The influence of $P2Y_{12}$ antagonists on vascular NO signalling

A Thesis submitted for the Degree of Doctor of Philosophy

by

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For to my fiancé Michał, who supported me along the way

and encouraged to stay focused on my writing.

Declaration

This work has not previously been accepted in substance for any degree and is not concurrently submitted in candidature for any degree.

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First begin
Taking in.
Cargo stored,
All aboard,
Think about
Giving out.
Empty ship,
Useless trip!

Never strain
Weary brain,
Hardly fit,
Wait a bit!
After rest
Comes the best.

Sitting still,
Let it fill;
Never press;
Nerve stress
Always shows.
Nature knows.

Critics kind,
Never mind!
Critics flatter,
No matter!
Critics curse,
None the worse.
Critics blame,
All the same!
Do your best.
Hang the rest!

Arthur Conan Doyle's poem: Advice To A Young Author

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Abstract

$P2Y_{12}$ antagonists are pharmacological agents used clinically in advanced stages of coronary artery disease in order to inhibit ADP-induced activation and aggregation of platelets and prevent deadly thrombotic events. Of the orally-prescribed $P2Y_{12}$ antagonists available clopidogrel is the most established, it exhibits an excellent safety track record and is a popular drug, and was accredited for years the second-best selling drug in the world. However, since clopidogrel was introduced to the market in 1997 many pleiotropic effects have been noticed, which suggest other off-target yet beneficial effects in addition to its anti-platelet effects.

The overall hypothesis being tested in this body of work was that $P2Y_{12}$ antagonists, clopidogrel in particular, have the positive influence on vascular NO signalling.

A vascular model was set up using isolated rabbit aortae in which clopidogrel enhanced NO donor-induced vasorelaxation. Although the precise mechanism was not found, the effect was independent of $P2Y_{12}$ receptors and possibly linked to decreased superoxide production and improved anti-oxidant/inflammatory status in vessels. This finding might be relevant for patients receiving concomitant therapy with organic nitrates and clopidogrel.

In vitro studies revealed novel S-nitrosation properties of $P2Y_{12}$ antagonists, surprisingly without the need for metabolism to their active form. Newly synthesized SNO derivatives of clopidogrel and prasugrel were more potent in inhibition of platelet aggregation and induction of vasodilation than their parental forms. Although the formation of drug-SNO species has to be confirmed *in vivo*, they have a potential to increase NO bioavailability in patients.

Clopidogrel administration to coronary artery disease patients resulted in upregulated plasma levels of nitrite and cGMP after 2 h-intake of a loading dose, which were further increased after 3 days of a maintenance therapy. This effect was never shown before in man and most likely reflects improved endogenous NO production, but also providing additional protection from the effects of nitrite at the same time.

Taken together, the results of this thesis clearly demonstrate the influence of clopidogrel on vascular response to NO as well as NO production, metabolism and bioavailability. It is important to identify these alternative pathways especially in the current era with alternative $P2Y_{12}$ antagonists that overcome some of the limitations of clopidogrel but may not share all the beneficial properties.

Commonly used abbreviations:

2Cs	Copper (I) chloride/cysteine
Ach	Acetylcholine
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
AUC	Area under curve
Ca^{2+}	Calcium ion
CAD	Coronary artery disease
cAMP	Cyclic adenine monophosphate
cGMP	Cyclic guanosine 3'-5'monophosphate
COX	Cyclooxygenase
Cu^{+}	Cuprous ion
Cu^{2+}	Cupric ion
Cys	Cysteine
DMSO	Dimethyl sulphoxide
EC	Endothelial cells
EC_{50}	Concentration required to achieve 50% effect
EDTA	Ethelene diamine tetra acetic
eNOS	Endothelial nitric oxide synthase
EPR	Electron paramagnetic resonance
g	Gravity
GPCR	G protein-coupled receptor
GSNO	*S*-nitrosoglutathione
H_2O	Water
H^{+}	Hydrogen ion
HCl	Hydrochloric acid
HUVEC	Human Umbilical Vein Endothelial Cells
iNOS	Inducible nitric oxide synthase
K^{+}	Potassium ion
L-NAME	L-Nitro-Arginine Methyl Ester
L-NMMA	NG-monomethyl-L-arginine
LTA	Light transmission aggregometry
MI	Myocardial infarction
N_2	Nitrogen
$NaNO_2$	Sodium nitrite
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NO_2^{-}	Nitrite anion
NO_3^{-}	Nitrate anion

NOA	Nitric oxide analyser
NOC9	MAHMA NONOate
NOS	Nitric oxide synthase
NO_x	Nitrate and nitrite anions
$O_2^{\bullet-}$	Superoxide
OBC	Ozone based chemiluminescence
ODQ	1H-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one
$ONOO^-$	Peroxynitrite
PAD	Peripheral artery disease
PAR	Protease-activated receptor
PBS	Phosphate buffer saline
PCI	Percutaneous Coronary Intervention
PE	Phenylepherine
PGI_2	Prostacyclin
ppp	platelet poor plasma
prp	platelet rich plasma
ROS	Reactive oxygen species
RSH	Reduced thiols
RSNO	S-nitrosothiol species
RT qPCR	Real time quantitative polymerase chain reaction
SD	Standard deviation
sGC	Soluble guanylate cyclase
SMC	Smooth muscle cells
SNO	S- Nitrosothiol
TAC	Total anti-oxidant capacity
Th	Thienopyridine
Th-SNO	Thienopyridine-nitrosothiol
TRAP	Thrombin receptor activating peptide
TXA_2	Thromboxane
VCl_3	Vanadium chloride

I. GENERAL INTRODUCTION

1. Vascular endothelium

The vascular endothelium forms the inner cellular lining of blood vessels in the entire vascular system, from the heart to the smallest capillary, with a surface area of 1-7 m^2 and a cumulative weight of 1 kg in humans[1]. This enormous interface facilitates the contact between blood stream and tissues. However, the vascular endothelium is more than a simple, passive transporter; it is a highly dynamic and heterogeneous organ that possesses vital secretory, synthetic, metabolic, and immunologic functions. This versatile role of vascular endothelium is affected by its unique semi-permeable structure in addition to the presence of membrane-bound receptors for numerous molecules including proteins, lipid transporting particles, metabolites and hormones. The specific structure and multiple receptors allow vascular endothelium to interact with blood cells from the outside as well as smooth muscle cells from the inside in order to mediate the whole range of physiological pathways including the control of vasomotor tone, blood cell trafficking, haemostatic balance, permeability, proliferation, survival, and innate and adaptive immunity[2] (Figure 1.1). For the same reasons the vascular endothelium is involved in most if not all cardiovascular disease states, either as a primary determinant of pathophysiology or as a victim of associated damage.

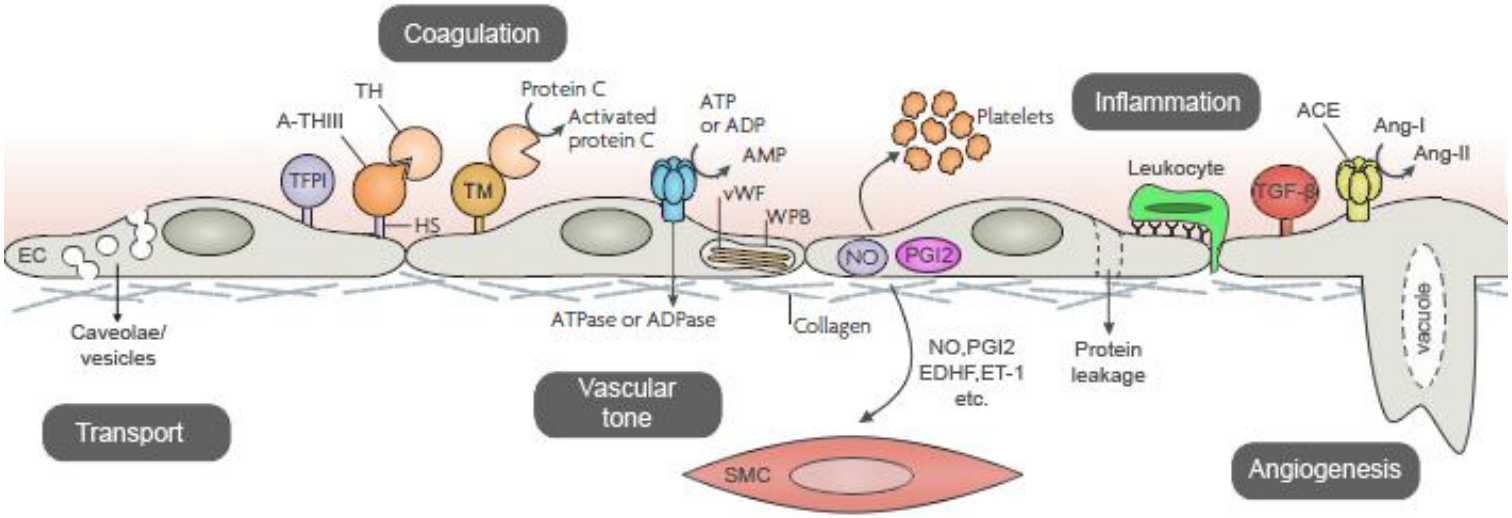


Figure 1.1. Major functions of vascular endothelium. The endothelial cells (1) govern the transport between blood and tissues using the caveolae/vesicle system and the intracellular junctions; (2) regulate the vascular tone by secretion of vasoactive substances that relax or constrict SMCs; (3) inhibit coagulation of blood by (i) control of thrombin generation, which otherwise activates the coagulation cascade, (ii) production of NO and PGI_2, which inhibit platelet activation, adhesion and aggregation and (iii) degradation, storage or physical separation of signals that activate (e.g. ADP) or facilitate the adhesion of platelets (vWf, collagen); (4) control the immunity and inflammation by facilitating the migration of leukocytes into the sites of vascular injury; (5) initiate new blood vessel formation (angiogenesis) which is essential for tissue growth, wound repair and cancer growth. Described in details in text. Adapted[3].

1.1 Major functions of vascular endothelium

1.1.1 Transport of plasma molecules

The primary role of endothelium is to form a semi-permeable membrane that retain blood cells and plasma proteins within the circulating blood stream and at the same time allow the passage of small solutes such as glucose, adrenaline and drugs between tissue and blood. The vascular permeability is regulated at two main levels, depending on the inflammatory status of cells. In the absence of a pathological stimulus, endothelial cells tightly monitor the transport of plasma molecules by means of non-specific and receptor-mediated endocytosis (within endothelial cells) and transcytosis (across endothelial cells). Some molecules (insulin or lipoproteins) are endocytosed to be used by the cell itself, others (transferrin, albumin) are transcytosed to reach the underlying cells, whereas others undergo both routes $(LDL)^{2}$. Under physiological conditions these processes require only the involvement of the caveolae/vesicle system, while the restrictive intracellular junctions stay impermeable to macromolecules (albumin, blood cells). However, upon the release of inflammatory mediators, the activation of vasodilatory pathways result in additional increased junctional permeability, allowing for example the passage of leukocytes and more intense transport of albumin[2, 4].

1.1.2 Maintenance of vascular tone

Endothelial cells regulate vascular tone by releasing vasodilating factors including nitric oxide (NO), prostacyclin (PGI_2), endothelium-derived

hyperpolarizing factor (EDHF) and others, as well as vasoconstrictors, including endothelin (ET_1), angiotensin-II (Ang-II), platelet-activating factor (PAF) and others (Table 1). These vasoactive mediators not only regulate the tone and growth of the underlying smooth muscle cells, but also regulate the reactivity of circulating leukocytes, erythrocytes, and platelets, and govern vascular permeability. Their biological effects are regulated by the localization of specific receptors on vascular and/or blood cells, through their rapid metabolism or gene transcription.

Agent	Vascular effects	Other effects
NO	Vasodilation Inhibition of SMC proliferation Inducement of angiogenesis	Inhibition of platelet adhesion, activation and aggregation; promotion of platelet disaggregation Inhibition of leukocyte adhesion Regulation of inflammation and apoptosis
PGI_2	Vasodilation Inhibition of SMC proliferation Inducement of angiogenesis	Inhibition of platelet adhesion, activation and aggregation; promotion of platelet disaggregation Inhibition of leukocyte adhesion Regulation of inflammation and apoptosis
EDHF	Vasodilation (esp. resistance vessels)	-
PAF	Vasoconstriction	Inducement of platelet activation and aggregation
ET_1[4]	Vasoconstriction (via ET_A receptor), main action Vasodilation (via ET_B receptor and by stimulation of NO, PGI_2 release)	Mitogenesis of SMC Activation of angiotensin-converting enzyme (ACE)
Ang-II[5]	Vasoconstriction (via AT_1 receptor), main action Vasodilation (via AT_2 receptor and by stimulation of NO release) Angiogenesis	Increase of sympathetic nervous system activity Maintenance of fluid homeostasis Inducement of leukocyte adhesion

Table 1. Principal vasoregulatory substances synthesized by endothelium, their effects on the vasculature and other processes. Vasoactive compounds not listed here, i.e. prostaglandins are discussed in text (see I. General Introduction, pages 41-43). Adapted[6].

1.1.3 Blood coagulation

A pivotal physiological function of the vascular endothelium is to maintain blood fluidity by providing an anti-coagulant surface and controlling the mechanisms that promote platelet activation, adhesion and result in blood clotting (Table 2). The anti-coagulant surface of endothelium is maintained thanks to the glycocalyx coating, a network of membrane-bound proteoglycans and glycoproteins integrated with endothelium- and plasma-derived soluble proteins[2, 6, 7]. Heparan sulfate (HS) proteoglycans represent 50–90% of the total amount of proteoglycans and they are bound to anti-thrombin III (A-THIII), which inhibits thrombin and its pro-coagulant actions[7]. Another soluble proteoglycan thrombomodulin (TM) that also binds thrombin, changes its substrate specificity from cleavage of fibrinogen and release of fibrin (mediator of blood clotting) to cleavage and activation of the anti-coagulant protein C[6].

Apart from providing the anti-coagulant surface, the endothelium also secretes NO and PGI_2 into plasma, which inhibit platelet activation, adhesion and aggregation. Moreover, it converts the platelet activators such as ADP and ATP into the inert AMP through the action of ADPases and ATPases[3]. Importantly, endothelial cells also store the active ultra-large von Willebrand factor (vWf) in Weibel-Palade bodies and become a physical barrier between platelets and the basal lamina, preventing the activation and adhesion of platelets to the endothelial surface by means of a direct contact with vWf and collagen[3]. Therefore, resting endothelial cells display anti-coagulant and anti-thrombotic properties. However, in the presence of thrombotic stimuli the endothelium becomes activated and cells change their status from being anti- to pro-coagulant (Table 2). This involves the induction of tissue factor (TF),

which accelerates the factor VII-dependent activation of factors X and IX, followed by an activation of other coagulation factors and a subsequent generation of thrombin, and fibrin, a prerequisite for the formation of a thrombus[8] (Figure 1.2).

Function	**Anti-coagulant, anti-thrombotic**	**Pro-coagulant, pro-thrombotic**
Control of thrombin generation and coagulation cascade	TFPIs HS/A-THIII TM/APC, protein S	TF, FIX, X, XII Thrombin, fibrin
Release of vasoactive agents	NO, PGI_2	TXA_2, ET-1
Degradation[a]/storage[b]/ separation[c] of platelet agonists	ADPase[a] Weibel-Pallade bodies[b] Integrity of endothelial membrane[c]	ADP[a] vWF[b] Collagen[c]
Activation/inhibition of the fibrynolytic system	t-PA, u-PA	PAI-1, PAI-2

Table 2. Regulation of coagulation and thrombosis by vascular endothelium. [a,b,c]corresponding factors. Only examples are resented. Abbreviations: APC (activated protein C), A-THIII (anti-thrombin III), HS (heparan sulphate proteoglycans), PAI (plasminogen activator inhibitor), TF (tissue factor), TFPIs (tissue factor pathway inhibitors), TM (thrombomodulin), t-PA (tissue-type plasminogen activator), u-PA (urokinase-type plasminogen activator), vWF (von Willebrand factor). Adapted[4, 6].

After the blood clot is formed, the endothelium is involved in the regulation of the fibrinolytic system, which converts the proenzyme plasminogen into active plasmin that degrades fibrin, ultimately leading to the digestion of the thrombus. The fibrynolytic system can be activated by tissue-type (t-PA) and urokinase-type (u-PA) plasminogen activators and inhibited by specific plasminogen activator inhibitors (PAI-1 and PAI-2) or α_2-antiplasmin[6]. Therefore, endothelial cells can control the life-span of a thrombus by increased or decreased secretion of t-PA and of PAI-1 from the vessel wall.

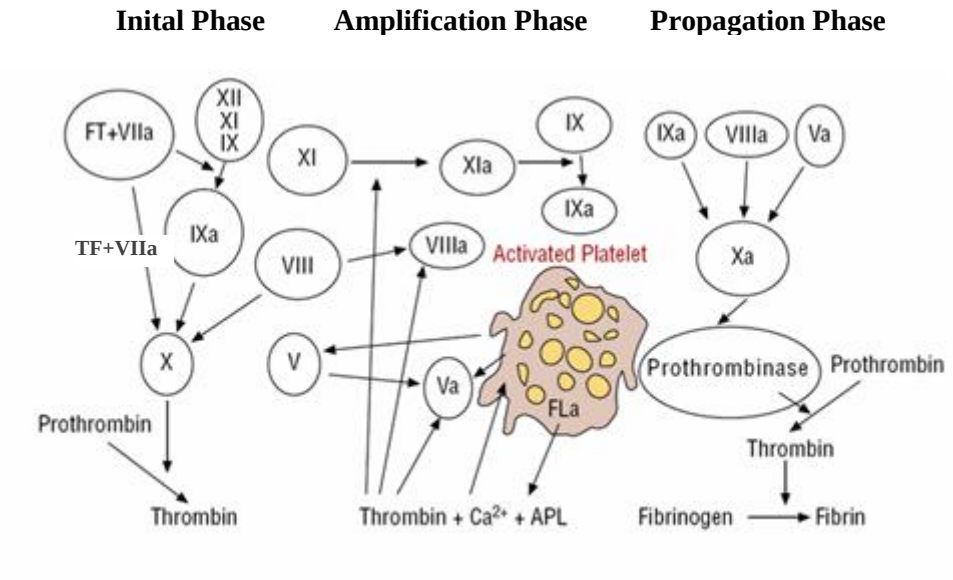


Figure 1.2. Coagulation cascade. Initial Phase – TF (Tissue factor) activates factor VII complex, which then triggers factor X, either directly or indirectly via factor IX, and transforms prothrombin into thrombin in small amounts. Amplification Phase - thrombin that has been formed, along with Ca^{2+} from the blood and APL (anionic phospholipids) derived from platelets, actively participates in a positive feedback process for the activation of factors XI, IX, VIII, and V, and, especially, to accelerate platelet activation. Propagation Phase - large quantities of activated factor X lead to the more conversion of prothrombin into thrombin and, through the action of thrombin, conversion of fibrinogen into fibrin. Adapted[8].

1.1.4 Immunity and inflammation

Endothelial cells play a crucial part in the regulation of immunity and inflammation in the first instance by antigen presentation to T cells and recruitment of inflammatory cells to the inflamed sites and in the second instance by expression of adhesion molecules and release of vasoactive and chemotactic factors, which facilitate the adherence and migration of circulating cells into the affected area (Table 3).

Mediator class	Pro-inflammatory	Anti-inflammatory
Vasoactive agents	Ang-II, ET_1, PGI_2, PGE_2, TXA_2	NO, PGI_2, PGE_2, PGJ_2
Complement	C3a, C5a	C1q receptor
Adhesion molecules	E-selectin, P-selectin, ICAM-1, VCAM-1	$\alpha_v\beta_3$ integrin, TSP receptor, PS receptor
Cytokines	TNF-α, IL-1β, IL-6	TGF-β1, IL-10
Chemokines	IL-8, MCP-1, MIP-1α	-
Transcription factors	NF-κB, STAT, NF-AT, AP-1	PPARγ
Mechanical forces	Stretch	Shear stress

Table 3. Mediators of pro- and anti-inflammatory responses in vascular cells. Abbreviations: AP-1 (activator protein-1), ICAM-1 (intercellular adhesion molecule-1), IL (interleukin), LT (leukotriene), MCP-1 (monocyte chemotactic protein-1), MIP-1α (macrophage inflammatory protein-1α), NF (nuclear factor), PG (prostaglandin), PPARγ (peroxisome proliferator-activated receptor γ), PS (phosphatidylserine), STAT (signal transducers and activators of transcription), TGF-β1 (transforming growth factor-β1), TNF (tumour-necrosis factor), TSP (thrombospondin), VCAM-1 (vascular cell adhesion molecule-1). Only examples are presented. Adapted[9, 10].

Both leukocytes and platelets interact with damaged vessel surfaces through a multistep process including (i) the initial formation of reversible attachments, (ii) activation of the attached cells, (iii) development of stronger, irreversible adhesions and (iv) recruitment of other circulating cells[11] (Figure 1.3). The activation and aggregation of platelets result in the formation of a thrombus (see I. General Introduction, pages 51-54) while the adhesion of leukocytes promotes their movement to the inflammatory sites, which then leads to heat, redness, swelling, pain and loss of function (the five signs of inflammation)[10]. In fact, there is a clear relationship between inflammation and thrombosis. For example, the pro-coagulant thrombin is capable of stimulating multiple inflammatory pathways, and equally, activated inflammatory cells produce many molecules such as TF, thrombin, IL-6, IL-8, and monocyte chemotactic protein MCP-1 which are capable of activating coagulation[5, 12].

Additionally, platelet activation leads to pro-thrombotic and pro-inflammatory responses via release of secondary mediators (ADP, TXA_2 and thrombin) from dense granules and inflammatory mediators (e.g. IL-1, TGF-β, TNF-α, RANTES) from alpha granules[13].

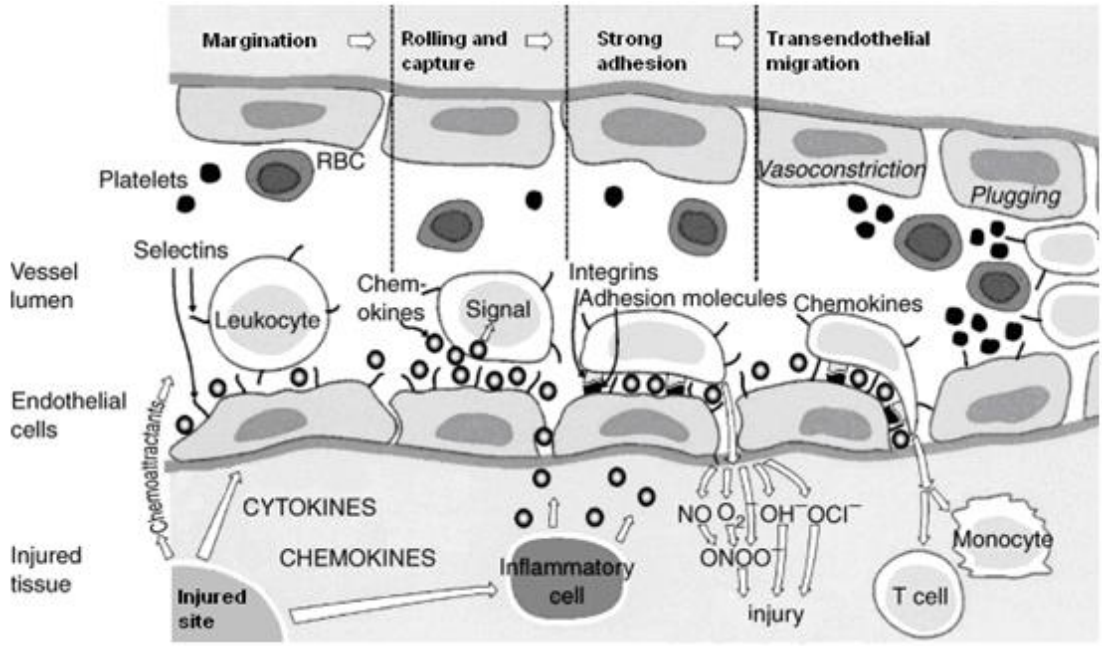


Figure 1.3. Sequential steps in leukocyte recruitment showing leukocyte margination, rolling, firm adhesion and transmigration. At first leukocytes are attracted to endothelium by inflammatory mediators (chemokines, cytokines) released from tissue macrophages. Then, the reversible binding of selectins (P-, E-, L-selectins) expressed on leukocytes and endothelium facilitate rolling and reversible capturing of leukocytes. This leads to the activation of integrins and subsequent binding to their adhesion molecules (VCAM-1 and ICAM-1) on endothelium. Under the influence of additional chemokines, leukocytes become strongly attached and flattened until they start their migration to the sites of injury. Adapted[14]

Finally after the injurious stimulus is removed, the endothelium promotes the suppression of (i) pro-inflammatory gene expression, (ii) leukocyte movement and activation, (iii) thrombus formation, followed by clearance of inflammatory cells by apoptosis and phagocytosis; all in order to restore normal tissue structure and

function. Hence, the controlled pro-inflammatory response to the vascular injury and infection, followed by the adequate anti-inflammatory responses and resolution of inflammation are crucial for the maintenance of the vascular homeostasis. Prolonged inflammation causes endothelial dysfunction associated with the pathogenesis of many cardiovascular diseases.

1.1.5 Angiogenesis

Endothelial cells possess a remarkable ability to divide rapidly in response to hypoxia and inflammation and as such, they play a pivotal role in angiogenesis (the growth of blood vessels). Angiogenesis is as important in the embryo for the development of the vascular system and organ growth as in the adult for wound healing and repair. In the adult, the vessel growth starts from the recruitment of endothelial progenitor cells (EPCs) from the bone marrow to sites of neovascularisation[15]. EPCs are incorporated into nascent vessels or stimulate new vessel growth by releasing pro-angiogenic factors. This process is known as vasculogenesis. Angiogenesis itself refers to endothelial budding and sprouting in response to angiogenic factors, which loosen their inter-endothelial junctions (e.g. Ang-2) and break down the surrounding ECM (e.g. plasminogen activators, matrix metalloproteinases), liberating matrix-bound angiogenic growth factors (e.g. FGF-2, VEGF, IGF-1, TGF-β, TNF-α)[15]. After the new vessel sprout is formed out of naked endothelial cells, it becomes stabilised by recruitment of SMCs and pericites, deposition of ECM and tightening of cell junctions in the process named arteriogenesis[15].

The abnormal or excessive stimulus for angiogenesis contributes to the pathogenesis of many malignant, inflammatory, infectious and immune disorders including cancer, atherosclerosis, asthma, diabetes, cirrhosis, AIDS and many others[15].

1.2 Endothelial dysfunction in cardiovascular diseases

Endothelial cells perform several functions required to maintain vascular homeostasis. They are capable of producing a variety of molecules: vasodilators and vasoconstrictors, procoagulants and anticoagulants, oxidants and anti-oxidants, inflammatory and anti-inflammatory mediators, angiogenic and anti-angiogenic factors in order to balance all needs of the vascular system (Figure 1.4)[5]. However, when endothelial cells are injured, they loose the capacity to maintain homeostasis, which leads to the so called “endothelial dysfunction” and development of vascular disease[5]. This is not to be confused with the term “endothelial activation/stimulation”, which usually refers to the changed status of endothelial cells from anti-coagulant or anti-inflammatory to pro-coagulant or pro-inflammatory in response to local infection. The symptoms of both endothelial activation and dysfunction are often similar in terms of the thrombus formation or the enhancement of leukocyte recruitment but the outcomes are different; the first is beneficial and associated with host defense while the other is pathological and linked to vascular diseases[16].

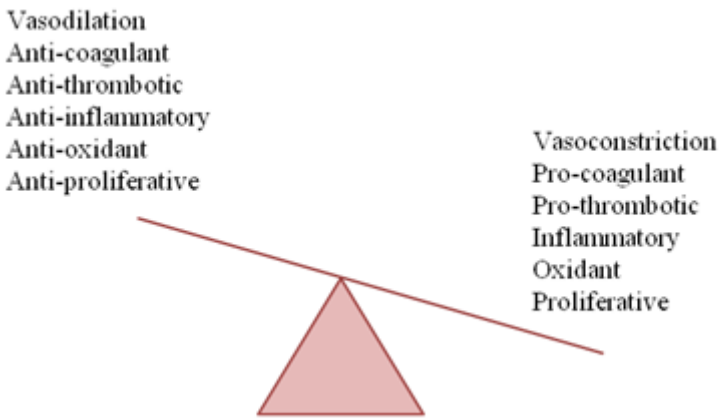


Figure 1.4. Equilibrium between the differing functions in vascular endothelium. The left side of the balance represents features of resting endothelial cells, which dominate in healthy endothelium. On the right side, there are main characteristics of activated endothelial cells which are engaged in response to the local infection. Adapted[5]

Endothelial dysfunction has been implicated in the pathophysiology of diverse cardiovascular diseases including all stages of atherosclerosis, thrombosis, hypertension, coronary artery disease (CAD), peripheral artery disease (PAD), diabetes, obesity, chronic heart and renal failure[17]. It has been not only associated with common cardiovascular risk factors but also correlated with disease progression and used as a prognostic tool for prospective cardiovascular events. Furthermore therapies known to reduce cardiovascular risk improve endothelial function while failure of the endothelium to respond is associated with higher risk[18, 19].

1.2.1 Reactive oxygen species and oxidative stress

Reactive oxygen species (ROS) are partially reduced and highly reactive O_2 metabolites such as superoxide ($O_2^{\bullet -}$) and $O_2^{\bullet -}$-derived hydroxyl radicals ($^{\bullet}OH$), hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$). They are created during mitochondrial oxidative phosphorylation and other electron transfer reactions in all

aerobic organisms and serve as signalling molecules in the regulation of signal transduction, gene expression and pathogen defense responses[20, 21]. In the vascular endothelium NADPH oxidases are considered a main source of $O_2^{\bullet-}$, although other enzymes such as cytochrome P450 (CYP450), xanthine oxidoreductase (XO), mitochondrial complex I and III, uncoupled eNOS, cyclooxygenase (COX), and lipooxygenase (LOX) also contribute.

Oxidative stress is defined as an imbalance between the production of ROS and their removal by naturally occurring anti-oxidant defenses of cells including enzymatic activity of catalase and superoxide dismutases (SODs), as well as direct actions of anti-oxidants like glutathione, vitamin E, β-carotene, ascorbate, urate and many others[22]. Increased cellular production of $O_2^{\bullet-}$ and H_2O_2 can facilitate formation of the more toxic and reactive $\cdot OH$ in the presence of divalent metal ions such as iron (Fenton reaction) or copper[23]. As $\cdot OH$ has a very short half-life and high reactivity, it can rapidly damage any surrounding macromolecules, including: (i) amino acids, potentially leading to protein inactivation/denaturation; (ii) carbohydrates, causing degradation; (iii) lipids, by interaction with polyunsaturated fatty acids of membrane phospholipids, leading to lipid peroxidation; and (iv) nucleic acids, resulting in possible mutations[20]. $ONOO^-$ reacts directly with sulphur-containing amino acids (cysteine and methionine), as well as those with an aromatic structure (e.g. tryptophan and tyrosine)[24]. Prolonged exposure to oxidative stress induces cell proliferation, hypertrophy, apoptosis and inflammation through activation of redox-sensitive transcriptional factors (e.g. NF-κB, AP-1) and leads to further damage.[23, 25] (Figure 1.5).

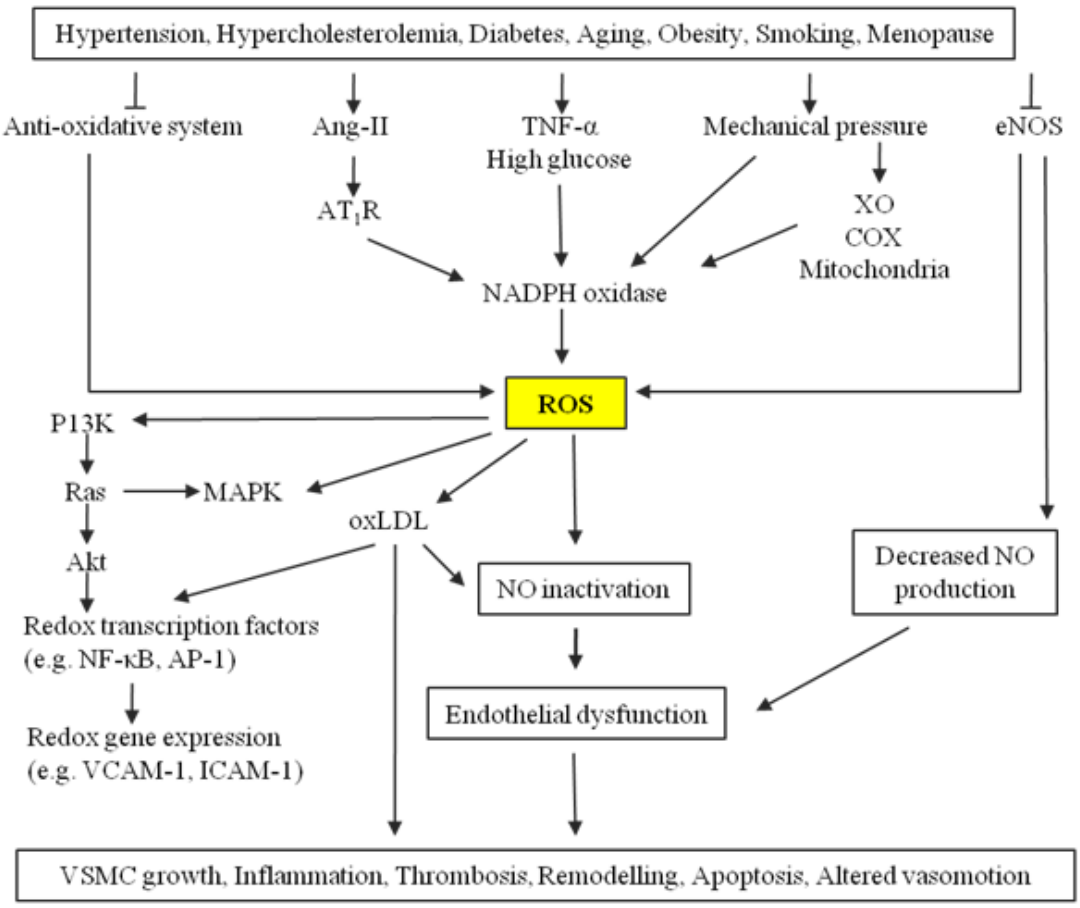


Figure 1.5. Main mechanisms contributing to ROS-related endothelial dysfunction and complications in cardiovascular diseases. Adapted[25].

Oxidative stress has been identified as a key determinant of endothelial dysfunction associated with cardiovascular disease[17, 19, 22, 25]. It has been linked, among others to 1) loss of NO bioavailability at various levels (see I. General Introduction, pages 15-18), 2) increased amounts of oxidized LDL-cholesterol molecules (LDL-ox), which change the phenotype of macrophages into foam cells and contribute to artherosclerotic plaque formation, 3) accumulation of advanced glycation end-products (AGEs), which contribute to diabetic vasculopathy, 4) activation and aggregation of platelets causing thrombus formation, 5) increased activation of Ang-II implicated in hypertension and chronic renal failure. Clinical

interventions to reverse oxidative stress and concomitant endothelial dysfunction are based in majority on lifestyle modification including exercise and diet change, as well as pharmacological therapy using anti-oxidant or lipid-lowering agents (e.g. statins), blockade of platelet activation and aggregation (e.g. aspirin) and inhibition of Ang-II (e.g. ACE inhibitors)[19].

1.2.2 NO bioavailability

NO is synthesized endogenously mainly from L-arginine in a complex reaction catalyzed by different members of the same family of enzymes called NO synthases (NOS). All NOS are heme- and flavin-containing enzymes that use NADPH, BH_4 and O_2 to convert L-arginine to L-cirtulline with concomitant release of NO[26]. NO is involved in fundamental processes of the cardiovascular system, such as vasodilation, neurotransmission, inflammation, thrombus formation and angiogenesis (see I. General Introduction, pages 22-24). Endothelial NOS (eNOS) is the main source of vascular NO and becomes uncoupled in the limited concentrations of essential co-factors (FAD, FMN, NADPH and BH_4) or the substrate L-arginine as demonstrated in diabetes, hypercholesterolaemia and other vascular diseases[26]. Uncoupling of eNOS switches off its oxygenase function producing NO and activates its reductase function producing $O_2^{\bullet-}$ and H_2O_2, which aggravates oxidative stress and endothelial dysfunction by lowering synthesis of NO and increasing production of ROS at the same time[26]. Oxidative stress also results in reduction of BH_4 and accumulation of asymmetric dimethylarginine (ADMA), a competitive inhibitor of eNOS, which has been identified as an independent risk factor and possible marker in patients with CAD[27]. eNOS gene expression and enzymatic activity may also be

negatively affected by altered eNOS phosporylation mediated by the phosphatidyl-inositol-3-kinase(PI3K)/ Akt pathway, dimerization, and intracellular localization to caveolae[25, 26].

