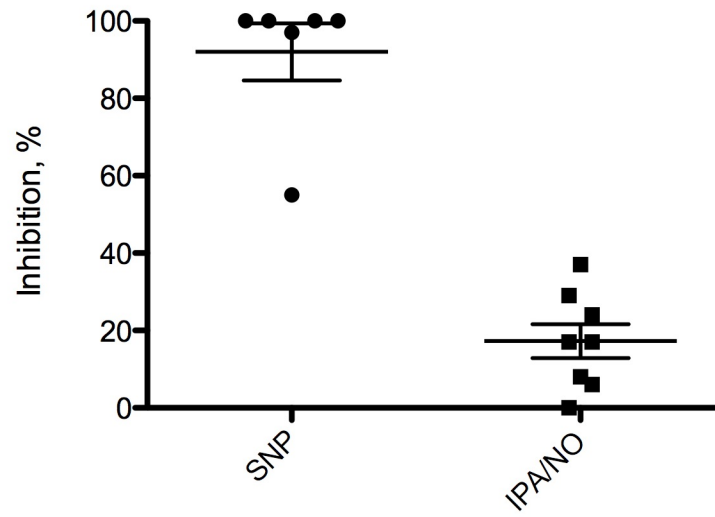


Figure 5.4 Differential inhibition ($p < 0.001$) of SNP ($10\mu\text{M}$) and IPA/NO ($10\mu\text{M}$) anti-aggregatory effect by CPTIO ($200\mu\text{M}$) in whole blood.

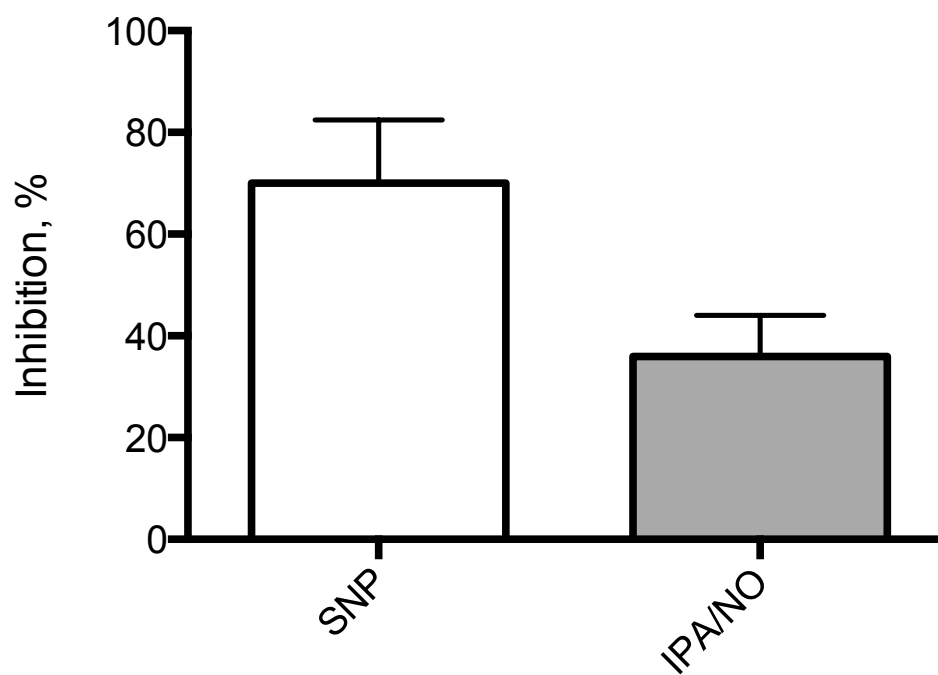


Figure 5.5 Inhibition of SNP (10 μ M; n=8) and IPA/NO (10 μ M; n=18) anti-aggregatory effects by ODQ (10 μ M), p=0.03 (un-paired *t* test) for differential inhibition, in platelet-rich plasma.

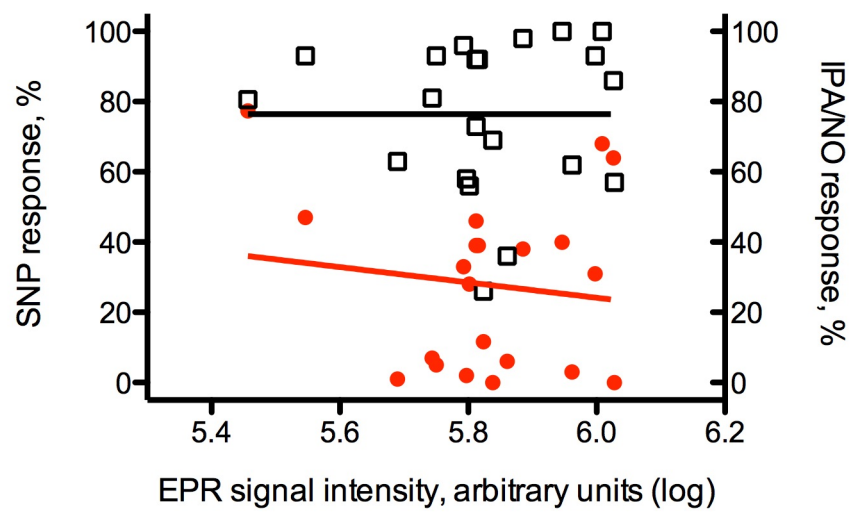


Figure 5.6 Relationship between baseline whole blood total ROS content (expressed as EPR signal intensity in arbitrary units) and platelet responsiveness to SNP (•) and IPA/NO (□) in whole blood. Neither relationship was statistically significant.

