Targeting the nitric oxide signalling pathway to modulate platelet function

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Declaration of originality

The nitrate/nitrite gas-phase chemiluminescence experiments were performed in collaboration with Dr Miranda Smallwood and Prof. Paul Winyard (Inflammation Research Group, University of Exeter Medical School). Genotyping experiments were performed by Charles River Laboratories (Margate, UK).

Otherwise, all work presented in this thesis is my own and expressed in my own words. Where the work of other authors is used (ideas, previous research, images) it is appropriately referenced and listed in the bibliography.

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Signature..Date...........18/11/2014.................................

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Abstract

Nitric oxide (NO) negatively regulates platelets and impaired NO signalling can lead to arterial thrombosis. The source of platelet-derived NO is unclear with recent proposals of NO synthase (NOS) independent NO sources, such as S-nitrosothiols (RSNOs) and inorganic nitrate/nitrite. Sildenafil citrate, a phosphodiesterase 5 (PDE5) inhibitor, enhances NO/cGMP signals in cells expressing PDE5 such as platelets. The aims of this study were to investigate the antiplatelet properties of sildenafil, its mechanism of action and to determine the upstream sources of NO affecting platelet function.

The functional effect of sildenafil was determined using a range of *in vitro* and *in vivo* platelet assays. The mechanism of action of sildenafil and upstream sources of NO/cGMP signals were assessed pharmacologically using established methods of *in vitro* and *in vivo* platelet aggregation. Bioconversion of nitrate to nitrite was determined using gas-phase chemiluminescence. The functional significance of NO/cGMP signalling events in platelets were investigated *in vivo* in W.T and eNOS$^{-/-}$ (a model of vascular dysfunction) mice.

Sildenafil exerted an antiplatelet effect by enhancing transient NO/cGMP signals generated by platelets independent of NOS activity *in vitro*. Inhibition of proposed mechanisms of NO release from RSNOs did not modify the inhibitory effect of sildenafil suggesting that RSNOs did not mediate platelet NO/cGMP signals. Nitrite was able to drive inhibitory cGMP signalling events in platelets *in vitro.* Furthermore, nitrate inhibited platelet function in eNOS$^{-/-}$ mice *in vivo* following enhanced bioconversion to nitrite, potentially as a compensatory mechanism due to impaired NO signalling.

In conclusion, inorganic nitrate/nitrite may critically regulate platelets following bioconversion to NO and dietary sources of nitrate/nitrite may generate compensatory NO during vascular disease. Furthermore, sildenafil may be beneficial in reducing the risk of platelet-driven cardiovascular disease by enhancing NO/cGMP signalling derived from both enzymic and inorganic sources and restoring impaired NO signalling during endothelial dysfunction.

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Firstly, I would like to thank Dr Michael Emerson, for all his guidance, support and advice over the last 4 years. Thank you for challenging me and making me work at my optimum whilst also helping me develop in confidence which will be invaluable throughout my career. I would also like to thank the rest of the group, Dr Antonia Solomon and (soon to be Dr!) Erica Smyth for all the good times but, on a serious note, for your teaching, support and advice.

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Awards, publications and presentations

GlaxoSmithKline Award for Young Investigator 2012, British Pharmacological Society Winter meeting (2012) - **Best student oral presentation**.

Best poster presentation, NHLI conference day, Imperial College London (2012) – **1st prize for best poster presentation**.

Apostoli GL, Solomon A, Smallwood MJ, Winyard PG & Emerson M (2014) Role of inorganic nitrate and nitrite in driving nitric oxide/cGMP-mediated inhibition of platelet aggregation *in vitro* and *in vivo*, *Journal of Thrombosis and Haemostasis*, **12**, 1880-1889.

Apostoli G, Solomon A & Emerson M (2013) Sildenafil reduces platelet activity *via* both NO synthase and NO synthase-independent pathways, Abstracts of the XXIV congress of the International Society on Thrombosis and Haemostasis. Journal of Thrombosis and Haemostasis, 11: 445-446 at http://onlinelibrary.wiley.com/doi/10.1111/jth.12284/abstract - **poster presentation and session moderator**.

Apostoli G, Solomon A & Emerson M (2012), Nitrate and nitrite exert inhibitory effects on platelet function *in vitro* and *in vivo* Proceedings of the British Pharmacological Society at http://www.pa2online.org/abstract/abstract.jsp?abid=30784&author=apostoli&cat=-1&period=52 – **oral presentation**.

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http://informahealthcare.com/doi/suppl/10.3109/09537104.2011.637373/suppl_file/09537104.2011.637373.pdf - **poster presentation**.

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Other conference presentations

NHLI postgraduate research day, Imperial College (June 2013 - **oral** and 2012 - **poster**), UK Platelet Meeting (Bath - October 2012 - **poster**), Euplan Conference (Maastricht, Netherlands - September 2012 - **poster**), Platelet Summer School (Birmingham - July 2011 - **poster**).

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Abbreviations

-/-	knock out (genetic modification)
μ	micro
μm	micro meter
μM	micromolar
ACD	acid citrate dextrose
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ANOVA	analysis of variance
APS	ammonium persulphate
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
COX	cyclo-oxygenase
CysNO	S-nitrosocysteine
DMSO	dimethyl sulphoxide
D-NAME	Nω-Nitro-D-arginine methyl ester hydrochloride
DTS	dense tubular system
ECM	extracellular matrix
ED	erectile dysfunction
eNOS	endothelial nitric oxide synthase
g	gravitational force
GP	glycoprotein
Gs	stimulatory GTP-binding protein
GSNO	S-nitrosoglutathione
GSNOR	S-nitrosoglutathione reductase
HCl	hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HSP27	heat shock protein 27

IBMX	3-Isobutyl-1-methylxanthine
IC_{50}	half maximal inhibitory concentration
iNOS	inducible nitric oxide synthase
IP	prostacyclin receptor
IP_3	inositol-1,4,5-trisphosphate
IP_3R	inositol-1,4,5-trisphosphate receptor
IRAG	inositol-1,4,5-trisphosphate receptor-associated cGMP kinase substrate
IVM	intravital microscopy
KCl	potassium chloride
LAT	linker for activation of T cells
L-AT	amino acid transporter system-L
L-NAME	Nω-Nitro-L-arginine methyl ester hydrochloride
m	milli
$MgCl_2$	magnesium chloride
MLCK	myosin light chain kinase
mM	millimolar
mTHB	modified tyrodes HEPES buffer
n	nano
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
$NaHPO_4$	sodium phosphate dibasic dodecahydrate
NaOH	sodium hydroxide
NC3Rs	The National Centre for the Replacement, Refinement and Reduction of Animals in Research
nM	nanomolar
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NO/sGC/cGMP	nitric oxide/soluble guanylyl cyclase/cyclic guanosine monophosphate
NOS	nitric oxide synthase

NOx	nitrogen oxides (nitrate, nitrite and S-nitrosothiols)
NSF	*N*-ethylmaleimide-sensitive factor
OCS	open canalicular system
ODQ	1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one
PAH	pulmonary arterial hypertension
PAR	protease activated receptors
PDI	protein disulphide isomerase
PGE	prostaglandin
PGI_2	prostacyclin
PI3K	phosphoinositide 3-kinase
PIP_2	phosphatidylinositol 4,5-bisphosphate
PIP_3	phosphatidylinositol (3,4,5)-trisphosphate
PKA	protein kinase A
PKB/Akt	protein kinase B / serine/threonine-specific protein kinase
PKC	protein kinase C
PKG	protein kinase G
PLC	phospholipase C
pNPP	*para*-nitrophenylphosphate
PRP	platelet rich plasma
PVDF	polyvinylidene fluoride
ROS	reactive oxygen species
RSNO	S-nitrosothiol
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SFK	src family kinases
sGC	soluble guanylyl cyclase
SH	sulphydryl group
sil	sildenafil citrate
SNP	sodium nitroprusside

TBS-T	Tris-Buffered Saline Tween-20
TCEP-HCl	Tris(2-carboxyethyl)phosphine hydrochloride
TEMED	tetramethylethylenediamine
TP	thromboxane receptor
TXA_2	thromboxane A_2
VASP	vasodilator stimulated phosphoprotein
VASP-P	phosphorylated vasodilator stimulated phosphoprotein
Veh	vehicle
vWF	von Willibrand factor
W.T	wild-type
WP	washed platelets

Chapter 1: Introduction

Platelets

Platelets (also known as thrombocytes) were first described in 1882 and are small (~2.5μm), anucleate cytoplasmic fragments that play a key role in haemostasis (Bizzozero, 1882). They are derived from megakaryocytes found in bone marrow (Djaldetti et al., 1979). Platelets are known to circulate in the blood for an average of 7-10 days (Stuart et al., 1975) before being phagocytosed and subsequently destroyed in the spleen and liver (Hjort and Paputchis, 1960; Neiman et al., 1987). They are a major blood component and have an important role in haemostasis. At sites of vessel injury, platelets interact with the vessel wall and with each other to form a haemostatic plug and avoid uncontrollable haemorrhage. However, inappropriate activation of platelets can drive a condition known as arterial thrombosis and cause potentially fatal ischemic events such as myocardial infarction and stroke (Antithrombotic Trialists' Collaboration, 2002; Dyken et al., 1973). Due to their central role in disease, platelets are an important drug target to prevent the occurrence of platelet-driven cardiovascular events and antiplatelet therapies are usually the first line of treatment for those most at risk. However, there is a need for new antiplatelet therapies because current drugs on the market are limited by their lack of efficacy and adverse events.

Platelets in the cardiovascular system

Platelets are involved in a diverse range of roles in the body. They have been implicated in immune responses (Mayadas et al., 1993), growth and tissue regeneration (Anitua et al., 2004; Intini, 2009) and tumour metastasis (Takagi et al., 2013).

Platelets are the first line of defence against bleeding. During vascular damage the underlying extracellular matrix (ECM) is exposed and triggers platelet adhesion, activation and aggregation through the stimulation of platelet surface receptors. Prothrombotic substances released from the platelet act as positive feedback mechanisms by inducing further platelet activation, recruiting more platelets from the blood stream and facilitating platelet-platelet interactions to form a haemostatic plug and control excessive bleeding (see 'Platelet activity' for more information of platelets in primary haemostasis).

In addition, activated platelets are involved in secondary haemostasis (Monroe and Hoffman, 2006; Rumbaut and Thiagarajan, 2010). They provide an efficient catalytic surface for the assembly of cofactor enzyme complexes (Heemskerk et al., 1997). This is essential to enable the occurrence of the propagation phase of coagulation. Platelet activation causes an increase in intracellular Ca^{2+} which activates the ATP-independent enzyme scramblase. This enzyme is able to 'flip' phosphatidylserine and other anionic phospholipids from the inner aspect of the lipid bilayer to the outer, creating a negative charge on the outer platelet membrane (Comfurius et al., 1996; Wolfs et al., 2005). Coagulation factors FIX, FVIII and FV are able to bind to the platelet surface in preparation for large-scale thrombin generation known as the amplification phase. In the propagation phase, FVIIIa/FIXa complex forms and activates FX (Monroe and Hoffman, 2006). FXa is able to associate with FVa on the platelet surface converting FII (prothrombin) to FIIa (thrombin) producing a localised burst of thrombin generation (Scandura and Walsh, 1996). Thrombin induces the formation of a stable clot by catalysing the reaction of fibrinogen to fibrin (forming a fibrin mesh) and recruiting more platelets from the circulation. Dissolution of the clot occurs after the blood vessel has healed and blood flow to the area is restored.

Conditions such as a low platelet count (thrombocytopenia), genetic mutations affecting platelet function (Nurden, 1999) or systemic bacterial infections (thrombocytopenia purpura) (Fitzgerald et al., 2006) can cause excessive bleeding problems and emphasise the importance of platelets in haemostasis.

Platelet morphology

This section will briefly discuss the complex structure and ultrastructure of platelets. Inactive platelets are thought to consist of 3 zones; the peripheral zone, the sol-gel zone and the organelle zone (Figure 1) (Werner and Morgenstern, 1980).

The peripheral zone is mainly associated with the adhesion and aggregation of platelets as it presents receptors for major agonists such as collagen, thrombin, adenosine diphosphate (ADP) and others. It consists of the platelet membrane, cytoskeleton (consisting of actin and myosin filaments) and a glycocalyx coating surrounding the cell fragment.

The sol-gel zone is responsible for the contraction and support of the channel networks of the platelet, the open canalicular system (OCS) and the dense tubular system (DTS) (Behnke, 1967). The OCS is formed by invaginations of the plasma membrane providing a larger surface area during platelet aggregation. This structure is a connected series of channels which enable plasma substances to enter the cytoplasm and platelet products to exit (White and Escolar, 1991). The DTS is a smooth endoplasmic reticulum membrane system present internally in the cytoplasm. The DTS is important for platelet function because it is a receptor-mediated calcium (Ca^{2+}) store (Cutler et al., 1978) essential for initiating morphological changes necessary for platelet aggregation (Ebbeling et al., 1992) and the DTS is a site of cyclooxygenase (COX) expression, an important enzyme involved in thromboxane A_2 (TxA_2) and prostaglandin synthesis (Gerrard et al., 1978, 1976; Laposata et al., 1987).

The organelle zone contains the dense body system consisting of mitochondria, glycogen granules (rare and unknown role - potentially an energy store; (White, 1999)), alpha granules (functions as a metabolic pool containing proteins involved in platelet aggregation and adhesion; (Gerrard et al., 1980; Harrison and Cramer, 1993)), dense granules (functions as a secretary pool containing substances essential for platelet-platelet and platelet-protein interactions; (Dale et al., 2002; de Korte et al., 1990)) and lysosomal granules (unknown role but thought to contain enzymes that resolve haemostatic plugs or assist with endosomal digestion; (Neiman et al., 1987)) (Werner and Morgenstern, 1980).

A

B
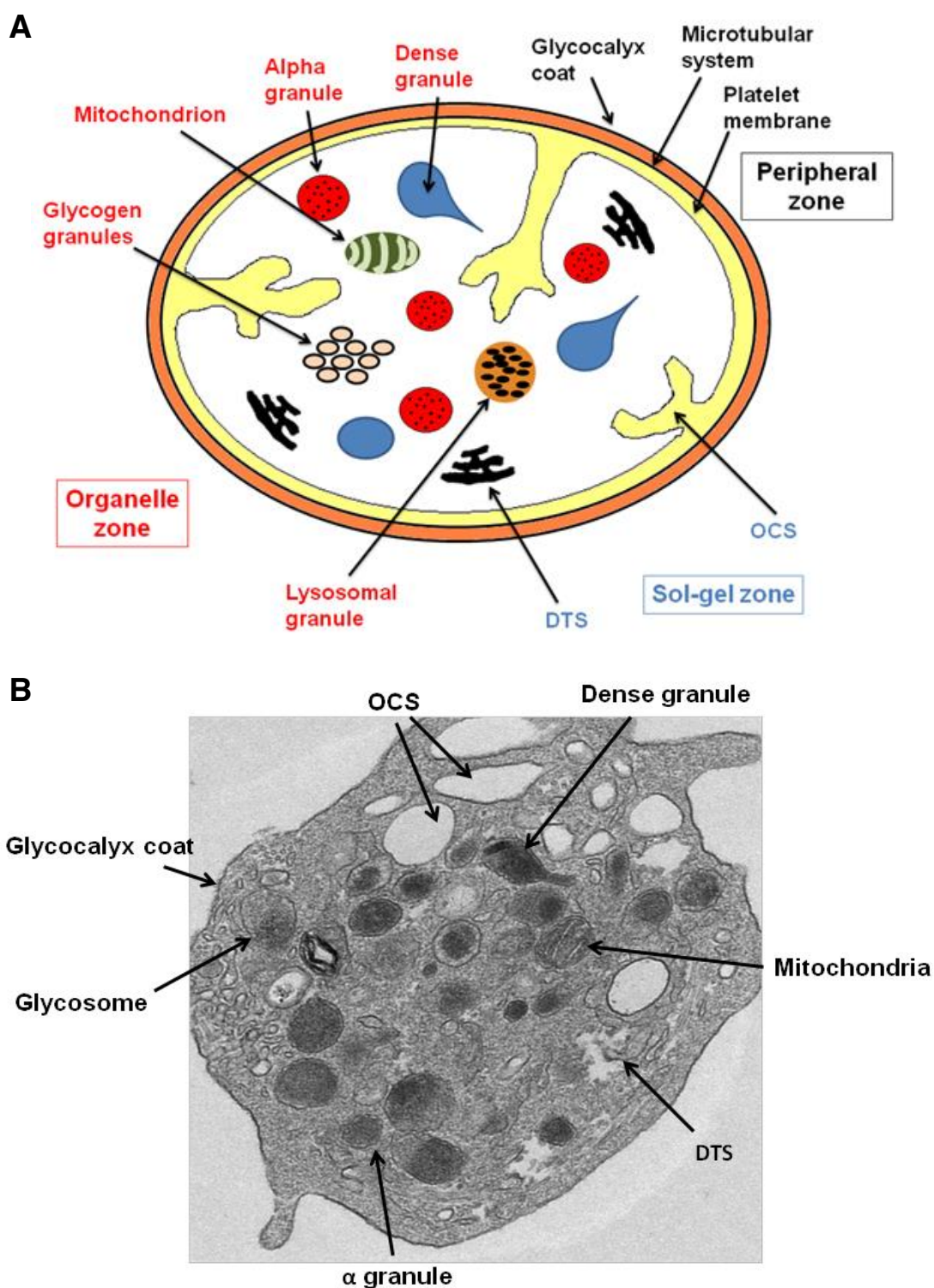
Figure 1: **Platelet morphology.**

A: Basic diagram demonstrating the structure and organisation of platelets. DTS-dense tubular system; OCS-open canalicular system. Adapted from (White, 1979).

B: Electron microscopy image of a platelet from our laboratory (Emerson, unpublished).

Platelet activity

Platelets are complex cells and are equipped with a plethora of receptors for many agonists, antagonists and ligands. Here I will discuss the process by which platelets are activated and recruited to the site of vessel injury and then I will briefly review the signal transduction mechanisms occurring after receptor occupancy. Under normal healthy conditions the platelet rarely interacts with the vessel wall. Platelet-ECM interaction induces a cascade of events known as platelet adhesion, activation and aggregation.

In the instance of a damaged blood vessel, the underlying ECM containing prothrombotic substances, such as collagen and von Willibrand Factor (vWF), become exposed and interact with platelet surface glycoprotein (GP) receptors. Under high shear flow rates ($>1000s^{-1}$) the platelet surface receptor GPIb-V-IX interacts with immobilised vWF (Yago et al., 2008) and possibly other candidates (Jurk et al., 2003) to induce tethering of the platelet to the damaged vessel wall. At lower shear flow rates ($<1000s^{-1}$) platelets are able to bind to additional prothrombotic molecules such as the potent adhesion mediator collagen. Collagen interacts with the tyrosine kinase linked receptor GPVI and integrin $\alpha_2\beta_1$ to induce platelet activation and downstream stable adhesion, forming a monolayer of platelets over the damaged vessel. Activation of the integrin $\alpha_{IIb}\beta_3$ into its high affinity state mediates firm adhesion by binding to immobilised vWF and fibrinogen (Savage et al., 1996). Other integrins such as $\alpha_5\beta_1$ and $\alpha_6\beta_1$ have been reported to have platelet adhesive properties, however their physiological relevance is unclear (Grüner et al., 2003).

Tyrosine kinase mediated signalling initiated by the occupancy of platelet surface receptors such as GPVI promotes platelet activation and shape change. Platelet activation induces release of prothrombotic substances, stored in granules or synthesised upon stimulation, to recruit more platelets to the damaged area, induce amplification of the platelet response and to facilitate platelet-platelet interactions. Secondary agonists (adenosine diphosphate (ADP) and TxA$_2$) released from the activated platelet and endogenous mediators generated by the coagulation cascade (thrombin) can stimulate a range of G-protein coupled receptors to induce further

activation of platelets and ultimately lead to the stabilisation of platelet aggregates. Platelet activation results in 'inside-out' signalling events, which cause integrins, such as $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$, to undergo a conformational change to their high affinity state – a process essential for stable adhesion to the vessel wall and platelet aggregation.

Platelet activation ultimately results in platelet aggregation. Platelet aggregation is a dynamic process which involves platelet-platelet interactions. Activation of platelets induces the emptying of platelet granules to provide essential components for platelet-platelet interactions such as fibrinogen and vWF. Platelets form divalent bonds with other platelets through integrin $\alpha_{IIb}\beta_3$ and, under high shear flow rates, GPIbα (Ruggeri et al., 2006). In addition, thrombin from the coagulation cascade cleaves fibrinogen to fibrin and, in combination with FXIII, forms a stable fibrin mesh (Dickneite et al., 2002). This not only supports the platelet aggregates but also traps blood cells from the circulation to form a stable clot.

Platelet agonists

Platelets have limited ability to synthesise proteins and therefore come well equipped with a plethora of receptors for many ligands. In turn, this makes the platelets highly receptive to their surrounding environment. This section will briefly review the major platelet agonists.

Collagen

There are 28 types of collagens expressed in humans, 7 present in the vessel wall. However, collagen type I and III are the major collagens interacting with platelets (Farndale, 2006; Saelman et al., 1994). Collagen is a potent platelet agonist that is present in the ECM and only interacts with blood cells upon vessel damage. The large blood-borne multimeric glycoprotein vWF can bind to exposed collagen fibres type I, III and IV (Flood et al., 2012), which enables collagen to indirectly interact with platelets *via* the adhesive glycoprotein GPIb-IX-V. Direct collagen receptors include the tyrosine kinase linked GPVI (Gibbins et al., 1997) and integrin $\alpha_2\beta_1$ (Inoue et al., 2003). The binding of platelets to collagen induces platelet activation, which results in the conformational change of integrins *via* 'inside-out' signalling and stable adhesion to the vessel wall.

Thrombin

Thrombin is one of the most potent platelet agonists. The serine protease is produced by cleavage of prothrombin (a liver synthesised protein) by the prothrombinase complex, activated factors Xa and Va (Monroe et al., 2002; Rosing et al., 1980). G-protein coupled protease activated receptors (PARs) are the most established receptors for thrombin on platelets (Kahn et al., 1998). Thrombin activates PARs by cleaving and exposing N-terminus which subsequently acts as a ligand by activating itself (Brass et al., 1992; Vu et al., 1991). PAR activation results in intracellular Ca^{2+} increases, platelet shape change, attenuation of inhibitory platelet responses and ultimately platelet aggregation. In humans there are 2 PAR receptors activated by thrombin; PAR1 and PAR4 (Kahn et al., 1999). PAR4 is only active at high concentrations of thrombin and therefore PAR1 is considered to be the main thrombin receptor (Kahn et al., 1998). See Figure 3 for PAR-1 and PAR-4 G-protein α subunit coupling and downstream events.

Another receptor involved in thrombin-induced platelet activation is the GPIb-IX-V complex, however its mechanism of activation is not well understood. It is known that patients lacking GPIb (Bernard-Soulier disease) have reduced platelet responses to thrombin (Jamieson and Okumura, 1978). Moreover, $GPV^{-/-}$ mice were hypersensitive to thrombin and developed larger thrombi in response to thrombin than W.T mice *in vivo* (Ramakrishnan et al., 2001, 1999).

Adenosine diphosphate (ADP)

ADP is a weak platelet agonist that results in platelet shape change and reversible aggregation (Born, 1962). It acts more as a 'secondary' agonist as it amplifies platelet responses and stabilises thrombus formation (Gachet, 2008). Upon activation, large amounts of ADP are released from platelet dense granules further activating surrounding platelets. ADP acts on the G-protein coupled purinergic (P2) receptors; $P2Y_1$ and $P2Y_{12}$ (Gachet et al., 1995; Wang et al., 2003). The activation of both is necessary to induce full platelet aggregation. $P2Y_{12}$ is the most abundant platelet P2 receptor (Wang et al., 2003) and the target of thienopyridine antiplatelet drugs (i.e clopidogrel) (Gachet, 2005). $P2Y_{12}$ is G_i-coupled and activation of this receptor results in amplification of the aggregatory response by inhibiting adenylate

cyclase activity and cAMP production (Yang et al., 2002). $P2Y_1$ accounts for 20-30% of total ADP binding sites on the platelet surface (Savi et al., 1998). This receptor is expressed on the platelet surface membrane, the membrane of α granules and the open canalicular system (Nurden et al., 2003). $P2Y_1$ is a G_q- coupled protein and therefore activates phospholipase C (PLC), protein kinase C (PKC) and Ca^{2+} mobilisation and influx from external pools to induce major platelet responses (Offermanns et al., 1997; Sage et al., 1990).

Thromboxane (TxA2)

TxA_2 is a prostanoid derived from cyclooxygenase (COX) mediated arachidonic acid metabolism. COX-1 is present in platelets and can synthesise TxA_2 upon platelet activation. TxA_2 is then released in a paracrine fashion exerting its activity as a secondary agonist and amplifying the platelet response. TxA_2 activates G-protein coupled thromboxane receptors (TP) on platelets. TP is coupled to G_{13} which results in platelet shape change (Moers et al., 2003) and G_q which increases cytosolic Ca^{2+} from intracellular stores (Offermanns et al., 1997). The most common antiplatelet therapy, aspirin, irreversibly inhibits COX-1 in platelets and in general attenuates excessive platelet aggregation by blocking platelet TxA_2 synthesis (see 'Common platelet drugs').

Signal transduction in platelets

The previous section discussed common platelet agonists and receptors by which they induce their activity. This section will briefly discuss platelet signalling pathways associated with receptor occupancy and the subsequent effect on platelet function.

Tyrosine kinase mediated signalling

Receptors GPVI and GPIb are associated with immunoreceptor tyrosine-based activation motifs (ITAMs), which induce platelet activation *via* protein tyrosine kinase phosphorylation events (Gibbins et al., 1996; Suzuki-Inoue et al., 2006; Wu et al., 2001). GPVI is currently the best characterised tyrosine kinase signal transduction event (schematic overview of tyrosine kinase signal transduction of GPVI is depicted in Figure 2). GPVI is a member of the immunoglobulin superfamily and has a mucin-like stalk, transmembrane region and short cytoplasmic tail (Horii et al., 2006). Src tyrosine kinases, Fyn and Lyn, are bound to the cytoplasmic tail *via* src homology 3

(SH3) domain (Suzuki-Inoue et al., 2002). The cytoplasmic tail of GPVI is associated with the Fc receptor γ (FcRγ) chain that bears the important signalling region, ITAM. Upon receptor occupancy, Fyn and Lyn are activated and phosphorylate ITAMs present on the FcRγ chain (Gibbins et al., 1996). The FcRγ chain acts as a docking site for the tyrosine kinase Syk *via* its src homology 2 (SH2) domain (Benhamou et al., 1993; Dangelmaier et al., 2005). Syk then phosphoryates and activates the 'Linker for Activation of T-cells' (LAT), which in turn act as a secondary docking site for kinases such as phosphoinositide 3-kinase (PI3K) and phospholipase Cγ2 (PLCγ2) (Gibbins et al., 1998; Gross et al., 1999). Membrane bound PLCγ2 hydrolyses phosphatidylinositol (4,5)-bisphosphate (PIP_2) to inositol (1,4,5)-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 initiates an increase in cytoplasmic Ca^{2+} by binding to IP_3 receptors (IP_3R) present on the DTS (El-Daher et al., 2000). DAG initiates the activation of protein kinase C (PKC) by inducing its translocation from the cytoplasm to the platelet membrane. Increases in intracellular Ca^{2+} and activated PKC initiate major platelet responses such as cytoskeletal assembly (Barkalow et al., 2003), secretion of storage granules (Konopatskaya et al., 2009), the expression of integrins $α_{IIb}β_3$ and $α_2β_1$ in their high affinity state (inside-out signalling) (Bennett and Vilaire, 1979), arachidonic acid mobilisation and phospholipid scrambling (creating a procoagulant surface for clotting factors to bind) (Heemskerk et al., 1997).
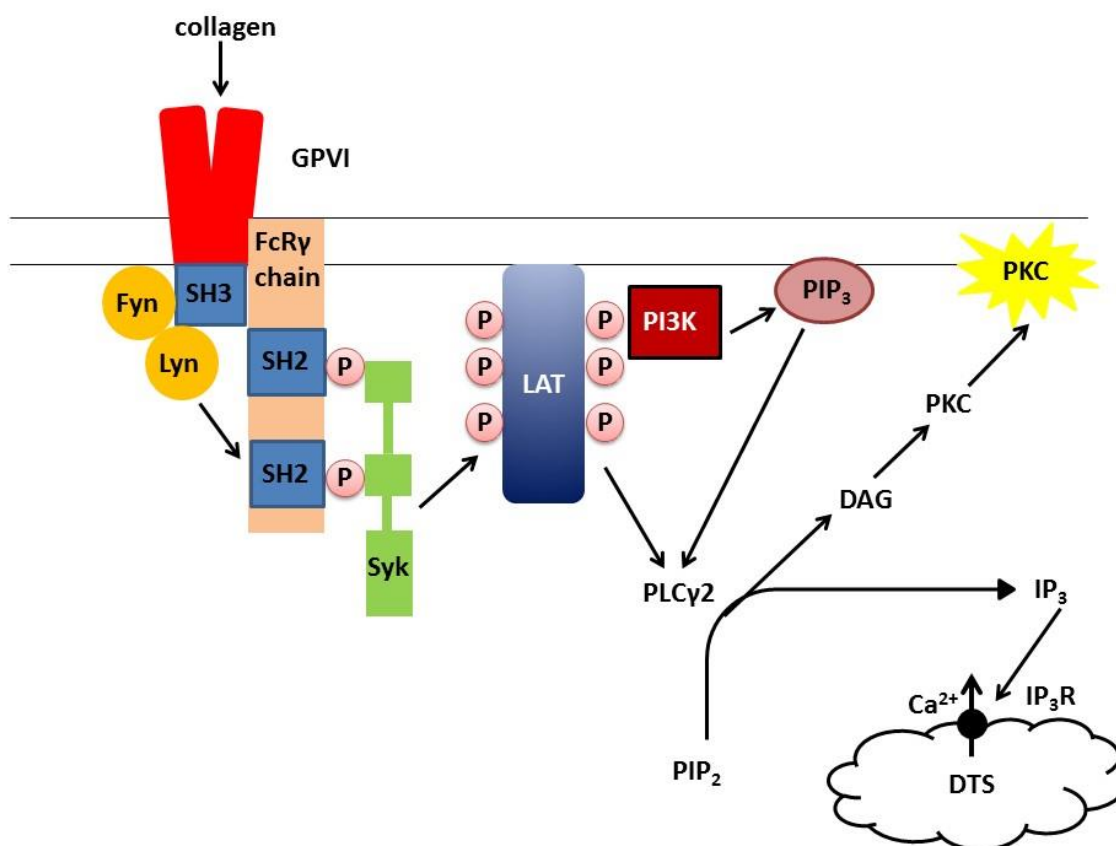
Figure 2: **Tyrosine kinase signal transduction.**

Upon receptor occupancy, src kinases Fyn and Lyn are activated and phosphorylate immunoreceptor tyrosine-based activation motifs present on the associated FcRγ chain. FcRγ acts as a docking site for the tyrosine kinase Syk *via* its src homology (SH) 2 domain. Activated Syk induces a signalling cascade and phosphorylates the 'Linker for Activation of T-cells' (LAT) which acts as a docking site for the recruitment of phosphoinositide 3-kinase (PI3K), phospholipase Cγ2 (PLCγ2) and adaptor proteins. Through the generation of phosphatidylinositol (3,4,5)-trisphosphate (PIP$_3$), among other products, PLCγ2 is recruited and activated. PLCγ2 hydrolyses phosphatidylinositol (4,5)-bisphosphate (PIP$_2$) to inositol (1,4,5)-trisphosphate (IP$_3$) and diacylglycerol (DAG). IP$_3$ induces calcium (Ca^{2+}) release *via* activation of IP$_3$ receptors (IP$_3$R) present on the dense tubular system (DTS). DAG stimulates protein kinase C (PKC) which translocates to the platelet membrane and becomes active. Increases in intracellular Ca^{2+} and PKC activation causes platelet activation and initiates major platelet responses. Image adapted from (Gibbins, 2004).

G-protein coupled receptors

Platelets express a number of G-protein coupled receptors that can induce stimulatory or inhibitory signals. Heterotrimeric G-proteins are composed of α, β and γ subunits, the former bound to guanine nucleotides. Upon activation, the α subunit becomes GTP-bound, dissociates from its β and γ subunits and interacts with downstream effectors (Shen et al., 2012). The α subunit classification determines the

target proteins affected by G-protein coupled receptor activation and downstream signalling events.

The α subunit G_q activates PLC which generates DAG and IP_3 and initiates major platelet responses through increased Ca^{2+} concentration. G_q is coupled to the thrombin receptors PAR-1 and PAR-4, ADP receptor $P2Y_1$ and TxA_2 receptor TP.

The α subunit G_{13} activates the Rho/Rho kinase pathway which regulates myosin light chain phosphatase and results in the phosphorylation of myosin light chain (MLC). Activated MLC enhances actin filament cross-linkage and results in platelet shape change and aggregation (Moers et al., 2003). G_{13} is coupled to the thrombin receptors PAR-1 and PAR-4, TP receptors and fibrinogen receptor $\alpha_{IIb}\beta_3$ (Gong et al., 2010) (also see 'Outside-in signalling').

The α subunit G_i inhibits adenylate cyclase activity and enhances platelet aggregation by lowering intraplatelet cAMP concentration (Jantzen et al., 2001). In addition, G_i has been shown to stimulate PI3K, which results in further Ca^{2+} release and platelet aggregation (Garcia et al., 2010).

The α subunit G_s is inhibitory and is detailed under 'Prostacyclin (PGI_2)' in the 'Physiological inhibitors of platelet function' subsection of this chapter.
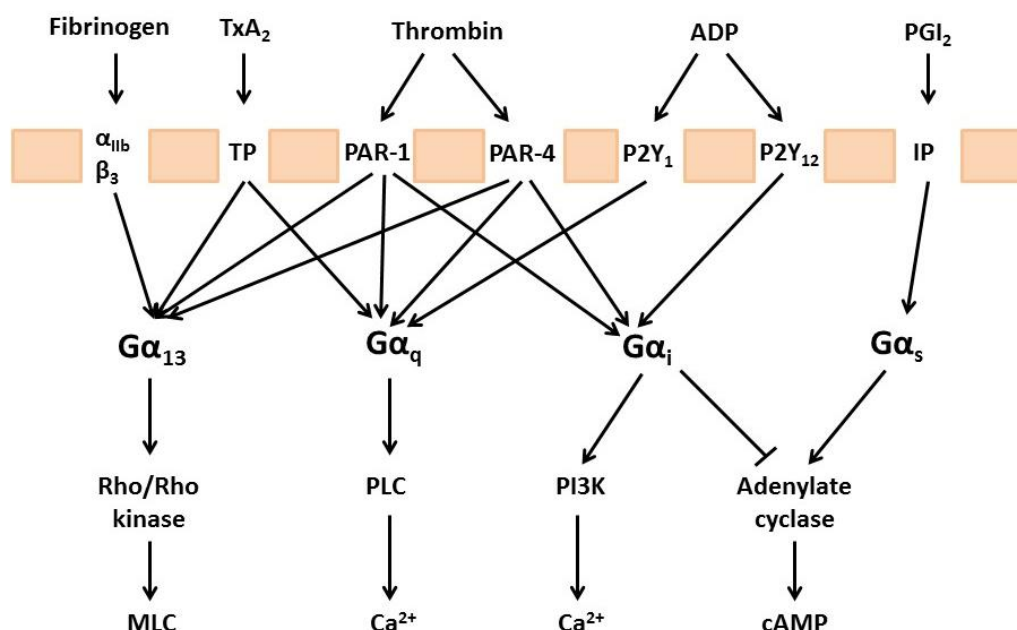
Figure 3: **G-protein coupled receptor signal transduction in platelets**.

Thromboxane (TxA$_2$; TP), thrombin (PAR-1 and PAR-4) and fibrinogen ($\alpha_{IIb}\beta_3$ by outside-in signalling) receptors are coupled to G$_{13}$ that activates Rho/Rho kinase mediated signalling, phosphorylates myosin light chain (MLC) and induces platelet shape change. P2Y$_1$ (by ADP) in addition to TxA$_2$ and thrombin receptors are coupled with G$_q$. G$_q$ activates phospholipase C (PLC) which elevates intracellular calcium (Ca^{2+}) concentration. Thrombin-stimulated PAR-1 and PAR-4 and ADP-stimulated P2Y$_{12}$ activates G$_i$ which stimulates phosphoinositide 3-kinase (PI3K; leading to increased Ca^{2+} concentration) and inhibits adenylate cyclase (attenuating inhibitory cyclic adenosine monophosphate (cAMP) signalling). And finally prostacyclin (PGI$_2$ *via* the prostacyclin receptor (IP)) is coupled to G$_s$ which stimulate inhibitory cAMP signalling through adenylate cyclase activation. Image adapted from (Broos et al., 2011).

Platelet integrins

Platelet integrins are essential for stable adhesion and platelet aggregation. Platelet integrins have bidirectional signalling. Platelet activation is necessary for high affinity integrin binding (inside-out signalling) and ligand binding induces intracellular signalling (outside-in signalling). This section will discuss the activity and function of integrin signalling.

Inside-out signalling

Platelet activation results in increased cytoplasmic Ca^{2+} concentration and PKC activation, which induces the conformational change of integrins from their low affinity to high affinity state. This process is known as inside-out signalling and activates integrins $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$. The activation of integrin $\alpha_{IIb}\beta_3$ was first identified in 1979 and is the best characterised (Bennett and Vilaire, 1979). Therefore inside-out signalling will be discussed in the context of the integrin $\alpha_{IIb}\beta_3$. PKC and 'Ca^{2+}

and diacylglycerol regulated guanine nucleotide exchange factor I' (CalDAG-GEFI; activated by Ca^{2+} and DAG) activate the small GTP binding protein Rap1b (Chrzanowska-Wodnicka et al., 2005). Rap1b forms an 'activation complex' by associating with Rap1-interacting adaptor molecule (RIAM) and talin. This complex activates $\alpha_{IIb}\beta_3$ by regulating cytoskeletal rearrangement and disrupting the interaction between α and β subunits to expose the ligand binding site (Di Minno et al., 1983). Kindlin3 has been reported to be essential for integrin $\alpha_{IIb}\beta_3$ activation by interaction with talin (Moser et al., 2008). The exact mechanism by which kindlin3 and talin interacts is unknown; however, the haematopoietic-restricted adapter protein ADAP has recently been reported to be involved (Kasirer-Friede et al., 2014).
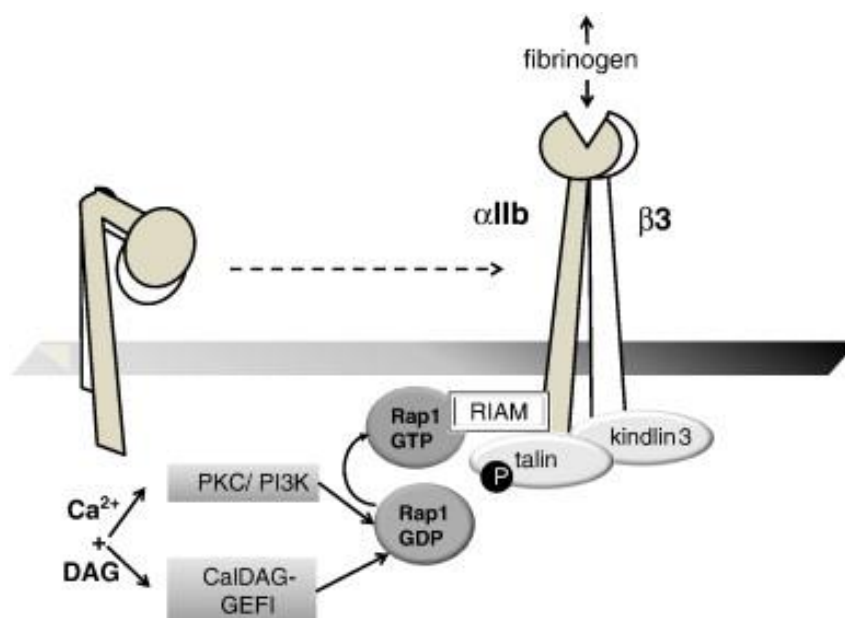
Figure 4: **Activation of integrin $\alpha_{IIb}\beta_3$ by inside-out signalling**.

Platelet activation induces intracellular calcium (Ca^{2+}) increase and diacylglycerol (DAG) synthesis which stimulates protein kinase C/phosphoinositide 3-kinase (PKC/PI3K) and 'Ca^{2+} and diacylglycerol regulated guanine nucleotide exchange factor I' (CalDAG-GEFI) activity. This activates Rap1 which forms a complex with Rap1-interacting adaptor molecule (RIAM) and talin. The 'activation complex' results in cytoskeletal rearrangements and induces a conformational change in integrin affinity from low to high. Kindlin3 is essential in integrin activation but the exact mechanism is unknown. Image taken from (Broos et al., 2011).

Outside-in signalling

Receptor occupancy of integrin $\alpha_{IIb}\beta_3$ triggers signalling known as 'outside-in'. The β_3 subunit bears the conserved integrin cytoplasmic tyrosine (ICY) domain, which can initiate intracellular signalling by tyrosine phosphorylation. The same conserved motif

has been described in many β subunits, which suggests that other integrins can also mediate outside-in signalling (Inoue et al., 2003; Phillips et al., 2001). Outside-in signalling mediated by $\alpha_{IIb}\beta_3$ is necessary for clot retraction and platelet aggregation. Genetically modified mice with point mutations in the ICY domain (DiYF mice) of $\alpha_{IIb}\beta_3$ exhibit an aggregatory and bleeding defect (Law et al., 1999). The β_3 subunit is associated with G-protein α subunit $G\alpha_{13}$ which stimulates SFKs (Gong et al., 2010; Suzuki-Inoue et al., 2007). SFKs promote RhoGAP (Rho GTPase-activating proteins) which accelerates the conversion of RhoGTP to the inactive RhoGDP and promotes cell spreading. Activation of calpain (calcium-dependent protease) cleaves the β_3 subunit and dissociates SFKs (mainly c-src) which promotes Rho activation and induces clot retraction (Flevaris et al., 2007) (Figure 5). In addition, SFKs have been shown to activate Syk and initiate signalling similar to that seen by GPVI tyrosine kinase activation (Boylan et al., 2008).
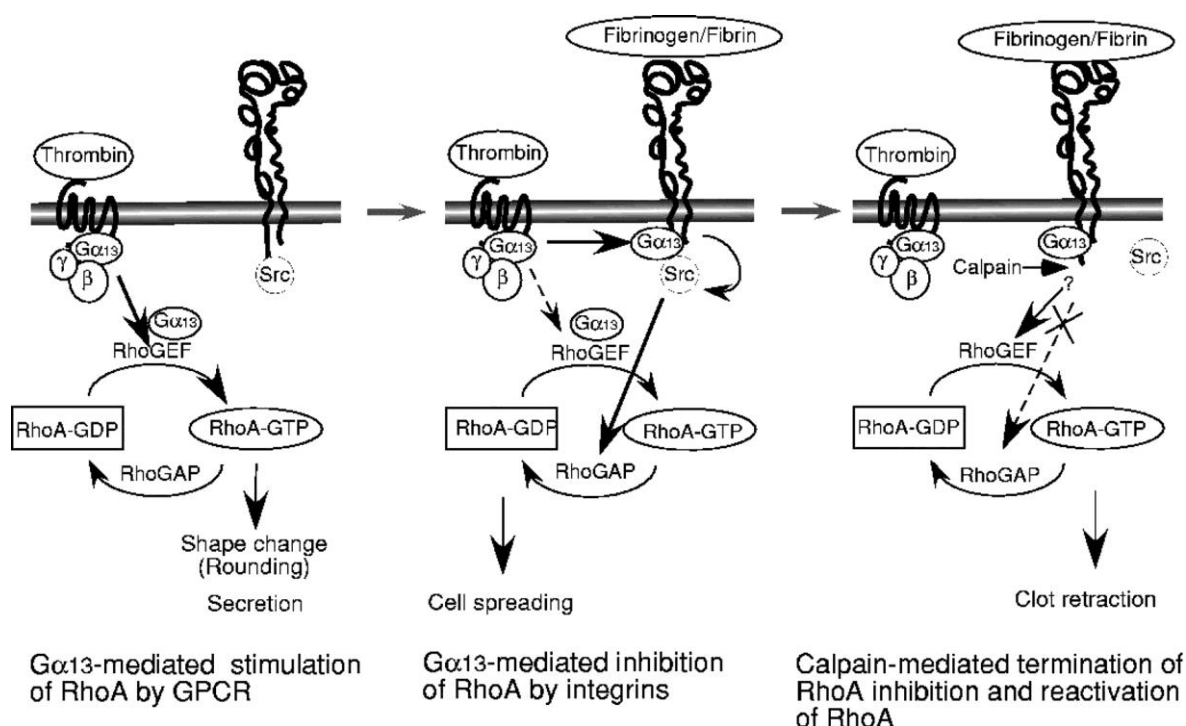
Figure 5: **Outside-in signalling controlling platelet spreading and clot retraction.**

Activation of G-protein $G\alpha_{13}$ promotes platelet shape change and granule secretion by stimulating RhoGEF (Rho guanine nucleotide-exchange factors). Occupancy of the integrin $\alpha_{IIb}\beta_3$ associates $G\alpha_{13}$ with the β_3 subunit, stimulating src family kinases (SFKs) such as c-src and stimulates RhoGAP (Rho GTPase-activating proteins). This accelerates the inactivation of RhoA-GTP and favours cell spreading. The calcium-dependent protease (calpain) acts as a molecular switch to cleave the src binding site of the β_3 subunit and stimulate RhoGEF which reactivates RhoA and induces clot retraction. Image taken from (Li et al., 2010).