Physiological levels of NO can also be dramatically reduced by its reaction with $O_2^{\bullet-}$, which leads to the formation of $ONOO^-$. $ONOO^-$ is a major mediator of the pathological effects associated with NO, particularly under conditions of severe oxidative stress and advanced disease (e.g. highly developed atherosclerosis or diabetes, sepsis), when the production of both $O_2^{\bullet-}$ and NO are augmented[26]. $ONOO^-$ like other reactive oxygen/nitrogen species (ROS/RNS) mediate the deleterious oxidation and nitration of proteins, lipids, or DNA and contribute substantially to oxidative stress and endothelial dysfunction[26] (Figure 1.6).

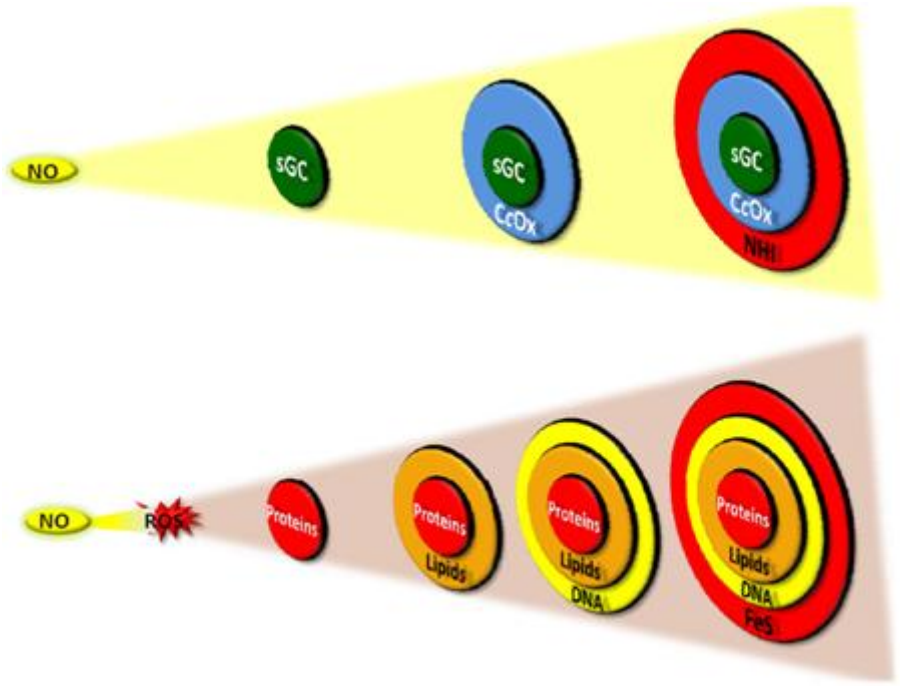


Figure 1.6. Cellular targets of NO and associated nitrogen oxides. The most sensitive target of NO is sGC, followed by cytochrome c oxidase (C*c*Ox) and non-heme iron (NHI; at the top). Reaction of NO with ROS changes target susceptibility (at the bottom). Under conditions of increased free radical production, NO forms oxidation products that react with proteins, lipids, DNA, and FeS centers predominantly over the targets shown at the top. Adapted[24]

Reduced NO availability as a result of decreased synthesis (by uncoupled or reduced eNOS regulation) or increased degradation (by reaction with $O_2^{\bullet-}$) is a hallmark of endothelium dysfunction[17, 25]. While some of the available therapies aimed at increasing NO bioavailability involve restoring the oxygenase function of eNOS by replenishing limited BH_4 factor and/or L-arginine, others focus on reducing the oxidative stress caused by $O_2^{\bullet-}$ and $ONOO^-$, which alters NO production, amount and function of its downstream receivers[28]. For example, high concentrations of vitamin C have been shown to restore NO-dependent responses in ApoE-deficient mice and in CAD patients due to its anti-oxidant properties to reduce oxidation of

BH_4[28]. Moreover, inhibition of upregulated vascular enzymes generating $O_2^{\bullet-}$ such as CYP450 improved endothelium-dependent NO-mediated vasodilation in CAD patients. Interestingly, a number of clinical trials have not confirmed the protective effects of vitamin E on atherosclerosis progression or major cardiovascular events[29]. The apparent lack of clinical usefulness of some anti-oxidant therapies is not clear. It has been suggested that because anti-oxidants, including vitamins, react with $O_2^{\bullet-}$ one billion times slower than NO, a reaction of $O_2^{\bullet-}$ with NO is thermodynamically preferred[29]. There are also studies indicating that vitamins do not penetrate into the vascular wall sufficiently, hence they may reach therapeutic levels in the plasma but not in tissues. Therefore, not ROS but the enzymes contributing to their upregulation have become more interesting targets for reducing oxidative stress and improving NO bioavailability.

1.3 Measurement of endothelial function

Assessment of endothelial function is a useful diagnostic and prognostic tool in the setting of cardiovascular risk. Since the endothelium carries out a great variety of functions, including control of vasomotor tone, coagulation of blood and inflammation, a number of biochemical markers have been used as indicators of endothelial dysfunction, such as selectins, ILs, ICAM-1, VCAM-1, C-reactive protein (CRP), TNF-α, interferon γ (IFN-γ), MCP-1, vWf, t-PA, PAI-1, microparticles, circulating endothelial cells etc. (Table 4).

Markers	Effects
Oxidative stress: TAC of plasma, PGF2α, ADMA, oxLDL, lipid peroxides, protein modifications (3-NT, 8-OHdG)	Inactivates NO, damages lipids, proteins and DNA, upregulates inflammatory and coagulation responses
NO pathway: ADMA, impaired FMD or vascular response to acetylcholine, low levels of NO metabolites	Limits NO bioavailability
Circulating endothelial cells	Marker for endothelial injury
Microparticles	Vesicles formed by cell membranes after endothelial activation
Angiogenic growth factors: VEGF, PlGF	Induce permeability, migration and angiogenesis
Cytokines: ILs, TNFα, MCP-1	Stimulate more ROS production and upregulation of inflammatory genes
Inflammation markers: vWF, E-selectin, VCAM-1, ICAM-1	Reflect extent of endothelial cell activation and damage
Platelet activation and aggregation: P-selectin, TXA_2/TXB_2	Cause thrombus formation at injured sites
Fibrynolytic pathway: PAI-1, tPA	Associated with endothelial injury

Table 4. Biomarkers reflecting endothelial dysfunction (injury). Only examples are presented. Abbreviations are explained in text. Adapted[23, 30, 31]

1.3.1 Oxidative stress

In terms of measuring oxidative stress, methods range from direct detection of ROS species with various electrodes, to stabilization of ROS using spin traps, or their effect on fluorescent probes, through measurement of oxidation (oxLDL, 8-OHdG, lipid peroxides) or nitrosation [(3-nitrotyrosine (3-NT)] end-products, and downstream markers of oxidation activity (ADMA, $PGF_{2\alpha}$, AGEs). ROS are extremely unstable and reactive molecules, which make the specific quantification of certain species ($O_2^{\cdot-}$, $\cdot OH$, H_2O_2, $ONOO^-$) in biological systems a real challenge. Furthermore, because different ROS have distinct half-lives, diffusion rates and rates

of reaction with other molecules, one factor controlling the outcome of ROS may be their site of production. Therefore, an observation of upregulated ROS is indicative only and should be accompanied by other measures, including relevant source enzymes (e.g NADPH, XO, uncoupled eNOS) and effectors of the oxidative signal.

An alternative measure of oxidative stress, which can be used in plasma, is to record the plasma total anti-oxidant capacity (TAC), also known as oxygen radical absorbance capacity (ORAC)[32, 33]. This method instead of measuring amount or outcome of ROS, provides information on how efficient different anti-oxidant defenses of various components of blood are in providing protection against ROS attack. Although TAC/ORAC is only the net effect of many different compounds and systemic interactions, it gives more biologically relevant information than that obtained from measuring individual anti-oxidants. It should be noted that results from TAC/ORAC can be interpreted in different ways depending on the time and level of oxidative stress. For example, the *in vivo* increase of oxidative stress in plasma could be due to a temporal imbalance of anti-oxidant responses, thus will be reflected as a reduction in TAC. However, a prolonged exposure to oxidative stress may lead to the chronic adaptation and upregulation of anti-oxidant defenses *in vivo*, which will be reflected by an increased TAC in this setting.

1.3.2 NO bioavailability

NO bioavailability can be determined by measuring the vasomotor response to physiological or pharmacological stimuli. Quantitative coronary angiography after intra-arterial infusion of acetylcholine is a widely used, although a quite invasive approach *in vivo*. Non-invasive measurements of flow-mediated dilation (FMD) of the

brachial artery by imaging with high-resolution ultrasound are more promising methods for wide application in humans. These also exhibit certain limitations due to significant methodological variation among operators and differences in baseline diameters/values of various individuals. *In vitro,* endothelial function may be assessed on isolated vessels, where isometric tension is recorded on a myograph for their responses to endogenous or exogenous NO donors.

Laboratory methods to determine NO bioavailability are based on the actual quantification of NO. Free NO is extremely difficult to measure due to its unstable nature and high reactivity with other molecules. There have been several methodologies developed using NO electrodes, NO spin traps [in conjunction with electron paramagnetic resonance (EPR)], or NO fluorescent probes allowing the capture and measurement of NO in real time or accumulated over time. However, all these techniques suffer from insufficient specificity of detection [e.g. diaminofluorescein (DAF fluorescent probe)] or inadequate sensitivity, which become particularly problematic in complex biological samples such as plasma where actual levels are typically around the detection limit. Another approach is to measure more stable metabolites of NO namely nitrite, nitrate and S-nitosothiols (RSNO). These are the primary oxidation metabolites of NO. However care must be taken in interpreting results since these are no longer perceived as being unnecessary bi-products of NO but have been shown to be potential ‘stores’ of NO that can circulate and can be re-cycled to exhibit biological function under certain conditions (see I. General Introduction, pages 25-28). NO metabolites can be measured using Griess reagent, HPLC or ozone-based chemiluminescence (OBC). While the Griess method has very poor sensitivity and HPLC involves laborious procedures, OBC is currently the most

specific and sensitive method to measure NO release from different NO metabolites with minimum processing of the samples required[34].

2. Nitric Oxide in the vasculature

NO is produced by a variety of mammalian cells, including endothelium, macrophages, neurons, cardiomyocytes, platelets, and fibroblasts. NO produced by the endothelium modulates vasomotor tone, inhibits platelet adhesion and aggregation, inhibits smooth muscle cell proliferation and governs vascular permeability. NO can also exhibit distinctly different and even opposing redox functions depending on the amount and microenvironmental conditions in which it is produced. Under physiological conditions at low concentrations of $O_2^{\bullet-}$, NO mediates anti-oxidant, anti-apoptotic and protective effects; it defends cells from oxidative stress by scavenging $O_2^{\bullet-}$, inhibits expression of adhesion molecules (VCAM-1, ICAM-1) and selectins (P-selectin, E-selectin), and inactivates Bad and caspase-9[26]. Conversely, excessive NO production in the presence of oxidative stress contributes and augments ROS-mediated tissue damage and lipid peroxidation by production of highly reactive $ONOO^-$ and direct inhibition of mitochondrial respiratory chain enzymes[26] (Figure 1.8).

2.1 NOS as a cellular source of NO

Different NOS isoforms: neuronal NOS (nNOS/NOS1), endothelial NOS (eNOS, NOS3) and inducible NOS (iNOS/NOS2) were named after the tissue in which they were first purified and cloned however, since then, they have been

identified elsewhere (Table 5). Both nNOS and eNOS are expressed constitutively, exhibit low basal activity and are stimulated by the increase in intracellular Ca^{2+} concentration and Ca^{2+}/calmodulin binding[26]. eNOS is highly expressed in endothelial cells and is a major source of plasma NO, generating NO in response to shear stress and other physiological stimuli. Furthermore, it has been shown that activity of eNOS can be regulated not only by Ca^{2+} levels, but also by numerous transcriptional and translational events affecting its mRNA stability, intracellular translocation and phosphorylation[26]. iNOS, on the other hand, is tightly bound to Ca^{2+}/calmodulin complex and does not depend on intracellular Ca^{2+} concentration. It is primarily expressed in response to immunological stimuli, is mainly regulated at the expressional level and generates large amounts of NO (100-1000-fold more NO than eNOS)[26]. The high amounts of NO produced by iNOS can have beneficial anti-microbial, anti-atherogenic and anti-apoptotic actions. However, aberrant iNOS induction has been associated with numerous human diseases such as asthma, arthritis, neurodegenerative diseases, tumor development or septic shock. Furthermore, a certain pattern of increased iNOS accompanied by reduced eNOS has been reported in atherosclerosis as well as response to ischaemia, hypercholesterolaemia and ROS[26].

In addition to the classical NOS-dependent production, NO can also be released from S-nitrosothiols and/or via reduction of nitrate and nitrite back to NO by mechanisms, which have not been fully elucidated (see I. General Introduction, pages 25-28).

NOS	Distribution	Regulation	Main functions
nNOS/ NOS1[35, 36]	Neurons Skeletal muscle Epithelial cells Endothelial cells Cardiomyocytes	Agonists of NMDA receptors Ca^{2+}/calmodulin mRNA↑ Phosphorylation	Neuroprotection and neurotransmission in NANC nerves ($nNOS^{-/-}$ -pain↓, memory↓) Regulation of cardiac function ($nNOS^{-/-}$, nNOS⊥ -cardiac contractlity↑) Peristalsis ($nNOS^{-/-}$-gastric dilation and stasis)
iNOS/ NOS2[26, 35, 37]	Most cell types in response to inflammation Pulmonary epithelial cells	Endotoxin and cytokines (IL-1, IL-2, TNF-α, IFN-γ) mRNA↑↑↑ (mostly transcriptionally regulated)	Regulation of immune response to pathogens ($iNOS^{-/-}$-leukocyte recruitment↑ in endotoxic shock) Angiogenesis ($iNOS^{-/-}$-tumors↑)
eNOS/ NOS3[26, 35]	Endothelial cells Cardiomyocytes Neurons, astrocytes Platelets Epithelial cells Skeletal muscle	Receptor-dependent agonists (thrombin, ADP, BK, substance P, His, Ach, VEGF) Ca^{2+}/calmodulin mRNA↑ (shear stress, VEGF, chronic exercise, TGFβ, statins, oestrogens) mRNA↓ (TNF-α, LPS) Phosporylation Intracellular trafficking (meristoylation and palmitoylation, interaction with caveolin-1 and -3, hsp90, CHIP etc.)	Vascular smooth muscle relaxation (eNOS⊥, $eNOS^{-/-}$-blood pressure↑) Inhibition of platelet aggregation (eNOS⊥-bleeding time↓) Regulation of vascular perme-ability ($eNOS^{-/-}$, eNOS⊥ -leukocyte recruitment↑) Inhibition of vascular SMC proliferation ($eNOS^{-/-}$-hyperplasia↑) Angiogenesis ($eNOS^{-/-}$-wound healing↓) Cardiac contractility (Ca^{2+} -spark frequency)

Table 5. Summary of NOS characteristics including their tissue distribution, regulation and main functions. The different roles of particular NOS isoforms are well documented in the literature and selected examples are listed in brackets. For a more in-depth view, the reader is directed to the citations. It should be noted that some NOS inhibitors may block more than one isoform and that gene knockout therapy may potentially upregulate the compensatory pathways, which altogether may confound some of the findings. Symbols: ⊥ - isoform specific inhibition, -/- -knockout mice, ↑ - enhanced, ↓ -reduced.

2.2 Inorganic nitrate and nitrite as alternative sources of NO

Nitrite (NO_2^-) and nitrate (NO_3^-) are the primary oxidation products of NO. Nitrate in the diet contributes ~70% of nitrate levels in plasma in humans. At normal O_2 levels, NO produced from eNOS and other sources is oxidized to NO_2^- and then to NO_3^- through a series of reactions and various NO metabolites intermediates (equations below).

$$2NO + O_2 \rightarrow 2NO_2$$
$$NO_2 + NO \rightarrow N_2O_3$$
$$N_2O_3 + H_2O \rightarrow 2NO_2^- + 2H^+$$
$$2NO_2 + H_2O \rightarrow NO_2^- + NO_3^- + 2H^+$$

However, under low O_2 conditions NO_2^- can be reduced back to NO thanks to enhanced activity of 'nitrite reductases', which act as oxygen sensors[38, 39]. These enzymes include among others deoxyhaemoglobin, xanthine oxidoreductase (XO), aldehyde oxidase (AO), eNOS, cytochrome P450 and the mitochondrial transport chain. The ability of NO_2^- to serve as an alternative source of NO in ischemic/hypoxic conditions has been appreciated in human physiology relatively recently. NO_2^- administration has been shown to vasodilate hypoxic tissue regions[40-42], reduce blood pressure[43] and provide cytoprotection in ischaemia/reperfusion injury[40, 44-46].

Lately, NO_3^- has been discovered to be another alternative source of NO via reduction to NO_2^- by nitrate reductase-containing bacteria residing in human saliva[47]. When nitrate rich food (green leafy vegetables, beetroot) is ingested, NO_3^- is reduced to NO_2^- over a few hours via the entero-salivary circulation, which is reflected by an increase in plasma levels of both metabolites[48-50]. Dietary supplementation of NO_3^-

and the subsequent increase in NO_2^- has been shown to provide vasodilation and reduction in blood pressure[48, 50, 51], inhibition of platelet aggregation[50, 51], protection against ischaemia/reperfusion injury[50] and reduction of oxygen cost in exercise[52-54].

Therefore, both NO_2^- and NO_3^- have a great potential in therapeutic treatment of cardiovascular diseases associated with endothelial dysfunction, reduced NO bioavailability and regional ischaemia as well as an improvement of exercise performance[50, 55, 56]. Current therapy of CAD, angina and heart failure involves application of organic nitrates and is limited to short-term treatment due to nitrate tolerance, increased oxidative stress and endothelial dysfunction, which occurs in chronic use[57]. Additionally, the efficacy of NO donors including organic nitrates depends on a healthy endothelium which is usually disturbed in cardiovascular diseases, which suggests that therapy with NO_2^- and/or NO_3^- might be a better alternative[46].

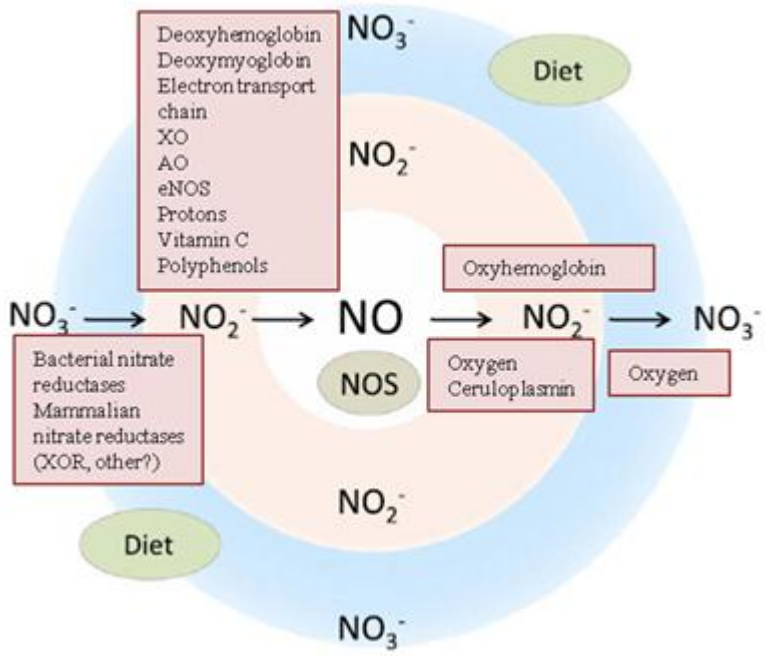


Figure 1.7. Proposed NO_3^--NO_2^--NO pathway. Adapted[58].

Although nitrite and dietary nitrate has demonstrated to have a range of beneficial vascular effects, there are still certain issues that remain to be explained and investigated. First of all, the interindividual variability in baseline plasma nitrite and nitrate seem to have an impact on the level of augmentation of these metabolites in plasma and the magnitude of vascular changes in response to the dietary nitrate. For example, dietary nitrate did not enhance running performance in elite cross-country skiers although earlier studies have reported improved exercise performance in healthy adults[59]. The elite athletes had lower baseline nitrite than normal individuals and the nitrite augmentation after dietary nitrate ingestion was also respectively smaller. In another study, dietary nitrate reduced blood pressure in males, which had lower baseline nitrite concentration but had no influence on females with higher baseline plasma concentrations[51]. Although these observations need confirmation, they suggest that the efficiency of NO_3^- and/or NO_2^- treatment may depend on initial plasma levels of these metabolites.

Another issue of concern are effects of nitrite beyond its reduction to NO, which may influence the vasculature and contribute to the observed NO-like effects. Alternative pathways of nitrite activity include the formation of nitrosothiols and nitrated fatty acids, as well as direct vasodilation via COX-mediated pathways[42, 46, 55, 60]. Although the majority of nitrite vascular effects are thought to be mediated by NO, the exact mechanism is not well defined. The difficulty in identifying a specific signalling pathway perhaps comes from a rapid redistribution of NO_2^- within different compartments of blood and tissues. For example, a low-dose infusion of nitrite has been shown to induce a prolonged vasodilator effect in the pulmonary vasculature even after plasma NO_2^- levels returned back to baseline[40].

The long-term effects of dietary nitrate supplementation have to be investigated in man in order to elucidate the implied interaction with other nutrients (e.g. polyphenols) and possible cancerogenic effect[55].

2.3 S-nitrosothiols as alternative sources and effectors of NO

S-nitrosothiols (RSNOs) are derivatives and storage forms of NO, where a nitrosonium moiety (NO^+) is covalently bound to the sulfhydryl group (SH) of proteins or low-molecular-mass thiols. NO^+ is a by-product of NO metabolism in blood (equation 1 below) and can also be formed from nitrite in acidic pH (equation 2 below). NO^+ reacts with SH groups forming RSNO (equation 3 below).

$$\begin{aligned} &2NO + O_2 \rightarrow 2NO_2 \rightleftarrows N_2O_4 \\ &NO + NO_2 \rightleftarrows N_2O_3 \\ &N_2O_3 + 2H^+ \rightleftarrows 2NO^+ + H_2O \end{aligned} \quad (1)$$

$$NO_2^- + 2H^+ \rightleftarrows NO^+ + H_2O \quad (2)$$

$$RSH + NO^+ \rightarrow RSNO + H^+ \quad (3)$$

RSNOs possess many of the biological activities of NO itself such as relaxation of smooth muscle, inhibition of platelet aggregation or regulation of cellular redox, apoptotic and inflammatory responses[61-64]. RSNOs execute these functions via two main mechanisms: 1) they can act as special NO donors providing protection and storage of the NO^+ group, which can be transferred to other RSNOs (transnitrosation) and released only when cleaved ; 2) they can induce post-translational modifications of cysteine residues on proteins (S-nitrosylation) and

therefore, contribute to the regulation of their function and signal transduction[65-67]. RSNOs are stable in acidic or alkaline conditions but can be cleaved very efficiently by transition metals (e.g. Cu, Hg), and even ascorbate. This property of RSNOs is used for their detection by 2 C's (mixture of CuCl and Cys) or $HgCl_2$ reagents (see III. General Methods, page 88).

The *in vivo* reduction mechanism of RSNOs back to NO (denitrosylation) is still the matter of some debate[65], although in SNO-proteins S-nitrosoglutathione reductase (GSNOR) and thioredoxin (Trx) were reported to play an important role[68]; GSNOR is not only required for physiological denitrosylation of GSNO but also governs S-nitrosylation of other proteins by influencing the cellular equilibrium between protein S-nitrosothiols and GSNO.

Over 100 proteins have been identified to be S-nitrosated in human and other mammal pathophysiologies, including those affecting NO bioavailability and function[68, 69]. For example, it has been shown that S-nitrosylation activates ariginase and inhibits eNOS and eNOS regulating proteins (i.e. HSP90), as well as inhibits the main target for NO, sGC, which taken together has detrimental effects on NO production and signalling. On the other hand, inhibitory S-nitrosylation of NADPH and PDE5 help to preserve NO bioavailability by inhibiting production of $O_2^{\bullet-}$ and increase levels of one of the main NO second messengers, cGMP[68]. Furthermore S-nitrosylation of caspase3, N-ethylmaleimide-sensitive factor (NSF) and NF-κB were taken to explain the anti-apoptotic, anti-thrombotic and anti-inflammatory properties of NO[68]. Despite these recent discoveries, it is not yet clear how RSNOs and NO work in concert with each other and whether S-nitrosylation of proteins only reflects NO activity or on its own is truly a crucial mechanism in cell signalling. Nevertheless, some of the exogenous RSNOs compounds (CysNO, GSNO, SNAC) have been used

as endothelial-independent NO donors in animal and human model systems (see I. General Introduction, pages 49-51).

It is important to distinguish between S-nitrosation and other thiol modifications, including S-glutathiolation and S-oxidation as different cysteine thiol adducts are regulated by different mechanisms depending on their microenvironments and the availability of reducing agents[70]. Interestingly, all thiol-mediated modifications of proteins are mediated by ROS/RNS and correlate with various stages of oxidative stress (Figure 1.8). Hence, not only S-nitrosation but also S-glutathiolation and S-oxidation have been implicated in both redox signalling and oxidative damage[70].

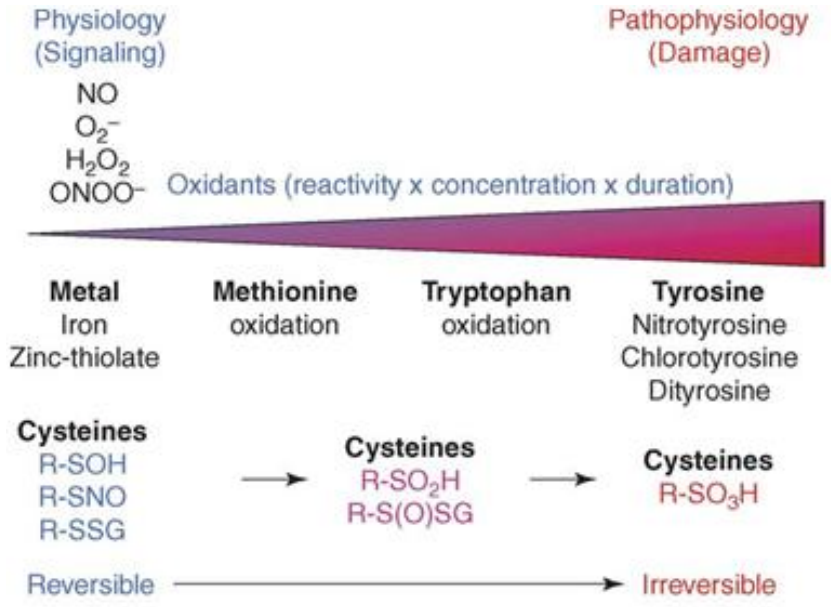


Figure 1.8. Influence of ROS/RNS on post-translational oxidative modification of thiols in pathophysiology. Adapted[70].

3. Smooth muscle contraction and relaxation

The middle layer of arteries, arterioles, venules and veins is composed mainly of vascular smooth muscle cells (SMC). SMC are wrapped around vessels so that their contractile tension (tone) regulates the vessel diameter[1].

3.1 The regulation of myosin light chain

The contractile state of smooth muscle is regulated by phosphorylation and activity of the myosin light chain (MLC)[71]. Phosphorylation of MLC is mediated by MLC kinase (MLCK) and leads to the stimulation of the myosin ATPase activity and smooth muscle contraction. MLC phosphatase (MLCP) reverses this process by catalyzing the dephosphorylation of MLC. The balance between MLC kinase and phosphatase activities is a critical determinant in the contractile status of smooth muscle and it can be affected by (i) Ca^{2+}, including the changing levels of intracellular calcium $[Ca^{2+}]_i$ and Ca^{2+} sensitization, (ii) second messengers: cAMP, cGMP, inositol 1,4,5-trisphosphate (IP_3), diacylglycerol (DAG) and/or protein kinases (i.e. PKA, PKG, PKC, rhoA kinase).

Upon elevation of $[Ca^{2+}]_i$ and increased formation of Ca^{2+}/Camodulin complex (Ca/CaM), MLCK binds Ca/CaM and is activated, which ultimately results in smooth muscle contraction. When $[Ca^{2+}]_i$ is lowered, the MLCK is less active, thereby favouring smooth muscle relaxation. Additionally, Ca/CaM regulates activities of other enzymes such as Ca^{2+}-dependent kinases, which influences the vasomotor tone by stimulation of the downstream signalling pathways or eNOS in endothelium, which catalyse production of NO and mediates endothelium-dependent relaxation.

Second messengers such as cAMP and cGMP change the MLC kinase/MLC phosphatase balance by activation of their relative kinases: PKA/PKG, which elicit multiple phosphorylations of cellular proteins that result in lowering of $[Ca^{2+}]_i$ (see I. General Introduction, pages 43-45). Furthermore, the vascular tone can be regulated through Ca^{2+}-sensitization, due to the inhibition of MLC phosphatase by rhoA kinase and PKC. Thanks to the phenomenon of Ca^{2+}-sensitization, the contraction of SMC is well maintained despite the substantial fall in $[Ca^{2+}]_i$, when Ca^{2+} is pumped back into sarcoplasmic reticulum (SR). Some protein kinases such as PKG can activate MLC phosphatase directly via inhibition of the inactivating RhoA pathway, ultimately resulting in dephosphorylation of the vasoconstricting MLC and relaxation of smooth muscle[1, 72].

3.2 Modulation of intracellular calcium (ion channels)

The process of smooth muscle contraction and relaxation is modulated by the intracellular level of calcium $[Ca^{2+}]_i$, and the sensitivity of contractile proteins to $[Ca^{2+}]_i$ in response to various stimuli. When calcium signalling is stimulated in a cell, Ca^{2+} enters the cytoplasm from one of two general sources: (i) it is released from intracellular stores, mainly from sarcoplasmic reticulum (SR) or (ii) it enters a cell from the extracellular space.

In the vascular smooth muscle cell membrane, Ca^{2+} influx is regulated by L-type voltage-operated Ca^{2+} channels (VOCC), receptor-operated Ca^{2+} channels (ROCC), and store-operated Ca^{2+} channels (SOCC)[73-75].

VOCC act as main Ca^{2+} channels in the vascular smooth muscle cell membrane and like other voltage-gated channels respond to changes in the membrane

potential. The resting membrane potential in artery ranges from -50 mV to -60 mV and is regulated by various members of K^+ channels. This negative membrane potential allows VOCC to be slightly open and provides sufficient Ca^{2+} influx that sustains the basal tone of the vessel. The opening of VOCC increases significantly with a depolarization (more positive potentials caused by inhibition and closure of K^+ channels), leading to Ca^{2+} influx and contraction. Conversely, a hyperpolarization (more negative potentials caused by activation and opening of K^+ channels) closes VOCC, decreases the $[Ca^{2+}]_i$, and causes relaxation.

ROCC mediate signals without the necessity of a change in membrane potential. They are non-selective cation channels typically activated by inositol lipid signalling, which is initiated by the activation of G protein-coupled phospholipase C (PLC). PLC catalyzes the cleavage of phosphatidylinositol 4,5-biphosphate (PIP_2) to form two second messengers IP_3 and DAG. IP_3 diffuses away from the membrane and binds to specific IP_3 receptors (IP_3R) on SR to allow Ca^{2+} release from the intracellular stores. Apart from IP_3R, ryanodine receptors (RyR) also mediate Ca^{2+} release from SR[1]. DAG remains in the plasma membrane where it activates PKC, which contributes to Ca^{2+}-sensitization.

SOCC mediates store-operated Ca^{2+} entry and thus plays a critical role to refill Ca^{2+} in SR and to maintain Ca^{2+} homeostasis. The molecular identity of SOCC and the mechanism linking SR depletion to the SOC channels are not clear. However it is believed that SOCC are composed of transient receptor potential (TRP) proteins and their activation can be independent of IP_3 production[75-77].

Ca^{2+} efflux to the extracellular space is regulated by other families of proteins: the plasma membrane Ca^{2+} ATPase (PMCA) and the Na^+/Ca^{2+} exchanger (NCX)[78, 79]. The NCX is a large-capacity, low-affinity carrier, which is mostly found in excitable

cells, where it ejects the bulk amounts of Ca^{2+}. The PMCA is a lower capacity system and has a high affinity for Ca^{2+} even at the very low $[Ca^{2+}]_i$, thereby maintaining Ca^{2+} at its normally very low levels in resting cells. Uptake into the SR is mediated by the sarco- and endoplasmic reticulum Ca^{2+} ATPase family (SERCA)[80].

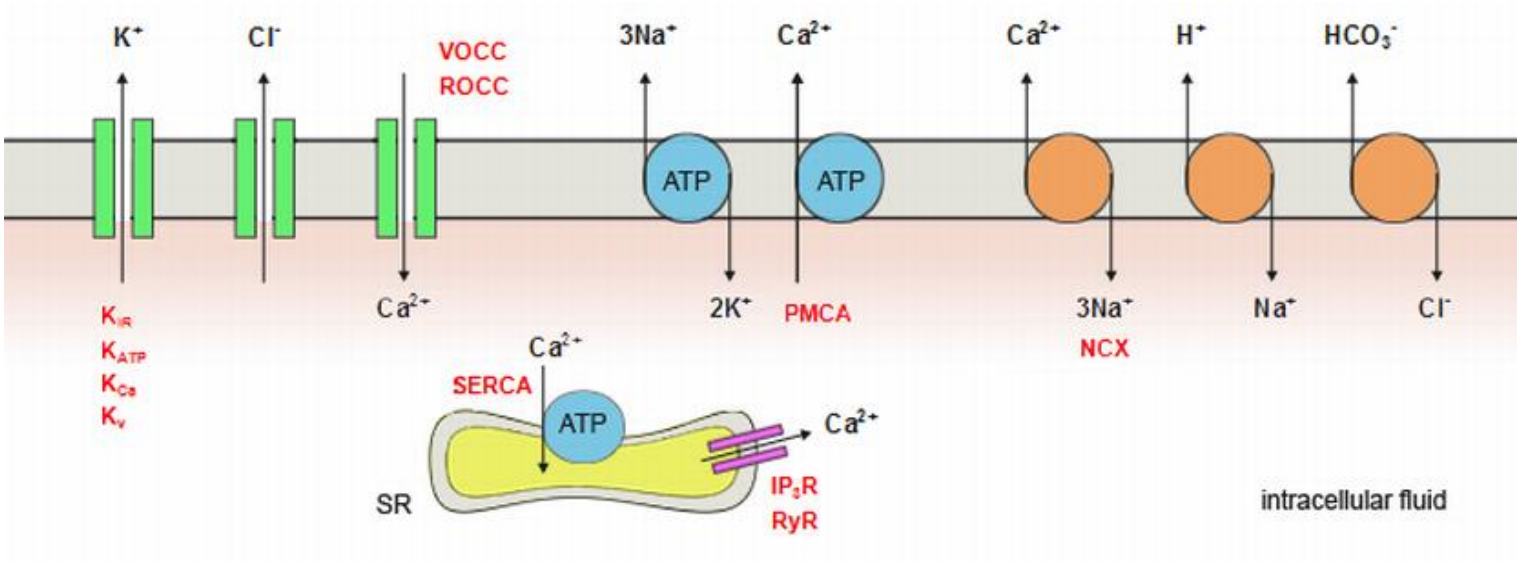


Figure 1.9. Principal ion channels (green), ion pumps (blue) and exchangers (orange) in the cellular membrane and sarcoplasmic reticulum (SR) of vascular cells. K^+ channels generate while Cl^- and Ca^{2+} channels modulate the negative membrane potentials. VOCC and ROCC mediate contraction by transmitting extracellular Ca^{2+} into the cell and IP_3R and RyR channels contribute to high levels of $[Ca^{2+}]_i$ by emptying SR stores of Ca^{2+}. Na^+-H^+ exchanger fight intracellular acidosis during sustained contraction. The Ca^{2+} ATPase (PMCA) and the Na^+/Ca^{2+} exchanger mediate relaxation by transmitting $[Ca^{2+}]_i$ outside the cell. SERCA pumps refill SR stores with Ca^{2+}. Na^+-K^+-ATPase pumps and HCO_3-Cl^- exchangers maintain the respective high intracellular K^+ and Cl^- concentrations. Adapted[1].