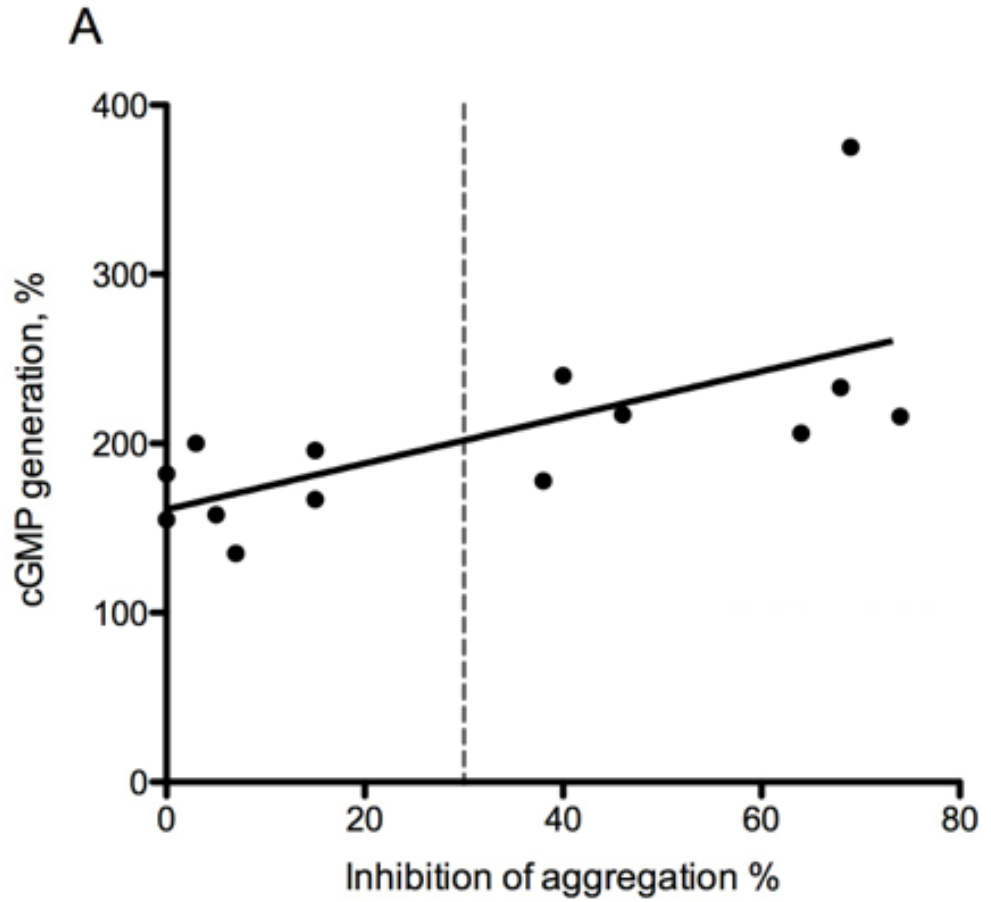


Figure 5.7 Relationship ($r=0.67$, $p<0.01$) between effects of SNP ($10 \mu\text{M}$) on platelet aggregation and cGMP generation (as a measure of sGC activation) in platelet-rich plasma.

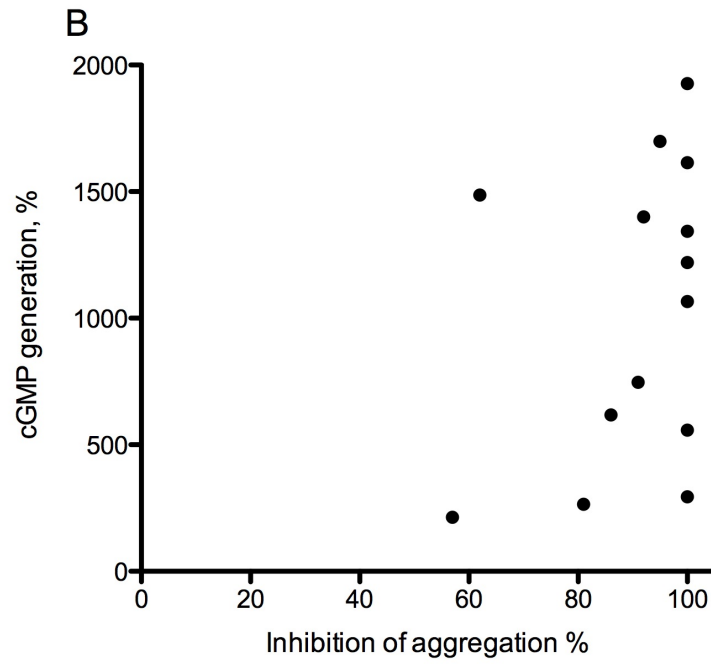


Figure 5.8 Relationship between effects of IPA/NO (10 μ M) on platelet aggregation and cGMP generation (as a measure of sGC activation) in platelet-rich plasma. There was no significant relationship.