Physiological inhibitors of platelet function

Nitric oxide (NO)

NO is synthesised in the vascular endothelium by an enzyme known as endothelial nitric oxide synthase (eNOS) (further detail in 'Enzymic NO sources – nitric oxide synthases' later in this chapter). NO produced by eNOS is released in a paracrine fashion and diffuses into surrounding cells such as vascular smooth muscle cells and platelets (Azuma et al., 1986; Palmer et al., 1987). More recently, other sources of NO affecting platelet function have been elucidated, however this will be addressed later in this chapter. This section will focus on NO signalling in platelets.

cGMP-dependent signalling

NO acts *via* the NO/sGC/cGMP (nitric oxide/soluble guanylate cyclase/cyclic guanosine monophosphate) pathway (Figure 6). The lipophilic nature of NO allows the molecule to diffuse through the platelet membrane and bind to the haem moiety of sGC enzyme (Ignarro et al., 1982). The histidine ligand is displaced and the enzymatic activity of sGC increases by 200-fold (Lawson et al., 2000). sGC catalyses the conversion of GTP to cGMP which can then regulate 2 sets of protein effectors, protein kinase G (PKG; mainly PKGIβ) and phosphodiesterases (PDEs). PDEs act as feedback regulators, promoting or terminating the signal. PKG causes the majority of the effects of NO by phosphorylating a number of proteins that regulate platelet activity. This was established using PKG knockout (PKG$^{-/-}$) mice, as they had increased platelet sensitivity and a lack of response to NO stimulation (Massberg et al., 1999). Platelet functions inhibited by NO include Ca^{2+} mobilisation, cytoskeletal rearrangement, secretion of secondary agonists and platelet adhesion.
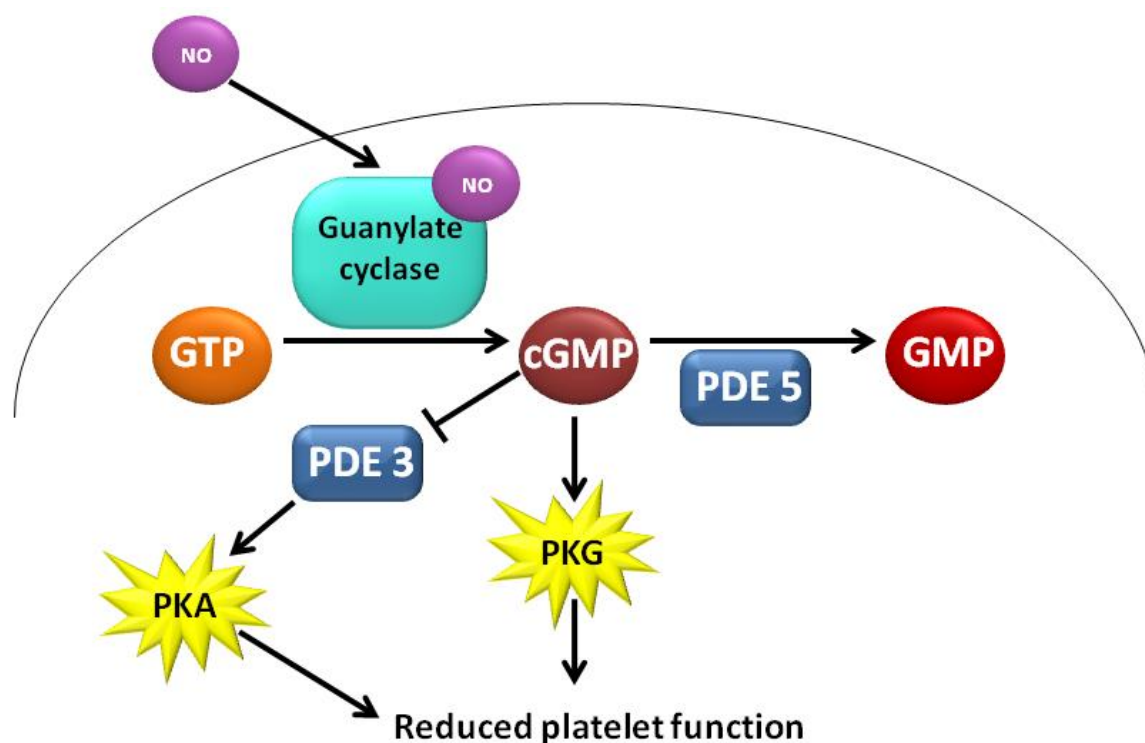
Figure 6: **Schematic diagram of nitric oxide signalling in platelets *via* the NO/sGC/cGMP pathway.**

NO is able to diffuse into the platelet and activate soluble guanylate cyclase. sGC catalyses the conversion of GTP to cGMP which can in turn phosphorylate and activate PKG. cGMP is also able to inhibit PDE3 activity which increases the concentration of cAMP and the phosphorylation of PKA. Both PKG and PKG can phosphorylate a range of targets which reduce platelet activity and negatively regulate platelet function. The effect of cGMP is terminated *via* the PDE5 enzyme which breaks down cGMP to its inactive form and therefore acts as a negative feedback regulator.

NO-nitric oxide; sGC-soluble guanylate cyclase; GTP-guanosine triphosphate; cGMP-cyclic monophosphate; PKG-protein kinase G; PKA-protein kinase A; PDE3-phosphodiesterase 3; PDE5-phosphodiesterase 5; GMP-guanosine monophosphate.

Ca²⁺ mobilisation

Activated PKG phosphorylates the IP₃receptor IP₃R (Cavallini et al., 1996) and inositol-1,4,5-trisphosphate receptor-associated cGMP kinase substrate (IRAG) (Antl et al., 2007) which decreases the release of Ca^{2+} from intracellular stores (Trepakova et al., 1999).

Cytoskeletal rearrangement

NO inhibits platelet shape change by targeting the actinomyosin contractile machinery. Activated PKG phosphorylates vasodilator activated phosphoprotein (VASP) at ser239, ser157 (preferred by PKA) and thr278 (weakly phosphorylated by

both PKG and PKA) which prevents binding to F-actin and suppresses actin polymerisation (Bachmann et al., 1999; Massberg et al., 2004; Smolenski et al., 1998). PKG regulates the phosphorylation state of MLC and is therefore able to modify actin-myosin interactions (Roberts et al., 2009). However, it is unclear whether NO signalling inhibits MLC kinase or activates MLC phosphatase (Somlyo, 2007). Heat shock protein 27 (HSP27) is also a target of PKG which reduces actin polymerisation and contributes to the inhibitory effect of NO (Butt et al., 2001).

Secretion of secondary agonists

Secondary agonists are agonists that enhance platelet recruitment, such as ADP and TxA$_2$. NO decreases ADP signalling by reducing Ca^{2+} mobilisation and preventing activation of PKC, thereby inhibiting dense granule secretion and release of ADP (Durante et al., 1992; Morrell et al., 2005). Activated PKG directly phosphorylates TP receptors and prevents TxA$_2$ activation of downstream signalling events (Wang et al., 1998).

Platelet adhesion and aggregation

NO/cGMP signals are able to block platelet functions that occur after platelet activation. For example, NO exerts no effect on GPVI and GPIb binding (which are able to bind when platelets are inactivated); however, NO is able to block platelet adhesion and aggregation *via* the integrins $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ which are only present in the high affinity state after platelet activation (Graaf et al., 1992; Horstrup et al., 1994; Roberts et al., 2008).

Does NO-cGMP have a stimulatory effect on platelet function?

Agonist-stimulated cGMP increase upon platelet activation raised suspicion that cGMP may have a simulatory role in platelet aggregation (Wu et al., 1993). Du and colleagues found that NO-cGMP had a stimulatory effect on platelet function however this effect was time and concentration-dependent (i.e. stimulatory at low concentrations of NO-cGMP) (Li et al., 2003b). Further investigation identified that PKG inhibitors (KT5823 and Rp-pCPT-cGMP) were able to reverse the platelet secretion-dependent secondary wave of platelet aggregation induced by the vWF modulator ristocetin (Stojanovic et al., 2006). They also found that Akt$^{-/-}$ (mice lacking the serine/threonine protein kinase, Akt) and PKG$^{-/-}$ mice had reduced platelet

secretion and aggregation compared to WT mice (Li et al., 2003b; Stojanovic et al., 2006) and concluded that platelet agonists were able to stimulate NO-cGMP synthesis *via* eNOS through PI3K-Akt signalling. However, this doesn't explain how NO-cGMP signalling can then stimulate and promote platelet secretion and aggregation which remains unresolved. Gambaryan and colleagues have fiercely debated against the stimulatory role of cGMP by 1) disproving that platelets contain eNOS (Gambaryan et al., 2008), 2) demonstrating that $sGC^{-/-}$ mice have normal platelet aggregation responses but are insensitive to NO-induced platelet inhibition (Dangel et al., 2010) and 3) establishing that thrombin does not induce sGC activation, increases in cGMP or VASP phosphorylation (Gambaryan et al., 2012). To date only one research team has identified a stimulatory role for NO-cGMP in platelets and therefore it is generally accepted that NO-cGMP has an inhibitory effect on platelet function.

cGMP-independent signalling

Other cGMP-independent pathways have previously been identified such as reversible protein S-nitrosation and irreversible protein nitration.

Protein S-nitrosation is a posttranslational modification by which NO is covalently attached to the sulphur moiety of a cysteine thiol group (-SH) forming what is known as an S-nitrosothiol (RSNO) (Stamler et al., 1992). *N*-ethylmaleimide-sensitive factor (NSF) (Matsushita et al., 2003; Morrell et al., 2005) and integrin $\alpha_{IIb}\beta_3$ (Oberprieler et al., 2007) are known to be subjected to S-nitrosation which consequently inhibits platelet activity. Interestingly, NO synthesis has been reported to be regulated by S-nitrosation which in turn will impact platelet function. The enzyme responsible for L-arginine catabolism, arginase, can be activated by S-nitrosation at cysteine 303 resulting in decreased bioavailability of L-arginine and therefore NO (Santhanam et al., 2007). NOS itself can undergo auto-S-nitrosation which can negatively regulate NO synthesis (Patel et al., 1996; Ravi et al., 2004).

Protein nitration is an irreversible post-translational protein modification where a nitro group ($-NO_2$) is added to protein tyrosine residues to form 3-nitrotyrosine. Platelet proteins, such as α-actinin, can be nitrated by peroxynitrite resulting in inhibition of actinomyosin contractile machinery and platelet aggregation (Marcondes et al.,

2006). Although not a direct effect on platelets, fibrinogen can be subjected to nitration resulting in accelerated fibrin mesh formation *in vivo* and *in vitro* which then acts by stabilising platelet clots (Parastatidis et al., 2008).

Prostacyclin (PGI$_2$)

Prostacyclin (also known as prostaglandin I$_2$) is a member of the eicosanoid family and a product of the COX pathway. In platelets, prostacyclin binds to the prostaglandin I$_2$ (IP) receptors (Dutta-Roy and Sinha, 1987) which are coupled to the G-protein receptor, G$_s$. G$_s$ is converted to its active GTP-bound form which activates adenylate cyclase (AC) and catalyses the conversion of ATP into cAMP (Figure 7; Paul et al., 1998). cAMP stimulates two effector proteins, PDEs and protein kinase A (PKA type I or PKA type II) (Fetalvero et al., 2007). PKA inhibits platelet function by targeting virtually the same protein targets as PKG causing inhibition of Ca^{2+} mobilisation, modulation of actin cytoskeletal dynamics, decrease in platelet adhesion, granule secretion and aggregation (Smolenski, 2012). To date, there are a few exceptions as PKA has been shown to phosphorylate G$_{13}$ (inhibits RhoA which promotes MLC phosphatase activity (Manganello et al., 2003)), GPIbβ (involved in cell adhesion (Wardell et al., 1989)), filamin-A (provides protection against proteolysis (Chen and Stracher, 1989)) and caldesmon (unknown function – potentially acting as a calmodulin binding protein and inhibiting shape change (Hettasch and Sellers, 1991)) which has not currently been associated with PKG.
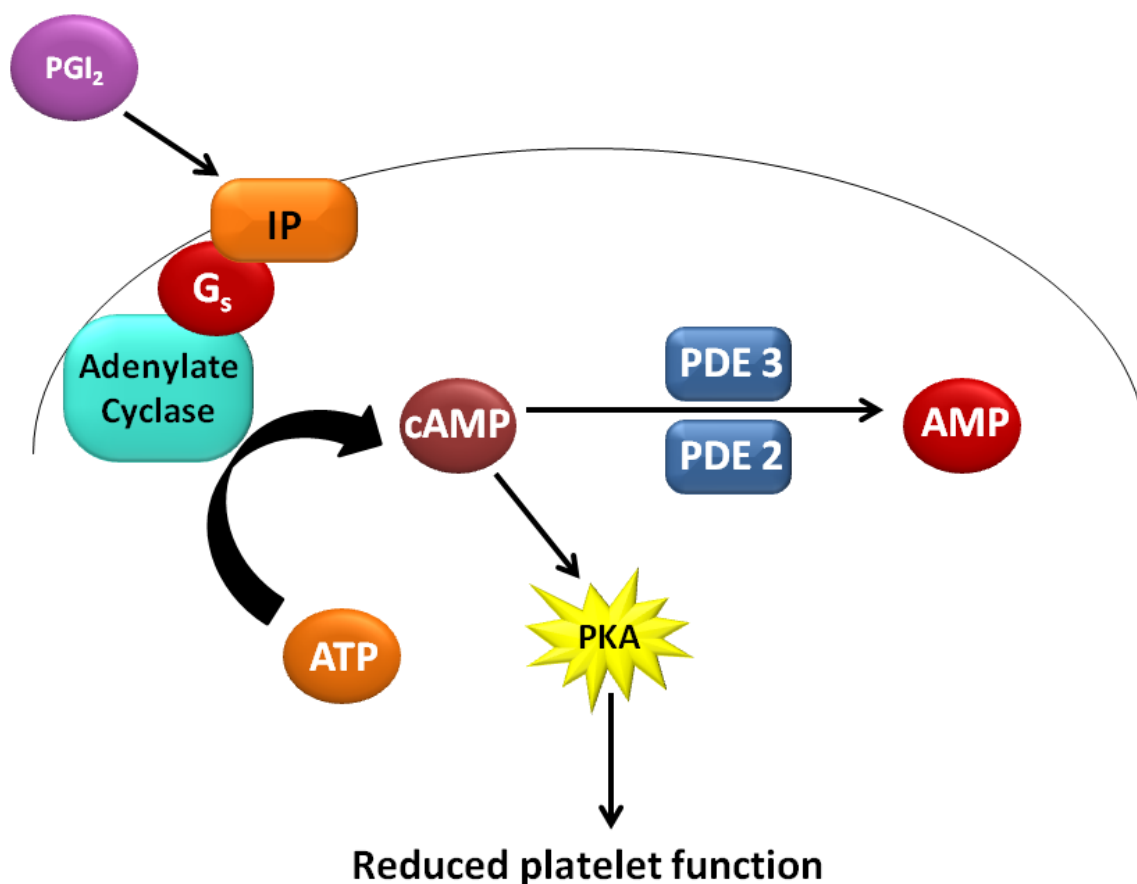
Figure 7: **Schematic diagram of prostacyclin signalling in platelets *via* the IP receptor-mediated signalling.**

PGI_2 released from the vascular endothelium binds to the IP receptor which in turn activates adenylate cyclase. AC catalyses the conversion of ATP to cAMP which can stimulate the activation of PKA. Activation of PKA enables phosphorylation of a range of targets which reduce platelet activity and negatively regulate platelet function. Activation of PDE2 and PDE3 terminates this pathway by breaking down cAMP to its inactive form and therefore acting as a negative feedback regulator.

PGI_2-prostacyclin; IP-prostacyclin receptor; G_s-stimulatory GTP-binding protein; AC-adenylate cyclase; ATP-adenosine diphosphate; cAMP-cyclic adenosine monophosphate; PKA-protein kinase A; PDE2/3-phosphodiesterases; AMP-adenosine monophosphate.

Platelet function in cardiovascular disease

Platelets play a pivotal role in arterial thrombosis, which is a common cause of fatal ischaemic conditions such as myocardial infarction and stroke. Arterial thrombosis is characterised by the inappropriate activation of platelets that lead to the formation of platelet aggregates and blockade of blood vessels. The exact causes of the inappropriate activation of platelets are unclear; however, hypertension (increased shear stress promotes platelet aggregation) and damaged vascular endothelium (reduced production of platelet inhibitors) are believed to be major contributors (Löwenberg et al., 2010). The vascular endothelium is known to regulate platelet function by synthesising and releasing inhibitors of platelet function such as NO and PGI_2 (Nucci et al., 1988) and therefore damage to the vascular endothelium results in the enhanced ability of platelets to aggregate. Atherosclerosis is the formation of fatty deposits in blood vessels (atherosclerotic plaques) which attenuate blood flow and cause endothelial dysfunction (Anderson, 2003). Platelets play a critical role in atherogenesis by releasing inflammatory and immune modulating factors upon activation (Huo et al., 2003). This enables interaction with leukocytes and endothelial cells which induces chronic inflammation and cell proliferation. In fact, P-selectin deficiency in a mouse model of atherosclerosis significantly delayed lesion formation in the aorta (Burger and Wagner, 2003) and highlighted the significant role platelets play in atherosclerotic plaque formation. High shear stress can rupture atherosclerotic plaques and instigate arterial thrombosis by exposing the underlying ECM and initiating platelet adhesion, activation and aggregation (Broos et al., 2011). Overall, an imbalance in positive and negative regulators of platelets can lead to arterial thrombosis which is a major cardiovascular complication that accounts for over 25% of all deaths worldwide (World Health Organisation, 2010). This highlights the need for antiplatelet therapies to reduce the inappropriate activation of platelets and, in turn, reduce the risk of platelet-driven cardiovascular events.

Common platelet drugs

Antiplatelet therapy is the most commonly prescribed medicine to prevent and manage arterial thrombosis. These drugs target common pathways of platelet aggregation such as the synthesis and release of TxA_2 (aspirin), ADP-induced platelet activation (thienopyridines such as clopidogrel) and the activation of integrin

$\alpha_{IIb}\beta_3$ receptor (abciximab). Aspirin is the most commonly prescribed antiplatelet therapy. A meta-analysis of 145 randomised clinical trials showed reduced risk of vascular events and death in 25% of high risk patients (Antithrombotic Trialists' Collaboration, 2002). Aspirin irreversibly antagonises COX-1 by acetylating serine 529 (Roth et al., 1975) which inhibits the synthesis of TxA_2 and amplification of the platelet aggregatory response. Thienopyridines (clopidogrel, ticagrelor, prasugrel) selectively and irreversibly inhibit $P2Y_{12}$ receptors and therefore target and reduce secondary agonist platelet activation. This drug is usually coadministered with aspirin in patients at high risk of thrombosis (e.g acute coronary syndromes and post-percutaneous coronary intervention) (Nagakawa et al., 1990). Integrin $\alpha_{IIb}\beta_3$ inhibitors (abciximab, eptifibatide and tirofiban) are potent antiplatelet drugs that inhibit platelet surface integrin $\alpha_{IIb}\beta_3$ receptors (Ostrowska et al., 2014). These drugs are only given as a short term treatment due to their intravenous administration (Coller, 2001).

Current antiplatelet drugs can induce severe adverse effects such as increased incidence of bleeding, a multitude of effects on the gastrointestinal tract and hepatic dysfunction (Burger et al., 2005). A considerable number of patients still experience cardiovascular events and, due to clinical (pharmacodynamics, poor compliance, unsuitable dose; Biondi-Zoccai et al., 2006), genetic (polymorphisms of cytochrome P450 and target molecules; Jia et al., 2013; Lau et al., 2004) and cellular factors (compensatory mechanisms, pharmacokinetic issues; Halushka et al., 1981), drug resistance has been known to occur in some patients (Angiolillo et al., 2008; Zimmermann and Hohlfeld, 2008). Some of the more potent antiplatelet therapies (such as abciximab) have unsuitable administration routes and safety profiles and therefore can only be taken short-term and under surveillance by a healthcare professional (Gonzalez, 1998). In addition, oral administration of integrin $\alpha_{IIb}\beta_3$ inhibitors has been disappointingly unsuccessful (Chew et al., 2001). These issues highlight the need to find new antiplatelet therapies that will reduce inappropriate platelet activation with minimal adverse effects.

Nitric oxide in cardiovascular function and disease

NO is implicated in many biological processes such as immunology, muscle contraction and neurotransmission. However, a major role of NO is in the cardiovascular system. NO is a small gaseous molecule made up of 1 nitrogen and 1 oxygen atom covalently bound with a half-life of only a few seconds (Lim et al., 2006). NO was first acknowledged in the 1980s as the endothelium-derived relaxing factor (EDRF) due to acetylcholine initiating vasodilation only in the presence of the vascular endothelium (Furchgott and Zawadzki, 1980; Ignarro et al., 1987). NO is produced endogenously by a group of enzymes known as nitric oxide synthases (NOSs). Recent research has uncovered other sources of NO generation such as the bioconversion of dietary inorganic nitrates (Lundberg et al., 2008) and metabolism of S-nitrosothiols (Singh et al., 1996). The physiological importance of these mechanisms in regulating cardiovascular function is unclear (see 'The source of NO' for more information regarding the generation of NO).

NO is known for its cardioprotective properties. NO generation by eNOS within endothelial cells is released into the surrounding tissues such as vascular smooth muscle and the blood. NO activity on vascular smooth muscle cells induces vasodilation (Huang et al., 1995). This enables 'fine tuning' of vessel tone which, in turn, improves cardiac function. NO has been reported to reduce leukocyte adherence to the vessel wall (Kubes et al., 1991; Lefer et al., 1999) which potentially accounts for its protective effect against atherogenesis (Kuhlencordt et al., 2001). Most important for this thesis is the effect of NO on platelet function. NO can inhibit platelet aggregation *in vitro* in whole blood (Yoshimoto et al., 1999) and *in vivo* (Emerson et al., 1999; Freedman et al., 1999; Moore et al., 2010). Therefore NO counteracts atherogenesis and platelet hypersensitivity that could potentially lead to arterial thrombosis.

Due to the cardioprotective effects of NO, defects in its signalling have been linked to cardiovascular disease. In fact an impairment of the NOS pathway is thought to be one of the earliest events in atherogenesis (Kuhlencordt et al., 2001; Napoli and Ignarro, 2001). In vascular disease endothelial NO production is impaired, possibly by an increase in oxidative stress and uncoupling of the eNOS enzyme

(Forstermann and Munzel, 2006). eNOS knockout (eNOS$^{-/-}$) mice have been shown to have hypertension (Huang et al., 1995), hypercoagulability (Freedman et al., 1999), increased susceptibility to atherosclerosis (Kuhlencordt et al., 2001) and increased leukocyte-endothelial interactions (Lefer et al., 1999) compared to the wild type (W.T) control, which are all contributors to cardiovascular disease. Overall, there is a significant body of research indicating that the bioavailability of NO can be reduced in cardiovascular disease (Chirkov et al., 2002; Ekmekçi et al., 2013; Erdmann et al., 2013; Forstermann et al., 1988; Kuhlencordt et al., 2001; Ludmer et al., 1986; Pieper, 1999; Raitakari et al., 2001; Schmidt et al., 2012; Yamashiro et al., 2010) and this demonstrates the appeal of developing drugs that can target NO synthesis, regulation and signalling pathway (as reviewed Huang, 2009; Napoli and Ignarro, 2001).

The source of NO affecting platelet function

As mentioned previously, NO is a major negative regulator of platelet function. This section will cover the sources of NO that are potentially able to modify platelet function.

Enzymic NO sources – nitric oxide synthases

NO is produced by the catalytic conversion of the amino acid L-arginine to L-citrulline by NOS enzymes (Boucher et al., 1992). These enzymes are dimeric flavoproteins which use tetrahydrobiopterin (BH$_4$) as a cofactor and contain a haem group as shown in Figure 8 (Pollock et al., 1991). NO synthesised by NOS is released in a paracrine fashion and diffuses into nearby cells to exert its biological effect (Azuma et al., 1986; Radomski et al., 1987).
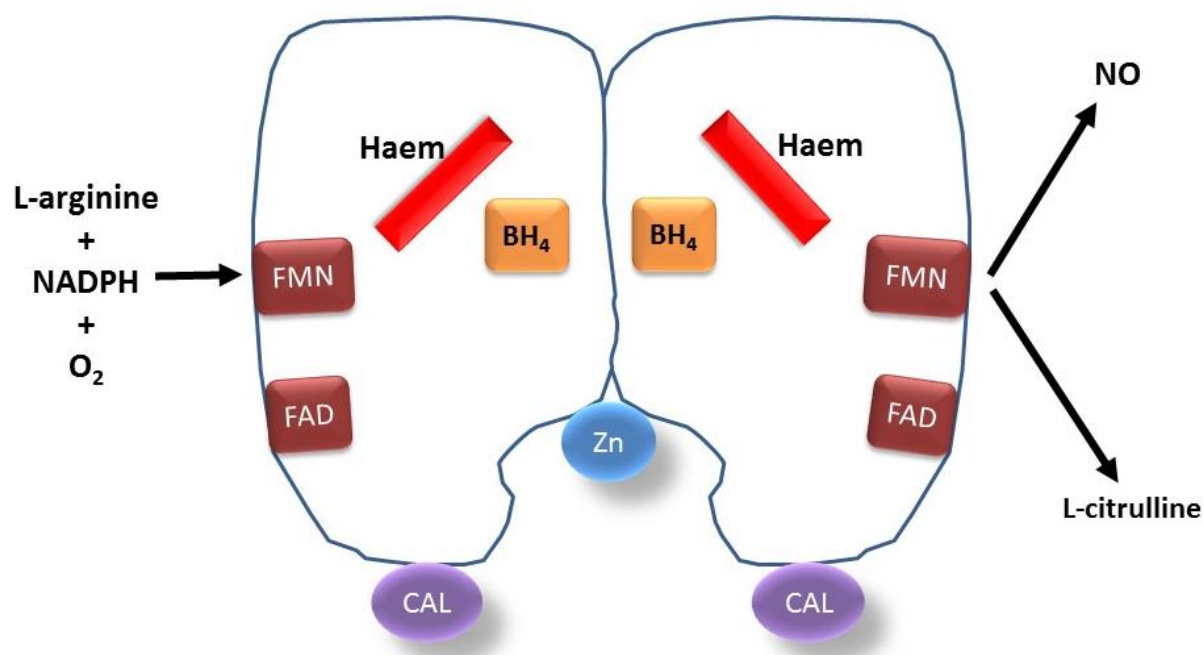
Figure 8: **Schematic diagram of the NOS enzyme.**

NOS enzymes metabolise the conversion of L-arginine and O_2 to L-citrulline and NO, a reaction facilitated by cofactors such as nicotinamide adenine dinucleotide phosphate (NADPH). They are dimeric enzymes that bind to cofactors such as haem groups, tetrahydrobiopterin (BH_4), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and a zinc atom (Zn). On calcium-calmodulin binding (CAL) the enzyme is able to catalyse the reaction to produce NO. Image adapted from (Vallance and Leiper, 2002).

There are 3 known NOS isoforms expressed constitutively or induced when necessary. The isoform inducible NOS (iNOS) is synthesised in response to pathological stimuli and generates NO independent of intracellular Ca^{2+} concentrations (Huang et al., 1998). In contrast, endothelial (eNOS) and neuronal (nNOS) are constitutively expressed and are dependent on calcium-calmodulin binding for the synthesis of NO to occur (Bredt and Snyder, 1990).

NOS provides a major source of NO that affects platelet function. Administration of NOS inhibitors significantly enhanced platelet aggregation *in vivo* in W.T mice (Emerson et al., 1999; Moore et al., 2010). The eNOS isoform is potentially the main contributor to platelet NO. Previous studies reported hypercoagulability (Freedman et al., 1999) and increased platelet aggregation (Moore et al., 2011) in eNOS $^{-/-}$ mice compared to W.T mice. Previous reports have suggested that nNOS can be expressed in arterial vessels after hypoxic events (Ward et al., 2005) and therefore

may contribute to NO signalling in ischaemic conditions such as atherosclerosis. However, work from our group has shown that other forms of NOS (iNOS and nNOS) have negligible effects on *in vivo* platelet aggregation (Moore et al., 2011).

The presence of platelet NOS

Platelets have been reported to generate NO *via* their own form of NOS. Researchers have reported the presence of the L-arginine/NO pathway (Freedman et al., 1997; Radomski et al., 1990) and NO release from platelets *in vitro* (Malinski et al., 1993; Zhou et al., 1995). Furthermore, the hypercoagulability seen in eNOS^{-/-} mice was restored by the transfusion of W.T platelets which suggested that platelets were generating NO by a form of eNOS (Freedman et al., 1999). eNOS protein and mRNA expression has been reported in some publications (Aytekin et al., 2012; Berkels et al., 1997; Mehta et al., 1995; Patel et al., 2006) however, the presence of eNOS in platelets is a subject of fierce debate. Contrary to the above studies, other research groups have reported a lack of eNOS mRNA and protein expression in platelets (Gambaryan et al., 2008; Ozuyaman et al., 2005; Tymvios et al., 2009). In fact, Gambaryan *et al.* (Gambaryan et al., 2008) addressed the issue of contradiction and have published that some commercially available eNOS antibodies inappropriately detect eNOS expression in eNOS^{-/-} mice. Gambaryan *et al.* (2008) emphasise the need to have appropriate positive and negative controls when detecting protein expression, a method adopted by Tymvios *et al.* (Tymvios et al., 2009). All things considered, it is unclear whether platelets generate NO *via* their own form of NOS.

Non-enzymic NO sources

S-nitrosothiols

S-nitrosothiols (also termed thionitrites and RSNOs) are a class of compounds that contain a nitroso group attached to the sulphur atom of sulphydryl compounds collectively known as thiols (chemical structure RSNO). These compounds can be found endogenously as large (S-nitrosoalbumin and S-nitrosohaemoglobin) or small (S-nitrosocysteine (CysNO) and S-nitrosoglutathione (GSNO)) molecules (Gow et al., 2002). RSNOs are produced by the addition (S-nitrosation) or transfer (transnitrosation) of a nitroso group to a reduced sulphydryl group (Figure 9).

$$RSH \xrightarrow[\text{nitrosation}]{NO_x} RSNO \xrightarrow[\text{denitrosation}]{e^-} NO + RSH$$
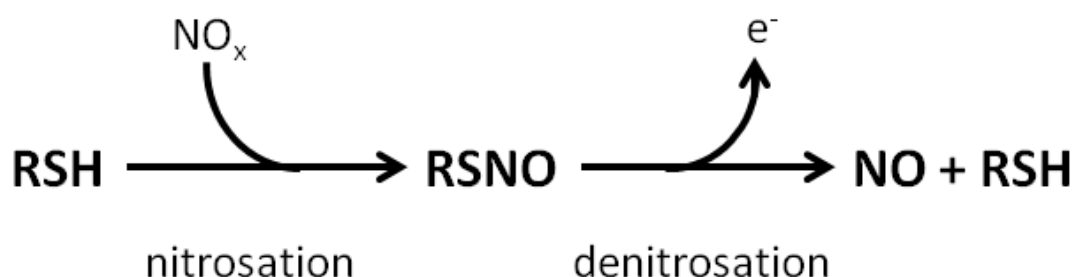
Figure 9: **Schematic diagram of RSNO formation (nitrosation) and metabolism (denitrosation).**

Denitrosation occurs by reduction (removal of an electron) and therefore breakage of the disulphide bond.

RSH – thiol; NO_x – nitrogen oxides; RSNO – S-nitrosothiol; e^- - electron; NO – nitric oxide.

RSNOs have been suggested to act as active intermediates of NO signalling by preserving NO bioavailability and compartmentalising its activity (Matsumoto and Gow, 2011; Myers et al., 1990; Stamler et al., 1992). In fact, RSNOs were a proposed candidate for EDRF (Myers et al., 1990). Therefore RSNOs have attracted research into their therapeutic potential (Richardson and Benjamin, 2002). RSNOs can activate cGMP-independent (Crane et al., 2005) and cGMP-dependent NO signalling (Bell et al., 2007; Ignarro et al., 1980) and subsequent research has validated that RSNOs can act as NO 'reservoirs' (Al-Ani et al., 2006; Chvanov et al., 2006; Diesen et al., 2008; Shah et al., 2007; Singh et al., 1996). Previous work established that spontaneous decomposition of RSNOs and NO production did not correlate with biological activities suggesting the presence of cell-specific mechanisms. Therefore RSNOs cannot be considered to be typical NO donors (Gordge et al., 1998; Mathews and Kerr, 1993). RSNOs are believed to contribute to NO signalling *via* an array of mechanisms that either transport RSNOs into the cell (Riego et al., 2009) or catabolism of the compound by enzymatic or non-enzymatic mechanisms which NO can elicit its activity by diffusing into the cell (Freedman et al., 1995; Root et al., 2004).

A large body of research has proven that RSNO compounds have the ability to inhibit platelet adhesion (Irwin et al., 2009), granule secretion (Morrell et al., 2005), fibrinogen binding (Simon et al., 1993) and aggregation (Mellion et al., 1983; Priora

et al., 2011). The effect on platelet function was proven to be mediated *via* cGMP-dependent and independent NO-related pathways (Irwin et al., 2009; Mathews and Kerr, 1993; Sogo et al., 2000). The inhibitory effect of RSNO compounds on platelets has also been identified *in vivo* (Miller et al., 2003; Vilahur et al., 2004). In fact, GSNO selectively stimulates inhibitory NO signalling in platelets whilst preserving vascular tone *in vivo*, which has brought interest of these compounds as anti-thrombotic agents (de Belder et al., 1994; Xiao and Gordge, 2011). RSNOs are known to be present in platelets and have been suggested to deliver NO by various mechanisms (Hirayama et al., 1999; Mathews and Kerr, 1993). Metabolising enzymes such as the thiol isomerase protein disulphide isomerase (PDI) and membrane transporters such as the amino acid transporter system-L (L-AT) have been proposed as possible mechanisms of RSNO NO-related activity in platelets (Gordge and Xiao, 2010).

Amino acid transporter system-L

L-AT belongs to a family of proteins known as the heterodimeric amino acid transporters. Collectively, they are responsible for the uptake of amino acids from the diet to a wide range of cells in the body (Wagner et al., 2001). It has also been identified that L-AT can transport RSNOs across plasma membranes (Li and Whorton, 2005; Zhang and Hogg, 2004). The ability of L-AT to transport low-molecular weight RSNOs such as CysNO from the extracellular to intracellular space has been well characterised in many cell types (Satoh et al., 1997; Zhang and Hogg, 2005, 2004). Riego *et al.* (Riego et al., 2009) demonstrated that CysNO could increase intracellular cGMP, potentially by stimulation of sGC, by L-AT mediated transport. In platelets, CysNO has been reported to significantly inhibit platelet aggregation which coincided with an increase in sGC stimulation (Mathews and Kerr, 1993); however, the involvement of L-AT-mediated RSNO transport in platelets has not been fully defined.

Protein disulphide isomerase

PDI is primarily an endoplasmic reticulum (ER) protein that regulates disulphide bond modification and therefore can exchange, oxidise or reduce disulphide bonds between thiols. Recent identification has established thiol isomerase expression in

locations other than the ER (Turano et al., 2002; van Nispen Tot Pannerden et al., 2009). For example, inactivated platelets have been identified to express PDI and other thiol isomerases on their cell surface and activated platelets have the ability to release these enzymes from intracellular stores (Chen et al., 1995, 1992; Holbrook et al., 2010). Thiol isomerases regulate platelet function, predominantly *via* integrin-mediated platelet activation (as reviewed in (Cho, 2013)). In fact, PDI function is required for thrombus formation but has limited effect on haemostasis suggesting that PDI may potentially be a good target for antithrombotic therapy (Jasuja et al., 2012; Kim et al., 2013). Blockade of platelet PDI has been reported to inhibit platelet function by $\alpha_{IIb}\beta_3$ (Kim et al., 2013; Manickam et al., 2008) and $\alpha_2\beta_1$ integrin activation (Lahav et al., 2003, 2000) independent of platelet activation. In addition, PDI has been reported to positively stimulate platelets by regulating thrombin-mediated thrombin generation (Jurk et al., 2011) and potentially vWF-GPIbα binding (Burgess et al., 2000).

In contrast to PDI inhibition as a potential antithrombotic target, its reducing ability has been identified as a method of RSNO denitrosation and a mechanism of NO delivery. Previous research validated that inhibition of PDI decreased GSNO-stimulated NO release in WP (Root et al., 2004). Furthermore, the free radical NO was proven to be a product of PDI-GSNO interaction (Sliskovic et al., 2005). Most interestingly, Gordge and colleagues have identified that PDI inhibition reversed the increase in intraplatelet cGMP seen after stimulation with GSNO (Bell et al., 2007; Shah et al., 2007) which provided evidence for the involvement of PDI in NO delivery from administered RSNOs. However, the involvement of PDI in releasing NO from endogenous RSNOs has not been elucidated.

Although the previous work regarding RSNO-derived NO looks promising, the ability of endogenous RSNOs to modulate platelet function by generating NO is unclear and the mechanisms by which NO generation occur are unknown.

Inorganic nitrate and nitrite

Inorganic nitrate, and to some extent nitrite, is present in our diet and found in high concentrations in vegetables (rocket, celery, beetroot) and cured meats. Until the last decade, oxidative metabolites nitrite (NO_2^-) and nitrate (NO_3^-) were considered to be

inert by-products of the eNOS-derived NO pathway. Recent research has reported the bioconversion of nitrate to nitrite and NO as an alternative and potentially valuable source of bioactive NO (as reviewed in (Gilchrist et al., 2010; Kapil et al., 2010)). For centuries, organic nitrates have been known to reduce blood pressure and platelet activity. However, only recently has it been identified that inorganic nitrate from our diet can have vasodilatory and antiplatelet properties (Larsen et al., 2006; Lundberg and Govoni, 2004; Webb et al., 2008b). Dietary inorganic nitrate can reduce *ex vivo* platelet aggregation in healthy human volunteers (Larsen et al., 2006; Richardson et al., 2002; Velmurugan et al., 2013; Webb et al., 2008b). Moreover, W.T mice on a low nitrate-containing diet exhibited a significant increase in *ex vivo* platelet aggregation compared with W.T mice on a normal diet (J. Park et al., 2013). So far, however, there have been no studies investigating the impact of nitrate bioconversion to NO on platelet function *in vivo* and therefore the contribution of NO derived from dietary nitrate in the presence of endogenous NO sources remain unknown.

Mechanism of nitrate mediated bioactivity

Once ingested, nitrate is rapidly absorbed with a high bioavailability of ~100% (van Velzen et al., 2008). Potentially due to specific transporters (Qin et al., 2012), nitrate is retained in the salivary glands and secreted in the saliva. Nitrate-reducing bacteria present on the dorsal surface of the tongue can reduce nitrate to nitrite which is subsequently absorbed into the blood (Govoni et al., 2008). However, the essential involvement of bacteria has been debated as the same research group identified an increase in nitrate-induced plasma nitrite concentration in germ-free mice (Jansson et al., 2008). Nitrite is subsequently reduced to bioactive NO however the mechanism(s) behind nitrite reduction has not been verified. Many possible mechanisms have been reported such as the reduction of nitrite to NO due the acidic environment of the stomach (Benjamin et al., 1994) and in hypoxic conditions (Maher et al., 2008; Webb et al., 2004), the presence of nitrite reducing enzymes (Jansson et al., 2008), the reducing ability of haem proteins (Cosby et al., 2003) and reducing agents such as vitamin C (Gago et al., 2007).

The exact mechanism by which nitrate can inhibit platelet aggregation is unclear. Original experimentation correlated an increase in gastric, but not plasma, S-nitrosothiol concentration with significantly reduced platelet aggregation *ex vivo* in healthy human volunteers after ingestion of nitrate (Richardson et al., 2002). Further studies identified that it was the increase in plasma nitrite that correlated with a decrease in *ex vivo* platelet aggregation and reduced blood pressure (Larsen et al., 2006; Velmurugan et al., 2013; Webb et al., 2008b). In fact, interruption of the enterosalivary circulation (*via* spitting) reversed the increase in plasma nitrite and the inhibitory effect on platelets after ingestion of beetroot juice (a source of nitrate) (Webb et al., 2008b). The nitrate-mediated plasma nitrite concentration and reduced platelet activity have also been observed in mice (Jansson et al., 2008; J. Park et al., 2013). The mechanism by which plasma nitrite was able to inhibit platelet function is more elusive. Previous research has identified that the inhibitory effect of nitrite on platelet aggregation is dependent on the presence of erythrocytes (Srihirun et al., 2012; Velmurugan et al., 2013). Therefore, currently it is believed that nitrite is reduced to NO at the level of the erythrocyte and not the platelet.

Inorganic nitrate/nitrite in cardiovascular disease

A previous study identified that participants with atrial fibrillation had decreased plasma nitrite and platelet cGMP concentrations compared to age matched controls which suggested that the nitrate/nitrite/NO pathway could be dysregulated in cardiovascular disease (Minamino et al., 1997). Interestingly, dietary supplementation with nitrate reversed the metabolic syndrome seen in eNOS$^{-/-}$ mice, an animal model of vascular dysfunction (Carlström et al., 2010). Previous studies have reported that eNOS$^{-/-}$ mice have enhanced platelet activity (Freedman et al., 1999; Moore et al., 2010). It was recently reported that eNOS$^{-/-}$ mice have significantly lower nitrate and nitrite plasma concentrations however this only corresponded with a trend in enhanced platelet aggregation *ex vivo* (J. Park et al., 2013). The effect of nitrate on human platelet aggregation during vascular disease is unknown. In summary, the effects of nitrate on *in vivo* platelet function during endothelial dysfunction are unknown.

Phosphodiesterases in platelets

Phosphodiesterases are enzymes that hydrolyse cAMP and cGMP to cease their activity. There are 11 phosphodiesterase (PDE) enzyme families found in humans. In platelets there are 3 PDE isoenzymes, PDE2, PDE3, and PDE5 (Hidaka and Asano, 1976) that have been identified *via* their substrates, kinetics and sensitivity to inhibitors (Gresele et al., 2011).

Phosphodiesterase 2

To date, one gene has been identified with 3 splice variants (PDE2A1, PDE2A2 and PDE2A3) (Martins et al., 1982). PDE2A is capable of hydrolysing both cAMP and cGMP, but binds with a low affinity (Martins et al., 1982). It was originally named cGMP-stimulated phosphodiesterases because cGMP promotes the hydrolysis of cAMP by PDE2 (Figure 10). cGMP binding to PDE2 stimulates the hydrolysis of cAMP by 10-fold (Grant et al., 1990). The enzyme has shown to be localised in cardiac myocytes, endothelial cells, neurons, the adrenal medulla and platelets (Haslam et al., 1999). PDE2A is a homodimer of 105kDa subunits containing allosteric regulatory sites and catalytic sites (Stroop and Beavo, 1991).
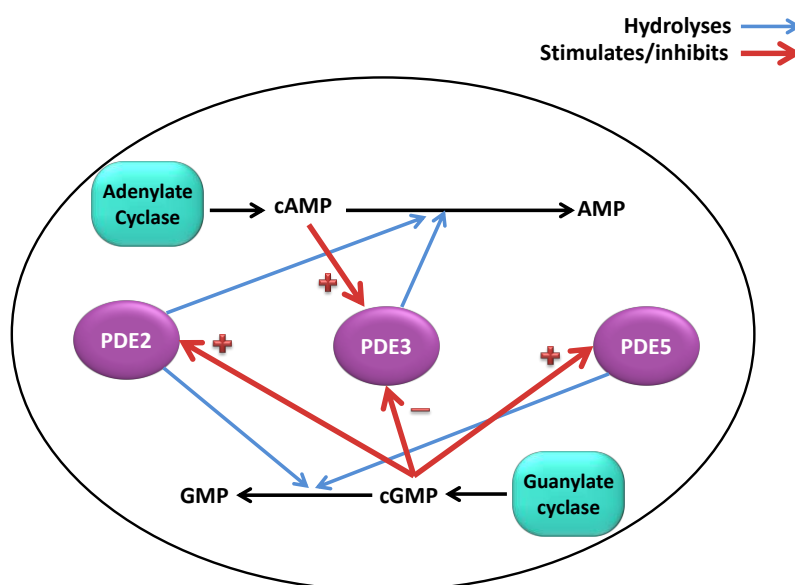
Figure 10: **Schematic diagram of platelet phosphodiesterase crosstalk.**

The blue arrows indicate cyclic nucleotide hydrolysis and the red arrows indicate enzyme modification (positive and negative stimulation) by cyclic nucleotides. Details' regarding how modulation occurs is in the text.

cAMP-cyclic adenosine monophosphate; AMP-adenosine monophosphate; cGMP-cyclic guanosine monophosphate; GMP-guanosine monophosphate; PDE-phosphodiesterase.

Phosphodiesterase 3

Two PDE3 genes have been identified, PDE3A (implicated in cardiac function and fertility; Choi et al., 2001; Masciarelli et al., 2004) and PDE3B (implicated in lipolysis; Reinhardt et al., 1995). PDE3A is found in cardiac muscle, vascular smooth muscle cells, oocytes and platelets (Bender and Beavo, 2006; Grant and Colman, 1984). PDE3 is able to hydrolyse both cGMP and cAMP, however the V_{max} for cAMP is 4-10 fold higher than that for cGMP (Grant and Colman, 1984). In fact, PDE3 was originally named cGMP-inhibited cAMP phosphodiesterases as cGMP competitively binds with cAMP thereby inhibiting its hydrolysis (Grant and Colman, 1984; Tang et al., 1997) (Figure 10). The enzyme is a dimer containing 61kDa subunits. Unique to other phosphodiesterases, PDE3 has a 44 amino acid insert which could be involved in membrane association and inhibitor specificity (Tang et al., 1997). Inhibition of PDE3A potentially could be cardiotonic, antithrombotic and vasodilatory however little clinical success has arisen from these inhibitors (as reviewed in Bender and Beavo, 2006).

Phosphodiesterase 5

As with PDE2, only one PDE5 gene has been identified with 3 splice variants (PDE5A1, PDE5A2 and PDE5A3) (Lin et al., 2000). However, it is unknown which splice variants are expressed in platelets. PDE5A1 and PDE5A2 cDNA have been identified in most tissues and PDE5A3 mainly in smooth muscle. However, PDE5 activity has only been identified in vascular smooth muscle and platelets (Lin, 2004). PDE5 is known to specifically hydrolyse cGMP (Francis et al., 1980; Wallis et al., 1999). Originally named cGMP-binding cGMP-specific phosphodiesterases (cG-BPDEs), cGMP must be bound to both allosteric binding sites to activate the enzyme and allow phosphorylation by PKG or PKA (Corbin et al., 2000; Mullershausen et al., 2003; Rybalkin et al., 2003) (Figure 10). PDE5 is a homodimer of 190kDa with a C-terminal catalytic domain and N-terminal allosteric regulatory domain (Sung et al., 2003; Figure 11).
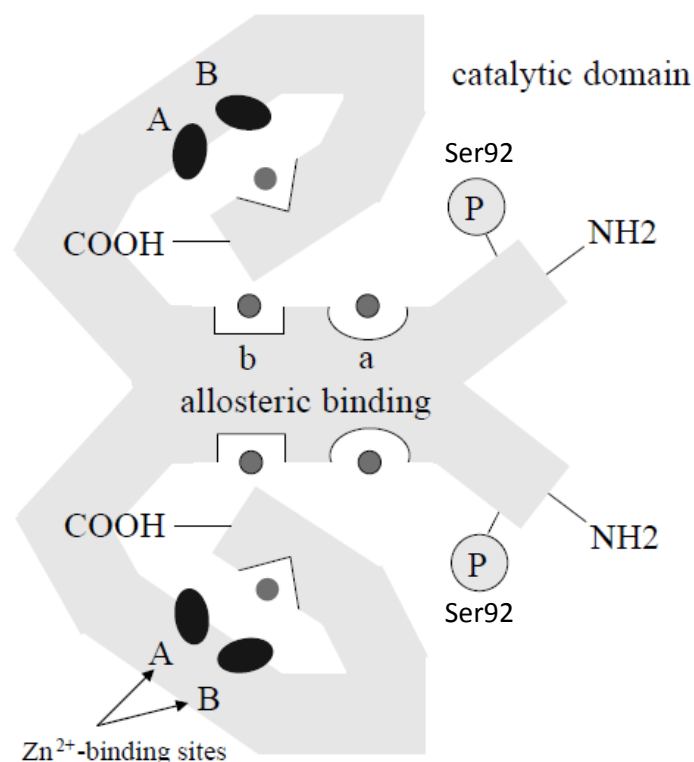
Figure 11: **Schematic representation of phosphodiesterase 5 (PDE5) structure.**

Allosteric binding domains a and b bind cGMP (also known as GAF domain). This allows for phosphorylation of Ser92 by PKG. Taken from (Puzzo et al., 2008).

P-phosphorylation; cGMP-cyclic guanosine monophosphate; PKG-protein kinase G.

Phosphodiesterase 5 (PDE5) inhibitors

PDE5 inhibitors selectively enhance NO signalling by inhibiting negative feedback mechanisms. PDE5 inhibitors are the drug of choice for the treatment of erectile dysfunction (ED) and the class includes sildenafil (Viagra®), vardenafil (Levitra®) and tadalafil (Cialis®) (Toque et al., 2008). This section will discuss the mechanism of action, pharmacokinetics and therapeutic uses of PDE5 inhibitors.