As discussed above, ion channels provide the major source of $[Ca^{2+}]_i$ that activates contractile proteins in SMC. On the contrary, the rise in $[Ca^{2+}]_i$ in EC stimulates the synthesis and release of vasoactive substances: NO, PGI_2 and endothelium-derived hyperpolarization factor (EDHF) which all induce endothelium-dependent vasorelaxation via various mechanisms (see I. General Introduction, pages 43-48). As in SMC, EC express K^+ channels, which contribute to the determination and regulation of endothelial membrane potential whereas they generally (in conduit arteries) lack voltage-gated Ca^{2+} channels[81]. Therefore, a major mode of Ca^{2+} entry to these cells is mediated by various types of ROCC and SOCC while Ca^{2+} efflux is based on the activity of PMCA and to lesser extent on NCX. Intracellular stores of Ca^{2+} in endothelial SR are emptied by IP_3R and RyR and refilled by SERCA[81].

3.3 Signalling via G-protein-coupled receptors

G proteins are signal transducers, attached to the cell surface plasma membrane, that connect receptors to effectors and thus to intracellular signalling pathways. GPCRs are seven-transmembrane receptors which communicate signals from a large number of (i) biogenic amines: noradrenaline, dopamine, 5-hydroxytryptamine (5-HT), histamine, acetylcholine; (ii) amino acid and ions: glutamate, calcium, gamma-aminobutyric acid (GABA); (iii) lipids: lysophosphatidic acid (LPA), sphingosine-1-phosphate (S1P), prostaglandins, leukotrienes; (iv) peptides and proteins: chemokines, angiotensin, thrombin, endothelin, bradykinin and (v) others: light, odorants, nucleotides[82, 83].

In the G protein's unactivated state, the G protein is bound to GDP and exists as a heterotrimer consisting of α, β, and γ subunits. Upon receptor activation, the G

protein exchanges GDP for GTP, causing the dissociation of the GTP-bound α and βγ subunits and triggering diverse signalling cascades. On the basis of sequence and signalling similarity, the Gα subunits have been divided into four families ($G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$, $G\alpha_{12/13}$) and this classification has served to define both receptor and effector coupling[82] (Table 6).

Main signal transduction pathways involving GPCRs include the cAMP signal pathway and the IP_3 signal pathway. Typically $G\alpha_s$ stimulates adenylyl cyclase and increases levels of cAMP, whereas $G\alpha_{i/o}$ inhibits adenylyl cyclase and lowers cAMP levels. Members of the $G\alpha_{q/11}$ family bind to and activate PLC, which cleaves PIP_2 into DAG and IP_3. Besides the regulation of these classical second-messenger generating systems, Gβγ subunits and Gα subunits such as $G\alpha_{12/13}$ and $G\alpha_{q/11}$ can also control activities of other intracellular signal-transducing molecules, including small GTP-binding proteins of the Ras and Rho families and members of the mitogen-activated protein kinases (MAPK) including extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK), p38 and ERK5[82, 84]. These proteins regulate various cellular activities, such as cell growth, differentiation and survival.

G protein	Receptors (examples)	Main signalling pathway
$G\alpha_s$	B-adrenergic Dopamine D1, D5 Adenosine A2 Serotonin 5-HT4, 6, 7 Glucagon receptors	AC↑→cAMP↑
$G\alpha_{i/o}$	α2-adrenergic Muscarinic M2, M4 Adenosine A1, A3 Serotonin 5-HT1, 5 Purinergic $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$	AC↓→cAMP↓
$G\alpha_{q/11}$	α1-adrenergic Muscarinic M1, M3, M5 Serotonin 5-HT2 Purinergic $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ PAR4 TP	PLC↑→IP_3↑, DAG↑
$G\alpha_{12/13}$	PAR1 TP	Rho↑→MAPK↑

Table 6. A recent classification of G-protein-coupled receptors (GPCR) based on the homology of Gα subunits. Adapted[85].

The final cellular response after the activation of particular GPCR depends on the specificity of agonist and the distribution of receptors. Some ligands with a broad specificity are able to activate more than one G protein. For example, epinephrine, a nonselective agonist of all adrenergic receptors, signals through (i) the β-adrenergic receptor coupled to $G\alpha_s$, causing an increased heart rate, (ii) the α1-adrenergic receptor to $G\alpha_{q/11}$, leading to vasoconstriction and (iii) the α2-adrenergic receptor to $G\alpha_{i/o}$, opening K^+ channels[86]. Despite the broad specificity of some agonists, their overall effect also depends on the tissue distribution of their receptors. For example, the activation of endothelial cell $G\alpha_{i/o}$–coupled M3 muscarinic receptors by acetylcholine results in a release of endothelium-derived relaxing factors, which relax vascular smooth muscle pre-contracted with norepinephrine. Removal of the

endothelium eliminates the relaxant effect and reveals contraction caused by direct action of acetylcholine on vascular smooth muscle mediated primarily by M3-muscarinic receptors coupled to $G\alpha_{q/11}$-proteins[87].

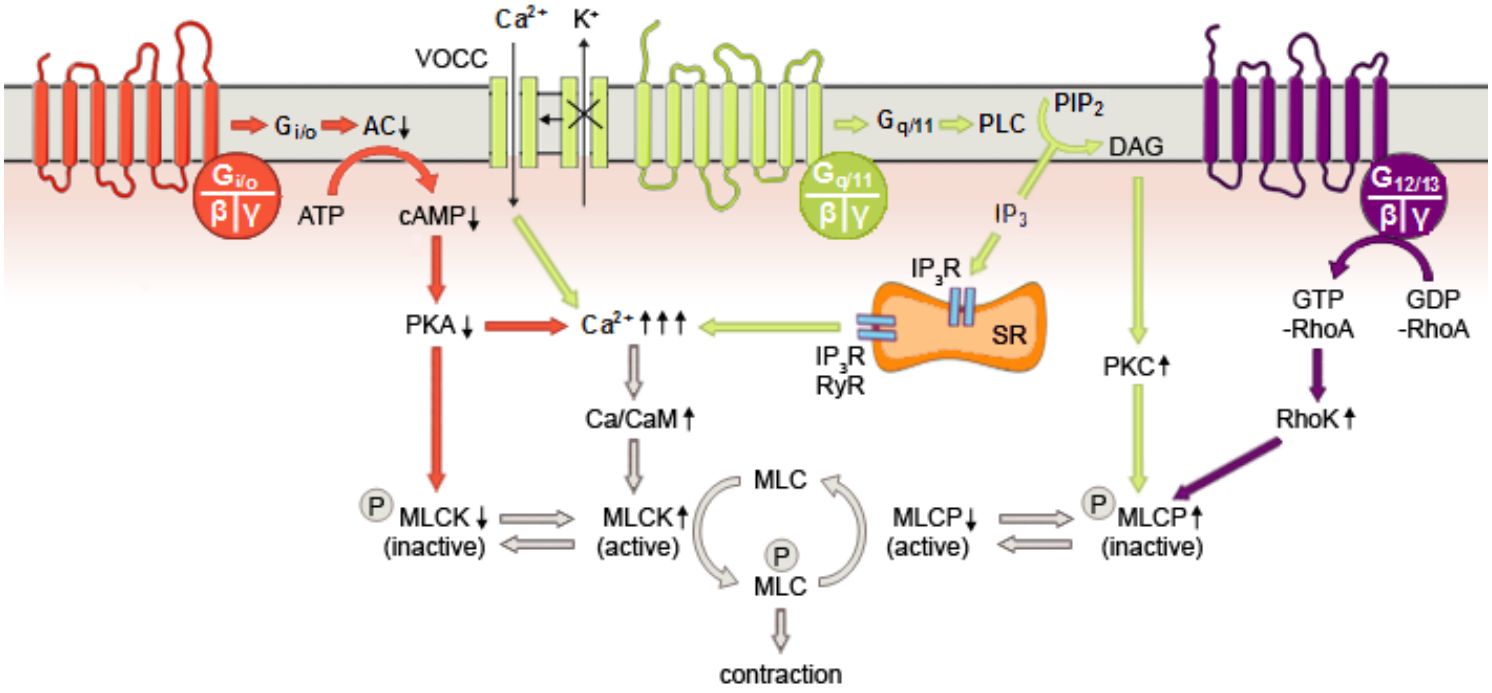


Figure 1.10. GPCR-mediated vasoconstriction of SMCs. The central part leading to constricting MLC lies in the raise of $[Ca^{2+}]_i$, which by binding to Cam (Ca-Cam), activates MLCK. Depending on which GPCR is activated by agonist, $[Ca^{2+}]_i$ can by increased by multiple mechanisms. In red- stimulation of $G\alpha_{i/o}$ subunit leads to the inhibition of AC/cAMP/PKA pathway, which evokes a rise in $[Ca^{2+}]_i$. In green- activation of $G\alpha_{q/11}$ triggers PLC/PIP_2/IP_3 cascade and release of stored Ca^{2+} in SR. Additionally, closing K^+ channels causes the membrane depolarization and opening of VOCC, which transmit the extracellular Ca^{2+} into cells. In purple- the sustained constriction is maintained by phosphorylation=inhibition of MLCP by PKC and/or RhoK (Ca^{2+} sensitisation).

3.4 Prostaglandins and vascular tone

Prostaglandins (PGs) are a major product of the arachidonic acid (AA) cascade generated by cyclooxygenase (COX) enzymes. AA is a plasma fatty acid derived from the cleavage of dietary linoleic and linolenic acids by phospholipase 2 (PLA_2). In endothelial cells, PLA_2 activation is a Ca^{2+}- dependent step. COX enzymes utilize AA to catalyze the formation of short-lived intermediates prostaglandin G2 and prostaglandin H2, which are then transformed to different prostaglandins: (i) PGD_2, PGE_2 and $PGF_{2\alpha}$ via PG isomerases, (ii) PGI_2 via PGI_2 synthase (PGIS) or (iii) TXA_2 via TXA_2 synthase (TXS)[88]. COX enzymes are present in two isoforms: COX-1 and COX-2 and they prefentially co-localise with particular isomerases/synthases to determine the final PG produced (Figure 1.11). Therefore, COX-1 usually couples with TXS, PGF isomerases and the cytosolic PGE_2 isomerase while COX-2 typically links to PGIS and the microsomal PGE isomerase[88]. Conventionally, COX-1 is regarded as a constitutive enzyme with house-keeping functions, whereas COX-2 is considered the inducible form with a particular role in inflammation. Indeed, it has been shown that COX-1 is constitutively expressed in endothelial and smooth muscle cells *in vitro*, while the expression of COX-2 is increased by growth factors, cytokines and LPS[89]. However, it is now apparent that COX-2 is the dominant source of PGI_2 produced even under physiological conditions and that both COX isoforms are upregulated in foam cells and in SMC in atherosclerotic plaque[89].

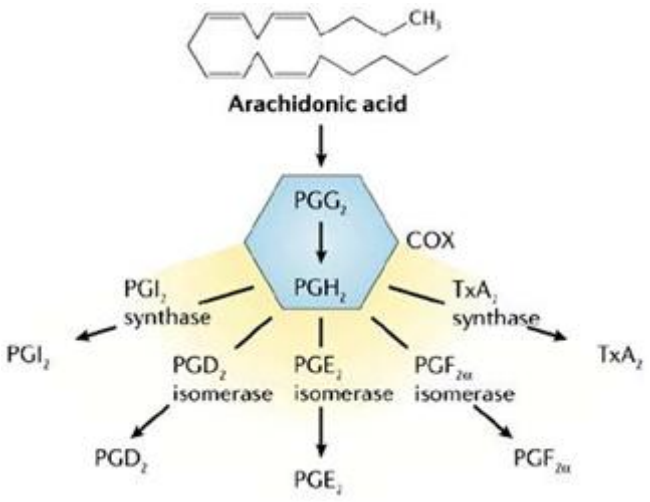


Figure 1.11. COX pathway. COX enzymes metabolize arachidonic acid (AA) to PGI_2, PGD_2, PGE_2, $PGF_{2\alpha}$ and TXA_2 via tissue specific isomerases. Adapted[89].

While PGI_2 is a main vasodilator (see I. General Introduction, page 45), TXA_2 is a major vasoconstrictor of the COX pathway, acting via the TP receptor and the classical $G\alpha_{q/11}$-dependent signalling (see I. General Introduction, pages 36-41). TXA_2 is mostly known as a main metabolite of COX-1 activity in platelets, stimulating activation of new platelets and increasing platelet aggregation (see I. General Introduction, pages 51-54). However, it has been shown that either COX isoform may generate TXA_2 and TXS mRNA can also be found in tissues other than platelets[88].

Other prominent eicosanoids such as PGE_2, PGD_2 and $PGF_{2\alpha}$ may also contribute to a lesser extent to the regulation of vascular tone[88]. PGE_2, like PGI_2 is a dominant product of COX-2 under physiological conditions *in vivo*. PGE_2 may act via four receptor subtypes: G_q-coupled EP1 and EP3, causing increase of $[Ca^{2+}]_i$ in SMC and vasoconstriction and/or G_s-coupled EP2 and EP4, leading to decrease of $[Ca^{2+}]_i$ in SMC and vasodilatation (see I. General Introduction, pages 36-41). The contrasting biological effects of these receptors come from their varied tissue distribution, as well as different concentrations of PGE_2. PGD_2 is produced largely by activated mast cells

and causes vasodilatation while $PGF_{2\alpha}$ is a vasoconstrictor and its enhanced formation has been associated with oxidative stress and cardiovascular diseases (see I. General Introduction, page 19).

3.5 Endothelium-dependent vasorelaxation

Vascular smooth muscle relaxation (vasorelaxation, vasodilation) is caused by either removal/inhibition of the contractile stimulus or by direct action of an agonist that enhances the relaxation mechanism. Depending on the origin of this agonist, the relaxation of SMC is dependent or independent on the endothelium. Regardless of the stimulus, decreased $[Ca^{2+}]_i$ in SMC and increased MLC phosphatase activity is a pre-requisite for vasorelaxation (see I. General Introduction, page 31-32).

Endothelium-dependent vasorelaxation is mediated primarily by three different mediators: NO, PGI_2 and EDHF released by endothelium which then diffuses to smooth muscle.

3.5.1 NO and vascular tone

The main receptor for NO is an enzyme sGC, which catalyses the conversion of guanosine triphosphate (GTP) to the second messanger cGMP. NO-induced activation of sGC depends on the presence of a reduced Fe^{2+} haem moiety and results in a 200-fold increase of the activity of sGC[90]. Accumulation of cGMP leads primarily to the activation of the cGMP-dependent protein kinase I (PKGI), and also cGMP-gated channels, cyclic nucleotide phosphodiesterases (PDEs) and cGMP-dependent protein kinase II (PKGII)[90]. Many targets of PKGI activity have been

identified including: (i) activation of K^+ channels, (ii) inhibition of L-type Ca^{2+} channels, (iii) activation of the Ca^{2+} Mg^{2+} ATPase and (iv) the Na^+/Ca^{2+} exchanger, (v) activation of SERCA, (vi) inhibition of the sarcoplasmic reticulum IP_3 receptor or the PLC-dependent formation of IP_3 and (vii) activation of the MLC phosphatase[90]. Generally speaking, the activation of NO/sGC/cGMP/PKG pathway in vascular cells initiates a cascade of phosphorylation reactions, which result in lowering the intracellular levels of Ca^{2+} and ultimately, in the relaxation of the smooth muscle cells. The level of cellular cGMP is determined largely by the balance between its synthesis by sGC and breakdown by PDEs, although the cGMP-gated channels may also play a role by exporting cGMP from some cells[90].

It should be acknowledged that NO may contribute independently of sGC and PKG to lower cytosolic Ca^{2+} levels via (i) direct S-nitrosation and thus activation of K^+ channels; (ii) reaction with superoxide ($O_2^{\cdot-}$) with the formation of peroxynitrite ($ONOO^-$), which can bind to glutathione (GSH) and activate SERCA and (iii) direct inhibition of CYP450 which can produce 20-HETE, an inhibitor of large conductance Ca^{2+}-dependent K^+ channel (BK_{Ca})[91]. Interestingly, cGMP-independent events contribute proportionately more to the overall vasorelaxation induced when comparing the effect of NO donors to that of endogenous NO[92]. Moreover, there is a differential involvement of cGMP-dependent and -independent vasodilation between various NO donors. For NO donors belonging to RSNO group, cGMP-independent effects might be explained by the particular metabolism of RSNO involving transnitrosation and S-nitrosylation of proteins[93] (see I. General Introduction, pages 28-30). For other NO donors, which generate NO spontaneously a possible explanation for the discrepancies between endogenous and exogenous NO, is that the site of NO generation has an influence on the proportion of vasorelaxation mediated

by cGMP-independent mechanisms. In other words, NO generated within the tissue has more ready access to sGC whereas NO delivered from outside the cell may more easily access non-sGC sites of action.

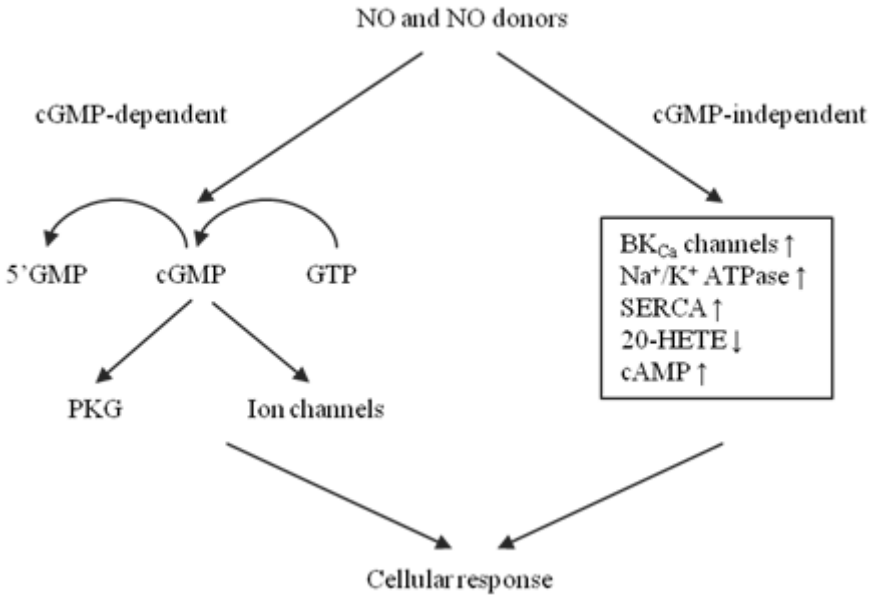


Figure 1.12. Cellular responses to NO and NO donors including cGMP-dependent and cGMP-independent mechanisms. Adapted[92].

3.5.2 PGI_2 and vascular tone

Vasorelaxation induced by PGI_2, the main vascular product of COX-2 activity, is determined by specific vascular smooth muscle cell receptors known as the isoprostenoid (IP) receptors. The IP receptor is a seven transmembrane receptor which couples primarily to G_s to activate AC and increase cAMP levels[88]. Accumulation of cAMP leads mainly to the activation of the cAMP-dependent protein kinases I and II (PKAI and PKAII) together with the GTP-exchange protein EPAC, the cAMP-gated ion channels and PDEs[90]. PKA phosphorylates many target proteins, including (i) K^+

channels, (ii) L-type Ca^{2+} channels, (iii) SERCA2, (iv) RyR, (v) MLCK and (vi) eNOS which lead eventually to the lowering of cytosolic Ca^{2+} levels and relaxation of SMC[94-96].

3.5.3 Crosstalk between NO and PGI_2 pathways and vascular tone

Some of the phosphorylation substrates of PKA are shared with PKG, making a link between the PGI_2/AC/cAMP/PKA and NO/sGC/cGMP/PKG systems. In fact, there are many well documented examples of cross-activation between these two signalling pathways, not only on the level of i) activated protein kinases, but also ii) between cAMP and cGMP catalytic sites of PDEs, or even iii) the expression and activity of COX and NOS enzymes themselves[90, 97, 98].

It has been shown that cGMP and cAMP compete for catalytic sites of certain PDEs, which are able to hydrolyze both cyclic nucleotides. For example, cAMP-dependent PDE 3 (PDE3), which normally accelerates the degradation of cAMP, can be inhibited by the modest elevation of cGMP[90]. In this way, cGMP can enhance the cAMP signalling. However, the much higher levels of cGMP can bind to the allosteric site and thus, act as an activator of another cAMP-dependent PDE i.e. PDE2 reducing the cAMP signalling[90]. On the other hand, the elevation of cAMP is unlikely to affect more cGMP-specific PDEs like PDE5, PDE6 or PDE9[90].

In recent years it has been suggested that NO and PGs may regulate their own biosynthesis and NOS/COX activity. NO proved to increase COX activity by different means, for example by (i) reaction with the superoxide, which otherwise inactivates COX, (ii) S-nitrosylation and activation of COX and iii) the regulation of NF-κB, which is a potent inducer of COX-2[12, 97, 99-102]. In addition, the potential role of an

iron-heme center of COX enzyme was also indicated[97, 102]. It is known that COX contains an iron-heme center at the active site and that NO interacts with iron containing enzymes leading to either a stimulation (in the case of sGC) or inhibition (e.g. aconitase) of the enzymatic activity.

In terms of vasodilation, the majority of studies reveal the compensatory role of NO towards the inhibited COX activity[103, 104]. For example, it was shown that *in vivo*, inhibition of COX using non steroidal anti-inflammatory drugs (NSAIDs) is without effect on basal blood pressure in healthy mice suggesting that NO compensates for the loss of vasodilatory PGs. However, when L-NAME was present together with NSAIDs, a significant enhancement of blood pressure and phenylephrine-induced constriction were found[103] (Figure 1.13). Similarly, aortic rings from $COX2^{-/-}$ mice constricted significantly more than WT mice when NO generation was blocked[103].

Accordingly, there are studies using $eNOS^{-/-}$ mice, showing that COX-2-derived PGs compensate for loss of NO in regulation of coronary hemodynamics and flow-induced dilatation *in vivo*. Furthermore, although L-NAME was shown to increase blood pressure in WT mice, when L-NAME was co-administered with NSAIDs, the resulting blood pressure elevation was much higher and more prolonged[103].

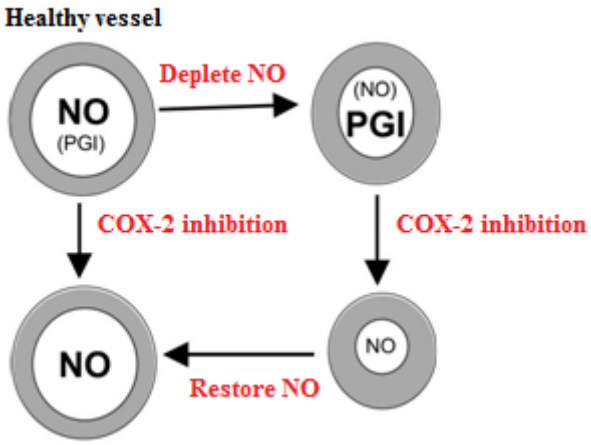


Figure 1.13. Crosstalk between NO and prostacyclin. Healthy large vessels maintain tone predominantly using NO. If NO is depleted, COX-2–derived prostanoids (e.g. PGI) compensate. Then, inhibition of COX-2 leads to constriction because PGI synthesis is blocked and may predispose to cardiovascular side effects. Repletion with NO prevents the increase in tone mediated by COX-2 blockade. Adapted[103].

3.5.4 EDHF and vascular tone

The precise nature of the EDHF has not been defined, but is broadly considered as a substance or a change in membrane potential generated by EC and transmitted to SMC via myoendothelial gap junctions[105]. Spreading to the smooth muscle cell, EDHF leads to the hyperpolarization of the membrane, and as a consequence, to the relaxation of SMC without the increase of intracellular concentration of cyclic nucleotides – cGMP or cAMP, which is characteristic of the classical vasodilators[106]. Hence, *in vitro* EDHF-dependent relaxation of vessels is defined as the residual relaxation in the presence of inhibitors for NO and PGI_2. However, this raises the question of whether EDHF exists when NO is present or whether it is purely a compensatory mechanism. The most popular candidates for EDHF include epoxyeicosatrienoic acids (EETs), potassium ions (K^+), and hydrogen

peroxide (H_2O_2). Although there is still a lot of controversy around the nature of EDHF, there is a common agreement that it is associated with the activation of two populations of endothelial Ca^{2+}- dependent potassium channels: the small-conductance K_{Ca} (SK_{Ca}) and the intermediate-conductance K_{Ca} (IK_{Ca}) potassium channels[107]. Endothelium-dependent hyperpolarization is more significant in the regulation of the vascular tone of small, resistance vessels (<300 μm), thereby playing a particularly important role in the microcirculation[108].

EDHF has been shown to accrue greater significance in terms of overall vasodilation as the size of the blood vessels decreases. In this sense it is inversely related to NO production by NOS. Thus in smaller vessels, EDHF is thought to be the main vasodilator, a factor that might be particularly important in diabetes with the associated pathologies of neuropathy and retinopathy.

3.6 Endothelium-independent vasorelaxation (NO donors)

Endothelium-independent vasorelaxation is mostly triggered by stimulation of $G\alpha_s$-dependent activation of the AC/cAMP/PKA pathway by an agonist (i.e. adenosine, histamine, adrenaline) or by delivery of exogenous NO with NO donors. Different NO donors have often different profiles of action depending on the mechanism of NO generation and delivery[65] (Table 7).

Class	Examples	Mechanism of NO generation
Organic nitrate	GTN ISDN	Tissue activation
Metal nitrosyl	sodium nitroprusside	Tissue activation
Syndonimine	SIN-1	Spontaneous generation of NO
NONOate amines	DEA/NO DETA/NO MAHMA/NO Sper/NO	Spontaneous generation of NO
S-nitrosothiols	CysNO GSNO SNAC SNAP CapSNO	NO generation is catalysed by Cu^{+} ions but S-nitrosothiols can also act via other mechanisms (see I. General Introduction, pages 28-30)

Table 7. Classification of different NO donors. Abbreviations: CapSNO (S-nitrosocaptopril), CysNO (*S*-nitrosocysteine), DEA/NO (2-(*N*,*N*-diethylamino)diazen-1-ium-1,2-diolate), DETA/NO (*Z*-1-[*N*-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate), GSNO (*S*-nitroso-glutathione), GTN (glyceryl trinitrate), ISDN (isorbide dinitrate), MAHMA/NO (*Z*-1-*N*-methyl-N-[6-(N-methylammoniohexyl) amino]diazen-1-ium-1,2-diolate), SIN-1 (3-morpholino-sydnonimine), SNAC (*S*-nitroso-N-acetylcysteine), SNAP (*S*-nitroso-N-acetyl-DL-penicillamine), Sper/NO (*Z*-1-{*N*-[3-aminopropyl]-*N*-{4-{3-aminopropylammonio)butyl]-amino} diazen-1-ium-1,2-diolate). Adapted[92].

The use of some NO donors has shown certain limitations or undesired side effects. For example, organic nitrates were successfully used in prevention and treatment of angina pectoris until it was discovered that patients develop tolerance to long-acting nitrates due to progressive decrease in availability of reduced thiol groups and/or increased vascular superoxide production. Another tissue-activated-NO donor sodium nitroprusside used in the treatment of hypertensive emergencies was associated with an accumulation of cyanide in the body. In turn, the liberation of NO from syndonimine was accompanied by concomitant release of superoxide. Furthermore, despite the fact that NONOate amines are very efficient in generation of

NO, they are also very short-lived NO donors and for that reason not always suitable in clinical practice. Probably the most promising class of NO donors with some additional therapeutic potential are S-nitrosothiols (RSNO)[109]. RSNOs mediate many of their biological activities of NO not only by donating a NO group but also by nitrosation and S-nitrosylation of proteins (ion channels, receptors, enzymes), thus bypassing the generation of free NO (see I. General Introduction, pages 28-30). Some RSNOs have been successfully used in clinical practice[110], including i) SNAC for prevention of contrast-induced nephropathy, treatment of paracetamol poisoning, and thinning of respiratory secretions; ii) CapSNO for the treatment of hypertension and heart failure and iii) SNAP for the treatment of rheumatoid arthritis. RSNO are less subject to neutralization of the NO moiety, and can therefore be considered capable of remote NO delivery. This was first demonstrated with arterial infusion of GSNO to result in downstream vasodilation.

4. Anti-platelet therapy

4.1 Pathophysiology of atherothrombosis

Platelets are anuclear blood cells that circulate in a human's cardiovascular system mostly in quiescent form thanks to i) the anti-coagulant and anti-thrombotic surface of endothelium, ii) secretion of NO by endothelium and platelets and PGI_2 by endothelium, iii) degradation, storage or separation of platelet agonists in endothelial Weibel-Pallade bodies and in platelet storage granules and iv) inhibition of the fibrynolytic system (see I. General Introduction, pages 5-7). During acute coronary syndromes (ACS) and percutaneous coronary interventions (PCI) vascular

endothelium is activated or damaged due to atherosclerotic plaque rupture or surgical procedures exposing the subendothelial matrix. This leads to release of pro-thrombotic factors and localized platelet activation and adhesion. Initial contact of platelets with vascular endothelium is mediated by primary agonists: vWf and collagen, which interact with their respective GPCR and stimulate the extracellular release of secondary agonists: ADP, TXA_2 and thrombin into the blood[111]. These secondary mediators cause platelets to change shape, stick to each other and secrete the contents of their storage granules. Platelet activation triggers a conformational change in glycoprotein IIb/IIIa (GBIIb/IIIa) receptors (integrin αIIbβ3), which increases their affinity for fibrinogen and vWF, causing platelet aggregation and thrombus formation[112] (Figure 1.14). Importantly, the activation of GBIIb/IIIa promotes coagulation and fibrynolytic pathways leading to thrombus stabilization and subsequent platelet fibrin clot formation (see I. General Introduction, pages 5-7).

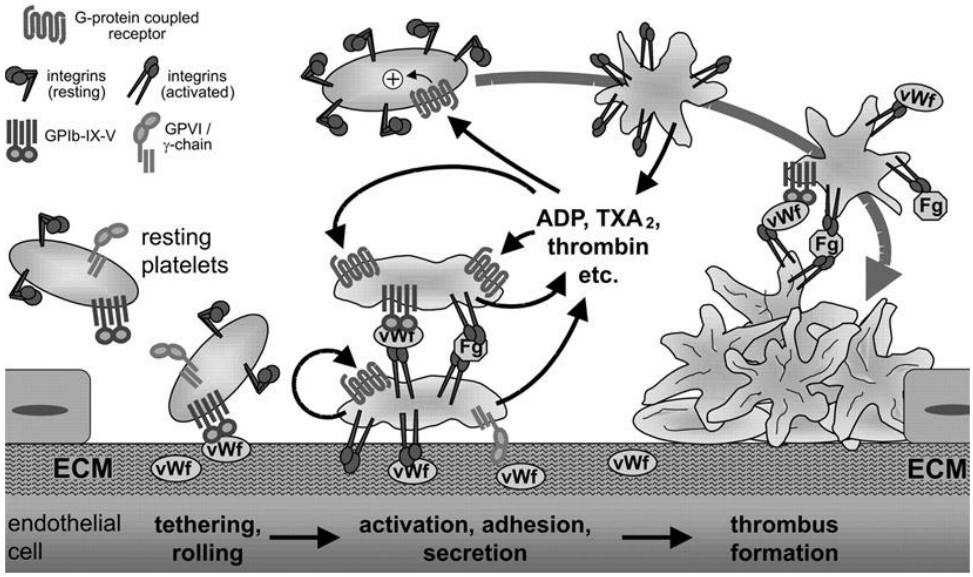


Figure 1.14. Stages in thrombus and plug formation. At a site of vascular injury, thrombus formation is initiated by the exposure of collagen and vWf in the vessel wall situated in extracellular matrix (ECM). Rolling platelets are activated and adhere to the collagen matrix by interaction between GPIb-IX-V receptor with vWf and GPVI with collagen. The secretion of ADP, TXA_2 and thrombin from platelet storage granules serves to amplify ongoing platelet activation. These events switch on intracellular signalling leading to inside-out activation of resting β-integrins recruiting circulating platelets in developing platelet aggregates. Fibrinogen and vWf bound to activated integrins contribute to thrombus stabilisation and fibrin clot formation. Adapted[111]

The thrombus may resolve locally and is an essential part of vascular homeostasis or alternatively it may embolize (migrate) to a distal part of the body and can often be the cause of severe ischemic events such as myocardial infarction (MI), ischemic stroke (IS), transient ischemic attack (TIA), pulmonary embolism, acute ischemia of limb and vascular death. That is why atherothrombosis is a common consequence and the ultimate cause of death in numerous cardiovascular diseases (CVD), including peripheral artery disease (PAD), coronary artery disease (CAD), atrial fibrylation, atheroslerosis, hypercoagulability, periprocedular vascular injury etc. In order to improve outcomes of CVD patients numerous anti-platelet and anti-

thrombotic therapies directed at modulating the key components of platelet activation and aggregation and thrombus generation (including blood coagulation) have been developed[113].

4.2 Targets for anti-platelet therapies

Current anti-platelet therapies target primarily COX-1 and platelet surface receptors such as TXA_2, $P2Y_{12}$, GBIIb/IIIa receptors and proteinase-activated receptor 1 (PAR1)[4] (Figure 1.15).

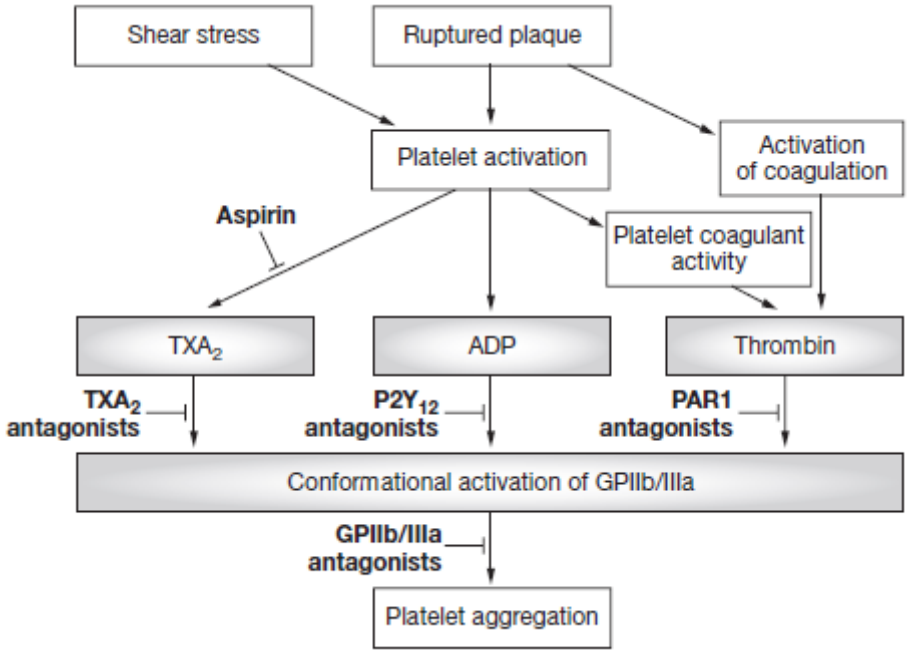


Figure 1.15. Main targets of anti-platelet therapies. Platelet aggregation can be inhibited by drugs blocking 1) COX-1 activity and TXA_2 formation (e.g. aspirin), 2) the action of soluble agonists (e.g. TXA_2, $P2Y_{12}$, PAR1 antagonists) or the binding of fibrinogen and other ligands to GPIIb/IIIa receptors. Adapted[4]

TXA_2 is generated from its precursor arachidonic acid (AA) through the activation of COX-1 pathway (see I. General Introduction, page 41). The release of

TXA_2 from platelets causes amplification of their activation via TXA_2/prostaglandin H2 (PGH2) receptor (TP) coupled to G_q and $G_{12/13}$ (see I. General Introduction, pages 36-41)[111]. TXA_2 actions can be inhibited at the level of COX-1 or through direct inhibition of TP receptors. TP receptors are also expressed in other cell types such as smooth muscle cells, macrophages, and monocytes[114].

