A STUDY OF THE MECHANISMS OF ACTION OF NITRIC OXIDE IN PLATELETS: THERAPEUTIC POTENTIAL OF NOVEL ANTITHROMBOTIC AGENTS

Michael Stuart Crane

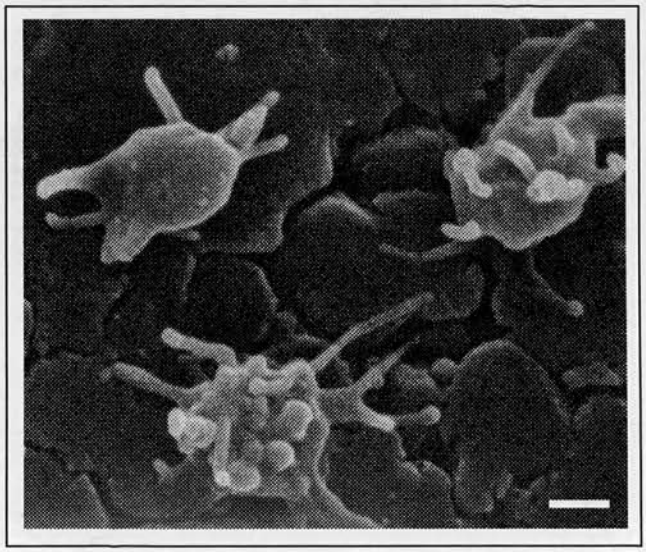

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Image: Scanning electron micrograph showing activated platelets adhering to a disc composed of Co^{2+}-exchanged zeolite-A and polytetrafluoroethylene. The scale bar represents 1 μm.

DECLARATION

I hereby declare that this thesis has been composed entirely by me, that all the work within it, unless clearly stated, is my own, and that it has not been submitted for any other degree or qualification.

Michael Crane

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Well as I sit here, with a beer in my hand, I would really like to give thanks to some of the people that have helped me along the way. I have to admit, on reflection, thesis writing is probably not one of my most favourite pastimes, but without this bunch, it would have been made a whole lot more difficult.

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ABSTRACT

Nitric oxide (NO) is a critical free radical messenger that is synthesised by a wide variety of cell types within the body. NO has numerous functions throughout the cardiovascular system, including vasodilation, inhibition of smooth muscle proliferation and inhibition of platelet aggregation and adhesion. Many of the cellular effects of NO are known to be caused through NO-mediated stimulation of the intracellular enzyme soluble guanylate cyclase (sGC), leading to increased 3',5'-cyclic guanosine monophosphate (cGMP) production. However, it has recently become clear that NO can inhibit platelet activation via mechanisms independent of cGMP generation. Interestingly, the ability of NO to induce cGMP-independent effects may be related to the alternative redox species that NO is able to adopt under physiological conditions, including the formation of S-nitrosothiols.

An important aim of this thesis was to investigate the role of cGMP-independent mechanisms in NO-mediated inhibition of platelet function. In particular, emphasis was given to the impact of different NO-related species on cGMP-independent antiplatelet effects and the characterisation of potential intracellular targets for NO. Furthermore, the role of S-nitrosothiol reservoirs in prolonging NO-mediated antiplatelet activity and their mechanisms of formation were also assessed. A final aim of this thesis was to assess the therapeutic potential of novel NO donor materials as agents to reduce platelet adhesion to prosthetic graft conduits that are sometimes used for peripheral ischaemia.

Ex vivo aggregometry and intracellular Ca^{2+} measurements combined with experiments utilising a NO-sensitive electrode were adopted to investigate NO-

mediated cGMP-independent inhibition of platelet activation. Data obtained following pharmacological inhibition of sGC indicated that NO radical is the species required to elicit cGMP-independent antiplatelet effects. Furthermore, results revealed that plasma factors that cause extracellular generation of NO from NO-donors induce cGMP-independent antiplatelet effects to these donors. Further experiments were performed to identify possible intracellular targets for NO-mediated cGMP-independent antiplatelet effects. Platelet cyclooxygenase and the prevention of Ca^{2+} signalling events were both identified as likely intracellular targets for NO-mediated cGMP-independent inhibition of platelet function.

To explore mechanisms involved in the formation of an antiplatelet NO reservoir, platelets were treated with a bolus dose of NO donor. Aggregometry data in conjunction with chemiluminescent S-nitrosothiol measurements indicated that low molecular weight thiols present in PRP may play an important role in the formation and activation of an S-nitrosothiol NO reservoir.

Final experiments aimed to assess the therapeutic potential of novel NO donors as antiplatelet surfaces showed that the pre-treatment of prosthetic graft with a novel S-nitrosothiol, S-nitroso-*N*-valerylpenicillamine (SNVP), can reduce platelet adhesion, while NO-loaded zeolites may also form a high capacity NO-store with potent antiplatelet activity.

Taken together, these data indicate novel NO signalling pathways that may occur both in the plasma environment and within platelets, further defining the inhibitory role of NO on platelet function. The pre-treatment of prosthetic grafts with SNVP and/or construction of grafts or stents with zeolites may prove to be potentially useful in the clinical setting as potent antiplatelet surfaces.

PUBLICATIONS

FULL PAPERS:

Crane, M.S., Ollosson, R., Moore, K.P., Rossi, A.G., Megson, I.L. 'Novel role for low molecular weight plasma thiols in nitric oxide mediated control of platelet function' (2002). *J Biol Chem*, **277** (49) 46858-46863.

Crane, M.S., Rossi, A.G., Megson, I.L. 'A potential role for extracellular nitric oxide generation in cGMP-independent antiplatelet effects: biochemical and pharmacological considerations' *Brit J Pharmacol*, In Press.

Wheatley, P.S., Butler, A.R., Crane, M.S., Rossi, A.G., Megson, I.L., Morris, R.E. 'Zeolite A is a dynamic store for the biologically active free radical nitric oxide (NO): preparation of tuneable NO-generating zeolites with anti-platelet properties'. Paper in preparation for *J Am Chem Soc.*

Crane, M.S., Tambyraja, A., Butler, A.R., Rossi, A.G., Megson, I.L. 'S-Nitrosothiols – A means of preventing thrombosis in prosthetic bypass grafts?' Paper in preparation for *Br J Surg.*

ABSTRACTS:

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PRESENTATIONS

ORAL:

Annual meeting for the British Pharmacological Society, Universities of Glasgow and Strathclyde:

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ABBREVIATIONS

Abbreviation	Definition
AA	Arachidonic acid
ADMA	Asymmetric dimethylarginine
ADP	Adenosine-5'-diphosphate
AM	Acetoxymethyl ester
ANOVA	Analysis of variance
ASA	Aspirin
ASC	Ascorbate
ATP	Adenosine 5'-triphosphate
AUC	Area under curve
BH_4	Tetrahydrobiopterin
CaM	Calmodulin
cAMP	Cyclic adenosine- 3',5'-monophosphate
CCE	Capacitative Ca^{2+} entry
cGMP	Cyclic guanosine- 3',5'-monophosphate
CIF	Ca^{2+} influx factor
CNG	Cyclic nucleotide-gated
CNS	Central nervous system
Coll	Collagen
COX	Cyclooxygenase
CP	Caeruloplasmin
CPB	Cardiopulmonary bypass
Cys	Cysteine
Cys-gly	Cysteinyl-glycine
DAG	1,2,-Diacylglycerol
DEA	Diethylamine
DEA/NO	Diethylamine diazeniumdiolate
DMS	Demarcation membrane system
DMSO	Dimethylsulphoxide
DNH	7-dimethylaminonaphthalene-1,2-dicarboxylic acid hydrazide
DTS	Dense tubular system
EC_{50}	Concentration causing 50% of maximum response
EDTA	Ethylenediaminetetraacetic acid
EDRF	Endothelium-derived relaxing factor
5'6'-EET	5'6'-Epoxyeicosatetraenoic acid
EGTA	Ethyleneglycoltetraacetic acid
EHNA	Erthyro-9-(2-hydroxyl-3-nonyl)adenine
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy

Abbreviation	Definition
eNOS	Endothelial nitric oxide synthase
ePTFE	Expanded polytetrafluoroethylene
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
GP	Glycoprotein
GPL	Glycerophospholipids
GSH	Glutathione
GSNO	S-nitrosoglutathione
GTN	Glyceryl trinitrate
GTP	Guanosine-5'-triphosphate
Hb	Haemoglobin
HIT	Heparin-induced thrombocytopaenia
H_2O_2	Hydrogen peroxide
HSA	Human serum albumin
Hsp 27	Heat shock protein 27
5'-HT	5'-hydroxytryptamine (serotonin)
IBMX	3-Isobutyl-1-methyl xanthine
Ig	Immunoglobulin
IH	Intimal hyperplasia
iNO	Inhaled nitric oxide
iNOS	Inducible nitric oxide synthase
IP_3	Inositol 1,4,5-trisphosphate
IP_3R	Inositol 1,4,5-trisphosphate receptor
ISDN	Isosorbide dinitrate
KPE	Potassium phosphate-EDTA
L-arg	L-arginine
LDL	Low density lipoprotein
LMW	Low molecular weight
LMW-SNO	Low molecular weight S-nitrosothiol
L-NMMA	N^{ω}-monomethyl-L-arginine
MAHMA/NO	Methylamine hexamethylene methylamine diazeniumdiolate
MAP	Mitogen-activated protein
MLCK	Myosin light-chain kinase
MOTA	Mesoionic oxatriazoles
MP	Myosin phosphatase
mtNOS	Mitochondrial nitric oxide synthase
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate)
NANC	Non-adrenergic, non-cholinergic

Abbreviation	Definition
NEM	N-ethylmaleimide
NF-κB	Nuclear transcription factor κB
NHA	N^{ω}-hydroxy-L-arginine
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NO^+	Nitrosonium ion
NO^-	Nitroxyl ion
NO_2^-	Nitrite
NO_3^-	Nitrate
NO_2	Nitrogen dioxide
N_2O_3	Dinitrogen trioxide
N_2O_4	Dinitrogen tetraoxide
NO_x	Nitric oxide-related species
NONOate	Diazeniumdiolate
NOS	Nitric oxide synthase
ns	Not significant
O_2^-	Superoxide ion
OCS	Open cannalicular system
ODQ	1-*H*-[1,2,4]oxodiazolo[4,3-a]quinoxalin-1-one
·OH	Hydroxyl radical
$ONOO^-$	Peroxynitrite
ONOOH	Peroxynitrous acid
P	Platelet
PA	Platelet aggregate
PAF	Platelet activating factor
PDE	Phosphodiesterase
PDI	Protein disulphide isomerase
PECAM-1	Platelet-endothelial cell adhesion molecule-1
$PGE_1/D_2/G_2/H_2$	Prostaglandin $E_1/D_2/G_2/H_2$
PGI_2	Prostacyclin
PIP_2	Phosphatidylinositol 4,5,-bisphosphate
PKA/C/G	Protein kinase A/C/G
PLA_2/C	Phospholipase A_2/C

Abbreviation	Definition
PMCA	Plasma membrane Ca^{2+} ATPase
PPP	Platelet poor plasma
PRP	Platelet rich plasma
PTFE	Polytetrafluoroethylene
RGD	Arginine-glycine-aspartate
RLU	Relative luminescence units
ROS	Reactive oxygen species
R-SH	Thiol (reduced)
R-SNO	S-nitrosothiol
R-SS-R	Disulphide
SCB	Sodium cacodylate buffer
S.E.M.	Standard error of the mean
SERCA	Sarco-endoplasmic reticulum Ca^{2+} ATPase
sGC	Soluble guanylate cyclase
SIN-1	3-morpholinosydnonomine
SNAP	S-nitroso-*N*-acetyl-D,L-penicillamine
SNOC	S-nitrosocysteine
SNP	Sodium nitroprusside
SNVP	S-nitroso-N-valeryl-D,L-penicillamine
SOD	Superoxide dismutase
Sol	Solution (H_2O soluble)
SPER/NO	Spermine diazeniumdiolate
TCA	Trichloroacetic acid
TP	Thromboxane A_2 receptor
tPA	Tissue-type plasminogen activator
TRPC1/6	Transient receptor potential channel 1/6
TxA_2/B_2	Thromboxane A_2/B_2
Tyr	Tyrosine
VASP	Vasodilator-stimulated phosphoprotein
VP	Valeryl-D,L-penicillamine
vWF	von Willebrand Factor
WP	Washed platelets
Z/PTFE	Zeolite/polytetrafluoroethylene

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CHAPTER ONE

INTRODUCTION

1.1 INTRODUCTION

Platelets are tiny cell fragments that circulate in the bloodstream, where they maintain vascular haemostasis. In the healthy vascular system, platelets are quiescent until they encounter an area of vascular damage, whereupon they rapidly change their shape, adhere and aggregate, forming a plug to prevent blood loss (Gear, 1994; Packham & Mustard, 1984; Zucker & Nachmias, 1985). Endothelial cells that line blood vessels provide an antithrombogenic surface and secrete a number of mediators that prevent inappropriate adhesion and activation (Gordon, 1985; Mason *et al.*, 1977; Pearson, 1999). Of these, the free radical nitric oxide (NO) is arguably the most important. Although originally described as an endothelium-derived relaxing factor and potent antiplatelet agent, NO is now recognised to be a crucial mediator not only in the cardiovascular system, but also the immune and nervous systems (Moilanen & Vapaatalo, 1995; Moncada *et al.*, 1991; Zhang & Snyder, 1995).

Although platelet activity is essential to maintain vascular viability and prevent blood loss, inappropriate platelet activation and deposition in vessels is a major cause of thrombosis, leading to heart attacks and strokes (Eisenberg & Ghigliotti, 1999; Harker, 1998) and increased platelet activation is observed in a number of cardiovascular conditions, including vascular disease (Furman *et al.*, 1998; Reininger *et al.*, 1999; van Zanten *et al.*, 1994). An important contributing factor in many of these disease states is a lack of NO bioavailability (Busse & Fleming, 1996; Poredos, 2002; Vanhoutte, 1998), ultimately leading to a more systemically active population of platelets. One possible means to alleviate this problem is to supplement endogenous levels of NO by

administration of NO-donor drugs; indeed, organic nitrates have been used in clinical medicine since the late 19th century. However, organic nitrates have some major disadvantages, the most notable of which is that they cause tolerance and are poor inhibitors of platelet activation (Chirkov *et al.*, 1997; Gerzer *et al.*, 1988; Mangione & Glasser, 1994). In more recent times, several new classes of NO donor drugs have been developed, each with different chemical properties and distinct NO-release mechanisms (Megson, 2000; Megson & Webb, 2002). In addition to offering greatly increased therapeutic potential, these new NO donors are excellent investigative tools, which can be used to great advantage in deciphering complex NO signalling pathways. The aim of this thesis is to characterise the mechanism of action of some novel NO-donor drugs in human platelets, with respect not only to their pharmacological potential, but also as tools to further our understanding of NO-dependent physiological and pharmacological mechanisms.

1.2 PLATELETS

1.2.1 PLATELET FORMATION

Platelets are formed from highly specialized progenitor cells called megakaryocytes, themselves products of haematopoietic stem cells (Golde, 1991; Ogawa, 1993). In humans, each megakaryocyte can form ~10^3 platelets, generating a total of ~10^{11} platelets each day (Hartwig & Italiano, 2003). During development, megakaryocytes undergo numerous DNA replications and organisational events before the production of an elaborate series of membrane channels called the demarcation membrane system

(DMS; Behnke, 1968a; Behnke, 1968b; Nurden *et al.*, 1997). The exact function of the DMS is unclear, but it may serve as a membrane reserve for the formation of proplatelets (Radley & Haller, 1982), which are long 'pseudopod-like' extensions driven out by microtubule forces (Handagama *et al.*, 1987; Tablin *et al.*, 1990). Platelet formation occurs at the tips of proplatelets, where microtubule coiling and sliding drives platelet release (Hartwig & Italiano, 2003; Italiano & Shivdasani, 2003). Thrombopoietin is the major hormonal regulator of platelet production (Bartley *et al.*, 1994; de Sauvage *et al.*, 1994; Kaushansky *et al.*, 1994), although NO can also stimulate their production from megakaryocytes (Battinelli *et al.*, 2001).

1.2.2 PLATELET STRUCTURE

1.2.2.1 RESTING STATE

Resting state platelets are a rounded discoid shape with dimensions of approximately 3 μm x 0.5 μm (figure 1.1-A). The platelet surface is largely featureless, except for the openings of the open canalicular system (OCS; White & Clawson, 1980a; White & Clawson, 1980b), a complex membranous network that runs throughout the platelet, vastly increasing the platelet surface area. Platelets lack a nucleus and replicative machinery, but they contain many of the usual cellular organelles such as mitochondria and lysosomes. Despite their lack of nuclei, platelets are known to contain messenger RNA and are reported to be capable of synthesising a wide variety of proteins (Bugert *et al.*, 2003; Lindemann *et al.*, 2001). In addition, they are packed with secretory granules, broadly classified into two types: α-granules (0.2-0.4 μm diameter) and dense granules

(~0.15 μm diameter; White & Clawson, 1980a). α-granules generally store large adhesive proteins and tissue growth factors, while dense granules contain Ca^{2+} and activators of platelet function (table 1.1; Harrison & Cramer, 1993; McNicol & Israels, 1999). Many of these factors play a critical role in the adhesion of platelets to the subendothelium and their subsequent activation following tissue injury (chapters 1.2.3 & 1.2.4).

α-granules		Dense granules
• Fibrinogen	• Platelet-derived growth factor	• Ca^{2+}
• Vitronectin	• Endothelial cell growth factor	• Adenosine diphosphate (ADP)
• Albumin	• Coagulation factors V and VIII	• Adenosine triphosphate (ATP)
• von Willebrand factor	• IgG	• Serotonin (5-HT)
• von Willebrand antigen	• IgA	• Pyrophosphate
• Thrombospondin	• IgM	
• β-thromboglobulin	• Neutrophil-activating protein II	

Table 1.1 **Contents of platelet granules. Adapted from McNicol & Israels, 1999.**

In addition, platelet granules contain large numbers of adhesive molecules such as glycoprotein (GP) IIb/IIIa (Nurden *et al.*, 1996; Suzuki *et al.*, 1992; Youssefian *et al.*, 1997), P-selectin (Israels *et al.*, 1992; Stenberg *et al.*, 1985), and GP IV (Berger *et al.*, 1993); chapter 1.2.3). Platelets also possess a dense tubular system (DTS), the equivalent of the smooth endoplasmic reticulum in other cells, which is tightly associated with the OCS (White & Clawson, 1980b) and is the site at which a major proportion of Ca^{2+} is sequestered (Horiguchi *et al.*, 1998).

1.2.2.2 ACTIVATED STATE

Platelet activation is associated with a rapid shape change from a discoid shape to a larger spherical conformation before finally adopting an irregular shape characterised by extensions of pseudopodia (fig 1.1-B; Hartwig, 1992). Cytoskeletal actin reorganisation is crucial to drive platelet spheration and the extension of filopods (Fox *et al.*, 1984; Hartwig, 1992; Jennings *et al.*, 1981; Nachmias, 1980; Nachmias *et al.*, 1980). During activation, platelets also degranulate, releasing their contents primarily into the OCS (Hols *et al.*, 1985), and thereby into the extracellular environment. Platelet shape change is important in adhering to sites of vessel injury, and in recruiting additional platelets for thrombus development (Kuwahara *et al.*, 2002).

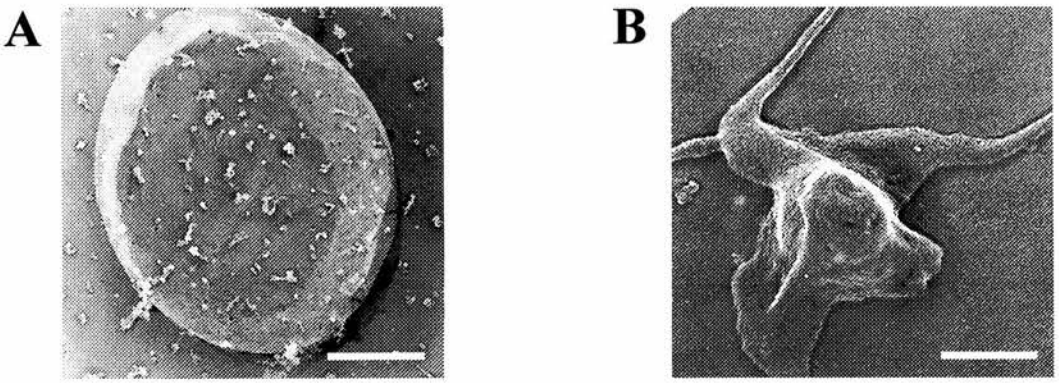


Figure 1.1 **Micrograph of a resting (A) and active (B) platelet. The scale bar represents 1 μm. Original source from Lind, 1994, taken with permission from www.images.md.**

1.2.3 PLATELET ADHESION

Platelets bind to the subendothelial matrix at sites of injury (Sixma *et al.*, 1979), a process which is tightly controlled through a number of receptor-ligand interactions

(table 1.2). Platelet adherence is a two-step process, where they undergo an initial 'rolling' event prior to stable adhesion (Moroi *et al.*, 1997; Savage *et al.*, 1996).

Receptor	Ligand(s)
GP Ib/V/IX	Von Willebrand Factor (vWF)
GP Ia/IIa ($\alpha_2\beta_1$)	Collagen
GP Ic/IIa ($\alpha_5\beta_1$, $\alpha_6\beta_1$)	Fibronectin, Laminin
GP IIb/IIIa ($\alpha_{IIb}\beta_3$)	vWF, Fibronectin, Vitronectin
$\alpha_v\beta_3$	vWF, Fibronectin, Vitronectin
GP IV	Collagen
Platelet-endothelial cell adhesion molecule 1 (PECAM-1)	PECAM-1

Table 1.2 **Some of the main receptors and ligands involved in platelet adhesion.**

In vessels that have a high shear stress, von Willebrand Factor (vWF) is essential in providing a direct link between the subendothelium and the platelet (Sakariassen *et al.*, 1979; Sixma *et al.*, 1984; Turitto *et al.*, 1985). vWF is a complex multimeric glycoprotein composed of identical disulphide-linked units with a molecular mass between 540-10,000 kDa (Dent *et al.*, 1991; Ruggeri, 1999; Ruggeri, 2001). vWF is stored in α-granules (table 1.1) and is also synthesised by endothelial cells (Mayadas & Wagner, 1991). It undergoes a conformational unravelling when attached to cellular surfaces with high shear (Siedlecki *et al.*, 1996) that may aid its binding to the subendothelium. In vessels with lower shear rates, vWF is less important because other adhesive molecules such as vitronectin and fibronectin may form bonds strong enough to withstand the lower shear forces (Savage *et al.*, 1998; Savage *et al.*, 1996). Collagen is another strongly adhesive component of the vessel wall (Baumgartner, 1977; Baumgartner *et al.*, 1977; Saelman *et al.*, 1994). Specific roles for platelet adhesion

receptors have been defined in many cases: for example, GP Ib/V/IX is important in the initial 'loose' tethering of platelets to the subendothelium during the rolling phase (Moroi *et al.*, 1997; Savage *et al.*, 1996), while anchoring to GP IIb/IIIa or GP Ia/IIa allows tight adhesion of platelets to the matrix (Kunicki *et al.*, 1988; Kuwahara *et al.*, 2002; Nieuwenhuis *et al.*, 1985).

1.2.4 PLATELET ACTIVATION

During adhesion, platelets become activated by a number of factors (table 1.3); they change shape, release their granule contents (table 1.1) and undergo signalling events that result in the exposure of the active conformation of the GP IIb/IIIa receptor (see figure 1.2 for summary). Although high concentrations of platelet activators may induce sufficient intracellular signals to complete platelet activation, lower levels of these agents require the positive feedback generated by platelet degranulation and thromboxane A_2 (TxA_2) synthesis (chapter 1.2.4.3) to drive exposure of GPIIb/IIIa and complete platelet activation (Blockmans *et al.*, 1995; Willoughby *et al.*, 2002). GPIIb/IIIa is a member of the integrin receptor family, containing receptors for proteins with the tripeptide sequence arginine-glycine-aspartate (RGD), including fibrinogen, vWF, vitronectin and fibronectin (Phillips *et al.*, 1988; Plow *et al.*, 2000; table 1.3). Platelets possess ~ 80,000 copies of GPIIb/IIIa (Wagner *et al.*, 1996), the highest density for any platelet membrane protein. Under basal conditions, GPIIb/IIIa has low affinity for fibrinogen (Shattil *et al.*, 1985; Sims *et al.*, 1991). However, when platelets become activated, GPIIb/IIIa undergoes a conformational change that greatly increases its

affinity for the RGD epitope (inside-out signalling; Hato *et al.*, 1998; Phillips *et al.*, 1991; Sims *et al.*, 1991). In addition, ligand binding induces clustering of GPIIb/IIIa (Isenberg *et al.*, 1987; Simmons *et al.*, 1997), which activates intracellular signals to cause further platelet activation (outside-in signalling; Banfic *et al.*, 1998; Fox *et al.*, 1993; Haimovich *et al.*, 1993; Huang *et al.*, 1993).

	Receptor(s)	Ligand(s)
G-protein coupled receptors	P_2Y_1, P_2Y_{12} receptors	ADP
	TP receptor	TxA_2
	Protease-activated receptor (PAR) 1, PAR4	Thrombin
	$5\text{-}HT_{2A}$- receptors	5-HT
	Platelet activating factor (PAF) receptor	PAF
Leucine-rich repeat	GP Ib/V/IX	vWF, thrombin, P-selectin
Immuno-globulin	GP VI/F_cR γ-chain	Collagen
	Platelet-Endothelial Cell Adhesion Molecule-1 (PECAM-1)	PECAM-1
Integrin	GP Ia/IIa ($\alpha_2\beta_1$)	Collagen
	GP IIb/IIIa ($\alpha_{IIb}\beta_3$)	Fibrinogen, vWF, fibronectin, vitronectin.
Scavenger	GP IV	Thrombospondin, collagen

Table 1.3 **Some of the main receptors and ligands involved in platelet activation.**

1.2.4.1 INTRACELLULAR MESSENGERS

Activation of phospholipase C (PLC) is an important signalling event in platelet aggregation by numerous agonists including thrombin, ADP, PAF, TxA_2, thrombin and collagen (Blockmans *et al.*, 1995). ADP, thrombin and TxA_2 activate PLCβ through $G_{\alpha q}$ signalling (Offermanns, 2000; Offermanns *et al.*, 1997), while activation of PLCγ2 is absolutely essential for platelet activation by collagen (Wang *et al.*, 2000; Wonerow *et al.*, 2003). Furthermore, GP Ib/V/IX and GP IIb/IIIa receptor occupation also stimulates PLCγ2 activation (Marshall *et al.*, 2002; Wonerow *et al.*, 2002). PLC converts

phosphatidylinositol 4,5-bisphosphate (PIP_2) to the intracellular messengers 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3; Fukami, 2002; fig 1.2).

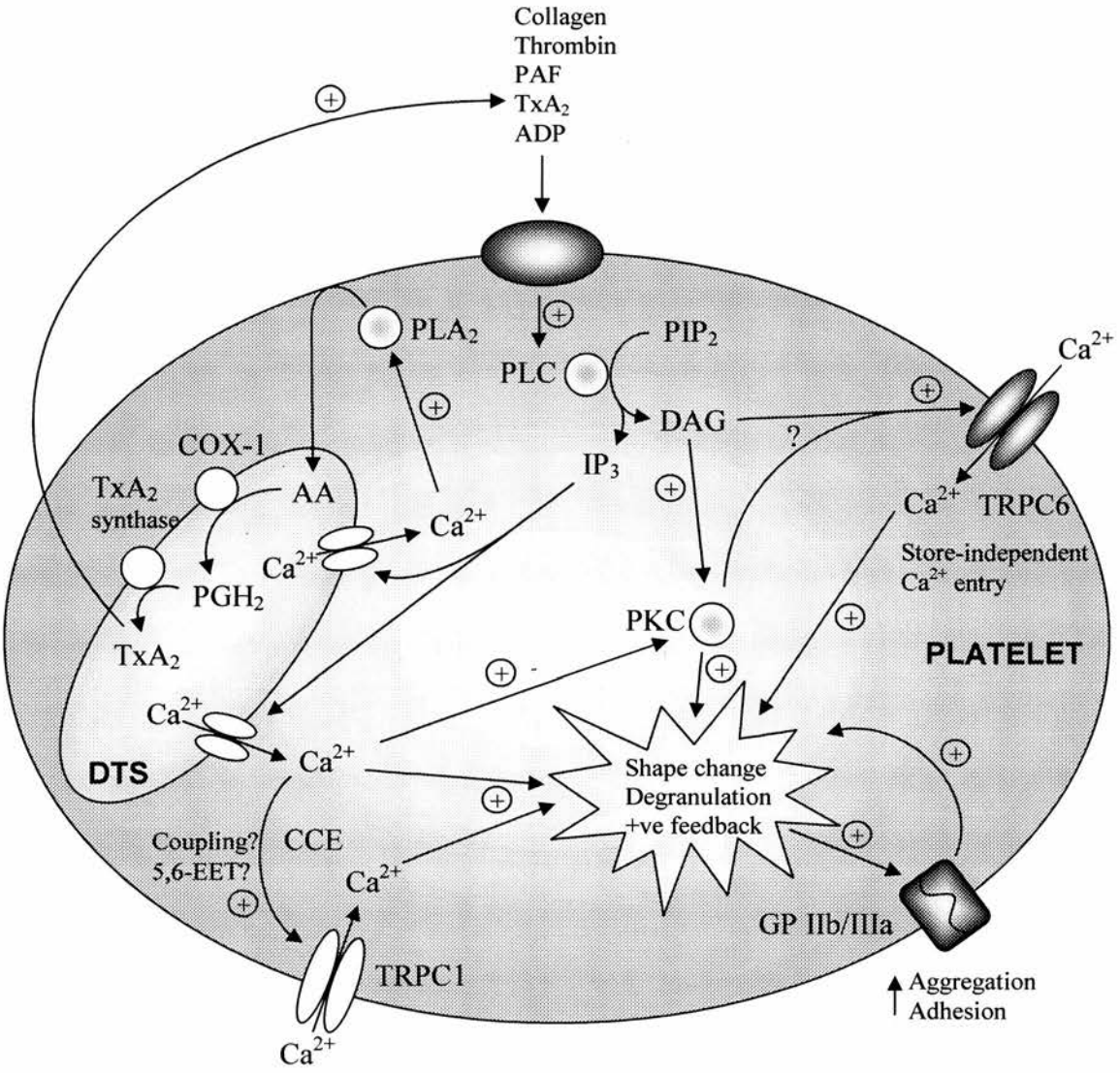


Figure 1.2 **Overview showing the complexity of platelet activation. Abbreviations: 5,6-EET, 5,6-Epoxyeicosatetraenoic acid; AA, arachidonic acid; CCE, capacitative Ca^{2+} entry; COX, cyclooxygenase; DAG; diacylglycerol; IP_3, inositol 1,4,5-trisphosphate; PGH_2, prostaglandin H_2; PIP_2, phosphatidyl inositol 4,5-bisphosphate; PKC, protein kinase C; PLA_2; phospholipase A_2; PLC phospholipase C; TRPC, transient receptor potential channel.**

IP_3 mediates Ca^{2+} release from intracellular stores (Ferris & Snyder, 1992; Furuichi & Mikoshiba, 1995) through platelet IP_3 receptors (type I) located on the membranes of the DTS (El-Daher *et al.*, 2000). DAG activates protein kinase C (PKC), which is essential for platelet shape change and degranulation (Dandona *et al.*, 1996; Rozenvayn & Flaumenhaft, 2003).

1.2.4.2 CALCIUM SIGNALLING

An increase in platelet intracellular Ca^{2+} is required for platelet activation (Davies *et al.*, 1989; Sargeant & Sage, 1994). Resting platelet intracellular Ca^{2+} levels (50-150 nM) are maintained by Ca^{2+} pumps on the external membrane, including the plasma membrane Ca^{2+} ATPase (PMCA; Dean *et al.*, 1997; Monteith *et al.*, 1998; Paszty *et al.*, 1998) and Ca^{2+}/Na^{+} exchanger (Kimura *et al.*, 1999; Rengasamy *et al.*, 1987), and on the DTS, including the sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA; Bokkala *et al.*, 1995; Martin *et al.*, 2002). Following platelet activation and the release of Ca^{2+} from the DTS, extracellular Ca^{2+} entry occurs (Rink & Sage, 1990; Rink *et al.*, 1982). In platelets, Ca^{2+} entry is controlled via two distinct mechanisms: (1) store operated Ca^{2+} entry or capacitative Ca^{2+} entry (CCE), and (2) store-independent Ca^{2+}entry.

1.2.4.2.1 CAPACITATIVE CALCIUM ENTRY (CCE)

In CCE, depletion of intracellular Ca^{2+} stores by IP_3 induces a signalling event that opens plasma membrane Ca^{2+} channels, thereby linking Ca^{2+} entry to the filling state of the intracellular stores (Parekh & Penner, 1997; Putney, 1986). There are many possible mechanisms for coupling between Ca^{2+} stores and Ca^{2+} entry channels (Putney *et al.*,

2001), including direct conformational coupling of the endoplasmic reticulum and plasma membrane through the IP_3-receptor (Berridge, 1990; Irvine, 1990), or through the release of a diffusible cytosolic messenger (calcium influx factor; CIF) upon Ca^{2+} store depletion (Putney, 1990; Randriamampita & Tsien, 1993). Interestingly, evidence suggests that CIF is a cytochrome P450 metabolite (Alonso *et al.*, 1991; Alvarez *et al.*, 1991; Xie *et al.*, 2002) and recently 5'6'-epoxyeicosatetraenoic acid (5,6-EET), a product of cytochrome P450 epoxygenase activity, has been identified as a component of CIF (Graier *et al.*, 1995; Rzigalinski *et al.*, 1999). Regardless of the mechanism involved, the transient receptor potential channel (TRPC) 1 on the plasma membrane is involved in CCE in platelets (Rosado *et al.*, 2002; Rosado & Sage, 2001).

1.2.4.2.2 STORE-INDEPENDENT CALCIUM ENTRY

A store-independent Ca^{2+} entry pathway has been identified in human platelets (Hassock *et al.*, 2002; Rosado & Sage, 2000). Although this mechanism can be activated by DAG, it is unclear whether this is through a direct effect of DAG on Ca^{2+} channels (Hassock *et al.*, 2002; Hofmann *et al.*, 1999), or via DAG-mediated activation of PKC (Rosado & Sage, 2000). TRPC6 has been identified in human platelets, and is involved in store-independent Ca^{2+} entry (den Dekker *et al.*, 2001; Hassock *et al.*, 2002).

1.2.4.3 THROMBOXANE SYNTHESIS

Increased platelet Ca^{2+} levels activate cytosolic phospholipase A_2 (PLA_2) in platelets (Clark *et al.*, 1995; Kramer *et al.*, 1993). PLA_2 catalyses the hydrolysis of membrane glycerophospholipids, resulting in the generation of arachidonic acid (AA; Clark *et al.*,

1995; McKean *et al.*, 1981). Following its synthesis, AA concentrates in the DTS (Laposata *et al.*, 1987), where it is converted via the membrane haemoprotein COX-1 to prostaglandin (PG) H_2 (Carey *et al.*, 1982; Smith *et al.*, 1996b). COX-1 is homodimeric (Garavito & DeWitt, 1999; M_r ~ 70 kDa) and plays a vital role in platelet activation because it catalyses the first committed step in prostanoid synthesis (Smith *et al.*, 1996b). Subsequent conversion of PGH_2, primarily to TxA_2 via the action of TxA_2 synthase then occurs (Needleman *et al.*, 1976a; Needleman *et al.*, 1976b), which along with granule release maintains a positive feedback loop to drive platelet aggregation (Blockmans *et al.*, 1995).

1.2.4.4 ENDOGENOUS INHIBITORS OF PLATELET ACTIVATION

A number of factors can inhibit platelet activation (table 1.4), the most notable of which are prostacyclin (PGI_2) and NO, which act synergistically to prevent platelet activation (Radomski *et al.*, 1987a). PGI_2, a product of the vascular endothelium, has long been known to be a potent inhibitor of platelet aggregation (Moncada *et al.*, 1976; Whittaker *et al.*, 1976).

'Receptor(s)'	Ligand(s)
Soluble guanylate cyclase (sGC)	NO
PGI_2 receptor	PGI_2, PGE_1
PGD_2 receptor	PGD_2
A_2 receptor	Adenosine

Table 1.4 **Some of the main 'receptors' and ligands involved in inhibition of platelet activation.**

PGI_2 inhibits platelet aggregation via activation of adenylate cyclase, leading to cyclic-3'5'-adenosine monophosphate (cAMP) synthesis and activation of protein kinase A (PKA; Mustard *et al.*, 1980; Wise, 2003). The use of PGI_2 in the preparation of washed platelets prevents activation and prolongs platelet viability (Blackwell *et al.*, 1982; Read *et al.*, 1985). However, PGI_2 is a weak inhibitor of platelet adhesion (Krishnamurthi *et al.*, 1984; Radomski *et al.*, 1987d), while NO is an efficient inhibitor of both platelet adhesion and aggregation (Radomski *et al.*, 1987b; Radomski *et al.*, 1987c). PGD_2 is also able to prevent platelet aggregation (Nishizawa *et al.*, 1975), although it is considerably less efficient than PGI_2 (Whittle *et al.*, 1978). Similarly, adenosine has also long been known to inhibit platelet aggregation (Born *et al.*, 1964; Caen *et al.*, 1972) through G-protein-mediated activation of adenylate cyclase and production of cAMP (Paul *et al.*, 1990).

1.2.5 PLATELET PHYSIOLOGY

A role for platelets in haemostasis has been implied for well over 100 years (Bizzozero, 1881; Bizzozero, 1882). They adhere and aggregate at sites of vascular damage, thereby plugging breaches in the vascular wall and preventing excessive blood loss. In addition to this role, platelets are also active participants in inflammation and host defence against infection (Mannaioni *et al.*, 1997; Weyrich *et al.*, 2003). Platelets release numerous bactericidal factors (Hirsch, 1960; Weksler & Nachman, 1971; Yeaman *et al.*, 1992), and can bind and internalise various micro-organisms including bacteria (Clawson & White, 1971a; Clawson & White, 1971b), pathogenic fungi (Christin *et al.*,

1998; Kennedy *et al.*, 1992; Robert *et al.*, 2000) and viruses (Bik *et al.*, 1982; Davis & Zauli, 1995). Platelet adhesion and activation is important in assisting leukocyte function and in the initiation of vascular repair through the wide variety of mediators released during activation (table 1.1; Anitua *et al.*, 2004).

1.2.6 PLATELET PATHOPHYSIOLOGY

Platelets are a crucial element in haemostasis, and dysfunction can lead to excessive bleeding on one hand, or increased thrombosis on the other. Furthermore, inappropriate platelet activity may contribute to various inflammatory conditions, exacerbating tissue damage. Therefore, platelets are a legitimate therapeutic target in a number of disorders.

1.2.6.1 REDUCED PLATELET ACTIVITY

Reduced platelet function may be caused by genetic defects in genes that code for proteins involved in platelet adhesion, activation or production. Bernard-Soulier syndrome is an autosomal recessive disorder (Lopez *et al.*, 1998; Nurden & Nurden, 2001) characterised by reduced platelet adhesion, due to a genetic defect in the GP1b/V/IX receptor (Kunishima *et al.*, 1994; Sae-Tung *et al.*, 1996; Simsek *et al.*, 1994). Glanzmann-thrombasthenia, which is due to a defect in GPIIb/IIIa (Basani *et al.*, 1996; Grimaldi *et al.*, 1998; Ruan *et al.*, 1999), is associated with the complete absence of platelet aggregation, and is associated with purpura, epistaxis and gastrointestinal bleeding (French & Seligsohn, 2000; Nurden & Nurden, 2001). The main treatment for these disorders is transfusion, although gene therapy may be of possible use in the future

(Wilcox *et al.*, 2000). Reduced platelet function may also be acquired via other haematological defects such as myeloproliferative disorders (Landolfi *et al.*, 1997) or extended drug therapy (e.g. heparin; Despotis *et al.*, 1996).

1.2.6.2 INCREASED PLATELET ACTIVITY

Increased platelet reactivity has been observed in patients with diabetes (Knobler *et al.*, 1998; Mandal *et al.*, 1993; Tomaselli *et al.*, 1990), hypertension (Chen *et al.*, 1984; Lande *et al.*, 1987; Thomas *et al.*, 1992), stable/unstable angina (Kabbani *et al.*, 2001; Kusui *et al.*, 1989; Smitherman *et al.*, 1981) and peripheral/coronary artery disease (Furman *et al.*, 1998; Reininger *et al.*, 1999; van Zanten *et al.*, 1994). In diabetics, hyperglycaemia may increase vWF-dependent platelet activation at high shear rates (Gresele *et al.*, 2003), possibly through a glucose-mediated rise in plasma osmolarity leading to interference of the platelet membrane and increased reactivity (Keating *et al.*, 2003). Oxidised low density lipoprotein (LDL), a key mediator in the progression of atherosclerosis (Navab *et al.*, 1996), directly activates platelets (Aviram, 1989; Aviram *et al.*, 1989). Thrombotic disorders are also increased in hyperhomocysteinemia (Bos & den Heijer, 1998; Coppola *et al.*, 2000; de Jong *et al.*, 1998), which is an independent predictor of atherosclerosis and coronary artery disease (Kang *et al.*, 1986; Montalescot *et al.*, 1997; Yoo *et al.*, 1999). Homocysteine has been hypothesised to increase platelet activation through enhanced vessel and platelet TxA_2 synthesis (Bagi *et al.*, 2001; Ungvari *et al.*, 2000). Furthermore, increased platelet reactivity is also associated with risk factors for atherosclerosis including smoking (Schmidt & Rasmussen, 1984; Schmidt *et al.*, 1990). A common factor in many disease states that are linked to platelet

hyperactivity is the manifestation of endothelial dysfunction (chapter 1.3.8.2), a condition characterised by a reduction in NO bioavailability. As mentioned, platelets also play an active role in the progression of inflammatory conditions, including atherosclerosis. Platelet adhesion to the endothelium coincides with inflammation-induced gene expression and precedes the development of atherosclerotic lesions in ApoE deficient mice (Massberg *et al.*, 2002). Indeed, adhered platelets instigate and propagate lesion formation through P-selectin-mediated leukocyte recruitment (Burger & Wagner, 2003; Huo *et al.*, 2003). In this situation, platelets are at the heart of a self-propagating system in which platelet adhesion and activation drive inflammation, creating positive feedback to further increase platelet activation, deposition and tissue damage.

1.2.7 ANTIPLATELET THERAPY

Aspirin has been the major antiplatelet agent of choice for many years. Secondary prevention with antiplatelet agents, primarily aspirin, reduces the risk of vascular occlusion by 25% (ATC, 1994); furthermore aspirin treatment for the primary prevention of coronary heart disease decreases cardiovascular events by 15% (Sanmuganathan *et al.*, 2001). Aspirin primarily exerts its effect via acetylation of the substrate pocket of COX, thereby inactivating the enzyme and leading to an inability of platelets to synthesise TxA_2 (Schror, 1997; Vane *et al.*, 1990), although other mechanisms such as increasing NO bioavailability exist (Awtry & Loscalzo, 2000; Lopez-Farre *et al.*, 1995). However, limitations of aspirin include gastrointestinal

haemorrhage (Derry & Loke, 2000), and aspirin resistance, which can occur in up to 40% of patients (Gum *et al.*, 2001). Other commonly used antiplatelet agents include the ADP receptor (P_2Y_{12}) antagonist clopidogrel, and GPIIb/IIIa inhibitors, such as abciximab and tirofiban. Clopidogrel compared favourably to aspirin in terms of reducing stroke and myocardial infarction in a trial involving patients at risk of ischaemic events (CAPRIE, 1996). Furthermore, the combination of aspirin and clopidogrel can result in a synergistic inhibition of platelet activation, (Moshfegh *et al.*, 2000), and is of benefit in preventing acute coronary syndromes in patients with unstable angina (Peters *et al.*, 2003). Intravenous GPIIb/IIIa inhibitors are also useful in preventing acute coronary syndromes in patients undergoing percutaneous coronary intervention (Bhatt & Topol, 2000), however oral GPIIb/IIIa antagonists can increase mortality (Chew *et al.*, 2001), possibly via activation of the GPIIb/IIIa receptor (Cox *et al.*, 2000), resulting in the generation of outside-in signals (Du & Ginsberg, 1997; Haimovich *et al.*, 1993). NO donor drugs offer an alternative to these existing therapies, with potential use in the treatment of a wide range of vasculopathies (chapter 1.3.9).

1.3 NITRIC OXIDE

The discovery of NO as a crucial signalling molecule in the cardiovascular system led to the 1998 Nobel award to Robert F Furchgott, Louis J Ignarro, and Ferid Murad. Their seminal work identified the central role of the endothelium in vascular function and delineated the pathway of production of NO from the endothelium to its activation of the intracellular enzyme, soluble guanylate cyclase.

1.3.1 EDRF & NITRIC OXIDE

In the 1980s, the importance of the vascular endothelium in eliciting relaxation to a wide range of agonists including acetylcholine, bradykinin, and thrombin was realised (Busse *et al.*, 1985; Furchgott, 1984; Furchgott & Zawadzki, 1980). These agonists were observed to cause the release of an endothelium-derived relaxing factor (EDRF) that, as well as inducing a profound vasodilatation (Furchgott, 1984; Furchgott & Zawadzki, 1980), induced a potent inhibition of platelet aggregation and adhesion (Azuma *et al.*, 1986; Busse *et al.*, 1987; Radomski *et al.*, 1987a; Radomski *et al.*, 1987c). EDRF was observed to have a short biological half life (~ 6 seconds; Cocks *et al.*, 1985; Griffith *et al.*, 1984), and to stimulate soluble guanylate cyclase (Rapoport *et al.*, 1983; Rapoport & Murad, 1983), resulting in 3',5'-cyclic guanosine monophosphate (cGMP) production. Subsequently, EDRF was found to have chemical and pharmacological properties that were indistinguishable from the free radical, NO (Furchgott, 1988; Ignarro *et al.*, 1988; Moncada *et al.*, 1988; Palmer *et al.*, 1987).

1.3.2 SYNTHESIS OF NITRIC OXIDE

In mammals, NO is synthesised by isoforms of the enzyme, nitric oxide synthase (NOS) (Griffith & Stuehr, 1995; Knowles & Moncada, 1994). NOS isozymes catalyse the formation of NO and L-citrulline from the substrates L-arginine and O_2 in the presence of the electron donor, nicotinamide adenine dinucleotide phosphate (NADPH; fig 1.3; (Marletta, 1993). Studies in the early 1990s isolated 3 major NOS isoforms: neuronal NOS (nNOS or NOS I; Bredt *et al.*, 1991b; Bredt & Snyder, 1990), inducible NOS

(iNOS or NOSII; Hevel *et al.*, 1991; Lyons *et al.*, 1992; Stuehr *et al.*, 1991; Xie *et al.*, 1992), and endothelial NOS (eNOS or NOSIII; Janssens *et al.*, 1992; Lamas *et al.*, 1992; Nishida *et al.*, 1992; Sessa *et al.*, 1992). In addition to these isoforms, a 4th NOS isoform has been identified in mitochondria (mtNOS; Lacza *et al.*, 2003; Tatoyan & Giulivi, 1998), although this may be a splice variant of nNOS or iNOS (Eissa *et al.*, 1996; Elfering *et al.*, 2002; Kanai *et al.*, 2001).