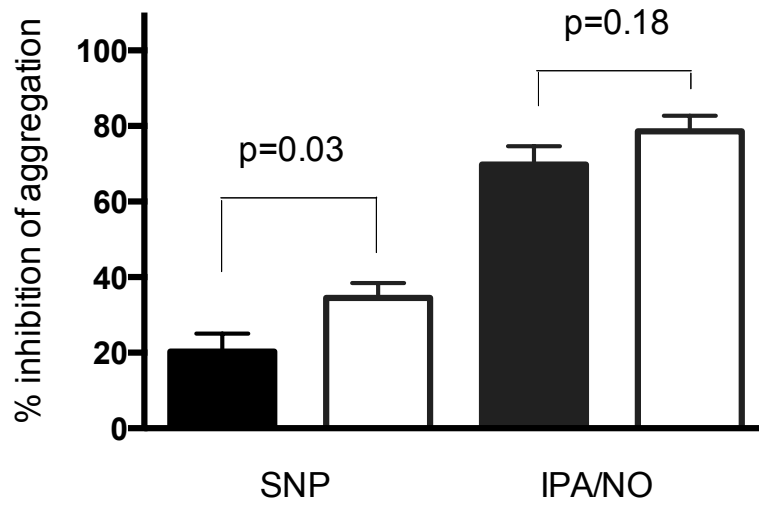


Figure 5.9 Anti-aggregatory effects of SNP (10 μ M) and IPA/NO (10 μ M) in whole blood of subjects with (closed bars; n=21) or without (open bars; n=36) diabetes mellitus.

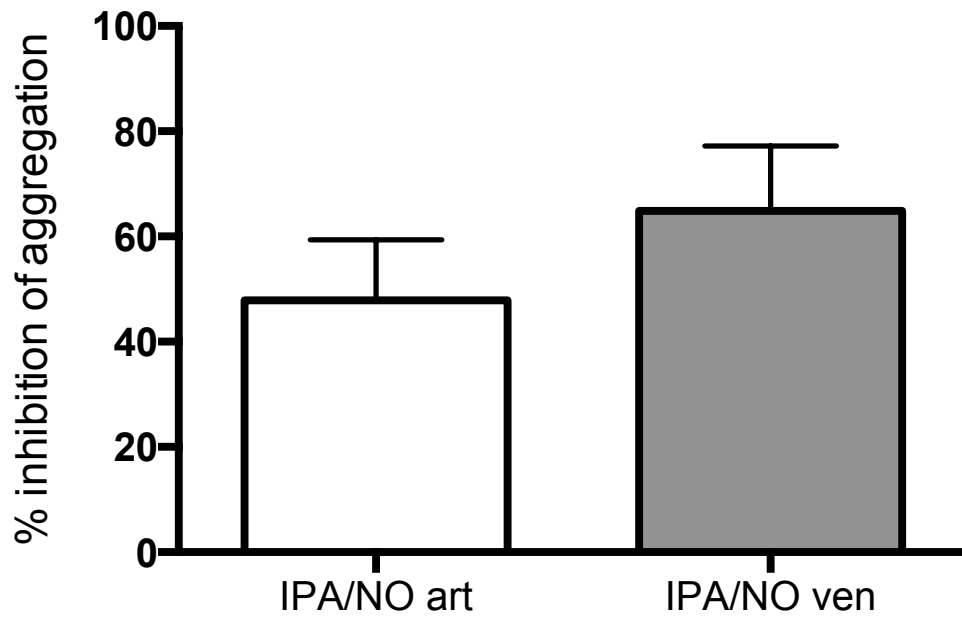
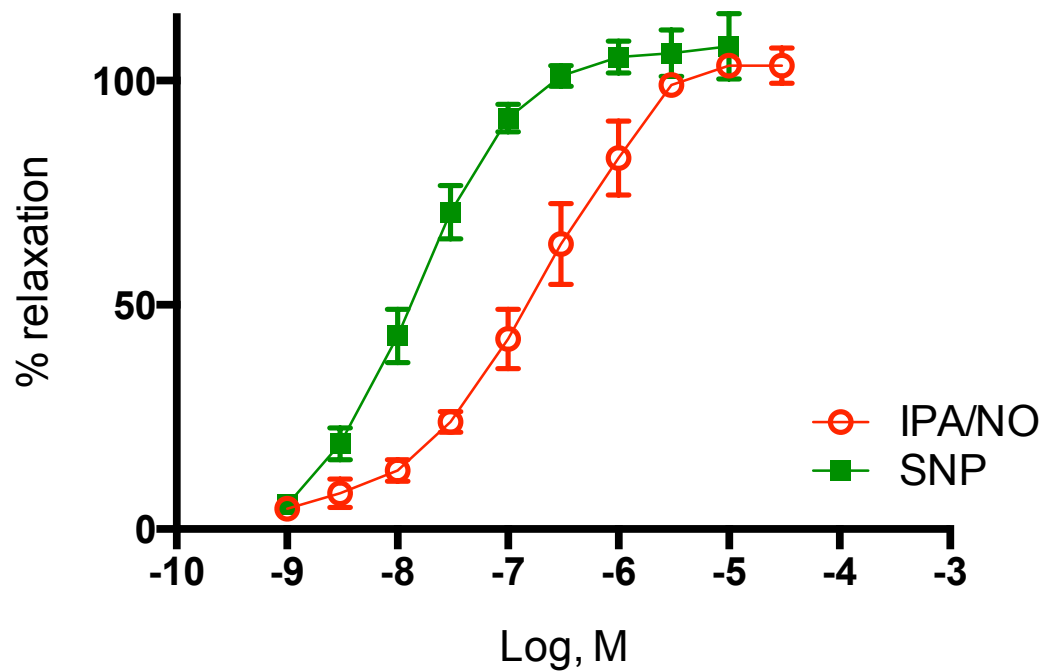
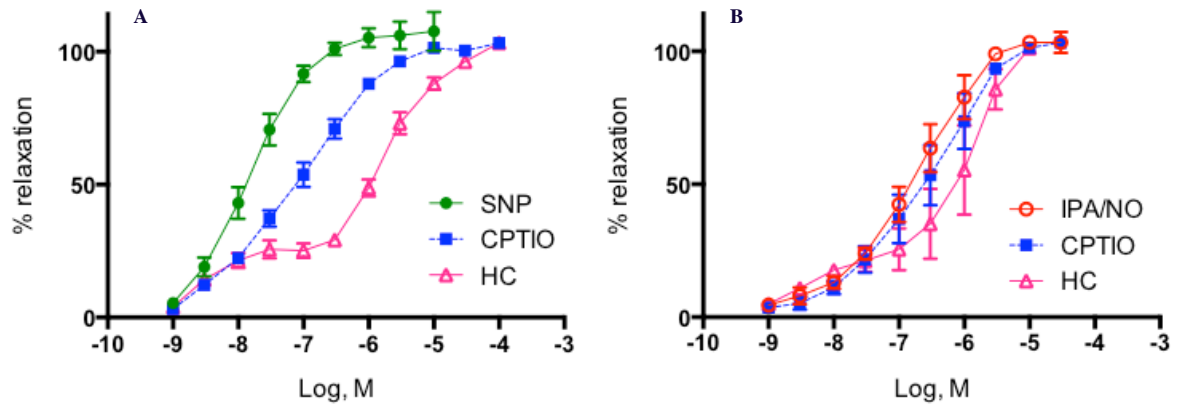