Sildenafil

Sildenafil (originally called UK-92,480) was the first PDE5 inhibitor developed by Pfizer European research laboratories in Sandwich, UK, which was originally investigated as a treatment for hypertension and angina (Jackson et al., 2005). After unsuccessful clinical trials it was noted that sildenafil was an effective treatment for ED (Boolell et al., 1996; Terrett et al., 1996); a condition known to affect 1 in 10 men

worldwide. Sildenafil, under the trade name Viagra®, received approval from the Food and Drug Agency (FDA) and European Medicines Evaluation Agency (EMEA) in 1998 (Ghofrani et al., 2006). Viagra® is now the most widely used drug for the treatment of ED.

Mechanism of action

The structure of sildenafil is very similar to that of cGMP as it bears the same guanine ring (Figure 12). Corbin and colleagues demonstrated that sildenafil competitively binds to the catalytic site on the PDE5 enzyme and therefore inhibits the breakdown of cGMP to its inactive form GMP (Corbin et al., 2003).
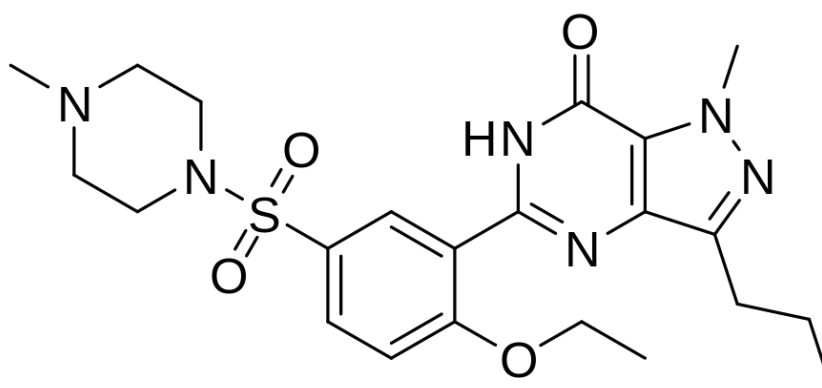
Figure 12: **Basic chemical structure of sildenafil.**

Sildenafil is a heterocyclic compound with a pyrazolo-pyrimidinone nucleus. It is structurally very similar to cGMP because it has the same pyrazolo-pyrimidinone nucleus (5 member pyrazolo ring and 6 member pyrimidinone ring) as the guanine ring present in cGMP (Francis and Corbin, 2005).

As demonstrated in the signalling pathway, sildenafil selectively inhibits PDE5 enzymes, blocking the breakdown of cGMP and therefore prolonging its activity and amplifying the NO signal (Corbin et al., 2003; Terrett et al., 1996; Figure 13). Other mechanisms of action have been identified as sildenafil is thought to improve NO signalling by activating phosphatidylinositol 3-kinase (PI3K)/Akt and thereby activating eNOS by phosphorylation (Musicki et al., 2005). Most interestingly, a recent study by Bivalacqua et al. (2013) has proven that sildenafil is able to reduce oxidative/nitrosative stress in cardiovascular disease by reversing the uncoupling of eNOS and restoring NO signalling. The mechanism by which this occurs is to be determined.
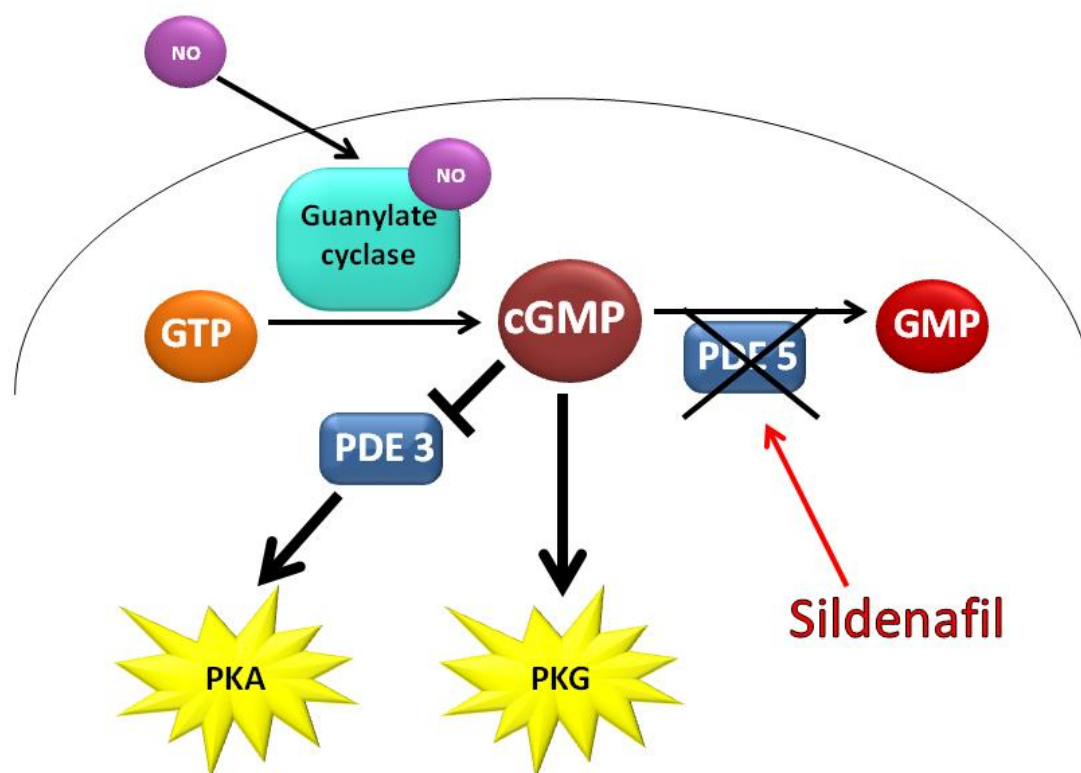
Figure 13: **Schematic diagram of the mechanism of action of sildenafil.**

Sildenafil inhibits PDE5 which stops the breakdown of cGMP to its inactive form and therefore enhances NO signalling by prolonging the activity of cGMP. cGMP increases the activation of PKA and PKG which can phosphorylate many targets and induce an inhibitory response.

NO-nitric oxide; GTP-guanosine triphosphate; cGMP-cyclic monophosphate; PKG-protein kinase G; PDE5-phosphodiesterase 5; GMP-guanosine monophosphate; PKA-protein kinase A; PDE3-phosphodiesterase 3.

Pharmacokinetics

Sildenafil is a relatively lipophilic drug that is completely absorbed from the gastrointestinal tract after oral administration (Boolell et al., 1996). Maximal plasma concentrations of ~200-610 ng mL^{-1} (~0.5 - 1µM) are reached at ~1.5 hours after a single oral dose of sildenafil and ~96% of the drug is bound to plasma proteins (Muirhead et al., 2002; Walker et al., 1999). The majority of sildenafil is metabolised before it is excreted (Walker et al., 1999). Sildenafil is metabolised by N-demethylation by the cytochrome P450 enzymes CYP2C9 and CYP3A4 present in the liver (Hyland et al., 2001; Warrington et al., 2000). Sildenafil has a terminal elimination half-life of ~4 hours (Muirhead et al., 2002).

Therapeutic uses

Sildenafil will exert an effect where PDE5 is expressed and therefore its therapeutic use has concentrated on its effect on vascular beds. In fact, sildenafil may potentially become the treatment of choice for a range of conditions that result in regional blood supply deficiencies (Ghofrani et al., 2006).

Sildenafil was first approved for the use in erectile dysfunction (ED) in 1998. The PDE5 inhibitor was the first oral therapy that acts by enhancing natural endogenous signalling. Erectile function is dependent on a psychogenic stimulus which releases NO from non-adrenergic non-cholinergic nerves causing smooth muscle relaxation and increase in blood flow in the corpus cavernosum (Rajfer et al., 1992). The increase in blood flow induces shear stress which stimulates more NO production by eNOS (Hurt et al., 2002). In cardiovascular disease, eNOS function is impaired which can lead to ED and therefore Viagra® can enhance NO signalling by inhibiting the breakdown of cGMP and combating ED (Ballard et al., 1998).

Following this, PDE5 was found to be highly expressed in lung tissue (Corbin et al., 2005). In 2005, sildenafil was approved by the FDA and EMEA for the symptomatic relief of Pulmonary Arterial Hypertension (PAH) under the trade name Revatio® (Ghofrani et al., 2006). PAH is a fatal condition that is caused by increased vascular resistance in the pulmonary arteries and may lead to right ventricular heart failure. Revatio® administration enhances NO signalling, cGMP concentration and smooth muscle relaxation and therefore reduces pulmonary vascular resistance and provides more surface area for gas exchange (Corbin et al., 2005; Rubin et al., 2011).

Other potential uses for sildenafil that are currently under research are for the treatment of Raynauds Phenomenon (Fries et al., 2005), altitude sickness (Ghofrani et al., 2004; Richalet et al., 2005), Chronic Obstructive Pulmonary Disease (COPD) (Alp et al., 2006) and for the prevention of ischaemia-reperfusion injury (Gori et al., 2005; Ockaili et al., 2002).

Other PDE5 inhibitors

Since the development of sildenafil, 2 more PDE5 inhibitors have been developed and approved for use in ED, vardenafil hydrochloride and tadalafil (Figure 14).
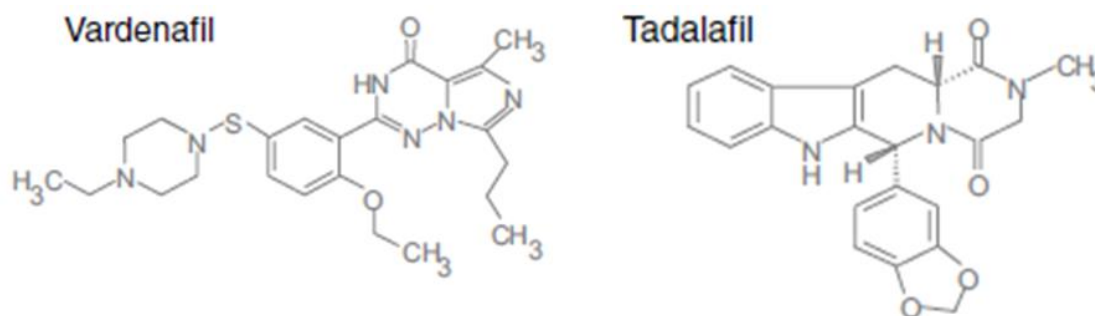
Figure 14: Chemical structures of phosphodiesterase 5 inhibitors.

A: vardenafil hydrochloride B: tadalafil. Adapted from (Wright, 2006).

Vardenafil is similar in structure to sildenafil and hence has very similar pharmacokinetics, efficacy and toxicity profiles (Klotz et al., 2001). The active metabolite of vardenafil only accounts for 7% of total pharmacological activity (compared to sildenafil's 20%) and therefore may be more beneficial in patients with slower metabolism (Bischoff, 2004). It is worth noting that vardenafil may have additional activities by directly blocking store-operated Ca^{2+} channels (Toque et al., 2008) however the pharmacological and physiological importance of this is yet to be established.

Tadalafil was developed as a long-term treatment of ED to allow more spontaneous erectile function by providing the patient with a longer therapeutic window of activity. Tadalafil has a very different chemical structure which is reflected in its different pharmacokinetic properties compared to sildenafil and vardenafil. The most prominent characteristic of tadalafil is that it has a half-life of 17.5 hours (Young et al., 2005).

The effect of PDE5 inhibition on platelets has been somewhat controversial. There are few studies that investigate the effects of vardenafil and tadalafil on platelet function and therefore I used the best characterised PDE5 inhibitor sildenafil citrate in this study.

Could sildenafil be used as an antiplatelet drug?

NO is an important physiological inhibitor of platelet function. Deficiency in NO signalling can cause an increase in platelet activity and the risk of arterial thrombosis (Erdmann et al., 2013; Freedman et al., 1999; Minamino et al., 1997; Moore et al., 2010). Therefore enhancing the NO pathway may be a good therapeutic approach to reduce the risk of platelet-driven cardiovascular events. The PDE5 inhibitor sildenafil improves NO signalling by antagonising the breakdown of cGMP and amplifying the NO signal. Despite the fact that platelets contain high concentrations of PDE5 enzyme, the effect of sildenafil on platelets has been largely ignored. The current literature has reported a prothrombotic effect, inhibitory effect and lack of effect of sildenafil on platelet function. This section will review the previous work and controversies surrounding the effect of sildenafil on platelets.

Since sildenafil has been approved for the treatment of ED there have been concerns regarding increased risk of ischaemic cardiovascular complications in patients taking sildenafil (Arora et al., 1999; Kekilli et al., 2005; Morgan et al., 2001). However, these were case reports based on individual observations and did not take into account that ED is usually caused by underlying vascular disease. Since, a series of clinical trials has proven that sildenafil does not increase patient risk of cardiovascular events, however this debate appears to remain unsolved (Jackson et al., 2006). A few studies have supported the pro-thrombotic effect of sildenafil by demonstrating that NO/PKG activity has a biphasic stimulatory effect on platelets *in vitro* (Blackmore, 2011; Li et al., 2003b). However, further investigations have identified that the stimulatory effect only occurs in certain conditions such as low cGMP concentrations (Stojanovic et al., 2006) and other groups have heavily disputed that cGMP can be stimulatory (Gambaryan et al., 2012, 2008).

Some studies have identified a lack of effect of sildenafil on platelet function. Sildenafil administration had no adverse effect on bleeding time (Morales et al., 1998) and, the authors concluded, no effect on platelet function *in vivo*. However, the relevance of bleeding time to platelet function is debatable. The majority of *in vitro* studies have reported that sildenafil enhances NO-induced inhibition of platelet aggregation but has no inhibitory effect on platelet activity in the absence of a NO

donor (Dunkern and Hatzelmann, 2005; Gudmundsdóttir et al., 2005; Schwarz et al., 2007; Toque et al., 2008; Wallis et al., 1999; Wilson et al., 2008). This highlights the need to investigate the effects of sildenafil on platelet function *in vivo* in the presence of endogenous physiological inhibitors such as NO produced from the vascular endothelium.

In contrast to the above findings, a significant body of research that has identified the antithrombotic and cardioprotective effects of NO/cGMP signalling (Carlström et al., 2010; Emerson et al., 1999; Forstermann and Munzel, 2006; Freedman et al., 1999; Huang, 2009; Tymvios et al., 2009). Clinical studies have reported the inhibitory effect of sildenafil on platelets *ex vivo*. Berkels *et al.* (Berkels et al., 2001) demonstrated that orally administered sildenafil in healthy human subjects significantly inhibited collagen-induced, but not ADP-induced, platelet aggregation *ex vivo*. In support of this, Halcox *et al.* (Halcox et al., 2002) identified that sildenafil inhibited surface expression of activated integrin $\alpha_{IIb}\beta_3$ in unstimulated and ADP-stimulated platelets *ex vivo*. Another interesting observation was that PAH patients treated with sildenafil exhibited decreased platelet function. The study has shown that 17 out of 21 patients participating in research had abnormal PFA-100 closure times, an *in vitro* measure of platelet function (Ma et al., 2011). Other markers of platelet inhibition such as *in vitro* intracellular Ca^{2+} studies have shown that pre-treatment of platelets with sildenafil can inhibit thrombin-induced intracellular Ca^{2+} mobilisation (Wilson et al., 2008), once again supporting that sildenafil has an inhibitory effect on platelets.

Although the majority of research suggests sildenafil has an inhibitory effect on platelet function, the effect of sildenafil on platelet function *in vitro* and *in vivo* is unclear. Given that the therapeutic uses of sildenafil are broadening, there is a crucial need to fully understand the effect of sildenafil on platelet function. The study presented here will investigate the antithrombotic potential of sildenafil by assessing its effect on platelet function *in vitro* in isolated platelets and *in vivo* in the presence of an intact vascular endothelium.

Objectives and aims of this thesis

Inappropriately activated platelets are a major cause of fatal ischaemic cardiovascular events. Current antiplatelet therapies are limited by the variability of the patient response and adverse events. Imbalances between positive and negative regulators of platelet function are believed to be the pathogenesis behind these 'hypersensitive' platelets. NO, a major negative regulator of platelet function, is impaired in cardiovascular disease potentially due to damage of the endothelial cells. This thesis investigated the therapeutic potential of targeting and enhancing NO signalling in platelets to prevent platelet-driven cardiovascular events. The broad hypothesis of this thesis was that enhanced NO/cGMP signalling in platelets would exert an antiplatelet effect during vascular dysfunction whilst preserving normal platelet function in vascular health. The PDE5 inhibitor, sildenafil citrate, would have therapeutic potential as an antithrombotic agent by enhancing NO/cGMP signalling generated by enzymic and non-enzymic NO sources.

The aims of this thesis were to:-

- Determine the effect of sildenafil citrate on platelet function *in vitro* and *in vivo*.
- Investigate the mechanism of action of sildenafil on platelet activity.
- Investigate the upstream sources of NO modifying platelet function by using sildenafil as a pharmacological tool.
- Determine the functional significance of NO/cGMP platelet signalling during vascular health and in endothelial dysfunction.

This study was performed using a range of *in vivo* and *in vitro* techniques to investigate functional, molecular and biochemical effects. A range of pharmacological interventions and animal models was utilised to further dissect mechanistic pathways and therapeutic potential.

Chapter 2: Materials and Methods

Materials

Compound	Supplier
111Indium Oxine (In^{111})	GE healthcare (Bucks, UK)
1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ)	Sigma-Aldrich (Poole, UK)
3-isobutyl-1-methylxanthine (IBMX)	Sigma-Aldrich (Poole, UK)
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Sigma-Aldrich (Poole, UK)
Acid citrate dextrose (ACD)	Sigma-Aldrich (Poole, UK)
Acrylamide-Bis (30%, 1:37.5)	Sigma-Aldrich (Poole, UK)
Adenosine diphosphate (ADP)	Sigma-Aldrich (Poole, UK)
Ammonium persulfate (APS)	Sigma-Aldrich (Poole, UK)
Anti-rabbit horseradish peroxidase-conjugated antibody	Dako (Cambridgeshire, UK)
Apyrase	Sigma-Aldrich (Poole, UK)
Ascorbic acid	Sigma-Aldrich (Poole, UK)
Bacitracin	Sigma-Aldrich (Poole, UK)
Bovine serum albumin (BSA)	Sigma-Aldrich (Poole, UK)
Citric acid	Sigma-Aldrich (Poole, UK)
Collagen (type I)	Takeda Pharmaceuticals International (Linz, Austria)
D-arginine	Sigma-Aldrich (Poole, UK)
DC protein reagent assay	Bio-rad (Hercules, USA)
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich (Poole, UK)
D-Leucine	Sigma-Aldrich (Poole, UK)
ECL plus detection kit	Thermoscientific (Basingstoke, UK)
GAPDH antibody	Santa Cruz (California, USA)
Glucose	Sigma-Aldrich (Poole, UK)
Glycine	Sigma-Aldrich (Poole, UK)
Haemoglobin (from bovine blood)	Sigma-Aldrich (Poole, UK)
Hydrochloric acid	Sigma-Aldrich (Poole, UK)
Hydroxocobalamin hydrochloride	Sigma-Aldrich (Poole, UK)
Iloprost	Cayman Chemicals (Washington, USA)
Lactate dehydrogenase assay kit	Roche (West Sussex, UK)
L-arginine	Sigma-Aldrich (Poole, UK)
L-Leucine	Sigma-Aldrich (Poole, UK)
Magnesium chloride (MgCl$_2$)	Sigma-Aldrich (Poole, UK)
Mercury dichloride	Sigma-Aldrich (Poole, UK)
Methanol	Sigma-Aldrich (Poole, UK)
Methyl acetate	Sigma-Aldrich (Poole, UK)
Milk powder (non-fat)	Marvel (Knighton, UK)
Nitrate/Nitrite Colorimetric Assay Kit	Cayman Chemicals (Washington, USA)
Nu-PAGE LDS sample buffer	Life Technologies (Paisley, UK)
Nω-Nitro-D-arginine methyl ester hydrochloride (D-NAME)	Sigma-Aldrich (Poole, UK)

Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME)	Sigma-Aldrich (Poole, UK)
Para-nitrophenylphosphate (pNPP)	Sigma-Aldrich (Poole, UK)
Phosphatase inhibitor cocktail 1	Sigma-Aldrich (Poole, UK)
Phosphatase inhibitor cocktail 3	Sigma-Aldrich (Poole, UK)
p-nitrophenylphosphate	Invitrogen (Paisley, UK)
Potassium chloride (KCl)	Sigma-Aldrich (Poole, UK)
Protease inhibitor - cOmplete ULTRA Tablets, Mini, EASYpack	Roche (West Sussex, UK)
RIPA buffer	Sigma-Aldrich (Poole, UK)
Sildenafil citrate	Pfizer (Peapack, NJ, USA)
Sodium bicarbonate (NaHCO$_3$)	Sigma-Aldrich (Poole, UK)
Sodium chloride (NaCl)	Sigma-Aldrich (Poole, UK)
Sodium dodecyl sulphate (SDS)	Sigma-Aldrich (Poole, UK)
Sodium hydroxide (NaOH)	Sigma-Aldrich (Poole, UK)
Sodium nitrate	Sigma-Aldrich (Poole, UK)
Sodium nitrite	Sigma-Aldrich (Poole, UK)
Sodium nitroprusside (SNP)	Sigma-Aldrich (Poole, UK)
Sodium phosphate dibasic dodecahydrate (NaHPO$_4$)	Sigma-Aldrich (Poole, UK)
Tetramethylethylenediamine (TEMED)	Sigma-Aldrich (Poole, UK)
Thrombin	Sigma-Aldrich (Poole, UK)
Tris(2-carboxyethyl)phosphine hydrochloride (TCEP HCl)	Sigma-Aldrich (Poole, UK)
Trisodium citrate	Sigma-Aldrich (Poole, UK)
Triton-X	Sigma-Aldrich (Poole, UK)
Trizma base	Sigma-Aldrich (Poole, UK)
Tween 20	Sigma-Aldrich (Poole, UK)
Urethane	Sigma-Aldrich (Poole, UK)
VASP antibody	Cell Signaling (Hertfordshire, UK)
VASP-P(239) antibody	Cell Signaling (Hertfordshire, UK)

Equipment

Equipment and software	Supplier
Aggrolink software	Chronolog Corp (Havertown, USA)
Chronolog optical aggregometer	Chronolog Corp (Havertown, USA)
Chronolog siliconised stir bars	Chronolog Corp (Havertown, USA)
Ettan DIGE Imager	Amersham Biosciences (Piscataway, USA)
Glass cuvettes	LabMedics (Oxfordshire, UK)
Graphpad Prism 5.0 software	GraphPad (CA, USA)
Image Quant software	GE healthcare (Bucks, UK)
ImageJ software	National Institutes of Health (Maryland, USA)

KC4 data analysis software	BioTek (Bedfordshire, UK)
Microsoft office Excel	Microsoft (Reading, UK)
Mini-PROTEAN tetra-cell western blotting kit	Bio-Rad (Hercules, USA)
Polyvinylidene fluoride membrane (PVDF)	Bio-Rad (Hercules, USA)
Sievers Nitric Oxide Analyzer-280i	Analytix Ltd (Bolden, UK)
Single point extended area ratio (SPEAR) detector	eV Products (Saxonburg, USA)
Specialist radioactive count software	Mumed Systems (London,UK)
Sysmex F-820 Haematology Analyser	Sysmex UK Ltd (Milton Keynes, UK)
Trans-Blot SD semi-dry electrophoretic transfer cell	Bio-Rad (Hercules, USA)
UCS-20 spectrometer	Spectrum Techniques (TN, USA)
Whatman 3MM chromatography paper	Thermo Fisher Scientific (Loughborough, UK)

Buffers

Buffer	Compound	Molarity
modified tyrodes-HEPES buffer (mTHB)	Sodium chloride	133.47mM
	Potassium chloride	2.68mM
	$NaHPO_4$	335µM
	Sodium bicarbonate	11.9mM
	HEPES	19.97mM
	$MgCl_2$	840.2µM
	Glucose	5mM
Acid citrate solution	Citric acid	41.64mM
	Trisodium citrate	74.8mM
Tyrodes (*in vivo*)	Sodium chloride	136.89mM
	Potassium chloride	2.68mM
	$NaHCO_3$	11.9mM
	Glucose	5.55mM
Running buffer	Trizma base	25.01mM
	Glycine	191.82mM
	SDS	3.47mM
TBS-T	Trizma base	50mM
	NaCl	48.66mM
	Tween 20 (NB: pH to 7.4 before addition)	1%
Semi-dry transfer buffer	Trizma base	25.01mM
	Glycine	191.82mM
	Methanol	2%
	SDS	3.47mM
Adhesion buffer	Trisodium citrate	0.1M
	Triton-X	0.1%
	HCl	pH 5.4

Methods

Mice

Male C57BL/6 mice weighing 20-25g were purchased from Harlan (Bicester, UK) and left for 1 week to acclimatise to their environment before any procedures were carried out. eNOS knockout mice (NOS-3$^{-/-}$, i.d: 0026847) purchased from Jackson Laboratory (ME, USA) were bred in-house and weighed ~20-25g before any procedures were carried out. All mice were kept in 12 hour light/dark cycles and provided regular rodent chow and water *ad libitum*. All procedures were conducted under the Home Office project license PPL 70/7190 and approved by the Ethical Review Panel at Imperial College London and procedures were refined in association with the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs).

Platelet preparation

In vitro

Human: Blood was collected from aspirin-free healthy human volunteers *via* venepuncture from the antecubital fossa. Blood was obtained using a 21 gauge butterfly cannula attached to a 60mL syringe containing 1:9 volume acid citrate solution. Informed consent was obtained from donors before procedures were carried out. Procedures were approved by the National Research Ethics Service (REC reference number: 07/H0708/72).

Blood was centrifuged at 100 g for 20 minutes to separate the platelet rich plasma (PRP). To produce a washed platelet (WP) solution, PRP was collected, transferred into a new falcon tube containing acid citrate solution (1:100) and 175nM PGE$_1$ and centrifuged at 1400 g for 10 minutes. The supernatant (platelet poor plasma) was discarded and the pellet resuspended in mTHB. The final centrifugation was repeated with the addition of acid citrate solution (1:20) and 175nM PGE$_1$. The platelet count was determined using a Sysmex F-820 Haematology Analyser (Milton Keynes, UK) and was diluted in mTHB to obtain an overall washed platelet count of 2.5 x 10^5 µL^{-1}. The platelets were then left to rest at room temperature for 30 minutes.

In vivo

Mouse: Donor mice were anaesthetised with an i.p. administration of urethane (10mL kg^{-1} of 25% (w/v)). Cardiac puncture was performed using a 15G needle on a 2mL syringe containing 200μL ACD (Sigma Aldrich, UK) to avoid blood clotting (as shown in Figure 15). The mice were terminated by cervical dislocation.

Blood was centrifuged at 300 *g* for 3 minutes. The supernatant was transferred to a new eppendorf and the PRP and remaining blood was centrifuged for a further 300 *g* for 3 minutes with the addition of 400μL of tyrodes/ACD/PGE$_1$ (720:10:1) buffer. The supernatant from the remaining blood was pipetted into a new eppendorf and centrifuged for a further 2 minutes at 200 *g*. The combined PRP was centrifuged at 1500 *g* for 7 minutes to obtain a platelet pellet then resuspended in tyrodes/ACD/PGE$_1$ buffer (250μL per mouse donor).

The platelets were incubated with 1.8MBq Indium Oxine (In^{111}) for 10 minutes. To remove excess radioactivity the platelets were centrifuged at 1500 *g* for 5 minutes. The pellet was washed gently with tyrodes and then resuspended in 250μL of tyrodes per mouse donor. The radiolabelled platelets were then left to rest at room temperature for 30 minutes.

In vivo methods

In vivo *measurement of platelet aggregation*

Recipient mice (~25g) were anaesthetised with an i.p. administration of urethane (10mL kg^{-1} of 25% (w/v)) and placed supine on a heat mat. Minor surgery was performed to expose the left femoral vein into which 220μL of the radiolabelled platelets were injected using a 29G insulin syringe (Figure 15). The circulating radioactive platelets were left to equilibrate for 15 minutes prior to any subsequent experimental procedures. Radiolabelled platelets were monitored in the pulmonary circulation using a single point extended area ratio (SPEAR) detector (eV Products, Saxonburg, PA) placed over the thorax as demonstrated in Figure 15. As previously shown, thrombi generated in the circulation accumulates in the small vessels of the pulmonary circulation which results in an increase in radioactive counts in the SPEAR probe (Tymvios et al., 2008). Radioactivity (γ counts) was recorded

continuously using a UCS-20 spectrometer connected to a laptop. Specialised software supplied by Mumed Systems (London, UK) was used to measure changes in radioactive counts over time (Figure 15). For administration of compounds, the right femoral vein was exposed and kept moist using tyrodes to avoid collapsing of the vein. The mice were terminated by cervical dislocation after the experiment.
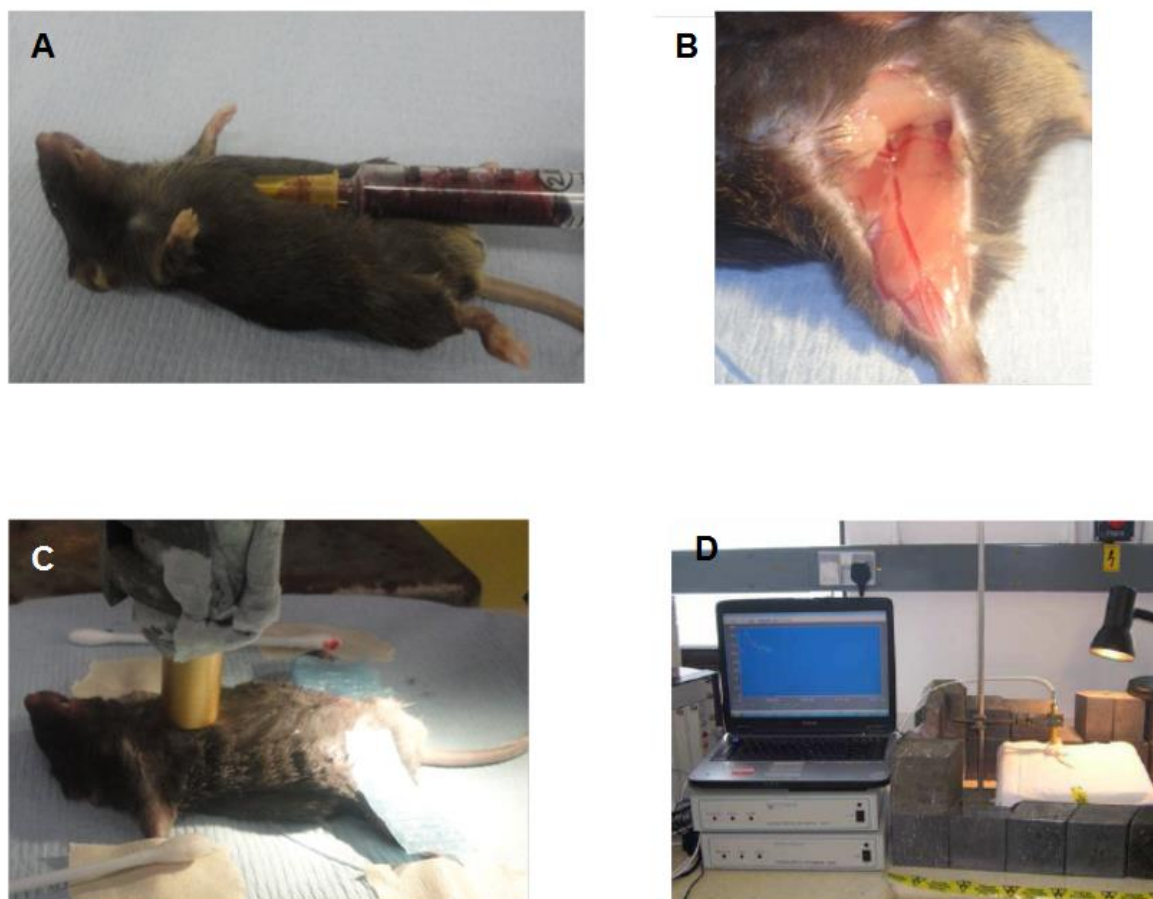
Figure 15: *In vivo* **measurement of platelet function in a model of pulmonary thromboembolism**.

A) Cardiac puncture for blood collection from anaesthetised donor mice. B) Exposure of the femoral vein for injection of In^{111} radiolabelled washed platelets or drug administration. C) Placement of probes over the thoracic region and abdomen. D) The set up of the equipment used to monitor the radiolabelled platelets.

Plasma and salivary gland extractions

After drug treatment, mice (~25g) were anaesthetised with an i.p. administration of urethane (10mL kg^{-1} of 25% (w/v)). Cardiac puncture was performed using a 15G needle on a 2mL syringe containing 200µL ACD (Sigma Aldrich, UK) to avoid blood clotting. The blood was centrifuged for 2 minutes at 15700 *g* and the plasma

removed and snap frozen in liquid nitrogen. Meanwhile the mouse was placed on its dorsal side, tail facing the investigator. A midline incision was made along the neck below the jaw (see Figure 16). Blunt dissection was used to expose the salivary glands. The submandibular and major sublingual glands were removed and snap frozen in liquid nitrogen (Jonjic, 2001). The samples were stored in -80°C until analysis was carried out.
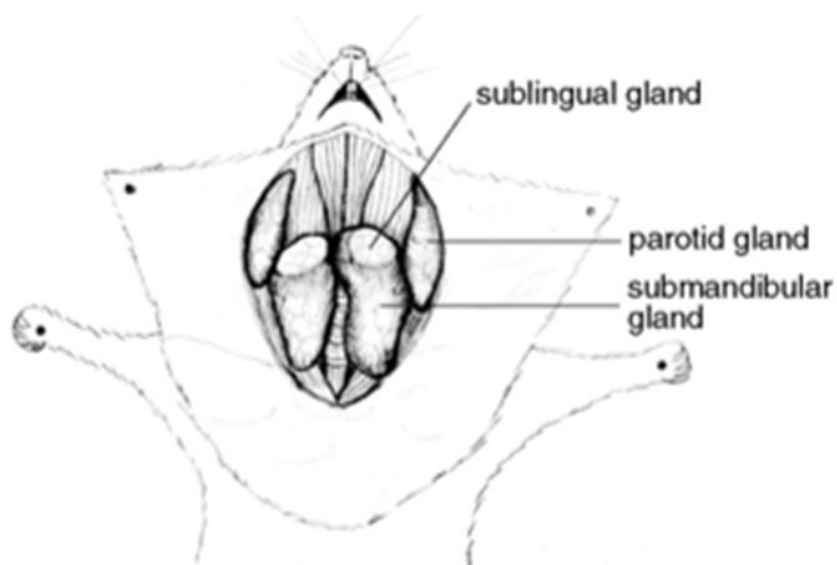
Figure 16 : Diagram of salivary gland dissection (Jonjic, 2001)

In vitro methods

Optical platelet aggregometry

Aliquots of human PRP or WP (450μL) were placed in an optical platelet aggregometer in siliconised glass cuvettes under stirring conditions (~1200rpm). Platelet agonists (50μL) were administered and the extent of platelet aggregation was measured by light transmission (Born, 1962). Changes in optical density were recorded using AggroLink software (Chrono-log Corporation, Havertown, PA, USA).

Western blotting

Protein preparation

WP were incubated with test drugs and centrifuged for 1 minute at 13,700 *g* at 4ºC. The pellet was resuspended in 100μL of lysis buffer (RIPA buffer with 1:1000 phosphatase cocktails 1 and 3, 50μM Tris (2-carboxyethyl) phosphine hydrochloride (TCEP HCl), 2x protease inhibitor (cOmplete Protease Inhibitor Cocktail Tablets, Roche)). These samples were left on ice over 1 hour, vortexing occasionally. Centrifugation was repeated and the supernatant collected. These samples were then stored at -80ºC until further analysis. Protein content of samples were analysed using *DC* protein assay kit (Bio-rad, Hercules, USA) and were compared to known concentrations of BSA in lysis buffer (0-10μg/mL). Sample densities were measured at 750nm on a 96-well plate reader (PowerWave HT Microplate Spectrophotometer, Bio-Tek, Bedfordshire, UK). For SDS-PAGE, samples were diluted to 1.5mg mL^{-1} and Nu-PAGE lithium dodecyl sulphate (LDS) sample buffer (1:4) was added.

SDS-PAGE

20μg of protein was run on a 10% acrylamide gel (resolving gel: 10% acrylamide-bis (1:37.5), 0.375M Tris pH 8.8, 40.4% H_2O, 0.1% SDS, 0.04% APS and 0.002% TEMED. Stacking gel: 4% acrylamide-bis (1:37.5), 0.124M Tris pH6.8, 60.4% H_2O, 0.1% SDS, 0.02% APS and 0.002% TEMED) at 70V through stacking gel and then increased to 100V for around 1.5 hours. The gels were transferred onto PVDF membrane *via* semi-dry transfer method using trans-blot SD transfer cell (Bio-rad, Hercules, USA) at 80mA for around 1.5 hours.

Protein visualisation

The membranes were incubated in 5% non-fat milk in TBS-T at room temperature for 1 hour. Following, the membranes were incubated in a falcon tube containing 5mL of the appropriate primary antibody at 4°C overnight. The membranes were then washed in TBS-T for 5 minutes 4 times and incubated in a falcon tube containing 5mL of secondary anti-rabbit horseradish peroxidase-conjugated antibody (HRP antibody) at room temperature for 1 hour. The washings in TBS-T were repeated and the blots were visualised using an enhanced chemiluminescence detection kit (ECL plus kit;Thermoscientific, Basingstoke, UK) on a charge-coupled device imager (Ettan DIGE Imager, Amersham Biosciences Corp, Piscataway, USA).

Nitrate/nitrite colorimetric assay

mTHB and washed platelets were incubated with the appropriate drugs and snap frozen in liquid nitrogen. Samples were stored in -80°C until analysis. Nitrate/nitrite measurements were performed using the Cayman Chemicals nitrate/nitrite colorimetric assay kit (Michigan, USA). In this kit nitrite is measured by the use of Griess reagents which convert nitrite to the deep purple compound azo. The absorbance of this compound was measured at 540nm. Total nitrate/nitrite concentrations were measured by using nitrate reductase to reduce nitrate to nitrite before adding the Griess reagents.

Nitrate/nitrite gas-phase chemiluminescent assay

Mouse salivary glands were homogenised with phosphate-buffered saline (PBS) using a Mixer Mill MM 400 homogeniser for 3 minutes at a fibrational frequency of 30Hz. Salivary gland solution or plasma was deproteinised by the addition of NaOH (0.5M – 5 minute incubation at room temperature) and $ZnSO_4$ (10% w/v – sample vortexed and incubated for 15 minutes). After deproteinisation, samples were centrifuged at 17,500 g for 5 minutes and the supernatant was extracted and analysed for nitrate/nitrite concentration. Nitrate/nitrite concentrations were measured using a Sievers nitric oxide analyser. Samples were refluxed in vanadium III chloride (0.1M) and HCl (1M) at 95ºC (nitrate analysis) or in sodium iodide (0.3M) and glacial acetic acid at 35ºC (nitrite analysis). In these conditions, nitrate and nitrite were reduced to NO. The interaction of NO with O_3 caused light emission which was

detected using chemiluminescence. Nitrate/nitrite concentrations were determined using standard concentration curves.

Clot retraction

In sterile glass test tubes, 4:17 PRP to mTHB, desired test drug and 5µL of the donors red blood cells (obtained from centrifugation) were added to a total of 950µL and incubated before the addition of 2.5U mL^{-1} of thrombin. Sealed pasteurised glass pipettes were placed in the test tubes and left for 2 hours. The clots were weighed and given as a percentage of the total weight.

Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) release is a measure of membrane integrity and therefore an indirect measure of cytotoxicity. WP were prepared as stated previously and made up to a concentration of $5x10^4$µL^{-1}. Platelets were incubated with the drug of interest for 30 minutes and the amount of LDH released was measured using the LDH cytotoxcity detection kit (Roche, West Sussex, UK). Lysis buffer provided by the kit was added to platelets as a positive control and unstimulated platelets were used as a negative control. LDH concentration in the platelet samples is measured *via* an enzymatic test. Released LDH reduces NAD$^+$ to NADH/H$^+$ by the conversion of lactate into pyruvate. The kit contains the catalyst diaphorase which transfers the proton (H$^+$) to reduce the tetrazolium salt INT to formazan salt which is red in colour. Absorbance can be read at 490nm on an optical plate reader.

Collagen adhesion assay

Prior to the experiment, 96 well plates were incubated with 50µL of 50µg mL^{-1} equine collagen type I overnight at 4ºC on a shaker table (leaving at least 3 wells collagen-free). Excess collagen was washed off using mTHB. Non-specfic binding was blocked using 100µL of 1% bovine serum albumin (BSA) at room temperature for 1 hour and then washed twice using mTHB. WP were prepared as detailed above. Apyrase (1U mL^{-1}) and indomethacin (10uM) was added to avoid secondary agonist ADP and thromboxane signalling, respectively and the platelets were left to incubate for 30 minutes at 37ºC. The washed platelets were incubated with the drug of interest and then added to the wells (50µL) in triplicate. The 'adhered' concentration curve was also added (50µL) in triplicate at this time, ensuring the collagen-free

wells were left blank. The plate was incubated for 45 minutes at 37ºC before the non-adherent platelets were washed off twice using mTHB. The 'total' concentration curve was added in triplicate at this point. A mixture of adhesion buffer plus 0.01% of the chromogenic substrate *para*-nitrophenylphosphate (pNPP) was added at 50μL in all wells. The plate was left to incubate for 45 minutes at 37ºC and the reaction was stopped using 100μL of 2M sodium hydroxide (NaOH). The plate was read at 405nm using an optical plate reader.

Data and statistical analysis

All data were expressed as mean ± standard error of the mean (SEM). *In vivo* platelet aggregation data was expressed as the percentage increase in maximal radioactive counts from the baseline recording (% aggregation), arbitrary values of area under the curve (AUC) or time taken (seconds) for response to return to baseline (duration of response). *In vitro* platelet aggregation data were arbitrary 'area under the curve' values generated by the Aggrolink software (version 5.2.1, Chrono-log, Havertown, USA). All graphs and statistical tests were carried out using GraphPad Prism 5.0 software package (GraphPad, CA, USA). Data was displayed as normalised values for clarity. All statistical tests were performed on raw data. Where statistical comparisons were made, it was determined whether the data was normally distributed using an F-test. Following this, appropriate parametric or non-parametric statistical tests were used to compare mean values (statistical tests are detailed in the figure legends). P-value < 0.05 was considered to denote statistical significance.

Chapter 3: Establishing agonist concentrations

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Objectives and aims

Before assessing the inhibitory effects of compounds on platelet aggregation it was necessary to determine suitable concentrations of platelet agonists. Submaximal agonist concentrations that cause 75% of maximal platelet aggregation (EC_{75}) are necessary to determine the inhibitory effect of a compound on platelet function. If the chosen agonist concentration is too low or too high it may mask the inhibitory effect of the compound and therefore it is important to use the appropriate agonist concentration in subsequent experiments. To accurately determine submaximal agonist concentrations, I performed platelet agonist concentration responses *in vitro* and *in vivo* using optical platelet aggregometry and *in vivo* measurement of radiolabelled platelet aggregation.

In vivo measurement of platelet aggregation was dependent on the radiolabelling of platelets. To confirm that platelets would be detected *in vivo*, I assessed my ability to successfully radiolabel platelets before carrying out further experiments.

The following experiments were therefore conducted:

- Agonist concentration-responses (collagen, thrombin and ADP) using optical platelet aggregometry.
- Quantification of platelet radiolabelling and detection of radioactivity *in vivo* after administration of platelets into a recipient mouse.
- Collagen dose-response *in vivo* in mice.

Methods

Optical platelet aggregometry

Agonist concentration responses

PRP or WP were analysed in an optical platelet aggregometer (stirring at 1200rpm). After baseline recording for 1 minute, collagen (0.05-50µg mL^{-1} for PRP and 0.78-50µg mL^{-1} for WP), ADP (0.3-30µM) or thrombin (0.008-0.5U mL^{-1}) was added to the cuvette and platelet aggregation was recorded for 4 minutes. The data were collected using Aggrolink software (Chronolog Corp,Havertown, USA) and analysed using GraphPad prism 5 (GraphPad, CA, USA).

In vivo measurement of platelet aggregation

Measuring platelet radiolabelling

In vivo methodology was carried out as detailed in chapter 2. In brief, isolated platelets were prepared using blood from donor mice and radiolabelled using a gamma emitter, indium oxine (In^{111}). The radiolabelled platelets were then administered into an anaesthetised recipient mouse. During this process, platelet radioactivity was recorded after initial addition of In^{111} (during the 10 minute incubation), after resuspension of radiolabelled platelets (during the 30 minute incubation) and in the mouse thoracic cavity after administration using a SPEAR detector. The data was recorded using UCS-20 spectrometer and specialist radioactive counting software and analysed using Excel and GraphPad prism 5.0.

Collagen dose response

C57BL/6 male mice (~25g) were anaesthetised and administered radiolabelled platelets as described in chapter 2. 50µL of collagen (25, 50, 75µg kg^{-1}) was administered into the femoral vein and the aggregation response was measured for 10 minutes. Changes in radioactive counts were recorded and analysed as mentioned previously.