1.3.2.1 NOS LOCALISATION

While nNOS and eNOS are expressed constitutively, iNOS expression is induced in response to infection or inflammatory stimuli (Geller *et al.*, 1993; Griffith & Stuehr, 1995; Koprowski *et al.*, 1993; Szabo & Thiemermann, 1995). nNOS is found in both central and peripheral (non-adrenergic non cholinergic; NANC) neurones (Bredt *et al.*, 1991a; Bredt *et al.*, 1991b; Chakder *et al.*, 1997), skeletal muscle (Silvagno *et al.*, 1996), and in kidney macula densa cells (Wilcox *et al.*, 1992). iNOS was originally isolated in macrophages (Hevel *et al.*, 1991; Stuehr *et al.*, 1991), but is now known to be inducible in almost all cell types, including vascular smooth muscle and endothelial cells (Koide *et al.*, 1993; MacNaul & Hutchinson, 1993). While eNOS is highly expressed in the vascular endothelium (Forstermann *et al.*, 1993), it is also expressed by various epithelial tissues (Tseng *et al.*, 1996; Xue *et al.*, 1996) and skin cells (Sakai *et al.*, 1996; Shimizu *et al.*, 1997). Platelets also express eNOS (Muruganandam & Mutus, 1994; Radomski *et al.*, 1990a; Radomski *et al.*, 1990b; Sase & Michel, 1995), which in combination with endothelium-derived NO plays an important role in modulating thrombus formation.

1.3.2.2 NOS STRUCTURE AND CATALYTIC MECHANISM

NOS enzymes exist as homodimers, with each monomer containing an oxygenase domain linked to a reductase domain by a Ca^{2+}/calmodulin-binding region (fig 1.4; Alderton *et al.*, 2001). Within the reductase domain are the binding sites for flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD) and NADPH (Ghosh & Stuehr, 1995; Richards & Marletta, 1994), while the oxygenase domain contains the binding sites for L-arginine (L-Arg) and tetrahydrobiopterin (BH_4) along with the haem prosthetic group (Crane *et al.*, 1998; Raman *et al.*, 1998). Electrons donated from NADPH flow through the flavin cofactors to the haem group (fig 1.4), reducing ferric (Fe^{3+}) haem to the ferrous (Fe^{2+}) form and allowing the binding of O_2 (Abu-Soud *et al.*, 1997; Adak *et al.*, 1999; Poulos *et al.*, 1999). Bound calmodulin (CaM) has been proposed to be required for electron flow across the dimer interface to the haem group (Abu-Soud *et al.*, 1994; Matsuda & Iyanagi, 1999). It has been proposed that electrons flow from the reductase domain in one subunit to the oxygenase domain in the other subunit, in a head-to-tail fashion (Siddhanta *et al.*, 1996; fig 1.4). CaM is permanently bound to iNOS (Cho *et al.*, 1992), while an increase in intracellular Ca^{2+} levels is required for CaM binding to eNOS and nNOS (Griffith & Stuehr, 1995; Marletta, 1993). Thus, increased intracellular Ca^{2+} is not required for iNOS, but is essential for eNOS and nNOS activity.

The first step in NO synthesis is a 2-electron oxidation of arginine to N^{ω}-hydroxy-L-arginine (NHA). BH_4 is essential for NHA formation, and may function as a redox active electron donor in this process (Bec *et al.*, 1998; Hurshman *et al.*, 1999;

Witteveen *et al.*, 1999). BH_4 also has numerous other roles in NOS function, including increasing the coupling between NAPDH oxidation and NO synthesis (Vasquez-Vivar *et al.*, 1999; Vasquez-Vivar *et al.*, 1998) and promoting NOS dimer stabilisation (Baek *et al.*, 1993; Klatt *et al.*, 1995). NO is formed when NHA undergoes a further 3-electron oxidation catalysed at the haem centre of the NOS enzyme (Alderton *et al.*, 2001; Korth *et al.*, 1994).

1.3.2.3 NOS REGULATION

As mentioned previously, cellular output of NO may be increased through upregulation of NOS protein expression or by an increase in intracellular Ca^{2+}. The latter effect is of particular importance within platelets given that the induction of platelet activation and Ca^{2+} signalling events will also result in the activation of eNOS and the generation of NO. Indeed, platelet-derived NO plays a prominent role in reducing platelet recruitment following activation (Freedman *et al.*, 1997; Freedman *et al.*, 1999). In addition to protein expression and Ca^{2+} effects, eNOS activity is both positively (Butt *et al.*, 2000; Chen *et al.*, 1999; Dimmeler *et al.*, 1999; Wang *et al.*, 2004) and negatively (Chen *et al.*, 1999; Harris *et al.*, 2001; Michell *et al.*, 2001) regulated by phosphorylation. Activation of eNOS by phosphorylation can increase eNOS output and significantly lower Ca^{2+} sensitivity (Boo *et al.*, 2003; Dimmeler *et al.*, 1999; Fulton *et al.*, 1999), an important component in the activation of NO production by shear stress (Boo *et al.*, 2002; Fisslthaler *et al.*, 2000; Li *et al.*, 2004). eNOS activity is also controlled by N-myristoylation and palmitoylation, which aids the targeting of eNOS to caveolae domains on the membrane surface (Garcia-Cardena *et al.*, 1996; Liu *et al.*, 1996).

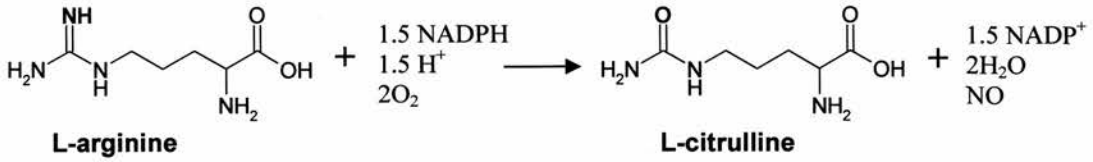


Figure 1.3 **Structure of L-arginine and L-citrulline, indicating the reaction stoichiometry for the formation of NO.**

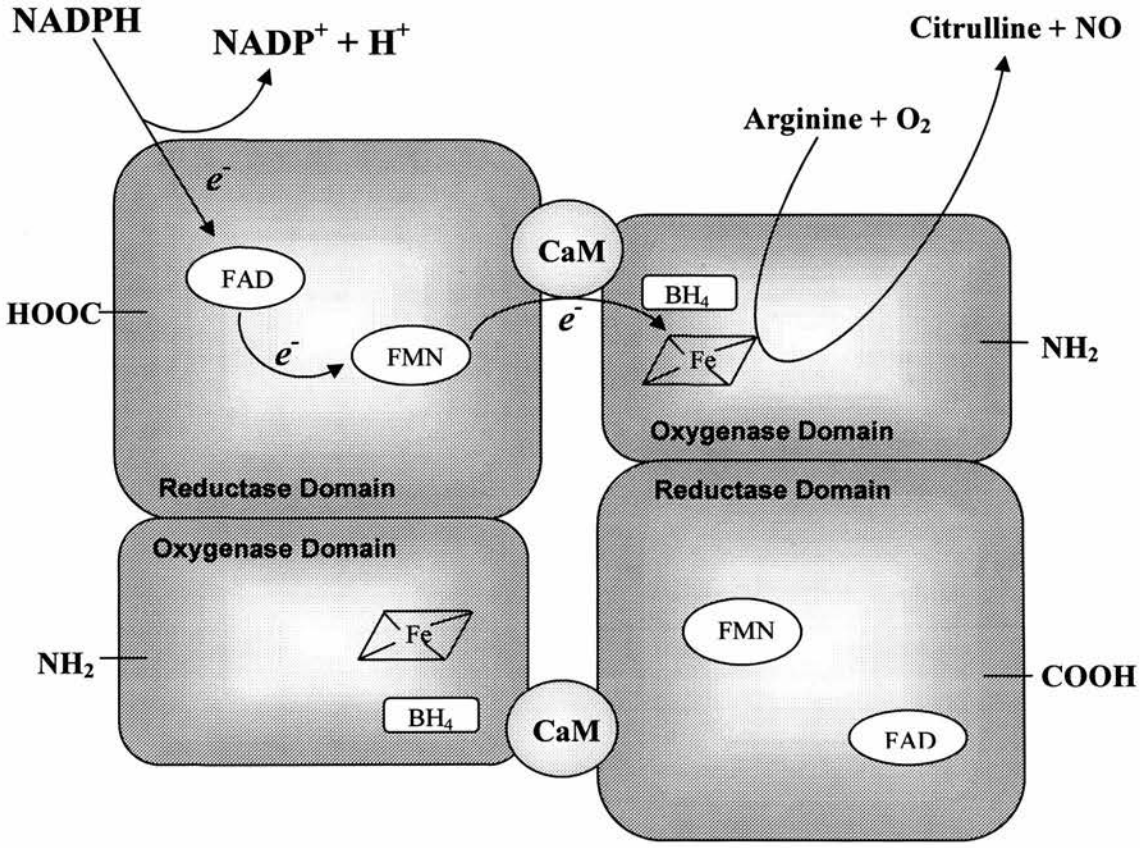


Figure 1.4 **General structure of the NOS enzyme, showing the flow of electrons from NADPH to haem. Adapted from Alderton *et al.*, 2001.**

In addition to their presence in the endothelium, caveolae have been identified in platelets (Jayachandran & Miller, 2003). Localisation of eNOS to caveolae inhibits

eNOS activity (Feron *et al.*, 1996; Ju *et al.*, 1997); agonists such as bradykinin cause dissociation and translocation of eNOS to the cytosol (Prabhakar *et al.*, 1998), allowing CaM to bind to eNOS (Michel *et al.*, 1997a; Michel *et al.*, 1997b) and activate NO synthesis.

1.3.3 SOLUBLE GUANYLATE CYCLASE

Following its synthesis by NOS, the classically defined 'receptor' for NO is soluble guanylate cyclase (sGC). sGC catalyses the conversion of guanosine 5'- triphosphate (GTP) to 3',5'-cyclic guanosine monophosphate (cGMP), which causes downstream signalling events via protein kinase G (PKG), phosphodiesterases (PDEs) and ion channels (Lucas *et al.*, 2000; Waldman & Murad, 1987). In addition to cGMP-dependent effects, it is clear that NO also signals through a number of cGMP-*independent* signalling pathways, which are discussed in a later section (chapter 1.3.5).

1.3.3.1 sGC STRUCTURE AND FUNCTION

sGC is expressed in the cytoplasm of almost all mammalian cells including platelets (Chhajlani *et al.*, 1989; Collier & Vallance, 1989) and exists as a heterodimer consisting of α and β subunits (Harteneck *et al.*, 1990; Kamisaki *et al.*, 1986). Several different isotypes of sGC are expressed within cells, the most common types being $\alpha 1$ and $\beta 1$ (Koesling & Friebe, 1999). sGC monomers are divided into 3 functional domains (fig 1.5): a C-terminal catalytic domain, a central dimerisation region and an N-terminal haem binding domain (Andreopoulos & Papapetropoulos, 2000; Schulz *et al.*, 1991).

The catalytic and dimerisation domains in sGC display high homology with particulate guanylate cyclase (Thorpe & Garbers, 1989; Wilson & Chinkers, 1995), which synthesises cGMP in response to natriuretic peptides such as atrial natriuretic peptide (Tremblay *et al.*, 2002). Interestingly, a proportion of platelet sGC is associated with the membrane fraction, and subsequent activation has been observed to increase the membrane-bound fraction (Kempfert & Behrends, 2003; Zabel *et al.*, 2002). Furthermore, membrane-bound sGC has greater sensitivity towards NO (Zabel *et al.*, 2002), suggesting that redistribution of sGC to membranes may increase cellular responsiveness to NO. The haem element of sGC is responsible for transmitting NO-dependent activation of cGMP synthesis (Craven & DeRubertis, 1978; Craven *et al.*, 1979). The haem-binding domain is co-ordinated by His105, which is present on the β-subunit (Wedel *et al.*, 1994; Zhao & Marletta, 1997), although many other residues including Cys78 & 214 (Friebe *et al.*, 1997), and Tyr 125 and Arg 139 (Schmidt *et al.*, 2004) on the β-subunit are also important for haem binding and the activation of sGC by NO. Interestingly, a number of compounds that activate sGC via an NO-independent mechanism have also been described. These compounds include YC-1, BAY 41-2272 and BAY 58-2667, all of which are potent antiplatelet agents (Stasch *et al.*, 2001; Stasch *et al.*, 2002; Wu *et al.*, 1995). In addition to the haem-Fe, sGC also binds Cu^{2+}, which may act as a co-enzyme for enzyme activity (Schrammel *et al.*, 1996), possibly via catalysing the release of NO from S-nitrosothiols (Singh *et al.*, 1996; chapter 1.3.6.4). NO radical, and not nitrosonium (NO^+) or nitroxyl (NO^-) is the only proven NO-related species capable of direct sGC activation (Dierks & Burstyn, 1996). The binding of NO

to the ferrous haem of sGC results in the formation of a nitrosyl-haem complex, which breaks the axial bond between haem and His105 (Deinum *et al.*, 1996; Ignarro *et al.*, 1982; Stone *et al.*, 1995). The resulting conformational change causes a ~400 fold activation of sGC (Stone & Marletta, 1996), and a reduction in the K_m of sGC for GTP (Wolin *et al.*, 1982).

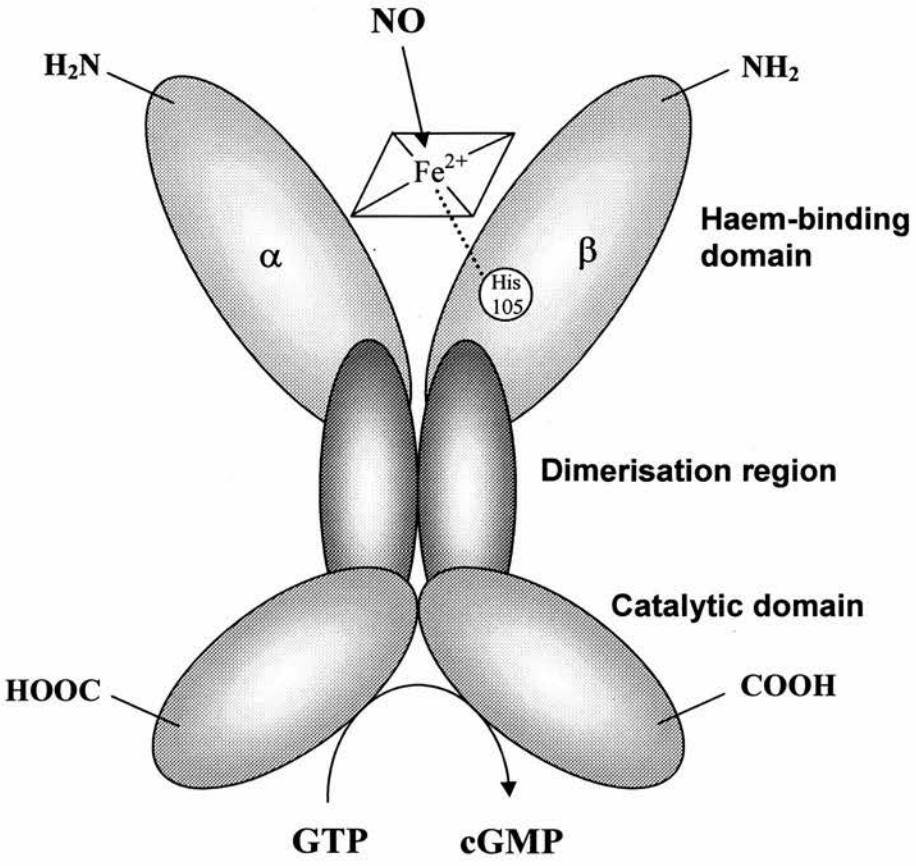


Figure 1.5 **Structure of sGC showing the 3 main domains and His 105 which provides an axial ligand to the haem group. Adapted from Hobbs, 1997.**

There is considerable debate as to the half-life of NO-sGC signalling. Purified nitrosyl-sGC has a half life between 5 s and 3 min at 37 °C (Brandish *et al.*, 1998; Kharitonov *et al.*, 1997; Margulis & Sitaramayya, 2000), the deactivation rate increasing in the

presence of factors such as GTP, oxy-haemoglobin and low molecular weight thiols including glutathione (GSH; Brandish *et al.*, 1998; Margulis & Sitaramayya, 2000). However, in intact cells, the deactivation rate of sGC has been estimated to have a half-life of 190 ms (Bellamy & Garthwaite, 2001), and sGC has been observed to undergo rapid desensitisation in experiments performed in human platelets (in the order of seconds), with a recovery half-life of ~1.5 min (Bellamy *et al.*, 2000). Thus sGC seems well equipped to rapidly modulate its activity in response to changing NO transients.

1.3.4 TARGETS FOR cGMP

There are two main cellular effectors for cGMP in platelets, namely protein kinase G (PKG) and phosphodiesterases (PDEs). In addition to PKG and PDEs, cGMP also signals through non-selective, voltage gated cyclic nucleotide-gated (CNG) channels, which are important in phototransduction in rod photoreceptors and signal transduction throughout the brain (Matulef & Zagotta, 2003). However, CNG channels have not been identified in platelets to date.

1.3.4.1 PROTEIN KINASE G

PKG is the primary effector for cGMP signals in platelets. Binding of cGMP to PKG activates adenosine 5'-triphosphate (ATP)-dependent phosphorylation of key signalling proteins on serine or threonine residues (Hofmann *et al.*, 1992). Mammals possess two different isoforms of PKG (Jarchau *et al.*, 1994; Lohmann *et al.*, 1997; Wernet *et al.*, 1989). PKG I is a 76 kDa soluble form that exists as a homodimer and is widely

expressed throughout the body, including platelets (Eigenthaler *et al.*, 1992; Keilbach *et al.*, 1992). Splicing of PKG I gives rise to α and β isoforms (Pfeifer *et al.*, 1999); platelets primarily contain the β isoform (Eigenthaler *et al.*, 1992). PKG II is a particulate form that is heavily expressed in the intestine (Jarchau *et al.*, 1994; Markert *et al.*, 1995), where it plays a role in mediating fluid homeostasis (Vaandrager *et al.*, 1997). Platelets lacking functional PKG I demonstrate increased adhesion and aggregation following ischaemia-reperfusion (Massberg *et al.*, 1999). PKG is a potent inhibitor of Ca^{2+} signalling in human platelets (Geiger *et al.*, 1992; Heemskerk *et al.*, 1994; Menshikov *et al.*, 1993) and has been demonstrated to phosphorylate platelet IP_3 receptors (Cavallini *et al.*, 1996; El-Daher *et al.*, 2000) resulting in the inhibition of Ca^{2+} efflux from the DTS. cGMP analogues also inhibit IP_3 production in platelets, probably through PKG activation (Nakashima *et al.*, 1986; Takai *et al.*, 1981). The inhibition of Ca^{2+} signalling in platelets has far-reaching consequences due to the Ca^{2+} requirement of many of the proteins needed for platelet activation. Furthermore, PKG can phosphorylate platelet TxA_2 receptors (Wang *et al.*, 1998), resulting in receptor desensitisation (Reid & Kinsella, 2003) and preventing further positive feedback and Ca^{2+} signalling events. In addition to Ca^{2+} signalling, PKG can phosphorylate proteins involved with the cytoskeleton. A major phosphorylation target for PKG (and PKA) in platelets is the vasodilator-stimulated phosphoprotein (VASP; Butt *et al.*, 1994; Halbrugge *et al.*, 1990; Meinecke *et al.*, 1994; Smolenski *et al.*, 1998). VASP is present in high concentrations in human platelets (Eigenthaler *et al.*, 1992), and is associated with microfilaments at areas of focal contact (Reinhard *et al.*, 1992), where it regulates

actin polymerisation and organisation (Reinhard *et al.*, 2001). Phosphorylation of VASP decreases its ability to nucleate and interact with actin filaments (Harbeck *et al.*, 2000), and correlates with GPIIb/IIIa receptor inhibition in intact human platelets (Horstrup *et al.*, 1994). Furthermore, studies utilising VASP knockout mice indicate that VASP increases susceptibility to cGMP-mediated inhibition of platelet aggregation (Aszodi *et al.*, 1999), and that it is absolutely essential for NO-mediated inhibition of platelet adhesion (Massberg *et al.*, 2004). Heat shock protein 27 (Hsp27), which is also involved in the organisation of the platelet cytoskeleton, is also phosphorylated by PKG in platelets (Butt *et al.*, 2001). Hsp27 polymerises actin following activation by the mitogen-activated protein (MAP) kinase pathway following platelet activation, and phosphorylation of Hsp27 by PKG reduces this actin polymerisation (Butt *et al.*, 2001). An additional target for PKG in platelets is the small GTPase Rap1b (Reep & Lapetina, 1996). Rap1b potentiates GPIIb/IIIa mediated interactions in platelets and megakaryocytes (Bertoni *et al.*, 2002; de Bruyn *et al.*, 2003), and has been identified as a key mediator in GPVI-mediated platelet aggregation (Larson *et al.*, 2003). Phosphorylation of Rap1b by PKG correlates with the inhibition of collagen-induced platelet aggregation (Reep & Lapetina, 1996). Other potential substrates for PKG include myosin light chain kinase (MLCK; Nishikawa *et al.*, 1984) and myosin phosphatase (MP; Nakamura *et al.*, 1999), which are involved in the platelet shape change response (Bromberg *et al.*, 1985; Daniel *et al.*, 1984). However, PKG-mediated phosphorylation of MLCK or MP has yet to be demonstrated in intact platelets.

1.3.4.2 PHOSPHODIESTERASES

PDEs catalyse the hydrolysis of the cyclic nucleotides cGMP and cAMP to their corresponding inactive 5'-nucleotides, and therefore also influence intracellular cGMP levels. Mammals possess at least 11 different PDE isozymes (Beavo, 1995; Fawcett *et al.*, 2000; Soderling & Beavo, 2000), 3 of which are important in platelets; types 2, 3 and 5 (Haslam *et al.*, 1999; Hidaka & Asano, 1976). Selective PDE inhibitors for these isozymes are available and many are potent antiplatelet agents (table 1.5; Berkels *et al.*, 2001; Holbrook & Coker, 1989; Kimura *et al.*, 1985; Muller *et al.*, 1990). PDE2 hydrolyses both cGMP and cAMP, and its activity is stimulated by cGMP (Martins *et al.*, 1982; Mumby *et al.*, 1982). PDE3 selectively hydrolyses cAMP, and cGMP inhibits enzyme activation (Degerman *et al.*, 1997), while PDE5 binds and selectively hydrolyses only cGMP (Haslam *et al.*, 1999; Thomas *et al.*, 1990).

PDE isozyme	Inhibitors
PDE 2	Erythro-9-(2-hydroxyl-3-nonyl)adenine (EHNA)
PDE 3	Cilostazol, Enoxamone, Milrinone, Siguazodan
PDE 5	Dypyridamole, MY-5445, Sildenafil, Zaprinast

Table 1.5 **Selective inhibitors of PDE isozymes found in platelets. Adapted from Essayan, 1999.**

The ability of cGMP to inhibit PDE3 and increase cAMP levels demonstrates a degree of crosstalk between the cAMP/cGMP systems, and may explain the synergism observed between cGMP and cAMP elevating agents in smooth muscle relaxation and the inhibition of platelet activation (Maurice & Haslam, 1990a; Maurice & Haslam, 1990b). Interestingly, PKG has recently been demonstrated to phosphorylate PDE5 and

increase enzyme activation in platelets (Corbin *et al.*, 2000; Mullershausen *et al.*, 2003). Thus, PKG-mediated phosphorylation of PDE5 may be a mechanism providing long term feedback to modulate platelet responses to NO.

1.3.5 NO-MEDIATED cGMP-INDEPENDENT SIGNALLING

Several lines of evidence indicate that NO can inhibit platelet activation via a mechanism that does not rely on an increase in intracellular cGMP concentration (Gordge *et al.*, 1998; Gordge *et al.*, 1995; Sogo *et al.*, 2000b; Trepakova *et al.*, 1999; Tsikas *et al.*, 1999a; fig 1.6). Inhaled NO has been demonstrated to inhibit human platelet aggregation via a cGMP-independent mechanism (Beghetti *et al.*, 2003). Moreover, a stimulatory role for PKG in platelet activation has been identified (Li *et al.*, 2003). Despite the clear existence of cGMP-independent antiplatelet effects of NO, very few molecular targets have been conclusively proven in human platelets. A tool that has significantly aided the investigation of cGMP-independent effects is 1-*H*-[1,2,4]oxodiazolo[4,3-*a*]quinoxalin-1-one (ODQ), a potent and relatively selective inhibitor of sGC (fig 1.7; Moro *et al.*, 1996). ODQ rapidly oxidises the ferrous haem of sGC to the ferric form (Zhao *et al.*, 2000), resulting in a persistent inhibition of sGC that may only be partially overcome by a 100-fold excess of the NO donor S-nitroso-N-acetylpenicillamine (SNAP; Moro *et al.*, 1996).

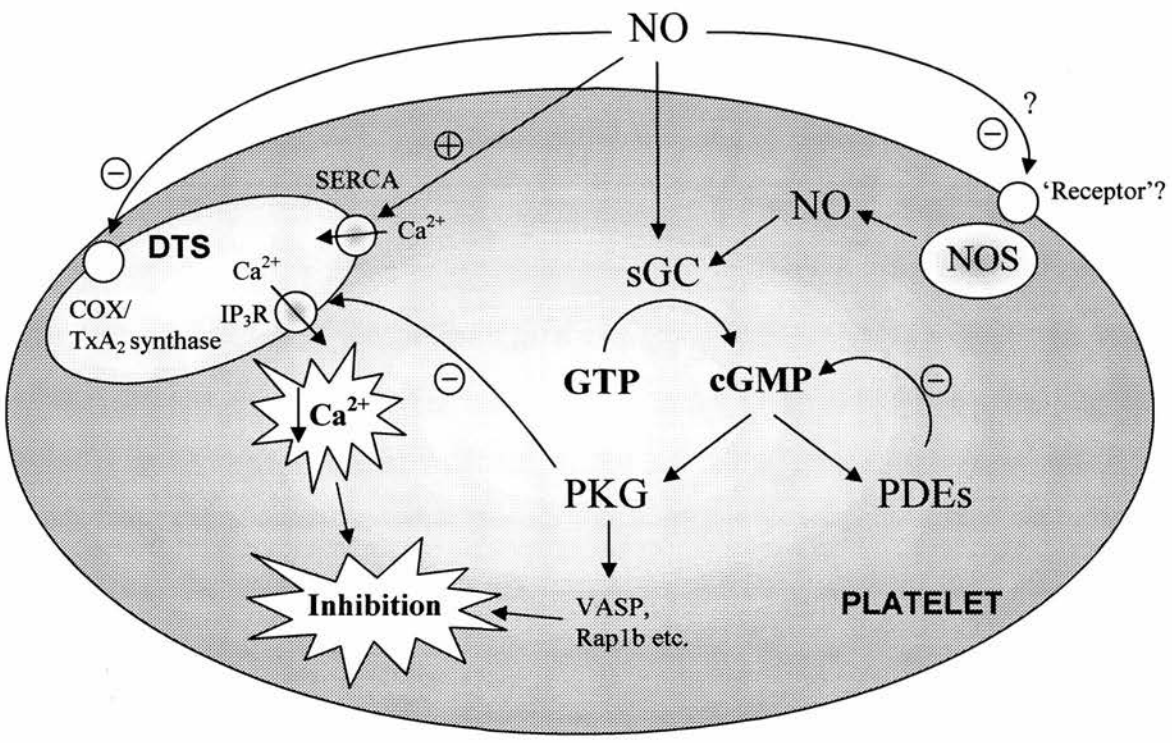


Figure 1.6 **Mechanisms involved in NO-mediated inhibition of platelet activation. Abbreviation: IP_3R, IP_3 receptor.**

ODQ offers significant advantage over other previously used sGC inhibitors such as methylene blue and LY 83583 which can generate superoxide anions and interfere with prostanoid biosynthesis (Hasegawa *et al.*, 2004; Martin *et al.*, 1989; Wolin *et al.*, 1990). Using ODQ, NO has been shown to increase the refilling of Ca^{2+} back to the DTS via acceleration of SERCA through a cGMP-independent mechanism (Homer & Wanstall, 2002; Pernollet *et al.*, 1996; Trepakova *et al.*, 1999). Although recent studies performed in vessels suggest that the NO-mediated chemical modification of SERCA involves peroxynitrite-dependent S-glutathiolation of a critical cysteine residue in the protein (Adachi *et al.*, 2004), it is unclear whether a similar mechanism exists in platelets.

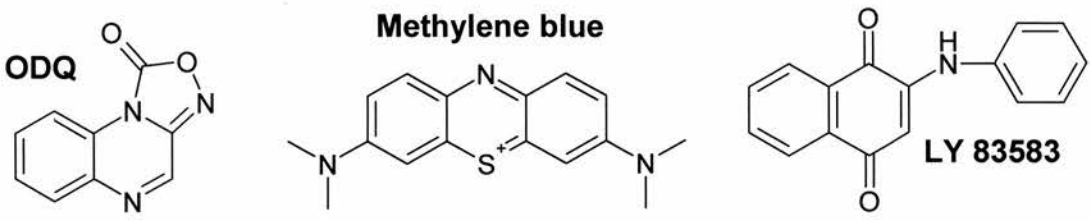


Figure 1.7 **Structures of the sGC inhibitors ODQ, methylene blue and LY 83583.**

Furthermore, while one group has reported that NO exerts a biphasic effect on SERCA (Pernollet *et al.*, 1996), where low concentrations of NO stimulated SERCA activity and high concentrations had the opposite effect, another group have reported only an acceleratory effect of NO on SERCA activity (Trepakova *et al.*, 1999). S-nitrosocysteine has been reported to inhibit collagen-induced TxA_2 synthesis in platelets via a cGMP-independent mechanism (Tsikas *et al.*, 1999a). This effect may be explained via NO-mediated inhibition of COX-1 (Kanner *et al.*, 1992; Tsai *et al.*, 1994) or TxA_2 synthase (Wade & Fitzpatrick, 1997). However, it is likely that other cGMP-independent pathways exist in platelets (Gordge *et al.*, 1998; Sogo *et al.*, 2000b; Trepakova *et al.*, 1999). Interestingly, a correlation exists between the amount of NO released in the extracellular environment and cGMP-independent inhibition of platelet aggregation (Sogo *et al.*, 2000b), a finding extended to NO-mediated vasodilatation (Homer *et al.*, 1999; Miller *et al.*, 2004). These data highlight a possible role for an extracellular plasma membrane-located target for cGMP-independent inhibition of platelet activation by NO (Gordge *et al.*, 1998; Sogo *et al.*, 2000b).

1.3.6 NO BIOCHEMISTRY

It is becoming increasingly evident that the chemistry and biochemistry of NO is central to its role as a mediator in the cardiovascular, nervous and immune systems. NO signalling in a particular tissue or organ can be dramatically altered in response to different physiological or pathophysiological conditions, which is highly dependent on the interaction of NO with numerous biological species.

1.3.6.1 REACTION WITH HAEM

Arguably the most important biological reaction of NO is with haem, resulting in sGC activation and the generation of cGMP (chapter 1.3.3). In addition to sGC haem, NO also reacts rapidly with haem groups on haemoglobin and myoglobin (Doyle & Hoekstra, 1981; Herold *et al.*, 2001; Herold & Rehmann, 2001). NO can react with both oxy and deoxy-haemoglobin, resulting in the generation of methaemoglobin and nitrate (NO_3^-), or iron-nitrosyl-haemoglobin respectively. Although the reaction between NO and oxy-haemoglobin is a likely route of NO inactivation (Kosaka *et al.*, 1989; Schechter & Gladwin, 2003), recent evidence suggests that the NO-deoxyhaemoglobin reaction may be important in the preservation of NO-bioactivity, via the formation of S-nitrosohaemoglobin (Datta *et al.*, 2004; Jia *et al.*, 1996; Luchsinger *et al.*, 2003). Other haemoproteins that NO has been shown to interact with and inhibit include cytochrome P450 (Khatsenko *et al.*, 1993; Oyekan, 2002; Quaroni *et al.*, 1996), NOS (Buga *et al.*, 1993; Hurshman & Marletta, 1995), and COX (Goodwin *et al.*, 1998; Goodwin *et al.*, 1999a; O'Donnell *et al.*, 2000).

1.3.6.2 REACTION WITH MOLECULAR OXYGEN

In physiological solutions at low concentrations of NO (nM), the reaction between NO and molecular oxygen (O_2) is relatively slow (pseudo 2nd order; rate constant ~ 4 x 10^6 $M^{-2}sec^{-1}$) and yields nitrogen dioxide NO_2 (equation 1; Lewis & Deen, 1994; Wink *et al.*, 1994). Although NO_2 can react with water to generate nitrite (NO_2^-) and nitrate (NO_3^-; equation 2; Butler *et al.*, 1995), it preferentially reacts with NO to generate N_2O_3 (equation 3; Espey *et al.*, 2001; Wink *et al.*, 1993).

$$NO + O_2 \longrightarrow NO_2 \quad (1)$$

$$2NO_2 + H_2O \longrightarrow NO_2^- + NO_3^- + 2H^+ \quad (2)$$

$$NO_2 + NO \longrightarrow N_2O_3 \quad (3)$$

Given the low concentrations of NO generated under physiological conditions and the slow rate of NO autooxidation, the contribution of N_2O_3 to NO physiology has previously been questioned. However, it is now clear that N_2O_3 formation may be accelerated in the hydrophobic interiors of proteins and membranes (Goss *et al.*, 1999; Liu *et al.*, 1998a; Nedospasov *et al.*, 2000), which greatly concentrate reacting NO species. N_2O_3 has been the subject of much interest because it is a powerful S-nitrosating agent (chapter 1.3.6.4); however it also reacts with water, resulting in the generation of NO_2^- (equation 4).

$$N_2O_3 + H_2O \longrightarrow 2NO_2^- + 2H^+ \quad (4)$$

1.3.6.3 REACTION WITH SUPEROXIDE

Superoxide (O_2^-), a reactive oxygen species (ROS), is generated by mammals through a number of different enzymes including NAD(P)H oxidases, xanthine oxidases,

cyclooxygenase, NOS and enzymes of the respiratory chain (Griendling *et al.*, 2000; Lander, 1997; Porasuphatana *et al.*, 2003; Turrens, 1997). Although O_2^- is usually removed via the action of superoxide dismutase (SOD) and catalase (equation 5), excess O_2^- generation can lead to endothelial dysfunction (chapter 1.3.8.2). O_2^- reacts with NO at almost diffusion limited rates (rate constant: 6.7 x 10^9 $M^{-1}s^{-1}$; Huie & Padmaja, 1993; Jourd'heuil *et al.*, 2001), resulting in the generation of peroxynitrite ($ONOO^-$; equation 6).

$$O_2^- + 2H^+ \xrightarrow{SOD} O_2 + H_2O_2 \xrightarrow{catalase} O_2 + H_2O \quad (5)$$

$$O_2^- + NO \longrightarrow ONOO^- \quad (6)$$

Peroxynitrite is a potent cytotoxic agent (Radi *et al.*, 1991a; Radi *et al.*, 1991b; Szabo, 1996), and undergoes rapid decomposition via peroxynitrous acid (equation 7) to form NO_2 and hydroxyl radicals ($OH^·$; equation 8; Butler *et al.*, 1995; Kharitonov *et al.*, 1994; Wink *et al.*, 1993).

$$ONOO^- + H^+ \longrightarrow ONOOH \quad (7)$$

$$ONOOH \longrightarrow NO_2 + HO^· \quad (8)$$

Under physiological conditions, $ONOO^-$ can also react with CO_2, ultimately generating further NO_2 and NO_2^- (Espey *et al.*, 2002; Jourd'heuil *et al.*, 1999). Thus, multiple mechanisms exist for the conversion of NO to NO_2 and subsequently N_2O_3.

1.3.6.4 REACTION WITH THIOLS

NO itself is a poor nitrosating agent: the principal NO-derived species involved in the biological nitrosation of thiols is N_2O_3 (Keshive *et al.*, 1996; Kharitonov *et al.*, 1995). N_2O_3 reacts with thiols (general formula R-SH) to generate S-nitrosothiols (RSNO;

equation 9). RSNOs undergo rapid transnitrosation reactions (equation 10; (Hogg, 1999; Liu *et al.*, 1998b), shown to occur *in vivo* (Scharfstein *et al.*, 1994), resulting in the transfer of an NO^+ moiety from one thiol to another. Furthermore, RSNOs are extremely susceptible to catalysis by agents including Cu^+ and light (Gorren *et al.*, 1996; Sexton *et al.*, 1994; Singh *et al.*, 1996), resulting in the liberation of NO (equation 11).

$$N_2O_3 + RSH \longrightarrow RSNO + H^+ + NO_2^- \quad (9)$$

$$R_1SNO + R_2SH \longrightarrow R_1SH + R_2SNO \quad (10)$$

$$2RSNO \xrightarrow[h\nu]{Cu^+} 2RSSR + 2NO \quad (11)$$

The interaction of NO with protein thiols (such as cys 34 of albumin) results in the generation of species with considerably longer half-life than NO-itself (Stamler *et al.*, 1992a; Stamler *et al.*, 1992c). Thus, species such as S-nitrosoalbumin are candidates for a potential 'NO reservoir' that may prolong NO bioactivity. Furthermore, low molecular weight (LMW) thiols such as cysteine form unstable RSNOs (Mathews & Kerr, 1993) that are prone to rapidly decompose with the subsequent generation of NO. Therefore, transnitrosation reactions resulting in the transfer from protein-bound NO to LMW thiols may result in the potentiation of RSNO activity and the rapid release of NO (Scharfstein *et al.*, 1994).

1.3.6.5 REACTION WITH TYROSINE

$ONOO^-$ is a potent nitrating species, resulting in the addition of an NO_2^+ equivalent to a site of electron density, often on protein tyrosine residues (Sawa *et al.*, 2000; van der Vliet *et al.*, 1995). The ability of $ONOO^-$ to nitrate critical tyrosine residues and interfere with phosphorylation events is a mechanism implicated in the antiplatelet

activity of $ONOO^-$ (Low *et al.*, 2002; Mondoro *et al.*, 1997). Furthermore, $ONOO^-$-mediated nitration of tyrosine residues in COX may inhibit platelet COX activity (Boulos *et al.*, 2000), although it is clear that NO itself is also able to nitrate COX (Goodwin *et al.*, 1998).

1.3.6.6 OTHER REACTIONS

NO can react with non-haem iron-sulphur centres such as those found in the respiratory chain, leading to inhibition of respiration (Brown & Cooper, 1994; Butler & Megson, 2002; Welter *et al.*, 1996). NO is also a potent inhibitor of lipid peroxidation and interacts with lipid-derived radicals at diffusion-limited rates to terminate damaging lipid peroxidation propagation reactions (Maricq & Szente, 1996; O'Donnell *et al.*, 1997; Rubbo *et al.*, 1994). However, both $ONOO^-$ and NO_2 can oxidise and nitrate unsaturated lipids (D'Ischia *et al.*, 1999; Gallon & Pryor, 1994; O'Donnell & Freeman, 2001; Patel & Block, 1986), which may play a role in the pathogenesis of inflammatory conditions such as atherosclerosis (Lusis, 2000; Maxwell & Lip, 1997).

1.3.7 NO PHYSIOLOGY

NO functions not only as an important inhibitor of platelet function but also as a key signalling molecule throughout the cardiovascular system. Furthermore, NO has far-reaching roles throughout both the nervous and immune systems, with wide physiological implications on bodily function.

1.3.7.1 PLATELETS

The basal release of NO is critical in modulating platelet reactivity *in vivo* (Golino *et al.*, 1992; Yao *et al.*, 1992). Importantly, NO derived from both the endothelium and platelets contributes to the antiplatelet effect (Freedman *et al.*, 1997; Freedman *et al.*, 1999; Simon *et al.*, 1995). Platelet-derived NO in particular seems to play a role to prevent further platelet recruitment following activation (Freedman *et al.*, 1997; Freedman *et al.*, 1999), in line with the known Ca^{2+}-mediated activation of platelet NOS during the aggregation response. Indeed, platelets from patients with acute coronary syndromes generate less NO than those with stable angina (Freedman *et al.*, 1998), implying that platelet NO production protects against thrombosis. Similarly, the importance of endothelium-derived NO in preventing inappropriate platelet adhesion and aggregation is underpinned in endothelial dysfunction (chapter 1.3.8.2), which is a root cause in many disease states associated with excessive thrombosis.

1.3.7.2 BLOOD VESSELS

As mentioned above, NO accounts for at least some of the properties of EDRF and relaxes vascular smooth muscle leading to a profound vasodilation (Busse *et al.*, 1985; Furchgott, 1984; Furchgott & Zawadzki, 1980; Palmer *et al.*, 1987). As in platelets, NO induces vasodilation via both cGMP-dependent and independent mechanisms (Homer *et al.*, 1999; Miller *et al.*, 2004; Rapoport *et al.*, 1983; Rapoport & Murad, 1983). Infusion of NOS inhibitors such as N^{ω}-monomethyl-L-arginine (L-NMMA) in humans causes vasoconstriction, particularly in arterial conduits, indicating that tonic NO production by

the endothelium contributes to basal vascular tone (Haynes *et al.*, 1993; Vallance *et al.*, 1989a; Vallance *et al.*, 1989b). Interestingly, resting platelets may also synthesise sufficient quantities of NO to significantly affect vascular tone and modulate vascular blood flow (Zhou *et al.*, 1995). It is therefore unsurprising that reduced NO bioavailability has been identified as a contributory factor in the pathogenesis of hypertension (Linder *et al.*, 1990; Panza *et al.*, 1993) although controversy still reigns as to whether such endothelial dysfunction is a cause or effect of hypertension.

1.3.7.3 THE HEART

Cardiomyocytes can express all 3 isoforms of NOS (Balligand *et al.*, 1994; de Belder *et al.*, 1993; Xu *et al.*, 1999). The effects of NO on the heart are complicated and dependent on the underlying activity of the heart. In unstimulated hearts, NO induces a biphasic effect, exerting a positive inotropy at low concentrations, while causing a negative inotropy at higher concentrations (Kojda *et al.*, 1996; Mohan *et al.*, 1996; Wegener *et al.*, 2002a). Interestingly, the positive inotropic effect may be mediated via cGMP-independent mechanisms (Campbell *et al.*, 1996; Paolocci *et al.*, 2000), while the negative inotropic effect is likely mediated through the activation of PKG (Wegener *et al.*, 2002b). In hearts stimulated by β-adrenergic agonists, NO-dependent negative inotropic effects are more evident (Gyurko *et al.*, 2000), and may form a negative feedback system to regulate contractility via β_3-mediated stimulation of eNOS (Barouch *et al.*, 2002; Kanai *et al.*, 1997). In addition to inotropic effects, NO also increases cardiac relaxation (Grocott-Mason *et al.*, 1994; Layland *et al.*, 2002), decreases heart

rate (Balligand *et al.*, 1993; Choate *et al.*, 2001), and through its vasoactive properties is able to modulate cardiac preload and afterload.

1.3.7.4 KIDNEYS

NO plays a central role in the kidney, regulating glomerular filtration, sodium haemostasis and blood pressure. (Blantz *et al.*, 2002; Cowley *et al.*, 2003; Kone & Baylis, 1997). NO is important in maintaining adequate circulation within the renal medulla (Mattson *et al.*, 1992; Navar *et al.*, 1996), a reduction of which can lead to the development of hypertension (Mattson *et al.*, 1994). It is clear that one of the central roles of NO within the kidney is to counteract the renin-angiotensin system (De Nicola *et al.*, 1992; Sigmon *et al.*, 1992). NO is able to inhibit Na^+ reabsorption throughout kidney tubules (Liang & Knox, 2000; Ortiz & Garvin, 2001), and has been shown to decrease the sensitivity of tubuloglomerular feedback (Thomson & Deng, 2003; Thorup & Persson, 1994), a process which is notably enhanced in spontaneously hypertensive rats (Welch *et al.*, 2000).

1.3.7.5 THE IMMUNE SYSTEM

NO can prevent inflammatory cell adhesion, activation and chemotaxis (Chello *et al.*, 1998; May *et al.*, 1991; Sato *et al.*, 1996; Thomazzi *et al.*, 2004). Following infection, iNOS expression is upregulated and NO production is substantially increased (Nicholson *et al.*, 1996; Ochoa *et al.*, 1991; Stenger *et al.*, 1996; Wheeler *et al.*, 1997). Upregulation of iNOS primarily occurs in inflammatory cells, although numerous cell types have also been shown to express iNOS under inflammatory conditions (Rao,

2000). Although NO itself is antimicrobial (Assreuy *et al.*, 1994; Fernandes & Assreuy, 1997), the cytotoxic effects of NO are often mediated via the generation of other intermediates such as S-nitrosothiols (Incze *et al.*, 1974) or $ONOO^-$ (De Groote *et al.*, 1995; Vazquez-Torres *et al.*, 1996), resulting from the simultaneous generation of O_2^- and NO by inflammatory cells (Rodenas *et al.*, 1995). Numerous mechanisms of NO-mediated cytotoxicity have been identified, including direct damage of DNA (Maragos *et al.*, 1993; Wink *et al.*, 1991) and inhibition of respiration (Castro *et al.*, 1994; Hausladen & Fridovich, 1994). NO is also involved in the inflammatory process, but its role is complex. Although NO may contribute to vasodilation and vascular leakage (Vallance & Moncada, 1994), it may also decrease leukocyte recruitment by decreasing the expression of various cell adhesion molecules on endothelial cells (Khan *et al.*, 1996; Spiecker *et al.*, 1997). These effects may be mediated through NO-dependent effects on the activity of nuclear transcription factor κB (NF-κB; Lander *et al.*, 1993; Park *et al.*, 1997; Peng *et al.*, 1995), an important regulator of inflammatory gene expression (Ghosh *et al.*, 1998; May & Ghosh, 1998). In chronic inflammation, as occurs in conditions such as atherosclerosis, NO, and particularly iNOS, may have a positive or negative effect on the inflammatory process (Detmers *et al.*, 2000; Rikitake *et al.*, 1998; Russell *et al.*, 1995), which is likely to be dependent on the relative conversion of NO to cytotoxic $ONOO^-$ (chapter 1.3.6.3; Buttery *et al.*, 1996; Luoma *et al.*, 1998).

1.3.7.6 THE NERVOUS SYSTEM

NO functions as a neurotransmitter in both peripheral and central nerves. In the periphery, NO constitutes an important effector of inhibitory NANC neurones (Bult *et al.*, 1990; Li & Rand, 1989), which induce relaxation of smooth muscle in blood vessels (Cederqvist *et al.*, 1991; Persson *et al.*, 1991) and tissues, including the gut and penis (Boeckxstaens *et al.*, 1991a; Boeckxstaens *et al.*, 1991b; Desai *et al.*, 1991; Kim *et al.*, 1991). In the central nervous system (CNS), nNOS is highly expressed, particularly in the cerebellum and hypothalamus (Schilling *et al.*, 1994; Vanhatalo & Soinila, 1995). NO seems to have a number of roles within the CNS, including the central control of the cardiovascular system (Chikada *et al.*, 2000), and in long term potentiation and memory formation (Bohme *et al.*, 1993; Holscher & Rose, 1992). NO may also function as a neuroprotector through its antioxidant effects (Mohanakumar *et al.*, 2002). While many of the effects of NO in the nervous system are mediated through the sGC-cGMP signalling axis, NO can also regulate signalling through modulation of the numerous ion channels via S-nitrosation reactions (Ahern *et al.*, 2002).

1.3.7.7 OTHER ORGANS AND TISSUES

As mentioned, NO is an important regulator of gut motility, primarily mediated via NANC neurones. NO enhances gastric accommodation and emptying (Konturek *et al.*, 1995), while scavengers of NO increase oesophageal peristalsis (Murray *et al.*, 1995). In the liver, basal NO has been demonstrated to be hepatoprotective via a mechanism not solely dependent on its vasorelaxation properties (Cottart *et al.*, 1999). Similarly, NO released from epithelial cells within the lungs is bronchoprotective (Ricciardolo *et al.*,

1996) and prevents agonist-induced bronchoconstriction (Figini *et al.*, 1996; Taylor *et al.*, 1998; Yoshihara *et al.*, 1998). Many of the protective roles of NO are likely to be mediated via antioxidant mechanisms (Wink *et al.*, 2001). Other important physiological roles of NO include the modulation of salivary secretion (Looms *et al.*, 2002) and a wide variety of reproductive functions (Dixit & Parvizi, 2001).