Figure 5.10 Anti-aggregatory effects of IPA/NO (10 μ M) in arterial vs venous whole blood (n=6), $p < 0.01$ (paired t -test).



	IPA/NO	SNP
Log EC ₅₀	-6.7 ± 0.1	-7.9 ± 0.1

Figure 5.11 Combined concentration-response curves for the HNO donor IPA/NO (n=5) and the NO donor SNP (n=4) on saphenous veins in organ bath.



	SNP	SNP+ CPTIO	SNP+HC
LogEC ₅₀	-7.9±0.1	-7.1±0.1	-5.7±0.1

	IPA/NO	IPA/NO + CPTIO	IPA/NO + HC
LogEC ₅₀	-6.7±0.1	-6.5±0.2	-5.6±0.7

Figure 5.12 Effects of NO scavengers CPTIO (200 μM) and hydroxycobalaminine (HC, 100 μM) on vasodilator actions of (A) SNP (n=4) and (B) IPA/NO (n=5) in saphenous veins.

A) one-way ANOVA for EC₅₀: p=0.03; with post hoc comparisons (Bonferroni):

p=NS for EC₅₀ of SNP vs CPTIO

p=0.03 for EC₅₀ of SNP vs HC

B) one-way ANOVA for EC₅₀: p=0.3

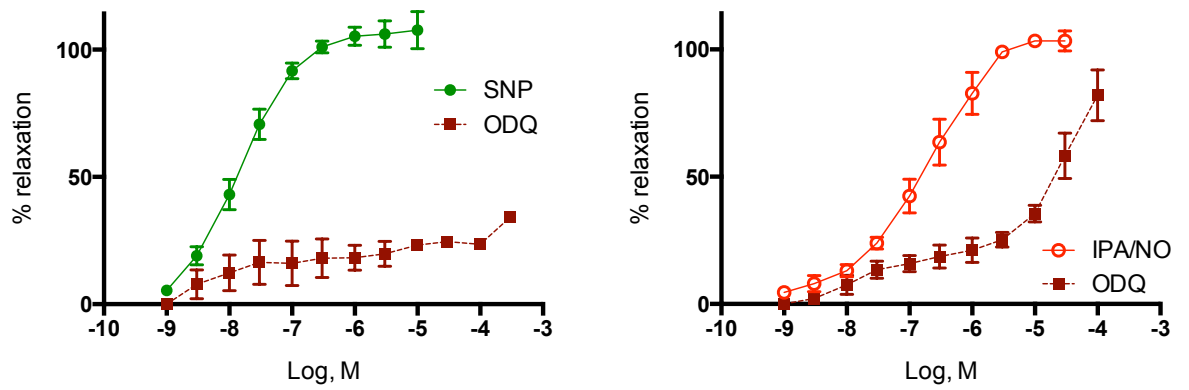


Figure 5.13 Effects of incubation of sGC inhibitor ODQ (10 μ M) on vasodilator properties of SNP (n=3) and IPA/NO (n=3) in saphenous vein rings *in vitro*; p<0.0001 for inhibition by ODQ on both SNP and IPA/NO.