Results

In vitro concentration-response to collagen, thrombin and ADP

In order to determine submaximal concentrations of platelet agonists to use in subsequent *in vitro* experiments, *in vitro* concentration-response experiments were carried out. Collagen induced concentration-dependent platelet aggregation in WP and PRP. Collagen was ~9 times more potent in PRP than WP. The EC_{75} for collagen was 5.62μg mL^{-1} in WP and 0.63μg mL^{-1} in PRP (see Figure 17A and Figure 17B). Thrombin promotes blood clotting by cleaving fibrinogen into fibrin, facilitating the formation of a fibrin mesh (Greenberg et al., 1985). To investigate the effect of thrombin directly on platelet function (and not due to secondary fibrin mesh formation), the effect of thrombin was investigated in WP. Thrombin caused concentration-dependent platelet aggregation in WP. The EC_{75} value of thrombin was 0.05U mL^{-1} (see Figure 17C). ADP was investigated in PRP because of the impaired aggregation response of ADP in WP due to the reduced presence of fibrinogen (Solum, 1970) and platelet desensitisation (Ardlie et al., 1971). ADP caused concentration-dependent platelet aggregation in PRP. The EC_{75} value of ADP was 0.91μM (see Figure 17D).
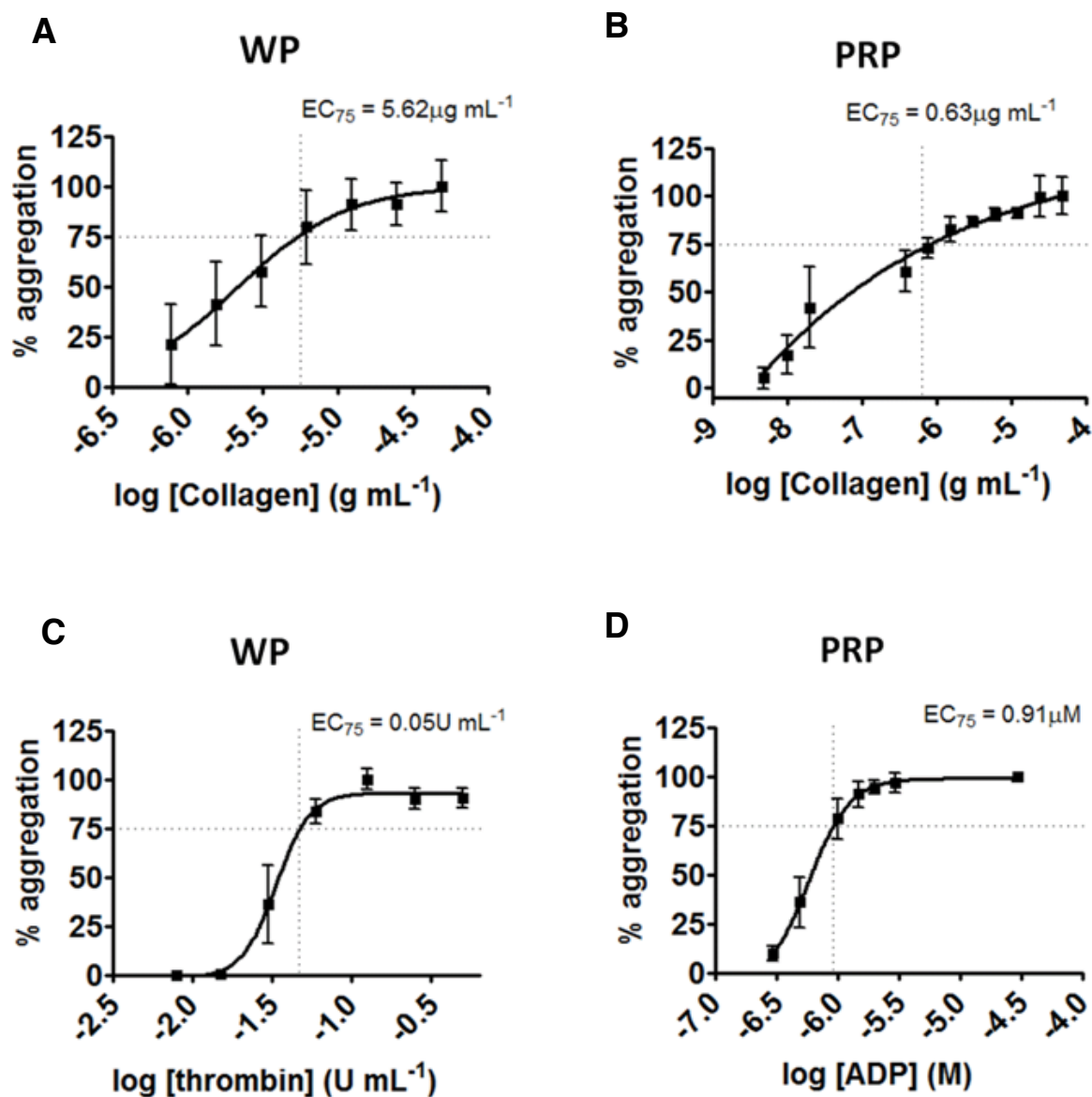
Figure 17: *In vitro* concentration-response curves of a variety of platelet agonists

Platelet preparations were stimulated with a range of agonist concentrations *in vitro* using optical platelet aggregometry. The Aggrolink software calculated area under the curve (arbitrary units) and this was used as a measure of platelet aggregation. EC_{75} values were generated using Graphpad prism 5.0 and the data expressed as mean ± SEM. Human washed platelets were stimulated with (A) collagen (0.78-50µg mL^{-1}), n=5, EC_{75} = 5.62µg mL^{-1} or (C) thrombin (0.008-0.5U mL^{-1}), n=5, EC_{75} = 0.05U mL^{-1}. Human platelet-rich plasma was stimulated with (B) collagen (0.05-50µg mL^{-1}), n=4, EC_{75} = 0.63µg mL^{-1} or (D) ADP (0.3-30µM), n=5, EC_{75} = 0.91µM. WP-washed platelets; PRP-platelet-rich plasma; ADP-adenosine diphosphate.

Efficiency of radiolabelling platelets

In order to demonstrate my ability to successfully radiolabel platelets and detect them *in vivo* in the murine circulation, platelet radioactivity and *in vivo* baseline radioactive counts were measured. Mouse platelets were radiolabelled by 64.4% of total radioactivity added. A baseline radioactive count of ~40,000 per 8 seconds was achieved *in vivo* (see Table 1).

Table 1: **Efficiency of radiolabelling platelets.**

Radioactivity was recorded after initial administration of the In^{111} (incubation period), after centrifugation, washing and resuspension of platelets in tyrodes (during resting period) and after administration in anaesthetised recipient mice (baseline radiolabelled platelets detected *in vivo*). N=6.

N=6	counts per 8 seconds
Counts upon initial addition of In^{111}	102975 ± 4271
Counts following platelet labelling	66158 ± 2165
% of radioactive labelling	64.4 ± 0.0%
Basal counts *in vivo*	39857 ± 4933

In vivo dose-response to collagen

To determine the submaximal dose of collagen to use in subsequent *in vivo* experiments, an *in vivo* collagen dose-response was carried out. Collagen 25-75 µg kg^{-1} induced a dose-dependent increase in radioactive counts within the pulmonary circulation; Figure 18A is an example trace of this effect. The collagen response was measured by recording the percentage increase in radioactive counts (Figure 18B), area under the curve (AUC) of the response (Figure 18C) and duration of the response (Figure 18D). AUC and duration of response demonstrated positive linear trends in radioactivity whereas the percentage increase in radioactive counts was maximal at a concentration of 50µg kg^{-1}.
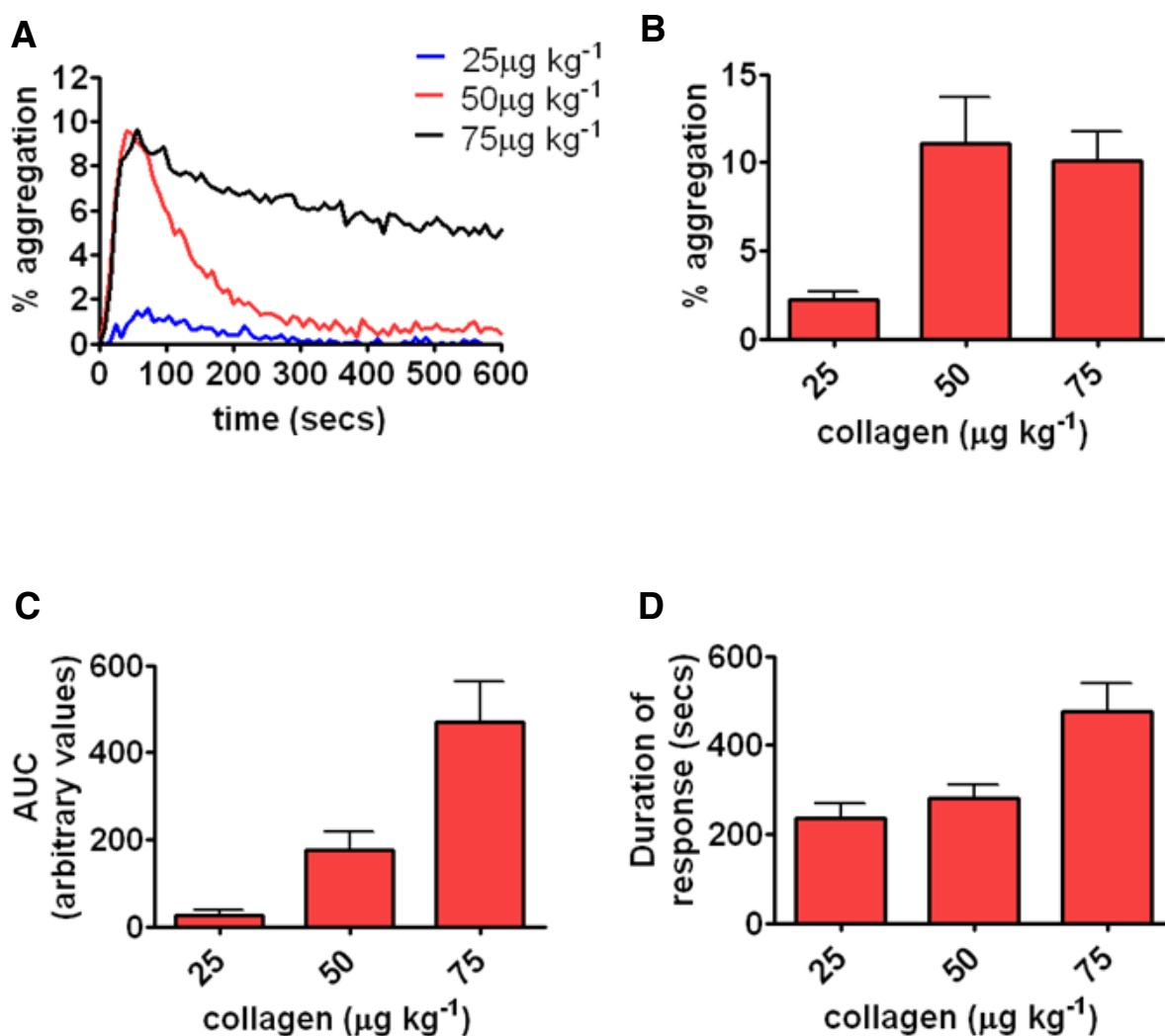
Figure 18: *In vivo* **dose response to collagen**.

Isolated platelets from donor mice were radiolabelled with In^{111} and administered to anaesthetised recipient mice. Radioactive counts were measured using a probe over the thoracic cavity. Collagen (25, 50 and 75µg kg^{-1}) was administered i.v and changes in radioactive counts were recorded over 10 minutes. (A) Mean trace of collagen response (percentage increase from the baseline radioactive counts) *vs* time (seconds). Data expressed as mean (error bars omitted for clarity). (B) The maximum percentage increase from baseline radioactive counts. (C) Area under the curve (arbitrary values). (D) The time it takes for the radioactive counts to return to baseline (seconds). Data expressed as mean ± SEM. N=5.

Discussion

The aim of this chapter was to assess the efficacy of radiolabelling platelets and to determine appropriate concentrations of platelet agonists to take forward into further experimentation. Platelet agonists were investigated in platelet aggregation assays *in vitro* using optical platelet aggregometry (Born, 1962) and *in vivo* using a well established model of measuring murine platelet aggregation (Apostoli et al., 2014; Moore et al., 2010; Smyth et al., 2014; Solomon et al., 2013; Tymvios et al., 2008).

The submaximal concentrations of platelet agonists in optical platelet aggregometry were determined using concentration-response curves. The submaximal concentrations (EC_{75}) were used as a guide for agonist concentrations to use in future experiments. Based on these results, collagen at a concentration of 5µg mL^{-1} was used optical platelet aggregometry using WP. A higher concentration of thrombin (0.1U mL^{-1}) was chosen for subsequent experiments because thrombin is a potent agonist and has a very steep concentration response curve. These agonist concentrations are consistent with the current literature (Antl et al., 2007; Gambaryan et al., 2010; Moore et al., 2010; Solomon et al., 2013).

Before carrying out *in vivo* experimentation it was necessary to assess the ability of platelets to be radiolabelled. The results showed that platelets were efficiently radiolabelled and a stable baseline radioactive count could be detected *in vivo*. Based on these results, subsequent *in vivo* experiments to record platelet agonist responses could be performed. *In vivo* collagen dose-responses were performed to determine a suitable agonist concentration to assess *in vivo* platelet aggregation. We have previously published that increases in radioactive counts upon administration of platelet agonists in our model represents the formation of platelet aggregates accumulating in the small vessels of the pulmonary vasculature (Moore et al., 2010; Tymvios et al., 2008). Due to the complexitiy of *in vivo* experimentation, more than one parameter was considered when deciding on a suitable agonist concentration. Although 50µg kg^{-1} of collagen induced a maximal platelet response, it only caused ~50% response for the other parameters (AUC and duration of response) and therefore was chosen for subsequent experimentation. These results have been

previously observed within my group who established this technique (Moore et al., 2011, 2010; Tymvios et al., 2008).

In conclusion, this chapter determined appropriate concentrations to use in subsequent platelet aggregation experiments.

Chapter 4: The functional effect of sildenafil on platelets

Objectives and aims

The effect of sildenafil on vascular smooth muscle cells has been well established (Boolell et al., 1996). However, the effect of sildenafil on platelets has been largely ignored despite the fact that platelets contain high concentrations of PDE5 enzyme (Schwarz et al., 2007; Wallis et al., 1999). Although previous studies have shown that sildenafil can enhance NO-mediated inhibition of platelet aggregation, the ability of sildenafil to directly modulate the aggregation of isolated platelets is unknown.

In this chapter the ability of sildenafil to modulate platelet aggregation to a range of agonists will be investigated using optical platelet aggregometry. VASP is commonly used as a biomarker of cyclic nucleotide signalling in platelets. In order to link the functional events with platelet signalling, the effect of sildenafil on the phosphorylated status of VASP (VASP-P) will be investigated. To determine the broader functional role of sildenafil on platelets, experiments will be extended to other *in vitro* functional assays such as platelet adhesion and clot retraction.

Finally, although sildenafil has been shown to reduce bleeding time and inhibit platelet aggregation *ex vivo* (Berkels et al., 2001), the direct effects on *in vivo* platelet aggregation are unclear and will also be investigated.

Aims of this chapter were to:

- Assess the functional effect of sildenafil on platelets using optical aggregometry using a range of platelet agonists.
- Establish the effect of sildenafil on the phosphorylation status of VASP at Serine239 (VASP-P(239)).
- Investigate the effect of sildenafil on clot retraction, a simple measure of integrin $\alpha_{IIb}\beta_3$ function.
- Investigate the effect of sildenafil on platelet adhesion to collagen.

- Assess the effect of sildenafil on *in vivo* platelet aggregation in the presence of an intact vascular endothelium.

Methods

Optical platelet aggregometry

Sildenafil on platelet-rich plasma and washed platelets

Human PRP or WP were incubated with sildenafil (10, 100 or 1000nM), vehicle control (mTHB containing 0.01% (v/v) DMSO) or mTHB for 5 minutes before stimulation with a range of ADP concentrations (0.3-30µM), thrombin (0.1U mL^{-1}) or collagen (5µg mL^{-1}). Platelet aggregation was recorded for 4 minutes.

Lactate dehydrogenase assay

Effect of DMSO on platelets

Diluted human WP were incubated with a range of dimethyl sulphoxide (DMSO) percentage concentrations (0.01-5%) or mTHB for 30 minutes prior to the assay.

Clot retraction

Effect of sildenafil on clot retraction

Diluted human PRP aliquots were incubated with mTHB, sildenafil (1, 10µM), vehicle control (mTHB containing 0.5% (v/v) DMSO) or SNP (10µM) for 5 minutes before the stimulation of thrombin (1U mL^{-1}).

Adhesion assays

Sildenafil and SNP concentration responses

Human WP were incubated with sildenafil (1, 10, 100 or 1000nM), sodium nitroprusside (SNP; 1, 10, 100 or 1000nM) or their respective vehicles (sildenafil; vehicle control (mTHB containing 0.01% (v/v) DMSO), SNP; mTHB vehicle) for 10 minutes before carrying out the adhesion assay.

Western blotting

Sildenafil concentration response on VASP-P(239)

Human WP were incubated with sildenafil (10, 100 or 1000nM), vehicle control (mTHB containing 0.01% (v/v) DMSO) or positive control SNP (1µM) for 5 minutes before carrying out the sample preparation. The protein content of the samples were

standardised, run on an SDS-PAGE gel and transferred onto a PVDF membrane as detailed in chapter 2. The primary antibodies used were rabbit anti-VASP (1:1000), rabbit anti-phospho-VASP (Ser239) (1:1000) and the housekeeping protein rabbit anti-GAPDH (1:500) all left to incubate overnight at 4ºC. The secondary antibody used for all the above primary antibodies was anti-rabbit HRP antibody (1:2000) left to incubate for 1 hour at room temperature before protein visualisation as detailed in chapter 2.

In vivo measurement of platelet aggregation

The effect of sildenafil in vivo in W.T mice

C57BL/6 male mice (~25g) were anaesthetised and radiolabelled platelets were administered as described in chapter 2. Sildenafil (50µg kg^{-1}) or vehicle control (mTHB containing 0.1% (v/v) DMSO) was administered into the femoral vein of the recipient mouse. After 5 minutes, 50µg kg^{-1} collagen (50µL) was intravenously administered and the aggregation response was measured for 10 minutes.

Results

Sildenafil reduced platelet aggregation *in vitro*

Sildenafil alone had no significant effect on ADP-induced platelet aggregation *in vitro* in PRP (Figure 19A). Sildenafil demonstrated significant ($P<0.05$) concentration-dependent inhibition of collagen (Figure 19B and Figure 19C) and thrombin (Figure 19D and Figure 19E) induced platelet aggregation in WP compared to vehicle treated platelets expressed as representative traces (Figure 19B and Figure 19D) and graphs of mean responses (Figure 19C and Figure 19E). Sildenafil caused no significant inhibitory effect on collagen-induced platelet aggregation at 10nM (~10% inhibition). However sildenafil caused significant inhibition of collagen-induced platelet aggregation at 100nM (~30% inhibition) and 1µM (~35% inhibition) (Figure 19C). Sildenafil caused no significant inhibitory effect on thrombin-induced platelet aggregation at 10nM (~5% inhibition) and 100nM (~15% inhibition) but did cause significant inhibition at a concentration of 1µM (~25% inhibition) (Figure 19E).
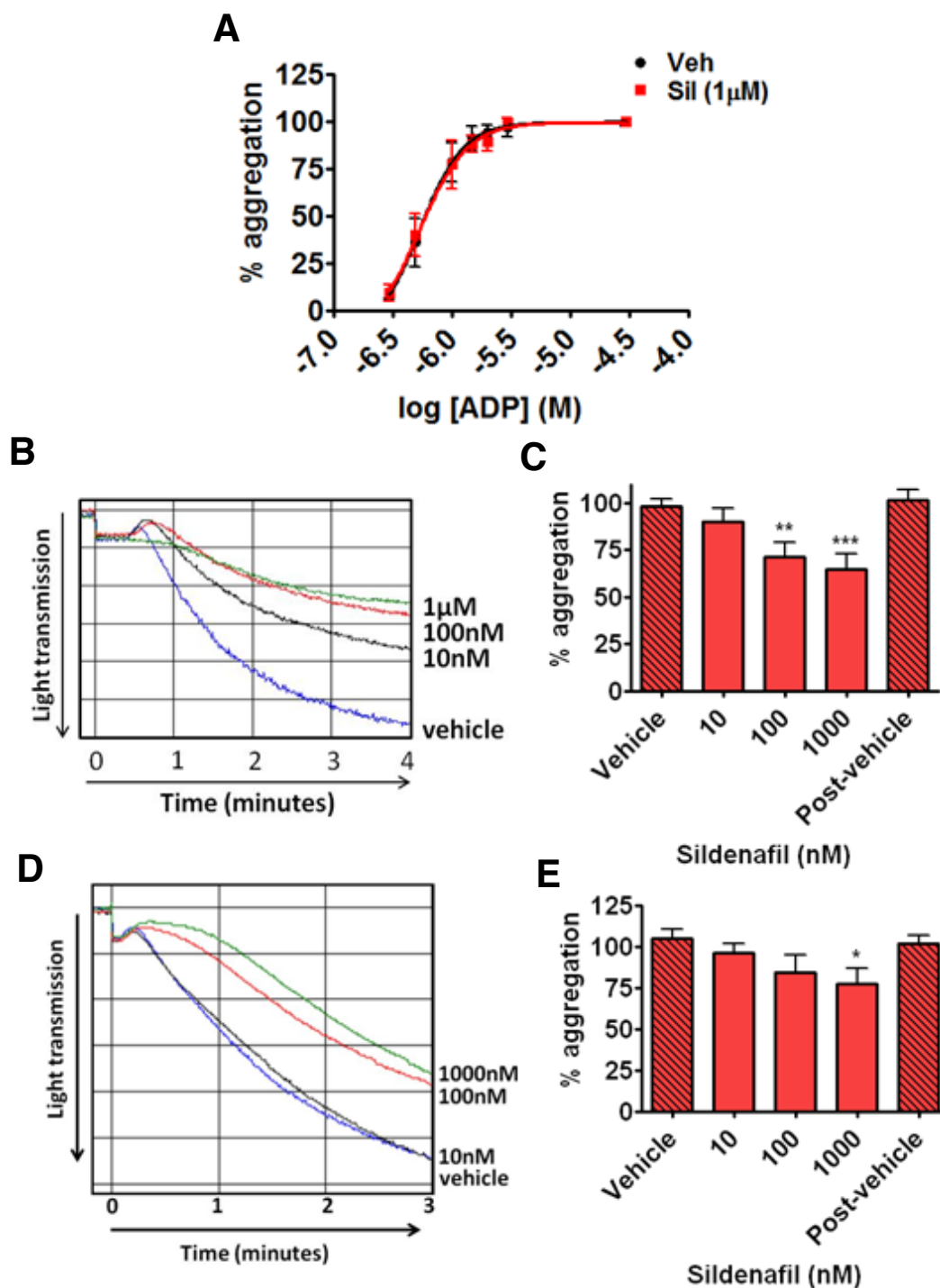
Figure 19: **Sildenafil reduced platelet aggregation *in vitro*.**

Platelet preparations (A: platelet-rich plasma (PRP) B-E: washed platelets (WP)) were pre-incubated with vehicle (veh, DMSO, 0.01%) or sildenafil citrate (sil, 10 nM - 1 µM) for 5 minutes before stimulation with (A) adenosine diphosphate (ADP, 0.3-30µM), (B-C) collagen (5 µg mL^{-1}) and (D-E) thrombin (0.1 U mL^{-1}). Platelet aggregation was analysed in an optical platelet aggregometer. (A) Vehicle EC_{50} = 0.585 µM, sildenafil EC_{50} = 0.447 µM. An F-test was used to identify statistical significance between the EC_{50} values. Ns = non-significant (B, D) Example traces representative of 7-8 independent experiments. (C,E) Data expressed as mean±S.E.M. Repeated measures one way ANOVA with Dunnett's post-hoc test. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

DMSO (0.01%) was used as a vehicle for sildenafil. The cytotoxic effect of DMSO was investigated using an LDH assay to determine membrane integrity. The positive control (platelets treated with lysis buffer) caused maximum LDH release. Concentrations of DMSO up to 5% caused no significant increase in LDH release compared to the negative control (untreated platelets) (Figure 20).
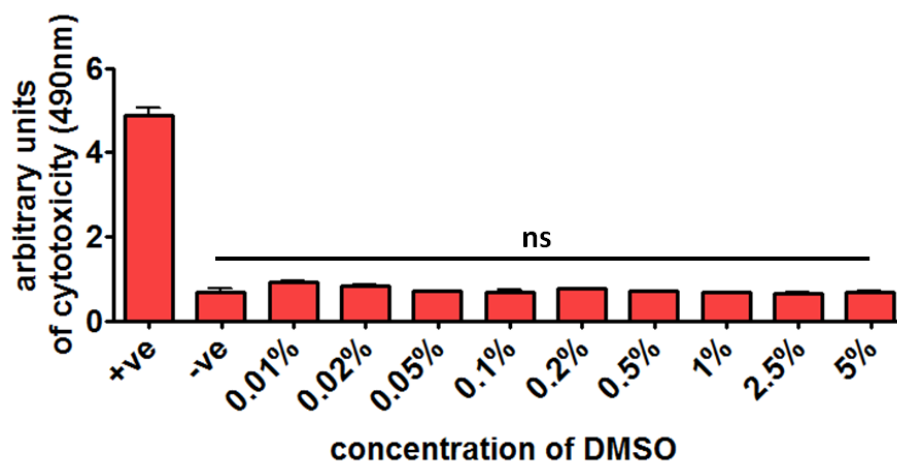
Figure 20: **DMSO concentration-response on lactate dehydrogenase (LDH) release**.

Human washed platelets were pre-incubated with DMSO (0.01-5%) and cytotoxicity was measured as a release of LDH from the platelets. The negative control (-ve) contained washed platelets alone and the positive control (+ve) contained washed platelets with lysis buffer to produce maximum LDH release. Repeated measures one way ANOVA with Dunnett's post-hoc test, DMSO concentration *vs* negative control. ns=non-significant. Data is expressed as mean±SEM. N=6.

Sildenafil increased the phosphorylation of VASP-P(239)

To associate the antiplatelet effect of sildenafil with signalling events, the effect of sildenafil on VASP phosphorylation at Serine239 was investigated. VASP-P(239) was chosen because it is one of the best characterised sites of PKG phosphorylation *in vitro*. Figure 21A shows a representative blot of total VASP, VASP-P(239) and the loading control GAPDH. Figure 21B shows a bar chart of the mean densitometry data which expresses the percentage of VASP-P(239) compared to total VASP. The positive control SNP (1µM) induced significant VASP phosphorylation compared to vehicle treated platelets (Figure 21A and Figure 21B). Sildenafil induced a concentration-dependent increase in VASP-P(239) which was statistically significant at a concentration of 1µM compared to vehicle treated platelets (Figure 21A and Figure 21B).
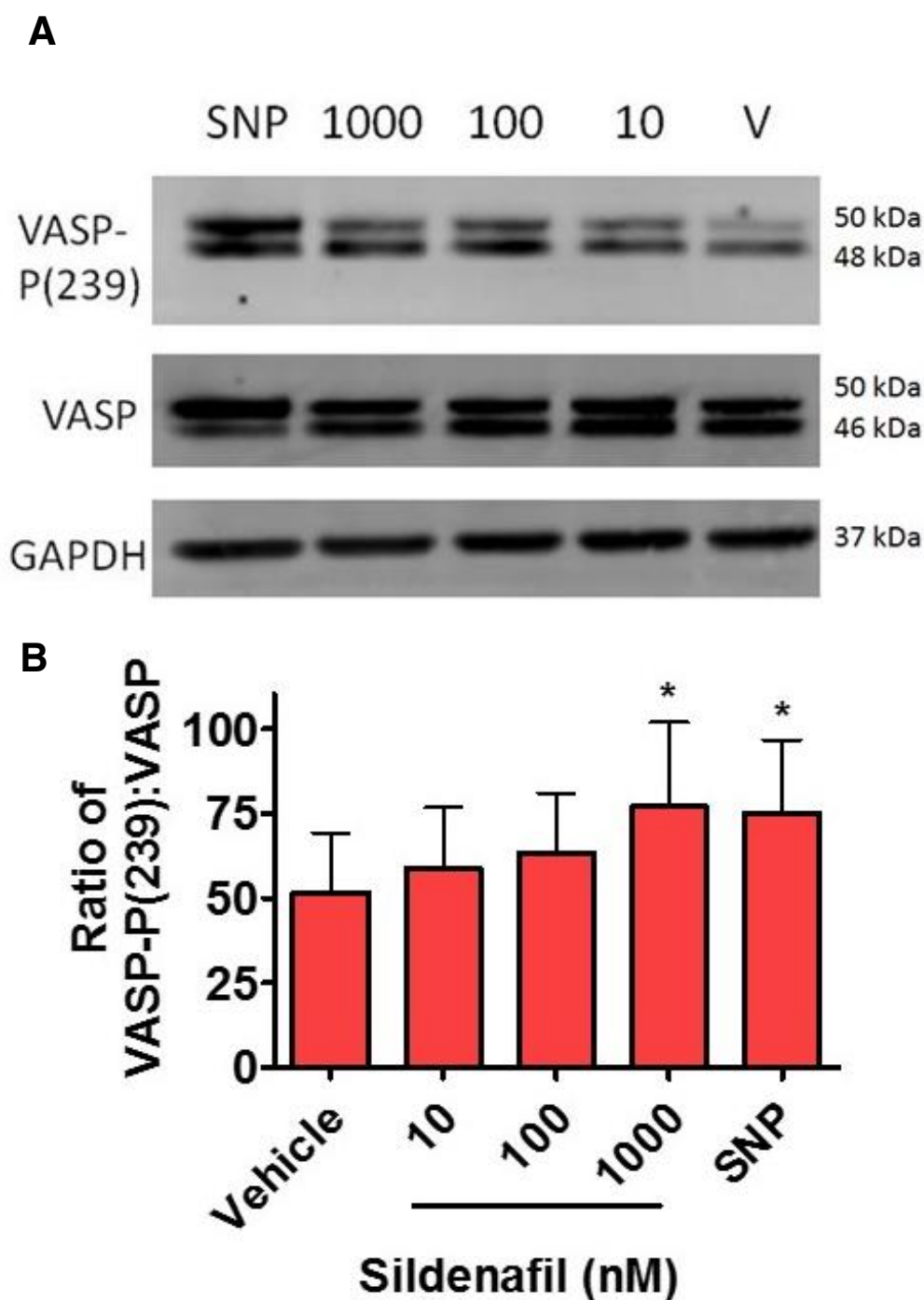
Figure 21: **Sildenafil increased the phosphorylation of VASP-P(239)**.

Vehicle (DMSO, 0.01%), sildenafil citrate (sil, 10-1000nM) and sodium nitroprusside (SNP, 1µM) were pre-incubated with human washed platelets for 5 minutes. Sildenafil concentration dependently induced VASP-P(239) phosphorylation, data presented as (A) Western blot representative of 5 independent experiments and (B) ratio of VASP-P(239) compared to total VASP. N=5. Data expressed as mean±SEM. Repeated measures one way ANOVA with Dunnett's post-hoc test. *$P<0.05$, compared to vehicle treated.

Sildenafil had no effect on collagen adhesion under static conditions

Increasing concentrations of sildenafil (1-1000nM) had no significant effect on platelet adhesion to collagen under static conditions (Figure 22A). Similarly, SNP (0.01-100µM) also caused no significant effect on platelet adhesion to collagen compared to vehicle-treated platelets (Figure 22B).

Sildenafil had no effect on clot retraction

The treatment of PRP with SNP demonstrated an increased trend in clot weight (therefore a decrease in clot retraction) however sildenafil treatment had no effect (Figure 23). This was shown as typical images of the clots (n=1) (Figure 23A) and as a bar chart (Figure 23B).
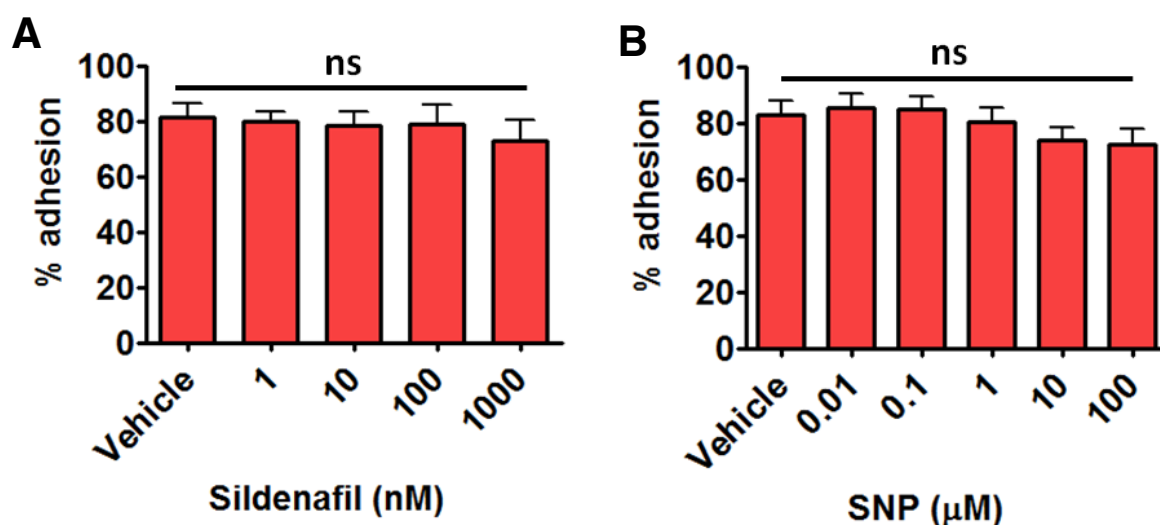
Figure 22: **Sildenafil had no effect on collagen adhesion in static conditions**.

Human washed platelets were preincubated with vehicle (0.01% DMSO or mTHB), sildenafil (1-1000nM) or the NO donor sodium nitroprusside (SNP – 0.01-100µM) before carrying out collagen platelet adhesion assays. (A) sildenafil concentration response (B) SNP concentration response. Repeated measures ANOVA with Dunnett's post-hoc test vs vehicle. ns=non significant. Data is expressed as mean±SEM. N=4.
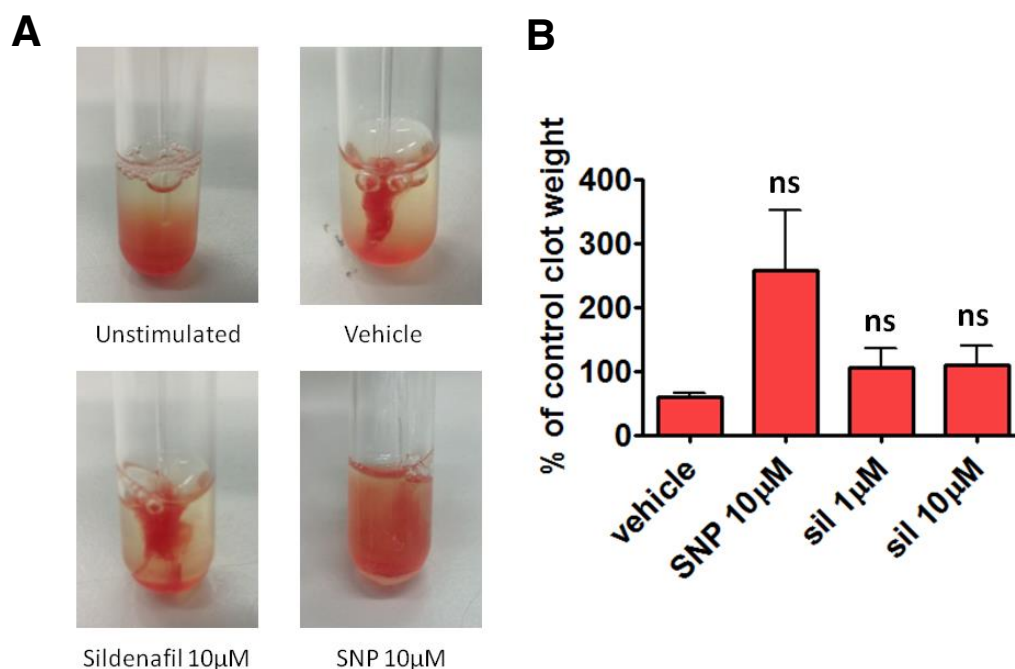
Figure 23: **Sildenafil had no effect on clot retraction**.

Platelet-rich plasma was incubated with the negative control mTHB, the positive control sodium nitroprusside (SNP - 10µM), vehicle (DMSO – 0.5%) or sildenafil (1µM or 10µM) before the addition of thrombin. (A) Photos of the clot representative of 3 independent experiments. (B) Mean percentage of clot weight compared to the control. Data is expressed as mean±SEM. N=3. Repeated measures ANOVA with Dunnett's post-hoc test vs vehicle. Statistical test performed on raw values (clot weight).

<u>Sildenafil reduced platelet aggregation *in vivo* in W.T mice</u>

The effect of sildenafil on *in vivo* platelet aggregation was determined. Sildenafil at a concentration of 50µg kg^{-1} significantly reduced *in vivo* collagen-induced platelet aggregation compared to vehicle as demonstrated by an example trace (Figure 24A), mean peak response as a percentage increase from baseline counts (Figure 24B) and duration of response in seconds (Figure 24D). The area under the curve (AUC) showed a non-significant trend towards inhibition of platelet aggregation (Figure 24C).
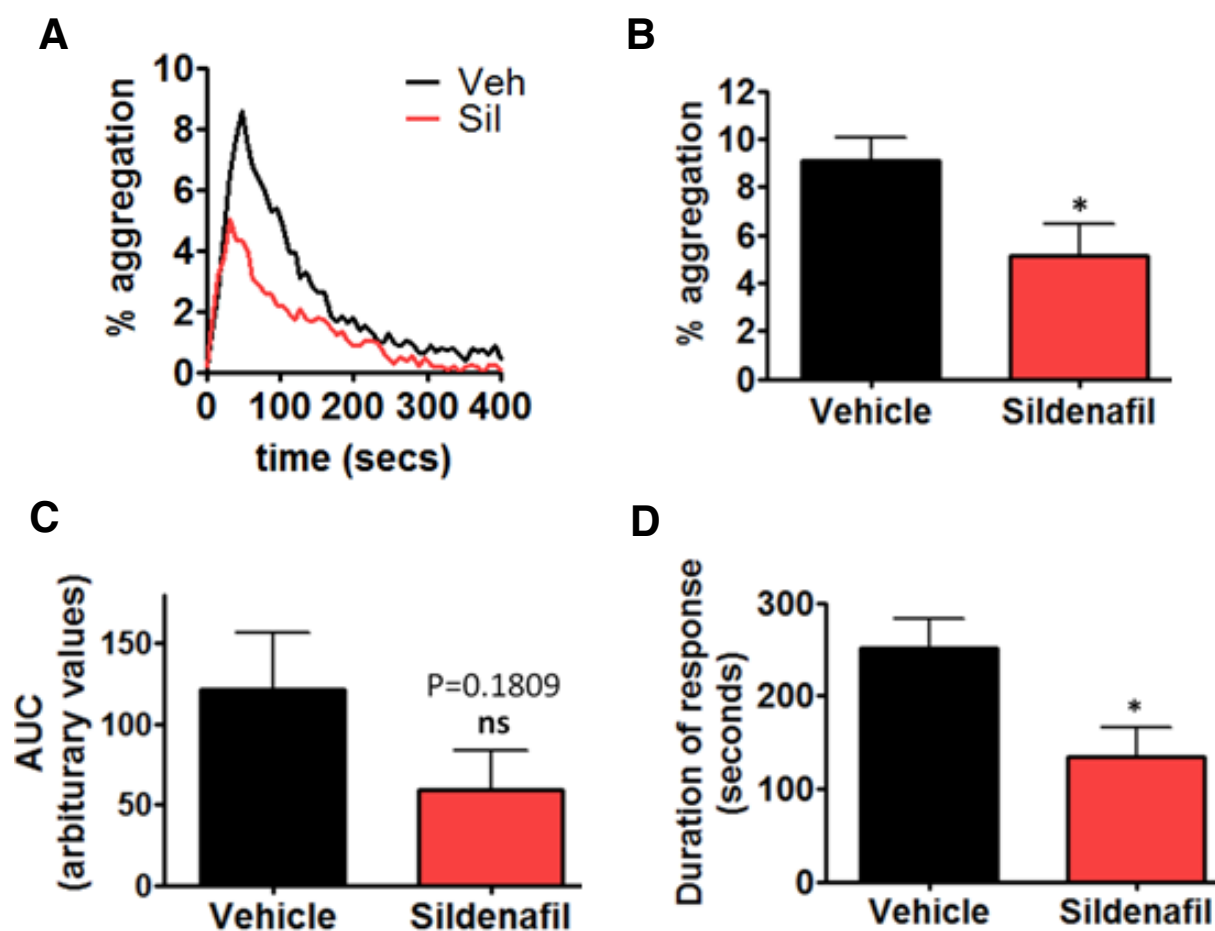
Figure 24: **Sildenafil reduced platelet aggregation *in vivo* in W.T mice**.

Sildenafil (50 µg kg^{-1}) or vehicle (DMSO, 0.1%) were administered to C57BL/6 mice 5 minutes before collagen (50 µg kg^{-1}). Platelet aggregation was measured as changes in radioactive counts in the pulmonary vasculature. (A) Mean trace of collagen response expressed as percentage increase from baseline, error bars omitted for clarity. (B) Maximum percentage increase from baseline (C) area under the curve and (D) the time it takes for the response to return to baseline was expressed as mean ± SEM. Unpaired Student's *t*-test. ns= non-significant, *P<0.05 compared to vehicle treated. N=6.

Discussion

Prior to this study, the direct effect of sildenafil on platelets *in vitro* and *in vivo* was unclear. This chapter investigated the effect of sildenafil on platelets *in vivo* and *in vitro* using a range of functional and molecular techniques.

I first wanted to establish the effect of sildenafil on platelet aggregation *in vitro*. Wallis and colleagues demonstrated that in PRP sildenafil had no effect on ADP-induced platelet aggregation *in vitro* in the absence of the NO donor sodium nitroprusside (SNP) (Wallis et al., 1999). My data is in agreement with this study, as sildenafil had no significant effect on ADP-induced platelet aggregation *in vitro* in PRP. However, in WP sildenafil caused significant concentration-dependent inhibition of platelet aggregation by collagen and thrombin. Previous literature suggested that sildenafil only exerted an inhibitory effect on platelets *in vitro* in the presence of an external source of NO (Berkels et al., 2001; Gudmundsdóttir et al., 2005; Wallis et al., 1999), however all these studies were carried out in PRP and not isolated platelets. Sildenafil has previously been proven to be highly plasma protein bound which may have reduced the concentration of sildenafil able to act directly on platelets in PRP preparations (Walker et al., 1999). In addition, PRP contains substances known to enhance platelet aggregation (for example fibrinogen, vWF and enzymes such as thrombin (Born and Cross, 1964; Cazenave et al., 2004)) and lacks potential sources of negative regulation (for example NO produced by erythrocytes (Kleinbongard et al., 2006; Srihirun et al., 2012; Webb et al., 2008a)) and therefore promotes platelet aggregation. This highlights the necessity to investigate the effect of sildenafil on platelet signalling in isolated platelets and then translate results *in vivo* to investigate the physiological relevance. Overall, it was established using Figure 19 that sildenafil was able to inhibit platelet function *in vitro* but the physiological relevance of this *in vivo* remained to be determined.

The solvent DMSO was used as a vehicle due to the poor water solubility of sildenafil. Due to concerns of the safety and toxicity of DMSO (Galvao et al., 2013), a lactate-dehydrogenase (LDH) assay was used as a test of cytotoxicity. DMSO concentrations up to 5% had no significant effect on platelet LDH release compared to the negative control and therefore the solvent did not damage platelet membrane

integrity. The results from the LDH assay and platelet aggregation studies (vehicle-treated platelets exhibited full aggregatory response compared to control platelets) implied that DMSO concentrations used in this study did not cause cytotoxicity. Other studies using sildenafil have used similar concentrations of DMSO and, in agreement with this study, have not seen any evidence of DMSO cytotoxicity in their experiments (Dunkern and Hatzelmann, 2005; Gudmundsdóttir et al., 2005). It is unlikely that the vehicle DMSO caused any non-specific effects throughout this thesis.

VASP-P(239) has been identified as a molecular marker of NO/cGMP signalling events (Ibarra-Alvarado et al., 2002) and is one of the best characterised sites of PKG phosphorylation *in vitro* (Smolenski et al., 1998). In this study, VASP-P(239) was used as a biomarker of platelet signalling. Sildenafil, similar to the positive control SNP, caused a significant increase in VASP phosphorylation at Serine239 compared to the vehicle treated platelets. This confirms that sildenafil was able to inhibit platelet signalling events and indicated that sildenafil-mediated inhibition of platelet aggregation is, at least in part, due to protein kinase activity. VASP-deficient mice are resistant to the inhibitory effects of NO on platelet adhesion (Massberg et al., 2004) which supports my finding that sildenafil and SNP reduced platelet aggregation *via* the NO/sGC/cGMP pathway. However the mechanism behind sildenafil-mediated inhibition cannot be deciphered from this result because both PKG and PKA are known to phosphorylate VASP at Serine239 (Burkhardt et al., 2000). In addition, VASP phosphorylation at Serine239 has previously shown to be reversed in the presence of a PKA inhibitor (Li et al., 2003a). This suggested that there is a complex interplay between cGMP and cAMP signalling and therefore the involvement of cAMP signalling in sildenafil-mediated platelet inhibition is to be further investigated.

To determine the inhibitory effect of sildenafil on the adhesive ability of platelets, I investigated platelet adhesion to collagen in the presence and absence of sildenafil. Figure 22 of this study showed that sildenafil had no significant effect on collagen-induced platelet adhesion. Similarly, SNP also had no effect on collagen-induced platelet adhesion which suggested that NO/cGMP signalling had no effect on the

adhesive properties of platelets. Therefore NO/cGMP signalling has a specific inhibitory effect on platelet aggregation, potentially due to affecting activation-dependent platelet function. In support of this finding, Michelson *et al.* (Michelson et al., 1996) identified that the inhibitory effect of NO was activation-dependent and had limited effect on GPIb-IX signalling and previous findings have reported that sildenafil was able to inhibit platelet activation (Dunkern and Hatzelmann, 2005; Halcox et al., 2002). Interestingly, Wu *et al.* (Wu et al., 1997) previously demonstrated that cGMP-elevating agents could inhibit platelet adhesion to collagen in static conditions however high concentrations of SNP were unable to cause significant inhibition of platelet adhesion. A subsequent study by Roberts *et al.* (Roberts et al., 2008) identified that the inhibitory effect of NO on collagen-induced platelet adhesion was reversed in the presence of apyrase and indomethacin and hence could only affect the activation-dependent component of adhesion. Due to the addition of apyrase and indomethacin in experimentation, my results support those published by Roberts *et al.* (Roberts et al., 2008). Unfortunately, this study was limited due to investigation of sildenafil on platelet adhesion in WP and under static conditions. Further investigations could include determining the effect of sildenafil on platelet adhesion in a more physiological setting such as under flow conditions (Graaf et al., 1992) and *in vivo* (Massberg et al., 2004). This study was also limited by the lack of a positive control to prove that static platelet adhesion could be inhibited. A positive control would be necessary to accurately conclude the effect of sildenafil on static platelet adhesion. Overall, my data suggests that sildenafil had a specific inhibitory effect on platelet aggregation and therefore is able to modify activation-dependent platelet function.

Clot retraction is platelet-driven and characterised by the contraction of a fibrin clot to pull the edges of a wound together and therefore a simple method of assessing the efficiency of 'outside-in' signalling through the platelet integrin $\alpha_{IIb}\beta_3$ (Tucker et al., 2012). Previous work has identified that NO inhibits the activation of platelet integrin $\alpha_{IIb}\beta_3$ and myosin light chain (Roberts et al., 2009) and therefore the effect of sildenafil on clot retraction was investigated. The positive control SNP demonstrated a non-significant trend in clot retraction creating a larger, heavier clot. Interestingly, sildenafil had no effect on clot retraction which suggests that sildenafil has no effect

on integrin $\alpha_{IIb}\beta_3$ signalling and may not affect the platelet component of wound healing.

Due to the limited effect on platelet collagen adhesion and clot retraction, subsequent experiments focussed on the effect of sildenafil on platelet aggregation. The ability of sildenafil to have a specific negative impact on platelet aggregation may be beneficial in preserving normal platelet activity whilst reducing platelet hypersensitivity in the treatment of thrombotic risk however further experimentation will be necessary to determine this.

Finally, I wanted to determine the importance of the inhibitory effect of sildenafil on platelet aggregation *in vivo*. To achieve this, I investigated the effect of sildenafil in a mouse model of platelet aggregation. The animal model used in this study enabled the assessment of platelet aggregation *in vivo* in the presence of an intact vascular endothelium and platelet mediators such as eNOS-derived NO. This was an important factor in this study due to the involvement of the vascular endothelium in modulating platelet function (Moore et al., 2010; Tymvios et al., 2009, 2008). The *in vivo* results showed that sildenafil was able to cause significant inhibition of collagen-induced platelet aggregation compared to the vehicle control in W.T mice. This effect was specific to platelet aggregation and was not dependent on vessel tone (Moore et al., 2010). Previous research has reported that sildenafil had an inhibitory effect on platelet function *in vivo* by increasing bleeding time 1 hour post administration. Interestingly, the increased bleeding time did not correlate with *ex vivo* platelet aggregation which was maximally inhibited 4 hours post administration, several hours after bleeding time returned to normal. This highlighted the inability to specifically measure *in vivo* platelet activity using this method. Other research has also reported the inhibitory effect of sildenafil on platelet aggregation *ex vivo* (Halcox et al., 2002). The work presented in this thesis is the first to demonstrate that sildenafil could exert an inhibitory effect on *in vivo* platelet aggregation in the presence of endogenous physiological inhibitors without the need to induce vascular or haemostatic factors. The work in this chapter suggests that sildenafil demonstrates therapeutic benefit as an antithrombotic agent however the

physiological relevance in cardiovascular disease and in humans is to be further investigated.