1.3.8 PATHOPHYSIOLOGICAL IMPACT OF NO

Due to the participation of NO in numerous physiological processes, a significant reduction or increase in NO synthesis or bioavailability can contribute to numerous pathophysiological events.

1.3.8.1 INCREASED NO PRODUCTION

Enhanced NO synthesis has been implicated in the pathogenesis of septic shock (Fleming *et al.*, 1990; Thiemermann *et al.*, 1993), a condition characterised by the rapid onset of hypotension in response to severe infection that can lead to systemic vascular collapse (Parrillo *et al.*, 1990). Increased NO synthesis appears to be mediated via an early activation of eNOS, followed by induction of iNOS (Fleming *et al.*, 1990; Kilbourn *et al.*, 1990; Sato *et al.*, 1995). Enhanced NO production may also be involved in the pathophysiology of anaphylactic shock and haemorrhagic shock (Amir & English, 1991; Zingarelli *et al.*, 1992). In the central nervous system, excess NO generation may be responsible for glutamate-induced neuronal death (Dawson *et al.*, 1991; Yamauchi *et al.*, 1998), while a pathophysiologic role for eNOS-derived NO in portal hypertension

has also been identified (Sieber & Groszmann, 1992; Theodorakis *et al.*, 2003). Although iNOS expression is increased in numerous cardiovascular conditions including atherosclerosis (de Belder *et al.*, 1995; Dusting, 1996), these conditions are also often associated with increased synthesis of oxygen-derived radicals such as O_2^- (Kojda & Harrison, 1999), resulting in the formation of $ONOO^-$ and destructive hydroxyl radicals (chapter 1.3.6.3; Kontos & Hess, 1983). Indeed, a likely source for increased O_2^- is NOS itself, which under reduced substrate or cofactor availability can generate O_2^- (Miller *et al.*, 2000a; Stroes *et al.*, 1998; Xia *et al.*, 1998). Thus, an increase in iNOS expression can cause a paradoxical depression of NO bioavailability.

1.3.8.2 REDUCED NO PRODUCTION/ BIOAVAILABILITY

Endothelial dysfunction is often defined by a reduction in the bioavailability of NO in the vasculature as a result of reduced NO synthesis and/or accelerated NO destruction. Endothelial dysfunction has been observed in numerous cardiovascular conditions including atherosclerosis and its associated risk factors (Bellamy *et al.*, 1998; Bossaller *et al.*, 1987; Drexler & Zeiher, 1991; Pepine *et al.*, 1998), diabetes (Cosentino & Luscher, 1998; Johnstone *et al.*, 1993), heart failure (Drexler *et al.*, 1992; Katz *et al.*, 1993), and hypertension (Linder *et al.*, 1990; Panza *et al.*, 1993). Furthermore, platelet synthesis of NO predicts coronary syndromes such as unstable angina and myocardial infarction (Freedman *et al.*, 1998), of which thrombosis is a major precipitating cause (Rentrop *et al.*, 1981; Vetrovec *et al.*, 1982). Numerous mechanisms have been identified that might contribute to reduced NO bioavailability. In diabetes, decreased NO may be a direct consequence of the reduced ability of glycated red blood cell

haemoglobin to deliver NO (James *et al.*, 2004). Reduced eNOS expression is observed in patients with atherosclerosis (Oemar *et al.*, 1998), and decreased endogenous levels of L-arg and BH_4 are implicated in the development of endothelial dysfunction (Drexler *et al.*, 1991; Tiefenbacher *et al.*, 2000). Synthesis of NO can also be reduced by asymmetric dimethylarginine (ADMA), an endogenous inhibitor of NOS, which is increased in atherosclerotic disease (Boger *et al.*, 1998; Miyazaki *et al.*, 1999). As mentioned above, reduced levels of L-Arg and BH_4 can also lead to the generation of O_2^- by NOS. This, in combination with increased generation of O_2^- by enzymes such as NAD(P)H oxidase (Meyer & Schmitt, 2000), not only reduces NO bioavailability but results in the formation of $ONOO^-$, which chemically denudes the endothelium, leading to the exposure of a pro-thrombotic surface. Moreover, high concentrations of O_2^- and $ONOO^-$ directly activate platelets (Brown *et al.*, 1998; Leo *et al.*, 1997). These observations, coupled with data from animal thrombosis models showing that modulation of endogenous NO synthesis markedly modifies platelet responses *in vivo* (Stagliano *et al.*, 1997; Yao *et al.*, 1992) emphasise the importance of a fully functional NO generating system in maintaining normal platelet function and haemostasis.

1.3.9 NITRIC OXIDE THERAPY

Drugs that deliver NO provide an obvious means of treating thrombotic disorders and other conditions characterised by endothelial dysfunction and a lack of NO bioavailability. In addition, NO-donor drugs are excellent investigative tools for delineating complicated NO-mediated signalling pathways. Recent approaches in the

development of NO-donor drugs have led to the development of a number of novel NO-based therapies that may prove useful antiplatelet agents.

1.3.9.1 INHALED NITRIC OXIDE

Inhaled NO (iNO) inhibits platelet aggregation and prolongs bleeding *in vivo* (Beghetti *et al.*, 2003; Gries *et al.*, 1998; Gries *et al.*, 2000). Interestingly, iNO appears to inhibit platelet activation via a cGMP-independent mechanism (Beghetti *et al.*, 2003). Furthermore, it also prevents platelet P-selectin expression, an effect likely to account for its ability to inhibit platelet-leukocyte interaction (Gries *et al.*, 1998; Gries *et al.*, 2003). Animal-based studies indicate that iNO may be useful in the treatment of conditions such as acute pulmonary embolism and acute lung inflammation (Gries *et al.*, 1997; Kermarrec *et al.*, 1998). However, iNO is rapidly inactivated by haemoglobin, and can lead to methaemoglobinaemia (Weinberger *et al.*, 2001), although this risk is preventable by close monitoring. Indeed, the rapid inactivation of iNO by oxy-haemoglobin may explain why some groups have found that iNO has only modest, or no effect on platelet function (Albert *et al.*, 1999a; Albert *et al.*, 1999b; Albert *et al.*, 1996).

1.3.9.2 ORGANIC NITRATES

Organic nitrates have been in clinical use for over 100 years. Glyceryl trinitrate (GTN) and isosorbide dinitrate (ISDN; fig 1.8) are commonly used in the treatment of angina and heart failure (Vlay & Cohn, 1985). Nitrates dilate veins, collaterals and coronary arteries, reducing cardiac preload and increasing oxygenation of the heart respectively (Parker & Parker, 1998). *In vivo* administration of GTN and ISDN inhibits platelet

aggregation and adhesion in patients with angina and myocardial infarction (Diodati *et al.*, 1990; Gebalska *et al.*, 2000) and reduces platelet deposition in pig arteries following balloon angioplasty (Lam *et al.*, 1988) but not in rabbits (Miller *et al.*, 2003). Nitrates such as GTN inhibit platelet activation through a mechanism completely dependent on activation of sGC (Sogo *et al.*, 2000b). One of the main caveats with organic nitrates is tolerance (Mangione & Glasser, 1994), which can prevent the antiplatelet action of nitrates even at very low doses (Chirkov *et al.*, 1997). Nitrate tolerance is primarily due to the fact that these compounds require bioactivation, although the cellular mechanisms responsible are still not clear. Previous evidence has implied a role for enzymes such as glutathione-S-transferase and cytochrome P450 reductase (McGuire *et al.*, 1998; Simon *et al.*, 1996) and for thiols, which may be required as a cofactor (Fung *et al.*, 1992). However, more recent data indicate that tolerance may be at least partly due to nitrate-mediated inhibition of vascular aldehyde dehydrogenase and increased generation of reactive oxygen species within mitochondria (Chen *et al.*, 2002; Sydow *et al.*, 2004). Nevertheless, mechanisms independent of nitrate bioactivation may also be responsible for tolerance, including plasma volume expansion, upregulation of PDE 1A1 activity, increased vascular synthesis of endothelin-1, and desensitisation of sGC (Artz *et al.*, 2002; Kim *et al.*, 2001; Klemsdal *et al.*, 1996; Munzel *et al.*, 1995).

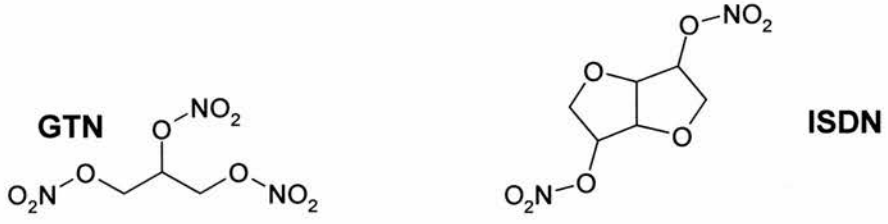


Figure 1.8 **Structure of the organic nitrates GTN and ISDN.**

Platelets also appear to be poor at metabolising organic nitrates to their active form (Weber *et al.*, 1996; Weber *et al.*, 1993), which may explain why nitrates only demonstrate weak antiplatelet activity in some studies (Drummer *et al.*, 1991; Gerzer *et al.*, 1988).

1.3.9.3 SODIUM NITROPRUSSIDE

Sodium nitroprusside (SNP; fig 1.9) has long been known to be an inhibitor of platelet activation (Glusa *et al.*, 1974; Saxon & Kattlove, 1976). Similarly to organic nitrates, SNP requires biological factors to generate NO *in vivo* (Butler & Megson, 2002; Rochelle *et al.*, 1994), and inhibits platelet function via a mechanism dependent on cGMP formation (Kawabata, 1996; Sogo *et al.*, 2000b). The exact molecular candidate responsible for bioactivation of SNP is unclear, but it may involve a membrane bound NADH/NADPH oxidoreductase (Kowaluk *et al.*, 1992; Mohazzab *et al.*, 1999). Although SNP may also induce tolerance (Papapetropoulos *et al.*, 1996; Zhang *et al.*, 1993), its clinical use as an antiplatelet agent is mainly limited by difficulties in dose titration and the risk of cyanide poisoning (Friederich & Butterworth, 1995; Smith & Kruszyna, 1974).

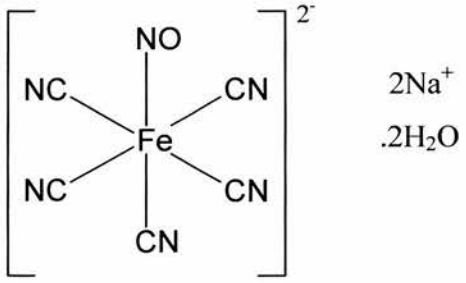


Figure 1.9 **Structure of sodium nitroprusside.**

1.3.9.4 SYDNONOMINES & MESOIONIC OXATRIAZOLES

Sydnonomines and mesoionic oxatriazoles (MOTA) are two closely related classes of NO donors with similar chemical characteristics. 3-morpholinosydnonomine (SIN-1; fig 1.10), the active metabolite of molsidomine (Reden, 1990), was originally believed to be a pure NO donor drug. However, SIN-1 is now known to simultaneously generate O_2^- (Feelisch *et al.*, 1989; Hogg *et al.*, 1992); given the near instantaneous rate of reaction between NO and O_2^- (chapter 1.3.6.3), SIN-1 is now more correctly regarded as a $ONOO^-$ generator. From a mechanistic standpoint, SIN-1 undergoes an initial hydroxylation reaction followed by O_2-mediated conversion to its metabolite SIN-1C, resulting in the formation of NO and O_2^- (Feelisch *et al.*, 1989; Noack & Feelisch, 1989). SIN-1 is considerably more potent at inhibiting platelet activation than nitrates (Bult *et al.*, 1995; Gerzer *et al.*, 1988), and has been shown to act by increasing platelet cGMP synthesis (Gerzer *et al.*, 1988; Karrenbrock *et al.*, 1990). Interestingly, $ONOO^-$ itself exerts dual effects on platelets, inhibiting activation at lower concentrations, while inducing activation at higher concentrations (Brown *et al.*, 1998).

SIN-1 **GEA-3162**

Figure 1.10 **Structures of SIN-1 and GEA-3162.**

The conversion of $ONOO^-$ back to NO appears to be a critical step in the inhibitory action of $ONOO^-$ (Brown *et al.*, 1998; Moro *et al.*, 1994), although $ONOO^-$-mediated

nitration of platelet tyrosine residues may also play a role in the inhibitory action (Low *et al.*, 2002). Nevertheless, SIN-1 has been shown to reduce platelet adhesion and thrombosis in a pig model of balloon angioplasty (Groves *et al.*, 1993).

MOTA such as GEA 3162 (fig 1.10) and GEA 3175 have also been shown to increase cGMP and inhibit platelet function (Grenegard *et al.*, 1996; Kankaanranta *et al.*, 1996). The mechanism of NO generation by these compounds is not clear, but may be accelerated by plasma factors (Kankaanranta *et al.*, 1996). Similarly to SIN-1, MOTA also seem to generate O_2^- concurrently with NO (Schmidt *et al.*, 2001; Taylor *et al.*, 2004), which may again limit their clinical potential, given the likely deleterious effects of $ONOO^-$ in atherogenesis (Demiryurek *et al.*, 1998; White *et al.*, 1994).

1.3.9.5 DIAZENIUMDIOLATES

Diazeniumdiolates (or NONOates) have the general formula $X[N(O)NO]^-$ and constitute a class of NO donor that are synthesised by exposing nucleophiles to pressurised NO gas (Keefer *et al.*, 1996; Morley & Keefer, 1993). One of the major advantages of the diazeniumdiolates is their highly predictable NO donating properties. They do not require bioactivation, but spontaneously release NO at a rate dependent on pH, temperature and the nature of the nucleophile (X; Davies *et al.*, 2001; Morley & Keefer, 1993). A further benefit of the diazeniumdiolates is that they do not induce tolerance (Hinz & Schroder, 1998; Homer & Wanstall, 1998). In platelets, the ability of diazeniumdiolates to inhibit aggregation correlates with their predicted rate of NO release (Raulli, 1998). Diethylamine diazeniumdiolate (DEA/NO) and methylamine hexamethylene methylamine diazeniumdiolate (MAHMA/NO; fig 1.11) are two

examples with short half-lives at physiological temperature and pH (1-2 min; Homer & Wanstall, 1998; Morley & Keefer, 1993). Although DEA/NO and MAHMA/NO can both increase cGMP, both donors have also been shown to inhibit platelet activation by a cGMP-independent mechanism (Homer & Wanstall, 2002; Sogo *et al.*, 2000b). Another commonly used compound in this class, spermine diazeniumdiolate (SPER/NO; fig 1.11), has also been shown to inhibit platelet aggregation, although it is considerably less potent on account of its longer half-life under physiological conditions (~ 40 min; Diodati *et al.*, 1993; Morley & Keefer, 1993). Although few *in vivo* studies on the antiplatelet activity of the diazeniumdiolates have been performed, results so far indicate a potential role for them as antiplatelet agents *in vivo* (Homer & Wanstall, 2003; Saavedra *et al.*, 1996).

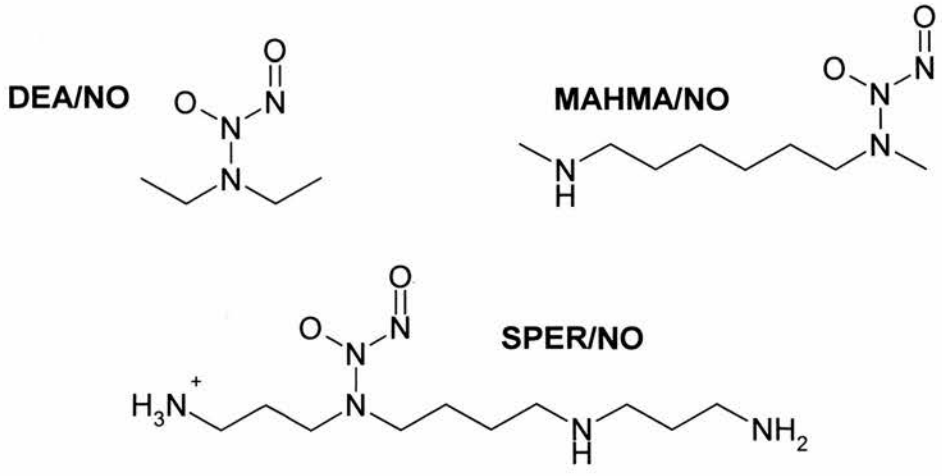


Figure 1.11 **Structures of DEA/NO, MAHMA/NO and SPER/NO.**

1.3.9.6 S-NITROSOTHIOLS

As mentioned previously, some RSNOs including S-nitrosoalbumin and S-nitrosoglutathione (GSNO; fig 1.12), are formed endogenously. RSNOs may also be synthesised via S-nitrosation of reduced thiols (Williams, 1985). RSNOs have received much interest as antiplatelet agents, owing to the fact that they exert some degree of selectivity for platelets over blood vessels (de Belder *et al.*, 1994; Ramsay *et al.*, 1995; Vilahur *et al.*, 2004). In the clinical setting, GSNO has been shown to reduce embolisation in patients undergoing carotid endarterectomy and angioplasty (Kaposzta *et al.*, 2001; Kaposzta *et al.*, 2002). The inhibition of platelet aggregation and adhesion mediated by RSNOs has been shown to correlate with increased cGMP formation (Lieberman *et al.*, 1991; Mellion *et al.*, 1983; Mendelsohn *et al.*, 1990; Radomski *et al.*, 1992; Simon *et al.*, 1993). However, it appears that cGMP-independent mechanisms also play an important role in the inhibition of platelet function by some of these compounds (Gordge *et al.*, 1998; Sogo *et al.*, 2000b; Tsikas *et al.*, 1999a). Recently, a number of novel analogues of the well characterised RSNO, S-nitroso-N-acteyl-D,L-penicillamine (SNAP; fig 1.12), have been described (Megson *et al.*, 1997; Megson *et al.*, 1999). These novel RSNOs may offer considerable advantage over SNAP and other RSNOs, on account of their improved stability and selectivity for areas of endothelial damage (Megson *et al.*, 1997; Miller *et al.*, 2003; Sogo *et al.*, 2000c). Indeed, S-nitroso-N-valeryl-D,L-penicillamine (SNVP; fig 1.12) has been shown to exert a prolonged and selective antiplatelet effect in rabbits following balloon angioplasty (Miller *et al.*, 2003).

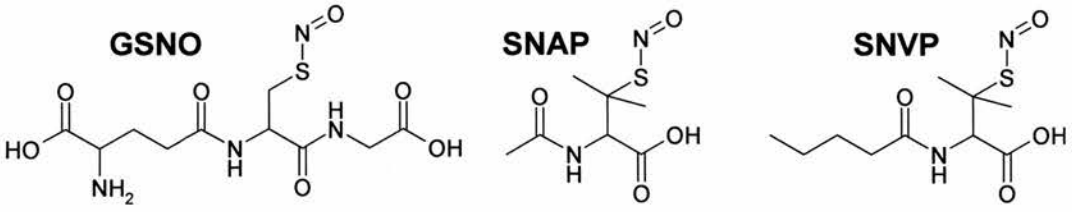


Figure 1.12 **Structures of GSNO, SNAP and SNVP.**

Despite the improved stability of novel S-nitrosothiols such as SNVP compared to exisiting S-nitrosothiols, the ability of SNVP to readily undergo transnitrosation reactions with other cysteine-containing compounds (equation 10; chapter 1.3.6.4; (Megson *et al.*, 1999) will mean that unstable low molecular weight S-nitrosothiols such as S-nitrosocysteine are rapidly formed. Therefore, within biological media such as plasma, SNVP is likely to be highly unstable and give rise to substantial quantities of NO (radical) and nitrite (chapters 1.3.6.2-4). Indeed, in some instances even SNVP diluted in phosphate buffer may be unstable at -80 °C in the presence of metal ion chelator (EDTA; James P.E., personal communication). It is therefore important to note that as a class of molecule, S-nitrosothiols are chemically unstable and decompose at a rate that is highly dependent upon the environment in which they are placed.

1.3.9.7 HYBRID NITRIC OXIDE DONORS

A recent development in NO-based therapy is the addition of NO-donating moieties to existing pharmaceutical agents. NCX-4016, a NO-releasing derivative of aspirin, has been shown to maintain the platelet inhibitory effects of aspirin, whilst demonstrating a gastrosparing effect compared to the parent compound (Fiorucci *et al.*, 2003; Lechi *et*

al., 1996; Wallace *et al.*, 1995). Importantly, both the aspirin and NO containing moieties contribute to its biological effects (Wallace *et al.*, 2002). Further advances in NO-hybrid therapy include the S-nitrosation of tissue plasminogen activator (tPA), an endogenous fibrinolytic agent, and vWF fragment, an inhibitor of platelet adhesion to vWF (Dardik *et al.*, 2000; Delyani *et al.*, 1996; Gurevitz *et al.*, 2000; Stamler *et al.*, 1992b). S-nitrosation of tPA bestowed antiplatelet activity upon the enzyme, while it also markedly increased the antiplatelet activity of vWF fragment.

1.3.9.8 NITRIC OXIDE DONORS AS A COATING FOR GRAFTS, STENTS & EXTRACORPOREAL CIRCUITS

Another recent advancement in NO-based therapy is the generation of modified prosthetic materials with an inbuilt capacity to donate NO. Although many prosthetic graft surfaces such as those made from Dacron or expanded polytetrafluoroethylene (ePTFE) are generally regarded to have minimal prothrombotic activity, they firmly remain second choice behind autologous vein for the bypass of occluded arteries in patients with peripheral artery disease on account of higher failure rates (Bergan *et al.*, 1982; Londrey *et al.*, 1991; Van de Pavoordt *et al.*, 1986). The production of NO generating prosthetic grafts is therefore a potential means to reduce platelet deposition and increase graft patency. Furthermore, NO releasing materials may also have a use as a coating for stents or extracorporeal circuits such as those utilised in cardiopulmonary bypass (CPB) or dialysis. Procedures such as CPB require systemic heparinisation (Frederiksen, 2000) and are associated with platelet degranulation and the presence of 'spent' platelets within the bloodstream (Harker *et al.*, 1980; Rinder *et al.*, 1991; Rinder

et al., 1994), while the use of stents following balloon angioplasty may increase thrombosis (Schatz *et al.*, 1991). Although an obvious limitation to the application of NO-producing surfaces will be their temporary duration of action, there may still be significant benefit in reducing platelet adhesion in the perioperative period. Moreover, this caveat is unlikely to limit their use in short-term procedures such as CPB or dialysis.

To date, numerous approaches to the production of NO-generating surfaces have been adopted. These include the incorporation of SNP into polyelectrolyte microlayers (Thierry *et al.*, 2003), NO-releasing silicone rubbers (Zhang *et al.*, 2002), diazeniumdiolate-containing polymers (Batchelor *et al.*, 2003; Mowery *et al.*, 2000; Parzuchowski *et al.*, 2002), and the coating of surfaces with S-nitrosated albumin (Maalej *et al.*, 1999). Indeed, novel S-nitrosothiols including SNVP may also be useful antiplatelet coatings for artificial surfaces, given their increased lipophilicity above other commonly used S-nitrosothiols such as GSNO (Megson *et al.*, 1999). However, alternative approaches to NO donating materials also merit further investigation, in order that the NO-donating properties of these materials may be optimised. In this arena, one possible alternative is the use of zeolites, nanoporous solids that are often used in catalytic converters to remove atmospheric pollutants, including NO (Pontikakis *et al.*, 2001; Yahiro & Iwamoto, 2001). However, their potential as a store of NO that may be applied for use in prosthetic grafts and extracorporeal circuits has yet to be assessed.

1.4 PROJECT AIMS

Against this background, the main aim of this thesis is to further characterise the antiplatelet effects of NO and NO donor drugs, and to assess their therapeutic potential as antiplatelet agents.

The primary hypotheses that will be addressed are:

- Extracellular NO generation is required for cGMP-independent inhibition of platelet activation, and that this response may be modulated by plasma antioxidants
    - NO inhibits platelet Ca^{2+} signalling via a cGMP-independent mechanism
    - COX is an important cGMP-independent target for NO in platelets
- Plasma thiols, such as albumin and low molecular weight thiols, play a crucial role in prolonging the antiplatelet effect of NO
- SNVP is an NO-donor that may be used to coat artifical grafts to prevent platelet activation
- Zeolites are a novel means of storing NO and can be used to generate surfaces with potent antiplatelet properties

CHAPTER TWO

METHODS

2.1 PREPARATION OF PLATELETS

2.1.1 PLATELET RICH PLASMA & WASHED PLATELETS

Permission to take blood from normal volunteers was granted by the Royal Infirmary of Edinburgh Research Ethics Committee (1702/95/3/11) and consent was obtained from each donor. Venous blood was drawn through a 19 gauge needle from the antecubital fossa of healthy human volunteers aged between 21 and 63. Volunteers had not taken medication known to affect platelet function for at least 10 days prior to sampling. Blood was drawn into 50 ml Falcon tubes containing sodium citrate (0.38% final concentration) and centrifuged (120 *g*; 20 min; fig 2.1). Platelet rich plasma (PRP) was aspirated and the remaining fraction centrifuged (1200 *g*; 10 min) to obtain platelet poor plasma (PPP). Washed platelets (WP) were prepared by centrifugation of PRP (1200 *g*; 10 min; fig 2.1) in the presence of prostacyclin (PGI_2; 300 ng/ml), and the platelet pellet resuspended in an equal volume of modified HEPES-tyrode buffer (pH 7.4) containing (in mM): 137 NaCl, 2.7 KCl, 1.05 $MgSO_4$, 0.4 NaH_2PO_4, 1.8 $CaCl_2$, 12.5 $NaHCO_3$, 5.6 glucose, 10 HEPES, and 10.9 trisodium citrate. Following a secondary centrifugation (1200 *g*; 10 min) in the presence of 300 ng/ml PGI_2, platelets were resuspended in an equal volume of PGI_2-free HEPES-tyrode. Platelet count was determined using a Coulter A^c.T 8 Haematology Analyser (Coulter Electronics, Luton, U.K.), and standardised to 250 x 10^9 L^{-1} via dilution with PPP (PRP) or HEPES-tyrode (WP).

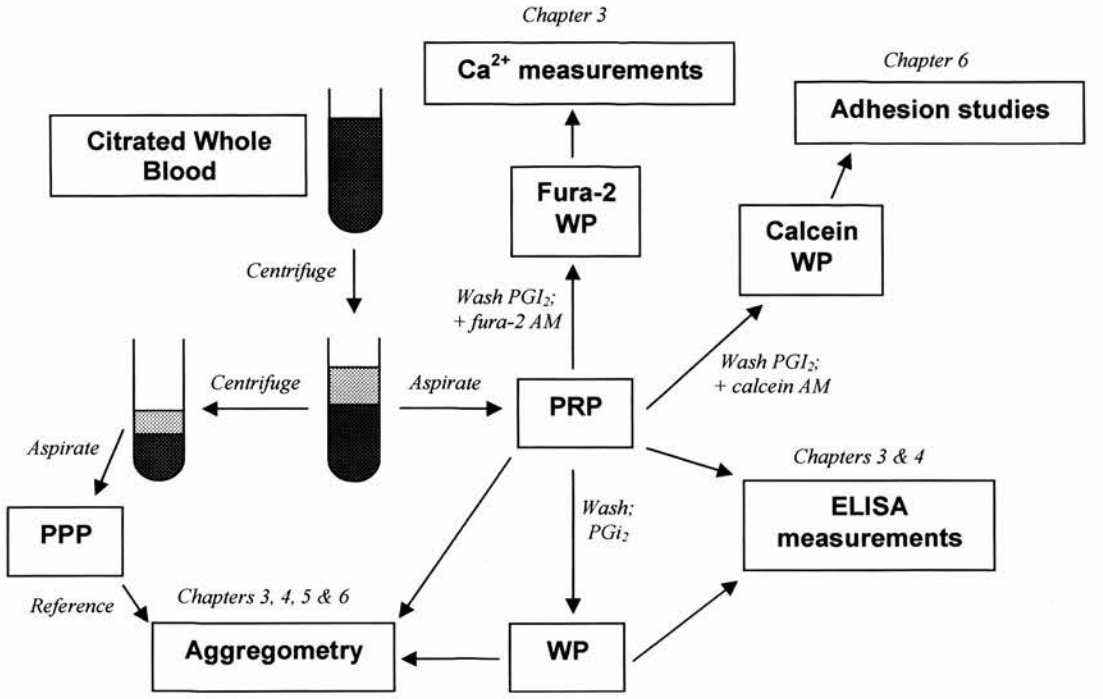


Figure 2.1 **Schematic diagram showing the main phases of platelet preparation and their use within this thesis. Abbreviations: AM, acetoxymethyl ester; ELISA, enzyme-linked immunosorbent assay.**

2.1.2 PREPARATION OF FLUORESCENT INDICATOR LABELLED PLATELETS

Platelets were labelled with acetoxymethyl (AM) ester derivatives of the fluorescent indicators fura-2 and calcein for Ca^{2+} measurements and platelet adhesion studies respectively (fig 2.1). Intracellular cleavage of the AM functional group by esterases traps these indicators within cells (Haworth & Redon, 1998; Liminga *et al.*, 2000). Briefly, aliquots (2 ml) of PRP were centrifuged in the presence of PGI_2 (300 ng/ml) and resuspended in HEPES-tyrode (0.25ml). Fura-2 AM (2 μM) or calcein-AM (10 μM) were added and the suspension incubated at room temperature for 30-min. Pluronic F-

127 (final concentration 0.1%), a mild detergent, was also co-incubated with platelets loaded with calcein to prevent the formation of micelles that occurs with fluorescent indicators (Yates *et al.*, 1992), which can decrease loading efficiency. Following incubation, platelets were diluted to their original volume with HEPES-tyrode, PGI_2 added (300 ng/ml) and the mixture centrifuged (1200 x *g*, 10 min). The supernatant was aspirated and discarded to remove extracellular fluorescent marker prior to resuspenion in HEPES-tyrode. Platelet count was determined and standardized to 250 x 10^9/ L via dilution with HEPES-tyrode as described previously.

2.2 NO ELECTRODE MEASUREMENTS

NO generation was measured using an isolated NO electrode (World Precision Instruments, Stevenage, U.K.). Data were captured by an Apollo 4000 Free Radical Analyser (World Precision Instruments) or via a MacLab 4e analogue digital converter (AD Instruments, Sussex, U.K.). The electrode was calibrated using DEA/NO (100-1600 nM) in phosphate buffer (pH 4.0; fig 2.2.a). DEA/NO decomposition is extremely rapid at pH ≤ 5.0 (Davies *et al.*, 2001). Using this calibration, the limit of detection of the NO electrode was found to be ~ 10 nM NO. The change in peak NO signal was measured and used to generate the calibration graph (fig 2.2.b). Stirred (600 rpm) aliquots (2 ml) of PRP or WP were equilibrated to 37°C for a minimum of 15 min, until a stable baseline was achieved. Precise experimental protocols are as outlined in the experimental chapters.

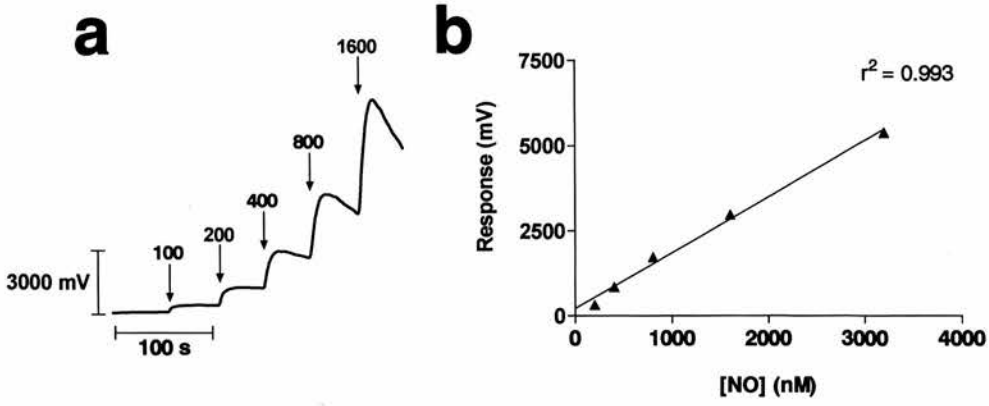


Figure 2.2 **Example calibration trace (a) and graph (b) for the NO electrode. Additions of DEA/NO (in nM) are indicated.**

2.3 AGGREGOMETRY

Aggregometry was carried out using either a two channel (Chronolog Ca560, Labmedics, Stockport, U.K.) or four-channel platelet aggregometer (Chronolog 470 VS) at 37°C with stirring (1000 rpm). Aggregation was recorded as a change in turbidity (light transmission) in PRP or WP measured against a PPP or HEPES-tyrode reference respectively (fig 2.1). Data were captured via an analogue digital converter (MacLab 4e, AD Instruments, Sussex, U.K.) and recorded using MacLab Chart v3.3.7. Aliquots (0.5 ml) of PRP or WP were equilibrated in the aggregometer at 37°C for 10-min before the addition of any drugs. Inhibition of platelet aggregation by various NO-donors was assessed by incubating PRP or WP with the donors prior to stimulating platelet aggregation with peri-maximal concentrations of collagen (type I fibrils; 2.5 μg/ml), U46619 (a TxA_2 analogue; 8 μM), arachidonic acid (AA; 100 μM) or PGH_2 (100 ng/ml). These concentrations of agonists were used in order that any inhibitory effect of

the NO donors would be clearly observed. Aggregation was measured for 5-min following the addition of agonist, and the peak change in response noted. Specific protocols are as described in the experimental chapters.

2.4 FLUORESCENCE STUDIES

All fluorescence measurements detailed below were made using a Perkin Elmer LS50B luminescence spectrometer (Perkin Elmer, Berkshire, U.K.).

2.4.1 MEASUREMENT OF INTRACELLULAR Ca^{2+} LEVELS

To study platelet Ca^{2+} signalling events, aliquots (1.5 ml) of Fura-2 loaded platelets (fig 2.1) were equilibrated to 37°C in the spectrometer for 10 min or until a stable baseline was achieved. The spectrometer was set at excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. Following equilibration, a baseline was recorded for 1-min prior to the addition of NO donor and/or agonist, and the response recorded for a further 5-min. To calculate the intracellular Ca^{2+} levels, ratio values were converted to Ca^{2+} concentrations using FL WinLab software (Perkin Elmer, Berkshire, U.K.), according to the Grynkiewicz equation (Grynkiewicz *et al.*, 1985):

$$[Ca^{2+}]_t = K_d \times \frac{(R_t - R_{min})}{(R_{max} - R_t)} \times SFB$$

Where:

- $[Ca^{2+}]_t$ = Ca^{2+} concentration at time point t
- K_d = Dissociation constant of fura-2 at 37 °C
- R_t = Fluorescence intensity ratio at time t
- $R_{max/min}$ = Fluorescence intensity ratio of completely Ca^{2+} bound/unbound Fura-2
- SFB = Ratio of the fluorescence intensities for Ca^{2+}-bound/unbound Fura-2 at λ_{340}

Platelets were solubilised in 1% Triton-X-100 to calculate R_{max}, followed by Ca^{2+} chelation with 20 mM EGTA to determine R_{min}. The specific protocol is as described in chapter 3.

2.4.2 ADHESION STUDIES

Calcein-labelled platelets (fig 2.1) were used to determine platelet adhesion to expanded polytetrafluoroethylene (ePTFE) prosthetic graft (6mm; Gore-Tex®, Arizona, U.S.A.). To calibrate platelet loading of calcein AM, aliquots (18 μl) of platelets were solubilised in Triton-X-100 (1% final concentration) and the fluorescence measured using an excitation wavelength of 494 nm and an emission wavelength of 517 nm. In all cases, a linear correlation was observed between platelet count and fluorescence measured (fig 2.3).

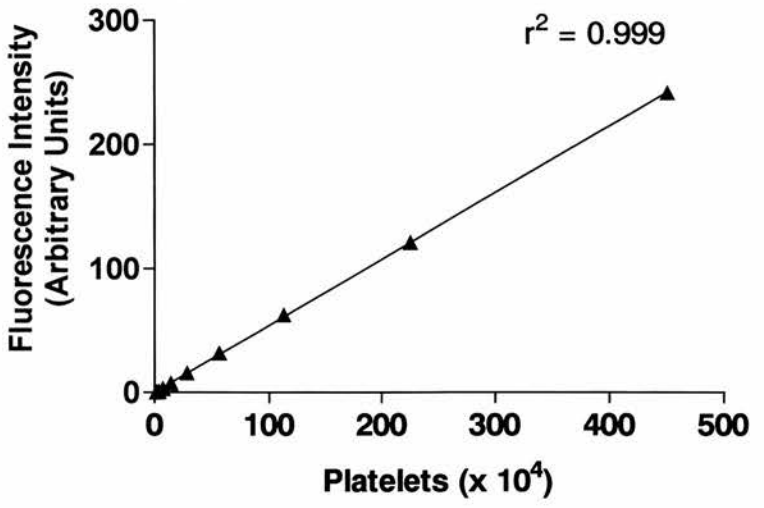


Figure 2.3 **Calibration for platelet loading of calcein-AM.**

To examine the effect of NO donors on platelet deposition on ePTFE graft, segments (3.0 cm) of prosthetic graft were bathed in either HEPES-tyrode or NO donor for 30-min prior to mounting in an open tubing circuit maintained at 37 °C connected to a 50 ml digital syringe pump (Vickers Medical Treonic IP3 Digital Syringe Pump, Stirling, U.K.). Following mounting, the prosthetic graft was perfused with 15 ml HEPES-tyrode at a flow rate of 100 ml/hour, followed by 10 ml of calcein-labelled platelets. The graft was then perfused with a further 15 ml of HEPES-tyrode to flush out non-adhering platelets prior to its removal and placement in a HEPES-tyrode solution containing 1% Triton-X-100 to lyse adhered platelets. The number of adhered platelets was then measured by fluorescence as outlined above.

2.5 SPECTROPHOTOMETRIC MEASUREMENTS

2.5.1 MEASUREMENT OF HAEMOGLOBIN

The haemoglobin content of PRP, PPP and WP was determined using an assay kit (Sigma Diagnostics, Dorset, U.K.) based on the haemoglobin catalysed oxidation of 3,3',5,5'-tetramethylbenzidine by hydrogen peroxide, with absorbance measured at 600 nm (Lijana & Williams, 1979; Standefer & Vanderjagt, 1977). Aliquots (10 μl) of PRP, PPP or WP were used, and the concentration of haemoglobin calculated using a 15 mg/dl haemoglobin standard (Sigma Diagnostics, Dorset, U.K.) as a reference (assay sensitivity: 100 nM haemoglobin). All tests were performed in duplicate.

2.5.2 MEASUREMENT OF PLASMA THIOLS

The reduced thiol content of plasma and solutions of human serum albumin (HSA) ± low molecular weight thiols (LMW) were quantified via reaction with 5,5'-Dithiobis(2-nitrobenzoic acid; DTNB) and colorimetric determination at 412 nm by a well established method (Ellman, 1959). Briefly, 0.1 ml samples were incubated in 0.8 ml potassium phosphate-EDTA buffer (KPE buffer) containing (in mM): 16 KH_2PO_4, 84 K_2HPO_4, and 5 EDTA, pH 7.5. The reaction was started with the addition of 0.1 ml of 2 mg/ml DTNB, and the resulting mixture incubated for 1 hr (room temperature) prior to spectrophotometric determination at 412 nm against a KPE buffer reference.

2.6 CHEMILUMINESCENT MEASUREMENTS

2.6.1 CYCLOOXYGENASE ASSAY

The effect of NO donors on COX-1 activity *in vitro* was assessed via a luminometry assay based on COX-1 catalysed luminescence of the luminol derivative 7-dimethylaminonaphthalene-1,2-dicarboxylic acid hydrazide (DNH; Forghani *et al.*, 1998). Luminescence was measured by a Lumac Biocounter M2500 (Lumac, Landgraaf, Netherlands). Samples were performed in 0.5 ml reaction mixtures. Briefly, COX-1 (100 U/ml; Sigma; ovine, purified from ram seminal vesicle) were pre-incubated with an equimolar concentration of hematin for 10-min at room temperature in HEPES-tyrode. Hematin was added to COX-1 to replace haem that is lost during the purification of COX-1 (Malkowski *et al.*, 2000). Following incubation, 17.2 µl DNH solution

(Cayman Chemicals) was added, and the reaction mixture placed into the luminometer. Arachidonic acid (100 μM) was injected via the luminometer, and the luminescent signal immediately integrated for 10-sec to measure product formation (Forghani *et al.*, 1998). To examine the effect of NO on COX-1 activity, NO donors were pre-incubated with the COX-1 enzyme reaction mixture for 1-min prior to assay, as outlined in chapter 4.

2.6.2 MEASUREMENT OF S-NITROSOTHIOLS

S-nitrosothiols (RSNOs) were quantified at University College London, Royal Free Campus in a collaboration with Professor Kevin Moore and Richard Ollosson via a method based on copper/iodide-induced cleavage of the S-NO bond and measurement by chemiluminescence (Marley *et al.*, 2000). To establish baseline RSNO levels, 0.5 ml aliquots of PRP or WP were transferred to vials containing N-ethylmaleimide (NEM) and EDTA (final concentration 5 mM and 2 mM respectively) dissolved in phosphate buffered saline. Samples were centrifuged (1800 *g*; 5 min), and the supernatant aspirated. Acidified sulfanilamide (2.5% dissolved in 0.1 M HCl) was added to the supernatant to remove nitrite before storage at –70 °C prior to RSNO detection. RSNO formation following NO-donor treatment was also determined, each time using NEM/ EDTA to stop the reaction. Samples were centrifuged, the supernatant and pellet treated with acidified sulfanilamide and stored at –70 °C as before. For RSNO detection, 8 ml glacial acetic acid and 2 ml KI (50 mg/ml) were added to a reaction vessel maintained at 70 °C and constantly purged under a stream of N_2. Following equilibration, $Cu(II)SO_4$

(200 mM) was added for 1-min, prior to the injection of the RSNO sample through a Hamilton® syringe. RSNO-derived NO released in the purge vessel was measured by its chemiluminescent reaction with ozone by a Sievers Nitric Oxide Analyser (NOA 280, Sievers, Colorado, U.S.A.). Data collection and analysis was performed using NO Analysis software (Sievers) and RSNO concentrations calculated against a sodium nitrite standard (fig 2.4). Although there is an inherent danger in extrapolating RSNO concentrations from a sodium nitrite standard, previous evidence indicates that the efficiency of NO cleavage from RSNOs in this system is extremely high and results in the generation of a chemiluminescent signal that is 99% of that achieved with an equimolar nitrite standard (Marley *et al.*, 2000). Therefore, the use of a sodium nitrite standard within this system is a useful indicator for approximating RSNO concentrations.

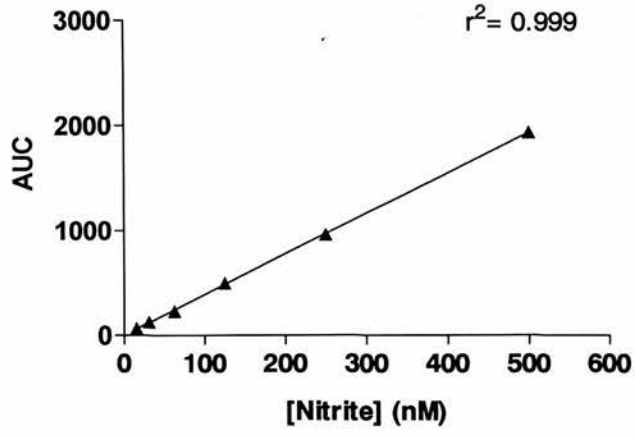


Figure 2.4 **Sodium nitrite calibration for RSNO determination. Abbreviation: AUC, area under curve.**

2.7 ENZYME-LINKED IMMUNOSORBENT ASSAYS (ELISA)

2.7.1 MEASUREMENT OF cGMP

Samples (0.5 ml) of PRP or WP (fig 2.1) for cGMP analysis were initially treated with 300 µl of 10% trichloroacetic acid (TCA) to lyse platelets and precipitate proteins. The resulting mixture was centrifuged (2000 x *g*; 10-min) and the supernatant aspirated and diluted 1:10 in 0.1 N HCl before storage (-20 °C; < 2 weeks) prior to cGMP ELISA (low pH, R&D systems, Abington, U.K.). The non-acetylated form of ELISA was used, and samples assayed in duplicate using a Mulitskan Ascent plate reader (Thermo Labsystems, South Trentham, U.K.) with Ascent v 2.6 software (Thermo Labsystems), reading at an absorbance of 405 nm. An example calibration is included (fig 2.5). In all experiments, platelets were pre-treated with the PDE inhibitor 3-Isobutyl-1-methyl xanthine (IBMX; 1 mM; 20-min) to inhibit metabolism of cGMP, prior to NO donor treatment and cell lysis with TCA.

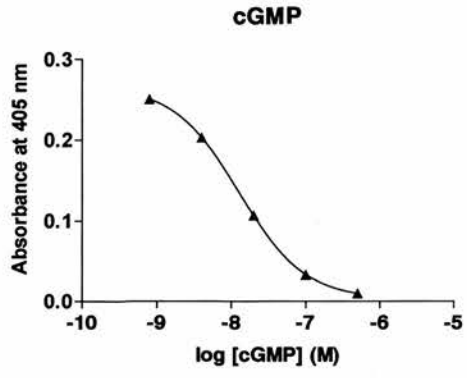


Figure 2.5 **Calibration for cGMP ELISA.**

2.7.2 MEASUREMENT OF THROMBOXANE B_2 (TxB_2)

TxB_2 is generated by non-enzymatic hydration of TxA_2 (Viinikka & Ylikorkala, 1980) and is the accepted index of TxA_2 formation, given the short half-life of TxA_2 under physiological conditions (~ 37 sec; Smith, 1989). Aliquots (0.5 ml) of WP were assayed for TxB_2 formation by ELISA (R&D Systems; fig 2.1) using a Multiskan Ascent with Ascent software v 2.6, measuring absorbance at 405 nm as described above. A sample calibration is included (fig 2.6). Samples of WP treated with NO donors or aspirin (chapter 4) were stimulated with either arachidonic acid (100 μM) or PGH_2 (100 ng/ml) at 37 °C for 5-min prior to treatment with indomethacin (100 μM) or ozagrel (20 μM) respectively to prevent further TxB_2 generation. Following 5-min incubation, WP were frozen in liquid nitrogen and stored at -70 °C prior to assay.

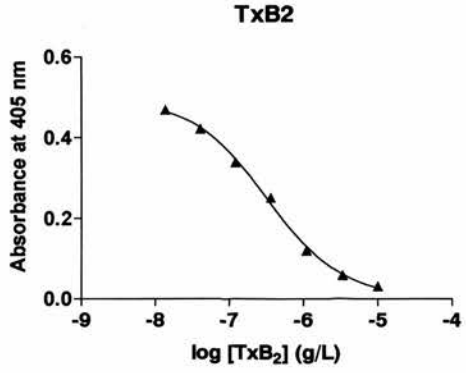


Figure 2.6 **Calibration for TxB_2 ELISA.**

2.8 ELECTRON MICROSCOPY

Scanning electron microscopy was performed to examine platelet deposition on zeolite/PTFE discs. Following the incubation of zeolite/PTFE discs with platelets,

samples were fixed in 3 % glutaraldehyde in 0.1 M sodium cacodylate buffer (SCB; pH 7.4) overnight. Following a 1 hour wash in glutaraldehyde-free SCB, discs were post-fixed in osmium tetroxide in SCB for 2 hours before undergoing dehydration in graded acetone (50-100%; 10 min intervals) and critical point drying with CO_2 (E3000 SII CPD, Polaron Equipment Ltd, Watford U.K.). Samples were then coated with gold-palladium alloy (SC500 Sputter Coater, Emscope Laboratories Ltd, Kent, U.K.) prior to examination under a scanning electron microscope (Phillips 505, Eindhoven, Netherlands). The preparation of the discs for scanning electron microscopy and development of negatives was kindly carried out by Steve Mitchell in the Royal School of Veterinary Studies.