Chapter 6: Summary and future directions

Nitric oxide (NO) is a critically important biological messenger in the cardiovascular system, maintaining, a normal endothelium and an anti-thrombotic intravascular milieu. NO is a key regulator of processes of vascular homeostasis including vasorelaxation, anti-aggregatory actions and vessel permeability.

NO acts in different ways to regulate vascular tone. Healthy endothelial cells constantly produce NO to create a 'basal' vasorelaxation. NO diffuses from endothelial cells directly across the membranes of the underlying vascular smooth muscle cells. This in turn, increases production of cGMP via activation of sGC. Increased cGMP production leads to activation of a cGMP-dependent protein kinase and decrease in intracellular Ca^{2+} , which results in vasorelaxation.

Vascular NO release may increase under influence of shear stress or a number of biochemical stimuli including ADP, thrombin, acetylcholine and serotonin. Inhibition of NO production can predispose to pathophysiological processes, e.g. hypertension or stroke, reflecting attenuation of the effects of NO on thrombotic regulation.

Inhibition of platelet aggregation is one of the most important physiological actions of NO. In the scenario of endothelial dysfunction, when there is a deficiency in the production of endothelial NO, one should expect increased platelet aggregation. This is the 'dark side' of platelet pathophysiology. Interestingly, in many cardiovascular disease states the primary problem is not impairment of NO production. This is due to the phenomenon of NO resistance: a decrease in responsiveness to NO, which may be explained by reduction in sGC sensitivity to NO and/or by "scavenging" of NO by superoxide. Given that impaired NO signaling is often engendered by NO resistance, this phenomenon is a major therapeutic target in many forms of heart disease. Several studies have confirmed amelioration of NO resistance by ACE inhibitors (Chirkov et al. 2004; Willoughby, S. R. et al. 2012), insulin (Worthley et al. 2007), perhexiline (Willoughby, S. R. et al. 2002) and statins (Stepien et al. 2003). However, until the current study, there were no reports on circumvention of this phenomenon. Circumvention of NO resistance that accompanies many acute cardiovascular disease states (including

acute myocardial infarction and acute heart failure) would be a “holy grail” in the treatment of cardiac emergencies.

Recent data have implicated nitroxyl as another nitrogen oxide capable of activating sGC. *In vivo*, nitroxyl is thought to exist in its protonated form (HNO); it has no unpaired electron, and because of that, is resistant to scavenging by superoxide under conditions of increased superoxide release. Therefore, HNO donors might be expected to induce continued sGC stimulation despite the oxidative stress commonly associated with cardio-vascular diseases.

Nitrite was also considered as a means of circumventing NO resistance in our study.

Since application of nitrite in the early 20th century as an agent for treatment of angina pectoris and arterial hypertension, its use has decreased due to case reports of cardiovascular collapse. Subsequent reports of its possible carcinogenicity further reduced the initial interest to nitrite in clinical practice (with the exception of its role as an antidote for cyanide poisoning). Until recently, nitrite was regarded as an inert end product of endogenous NO metabolism. However, in the last decade nitrite has triggered substantial scientific interest, and is now regarded as an additional facet of nitric oxide signaling pathway in physiology with important therapeutic implications.

The experimental studies presented in this thesis address the phenomenon of NO resistance and its impact on tissue responsiveness to nitrite, in the context of circumventing this problem with nitroxyl donors. New insights into the phenomenon of hypoxic potentiation of nitrite physiological effects, both in human platelets and vessels, are also evaluated in this thesis.

Chapter 1 summarizes current knowledge on NO physiology and pathophysiology, describes the phenomenon of NO resistance with its deleterious impact on cardiovascular health. This chapter also introduces the data accumulated in the literature so far on physiological effects of nitrite and the phenomenon of hypoxic potentiation of nitrite effects. Given that the current studies were performed utilizing human platelets and blood vessels, the chapter also provides an overview

of platelet physiology and vascular endothelial function. It also introduces nitroxyl, the redox sibling of NO, and substantiates a need for experimental studies in this direction.

Chapter 2 deals with the methodology utilized in the current studies.

In particular it describes the following techniques used in the work on this thesis:

- Organ bath for *in vitro* study of vasodilator effects of nitrite
- Platelet aggregometry (whole blood and platelet-rich plasma)
- Electronparamagnetic resonance (EPR) spectroscopy for measurement of ROS

Chapter 3. Nitrite vasodilator effects *in vitro*: impact of hypoxia.

The following hypotheses were tested in this study:

Primary: In human saphenous vein, vasodilator effect of nitrite is subject to the phenomenon of hypoxic potentiation.

Secondary: Vasodilator effects of NO_2^- are equally potentiated by hypoxia in veins and arteries. Prolonged administration of NO_2^- does not result in induction of tolerance, and there is no cross-tolerance between NO_2^- and GTN.