The conclusion of this chapter is that sildenafil had an inhibitory effect on platelet aggregation *in vitro* and *in vivo* which was mediated in part *via* intracellular signalling indicative of protein kinase activity. Sildenafil-mediated platelet inhibition preserved activation-independent platelet activities such as adhesion and clot retraction which suggests that sildenafil may be beneficial as an antithrombotic agent. Contrary to previous studies, the activity of sildenafil occurred in the absence of exogenous physiological inhibitors, therefore the mechanism of action in platelets was determined in Chapter 5.

Chapter 5: Mechanism of action of sildenafil on platelets

Objectives and aims

Chapter 4 established that sildenafil exerted an inhibitory effect on platelet aggregation *in vitro* and *in vivo.* The mechanism of action of sildenafil in vascular smooth muscle cells is well established (Boolell et al., 1996; Terrett et al., 1996). Although it has been proven that sildenafil can enhance NO-mediated inhibition of platelet aggregation (Gudmundsdóttir et al., 2005) the mechanism of action by which sildenafil directly inhibits platelet aggregation was not known. The overall aim of this chapter was to explore the mechanism of action by which sildenafil directly inhibits platelet aggregation in the absence of exogenous NO. Specifically, I aim to explore the issues of cGMP/cAMP crosstalk and determine the dependence of the effects of sildenafil upon sGC, endogenous NO and NOS.

The aims of this chapter were to:

- Investigate the ability of sildenafil to modify cGMP- or cAMP-mediated inhibition of platelet aggregation using optical platelet aggregometry.
- Establish whether sildenafil-mediated inhibition of platelet aggregation *in vitro* was dependent upon:
  - sGC activation.
  - presence of NO.
  - NOS activity.
- Investigate the effect of sildenafil on *in vivo* platelet aggregation in mice lacking functional eNOS.

Methods

Optical platelet aggregometry

Effect of sildenafil on sodium nitroprusside- or iloprost-mediated platelet inhibition of platelet aggregation

Human WP were incubated with sildenafil (10nM), vehicle (mTHB containing 0.01% (v/v) DMSO) or mTHB for 5 minutes before the addition of SNP (0.01-100µM), iloprost (0.1-1000pM) or their respective vehicles (mTHB or mTHB containing 0.01% (v/v) methyl acetate). WP were incubated for a further 5 minutes before stimulation with collagen (5µg mL^{-1}). Platelet aggregation was recorded for 4 minutes.

Sildenafil concentration response in the presence of modulators of the NO pathway

Human WP were incubated with sGC inhibitor ODQ (10µM), vehicle (mTHB or mTHB containing 0.05% (v/v) of DMSO), the NO scavengers hydroxocobalamin (100µM) or haemoglobin (5µM), the NOS substrate L-arginine (1mM) or its inactive isomer D-arginine (1mM) for 5 minutes before the addition of sildenafil (10-1000nM), vehicle control (mTHB containing 0.01% (v/v) DMSO) or NO donor SNP (1µM). WP were incubated for a further 5 minutes before stimulation with collagen (5µg mL^{-1}). Platelet aggregation was recorded for 4 minutes.

Sildenafil concentration response in the presence of L-NAME

Human WP were incubated with the non-selective NOS inhibitor L-NAME (100µM), its inactive isomer D-NAME (100µM) or mTHB for 10 minutes before the addition of sildenafil (10-1000nM) or vehicle control (mTHB containing 0.01% (v/v) DMSO). WP were incubated for a further 5 minutes before stimulation with collagen (5µg mL^{-1}). Platelet aggregation was recorded for 4 minutes.

Lactate dehydrogenase assay

SNP concentration response

Diluted human WP were incubated with SNP (1-100µM), mTHB or thrombin (0.1U mL^{-1}) for 30 minutes prior to the assay.

<u>Western blotting</u>

The effect of sildenafil on VASP(239) phosphorylation in the presence of ODQ and L-NAME

Human WP were incubated with ODQ (10µM), L-NAME (100µM) vehicle control (mTHB containing 0.05% (v/v) DMSO) or mTHB (negative control) for 5 minutes before the addition of sildenafil (10nM), vehicle control (mTHB containing 0.01% (v/v) DMSO) or SNP (1µM – positive control). WP were then incubated for a further 5 minutes before carrying out the sample preparation. The samples were quantified, run on an SDS-PAGE gel and transferred onto a PVDF membrane as detailed in chapter 2. The primary antibodies used were rabbit anti-VASP (1:1000), rabbit anti-phospho-VASP (Ser239) (1:1000) and the housekeeping protein rabbit anti-GAPDH (1:500) all left to incubate overnight at 4ºC. The secondary antibody used for all the above primary antibodies was anti-rabbit HRP antibody (1:2000) left to incubate for 1 hour at room temperature before protein visualisation as detailed in chapter 2.

<u>*In vivo* measurement of platelet aggregation</u>

The effect of sildenafil in vivo in eNOS$^{-/-}$ mice

eNOS$^{-/-}$ mice (~25g) were anaesthetised and radiolabelled platelets were administered as described in chapter 2. Sildenafil (50µg kg^{-1}) or DMSO vehicle (0.2%) was administered into the femoral vein of the recipient eNOS$^{-/-}$ mouse. After 5 minutes, 50µg kg^{-1} collagen (50µL) was intravenously administered and the aggregation response was measured for 10 minutes.

Results

Sildenafil enhanced inhibition of platelet aggregation mediated by cGMP, but not cAMP, signalling

Sildenafil at a concentration of 10nM (previously shown to not significantly inhibit platelet function - Figure 19C) was used as a tool to investigate whether PDE5 antagonism modified cGMP- and cAMP-mediated inhibition of platelet aggregation.

SNP (0.01-100µM) caused a concentration-dependent inhibitory effect on collagen-induced platelet aggregation. The presence of sildenafil significantly shifted the SNP concentration response curve to the left reducing the IC_{50} from 170.0nM to 20.9nM (Figure 25A). An LDH assay was performed to assess the cytotoxic effect of SNP on platelets. The positive control (platelets treated with lysis buffer) caused maximum LDH exposure. SNP (1-100µM) did not cause a significant increase in LDH release compared to the negative control (untreated platelets) (Figure 25B).

The prostacyclin mimetic iloprost (0.1-1000pM) caused a concentration-dependent inhibitory effect on collagen-induced platelet aggregation. Figure 25C shows that there was no significant effect on the concentration-response of iloprost in the presence and absence of sildenafil. Sildenafil did not significantly modify the IC_{50} value (sildenafil treated – 0.15nM, vehicle treated – 0.19nM) or shift the inhibitory curve (Figure 25C).

<u>The soluble guanylyl cyclase inhibitor ODQ reversed sildenafil-induced inhibition of</u> <u>*in vitro* platelet aggregation</u>

ODQ alone had no significant effect on collagen-induced platelet aggregation (Figure 26B). SNP significantly inhibited collagen-induced platelet aggregation which was reversed in presence of ODQ and therefore validated that ODQ inhibited sGC activity (Figure 26A). ODQ significantly reversed sildenafil-induced inhibition of platelet aggregation (Figure 26B).

To associate the platelet aggregation results with signalling events, sildenafil-induced VASP phosphorylation in the presence of ODQ was investigated. As shown in Figure 27, sildenafil and the positive control SNP caused a significantly increase in VASP-P(239) compared to vehicle treated platelets. In support of the functional aggregation data, sildenafil in the presence of ODQ caused no significant increase in VASP-P(239) compared to vehicle treated platelets. Data is expressed as a representative blot showing the expression of VASP-P(239), total VASP and loading control GAPDH (Figure 27A) and a graph of the mean densitometry data of the percentage of VASP-P(239) compared to total VASP (Figure 27B).

A

B

C
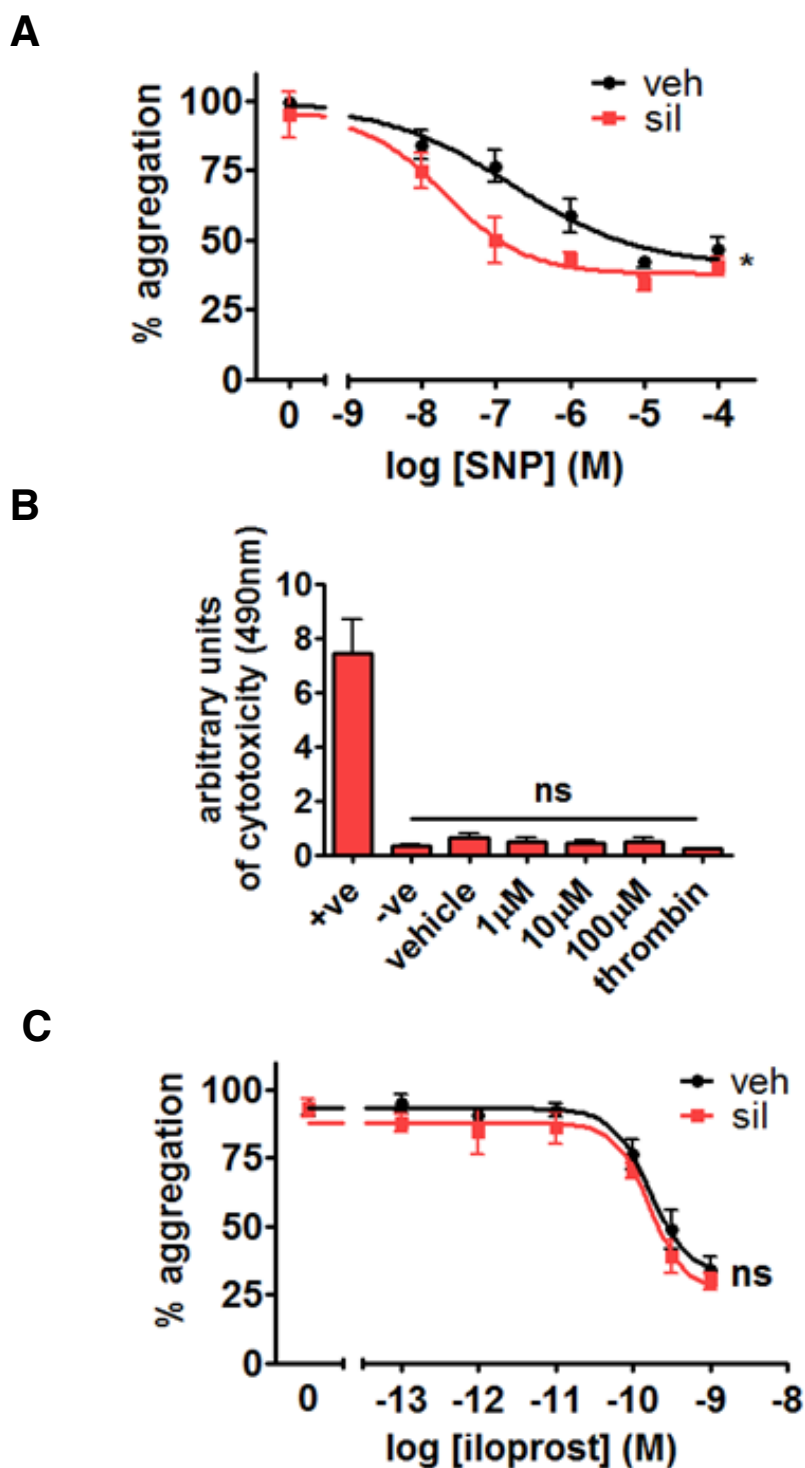
Figure 25: **Sildenafil enhanced inhibition of platelet aggregation mediated by cGMP, but not cAMP, signalling**.

Human washed platelets were pre-incubated with sildenafil (10nM) or vehicle (mTHB containing 0.01% (v/v) DMSO) and (A) SNP (0.01-100μM) or (C) iloprost (0.1-1000pM) before stimulation with collagen (5μg mL^{-1}) in optical platelet aggregometry. N=5. An F-test was used to identify statistical significance between IC$_{50}$ values. Ns= non-significant, *P<0.05. (B) Human washed platelets were pre-incubated with mTHB, SNP (1-100μM) or thrombin (0.1U mL^{-1}) prior to performing the lactate dehydrogenase (LDH) assay. N=7. Repeated measures ANOVA with Dunnett's post-hoc test. All data is expressed as mean±SEM. Veh-vehicle; sil-sildenafil; SNP-sodium nitroprusside.

A

B
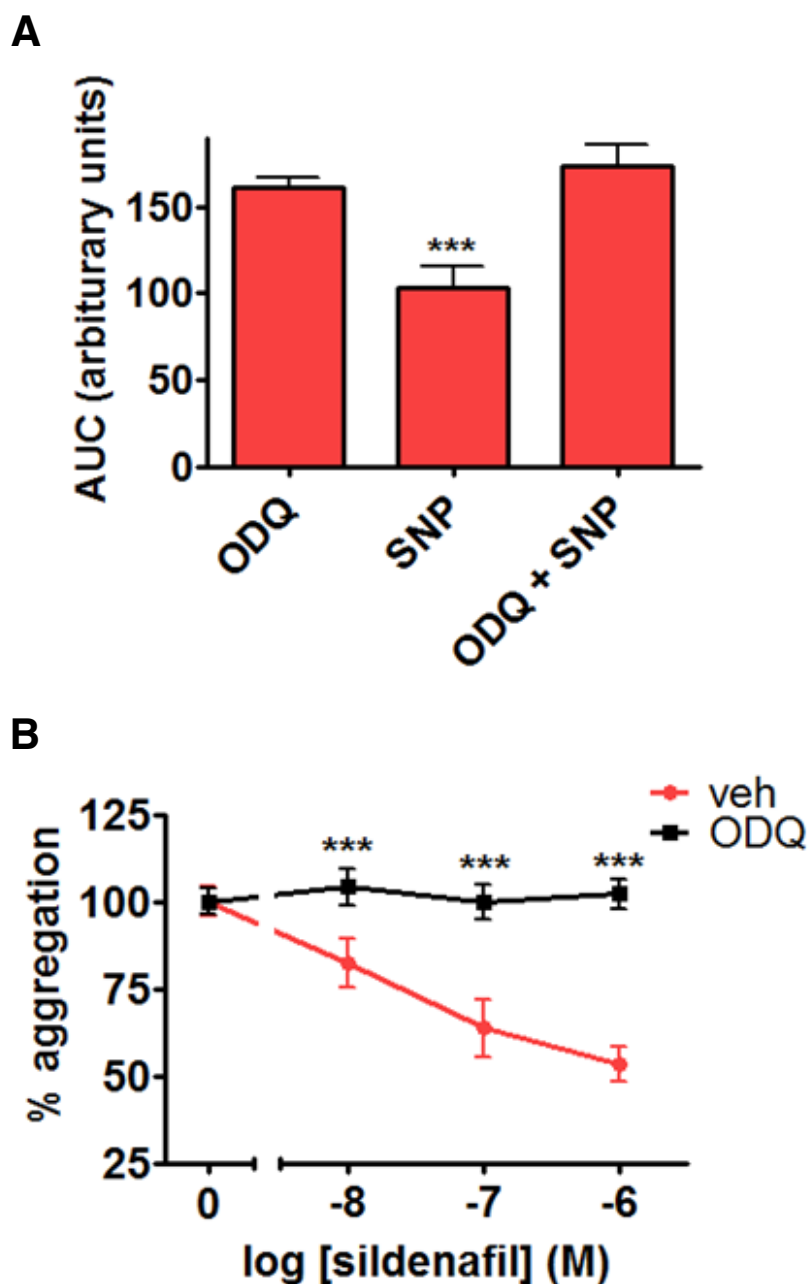
Figure 26: **The soluble guanylyl cyclase inhibitor ODQ reversed sildenafil-induced inhibition of *in vitro* platelet aggregation.**

Human washed platelets were pre-incubated with ODQ (10µM) or vehicle (veh, DMSO 0.05%) and sodium nitroprusside (SNP, 1µM), sildenafil (sil, 10-1000nM) or vehicle (veh, DMSO 0.05%) before stimulation with collagen (5µg mL^{-1}) in optical platelet aggregometry. (A) Control data. ODQ reversed SNP-induced inhibition of platelet aggregation. Repeated measures one-way ANOVA with Bonferroni post-hoc test. (B) ODQ reversed sildenafil-induced inhibition of platelet aggregation. Repeated measures two-way ANOVA with Bonferroni post-hoc test ***P<0.001. N=7. All data is expressed as mean ± SEM.
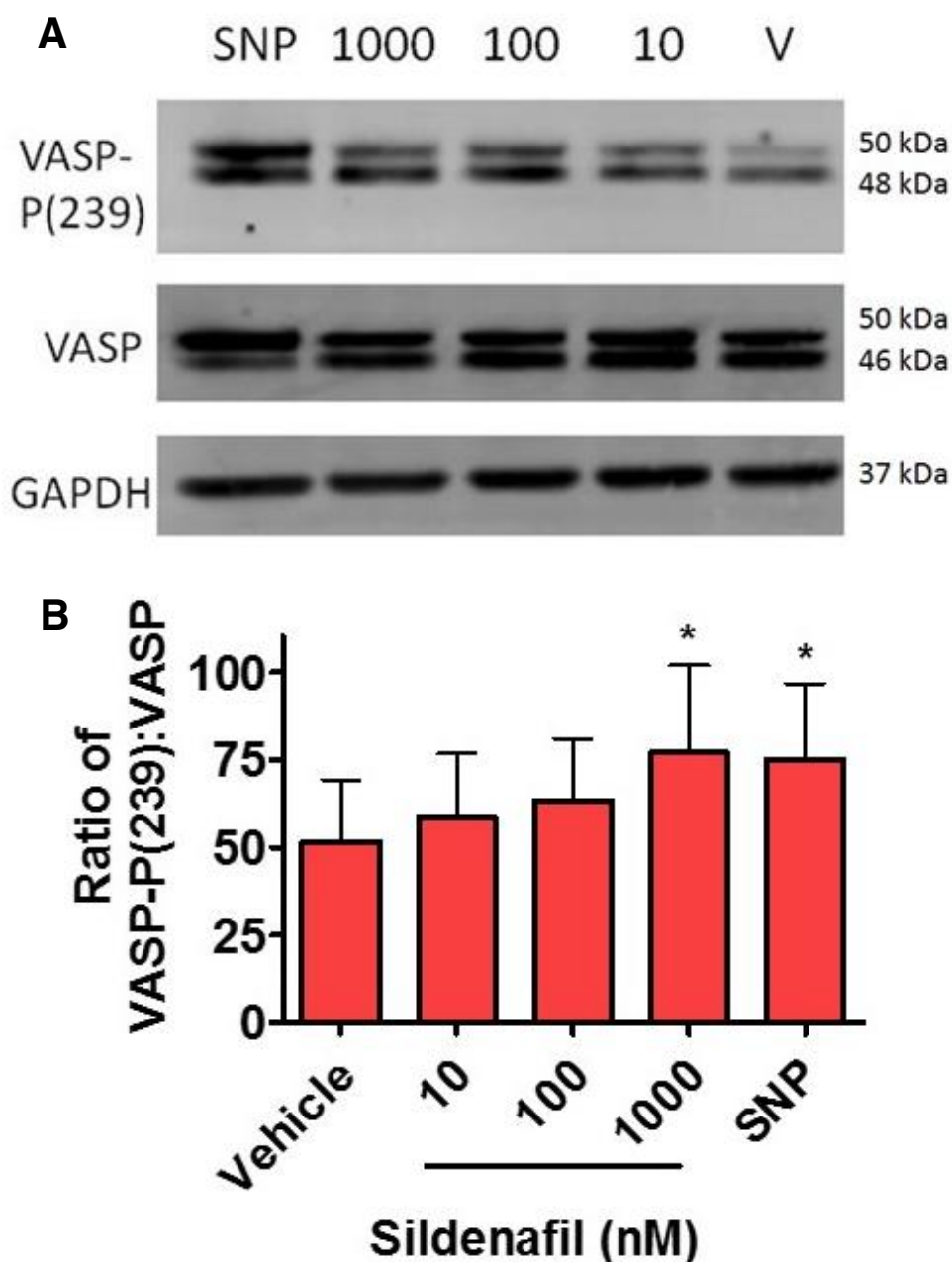
Figure 27: **Western blot showing VASP-P(239) of sildenafil in the presence and absence of ODQ and L-NAME**.

In human washed platelets sildenafil (sil, 100nM) induced VASP-P(239) phosphorylation was abolished by ODQ (10µM) whereas L-NAME (100µM) had no effect, data presented as (A) Western blot representative of 5 independent experiments and (B) ratio of VASP-P(239) compared to total VASP. N=5. Data expressed as mean±SEM. One way ANOVA with Bonferroni post-hoc test. *$P<0.05$, **$P<0.01$ compared to vehicle treated, ns = not significant compared to sildenafil treated. Sodium nitroprusside (SNP, 1µM) was used as a positive control. Veh-vehicle; ODQ-1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; L-NAME-Nω-Nitro-L-arginine methyl ester hydrochloride.

NO scavengers reversed sildenafil-induced inhibition of platelet aggregation

NO scavengers, hydroxocobalamin (HXB - internal NO scavenger) and haemoglobin (Hb – external NO scavenger), were used to assess the dependence of the antiplatelet effect of sildenafil on the presence of NO. HXB and Hb did not significantly modify collagen-induced platelet aggregation (Figure 28C). The positive control SNP significantly inhibited collagen-induced platelet aggregation and this inhibition did not occur in the presence of HXB or Hb (Figure 28A and Figure 28B). Sildenafil-mediated inhibition of platelet aggregation was significantly reversed in the presence of HXB and Hb. As seen in Figure 28C, the NO scavengers significantly reversed the antiplatelet effect of sildenafil at 100nM and 1µM concentrations.

The non-selective NOS inhibitor L-NAME had no effect on sildenafil-induced inhibition of platelet aggregation

L-NAME was used to investigate the dependence of the antiplatelet effect of sildenafil on NOS activity. L-NAME and its inactive isomer D-NAME had no significant effect on platelet aggregation compared to vehicle treated platelets (Figure 29A). Sildenafil exerted an inhibitory effect on platelet aggregation in the presence of L-NAME which was demonstrated by the lack of significance using a two-way ANOVA statistical test (Figure 29B).

In order to associate the aggregation results with platelet signalling, sildenafil-induced VASP phosphorylation in the presence of L-NAME was investigated (Figure 27). Sildenafil significantly increased VASP phosphorylation at Serine239 compared to vehicle treated platelets. The presence of L-NAME had no significant effect on sildenafil-mediated VASP-P(239) compared to sildenafil treated platelets. Data is expressed as a representative blot showing the expression of VASP-P(239), total VASP and loading control GAPDH (Figure 27A) and a graph of the mean densitometry data of the percentage of VASP-P(239) compared to total VASP (Figure 27B).
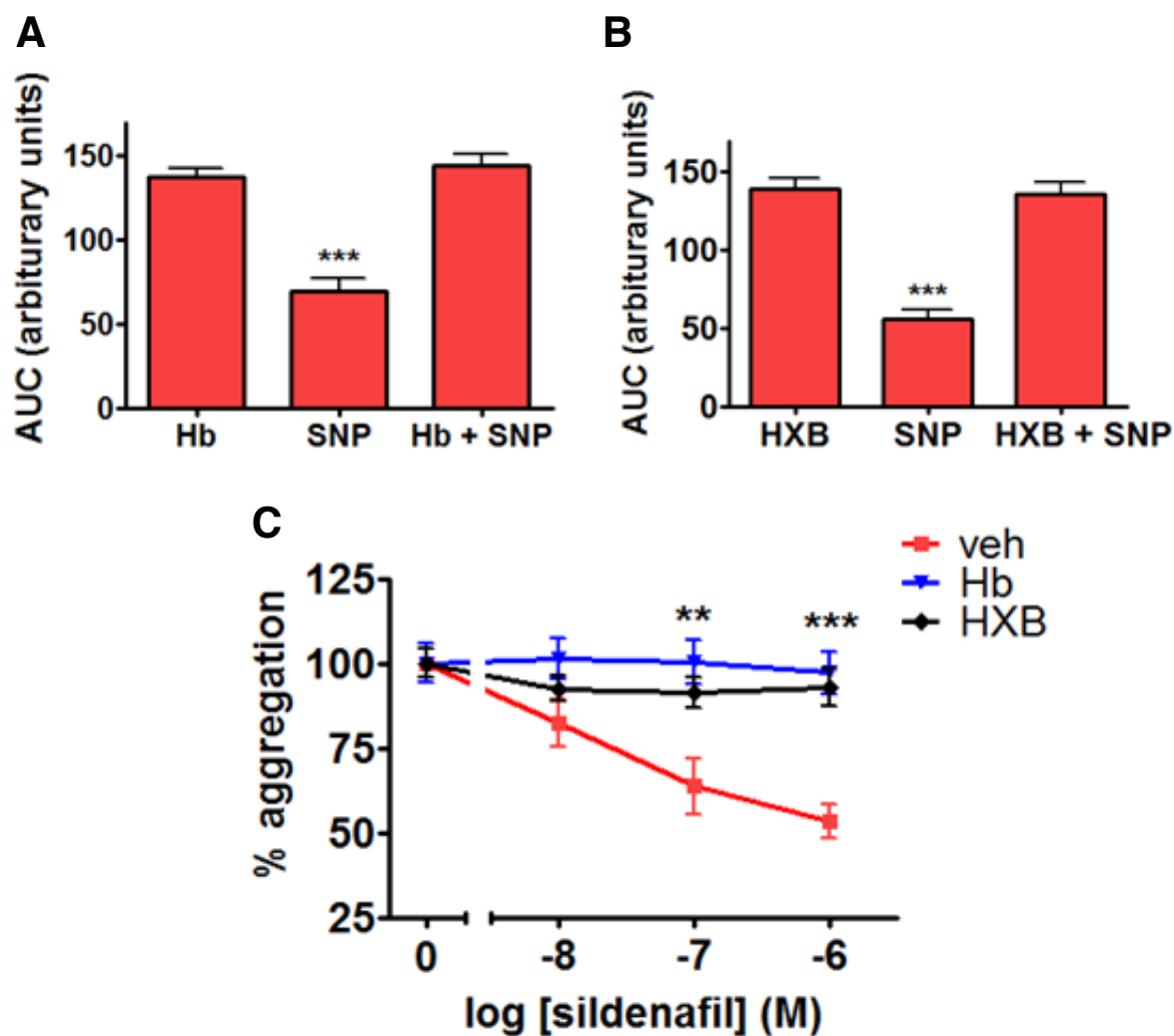
Figure 28: **NO scavengers reversed sildenafil-induced inhibition of platelet aggregation**.

Human washed platelets were pre-incubated with haemoglobin (Hb, 5μM), hydroxocobalamin (HXB, 100μM) or vehicle (veh, mTHB) and sodium nitroprusside (SNP, 1μM), sildenafil (sil, 10-1000nM) or vehicle (veh, DMSO 0.01%) before stimulation with collagen (5μg mL^{-1}) in optical platelet aggregometry. (A, B) Control data. Hb (A) and HXB (B) reversed SNP-induced inhibition of platelet aggregation. Repeated measures one-way ANOVA with Bonferroni post-hoc test. (C) Hb and HXB reversed sildenafil-induced inhibition of platelet aggregation. Repeated measures two-way ANOVA with Bonferroni post-hoc test. **P<0.01, ***P<0.001. N=7. All data is expressed as mean ± SEM.
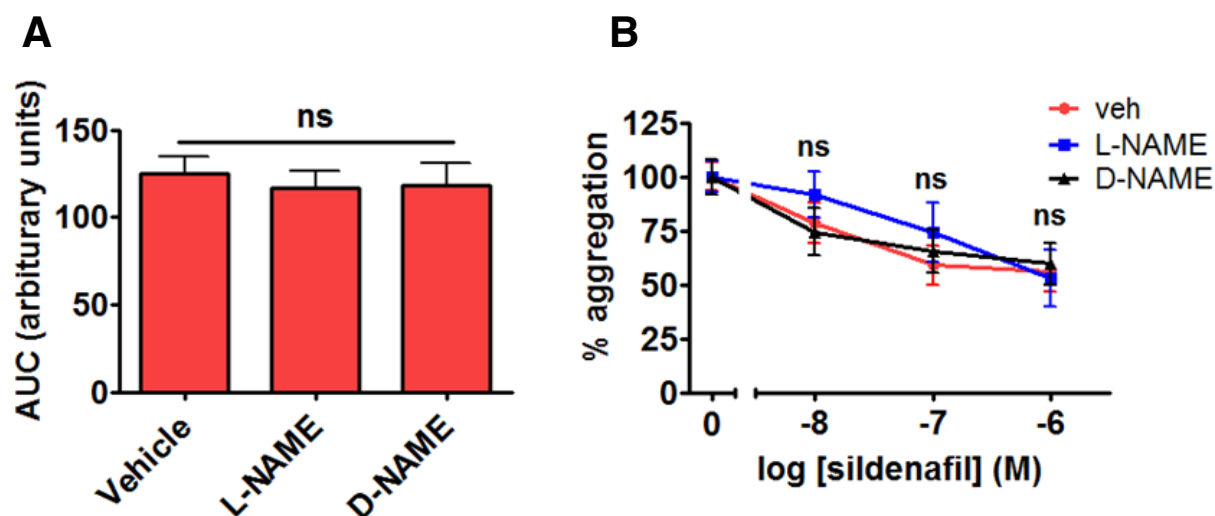
Figure 29: **The non-selective NOS inhibitor L-NAME had no effect on sildenafil-induced inhibition of platelet aggregation.**

Human washed platelets were pre-incubated with L-NAME (100µM), D-NAME (100µM) or vehicle (veh, mTHB) and vehicle (DMSO 0.01%) or sildenafil (10-1000nM) before stimulation with collagen (5µg mL^{-1}) in optical platelet aggregometry. (A) Control data. L-NAME and D-NAME had no significant (ns) effect on platelet aggregation. Repeated measures one-way ANOVA with Dunnett's post-hoc test. (B) L-NAME and D-NAME had no significant effect on sildenafil-induced inhibition of platelet aggregation. Repeated measures two-way ANOVA with Bonferroni post-hoc test. ns=non significant. N=7. All data is expressed as mean ± SEM. L-NAME-Nω-Nitro-L-arginine methyl ester hydrochloride; D-NAME-Nω-Nitro-D-arginine methyl ester hydrochloride.
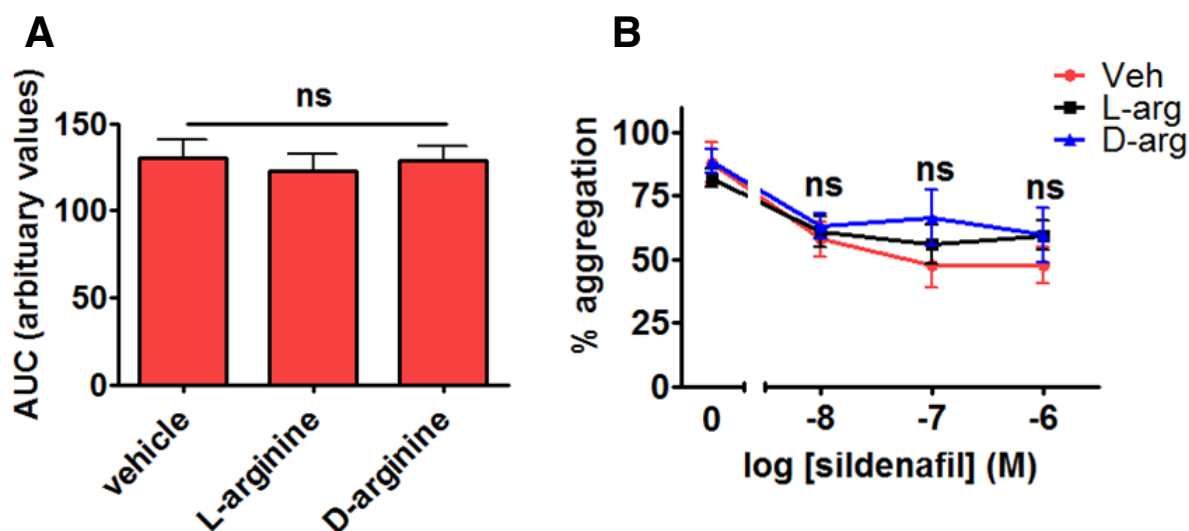
Figure 30: **NOS substrate L-arginine had no effect on sildenafil-induced inhibition of platelet aggregation**.

Human washed platelets were pre-incubated with L-arginine (L-arg, 1mM), D-arginine (D-arg, 1mM) or vehicle (veh, mTHB) and vehicle (DMSO 0.01%) or sildenafil (10-1000nM) before stimulation with collagen (5µg mL^{-1}) in optical platelet aggregometry. (A) Control data. L-arginine and D-arginine had no significant (ns) effect on platelet aggregation. Repeated measures one-way ANOVA with Dunnett's post-hoc test. (B) L-arginine and D-arginine had no significant effect on sildenafil-induced inhibition of platelet aggregation. Repeated measures two-way ANOVA with Bonferroni post-hoc test. ns=non significant. N=7. All data is expressed as mean ± SEM.

The NOS substrate L-arginine had no effect on sildenafil-induced inhibition of platelet aggregation

L-arginine, a rate limiting substrate for NOS-derived NO synthesis, was used to further investigate the dependence of the antiplatelet effect of sildenafil on NOS activity. L-arginine and its inactive isomer D-arginine had no significant effect on platelet aggregation compared to vehicle treated platelets (Figure 30A). L-arginine also had no significant effect on sildenafil-mediated inhibition of platelet aggregation compared to vehicle or D-arginine treated platelets at sildenafil concentrations of 10nM, 100nM and 1µM (Figure 30B).

Sildenafil had no effect on collagen-induced platelet aggregation *in vivo* in eNOS$^{-/-}$ mice

In order to assess the *in vivo* relevance of *in vitro* findings in isolated platelets, the effect of sildenafil on platelet aggregation was investigated *in vivo* in eNOS$^{-/-}$ mice. Firstly, the mice were genotyped to ensure they lacked functional eNOS gene. eNOS$^{-/-}$ and W.T mice displayed bands at 2 different sites, eNOS$^{-/-}$ mice at 258 bp (non-functional eNOS genotype) and W.T mice at 337 bp (functional eNOS genotype). The W.T control (W.T) matched that of Charles Rivers W.T control (C). A DNA mix from W.T and eNOS$^{-/-}$ mice (HET) confirmed that WT and mutant PCR fragments could be detected in the same polymerase chain reaction experiment. No bands were present in the water control (H_2O) (Figure 31).

In vivo, sildenafil had no significant effect on the peak response, AUC or duration of response of collagen-induced platelet aggregation in eNOS$^{-/-}$ mice compared to vehicle treated eNOS$^{-/-}$ mice (see Figure 32).
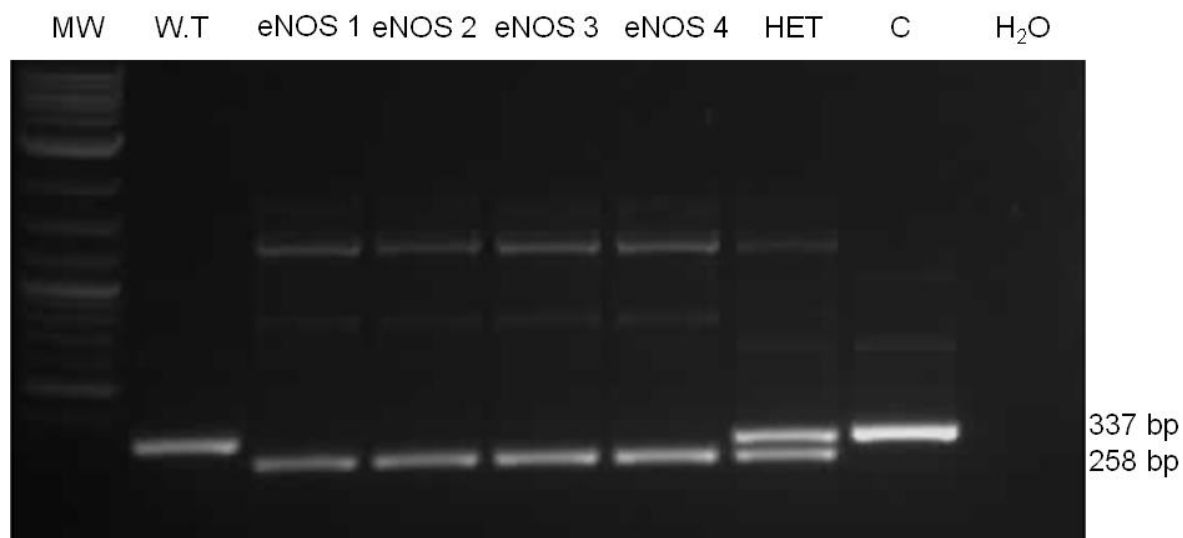
Figure 31: **Genotyping of eNOS$^{-/-}$ mice.**

337 bp correlates to functional eNOS genotype. 258 bp correlates to inactive eNOS genotype. MW= DNA ladder. W.T= Our wild type control. eNOS (1-4)= samples from the eNOS$^{-/-}$ mice used. HET= heterozygous control. Due to homozygous breeding, a heterozygous control wasn't present and therefore a DNA mix from both W.T and knockout mice were used to confirm WT and mutant PCR fragments could be detected in the same PCR reaction. C= Charles River Laboratories W.T control. H$_2$O= water control to show no contamination of the master mix. Courtesy of Charles River Laboratories (Margate, UK).
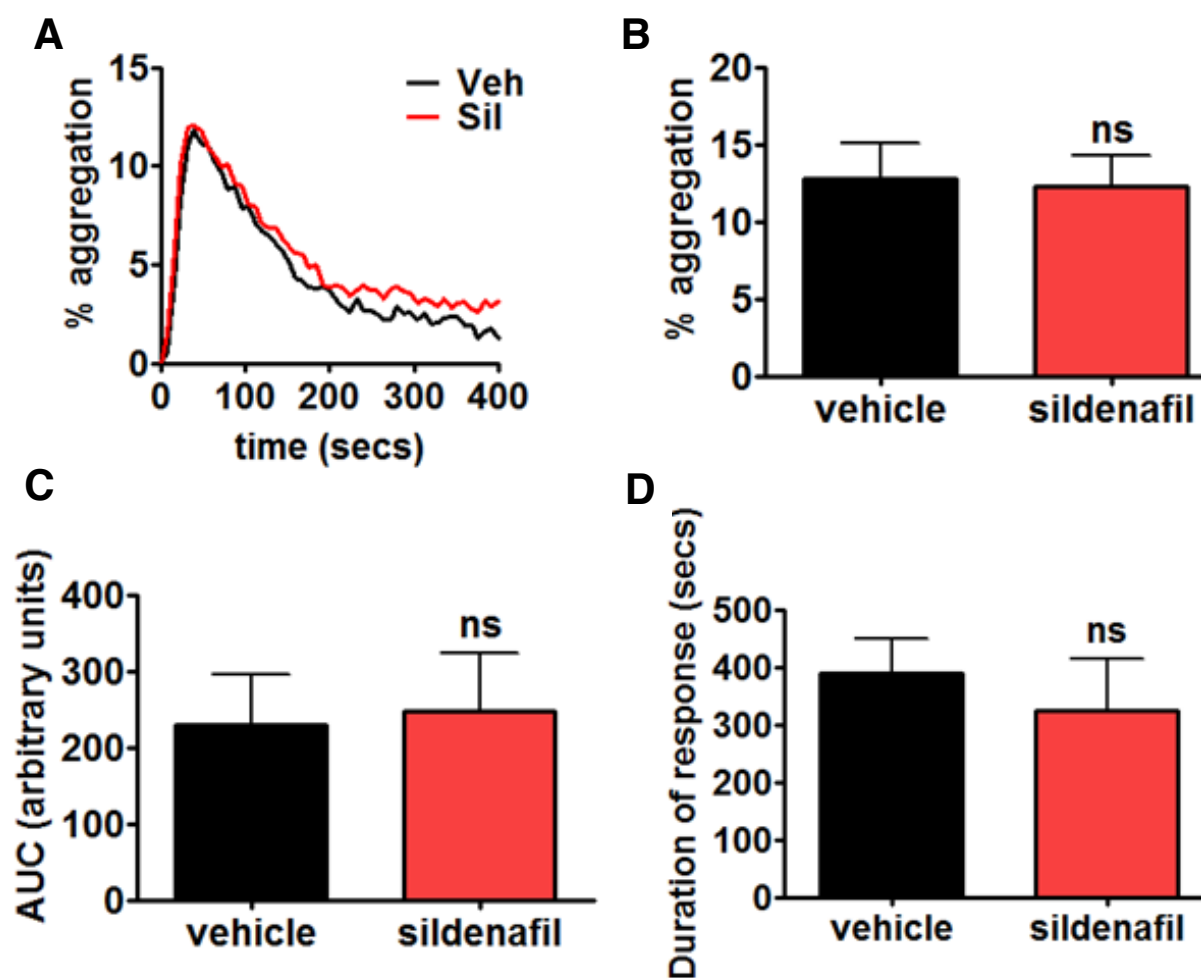
Figure 32: **Sildenafil had no effect on collagen-induced platelet aggregation *in vivo* in eNOS$^{-/-}$ mice.**

Sildenafil (50 µg kg^{-1}) or vehicle (DMSO, 0.1%) was administered to eNOS$^{-/-}$ mice 5 minutes before collagen (50 µg kg^{-1}). Platelet aggregation was measured as changes in radioactive counts in the pulmonary vasculature. (A) Mean trace of collagen response expressed as percentage increase from baseline, error bars omitted for clarity. (B) Maximum percentage increase from baseline (C) area under the curve and (D) the time it takes for the response to return to baseline was expressed as mean ± SEM. Unpaired Student's *t*-test. ns= non-significant compared to vehicle treated. N=4.

Discussion

Chapter 4 showed that sildenafil exerted an inhibitory effect on platelet aggregation *in vitro* and *in vivo*. Sildenafil was able to modify platelet function in the absence of the vascular endothelium and exogenous physiological inhibitors such as NO. The aim of this chapter was to investigate the mechanism of action of sildenafil on platelet aggregation.

SNP has been established to solely activate sGC and induce cGMP-dependent NO signalling (Dangel et al., 2010; Gudmundsdóttir et al., 2005) hence it was used as a positive control in subsequent experiments. SNP is an intracellular sGC/cGMP-dependent NO donor that spontaneously releases NO upon diffusing into the cell (Sogo et al., 2000). SNP-induced cytotoxicity was an experimental concern because it has been reported that 5 cyanide molecules are released for every NO moiety within the cell (Friederich and Butterworth, 1995). Experimentation in this study showed no signs of SNP cytotoxicity because the inhibitory effect on platelet aggregation was reversed in the presence of ODQ (sGC inhibitor - Figure 26A) and the NO scavenger haemoglobin (Hb - Figure 28A) and there was no significant damage to platelet membrane integrity (Figure 25B). Therefore I have shown that SNP, at concentrations up to 100µM, was a relatively non-toxic drug to use as a positive control in subsequent *in vitro* experiments.

Given the reported crosstalk between cyclic nucleotides and PDEs (Burkhardt et al., 2000; Dunkern and Hatzelmann, 2005; Grant et al., 1990; Li et al., 2003a) the effect of sildenafil on the functional effect of cGMP and cAMP-mediated inhibition of platelet aggregation was determined. In agreement with previous research, sildenafil (10nM) enhanced the inhibitory effect of the NO donor SNP (Gudmundsdóttir et al., 2005; Wallis et al., 1999; Wilson et al., 2008). This result was supported by the western blot data in Chapter 4 which showed that sildenafil increased VASP-P(239) expression, a biomarker of PKG activity. In contrast, sildenafil (10nM) had no effect on iloprost-mediated (and therefore cAMP stimulated) inhibition of platelet aggregation. This provided evidence that sildenafil acted *via* the cGMP, but not cAMP pathway. Unfortunately, in this study it was not possible to determine biochemical cGMP/cAMP concentrations in platelets in the presence and absence of

sildenafil potentially due to subtle changes in cyclic nucleotide concentrations. In other studies, researchers have amplified the cGMP signal with high concentrations of PDE inhibitors (Velmurugan et al., 2013), an approach which could not be used in this study as it would mask the detection of subtle changes in cGMP concentration. However, sildenafil is a well-established PDE5 inhibitor known to enhance cGMP signalling which further validates these results (Boolell et al., 1996; Corbin et al., 2003; Corbin and Francis, 1999). Previous studies have shown that the inhibitory effect of sildenafil on platelet activation was partly due to PDE crosstalk and inhibition of PDE3 (Dunkern and Hatzelmann, 2005). Using Figure 25 of this study, it was shown that sildenafil did not enhance iloprost-induced inhibition of platelet aggregation and therefore did not functionally effect cAMP signalling in platelets. This work provided evidence that sildenafil selectively enhances NO/cGMP signalling with no measurable functional effect on prostacyclin-mediated inhibition despite the suggested downstream crosstalk between these pathways.

Further investigations determined whether sildenafil enhanced cGMP signalling *via* sGC activation. This was achieved *in vitro* by pre-treating platelets with a selective irreversible sGC inhibitor ODQ. ODQ completely reversed the inhibitory effect of SNP on platelet aggregation which validated that ODQ inhibited sGC activity (Garthwaite et al., 1995). Sildenafil did not exert an inhibitory effect on platelet aggregation in the presence of ODQ which demonstrated the dependence of sildenafil on sGC activation. This was further proven using molecular techniques which demonstrated that sildenafil-induced increase of VASP-P(239), a marker of protein kinase activity in platelets, did not occur in the presence of ODQ (Figure 27). Thus, sildenafil exerted an inhibitory effect on collagen-induced platelet aggregation *via* the sGC/cGMP pathway. This finding was supported by other work that established the ability of ODQ to reverse cGMP-mediated accumulation (Gudmundsdóttir et al., 2005; Lies et al., 2013; Zhao et al., 2000). Overall, I have suggested that sildenafil caused an antiplatelet effect by enhancing intracellular cGMP signalling in platelets *via* sGC activation.

Due to sildenafil modifying platelet function in an isolated platelet preparation in the absence of exogenous sources of NO, it was investigated whether the inhibitory

effect of sildenafil on platelet aggregation was dependent on the presence of NO. Two distinct NO scavengers, an intracellular (hydroxocobalamin (HXB)) and extracellular (haemoglobin (Hb)), were used. However, it is known that Hb has high affinity for NO and could potentially compete with sGC by drawing the SNP-derived NO outside the cell, acting as an intracellular NO scavenger (Sogo et al., 2000). This effect was apparent in this study as Hb was able to reverse SNP-mediated inhibition of platelet aggregation, an effect that was proposed to be solely intracellular (Bates et al., 1991; Sogo et al., 2000). The inhibitory effect of sildenafil on platelet aggregation was NO dependent and did not occur in the presence of either NO scavenger. However, the impact of intracellular or extracellular NO source on sildenafil activity was unclear. The NO-dependence of sildenafil further proves that NO caused stimulation of sGC (Dangel et al., 2010) which generated transient cGMP signals in platelets (Gambaryan et al., 2013). In agreement with the results presented here, other researchers have established that sildenafil inhibits platelet function by enhancing NO/sGC/cGMP signalling initiated by the addition of NO donors (Berkels et al., 2001; Gudmundsdóttir et al., 2005; Wallis et al., 1999). I have shown that sildenafil caused a NO/sGC dependent antiplatelet effect in isolated platelets in the absence of exogenously applied NO donors. Therefore I conclude that platelets have the intrinsic ability to generate NO/cGMP signals in platelets that can be enhanced by the PDE5 inhibitor sildenafil.