ADP controls platelet activation via two platelet receptors $P2Y_1$ (G_q coupled) and $P2Y_{12}$ (G_i coupled; see I. General Introduction, pages 36-41)[111]. $P2Y_1$ receptors mediate changes in platelet shape, calcium mobilization and initiation of reversible aggregation whereas $P2Y_{12}$ receptors amplify aggregation by inhibition of AC and decrease in cAMP. Although sustained aggregation requires activation of both receptors, the $P2Y_{12}$ receptor is considered a better target because of its less uniform distribution in other tissues and a dominant role in platelet aggregation[115]. $P2Y_{12}$ receptors are primarily present in platelets and only minor expression has been found in endothelial cells, brain, glial cells and smooth muscle cells[116]. In turn, $P2Y_1$ receptors are ubiquitously expressed in many tissues including heart, blood vessels, smooth muscle cells, neural tissue, testis, prostate, ovary, and platelets[114].

Thrombin acts predominantly via protease-activated receptors 1 and 4 (PAR1 and PAR4), which couple to G_q, $G_{12/13}$ and in some cases also to G_i (see I. General Introduction, pages 36-41)[111]. Early traces of thrombin generated during the initiation phase of coagulation by tissue factor (TF) cleave a portion of PARs, which activates them and triggers multiple signal transduction pathways that modulate thrombosis, coagulation and inflammation. PARs are widely distributed in the vascular system and occur on platelets, endothelial cells, leukocytes and vascular smooth muscle cells[115]. PAR1 is more potent when activated than PAR4, and thus PAR1 is the preferred target for developing therapies[115].

Experimental anti-platelet therapies target other platelet receptors including integrins αIIbβ3 and α2β1, P-selectin, prostaglandin E2 (EP3), 5-hydroxytryptamine 2A ($5HT_{2A}$) receptors as well as intracellular proteins such as PDEs and phosphoinositide 3-kinase (PI3K) and endothelial products i.e. NO[113].

4.3 Dual anti-platelet therapy

Because platelet activation is determined by numerous signalling pathways, a multidrug treatment has been proposed to provide higher efficacy than individual therapies in the prevention of atherothrombotic complications[117]. Aspirin is the first anti-platelet agent to be evaluated and remains the main component of a dual anti-platelet therapy. Aspirin is known to irreversibly acetylate Ser529 of COX-1 inhibiting the platelet TXA_2 production for the lifespan of platelets, although other COX-1 independent actions have also been recognized[113]. In high risk patients, aspirin reduces the risk of vascular death by ~15 % and non-fatal vascular events by ~30 % as shown by meta-analysis of over 100 randomized trials[113]. However, 10-20 % of aspirin–treated patients have recurrent vascular events in 5 years after their incident event[115]. Furthermore, aspirin therapy is associated with upper-GI toxicity and hemorrhage and ~5 % people are intolerant to the drug[118]. Altogether, it stimulated for the development of better anti-platelet drugs.

4.3.1 $P2Y_{12}$ antagonists

$P2Y_{12}$ antagonists are very attractive agents for inhibition of platelet activation and aggregation because they block the ADP-related amplification of platelet activation including not only aggregation but also further procoagulant response and secretion from alpha- and dense- granules[119]. Unlike GPIIb/IIIa antagonists, they do not provide a complete blockage of platelet aggregation leading to intolerable bleeding. Initial $P2Y_{12}$ antagonists that have been discovered belong to the group of thienopyridines and were named sequentially ticlopidine, clopidogrel and prasugrel. Thienopyridines require a hepatic metabolism to generate active metabolites, which bind irreversibly to the active site of the $P2Y_{12}$ receptor for the lifetime of platelet[120]. Due to certain limitations of these drugs, a new generation of $P2Y_{12}$ inhibitors has been developed including ticagrelor, cangrelor and elinogrel, which interact reversibly with the allosteric site of the $P2Y_{12}$ receptor[121]. Since cangrelor and elinogrel require intravenous administration, their use is restricted to hospitalized patients and they are not discussed here. This thesis focuses on the main lines of anti-platelet therapy administered to the out-patient population.

4.3.1.1 Ticlopidine

Ticlopidine alone or in combination with aspirin was shown to be beneficial and superior to oral anticoagulants in preventing stent thrombosis (Innovative Stratification of Arrhythmic Risk-ISAR, Stent Anticoagulation Restenosis Study-STARS)[115], more effective than aspirin or placebo in the secondary prevention of stroke (Ticlopidine Aspirin Stroke Study-TASS, Canadian American Ticlopidine

Study-CATS)[115] and more effective than placebo in patients with mixed atherothrombosis (Swedish Ticlopidine Multicentre Study-STIMS)[115]. However, ticlopidine also has been associated with unfavorable side effects such as neutropenia, bone marrow aplasia and thrombotic thrombocytopenic purpura and therefore largely replaced by clopidogrel, which offered better safety profiles (Clopidogrel Aspirin Stent International Cooperative Study-CLASSICS, Ticlid or Plavix Post-Stents-TOPPS)[115, 122, 123].

4.3.1.2 Clopidogrel

In the Clopidogrel Versus Aspirin in Patients at Risk of Ischaemic Events (CARPIE) study clopidogrel monotherapy was modestly more effective than aspirin in reduction of the composite end point of MI, IS or vascular death with most of the benefit observed in patients with PAD[117, 124]. Since that trial, a number of studies have evaluated the efficacy and safety of dual anti-platelet therapy with clopidogrel and aspirin in several settings including in patients with ACS (Clopidogrel as Adjunctive Reperfusion Therapy-Thrombosis in Myocardial Infarction-CLARITY-TIMI[125], Clopidogrel and Metoprolol in Myocardial Infarction Trial-COMMIT[126], Clopidogrel in Unstable Angina to Prevent Recurrent Events-CURE[127]), undergoing PCI (Clopidogrel for the Reduction of Events During Observation-CREDO[128], PCI-CLARITY[129], PCI-CURE[130]) and those with stroke (Management of Atherothrombosis with Clopidogrel in High-Risk Patients-MATCH[131]) and mixed atherothrombotic disorders (Clopidogrel for High Atherothrombotic Risk and Ischemic Stabilization-CHARISMA[132]). In general a clear benefit of clopidogrel in combination with aspirin was observed in a wide range of patients with ACS and

across the spectrum of patients undergoing coronary stenting as a secondary prevention strategy. However, current data do not support dual anti-platelet therapy with clopidogrel in the primary prevention or treatment of cerebrovascular disease[117]. Major clinical trials of clopidogrel are summerised in Table 8.

Trial	Clopidogrel therapy (n)	Comperator therapy (n)	End points	Findings
ACS				
CLARITY-TIMI[a,125]	300 mg LD, 75 mg MD (1752) for 30 days	Placebo (1739)	Composite of occluded infarct artery on angiography, or death or recurrent MI before angiography	36 % reduction in primary outcome with clopidogrel and 20 % reduction in CV death, MI or ischemia that required urgent revascularization, no increase in TIMI major or total bleeding.
COMMIT[a,126]	75 mg MD for 4 weeks (22961)	Placebo (22891)	First: composite of death, reinfarction or IS; second: death from any cause during scheduled treatment period	Improved outcomes with clopidogrel (first: RRR= 9 %; second: RRR=7%), no increase in bleeding.
CURE[a,127]	300 mg LD, 75 mg MD for 3-13 months (6259)	Placebo (6303)	First: composite of vascular death, nonfatal MI or IS; second: composite of first primary outcome or refractory ischemia	Clopidogrel more effective than placebo in reducing both outcome measures (first: RR=0.8; second: RR=0.86) but increased bleeding.
DISPERSE-2[a,133]	300 mg LD, 75 mg MD for 12 weeks (327)	90 (334) or 180 (323) mg/bid ticagrelor MD	The Kaplan-Meier rate of major or minor bleeding through 4 weeks	No difference in major bleeding but an increase in minor bleeding at the higher dose of ticagrelor.
PLATO[a,134]	300-600 mg LD, 75 mg MD for 12 months (9333)	180 mg ticagrelor LD, 90 mg/bid MD ticagrelor (9291)	First: composite of vascular death, MI or IS; second: composite of first primary outcome and/or death from any cause, ischemia, TIA, or other arterial thrombotic event.	Improved outcomes with ticagrelor (first: HR=0.84; second: HR=0.84-0.88) but higher rate of non-CABG-related major bleeding according to study and TIMI criteria and higher incidence of dyspnea and ventricular pauses in the first week.

PCI and/or stent				
CREDO[a,128]	300 mg LD pre-PCI, 75 mg MD to day 28; 75 mg MD day 29–12 months (1053)	Placebo 3–24 h pre-PCI , 75 mg MD clopidogrel to day 28; placebo day 29–12 months (1063)	First: 1-year composite of death, MI, IS; second: 28-day composite of death, MI, urgent revascularization	Improved primary outcome with clopidogrel (RRR=26.9 %). Clopidogrel pretreatment did not improve the secondary point. No significant increase in major bleeding.
PCI-CLARITY[a,129]	300 mg LD, 75 mg MD (933)	Placebo (930)	Composite of vascular death, recurrent MI, IS (measured from PCI to 30 days after randomization)	Improved outcomes with clopidogrel (OR 0.54); no significant increase in TIMI major or minor bleeds.
PCI-CURE[a,130]	300 mg LD, 75 mg MD (1313)	Placebo (1345)	Composite of vascular death, MI or urgent revascularization, within 30 days of PCI	Clopidogrel more effective than placebo (31 % reduction in cardiovascular death or MI); no difference in major bleeding.
TRITON-TIMI[a,98]	300 mg LD, 75 mg MD (6795) for 15 months	60 mg prasugrel LD, 10 mg MD prasugrel (6813)	Composite of vascular death, MI, IS	Prasugrel more effective than clopidogrel (HR=0.81) but associated with increased major and fatal bleedings.
CLASSICS[a,123]	300 mg LD, 75 MD (345), or 75 mg MD (335)	250 mg/bid ticlopidine (340)	Major bleeds, neutropenia, thrombocytopenia, drug discontinuation for noncardiac event	Clopidogrel had superior safety and tolerability to ticlopidine.
TOPPS[a,122]	300 mg LD, 75 mg MD (494)	500 mg LD ticlopidine, 250 mg bid ticlopidine (522)	Failure to complete 2 weeks of treatment	Clopidogrel was better tolerated than Ticlopidine.

Other CVD (mixed atherothrombotis, stroke)				
CHARISMA and posthoc analysis[a,132]	75 mg MD for 28 moths (7802; posthoc: 4735)	Placebo (7801; posthoc: 4743)	First: composite of first occurrence of MI, IS or vascular death; second: composite of first primary outcome or hospitalization for unstable angina, TIA, or revascularization procedure	Clopidogrel caused no significant reduction in primary outcome in patients with clinically evident CVD (CAD, PAD, CBVD) or multiple risk factors and significant reduction of the rates of the secondary end point (RR=0.92). Significant increase in moderate but not severe bleeding according to Gusto definition. Posthoc analysis revealed 17 % reduction in primary outcome with clopidogrel in patients with documented prior MI, IS or symptomatic PAD; non-significant increase in moderate or severe bleeding.
MATCH[131]	75 mg MD (3797)	75 mg clopidogrel and 75 mg aspirin (3802)	Composite of MI, IS, vascular death or rehospitalization for acute ischemic events	No significant reduction in primary outcome; more common major and life-threatening bleedings with dual therapy in patients with recent IS or TIA.
CARPIE[124]	75 mg MD for median of 1.9 year (9599)	325 mg aspirin (9586)	Composite of first occurrence of MI, IS or vascular death	Improved outcomes with clopidogrel (RRR=8.7 %) in patients with symptomatic atherosclerotic disease; in PAD patients (RRR=23.8%); no difference in rate of bleeding disorder but greater rate of GI bleeding with aspirin.

Table 8. Key trials of clopidogrel therapy in comparison to placebo/aspirin/other P2Y12 antagonists (>500 patients). [a]Both treatment arms received aspirin. Abbreviations (except for trial acronyms): ACS (acute coronary syndrome), bid (twice daily), CABG (coronary artery bypass graft), CAD (coronary artery disease), CBVD (cerebrovascular disease), CVD (cardiovascular disease), HR (hazard ratio), IS (ischemic stroke), LD (loading dose), MD (maintenance dose), MI (myocardial infarction), n (number of patients), OR (odds ratio), PAD (peripheral vascular disease), PCI (percutaneous coronary intervention), RR (relative risk), RRR (relative risk reduction), TIA (transient ischemic attack), TIMI (thrombosis in myocardial infarction).

Despite the beneficial actions of clopidogrel, its clinical utility is compromised by a delayed onset and offset of platelet inhibition and variable responses including nonresponsiveness to the drug occurring in up to 30% of patients. Introduction of 600 mg loading dose shortened the delay in onset of action in comparison to 300 mg loading dose from 4-6 h to 2-4 h after administration[135]. A delayed offset of clopidogrel action is caused by the irreversible nature of its anti-platelet effects and increases a bleeding risk in patients who require an urgent surgery within 5 days of stopping treatment[127]. However, inhibition of platelets's aggregation for their lifespan has advantages for patients who miss one or more doses of the drug.

A heterogenous patients' response to clopidogrel treatment is primarily explained in terms of the inter-individual variability in active metabolite generation. Because clopidogrel is metabolized in a 2-step process dependent on the CYP450 liver enzymes[136], genetic polymorphisms affecting these enzymes (e.g. in CYP2C19[137, 138]) and coadministration of drugs metabolized by them (e.g. lipophilic statins[139] and proton pump inhibitors (PPIs)[140]) have shown to limit the formation of the clopidogrel active metabolite. However, there is no consistent clinical evidence confirming an adverse interaction between PPIs and clopidogrel affecting patient outcomes[141, 142]. Furthermore other mechanisms have been proposed to contribute such as the individual variability in absorption of clopidogrel and baseline platelet reactivity, inadequate dosing and cigarette smoking[115]. Nonresponsiveness to clopidogrel has been partially overcome by replacing 300 mg with 600 mg loading dose but is still common in ~20 % population[143]. These limitations of clopidogrel therapy led to development of new $P2Y_{12}$ antagonists.

4.3.1.3 Prasugrel

The third generation-thienopyridine, prasugrel, requires a single step activation and is not influenced by functional variability of CYP450 enzymes[144]. Thanks to more efficient metabolism than clopidogrel, prasugrel treatment was associated with more consistent, faster and potent inhibition of platelet aggregation[144]. In the Trial to Assess Improvement in Therapeutic Outcomes by Optimizing Platelet Inhibition with Prasugrel (TRITON-TIMI) prasugrel was more efficient than clopidogrel in treatment of patients with ACS undergoing PCI but was associated with a higher occurrence of life-threatening and fatal bleeding[98]. Results of this trial supported a concept that a superior $P2Y_{12}$ antagonist improves the efficacy of dual anti-platelet therapy in ACS patients treated with PCI at expense of increased bleeding and therefore its use should be limited to treatment of high-risk groups. This led to investigation of alternative non-competitive and reversible inhibitors of $P2Y_{12}$ receptors such as ticagrelor, which would maintain receptors fully functional upon dissociation of drug and hopefully prevent excess bleeding.

4.3.1.4 Ticagrelor

Ticagrelor does not belong to thienopyridine family. It does not require metabolic activation for its anti-platelet activity but it does have an active metabolite, which is equally potent in inhibition of $P2Y_{12}$ receptors as the parental drug. Ticagrelor works through affecting the allosteric site of the $P2Y_{12}$ receptor, thus preventing ADP-induced signalling in platelets, rather than the actual binding of ADP to the receptor as influenced by the thienopyridines. This interaction is reversible and

has a half-life of 7-12 h, which imposes twice-daily dosing in order to maintain the inhibitory effect[145].

Ticagrelor provided a greater and faster inhibition of platelet aggregation both at post loading and at steady state than clopidogrel in ACS patients (Dose Confirmation Study Assessing Anti-Platelet Effects of AZD6140 versus Clopidogrel in NSTEMI 2-DISPERSE-2[133], ONSET/OFFSET[79]). Furthermore the pharmacokinetics of ticagrelor were unaffected by prior clopidogrel dosing (Response to Ticagrelor in Clopidogrel Nonresponders and Responders and Effect of Switching Therapies-RESPOND[146]). In the PLATelet inhibition and patient Outcomes (PLATO) trial, high risk patients with ACS had a 16 % lower rate of the composite end point (vascular death, MI and IS) than those receiving clopidogrel[134]. Interestingly, a reduction in the rate of death from any cause was observed with ticagrelor despite only a moderate decrease in the risk of MI. This led to a speculation that beneficial effects of ticagrelor might be partially attributed to its off-target effects including an inhibition of adenosine uptake by erythrocytes *in vitro*. Increased adenosine plasma concentration caused by ticagrelor among other effects could potentially prevent sudden cardiac death, reduce infarct size or inhibit tumor growth[147]. The higher incidence of dyspnea and more frequent early ventricular pauses with ticagrelor reported in the PLATO study seem to support the adenosine-like effects of the drug. Importantly, there was no difference in the rate of overall major bleeding comparing ticagrelor with clopidogrel therapy, although the rate of non-procedure-related bleeding was increased with ticagrelor.

	Clopidogrel	**Ticagrelor**
Administration	600 mg LD, 75 mg MD daily	180 mg LD, 90 mg MD twice daily
Mechanism of action	Prodrug, irreversible	Active drug, reversible
Max. inhibition of platelet aggregation	40-60	85-95
Onset of action	2-4 h (600 mg)	2-4 h
Offset of action	5-7 days	3-5 days
Half-life	~11 days	7-12 h
Time to steady state	3-7 days	2-3 days
Pharmacologic interactions	Proton-pump inhibitors, calcium channel blockers	CYP3A4/5 inhibitors: rifampicin, ketoconazol, diltiazem

Table 9. Main pharmacologic characteristics of clopidogrel and ticagrelor. Adapted[145].

II. THESIS AIMS:

The overall objective of this body of work is to investigate off-target effects of thienopyridine and non-thienopyridine anti-platelet agents on vascular homeostasis. Specifically, these studies shall aim to:

1. Study direct influences of parental forms of clopidogrel (pro-clopidogrel) on the responsiveness of rabbit aortae to nitric oxide (NO). In addition, the potential anti-oxidant and anti-inlammatory effects of pro-clopidogrel on vessels will be examined using electron paramagnetic resonance (EPR) and real time quantitative PCR (RT qPCR).

2. Investigate the ability of thienopyridines and ticagrelor to make biologically active nitrosothiols *in vitro*. The properties of newly synthesized SNO derivatives will be tested in terms of inhibition of platelet aggregation, relaxation of rabbit aortae and transnitrosation reactions.

3. Determine the influence of a loading dose and maintenance therapy with clopidogrel on plasma markers of NO production, metabolism and bioavailability in coronary artery disease (CAD) patients. The relationship between vascular, oxidative and platelet-derived effects of clopidogrel will be also examined.

III. GENERAL METHODS

1. Animal sacrifice

Male New Zealand White rabbits (2-2.5 kg) were terminally anesthetized by intravenous injection of sodium phenobarbitone (120 mg/kg; Merial Ltd.). The descending aorta was then excised and immersed in chilled Krebs buffer (containing 109.2 mM NaCl, 2.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4 \cdot 7\ H_2O$, 25.0 mM $NaHCO_3$, 11.0 mM D (+) Glucose, 1.5 mM $CaCl_2 \cdot 2H_2O$, pH 7.4). The aorta was cleaned of adventitial tissue and cut into 2-mm-wide ring segments.

$P2Y_{12}^{-/-}$ male mice and their genetically matched wild-type strain $P2Y_{12}^{+/+}$ (20-25 g) on a C57BL/6 background were kindly provided by Sheila Francis (The University of Sheffield). Genotyping of $P2Y_{12}$ mice was performed elsewhere using polymerase chain reaction (PCR)[148]. Mice were sacrified by cervical dislocation and their descending aortas were excised and treated as above.

All procedures were carried out according to UK Schedule 1 legislation under guidelines issued by Cardiff University.

2. Isometric tension studies

Rabbit aortic rings were mounted in a 8-channel tissue organ bath system (custom built) and isometric tension was recorded on a PowerLab 8/SP data acquisition system (ADInstruments). Mouse aortic rings were mounted in a 4-channel multi myograph system (DMT-610M) with in-built Powerlab data acquisition system (ADInstruments). Vessel segments (rabbit and mouse) were equilibrated for 30 min in

Krebs buffer at 37 °C and continuously bubbled with 5 % CO_2 and 95 % O_2. They were then gradually stretched to a resting tension of 2 g (rabbits) or 4 mN (mouse), which was maintained throughout the experiment. Arterial integrity was assessed first by constriction of vessels with phenylephrine (PE; 1 μM), followed by stimulation with acetylcholine (Ach; 10 μM). This was repeated two more times in order to achieve the maximal responses of each of the studied vessels.

2.1 Measurement of vascular responses to PE, Ach and NO donors

In the rabbit vessel preparation, cumulative dose response curves were initially performed for PE (1 nM-10 μM). PE (1 μM) produced 70-85 % of maximal response and this dose was then used to pre-constrict vessels for subsequent vasodilation experiments as it provided a greater range over which relaxation/contriction responses could be measured. Cumulative dose response curves to Ach (1 nM-10 μM), S-nitrosoglutathione (GSNO, 1 nM-10 μM; Enzo Life Sciences), MAHMA NONOate (NOC9, 100 pM-1 μM; Enzo Life Sciences) or isosorbide dinitrate (ISDN, 0.42 nM-4.24 μM; Heath Hospital) were performed on aortic rings following pre-constriction with PE. NOC9 was used in comparison to GSNO as it releases NO very quickly ($t_{1/2}$=2.5 min) in neutral pH and does not depend on prior tissue metabolism. ISDN was used as the most commonly used NO donor in the clinical setting.

In the mouse vessel preparation, cumulative dose responses to PE (1 nM-1 μM) had to be performed before each experiment in order to achieve a stable constriction. Cumulative dose response curves to Ach and GSNO were performed, as for rabbit vessels.

Concentration-response curves to agonists were fitted using a standard sigmoid dose-response curve (Y=Bottom + (Top-Bottom)/(1+10^((LogEC50-X)))) with a Hill Slope of 1.0 and no constraints. Doses producing 50 % response [$\log_{10}(EC_{50})$] were determined. Additionally, EC_{50}Ratio ($EC_{50}R$) was computed in chosen samples to report the change of EC_{50} in relation to corresponding controls (Figure 2.1).

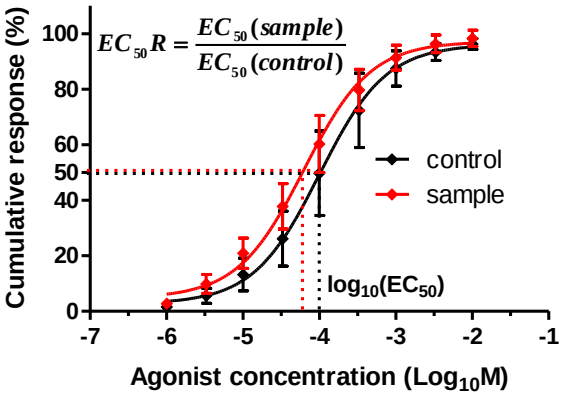


Figure 2.1. Evaluation of aortic rings response to cumulative doses of agonists. When different relaxants were tested, vessels were firstly pre-constricted with PE and the [$\log_{10}(EC_{50})$] of agonists was calculated as a percentage relative to PE-induced constriction. When a constriction response to PE was tested, the highest effective PE concentration reflected 100 % response.

2.2 Measurement of relaxant responses to thienopyridine-SNO (Th-SNO)

In the rabbit vessel preparation, aortic rings were pre-constricted with 1 μM PE and a single dose of thienopyridine, thienopyridine-SNO (Th-SNO) mixture or nitrite was added. Drug preparation is described in detail in IV. Results (page 145). Briefly, 10 mM thienopyridine tablet solutions were mixed with varied concentrations of nitrite (1-10 mM) in order to make SNO derivatives. Parallel biochemical analysis

by Cu^{+}/Cys (2Cs) ozone based chemiluminescence (OBC) revealed that only ~1 % of Th-SNO mixture contains Th-SNO. Therefore, in order to account for un-reacted reagents, the corresponding thienopyridine and nitrite solutions used for the reaction were also checked separately for vasodilatory capacity.

The maximal relaxation (Rmax) of Th-SNO mixture was calculated as a percentage relative to PE-induced constriction. Nitrite-induced vasodilation was determined over the same time and subtracted from Rmax of Th-SNO mixture in order to control for un-reacted nitrite and report Th-SNO-induced vasorelaxation accurately (Figure 2.2). Native thienopyridine solutions did not induce significant relaxation of pre-constricted rings.

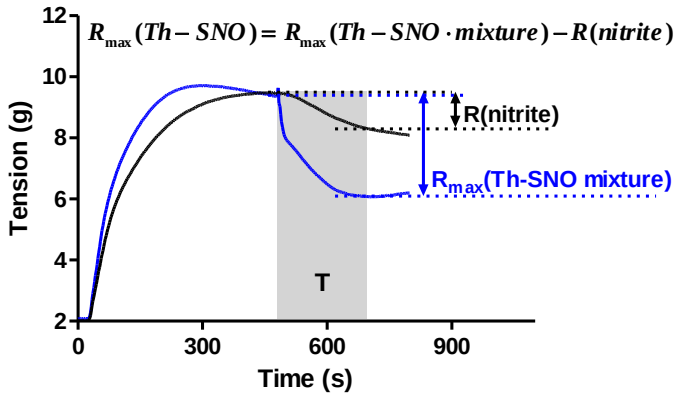


Figure 2.2 Evaluation of relaxation of pre-constricted aortic rings to single doses of Th-SNO. Rmax of Th-SNO mixtures in time (T) were corrected by unreacted nitrite by subtraction of nitrite-induced relaxation over the same time (T).

3. Electron paramagnetic resonance (EPR)

Electron paramagnetic resonance (EPR) spectroscopy is the only technique capable of direct detection and identification of free radicals. The method is not

sensitive enough to detect short-lived species such as superoxide anion radical ($O_2^{\bullet-}$) directly in biological samples, but spin trapping is used to stabilize radicals by creating longer-lived radical adduct species. The spin traps 5,5-dimethyl-1-pyrroline-Noxide (DMPO), and 5-(diethoxyphosphoryl)-5-methyl-1- pyrroline-N-oxide (DEPMPO) are widely used for detection of $O_2^{\bullet-}$, although DEPMPO has been preferred due to higher sensitivity, specificity and stability[129, 149, 150].

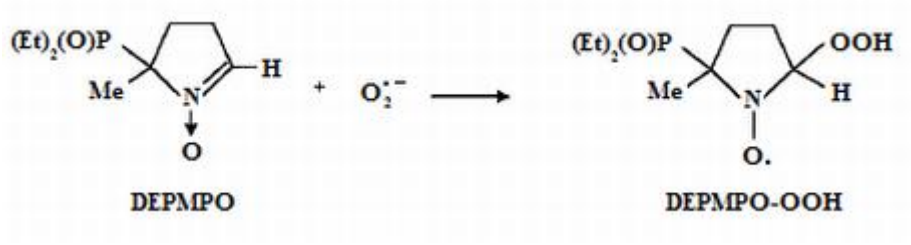


Figure 2.3. Structure of DEPMPO and its superoxide adduct DEPMPO-OOH. Adapted[151]

Vascular superoxide formation was determined by spin trapping of $O_2^{\bullet-}$ released from rabbit aortic rings incubated in Krebs buffer with 100 mM DEPMPO (Axxora Uk Ltd.). Typically, 2 aortic rings were kept for 5 min in 100 µl buffer in one well of a 96-well plate. Samples containing DEPMPO- $O_2^{\bullet-}$ adducts were loaded into a 0.8-1.1 mm i.d. glass capillary (Fisher Scientific UK), sealed with Crytoseal and placed into a quartz tube holder and into the cavity of a Bruker e-scan EPR spectrophotometer operating at 9.5 GHz. Typically 21 s scans were taken for up to 10 min (typical recording conditions were: scan range 150 G, modulation amplitude 1.01 G, microwave power 15.21 mW). Standards of superoxide were made for reference by mixing 0.3 mM hypoxanthine and 0.08 U/mg of xanthine oxidase (Figure 2.4). Potassium superoxide (KO_2) was also used as a positive control (Figure 2.5).

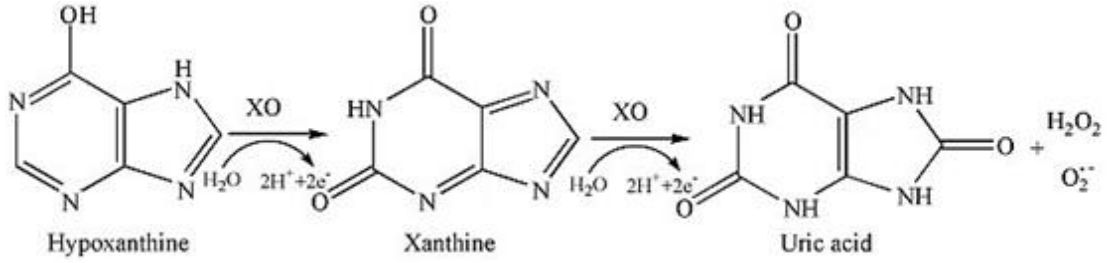


Figure 2.4. Two step enzymatic process of hypoxanthine generation of $O_2^{\bullet-}$ catalysed by xanthine oxidase. Adapted[152]

EPR spectra were smoothed using polynomial fitting (2nd order) with adjacent averaging. The area under curve (AUC) of all component peaks of the EPR spectrum was analysed individually using GraphPad Prism and $\log_{10}$(AUC) was used to report the relative amounts of superoxide.

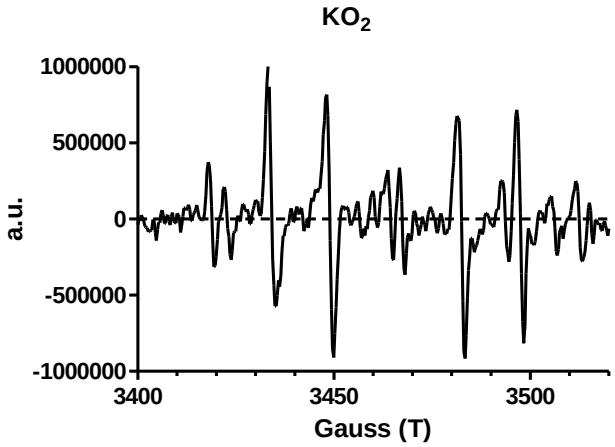


Figure 2.5. Representative trace of DEPMPO-$O_2^{\bullet-}$ spin adducts of potassium superoxide. EPR spectra was smoothed using polynomial fitting (2nd order) with adjacent averaging. The presence of the characteristic 1:2:2:1 splitting of the DEPMPO-$O_2^{\bullet-}$ adducts is visible.

4. Real time quantitative PCR (RT qPCR)

4.1 Primer design

Primers were designed using Primer3 and BLAST tools so as to be specific to the PCR templates and to be separated by at least one intron on the corresponding genomic DNA. The amplicon size was planned to be between 50 and 250 bp. The list of sense and antisense primers for rabbit mRNA are presented in Table 10.

mRNA	Sequence (5' – 3')	Conc. (nM)
COX-2	Sense CGATGACTGCCCAACGCCCA	230
	Antisense GGCGCAGTTTATGCTGTCTGTCCA	230
GAPDH	Sense CGCGTCCCCGAGACACGATG	460
	Antisense TGGTGACCAGGCGCCCAATG	460
HPRT-1	Sense CGCAGCCCCAGCGTTGTGAT	920
	Antisense CCCTTGAGCACACAGAGGGC	920
IL-1 β	Sense TGCAGGCTCCAGGATGCACA	690
	Antisense TGAGGCCCAAGGCCACAGGT	690
IL-6	Sense AAGACGGATGCTTCCCGCCG	690
	Antisense GCAACGGCTGGCTTGAGGGT	690
iNOS	Sense CAGAGCAGTACAAGCTCAC	690
	Antisense GGATCTCAGCCTCATGGTG	690
TNF-α	Sense GCCACCACGCTCTTCTGCCT	690
	Antisense GAGCTGGCCCTCCACTTGCG	690
VCAM-1	Sense TGAGGAGCGAGGGGACCACG	460
	Antisense TCCAACCTCCAAAGGGCCACTCA	460

Table 10. List of sense and antisense rabbit primers used in RT-qPCR.

4.2 Primer optimization

In order to find the optimal concentrations of primers, 15 combinations of varying concentrations (230-920 nM) of the sense and antisense primers were checked. Primer combinations with the lowest threshold cycle values (C_t) and the highest fluorescence signal (ΔRn) were chosen (Figure 2.6) to give the most sensitive and reproducible assays.

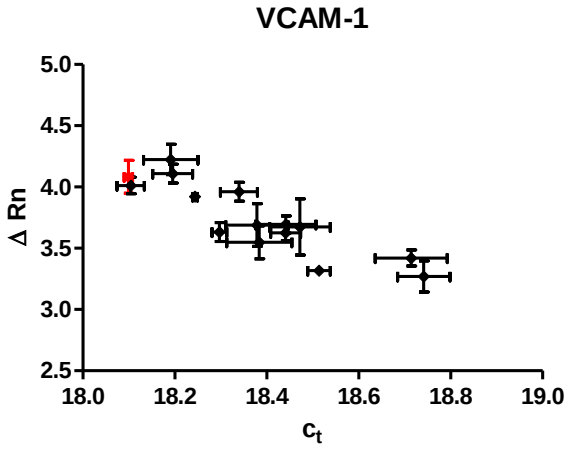


Figure 2.6. Primer optimization. In red – chosen combination of primers.

4.3 Extraction of RNA

Extraction of RNA from rabbit aortic rings was performed using Trizol reagent, following modified Chomczynski method, which performs well with 50-100 mg of tissue (one rabbit ring weighs approximately 70-80 mg).

4.3.1 Vessel homogenization

Each rabbit aortic ring was transferred from -80 °C into 500 µl Trizol, which is a mixture of phenol and guanidine isothiocyanate, and were homogenized using an

Ultra Turrax T25 homogenizer until completely disrupted. After 5 min incubation at room temperature, which permits the complete dissociation of nucleoprotein complexes, lysates were centrifuged at 12000 x g for 10 min at 4 °C to remove insoluble material.

4.3.2 Removal of DNA and proteins

100 µl chloroform was added to the supernatants, containing rabbit RNA, which then was shaken vigorously by hand for 15 s, incubated for 3 min at room temperature and centrifuged 12000 x g for 15 min at 4 °C. This allowed separation of the mixture into: i) a lower red phase, containing protein and lipid; ii) a white interphase, containing DNA and iii) a colourless upper phase, containing RNA.

4.3.3 RNA precipitation

After removal of DNA and proteins, RNA was carefully transferred to fresh tubes, and equal amounts of 70-75 % ethanol were added and vortexed to precipitate RNA. The RNA precipitate was usually not visible by naked eye.

4.3.4 RNA wash and re-dissolving

In order to improve the quality of extracted rabbit RNA, further steps were performed on columns provided by PureLink RNA mini kit (Invitrogen) as per the manufacturer's protocol. RNA precipitate was retained on columns and washed

several times with buffers, helping to remove solvents used for RNA extraction. Finally, RNA was eluted from columns using 30 µl RNase-free water.

4.3.5 Quantity and quality assessment of RNA

The concentration and quality of isolated RNA were measured using the NanoDrop Spectrophotometer 1000. RNA has its absorption maximum at 260 nm and the absorbance ratios at 260/280 nm and 260/230 nm were used to assess its purity. Both ratios should be approximately 2.0-2.1. The RNA extraction method described here gave 70-180 ng/µl rabbit RNA. 260/280 ratio was 1.8-2.1, confirming samples were free of DNA impurities. The 260/230 ratio was more variable (0.3-2.1), and where the reading was below 1.0, this was taken to reflect there was a considerable contamination with protein or solvents, and the RNA preparation was further purified. Around 10 % samples required this purification step.

4.3.6 Purification of RNA

In order to purify RNA from protein and/or solvent impurities and improve 260/230 ratio, 3 µl of 3 M sodium acetate and 60 µl of 100 % ethanol were added to 30 µl RNA, vortexed and kept on ice for 15 min at -80 °C. After thawing, the mixture was centrifuged 12000 x g for 10 min at 4 °C, supernatant removed and 150 µl of 70 % ethanol cooled to -20 °C was added. Then the mixture was vortexed, centrifuged 12000 x g for 2 min at 4 °C and the RNA pellet was dried in the air. Finally, RNA precipitate was re-dissolved in RNase-free water and measured once again on the NanoDrop.