2.9 MATERIALS

General laboratory reagents (buffer salts, EDTA, TCA etc.) were supplied by Fisher. Other reagents were diluted in either HEPES-tyrode, phosphate buffered saline (PBS), 0.01 M NaOH, dimethyl sulphoxide (DMSO), or H_2O (table 2.1). Some reagents were supplied as a water soluble solution (Sol). Company locations are detailed in table 2.2.

2.10 STATISTICS

Statistical analyses are as stated throughout the experimental chapters. Data were analysed using GraphPad Prism software v3.03 (GraphPad Software, San Diego, U.S.A). Degrees of significance are abbreviated as follows: *** = $P<0.001$; ** = $P< 0.01$; * = $P<0.05$. In all cases, $P>0.05$ was not considered to be statistically

significant (ns; not significant). Where expressed, data are in the form mean ± standard error of the mean (S.E.M.).

Reagent	Vehicle	Supplier
Arachidonic Acid (AA)	DMSO[1]	Sigma
Caeruloplasmin (CP; human)	Sol	Sigma
Calcein AM	DMSO	Molecular Probes
Collagen (type I; fibrils; equine)	Sol (suspension)	Labmedics
COX-1; ovine	HEPES-tyr	Sigma
L-Cysteine (cys)	PBS	Sigma
Cysteinyl-Glycine (cys-gly)	PBS	Sigma
DEA	0.01 M NaOH[2]	Sigma
DEA/NO	0.01 M NaOH[2]	Alexis
DTNB	DMSO	Sigma
Fura-2 AM	DMSO	Sigma
Glutathione (GSH)	PBS	Sigma
Haemoglobin (Hb; bovine)	H_2O[3]	Sigma
Hematin (bovine)	DMSO	Sigma
Human serum albumin (HSA)	HEPES-tyr	Sigma
Indomethacin	DMSO	Sigma
DNH	Sol	Cayman
NEM	Ethanol	Sigma
ODQ	DMSO	Tocris Cookson
Ozagrel	PBS	Sigma
PGH_2	Sol	Cayman
PGI_2	PBS	Sigma
Pluronic F-127	DMSO	Molecular Probes
SNP	HEPES-tyr	Sigma
SNVP	HEPES-tyr	Synth[4]
Sulfanilamide	0.1 M HCl	Sigma
Triton-X-100	HEPES-tyr	Sigma
U46619	HEPES-tyr	Sigma
VP	HEPES-tyr	Synth[4]
Zeolite/PTFE discs	-	Synth[5]

Table 2.1 **Suppliers and vehicles for reagents.**

Supplementary information for table 2.1:

[1]For the COX chemiluminescent assay, AA was converted to the sodium salt via the addition of NaOH (O'Donnell *et al.*, 2000), followed by dilution in HEPES-tyrode and pH adjustment to 7.4.

[2]Aliquots of DEA or DEA/NO stored in 0.01 M NaOH were pre-diluted in PBS prior to its addition to platelet suspensions.

[3]Met-haemoglobin was reduced to the ferro form by sodium dithionite (57.4 μM), and excess dithionite removed by dialysis (Martin *et al.*, 1985). Spectrophotometry indicated that Hb existed primarily in the oxygenated (Fe II) form.

[4]SNVP and VP were kindly donated by Dr A.R. Butler and Dr F.A. Mazzei, University of St. Andrews. Synthesis was via a published method (Megson *et al.*, 1999; Miller *et al.*, 2000b).

[5]Zeolite/PTFE discs were synthesised by Dr P.S. Wheatley and Prof R.E.Morris, University of St. Andrews. Briefly, cobalt zeolite-A was prepared from as-synthesised sodium zeolite-A via ion exchange in cobalt-acetate. Following filtration, the zeolite was ground with PTFE (75% Zeolite: 25% PTFE) and pressed into 5 mm discs (~ 20 mg) under 2 tons for 30 s. Discs were then dehydrated and exposed to 3 atm of a mixture of NO and Helium (10 % NO: 90 % He) and stored in sealed Schlenk tubes prior to use.

Company	Location
Alexis Biochemicals	Nottingham, U.K.
Cayman Chemical Company	Michigan, U.S.A
Fisher	Loughborough, U.K.
Labmedics	Salford, U.K.
Molecular Probes	Paisley, U.K.
Tocris Cookson	Bristol, U.K.
Sigma Aldrich	Poole, U.K.

Table 2.2 **Companies and locations.**

CHAPTER THREE

A POTENTIAL ROLE FOR EXTRACELLULAR NITRIC OXIDE GENERATION IN cGMP-INDEPENDENT ANTIPLATELET EFFECTS

3.1 INTRODUCTION

NO has long been recognized to inhibit platelet activation by increasing the synthesis of cyclic-3'5'-guanosine monophosphate (cGMP) via stimulation of the enzyme soluble guanylate cyclase (sGC; Busse *et al.*, 1987; Mellion *et al.*, 1981). Activation of G-kinase by cGMP inhibits platelet function through phosphorylation of key proteins including vasodilator-stimulated phosphoprotein (VASP; Butt *et al.*, 1994; Halbrugge *et al.*, 1990), thromboxane A_2 (TxA_2) receptors (Wang *et al.*, 1998) and proteins involved in the Ca^{2+} signalling pathway (Busse *et al.*, 1987; Cavallini *et al.*, 1996; Kawahara *et al.*, 1984; Matsuoka *et al.*, 1989; McDonald & Murad, 1995; Nakashima *et al.*, 1986). An elevated cytosolic Ca^{2+} concentration following agonist stimulation is a critical signalling event required for platelet shape change and aggregation (Blockmans *et al.*, 1995; Gerrard *et al.*, 1978; Murer, 1985; Rink, 1988). Recently, several cGMP independent signalling mechanisms have been identified (Ahern *et al.*, 2002; Gordge *et al.*, 1998; Homer & Wanstall, 2002; Thyagarajan *et al.*, 2002; Trepakova *et al.*, 1999; Tsikas *et al.*, 1999a; White *et al.*, 2002). In platelets, NO accelerates sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA)-dependent refilling of internal Ca^{2+} stores (Homer & Wanstall, 2002; Trepakova *et al.*, 1999), and the unstable S-nitrosothiol, S-nitrosocysteine, inhibits agonist-induced TxA_2 synthesis in human platelets (Tsikas *et al.*, 1999a). Furthermore, the importance of cGMP-independent mechanisms is underpinned by the recent discovery that protein kinase G has an excitatory role in platelet activation (Li *et al.*, 2003).

An important aspect in assessing cGMP-independent mechanisms is identification of the exact NO-related (NO_x) species responsible for the effect. However, studies in platelets are complicated by a number of factors. Firstly, blood plasma contains antioxidants such as ascorbate (~100 μM; Esteve *et al.*, 1997) and low molecular weight thiols (10-20 μM; Mansoor *et al.*, 1992), which can catalyse the release of NO from S-nitrosothiols (Ignarro *et al.*, 1981; Singh *et al.*, 1996). Secondly, NO and its higher oxides can interact with plasma proteins such as albumin and haemoglobin, resulting in the formation of S-nitrosated proteins with considerably different properties to NO itself (Crane *et al.*, 2002; Gow *et al.*, 1997; Gow *et al.*, 1999; Kharitonov *et al.*, 1995; Pawloski *et al.*, 1998; Scharfstein *et al.*, 1994; Simon *et al.*, 1993; Stamler *et al.*, 1992a). Plasma is also an abundant source of the Fe^{2+}/Cu^{2+} transporting protein caeruloplasmin (CP), which catalyses S-nitrosothiol formation and decomposition (Dicks & Williams, 1996; Inoue *et al.*, 1999). Thirdly, NO can react with superoxide (O_2^-) at almost diffusion limited rates, leading to the generation of peroxynitrite ($ONOO^-$; chapter 1.3.6.3; Espey *et al.*, 2002; Jourd'heuil *et al.*, 2001). $ONOO^-$ has been reported to exert both inhibitory and excitatory effects in platelets (Brown *et al.*, 1998; Moro *et al.*, 1994). In the case where the simultaneous generation of NO and O_2^- is desired to create $ONOO^-$, plasma may contain enough antioxidants to remove at least a proportion of O_2^- before it has the opportunity to react with NO. Finally, endogenous pathways exist for the conversion of $ONOO^-$ to potent nitrosating species such as N_2O_3, which can lead to formation of S-nitrosothiols and NO (Espey *et al.*, 2002; Mayer *et al.*, 1998).

To date, a number of studies in both platelets and blood vessels have indicated a correlation between the amount of NO (radical) released by an NO donor, and the level of cGMP-independent activity observed (Homer *et al.*, 1999; Miller *et al.*, 2004; Sogo *et al.*, 2000b). The aim of the studies described in this chapter was to perform systematic experiments to test the hypothesis that *extracellular* generation of NO, but not $ONOO^-$ or S-nitrosothiols is the most important determinant for cGMP-independent inhibition of platelet activation. Furthermore, we hypothesised that extracellular generation of NO instils antiplatelet effects via inhibition of Ca^{2+} mobilisation. To test this hypothesis, the cGMP independent effects of the predictable NO donor DEA/NO (chapter 1.3.9.5; (Davies *et al.*, 2001), the relatively stable S-nitrosothiol SNVP (chapter 1.3.9.6; Megson *et al.*, 1999) and the $ONOO^-$ generator SIN-1 (chapter 1.3.9.4; Feelisch *et al.*, 1989) were tested. Furthermore, plasma proteins CP and SOD were used as membrane impermeant tools to specificially elicit the extracellular release of NO from SNVP and unmask that from SIN-1 respectively, to probe the role of extracellular NO in cGMP-independent inhibition of human platelet activation.

3.2 METHODS

3.2.1 cGMP MEASUREMENTS

cGMP measurements were performed to assess the concentration of the sGC inhibitor ODQ (chapter 1.3.5) required to completely inhibit platelet cGMP synthesis to a maximal concentration of DEA/NO (10 μM). Platelet rich plasma (PRP) and washed platelets (WP) were prepared as described in the methods section (chapter 2.1.1). Aliquots (0.5 ml) of PRP or WP were equilibrated in the platelet aggregometer and incubated with the phosphodiesterase inhibitor 3-Isobutyl-1-methyl xanthine (IBMX; 1 mM) for 20-min prior to the addition of DEA/NO (10 μM). To assess the inhibitory action of ODQ on cGMP formation, WP or PRP were pre-incubated with ODQ (20 μM or 100 μM) for 15-min prior to the addition of DEA/NO. In all cases, DEA/NO was incubated in the platelets for 30 s prior to the addition of 300 μl of 10% trichloroacetic acid to lyse platelets and precipitate the proteins. The 30 s time-point was used so that cGMP measurement occurred shortly after the peak cGMP synthesis (Bellamy *et al.*, 2000). The resulting mixture was then centrifuged and the supernatant aspirated and stored (-20 °C; < 2 weeks) prior to cGMP ELISA as described in the methods section (chapter 2.7.1; n = 5).

3.2.2 NO ELECTRODE MEASUREMENTS

NO generation was measured using a calibrated NO electrode described in the methods section (chapter 2.2). Nitric oxide-related (NO_x) donors DEA/NO (3 μM), SNVP (100

μM) or SIN-1 (100 μM) were added to 2 ml aliquots of PRP or WP and the NO generation recorded for 6-min. In experiments involving sGC inhibition, PRP was treated with a supramaximal concentration of ODQ (20 μM) for 15-min, as determined in the preliminary cGMP measurements, before the addition of NO_x donor. In further experiments, the effect of plasma factors on the release of NO from SNVP and SIN-1 was investigated. WP were incubated with levels of caeruloplasmin (CP) that approximate plasma concentrations (0.4 g/L; Prakasam *et al.*, 2001; Ravin, 1961) for 1-min prior to the addition of SNVP (100 μM). CP, a membrane impermeant protein that has previously been shown to catalyse the release of NO from S-nitrosothiols (Dicks & Williams, 1996), was incubated specifically with SNVP to investigate the effect of generating NO from SNVP in the extracellular compartment. Similarly, WP was incubated with superoxide dismutase (SOD, 500 U ml^{-1}), ascorbate (ASC, 100 μM), and human serum albumin (HSA, 4%) for 1-min prior to the addition of SIN-1 (100 μM) to examine the effect of plasma factors on the generation of NO by SIN-1 (n= 4 for all experiments).

3.2.3 AGGREGOMETRY

Aggregometry was carried out as described in the methods section (chapter 2.3). Aliquots (0.5 ml) of PRP or WP were equilibrated in the aggregometer at 37°C before the addition of DEA/NO (1 nM-10 μM), SNVP (10 nM-100 μM) or SIN-1 (3 nM-300 μM). Following incubation of drug for 1-min, U46619 (a TxA_2 analogue; 8 μM) was added to the platelets to induce aggregation, and the response measured for 5-min.

U46619 was used in these experiments because it activates a signalling pathway downstream of platelet TxA_2 synthesis, minimising the effect of cGMP-independent inhibition of endogenous TxA_2 synthesis (Tsikas *et al.*, 1999a). In experiments designed to investigate cGMP-independent mechanisms, ODQ (20 μM) was pre-incubated with platelets for 15-min before the addition of DEA/NO, SNVP or SIN-1. In further experiments, aliquots of WP pre-treated with ODQ were incubated with various plasma factors to determine the effect of releasing NO on cGMP-independent inhibition of platelet activation. In these experiments, CP (0.4 g L^{-1}) was added to WP for a 1-min period prior to SNVP (0.1-100 μM), which was added 1-min prior to U46619. As described previously, CP was incubated specifically with SNVP to investigate the effect of generating NO from SNVP in the extracellular compartment. Similarly, WP pre-incubated with ODQ were treated with supra-maximal SOD (500 U ml^{-1}) for 1-min before the addition of SIN-1 (3 nM-300 μM), 1-min prior to U46619. Supramaximal concentrations of SOD were added to WP to remove superoxide generated in the extracellular compartment by SIN-1, thereby preventing its rapid reaction with NO and thus allowing investigation of the antiplatelet effects of extracellular NO derived from SIN-1 (n=6 for all experiments).

3.2.4 Ca^{2+} STUDIES

Fura-2 labelled WP were prepared as described in the methods section (chapter 2.1.2) and intracellular Ca^{2+} measured via fluorescence as described previously (chapter 2.4.1). Aliquots (1.5 ml) of Fura-2 loaded WP were equilibrated at 37°C before the addition of

DEA/NO (10 μM), SIN-1 (100 μM), or SNVP (100 μM). After a 1-min incubation period, U46619 (8 μM) was added and the response measured for 5-min. These experiments were also repeated in platelets pre-incubated with ODQ (20 μM) for 15 min. Analogous to aggregometry experiments, aliquots of WP pre-treated with ODQ were incubated with CP (0.4 g L^{-1}) for a 1-min period prior to the addition of SNVP (100 μM), which was added 1-min prior to U46619. Similarly, ODQ-treated WP pre-incubated with SOD (500 U ml^{-1}) for 1-min before the addition of SIN-1 (100 μM), 1-min prior to U46619 (n = 4-6 for all experiments).

3.2.5 HAEMOGLOBIN MEASUREMENTS

The haemoglobin content of PRP and WP was determined by spectrophotometry as described in the methods section (chapter 2.5.1; n=3-5).

3.3 RESULTS

3.3.1 cGMP MEASUREMENTS

Incubation of platelets with DEA/NO (10 μM) caused a significant ($P < 0.01$; one-way ANOVA followed by Dunnett's multiple comparison test) ~ 2-fold increase in platelet cGMP levels in both PRP and WP (in pmol/10^8 platelets: PRP control: 22.4 ± 4.9, + DEA/NO: 42.5 ± 4.3; WP control: 47.3 ± 3.0, + DEA/NO: 97.2 ± 9.8). Pre-incubation of platelets with ODQ at both concentrations tested (20 μM and 100 μM) completely prevented the DEA/NO-induced increase in cGMP in PRP and WP (in pmol/10^8 platelets: PRP: + 20 μM ODQ: 21.9 ± 2.7, + 100 μM ODQ: 22.3 ± 3.2; WP: + 20 μM ODQ: 43.2 ± 2.1, + 100 μM ODQ: 41.1 ± 2.9). In both cases, no significant difference to baseline cGMP levels was observed ($P > 0.05$; one-way ANOVA followed by Dunnett's multiple comparison test). Since 20 μM ODQ was sufficient to completely prevent DEA/NO-induced cGMP accumulation, this concentration was used in subsequent experiments to reduce non specific effects caused by vehicle (DMSO).

3.3.2 GENERATION OF NO IN PRP & WP BY DEA/NO, SNVP & SIN-1

Addition of DEA/NO (3 μM), SNVP (100 μM) and SIN-1 (100 μM) to PRP at concentrations with maximal antiplatelet activity resulted in measurable NO generation from each compound (fig 3.1.a). In all samples tested, there was a short lag phase (60-

90 s) before NO was detected. In a parallel series of experiments, DEA/NO (3 μM), SNVP (100 μM) or SIN-1 (100 μM) was added to WP. High levels of NO were detected with DEA/NO (fig 3.1.b), while only a transient, low level of NO was detected with SNVP (< 50 nM; fig 3.1.b inset). NO was not detected with SIN-1, even under conditions of maximum electrode sensitivity (threshold ~ 10 nM; fig 3.1.b inset).

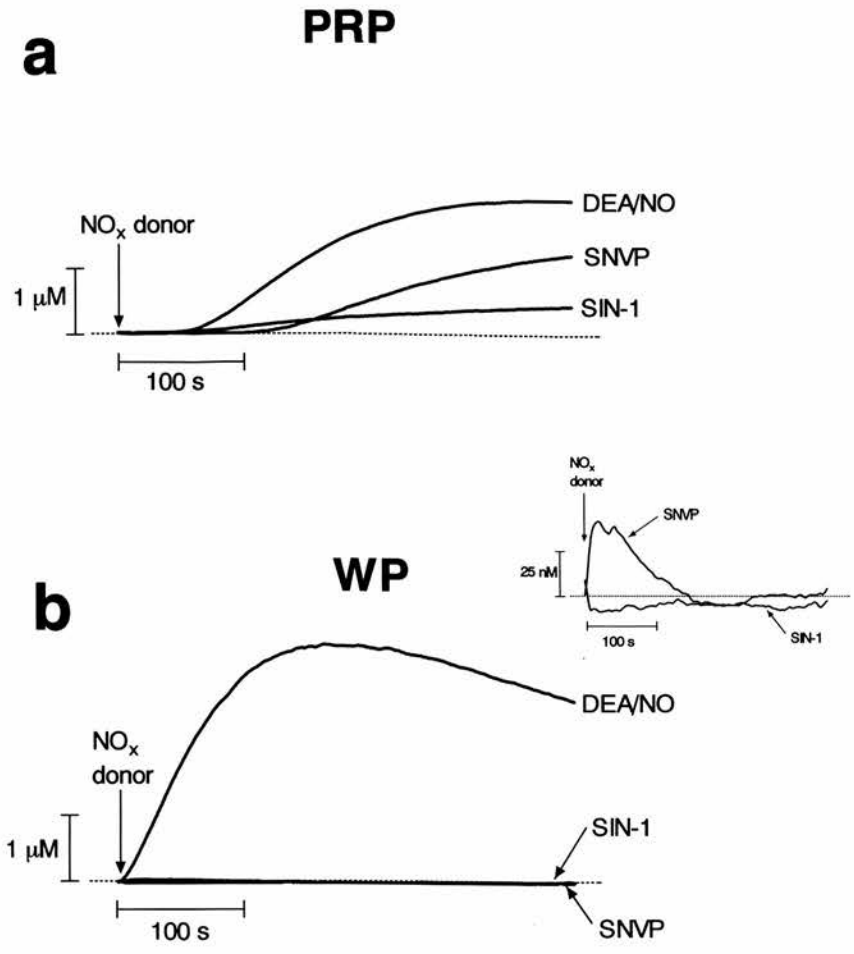


Figure 3.1 **Generation of NO by DEA/NO, SNVP and SIN-1 in PRP and WP. Platelets were equilibrated at 37°C before the addition of DEA/NO (3 μM), SNVP (100 μM) or SIN-1 (100 μM) to PRP (a) or WP (b). Experiments involving the addition of SNVP (100 μM) and SIN-1 (100 μM) to WP are also shown on a smaller scale (inset). Data shown are the mean of 4-5 experiments.**

3.3.3 DEA/NO-MEDIATED INHIBITION OF PLATELET AGGREGATION IN PRP & WP

DEA/NO (1 nM-10 μM) inhibited U46619-induced platelet aggregation in PRP and WP in a concentration dependent manner (fig 3.2.a.i-iii). DEA/NO was approximately 100-fold more potent in WP compared to PRP. Pre-incubation of the sGC inhibitor (ODQ; 20 μM) with PRP for 15-min did not affect DEA/NO-mediated inhibition of platelet aggregation (control IC_{50}: 131 nM, +ODQ: 340 nM; $P > 0.05$; two-way ANOVA). However in WP, ODQ inhibited DEA/NO-mediated inhibition of platelet aggregation, causing a right-shift of the concentration response curve (control IC_{50}: 6.9 nM, +ODQ: 1.43 μM).

3.3.4 SNVP-MEDIATED INHIBITION OF PLATELET AGGREGATION IN PRP & WP

SNVP (10 nM-100 μM) also caused a concentration dependent inhibition of U46619-induced platelet aggregation in PRP and WP (fig 3.2.b.i-ii). Similarly to DEA/NO, SNVP (10 nM-100 μM) also inhibited platelet aggregation in WP at substantially lower concentrations than in PRP. Incubation of platelets with ODQ did not affect the inhibition of U46619-induced aggregation in PRP (control IC_{50}: 20 μM, +ODQ: 26 μM; $P > 0.05$; two-way ANOVA). However, ODQ abolished SNVP-mediated inhibition of aggregation in WP at all concentrations of SNVP tested (control IC_{50}: 270 nM).

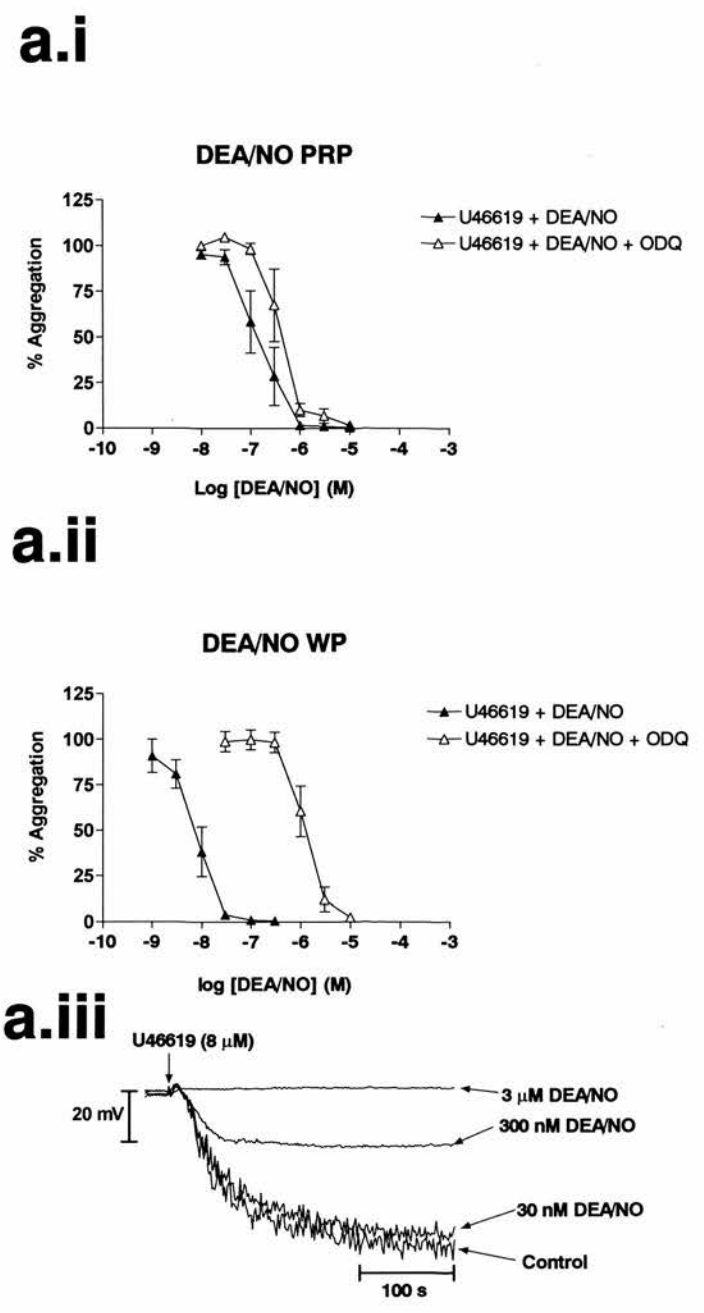
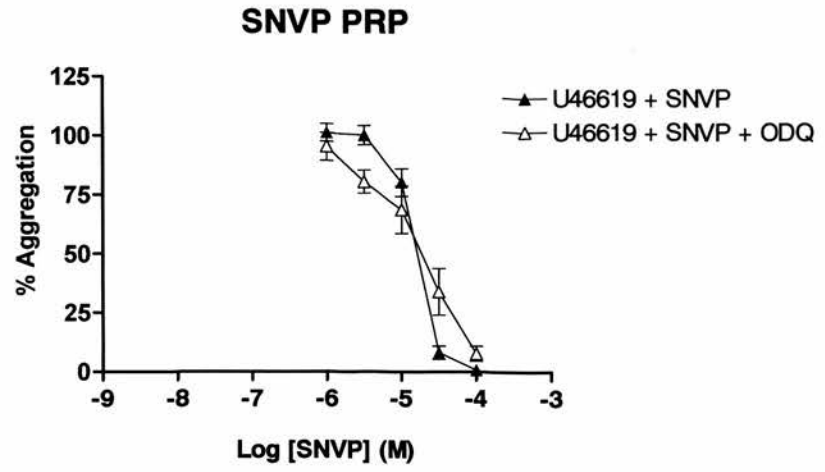


Figure 3.2.a **Inhibition of platelet aggregation by DEA/NO in PRP (i) and WP (ii) in the presence and absence of ODQ. Platelets were equilibrated to 37°C before treatment with DEA/NO for 1-min prior to stimulation with U46619 (8 μM). ODQ (20 μM) was pre-incubated with platelets for 15-min before the addition of DEA/NO, followed by U46619 1-min later. A representative trace of experiments conducted in PRP (control) is included (n= 6).**

b.i



b.ii

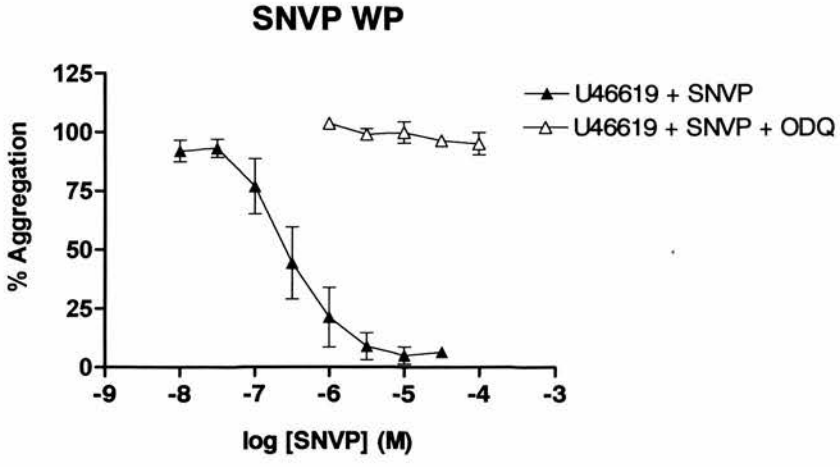
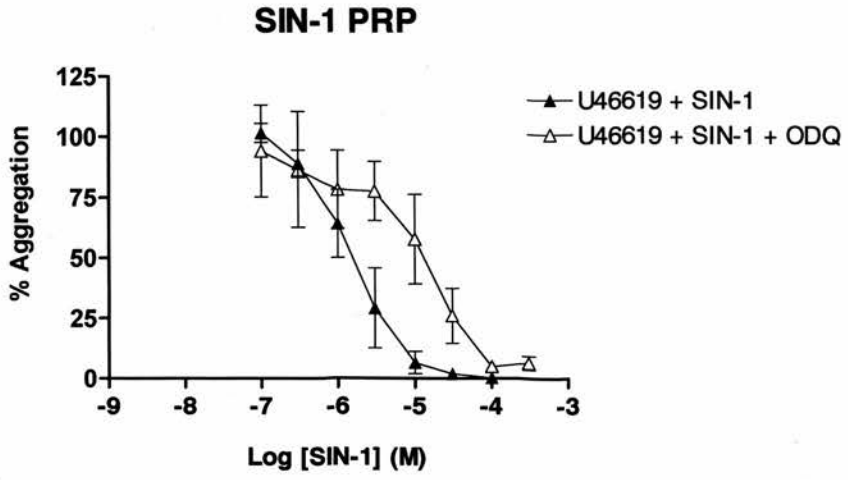


Figure 3.2.b **Inhibition of platelet aggregation by SNVP in PRP (i) and WP (ii) in the presence and absence of ODQ. Platelets were equilibrated to 37°C before treatment with SNVP for 1-min prior to stimulation with U46619 (8 μM). ODQ (20 μM) was pre-incubated with platelets for 15-min before the addition of SNVP, followed by U46619 1-min later (n=6).**

c.i



c.ii

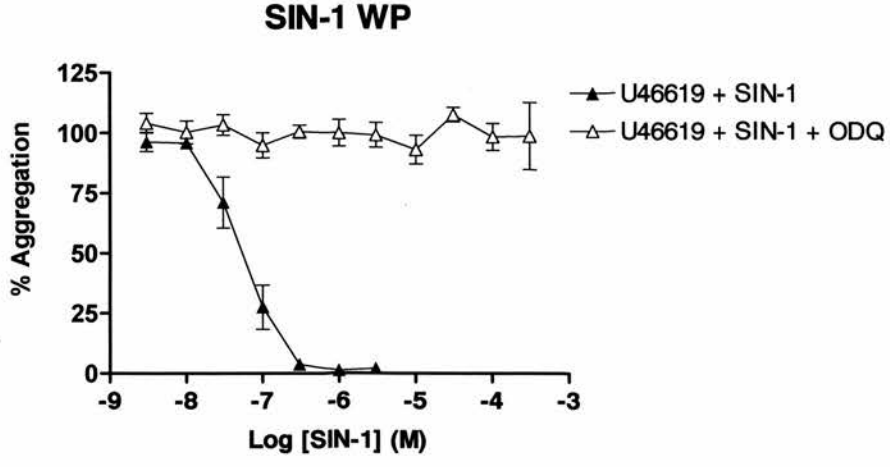


Figure 3.2.c **Inhibition of platelet aggregation by SIN-1 in PRP (i) and WP (ii) in the presence and absence of ODQ. Platelets were equilibrated to 37°C before treatment with SIN-1 for 1-min prior to stimulation with U46619 (8 μM). ODQ (20 μM) was pre-incubated with platelets for 15-min before the addition of SIN-1, followed by U46619 1-min later (n=6).**

3.3.5 SIN-1-MEDIATED INHIBITION OF PLATELET AGGREGATION IN PRP & WP

SIN-1 (3 nM -300 μM) also inhibited U46619-induced platelet aggregation in PRP and WP in a concentration-dependent manner (fig 3.2.c.i-ii). SIN-1 was a considerably more potent inhibitor of platelet aggregation in WP compared to PRP. Incubation of ODQ caused a significant (~10-fold) rightward shift in the concentration response curve for SIN-1 in PRP (control IC_{50}: 1.4 μM, +ODQ: 17 μM; $P < 0.05$; two-way ANOVA). In WP, ODQ abolished SIN-1 mediated inhibition of platelet aggregation at all concentrations tested (control IC_{50}: 54 nM).

3.3.6 EFFECT OF PLASMA FACTORS ON THE GENERATION OF NO BY SNVP AND SIN-1

In experiments designed to establish the effect of plasma factors and extracellularly generated NO on SNVP and SIN-1, WP was reconstituted with CP at a level that approximates its concentration in plasma (0.4 g/L; Prakasam *et al.*, 2001; Ravin, 1961). CP was used specifically in combination with SNVP in these experiments because it is a membrane impermeant protein and will catalyse NO generation from S-nitrosothiols within the extracellular compartment (Dicks & Williams, 1996). CP was observed to greatly enhance the release of NO from SNVP (fig 3.3.a). Similarly, SOD was utilised only in combination with SIN-1 to investigate the effect of removing superoxide, thereby allowing NO generated by SIN-1 in the extracellular compartment to persist.

Incubation of WP with SOD (500 U ml^{-1}) prior to the addition of SIN-1 resulted in measurable NO generation (fig 3.3.b). Furthermore, incubation of WP with the plasma components ascorbate (100 μM) and HSA (4%) prior to SIN-1 also resulted in measurable NO generation from SIN-1 (fig 3.3.b).

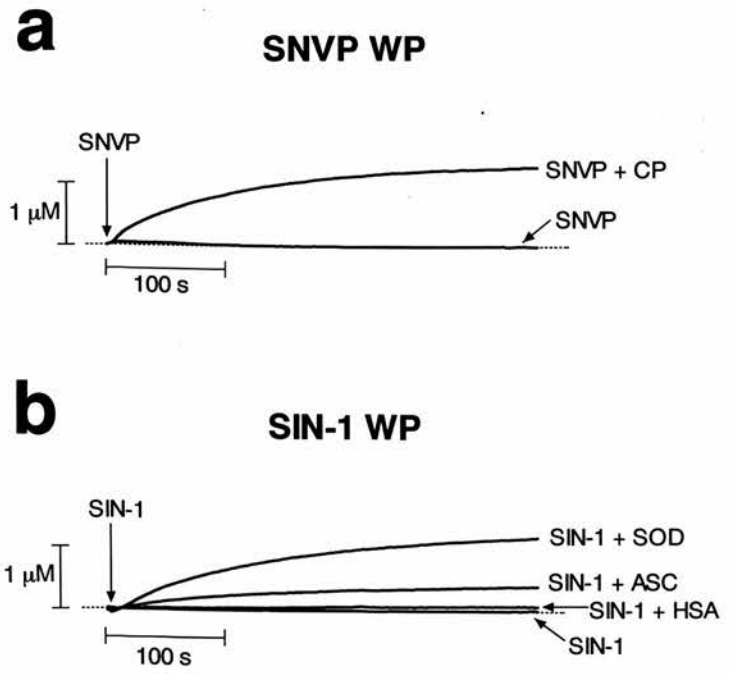


Figure 3.3 **Effect of plasma factors on the generation of NO from SNVP and SIN-1 in WP. Platelets were equilibrated to 37°C before the addition of SNVP (100 μM; a) or SIN-1 (100 μM; b). In experiments involving SNVP, WP were pre-incubated with CP (0.4 g/L) for 1-min prior to the addition of SNVP. In SIN-1 experiments, WP were pre-incubated with SOD (500 U/ml), ASC (100 μM) or HSA (4%) for 1-min before the addition of SIN-1. Data shown are the mean of 4 experiments.**

3.3.7 EFFECT OF CP & SOD ON SNVP & SIN-1-MEDIATED INHIBITION OF PLATELET AGGREGATION

To assess the effect extracellular NO generation from SNVP on cGMP-independent antiplatelet effects, WP were reconstituted with CP (0.4 g L^{-1}) in the presence of ODQ prior to treatment with SNVP. In the presence of ODQ and CP, SNVP caused a concentration-dependent inhibition of platelet aggregation (fig 3.4.a; IC_{50}: 3.3 μM). Similarly, to investigate the effect of preventing superoxide generated by SIN-1 reacting with NO in the extracellular compartment on cGMP-independent inhibition of platelet aggregation, WP were reconstituted with SOD (500 U/ml) in the presence of ODQ before treatment with SIN-1. In these conditions, SIN-1 concentration-dependently inhibited platelet aggregation (fig 3.4.b; IC_{50}: 3.0 x 10^{-5} M).

3.3.8 EFFECT OF NO_X DONORS ON Ca^{2+} SIGNALLING IN FURA-2 LOADED WP

Addition of U46619 to fura-2 loaded WP caused an expected rapid Ca^{2+}spike, followed by a sustained elevation of intracellular Ca^{2+}levels. Pre-incubation of WP with DEA/NO (10 μM) inhibited this Ca^{2+}signalling, an effect that was not blocked by ODQ (fig 3.5.a.i; $P > 0.05$; Student's paired t-test). Incubation of WP with SNVP (100 μM) also inhibited U46619-induced Ca^{2+}signalling, but this effect was blocked by ODQ (fig 3.5.a.ii, $P < 0.01$; one-way ANOVA followed by Dunnett's multiple comparison test).

Pre-incubation of ODQ-treated WP with CP 1-min prior to SNVP reversed the antagonistic effect of ODQ on SNVP ($P<0.01$; one-way ANOVA followed by Dunnett's multiple comparison test), to a similar level observed with the S-nitrosothiol alone (fig 3.5.a.ii). Similarly, incubation of WP with SIN-1 (100 μM) resulted in an inhibitory effect on U46619-induced Ca^{2+} signalling that was reversed by ODQ (fig 5.a.iii; $P < 0.01$; one-way ANOVA followed by Dunnett's multiple comparison test). Pre-incubation of ODQ-treated WP with SOD also prevented the inhibitory action of ODQ on SIN-1 (fig 3.5.a.iii; $P<0.01$; one-way ANOVA followed by Dunnett's multiple comparison test). Summary data showing this trend are also presented (fig 3.5.b).

3.3.9 EFFECT OF ODQ ON NO GENERATION BY NO_X DONORS IN PRP

In experiments investigating the effect of ODQ (20 μM) on NO generation by the NO_x donors in PRP, ODQ was observed to substantially reduced the length of the lag phase observed with all three donor drugs, without altering the maximum concentration of NO detected (fig. 3.6.a-c).

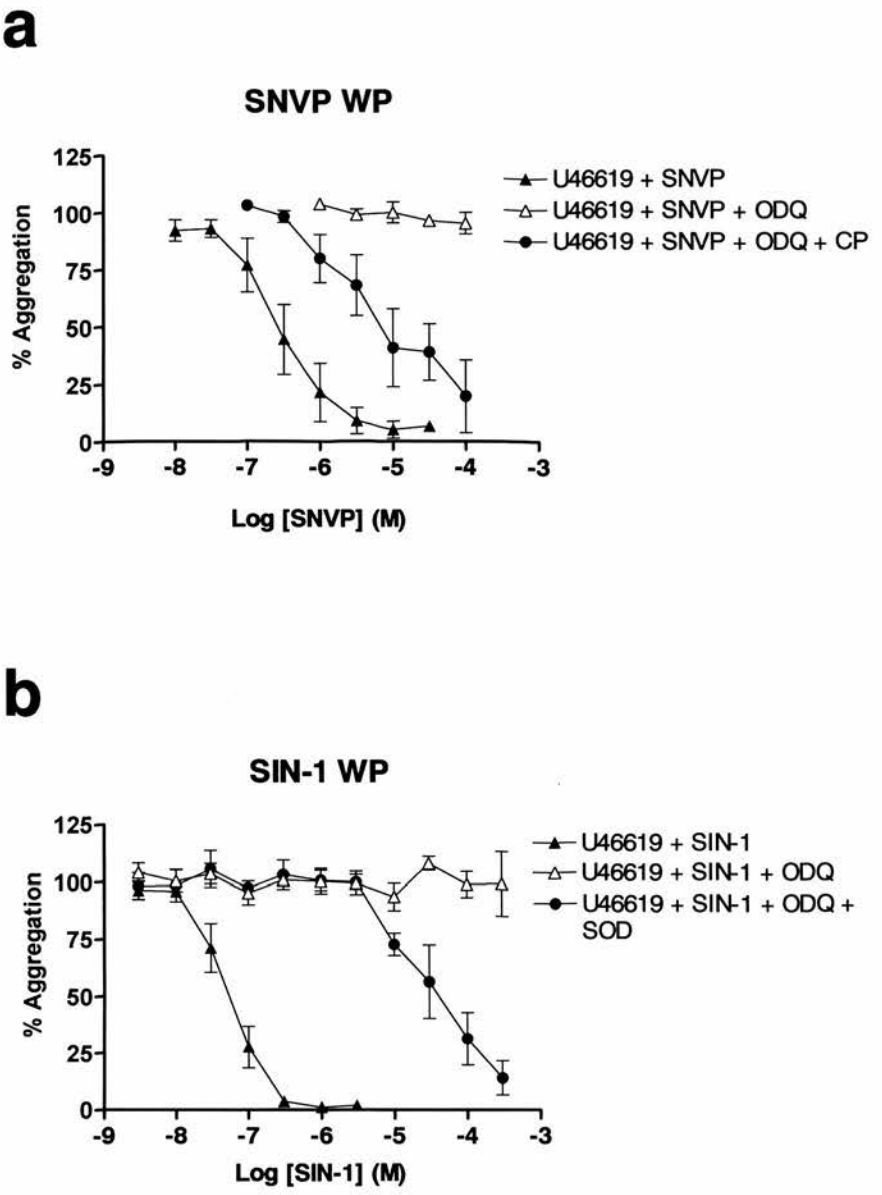


Figure 3.4 **Effect of plasma factors on cGMP-independent inhibition of platelet aggregation by SNVP and SIN-1 in WP. Platelets equilibrated to 37°C were pre-incubated with ODQ (20 μM) for 14-min before the addition of CP (0.4 g/L) 1-min prior to the addition of SNVP (a). SOD (500 U/ml) was also added to ODQ-treated WP 1-min before the addition of SIN-1 (b). After incubation with SNVP or SIN-1 for 1-min, U46619 (8 μM) was added to induce aggregation. Previous data are added as a comparison (n=6).**

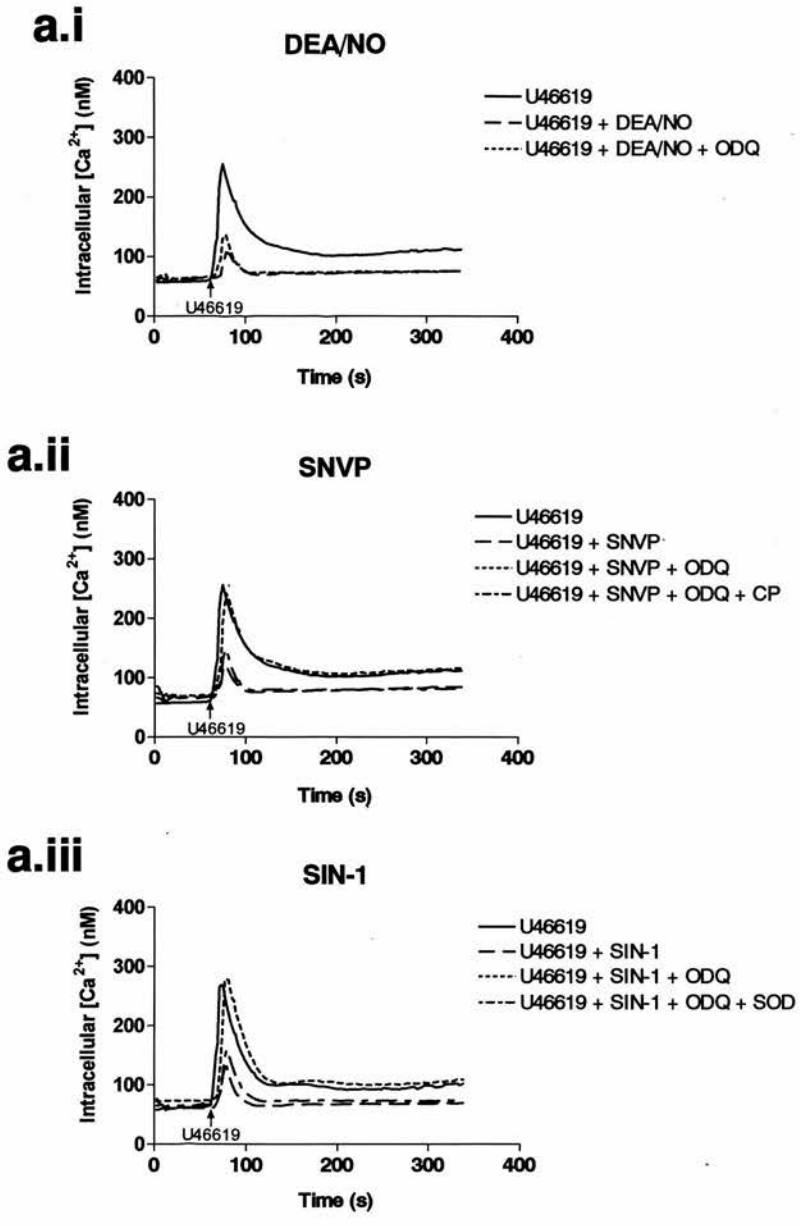


Figure 3.5.a **Representative traces showing the effect of DEA/NO, SNVP and SIN-1 on Ca^{2+}signalling in Fura-2 labelled WP. Platelets loaded with Fura-2 were equilibrated to 37°C before the addition of DEA/NO (10 μM – a.i), SNVP (100 μM – a.ii) or SIN-1 (100 μM – a.iii). In experiments involving ODQ, WP were pre-incubated with ODQ (20 μM) for 15-min before the addition of NO_x donor. In other experiments, ODQ-treated WP were reconstituted with CP (0.4 g/L) or SOD (500 U/ml) before the addition of SNVP or SIN-1 respectively.**

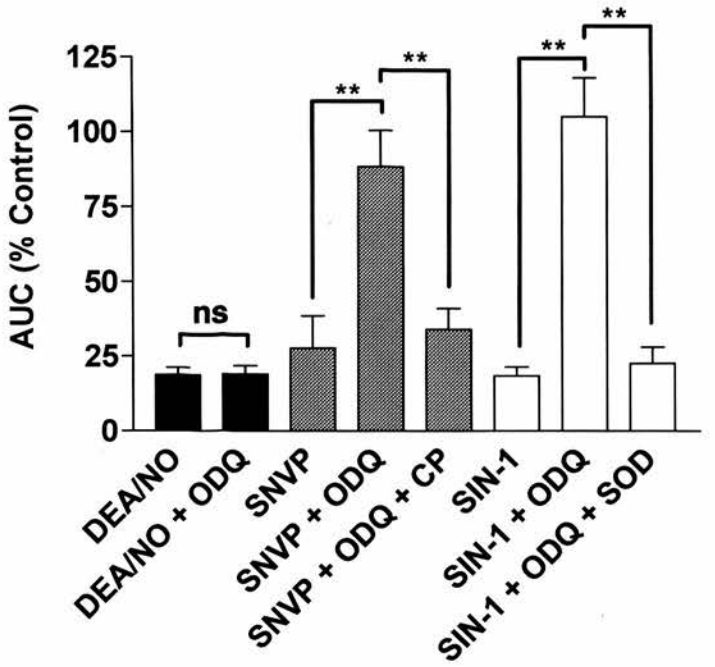


Figure 3.5.b **Summary data showing the effect of NO_x donors on U46619-induceCa^{2+} signalling in Fura-2 labelled WP. Data were obtained by measuring the area under curve (AUC; ns = P>0.05; ** = P<0.01; n = 4-6).**

3.3.10 HAEMOGLOBIN MEASUREMENTS

The haemoglobin concentration in PRP was 0.35 ± 0.03 μM, while no haemoglobin was detected in WP (limit of detection: 100 nM).