Major findings:

- i. Nitrite is a potent vasodilator. When compared to standard NO donor GTN:
 - nitrite vasodilator effects are significantly potentiated in hypoxia in human saphenous veins. However, in human internal mammary arteries, nitrite-induced vasodilation is not potentiated under hypoxia.
 - Hypoxia *per se* causes significant relaxation of saphenous vein basal tone.
 - GTN vasodilator effects are not potentiated in hypoxia.
 - Prolonged exposure of human saphenous vein to nitrite does not cause tolerance or cross-tolerance to GTN

- ii. Nitrite effects in saphenous veins are substantially inhibited by ODQ, suggesting that they are largely mediated by soluble guanylate cyclase.
- iii. Haemoglobin, myoglobin and red blood cells significantly increase hypoxic potentiation of nitrite vasodilator effects in human saphenous veins.
- iv. Hypoxic potentiation of nitrite is diminished when saphenous vein intrinsic myoglobin is blocked by ferricyanide.
- v. The xanthine oxidase inhibitor allopurinol does not affect vasodilator actions of nitrite.

The results of this work provide further understanding on hypoxic potentiation of vasodilatory actions of NO_2^- . For the first time we show significant potentiation of vasodilator properties of NO_2^- by hypoxia in human saphenous veins *in vitro*.

Experiments to evaluate mechanistic aspects of nitrite vasodilator effects under hypoxia *in vitro*, therefore revealed: -

- nitrite vasodilator effects are sGC-mediated
- reduction of vascular endogenous nitrite is carried by intrinsic Mb
- xanthine oxidase does not play a role as nitrite reductase in human saphenous veins
- vascular Mb and Hb (within red blood cells) are the main nitrite reductases in human saphenous veins
- hypoxic potentiation of NO_2^- vasodilator effects does not occur in human internal mammary artery

These data, along with the results of platelet work presented in chapter 4 of this thesis, provide a better understanding of the phenomenon of hypoxic potentiation of NO_2^- effects in different tissues and settings. This *in vitro* study has shown significant venodilator effect of NO_2^- under hypoxia. Furthermore, this was specific for NO release from NO_2^- - there was no significant potentiation of vasodilator properties of GTN under hypoxia.

The phenomenon of hypoxic potentiation of NO_2^- effects is of considerable clinical interest: the concept of being able to deliver a vasodilator (and also anti-

aggregatory) stimulus selectively to hypoxic regions has a potential clinical application in all conditions in which hypoxia occurs in the presence of intact mechanisms for NO/sGC signaling. In a setting of impaired NO generation via NOS in hypoxic tissue, nitrite reduction to NO can theoretically restore vascular haemostasis and prevent thrombo-vascular events. From this point of view, nitrite might find beneficial use in stroke medicine, treatment of acute and chronic cardiovascular diseases: acute myocardial infarction, coronary interventions, heart failure, peripheral vascular disease, and organ transplantation.

Although the data on asymmetry of hypoxic potentiation of NO_2^- effect are currently limited to a comparison between saphenous vein (extensive potentiation) and internal mammary artery (apparently no potentiation), this finding is of potential physiological interest. For example, the “diving reflex” includes sympathetic and parasympathetic discharges triggered by cold stimulation of trigeminal nerve afferents and maintained by hypoxia, inducing intensive arterial constriction in all but cerebral, coronary and adrenal circulations (Campbell, Gooden & Horowitz 1969). NO_2^- -induced hypoxic potentiation would alternate this vasoconstriction. Therefore it would be of interest to see whether NO_2^- hypoxic potentiation in arteries is most intense in the cerebral, coronary and adrenal circulations.

Future directions

Nitrite has been used for long time in clinical practice, e.g. in the treatment of cyanide poisoning, and the safety profile of its intravenous use has been evaluated. However, virtually nothing is known of its therapeutic efficacy in acute cardiovascular disease states in humans. More nitrite studies including clinical human trials need to be carried out. These should include:

- Evaluation of effects of nitrite on coronary haemodynamics in the catheterization laboratory. The critical issues here concern the potential advantages of utilization of nitrite in improving myocardial salvage in patients with severe haemodynamic compromise and associated regional

hypoxia. Issues of particular relevance include the potential utility of selective vasodilatation in hypoxic regions as a means of avoiding “coronary steal” in such patients. In this regard, comparisons with adenosine- and GTN-induced dilatation would be relevant. It is also possible that the benefit of selective NO release into hypoxic, ischaemic areas might include more effective activation of post-conditioning pathways (Cour et al. 2011), rather than just improvement of regional flow.

- Clinical trials of utilization of intravenous nitrite in the treatment of acute coronary syndromes and heart failure. Currently, patients are often treated with intravenous GTN on presentation to emergency departments. Acute coronary syndromes are associated with myocardial hypoxia and hence potential tissue NO deficiency. As it was shown in the current study, nitrite is a potent vasodilator in hypoxia when compared to GTN. Importantly, nitrite does not cause tolerance or cross-tolerance to GTN and this could be very useful clinically when the infusion is used for more than 24 hours. GTN tolerance is the main limiting factor of prolonged GTN utility currently.
- The potential therapeutic role of nitrite infusions as a component of treatment of critical peripheral vascular disease, with associated acute limb ischaemia, also represents an attractive area for future clinical evaluation.
- Nitrite-coated stents and balloons, whether utilized in the treatment of coronary artery or peripheral artery disease should be tested in animal models. This idea of local release of nitrite to the site of endothelial dysfunction warrants testing in models of vascular stenosis/occlusion with associated hypoxia.
- Currently there are only small studies available on the use of nitrite in pulmonary hypertension (e.g. inhalation of nitrite aerosol): - results are promising. The idea of delivering nitrite locally to the pulmonary circulation needs to be evaluated. This could be achieved with slow nitrite infusions via specialized long-term catheters similar to those used in oncology for chemotherapeutic agents.