4.4 cDNA conversion and RT qPCR conditions

500 ng of total RNA was reverse transcribed with random primers into cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) under the following conditions: 10 min at 25 °C, 120 min at 37 °C, 5 s at 85 °C and then stored at 4 °C for further analysis.

The cDNA was diluted 20-fold prior to PCR amplifications, which were performed in a total volume of 10 µl using Fast SYBR® Green Master mix (Applied Biosystems). Negative controls were included, in order to check the possible contamination of cDNAs or assay reagents by genomic DNA. For this purpose, instead of cDNA, either mRNA or water was added to the reagent mix.

All PCR amplifications were performed in triplicate under the following conditions: 2 min at 95 °C, followed by a total of 35 cycles (10 s at 95 °C, 10 s at 60 °C and 26 s at 72 °C) and the melting curve analysis. Fluorescence was measured through the slow heating phase and C_t-values (intersections of the threshold with amplification plots) obtained by the LightCycler analysis software were collected for the analysis (see below).

4.5 Method of calculation

The relative expression of a target gene in relation to a reference (house-keeping) gene was calculated for each sample and normalized against a calibrator on the basis of $\Delta\Delta C_t$ method[153]. This method accounts for sample to sample variations caused by differences in the initial quality and quantity of the nucleic acid and

provides a constant calibrator point between PCR runs. The calibrator was prepared from untreated, fresh tissues.

Results were expressed as the target/reference ratio of each sample normalized by the target/reference ratio of the calibrator (equation below). PCR efficiency (E) differences were corrected to 1.95 in order to achieve exact results. C_t values were determined automatically by the sequence detection system (SDS) software (Figure 2.7).

$$\mathbf{Ratio(R)} = \frac{(E_{target})^{\Delta C_t \text{(calibrator-sample)}}}{(E_{ref})^{\Delta C_t \text{(calibrator-sample)}}}$$

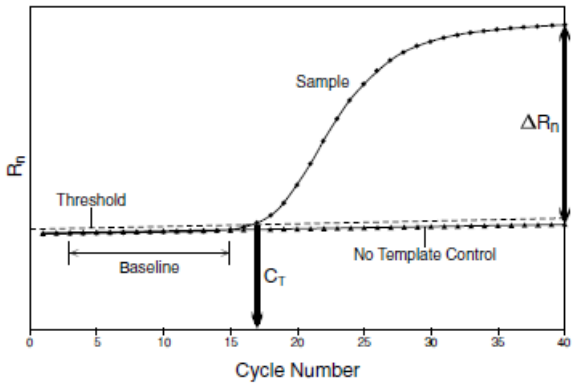


Figure 2.7. Calculation of Ct values by SDS software. The threshold is set above the baseline and within the exponential growth phase of the amplification curve. The intersection of the threshold with the amplification plot defines the C_t in real-time PCR assays.

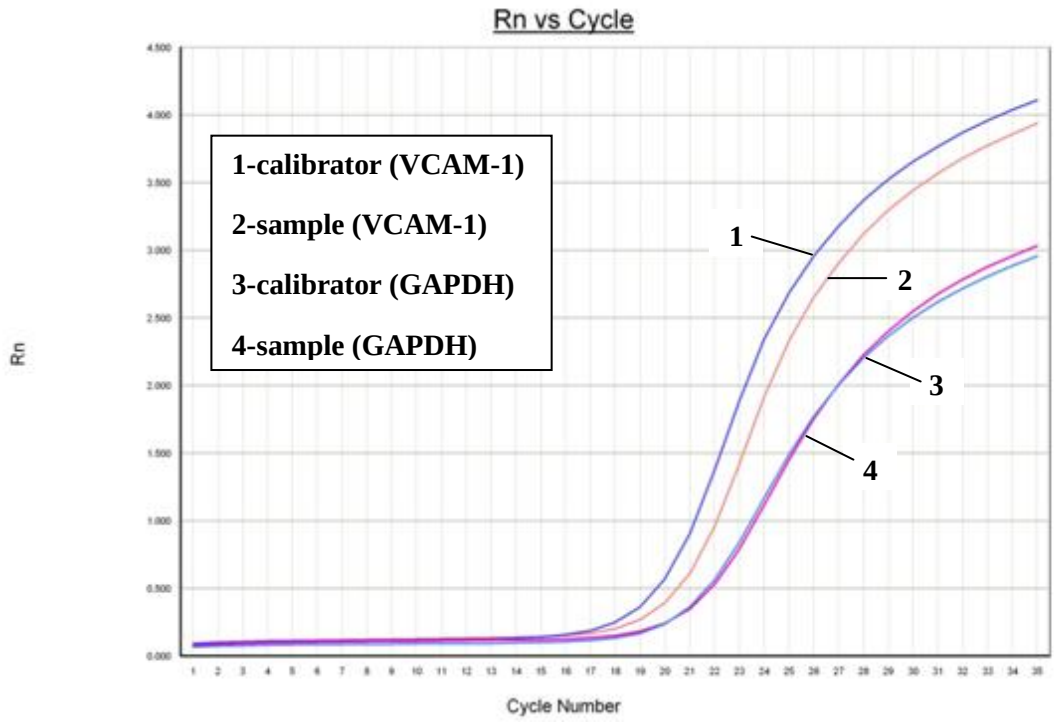


Figure 2.8. Typical amplification plots of target (e.g. VCAM-1) and reference (GAPDH) genes in the sample and the calibrator.

4.5.1 Choice of house-keeping gene

Relative RNA levels from test samples were calculated normalized against one or two reference housekeeping genes GAPDH and HPRT-1 (Figure 2.9). Because results coming from either analysis were very similar, all experimental samples were subsequently normalized only against GAPDH.

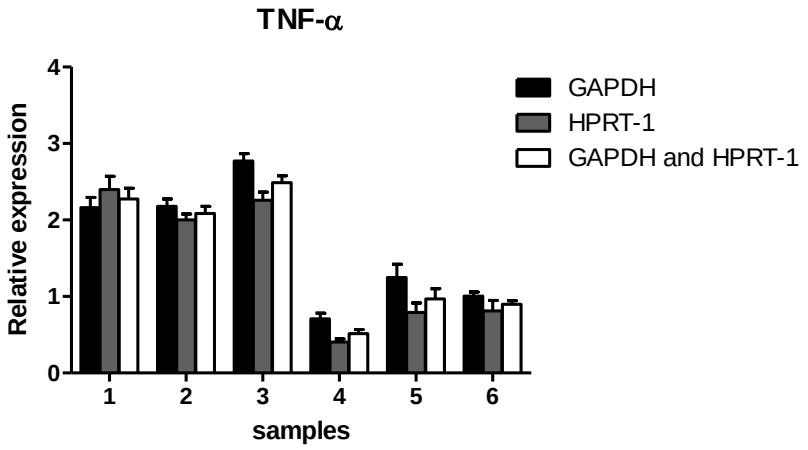


Figure 2.9. Comparison of results calculated against one or two house-keeping genes.

4.5.2 Melting curve analysis

The specificity of the amplified product (amplicon) was verified after each experiment by the analysis of the melting curve. When a particular RT qPCR reaction was heated slowly, only one peak at the characteristic melting temperature should be revealed (Figure 2.10). Melting temperature is dependent on the guanine/cytosine (GC)-content and the size of amplicon. Primer-dimers and other nonspecific products or impurities would cause additional peaks.

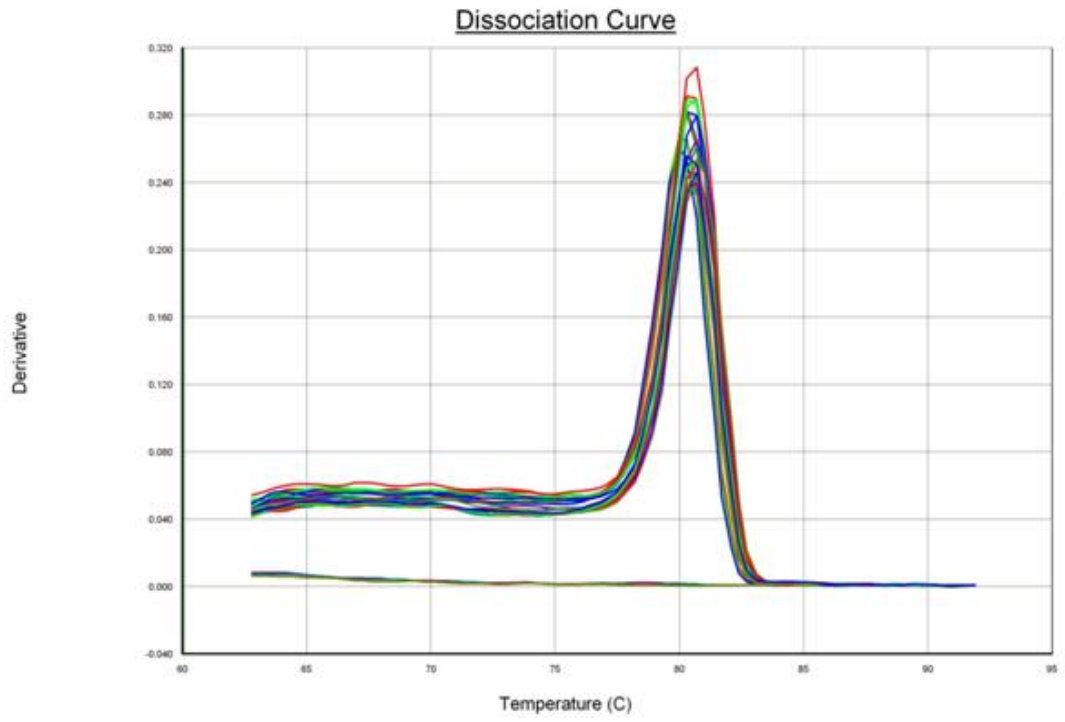


Figure 2.10. Typical melting curve plots of GAPDH measured after one experiment.

5. Nitric oxide analysis (NOA) using ozone-based chemiluminescence (OBC)

NO metabolites: nitrite, nitrate and RSNO were measured using ozone-based chemiluminescence (NO analyzer, NOA280i, Sievers) as has been described previously[34, 154]. Typically, NO was cleaved from the species of interest by the use of specific cleavage reagents, it was then carried by inert N_2 gas through the NaOH trap to the NOA, where it reacted with ozone (equation below). Energy released from this reaction in a form of a photon (*hv*) was amplified in a photo-multiplier tube (PMT) and was recorded as a potential difference (mV, Figure 2.11).

$$NO^{*} + O_3 \rightarrow NO_2^{*} + O_2 \rightarrow NO_2 + O_2 + hv$$

Data was acquired as a series of peaks by the NOAnalysis Software (Liquid) and each peak was analyzed in terms of area under curve (AUC) using the Liquid

analysis or Origin software. The amount of NO metabolites was determined from relevant standard curves performed on a daily basis.

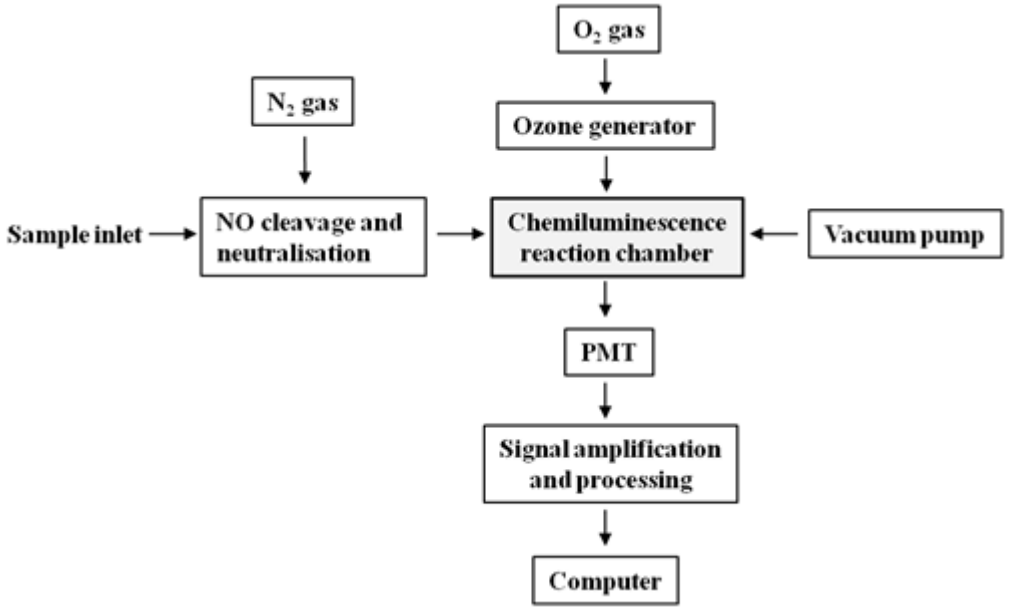


Figure 2.11. NO analysis (NOA) using ozone-based chemiluminescence (OBC). A vacuum pump draws ozone and sample into the chemiluminescence reaction chamber. In the reaction chamber, NO previously released from the sample and neutralized reacts with ozone. The emitted energy is detected and amplified by PMT, which is measured and displayed on the computer as a potential difference between PMT's plates (mV). Adapted[154].

5.1 Measurement of NO metabolites in plasma

Platelet poor plasma (ppp) for NO metabolite analysis was isolated from K_3EDTA vacutainer bottles by centrifugation immediately after blood collection (1500 g, 10 min, 4 °C), then snap frozen in liquid nitrogen and stored at -80 °C. Frozen samples were used for the analysis within six months after collection. It has been shown previously that levels of plasma nitrite and protein-bound NO remain unchanged up to 6 months[34]. After that time plasma RSNO and nitrite are gradually oxidized to nitrate.

Immediately prior to NO analysis, plasma samples were thawed for 3 min in a water bath at 37 °C.

5.1.1 Plasma nitrite and S-nitrosothiols

NO was cleaved from plasma nitrite (equation 1 below) and S-nitrosothiols (RSNO; equations 2 below) by tri-iodide reagent containing 28.5 mM I_2, 66.9 mM KI and 0.78 M glacial acetic acid. The NOA setup for plasma nitrite and RSNO measurement in conjunction with the tri-iodide reagent is shown in Figure 2.12. The intra- and inter-assay coefficients of variation (CV) were 7 % and 17 %, respectively (See Shawmendra Bundhoo MD 2011).

$$2HNO_2 + 2I^- + 2H^+ \rightarrow 2NO + I_2 + 2H_2O \quad (1)$$

$$I_2 + I^- \rightarrow I_3^-$$
$$I_3^- + 2RSNO \rightarrow 3I^- + RS - SR + 2NO^+ \quad (2)$$
$$2NO^+ + 2I^- + 2H^+ \rightarrow 2NO + I_2 + 2H_2O$$

Figure 2.12. NOA setup for plasma nitrite and/or RSNO measurement. 200 µl sample (+ few drops of Antifoam^{TM}) is injected through the rubber septum (1) into a specially constructed purge vessel with 5 ml tri-iodide reagent (2). The temperature of the reagent is maintained at 50 °C by a beaker of water (3) kept on a hot plate (4) linked to IKATRON® ETS-D4 fuzzy (5). Released NO is carried in an inert N_2 gas stream controlled by a flowmeter (6; flow: 200 cm^2/min) to a round-bottom flask with 1 M NaOH (7). NaOH protects NOA from damage by hot acid vapour and ensures that N-oxide contaminants are not converted to NO (no false positives). Neutralized NO vapour is directed to NO analyser (8).

A standard curve was constructed using water and different concentrations of sodium nitrite 62.5 nM, 125 nM, 250 nM, 500 nM and 1000 nM (Figure 2.13).

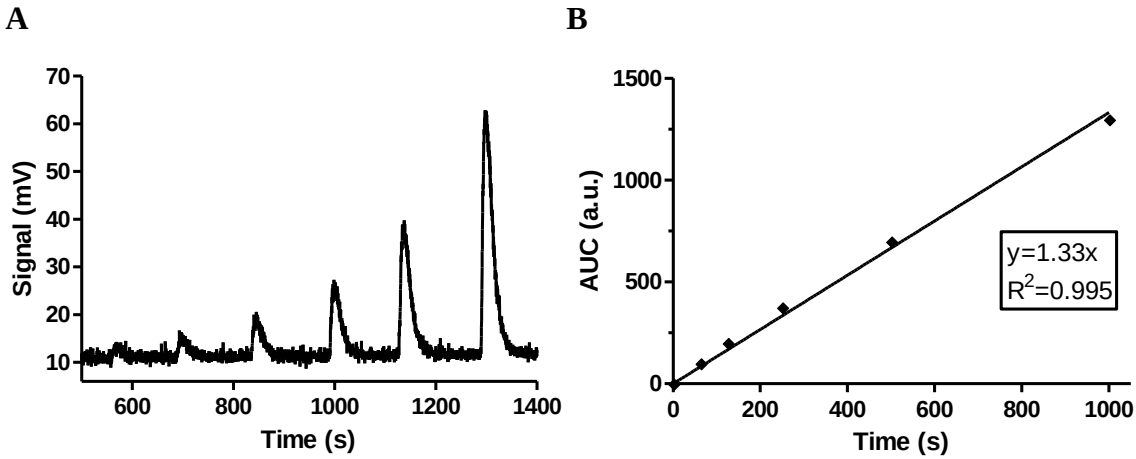


Figure 2.13. Analysis of standard curve for plasma nitrite and RSNO measurement. Typical signals of water and 62.5 nM, 125 nM, 250 nM, 500 nM and 1000 nM sodium nitrite are shown (left). The corresponding AUC of detected peaks (corrected by AUC of water) were used to calculate the slope coefficient of the standard curve (right).

In order to distinguish between nitrite and RSNO, the same sample was run before and after a pre-treatment with 5 % acidified sulphanilamide (290 mM), which binds nitrite ions and allows the selective measurement of the residual plasma RSNO (Figure 2.14).

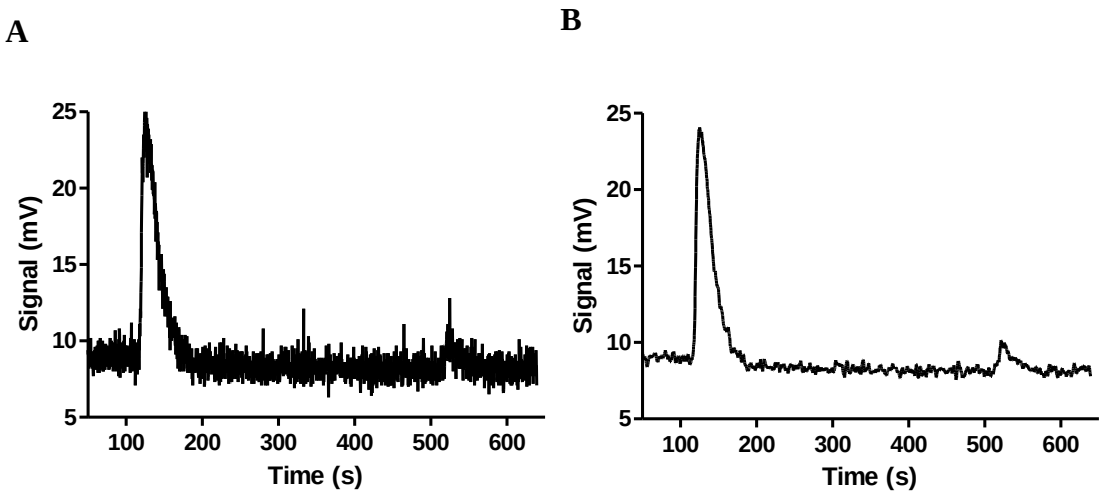


Figure 2.14. Typical plasma nitrite and RSNO signals before (left) and after (right) the adjacent smoothing. Plasma nitrite peaks were well defined and the corresponding AUC was determined using in-built Liquid analysis. Plasma RSNO peaks were smaller and less clear and therefore the Origin 7 software was used for the appropriate smoothing and calculation of AUC.

5.1.2 Plasma nitrate

NO was cleaved from plasma nitrate by vanadium chloride (equation below) reagent containing 49.9 mM VCl_3 and 0.8 M HCl. The reagent was filtered through a 0.22 µm filter before use, revealing a turquoise blue colour. The NOA setup for plasma nitrate measurement in conjunction with VCl_3 reagent is shown in Figure 2.15. The intra- and inter-assay coefficients of variation (CV) were 4 % and 14 %, respectively (See Shawmendra Bundhoo MD 2011).

$$2VCl_3 + 4HCl + NO_3^- \rightarrow 2VCl_5 + 2H_2O + NO$$

Figure 2.15. NOA setup for plasma nitrate measurement. 20 µl sample is injected through the rubber septum (1) into a 2-neck round bottom flask with 30 ml vanadium chloride reagent (2). The temperature of the reagent is maintained at 85 °C by a beaker of water (3) kept on a hot plate (4) linked to IKATRON® ETS-D4 fuzzy (5). Released NO is carried in an inert N_2 gas stream controlled by a flowmeter (6; flow: 200 cm^2/min) and directed through a condenser (7) to a round-bottom flask with 1 M NaOH (8). The water flow in the condenser was unconventional (*). The condenser and NaOH protects NOA from damage by very hot acid vapour. Neutralized NO vapour is directed to NOA analyser (9).

A standard curve was constructed using water and different concentrations of sodium nitrate 6.25 µM, 12.5 µM, 25 µM, 50 µM and 100 µM (Figure 2.16). In addition to nitrate ions, VCl_3 reagent also reduces both NO_2^- and RSNO to NO, thus providing a "total" plasma measure of nitrate+nitrite+RSNO. Therefore, the amount of nitrite and RSNO obtained by tri-iodide analysis was subtracted from that observed with VCl_3 reagent in order to reflect an accurate nitrate value.

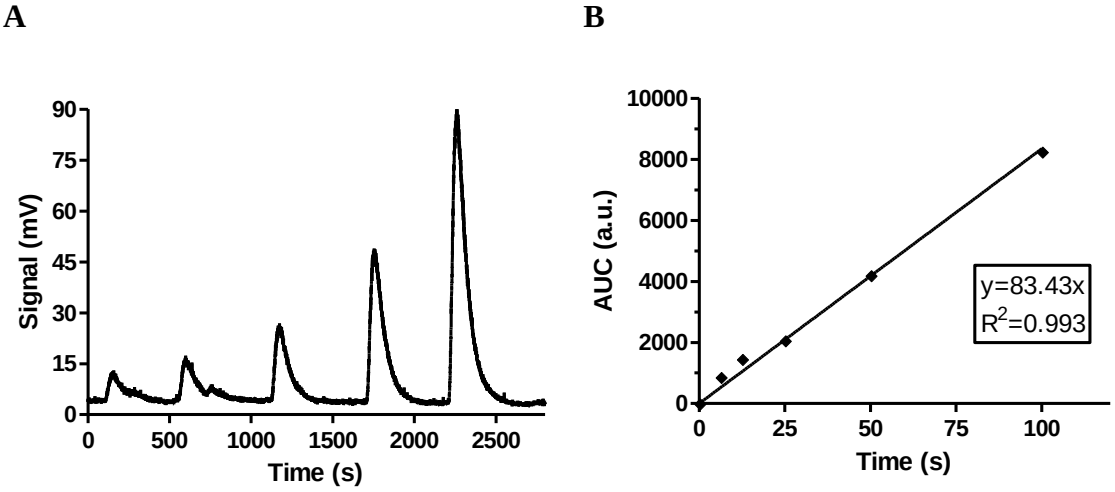


Figure 2.16. Analysis of standard curve for plasma nitrate measurement. Typical signals of 6.25 µM, 12.5 µM, 25 µM, 50 µM and 100 µM sodium nitrate are shown (left). The corresponding AUC of detected peaks (corrected by AUC of water) were used to calculate the slope coefficient of the standard curve (right).

5.2 Measurement of RSNO in Th-SNO preparations

The RSNO was cleaved from thienopyridine-SNO (Th-SNO) preparations (equations below) by 2 C's reagent containing 0.1 mM CuCl and 0.97 mM cysteine. Despite the reagent, the NOA setup was the same as that used for tri-iodide (Figure 2.12). The intra- and inter-assay coefficients of variation (CV) were 7 % and 18 %, respectively (See Shawmendra Bundhoo MD 2011).

$$RS-NO+Cu^{+}+H^{+}\rightarrow RSH+NO+Cu^{2+}$$
$$2RSH+2Cu^{2+}\rightarrow RS-SR+2Cu^{+}+2H^{+}$$

A standard curve was constructed using water and different concentrations of nitroso-N-acetylcysteine (NACSNO) 250 nM, 500 nM, 1000 nM, 2000 nM and 4000 nM. NACSNO was prepared fresh by mixing 1 M acidified N-acetylcysteine (NAC) and 1.1 M sodium nitrite in the dark. Before NOA analysis, the precise concentration of NACSNO was measured by UV spectrophotometer (Figure 2.17).

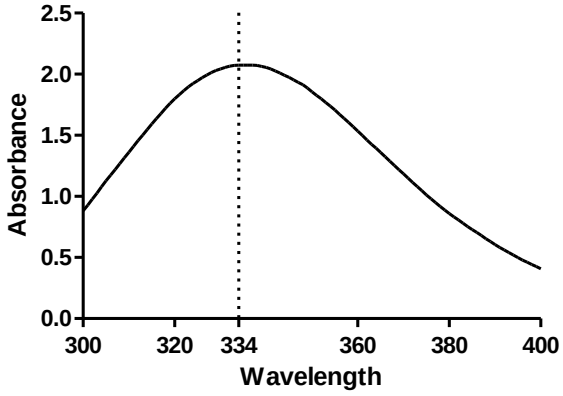


Figure 2.17. Spectrophotometric detection of NAC-SNO. The maximal absorbance of NACSNO at 334 nm was used to calculate the amount of NACSNO. For example, the absorbance 2.073 is equivalent to 0.57 M of NAC-SNO after dilution correction (2.073/727*200=0.57).

It has been shown that the neutrality of 2 C's reagent ensures that other metabolites such as nitrites and nitrates remain undetected in biological samples, providing specificity. However, considering the high concentrations of reagents used to make Th-SNO, the above method was validated for our purposes (see IV. Results, page 149-151).

6. ELISA

All ELISAs were performed using commercially available kits. The absorbance was measured on the FLUOstar OPTIMA (BMG Labtech) or Multiscan EX (LABsystems) depending on the filter required.

6.1 Rabbit aortic rings

6.1.1 Vessel homogenization

Previously frozen vessel segments were weighed and/or cut into smaller pieces and homogenized with 1.4 mm stainless blend beads (Next Advance, SSB14B) using BBX24- Bullet Blender®. Typically, 4 rabbit aortic rings were mixed with 150 µl homogenization buffer and 100 mg beads and homogenised for 10 min at speed 10. Homogenization buffer contained 100 µM IBMX. In a few cases the homogenisation was not efficient, samples were additionally homogenised using a glass homogeniser. Tissue homogenates were centrifuged at 12000 x g for 15 min at 4 °C to remove the cellular debris and tissue supernatants were stored at -20 °C.

The different samples were standardized to total protein content measured by Bradford protein assay (cGMP) or by the weights of the aortic rings (cAMP).

6.1.2 Bradford protein assay

The total protein prior to cGMP measurement in rabbit aortic rings was quantified by the Pierce Coomassie® Protein Assay Reagent (23200) using a

modified Bradford Coomassie® Dye-protein binding colorimetric method. When Coomassie® Dye binds protein in an acidic medium, an immediate shift in absorption maximum occurs from 465 nm to 595 nm with a concomitant colour change from brown to blue.

Tissue supernatants and BSA standards (25-2000 µg/ml) were loaded on a 96-well plate (Greiner Bio-one, 655101) in duplicate followed by addition of Coomassie® Reagent, 10 min incubation on a bench top and readout at 595 nm. BSA standards were prepared in 0.9 % NaCl in homogenisation buffer.

The above method resulted in 800-1400 µg/ml total protein from 4 rabbit aortic rings.

6.1.3 cGMP

cGMP was quantified using cGMP Direct Immunoassay Kit (Abcam®, ab65356) according to the manufacturer instructions. A standard curve was constructed by plotting the mean absorbance for each standard on the y axis against the pmol cGMP/50 µl on the x axis and the best fit curve was adjusted using GraphPad® software (Figure 2.18).

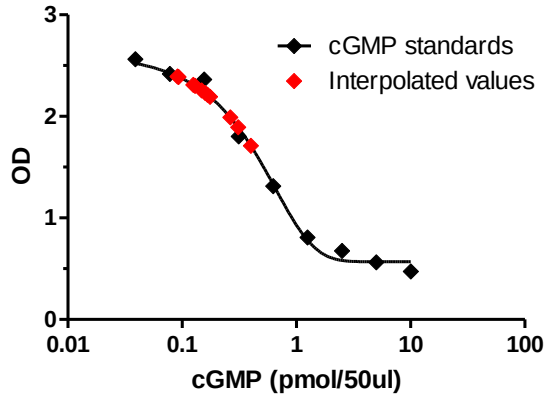


Figure 2.18. Standard curve of cGMP (tissue samples).

6.1.4 cAMP

cAMP was quantified using cAMP Enzyme Immunoassay Kit (R&D systems; KGE002B) according to the manufacturer instructions. OD was determined at 450 nm and 550 nm for correction of optical imperfections in the plate. Readings at 550 nm were subtracted from the readings at 450 nm as recommended by the manufacturer. A standard curve was constructed by plotting the mean absorbance for each standard on a linear y axis against the concentration on a logarithmic x axis and the best fit curve was adjusted using GraphPad® software (Figure 2.19).

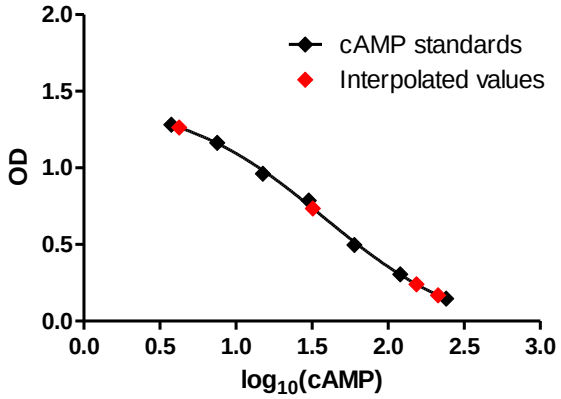


Figure 2.19. Standard curve of cAMP.

6.2 Plasma samples

Platelet poor plasma (ppp) was prepared in the same way as for NO metabolites measurement (see III. General Methods, page 83).

6.2.1 cGMP

cGMP was quantified using cGMP Enzyme Immunoassay Kit (R&D systems; KGE003) according to the manufacturer's instructions. Ppp was diluted 20x in assay buffer. It was essential to incubate immunological complexes on a horizontal orbital microplate shaker (0.12'' orbit) set at 500±50 rpm, otherwise the signal amplitude was very low (Figure 2.20).

OD was determined at 450 nm and 550 nm for correction of optical imperfections in the plate. Readings at 550 nm were subtracted from the readings at 450 nm as recommended by the manufacturer. A standard curve was constructed by plotting the mean absorbance for each standard on a linear y axis against the concentration on a logarithmic x axis and the best fit curve was adjusted using GraphPad® software.

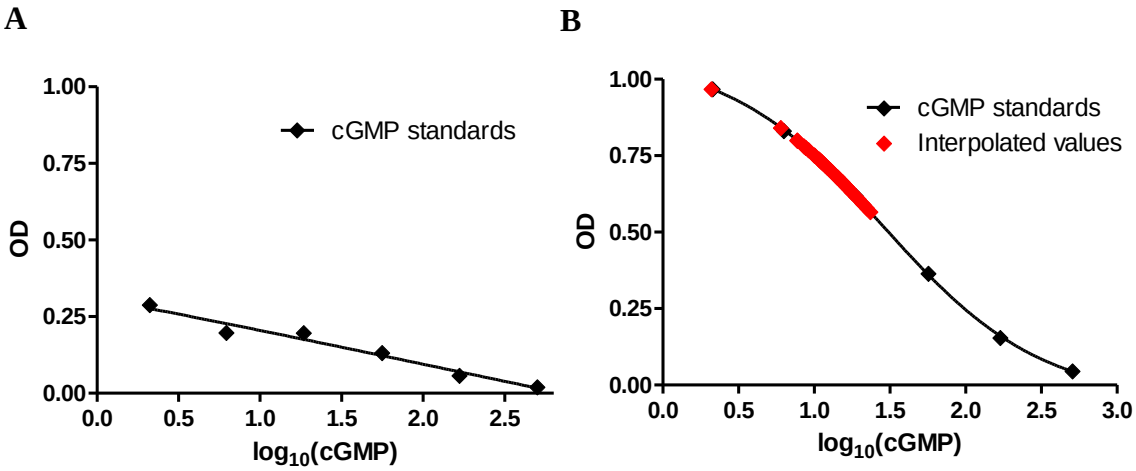


Figure 2.20. Standard curve of cGMP (plasma samples). Inappropriate mixing (A) versus appropriate mixing (B).

6.2.2 Soluble P-selectin

Soluble P-selectin (sP-selectin) was quantified using a sP-selectin Enzyme Immunoassay Kit (R&D systems; BBE6) according to the manufacturer's instructions. Ppp was diluted 15x in assay buffer. OD was determined at 450 nm and 650 nm for correction of optical imperfections in the plate. Readings at 650 nm were subtracted from the readings at 450 nm as recommended by the manufacturer. A standard curve was constructed by plotting the mean absorbance for each standard on the y axis against the concentration on the x axis and the best fit curve was adjusted using GraphPad® software (Figure 2.21).

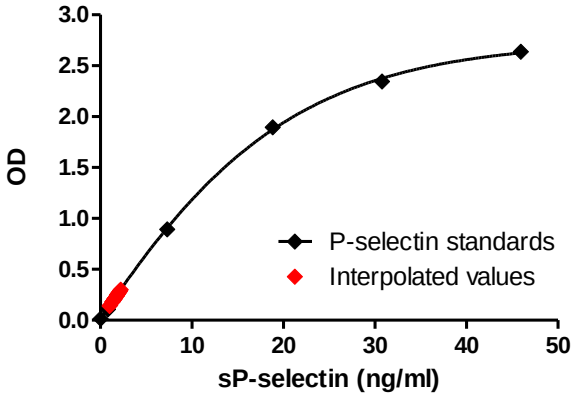


Figure 2.21. Standard curve of sP-selectin.

6.2.3 3-Nitrotyrosine

3-Nitrotyrosine (3-NT) was quantified using a 3-NT Enzyme Immunoassay Kit (Hycult® biotech; HK501-02) according to the manufacturer's instructions. Ppp was diluted 5x in assay buffer. OD was determined at 450 nm and 550 nm for correction of optical imperfections in the plate. Readings at 550 nm were subtracted from the readings at 450 nm as recommended by the manufacturer. A standard curve was constructed by plotting the mean

absorbance for each standard on a linear y axis against the concentration on a logarithmic x axis and the best fit curve was adjusted using GraphPad® software (Figure 2.22).

We experienced difficulties in measuring 3-NT in 8 plasma samples. Although 5x dilution of plasma was optimal for most samples, some had very high OD, which was outside the standard curve. In turn, when higher plasma dilution was used, other samples had too low OD and could not be interpolated. Overall, a greater range of readings was obtained in comparison to other ELISA assays.

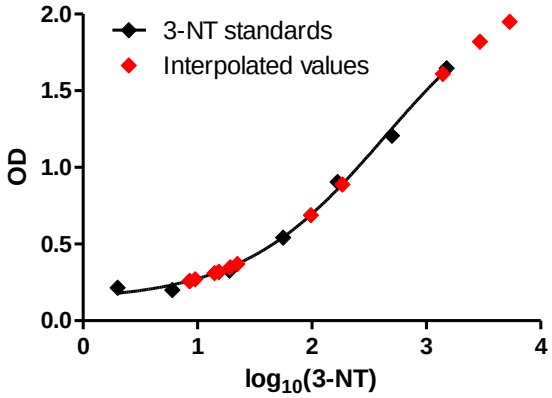


Figure 2.22. Standard curve of 3-Nitrotyrosine (3-NT). Some interpolated values were bigger than the highest 3-NT standard. However, increasing the plasma dilution up to 10x resulted in the OD lower than the lowest 3-NT standard in other samples.