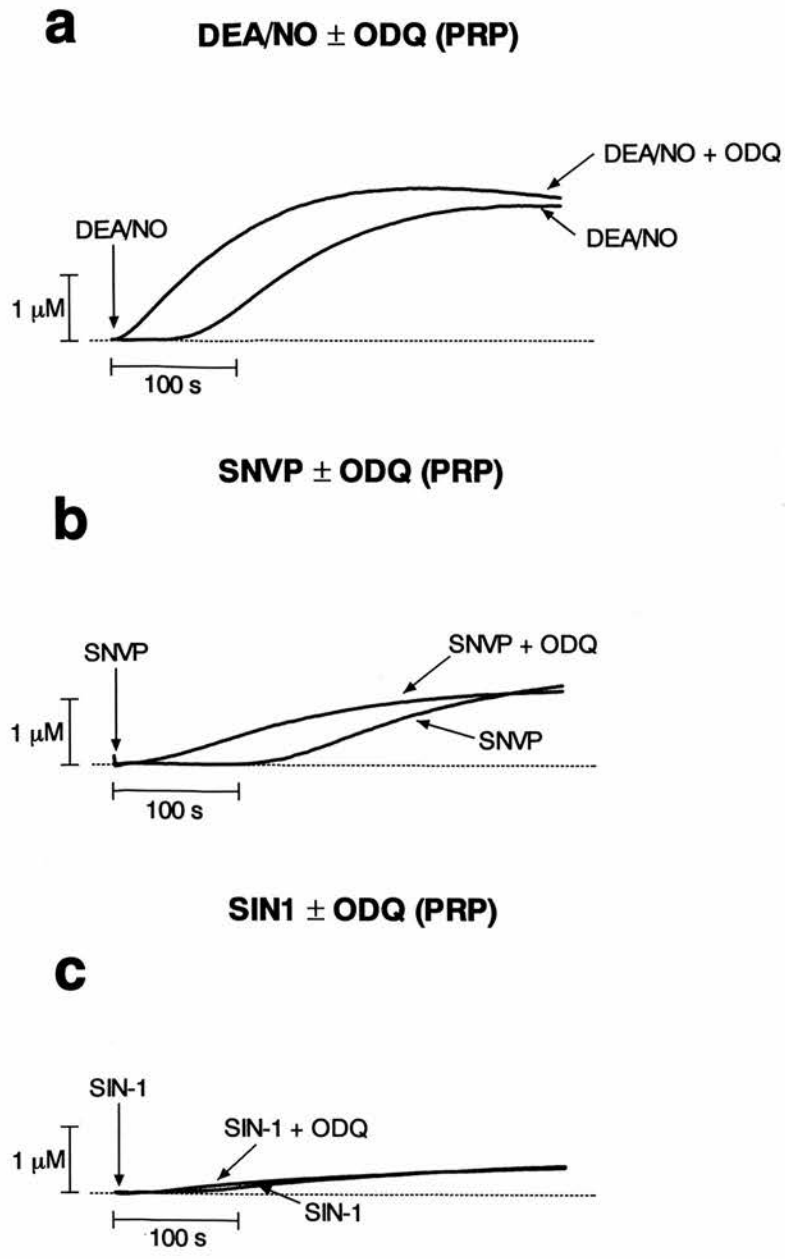


Figure 3.6 **Effect of ODQ incubation on NO generation by DEA/NO, SNVP and SIN-1 in PRP. Platelets were equilibrated to 37°C before the addition of DEA/NO (3 μM; a), SNVP (100 μM; b) or SIN-1 (100 μM; c). In experiments involving ODQ, PRP was treated with ODQ (20 μM) for 15-min before the addition of DEA/NO, SNVP or SIN-1. Data shown are the mean of 4 experiments.**

3.4 DISCUSSION

These results suggest that NO-mediated cGMP-independent antiplatelet effects are reliant on the generation of detectable (>30 nM) NO (radical) in the extracellular compartment. DEA/NO inhibited platelet aggregation via cGMP-independent mechanisms in both PRP and WP, implying a role for exogenous NO in cGMP-independent inhibition of activation. SNVP, however, inhibited platelet aggregation via a cGMP-independent mechanism in PRP, but inhibition in WP was entirely dependent on cGMP. Analysis of NO generation by a high concentration of SNVP (100 μM) using an isolated electrode revealed that SNVP generated significant amounts (~ 1 μM) of NO in PRP sustained throughout a 5-min period, but in WP only generated a small transient increase in extracellular NO (~ 30 nM) that persisted for only ~ 2-min. Reconstitution of WP with the copper-containing protein, CP, at similar levels to those found in plasma elicited extracellular release of NO from SNVP and conferred cGMP-independent inhibition of platelet aggregation to SNVP. Incubation of platelets with SIN-1 revealed a similar trend to that observed with SNVP, with cGMP-independent inhibition of aggregation only observed in PRP and not WP. SIN-1 only generated detectable NO in PRP, but incubation of WP with SOD resulted in detectable generation of extracellular NO from SIN-1, and also caused cGMP-independent inhibition of aggregation. Experiments with Fura-2 loaded platelets demonstrated that both CP and SOD conferred cGMP-independent inhibition of Ca^{2+} signalling by SNVP and SIN-1 respectively, indicating that the cGMP-independent target(s) play a role in the regulation of platelet Ca^{2+}signalling. Taken together, these data suggest a potential requirement for

exogenous NO in the generation of cGMP-independent inhibition of platelet activation through inhibitory effects on Ca^{2+}mobilisation.

3.4.1 ODQ-MEDIATED INHIBITION OF sGC

Confidence in the inhibitory effects of ODQ on sGC is essential to facilitate interpretation of the data derived from this study. Initial experiments were performed to assess the concentration of ODQ required to prevent cGMP formation to a maximal concentration of DEA/NO (10 μM). Previous data have shown that 100:1 excess of the NO donor SNAP can result in a partial reversal of ODQ-mediated inhibition of sGC (Moro *et al.*, 1996). In these experiments, a theoretical maximum of 20 μM NO will be released by 10 μM DEA/NO, which is equivalent to the concentration of ODQ used here, and unlikely to be sufficiently high to overcome ODQ-mediated inhibition. cGMP measurements revealed that 20 μM ODQ was sufficient to completely prevent NO-mediated cGMP formation and that no added benefit was observed when the ODQ concentration was increased to 100 μM. Furthermore, the observation that 20 μM ODQ was sufficient to completely prevent SNVP and SIN-1-mediated inhibition of aggregation in WP at concentrations 1000-fold greater than that required to inhibit aggregation confirms the notion that these NO-donors do not release sufficient NO intracellularly to overcome ODQ-mediated inhibition of sGC. These experiments therefore support the hypothesis that the inhibition of aggregation observed in the presence of 20 μM ODQ represent genuine cGMP-independent responses.

There is an issue regarding the specificity of ODQ, and it now seems clear that ODQ is able to oxidise the haem group of other haemoproteins including haemoglobin (Zhao *et al.*, 2000), a finding supported by data here (fig 3.6). Indeed, it is therefore plausible that ODQ could oxidise the haem centres of platelet COX-1 or TxA_2 synthase, and therefore impede platelet TxA_2 synthesis and the positive feedback response that promotes aggregation. However, previous data obtained within this laboratory indicate that ODQ does not prevent collagen-induced platelet aggregation, while results presented later in this thesis indicate that pre-incubation of platelets with the COX-1 inhibitor aspirin completely inhibits collagen-induced platelet aggregation in calcein-labelled WP (chapter 6.3.2 & fig 6.3). These observations therefore support the hypothesis that ODQ does not significantly interfere with platelet prostanoid synthesis, and provides further support for its use as a sGC inhibitor in experiments outlined here.

3.4.2 NO_x DONORS, EXTRACELLULAR NO & cGMP-INDEPENDENT INHIBITION OF PLATELET AGGREGATION

The NO_x donors used in this study were purposefully selected to allow examination of the NO_x species responsible for the cGMP-independent antiplatelet effect. DEA/NO hydrolyses in physiological solutions with a half-life of ~ 2 min at physiological temperature and pH (Davies *et al.*, 2001). Importantly, biological factors are not required to drive DEA/NO hydrolysis, therefore DEA/NO will generate equivalent amounts of NO in both PRP and buffer. SNVP, a more stable and lipophilic analogue of

the well-recognized S-nitrosothiol, SNAP, was chosen for these studies because it is relatively stable but nevertheless undergoes transnitrosation reactions (Megson *et al.*, 1999): it is a useful tool in establishing a role for transnitrosation in cGMP-independent effects. SIN-1 was originally believed to be a NO donor, but is now known to generate O_2^- concurrently with NO (Feelisch *et al.*, 1989; Noack & Feelisch, 1991; Taylor *et al.*, 2004). The reaction rate between NO and O_2^- is near diffusion limited (6.7×10^9 $M^{-1}s^{-1}$; chapter 1.3.6.3; Huie & Padmaja, 1993), making SIN-1 an effective and convenient $ONOO^-$ donor.

Our results indicate that in WP, all three NO_x donors stimulate sGC, inhibit Ca^{2+} signalling and prevent platelet aggregation. The EC_{50} for DEA/NO in WP was ~ 10 nM (fig 3.2.a.ii.), indicating that low nM concentrations of NO (≤ 20 nM) are sufficient to stimulate sGC. A key finding in these experiments is that cGMP-independent antiplatelet effects are only observed in conditions where extracellular NO is detectable. Thus, while all NO_x donors generated extracellular NO in PRP and induced cGMP-independent inhibition of aggregation, only DEA/NO generated substantial and sustained levels of NO and induced cGMP-independent antiplatelet effects in WP (EC_{50} ≤ 2 μM NO; figs 3.1 & 3.2). At this juncture, it is therefore unclear whether the absolute NO concentration or the site of its release is the important factor for cGMP-independent antiplatelet effects. This supposition may be answered most clearly via analysis of the data obtained for SIN-1. In WP, SIN-1 exhibited only cGMP-dependent inhibition of platelet aggregation (EC_{50} ~ 50 nM; fig 3.2.c.ii) and even at high concentrations (100 μM) did not generate detectable levels of extracellular NO (limit of detection ~ 10 nM;

fig 3.1.b). Thus, 50 nM SIN-1 is sufficient to generate the low nM amounts of intracellular NO required to stimulate sGC. Assuming that the addition of maximal concentrations of SIN-1 (300 μM) to WP results in a similar-fold increase in intracellular NO (i.e. a 6000-fold increase of intracellular NO to levels in the μM range), the manifestation of cGMP-independent effects would be expected if the concentration of NO is the sole limiting factor. However, cGMP-independent effects with SIN-1 are only observed when extracellular NO is detected (i.e. in PRP or when extracellularly generated NO is unmasked by the membrane-impermeant protein SOD). These data therefore support the hypothesis that the site of NO generation is an important factor governing cGMP-independent antiplatelet effects. This premise is further supported by the SNVP data. Although the addition of SNVP to WP resulted in a small, transient increase in extracellular NO (~ 30 nM from 100 μM SNVP; fig. 3.1.b), this was insufficient to elicit cGMP-independent effects, suggesting that concentrations of extracellular NO exceeding 30 nM are required for cGMP-independent antiplatelet effects. The addition of the plasma protein CP induced the release of extracellular NO and stimulated cGMP-independent inhibition of platelet aggregation, implying a role for extracellular NO in cGMP-independent antiplatelet effects. However, it is important to acknowledge that NO can rapidly move across biological membranes, and the experimental design adopted here cannot ascertain the origin of the NO, whether it is from extracellular, intracellular or both sources. It is thus difficult to conclusively determine a role for extracellular NO in cGMP-independent antiplatelet effects.

3.4.3 $ONOO^-$ AND PLATELET ACTIVATION

Interestingly, high concentrations of $ONOO^-$ (≥ 150 μM) can *activate* human WP (Brown *et al.*, 1998; Moro *et al.*, 1994). The concentrations of SIN-1 we have used here are likely to result in the formation of much smaller concentrations of $ONOO^-$ (a 1 mM SIN-1 solution has been demonstrated to release ~ 7 μM/ min O_2^- and ~ 4 μM/ min NO in PBS at 37°C, pH 7.2; Hogg *et al.*, 1992). The use of SIN-1 and the comparatively low concentrations of $ONOO^-$ that it generates may explain why others that have used preformed $ONOO^-$ have found that it induces cGMP-independent inhibition of platelet aggregation (Low *et al.*, 2002). Our results suggest that at low concentrations, $ONOO^-$ exerts anti-platelet effects via stimulation of sGC and production of cGMP, in agreement with previous observations that $ONOO^-$ mediated cGMP accumulation can be achieved through intracellular conversion to S-nitrosothiols and NO (Mayer *et al.*, 1998; Mayer *et al.*, 1995).

3.4.4 cGMP-INDEPENDENT INHIBITION OF PLATELET Ca^{2+} SIGNALLING

These results indicate that NO inhibits Ca^{2+} signalling via a cGMP-independent mechanism. To date, numerous potential targets for cGMP-independent inhibition have been established. There is convincing evidence that NO can activate the platelet sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA; Homer & Wanstall, 2002; Trepakova *et al.*, 1999). Data presented here suggest that the cGMP-independent effects

of NO do indeed impact on Ca^{2+} trafficking (fig 3.5), in agreement with earlier findings. However, the apparent role for extracellular NO in cGMP-independent inhibition of platelet activation might suggest that NO-mediated modification of cell surface components is a more likely target than an intracellular component that has to compete for NO with high affinity sGC found throughout the cytoplasm. This discrepancy may be explained by the observation that SERCA is located in the dense tubular system (Horiguchi *et al.*, 1998), in close proximity to the open cannalicular system and plasma membrane (chapter 1.2.2.1). Therefore in platelets, SERCA may be in an ideal position proximal to the outer surface of the platelet to detect NO generated in the extracellular environment and respond to increases in NO by enhancing sequestration of Ca^{2+} back within the dense tubular system. However, other cGMP-independent mechanisms including the interaction of NO with platelet surface thiols may also play a role as previously implied (Gordge *et al.*, 1998; Sogo *et al.*, 2000b).

3.4.5 PHYSIOLOGICAL & PHARMACOLOGICAL IMPLICATIONS

The physiological implications of data presented here are unclear. Concentration-response curves in PRP ± ODQ are difficult to compare on a quantitative level on account of the fact that ODQ will oxidise residual haemoglobin present in PRP (fig 3.6; Zhao *et al.*, 2000), reducing its ability to bind NO and thereby effectively increasing the NO dose received by these platelets. Indeed, results presented here demonstrate that washing procedure results in the removal of haemoglobin, indicating that the majority of

haemoglobin present within PRP was cell-free. Although there is controversy regarding the fate of NO following its interaction with haemoglobin, evidence indicates that while red blood cells may conserve NO and increase its delivery within hypoxic tissues (Datta *et al.*, 2004; Jia *et al.*, 1996) cell-free haemoglobin inactivates NO (Nakai *et al.*, 1996; Olson *et al.*, 2004; Reiter *et al.*, 2002). The observation that WP, which are devoid of haemoglobin, demonstrate increased sensitivity to the NO_x donors agrees with these findings. It is interesting that cell-free haemoglobin is present within blood *in vivo* (Lentener, 1984) and will represent a significant barrier for NO-mediated platelet effects, irrespective of the NO source. The fact that physiological concentrations of plasma constituents such as CP and ascorbate or HSA accelerate the release of NO from SNVP and SIN-1 respectively (fig 3.3) and that haemoglobin-mediated scavenging of NO will likely have to be overcome before any antiplatelet effects are observed will mean that cGMP-independent effects of these drugs are likely to be evoked.

3.4.6 SUMMARY

In summary, our data suggest a requirement for the extracellular generation of NO (> 30 nM) to stimulate cGMP-independent inhibition of platelet activation. Plasma antioxidants and proteins such as CP can evoke cGMP-independent antiplatelet activities on S-nitrosothiols and $ONOO^-$ generators by accelerating the extracellular release of NO from these compounds, suppressing platelet Ca^{2+} signalling events and inhibiting platelet function (see fig 3.7 for summary).

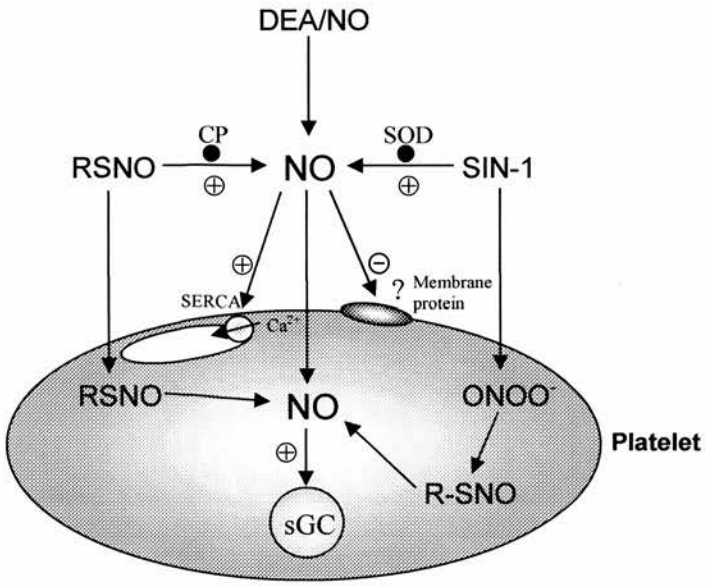


Figure 3.7 **Summary of proposed mechanism for the requirement of extracellular NO for cGMP-independent effects. NO inhibits platelet activation via cGMP-independent mechanisms, possibly involving acceleration of SERCA-mediated sequestration of Ca^{2+} into the platelet DTS or via other unspecified effects on the platelet membrane. Plasma antioxidants prevent the reaction between NO and O_2^-, and catalyse the release of NO from S-nitrosothiols, thereby accentuating cGMP-independent antiplatelet effects. NO produced in the intracellular environment by the intracellular conversion of S-nitrosothiols and peroxynitrite primarily binds to sGC, resulting in cGMP-dependent inhibition of platelet activation.**

CHAPTER FOUR

EFFECT OF NITRIC OXIDE ON THE THROMBOXANE A_2 PATHWAY

4.1 INTRODUCTION

A critical early signalling event in platelet aggregation is the activation of the arachidonic acid (AA)/ thromboxane A_2 (TxA_2) signalling pathway, which forms a positive feedback system to drive platelet shape change and the aggregation response (chapter 1.2.4.; Blockmans *et al.*, 1995; Zucker & Nachmias, 1985). Physiological agonists such as collagen activate the pathway by increasing intraplatelet Ca^{2+} levels (fig 4.1), resulting in phospholipase A_2 (PLA_2) activation and synthesis of AA from glycerophospholipids (GPL) present within the platelet membrane (Clark *et al.*, 1995; Kramer *et al.*, 1993). Intracellularly synthesised, or exogenously added AA induces platelet aggregation via its sequential conversion to prostaglandin H_2 (PGH_2) and TxA_2 via the membrane-bound enzymes COX-1 and TxA_2 synthase respectively (chapter 1.2.4.3; fig 4.1; Carey *et al.*, 1982; Needleman *et al.*, 1976b; Smith *et al.*, 1996b). TxA_2 receptor (TP) stimulation then activates phospholipase C, leading to the liberation of Ca^{2+} from the dense tubular system (DTS), activation of protein kinase C (PKC) and the initiation of platelet shape change and aggregation (chapter 1.2.4; Blockmans *et al.*, 1995; Dandona *et al.*, 1996; Ferris & Snyder, 1992; Fukami, 2002; Jackson *et al.*, 2003).

At the molecular level, both COX-1 and TxA_2 synthase are haemoproteins (M_w ~ 70,000 and ~ 58,000 respectively) and are located within the DTS (Carey *et al.*, 1982; Needleman *et al.*, 1976b; Nusing *et al.*, 1990; Smith & Marnett, 1991). COX-1 catalyses a two-step conversion of AA to PGH_2: the first (cyclooxygenase) step involves conversion of AA to the hydroperoxide PGG_2, while the second phase involves the

reduction of PGG_2 to PGH_2, which occurs at a distinct peroxidase catalytic site (Marnett *et al.*, 1999; Smith *et al.*, 1996b).

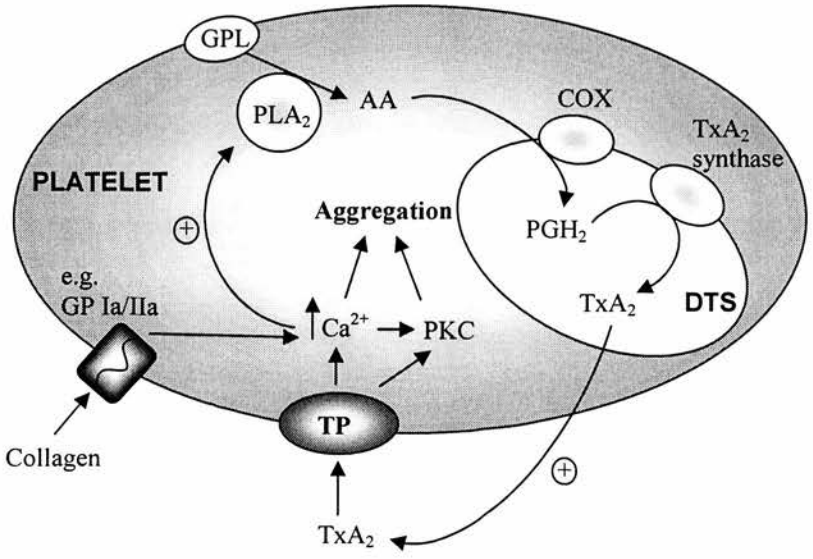


Figure 4.1 **Overview of the arachidonic acid signalling pathway in platelets. For details, see text.**

However, the haem prosthetic group is an absolute requirement for both the cyclooxygenase and peroxidase activities of COX-1 (Ogino *et al.*, 1978). Similarly, haem is essential for the conversion of PGH_2 to TxA_2 by TxA_2 synthase (Hecker & Ullrich, 1989). The importance of COX-1 and TxA_2 synthase to platelet activation is underlined by the ability of non-steroidal anti-inflammatory drugs such as aspirin (ASA) and TxA_2 synthase/receptor antagonists (e.g. ridogrel) to inhibit platelet activation to a wide range of agonists (De Clerck *et al.*, 1989; Gallus, 1985; Hoet *et al.*, 1990; Smith *et al.*, 1980).

Numerous lines of evidence indicate that NO and its related species (NO_x) modulate prostaglandin biosynthesis (Goodwin *et al.*, 1999a; Goodwin *et al.*, 1999b). However, a concensus over the effects of NO on prostaglandin synthesis has yet to be

reached. NO has been shown to either directly stimulate (Davidge *et al.*, 1995; Hajjar *et al.*, 1995; Maccarrone *et al.*, 1997) or inhibit COX-1 activity (Kanner *et al.*, 1992; Tsai *et al.*, 1994), while other evidence suggests that NO does not affect COX activity (Curtis *et al.*, 1996). Furthermore, while NO-mediated nitrosation of the catalytic domain of COX-1 has previously been implicated as a mechanism resulting in the activation of COX-1 (Hajjar *et al.*, 1995), the S-nitrosothiol, S-nitrosocysteine (SNOC), has been shown to inhibit TxA_2 synthesis in human platelets (Tsikas *et al.*, 1999a). In addition, NO has been shown to directly nitrate the catalytically active tyrosine 385 residue of COX-1 (Goodwin *et al.*, 1998), while $ONOO^-$, generated by the reaction between NO and O_2^- (equation 5, chapter 1.3.6.3), may activate PG biosynthesis via the provision of peroxides that are required for the initial activation of COX (Landino *et al.*, 1996). Finally, NO has also been shown to inhibit the activity of TxA_2 synthase in intact platelets (Wade & Fitzpatrick, 1997), adding a further level of complexity.

Given the unclear effects of NO, S-nitrosothiols and $ONOO^-$ on the AA/TxA_2 signalling pathway, the aim of this series of experiments was to examine the effect of different NO_x species on COX activity using a novel, commercially available *in vitro* chemiluminescent assay. Utilising a similar approach to that adopted in chapter 3, we performed experiments to test the hypothesis that NO inhibits COX-1 activity by examining the effect of the NO donor DEA/NO, the stable S-nitrosothiol SNVP and the $ONOO^-$ generator SIN-1 on COX-1 activity. Similarly, we extended this study to examine the effect of the Cu^{2+}-containing protein caeruloplasmin (CP) and superoxide dismutase (SOD), found within human plasma (Prakasam *et al.*, 2001; Sun *et al.*, 1988), to modulate the effects of SNVP and SIN-1 on COX-1 activity respectively. A further

aim of these experiments was to examine the effect of NO on the AA/ TxA_2 pathway in intact platelets. To this end, the effect of DEA/NO on AA and PGH_2-induced platelet activation and TxA_2 formation by means of *ex vivo* aggregometry and ELISA techniques was assessed.

4.2 METHODS

4.2.1 MEASUREMENT OF COX-1 ACTIVITY

The activity of ovine COX-1 purified from ram seminal vesicles (Sigma Aldrich) was assayed *in vitro* via a chemiluminescent method (Cayman chemical) based on the COX-catalysed luminescence of a cyclic naphthalene hydrazide derivative (7-dimethylaminonaphthalene-1,2-dicarboxylic acid hydrazide; DNH; chapter 2.6.1.; (Forghani *et al.*, 1998). To examine the effect of NO on COX-1 activity, DNH was added to hematin-reconstituted COX-1 (chapter 2.6.1) prior to the addition of DEA/NO (0.1-10 μM) or its NO-free parent nucleophile diethylamine (DEA; 10 μM) for 1-min at room temperature. Following incubation of DEA/NO or DEA, AA was injected via the luminometer and luminescence measured as described in the methods section (chapter 2.6.1). To serve as controls, COX-1 activity in the absence of hematin was assayed, as was the activity of COX-1 incubated in the presence of ASA (200 μM) for 15-min at room temperature. In further experiments, the effect of SNVP (30 μM) ± CP (0.4 g/L) and SIN-1 (30 μM) ± SOD (1000 U/ml) on COX-1 activity was also assessed to examine the effect of S-nitrosothiols and $ONOO^-$ on enzyme activity respectively (n=6 for all experiments). CP and SOD were utilised in these experiments simply to generate NO and/or unmask NO produced by SNVP and SOD respectively, akin to chapter 3, and were added immediately prior to the addition of SNVP and SIN-1 to the reaction mixture. The concentration of CP in plasma is ~ 0.4 g/L (Prakasam *et al.*, 2001) and previous data had shown this concentration capable of catalysing the release of NO from

SNVP (chapter 3, fig 3.3.a). 1000 U/ml SOD, a concentration supramaximal to that found in plasma (5-25 U/ml; Aydin *et al.*, 2004; Sun *et al.*, 1988), was used in order to prevent the rapid reaction between O_2^- and NO (chapter 1.3.6.3) derived from SIN-1.

4.2.2 AGGREGOMETRY

WP were prepared and aggregometry performed as described in the methods section (chapters 2.1.2 & 2.3 respectively). To examine the effect of NO on AA and PGH_2-mediated platelet aggregation, WP were incubated with DEA/NO (10 μM) or its NO-free parent nucleophile, DEA (10 μM), for 1-min prior to activation of platelets with either AA (100 μM) or PGH_2 (100 ng/ml). Platelet aggregation was then measured for 5-min as described (chapter 2.3). In further experiments, WP were pre-incubated with ODQ (20 μM) for 15-min prior to the addition of DEA/NO (10 μM) for 1-min following platelet activation with AA or PGH_2 as described above. Finally, the ability of ASA (200 μM; pre-incubated in WP for 5-min) to inhibit AA-induced platelet aggregation was also assessed (n=6 for all aggregometry experiments).

4.2.3 MEASUREMENT OF AA AND PGH_2-INDUCED TXA_2 FORMATION

AA and PGH_2-induced TxA_2 formation was estimated via an ELISA for the detection of TxB_2 (chapter 2.7.2), a marker for TxA_2 formation on account of its rapid formation from the non-enzymatic hydration of TxA_2 (Smith, 1989; Viinikka & Ylikorkala, 1980).

The same samples as had been used for the aggregometry (chapter 4.2.2) were processed for the TxB_2 assay. Briefly, following a 5-min incubation of WP with AA or PGH_2, indomethacin (100 μM; a COX-1 inhibitor; Smith *et al.*, 1994) or ozagrel (20 μM; a TxA_2 synthase inhibitor; Naito *et al.*, 1983) was added respectively for 5-min to prevent further TxA_2 formation. Samples were then immediately frozen in liquid nitrogen and stored at -70 °C prior to TxB_2 ELISA as described (chapter 2.7.2; n=5-6 for TxB_2 measurements).

4.3 RESULTS

4.3.1 *EFFECT OF ASA AND HEMATIN ON COX-1 ACTIVITY*

Addition of AA (100 μM) to hematin-reconstituted COX-1 in the presence of the chemiluminescent substrate DNH resulted in the generation of a chemiluminescent signal (fig 4.2.a). Incubation of ASA (200 μM) for 15-min significantly inhibited COX-1 activity (fig 4.2.a; $P < 0.01$; Dunnett's multiple comparison test following one-way ANOVA). Incubation of AA with COX-1 in the absence of hematin also prevented AA-stimulated COX-1 activity (fig 4.2.a; $P < 0.01$; Dunnett's multiple comparison test following one-way ANOVA).

4.3.2 *EFFECT OF DEA/NO ON COX-1 ACTIVITY*

Incubation of DEA/NO (0.1-10 μM) with hematin-reconstituted COX-1 resulted in a concentration-dependent inhibition of COX-1 activity (fig 4.2.b). While incubation of COX-1 with both 1 and 10 μM DEA/NO significantly inhibited COX-1 activity ($P<0.01$; Dunnett's multiple comparison test after one-way ANOVA) incubation with 0.1 μM DEA/NO was without effect ($P > 0.05$; Dunnett's multiple comparison test following one-way ANOVA). Furthermore, incubation of COX-1 with the NO-free nucleophile DEA (10 μM) did not affect COX-1 activity ($P > 0.05$; Dunnett's multiple comparison test following one-way ANOVA).

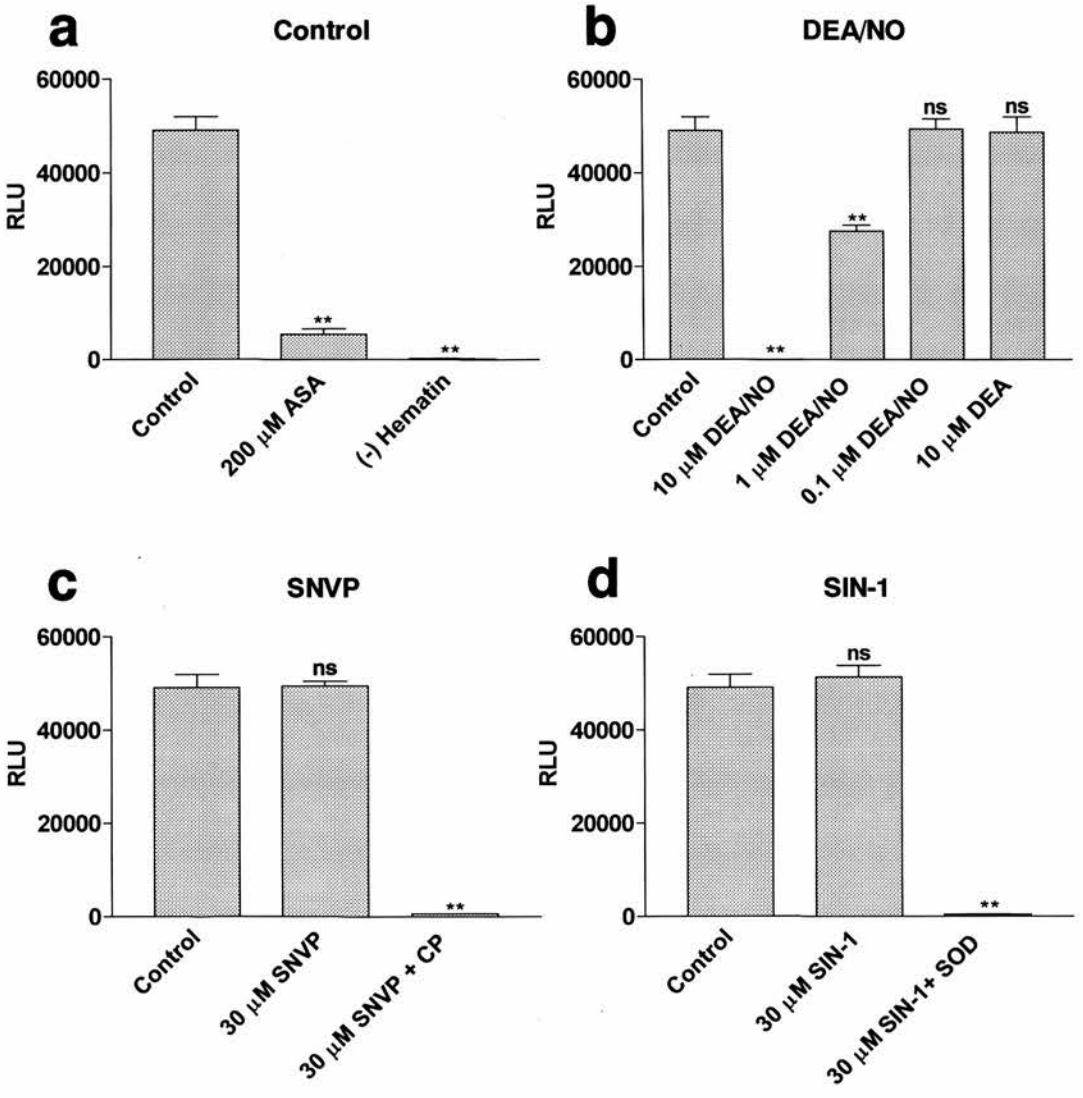


Figure 4.2 **Effect of ASA, hematin, and NO_x donors on AA-stimulated COX-1 activity. A control response was generated via the addition of AA (100 μM) to hematin-reconstituted COX-1 in the presence of the chemiluminescent substrate, DNH. The effect of ASA (200 μM), pre-incubated with COX-1 for 15-min and the effect of omission of hematin from the reaction mixture was investigated (a). The ability of the NO_x donors DEA/NO (0.1-10 μM), SNVP (30 μM) ± CP (0.4 g/L) and SIN-1 (30 μM) ± SOD (1000 U/ml) to inhibit COX-1 activity following a 1-min incubation was also assessed (b-d). The effect of DEA (10 μM), incubated with COX-1 for 1-min was also investigated (b; ns = P>0.05; ** = P<0.01; n=6). Abbreviation: RLU; relative luminescence units.**

4.3.3 *EFFECT OF SNVP ± CP ON COX-1 ACTIVITY*

Incubation of SNVP (30 μM) with hematin-reconstituted COX-1 did not significantly affect COX-1 activity (fig 4.2.c; P>0.05; Dunnett's multiple comparison test following one-way ANOVA). Co-incubation of CP (0.4 g/L), which catalyses the generation of NO from SNVP (fig 3.3.a.; chapter 3.3.6), significantly inhibited AA-induced COX-1 activity (P < 0.01; Dunnett's multiple comparison test after one-way ANOVA).

4.3.4 *EFFECT OF SIN-1 ± SOD ON COX-1 ACTIVITY*

Incubation of SIN-1 (30 μM) with COX-1 did not affect COX-1 activity (fig 4.2.d; P>0.05; Dunnett's multiple comparison test following one-way ANOVA). Co-incubation of a high concentration of SOD (1000 U/ml), to remove superoxide and therefore unmask NO generated by SIN-1, significantly inhibited AA-induced COX-1 activity (fig 4.2.d; P<0.01; Dunnett's multiple comparison test following one-way ANOVA).

4.3.5 *EFFECT OF DEA/NO ON AA-INDUCED AGGREGATION*

Incubation of WP with the NO-free nucleophile, DEA, prior to stimulation with AA resulted in an aggregation response that was 93.4 ± 9.5 % of the control AA-induced aggregation response (P>0.05; Student's paired t-test). Addition of DEA/NO (10 μM) or ASA (200 μM) to WP inhibited AA-induced platelet aggregation compared to DEA

(fig 4.3; P < 0.01; Dunnett's multiple comparison test following one-way ANOVA). Pre-incubation of ODQ (20 μM) with platelets for 15-min prior to the addition of DEA/NO did not reverse the aggregation response to levels observed with DEA (P > 0.05; Dunnett's multiple comparison test after one-way ANOVA).

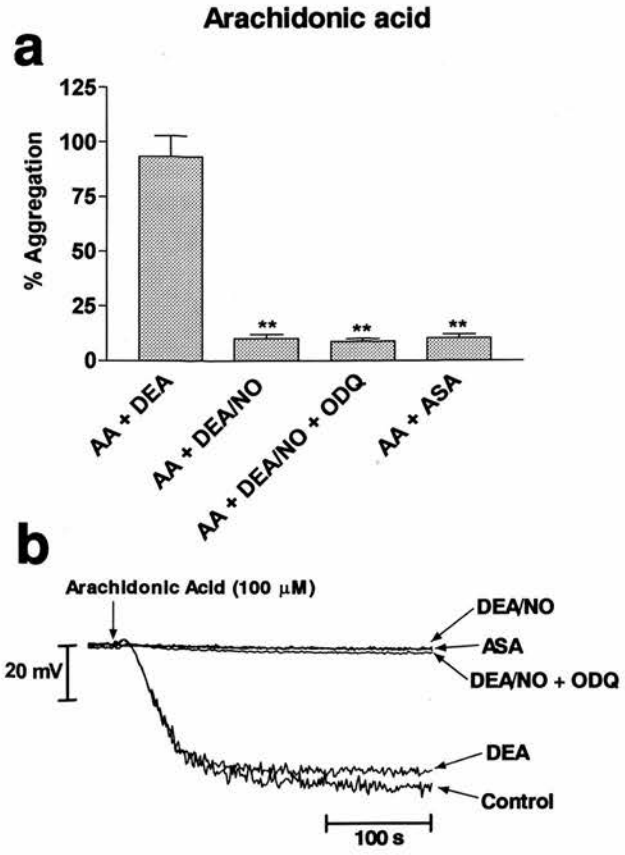


Figure 4.3 **Effect of DEA, ASA and DEA/NO ± ODQ on AA-induced platelet aggregation. DEA/NO (10 μM) or DEA (10 μM) was incubated with WP for 1-min prior to the addition of AA (100 μM) to induce aggregation. Where ODQ was used, it was pre-incubated with WP for 15-min prior to the addition of DEA/NO, followed by the addition of AA 1-min later. ASA (200 μM) was pre-incubated for 5-min with WP before the addition of AA. Summary data (a) and a representative trace (b) are included (** = P<0.01; n=6).**

4.3.6 *EFFECT OF DEA/NO ON PGH_2-INDUCED AGGREGATION*

Addition of DEA to WP for 1-min prior to stimulation with PGH_2 (100 ng/ml) resulted in an aggregation response that was 103.7 ± 9.2 % of the control aggregation response ($P>0.05$; Student's paired t-test). DEA/NO (10 μM) significantly inhibited PGH_2-induced platelet aggregation compared to DEA (fig 4.4; $P<0.01$; Dunnett's multiple comparison test following one-way ANOVA). Pre-incubation of ODQ (20 μM) for 15-min prior to the addition of DEA/NO did not reverse aggregation to levels observed with DEA ($P>0.05$; Dunnett's multiple comparison test following one-way ANOVA).

4.3.7 *EFFECT OF DEA/NO ON AA-INDUCED TXB_2 FORMATION*

Stimulation of platelets with AA (100 μM) resulted in the formation of TxB_2 (145.5 ± 16.0 ng/10^8 platelets). Incubation of WP with DEA (10 μM) did not significantly affect AA-induced TxB_2 formation (fig 4.5.a.; $P>0.05$; Dunnett's multiple comparison test following one-way ANOVA) while DEA/NO (10 μM) inhibited AA-induced TxB_2 formation in both the presence and absence of 20 μM ODQ (fig 4.4.a.; $P<0.01$; Dunnett's multiple comparison test following one-way ANOVA). Incubation of WP with ASA (200 μM) also resulted in a significant inhibition of TxB_2 formation (fig 4.5.a.; $P<0.01$; Dunnett's multiple comparison test following one-way ANOVA).

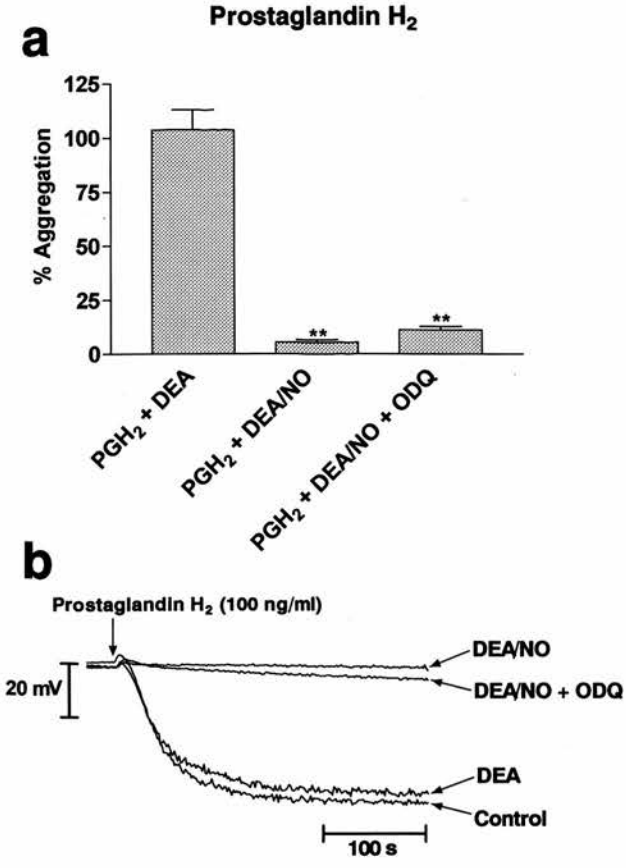


Figure 4.4 **Effect of DEA and DEA/NO ± ODQ on PGH_2-induced platelet aggregation. DEA/NO (10 μM) or DEA (10 μM) was incubated with WP for 1-min prior to the addition of PGH_2 to induce aggregation. Where ODQ was used, it was pre-incubated with WP for 15-min prior to the addition of DEA/NO, followed by the addition of PGH_2 1-min later. Summary data (a) and a representative trace (b) is included (** = P<0.01; n=6).**

4.3.8 *EFFECT OF DEA/NO ON PGH_2-INDUCED TXB_2 FORMATION*

Addition of PGH_2 (100 ng/ml) to WP also resulted in TxB_2 formation that was significantly increased (~ 2-fold) compared to AA-stimulated platelets pre-incubated with ASA (PGH_2-induced TxB_2 formation: 12.5 ± 1.7 ng/10^8 platelets vs 5.3 ± 1.4

ng/10^8 TxB_2 formed for ASA-treated AA-stimulated platelets; P<0.05; Student's unpaired t-test). Incubation of WP with DEA (10 μM), DEA/NO (10 μM) or DEA/NO + ODQ (20 μM) did not affect PGH_2-induced TxB_2 formation (fig 4.5.b; P>0.05; Dunnett's multiple comparison test following one-way ANOVA).

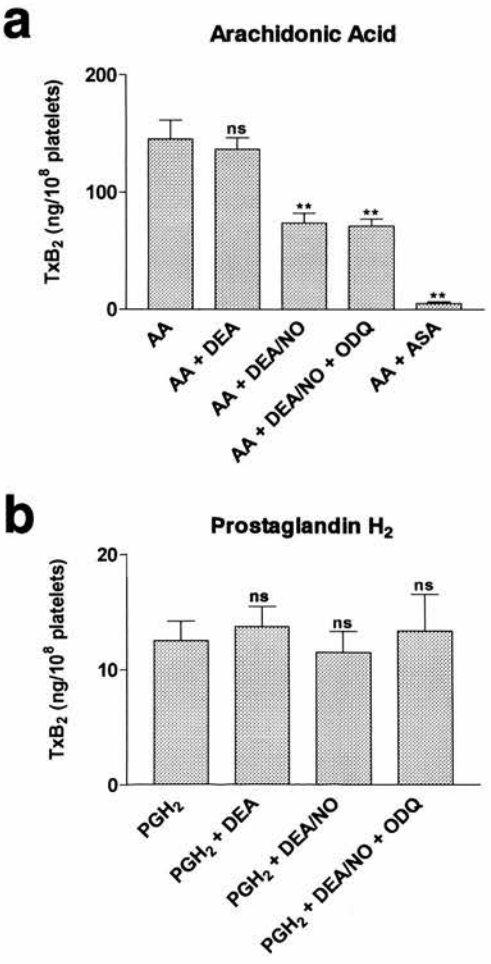


Figure 4.5 **Effect of DEA, ASA and DEA/NO ± ODQ on AA and PGH_2-induced TxB_2 formation. DEA/NO (10 μM) or DEA (10 μM) was incubated with WP for 1-min prior to the addition of AA (100 μM) or PGH_2 (100 ng/ml) to induce aggregation. Where ODQ was used, it was pre-incubated with WP for 15-min prior to the addition of DEA/NO, followed by the addition of AA 1-min later. ASA (200 μM) was pre-incubated for 5-min with WP before the addition of AA. Following a 5-min incubation of AA or PGH_2, indomethacin (100 μM) or ozagrel (20 μM) were added respectively for 5-min prior to freezing and TxB_2 ELISA (** = P<0.01; n=5-6).**

4.4 DISCUSSION

Taken together, these results indicate an inhibitory role for NO on COX-1 activity. Experiments performed *in vitro* utilising a chemiluminescence COX-1 assay indicated that DEA/NO inhibits COX-1 in a concentration-dependent manner. The effects of DEA/NO may be attributed to the generation of NO because the NO-free parent nucleophile, DEA, was ineffectual. Further experiments revealed that the relatively stable S-nitrosothiol, SNVP (30 μM), was also without effect on COX-1 activity. However, when SNVP was co-incubated with a physiological concentration of the copper-containing plasma protein CP (0.4g/L), an inhibitory effect on COX-1 was observed. Similarly, while the incubation of the $ONOO^-$ generator, SIN-1 (30 μM), did not affect COX-1 turnover, co-incubation of SIN-1 with SOD (1000 U/ml) prevented COX-1 activity, suggesting that NO (radical) inhibits COX-1 activity *in vitro*. Experiments performed in WP revealed that DEA/NO (10 μM) inhibited both AA and PGH_2-evoked platelet aggregation compared to DEA (10 μM). Furthermore, pre-incubation of WP with ODQ (20 μM) did not affect DEA/NO-mediated inhibition of aggregation by either agonist, indicating the inhibitory response was cGMP-independent. Analysis of TxB_2 formation indicated that DEA/NO inhibited AA-induced TxB_2 formation, but not PGH_2-evoked TxB_2 formation, suggesting that COX-1 was the site of NO-mediated inhibition of TxB_2 synthesis. As observed with the aggregometry data, ODQ (20 μM) did not reverse DEA/NO-mediated inhibition of AA-induced TxB_2 synthesis, indicating that the inhibitory effect was cGMP-independent. Taken together

these data indicate a direct inhibitory role for NO (radical), but not necessarily S-nitrosothiols or $ONOO^-$ on COX-1 activity.

4.4.1 ASSAY OF COX-1 VIA CHEMILUMINESCENCE

COX-1 enzyme activity was assayed utilising a chemiluminescent technique based on the COX-1 peroxidase-site catalysed oxidation of a luminol derivative DNH (Forghani *et al.*, 1998). This assay is a sensitive, real-time indicator of COX-1 activity, with the cyclooxygenation step rate-limiting for the generation of luminescence (Forghani *et al.*, 1998). Importantly, the haem prosthetic group is absolutely required for both the cyclooxygenase and peroxidase activities of COX-1 (Ogino *et al.*, 1978). During purification of COX-1, dissociation of the haem cofactor occurs (Malkowski *et al.*, 2000) and therefore haem must be added for enzyme activity. Our results indicate that the presence of hematin is required for enzyme activity (fig 4.2.a) in agreement with these findings. Importantly, control experiments also indicated that incubation of COX-1 with ASA (200 μM) for 15-min inihibited the chemiluminescent signal (fig 4.2.a), in line with the known inhibitory action of ASA on COX-1 (Gallus, 1985; Schror, 1997). These data therefore support the use of the chemiluminescent assay as a measure of COX-1 activity. In addition, although ovine COX-1 was used in these *in vitro* experiments because it is readily available, amino acid sequence homology between the human and sheep form is extremely high (91 %; Yokoyama & Tanabe, 1989) and therefore NO-mediated effects on sheep COX-1 are likely directly applicable to the human isoform. Nevertheless, there is an inherent danger with extrapolation of results

between species and cell types, and further experiments would be required to confirm these results in human platelet COX-1.