Chapter 4. Anti-aggregatory effects of nitrite in normoxia and hypoxia

The following hypothesis were tested in this study:

Primary: The anti-aggregatory effect of NO_2^- is subject to the phenomenon of hypoxic potentiation in humans.

Secondary: NO_2^- is a more potent inhibitor of platelet aggregation in venous vs arterial blood and its effects are NO-mediated.

Major findings:

1. Anti-aggregatory effects of nitrite are:
 - NO-mediated and correlate with those of the NO donor SNP.
 - Subject to ‘NO resistance’.
2. Anti-aggregatory actions of nitrite are more potent in venous relative to arterial blood. This is consistent with lower pO_2 in venous blood and higher deoxyhaemoglobin fraction. Deoxyhaemoglobin is the primary nitrite reductase in blood. In contrast, effects of SNP do not differ between arterial and venous blood.
3. Exposing human subjects to brief mild hypoxia causes deoxyhaemoglobin content to rise in the arterial blood.
4. There is no difference in levels of reactive oxygen species (ROS) in arterial vs venous blood.
5. Under normoxic conditions, anti-aggregatory effects of nitrite are more pronounced in whole blood than in platelet-rich plasma. SNP, on the other hand, being a direct NO donor, is similarly potent in whole blood and platelet-rich plasma.
6. Evidence of bioactivity of endogenous nitrite:
 - Hypoxia potentiates anti-aggregatory effects of nitrite both in whole blood and in platelet-rich plasma.

- Platelets in arterial blood show significantly greater aggregatory responses to ADP in comparison to those in venous blood: more potent bioconversion of intrinsic nitrite in venous relative to arterial blood, despite generally higher contents of nitrite in the arterial blood.
- In platelet-rich plasma both deoxyhaemoglobin and deoxymyoglobin exert significant anti-aggregatory effects in the absence of added nitrite.

The results of this work provide further mechanistic insights on hypoxic potentiation of anti-aggregatory actions of NO_2^- . Utilizing SNP as a comparator NO donor, we demonstrated significantly more potent anti-aggregatory effects of NO_2^- in human venous vs arterial whole blood, and that this difference was directly correlated with deoxyHb concentration. This is supported by *in vitro* platelet work with artificially induced different hypoxia levels in a custom-made hypoxic chamber. Additionally we have demonstrated that tissue responsiveness to NO_2^- , like that of SNP, is constrained in the presence of NO resistance.

In the current experiments it was documented that in the absence of additional NO_2^- , ADP-induced aggregation was significantly greater in arterial than in venous blood. This raised the possibility that endogenous NO_2^- might be selectively limiting platelet aggregation in venous blood (Srihirun et al. 2012), supporting this hypothesis and consistent with the previously reported findings of Park et al (Park et al. 2013) in murine platelets. When experiments were performed in platelet-rich plasma under hypoxia, additional evidence of the impact of endogenous NO_2^- was evident in that addition of deoxyHb or deoxyMb exerted anti-aggregatory effects in the absence of exogenous NO_2^- .

As regards mechanism(s) underlying both hypoxic potentiation of anti-aggregatory effects of NO_2^- and accentuation of NO_2^- effects in venous blood, we demonstrated that no similar changes occurred with SNP, either regarding arterio-venous differences or effects of *in vitro* hypoxia. Experiments in platelet-rich plasma suggested that both deoxyHb and deoxyMb might be contributors to the potentiation of anti-aggregatory effects of NO_2^- in venous blood and under hypoxia.

Correlation data suggested that increases in deoxyHb concentrations during progressive hypoxia might play a primary role.

The whole phenomenon of hypoxic potentiation of NO_2^- effects is of considerable clinical interest: the concept of being able to deliver a vasodilator and anti-aggregatory stimulus selectively to hypoxic regions has a potential clinical application in conditions in which hypoxia occurs in the presence of intact mechanisms for NO/sGC signaling. Conversely, it is possible that the presence of arterio-venous shunting (for example in haemodialysis patients) may attenuate venodilator/anti-aggregatory effects of NO_2^- by virtue of induction of increased oxygen content in venous blood. These areas remain to be explored with these specific mechanisms in mind.

Future directions

Anti-aggregatory actions of nitrite need to be evaluated in conjunction with the proposed several future nitrite studies outlined above – in chapter 3, for example studies of nitrite in acute coronary syndromes and heart failure, peripheral vessels disease, and pulmonary hypertension. The main idea of being able to deliver NO into the hypoxic tissue is of significant importance. In acute vascular thrombosis nitrite potentially can improve clinical outcomes when delivered to hypoxic tissue lacking endogenous NO production via the classical L-arginine pathway.

Chapter 5. Nitroxyl effects in vasculature and platelets: circumvention of nitric oxide resistance.