7. Light transmission aggregometry (LTA)

Light transmission aggregometry (LTA) is a laboratory-based technique allowing the evaluation of platelet aggregation in response to agonists[155]. When platelets aggregate, the light absorbance (A) is reduced in time. As a result, the light transmittance (T) increases and can be calculated from the absorbance (equation below), reflecting the degree of platelet aggregation.

$$T = 10^{(2-A)}$$

In our setting, the 96-well plate was loaded with different agonists/antagonists and platelet rich plasma (prp) before it was placed in the spectrophotometer (FLUOstar OPTIMA) for 10 min at 37 °C. Absorbance was measured at 620 nm from the bottom of the plate every 2 min after a 30 s linear shaking of the plate.

7.1 Th-SNO preparations

In order to test aggregatory capacity, thienopyridine-SNO (Th-SNO) was prepared by mixing thienopridine (Th) and nitrite under appropriate conditions (see IV. Results, page 145) and the 96-well plate was loaded first with platelet agonist ADP (20 μM) or PBS as a negative control. Then various concentrations of Th (10 μM-10 mM), Th-SNO (10 μM-10 mM) or nitrite (1-1000 mM) were added. GSNO (0.1-100 μM) was used as a positive control, which reflected 100 % RSNO yield. Prp of healthy subjects was isolated from trisodium citrate Vacutainer bottles by centrifugation (100 g, 10 min, 25 °C) and added to the plate. The increase in transmittance over 10 min was calculated relative to that of ADP-induced aggregation.

7.2 Plasma samples

In order to assess the influence of a 600 mg clopidogrel loading dose on platelet aggregation in coronary artery disease (CAD) patients, a 96-well plate was first loaded with platelet agonists ADP (20 μM) and Thrombin Receptor Activated Peptide (TRAP; 10 μM) or PBS as a negative control. Then prp was isolated from CAD patients in the same way as from healthy subjects (see above) and added to the plate. The increase in transmittance over 10 min was calculated separately relative to that of ADP- and TRAP-induced aggregation.

8. Measurement of reduced thiols (RSH)

The content of reduced thiols (RSH) in chemical and biological preparations was measured using a ThioStar Fluorescent Thiol Detection Reagent (Bioquote) specific for reduced thiols. Fluorescence was recorded by the FLUOstar OPTIMA at the excitation 380 nm and the emission 510 nm. The corresponding concentrations of free thiols were calculated from a standard curve for reduced glutathione (GSH; Figure 2.23).

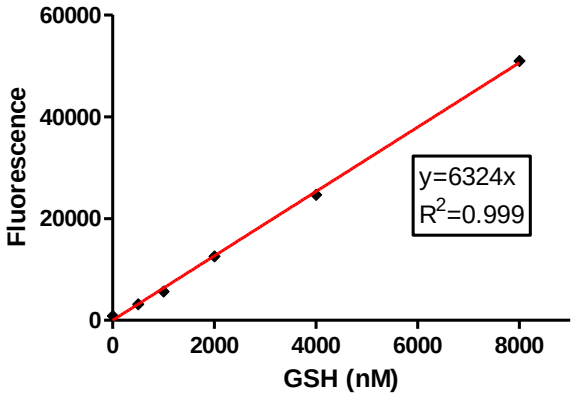


Figure 2.23. Standard curve of GSH used for estimation of RSH in the sample.

8.1 Thienopyridine tablets

Tablets of the commercially available clopidogrel sulphate, prasugrel, ticlopidine and ticagrelor were crushed and dissolved in HPLC water to a final concentration 8.35 mM. After a 10 min incubation at 37 ºC, pH was adjusted to pH=7-8 and samples were loaded onto a 96-well plate in triplicate prior to addition of 2.5 μg/ml ThioStar probe. Fluorescence was recorded 5-15 min after addition of the probe.

8.2 Plasma samples

Platelet poor plasma samples were diluted 1000x in PBS and added in duplicate to a 96-well plate, followed by addition of 2.5 μg/ml ThioStar Reagent. Fluorescence was recorded every 5 min after addition of the probe until fluorescent signals reached plateau (Figure 2.24).

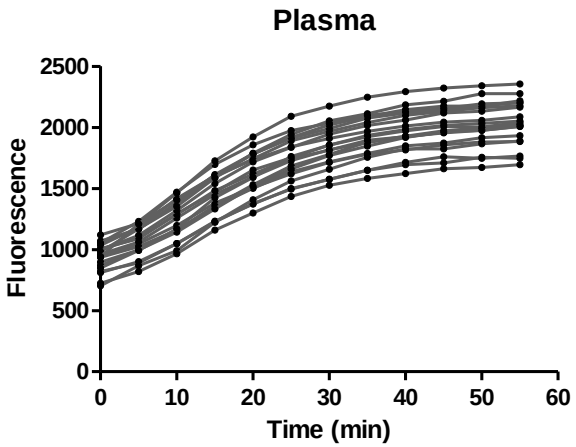


Figure 2.24. Traces of fluorescence signal of plasma samples loaded with ThioStar Reagent changing in time.

9. Measurement of total anti-oxidant capacity (TAC)

The principle of the TAC assay, also known as oxygen radical absorbance capacity (ORAC) assay has been described in detail elsewhere[32, 33]. Briefly, it is based on the *in vitro* inhibition by anti-oxidants of the free radical damage to a fluorescent probe. This inhibition is observed as a preservation of the fluorescent signal over time and is quantified by calculating the resultant difference in AUC. It is usually reported in comparison to a standard anti-oxidant preparation.

Samples were diluted in PBS and added in triplicate to a 96-well plate loaded with sodium fluorescein (10 nM). 70 μM 4-hydroxy-TEMPO (tempol; Sigma) was used as a standard anti-oxidant on each plate. Then, either water (blank) or 2,2'-azobis-2-methyl-propanimidamide (AAPH, 240 mM) were added prior to measurement. AAPH starts producing peroxyl radicals immediately after being dissolved, which is accelerated by raising the temperature.

Fluorescence measurement was performed on the FLUOstar OPTIMA for 60-90 min at 37 °C with 485 nm excitation and 520 nm emission. Fluorescence was measured (from the top) every minute after a short orbital shaking of the plate.

9.1 Anti-oxidants

Different concentrations 12.5 – 200 μM of the standard anti-oxidant chemicals (tempol; Sigma) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox; Sigma), and clopidogrel sulphate (LKT Laboratories, Inc) were prepared in PBS and used in the TAC assay to compare anti-oxidant capacity of samples.

9.2 Plasma samples

Although TAC assay is commonly used to assess the anti-oxidant power of various compounds, this method is not well described in plasma. Plasma is a good buffering system thanks to a rich pool of anti-oxidant proteins, which provide a very condense milieu. Hence, in order to measure its anti-oxidant properties, it requires high dilution. After running a series of plasma dilutions 10-4000x, we chose 1000x dilution because it showed the most similar fluorescence profile to tempol in terms of time of protection against AAPH (Figure 2.25).

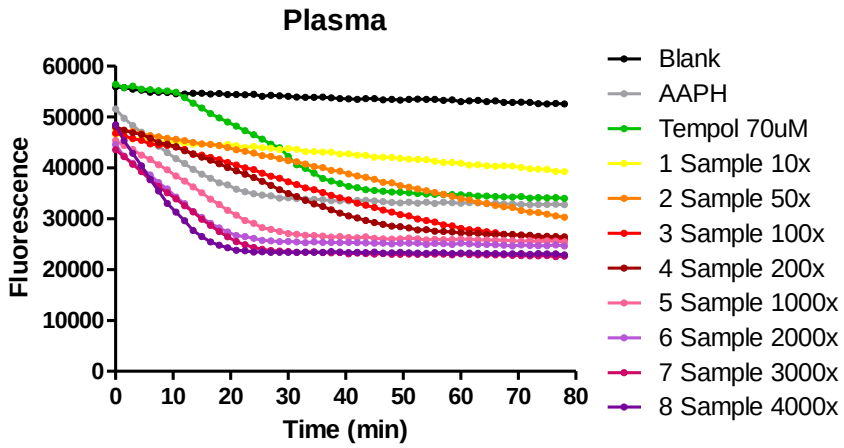


Figure 2.25. Fluorescence profiles of different plasma dilutions after addition of AAPH. Note that dilutions above were added and further diluted 8x on the 96-well plate.

9.3 Method of calculation

Differences between AUC of protected and unprotected loss in fluorescence were calculated (equation 1 below) to assess the anti-oxidant protection of tempol, trolox and clopidogrel. In case of plasma samples, despite previous adjustment for plasma dilution, the baseline fluorescence and the fluorescent profile were different from the standard in water. Therefore, plasma samples were assessed differently (equation 2 below) to avoid negative numbers (Figure 2.26).

$$TAC_{sample} = AUC_{sample+AAPH} - AUC_{AAPH} \quad (1)$$

$$TAC_{plasma} = \frac{AUC_{plasma+AAPH}}{AUC_{tempol-AAPH}} * 100\% \quad (2)$$

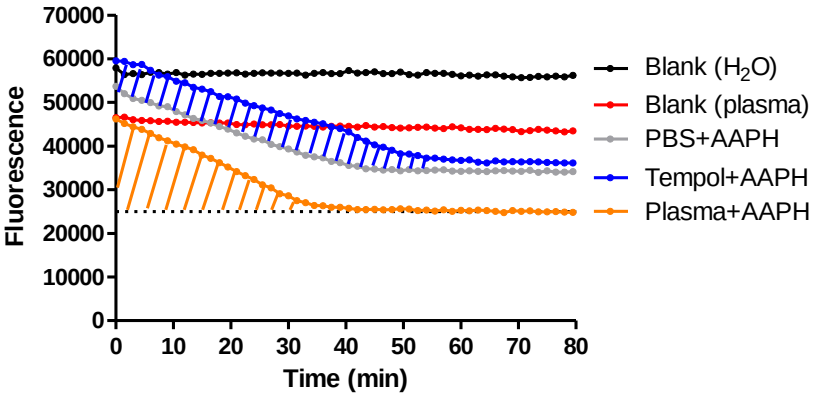


Figure 2.26. Fluorescence profiles of plasma (1000x) and 70 µM tempol before or after addition of AAPH. In blue: $AUC_{tempol\text{-}AAPH}$ and in yellow: $AUC_{plasma+AAPH}$.

IV. RESULTS

Part 1: Direct influence of clopidogrel on vascular NO homeostasis

Hypothesis: ***Parental form of clopidogrel primes vascular tissue to exhibit enhanced vasodilation.***

Specific aims of this chapter were:

- To determine whether the parental form of clopidogrel (pro-clopidogrel) changes the responsiveness of vessels to endogenous or exogenous NO.
- To investigate the potential anti-oxidant and anti-inflammatory effects of pro-clopidogrel on vascular tissue.

1. Introduction

Inhibition of $P2Y_{12}$ receptors on platelets has other consequences for the whole cardiovascular system. Activated platelets release a range of cytokines and chemokines, which attract immune cells, including leukocytes to inflammatory sites on vascular endothelium and aggravate inflammatory responses. In patients with endothelial dysfunction and atherosclerosis the non-resolved inflammation leads to amplification of thrombosis and causes further damage to the vasculature. That is why the inhibition of platelet activation and aggregation attenuates excessive inflammation, which helps to restore the vascular function.

Clopidogrel therapy has been associated with numerous anti-inflammatory effects[156-162]. These studies have demonstrated that: patients undergoing coronary arterial stenting had significantly lower levels of P-selectin, IL-1α, IL-2, IL-6, IL-13, TNF-β and TNF-α after receiving a maintenance dose of clopidogrel for at least 6 months[156]; plasma levels of hsCRP, sCD40L and RANTES and urinary excretion of 8-iso-prostaglandin F2α were decreased in CAD patients taking clopidogrel for at least 5 weeks[157]; platelet-leukocyte interactions, monocyte activation and plasma levels of RANTES were reduced in patients with type 2 diabetes mellitus receiving a maintenance dose of clopidogrel for 28 days[160]. Further anti-inflammatory effects of clopidogrel were also observed in animal models. For instance, tissue levels of CD40L and TF were lower in rabbit ischemic coronary artery after infusion of clopidogrel[159] and serum and tissue levels of P-selectin, ICAM-1, VCAM-1 and MCP-1 were decreased by clopidogrel treatment in rabbits with early signs of athelosclerosis[158].

Since 1997, when clopidogrel was introduced into the market, numerous pleiotropic effects of the drug have been reported and some of them have not been fully explained by differences in drug metabolism between individuals. In *in vitro* studies, clopidogrel increased intracellular levels of $[Ca^{2+}]_i$ and nitrite in the immortalized HUVEC cell line ECV304

without an influence on viability of cells[163] implying an influence on NOS activity. Furthermore, clopidogrel exhibits anti-platelet actions when pre-incubated with whole blood prior to addition of ADP, an effect not seen in platelet-rich plasma[164]. The authors of the latter study speculated that blood cells could biotransform clopidogrel into its main active metabolite. Others have observed that clopidogrel reduces ectonucleotidase activity of HUVECs, impairing the ability of vascular endothelial cells to prevent platelet aggregation by inhibition of ADP and AMP hydrolysis[165]. It is important to appreciate that all these *in vitro* effects were observed for the pro-clopidogrel form (without metabolism), which is not purported to inhibit $P2Y_{12}$ receptors directly without conversion to its active metabolite.

In animal models and humans, clopidogrel has been shown to influence vascular tone[166, 167], improve vascular function and increase release of endothelium-dependent relaxants[168]. Namely, clopidogrel (i) normalized increased PE-induced constriction and impaired Ach-induced relaxation in small mesenteric arteries from AngII-hypertensive rats[166], (ii) attenuated arterial constriction in rabbit and rat aortic rings[167], (iii) improved Ach-induced forearm blood flow (FBF) in CAD patients[157], (iv) increased flow-mediated dilation (FMD) in CAD patients[169] and those undergoing PCI[170] and (v) was associated with increased NO bioavailability[157, 159, 171] and release of prostaglandins[172]. The origin of vasomodulatory actions of clopidogrel is poorly understood and is the focus of the work in this chapter.

$P2Y_{12}$ receptors have been identified on endothelial and smooth muscle cells of human aorta and coronary arteries[173, 174], and it has been suggested that ADP-stimulated responses via $P2Y_{12}$ receptor activation might not be limited solely to platelets. This is in accord with studies on isolated guinea pig heart, where clopidogrel produced an eNOS-dependent increase in coronary flow *ex vivo*[171] and in CAD patients, where a loading dose of clopidogrel increased FMD of the brachial artery without concomitant inhibitory effects on platelet aggregation[169]. On the other hand, it was shown in small mesenteric arteries from AngII-

hypertensive rats that there is rather low expression of $P2Y_{12}$ receptors and that vascular responses to the $P2Y_{12}$ receptor agonist 2-MeS-ADP were not influenced by clopidogrel. This suggests a minor contribution of vascular $P2Y_{12}$ receptors in clopidogrel-mediated effects on vascular function in this model[166]. Additionally, studies using $P2Y_{12}$ KO mice revealed that the platelet $P2Y_{12}$ receptor, introduced with bone marrow transplantation, influences the vessel wall response to arterial injury and thrombosis to a greater extent than vascular $P2Y_{12}$ receptors[148].

The active metabolite of clopidogrel constitutes only less than 0.01% of the parent drug[139]. Despite this, treatment with clopidogrel provides numerous beneficial effects on the maintenance of vascular homeostasis and clopidogrel remains the most commonly prescribed $P2Y_{12}$ antagonist worldwide. This disproportion suggests alternative but important complementary pathways of clopidogrel action.

This study was designed to investigate in detail the capacity of the pro-drug clopidogrel to influence vascular function *in vitro* using isolated rabbit vessel preparations. Model systems were set up to examine possible differential effects of clopidogrel on the vascular responses to endogenous and exogenous sources of NO and their dependence on vascular $P2Y_{12}$ receptor mediated pathways, and the role(s) of documented anti-oxidant and anti-inflammatory properties of parental clopidogrel in this process.

2. Methods

2.1 Pre-incubation of aortic rings with clopidogrel

Prior to any experiments, aortic rings isolated from rabbits or mice were incubated in Krebs buffer (for composition see III. General Methods, page 68) with or without purified clopidogrel (Axxora Uk Ltd.); the material was relatively soluble and was dissolved directly in the buffer. Typically, 40 ml of Krebs buffer was warmed up to 37 °C in the water bath and oxygenated with gas (95% O_2/5% CO_2) before immersion of 2-6 aortic rings. As vessels settled at the bottom of the 50 ml tube, narrow gauge tubing supplying the gas was fixed at a position approximately half-way through the volume of the buffer to provide sufficient aeration but ensuring not to disturb the vascular integrity (Figure 3.1). It was essential that gas bubbles were gentle and did not cause vessels to agitate excessively.

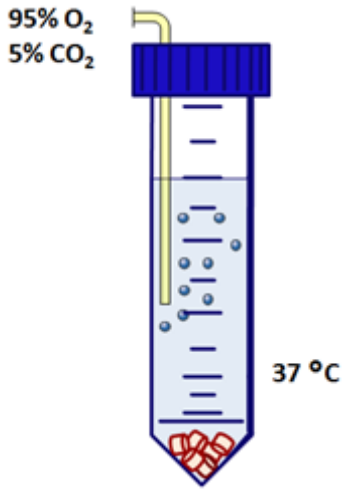


Figure 3.1. Setup for pre-incubation of aortic rings in Krebs buffer +/- clopidogrel.

Aortic rings pre-incubated in Krebs buffer with or without clopidogrel were used immediately for the isometric tension or EPR studies. Alternatively, they were snap frozen in

liquid nitrogen and stored in -80 °C for the cGMP and cAMP measurements by ELISA, and for VCAM-1, TNF- α and IL-6 mRNA relative gene expression by real time qPCR (RT qPCR). Samples intended for cAMP and cGMP analysis were incubated for 15 min with IBMX (PDE inhibitor; 100 μM) before freezing. A simplified scheme of experimental protocol is presented in Figure 3.2; for detailed method description please refer to II Methods.

2.1.1 Denudation.

In experiments with denuded vessels, the endothelium was carefully removed by gently rubbing the internal surface of the tissue ring with a wooden applicator before or after the pre-incubation stage. Removal of endothelium was confirmed by the absence of a vasodilator response to Ach (10 μM) in pre-constricted vessels.

2.1.2 Use of pharmacological inhibitors

Pharmacological inhibitors were used at the following concentrations: L-NMMA (eNOS inhibitor; 10 μM; Enzo Life sciences), indomethacin (COX inhibitor; 10 μM), CGS-15943 (A2 receptor inhibitor; 1 μM) and ODQ (sGC-inhibitor; 10 μM). Concentrations of L-NMMA, indomethacin and ODQ were chosen on the basis of work of others[42] (Shawmendra Bundhoo MD 2011 and Andrew Pinder PhD 2009) to maximally inhibit their target molecules in given conditions. The concentration of CGS-15943 was selected on the basis of published data[175] and additionally checked for efficiency against blocking adenosine receptor agonist (NECA); 1 μM CGS-15943 decreased the vascular total relaxation to NECA by 73.85% (+CGS-15943 Rmax=18.72±0.25% vs. -CGS-15943 Rmax=71.60±1.75%, n=1).

Stock solutions of L-NMMA (in water) , indomethacin (in DMSO) and CGS-15943 (in DMSO) were diluted in the pre-incubation buffer and were present for the whole pre-incubation period, whereas ODQ was added to tissue baths for 0.5 h before pre-constricting with PE.

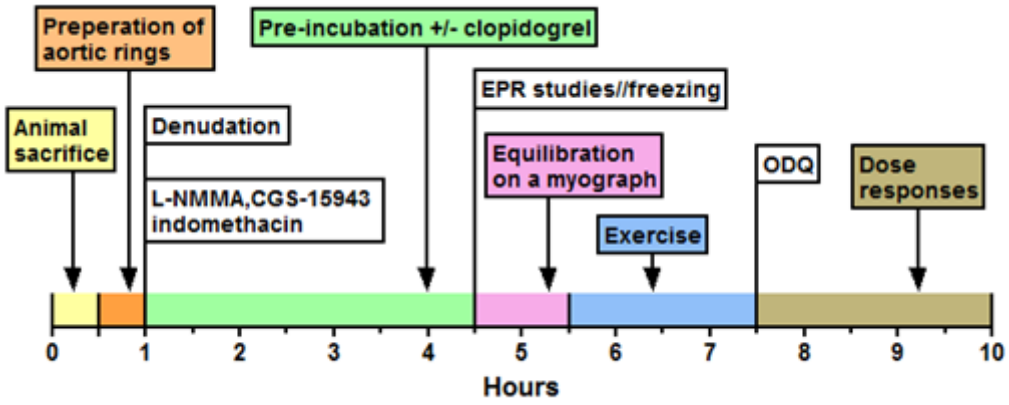


Figure 3.2. Time scale of experiments in isolated aortic rings. Exercise – a period when aortic rings were constricted with PE and relaxed with Ach for three times to achieve the maximal responses.

2.2 Data analysis and statistics

The results are expressed as mean±SD and "n" represent the number of independent experiments. In all studies, data was obtained from 2-4 aortic rings of individual animals and matched with corresponding controls to give an "n" of 1.

Concentration-response to agonists were fitted by nonlinear regression curves (sigmoidal dose-response) and concentrations producing 50 % response [$\log_{10}(EC_{50})$] were determined. EC_{50}Ratio (EC_{50}R) with a 95 % confidence interval was used to report the change of EC_{50} by clopidogrel in relation to corresponding controls and to compare this change between different experimental settings (e.g. in the presence of inhibitors). EC_{50}R was

computed using the EC_{50} Shift function in GraphPad Prism®, according to the formula: $EC_{50}R = EC_{50}sample / EC_{50}control$ (see III. General Methods, pages 69-70).

EPR spectra were smoothed using polynomial fitting (2nd order) with adjacent averaging. The area under curve (AUC) of all component peaks of the EPR spectra was analysed individually and $log_{10}(AUC)$ was computed to compare the relative amounts of superoxide.

Differences between groups were analysed using 2-tailed student's *t*-test unless stated otherwise. Paired analysis was used to compare a single treatment of aortic rings with matched controls on the same experimental day while the unpaired analysis was used to compare results coming from different experimental days. A normal distribution between group differences was assumed on the basis of the interval data type and similar data size.

All analysis was performed using GraphPad Prism® (version 5) software.

3. Results

3.1 Pre-incubation of vessels with clopidogrel enhances vasodilation to NO donors

3.1.1 Model development - rabbit aortic rings

An *in vitro* model of isolated rabbit aorta was developed in order to study the potential direct vascular effects of clopidogrel. Simple addition of clopidogrel to organ baths did not induce any immediate changes in contractile or relaxation responses of mounted vessels (as previously shown[167]). Therefore, aortic rings were pre-incubated in oxygenated Krebs buffer at 37 °C with or without clopidogrel (1000 µM) for 2, 3.5 and 6 h prior to isometric tension studies. After 2 h pre-incubation, responses to Ach or the exogenous NO donor GSNO were still unaffected by clopidogrel. Pre-incubation for 3.5 h caused an enhanced relaxation to GSNO in comparison to controls [$\log_{10}(EC_{50})$=-7.22±0.21 vs. $\log_{10}(EC_{50})$=-7.00±0.29, paired t-test, n=4, p=0.021] but not to Ach [$\log_{10}(EC_{50})$=-6.80±0.21 vs. $\log_{10}(EC_{50})$=-6.91±0.21, paired t-test, n=4, p=0.230; Figure 3.3 and 3.4]. This effect was not maintained over time; after 6 h pre-incubation, there was a substantial loss of vascular response to Ach compared to controls [$\log_{10}(EC_{50})$=-6.45±0.05 vs. $\log_{10}(EC_{50})$=-6.73±0.07, n=1] and also the enhanced response to GSNO was smaller than at 3.5 h [$\log_{10}(EC_{50})$=-6.92±0.05 vs. $\log_{10}(EC_{50})$=-6.84±0.01, n=1].

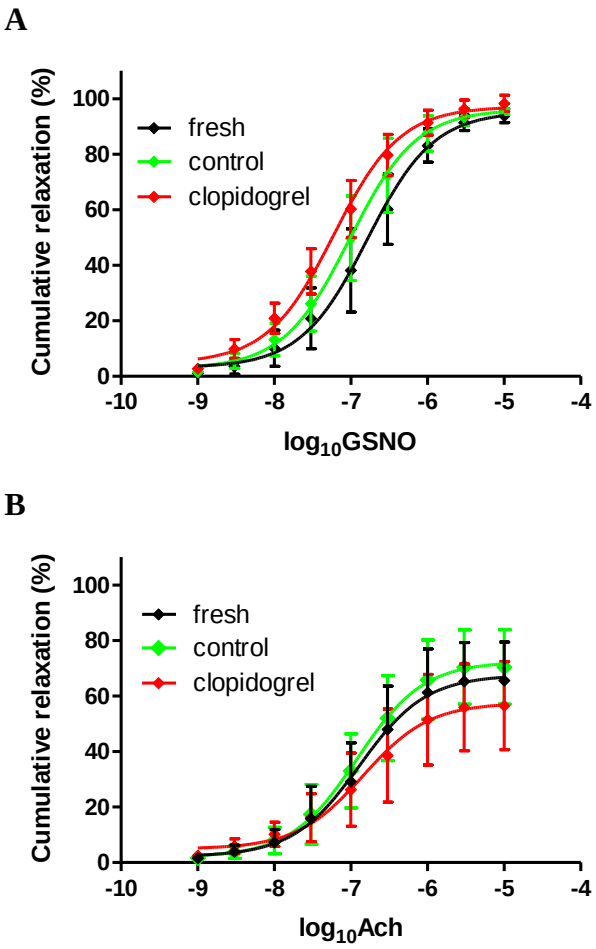


Figure 3.3. Concentration response curves to GSNO (A) and Ach (B) after pre-incubation of rabbit aortae for 3.5 h with clopidogrel (1000 µM). "Fresh" represents responses of aortic rings without the pre-incubation and "control" represent responses of aortic rings pre-incubated in Krebs buffer; n=4.

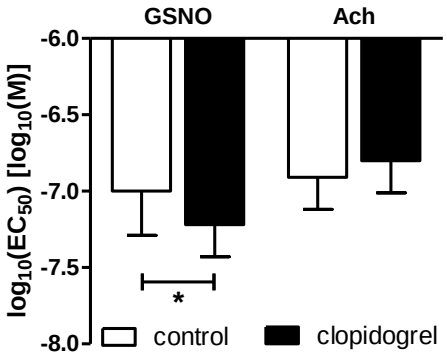


Figure 3.4. Influence of clopidogrel (1000 µM) on the GSNO- and Ach-induced relaxation of rabbit aortae. Paired 2-tailed student's *t*-test, n=4, *p<0.05.

The temperature at which vessels were kept during the pre-incubation period was crucial. In separate studies, we noticed that by lowering the temperature to 32 °C, we could prolong the time of incubation up to 6 h in order to increase the vascular response to GSNO even further without inducing significant damage to endothelium (see Shawmendra Bundhoo MD 2011). However, in order to provide the most physiological conditions, aortic rings were pre-incubated with or without clopidogrel at 37 °C for only 3.5 h for the majority of the studies presented in this thesis, since this provided maximal influence on GSNO responses without affecting Ach/PE and vessel integrity.

3.1.2 The influence of clopidogrel on constriction to PE

The cummulative concentration response to PE was tested; EC_{50} was not affected in the clopidogrel-treated group compared to controls [$\log_{10}(EC_{50})$=-6.76±0.02 vs. $\log_{10}(EC_{50})$=-6.83±0.02, paired t-test, n=4, p=0.069]. Although a trend towards decreased response to PE in clopidogrel-treated vessels was observed, this is unlikely the reason for the observed enhancement in vasodilation to GSNO (Figure 3.5).

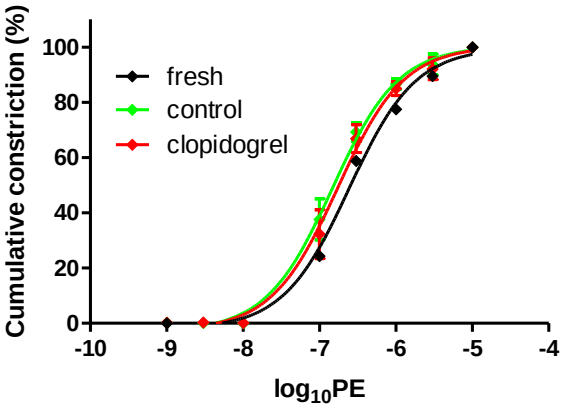


Figure 3.5. PE-induced constriction of rabbit aortae pre-incubated +/- clopidogrel. "Fresh" represents responses of aortic rings without the pre-incubation and "control" represent responses of aortic rings pre-incubated in Krebs buffer; n=4. A comparison of log(EC_{50}) is presented on page 120.

3.1.3 Comparison of different NO donors

Clopidogrel enhanced vessel response to GSNO, an NO donor which belongs to the group of S-nitrosothiols, relaxing smooth muscle via the SNO moiety. In real terms, it is an NO^+ moiety which is released from nitrosothiols following cleavage of the S-NO bond (such as with *hv* or metal ion). In order to assess whether our observations were specific to GSNO or S-nitroso-metabolism by tissue in particular, other NO donors such as NOC9 and ISDN were also tested (Figure 3.6). Clopidogrel enhanced NOC9-induced relaxation responses in comparison to controls [$\log_{10}(EC_{50})$=-8.00±0.03 vs. $\log_{10}(EC_{50})$=-7.90±0.04, paired t-test, n=4, p=0.013]. On the other hand, clopidogrel did not influence the relaxation of rabbit aortic rings to ISDN [$\log_{10}(EC_{50})$=-6.67±0.03 vs. $\log_{10}(EC_{50})$=-6.71±0.03, paired t-test, n=4, p=0.426].

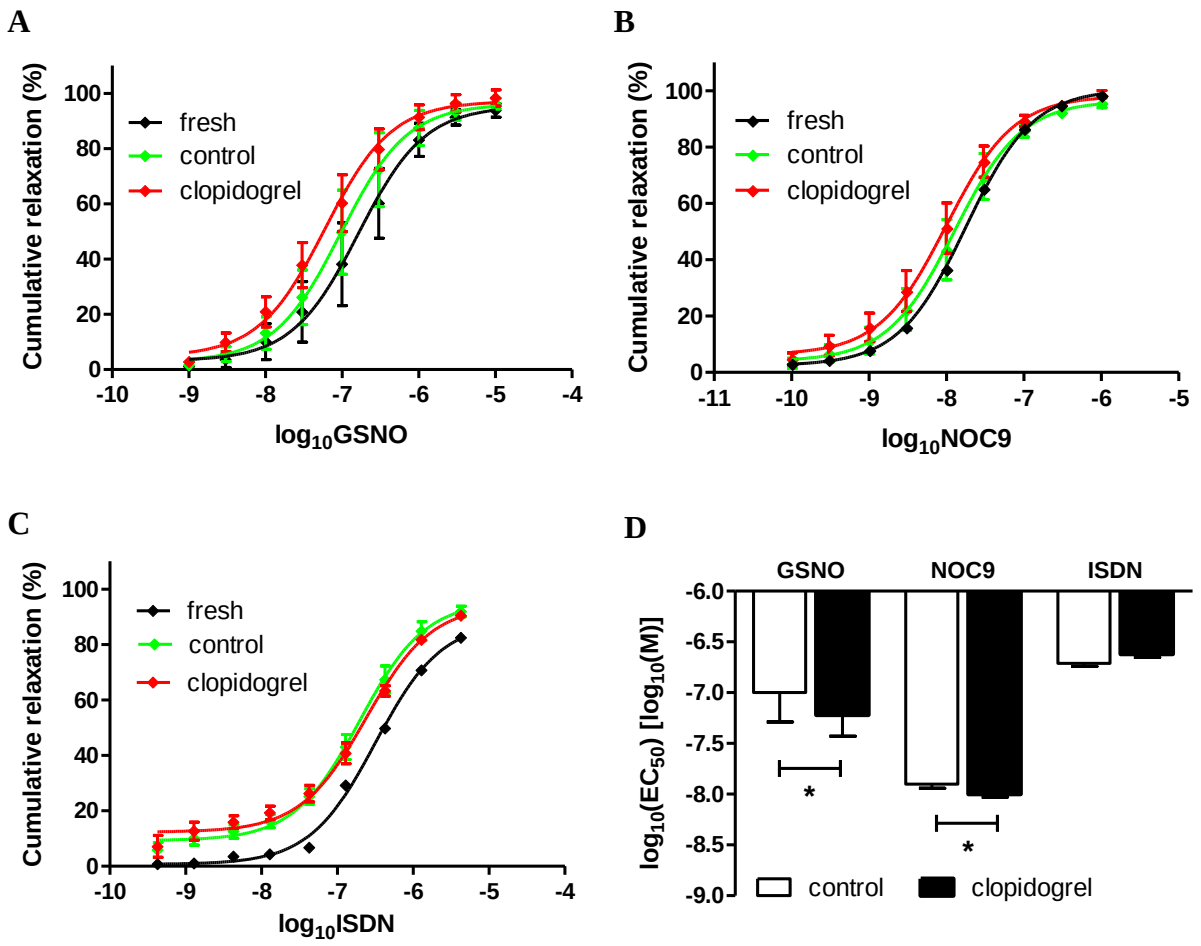


Figure 3.6. GSNO-, NOC9-and ISDN-induced relaxation of rabbit aortae +/-clopidogrel. (A) concentration response curves to GSNO (graph is reused for comparison); (B) concentration response curves to NOC9; (C) concentration response curves to ISDN; (D) Comparison of log(EC_{50}) of GSNO, NOC9 and ISDN**.** "Fresh" represent responses of aortic rings without the pre-incubation and "control" represent responses of aortic rings pre-incubated in Krebs buffer. Paired 2-tailed student's *t*-test, n=4, *p<0.05.

It may be of importance that ISDN exhibited a different relaxation profile compared to GSNO and NOC9 (Figure 3.7). ISDN needs to be metabolized in the smooth muscle before it begins to take an effect, which influences not only time (approx. 42 min for total vasorelaxation to ISDN vs. approx. 27 min in case of GSNO or NOC9) but also the effectiveness of relaxation [$\log_{10}(EC_{50})$ ISDN=-6.71±0.03 vs. $\log_{10}(EC_{50})$ GSNO=-7.09±0.24 vs. $\log_{10}(EC_{50})$ NOC9=-7.90±0.04]. This might explain, in part, the reason why enhanced relaxation was not observed in the case of ISDN.

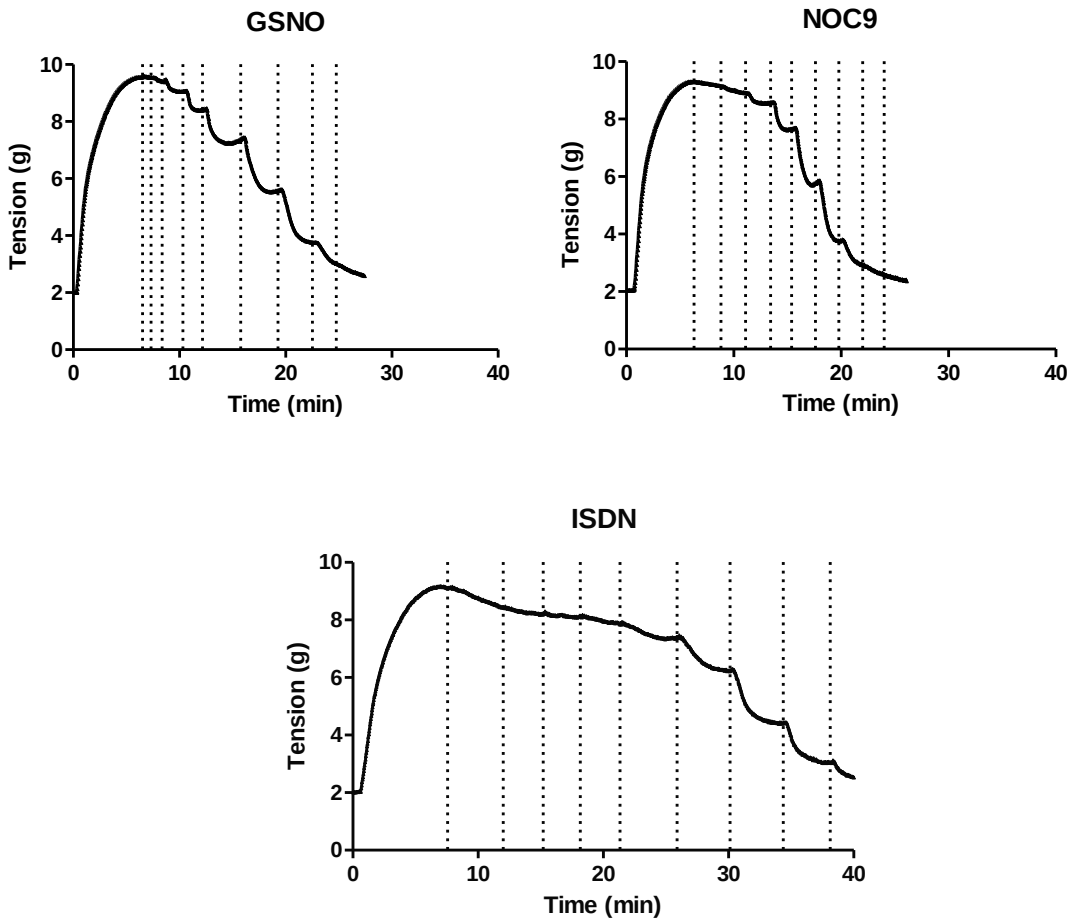


Figure 3.7. Relaxation profiles to GSNO, NOC9 and ISDN in rabbit aortae.