4.4.2 INHIBITION OF COX-1 ACTIVITY BY NO DONORS IN VITRO

Data obtained from *in vitro* experiments suggest that NO is a potent inhibitor of COX-1 activity (fig 4.2). This conclusion is supported by a number of observations. Firstly, the NO donor, DEA/NO, produced a concentration-dependent inhibition of chemiluminescence, whereas the NO-free nucleophile DEA did not. Secondly, the relatively stable S-nitrosothiol, SNVP, that nevertheless can undergo transnitrosation reactions (Megson *et al.*, 1999), did not inhibit COX-1 activity. However, when SNVP was incubated with the plasma protein, CP, which has previously been shown to catalyse the release of NO from SNVP and other S-nitrosothiols (chapter 3; fig 3.3.a; Dicks & Williams, 1996), an inhibition of COX-1 activity was observed. Finally, when the $ONOO^-$ generator SIN-1 was incubated with COX-1, no inhibitory effect on COX-1 activity was noted. However, as with SNVP, when NO generation was unmasked by the addition of SOD, inhibition of COX-1 activity was also observed. Previous data indicate that NO may either increase (Davidge *et al.*, 1995; Hajjar *et al.*, 1995; Maccarrone *et al.*, 1997) or decrease COX-1 activity (Kanner *et al.*, 1992; Tsai *et al.*, 1994). Interestingly, reports indicate that concentrations of NO as high as 2 mM are sufficient to result in only a small inhibition of COX-1 activity (Tsai *et al.*, 1994). Experiments here were conducted at room temperature, at which DEA/NO will have a half-life significantly

lower than the 2-min predicted at 37°C (Morley & Keefer, 1993). Given that DEA/NO was incubated with COX-1 for only a short period of time (1-min), these data suggest that low nM concentrations of NO may be sufficient to inhibit COX-1 activity. However, data presented here may be reconciled against these conflicting reports by evidence indicating that NO may act as a reducing substrate for peroxidase turnover (Curtis *et al.*, 1996; O'Donnell *et al.*, 2000). In this capacity, NO would be competing with the chemiluminescent substrate, DNH, for the provision of electrons to reduce the COX-1 haem following its oxidation by PGG_2 (fig 4.6). Thus, higher concentrations of NO supplied by DEA/NO may in fact out-compete DNH and prevent its oxidation and subsequent chemiluminescence. Indeed, in this sense NO would be expected to greatly stimulate COX-1 turnover, as occurs with other peroxidase reducing substrates (Kulmacz & Lands, 1983; Markey *et al.*, 1987), although this hypothesis would need to be confirmed by the measurement of COX-1 activity via alternative techniques such as oxygen electrode (Bambai & Kulmacz, 2000) or immunoassay (Reitz *et al.*, 1994). Nevertheless, data from experiments performed ex vivo indicate that DEA/NO inhibits TxA_2 formation via a cGMP-independent inhibition of COX-1 activity, albeit at higher concentrations (EC_{50}~ 10 μM).

The observation that SNVP did not affect COX-1-induced chemiluminescence argues against a role for S-nitrosothiols accelerating COX-1 turnover as previously implicated (Hajjar *et al.*, 1995), unless a specific endogenous S-nitrosothiol such as SNOC is required. Similarly, data obtained here did not indicate that $ONOO^-$ increased COX-1 activity as reported (Landino *et al.*, 1996). It is well known that peroxides are

required for the initial activation of COX; they are needed to oxidise the haem prosthetic group prior to the oxygenation of AA (fig 4.6; step 1; Kulmacz & Lands, 1983; Ohki *et al.*, 1979).

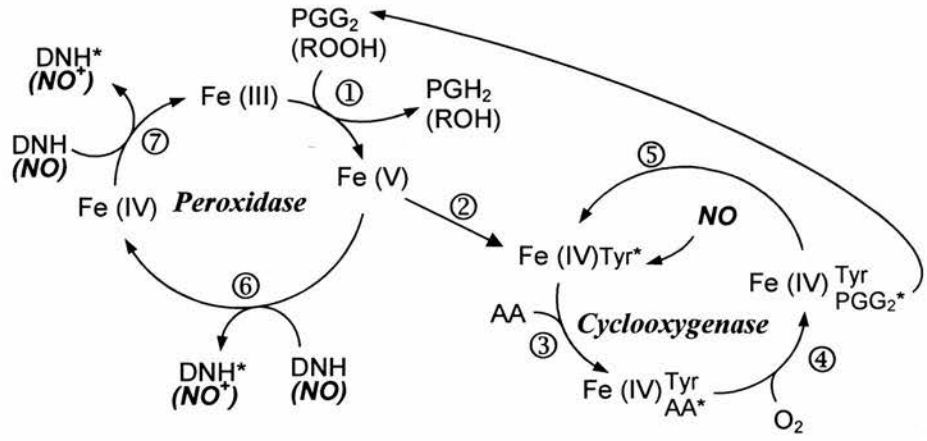


Figure 4.6 **Hypothetical mechanism for COX-1 catalysis including possible sites of NO interaction. (1) PGG_2, or other available hydroperoxides (ROOH), are required to initially oxidise resting COX-1 haem (Fe III) to Fe V, resulting in the generation of PGH_2, or equivalent hydroxyl compound (ROH). (2) Electron transfer from Tyr 385 results in the formation of a tyrosyl radical (Tyr*), which then oxidises AA and initiates the cyclooxygenase reaction cycle (3-5). PGG_2 is then reduced to PGH_2 at the peroxidase site on either the same enzyme or on a different COX-1 enzyme (1). (6-7) Reducing substrates are required to reduce Fe V to the resting state (Fe III) in order that the peroxidase site can catalyse the reduction of PGG_2 to PGH_2 (1). Reducing substrates can also reduce the tyrosyl radical, resetting the enzyme to the ferric state (not shown on diagram). In this assay, the chemiluminescent substrate, DNH, acts as the reducing substrate, resulting in the generation of excited DNH (DNH*) and the liberation of light. NO may interact with the COX-1 reaction mechanism also by acting as a reducing substrate, resulting in the generation of nitrosonium ion (NO^+). NO also interacts with the tyrosyl radical, ultimately resulting in the formation of nitrated tyrosine (chapter 4.4.4). Adapted from Tsai *et al.*, 1995.**

However, preparations of AA contain sufficient levels of hydroperoxide impurities (in the nM range; Kulmacz & Lands, 1983) within them to achieve activation of COX, thus requiring the presence of antioxidant systems to prevent COX-1 activation via these peroxides (Hemler *et al.*, 1979; Kulmacz & Lands, 1983). Therefore, it seems likely that

in this system, COX-1 is already turning over at a maximal rate and thus $ONOO^-$-mediated acceleration of COX-1 was not observed.

4.4.3 NO-MEDIATED INHIBITION OF PLATELET AGGREGATION AND TXA_2 SYNTHESIS

Data presented here indicate that DEA/NO (10 μM) inhibits both AA and PGH_2-induced platelet aggregation (figs 4.3 & 4.4). Furthermore, these data demonstrate that DEA/NO can inhibit AA, but not PGH_2-induced TxA_2 formation (fig 4.5), implying that NO inhibits COX-1 activity in intact human platelets. The hypothesis that the inhibitory effect of DEA/NO was a cGMP-independent response was confirmed by the lack of effect of 20 μM ODQ in reversing DEA/NO-mediated inhibition of platelet aggregation and TxA_2 formation. Importantly, previous data indicated that this concentration of ODQ effectively suppresses cGMP production from 10 μM DEA/NO (chapter 3.3.1). Although in the future it would be necessary to examine whether ODQ itself directly affects COX-1 or TxA_2 synthase activity, as discussed in chapter 3.4.1, ODQ does not affect collagen-induced platelet aggregation, whereas aspirin pre-treatment completely prevents this aggregation, supporting the hypothesis that ODQ does not significantly interefere with platelet TxA_2 synthesis. Interestingly, SNOC has previously been shown to inhibit TxA_2 synthesis in platelets by a cGMP-independent mechanism (Tsikas *et al.*, 1999a). Although results from *in vitro* experiments suggest that S-nitrosothiols do not affect COX-1 activity, the inhibitory activity of SNOC on TxA_2 activity may be explained by its highly unstable nature in physiological solutions (Mathews & Kerr,

1993), decomposing to generate high levels of NO. Results here indicate that DEA/NO (10 μM) is as effective as ASA (200 μM) in inhibiting AA-induced aggregation, although at the concentration tested it is clearly not as potent in preventing TxA_2 production. One possible explanation for this is that platelet concentration-response curves to agonists are typically steep, and it is therefore plausible that the ~2-fold reduction in TxA_2 production is sufficient to result in a near-complete inhibition of platelet aggregation. However, these results show that that incubation of platelets with a concentration of PGH_2 sufficient to induce aggregation results in ~10-fold decreased TxB_2 levels compared to those platelets stimulated with AA. This difference may be explained by the fact that PGH_2 is a potent agonist at TP receptors (Coleman *et al.*, 1994) and in this situation will be stimulating TxA_2-dependent signalling events independently of its conversion to TxA_2. However, the observation that DEA/NO was without effect on PGH_2-evoked TxA_2 synthesis, but caused near maximal inhibition of PGH_2-induced aggregation, strongly supports the existence of other downstream cGMP-independent routes for the inhibition of platelet aggregation (Homer & Wanstall, 2002; Trepakova *et al.*, 1999). Although it is difficult to firmly establish whether NO inhibits TxA_2 synthase activity given the relatively low proportion of AA that is converted from PGH_2 to TxA_2, PGH_2-induced TxA_2 formation in the control group resulted in a > 2-fold increase in TxA_2 over levels observed with AA-stimulated TxA_2 formation in the presence of ASA (PGH_2-induced TxB_2 formation: 12.5 ± 1.7 ng/10^8 platelets vs 5.3 ± 1.4 ng/10^8 TxB_2 formed for ASA-treated AA-stimulated platelets), indicating that PGH_2 induced TxA_2 formation over basal levels. Furthermore, these experiments were

performed in WP to remove basal levels of TxB_2 formed *in vivo*. These data, therefore, do not support the hypothesis that NO directly modulates TxA_2 synthase activity as suggested previously (Wade & Fitzpatrick, 1997), although it is likely to indirectly affect TxA_2 synthase activity via modulation of PGH_2 availability.

4.4.4 TARGETS FOR NO

A number of potential interactions between NO and COX-1 exist. As mentioned above, NO may act as a reducing substrate for COX-1 peroxidase activity (Curtis *et al.*, 1996; O'Donnell *et al.*, 2000), a finding at least partially supported by data presented here. However, by acting as a co-substrate to reduce COX-1 haem, NO is expected to support COX-1 activity. Indeed, in the absence of reducing substrates, reduction of PGG_2 to PGH_2 cannot occur; although this does not directly affect the cyclooxygenase cycle of COX-1, accumulation of PGG_2 (IC_{50} ~ 600 nM) leads to a rapid and permanent inactivation of COX-1, allowing only a few enzymatic cycles (Markey *et al.*, 1987; Wu *et al.*, 2003). Alternatively, NO may interact with sulphydryl groups present on COX-1 (Hajjar *et al.*, 1995). However, data presented here indicate that SNVP did not affect COX-1 activity, suggesting that nitrosation of sulphydryl groups on COX-1, at least by this S-nitrosothiol, does not play a role. Although we cannot rule out that a specific endogenous S-nitrosothiol such as S-nitrosoglutathione or SNOC will affect COX-1 activity, the use of such compounds, particularly SNOC, is unlikely to produce a clear result because these S-nitrosothiols are prone to spontaneously generate NO, thereby confusing S-nitrosothiol and NO (radical)-mediated effects. A further hypothesis is that

NO interacts with COX-1 haem, which has been demonstrated *in vitro* (Tsai et al., 1994). However, the K_d for the interaction of NO with native (ferric) COX-1 is ~ 1 mM (Tsai *et al.*, 1994), a concentration much greater than will be achieved in experiments here. Although NO interacts with artificially-reduced ferrous COX-1 haem with much greater efficiency (Tsai *et al.*, 1994), there is no evidence that ferrous COX-1 is formed *in vivo* (Goodwin *et al.*, 1999a) suggesting that interaction of NO with COX-1 haem is unlikely to be responsible for the inhibitory effect in intact platelets. Perhaps, therefore, the most likely route to inhibition of COX-1 is the interaction of NO with tyr 385, as has been shown to occur in the active site of both COX-1 and COX-2 (Goodwin *et al.*, 1998; Gunther *et al.*, 1997). Tyr 385 forms a tyrosyl radical following peroxide-induced oxidation of COX haem (fig 4.6; Dietz *et al.*, 1988; Tsai *et al.*, 1995), which is essential for the oxidation of AA to PGH_2 (Shimokawa *et al.*, 1990). Interestingly, the reaction of NO with the COX-1 tyrosyl radical results in the formation of 3-nitrosotyrosine, prior to undergoing a molecular rearrangement to the stable 3-nitrotyrosine (Goodwin *et al.*, 1998). Although $ONOO^-$ is the species usually implicated in the nitration of residues such as tyrosine (chapter 1.3.6.5; Sawa *et al.*, 2000; van der Vliet *et al.*, 1995), the generation of a tyrosyl radical permits a much more rapid reaction between NO and tyrosine in COX-1, as has been observed in other tyrosyl radical-dependent proteins (Lepoivre *et al.*, 1994; Lepoivre *et al.*, 1992; Szalai & Brudvig, 1996). Indeed, the observation here that $ONOO^-$ did not inhibit COX-1 activity *in vitro*, coupled with previous data indicating that SOD does not affect NO-mediated COX-1 nitration (Goodwin *et al.*, 1998) argue against a role for $ONOO^-$ in the modification of tyr 385 of COX-1 and inhibition of PGH_2 synthesis. Furthermore, reports indicate that high

concentrations of AA may out-compete NO for the tyr 385 radical (Goodwin *et al.*, 1998; Goodwin *et al.*, 1999a), explaining why only a partial effect of DEA/NO on COX-1 activity was observed in the *ex vivo* experiments performed here. In addition, the ability of NO to inhibit COX-1 activity may also be offset by its ability to act as a reducing substrate and accelerate enzyme turnover as described above. It seems likely, therefore, that the effect of NO on COX-1 activity in a particular system will be highly dependent on the presence of endogenous reducing substrates, AA levels and NO availability.

4.4.5 PHYSIOLOGICAL AND PHARMACOLOGICAL IMPLICATIONS

Although a relatively high concentration of DEA/NO was used in these experiments (10 μM; releasing a theoretical maximum of 20 μM NO), it is conceivable that sufficient NO may be generated within the local environment to inhibit COX-1 activity. From this standpoint it is interesting that following platelet activation and an increase in cytosolic Ca^{2+} levels, both COX-1 and platelet-NOS will be simultaneously activated. Furthermore, assuming that the inhibitory effect of NO is mediated via nitration of tyr 385, its effect is likely to be essentially irreversible, given the high stability of nitrated tyrosine (Shigenaga *et al.*, 1997; van der Vliet *et al.*, 1995). Therefore, it is possible that platelet-derived NO may reduce platelet TxA_2 synthesis through a covalent modification of the COX-1 active site. In addition to the physiological situation, these data imply that pharmacological concentrations of NO donors may inhibit platelet function via a cGMP-

independent inhibition of COX-1 synthesis. The observation that plasma components, including CP and SOD, can catalyse and/or unmask NO from S-nitrosothiols and $ONOO^-$ donors respectively (chapter 3; figs 3.1 & 3.3) imply that plasma and/or cellular components may also be able to induce COX-1 inhibition effects by these donors. However, the in vivo situation would be further complicated by cellular and cell-free haemoglobin, which would alter NO donor bioavailability.

4.4.6 SUMMARY

In summary, these data indicate that NO (radical) inhibits TxA_2 synthesis in intact platelets, most likely via an inhibitory effect on COX-1 activity. While these results support previous reports indicating that NO is a reducing substrate for COX-1 activity, further experiments are required to confirm this. Data obtained here do not support a role for S-nitrosothiols (at least SNVP) or $ONOO^-$ as inhibitors of COX-1 activity, nor do they support a previous finding that NO directly inhibits the TxA_2 synthase enzyme in intact platelets. A likely site for the NO-mediated inhibition of COX-1 activity is the catalytically active residue tyr 385 (Goodwin *et al.*, 1998).

CHAPTER FIVE

NOVEL ROLE FOR LOW MOLECULAR WEIGHT THIOLS IN NITRIC OXIDE-MEDIATED CONTROL OF PLATELET FUNCTION

5.1 INTRODUCTION

Despite the fact that NO is a powerful antiplatelet agent (Radomski *et al.*, 1987a; Radomski *et al.*, 1987d), its ability to prevent platelet activation is limited by its short half-life under physiological conditions (~3–10 sec; Cocks *et al.*, 1985; Griffith *et al.*, 1984). Such observations suggest that NO bioactivity should rapidly dissipate and only impact on cells within close diffusable range of the site of production (Lancaster, 1994; Lancaster, 1997). However, a number of studies suggest that NO can be incorporated into relatively stable endogenous reservoirs that modify its biological activity (Datta *et al.*, 2004; Jia *et al.*, 1996; Mulsch *et al.*, 1991; Scharfstein *et al.*, 1994; Stamler *et al.*, 1992a; Stamler *et al.*, 1992c; Stamler *et al.*, 1992d; Vedernikov *et al.*, 1992). S-nitrosothiols rank highly amongst the likely candidates for such a reservoir, on account of the relative abundance of suitable thiols in the biological environment (Jocelyn, 1972). A physiological role for S-nitrosothiols has been implicated following identification of endogenous S-nitrosothiols at relevant concentrations (Akaike *et al.*, 1997; Datta *et al.*, 2004; Goldman *et al.*, 1998; Marley *et al.*, 2000; Stamler *et al.*, 1992a; Tsikas *et al.*, 1999c), together with plausible pathways that could result in their formation (Gow *et al.*, 1997; Hogg *et al.*, 1996; Kharitonov *et al.*, 1995; Mayer *et al.*, 1998). In plasma, it has been shown that the majority of the S-nitrosothiol pool exists in the form of the high molecular weight species S-nitrosoalbumin (Marley *et al.*, 2001; Stamler *et al.*, 1992a; Tsikas *et al.*, 1999b). However, low molecular weight thiols such as glutathione are also present in plasma in the low micromolar range, and have previously been shown to potentiate the antiplatelet action of S-nitrosoalbumin (Simon

et al., 1993). Given the close proximity of platelets to the vascular endothelium, and the unique sensitivity of platelets towards S-nitrosothiol mediated inhibition, it is important to dissect the role of plasma-borne thiols in the modification of NO activity in platelets.

The aim of experiments conducted within this chapter was to test the hypothesis that the activity of a short-acting NO donor drug, diethylamine diazeniumdiolate (DEA/NO), is prolonged in the presence of plasma albumin through formation and subsequent activation of an S-nitrosoalbumin NO reservoir. Furthermore, these experiments explored the hypothesis that low molecular weight thiols have a unique role in both the formation and activation of an S-nitrosoalbumin reservoir, potentiating NO-mediated inhibition of platelet aggregation.

5.2 METHODS

5.2.1 NO ELECTRODE MEASUREMENTS

PRP, PPP and WP were prepared as described in the methods section (chapter 2.1.1). Samples (2 ml) of PRP and WP were pre-warmed to 37°C, before addition of DEA/NO (2 μM). NO concentration was measured for 30 min by a calibrated NO electrode as described in the methods section (chapter 2.2; n=6). In a different series of experiments, WP were reconstituted with 0.46 μM hemoglobin derived from red blood cell (RBC) lysate, prior to addition of DEA/NO (2 μM) and recording for 30-min (n=6).

5.2.2 HAEMOGLOBIN MEASUREMENTS

The haemoglobin content of PRP and PPP was quantified as described in the methods section (chapter 2.5.1; n=5).

5.2.3 AGGREGOMETRY

Platelet aggregometry was performed as described in the methods section (chapter 2.3). Aliquots (0.5 ml) of PRP and WP were equilibrated at 37°C before the addition of 2 μM DEA/NO (~IC_{80} for DEA/NO in PRP; Sogo *et al.*, 2000b). Platelet aggregation was then induced via the addition of collagen (2.5 μg/ml) 1-30 min later. Aggregation was monitored for 5 min, and the maximum response recorded. In a different series of experiments, WP were reconstituted with the low molecular weight (LMW) thiols

glutathione (GSH; 5 μM), cysteinyl-glycine (cys-gly; 10 μM) and cysteine (cys; 10 μM) to approximate plasma concentrations (Mansoor *et al.*, 1992). Thiol-reconstituted WP was also incubated in the absence and presence of 1 % human serum albumin (HSA); higher concentrations of HSA that approximate plasma levels (4%) were found to have non-specific effects in platelets, even after extensive dialysis. Platelets were incubated with DEA/NO (2 μM) before stimulation with collagen (2.5 μg/ml) 30 min later. In further experiments, WP reconstituted with GSH (5 μM) ± HSA (1%) were pre-incubated with donor RBC lysate to produce a final hemoglobin concentration of 0.46 μM. DEA/NO (2 μM) was added to WP for 30-min prior to addition of collagen (2.5 μg/ml) 30 min later. In control experiments, DEA/NO (2 μM) was added to WP 25 min before the addition of oxy-hemoglobin (10 μM). Platelets were then stimulated with collagen (2.5 μg/ml) 5 min later, and aggregation measured (n=8 for all experiments).

5.2.4 THIOL MEASUREMENTS

The reduced thiol content of plasma and HSA (1%)-reconstituted tyrodes ± the LMW thiols GSH (5 μM), cys-gly (10 μM) or cysteine (10 μM) was quantified as described in the methods section (chapter 2.5.2; n=5).

5.2.5 S-NITROSOTHIOL DETECTION

Baseline S-nitrosothiol formation was quantified via a chemiluminescent method described in the methods section (chapter 2.6.2). To determine S-nitrosothiol formation

after bolus NO injection, WP and PRP samples were prewarmed as before and 2 μM DEA/NO added. Aliquots (0.5 ml) of DEA/NO-treated WP or PRP were aspirated 1-30 min later and added to vials containing NEM/EDTA (5 mM and 2 mM respectively) to stop the reaction. Samples were then processed and quantified as described in the methods section (chapter 2.6.2; n=6).

5.3 RESULTS

5.3.1 HAEMOGLOBIN MEASUREMENTS

Haemoglobin (Hb) concentration in PRP was 0.46 ± 0.18 μM, and did not differ significantly from the hemoglobin concentration determined in PPP (0.39 ± 0.01 μM; P > 0.05; Student's paired t-test).

5.3.2 NO ELECTRODE STUDIES

Addition of 2 μM DEA/NO to WP resulted in a rapid increase in NO concentration, which reached a maximum of 3.2 ± 0.18 μM NO before it declined to basal levels within 20-25 min (fig 5.1). Administration of 2 μM DEA/NO to PRP showed that DEA/NO-derived NO was partially quenched in plasma, reaching a maximum extracellular concentration of 0.53 ± 0.11 μM. Addition of 2 μM DEA/NO to WP reconstituted with 0.46 μM hemoglobin derived from donor RBC lysate produced a profile matching that observed in PRP, with a maximum extracellular NO-concentration of 0.59 ± 0.05 μM.

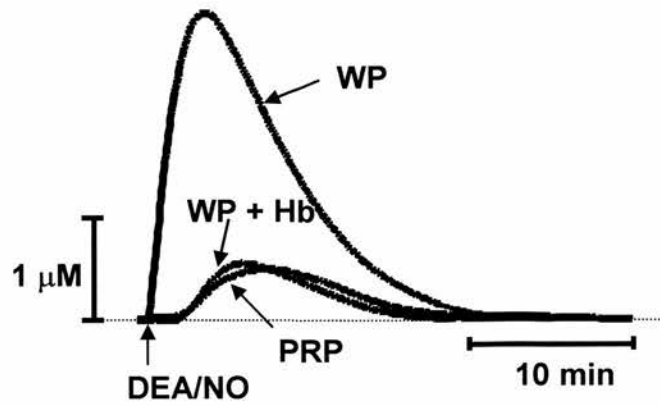


Figure 5.1 **Generation of NO by DEA/NO (2 μM) in WP, PRP, and WP reconstituted with autologous cell-free Hb (0.46 μM). Data are expressed as the mean of six experiments.**

5.3.3 EFFECT OF DEA/NO ON INHIBITION OF PLATELET AGGREGATION IN PRP & WP

Bolus administration of DEA/NO (2 μM) to PRP resulted in sustained inhibition of collagen-induced platelet aggregation that was maintained for at least 30 min (fig 5.2.a). In WP, however, inhibition of collagen-induced platelet aggregation by DEA/NO (2 μM) was attenuated at 20 min and abolished after 30 min (fig 5.2.a). The difference between inhibition of aggregation in PRP and WP was significant ($P<0.001$; two-way ANOVA followed by Bonferroni post-test). Representative traces from each time point in both PRP and WP are included (fig 5.2.b).

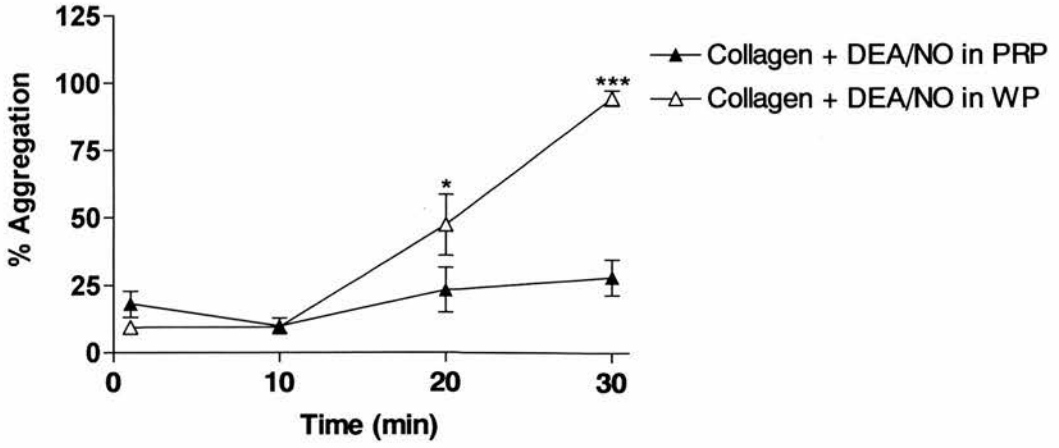


Figure 5.2.a **Inhibition of platelet aggregation by DEA/NO in WP and PRP. WP or PRP were equilibrated to 37°C before the addition of DEA/NO (2 μM). Platelet aggregation was then stimulated via the addition of collagen (2.5 μg/ml) 1-30 min later (*** = P<0.001; n=8).**

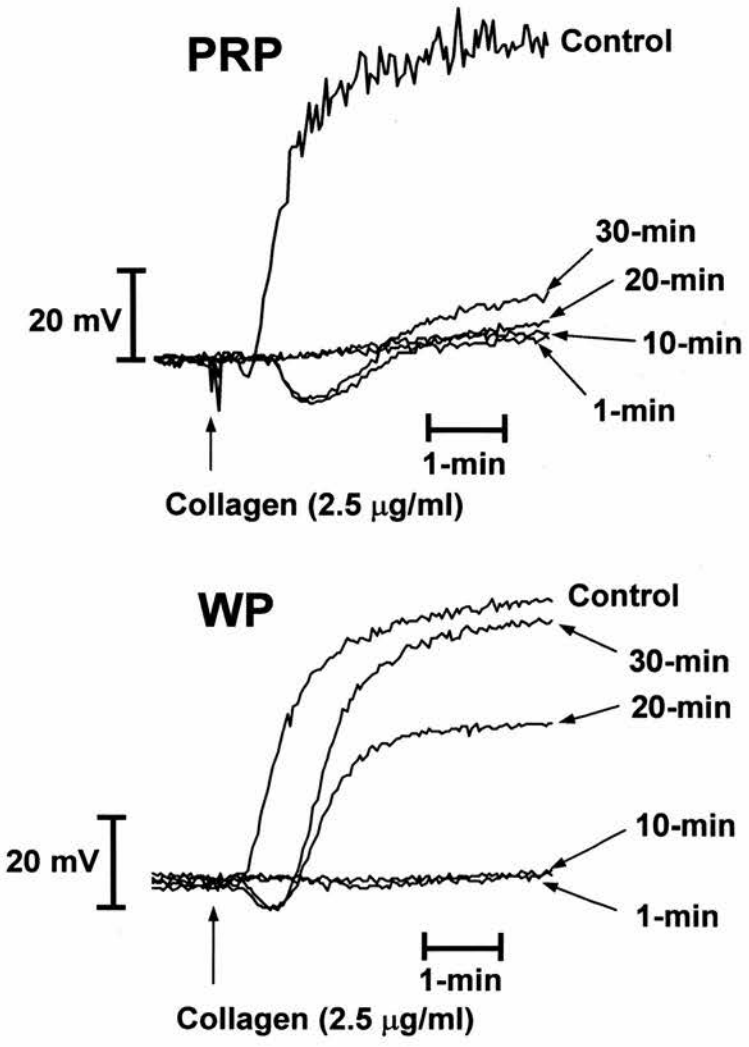


Figure 5.2.b **Representative traces showing DEA/NO (2 μM)-mediated inhibition in PRP and WP.**

5.3.4 EFFECT OF THIOLS ON DEA/NO-MEDIATED INHIBITION OF PLATELET AGGREGATION IN WP

Reconstitution of WP with the LMW thiols GSH (5 μM), cys-gly (10 μM), and cys (10 μM) did not alter the inhibition of platelet aggregation by DEA/NO after 30 min (fig 5.3;

P>0.05; one-way ANOVA followed by Dunnett's *post hoc* analysis). However, reconstitution of WP with 1% HSA resulted in a modest restoration of the inhibitory effect of DEA/NO after 30 min (fig. 5.3; P<0.01; one-way ANOVA followed by Dunnett's *post hoc* test). Co-incubation of WP with 1% HSA and either GSH, cys-gly or cys fully restored the inhibitory effect of DEA/NO after 30 min (fig 5.3; P<0.001; two-way ANOVA followed by Dunnett's *post hoc* analysis). In order to investigate the role of cell-free haemoglobin in the prolonged antiplatelet effect of DEA/NO, the ability of GSH ± HSA to prolong the DEA/NO-mediated antiplatelet activity in WP was assessed in the absence and presence of cell-free haemoglobin derived from donor red blood cells, at a concentration that was found to be present in PRP (0.46 μM). Inhibition of platelet aggregation by DEA/NO at 30 min in the presence of HSA and GSH was partially quenched by pre-incubation of 0.46 μM RBC-derived hemoglobin in WP (P<0.01; two-way ANOVA followed by Bonferroni post-test), although inhibition was still enhanced when compared to WP alone (fig 5.4).

5.3.5 EFFECT OF OXY-HB ON PROLONGED INHIBITION OF PLATELET AGGREGATION

To confirm that the prolonged (30 min) inhibition of platelet aggregation in PRP and WP was mediated by NO, the NO scavenger oxy-Hb (10 μM; bovine; reduced to Fe (II) by dithionite) was added to PRP and WP reconstituted with HSA alone, or with HSA and any of the LMW thiols. In each case, oxy-Hb abolished the prolonged inhibition observed (P<0.001; two-way ANOVA followed by Bonferroni post-test; fig 5.5).

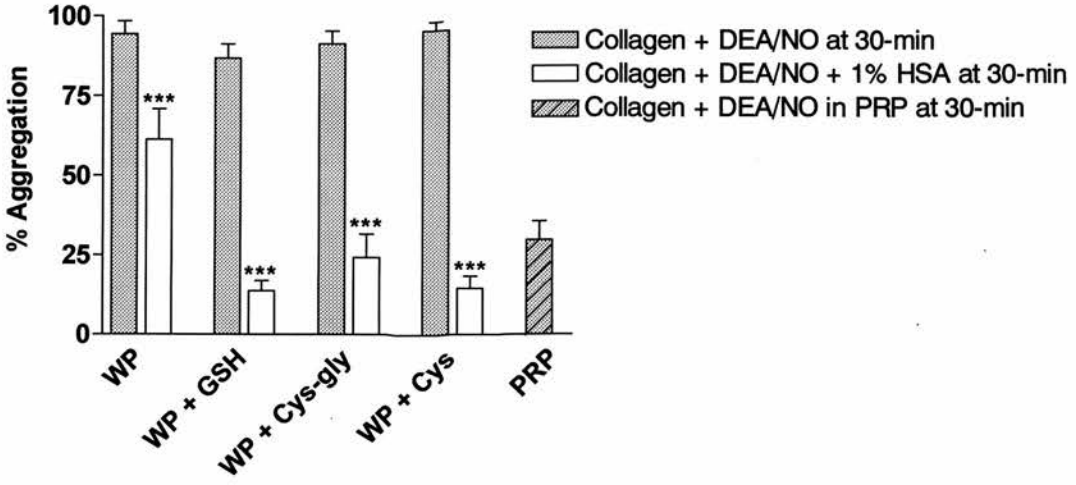


Figure 5.3 **Effect of LMW thiols and HSA on inhibition of platelet aggregation by DEA/NO. LMW thiols GSH (5 μM), cys-gly (10 μM), cys (10 μM) ± HSA (1%) were pre-incubated in WP before the addition of 2 μM DEA/NO. Platelet aggregation was then stimulated via the addition of collagen (2.5 μg/ml) 30 min later. PRP data are also added as a comparison (*** = P<0.001; n=8).**

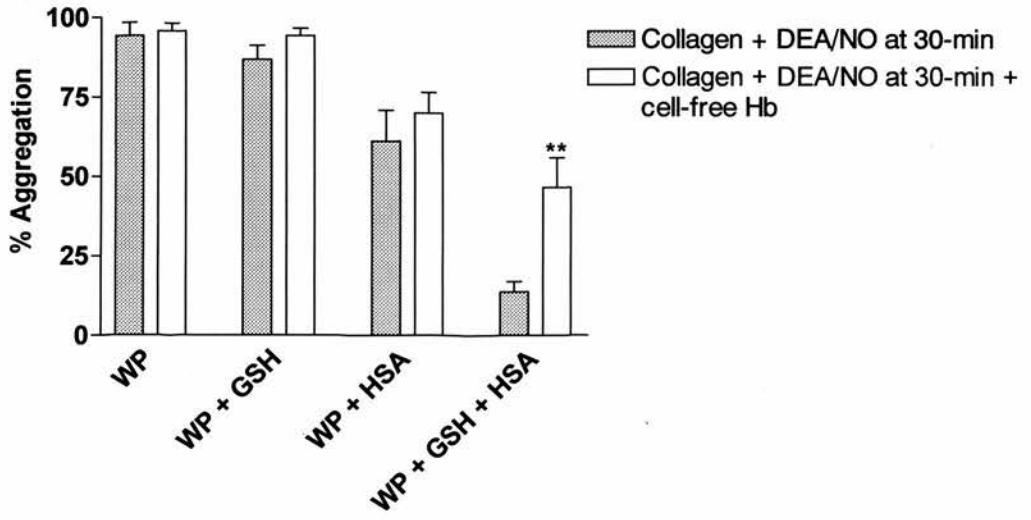


Figure 5.4 **Effect of cell-free Hb on DEA/NO mediated inhibition of platelet aggregation. WP ± RBC-derived Hb (0.46 μM) were incubated with GSH (5 μM) ± HSA (1%) before the addition of DEA/NO (2 μM). Aggregation was then induced by the addition of collagen (2.5 μg/ml) 30 min later (** = P<0.01; n=8).**

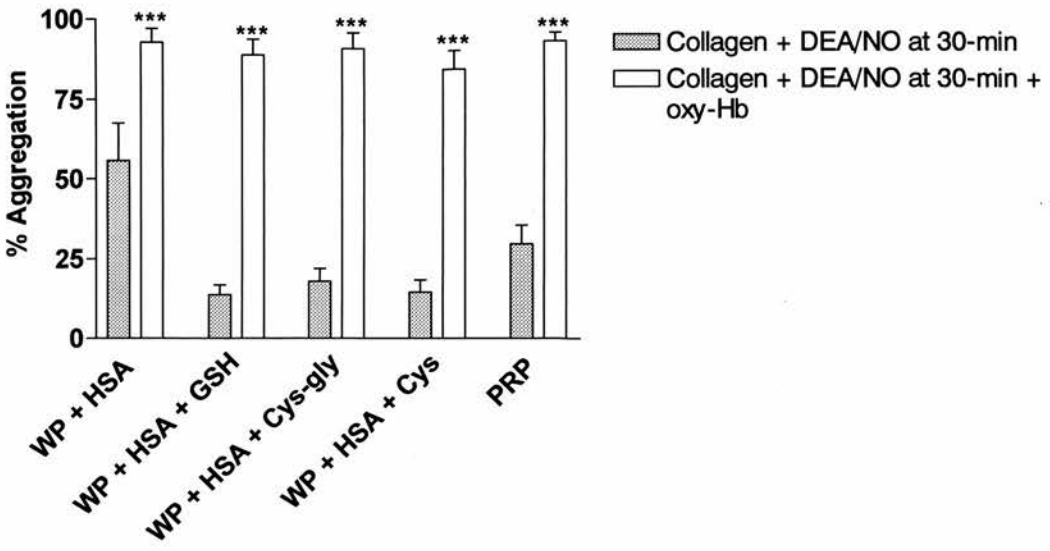


Figure 5.5 **Effect of oxy-Hb on DEA/NO mediated inhibition of platelet aggregation. WP reconstituted with HSA (1%) ± LMW thiols GSH (5 μM), cys-gly (10 μM), cys (10 μM) were pre-incubated with oxy-Hb (10 μM) before the addition of 2 μM DEA/NO. Platelet aggregation was then stimulated via the addition of collagen (2.5 μg/ml) 30 min later. The effect of Hb on PRP is also included (*** = P<0.001; n=8).**

5.3.6 THIOL MEASUREMENTS

The concentration of reduced thiol in plasma was 0.32 ± 0.01 mM. In HEPES-tyrode buffer containing1% HSA, thiol concentration was 0.11 ± 0.01 mM and did not differ significantly from 1% HSA containing GSH (5 μM; 0.12 ±0.01 mM), cys-gly (10 μM; 0.10 ± 0.01 mM) or cys (10 μM; 0.11 ± 0.01 mM).

5.3.7 S-NITROSOTHIOL DETECTION

Incubation of DEA/NO in PRP caused a rapid increase in S-nitrosothiol production which reached a maximum of 73.5 ± 15.4 nM after 10 min and diminished gradually

over the 30 min incubation period (fig. 5.6). Addition of DEA/NO to WP + 1% HSA resulted in a slower increase in S-nitrosothiol concentration, which reached a level close to that observed in PRP after 30 min (46.0 ± 8.8 nM). The presence of 5 μM GSH increased the formation of S-nitrosothiol approximately two-fold after a 30 min incubation of DEA/NO (104.5 ± 18.7 nM). There was no significant difference in S-nitrosothiol formation in WP + 1 % HSA compared to 1 % HSA alone (P>0.05; two-way ANOVA followed by Bonferroni post-test).

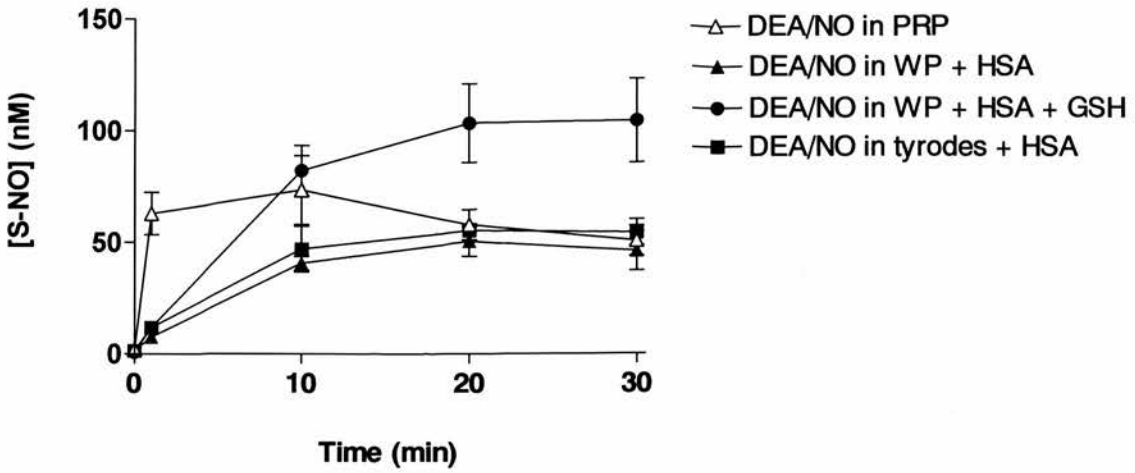


Figure 5.6 **S-nitrosothiol formation in PRP, 1% HSA HEPES-tyrode and reconstituted WP after treatment with DEA/NO. DEA/NO (2 μM) was added before the addition of NEM/ EDTA 1-30 min later to stop the reaction. Samples were then centrifuged, and the supernatant treated with acidified sulfanilamide (2.5% in 0.1M HCl) before S-nitrosothiol detection (n=6).**

5.4 DISCUSSION

These results clearly demonstrate that the biological activity of DEA/NO, a short acting NO-donor drug with a half-life of ~2 min at physiological temperature and pH, is significantly prolonged in platelet rich plasma (PRP) compared to washed platelets (WP), where activity closely mirrored NO concentration. Importantly, the prolonged inhibition of aggregation observed in PRP is mediated by NO, despite the clear decay of DEA/NO-derived NO to undetectable levels within the 30 min incubation period. Reconstitution of WP with human serum albumin (HSA) caused a partial restoration of DEA/NO-mediated inhibition after 30 min but, when combined with the low molecular weight (LMW) thiols GSH, cys-gly, or cys, the inhibitory action was fully restored to that seen in PRP, despite no tangible increase in total thiol content. Furthermore, the degree of inhibition of aggregation was associated with S-nitrosothiol formation in PRP and reconstituted platelets, indicating a crucial role for both protein and LMW thiols in prolonging the biological availability of NO.

5.4.1 GENERATION OF NO BY DEA/NO

NO was clearly detected in both WP and PRP treated with DEA/NO, a compound known to generate a maximum of two molar equivalents of NO upon hydrolysis (chapter 1.3.9.5). Importantly, while there was a clear divergence in the concentration of NO detected in PRP and WP, DEA/NO-derived NO declined to undetectable levels after a 20 min incubation period in both PRP and WP. As observed in chapter 3, there was a

delay in the appearance of NO in PRP after bolus injection of NO, indicating that plasma has some NO-scavenging ability. Analysis of Hb concentration revealed that PRP contained 0.46 μM Hb, with a potential capacity to scavenge ~1.5-2 μM NO, assuming all 4 heme groups are available for reaction with NO. Given that the delay in appearance of extracellular NO in PRP is approximately 2 min, during which time ~2 μM NO is released, these data indicate that Hb-mediated scavenging is responsible for the discrepancy between extracellular NO in PRP and WP. The concentration of Hb in PRP equated with that in PPP, indicating that the vast majority of Hb was cell-free. Although blood sampling and platelet isolation is likely to cause significant haemolysis *ex vivo*, cell-free Hb is known to limit the availability of NO *in vivo* (Reiter *et al.*, 2002).

5.4.2 PROLONGATION OF NO-MEDIATED INHIBITION BY PRP AND THIOLS

The ability of plasma components to prolong the antiplatelet effects of bolus DEA/NO is profound. While inhibition of platelet aggregation was sustained in PRP, substitution of plasma with HEPES-tyrode buffer resulted in a marked reduction in the duration of the inhibitory effect. Predictably, there was a close correlation between extracellular NO concentration and inhibition of platelet aggregation in WP, indicating that the degree of inhibition is closely defined by the extracellular NO concentration. In PRP, however, inhibition of aggregation was maintained, despite the progressive loss of extracellular NO from the system. The NO scavenger, oxy-Hb, abolished the sustained inhibitory effect in PRP, confirming that the effect was entirely NO-mediated. Given that human

plasma is an abundant source of reduced thiol (Jocelyn, 1972), and that the concentration of S-nitrosothiols in human plasma is relatively high (30-120 nM; Marley *et al.*, 2000; Tsikas *et al.*, 1999b; Tsikas *et al.*, 1999c), we hypothesised that thiols may have a role in the prolongation of NO bioactivity observed here. In human plasma, the single free cysteine residue present on serum albumin (cys 34) accounts for the majority of reduced thiol. However, LMW thiols are present in human plasma in the low micromolar range (Mansoor *et al.*, 1992), and S-nitrosothiols have previously been shown to undergo thiol-nitrosothiol exchange *in vivo* (Scharfstein *et al.*, 1994). Therefore, WP was reconstituted with albumin and LMW thiols to dissect thiol function on the antiplatelet activity of NO. These results clearly indicate that incubation of the LMW thiols GSH, cys-gly, and cys did not alter the duration of antiplatelet action of DEA/NO, but reconstitution with 1% HSA significantly prolonged inhibition of aggregation. Crucially, while DEA/NO-mediated aggregation was only partially restored with HSA, co-incubation of HSA with each of the LMW thiols completely restored the inhibitory action of DEA/NO at 30 min despite a negligible increase in the thiol pool. Furthermore, oxy-Hb completely reversed this inhibition, indicating that the LMW thiol/HSA effect is entirely NO-mediated.

5.4.3 LMW THIOLS & S-NITROSOTHIOL FORMATION

The correlation observed between S-nitrosothiol formation and inhibition of platelet aggregation strongly indicates that the role of thiols in prolongation of NO-mediated inhibition of platelet aggregation is through provision of a substrate for S-nitrosation.

Interestingly, our results indicate that there is a clear difference in the rate by which S-nitrosothiols are generated in PRP compared to thiol-reconstituted solutions. In PRP, S-nitrosothiol formation was very fast compared to that observed in reconstituted WP, with significant amounts being formed (~ 60 nM) after 1 min incubation with DEA/NO. Conversely, 1 min incubation of DEA/NO in HSA-reconstituted WP resulted in very low level S-nitrosothiol formation (<10 nM), which gradually increased to a maximum concentration of 50.5 ± 6.7 nM after 20-30 min. Despite rather different kinetics of formation of S-nitrosothiols in PRP and HSA-reconstituted WP, by 30 min, total S-nitrosothiol concentration is the same (~ 50 nM). However, inhibition of platelet aggregation is markedly different in PRP and HSA reconstituted WP after a 30 min incubation of DEA/NO. Previous data indicating that LMW thiols such as GSH can increase the antiplatelet action of S-nitrosoalbumin (Simon *et al.*, 1993) is supported by our results. These results suggest that S-nitrosoalbumin formed in reconstituted WP is an inefficient NO donor, and requires the presence of low molecular weight thiols such as those found in PRP to efficiently control physiological function, as has previously been proposed (Scharfstein *et al.*, 1994; Simon *et al.*, 1993; Stamler *et al.*, 1992a; Stamler *et al.*, 1992d). However, data obtained here emphasise an additional role for GSH and other LMW thiols in the formation of S-nitrosothiols. Co-incubation of GSH with HSA-reconstituted WP resulted in an increase in S-nitrosothiol concentration by approximately two fold. Furthermore, this increase was accompanied by a large augmentation of DEA/NO-mediated inhibition of platelet aggregation.