The following hypotheses were tested in this study:

Primary: The nitroxyl donor IPA/NO exerts potent anti-aggregatory effect. Nitroxyl circumvents NO resistance in human platelets when compared to the direct NO donor SNP.

Secondary: Nitroxyl exerts potent vasodilator properties in human saphenous veins.

Major findings:

1. On equimolecular basis, nitroxyl has more potent anti-aggregatory actions than SNP: cGMP accumulation in platelets is greater with nitroxyl than with SNP stimulation.
2. Anti-aggregatory effects of nitroxyl are:
 - more potent in platelet rich plasma relative to those in whole blood
 - more potent in venous relative to arterial whole blood.
3. Nitroxyl anti-aggregatory actions are partially sGC mediated and are only minimally NO-mediated.
4. Nitroxyl partially circumvents NO resistance: it demonstrates powerful anti-aggregatory actions regardless of NO responsiveness, e.g. responses in diabetics and non-diabetics are not significantly different, while with SNP diabetics are predominantly NO-resistant.
5. Nitroxyl vasodilator effects are:
 - potent in human saphenous veins
 - not NO-mediated but partially sGC-mediated.

NO resistance represents both an independent marker of increased cardiovascular risk and also a therapeutic target. NO resistance limits utility of organic nitrates, even on *de novo* basis, and potentially of nitrite. To date, all intervention studies have been directed at restoration of normal functioning of the NO/sGC cascade, a strategy which can be termed amelioration of NO resistance. This has been achieved via correction of hyperglycaemia, use of ACE inhibitors, perhexiline and possibly statins. However, the implications of such strategies relate to chronic disease management. A residual clinical problem is posed by the occurrence of NO resistance in association with cardiovascular emergencies such as acute heart failure and acute myocardial ischaemia/infarction. Under these circumstances a therapy

which is effective despite NO resistance is desirable. The current data suggest that HNO donors might to some extent solve this problem.

The results of this work provide evidence that the nitroxyl donor IPA/NO can partially circumvent NO resistance in human platelets. While there are theoretical reasons on the basis of previous animal studies to suggest that this would occur, this is the first demonstration of circumvention of NO resistance in humans.

Future directions

The current findings invite translation to the clinical setting and suggest that HNO donors may be particularly useful in acute cardiovascular disease states associated with NO resistance, such as acute heart failure. In this regard, the positive inotropic, but not chronotropic, effects of HNO donors demonstrated in the scenario of heart failure ((Paolocci et al. 2003), (Tocchetti et al. 2011)) are also attractive when combined with their vasodilatory and anti-aggregatory properties. Another clinically relevant finding is that HNO lacks cross-tolerance with organic nitrates (Irvine, Kemp-Harper & Widdop 2011). New HNO donors have been recently synthesized from acyl nitroso compounds and acyloxy nitroso compounds [(DuMond, Wright & King 2013), (Shoman et al. 2011)], and await appropriate translational investigations.

Based on the current evidence that has accumulated regarding nitroxyl donors, and once safe nitroxyl donors for human use are synthesized, the following studies could be proposed:

- Trial of nitroxyl intravenous therapy in patients with acute myocardial infarction, unstable angina, acute heart failure.
- Study of effects of nitroxyl therapy in patients with chronic heart failure in addition or instead of nitrate therapy.

- Study of effects of nitroxyl alone or in combination with intravenous insulin in patients presenting with hyperglycaemia and acute coronary syndrome, given that this represents a state par excellence of NO resistance.

The impact of the experiments described in this thesis therefore is essentially of two alternative strategies for pharmacotherapy of acute cardiovascular diseases, as summarized in Table 6.1. Interestingly, these strategies are conceptually quite different, with nitrite offering region-selective vasodilatation according to hypoxic distribution, and nitroxyl potentially exerting more generalized effects. The translation of these concepts to clinical reality remains attractive.

Table 6.1 Clinically relevant differences in pharmacological properties of nitroxyl donors, nitrite and organic nitrates.

Agent class	Examples	Pharmacological activity			Hyporesponsiveness		Hypoxic potentiation
		Vasodilator	Anti-aggregatory	+ve inotropic	<i>De novo</i> (resistance)	Acquired (tolerance)	
Organic nitrates	GTN	++	++		+++	+	
Non-nitrate NO donor	SNP	++	++		+++		
Nitroxyl donor	IPA/NO; CXL-1020	++	+++	+	+		
Nitrite	NaNO ₂	++	+		+++		+

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**Appendix: Published works related
and conducted towards this thesis**

Dautov, R.F., Ngo, D.T.M., Licari, G., Liu, S., Sverdlov, A.L., Ritchie, R.H., Kemp-Harper, B.K., Horowitz, J.D. & Chirkov, Y.Y. (2013) The nitric oxide redox sibling nitroxyl partially circumvents impairment of platelet nitric oxide responsiveness.
Nitric Oxide, v. 35(November), pp. 72-78

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It is also available online to authorised users at:

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Dautov, R.F., Stafford, I., Liu, S., Cullen, H., Madhani, M., Chirkov, Y.Y. & Horowitz, J.D. (2014)
Hypoxic potentiation of nitrite effects in human vessels and platelets.
Nitric Oxide, v. 40(August), pp. 36-44

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British Journal of Pharmacology, v. 169(3), pp. 659-670

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