3.2 Mechanism of enhanced GSNO-induced vasorelaxation by clopidogrel

3.2.1 Involvement of $P2Y_{12}$ receptors

Clopidogrel is purported to be a selective $P2Y_{12}$ receptor antagonist and only the active metabolite of clopidogrel formed in the liver can bind to the receptor. In our vascular model, the parental form of clopidogrel was used and therefore neither the presence of the active drug metabolism, nor activation of $P2Y_{12}$ receptors in rabbit aortae was expected. Nevertheless, in order to test whether the enhanced response of clopidogrel-stimulated tissue

to exogenous NO donors depends on the presence of $P2Y_{12}$ receptor in the vasculature, we carried out similar studies in $P2Y_{12}^{-/-}$ mice in comparison to wild type littermates.

3.2.1.1 Model development - mouse aortic rings

Aortic rings isolated from $P2Y_{12}^{+/+}$ (WT) and $P2Y_{12}^{-/-}$ (KO) mice were kept in oxygenated Krebs buffer with or without clopidogrel at 37 °C for 3.5 h (akin to the rabbit studies described above – see IV. Results, pages 110-112). However, the concentration of clopidogrel had to be lowered to 100 µM as the 1000 µM concentration limited vascular constriction responses up to 50 % (tested in WT mice). In comparison to controls 100 µM clopidogrel did not affect PE response both in WT [$\log_{10}(EC_{50})$=-7.14±0.02 vs. $\log_{10}(EC_{50})$=-7.08±0.03, paired t-test, n=6, p=0.263], and KO $P2Y_{12}$ mice [$\log_{10}(EC_{50})$=-7.05±0.02 vs. $\log_{10}(EC_{50})$=-6.97±0.03, paired t-test, n=6, p=0.077; Figure 3.8], respectively. Furthermore, the effect of clopidogrel on PE response was not different between phenotypes ($P2Y_{12}^{+/+}$ EC_{50}R PE vs. $P2Y_{12}^{-/-}$ EC_{50}R PE, unpaired t-test, n=6, p=0.527). Therefore, it was decided to pre-incubate mouse aortic rings with 100 µM clopidogrel in further studies.

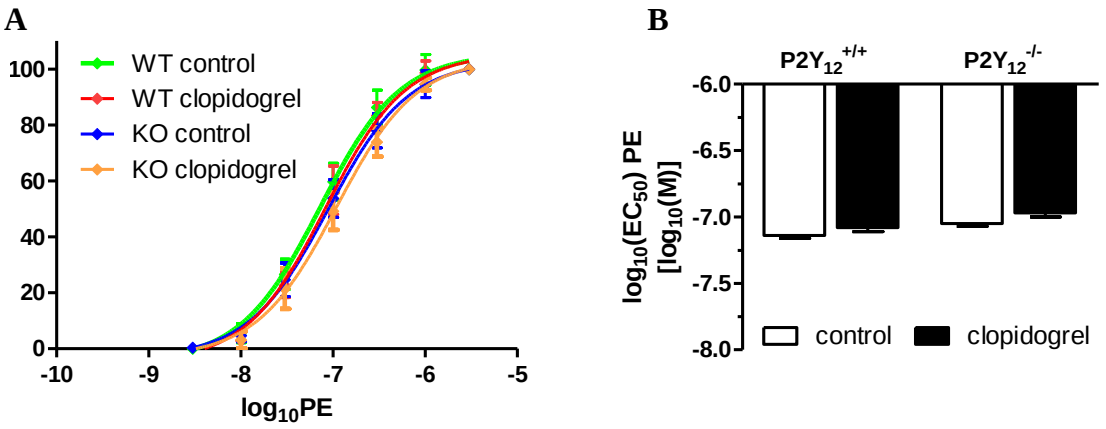


Figure 3.8. PE-induced constriction of aortae from $P2Y_{12}$ WT and KO mice pre-incubated +/- clopidogrel. (A) concentration response curves to PE; (B) comparison of log(EC_{50}) of PE. Paired 2-tailed student's *t*-test, n=6, p>0.05.

In terms of endothelium-dependent vasodilation, the Ach concentration response in mouse aorta was different to that in rabbit tissues; total relaxation to Ach was higher in mouse (mouse Rmax=95.4±7.0 % vs. rabbit Rmax=70.6±13.5 %), as well as Ach potency was higher [mouse $\log_{10}(EC_{50})$=-4.40±0.43 vs. rabbit $\log_{10}(EC_{50})$=-3.89±0.22; Figure 3.9].

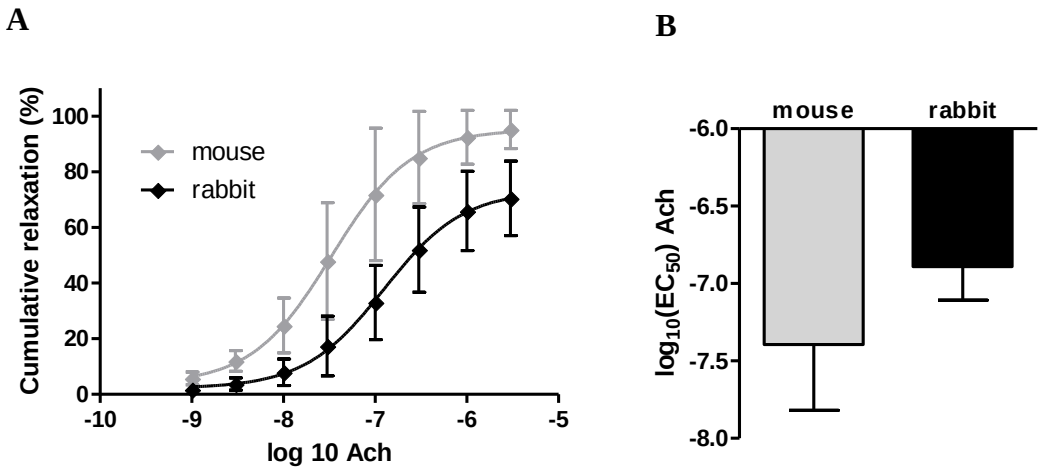


Figure 3.9. Ach-induced relaxation of mouse and rabbit aortae. (A) concentration response curves to Ach, n=4; (B) comparison of log(EC_{50}) of Ach.

In comparison to controls 100 μM clopidogrel significantly decreased vessel response to Ach both in WT [$\log_{10}(EC_{50})$=-7.48±0.08 vs. $\log_{10}(EC_{50})$=-7.37±0.06, paired t-test, n=6, p=0.006], and KO $P2Y_{12}$ mice [$\log_{10}(EC_{50})$=-7.52±0.08 vs. $\log_{10}(EC_{50})$=-7.36±0.07, paired t-test, n=6, p=0.014; Figure 3.10), respectively. The effect of clopidogrel on Ach response was not different between phenotypes ($P2Y_{12}^{+/+}$ EC_{50}R Ach vs. $P2Y_{12}^{-/-}$ EC_{50}R Ach, unpaired t-test, n=6, p=0.532).

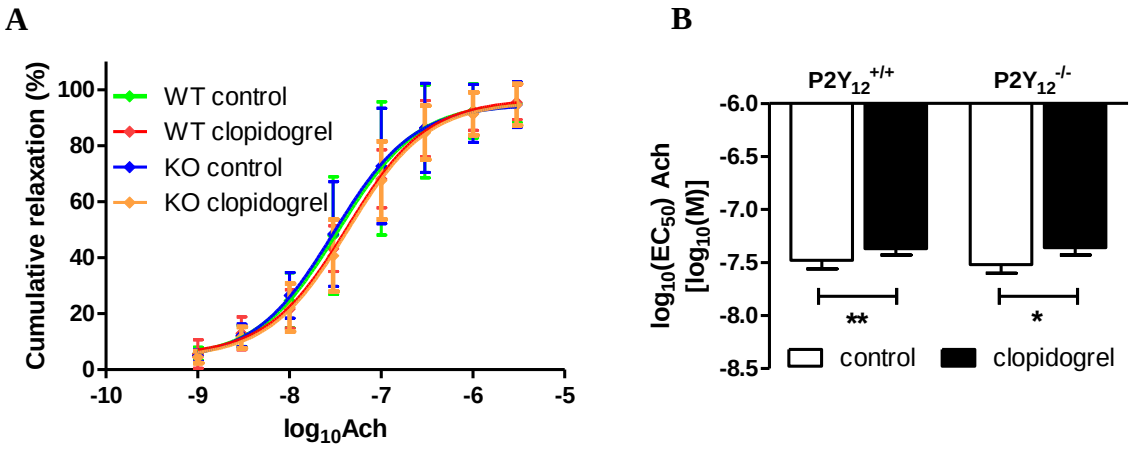


Figure 3.10. Ach-induced relaxation of aortae from $P2Y_{12}$ WT and KO mice pre-incubated +/- clopidogrel. (A) concentration response curves to Ach; (B) comparison of log(EC_{50}) of Ach. Paired 2-tailed student's *t*-test, n=6, *p<0.05, **p<0.01.

3.2.1.2 Enhanced response to GSNO

The primary question being addressed was whether clopidogrel enhanced responsiveness of mouse aortae to GSNO similarly to that observed for rabbit tissue. Studies in genetically matched WT strain mice revealed that clopidogrel increased significantly the GSNO response in mouse aortae compared to controls [$\log_{10}(EC_{50})$=-7.19±0.08 vs. $\log_{10}(EC_{50})$=-6.94±0.04, paired t-test, n=6, p=0.015; Figure 3.11]. Having confirmed this, parallel studies were carried out in $P2Y_{12}^{+/+}$ and $P2Y_{12}^{-/-}$KO mice to see if the presence of $P2Y_{12}$ receptor made an impact. Clopidogrel improved significantly the GSNO response in

KO mice compared to controls [$\log_{10}(EC_{50})$=-7.34±0.08 vs. $\log_{10}(EC_{50})$=-7.06±0.05, paired t-test, n=6, p=0.018] and the extent of improvement was similar to the WT strain ($P2Y_{12}^{+/+}$ EC_{50}R GSNO vs. $P2Y_{12}^{-/-}$ EC_{50}R GSNO, unpaired t-test, n=0.796), confirming that clopidogrel enhances vascular response to GSNO independent of $P2Y_{12}$ receptor interaction.

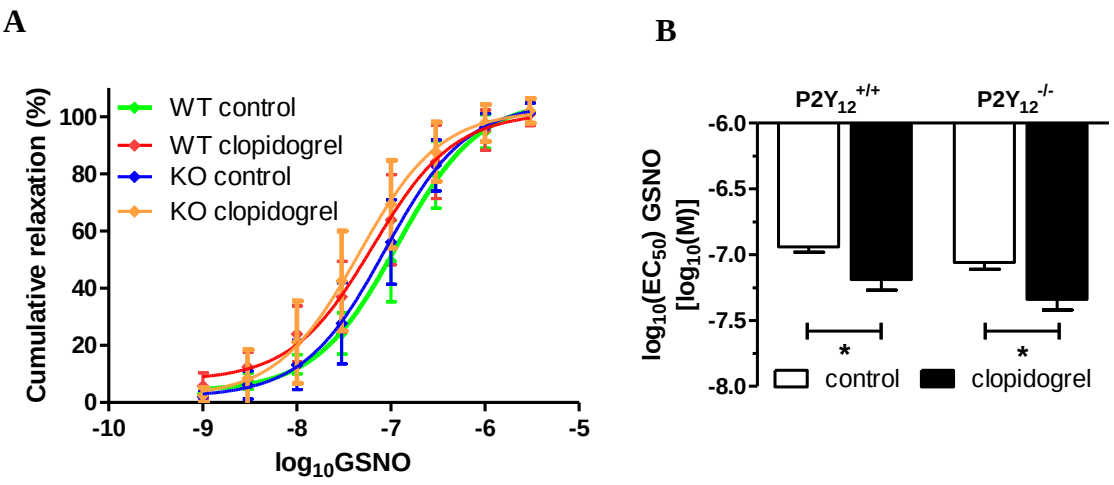


Figure 3.11. GSNO-induced relaxation of aortae from $P2Y_{12}$ WT and KO mice pre-incubated +/- clopidogrel. (A) concentration response curves to GSNO; (B) comparison of log(EC_{50}) of GSNO. Paired 2-tailed student's *t*-test, n=6, *p<0.05.

3.2.2 Involvement of endothelium

The site of interaction between clopidogrel and vessels was studied by assessing the role of vascular endothelium in our model. Rabbit aortic rings denuded of endothelium were incubated with and without clopidogrel and compared with endothelium intact aortic rings.

3.2.2.1 Influence of denudation on constriction

Denuded vessels showed no difference in PE-induced vasoconstriction in the clopidogrel-treated group in comparison to controls [$\log_{10}(EC_{50})$=-6.90±0.04 vs. $\log_{10}(EC_{50})$=-6.90±0.05, paired t-test, n=4, p=0.098; Figure 3.12]. The resultant $EC_{50}R$ was also not different from vessels with intact endothelium (-EC $EC_{50}R$ PE vs. +EC $EC_{50}R$ PE, unpaired t-test, n=4, p=0.118).

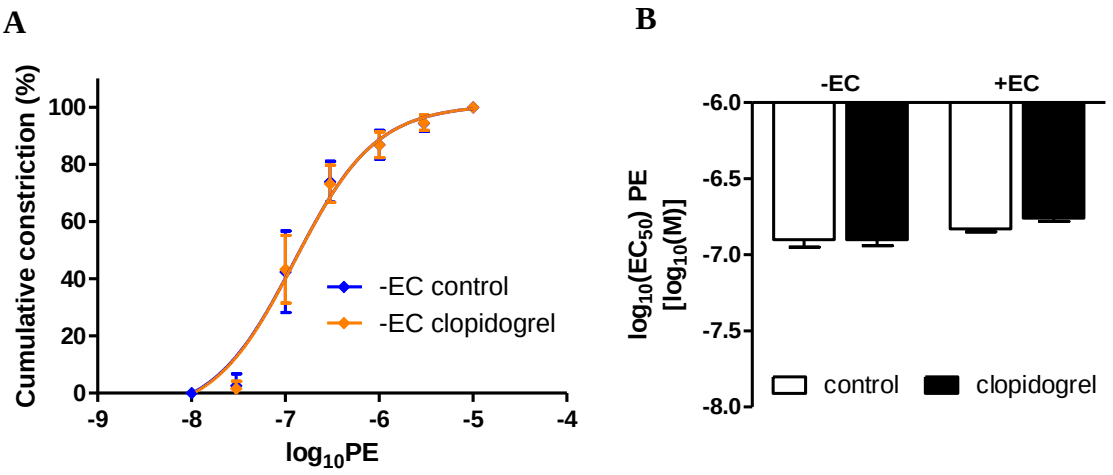


Figure 3.12. Influence of denudation of vessels on PE-induced constriction of rabbit aortae pre-incubated +/- clopidogrel. (A) concentration response curves to PE; (B) comparison of log(EC_{50}) of PE. Paired 2-tailed student's *t*-test, n=4, p>0.05. Concentration response curves to PE in the presence of endothelium (+EC) were presented on page 112 as they belonged to a separate experiment.

3.2.2.2 Influence of denudation on relaxation to GSNO

GSNO relaxes smooth muscle directly and is independent of endothelial production of NO. In line with this, relaxation of rabbit aorta to GSNO was not mediated by endothelium and when vessels were denuded after the pre-incubation with clopidogrel, the enhancement of GSNO-induced vasorelaxation remained. However, we were more interested in the role of endothelium in the clopidogrel "priming" effect *per se*, i.e. during the pre-incubation period.

Therefore, aortic rings, which were denuded prior to pre-incubation with clopidogrel were studied. These vessels showed no enhancement in GSNO-induced vasodilation by clopidogrel compared to controls [$\log_{10}(EC_{50})$=-6.94±0.06 vs. $\log_{10}(EC_{50})$=-6.93±0.04, paired t-test, n=4, p=0.865; Figure 3.13). Moreover, the resultant $EC_{50}R$ was significantly different than in vessels with intact endothelium, pointing to a crucial role of endothelium in the clopidogrel induced effect (-EC $EC_{50}R$ GSNO vs. +EC $EC_{50}R$ GSNO, unpaired t-test, n=4, p=0.025).

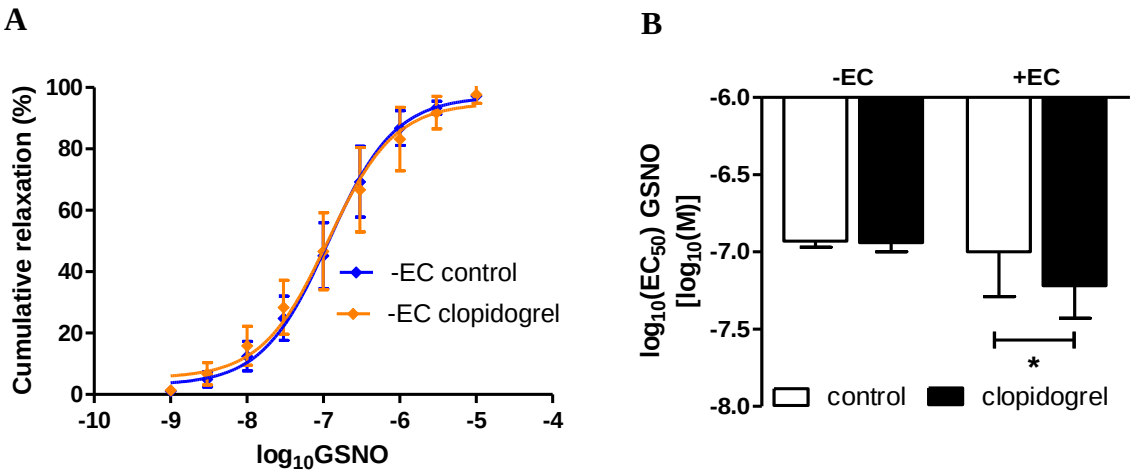


Figure 3.13. Influence of denudation of vessels on GSNO-induced relaxation of rabbit aortae pre-incubated +/- clopidogrel. (A) concentration response curves to GSNO; (B) comparison of log(EC_{50}) of GSNO. Paired 2-tailed student's *t*-test, n=4, *p<0.05. Concentration response curves to GSNO in the presence of endothelium (+EC) were presented on page 111 as they belonged to a separate experiment. Log(EC_{50}) of GSNO (+EC) is reused for comparison.

3.2.3 Involvement of endothelium-dependent relaxants

In order to further characterize the endothelium-dependent mechanism by which clopidogrel enhances GSNO-mediated vasodilatation, we assessed the involvement of eNOS and COX. The role of vascular adenosine pathways was also tested knowing of adenosine-like effects of ticagrelor[147]. Pharmacological blockers of eNOS (L-NMMA), COX (indomethacin) and A2 receptors (CGS-15943) were incubated with rabbit aorta with or without clopidogrel.

3.2.3.1 Influence of inhibitors on relaxation to GSNO in controls

Since, L-NMMA, indomethacin and CGS-15943 were incubated with rabbit aortic rings for 3.5 h, we checked first their influence on the GSNO-induced relaxation in control vessels (Figure 3.14). We found that GSNO-induced responses in controls were not changed in the presence of L-NMMA (+L-NMMA $\log_{10}(EC_{50})$=-6.37±0.08 vs. -L-NMMA $\log_{10}(EC_{50})$=-6.27±0.07, paired t-test, n=4, p=0.081), but were affected in the presence of CGS-15943 (+CGS-15943 $\log_{10}(EC_{50})$=-6.98±0.03 vs. –CGS-15943 $\log_{10}(EC_{50})$=-6.92±0.02, paired t-test, n=4, p=0.049) and indomethacin (+indomethacin $\log_{10}(EC_{50})$=-7.36±0.06 vs. -indomethacin $\log_{10}(EC_{50})$=-7.02±0.05, paired t-test, n=4, p=0.007).

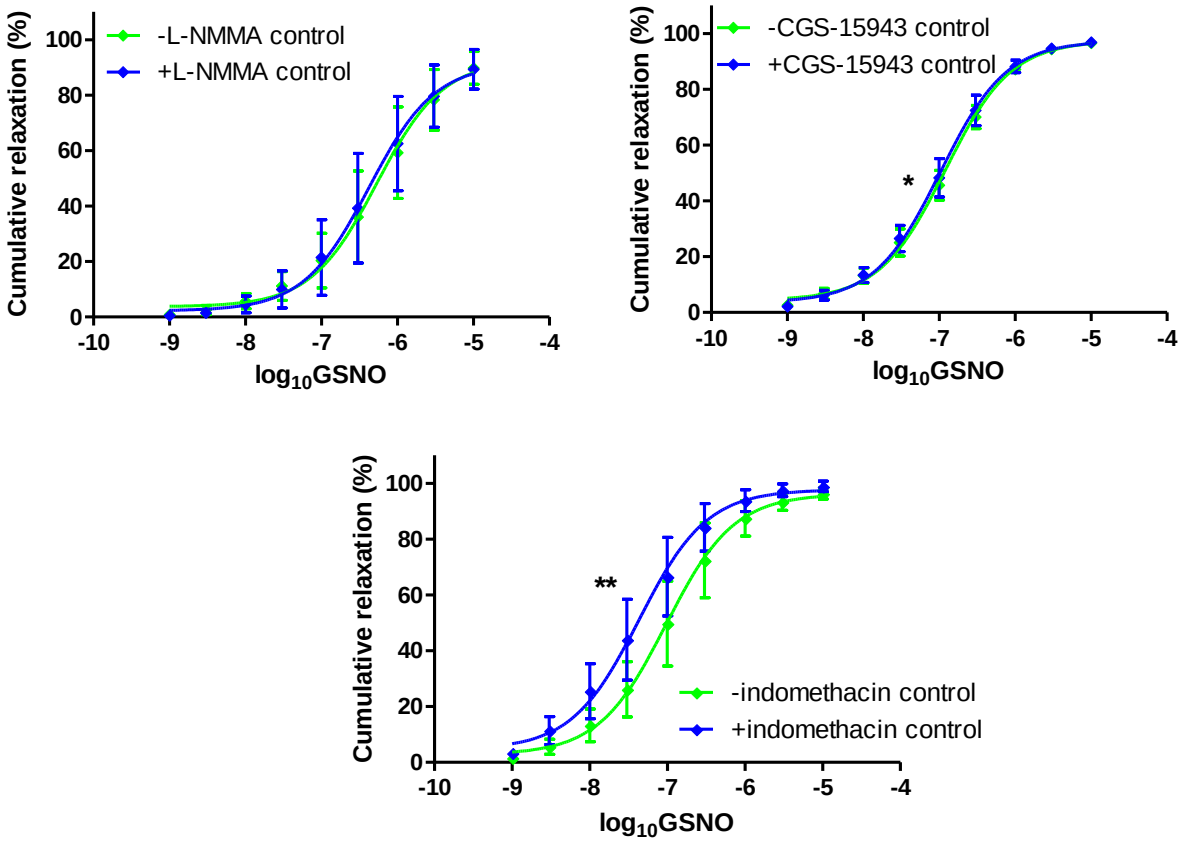


Figure 3.14. Concentration response curves to GSNO after pre-incubation of rabbit aortae +/- L-NMMA, +/- CGS-15943 and +/- indomethacin. Paired 2-tailed student's *t*-test, n=4, *p<0.05, **p<0.01.

3.2.3.2 Influence of inhibitors on relaxation to GSNO

We found that the presence of L-NMMA and CGS-15943 did not inhibit the enhanced response to GSNO in the clopidogrel-treated group compared to relative controls [$\log_{10}(EC_{50})$=-6.65±0.09 vs. $\log_{10}(EC_{50})$=-6.37±0.08, paired t-test, n=4, p=0.025; $\log_{10}(EC_{50})$=-7.09±0.04 vs. $\log_{10}(EC_{50})$=-6.98±0.03, paired t-test, n=4, p=0.054; Figure 3.15], pointing to the lack of involvement of eNOS and adenosine pathways. In the case of COX inhibition, these results were complicated by the fact that indomethacin had a large direct effect on controls (see IV. Results, page 122), and results from this experiment could not be interpreted in terms of confirming a real contribution of COX-dependent signalling in the GSNO-induced enhanced vasodilation by clopidogrel.

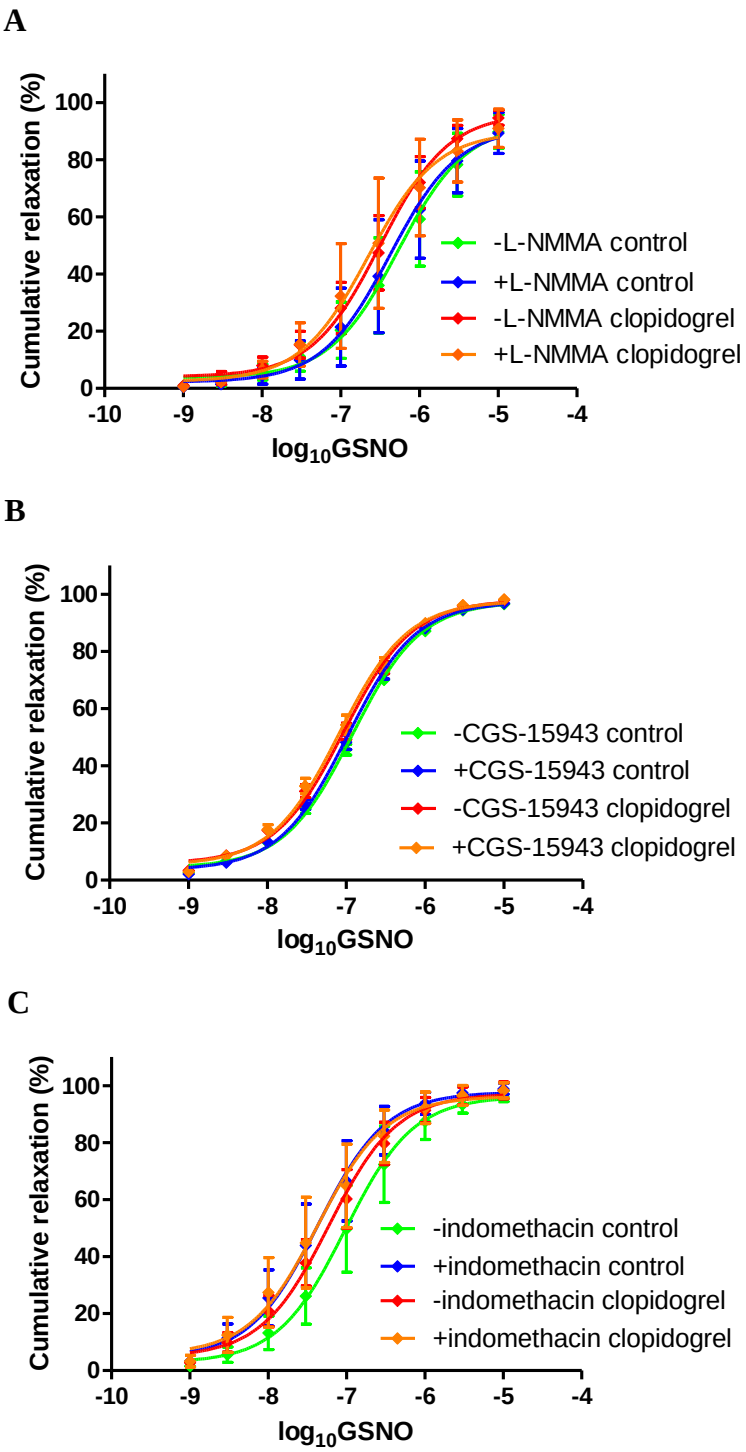


Figure 3.15. Concentration response curves to GSNO of rabbit aortae pre-incubated +/- clopidogrel and +/- L-NMMA (A), +/- CGS-15943 (B) and +/- indomethacin (C). Graphs contain controls presented before in Figure 3.14. Comparisons of all $\log_{10}(EC_{50})$ were not shown for simplicity, n=4.

In summary, the enhanced GSNO-induced response by clopidogrel was not different with and without inhibitors of eNOS and adenosine receptors (-L-NMMA EC_{50}R GSNO vs. +L-NMMA EC_{50}R GSNO, unpaired t-test, n=4, p=0.061; -CGS-15943 EC_{50}R GSNO vs. + CGS-15943 EC_{50}R GSNO, unpaired t-test, n=4, p=0.838; Figure 3.16).

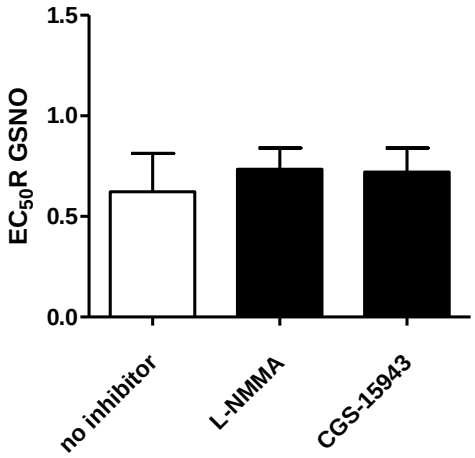


Figure 3.16. Influence of L-NMMA and CGS-15943 on GSNO-induced relaxation of rabbit aortae pre-incubated +/- clopidogrel. Clopidogrel-induced enhancement to GSNO was not different with or without these inhibitors. Unpaired 2-tailed student's *t*-test, n=4, p>0.05.

3.2.4 Involvement of sGC-cGMP-dependent relaxation

GSNO, like other exogenous NO donors, works mainly via sGC. However, there is well documented evidence for cGMP-independent mechanisms of action of NO and NO donors[92]. In order to address this issue, we added ODQ, a general blocker of sGC, to organ baths with mounted rabbit aortic rings, which were previously pre-incubated with or without clopidogrel.

ODQ did not prevent the enhanced response to GSNO in the clopidogrel-treated group in comparison to controls [$log_{10}(EC_{50})$=-7.52±0.17 vs. $log_{10}(EC_{50})$=-6.56±0.12, paired t-test, n=3, p=0.002; Figure 3.17], suggesting the involvement of NO/sGC-independent pathways. In fact, the enhanced response to GSNO by clopidogrel was 2-3-fold higher in the presence of ODQ. Noteworthy, the maximal residual relaxation to GSNO (Rmax%) remained unchanged by clopidogrel pre-treatment (21.25±3.59 % vs. 21.00±3.95 %, paired t-test, n=3, p=0.882).

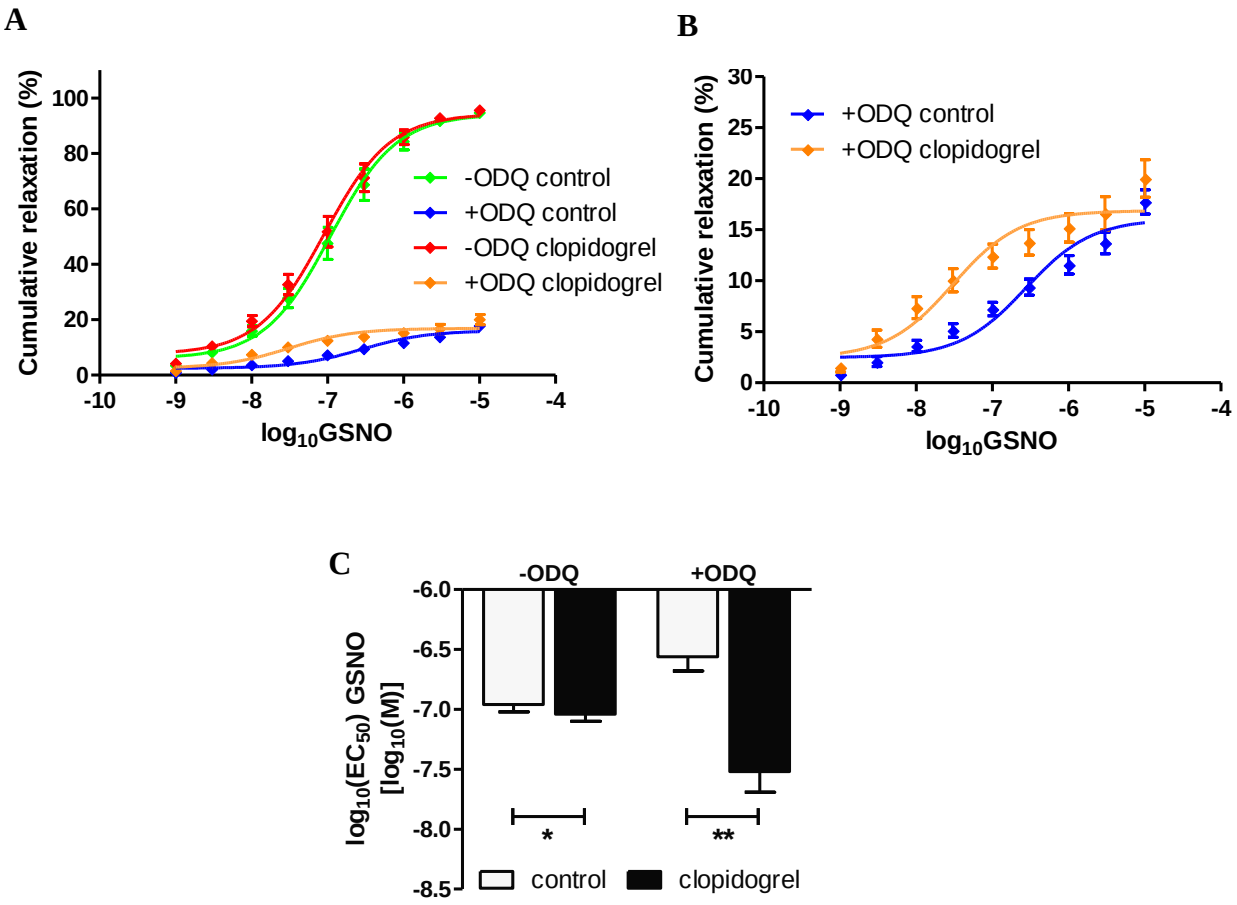


Figure 3.17. Influence of ODQ on GSNO-induced relaxation of rabbit aortae pre-incubated +/- clopidogrel. (A) concentration response curves to GSNO; (B) concentration response curves to GSNO in the presence of ODQ (reused and magnified); (C) comparison of log(EC_{50}) of GSNO. Paired 2-tailed student's *t*-test, n=3, *p<0.05, **p<0.005

3.2.5 The influence of clopidogrel concentration on relaxation to GSNO

Although all our analysis used rabbit aortic rings pre-incubated with 1000 μM clopidogrel, other concentrations of clopidogrel (10 μM and 100 μM) were also tested (Figure 3.18 and 3.19). This data was analysed using repeated measures ANOVA with a Bonferroni's multiple comparison test resulting in significant increases in response of vessels to GSNO in all clopidogrel groups when compared to controls [10 μM: $\log_{10}(EC_{50})$=-6.94±0.27 vs. $\log_{10}(EC_{50})$=-6.68±0.32, n=3, $p<0.01$; 100 μM: $\log_{10}(EC_{50})$=-6.87±0.33 vs. $\log_{10}(EC_{50})$=-6.68±0.32, n=3, $p<0.05$ and 1000 μM: $\log_{10}(EC_{50})$=-7.09±0.24 vs. $\log_{10}(EC_{50})$=-6.68±0.32, n=3, $p<0.001$]. There were no statistical differences between clopidogrel groups.