The ability of NOS to generate NO in platelets and drive sildenafil-mediated inhibition of platelet aggregation was explored. The presence of NOS in platelets has been a subject of controversy over years (Naseem and Riba, 2008). Platelets have been reported to produce their own NO which was thought to be generated *via* platelet NOS (Alves de Sá Siqueira et al., 2011; De Meirelles et al., 2007; Freedman et al., 1999, 1997). However, studies have proven that platelets do not contain NOS (Gambaryan et al., 2008; Ozuyaman et al., 2005; Tymvios et al., 2009) whereas other studies revealed no functional evidence of platelet-derived NO (Gudmundsdóttir et al., 2005; Wallis et al., 1999). Here I have shown that the antiplatelet effect of sildenafil occurred independently of NOS as a non-selective NOS inhibitor, L-NAME and NOS substrate, L-arginine did not modify the inhibitory effect of sildenafil on platelet aggregation. Therefore I conclude that platelets do not

generate endogenous NO/cGMP signalling through NOS activity. Drug concentrations and incubation times used in this study were based on previous publications that have investigated and successfully modified NOS activity. L-NAME has been shown to inhibit NOS with an IC_{50} of 0.81μM (Babbedge et al., 1993). We have previously shown that off-target effects of L-NAME occur at around 1mM (Tymvios et al., 2009) and therefore the L-NAME concentration 100μM was chosen to selectively and effectively inhibit NOS activity. L-arginine has been reported to stimulate NOS activity with an EC_{50} of 7.5μM (Tsai et al., 2005) and has been reported to stimulate NOS activity at concentrations 300μM and 1mM (Alfieri et al., 2014; Gambaryan et al., 2008). Therefore it was reasonably assumed that L-arginine at 1mM would stimulate NOS acitivity in my experiments. In support of my findings, other studies have reported the lack of NOS expression in platelets (Gambaryan et al., 2008; Tymvios et al., 2009). Therefore, the work in this chapter has established that platelets are able to generate NO/cGMP signals independent of NOS activity.

To determine the effect of NOS-independent NO/cGMP signals on platelet function *in vivo*, the effect of sildenafil was investigated in mice that did not express functional eNOS (eNOS$^{-/-}$ mice). Genotyping verified that the genetically modified mice used in this thesis lacked the expression of functional eNOS. Interestingly, sildenafil had no effect on *in vivo* collagen-induced platelet aggregation in eNOS$^{-/-}$ mice which suggested that the presence of eNOS was necessary for NO/cGMP signalling in platelets *in vivo*. Although this result supported work from our laboratory establishing eNOS as a critical regulator of platelet aggregation *in vivo* (Moore et al., 2011, 2010), it opposed my *in vitro* results demonstrating sildenafil's ability to enhance NOS-independent platelet NO/cGMP signals in the absence of a functional vascular endothelium. Previous studies have suggested the presence of NOS-independent NO synthesis (Gordge and Xiao, 2010; Lundberg et al., 2008) and these mechanisms have been proposed as long-term storage of NO (Lundberg and Govoni, 2004; Stamler et al., 1992). Therefore, it is possible that NO activity could be preserved *via* a long-term storage mechanism in platelets and sildenafil could be enhancing a NO/cGMP signal initiated by a storage pool of NO during *in vitro* experiments. This could potentially provide an explanation for the lack of effect of sildenafil *in vivo* in eNOS$^{-/-}$ mice because the platelets of these mice have never

been exposed to eNOS-derived NO which could be essential for long-term NO storage (for example, for S-nitrosothiol formation or nitrate/nitrite source). Additional experiments would be necessary to validate this theory.

In conclusion, sildenafil exerted an inhibitory effect on platelet aggregation by enhancing transient NO/cGMP signals generated by the platelet. Platelet NO/cGMP signals occurred independently of NOS activity *in vitro* but not *in vivo* and therefore the source of NO modulating platelet function upstream of cGMP was unknown. Chapters 6 and 7 of this thesis will further explore possible avenues for the NOS-independent NO source.

Chapter 6: S-nitrosothiol activity on platelets

<u>Objectives and aims</u>

Chapter 5 established that sildenafil exerted an antiplatelet effect *in vitro* by enhancing NO/cGMP signals generated by the platelet. These NO/cGMP signals occurred independently of NOS activity and therefore the source of platelet NO/cGMP signals was unknown.

Exogenous S-nitrosothiols (RSNOs) have been reported to exert an antiplatelet effect *via* the NO/sGC/cGMP pathway (de Belder et al., 1994; Mathews and Kerr, 1993; Xiao and Gordge, 2011). RSNOs are known to be present in platelets (Hirayama et al., 1999) however the ability of RSNOs to drive NO/cGMP signals in platelets is unknown.

The aim of this chapter is to investigate the ability of endogenous RSNOs to drive NO/cGMP signalling events in platelets and explain the inhibitory effect of sildenafil on platelet aggregation. This will be achieved by inhibiting suggested pathways of RSNO-derived NO release, L-AT (Riego et al., 2009) and PDI (Shah et al., 2007), in the presence and absence of sildenafil in optical platelet aggregometry. In addition, to directly investigate the ability of platelet RSNOs to drive NO/cGMP signalling events, mercury dichloride (forms a stable thiol-mercury bond and displaces NO moiety – known as the Saville reaction (Saville, 1958; Swift and Williams, 1997)) will be assessed in sildenafil-induced inhibition of platelet aggregation.

The aims of this chapter were to:

- Assess the effect of L-leucine, an L-AT inhibitor, on sildenafil-induced inhibition of platelet aggregation *in vitro*.
- Assess the effect of bacitracin, a PDI inhibitor, on sildenafil-induced inhibition of platelet aggregation *in vitro*.
- Examine the functional role of RSNOs on sildenafil-induced inhibition of platelet aggregation *in vitro* by displacing and releasing NO from the thiol using mercury dichloride ($HgCl_2$).

Methods

Optical platelet aggregometry

Sildenafil concentration response in the presence of L-leucine, bacitracin or Mercury dichloride ($HgCl_2$)

Human WP were incubated with L-leucine (1mM), D-leucine (1mM), bacitracin (0.5 or 1.75mM), $HgCl_2$ (100nM) or mTHB for 5 minutes before the addition of sildenafil (10-1000nM) or vehicle (mTHB containing 0.05% (v/v) DMSO). WP were incubated for a further 5 minutes before stimulation with collagen (5µg mL^{-1}). Platelet aggregation was recorded for 4 minutes.

$HgCl_2$ concentration response

Human WP were incubated with $HgCl_2$ (1nM - 100µM) or mTHB for 10 minutes before stimulation with collagen (5µg mL^{-1}). Platelet aggregation was recorded for 4 minutes.

Results

<u>L-leucine had no effect on sildenafil-induced inhibition of platelet aggregation</u>

L-leucine, a competitive L-AT inhibitor, and its inactive isomer D-leucine had no significant effect on collagen-induced platelet aggregation (Figure 33A). Sildenafil caused a concentration-dependent inhibitory effect on platelet aggregation. The presence of L-leucine or D-leucine had no significant effect on sildenafil-induced inhibition of platelet aggregation (Figure 33B).
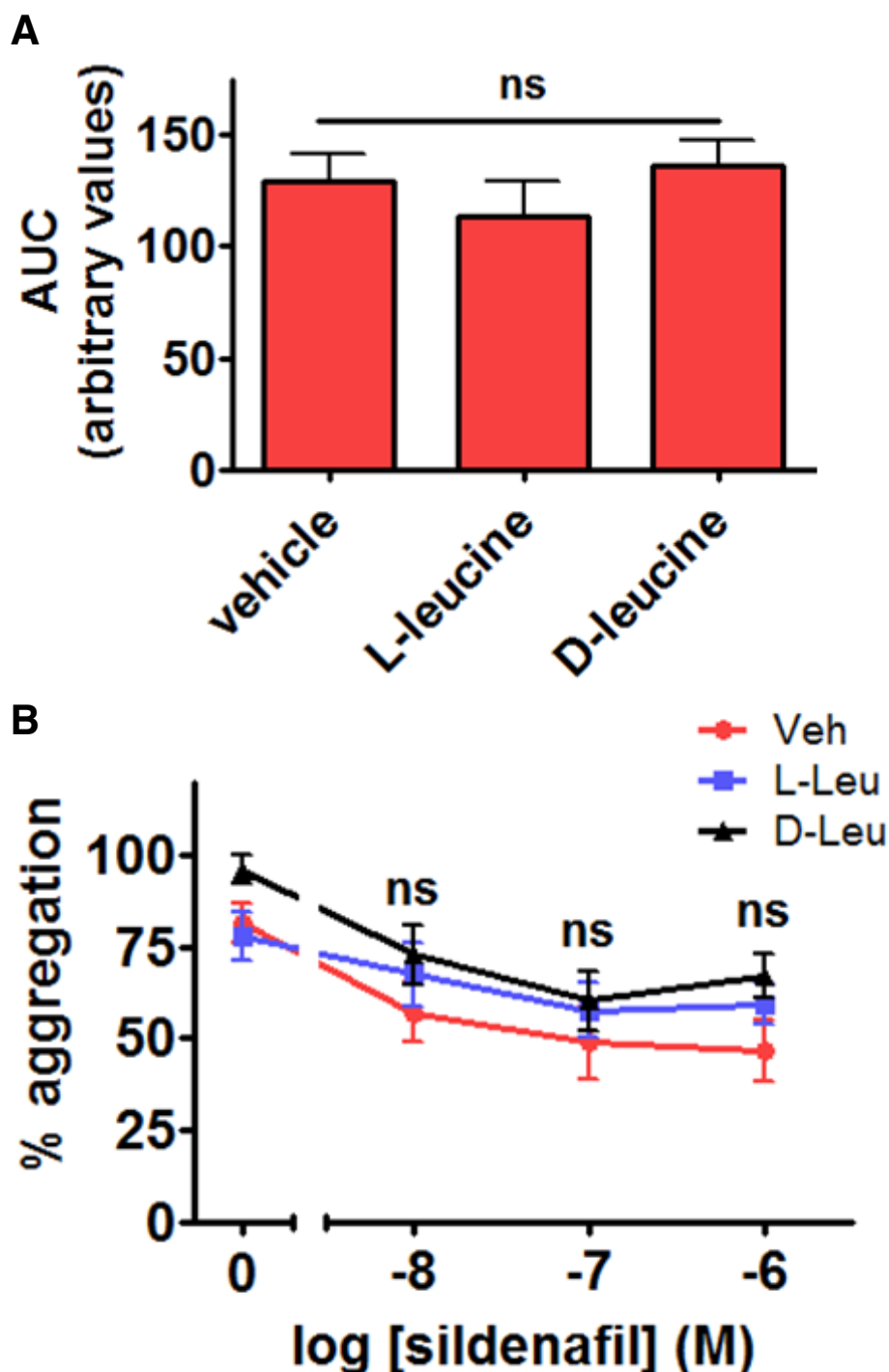
Figure 33: L-leucine had no significant effect on sildenafil-induced inhibition of platelet aggregation.

Human washed platelets were pre-incubated with L-leucine (L-leu; 1mM), D-leucine (D-leu; 1mM) or vehicle (saline) and sildenafil (sil, 10-1000nM) or vehicle (veh, DMSO 0.01%) before stimulation with collagen (5µg mL^{-1}) in optical platelet aggregometry. (A) Control data. L-leucine and D-leucine had no effect on collagen-induced platelet aggregation. Repeated measures one-way ANOVA with Dunnett's post-hoc test. (B) L-leucine and D-leucine had no effect on sildenafil-induced inhibition of platelet aggregation. Repeated measures two-way ANOVA with Bonferroni post-hoc test, ns= non-significant. N=6. All data is expressed as mean ± SEM.

<u>Bacitracin had no effect on sildenafil-induced inhibition of platelet aggregation</u>

Bacitracin, a PDI inhibitor, significantly inhibited collagen-induced platelet aggregation by ~30% at the concentration 1.75mM but had no significant effect at 0.5mM (Figure 34A). Bacitracin at the higher concentration (1.75mM) caused further reduction of sildenafil-mediated inhibition of platelet aggregation that was significant at 10nM, but was non-significant at 100nM and 1µM of sildenafil (Figure 34B). Bacitracin at the lower concentration of 0.5mM did not significantly modify the concentration-dependent inhibitory effect of sildenafil on platelet aggregation (Figure 34C).
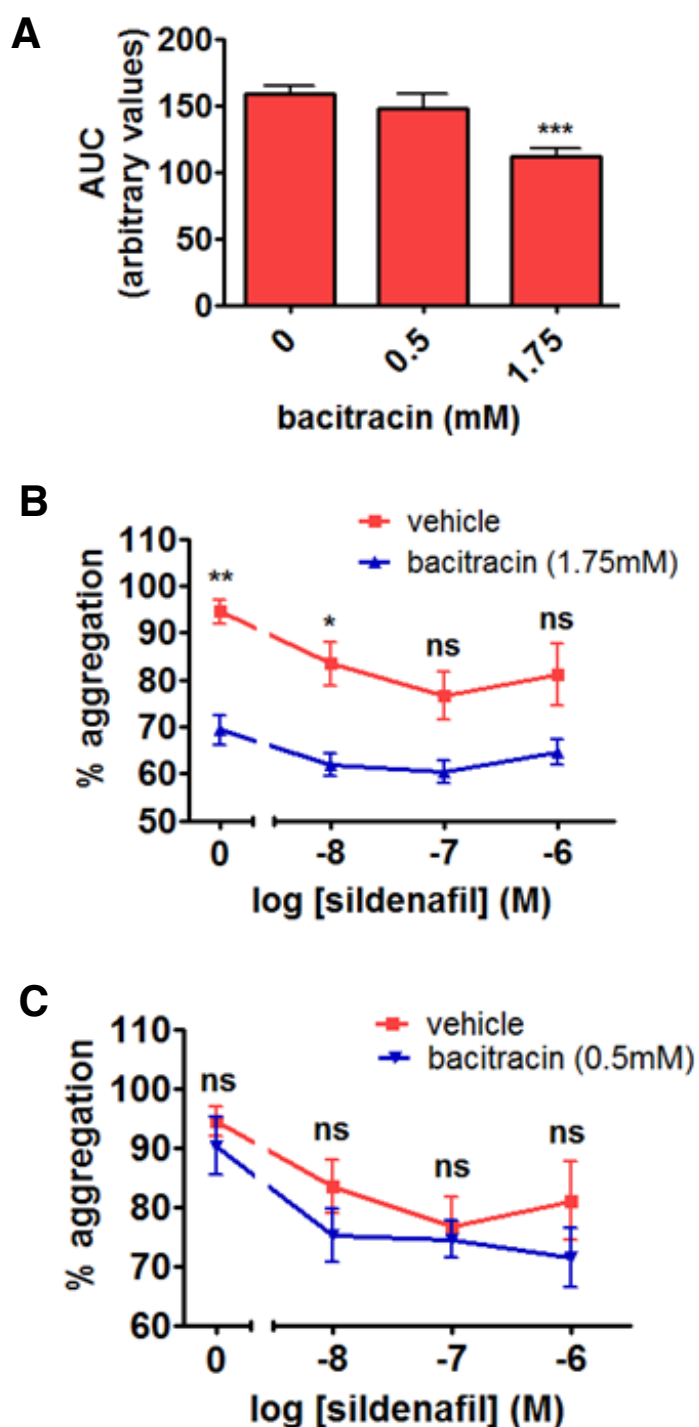
Figure 34: **Bacitracin had no significant effect on sildenafil-induced inhibition of platelet aggregation.**

Human washed platelets were pre-incubated with bacitracin (0.5 or 1.75mM) or vehicle (saline) and sildenafil (sil, 10-1000nM) or vehicle (veh, DMSO 0.01%) before stimulation with collagen (5µg mL^{-1}) in optical platelet aggregometry. (A) Control data. Bacitracin at 1.75mM, but not 0.5mM significantly inhibited collagen-induced platelet aggregation. Repeated measures one-way ANOVA with Dunnett's post-hoc test. (B) Bacitracin at 1.75mM significantly enhanced sildenafil-mediated inhibition of platelet aggregation at 10nM. (C) Bacitracin at 0.5mM had no effect on sildenafil-induced inhibition of platelet aggregation. Repeated measures two-way ANOVA with Bonferroni post-hoc test, ns= non-significant,*$P<0.05$, **$P<0.01$, ***$P<0.001$. N=8. All data is expressed as mean ± SEM.

Mercury dichloride ($HgCl_2$) had no effect on sildenafil-induced inhibition of platelet aggregation

To investigate the ability of RSNOs to drive NO/cGMP signals generated by the platelet, $HgCl_2$ was used to pharmacologically release the NO moiety from RSNOs (Saville, 1958). The effects of increasing concentrations of $HgCl_2$ on collagen-induced platelet aggregation were investigated to identify a subthreshold concentration of $HgCl_2$ to subsequently investigate the ability of sildenafil to enhance the NO signal generated by $HgCl_2$. $HgCl_2$ caused significant concentration-dependent inhibition of platelet aggregation at concentrations higher than 0.25µM (Figure 35A). To ensure the stability of the platelets throughout the experiment, vehicle-treated platelets were stimulated after the experiment (post-vehicle). 'Post-vehicle' platelets were able to produce ~100% aggregation of the vehicle treated platelets.

The pretreatment of platelets with 100nM of $HgCl_2$, a concentration that did not cause significant inhibition of platelet aggregation, had no significant effect on sildenafil-induced inhibition of platelet aggregation (Figure 35B).
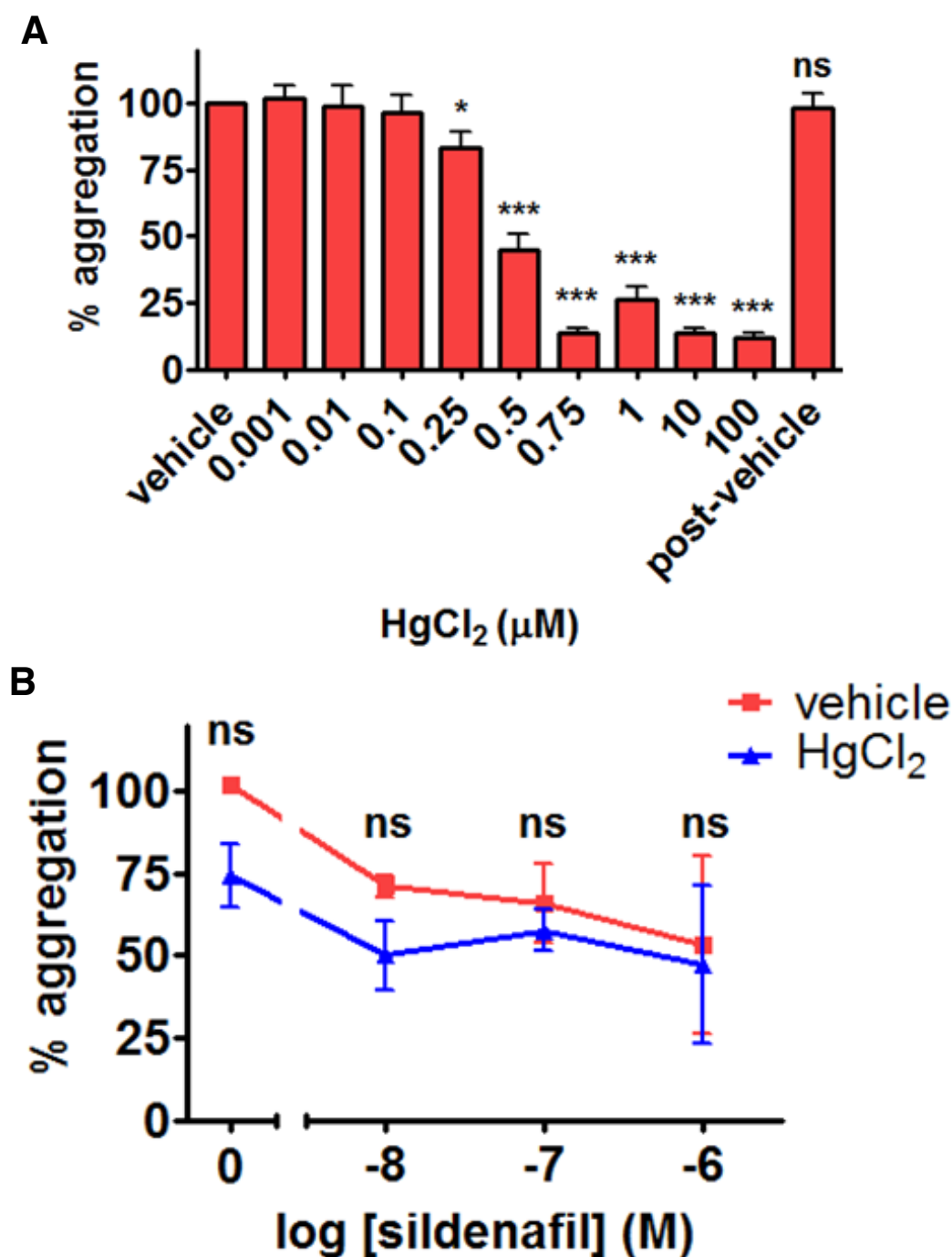
Figure 35: Mercury dichloride ($HgCl_2$) had no significant effect on sildenafil-induced inhibition of platelet aggregation.

Human washed platelets were pre-incubated with mercury dichloride ($HgCl_2$; 1nM-100µM) or vehicle (mTHB) and sildenafil (sil, 10-1000nM) or vehicle (veh, DMSO 0.01%) before stimulation with collagen (5µg mL^{-1}) in optical platelet aggregometry. (A) $HgCl_2$ caused a concentration-dependent significant inhibition of collagen-induced platelet aggregation. Repeated measures one-way ANOVA with Dunnett's post-hoc test. (B) $HgCl_2$ at 100nM had no effect on sildenafil-induced inhibition of platelet aggregation. Repeated measures two-way ANOVA with Bonferroni post-hoc test, ns= non-significant,*$P<0.05$, ***$P<0.001$. N=4. All data is expressed as mean ± SEM.

Discussion

Chapter 5 established that sildenafil exerted an antiplatelet effect by enhancing NO/cGMP signals generated by the platelet. These NO/cGMP signals occurred independently of NOS activity *in vitro* and therefore the source of platelet-derived NO was unknown. This chapter aimed to explore the role of platelet RSNOs in generating platelet NO/cGMP signals by targeting known mechanisms of RSNO-mediated NO release.

The amino acid transporter L-AT has been reported to transport RSNOs and stimulate sGC in a range of cells (Li and Whorton, 2005; Riego et al., 2009; Sandmann et al., 2005; Satoh et al., 1997). To investigate the involvement of L-AT in the generation of RSNO-derived NO/cGMP signals in platelets, sildenafil-mediated inhibition of platelet aggregation was investigated in the presence and absence of the L-AT inhibitor L-leucine. L-leucine, an L-AT substrate known to competitively inhibit L-AT RSNO transport (Riego et al., 2009), and its inactive isomer D-leucine had no significant effect on collagen-induced platelet aggregation (Figure 33A). L-leucine had no significant effect on sildenafil-induced inhibition of platelet aggregation (Figure 33B). L-leucine at a concentration of 1mM successfully inhibited L-AT activity by significantly attenuating intracellular RSNO concentration in neuron and macrophage cell lines (Nemoto et al., 2003; Zhang and Hogg, 2004) and therefore this concentration was used in this study to effectively and selectively inhibit L-AT activity. Unfortunately, this experiment was limited by the lack of a positive control to prove that L-leucine at a concentration of 1mM could inhibit L-AT transport in platelets. CysNO, a known substrate of L-AT, could be used to confirm the activity of L-AT in platelets and that the concentrations used here inhibited its function. Further experiments investigating molecular and biochemical measurements such as intracellular cGMP and RSNO/NO concentrations before and after L-leucine treatment would support this result and provide more convincing conclusions. Overall, my results suggested that L-AT had no effect on the ability of platelets to generate NO/cGMP signals. Bell *et al.* (Bell et al., 2007) reported that L-leucine did not significantly inhibit cGMP accumulation stimulated by an RSNO, GSNO in platelets which disputed the involvement of L-AT in RSNO-derived cGMP signalling events in platelets. In agreement with the exogenous application of

RSNOs on platelets, this chapter has established that L-AT is not involved in NO/cGMP signalling events in platelets driven by endogenous platelet RSNOs. In addition, previous studies reported that CysNO/L-AT-mediated effects were insensitive to NO scavengers (Riego et al., 2009; Zhang and Hogg, 2004). Previously I have shown that sildenafil-induced inhibition of platelet aggregation was sensitive to NO scavengers (see Chapter 5) which provides further evidence that L-AT was not involved in the antiplatelet effect of sildenafil. My results, in combination with previous literature, provide no evidence for the involvement of L-AT in RSNO-driven NO/cGMP signalling events in platelets. I have shown that L-AT activity was not essential for sildenafil-induced inhibition of platelet aggregation which established that L-AT was not involved in platelet NO/cGMP generation *via* potential NO release from RSNOs.

PDI has been shown to exert oxidoreductase activity and mediate denitrosation of exogenously applied RSNOs to release NO in platelets (Bell et al., 2007; Sliskovic et al., 2005). This chapter explored the ability of platelet PDI to undergo denitrosation of endogenous RSNOs to deliver NO into platelets and explain sildenafil-mediated inhibition of platelet aggregation. Previous studies have inhibited PDI denitrosation in human WP by the use of the cell impermeant potent PDI inhibitor bacitracin (Bell et al., 2007; Shah et al., 2007) and therefore this drug was used in the current study. Due to the integral activity of PDI in integrin-mediated platelet function, bacitracin was able to significantly inhibit collagen-induced platelet aggregation. This is in line with previous studies that have investigated the function of PDI and other thiol isomerases on platelet activity (Holbrook et al., 2012; Kim et al., 2013, 2013; Root et al., 2004). Bacitracin at the higher concentration (1.75mM) significantly inhibited platelet aggregation and masked the potential denitrosation activity of PDI. Therefore the dependence of platelet-derived NO/cGMP signals on PDI activity could not be established using bacitracin at the concentration of 1.75mM. The lower concentration of bacitracin (0.5mM) did not cause significant inhibition of platelet aggregation (Figure 34A) and was previously reported to significantly inhibit cGMP accumulation by GSNO stimulation (Bell et al., 2007). Therefore it was reasonably assumed that bacitracin at the concentration of 0.5mM was selective for the denitrosation activity of PDI in platelets. A sildenafil concentration-response was performed in the presence

and absence of bacitracin at the concentration of 0.5mM. Bacitracin had no significant effect on sildenafil-induced inhibition of platelet aggregation which suggests that PDI inhibition had no effect on the ability of platelets to generate NO/cGMP signals. Therefore my data provides no evidence to suggest that PDI was involved in the generation of NO/cGMP signals in platelets *via* potential NO release from RSNOs.

Future experiments associating the functional effects with biochemical analysis could be beneficial to further support my conclusions. The measurement of RSNO/NO concentrations before and after bacitracin treatment could give a clearer insight into the role of PDI in platelet NO signalling by demonstrating RSNO 'consumption' and NO 'production'. The selectivity of bacitracin for PDI has recently been disputed (Karala and Ruddock, 2010) and therefore the additional use of other PDI inhibitors (such as phenylarsine oxide (PAO) and the PDI and ERp57 antibody RL90) and PDI knockout mice will ensure PDI specificity and verify the conclusion that PDI is not involved in the generation of NO/cGMP signals in platelets. Previous studies have reported the involvement of PDI in the antiplatelet effect of RSNOs *via* NO/cGMP stimulation (Bell et al., 2007; Shah et al., 2007; Xiao and Gordge, 2011) which suggests that RSNOs were not involved in the antiplatelet effect of sildenafil in my study. However, previous studies only examined the effect of exogenous RSNOs and they did not associate their molecular and biochemical findings with functional studies. Therefore, I propose further investigation associating the aggregation results with molecular and biochemical analysis to verify the activity of PDI in the generation of NO/cGMP platelet signals (in detail under the heading 'Recommendations for future work' in chapter 8).

The final figure investigated the ability of platelet RSNOs to mediate platelet-derived NO/cGMP signals by pharmacologically releasing thiol-bound NO and investigating the functional impact of RSNOs on sildenafil-mediated inhibition of platelet aggregation. Thiols are also known as mercaptans, originating from the Latin corpus mercurium captāns meaning 'body capturing quicksilver' which is in reference to their high affinity to bind mercury (Ravichandran, 2004). Here it was investigated whether $HgCl_2$ could modify sildenafil-induced inhibition of platelet aggregation by binding to

platelet thiols and releasing NO from RSNOs (as shown in previous studies (Hirayama et al., 1999; Maejima et al., 2005; Sliskovic et al., 2005)). $HgCl_2$ exerted a concentration-dependent inhibition on collagen-induced platelet aggregation which, based on previous studies, was reasonably assumed to be due to an increase in NO stimulation (Saville, 1958; Swift and Williams, 1997). The inhibitory effect of sildenafil was investigated in the presence and absence of $HgCl_2$ at a concentration of 100nM, a concentration that did not significantly inhibit platelet aggregation. The presence of $HgCl_2$ had no significant effect on sildenafil-induced inhibition of platelet aggregation which suggested that RSNOs did not modify the effect of sildenafil on platelets. Collectively, my results have shown that RSNOs are not involved in the generation of NO/cGMP signals in platelets.

Future experiments investigating RSNO and NO concentrations of resting platelets and after pretreatment of $HgCl_2$ would further support the aggregation results from this chapter and provide further evidence of RSNOs involvement in the intrinsic ability of platelets to generate NO/cGMP signals. To my knowledge, this is the first study to have investigated the ability of platelet RSNOs to mediate endogenous NO/cGMP signals, however studies have confirmed the effects of exogenous RSNOs on mediating a NO/cGMP antiplatelet effect (Bell et al., 2007; Shah et al., 2007, 2003; Xiao and Gordge, 2011). This current study suggests that platelet-derived RSNOs do not modify platelet function by generating NO/cGMP signals, however further experimentation is necessary to validate this theory.

In conclusion, this chapter suggests that platelet RSNOs, in general or *via* mechanisms involving L-AT or PDI, are not involved in the generation of NO/cGMP signals in platelets *in vitro*. However, this was not an exhaustive investigation and I advise further experimentation to verify these findings.

Chapter 7: Nitrate/nitrite as a NO source in platelets

Objectives and aims

Chapter 5 established the ability of sildenafil to inhibit platelet aggregation by enhancing transient NO/cGMP signals generated by the platelet. Platelet NO/cGMP signals occurred independently of NOS activity *in vitro* and therefore the source of these signals remains unknown.

Inorganic nitrate and nitrite can be chemically reduced to NO *in vivo* and have been shown to exert antiplatelet effects *ex vivo* (Velmurugan et al., 2013; Webb et al., 2008b). However, the ability of nitrate/nitrite to generate NO/cGMP signals in the platelet is unknown. The overall aim of this chapter is to assess the ability of inorganic nitrate and nitrite to generate platelet-derived NO/cGMP signals and explain the inhibitory effect of sildenafil on platelet aggregation.

Detectable concentrations of nitrate and nitrite have been reported in the plasma (Govoni et al., 2008; Minamino et al., 1997), however the ability of intraplatelet nitrate/nitrite to generate platelet-derived NO/cGMP signals is unknown. Therefore, the concentration of nitrate and nitrite in WP will be investigated and the ability of platelet nitrate/nitrite (collectively known as nitrogen oxides (NO_x)) to be reduced to bioactive NO will be assessed. The effect of inorganic nitrate/nitrite will be investigated on platelet aggregation *in vitro* to ascertain the direct effect of nitrate/nitrite on platelet function.

Oral administration of nitrate has been reported to modestly reduce platelet aggregation *ex vivo* in healthy mice and humans (J. Park et al., 2013; Richardson et al., 2002; Velmurugan et al., 2013; Webb et al., 2008a). However, the ability of nitrate/nitrite to impact platelet function *in vivo* in the presence of an intact vascular endothelium (and therefore eNOS-derived NO) is unknown. Moreover, the impact of inorganic nitrate/nitrite on platelet function during endothelial dysfunction has not been identified. An additional aim of this chapter is to assess the ability of nitrate/nitrite to impact platelet function *in vivo* in W.T and eNOS deficient mice (as a model of endothelial dysfunction).

Finally, to confirm that the potential inhibitory effect of inorganic nitrate on *in vivo* platelet function is cGMP-mediated, the dual treatment of nitrate and sildenafil will be investigated on *in vivo* platelet aggregation in eNOS$^{-/-}$ mice.

The aims of this chapter were to:

- Identify the presence of nitrogen oxides (NO$_x$; umbrella term for nitrate and nitrite) in WP.
- Establish the ability of platelet NO$_x$ to be reduced to bioactive NO with the use of a mild reducing agent ascorbic acid.
- Assess the ability of nitrate and nitrite to mediate NO/sGC/cGMP-driven inhibition of platelet aggregation and modify sildenafil's activity on platelets *in vitro*.
- Investigate the ability of nitrate to inhibit *in vivo* platelet aggregation in healthy (W.T) mice and in an animal model of endothelial dysfunction (eNOS$^{-/-}$ mice).
- Investigate the ability of sildenafil to modify the effect of inorganic nitrate on platelet aggregation *in vivo* in eNOS$^{-/-}$ mice.

Methods

Nitrate/nitrite colorimetric assay

Nitrate/nitrite concentration of platelets

Human WP and mTHB were snap frozen in liquid nitrogen and nitrate/nitrite concentrations were analysed using the Griess reaction (using the Cayman colorimetric assay kit) as described in chapter 2.

Nitrate/nitrite concentration of platelets treated with ascorbic acid

Human WP were incubated with ascorbic acid (10µM and 1mM) or mTHB for 5 minutes. The samples were then snap frozen in liquid nitrogen and nitrate/nitrite concentrations were analysed using the Griess reaction (using the Cayman colorimetric assay kit) as described in chapter 2.

Optical platelet aggregometry

Effect of sildenafil and nitrate/nitrite

Human WP were incubated with sildenafil (10nM), vehicle control (mTHB containing 0.01% (v/v) DMSO) or mTHB for 5 minutes before the addition of sodium nitrate ($NaNO_3$ - 0.01-100µM), sodium nitrite ($NaNO_2$ - 0.01-100µM) or mTHB. The WP were incubated for a further 5 minutes before stimulation with collagen (5µg mL^{-1}). Platelet aggregation was recorded for 4 minutes.

Effect of sildenafil, nitrite and ODQ

Human WP were incubated with ODQ (10µM), vehicle control (mTHB containing 0.01% (v/v) DMSO) or mTHB then sildenafil (10nM), vehicle control (mTHB containing 0.01% (v/v) DMSO) or mTHB then $NaNO_2$ (0.01-100µM), SNP (1µM) or mTHB. Each drug addition was incubated for 5 minutes before the next. WP were analysed in an optical aggregometer and stimulated with collagen (5µg mL^{-1}). Platelet aggregation was recorded for 4 minutes.

Ascorbic acid concentration response

Human WP were incubated with ascorbic acid (0.1-1000µM) or mTHB for 5 minutes before stimulation with collagen (5µg mL^{-1}). Platelet aggregation was recorded for 4 minutes.

Effect of ascorbic acid and ODQ

Human WP were incubated with ODQ (10µM), vehicle control (mTHB containing 0.05% (v/v) DMSO) or mTHB for 5 minutes before the addition of ascorbic acid (0.1-1000µM) or mTHB. WP were incubated for a further 5 minutes before stimulation with collagen (5µg mL^{-1}). Platelet aggregation was recorded for 4 minutes.

<u>Western blotting</u>

Effect of nitrate and nitrite on VASP-P(239) in the presence of sildenafil and ODQ

Human WP were incubated with ODQ (10µM) or DMSO vehicle (<0.05%) then sildenafil (10nM) or vehicle control (mTHB containing 0.01% (v/v) DMSO) then NaNO$_3$ (100µM), NaNO$_2$ (100µM), SNP (1µM) or mTHB. Each drug was incubated for 5 minutes before the next and the samples were prepared as detailed in chapter 2. The samples were quantified, run on an SDS-PAGE gel and transferred onto a PVDF membrane as detailed in chapter 2. The primary antibodies used were rabbit anti-VASP (1:1000), rabbit anti-phospho-VASP (Ser239) (1:1000) and the housekeeping protein rabbit anti-GAPDH (1:500), all left to incubate overnight at 4ºC. The secondary antibody used for all the above primary antibodies was anti-rabbit HRP antibody (1:2000), left to incubate for 1 hour at room temperature before protein visualisation as detailed in chapter 2.

<u>*Ex vivo* measurement of nitrate and nitrite concentration.</u>

The effect of nitrate administration on salivary gland and plasma NO$_x$ concentration in W.T and eNOS$^{-/-}$ mice

C57BL/6 W.T and eNOS$^{-/-}$ mice (~25g) were treated with 100µL of NaNO$_3$ (1mmol/kg) or saline (w/v 0.9%) i.p. After 1 hour the mice were anaesthetised and plasma and salivary glands extracted (as detailed in chapter 2). Gas-phase chemiluminescence was performed on these samples as detailed in chapter 2.

In vivo measurement of platelet aggregation

The effect of nitrate administration on W.T or eNOS$^{-/-}$ mice

C57BL/6 or eNOS$^{-/-}$ mice (~25g) were treated with 100µL of NaNO$_3$ (1mmol/kg) or saline i.p for 30 minutes before they were anaesthetised and radiolabelled platelets administered as described in chapter 2. One hour after drug treatment, 50µg kg^{-1} collagen (50µL) was administered i.v and the aggregation response was measured for 10 minutes.

The dual effect of nitrate and sildenafil on eNOS$^{-/-}$ mice

eNOS$^{-/-}$ mice (~25g) were treated with 100µL of NaNO$_3$ (1mmol/kg) or saline and sildenafil citrate (10mg kg^{-1}) or DMSO (0.1%) i.p for 30 minutes before they were anaesthetised and radiolabelled platelets administered as described in chapter 2. One hour after drug treatment, 50µg kg^{-1} collagen (50µL) was administered i.v and the aggregation response was measured for 10 minutes.

Results

Platelets contain nitrate and nitrite which has the ability to be reduced to bioactive NO *in vitro*

mTHB buffer contained no detectable concentrations of NO_x. In contrast, WP contained ~30µM of NO_x, mainly nitrate but containing some nitrite (~5µM) (Figure 36A). The addition of a mild reducing agent, ascorbic acid caused a concentration-dependent reduction in platelet NO_x. Ascorbic acid at the concentration of 1mM significantly reduced nitrate/nitrite concentrations in platelets by approximately 80% compared to untreated platelets. A lower concentration of ascorbic acid (10µM) demonstrated a trend in reduced nitrate/nitrite concentrations in platelets (Figure 36B). Functionally, ascorbic acid caused significant concentration-dependent inhibition of collagen-induced platelet aggregation compared to vehicle treated platelets (1mM caused ~25% reduction in platelet aggregation; Figure 36C). The inhibitory effect of ascorbic acid was reversed in the presence of ODQ which exerted ~100% aggregation response compared to the control (Figure 36D).

A

B

C

D
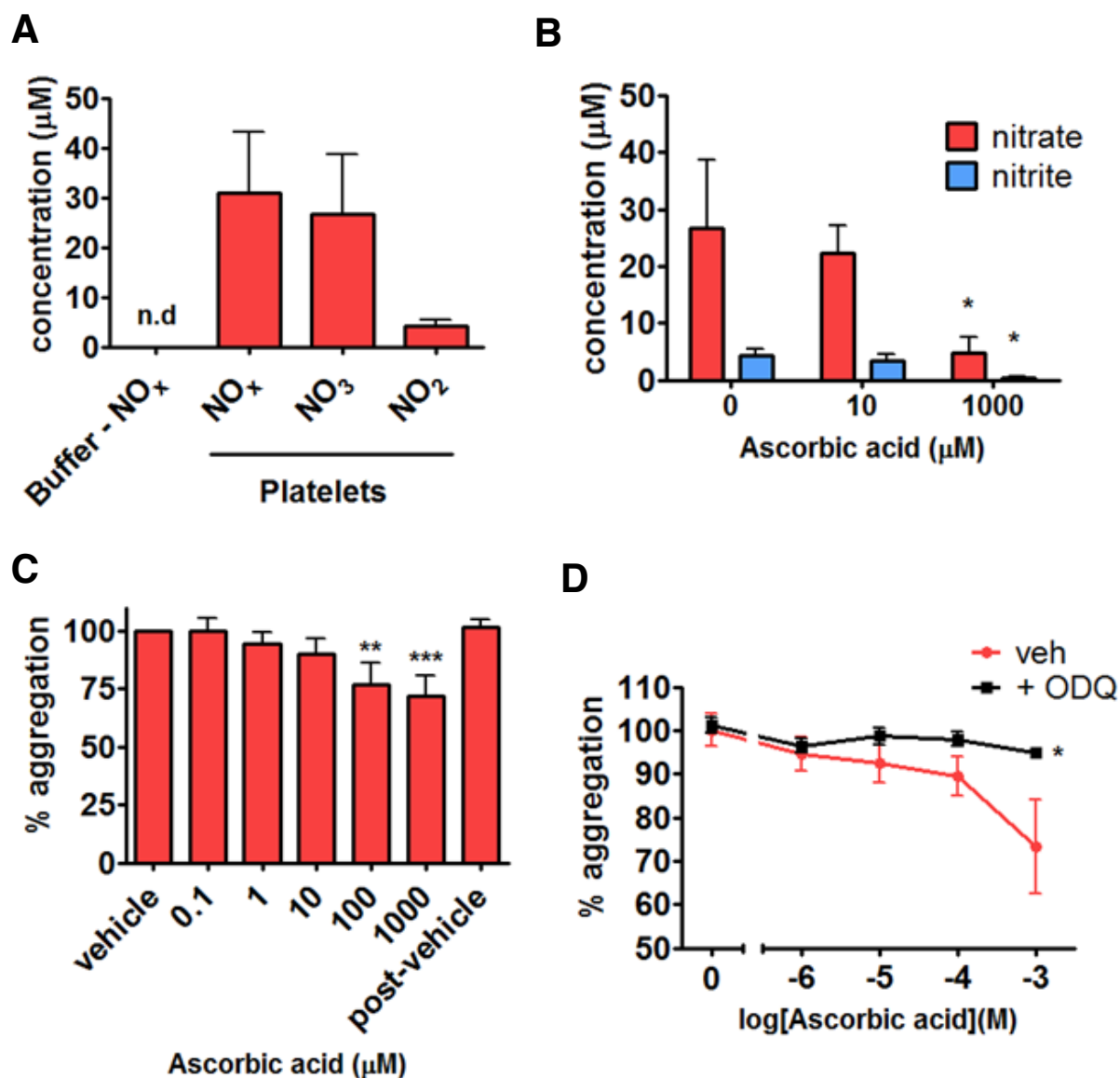
Figure 36: **Platelets contain nitrate and nitrite which has the ability to be reduced to bioactive NO *in vitro*.**

A-B: Nitrate and nitrite concentrations were measured in human washed platelets (A) untreated or (B) pre-incubated with vehicle (saline) or ascorbic acid (10, 1000 µM) for 5 minutes. Repeated-measured one-way ANOVA with Dunnett's post-hoc test. N=4. C: Collagen (5 µg mL^{-1}) induced *in vitro* platelet aggregation was inhibited in human washed platelets treated with ascorbic acid (100nM-1mM). Repeated-measures one-way ANOVA with Dunnett's post-hoc test. N=7. D: Ascorbic acid-induced inhibition of aggregation was reversed in the presence of ODQ (10 µM). Repeated-measures two-way ANOVA with Bonferroni post-hoc test. N=6. All data is expressed as mean±SEM. *$P<0.05$, **$P<0.01$, ***$P<0.001$ significance testing. NO_x-nitrogen oxides; NO_3-nitrate; NO_2- nitrite; N.d-not detectable; ODQ-1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one.
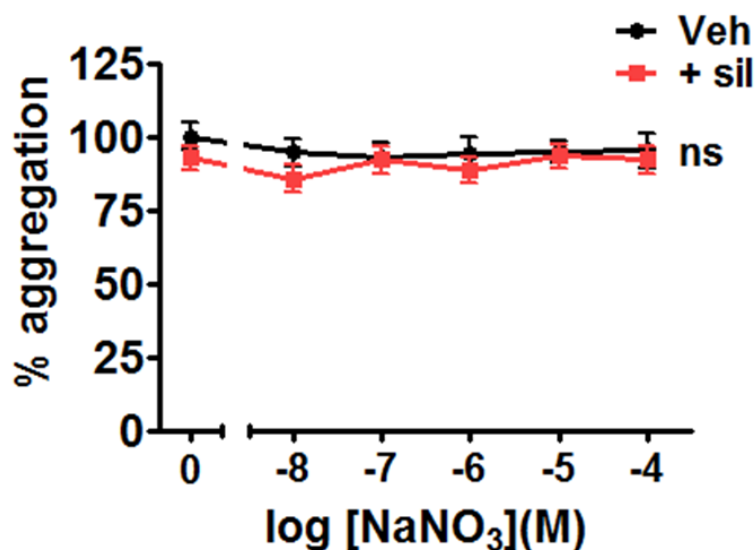
Figure 37: **Nitrate has no effect on platelet aggregation *in vitro*.**

Sodium nitrate (NaNO$_3$ - 10nM-100µM) in the presence and absence of a low concentration of sildenafil (10nM) had no effect on collagen (5µg mL^{-1}) induced platelet aggregation *in vitro*. Repeated measures two-way ANOVA with Bonferroni post-hoc test. ns=non-significant. N=6.

Nitrate has no effect on platelet aggregation *in vitro*

The pretreatment of platelets with nitrate at increasing concentrations (10nM-100µM) had no significant effect on collagen-induced platelet aggregation compared to vehicle-treated platelets. Nitrate in the presence of sildenafil also had no significant effect on collagen-induced platelet aggregation compared to vehicle-treated platelets (Figure 37).