5.4.4 MECHANISMS LEADING TO S-NITROSOTHIOL FORMATION

The mechanism for formation of S-nitrosothiols *in vivo* is a source of considerable debate; NO itself is a weak nitrosating agent, but higher oxides of NO such as N_2O_3 are potent nitrosating species (chapter 1.3.6.4; Espey *et al.*, 2001). The rate limiting step in the formation of N_2O_3 is the reaction of NO with molecular oxygen, which is pseudo second order ($k \sim 4 \times 10^6\ M^{-2}sec^{-1}$; Lewis & Deen, 1994; Wink *et al.*, 1994). Although originally thought too slow to account for endogenous levels of S-nitrosothiols, the reaction between NO and O_2 can be catalysed by caeruloplasmin (Inoue *et al.*, 1999), a Cu-containing protein abundant in plasma. Moreover, accelerated formation of S-nitrosothiols has been observed in the presence of biological membranes (Espey *et al.*, 2001; Liu *et al.*, 1998a) and in the hydrophobic core of proteins such as albumin (Rafikova *et al.*, 2002), which act as 'NO sinks' to concentrate nitrosating species. Whilst recognising that the pharmacological levels of NO used here are sufficiently high to facilitate significant formation of N_2O_3 that might subsequently nitrosate thiols, the results presented here with GSH and HSA confirm previous findings that the ability of albumin to catalyse S-nitrosothiol formation is greatly increased in the presence of low molecular weight thiols (Rafikova *et al.*, 2002). A modest increase (~x1.05) of thiol pool through addition of GSH to HSA-treated WP failed to significantly affect total thiol concentration, while causing a disproportionate increase in S-nitrosothiol formation (approximately two-fold). Our data demonstrate that the presence of platelets did not significantly alter S-nitrosothiol production, suggesting that plasma membrane mediated

acceleration does not play a part in this system. Given that we observed more rapid production of S-nitrosothiols in plasma than in reconstituted WP, these data suggest that S-nitrosothiol formation catalysed by plasma components like ceruloplasmin may be a key factor in the difference observed. Alternatively, the full complement of thiols in plasma may be required to provide an efficient pathway for the incorporation of NO into S-nitrosothiols. These results indicate that cell-free Hb at plasma concentrations has a net scavenging effect, implying that cell-free Hb functions to remove NO rather than to conserve NO bioactivity through the formation of additional S-nitrosated species. However, many pathways for S-nitrosothiol formation exist (Gow *et al.*, 1997; Mayer *et al.*, 1998), and may play a significant role in this system.

5.4.5 PATHOPHYSIOLOGICAL IMPLICATIONS

It is noteworthy that low serum GSH levels are an independent predictor of coronary heart disease (Morrison *et al.*, 1999) and that thiol supplementation in humans has been shown to cause an increase in both endothelium-dependent and independent relaxation (Creager *et al.*, 1997; Kugiyama *et al.*, 1998; Vita *et al.*, 1998), especially in subjects at risk of coronary artery disease (Kugiyama *et al.*, 1998). Furthermore, a number of potential mechanisms for the cardioprotective role of thiols have been identified, including scavenging of oxygen-derived free radical species (Vita *et al.*, 1998), and direct stimulation of NO synthase itself (Hofmann & Schmidt, 1995). These results imply that the bioavailability of LMW thiols may have a significant impact in the ability of plasma to form S-nitrosothiols and, therefore, prolong the antiplatelet action of

endothelium-derived NO (fig 5.7). Moreover, in light of evidence that S-nitrosoglutathione is relatively platelet selective (de Belder *et al.*, 1994), these data suggest that the ability of GSH and other LMW thiols to assist in S-nitrosothiol formation and delivery may be of crucial importance in the maintenance of haemostasis and might be compromised in coronary artery disease.

Data presented here also have important implications with respect to the potential for NO donor-mediated antithrombotic therapy. Formation of a durable plasma reservoir of NO that is slowly liberated through the action of LMW thiols suggests that prolonged antiplatelet activity might be afforded by delivery of short acting NO donor drugs that were previously considered too labile for this purpose.

5.4.6 SUMMARY

In summary, results presented here support the hypothesis that plasma thiols may play an important role to prolong the antiplatelet activity of NO. In particular, LMW thiol-mediated acceleration of S-nitrosoalbumin formation and delivery at the platelet surface may represent an efficient system to prolong NO bioactivity, which may have important consequences for NO derived from physiological and pharmacological means.

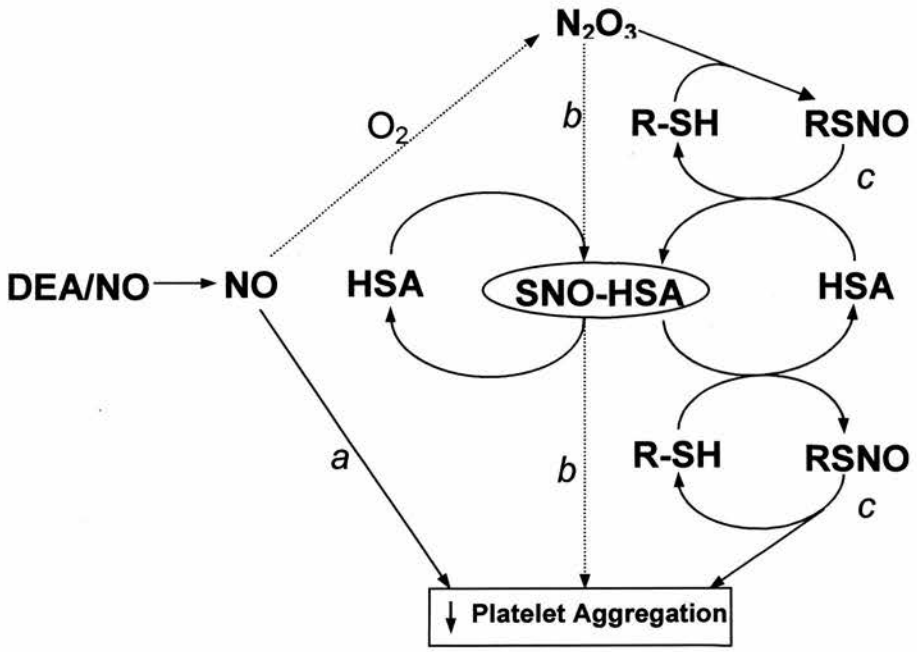


Figure 5.7 **Summary of proposed mechanism for the prolonged DEA/NO-mediated inhibition of aggregation observed in PRP and reconstituted WP. DEA/NO hydrolyses in aqueous solution to generate NO. NO diffuses into the platelet where it activates various cellular processes leading to inhibition of platelet aggregation (path *a*). Alternatively, DEA/NO-derived NO reacts with molecular oxygen to form nitrosating species such as N_2O_3, which react with the sulphydryl group on HSA to form relatively stable SNO-HSA. SNO-HSA inhibits aggregation via generation of NO at the platelet membrane surface (path *b*). In the presence of LMW thiols, N_2O_3 preferentially reacts with LMW thiols to form LMW S-nitrosothiols (RSNO). LMW S-nitrosothiols transnitrosate with HSA to form the S-nitrosoalbumin reservoir. Bioactive NO can be delivered to the platelet via a reverse of the previous process, leading to prolonged inhibition of aggregation (path *c*).**

CHAPTER SIX

THERAPEUTIC POTENTIAL OF NOVEL NITRIC OXIDE DONOR MATERIALS

6.1 INTRODUCTION

Bypass grafts are commonly used to improve blood flow in patients with peripheral ischaemia, with autologous vein the primary choice of graft (Bergan *et al.*, 1982; Londrey *et al.*, 1991; Van de Pavoordt *et al.*, 1986). However, a lack of vein availability and/or suitability occurs in up to 30 % of cases (Sayers *et al.*, 1998), requiring the need for prosthetic grafts such as those derived from Dacron or expanded polytetrafluoroethylene (ePTFE). One reason for the decreased patency of these prosthetic grafts compared to autologous vein is probably due to increased thrombogenicity of these artificial surfaces (Goldman *et al.*, 1982a; Goldman *et al.*, 1982b). Indeed, the role of platelet deposition in prosthetic graft failure is highlighted by the observation that antiplatelet agents such as aspirin (ASA) can increase graft patency (Adam *et al.*, 2001; Dorffler-Melly *et al.*, 2003a; Dorffler-Melly *et al.*, 2003b). However, ASA therapy has some disadvantages, most notably gastric toxicity (Derry & Loke, 2000) and the development of ASA resistance (Gum *et al.*, 2001), which may be associated with major adverse events in the long-term (Gum *et al.*, 2003). An alternative to aspirin therapy is to decrease the thrombogenicity of the graft surface itself. Given the potent ability of NO to prevent platelet adhesion (Radomski *et al.*, 1987c; Radomski *et al.*, 1987d), coating of surfaces with drugs with NO-donating capacity is a possible therapeutic strategy to reduce platelet deposition (Batchelor *et al.*, 2003; Fleser *et al.*, 2004; Maalej *et al.*, 1999; Mowery *et al.*, 2000; Parzuchowski *et al.*, 2002; Thierry *et al.*, 2003; Yoon *et al.*, 2002; Zhang *et al.*, 2002). Despite the fact that these surfaces are likely to be limited by the finite supply of NO that they are able to deliver, they

nevertheless merit investigation as tools to prevent platelet adhesion, at least in the short term.

S-nitroso-N-valeryl-D,L-penicillamine (SNVP) is an S-nitrosothiol that has previously been shown to cause prolonged and selective antiplatelet effects in regions of vascular injury (Miller *et al.*, 2003), a property that may be explained by the relatively high lipophilicity and stability of the S-nitrosothiol (Megson *et al.*, 1999). These intriguing properties may also make SNVP a useful NO donor for the coating of graft or extracorporeal circuits such as those used in dialysis or cardiopulmonary bypass. An alternative approach is the generation of materials with an inbuilt ability to donate NO. In a collaboration with Professor Russell Morris and Dr Paul Wheatley at the University of St. Andrews, ion-exchanged NO-loaded zeolite/PTFE discs were generated as described in methods chapter 2.9. Of the zeolite-A/PTFE discs (fig 6.1), Co^{2+}-exchanged discs were found to contain the highest capacity for NO when placed into a water saturated atmosphere as measured using an isolated NO-electrode (fig 6.2). Moreover, these discs were observed to be stable in a dry atmosphere.

The aim of this series of experiments was to examine a possible therapeutic role for novel NO-donors to reduce platelet activation and adhesion to artificial surfaces. In order to investigate the potential therapeutic application of these compounds, the antiplatelet ability of these materials were tested in a series of *ex vivo* experiments.

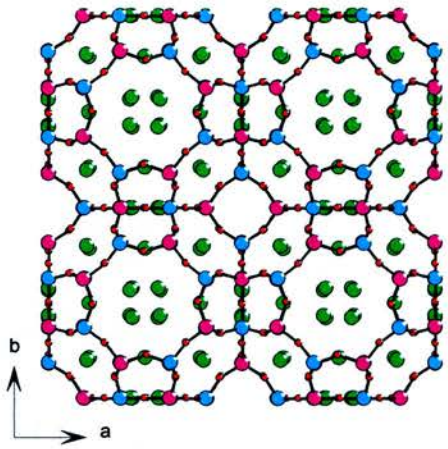


Figure 6.1 **Crystal structure of dehydrated Na-zeolite-A. The structure consists of alternating Si (blue) and Al (purple) centred tetrahedra with sodium cations (green) bound to the oxygen atoms (red) of the framework. The sodium cations can be readily exchanged with transition metal ions (e.g. Co^{2+}). For clarity, only the Al-O and Si-O bonds are drawn. Figure obtained from Professor Russell Morris, original data from Zhang *et al.*, 2003.**

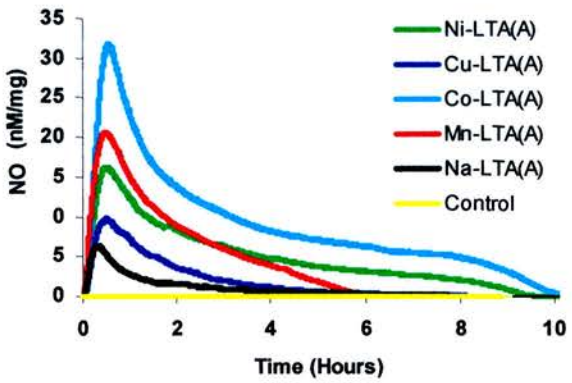


Figure 6.2 **Generation of NO by ion-exchanged zeolite-A (LTA) in water saturated vapour as measured using an isolated NO-electrode. The control is a Co^{2+}-exchanged zeolite that has not been exposed to NO, and results are standardised per mg of zeolite. Experiments were performed by Dr P Wheatley.**

Initially, experiments were conducted to test the hypothesis that pre-treatment of ePTFE graft with SNVP reduced adhesion of human platelets to the surface following drug washout. The effect of SNVP was compared to the well established NO donor sodium

nitroprusside (SNP; chapter 1.3.9.3) and also tested against the treatment of platelets with a maximal dose of aspirin. Secondly, experiments were performed to examine the antiplatelet effects of NO-loaded zeolites following the stimulation of platelet activation with a maximal concentration of the TxA_2 analogue U46619.

6.2 METHODS

6.2.1 MEASUREMENT OF PLATELET ADHESION TO PROSTHETIC GRAFT

Calcein-labelled platelets were prepared as described in the methods section (chapter 2.1.2) with platelet-calcein loading calibrated on a daily basis (chapter 2.4.2). To examine the ability of SNVP and SNP to reduce platelet adhesion to prosthetic graft, ePTFE graft (length: 3 cm; diameter 6 mm) was bathed in either HEPES-tyrode (control), SNVP (1 mM) or SNP (1 mM) for 30-min prior to mounting on the perfusion circuit maintained at 37°C (chapter 2.4.2). The effect of incubation of ePTFE with valeryl-penicillamine (VP), the NO-free parent compound of SNVP, was also tested. Following an initial perfusion with HEPES-tyrode (15 ml), calcein-labelled platelets (10 ml) were perfused (100 ml/hr) through the circuit before a final HEPES-tyrode washout (15 ml) to remove non-adhering platelets. Adhered platelets were removed and lysed by Triton-X-100 treatment (1% final concentration) and quantified by fluorimetry as described earlier (chapter 2.4.2). In further experiments, calcein-labelled platelets were treated with ASA (100 μM) for 1 hr prior to perfusion through the ePTFE graft following HEPES-tyrode washout. Platelet adhesion was then measured as before (chapter 2.4.2; n=5 for all experiments).

6.2.2 AGGREGOMETRY

To assess the function of calcein-labelled WP compared to normal WP, platelets were stimulated with a peri-maximal concentration of collagen (2.5 μg/ml) and aggregation measured for 5-min as described in the methods section (chapter 2.3; n=4). The effect of ASA (100 μM incubated for 1 hr) on collagen-induced platelet aggregation was also investigated (n=4). To assess the anti-aggregatory activity of zeolite-A, NO-loaded Co^{2+}-exchanged zeolite/PTFE discs were generated at the University of St. Andrews as described in the methods section (chapter 2.9). Discs were then suspended in platelet rich plasma (PRP; chapter 2.1.1) in the aggregometer in a stainless steel wire holder, with care taken to avoid interference of the light beam. Following a 1-min incubation of NO-loaded zeolite/PTFE discs, peri-maximal U46619 (8 μM) was added to activate platelets. Zeolite/PTFE discs not exposed to NO were used as a control alongside agonist-only treated PRP. The effect of the NO scavenger oxy-Hb (40 μM), incubated in PRP for 1-min prior to the zeolite/PTFE discs, was also examined to assess the involvement of NO from NO-loaded zeolite/PTFE discs on platelet function (n=5).

6.2.3 ELECTRON MICROSCOPY

Electron microscopy (EM) was performed to examine the ability of NO-loaded zeolite/PTFE discs to reduce platelet adhesion against control zeolite/PTFE discs. Control or NO-loaded zeolite/PTFE discs were incubated with PRP in the aggregometer for 1-min and 8 μM U46619 added. Discs were then removed and gently rinsed in PBS, prior to fixation and visualisation by electron microscopy (chapter 2.8; n=2).

6.3 RESULTS

6.3.1 EFFECT OF SNVP, SNP & ASPIRIN ON PLATELET ADHESION TO PROSTHETIC GRAFT

Pre-incubation of ePTFE graft with SNVP significantly reduced platelet adhesion to the graft compared to untreated ePTFE ($P<0.05$; Student's paired t-test; table 6.1). However, pre-treatment of ePTFE graft with either SNP or VP, or treatment of platelets with ASA was without effect ($P>0.05$; Student's paired t-test; table 6.1).

Treatment	Platelet adhesion (10^4 platelets / cm^2)	
	Untreated	Treated
SNP (graft)	20.9 ± 1.4	23.6 ± 3.2
VP (graft)	22.7 ± 4.2	22.8 ± 4.7
SNVP (graft)	22.7 ± 1.7	10.8 ± 1.3*
ASA (platelets)	19.7 ± 4.2	18.8 ± 5.0

Table 6.1 **Effect of SNP, SNVP, VP and aspirin on the adhesion of platelets to prosthetic graft. ePTFE was bathed in SNP, SNVP or VP for 30-min prior to mounting on a circuit and perfusion of platelets as described in the methods section. ePTFE bathed in HEPES-tyrode served as an untreated control. Platelets were also incubated with ASA (100 μM for 1 hr) prior to perfusion through HEPES-tyrode-bathed graft (* = $P<0.05$; n=5).**

6.3.2 EFFECT OF CALCEIN LOADING & ASPIRIN ON COLLAGEN-INDUCED PLATELET AGGREGATION

Stimulation of calcein-labelled WP with peri-maximal collagen resulted in a maximal aggregation response that was 96.9 ± 2.7 % of that observed in normal WP. Incubation

of calcein-labelled WP with ASA abolished collagen-induced platelet aggregation in these platelets (fig 6.3; $P < 0.001$; Student's paired t-test).

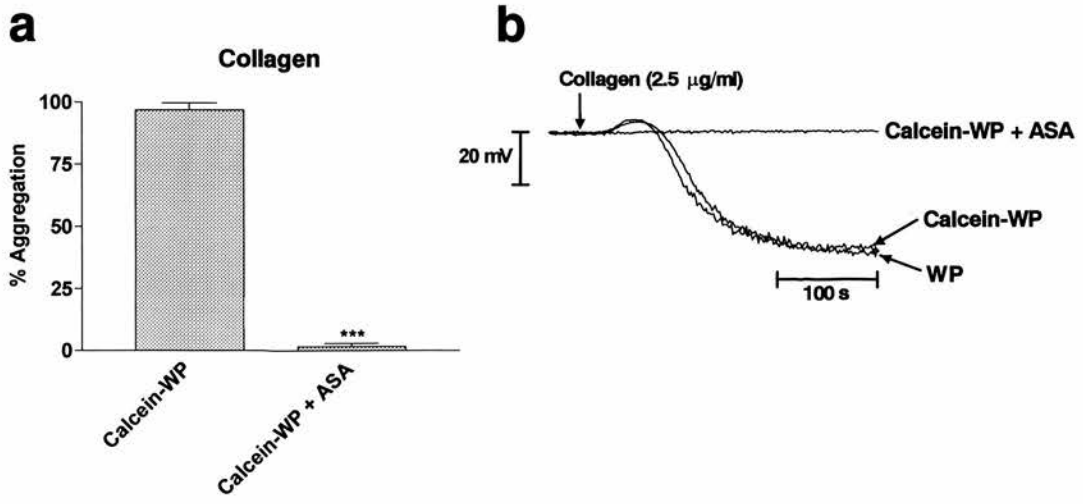


Figure 6.3 **Effect of calcein loading and aspirin on collagen-induced platelet aggregation. Platelets were equilibrated in the aggregometer before the addition of collagen and the response recorded for 5-min. Where used, ASA (100 μM) was incubated with platelets for 1 hr prior to the addition of collagen. Summary data (a) and a representative trace (b) are included (*** = P<0.001; n=4).**

6.3.3 EFFECT OF NO-LOADED ZEOLITE/PTFE DISCS ON PLATELET AGGREGATION

Incubation of NO-loaded zeolite/PTFE discs in PRP significantly inhibited U46619-induced platelet aggregation (fig 6.4; $P<0.01$; non parametric Kruskal-Wallis test followed by Dunn's multiple comparison for samples with different variances), while no significant inhibitory effect was observed when Hb was co-incubated with the NO-loaded zeolite/PTFE discs ($P>0.05$; Kruskal-Wallis test followed by Dunn's multiple comparison).

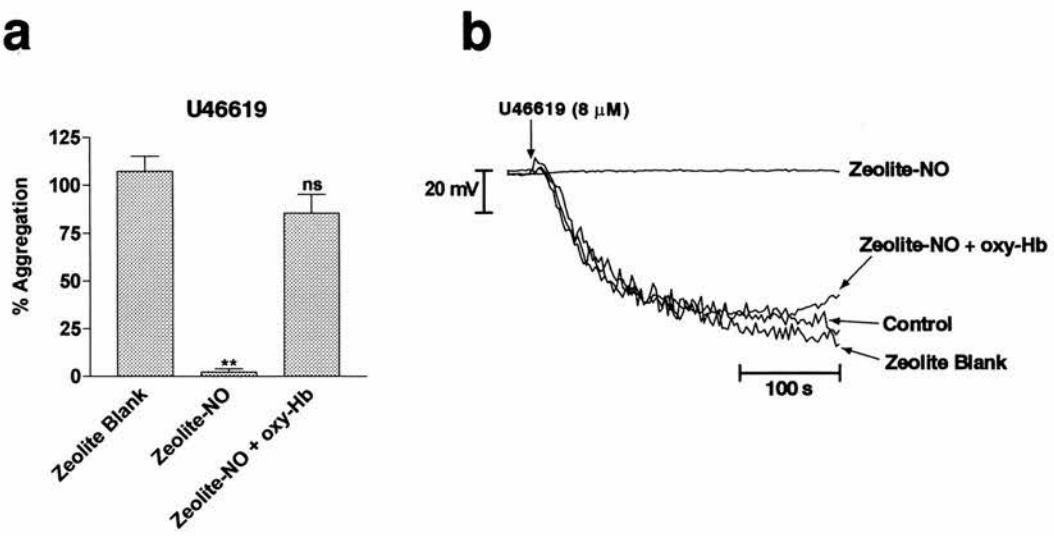


Figure 6.4 **Effect of NO exposure and Hb on the inhibition of U46619-induced platelet aggregation by zeolite/PTFE discs. Control and NO-exposed discs were incubated in PRP for 1-min prior to the addition of U46619. Where used, Hb (40 μM) was added to PRP 1-min before the addition of zeolite/PTFE disc, followed by the addition of U46619 1-min later. Summary data (a) and a representative trace (b) are included (** = P<0.01; n=5).**

6.3.4 VISUALISATION OF PLATELET ADHESION TO ZEOLITE/PTFE DISCS BY ELECTRON MICROSCOPY

Representative micrographs showing platelet adhesion to control (A) and NO-exposed (B) zeolite/PTFE discs are included (fig 6.5).

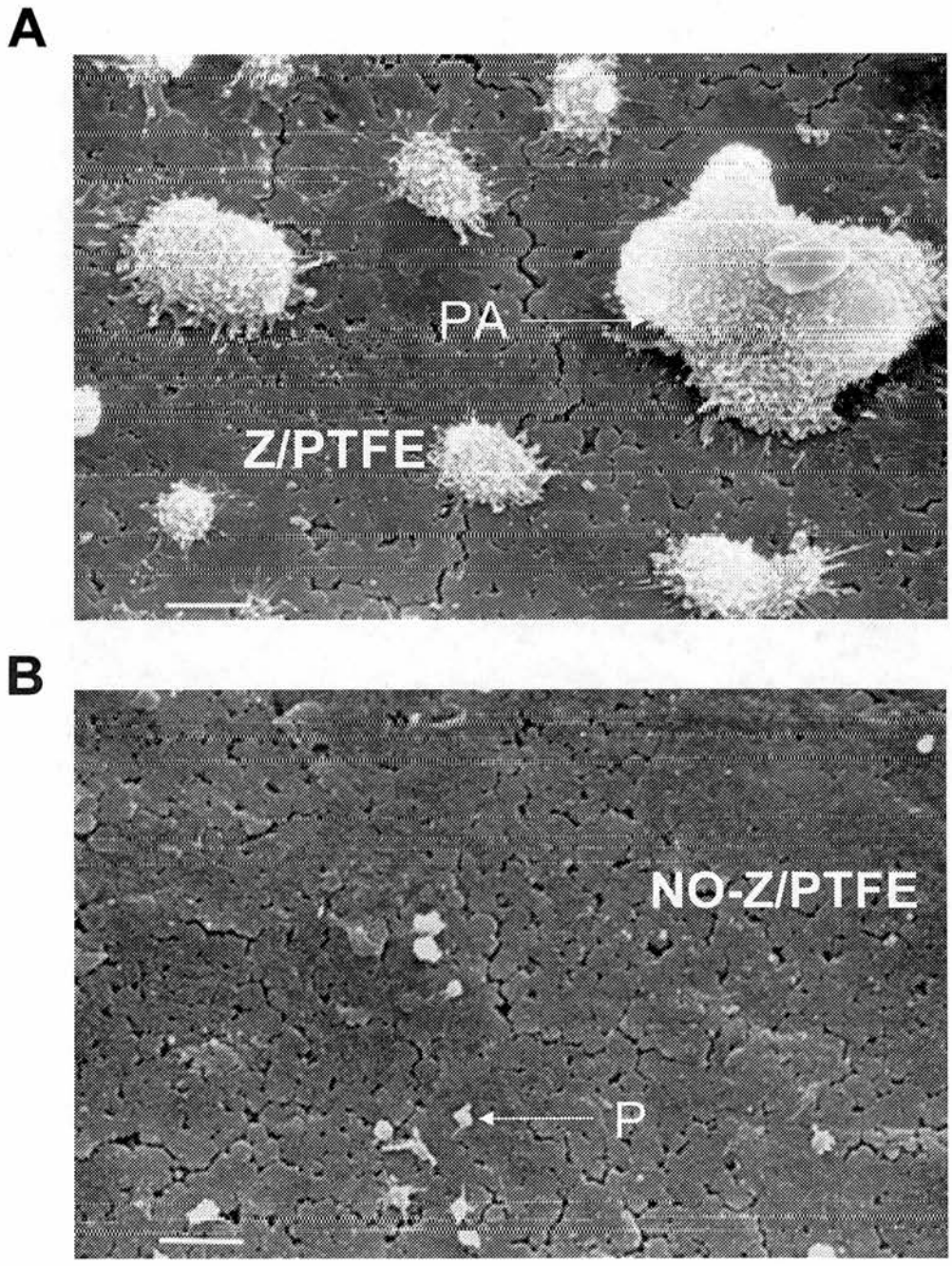


Figure 6.5 **Representative micrographs showing the effect of NO exposure on adhesion of platelets to zeolite/PTFE discs. Discs were pre-incubated with PRP for 1-min prior to the addition of U46619. The micrographs show platelet aggregates (PA) adhering to a control zeolite/PTFE disc (Z-PTFE; A) and of a platelet (P) adhering to an NO-loaded zeolite/PTFE disc (NO-Z/PTFE). The white bar represents 10 μm (n=2).**

6.4 DISCUSSION

These results show that in an *in vitro* perfusion system, platelet adhesion to ePTFE graft can be inhibited by incubating the ePTFE graft in SNVP, but not the well-established NO donor, SNP. Incubation of ePTFE graft with VP, the NO-free parent compound of SNVP, did not affect platelet adhesion to ePTFE graft, identifying a critical role for NO in the inhibition of platelet adhesion by SNVP. Moreover, incubation of platelets with ASA was also without effect on platelet adhesion to ePTFE graft, despite a clear ability of ASA to prevent collagen-induced platelet activation. Results obtained in these experiments also indicate a role for NO-loaded zeolite/PTFE materials as high capacity NO-stores that can prevent platelet aggregation following maximum stimulation of activation. Furthermore, electron microscopy revealed that exposure of zeolite/PTFE discs to NO reduced platelet adhesion to the discs.

6.4.1 CALCEIN-LABELLING AS A MARKER FOR PLATELET ADHESION

Preliminary studies confirmed calcein labelling as a reproducible technique to measure platelet adhesion, as observed in other studies (Alfon *et al.*, 2001; Bombeli *et al.*, 1998). The limit of detection was < 10,000 platelets (equivalent to 25-50 nl PRP), verifying high sensitivity of the technique. Calcein-labelling of platelets in this system has considerable advantages over other labelling techniques employed. These include labelling platelets with europium trichloride (Toes *et al.*, 1999), which may result in

platelet activation due to the hypotonic shock required to induce cellular uptake of europium, or radioactive-labelling with indium^{111} (Dewanjee *et al.*, 1981) or chromium^{51} (Ito *et al.*, 1989), which require large volumes of platelets and/or special handling due to the radioactivity involved. Data presented here indicate that the addition of collagen at a concentration that causes maximal aggregation in WP also elicited a maximal aggregatory response in calcein-labelled WP, indicating that calcein-labelled platelets are both functional and responsive to physiological stimuli.

6.4.2 SNVP-MEDIATED INHIBITION OF PLATELET ADHESION TO PTFE

Data presented here clearly indicate that the incubation of ePTFE graft in a solution (1 mM) of SNVP reduces platelet adhesion. This effect was found to persist despite an initial 15 ml (~ 10 min) washout period with HEPES-tyrode prior to the perfusion of platelets. Importantly, incubation of ePTFE graft with SNP was without effect on platelet deposition, indicating that the ability of SNVP to reduce platelet adhesion to ePTFE following washout is not a property shared by all NO donor drugs. SNP is a potent inhibitor of platelet activation in WP (Doni *et al.*, 1991) and has been shown to inhibit platelet aggregation via cGMP-dependent mechanisms (Sogo *et al.*, 2000b). In these experiments, SNP clearly does not persist on ePTFE graft following the washout protocol, rendering it incapable of preventing platelet adhesion to ePTFE. Interestingly, SNP has previously been shown to prevent platelet adhesion on endovascular stents when incorporated into polyelectrolyte multilayers (Thierry *et al.*, 2003). However, the

observation here that SNVP can inhibit platelet adhesion by incubating it with ePTFE graft for a short period of time means that such technically difficult procedures may be avoided by much simpler measures.

Results presented in table 6.1 indicate that incubation of platelets with ASA did not affect platelet adhesion to ePTFE graft, despite the observation that the concentration of ASA used was clearly sufficient to prevent collagen-induced platelet aggregation (fig 6.3). Previous data demonstrate a beneficial role for ASA treatment in patients with artificial prostheses, although it may be of little use in patients with autologous vein grafts (Dorffler-Melly *et al.*, 2003b; Mahmood *et al.*, 2003; Watson *et al.*, 2000). Although ASA did not affect platelet adhesion to ePTFE in this *in vitro* perfusion system, the effect of platelet activation was not investigated in these experiments. Furthermore, collagen deposition, which occurs on transplanted prosthetic grafts *in vivo* (Seeger *et al.*, 1990; Watase *et al.*, 1992), will not occur in this system, limiting any potential benefit of ASA treatment. Indeed, it is interesting to speculate that in a system with significant platelet activation, where presumably platelet deposition and recruitment will be greatly increased, the beneficial effects of SNVP (and ASA) may be considerably more pronounced.

A further important observation here is that VP, the un-nitrosated parent compound of SNVP, failed to affect platelet adhesion to ePTFE graft, indicating that the ability of SNVP to reduce platelet deposition can be attributed to the S-NO moiety of the compound. This finding was important in order to exclude the possibility that SNVP may induce unwanted non-specific effects on platelet function. Thus, the incubation of

ePTFE graft with SNVP results in the generation of a NO-releasing surface that can reduce platelet adhesion.

6.4.3 ROLE OF LIPOPHILICITY ON THE INHIBITION OF PLATELET ADHESION TO ePTFE

SNVP and other N-substituted analogues of SNAP have been shown to induce sustained vasodilation in human veins and arteries (Sogo *et al.*, 2000a; Sogo *et al.*, 2000c) and induce a prolonged and selective inhibition of platelet adhesion at sites of vascular injury (Miller *et al.*, 2003). Interestingly, the ability of SNAP analogues to exhibit prolonged effects in areas of damaged endothelium correlates with the relative lipophilicity of the compound by the length of the sidechain (Megson *et al.*, 1999). Moreover, another recently described lipophilic S-nitrosothiol, RIG 200, has also shown to be selective for areas of endothelial damage and induces a prolonged vasodilation in human vessels that persists for > 4 hr (Sogo *et al.*, 2000a; Sogo *et al.*, 2000c). One of the reasons we used SNVP for this study was its high lipophilicity and favourable stability (Megson *et al.*, 1999). It is interesting to speculate that the covalent modification of NO donors with lipophilic sidechains may facilitate their interaction with, and adsorption upon, prosthetic grafts such as those derived from ePTFE, presumably by increasing hydrophobic interactions. It remains an interesting question whether this 5-carbon valeryl functional group confers the prolonged antiplatelet properties of SNVP on ePTFE graft.

6.4.4 INHIBITION OF PLATELET FUNCTION BY NO-LOADED ZEOLITES

Data presented here clearly indicate a role for zeolites as high-capacity NO stores that have potent antiplatelet activity. Importantly, control zeolite discs did not exhibit antiplatelet activity, while the inhibitory activity of NO-loaded discs incubated in combination with the NO scavenger oxy-Hb was all but abolished, indicating that NO was responsible for the inhibitory action. Significantly, just a small NO-loaded zeolite/PTFE disc containing ~ 15 mg zeolite was sufficient to prevent platelet activation to a maximal concentration of the TxA_2 analogue U46619. Although the ability of zeolite/PTFE discs to inhibit platelet activation induced by other agonists (e.g. collagen) was not tested, the observation that these discs inhibit platelet activation via an NO-dependent mechanism supports the hypothesis that they will prove to have platelet-inhibiting activity against a wide range of physiological agonists via the inhibition of Ca^{2+} signalling and receptor function (chapters 1.3.4-5). Moreover, the observation that NO-loading of the zeolite/PTFE disc visibly prevented the adhesion of platelets to the surface of the disc supports a potential therapeutic role for zeolites as a coating to increase the biocompatibility of artificial surfaces.

6.4.5 THERAPEUTIC POTENTIAL OF NOVEL NO DONOR MATERIALS

The high levels of platelet deposition that occurs on prosthetic grafts makes them second choice behind autologous graft (Bergan *et al.*, 1982; Goldman *et al.*, 1982a; Goldman *et*

al., 1982b; Van de Pavoordt *et al.*, 1986). Although the infusion of NO donors may also be a useful therapeutic strategy to prevent platelet activation and adhesion following transplantation of artificial devices (Kaul *et al.*, 1996), unwanted systemic effects such as global vasodilation may be difficult to avoid (Homer & Wanstall, 2003). Therefore, the transplantation of NO generating prosthetic surfaces will ensure that the NO is delivered to the precise location where it is required.

The novel NO delivery methods described here have a wide variety of potential therapeutic applications for the prevention of platelet adhesion to prosthetic materials. While data here indicate a potential role for these materials as a coating for prosthetic graft, they may also be useful as a coating for stents. Despite the fact that stents reduce restenosis and the need for repeat revascularisation following percutaneous transluminal coronary angioplasty (PTCA; Serruys *et al.*, 1994b; SOS, 2002), their use is associated with the development of subacute thrombosis (Haude *et al.*, 1993; Schatz *et al.*, 1991; Schomig *et al.*, 1994; Serruys *et al.*, 1994a). Therefore, even NO donor-releasing materials with a finite capacity to donate NO may be potentially beneficial. However, given the presumption that the NO-releasing surfaces will only be able to prevent adhesion until their NO is exhausted, the use of these NO-delivery systems may be best suited as a coating for temporary-use extracorporeal circuits such as those employed in cardiopulmonary bypass (CPB) or dialysis. In these situations, NO generation would only be required for a period of hours, rather than months or years. Patients undergoing procedures such as CPB and dialysis require systemic heparinisation (Breiterman-White, 1995; Frederiksen, 2000), although recent progress has been made on the generation of heparin-coated surfaces for use on extracorporeal devices (Lin *et al.*, 2004; Wendel *et*

al., 1999; Wendel & Ziemer, 1999). However there are notable limitations associated with heparin use. Heparin does not prevent platelet activation induced by non-physiological surfaces (Rinder *et al.*, 1991; Rinder *et al.*, 1994) but conversely contributes to platelet activation and thrombocytopenia (Mehta & Mehta, 1982; Warkentin, 2004), which plays a role in the manifestation of haemostatic abnormalities observed following procedures such as CPB. Moreover, patients that are repeatedly exposed to heparin, such as those undergoing dialysis, are at risk of heparin-induced thrombocytopaenia (HIT; Luzzatto *et al.*, 1998; Yamamoto *et al.*, 1996). HIT is an immune-mediated disorder that results in the generation of antibodies against the heparin/platelet factor 4 complex, which interact on the platelet surface resulting in activation of both platelets and the coagulation cascade, leading to a high risk (up to 75 %) of thrombosis (Warkentin, 2003; Warkentin & Greinacher, 2003; Warkentin & Kelton, 1996). The coating of extracorporeal circuits with NO donating materials may therefore have substantial advantage over heparin within this arena. Indeed, preliminary studies are positive and have indicated that NO generating materials can prevent thrombus formation on a silicone graft in baboons (Smith *et al.*, 1996a) and remove the need for heparinisation following venovenous extracorporeal circulation in rabbits (Annich *et al.*, 2000).

6.4.6 LIMITATIONS & FUTURE DIRECTIONS

Despite the usefulness of the *in vitro* perfusion system adopted here to probe a potential role for SNVP in reducing platelet adhesion to ePTFE graft, there are caveats associated

with its use. One obvious limitation of this system is the slow flow rate used (0.1 L/hr; ~ 10^{-3} m/sec), which is likely to be substantially lower than that observed in equivalent diameter vessels *in vivo* (blood flow through brachial artery of ~ 5 mm diameter is ~ 1.5 L/hr; Newcomer *et al.*, 2004). Here, a low flow rate was used because the volume of platelets from each blood donor was limited. However, one alternative to reducing the flow rate is to run platelets through a closed-loop perfusion system, in which platelets would be repeatedly recycled through the ePTFE. Indeed, it would be interesting to investigate the ability of SNVP to prevent platelet adhesion in such a system, given that such procedures appear to activate platelets (Jung *et al.*, 2001).

As mentioned above, from a therapeutic standpoint, one of the main caveats of NO-coated materials is likely to be longevity, given that these materials are able to only release a finite supply of NO. Although this may not limit the potential of these materials as coatings for extracorporeal circuits where NO generation is only required for a period of hours, it may limit their application on stents and grafts where platelet accumulation continues over longer timeframes (Goldman *et al.*, 1982a; Wakefield *et al.*, 1989). Experiments performed here did not address the longevity of action of SNVP; it would be interesting to observe the effect of increasing the washout period prior to the perfusion of platelets to examine precisely for how long SNVP treatment may offer protection. Moreover, while data illustrated in figure 6.1 indicate that NO-loaded zeolites generate NO and may therefore offer some antiplatelet effect for up to 10 hr, it is intriguing to note that these NO-loaded zeolites appear to be stable within a dry atmosphere. Thus, it may be possible to create more stable and longer lasting derivatives by limiting the access of water to these NO-loaded zeolite materials, for

example, by the coating of zeolites with a hydrophobic layer. It seems clear that more work in this field is required to optimise the NO-generating properties of NO-releasing surfaces to maximise their potential therapeutic scope.

6.4.7 SUMMARY

In summary, these data indicate that SNVP can reduce adhesion to ePTFE graft via an NO-dependent mechanism, while NO-loaded zeolites can form a high-capacity NO-store that can prevent platelet activation and adhesion when combined with PTFE. Although more work is required to fully assess their NO-generating and platelet inhibiting capacity, these approaches show good promise and may be particularly useful in a therapeutic arena as a coating for temporary-use extracorporeal circuits such as used in CPB and dialysis.

CHAPTER SEVEN

DISCUSSION & FUTURE DIRECTIONS

7.1 NO DONORS AS A THERAPEUTIC STRATEGY FOR INHIBITING PLATELET FUNCTION

Although NO donors such as glyceryl trinitrate (GTN) and sodium nitroprusside (SNP) have been in clinical use for years, their value as antiplatelet agents is limited for reasons including weak antiplatelet activity and problems with dose-titration or tolerance (Drummer *et al.*, 1991; Mangione & Glasser, 1994; Smith & Kruszyna, 1974; Zhang *et al.*, 1993). Many compounds have been developed that can increase cellular cGMP levels via NO-independent mechanisms, including direct stimulators of sGC (Stasch *et al.*, 2002; Straub *et al.*, 2002; Wu *et al.*, 1995), inhibitors of PDE enzymes (Kim, 2003; Palacios *et al.*, 1995; Rabe *et al.*, 1995) and non-hydrolysable cGMP analogues including 8-bromo cGMP (Laustiola *et al.*, 1984). In the clinical setting, PDE inhibitors have met with success, at least for the treatment of conditions out-with platelet disorders. An obvious example is the PDE V inhibitor, sildenafil (Viagra^{TM}), which has demonstrated success rates of ≥ 95% for the treatment of erectile dysfunction (Carson *et al.*, 2002; Derry *et al.*, 2002). Recently, however, with the emergence of a number of novel NO donor drugs, it has become clear that many of the effects of NO may be mediated through signalling events that are not dependent on the stimulation of sGC and production of cGMP. In particular, cGMP-independent effects appear to play a prominent role in NO-mediated inhibition of platelet aggregation (Beghetti *et al.*, 2003;

Gordge *et al.*, 1998; Sogo *et al.*, 2000b). Furthermore, PKG, the primary target for cGMP in platelets, has a stimulatory role in platelet activation (Li *et al.*, 2003). Therefore, there may be significant benefit in investigating NO-mediated, cGMP-independent signalling events within platelets, in order that the most effective NO donor therapies are developed and that novel antiplatelet targets may be identified. In addition, investigation of the antiplatelet activities of novel NO donor drugs, including exploration into the effectiveness of new applications of NO-based therapies will provide invaluable information on physiological signalling pathways and future potential clinical uses respectively. However, the use of NO donor drugs as therapeutic agents has difficulties. These include the short half-life of NO (3-10 s; Cocks *et al.*, 1985; Griffith *et al.*, 1984), difficulty in generating stable drugs with a desirable duration of action, and the possibility that cGMP-independent effects may cause unwanted or detrimental events.

7.2 cGMP-INDEPENDENT SIGNALLING IN PLATELETS

Data presented within this thesis identify a key role for NO radical in the production of cGMP-independent antiplatelet effects by NO donors (Chapters 3 & 4). Given the potent ability of species such as S-nitrosothiols and $ONOO^-$ to modify protein cysteine and tyrosine residues respectively (Sawa *et al.*, 2000; Scharfstein *et al.*, 1994; van der Vliet *et al.*, 1995), one may have previously hypothesised that the formation of such

species would be important for cGMP-independent antiplatelet effects. However, the observation that cGMP-independent antiplatelet effects correlate with NO (radical) generation raises an intriguing question as to the chemical modification responsible for these inhibitory events. It is important to note that while data here indicate that SNVP is not sufficient to induce cGMP-independent antiplatelet effects, it does not denote that the cGMP-independent activity of NO is not mediated by S-nitrosation of critical protein cysteine residues. It may be the case that S-nitrosothiols such as SNVP cannot gain access to crucial modulatory cysteine sites, whereas NO may freely diffuse and interact with these residues. Alternatively, given the observation that NO is a relatively weak nitrosating agent (chapter 1.3.6.4), other smaller S-nitrosothiols such as S-nitrosocysteine (SNOC) may be important intermediates of cGMP-independent signalling. However, the use of low molecular weight S-nitrosothiols such as SNOC was avoided in these experiments because their high instability in physiological solutions (Mathews & Kerr, 1993) is unlikely to provide clarification between NO and S-NO-mediated effects.

At a molecular level, it seems clear that NO is able to accelerate the activity of platelet SERCA, thereby reducing intracellular Ca^{2+} levels (Homer & Wanstall, 2002; Trepakova *et al.*, 1999). This is a finding supported by data presented in chapter 3. By accelerating SERCA activity and decreasing Ca^{2+} availability, NO would be expected to be able to inhibit platelet aggregation to a wide variety of activating substances via cGMP-independent mechanisms. Indeed, this may make the development of NO-independent activators of platelet SERCA an attractive therapeutic option for the treatment of thrombotic disorders, provided that platelet-specific agents can be

generated. Interestingly, very recently, NO has been shown to activate vascular smooth muscle SERCA via $ONOO^-$-dependent modification (glutathiolation) of a critical cysteine residue (cys 674; Adachi *et al.*, 2004). Data presented in this thesis clearly indicate that in platelets, NO, rather than $ONOO^-$, is responsible for cGMP-independent antiplatelet effects (chapter 3), as evidenced by the lack of effect of SIN-1 to inhibit platelet function in WP in the presence of the sGC inhibitor, ODQ (fig 3.2.c). Interestingly, Adachi and colleagues observed that low levels (10-50 μM) of $ONOO^-$ accelerated SERCA, and that superoxide scavengers decreased NO-dependent relaxation and glutathiolation of cys 674, further supporting a role for $ONOO^-$ in acceleration of SERCA. In experiments performed in this thesis, levels of $ONOO^-$ are unlikely to reach 10-50 μM, even at the highest concentrations of SIN-1 used (300 μM). However, data here clearly show that when SOD was added to WP in the presence of SIN-1, cGMP-independent inhibition of aggregation was achieved. Therefore, while $ONOO^-$ may indeed cause cGMP-independent acceleration of SERCA, it appears that NO is more potent. The reason underlying the requirement for NO in cGMP-independent antiplatelet effects, as opposed to $ONOO^-$ in vascular smooth muscle, is unclear; these tissue-dependent effects of NO and $ONOO^-$ on SERCA require further characterisation. Although it is likely that NO may exert specific effects on different SERCA isoforms, both platelets and vascular smooth muscle express SERCA 2 and SERCA 3 (Anger *et al.*, 1993; Enouf *et al.*, 1992; Khan *et al.*, 2000; Kovacs *et al.*, 1997). It may be the case that alternative cGMP-independent mechanisms exert a more dominant role than SERCA-mediated effects in cGMP-independent inhibition of platelet activation.

Nevertheless, it is interesting to note that in SERCA-1 purified from skeletal muscle, NO is significantly more effective than $ONOO^-$ in modifying cysteine residues in the enzyme, primarily resulting in the formation of disulphide and S-nitrosated cysteine residues (Viner *et al.*, 2000).

A further cGMP-independent target for NO in platelets is COX-1, as characterised in chapter 4. Similarly to data described in chapter 3, NO (radical) was required for an inhibitory effect. It is interesting that data presented in chapter 3 suggests a role for the extracellular generation of NO for cGMP-independent effects, yet both SERCA and COX-1 are intracellular proteins. Certainly, it is difficult to unambiguously rule out the possibility that it is merely a high concentration of NO that is required to evoke cGMP-independent antiplatelet effects. If this is the case, it is perhaps less important from a physiological standpoint, although such high concentrations may be required to overcome the scavenging effect of cell-free haemoglobin, meaning that cGMP-independent effects are relevant in the physiological scenario. However, as discussed in chapter 3, both COX-1 and SERCA are located in the dense tubular system (Carey *et al.*, 1982) and as such may be in close proximity to the plasma membrane via its connections with the open cannalicular system. Pathways may exist for the transfer of NO from the plasma membrane to nearby intracellular targets, as has been proposed for the transport of S-nitrosothiols across membranes (Ramachandran *et al.*, 2001; Zai *et al.*, 1999; Zhang & Hogg, 2004). Nevertheless, the possibility that NO induces cGMP-independent modification of protein(s) present on the surface of platelets, including crucial cysteine residues, still remains. In this setting, it is interesting that sulphydryl-rich proteins such as protein disulfide isomerase (PDI) are

necessary for integrin activation, and that the treatment of platelets with membrane-impermeant agents that covalently modify cysteine residues inhibit platelet adhesion and aggregation (Essex *et al.*, 2001; Lahav *et al.*, 2002; Lahav *et al.*, 2003). Indeed, GP IIb/IIIa expresses external sulphydryl residues in its activated conformation (Lahav *et al.*, 2002; Yan *et al.*, 2000; Yan & Smith, 2000); moreover a cysteine-rich region in the extracellular domain of the β (IIIa) subunit may act as a redox 'switch' for integrin activation (Yan & Smith, 2000). The interaction of NO with cysteine at such sites may be a cGMP-independent route to the inhibition of platelet activation and would be in line with data presented here that indicate a requirement for the generation of extracellular NO for cGMP-independent antiplatelet effects.