To associate the aggregation results with signalling events, the effect of nitrate (100µM) in the presence and absence of sildenafil (10nM) and ODQ (10µM) on VASP-P(239) was investigated. Data is expressed as a representative blot of VASP-P(239), total VASP and protein loading control GAPDH (Figure 38A) and a bar chart showing the percentage of VASP-P(239) compared to total VASP (Figure 38B). Nitrate and sildenafil alone had no significant effect on VASP-P(239) phosphorylation compared to vehicle-treated platelets. SNP (positive control) induced significant VASP-P(239) phosphorylation compared to vehicle-treated platelets (Figure 38).
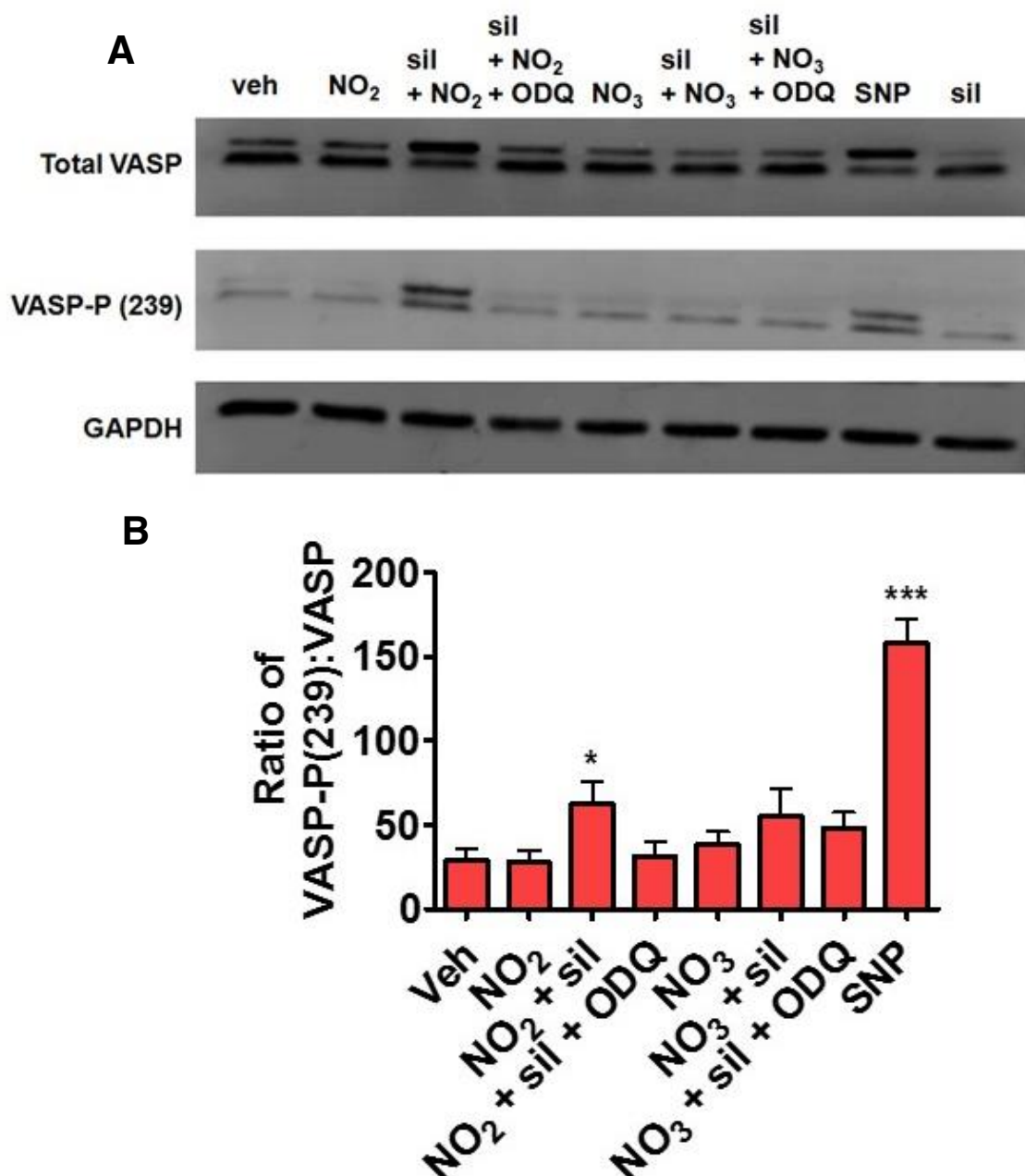
Figure 38: **Western blot showing VASP-P(239) expression after nitrate/nitrite treatment in the presence and absence of sildenafil and ODQ.**

Pre-incubation of platelets with sodium nitrite (NO_2, 100 µM) and sildenafil (sil, 10 nM) resulted in significant phosphorylation of VASP at serine 239 (VASP-P(239)) that was reversed in the presence of ODQ (10 µM). This effect was not seen with sodium nitrate (NO_3, 100 µM). Sodium nitroprusside (SNP, 1µM) was used as a positive control. Data presented as (A) Western blot representative of 5 independent experiments and (B) percentage of VASP-P(239) compared to total VASP. Data expressed as mean±SEM. One way ANOVA with Dunnett's post-hoc test. $P<0.05$, $P<0.001$ compared to vehicle treated. n=5.

Nitrite in the presence of a low concentration of sildenafil inhibited platelet aggregation *in vitro via* the sGC pathway

The pretreatment of platelets with nitrite (10nM-100µM) had no effect on collagen-induced platelet aggregation compared to the vehicle treated platelets. However, nitrite in the presence of sildenafil (10nM) induced significant concentration-dependent inhibition of collagen-induced platelet aggregation (up to 20% reduction) compared to nitrite treatment alone. The addition of the sGC inhibitor ODQ reversed the inhibitory effect of nitrite combined with sildenafil on platelet aggregation since ~100% aggregation was induced (Figure 39).

VASP phosphorylation was used as a biomarker of platelet signalling during drug treatments. Data was expressed as a representative blot of VASP-P(239), total VASP and protein loading control GAPDH (Figure 38A) and as a bar chart showing the mean percentage of VASP-P(239) compared to total VASP (Figure 38B). SNP (positive control) induced significant VASP-P(239) phosphorylation compared to vehicle treated platelets. Nitrite (100µM) in the presence of a low concentration of sildenafil (10nM) caused a significant increase in VASP phosphorylation at ser239 which was not seen in the presence of ODQ. Nitrite and sildenafil treatment alone had no significant effect on platelet VASP-P(239) when administered separately.
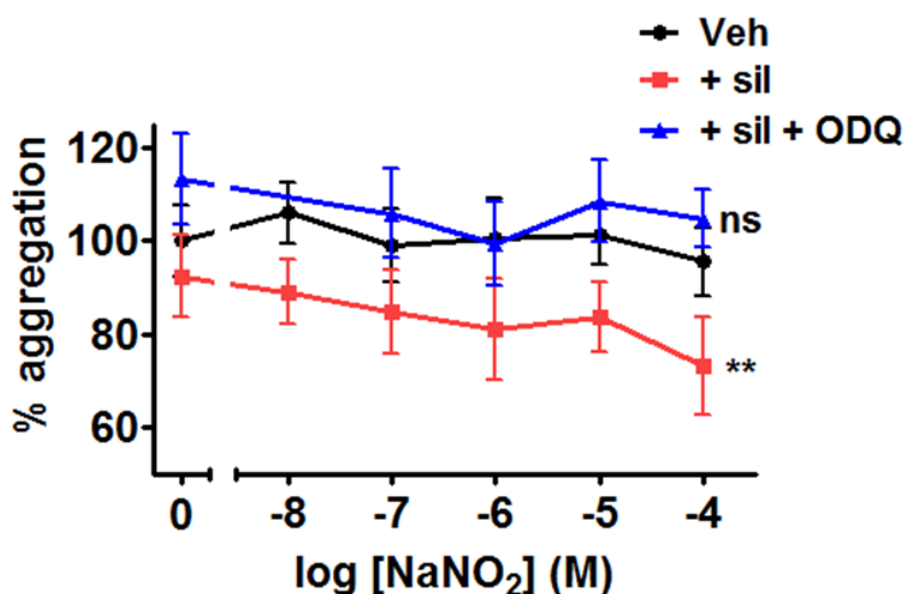
Figure 39: **Nitrite in the presence of a low concentration of sildenafil inhibited platelet aggregation** *in vitro* *via* **the sGC pathway.**

Effect of sodium nitrite (NaNO$_2$, 10nM-100µM) in the presence and absence of a low concentration of sildenafil (sil, 10nM) on collagen (5µg mL^{-1}) induced platelet aggregation. The significant inhibitory effect of nitrite + sildenafil was reversed in the presence of ODQ (10µM). Repeated measures two-way ANOVA with Bonferroni post-hoc test. ns=non-significant, **P<0.01. N=6.

In vivo administration of sodium nitrate increases nitrite concentrations in plasma and salivary glands of eNOS$^{-/-}$ mice

In W.T and eNOS$^{-/-}$ mice, nitrate (1mmol kg^{-1}) administration significantly increased the concentration of nitrate in the salivary glands (Figure 40A) and the plasma (Figure 40C) compared to saline-treated mice. Nitrate also induced a trend in increased salivary gland (Figure 40B) and plasma nitrite (Figure 40D) concentration compared to saline-treated in W.T mice however this was not statistically significant. Nitrate treatment caused a significant increase in salivary gland (~60%) (Figure 40B) and plasma (~30%) (Figure 40D) nitrite concentration compared to saline-treated in eNOS$^{-/-}$ mice.
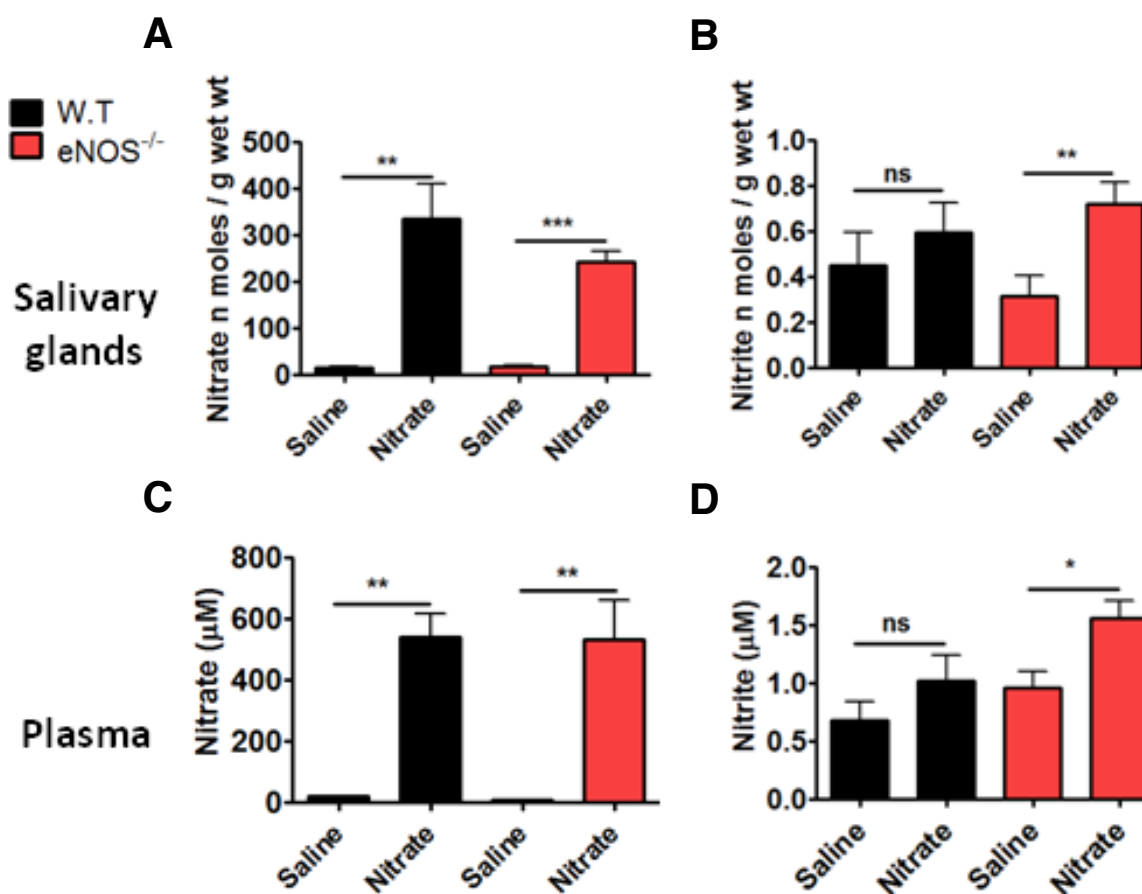
Figure 40: *In vivo* **administration of sodium nitrate increases nitrite concentrations in plasma and salivary glands of eNOS$^{-/-}$ mice**

Following treatment of mice with saline or sodium nitrate (1 mmol kg^{-1}) for 1 hour, nitrate concentrations were significantly increased in (A) salivary glands and (C) plasma in both wild-type (W.T) and eNOS$^{-/-}$ mice. Salivary gland (B) and plasma (D) nitrite concentration was significantly increased in eNOS$^{-/-}$, but not W.T. mice, following nitrate treatment. Data expressed as mean±S.E.M. Unpaired Student's *t*-test (A and C) or Mann-Whitney U-test (B and D) was performed depending on whether the data fitted a normal distribution (F-test). *P<0.05, **P<0.01, ***P<0.001, ns= non-significant (P>0.05), n=5-7. Result produced in collaboration with Dr Smallwood and Prof. Winyard, University of Exeter.

In vivo administration of nitrate significantly reduces platelet aggregation in eNOS$^{-/-}$ but not W.T mice

The effect of nitrate (1mmol kg^{-1}) upon *in vivo* platelet aggregation was investigated in W.T and eNOS$^{-/-}$ mice. In W.T mice nitrate had no significant effect on *in vivo* platelet aggregation compared to the saline-treated which is presented as an example trace (Figure 41A) and mean peak responses of 6 independent experiments (Figure 41B).

In eNOS$^{-/-}$ mice, nitrate caused a significant decrease (~33.3% reduction) in platelet aggregation compared to saline-treated mice. The results of the eNOS$^{-/-}$ mice are presented as an example trace of the response (Figure 41C) and the mean peak responses of 4 independent experiments (Figure 41D). Saline-treated eNOS$^{-/-}$ mice exhibited a trend in increased platelet aggregation compared to saline-treated W.T mice (P=0.0651). Nitrate treatment restored the elevated platelet response of eNOS$^{-/-}$ mice to normal W.T responses, which was around 15% aggregation from baseline.
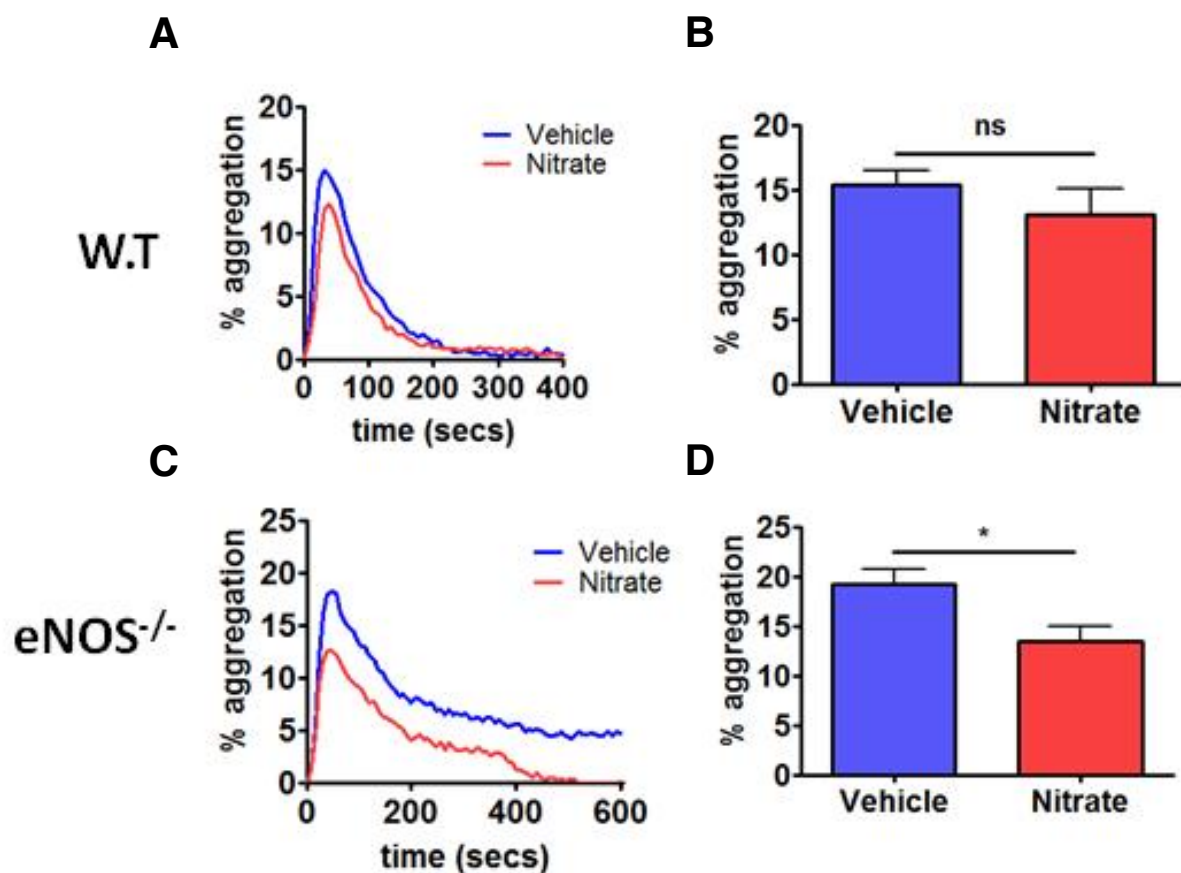
Figure 41: *In vivo* administration of nitrate significantly reduces platelet aggregation in eNOS$^{-/-}$ but not W.T mice.

Wild-type (W.T) and eNOS$^{-/-}$ mice were treated with saline or sodium nitrate (1mmol kg^{-1}) 1 hour prior to collagen (50 µg kg^{-1}) stimulation and radiolabelled platelet aggregation was measured as changes in radioactive counts in the pulmonary vasculature. (A,C) Mean trace of collagen response (percentage increase from the baseline radioactive counts) versus time in W.T (A) and eNOS$^{-/-}$ mice (C). Data expressed as mean (error bars omitted for clarity). (B,D) Maximum percentage increase from baseline radioactive counts in W.T (B) and eNOS$^{-/-}$ mice (D). Data expressed as mean ± S.E.M. Unpaired Student's *t*-test. *P<0.05, ns = non-significant (P>0.05), n=4-6.

Sildenafil enhanced nitrate-mediated inhibition of platelet aggregation *in vivo* in eNOS$^{-/-}$ mice

The effect of sildenafil and nitrate on *in vivo* platelet aggregation in eNOS$^{-/-}$ mice was investigated to establish that nitrate induced cGMP-mediated inhibition of platelet aggregation. As shown previously, nitrate caused a significant reduction (Figure 41C and Figure 41D) and sildenafil had no significant effect (Chapter 5, Figure 32) on collagen-induced platelet aggregation *in vivo* in eNOS$^{-/-}$ mice. The dual treatment of sildenafil and nitrate induced further inhibition of platelet aggregation compared to vehicle and nitrate treated mice. The results are presented as an example trace (Figure 42A) and mean peak response (Figure 42B). Data from Figure 42 is a sample size of 2 and therefore no statistics could be performed and the results are interpreted as a trend. Despite the small sample size, the dual treatment of sildenafil and nitrate caused considerable inhibition of platelet aggregation (~75% of vehicle treated) with tight error bars which further validates my interpretation.

A

B
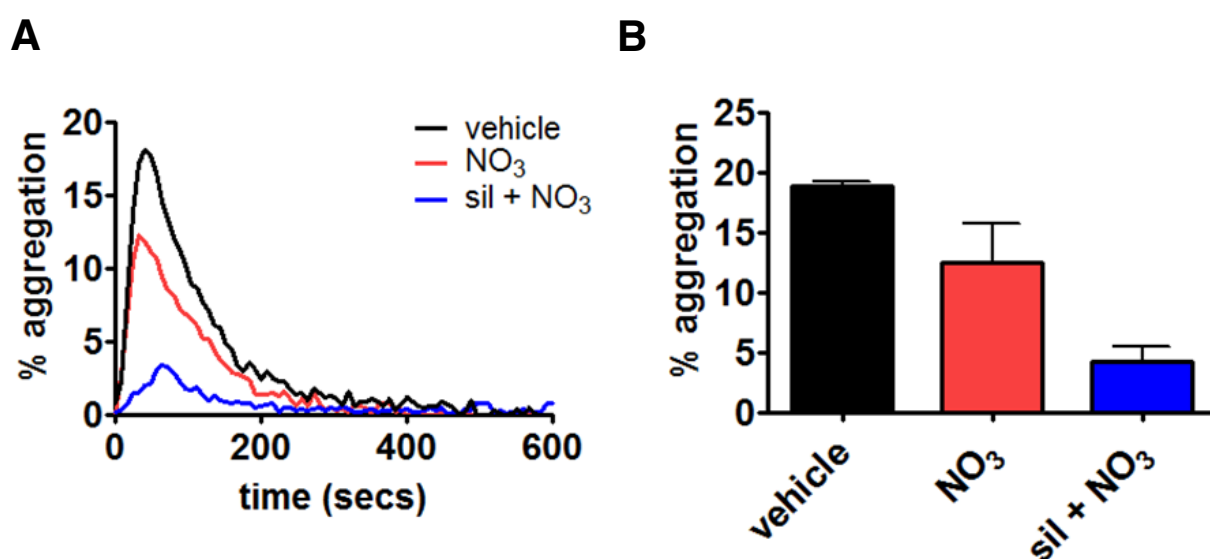
Figure 42: **Sildenafil demonstrated a trend in enhancing nitrate-mediated inhibition of platelet aggregation *in vivo* in eNOS$^{-/-}$ mice**

eNOS$^{-/-}$ mice were treated saline or sodium nitrate (NO$_3$, 1mmol kg^{-1}) and sildenafil (sil, 10mg kg^{-1}) or vehicle (DMSO, 0.1%) 1 hour prior to collagen (50 µg kg^{-1}) stimulation and radiolabelled platelet aggregation was measured as changes in radioactive counts in the pulmonary vasculature. (A) Mean trace of collagen response (percentage increase from the baseline radioactive counts) versus time. Data expressed as mean (error bars omitted for clarity). (B) Maximum percentage increase from baseline radioactive counts. Data expressed as mean ± SEM. N=2.

Discussion

This chapter explored the possibility that inorganic nitrate/nitrite could generate NO/cGMP signals in platelets which drive the inhibitory effect of sildenafil on *in vitro* platelet aggregation. The importance of nitrate-derived NO on *in vivo* platelet aggregation during eNOS deficiency and vascular health was also investigated.

First the presence of NO_x in WP was assessed by the use of a colorimetric nitrate/nitrite assay. It was established that nitrate and nitrite were present in WP preparations. Ascorbic acid has previously been shown to have reducing properties (Borsook and Keighley, 1933; Kashiba-Iwatsuki et al., 1996) and was used as a mild reducing agent in Figure 36. The pretreatment of platelets with ascorbic acid decreased the presence of platelet nitrate/nitrite and consequently exerted an inhibitory effect on *in vitro* collagen-induced platelet aggregation. Ascorbic acid-mediated inhibition of platelet aggregation was reversed in the presence of the sGC inhibitor ODQ. Collectively these results suggest that endogenous platelet nitrate and nitrite has the potential to be reduced to NO and exert an inhibitory effect on platelets by stimulating the NO/sGC/cGMP pathway. In support of the work presented here, other studies have reported similar concentrations of nitrate/nitrite in human plasma (J. W. Park et al., 2013; Velmurugan et al., 2013). It has previously been reported that ascorbic acid can inhibit platelet aggregation (Cordova et al., 1982; Wilkinson et al., 1999) and increase intraplatelet cGMP concentration (Raghavan et al., 2003; Schoepflin et al., 1977). However, this inhibitory effect was reported to be due to the antioxidant effect (improving NO signalling by scavenging ROS species) or stimulation of NOS (chemical stabilisation of tetrahydrobiopterin) and not a NO-related effect mediated by nitrate/nitrite reduction as suggested here. In addition to previous literature, this work provides further evidence that the inhibitory effect of ascorbic acid was NO driven. Overall, Figure 36 established that platelets contain nitrate and nitrite and demonstrated that in principle, platelet nitrate/nitrite had the ability to inhibit platelet activity by reduction to bioactive NO. However, nitrate/nitrite reduction to NO was mediated pharmacologically by the addition of a mild reducing agent and my data did not demonstrate the capability of platelets to reduce nitrate/nitrite to NO endogenously.

To investigate the ability of platelets to bioconvert nitrate/nitrite to NO, the direct effect of nitrate or nitrite on collagen-induced platelet aggregation was assessed. The addition of sodium nitrate *in vitro* in the presence and absence of a low concentration of sildenafil had no effect on NO/cGMP platelet signalling (no increase in VASP-P(239)) or effect platelet aggregation. In contrast, sodium nitrite in the presence of a low concentration of sildenafil caused a concentration-dependent inhibitory effect on platelet aggregation *in vitro.* Nitrite-induced inhibition of platelet aggregation was reversed in the presence of ODQ and the signalling events were verified using a biomarker of protein kinase activity VASP-P(239). These results suggest that the exogenous addition of nitrite, but not nitrate, was able to induce a transient cGMP inhibitory signal that was evident in the presence of a PDE5 inhibitor as a mechanism to stop rapid hydrolysation of cGMP. Therefore, the data from this chapter suggests that platelets have an endogenous capacity to reduce nitrite to bioactive NO. Other researchers have reported that nitrite can inhibit platelet aggregation by cGMP signalling, however this effect was dependent on the presence of erythrocytes which suggested that erythrocytes bear nitrite reductase activity and not platelets (Srihirun et al., 2012; Velmurugan et al., 2013). However, these experiments were performed in the absence of a PDE5 inhibitor and, as suggested in the results presented here, the inhibitory effect of nitrite might be masked by rapid hydrolysation of cGMP by PDE5. In other cell types, mammalian enzymes such as xanthine oxidase (Jansson et al., 2008; Zhang et al., 1998) and aldehyde dehydrogenase (Lin et al., 2013) can reduce nitrite but these enzymes are not present in the platelet proteome (Boyanova et al., 2012). Therefore, the mechanism by which platelets reduce nitrite to NO is unknown. Interestingly, rat liver mitochondria, an organelle also present in platelets, have been reported to reduce nitrite, but not nitrate, to bioactive NO (Kozlov et al., 1999) which suggests that mitochondria may be a potential candidate for nitrite reduction in platelets (see 'Recommendations for future work' in chapter 8). In summary, nitrite reduction may be a possible explanation for the endogenous ability of platelets to generate NOS-independent NO/cGMP signals, however the mechanism by which platelets metabolise nitrite is unclear.

To investigate the physiological relevance of my *in vitro* data, the effect of sodium nitrate on *ex vivo* nitrate and nitrite plasma and salivary gland concentration and *in vivo* platelet aggregation were assessed. Previous research has established that nitrate is produced endogenously by NOS at a concentration of $0.2 mmol\ kg^{-1}\ d^{-1}$ (Wickman et al., 2003). One nitrate-rich vegetable portion contains a higher concentration of nitrate than that produced by all forms of NOS over a day (Lundberg et al., 2008) therefore the concentration of $1 mmol\ kg^{-1}$ was chosen to investigate the effect of an external source of nitrate (*via* diet or supplementation) on platelet aggregation.

Salivary glands have been proven to play an important role in dietary nitrate bioconversion (see 'inorganic nitrate and nitrite' in Chapter 1: Introduction). Therefore salivary gland and plasma nitrate and nitrite concentrations were determined after the treatment of sodium nitrate in W.T and eNOS$^{-/-}$ mice to investigate the ability of these mice to bioconvert nitrate to nitrite. Gas-phase chemiluminescence was adopted in this experiment to ensure sensitive and accurate measurements of NO_x. The data presented here verified that sodium nitrate administration induced a significant increase in nitrate concentration in salivary glands and plasma of W.T and eNOS$^{-/-}$ mice compared to vehicle treated. Nitrate treatment significantly increased the concentration of nitrite in the salivary glands and plasma of eNOS$^{-/-}$ mice, an effect that was not seen in healthy W.T mice. This suggested that a mouse model of endothelial dysfunction had a greater capacity to bioconvert nitrate to nitrite *in vivo*. On the contrary, previous studies have shown that dietary nitrate increased the concentration of nitrite in whole blood and plasma of healthy participants and W.T mice (J. Park et al., 2013; Velmurugan et al., 2013). However, these previous studies chronically administered nitrate through the diet and used larger n numbers (increasing the statistical power to detect smaller differences) which potentially could have detected significance in my data. Results from this chapter establish that the lack of functional eNOS induces a greater capacity to bioconvert nitrate to nitrite and may suggest that inorganic nitrate/nitrite could compensate for impaired NO signalling during endothelial dysfunction.

To investigate the ability of inorganic nitrate to compensate for impaired NO signalling and modify platelet activity during endothelial dysfunction, the effect of nitrate on *in vivo* platelet aggregation in W.T and eNOS$^{-/-}$ mice was assessed. Similar to the effect of nitrate treatment on plasma nitrite concentration in W.T mice, nitrate had no significant effect on *in vivo* collagen-induced platelet aggregation in W.T mice. Collectively, my data suggests that nitrate has no effect on platelet function during vascular health. My results have contradicted other studies which have reported that dietary nitrate exerted a significant inhibitory effect on platelets in W.T mice and healthy participants (J. Park et al., 2013; Velmurugan et al., 2013). However both of these studies measured platelet aggregation *ex vivo* and not in the presence of endothelium-derived NO. Additionally, nitrate was administered chronically at high concentrations (~11.77mmol/L) which may have caused a significant difference between untreated and nitrate treated mice (J. Park et al., 2013). The present study examined the effects of nitrate on *in vivo* platelet aggregation in the presence of a healthy vascular endothelium and ascertained that nitrate-derived NO did not significantly modify platelet function in W.T mice. These results suggest that the bioconversion of nitrate to NO may be a redundant NO source in vascular health and consequently has no effect on platelet function.

Nitrate treatment significantly inhibited platelet aggregation in eNOS$^{-/-}$ mice which reversed the elevated thrombotic response to that seen in W.T mice. Although the data are only preliminary, sildenafil enhanced the inhibitory effect of nitrate on *in vivo* platelet aggregation in eNOS$^{-/-}$ mice, which suggests that nitrate-induced inhibition of platelet aggregation was at least partly cGMP-mediated. Collectively, the results from this chapter have determined that nitrate can exert a cGMP-mediated antiplatelet effect in eNOS$^{-/-}$ mice by bioconversion of nitrate to nitrite and potentially NO. My work bears similarities to the work of Carlström *et al.* (Carlström et al., 2010) who reported that dietary inorganic nitrate can compensate for the metabolic consequences of eNOS deficiency. Therefore, the results from this chapter suggest that nitrate-derived NO may compensate for impaired NO signalling during endothelial dysfunction and negatively regulate platelet function. To date there have been no investigations into the effect of inorganic nitrate on platelet function in patients with endothelial dysfunction and therefore the importance of this pathway in

human cardiovascular disease has not been established. The results presented in this chapter serve as a basis for future work to investigate the antiplatelet effect of inorganic nitrate in patients with endothelial dysfunction.

In conclusion, nitrite can generate transient NO/cGMP signalling events in platelets and could potentially drive the inhibitory effect of sildenafil on platelet function. My work suggests that nitrite can be reduced to NO by an unidentified nitrite reduction mechanism in platelets. In an *in vivo* setting, eNOS deficiency caused a greater capacity to bioconvert nitrate to nitrite and initiated NO/cGMP antiplatelet effects. This chapter suggests that inorganic nitrate/nitrite can compensate for impaired NO signalling during endothelial dysfunction whilst preserving normal platelet function in vascular health.

Chapter 8: General discussion

The importance of this study and objectives

Cardiovascular disease is the leading cause of death in the UK, with 152,000 incidences of stroke and 103,000 incidences of myocardial infarction occurring every year (British Heart Foundation, 2013). Platelets play a major role in cardiovascular disease and antiplatelet therapy is a commonly prescribed treatment to reduce the risk of ischaemic events (Angiolillo et al., 2008). Current antiplatelet therapies such as aspirin and thienopyridines have many limitations such as hypersensitivity, resistance and excessive bleeding which highlights a need to develop new drug targets (Michelson, 2010). NO is a major negative regulator of platelets and a significant body of research has reported reduced bioavailability of NO in cardiovascular disease (Naseem, 2005). Endothelial damage can lead to atherosclerosis, impaired eNOS function and increased risk of platelet-driven cardiovascular events due to the increased ability of platelets to activate. Previous studies investigating the expression of NOS in platelets have been controversial (Aytekin et al., 2012; Gambaryan et al., 2008; Gkaliagkousi et al., 2007; Naseem and Riba, 2008; Radomski et al., 1990; Tymvios et al., 2009). However, there is convincing evidence to suggest that platelets generate their own NO (Freedman et al., 1999, 1997; Malinski et al., 1993). Thus the exact source of NO affecting platelet function was unclear. The overall hypothesis of this study was that improved NO signalling in platelets could reduce the risk of platelet-driven cardiovascular disease. NO signalling can be amplified in cells containing PDE5 by antagonising the breakdown of cGMP with the use of the selective inhibitor sildenafil citrate. Platelets express high concentrations of PDE5 and for that reason, sildenafil may attenuate platelet function in cardiovascular disease by enhancing cGMP signalling and restoring impaired NO regulation.

The main objective of this thesis was to determine the therapeutic potential of enhancing NO/cGMP signals in platelets. This was achieved by assessing the antiplatelet effect of the PDE5 inhibitor sildenafil *in vitro* and *in vivo*. Following this, the upstream source driving cGMP signalling events in platelets was determined. Finally the functional significance of NO/cGMP signalling events in platelets was investigated during vascular health and endothelial dysfunction.

Summary of results

The effect of sildenafil on platelets

Sildenafil is a well-tolerated drug administered for the improvement of vascular function and approved for the treatment of ED and PAH (Muirhead et al., 2002; Rubin et al., 2011). Sildenafil is a selective PDE5 inhibitor and acts by enhancing NO signalling in cells expressing PDE5. A particularly appealing attribute of this drug is that it has limited effect on systemic blood pressure and therefore is an interesting candidate for antiplatelet therapy (Morales et al., 1998; Wallis et al., 1999). Current studies investigating the effect of sildenafil on platelet activity have been contradictory, potentially due to the inability to specifically investigate platelet function *in vivo* in the presence of an intact vascular endothelium. In this study, sildenafil exerted an antiplatelet effect which selectively targeted platelet aggregation by enhancing the NO/sGC/cGMP pathway. Sildenafil was able to exert an antiplatelet effect *in vitro* which demonstrated the presence of endogenous transient NO/cGMP signals upstream of PDE5 in platelets. The inhibitory effect of sildenafil was independent of NOS activity *in vitro*, but not *in vivo* in the absence of eNOS. This suggested the presence of an eNOS-dependent NO storage pool in platelets however further experimentation will be necessary to validate this.

The ability of endogenous S-nitrosothiols to modify platelet function

Exogenously applied RSNOs (CysNO and GSNO) can inhibit platelet function by stimulating the NO/sGC/cGMP pathway (Gordge and Xiao, 2010). The spontaneous release of NO from these drugs is much slower than their cellular effects and therefore suggested the presence of endogenous metabolising mechanisms able to drive NO release and bioactivity (Bell et al., 2007; Gordge et al., 1998; Mathews and Kerr, 1993). *In vivo* RSNOs have the selective ability to deliver NO/cGMP signals to platelets (de Belder et al., 1994; Xiao and Gordge, 2011), however the ability of endogenous RSNOs to generate NO and modify platelet function was unclear. Therefore this thesis investigated the ability of endogenous platelet RSNOs to generate NO/cGMP signalling events in platelets and explain the inhibitory effect of sildenafil on platelet function. The amino acid transporter L-AT is known to deliver RSNOs into many cell types (Riego et al., 2009; Zhang and Hogg, 2004). However,

in this study L-AT had no effect on the ability of platelets to generate NO/cGMP signals. Protein disulphide isomerase (PDI) has been reported to mediate the antiplatelet effects of exogenous RSNOs by its denitrosation activity (Bell et al., 2007; Xiao and Gordge, 2011). However, in this study PDI was not involved in the ability of platelets to generate NO/cGMP signals. Furthermore, the pharmacological release of NO from RSNOs using $HgCl_2$ did not modify sildenafil-mediated inhibition of platelet aggregation which suggested that endogenous RSNOs were not involved in NO/cGMP signalling events generated by the platelet. However, this work was only preliminary and further investigations are advised to support the conclusions drawn (see 'Recommendations for future work').

Nitrate/nitrite/NO cycling and the impact on platelets

Until recently, nitrate and nitrite were considered to be inert metabolites of NOS-derived NO or substances from the diet. Now it is widely accepted that inorganic nitrate can generate NOS-independent NO by bioconversion to nitrite and subsequently NO *in vivo* and exert vasodilatory and antiplatelet effects (Lundberg and Govoni, 2004; Velmurugan et al., 2013; Webb et al., 2008b). The ability of inorganic nitrate and nitrite to drive platelet-derived NO/cGMP signalling events was unknown and investigated in this thesis. Furthermore, the functional impact of *in vivo* nitrate bioconversion on platelet activity was investigated in the presence and absence of eNOS. This study confirmed that platelets contain detectable concentrations of nitrate and nitrite which had the potential to be reduced to bioactive NO. Nitrite exerted an antiplatelet effect *in vitro* by driving transient NO/cGMP signals in platelets. Therefore, this study provides evidence that nitrite mediates platelet-derived NO/cGMP signalling events and may explain the inhibitory effect of sildenafil on platelet function. *In vivo*, eNOS$^{-/-}$ mice exhibited a greater capacity to bioconvert nitrate to nitrite and exert a cGMP-mediated antiplatelet effect. My work suggests that inorganic nitrate can compensate for impaired NO signalling and reduce the risk of platelet-driven cardiovascular events whilst preserving normal platelet function in vascular health. This study supports the growing body of literature to suggest that inorganic nitrate may account, at least in part, for the beneficial effects of a diet high in vegetables on cardiovascular health.

Impact of these results

This work has furthered our knowledge on the somewhat controversial topic of platelet-derived NO. The novelty of this study includes the identification of the endogenous ability of platelets to generate NO/cGMP signals independently of NOS activity. Nitrite could potentially be an important regulator of platelet function due to its ability to drive transient NO/cGMP signalling events in platelets. This body of work has provided a greater insight into the regulation of platelets by NO and has opposed previous theories that platelets are solely regulated by the vascular endothelium or NO produced by platelet NOS.

This thesis further suggests that inorganic nitrate contributes to the beneficial effects of a vegetable-rich diet on cardiovascular health. In addition, this study emphasised the importance of nitrate-derived NO on *in vivo* platelet function during endothelial dysfunction. Increased intake of inorganic nitrate, by the diet or supplementation, may provide a compensatory source of NO and reduce the risk of arterial thrombosis, and potentially atherogenesis, in patients at risk of impaired NO signalling. Future research in this area should question and re-evaluate the current exceptionally low guidelines for acceptable daily intake of dietary nitrate ($3.7 \text{mg kg}^{-1} \text{d}^{-1}$) to allow for the beneficial effects on the cardiovascular system (Alexander et al., 2008).

The PDE5 inhibitor sildenafil citrate demonstrated therapeutic potential as an antithrombotic agent by enhancing NO/cGMP signalling derived from enzymic and inorganic sources *in vitro* and *in vivo*. I have shown that sildenafil was able to exert an antiplatelet effect at concentrations lower than those necessary for the symptomatic relief of ED. This study has highlighted the need to continue this research and perform clinical trials to assess the antithrombotic effect of low-dose long-term administration of sildenafil to treat the chronic condition that is arterial thrombosis. Similar to the effects of aspirin, I hypothesise that long-term treatment of sildenafil (similar to doses used in PAH) may reduce the risk of platelet-driven cardiovascular disease. Unlike aspirin, selectively enhanced NO signalling in platelets would target multiple pathways of platelet activation instead of just one and is therefore less likely to bear signs of drug resistance. Additionally, sildenafil may

even have a negative effect on platelet-driven atherogenesis. More investigations will need to be carried out to validate these hypotheses.

Overall, this thesis has highlighted the therapeutic potential of improving NO signalling to reduce the risk of platelet-driven cardiovascular events in vascular disease and compounds targeting the NO pathway may be the next generation of antithrombotic therapies. In the next section I will discuss proposed future studies to further our knowledge and translate these findings into clinical trials.

Recommendations for future work

The role of eNOS on platelet NO signalling

The inhibitory effect of sildenafil on platelet aggregation was NOS-independent *in vitro* but not *in vivo* (no effect on platelet aggregation in eNOS$^{-/-}$ mice) which suggested the potential of platelets to store NO produced from eNOS long-term. For example, RSNOs have been proposed as an intermediate of NO signalling by stabilising and extending the activity of NO (Ignarro et al., 1987; Stamler et al., 1992). Freedman *et al.* (Freedman et al., 1999) recognised that platelets from W.T mice were able to reverse the decreased bleeding time in eNOS$^{-/-}$ mice which highlighted the presence of platelet-derived NO. A future experiment could be to determine the ability of sildenafil to inhibit *in vivo* platelet aggregation of W.T platelets in eNOS$^{-/-}$ mice. This would provide evidence for non-enzymic NO generation from a long-term storage pool and assess the physiological impact of platelet-derived NO on platelet function. Our group previously reported that the non-selective NOS inhibitor L-NAME could significantly increase *in vivo* platelet aggregation compared to the vehicle treated (Moore et al., 2011; Tymvios et al., 2009), however it was unknown whether NO was still able to modify platelet function. It would be of interest to determine whether sildenafil could inhibit *in vivo* platelet aggregation of mice or donor platelets pre-treated with L-NAME. Collectively, these studies would explain the lack of effect of sildenafil on eNOS$^{-/-}$ mice, provide evidence for NOS-dependent NO storage in platelets and further disprove the presence of platelet NOS.

Investigation into the antiplatelet effect of RSNOs

This thesis provided no functional evidence to suggest that platelet RSNOs can stimulate endogenous NO/cGMP signalling events in platelets. However, the results presented here were preliminary and were limited by the inability to measure RSNO/NO concentration. Previous work has established the antiplatelet effect of exogenously applied RSNOs by the selective delivery of NO into the platelet and stimulating cGMP signals (Freedman et al., 1995; Riego et al., 2009; Xiao and Gordge, 2011). In addition, it has been established that RSNOs are present endogenously, more importantly in plasma (Rossi et al., 2001) and platelets (Hirayama et al., 1999). Therefore it is worth further investigating RSNOs as NO intermediates in platelet function.

To validate the role of endogenous RSNOs in mediating platelet NO/cGMP signals, I would determine the functional and biochemical effects of $HgCl_2$ on platelets. NO detection could be performed by fluorescence (4,5-diaminofluoreseine (DAF-2) reaction) (Kojima et al., 1998) or electrochemical methods (amperometric NO sensor) (Allen et al., 2005). RSNO/NO measurements would act as a control to verify that $HgCl_2$ is displacing NO and releasing it from its bound thiol. This work would identify whether RSNOs can release NO in principle, but would not prove if this occurs physiologically.

If platelets are able to release NO from RSNOs, I would further examine the role of PDI (the previously proposed mechanism by which therapeutic RSNOs exert their antiplatelet effects (Shah et al., 2007)) on platelet NO/cGMP signalling. As mentioned in Chapter 6, I would support the functional data with biochemical (RSNO/NO concentrations) and molecular (cGMP expression) analyses and use a positive control (such as GSNO) for verification.

The mechanism of action of nitrite on generating transient NO/cGMP signals in platelets

Nitrite was able to exert transient inhibitory NO/cGMP signals in platelets *in vitro* by unidentified mechanisms. I proposed that nitrite was reduced to NO before activating sGC and inducing cGMP accumulation. To validate this hypothesis, I would investigate the effect of nitrite and sildenafil on *in vitro* platelet aggregation in the

presence of a NO scavenger. To further support nitrite reduction to NO, the biochemical analysis of platelet NO concentration in the presence and absence of nitrite could be detected using an electrochemical NO sensor or DAF-2 chemiluminescence (Giustarini et al., 2004; Hunter et al., 2013).

Nitrite has been proven to react with thiols to produce RSNOs (Smith and Marletta, 2012), which have previously shown to inhibit platelet function (Miller et al., 2003; Vilahur et al., 2004). Therefore this lead to question the involvement of RSNOs in nitrite mediated NO/cGMP signalling in platelets. First I would detect whether nitrite can form RSNOs in platelets by using biochemical analysis. Then I would investigate proposed RSNO metabolism mechanisms identified in platelets (see above section 'Investigation into the antiplatelet effect of RSNOs').

Mitochondria and their role in platelet NO/cGMP signalling

Mitochondria have attracted much attention in the NO field due to their ability to produce NO_x (Benhar et al., 2008) and also as a target for NO activity (Broniowska et al., 2012; Maejima et al., 2005). It has been proven that mitochondria are able to produce NO, potentially by the reducing ability of cytochrome *c* oxidase (Castello et al., 2006; Kozlov et al., 1999). Platelets contain modest amounts of fully functioning mitochondria (White, 1979; Zharikov and Shiva, 2013). Hence, mitochondria may also be a candidate for RSNO metabolism/nitrite reductase activity in platelets which has not yet been investigated. The involvement of mitochondria in NO release by RSNOs or nitrite could be determined using the cytochrome *c* oxidase inhibitors myxothiazol and antimycin A (Arora et al., 2009; Castello et al., 2006; Kozlov et al., 1999).

Inorganic nitrate in human vascular disease

This thesis demonstrated that the absence of eNOS in mice induced a greater capacity to bioconvert nitrate to nitrite and, in turn, negatively impact platelet function. Previous work has established that inorganic nitrate can reduce blood pressure and exert an antiplatelet effect *via* cGMP signalling in healthy human participants (Larsen et al., 2006; Velmurugan et al., 2013; Webb et al., 2008b). However, the functional significance of nitrate bioconversion in humans with cardiovascular disease (and therefore impaired NO signalling) is unknown. It would

be interesting to assess the effects of inorganic nitrate on plasma NO_x concentration and *ex vivo* platelet aggregation in patients with known vascular impairment. This may provide insight into the functional impact of inorganic nitrate on platelet activity during vascular disease and establish if nitrate-derived NO can compensate for impaired NO signalling and reduce the risk of platelet-driven ischaemic events.

<u>Further investigating the therapeutic potential of sildenafil as an antithrombotic agent</u>
Here I have established that sildenafil significantly reduced platelet aggregation *in vitro* and *in vivo via* the NO/sGC/cGMP pathway. In support of this finding, other researchers have identified that sildenafil can reduce platelet function in healthy participants *ex vivo* (Berkels et al., 2001; Halcox et al., 2002). However, the functional significance of sildenafil-mediated inhibition of platelet aggregation in humans with vascular disease is unknown.

It was identified that sildenafil had no significant effect on *in vivo* platelet aggregation in eNOS$^{-/-}$ mice in this thesis. Unfortunately, the use of this genetically modified mouse model is limited by the complete absence of functional eNOS and therefore does not physiologically reflect the human scenario. It may be of interest to investigate the effect of sildenafil on *in vivo* platelet aggregation using a more physiologically relevant mouse model of impaired NO signalling such as the apolipoprotein E-deficient (apoE$^{-/-}$) mouse (Yamashiro et al., 2010). This will provide some insight into the use of sildenafil as an antithrombotic agent in disease states. Furthermore, platelets are known to play an integral role in the formation of atherosclerosis (Huo et al., 2003; Huo and Ley, 2004; Linden and Jackson, 2010), therefore in future the inhibitory effect of sildenafil on platelets could be investigated as a prevention of atherosclerotic development.

Dependent on the results of the proposed experiments in mouse models, it would then be necessary to investigate the potential antithrombotic effect of sildenafil in humans. Investigation into the effect of sildenafil on platelets in healthy participants may not be therapeutically relevant because this subgroup will have normal NO signalling and the NO/cGMP pathway may already be saturated. What would be interesting would be to determine the effect of sildenafil on platelet function (*ex vivo* platelet aggregation and bleeding time) in patients with impaired NO signalling such

as in vascular disease. Although these parameters are not fully representative of *in vivo* platelet function, this experiment may improve our understanding of the therapeutic potential of enhancing NO/cGMP signalling in these patients at risk of platelet-driven cardiovascular disease.

The combined effect of inorganic nitrate and sildenafil

Here I have suggested that sildenafil may substantially enhance the inhibitory effect of nitrate on platelets in preliminary investigations (n=2). This lead to concerns regarding the dual effect of sildenafil and inorganic nitrate on blood pressure and the effect of a diet high in inorganic nitrate on the incidence of sildenafil-induced adverse events. Currently there are major contraindications relating to the use of organic nitrates and sildenafil which can cause severe hypotension in some patients(Cheitlin et al., 1999; Francis and Corbin, 2005; Jackson et al., 2006). Hence I believe it would be necessary to further investigate the effects of inorganic nitrate and sildenafil use. I would investigate the effect of sildenafil and nitrate on *in vivo* murine platelet aggregation and blood pressure to determine the incidence of possible adverse events such as excessive bleeding and hypotension. This experiment would provide physiological insight into the dual treatment in a whole body system.

I would also look into performing a clinical trial to investigate the effect of sildenafil in healthy human participants consuming a low- and high-nitrate containing diet. I would determine blood pressure, *ex vivo* platelet aggregation and bleeding time parameters to further investigate the safety profile of sildenafil and this may lead to identifying a contributing factor of sildenafil-mediated patient variability. Depending on the results from these experiments, it may be worth investigating the effect of sildenafil and inorganic nitrate in patients with known vascular disease to determine if they are more at risk of adverse events such as hypotension and excessive bleeding.

Conclusions of this thesis

Platelets were able to generate their own NO/cGMP signals potentially by the reduction of nitrite to NO. Inorganic nitrate and nitrite can compensate for impaired NO signalling in the absence of eNOS and therefore may be critical negative regulators of platelet function during endothelial dysfunction. The PDE5 inhibitor sildenafil citrate (Viagra®) demonstrated therapeutic potential as an antithrombotic agent *in vitro* and *in vivo* by enhancing NO- and nitrite-mediated cGMP signalling in platelets. Overall, this thesis has highlighted the beneficial effect of enhancing NO/cGMP signalling in platelets to reduce the risk of platelet-driven cardiovascular events.

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Appendix: Publication

Georgina Apostoli
Targeting the NO signalling pathway to modulate platelet function
Platelet Biology, Molecular Medicine
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Role of inorganic nitrate and nitrite in driving nitric oxide–cGMP-mediated inhibition of platelet aggregation *in vitro* and *in vivo*

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Summary. *Background:* Nitric oxide (NO) is a critical negative regulator of platelets that is implicated in the pathology of thrombotic diseases. Platelets generate NO, but the presence and functional significance of NO synthase (NOS) in platelets is unclear. Inorganic nitrate/nitrite is increasingly being recognized as a source of bioactive NO, although its role in modulating platelets during health and vascular dysfunction is incompletely understood. *Methods:* We investigated the functional significance and upstream sources of NO–cGMP signaling events in platelets by using established methods for assessing *in vitro* and *in vivo* platelet aggregation, and assessed the bioconversion of inorganic nitrate to nitrite during deficiency of endothelial NOS (eNOS). *Results:* The phosphodiesterase 5 (PDE5) inhibitor sildenafil inhibited human platelet aggregation *in vitro*. This inhibitory effect was abolished by a guanylyl cyclase inhibitor and NO scavengers, but unaffected by NOS inhibition. Inorganic nitrite drove cGMP-mediated inhibition of human platelet aggregation *in vitro* and nitrate inhibited platelet function in eNOS$^{-/-}$ mice *in vivo* in a model of thromboembolic radiolabeled platelet aggregation associated with an enhanced plasma nitrite concentration as compared with wild-type mice. *Conclusions:* Platelets generate transient, endogenous cGMP signals downstream of NO that are primarily independent of NOS and may be enhanced by inhibition of PDE5. Furthermore, nitrite can generate transient NO–cGMP signals in platelets. The absence of eNOS leads to enhanced plasma nitrite levels following nitrate administration *in vivo*, which negatively impacts on platelet function. Our data suggest that inorganic nitrate exerts an antiplatelet effect during eNOS deficiency, and, potentially, that dietary nitrate may reduce platelet hyperactivity during endothelial dysfunction.

Keywords: nitric oxide; nitrites; pharmacology; platelets; thrombosis.

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Introduction

Platelet activation is governed by a variety of positive and negative stimuli that act to precisely regulate the process of hemostasis. Positive stimulators of platelets include subendothelial collagen, thrombin generated via the coagulation cascade, and ADP and thromboxane A$_2$, which are released from platelets themselves. The major negative regulators of platelets are prostacyclin (prostaglandin I$_2$ [PGI$_2$]) and nitric oxide (NO), which are generated by the vascular endothelium. An imbalance between positive and negative platelet stimuli contributes to the pathogenesis of thrombotic disorders such as myocardial infarction. NO is conventionally described as being generated by NO synthase (NOS) enzymes, which catalyze the conversion of L-arginine to L-citrulline, resulting in NO release [1]. Many of the effects of NO are mediated through activation of soluble guanylyl cyclase (sGC), subsequent cGMP production, and protein kinase activation, leading to further signaling events, including phosphorylation of vasodilator-stimulated phosphoprotein (VASP). The actions of cGMP are terminated by phosphodiesterase 5 (PDE5), which hydrolyzes active cGMP to inactive GTP. PDE5 is expressed at high levels in platelets, so that the effects of cGMP are transient, owing to its rapid hydrolysis. PDE5 inhibitors, e.g. sildenafil citrate, are used therapeutically in conditions associated with regional blood flow deficiency, such as erectile dysfunction and pulmonary arterial hypertension.

Sildenafil was reportedly associated with adverse cardiovascular events [2]; however, the risk was more recently shown to arise from the cardiovascular risk profile of patients with erectile dysfunction [3]. The focus can therefore shift towards the exploration of PDE5 inhibition in cardioprotection [3]. Sildenafil has also been shown to enhance NO-mediated inhibition of human aggregation *in vitro* [4] and to enhance collagen-induced aggregation *ex vivo* [5]. The ability of sildenafil to directly modulate platelet activation in the absence of exogenous or endothelial NO, as investigated by determining its effect upon isolated platelets *in vitro*, is poorly understood, although it had no effect on ADP-induced aggregation in platelet-rich plasma [6].

Endogenous NO derived from endothelial NOS (eNOS) in the vascular endothelium acts as a critical negative regulator of platelet function *in vivo* [7,8], and deficiency of eNOS is associated with endothelial dysfunction and the pathology of a range of cardiovascular diseases [9,10]. However, the intrinsic expression of eNOS in platelets remains contentious [11,12], and a number of studies have reported a lack of eNOS protein or mRNA in platelets, as well as a lack of a functional role of platelet-derived NO [13,14]. There is, however, considerable evidence that platelets generate NO [15,16], so that the source of endogenous NO in platelets is unclear. More recently, NO has been shown to be derived not only via NOS but also via reduction of inorganic nitrite [17]. In humans, dietary nitrate is concentrated and secreted by the salivary glands, and is reduced to nitrite by anaerobic bacteria on the tongue [18,19]. Nitrite is absorbed into the circulation and is chemically reduced to NO by a variety of mechanisms, including enzymatic processes that provide a mechanism for the localized delivery of NO to cells independently of NOSs [17,20]. Oral administration of nitrate lowered blood pressure [21] and modestly reduced platelet aggregation *ex vivo* in healthy volunteers [22], demonstrating the potential value of inorganic nitrate as a modulator of cardiovascular function. The ability of nitrate/nitrite to impact on platelets in the context of endothelial dysfunction *in vivo* is unknown.

We hypothesized that platelets generate transient NO–cGMP signals from upstream nitrate/nitrite. These signals may be amplified by PDE5 inhibition to reveal endogenous inhibitory signaling processes. Second, we hypothesized that, during endothelial dysfunction, exogenously administered inorganic nitrate acts as an alternative source of bioactive NO to counteract impaired eNOS activity by inhibiting platelet activation following bioconversion to nitrite.

Materials and methods

Materials

The materials used were as follows: indium-111 oxine (GE Healthcare, Amersham, UK); collagen (Takeda

Pharmaceuticals International, Linz, Austria); anti-VASP and anti-VASP-P(Ser239) (Cell Signalling, Hitchin, UK); iloprost (Cayman Chemicals, Washington, DC, USA); anti-rabbit horseradish peroxidase-conjugated antibody (Dako, Ely, UK); and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology, Dallas, TX, USA). Sildenafil citrate was kindly donated by Pfizer (Peapack, NJ, USA). All other materials were purchased from Sigma-Aldrich (Poole, UK).

Light transmission aggregometry

Blood was collected in acid–citrate–dextrose (ACD) (1 : 9, ACD : Blood) from aspirin-free volunteers aged 23–55 years and with an even sex distribution. Informed consent from all blood donors was obtained, and procedures were approved by the National Research Ethics Service. Platelet-rich plasma (PRP) was prepared by centrifugation at $100 \times g$ for 20 min. Washed platelets (WPs) were prepared by the addition of ACD (1 : 80, ACD : PRP) and prostaglandin E_1 (175 nM) to PRP, and centrifuged at $1400 \times g$ for 10 min. The pellet was resuspended in Tyrodes/HEPES buffer, and the final centrifugation step was repeated. WPs were resuspended to a platelet count of 250×10^3 μL^{-1} in tyrodes/HEPES buffer. Platelet preparations were incubated for 5 min with test compounds prior to stimulation with agonists, and aggregation was measured at 37 °C under stirring conditions in an optical aggregometer (Chrono-log Corporation, Havertown, PA, USA).

Western blotting

Human WPs were incubated with test compounds (as detailed for light transmission aggregometry) before centrifugation (15 700 $\times g$ for 1 min) and pellet resuspension in RIPA lysis buffer. Western blotting was performed as detailed previously [23]. The antibody concentrations used were as follows: rabbit anti-GAPDH, 1 : 500; anti-VASP, 1 : 1000; and anti-VASP-P(Ser239), 1 : 1000. Incubations were performed at 4 °C overnight.

Nitrate/nitrite colorimetric assay

Human WPs (500 μL) were incubated in the presence or absence of test compounds for 5 min before they were snap frozen and stored at − 80 °C. The nitrate/nitrite concentration was determined in supernatants with a nitrate/nitrite colorimetric assay kit (Cayman Chemicals, Ann Arbor, MI, USA).

Animals

Male C57BL/6 mice (20–30 g) were purchased from Harlan (Bicester, UK). eNOS knockout mice (eNOS$^{-/-}$, strain 0026847) were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and bred in-house. Protocols

involving the use of animals were licensed by the UK Home Office and approved by the Ethical Review Panel at Imperial College London. Procedures involving animals were conducted and are reported in accordance with ARRIVE guidelines [24].

Ozone chemiluminescence

Wild-type or eNOS$^{-/-}$ mice were pretreated with saline or sodium nitrate 1 h before they were anesthetized and plasma and salivary glands were extracted [25]. One hour has previously been shown to provide adequate time for increases in plasma nitrate/nitrite to occur following intraperitoneal or oral administration of inorganic nitrate [18,26,27]. All samples were snap frozen and stored at $-80\,°C$ until further analysis. Mouse salivary glands were homogenized with phosphate-buffered saline, with a Mixer Mill MM 400 homogenizer at a vibrational frequency 30 Hz (1800 min^{-1}) for 3 min. Salivary gland homogenates or plasma were deproteinized by incubation with sodium hydroxide (0.5 M) and zinc sulfate (10% w/v) for 15 min at room temperature. Samples were centrifuged (17 500 × g for 5 min), and the supernatant was extracted and analyzed for nitrate/nitrite concentration with a Sievers nitric oxide analyzer (280; Analytix, Boldon, UK). Samples were refluxed in vanadium (III) chloride (0.1 M) and hydrochloric acid (1 M) at 95 °C (nitrate analysis) or in sodium iodide (0.3 M) and glacial acetic acid at 35 °C (nitrite analysis). Nitrate/nitrite concentrations were detected according to ozone chemiluminescence, as previously reported [28].

In vivo platelet aggregation

Platelets were isolated from wild-type or eNOS$^{-/-}$ donor mice, and radiolabeled with 1.8 MBq of indium-111 oxine as previously described [29]. Radiolabeled platelets of the same genetic background were administered to anesthetized (urethane 25% w/v, 10 μL g^{-1}) recipient wild-type or eNOS$^{-/-}$ mice via the femoral vein, and platelet aggregation responses were measured as increases in platelet-associated counts in the pulmonary vascular bed following intravenous injection of collagen (50 μg kg^{-1}). In a typical experiment, five donor mice were bled, and the resulting platelet pool was evenly distributed into four recipient mice. The experimental protocol for sodium nitrate involved pretreatment of the recipient mice with saline (0.9% w/v) or sodium nitrate (1 mmol kg^{-1}, intraperitoneal) 1 h before collagen injection, and the experimental protocol for sildenafil involved the administration of vehicle (< 0.05% dimethylsulfoxide) or sildenafil (50 μg kg^{-1}, intravenous) 5 min before collagen injection.

Data analysis and statistics

All data were expressed as mean ± standard error of the mean. In vivo platelet aggregation data were expressed as the percentage increase in maximal radioactive counts from the baseline recording. In vitro platelet aggregation data were arbitrary 'area under the curve' values generated by AGGROLINK software (version 5.2.1; Chrono-log Corporation). All statistical tests were performed on raw data. Where statistical comparisons were made, Student's t-test, one-way ANOVA or a two-way ANOVA followed by a Bonferroni post hoc multiple comparison test were used to compare mean values. A P-value of > 0.05 was considered to denote statistical significance.

Results

Sildenafil inhibits platelet aggregation in vitro and in vivo

We investigated the presence of a functionally relevant, intrinsic NO–cGMP signaling cascade in isolated human platelets by enhancing transient cGMP signals via inhibition of PDE5. The selective PDE5 antagonist sildenafil (10–1000 nM) caused significant and concentration-dependent inhibition of collagen-induced (Fig. 1A,B) and thrombin-induced WP aggregation in vitro (Fig. 1C), but had no effect on ADP-induced aggregation (Fig. 1D). In vivo, collagen-induced platelet aggregation was significantly reduced following pretreatment of mice with 50 μg kg^{-1} sildenafil (Fig. 1E,F).

Sildenafil selectively amplifies endogenous NO-mediated signaling independently of NOS

Sildenafil (10 nM) significantly enhanced NO-mediated inhibition of platelet aggregation (Fig. 2A) but, in contrast, had no effect upon the inhibitory effect exerted by the PGI$_2$ mimetic iloprost (Fig. 2B). Sildenafil also induced a concentration-dependent increase in VASP-P(Ser239) phosphorylation (Fig. 1C–D) when applied to isolated platelets. Sildenafil-mediated inhibition of platelet aggregation was abolished in the presence of the sGC antagonist 1H-(1,2,4)-oxadiazolo[4,3-a] quinoxalin-1-one (ODQ) Fig. 2E) and the NO scavengers hemoglobin and hydroxocobalamin (Fig. 2F). In contrast, pretreatment of platelets with the NOS inhibitor L-NAME, its inactive isomer D-NAME and vehicle had no effect on sildenafil-mediated inhibition of aggregation (Fig. 2G). Similarly, sildenafil-mediated VASP-P(Ser239) phosphorylation was abolished by ODQ but not significantly affected by L-NAME (Fig. 2H–I).

Nitrite reduction drives cGMP-mediated inhibition of platelet aggregation

Having shown that isolated platelets generate inhibitory NO–cGMP signals that arise predominantly from sources other than NOS, we explored the ability of nitrate and nitrite to drive NO–cGMP-mediated inhibition of platelet aggregation. We first demonstrated the presence of both nitrate and nitrite in platelet extracts and undetectable levels in experimental buffers by using a colorimetric assay
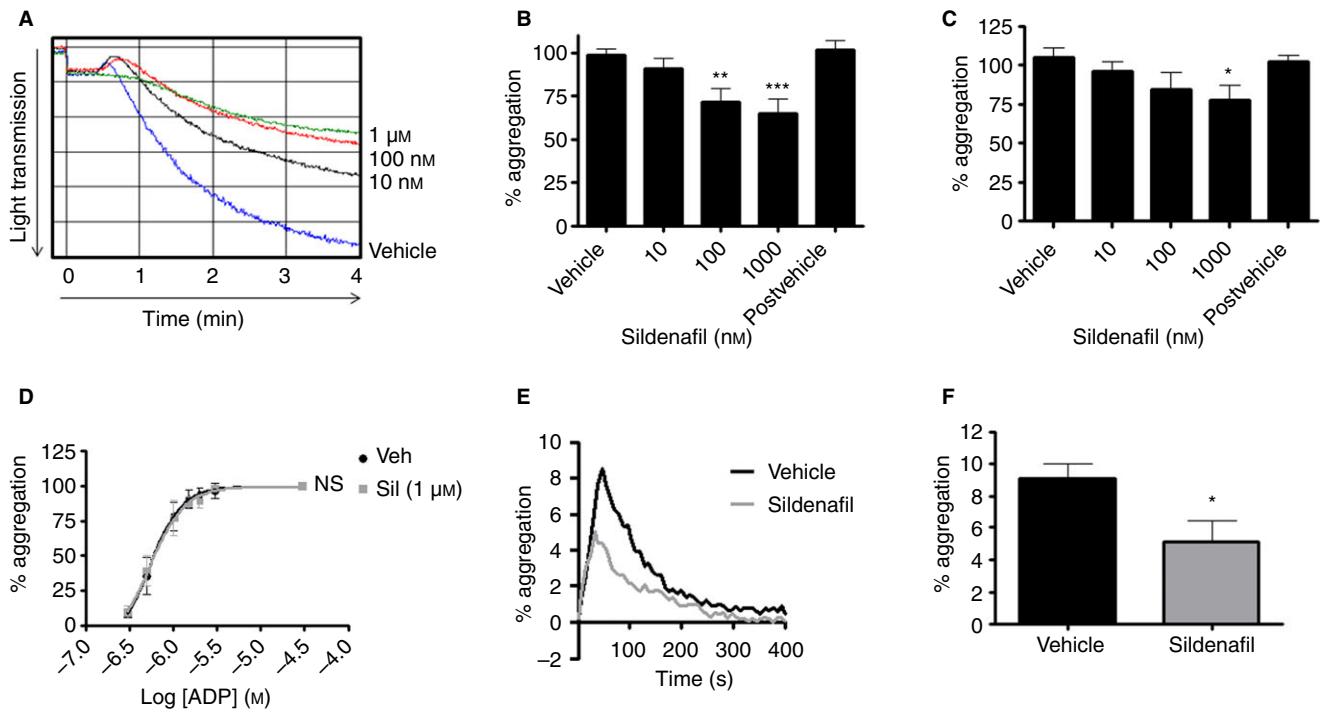
Fig. 1. Sildenafil inhibits platelet aggregation *in vitro* and *in vivo*. (A–D) Isolated human platelets were preincubated with vehicle or sildenafil citrate (10 nM to 1 μM) for 5 min before stimulation with (A, B) collagen (5 μg mL^{-1}), (C) thrombin (0.1 U mL^{-1}), and (D) ADP (0.3–30 μM). Platelet aggregation was measured as light transmission. (A) Example traces are representative of eight independent experiments. (B, C) Data are expressed as mean ± standard error of the mean (SEM), one-way ANOVA with Bonferroni *post hoc test*, *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ as compared with vehicle. (D) Vehicle EC$_{50}$ = 0.585 μM; sildenafil EC$_{50}$ = 0.447 μM. (E, F) Sildenafil (50 μg kg^{-1}) or vehicle was administered to mice 5 min before collagen (50 μg kg^{-1}). Platelet aggregation was measured as changes in radioactive counts in the pulmonary vasculature. (E) Mean trace of collagen response is expressed as percentage increase from baseline; error bars are omitted for clarity. (F) Maximum percentage increase from baseline is expressed as mean ± SEM, unpaired Student's *t*-test, *$P < 0.05$ as compared with vehicle, $n = 5$–8. Sil, sildenafil; Veh, vehicle.

(Fig. 3A). Washing of platelet suspensions with the mild reducing agent ascorbic acid lowered their nitrate and nitrite content, presumably because of chemical reduction to NO (Fig. 3B). Nitrate/nitrite depletion and any accompanying NO release were associated with inhibition of platelet aggregation (Fig. 3C). The inhibitory effect of nitrate/nitrite reduction by ascorbic acid was abolished by ODQ and hemoglobin (Fig. 3D).

Sodium nitrite (0.01–100 μM) caused concentration-dependent inhibition of aggregation in the presence of sildenafil (Fig. 3E), whereas equivalent concentrations of sodium nitrate had no effect (Fig. 3F). The inhibitory effect of nitrite was abolished by ODQ, and did not occur in the absence of sildenafil (Fig. 3E). Similarly, nitrite increased phosphorylation of VASP at Ser239 in the presence of sildenafil, an effect that was prevented by ODQ, whereas nitrate had no effect (Fig. 3G,H).

Inorganic nitrate inhibits platelet aggregation following enhanced bioconversion to nitrite during endothelial dysfunction in vivo

Sodium nitrate (1 mmol kg^{-1}) administration to mice led to increased concentrations of nitrate in salivary glands

(Fig. 4A) and increased plasma nitrate concentrations (Fig. 4B) in wild-type and eNOS$^{-/-}$ mice as measured by ozone chemiluminescence. There was also an accompanying increase in plasma nitrite that was not significant in wild-type mice but was significant in eNOS$^{-/-}$ mice as compared with saline-treated controls (Fig. 4C). Nitrate administration had no significant effect on subsequent collagen-induced platelet aggregation *in vivo* in wild-type mice, but significantly reduced platelet aggregation in eNOS$^{-/-}$ mice (Fig. 5A,B).

Discussion

Sildenafil has already been shown to amplify the inhibitory effect of exogenously applied NO on human platelet aggregation [4] and to reduce ADP-induced glycoprotein IIb–IIIa activation [30] and aggregation [5] *ex vivo*, indicating the ability of sildenafil to enhance the platelet inhibitory activity of exogenous and endothelial NO, respectively. The ability of sildenafil to enhance intrinsic endogenous NO signals in platelets is less well studied, although a lack of effect on ADP-induced aggregation has been reported [6]. We found a similar lack of effect when platelets were stimulated
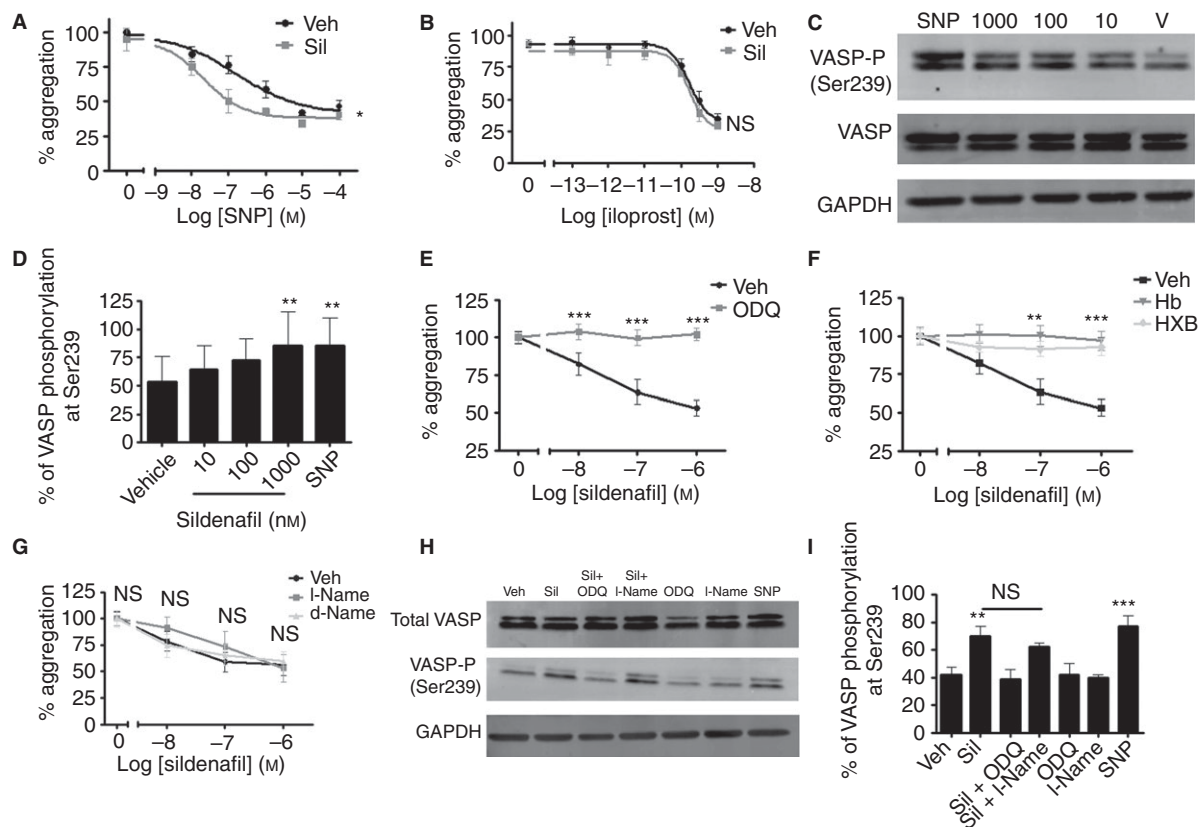
Fig. 2. Sildenafil selectively amplifies endogenous NO-mediated signaling independently of nitric oxide synthase. (A, B) Sildenafil (10 nM) significantly enhanced the inhibition of collagen-induced (5 µg mL^{-1}) washed human platelet aggregation mediated by (A) sodium nitroprusside (SNP) (0.01–100 µM) but not (B) iloprost (0.1–1000 pM). (C, D) sildenafil (10–1000 nM) and SNP (positive control) induced phosphorylation of vasodilator-stimulated phosphoprotein (VASP) at Ser239. Data are shown as (C) a western blot representative of four independent experiments, and (D) percentage of VASP-P(Ser239) as compared with total VASP. Data are expressed as mean ± standard error of the mean (SEM). (E–G) The inhibitory effect of sildenafil (10 nM to 1 µM) on collagen-induced aggregation was significantly inhibited by (E) 1H-(1,2,4)-oxadiazolo [4,3-a] quinoxalin-1-one (ODQ) (10 µM) and (F) hemoglobin (Hb) (5 µM) or hydroxocobalamin (HXB) (100 µM), whereas (G) L-NAME (100 µM) or D-NAME (100 µM) had no effect. (H, I) Sildenafil (100 nM)-induced VASP phosphorylation at Ser239 was abolished by ODQ, whereas L-NAME had no effect; data are presented as (H) a western blot representative of four independent experiments, and (I) percentage of VASP-P(Ser239) as compared with total VASP. Data are expressed as mean ± SEM, one-way ANOVA with Bonferroni *post hoc* test, *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ as compared with vehicle; NS, not significant ($P > 0.05$) as compared with sildenafil, $n = 4$–6. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; V, vehicle; Veh, vehicle; Sil, sildenafil.

with ADP in the absence of exogenous NO but, in contrast, found inhibition of collagen-induced and thrombin-induced aggregation in isolated platelet suspensions. This finding indicates not only the ability of sildenafil to directly modulate platelet activation in the absence of exogenous or endothelial NO, but also the presence of transient, endogenous signals upstream of PDE5 in platelets. The ability of sildenafil to modulate activation downstream of collagen and thrombin suggests modulation of pathways that were not triggered by the weaker agonist ADP. In addition, experiments with ADP in work published previously [6] and in the current study were conducted in the presence of plasma proteins, whereas experiments with collagen and thrombin were conducted in preparations lacking plasma proteins. The lack of effect of sildenafil in ADP experiments may therefore be partially pharmacokinetic, owing to interac-

tion of the relatively lipophilic sildenafil [31] with plasma proteins.

We confirmed that, in isolated platelets, sildenafil acts downstream of the NO–cGMP signaling cascade by showing that its inhibitory effects were completely abolished by the sGC antagonist ODQ and two distinct NO scavengers. We further confirmed that sildenafil mediates inhibitory signaling events in platelets by demonstrating phosphorylation of VASP at Ser239. Given the reported crosstalk between cyclic nucleotides and phosphodiesterases [32], particularly at the level of protein kinases [33,34], we investigated the selectivity of sildenafil upstream of these signaling events, and showed an ability of sildenafil to enhance the inhibitory effect of NO (Fig. 2A) but not PGI$_2$ (Fig. 2B). Sildenafil therefore selectively amplifies the inhibitory effect of NO while having no measurable functional effect on PGI$_2$-mediated
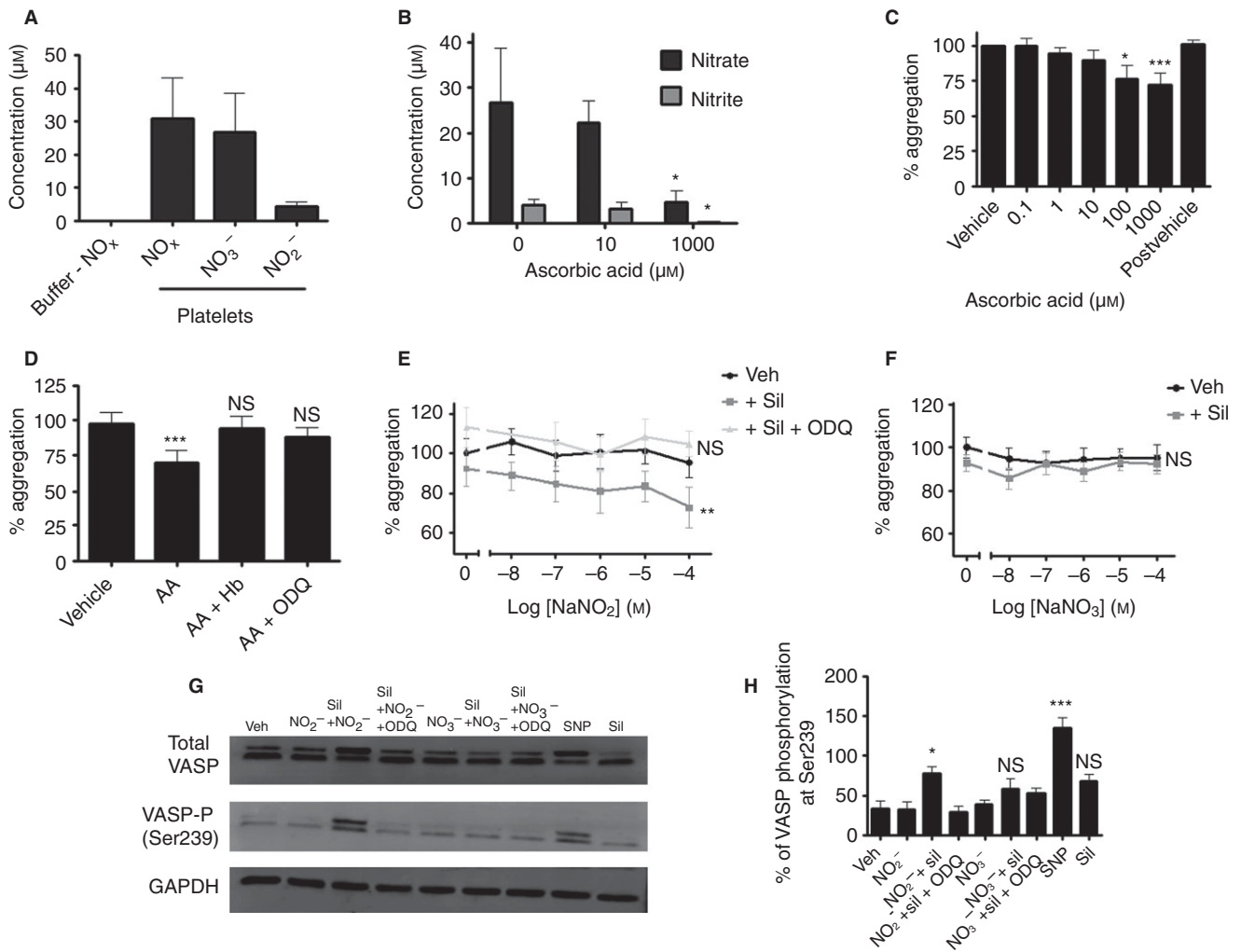
Fig. 3. Nitrite drives cGMP-mediated inhibition of platelet aggregation. (A, B) Nitrate and nitrite concentrations were measured in human platelets, (A) untreated or (B) preincubated with vehicle or ascorbic acid (AA) (10 μM and 1000 μM). Data are expressed as mean ± standard error of the mean (SEM), one-way ANOVA with Bonferroni *post hoc* test. (C) Collagen (5 μg mL^{-1})-induced aggregation was inhibited in nitrate/nitrite-depleted platelets treated with AA; one-way ANOVA with Bonferroni *post hoc* test. (D) AA-induced (2.5 mM) inhibition of aggregation was reversed in the presence of 1H-(1,2,4)-oxadiazolo[4,3-a] quinoxalin-1-one (ODQ) (10 μM) and hemoglobin (Hb) (5 μM); one-way ANOVA with Bonferroni *post hoc* test. (E, F) Sodium nitrite (NaNO$_2$, 0.01–100 μM) caused concentration-dependent inhibition of platelet aggregation in the presence of sildenafil (10 nM) that was reversed by ODQ (10 μM), an effect not seen with (F) sodium nitrate (NaNO$_3$, 0.01–100 μM). Data are expressed as mean ± SEM, two-way ANOVA with Bonferroni *post hoc* test. (G, H) Preincubation of platelets with NO$_2^-$ (100 μM) and sildenafil (10 nM) resulted in significant phosphorylation of vasodilator-stimulated phosphoprotein (VASP) at Ser239 that was reversed in the presence of ODQ (10 μM). This effect was not seen with NO$_3^-$ (100 μM). Sodium nitroprusside (SNP) (1 μM) was used as a positive control. Data are presented as (G) a western blot representative of four independent experiments, and (H) percentage of VASP-P(Ser239) as compared with total VASP. Data are expressed as mean ± SEM, one-way ANOVA with Bonferroni *post hoc* test, *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ as compared with vehicle, $n = 4$–7. NS, not significant; Sil, sildenafil; Veh, vehicle.

inhibition, despite downstream crosstalk between these pathways.

A number of groups, including ours, have previously suggested a lack of functional relevance of expression of NOS in human platelets [13,14]. This led us to consider the source of NO acting upstream of sildenafil to mediate inhibition of platelet aggregation. Studies with a NOS inhibitor (Fig. 2G) suggested that the effect of sildenafil occurred independently of NOS, suggesting alternative sources of bioactive NO. The validity of this conclusion depends upon effective blockade of NOS at the concentration of

L-NAME employed. L-NAME inhibits NOS with an IC$_{50}$ of 0.81 μM [35], and off-target effects in platelets emerge at approximately 500 μM to 1 mM [14]. Our working concentration of 100 μM can therefore be reasonably assumed to result in effective and selective inhibition of NOS activity in platelets, as previously reported [36]. Nonetheless, although we have data suggestive of NOS-independent inhibitory activity upstream of PDE5, we cannot entirely exclude the possibility that NO derived from NOS, if expressed in platelets, may have relevance under certain circumstances, albeit insignificant in the present study. We proceeded by
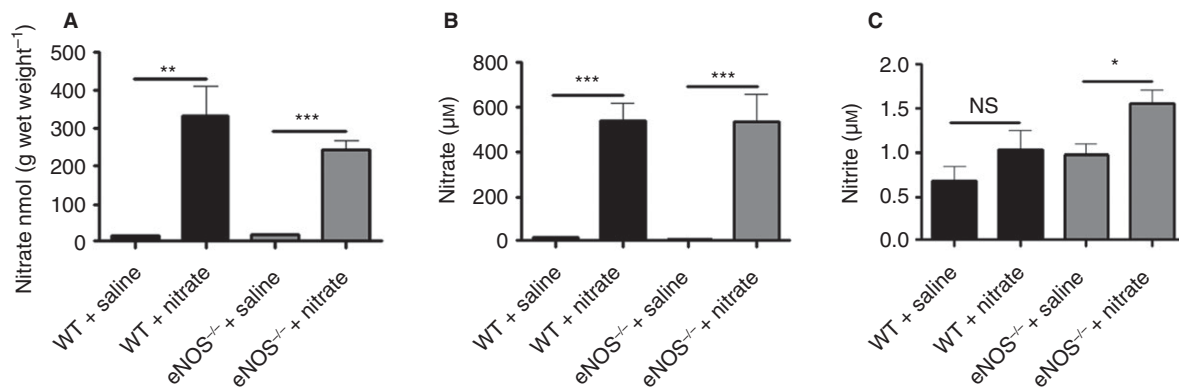
Fig. 4. Bioconversion of nitrate to nitrite is enhanced in the absence of endothelial nitric oxide synthase (eNOS). (A, B) Following treatment of mice with saline or sodium nitrate (1 mmol kg^{-1}, intraperitoneal) for 1 h, nitrate concentrations were significantly increased in (A) salivary glands and (B) plasma in both wild-type (WT) and eNOS$^{-/-}$ mice. (C) Plasma nitrite concentrations were significantly increased in eNOS$^{-/-}$ but not in WT mice following nitrate treatment. Data are expressed as mean $\pm$ standard error of the mean, unpaired Student's t-test, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; NS, not significant ($P > 0.05$), $n = 5$–7.
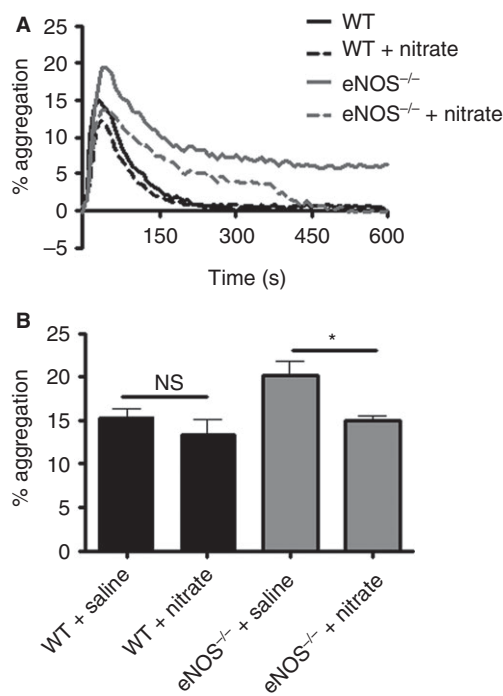

Fig. 5. Inorganic nitrate inhibits platelet aggregation during endothelial dysfunction *in vivo*. Wild-type (WT) and endothelial nitric oxide synthase (eNOS$^{-/-}$) mice were treated with saline or sodium nitrate (1 mmol kg^{-1}, intraperitoneal) 1 h prior to collagen (50 μg kg^{-1}), and radiolabeled platelet aggregation was measured as changes in radioactive counts in the pulmonary vasculature. (A) Mean trace of collagen response (percentage increase from the baseline radioactive counts) versus time. Data are expressed as mean (error bars omitted for clarity). (B) Maximum percentage increase from baseline radioactive counts. Data are expressed as mean $\pm$ standard error of the mean, unpaired Student's t-test, *$P < 0.05$; NS, not significant ($P > 0.05$), $n = 4$–6.

exploring the ability of inorganic nitrate and nitrite (administered as sodium nitrate and sodium nitrite) to modulate platelet activation and VASP phosphorylation.

In mammals, inorganic nitrate can be bioconverted to nitrite and subsequently reduced to NO by a variety of mechanisms [20,37,38]. We first confirmed that platelets contain nitrate/nitrite that could potentially be reduced to NO to explain the presence of endogenous, NOS-independent NO signals in platelets. Nitrate and nitrite levels in platelets were then successfully depleted by incubation of platelets with the mild and relatively non-toxic reducing agent ascorbic acid in buffer prepared with nitrate-free water. We hypothesized that reduced endogenous nitrite would generate an NO–cGMP signal that could be amplified by sildenafil to inhibit agonist-induced aggregation, and found evidence to support this when ascorbic acid induced ODQ-sensitive inhibition of aggregation. In fact, the inhibitory effect of ascorbic acid was entirely abolished by ODQ or NO scavenging, suggesting that, under the prevailing experimental conditions, ascorbic acid exerted functional effects that were entirely mediated via sGC and NO. Reducing agents exert a range of effects, including reduction of reactive oxygen species and peroxynitrite, and showing that reducing agents can drive inhibitory signaling, although indicating that, in principle, platelet function can be affected by reduced nitrite/nitrate, does not demonstrate an endogenous ability of platelets to reduce nitrate or nitrite to NO. We therefore investigated the functional impact of nitrate/nitrite in the absence of exogenous reducing agents to directly link nitrate/nitrite with platelet activity. Nitrite has previously been reported to directly inhibit platelet aggregation, although the concentrations required were higher than those found in plasma following nitrate administration to humans [21,39]. In contrast, lower, more relevant concentrations of nitrite were shown to have no effect on isolated platelets [22], reflecting either an inability of platelets to reduce nitrite or the generation of a transient signal without functional impact. The application of a fixed concentration of sildenafil in the current study revealed the ability of nitrite but not nitrate to generate ODQ-sensitive inhibitory signals

in platelets in the presence of sildenafil, suggesting an endogenous capacity to reduce nitrite. In line with earlier studies [22], nitrite had no effect on platelet aggregation in the absence of sildenafil, suggesting the generation of transient signals that are, under normal circumstances, rapidly hydrolyzed by PDE5. Although some evidence for sGC-independent effects of NO [40–42] has been reported, the effects of nitrite reported here are entirely sGC-dependent. This is in line with more recently reported data demonstrating the absolute dependence of NO-mediated signaling on sGC in platelets [43]. The mechanism by which platelets reduce nitrite remains unclear. In other cell types, such as vascular endothelial cells, nitrite is reduced enzymatically by xanthine oxidases [17,20] and aldehyde dehydrogenase [44]; however, these enzymes do not form part of the platelet proteome [45]. In addition, nitrite reduction is also suggested to occur in erythrocytes [22,46,47], implying an enhanced capacity to reduce nitrite in whole blood and *in vivo* as compared with isolated platelets or plasma. Therefore, alternative mechanisms in platelets, such as mitochondrial activity or the presence of as yet unidentified mediators with reducing capacity, may explain the efficacy of nitrite in isolated platelets reported here. Given the absence of many of the proposed nitrate reductase systems in platelets, we tentatively speculate that the most likely mechanism of nitrite reduction in platelets is a mitochondrial nitrite reductase, such as cytochrome *c* [48]. Demonstrating conclusively the role of mitochondrial components with critical roles in mitochondrial respiration and cellular metabolism in nitrite reduction and inhibition of platelet activation is likely to be challenging.

The presence of NOS in platelets has been contentious for some time now [11,12], and there is increasing evidence for a lack of importance of NOS-derived NO in regulating platelet function [13,14] (the primary source of NO affecting platelets physiologically being the vascular endothelium [7]). Nonetheless, platelets are widely reported to generate NO [15,16]. Our data provide one potential explanation for these apparently contradictory observations in addition to those previously suggested, such as NO production via S-nitrosothiols [49] and protein disulfide isomerases [50].

We also explored the *in vivo* relevance of our data obtained with isolated platelets. Sildenafil has previously been reported to improve coronary artery patency in a model of cyclic coronary occlusion [51]. The effect was suggested to be potentially platelet-mediated, but may also have resulted from coronary vasodilation. The model used in the current study was selected because it has previously been shown to measure platelet aggregation independently of any effect on vascular tone [7]. We can therefore conclude that, as well as exerting a direct inhibitory effect on platelets *in vitro*, sildenafil inhibits agonist-induced platelet aggregation *in vivo* via a direct effect on the platelet rather than via a secondary vascular effect. As *in vivo* preparations contain a fully functional vascular endothelium, it is reasonable to conclude that the effect of sildenafil on platelets *in vivo* is, at least in part, mediated via enhancement of NO derived from the vascular endothelium as well as via any direct platelet-mediated effect of nitrite.

Nitrate administration in humans has previously been shown to induce a fall in blood pressure and to inhibit *ex vivo* platelet aggregation [21,22]. In previously reported mouse studies, a lowering of plasma nitrite concentration was associated with enhanced platelet aggregation *ex vivo* [52]. Daily, 0.2 mmol kg^{-1} nitrate has been estimated to be produced endogenously by NOS [26,53]. One nitrate-rich vegetable portion contains more nitrate that that produced by all forms of NOS daily [54]. Therefore, the dose of 1 mmol kg^{-1} nitrate used in the current study reflects a realistic dose that could be achieved through dietary choices or supplementation. In the present study, the increase in plasma nitrite concentration following administration of nitrate to wild-type mice, although not significant, was similar to that reported previously in humans following consumption of high-nitrate beetroot juice ($\sim 0.2\ \mu$M) [21]. This increase in plasma nitrite concentration did not lead to a change in platelet aggregation *in situ* in our study, suggesting that NO was not a limiting factor in the context of a healthy vasculature. In eNOS$^{-/-}$ mice, however, nitrate administration led to an approximately five-fold greater increase in plasma nitrite concentration ($\sim 1\ \mu$M), indicating that bioconversion of nitrate to nitrite was certainly evident and indeed greater than that observed in wild-type mice. These data suggest that eNOS$^{-/-}$ mice may compensate for the absence of NO from conventional enzymatic sources by increasing NO generation from nitrate. Our data showing nitrate reduction in eNOS$^{-/-}$ mice also indicate that, although eNOS has been shown to mediate nitrite reduction in a previous study [55], this was not a primary mechanism of systemic reduction in our study. Our data raise the question of whether a similar switch in the physiologic source of NO from NOS to nitrite occurs in humans with vascular disease. This issue has not been addressed in the current study, and additional studies in humans and, in particular, patients with cardiovascular conditions associated with deficient eNOS activity are required to translate our mechanistic linking of eNOS with enhanced nitrite reduction to human pathology. If this translation is established, then nitrate/nitrite derived from the diet may become critical as a source of bioactive NO during endothelial dysfunction. We tested the functional relevance of the differential changes in plasma nitrite in wild-type and eNOS$^{-/-}$ mice by measuring platelet aggregation *in vivo*. Interestingly, the higher bioconversion of nitrate occurring in eNOS$^{-/-}$ mice was associated with a significant reduction in platelet aggregation, an effect not seen in wild-type mice. Our data therefore suggest that nitrate exerted a specific effect on platelet function under conditions of vascular dysfunction, namely eNOS deficiency,

whereas, under conditions of vascular health, associated with wild-type mice, normal platelet function was retained. These data are potentially of great interest, as they suggest targeted efficacy under conditions of endothelial dysfunction.

In summary, we have shown that platelets generate transient, endogenous cGMP signals that may be pharmacologically enhanced by inhibition of PDE5 activity. These signals are generated downstream of NO, but are primarily independent of NOS activity. Furthermore, nitrite is able to generate transient NO–cGMP signals in platelets that can be enhanced by sildenafil. The absence of eNOS leads to an enhanced capacity to bioconvert nitrate to nitrite, which, in turn, negatively impacts on platelet function. Our study adds to the increasing body of evidence suggesting that dietary nitrate may account, at least partly, for the beneficial effects of healthy diets, particularly those rich in green vegetables with high nitrate content. Furthermore, inorganic nitrate may potentially exert an antiplatelet effect specifically during endothelial dysfunction while allowing retention of normal platelet function in conditions of vascular health. Our study, combined with growing literature concerning the impact of dietary nitrate on cardiovascular health, suggests that the potential use of dietary nitrate supplementation in the primary prevention of platelet-driven cardiovascular events should be further explored.

Addendum

G. L. Apostoli design, conduct and analysis of experiments, and drafting of the manuscript. A. Solomon concept and design of experiments. M. J. Smallwood design, conduct and analysis of experiments. P. G. Winyard concept, design of experiments, and drafting of the manuscript. M. Emerson concept, design of experiments, drafting of the manuscript, and final approval of the manuscript.

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Disclosure of Conflict of Interests

P. G. Winyard reports receiving grants from James White Drinks Ltd, outside the submitted work. All other authors state that they have no conflict of interest.

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