7.3 NO & S-NITROSOTHIOL SIGNALLING

Data presented in chapter 5 indicate a role for endogenous S-nitrosothiols in prolonging the biological activity of NO in platelets. Although the ability of NO to interact with protein thiols such as cys 34 on human albumin has been previously demonstrated (Simon *et al.*, 1993; Stamler *et al.*, 1992a; Stamler *et al.*, 1992c), results presented here clearly illustrate that the presence of low molecular weight thiols such as cysteine or glutathione greatly increase the efficiency of the delivery system. This finding has considerable consequences for the use of NO donors as antiplatelet agents, implying that NO from donors such as the diazeniumdiolates (chapter 1.3.9.5) may have substantially longer biological activity than predicted by kinetics of NO generation *in vitro*. Indeed, given the profound ability of plasma factors such as CP to induce the release of NO from

S-nitrosothiols, including relatively stable analogues such as SNVP (chapter 3; Megson *et al.*, 1999), it would be interesting to compare the biological half-lives of these compounds. It may be the case that longer lasting diazeniumdiolates such as DETA/NO (t ½ ~ 20 hr; Mooradian *et al.*, 1995) may prove extremely effective long-term NO donors via generation of slow NO-release coupled with low molecular weight thiol-mediated nitrosothiol formation.

A further role for low molecular weight thiol-assisted S-nitrosothiol formation to prolong NO bioactivity may be to prevent NO degradation within the vasculature. Although, as discussed earlier, the interaction of NO with haemoglobin may result in either the cessation or prolongation of NO bioactivity, other mechanisms exist that may remove NO from the vascular system. While data presented in chapter 4 indicate that NO can inhibit COX-1 activity, they also agree with reports demonstrating that NO may act as a reducing substrate for peroxidase turnover (Curtis *et al.*, 1996; O'Donnell *et al.*, 2000). The rapid consumption of NO by reducing COX-1 haem in this manner has been previously proposed to represent a significant route for the disposal of bioactive NO (O'Donnell *et al.*, 2000). Given the lack of ability of SNVP to act in the same manner as NO, these data support the hypothesis that S-nitrosothiol formation may protect NO from COX-1-mediated consumption. Although the effect of other S-nitrosothiols (e.g. SNOC) were not investigated for reasons discussed earlier, it is reasonable to assume that endogenous S-nitrosothiols, particularly bulky adducts such as S-nitrosoalbumin, are equally protected from COX-1-mediated NO consumption. Indeed, it is possible that one of the reasons why COX-1 inhibitors such as aspirin are effective in the treatment of thrombotic disorders may be that, in addition to preventing the synthesis of the pro-

thrombotic mediator TxA_2, they also prevent the consumption of bioactive NO. Platelet-derived NO plays a crucial role in preventing platelet recruitment following activation (Freedman *et al.*, 1997; Freedman *et al.*, 1999) and aspirin has been demonstrated to prevent platelet recruitment *ex vivo* (Valles *et al.*, 2002). Furthermore, platelet adhesion and activation is increased in numerous cardiovascular conditions (Furman *et al.*, 1998; Reininger *et al.*, 1999; van Zanten *et al.*, 1994) and are more likely to have active COX-1 enzyme. Indeed, increased TxA_2 synthesis is observed in both hypertension and diabetes (Guimaraes *et al.*, 1998; Jain *et al.*, 1998; Minuz *et al.*, 2002). Therefore, a positive feedback loop may be created whereby increased platelet activation and COX-1 activity results in greater consumption of NO, which then precipitates further platelet recruitment and activation. It is interesting to speculate that if such a system were evident, then NO-aspirin hybrid drugs such as NCX-4016 may provide an additional benefit over the treatment of these conditions than with aspirin alone.

7.4 NOVEL NO-GENERATING BIOMATERIALS

Results presented in chapter 6 highlight a potential role for SNVP and NO-loaded zeolites as coatings/materials to reduce platelet deposition on prosthetic conduits. The design of prosthetic surfaces such as ePTFE to act as NO-delivery systems is a useful therapeutic strategy for a variety of reasons. Benefits include the targeting of NO to the exact area where it is required and also aversion of requirement for systemic anticoagulants such as heparin, which can ultimately lead to platelet activation. Furthermore, the laminar flow of blood through vessels (Aarts *et al.*, 1988; Wootton &

Ku, 1999) will force platelets towards the graft surface, meaning that surfaces that release NO are likely to be more efficacious than NO donors that distribute evenly throughout the bloodstream.

Results presented in chapter 6 indicate that zeolites can form a high capacity store of NO (radical). Given that these zeolites will form a source of extracellular NO, data from chapter 3 would argue that their antiplatelet effect is likely to include a cGMP-independent element. Moreover, it is also likely that low molecular weight thiols may catalyse S-nitrosoalbumin formation from NO derived from these zeolites, although whether sufficient NO would be generated to generate a significant antiplatelet action out-with the proximity of the graft is perhaps unlikely. More experiments are required to examine the mechanism and duration of the inhibitory action of NO-loaded zeolites.

The use of SNVP as an NO-generating coating for ePTFE graft is an attractive therapeutic strategy because ePTFE is a material with a proven track record for use as graft material. As discussed earlier, the use of SNVP-coated artificial surfaces and/or the construction of conduits with NO-loaded zeolites described in chapter 6 are likely to be limited to short-term inhibition of platelet adhesion and activation on prosthetic grafts or extracorporeal circuits including those used in cardiopulmonary bypass (CPB) and dialysis. However, if the synthesis of longer lasting preparations is possible, then the therapeutic potential of these materials may be greatly increased. For example, NO is a potent inhibitor of intimal hyperplasia (IH; Kown *et al.*, 2001), a process driven by a lack of endothelium (LoGerfo *et al.*, 1983; Spaet *et al.*, 1975), which may begin within 24 hr following vascular injury or transplant (Lemson *et al.*, 2000). IH has been demonstrated to occur on ePTFE grafts (Florian *et al.*, 1976), resulting in the appearance

of smooth muscle cells and collagen-generating fibroblasts (Watase *et al.*, 1992). IH is recognised as the main cause of thrombotic complications in the short to mid term following vascular intervention (Clowes, 1993), and is associated with activation of inflammatory genes and platelet and leukocyte deposition (Waltham & Harris, 2004) that result in progressive vessel stenosis. NO may prevent IH by inhibiting smooth muscle cell proliferation (Garg & Hassid, 1989; Janssens *et al.*, 1998) and by enhancing programmed cell death (Best *et al.*, 1999; Taylor *et al.*, 2003) and may therefore be of use to increase graft patency. Furthermore, prosthetic surfaces such as Dacron activate monocytes (van Aalst *et al.*, 2000); NO can prevent monocyte adhesion to both endothelial and vascular smooth muscle cells (Bath *et al.*, 1991; Osanai *et al.*, 2001), adding a further potential benefit of NO-eluting materials to improve graft viability. At any rate, the synthesis of NO-generating preparations that release NO over a period of weeks or months may prove beneficial, given evidence that ePTFE prostheses lose much of their thrombogenicity over a period of ~ 6 months due to endothelialisation (Ariyoshi *et al.*, 1997; Jeschke *et al.*, 1999). Indeed, some polyurethane prostheses demonstrate complete endothelialisation within a month of transplant (Jeschke *et al.*, 1999) and may prove particularly useful in combination with similar NO-delivery methods to those detailed here.

7.5 FUTURE DIRECTIONS

The main aim of experiments performed within this thesis was to investigate the mechanisms of action of existing and novel NO donor drugs on platelet aggregation and

adhesion in *in vitro* systems. Therefore, while these experiments have provided useful information on the antiplatelet signalling events mediated by NO and donor drugs within these artificial situations, the next logical step is to further study the potential of these drugs in an *in vivo* setting.

Chapters 3 and 4 indicate that NO donors including DEA/NO and SNVP, and $ONOO^-$ generators such as SIN-1, inhibit platelet activation via cGMP-independent mechanisms that involve the inhibition of platelet Ca^{2+} signalling and/or inhibition of TxA_2 formation. However, the ability of these compounds to affect platelet function *in vivo*, particularly via cGMP-independent mechanisms, is not clear. Although diazeniumdiolates such as MAHMA/NO may be more potent inhibitors of platelet function *in vivo* than S-nitrosothiols such as GSNO (Homer & Wanstall, 2003), the contribution of cGMP-independent signalling events to the inhibitory effect has not been discerned. It may be the case that short-lived NO donors such as MAHMA/NO induce long-term antiplatelet effects by permanently modifying critical proteins (e.g. SERCA), although the observation that platelets treated with a concentration of DEA/NO (2 μM) sufficient to induce cGMP-independent signals (chapter 3; fig 3.2.a.ii) recovered from DEA/NO-mediated inhibition (chapter 5; fig 5.2) suggests that cGMP-independent effects are fully reversible. Nevertheless, investigation into the temporal aspects of cGMP-independent effects merits further investigation. Moreover, further experiments are required to characterise whether the induction of cGMP-independent signals within platelets is a desirable event. Perhaps the best way to address these issues is to perform comparative experiments between NO donors that inhibit platelet activation via purely

cGMP-dependent effects (e.g. SNP) and ones that are known to stimulate cGMP-independent events (e.g. DEA/NO). Indeed, these experiments could also address whether novel NO donors including the diazeniumdiolates and S-nitrosothiols such as SNVP are useful as antiplatelet agents at concentrations that do not induce global vasodilation. On this front, results for the selective inhibition of platelet activation by SNVP (Miller *et al.*, 2003) and for a short-acting diazeniumdiolate (dimethylhexanediamine diazeniumdiolate; Kaul *et al.*, 1996) look promising.

The observation in chapter 5 that low molecular weight thiols accelerate S-nitrosoalbumin formation mean that the time-course of NO donor-mediated inhibition of platelet activation cannot be predicted by its observed half-life *in vitro*. However, it will be important to assess the ability of both cell-free and red blood cell haemoglobin to modulate the effects of these NO donor *in vivo*, to rule out the possibility that the prolonged inhibition of platelet activation is an *in vitro* artefact. The finding that infusion or supplementation of low molecular weight thiols enhances GTN and endothelium-dependent relaxation (Kugiyama *et al.*, 1998; Vita *et al.*, 1998) suggests that this pathway may play a role *in vivo*. Indeed, the supplementation of endogenous levels of low molecular weight thiols requires more investigation as a therapeutic strategy in conditions where levels of these thiols are decreased, such as coronary heart disease (Morrison *et al.*, 1999).

Results outlined in chapter 6 identify a potential therapeutic role for SNVP and NO-loaded zeolites as biocompatible agents to reduce platelet adhesion. It is clear that these materials require further scrutinisation before their full potential can be evaluated. These include trials designed to test the longevity of antiplatelet action and the ability of

these biomaterials to prevent platelet activation under physiological conditions *in vivo*. In addition, more work is required to establish the properties responsible for SNVP-mediated inhibition of platelet adhesion and to determine whether the chemical properties of SNVP may be improved upon to further decrease the thrombogenicity of artificial prostheses. For example, should the 5 C valeryl chain on SNVP prove essential for its retention within ePTFE, then increasing chain length may afford greater hydrophobic interaction and retention with such materials. On a similar note, it is worth comparing the effect of SNVP on other prosthetic materials (e.g. Dacron), with which SNVP may show greater efficacy. Further experiments are also required to investigate how practical zeolites are for the construction of grafts and whether or not they may be used to generate mechanically stable prostheses for long term use. In particular, the development of longer lasting NO-releasing surfaces and/or their combination with prosthetic surfaces that exhibit rapid endothelialisation is likely to have significant impact on the construction of biocompatible surfaces with improved long-term patency. Ultimately, it is important to assess the long-term therapeutic potential of novel NO donor drugs in conditions where increased platelet activity and/or reduced NO bioavailability is a problem. As mentioned, platelet activity is increased in coronary/peripheral vascular disease, atherosclerosis and hypertension (Furman *et al.*, 1998; Kabbani *et al.*, 2001; Lande *et al.*, 1987; Reininger *et al.*, 1999), usually in association with endothelial dysfunction and decreased NO bioavailability (Bossaller *et al.*, 1987; Linder *et al.*, 1990; Makin *et al.*, 2003; Werns *et al.*, 1989). It remains to be seen whether novel NO donors may afford protection against such conditions and improve patient outcome.

7.6 SUMMARY

In summary, data presented within this thesis characterise the inhibitory action of a number of NO donor drugs in human platelets and describes the antiplatelet action of novel NO-generating surfaces. In plasma, NO donors can inhibit platelet activation via cGMP-independent mechanisms provided sufficient extracellular NO radical is generated (see fig 7.1 for summary). Potential cGMP-independent targets within platelets include the Ca^{2+} pump, SERCA, and the prostaglandin generating protein, COX-1. Similarly, plasma low molecular weight thiols modulate the antiplatelet activity of NO by accelerating S-nitrosoalbumin formation and subsequent NO-delivery by means of transnitrosation reactions (fig 7.1). Finally, the coating of prosthetic graft materials with SNVP and/or the production of grafts and extracorporeal tubing with NO-loaded zeolites offer a potential means of increasing the biocompatibility of artificial prostheses and improving their function.

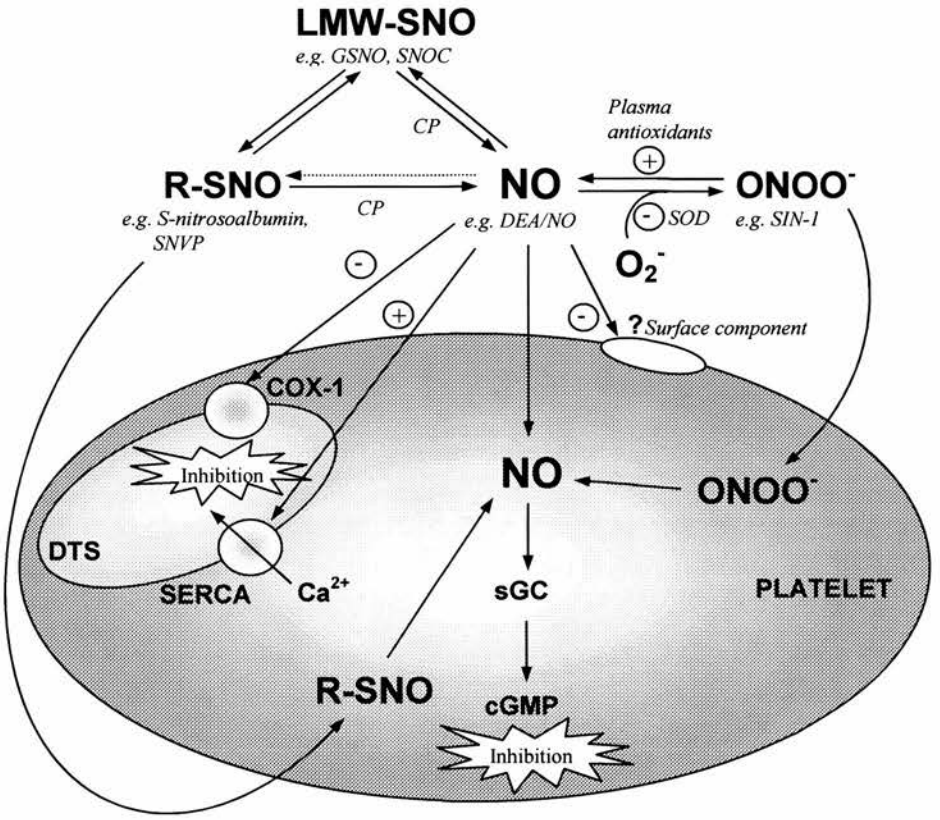


Figure 7.1 **Summary of the mechanisms proposed in this thesis. NO can inhibit platelet activation by binding to sGC resulting in cGMP-dependent inhibition of platelet activation. Alternatively, NO generated in the extracellular environment inhibits COX-1 or increases SERCA-dependent Ca^{2+} reuptake into the DTS, resulting in cGMP-independent antiplatelet effects. The interaction of NO with O_2^- results in $ONOO^-$ generation, although plasma antioxidants and SOD can prevent this reaction and favour the conversion of $ONOO^-$ back to NO. NO can also react with thiols resulting in the generation of S-nitrosothiols (R-SNO). In plasma however, NO preferentially reacts with low molecular weight thiols, resulting in the formation of low molecular weight S-nitrosothiols (LMW-SNO). LMW-SNO can undergo transnitrosation reactions with protein thiols such as cys 34 in serum albumin, resulting in the formation of stable S-nitrosothiol species that may prolong NO bioactivity. The acceleration of S-nitrosothiol decomposition by plasma factors such as CP, and the prevention of $ONOO^-$ formation by SOD, may induce cGMP-independent inhibition of platelet activation, provided sufficient extracellular NO is generated.**

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Novel Role for Low Molecular Weight Plasma Thiols in Nitric Oxide-mediated Control of Platelet Function*

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Nitric oxide (NO) is a powerful antiplatelet agent, but its notoriously short biological half-life limits its potential to prevent the activation of circulating platelets. Here we used diethylamine diazeniumdiolate (DEA/NO) as an NO generator to determine whether the antiplatelet effects of NO are prolonged by the formation of a durable, plasma-borne *S*-nitrosothiol reservoir. Preincubation of both platelet rich plasma (PRP) and washed platelets (WP) with DEA/NO (2 μM) for 1 min inhibited collagen-induced platelet aggregation by 82 ± 5 and 91 ± 2%, respectively. After 30 min preincubation with DEA/NO, NO was no longer detectable in either preparation, but aggregation remained markedly inhibited (72 ± 7%) in PRP. In contrast, the inhibitory effect in WP was almost completely lost at this time (5 ± 3%) but was partially restored (39 ± 10%) in WP containing human serum albumin (1%) and fully restored by co-incubation with albumin and the low molecular weight (LMW) thiols, glutathione, (5 μM), cysteinyl-glycine (10 μM), or cysteine (10 μM). This NO-mediated effect was not seen with LMW thiols in the absence of albumin and was associated with *S*-nitrosothiol formation. Our results demonstrate that LMW thiols play an important role in both the formation and activation of an *S*-nitrosoalbumin reservoir that significantly prolongs the duration of action of NO.

Nitric oxide (NO)[1] is a crucial free radical messenger with potent antiplatelet activity (1–5). NO synthesized in vascular endothelial cells and platelets is recognized to be a key mediator that protects against both atherogenesis and thrombosis (6). In platelets, NO primarily acts to stimulate soluble guanylate cyclase, ultimately resulting in a cyclic guanosine monophosphate (cGMP) and G kinase-mediated reduction in calcium mobilization (7, 8), although cGMP-independent inhibitory effects have also been identified (9). Under physiological conditions, the half-life of NO is short (~3–10 s) (10, 11), suggesting that NO bioactivity should rapidly dissipate and only impact on cells within close diffusible range of the site of production (12, 13). However, a number of studies suggest that NO can be incorporated into relatively stable endogenous reservoirs that modify its biological activity (14–19). *S*-Nitrosothiols rank high among the likely candidates for such a reservoir because of the relative abundance of suitable thiols in the biological environment (20). A physiological role for *S*-nitrosothiols has been implicated following identification of endogenous *S*-nitrosothiols at relevant concentrations (14, 21–24), together with plausible pathways that could result in their formation (25–28). In plasma, it has been shown that the vast majority of the *S*-nitrosothiol pool exists in the form of the high molecular weight species *S*-nitrosoalbumin (14, 29, 30). However, low molecular weight (LMW) thiols such as glutathione are also present in plasma in the low micromolar range and have previously been shown to potentiate the antiplatelet action of *S*-nitrosoalbumin (31). Given the close proximity of platelets to the vascular endothelium and the unique sensitivity of platelets toward *S*-nitrosothiol-mediated inhibition, it is important to dissect the role of plasma-borne thiols in the modification of NO activity in platelets.

Here, we tested the hypothesis that the activity of a short-acting NO donor drug, diethylamine diazeniumdiolate (DEA/NO), is prolonged in the presence of plasma albumin through formation and subsequent activation of an *S*-nitrosoalbumin NO reservoir. Furthermore, we explored the hypothesis that low molecular weight thiols have a unique role in both the formation and activation of an *S*-nitrosoalbumin reservoir, potentiating NO-mediated inhibition of platelet aggregation.

EXPERIMENTAL PROCEDURES

Materials—(Z)-1-(*N*,*N*-diethylamino)diazen-1-ium-1,2-diolate sodium salt (DEA/NO; Alexis Biochemicals, Lausen, Switzerland) was dissolved in 0.01 M NaOH and stored at −20 °C. DEA/NO was diluted in phosphate-buffered saline (pH 7.4) immediately before use. Bovine methemoglobin was reduced to the ferro (Fe^{2+}) form by sodium dithionite as previously described (32). Spectrophotometric analysis indicated that ferrohemoglobin existed primarily in the oxygenated form. Collagen reagent was purchased from Labmedics (Stockport, UK). All other chemicals were purchased from Sigma.

Platelet Preparation—Venous blood was drawn from the antecubital fossa of healthy volunteers (age 20–40 years) into citrated tubes (0.38% final concentration). Volunteers had not taken any medication known to affect platelet aggregation within the last 10 days. Platelet-rich plasma (PRP) and platelet poor plasma (PPP) were prepared as previously described (34). Washed platelets (WP) were prepared by centrifugation of PRP (1200 × *g*; 10 min) in the presence of PGI_2 (300 ng/ml), and the platelet pellet resuspended in an equal volume of modified HEPES-tyrode buffer containing (in mM): 137 NaCl, 2.7 KCl, 1.05 $MgSO_4$, 0.4 NaH_2PO_4, 1.8 $CaCl_2$, 12.5 $NaHCO_3$, 5.6 glucose, 10 HEPES, and 10.9

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[1] The abbreviations used are: NO, nitric oxide; cGMP, cyclic guanosine monophosphate; DEA/NO, diethylamine diazeniumdiolate; LMW, low molecular weight; PRP, platelet-rich plasma; PPP, platelet-poor plasma; WP, washed platelets; RBC, red blood cell; HSA, human serum albumin; NEM, *N*-ethylmaleimide; Hb, hemoglobin.

trisodium citrate. Following a secondary centrifugation (1200 × *g*; 10 min) in the presence of 300 ng/ml PGI_2; platelets were resuspended in an equal volume of PGI_2-free HEPES-tyrode. Platelet count was determined using a Coulter A^c.T 8 Hematology Analyzer (Coulter Electronics, Luton, UK) and standardized to 250×10^9 liter^{-1} via dilution with PPP (PRP) or HEPES-tyrode (WP).

NO Electrode Measurements—Samples (2 ml) of PRP and WP were prewarmed to 37 °C before addition of DEA/NO (2 μM). NO concentration was measured for 30 min by an isolated NO electrode (World Precision Instruments, Stevenage, UK). The electrode was calibrated using DEA/NO (0.1–3.2 μM) in phosphate buffer (pH 4.0); DEA/NO spontaneously decomposes at pH ≤ 5 (33). In a different series of experiments, WP were reconstituted with 0.46 μM hemoglobin derived from red blood cell (RBC) lysate, prior to addition of DEA/NO (2 μM) and recording for 30 min.

Hemoglobin Measurements—Plasma hemoglobin was quantified using an assay (Sigma Diagnostics) based on the hemoglobin catalyzed oxidation of 3,3′,5,5′-tetramethylbenzidine by hydrogen peroxide and colorimetric determination at 600 nm as described (34, 35).

Aggregometry—Aggregometry studies were performed via turbidometric analysis using a two-channel platelet aggregometer (Chronolog Ca560, Labmedics, Stockport, UK). Signals were processed by a MacLab/4e analogue-digital converter (AD Instruments, Sussex, UK) and displayed through Chart software (AD Instruments, Sussex, UK). Aliquots (0.5 ml) of PRP and WP were equilibrated at 37 °C before the addition of 2 μM DEA/NO (~IC_{90} for DEA/NO in platelets (9)). Platelet aggregation was then induced via the addition of collagen (2.5 μg/ml) 1–30 min later. Aggregation was monitored for 5 min, and the maximum response recorded. In a different series of experiments, WP were reconstituted with the LMW thiols glutathione (GSH; 5 μM), cysteinylglycine (Cys-gly; 10 μM), and cysteine (Cys; 10 μM) to approximate plasma concentrations (36). Thiol-reconstituted WP was also incubated in the absence and presence of 1% human serum albumin (HSA); higher concentrations of HSA that approximate plasma levels (4%) were found to have nonspecific effects in platelets, even after extensive dialysis. Platelets were incubated with DEA/NO (2 μM) before stimulation with collagen (2.5 μg/ml) 30 min later. In further experiments, WP reconstituted with GSH (5 μM) ± HSA (1%) were preincubated with donor RBC lysate to produce a final hemoglobin concentration of 0.46 μM. DEA/NO (2 μM) was added to WP for 30 min prior to the addition of collagen (2.5 μg/ml) 30 min later. In control experiments, DEA/NO (2 μM) was added to WP 25 min before the addition of oxy-hemoglobin (10 μM). Platelets were then stimulated with collagen (2.5 μg/ml) 5 min later, and aggregation measured.

Thiol Measurements—The reduced thiol content of plasma and HSA (1%)-reconstituted tyrodes ± the LMW thiols GSH (5 μM), Cys-gly (10 μM), or cysteine (10 μM) was quantified via reaction with 5,5′-dithiobis(2-nitrobenzoic acid) and colorimetric determination at 412 nm, as previously described (37).

S-Nitrosothiol Detection—Samples of PRP and WP were equilibrated in the aggregometer for 15 min. To establish baseline *S*-nitrosothiol levels, 0.5-ml aliquots of PRP or WP were transferred to vials containing *N*-ethylmaleimide (NEM) and EDTA (final concentration 5 mM and 2 mM, respectively). Samples were centrifuged (1800 × *g*; 5 min), and the supernatant aspirated. Acidified sulfanilamide (2.5% dissolved in 1 M HCl) was added to the supernatant, and the mixture stored at –70 °C prior to *S*-nitrosothiol detection. To determine *S*-nitrosothiol formation after bolus NO injection, WP and PRP samples were prewarmed as before and 2 μM DEA/NO added. Aliquots (0.5 ml) of DEA/NO-treated WP or PRP were aspirated 1–30 min later and added to NEM/EDTA to stop the reaction. Samples were centrifuged (1800 × *g*; 5 min), and the supernatant and pellet treated with acidified sulfanilamide and stored at –70 °C. *S*-Nitrosothiols were quantified by copper/iodide-induced cleavage of the S-NO bond and subsequent measurement by chemiluminescence as described (21).

RESULTS

Hemoglobin Measurements—Hemoglobin (Hb) concentration in PRP was 0.46 ± 0.18 μM (n = 5) and did not differ significantly ($p > 0.05$) from the hemoglobin concentration determined in PPP (0.39 ± 0.01 μM).

NO Electrode Studies—Addition of 2 μM DEA/NO to WP resulted in a rapid increase in NO concentration, which reached a maximum of 3.2 ± 0.18 μM (mean ± S.E.) NO before it declined to basal levels within 20–25 min (Fig. 1; n = 6). Administration of 2 μM DEA/NO to PRP showed that DEA/NO-derived NO was partially quenched in plasma, reaching a maximum extracellular concentration of 0.53 ± 0.11 μM. Addition of 2 μM DEA/NO to WP reconstituted with 0.46 μM hemoglobin derived from donor RBC lysate produced a profile matching that observed in PRP with a maximum extracellular NO concentration of 0.59 ± 0.05 μM (n = 6). Reconstitution of WP with 1% HSA ± GSH (5 μM) produced a NO trace similar to that observed with WP (results not shown).

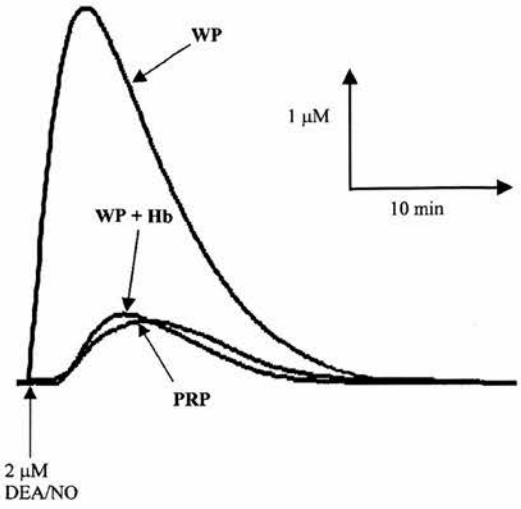

FIG. 1. **Generation of NO by DEA/NO in WP, PRP, and Hb reconstituted WP.** Platelets were equilibrated at 37 °C before the addition of 2 μM DEA/NO. Recording was stopped after a 30-min incubation of DEA/NO. Data are expressed as the mean of six experiments.

Effect of DEA/NO on Inhibition of Platelet Aggregation in PRP and WP—Bolus administration of DEA/NO (2 μM) to PRP resulted in sustained inhibition of collagen-induced platelet aggregation that was maintained for at least 30 min (Fig. 2*A*; n = 8). In WP, however, inhibition of collagen-induced platelet aggregation by DEA/NO (2 μM) was attenuated at 20 min and abolished after 30 min (Fig. 2*A*; n = 8). The difference between inhibition of aggregation in PRP and WP was significant ($p < 0.001$). Representative traces from each time point in both PRP and WP are included (Fig. 2*B*).

Effect of Thiols on DEA/NO-mediated Inhibition of Platelet Aggregation in WP—Reconstitution of WP with the LMW thiols GSH (5 μM), Cys-gly (10 μM), and Cys (10 μM) did not alter the inhibition of platelet aggregation by DEA/NO after 30 min (n = 8; $p > 0.05$). However, reconstitution of WP with 1% HSA resulted in a modest restoration of the inhibitory effect of DEA/NO after 30 min (Fig. 3; $p < 0.001$). Co-incubation of WP with 1% HSA and either GSH, Cys-gly, or Cys fully restored the inhibitory effect of DEA/NO after 30 min (Fig. 3; $p < 0.001$). Inhibition of platelet aggregation by DEA/NO at 30 min in the presence of HSA and GSH was partially quenched by preincubation of 0.46 μM RBC-derived hemoglobin in WP ($p < 0.01$), although inhibition was still significantly enhanced when compared with WP alone (Fig. 4; n = 8).

Effect of Hemoglobin on Prolonged Inhibition of Platelet Aggregation—Prolonged inhibition of platelet aggregation in WP reconstituted with HSA alone or with HSA and any of the LMW thiols was abolished by addition of the NO scavenger oxy-Hb (10 μM; $p < 0.001$, n = 8).

Thiol Measurements—The concentration of reduced thiol in plasma was 0.32 ± 0.01 mM (n = 5). In HEPES-tyrode buffer containing 1% HSA, thiol concentration was 0.11 ± 0.01 mM

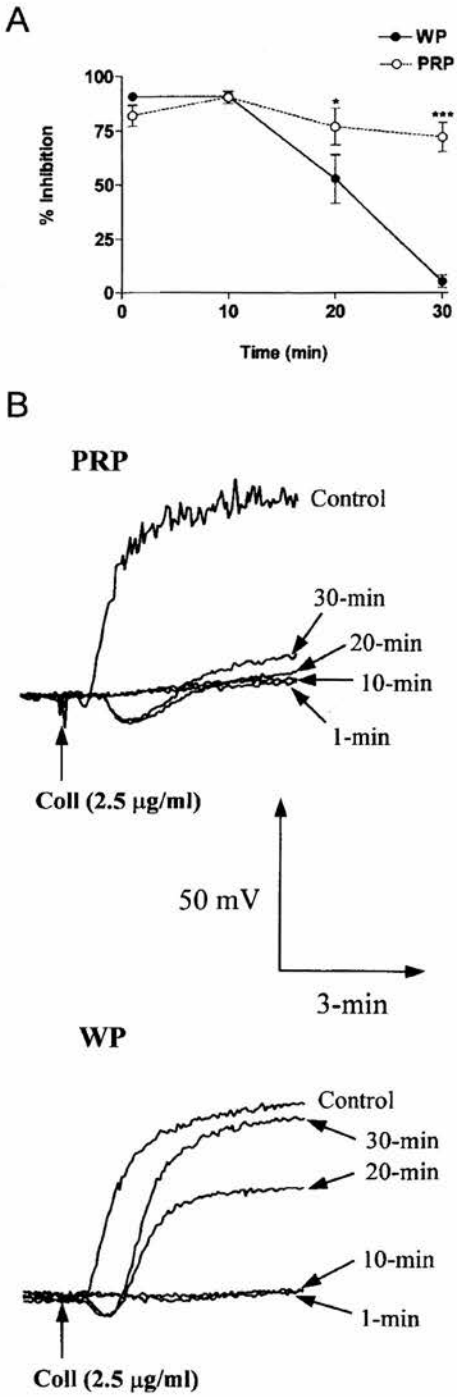

FIG. 2. **Inhibition of platelet aggregation by DEA/NO in WP and PRP.** WP or PRP were equilibrated to 37 °C before the addition of DEA/NO (2 μM). Platelet aggregation was then stimulated via the addition of collagen (2.5 μg/ml) 1–30 min later. Data are expressed as mean ± S.E. of eight experiments. (*, $p < 0.05$; ***, $p < 0.001$; *A*). Representative traces obtained from PRP and WP are also included (*B*).

($n = 5$) and did not differ significantly from 1% HSA containing GSH (5 μM; 0.12 ± 0.01 mM), Cys-gly (10 μM; 0.10 ± 0.01 mM), or Cys (10 μM; 0.11 ± 0.01 mM).

S-Nitrosothiol Detection—Incubation of DEA/NO in PRP caused a rapid increase in *S*-nitrosothiol production which reached a maximum of 73.5 ± 15.4 nM after 10 min and diminished gradually over the 30-min incubation period (Fig. 5; $n = 6$). Addition of DEA/NO to WP + 1% HSA resulted in a slower increase in *S*-nitrosothiol concentration, which reached a level close to that observed in PRP after 30 min (46.0 ± 8.8 nM). The presence of 5 μM GSH increased the formation of *S*-nitrosothiol ~2-fold after a 30 min-incubation of DEA/NO (104.5 ± 18.7 nM). There was no significant difference in *S*-nitrosothiol formation in WP + 1% HSA compared with 1% HSA alone ($p > 0.05$).

DISCUSSION

Our results clearly demonstrate that the biological activity of DEA/NO, a short-acting NO-donor drug with a half-life of ~2 min at physiological temperature and pH, is significantly prolonged in PRP compared with WP, where activity closely mirrored NO concentration. Importantly, the prolonged inhibition of aggregation observed in PRP is mediated by NO, despite the clear decay of DEA/NO-derived NO to undetectable levels within the 30-min incubation period. Reconstitution of WP with HSA caused a partial restoration of DEA/NO-mediated inhibition after 30 min, but when combined with the LMW thiols GSH, Cys-gly, or Cys, the inhibitory action was fully restored to that seen in PRP. Furthermore, the degree of inhibition of aggregation was associated with *S*-nitrosothiol formation in PRP and reconstituted platelets, indicating a crucial role for both protein and LMW thiols in prolonging the biological activity of NO.

NO was clearly detected in both WP and PRP treated with DEA/NO, a compound known to generate two molar equivalents of NO upon hydrolysis. Importantly, while there was a clear divergence in the concentration of NO detected in PRP and WP, DEA/NO-derived NO declined to undetectable levels after a 20-min incubation period in both PRP and WP. There was a delay in the appearance of NO in PRP after bolus injection of DEA/NO, suggesting that plasma has some NO scavenging ability. Analysis of Hb concentration revealed that PRP contained 0.46 μM Hb, with a potential capacity to scavenge ~1.5–2 μM NO, assuming that all four heme groups are available for reaction with NO. Given that the delay in appearance of extracellular NO in PRP is ~2 min, during which time ~2 μM NO is released, our data indicate that Hb-mediated scavenging is responsible for the discrepancy between extracellular NO in PRP and WP. The concentration of Hb in PRP equated with that in PPP, indicating that the vast majority of Hb was cell-free. The physiological relevance of these findings is, as yet, unclear because blood sampling and platelet isolation is likely to cause significant hemolysis. However, increased scavenging of NO by cell-free Hb might have important implications in conditions where hemolysis is increased *in vivo*, such as in subarachnoid hemorrhage (38).

The ability of plasma components to prolong the antiplatelet effects of bolus DEA/NO is profound. While inhibition of platelet aggregation was sustained in PRP, substitution of plasma with HEPES-tyrode buffer resulted in a marked reduction in the duration of the inhibitory effect. Predictably, there was a close correlation between extracellular NO concentration and inhibition of platelet aggregation in WP, indicating that the degree of inhibition is closely defined by the extracellular NO concentration. In PRP, however, inhibition of aggregation was maintained, despite the progressive loss of extracellular NO from the system. The NO scavenger, oxy-Hb, abolished the sustained inhibitory effect in PRP, confirming that the effect was entirely NO-mediated. Given that human plasma is an abundant source of reduced thiol (20) and that the concentration of *S*-nitrosothiols in human plasma is relatively high (30–120 nM) (21, 24, 30), we hypoth-

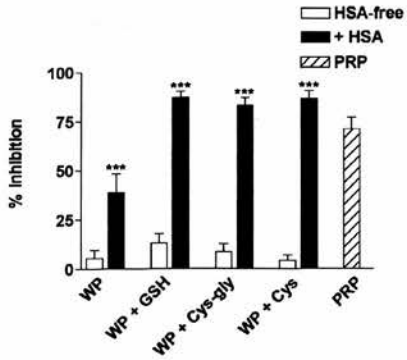

FIG. 3. **Effect of LMW thiols and HSA on inhibition of platelet aggregation by DEA/NO.** LMW thiols GSH (5 μM), Cys-gly (10 μM), and Cys (10 μM) ± HSA (1%) were preincubated in WP before the addition of 2 μM DEA/NO. Platelet aggregation was then stimulated via the addition of collagen (2.5 μg/ml) 30 min later. PRP data are also included for a comparison. Data are expressed as mean ± S.E. of eight experiments. (***, $p < 0.001$).

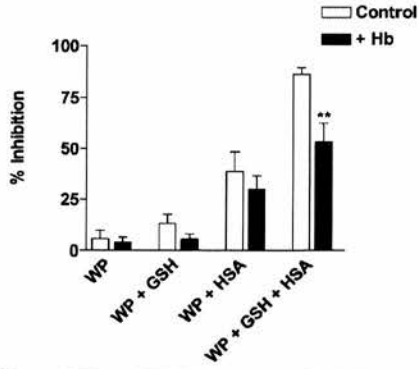

FIG. 4. **Effect of Hb on DEA/NO mediated inhibition of platelet aggregation.** WP ± RBC-derived Hb (0.46 μM) were incubated with GSH (5 μM) ± HSA (1%) before the addition of DEA/NO (2 μM). Aggregation was then induced by the addition of collagen (2.5 μg/ml) 30 min later. Data are expressed as mean ± S.E. of eight experiments. (**, $p < 0.001$).

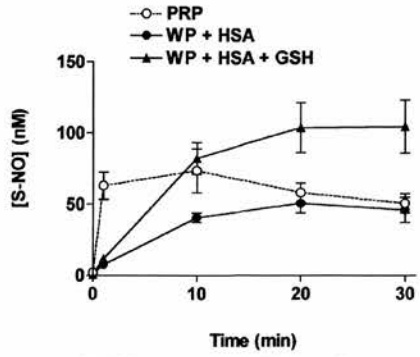

FIG. 5. ***S*-nitrosothiol formation in PRP and reconstituted WP after treatment with DEA/NO.** DEA/NO (2 μM) was incubated in PRP or reconstituted WP before the addition of NEM/EDTA 1–30 min later to stop the reaction. Samples were then centrifuged, and the supernatant treated with acidified sulfanilamide (2.5% in 1 M HCl) before *S*-nitrosothiol detection. Data are expressed as mean ± S.E. of six experiments.

esized that thiols may have a role in the prolongation of NO bioactivity observed here. In human plasma, the single free cysteine residue present on serum albumin (Cys-34) accounts for the majority of reduced thiol. However, LMW thiols are present in human plasma in the low micromolar range (36), and *S*-nitrosothiols have previously been shown to undergo thiol-nitrosothiol exchange *in vivo* (15). Therefore, WP was reconstituted with albumin and LMW thiols to dissect thiol function on the antiplatelet activity of NO. Our results clearly indicate that incubation of the LMW thiols GSH, Cys-gly, and Cys did not alter the duration of antiplatelet action of DEA/NO, but reconstitution with 1% HSA significantly prolonged inhibition of aggregation. Crucially, while DEA/NO-mediated aggregation was only partially restored with HSA, co-incubation of HSA with each of the LMW thiols completely restored the inhibitory action of DEA/NO at 30 min despite a negligible increase in the thiol pool. Furthermore, hemoglobin completely reversed this inhibition, indicating that the LMW thiol/HSA effect is entirely NO-mediated.

The correlation observed between *S*-nitrosothiol formation and inhibition of platelet aggregation strongly indicates that the role of thiols in prolongation of NO-mediated inhibition of platelet aggregation is through provision of a substrate for *S*-nitrosation. Interestingly, our results indicate that there is a clear difference in the rate by which *S*-nitrosothiols are generated in PRP compared with thiol-reconstituted solutions. In PRP, *S*-nitrosothiol formation was very fast compared with that observed in reconstituted WP, with significant amounts being formed (~60 nM) after 1 min of incubation with DEA/NO. Conversely, 1 min of incubation of DEA/NO in HSA-reconstituted WP resulted in very low level *S*-nitrosothiol formation (<10 nM), which gradually increased to a maximum concentration of 50.5 ± 6.7 nM after 20–30 min. Despite rather different kinetics of formation of *S*-nitrosothiols in PRP and HSA-reconstituted WP, by 30 min, total *S*-nitrosothiol concentration is the same (~50 nM). However, inhibition of platelet aggregation is markedly different in PRP and HSA reconstituted WP after a 30-min incubation of DEA/NO. Previous data indicating that LMW thiols such as GSH can increase the antiplatelet action of *S*-nitrosoalbumin (31) are supported by our results. We suggest that *S*-nitrosoalbumin formed in reconstituted WP is an inefficient NO donor and requires the presence of low molecular weight thiols such as those found in PRP to efficiently control physiological function as has previously been proposed (14, 15, 17, 31). However, data obtained here emphasize an additional role for GSH and other LMW thiols in the formation of *S*-nitrosothiols. Co-incubation of GSH with HSA-reconstituted WP resulted in an increase in *S*-nitrosothiol concentration by ~2-fold. Furthermore, this increase was accompanied by a large augmentation of DEA/NO-mediated inhibition of platelet aggregation.

The mechanism for formation of *S*-nitrosothiols *in vivo* is a source of considerable debate; NO itself is a weak nitrosating agent, but higher oxides of NO such as N_2O_3 are potent nitrosating species (39). The rate-limiting step in the formation of N_2O_3 is the reaction of NO with molecular oxygen, which is third order ($k \sim 4 \times 10^6\ M^{-2}\ s^{-1}$) (40, 41). Although originally thought too slow to account for endogenous levels of *S*-nitrosothiols, the reaction between NO and O_2 can be catalyzed by ceruloplasmin (42), a copper-containing protein abundant in plasma. Moreover, accelerated formation of *S*-nitrosothiols has been observed in the presence of biological membranes (39, 43) and in the hydrophobic core of proteins such as albumin (44), which act as "NO sinks" to concentrate nitrosating species. We recognize that the pharmacological levels of NO used here are sufficiently high to facilitate significant formation of N_2O_3 that might subsequently nitrosate thiols. However, our results with GSH and HSA confirm previous findings that the ability of albumin to catalyze *S*-nitrosothiol formation is greatly in-

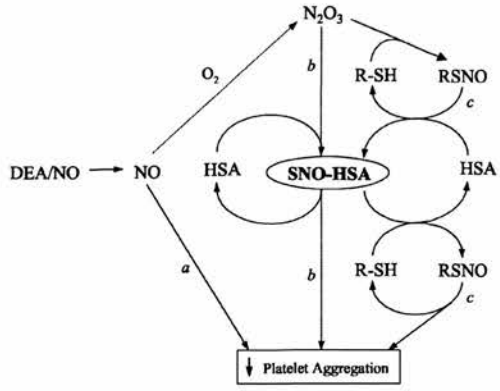

FIG. 6. **Summary of proposed mechanism.** DEA/NO hydrolyzes in aqueous solution to generate NO. NO diffuses into the platelet where it activates various cellular processes leading to inhibition of platelet aggregation (path *a*). Alternatively, DEA/NO-derived NO reacts with molecular oxygen to form nitrosating species such as N_2O_3, which react with the sulfhydryl group on HSA to form relatively stable SNO-HSA. SNO-HSA inhibits aggregation via generation of NO at the platelet membrane surface (path *b*). In the presence of LMW thiols, N_2O_3 preferentially reacts with LMW thiols to form LMW *S*-nitrosothiols (*RSNO*). LMW *S*-nitrosothiols transnitrosate with HSA to form the *S*-nitrosoalbumin reservoir. Bioactive NO can be delivered to the platelet via a reverse of the previous process, leading to prolonged inhibition of aggregation (path *c*).

creased in the presence of low molecular weight thiols (44). A modest increase (~5%) of thiol pool through addition of GSH to HSA-treated WP failed to significantly affect total thiol concentration, while causing a disproportionate increase in *S*-nitrosothiol formation (~2-fold). Our data demonstrate that the presence of platelets did not significantly alter *S*-nitrosothiol production, suggesting that plasma membrane-mediated acceleration does not play a part in this system. Given that we observed more rapid production of *S*-nitrosothiols in plasma than in reconstituted WP, we suggest that *S*-nitrosothiol formation catalyzed by plasma components like ceruloplasmin may be a key factor in the difference observed. Alternatively, the full complement of thiols in plasma may be required to provide an efficient pathway for the incorporation of NO into *S*-nitrosothiols. Our results indicate that cell-free Hb at plasma concentrations has a net scavenging effect, implying that cell-free Hb functions to remove NO rather than to conserve NO bioactivity through the formation of additional *S*-nitrosated species. We recognize that many pathways for *S*-nitrosothiol formation exist (26, 28), and thus may play a significant role in this system.

It is noteworthy that low serum GSH levels are an independent predictor of coronary heart disease (45) and that thiol supplementation in humans has been shown to cause an increase in both endothelium-dependent and -independent relaxation (46–48), especially in subjects at risk from coronary artery disease (46). Furthermore, a number of potential mechanisms for the cardioprotective role of thiols have been identified, including scavenging of oxygen-derived free radical species (48) and direct stimulation of NO synthase itself (49). Our results imply that the bioavailability of LMW thiols may have a significant impact on the ability of plasma to form *S*-nitrosothiols and, therefore, prolong the antiplatelet action of endothelium-derived NO (Fig 6). Moreover, in light of evidence that *S*-nitrosoglutathione is relatively platelet-selective (50), we suggest that the ability of GSH and other LMW thiols to assist in *S*-nitrosothiol formation and delivery may be of crucial importance in the maintenance of hemostasis and might be compromised in coronary artery disease.

Our results have important implications with respect to the potential for NO donor-mediated antithrombotic therapy. Formation of a durable plasma reservoir of NO that is slowly liberated through the action of LMW thiols suggests that prolonged antiplatelet activity might be afforded by delivery of short acting NO donor drugs that were previously considered too labile for this purpose.

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