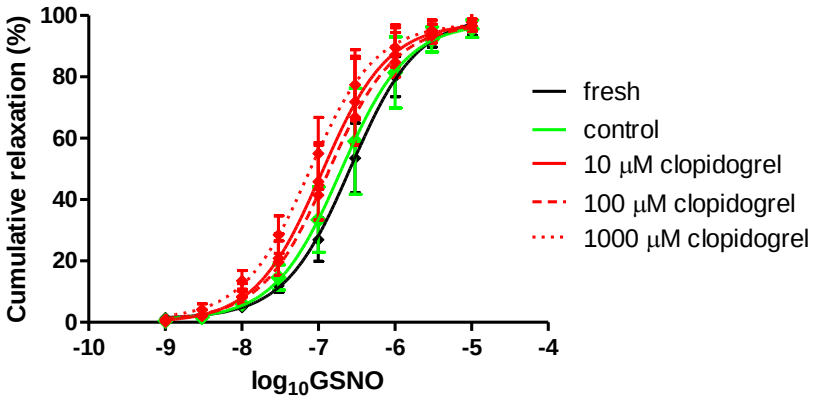


Figure 3.18. Concentration response curves to GSNO after pre-incubation of rabbit aortae for 3.5 h with clopidogrel (10-1000μM). "Fresh" represents responses of aortic rings without the pre-incubation and "control" represent responses of aortic rings pre-incubated in Krebs buffer, n=3.

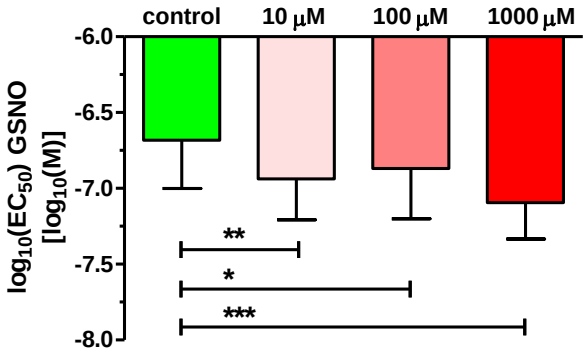


Figure 3.19. Influence of clopidogrel concentration on the GSNO-induced relaxation of rabbit aortae. Repeated measures ANOVA with a Bonferroni's multiple comparison test, n=3, *p<0.05, **p<0.01, ***p<0.001.

3.2.6 Involvement of cGMP- and cAMP-dependent pathways

In order to investigate further the effect of clopidogrel on downstream markers of vascular NO-signalling, we measured basal tissue levels of cGMP and cAMP with and without pre-incubation with clopidogrel (Figure 3.20). Rabbit aortic rings pre-incubated with clopidogrel did not show a significant change in intracellular levels of cGMP or cAMP, when compared with respective controls (pmol cGMP/mg protein=3.05±0.61 vs. 3.76±2.14, paired t-test, n=5, p=0.514; pmol cAMP/mg tissue=0.77±0.32 vs. 0.66±0.31, paired t-test, n=3, p=0.958). Taken together with the ODQ results presented above, this suggested that the enhanced vasorelaxation of rabbit aortae to GSNO by clopidogrel was not mediated by NO/sGC/cGMP-dependent pathways.

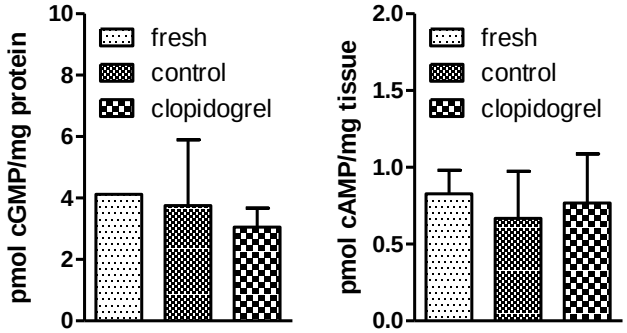


Figure 3.20. Basal levels of cGMP and cAMP in rabbit aortae pre-incubated +/- clopidogrel. "Fresh" represent responses of aortic rings without the pre-incubation and "control" represent responses of aortic rings pre-incubated in Krebs buffer. Paired 2-tailed student's *t*-test, n=3, p>0.05.

3.3 Anti-oxidant actions of clopidogrel

Clopidogrel therapy in humans has been recently associated with anti-oxidant and anti-inflammatory effects. We investigated the influence of the pro-drug clopidogrel on the oxidative status of rabbit aortic rings pre-incubated at 37 °C in oxygenated Krebs buffer for 3.5 h with or without 1000 μM clopidogrel. We used spin trapping techniques in conjunction with the DEPMPO probe to specifically capture superoxide released by these vessels to surrounding media over a 5 min period (see III. General Methods, pages 71-73).

3.3.1 Direct anti-oxidant properties of clopidogrel

Clopidogrel did not exhibit direct anti-oxidant properties. The amount of superoxide generated by a standard preparation of hypoxanthine/xanthine oxidase remained unchanged after incubation under similar conditions with 200 μM clopidogrel (Figure 3.21). Higher concentration of clopidogrel could not be used due to difficulties in finding the optimal

conditions for EPR scanning. The lack of a direct anti-oxidant effect of clopidogrel was confirmed by TAC assay (see IV. Results, page 191-193).

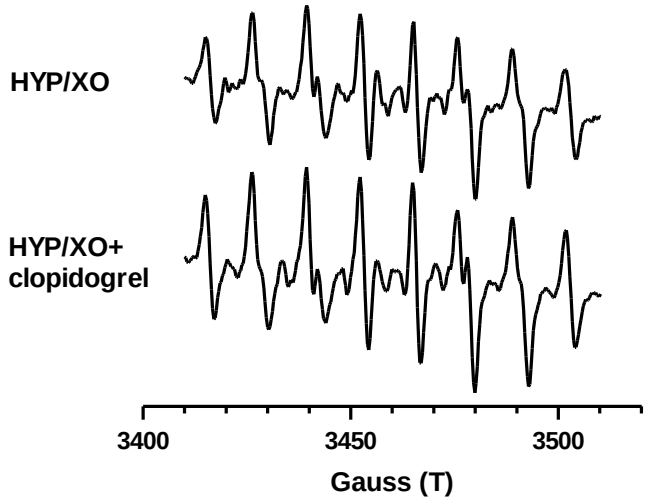


Figure 3.21. Representative trace of DEPMPO-superoxide spin adducts of hypoxanthine/xanthine oxidase (HYP/XO) +/- clopidogrel.

3.3.2 Influence of clopidogrel on superoxide production by vessels

Fresh vessels did not release detectable levels of superoxide (Figure 3.22), which is in agreement with low production of superoxide under physiological conditions. However, the 3.5 h pre-incubation of vessels triggered an increased extracellular production of superoxide, as reflected by the $\log_{10}$(AUC) of the EPR signal, characteristic of the trapped superoxide-DEPMPO adduct species (7.05±0.19 a.u.). Aortic rings pre-incubated for 3.5 h with clopidogrel released, in parallel, significantly lower amounts of superoxide into the surrounding buffer compared to controls (6.92±0.17 a.u. vs. 7.05±0.19 a.u., paired t-test, n=4, p=0.019), suggesting that clopidogrel provided a partial prevention of reactive oxygen intermediate generation in our model.

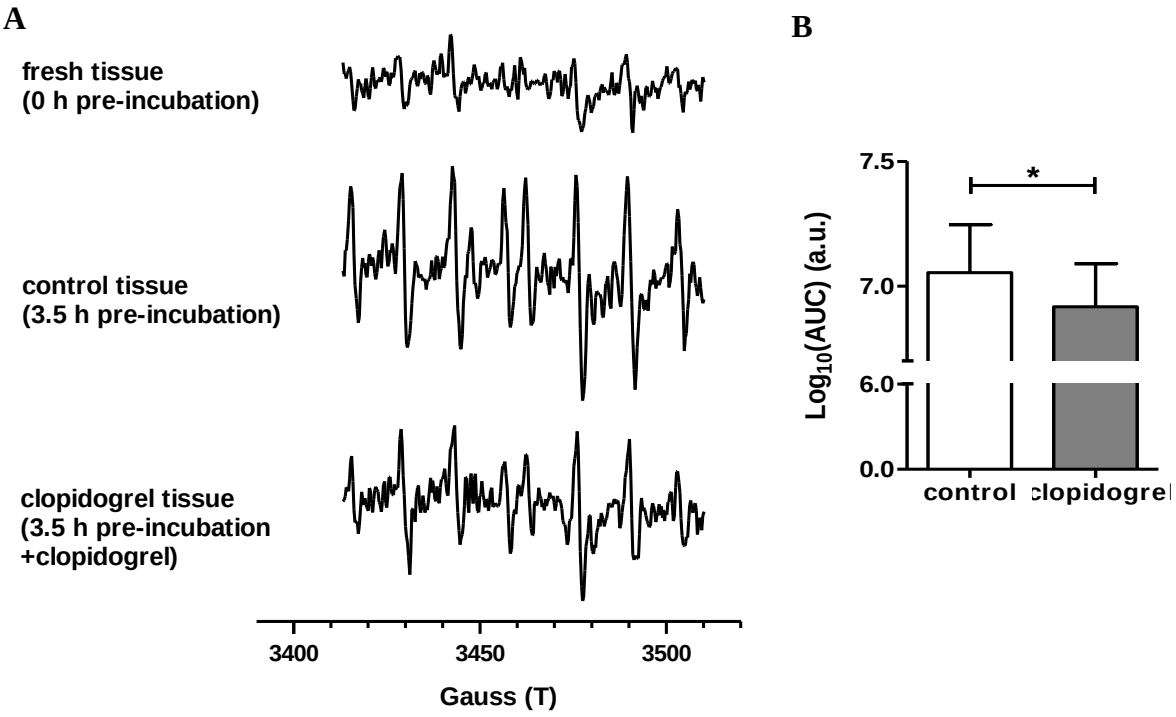


Figure 3.22. Indirect anti-oxidant properties of clopidogrel. (A) Representative traces of DEPMPO-superoxide spin adducts; (B) comparison of $\log_{10}$(AUC), representing amount of superoxide released by rabbit aortae pre-incubated +/- clopidogrel. Paired 2-tailed student's *t*-test, n=4, *p<0.05.

3.4 Anti-inflammatory actions of clopidogrel

In order to determine whether the anti-oxidant actions of clopidogrel observed were paralleled by changes in anti-inflammatory status, mRNA coding for VCAM-1, TNF-α, IL-6 and COX-2 was measured using RT qPCR after pre-incubation of rabbit aortic rings with or without 1000 μM clopidogrel.

Fresh aortic rings expressed low levels of VCAM-1, TNF-α, IL-6 and COX-2 mRNA and they were used as a reference for calculation of relative expression in vessels that were pre-incubated with or without clopidogrel. The 3.5 h pre-incubation of aortic rings in Krebs buffer caused an upregulation of all these genes (Figure 3.23), pointing to an increased inflammatory status of tissue. In the presence of clopidogrel during the pre-incubation, vessels

expressed significantly lower amount of TNF-α mRNA (5.07±2.53 vs. 7.58±2.96, paired t-test, n=3, p=0.014), unchanged amount of VCAM-1 mRNA (1.82±1.44 vs. 5.45±3.83, paired t-test, n=3, p=0.127), unchanged IL-6 mRNA (7.45±3.83 vs. 4.40±1.92, paired t-test, n=3, p=0.234) and significantly higher COX-2 mRNA (33.47±7.46 vs. 14.32±11.10, paired t-test, n=3, p=0.017) than corresponding controls. A trend towards lower expression of VCAM-1 mRNA and higher expression of IL-6 mRNA in the presence of clopidogrel has been observed.

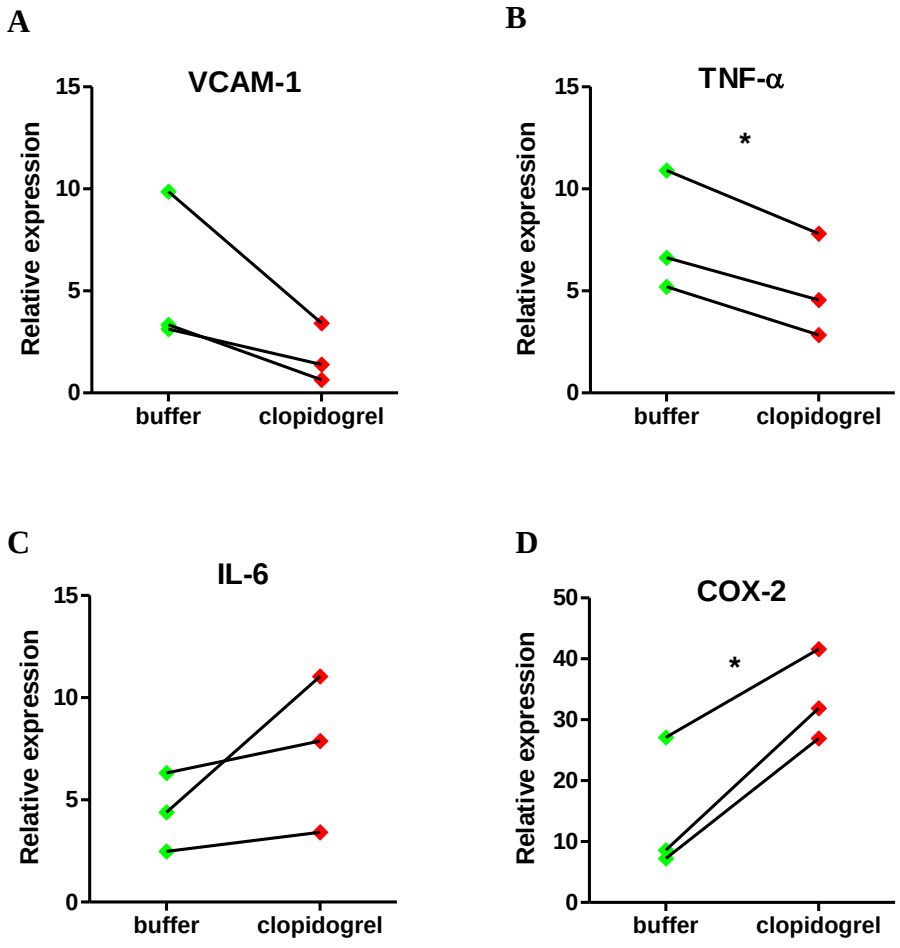


Figure 3.23. Anti-inflammatory properties of clopidogrel. The comparison of relative expression of VCAM-1 (A), TNF-α (B), IL-6 (C) and COX-2 (D) in rabbit aortae pre-incubated +/- clopidogrel, Paired 2-tailed student's *t*-test, n=3, *p<0.05.

4. Discussion

This study was designed to investigate the potential direct effects of a parental form of clopidogrel (pro-drug clopidogrel) on the vascular response to NO and anti-oxidant/anti-inflammatory status of vessels. We found that pre-incubation of rabbit aortic segments with the pro-drug clopidogrel enhanced the relaxation response to exogenous NO addition, while responses to stimulation of endogenous NO remained unaffected. This effect was independent of platelet and vascular $P2Y_{12}$ receptors, mediated by endothelium and limited to NO/sGC/cGMP-independent signalling pathways. Pre-incubation of vessels with clopidogrel reduced the extracellular release of superoxide by tissue and attenuated tissue TNF-α mRNA expression in this model, which may in part explain the enhanced response to exogenous NO following clopidogrel.

Clopidogrel has been associated with attenuated vascular constriction in rabbit and rat models[166, 167]. In the present study, cumulative concentration responses to PE were not affected.

Because clopidogrel has been shown to increase eNOS-dependent coronary flow in isolated heart of guinea pig[171] and FBF and FMD in patients[157, 161, 169], we expected to observe the augmented vasorelaxation to endogenous NO in our model. Instead, pre-incubation of rabbit aorta with pro-drug clopidogrel enhanced the responsiveness of the vessel only to exogenous NO donors (GSNO or NOC9) while response to Ach stimulation was not significantly affected. One possible explanation for this difference is the amount of NO reaching SMC; Ach-induced release of NO is limited by saturation of Ach receptors at the endothelial surface whereas free NO, coming from NO donors can act directly on the smooth muscle.

An important consideration is that GSNO has specific NO release mechanisms that may not be shared with other NO donors, and S-nitrosothiols have unique mechanisms of action in addition to the release of NO species involving transnitrosation and S-nitrosylation of proteins[66]. However, in the light of our data showing that clopidogrel also enhanced vasodilation to another NO donor NOC9, we may conclude that S-nitrosothiol signalling induced by GSNO was not critical to this process. In order to relate our finding to the clinical use of NO donors, ISDN was chosen because it has been used as a nitrosovasodilator for the treatment of angina pectoris[176] and heart failure[177]. Clopidogrel did not influence the relaxation of rabbit aortic rings to ISDN probably due to its slow bioconversion and the resulting extremely slow rate of relaxation induced in the isolated vessel model.

Given that clopidogrel is a $P2Y_{12}$ antagonist, most of its beneficial effects on the vasculature, including the inhibition of platelet aggregation, vasomodulatory actions and the anti-inflammatory and anti-oxidative properties, have been considered to be due to blockage of primarily platelet $P2Y_{12}$ and to lesser extent vascular $P2Y_{12}$ receptors by the active metabolite. Our study confirms for the first time that the pro-drug clopidogrel has a direct influence on the vascular endothelium beyond platelet and vascular $P2Y_{12}$ receptor-mediated pathways. Which endothelial receptors have been influenced by clopidogrel remains to be established.

Knowing that sGC is the main but not the only vascular receptor for NO, we investigated its role in our study using ODQ. We found that the enhanced GSNO-mediated vasorelaxation by clopidogrel was sustained and even bigger after blocking sGC with ODQ. It suggests that the pre-incubation of rabbit aortae with clopidogrel activated sGC-independent pathways and caused the general enhancement of GSNO-induced vasodilation. The possible involvement of sGC-independent mechanisms was confirmed by showing that basal cGMP levels were unchanged in clopidogrel-treated vessels. Further studies are required to identify

how the endothelium influences sGC/cGMP-independent pathways in smooth muscle and what sGC/cGMP-independent molecules are affected; the involvement of endogenous NO has been excluded.

Activation of sGC/cGMP-independent mechanisms by clopidogrel might be important considering different vascular responses to endogenous and exogenous NO stimuli observed in this study. It has been shown that cGMP-independent mechanisms become more prominent at higher concentrations of NO (μM-mM) and are generally more common for NO donors[92, 93]. In an attempt to explain this phenomenon, it has been suggested that NO generated within the tissue following receptor stimulation has more ready access to sGC, whereas NO delivered from outside the cell may more easily access non-sGC sites, such as K^+ channels[92].

Although, clopidogrel did not exhibit direct anti-oxidant properties, it attenuated the increased extracellular release of superoxide by rabbit aortic tissue. Typically, relatively low amounts of superoxide are produced, but under pathological or stress conditions, an imbalance between the rate of its generation and removal by cellular anti-oxidant defenses occurs. Excessive release of superoxide is involved either directly or via the generation of other radicals (H_2O_2, $ONOO^-$) in i) accelerated NO inactivation, (ii) alteration of intracellular redox signalling and (iii) oxidative damage of DNA, proteins and lipids, all leading to oxidative stress and endothelial dysfunction implicated in the development of various cardiovascular diseases[23]. In our model, the incubation of rabbit aorta under optimum laboratory conditions was associated with increased extracellular production of superoxide over time and paralleled by increased tissue levels of VCAM-1, TNF-α, IL-6 and COX-2 mRNA. Both excessive superoxide production and upregulated TNF-α gene were attenuated by clopidogrel, which is indicative of anti-oxidant and anti-inflammatory effects in this model. Similarly, a trend towards decreased VCAM-1 and increased IL-6 mRNA by clopidogrel may be interpreted as the initiation of tissue protective mechanisms, since IL-6

has been shown to suppress inflammation by inhibition of TNF production[178]. COX-2 is known as an inducible enzyme catalyzing the production of various prostanoids, which play a variety of biological functions including the regulation of inflammation (see I. General Introduction, pages 41-43). Prostanoids exert their effects via activation of GPCR on the cellular membranes or peroxisome proliferator-activated receptors (PPARs) in the nucleus. Depending on tissue-specific isomerases and the site of release, products of COX-2 activity may induce pro-inflammatory or anti-inflammatory responses. It is not known if the upregulation of COX-2 mRNA by clopidogrel was pro- or anti-inflammatory and further experiments are required for confirmation. However, other results presented might suggest anti-inflammatory and anti-oxidant effects on the basis of downregulated TNF-α mRNA and reduced formation of $O_2^{\bullet-}$ in the same tissues. Further studies using inhibitors of the enzymatic sources of superoxide are required to identify the anti-oxidant pathways stimulated by clopidogrel. Additionally, results from RT qPCR should be confirmed by protein-based techniques to confirm the anti-inflammatory properties of clopidogrel.

Clearly, less superoxide in the extracellular space translates into more NO available from exogenously applied NO donors and consequently, may provide an explanation for the enhanced vasodilation to GSNO observed following pre-incubation of vessels with clopidogrel (Figure 3.25).

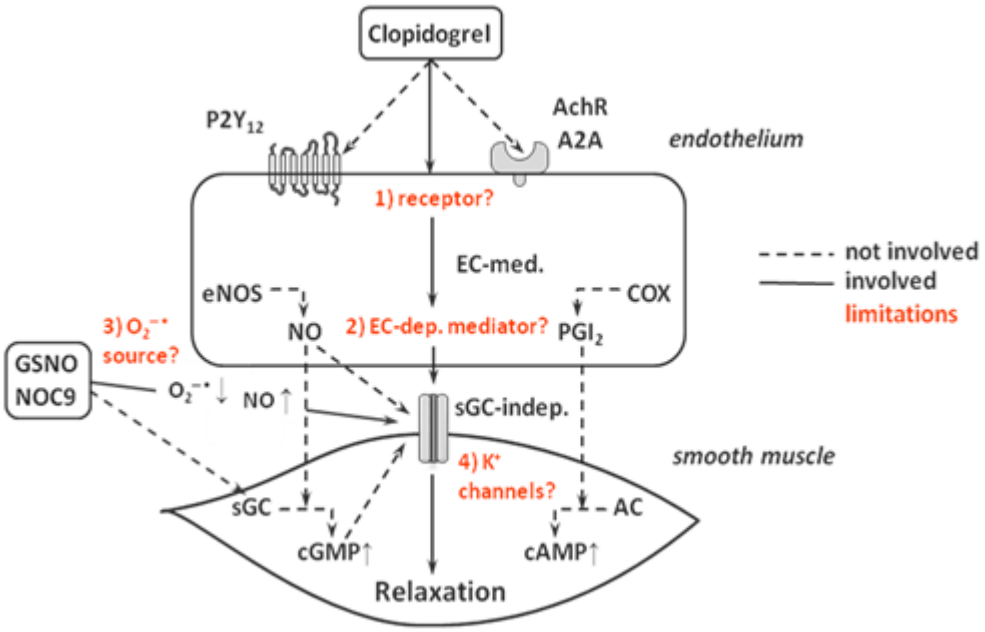


Figure 3.25. Summary of pathways involved and not involved in enhanced vascular response to GSNO by clopidogrel. The mechanism appears to be via EC-mediated (EC-med.) improvement in sGC-independent (sGC-indep.) pathways and/or reducing exogenous release of superoxide. In red, main limitations of the study have been outlined: 1) Although the involvement of $P2Y_{12}$ receptors has been eliminated, the actual receptor mediating clopidogrel actions on vascular endothelium has not been identified; 2) Knowing the crucial role of endothelium and lack of involvement of NO and PGI_2-dependent signalling, other EC-dependent mediators should be explored; 3) The exact source of $O_2^{-\bullet}$ is unknown; 4) The role of K^+ channels, which are involved in sGC-indep. pathways should be investigated.

The clinical relevance of our *in vitro* findings is not known. It is acknowledged that concentrations of clopidogrel used in experiments were at levels much higher than would be found physiologically and observed effects were relatively small. Peak plasma concentrations of clopidogrel metabolites 1-2 h after oral loading of clopidogrel (300-600 mg) in CAD patients were estimated as follows: (i) the active thiol metabolite 9-19 nM, (ii) the inactive carboxyl metabolite 50-140 μM and (iii) unchanged clopidogrel 50-140 nM[135]. The enhanced response of vessels to GSNO was observed when vessels were pre-conditioned with 10-1000 μM pro-drug clopidogrel and the bioconversion of parental form of clopidogrel to different metabolites was not determined.

In summary, we consider our findings important to fully understand the influence of clopidogrel on vascular homeostasis. Direct vasomodulatory as well as anti-oxidant and anti-inflammatory effects of clopidogrel observed in this study confirm that the drug has a positive impact on the vasculature beyond the inhibition of platelet aggregation. More research is required to identify the molecular pathways involved. This also provides impetus for similar studies with the newer anti-platelet agents, such as ticagrelor to investigate if these beneficial effects on vasculature translate to new generation drugs.

Summary:

- Pre-incubation of rabbit aortic segments with pro-drug clopidogrel enhanced the relaxation response to exogenous NO addition, while responses to stimulation of endogenous NO remained unaffected.

- This effect was independent of the platelet and vascular $P2Y_{12}$ receptors, mediated by endothelium and limited to NO/sGC/cGMP-independent signalling pathways.

- Extracellular release of superoxide and tissue TNF-α mRNA expression was attenuated by clopidogrel, which may in part explain the enhanced response to exogenous NO following clopidogrel.

Part 2: The ability of $P2Y_{12}$ antagonists to make biologically active nitrosothiols

Hypothesis: ***Thienopyridines can form S-nitrosothiol derivatives under appropriate conditions.***

Specific aims of this chapter were:

- To investigate whether the thienopyridine and non-thienopyridine anti-platelet agents participate in S-nitrosation reactions and the factors that control these reactions.
- To compare S-nitrosation between different forms of the same drug (salts of clopidogrel).
- To examine the biological properties of these SNO derivatives in terms of ability to inhibit platelet aggregation, to induce vasorelaxation and participate in transnitrosation reactions.

1. Introduction

$P2Y_{12}$ antagonists are a class of very successful agents utilized for the inhibition of platelet activation and aggregation by ADP. Their major use is in the prevention of thrombotic vascular events and ischaemic complications, often arising from percutaneous coronary intervention (PCI), and are recommended for patients with acute coronary syndromes (ACS) and/or undergoing coronary stenting as a part of a dual anti-platelet therapy[113, 115, 117, 119, 120]. $P2Y_{12}$ antagonists include 1) thienopyridines: ticlopidine, clopidogrel and prasugrel, which bind irreversibly to the active site of the ADP receptor, and 2) non-thienopyridines: ticagrelor, cangrelor and elinogrel which interact reversibly with the ADP receptor, but without blocking the ADP binding site. Thienopyridines (Th) bind covalently to $P2Y_{12}$ receptors by formation of disulfide bridges with the two extracellular Cys residues of the receptor (Cys17 and Cys270)[136, 179]. This interaction requires hepatic metabolism of the parental drugs to produce the active form which exhibits a critical reduced thiol group (Th-SH; Figure 4.1).

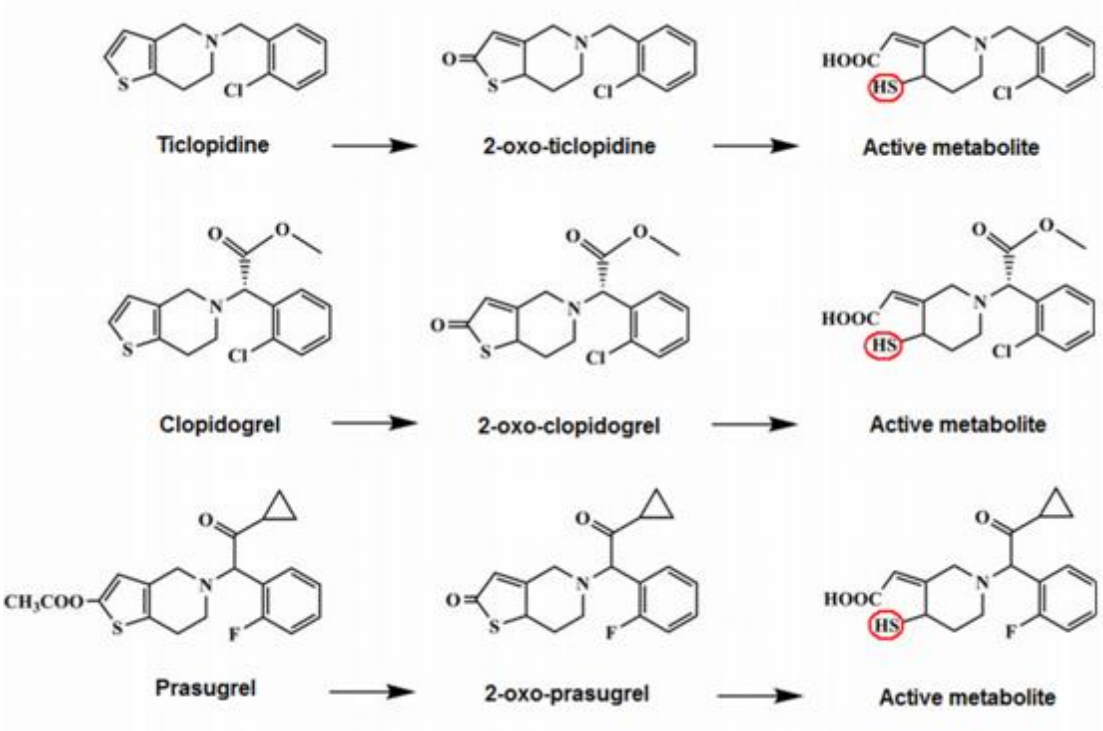


Figure 4.1. Chemical structures of ticlopidine, clopidogrel and prasugrel and their active metabolites. In red circles: critical thiol groups. Adapted from[180]

Reduced thiols (RSH) on proteins are particularly susceptible to oxidation and form oxidized thiols (RSSH), which can induce important structural changes for many proteins and regulate their function. In the presence of NO^+ or acidified nitrite (H^+ NO_2^-) RSH can be transformed into S-nitrosothiols (RSNO), which are key mediators in the NO signalling cascade[68] (equation 1 below).

$$RSH + NO^+ \rightarrow RSNO^+ + H^+ \quad (1)$$

$$Th - SH + H^+ + NO_2^- \rightarrow Th - SNO + H_2O \quad (2)$$

RSNO possess many of the biological activities of NO such as vasodilation, inhibition of platelet aggregation and regulation of cellular redox, apoptotic and inflammatory responses[31, 61-64]. RSNO also provide protection and storage of the NO^+

group, which can be transferred to proteins such as plasma albumin (transnitrosation) and travel in the circulation to sites distal to that of their origin[67]. The *in vivo* mechanism of release of NO from RSNO is still a matter of debate[65]. Recent studies have shown that extracellular CysSNO can be uptaken by endothelial and smooth muscle cells via L-type amino acid transporters (LAT-1, 2)[181, 182]. Additionally, RSNO can also induce post-translational modifications of cysteine residues in proteins (S-nitrosation/S-nitrosylation) and therefore, contribute to the regulation of their function and signal transduction[66, 68, 69]. Many proteins have been identified to be S-nitrosated in pathophysiology of human and other mammals, including those affecting NO bioavailability and function (see I. General Introduction, pages 28-30).

We initially hypothesized that thienopyridine tablets taken orally would form nitrosothiol derivatives (Th-SNO) only after conversion to the active form (containing free thiol) and in an environment where acidic conditions and nitrite could provide a theoretically suitable environment for the formation of RSNO species (equation 2 above). The formation of Th-SNO would have the potential to participate in all the reactions expected of native NO including direct inhibition of platelet aggregation. As such, it could also contribute to the observed variation in potency of inhibition of platelet aggregation between the active metabolites of different thienopyridines. According to the biochemistry of RSNO, the amount of Th-SNO formed would depend on the concentration of respective Th-SH, the degree of stomach acidity and available nitrite for the reaction.

Ticagrelor was not expected to form S-nitrosothiol derivatives because it does not form metabolites containing a reduced thiol moiety (Figure 4.2).

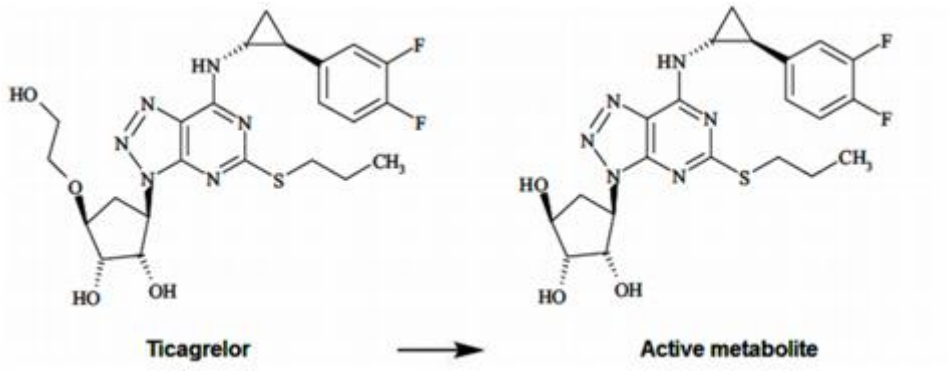


Figure 4.2. Chemical structure of ticagrelor and its active metabolite. Adapted from[183]

It was decided to test our hypothesis *in vitro* using pharmaceutical grade tablets of thienopyridines and ticagrelor under optimum conditions expected for the formation of RSNO. –SNO species were measured mainly by ozone-based chemiluminescence and Cu^{+}/Cys (2Cs) reagent and additionally by spectrophotometry and mass spectrometry. Where drug-SNO was formed, it was further examined for biological activity in terms of inhibition of platelet aggregation, vasorelaxation and transnitrosation.

2. Methods

2.1 Drug preparation

Ticlopidine *(Ticlopidin-ratiopharm*^{TM} Ratiopharm GmbH, Ulm, Germany) tablets containing 250 mg ticlopidine hydrochloride, clopidogrel (*Plavix* ^{TM} Bristol-Myers Squibb, UK) tablets containing 75 mg clopidogrel hydrogen sulphate, prasugrel (*Efient* ^{TM} Daiichi Sankyo UK) tablets containing 10 mg prasugrel hydrochloride and ticagrelor (*Brilique*^{TM} AstraZeneca, London, UK) tablets containing 90 mg ticagrelor were used in these studies. Purified clopidogrel sulphate (LKT Laboratories, Inc) was supplied as 98.6 % pure.

Crushed tablets or purified clopidogrel sulphate were dissolved in HPLC/double distilled water to yield a 10 mM suspension unless stated otherwise. Drug preparations were kept in dark and additionally double-filtered through a paper filter (#42) and a 0.22 μm filter for platelet aggregation studies. The content of reduced thiols (drug-SH) was determined using ThioStar™ Fluorescent Thiol Detection Reagent (see III. General Methods, page 97).

2.2 Drug-SNO production

Typically, 1 ml drug suspension was acidified with 1 M HCl, mixed with 1ml $NaNO_2$ and incubated at 37 °C in the water bath for 10 min. The reaction was carried out in a glass brown bottle prior to neutralization with 1 M NaOH and immediate analysis by Cu^{+}/Cys (2Cs) ozone based chemiluminescence (see III. General

Methods, page 88). Some drug-SNO preparations were also analysed by spectrophotometry (Jenway 6700) before and/or after addition of 8.9 mM $HgCl_2$.

2.3 Biochemical analysis

Drug-SNO formation was tested in terms of its dependence on 1) pH, 2) NO_2^- (nitrite) concentration and 3) time of incubation with nitrite. pH range 2-7 was adjusted using appropriate addition of HCl and measured by narrow gauge pH paper (Fisherbrand™) confirmed by pH meter, with accuracy to the nearest 0.5/0.1, respectively. Initially, high concentrations of nitrite 1-1000 mM were used and then a lower, more physiological range (0.01-1 mM) was tested. In order to assess the influence of incubation time on the formation of drug-SNO, 10 mM drug suspensions were kept with 10.1 mM nitrite for 10-120 min.

It should be mentioned that mass spectrometry of the purified clopidogrel sulphate preparation before and after addition of nitrite was also performed in collaboration with Dr Keith Morris in the University of Wales (UWIC) and these data are presented in the end of results section (pages 168-169).

2.4 Biological analysis

In order to analyse the biological function of chemically formed clop-SNO and pras-SNO, their ability 1) to influence platelet aggregation was investigated using light transmission aggregometry (LTA) and 2) to induce vasorelaxation was tested using isometric tension studies on isolated rabbit aortae. Furthermore, transnitrosation properties of clop-SNO to purified bovine serum albumin (BSA) and plasma protein

were analysed by a colleague (Narudeen Hassan) and these results are presented in the end of results section for reference (pages 167-168). Each biological assay was accompanied with 2 Cs analysis, which allowed the accurate assessment of RSNO concentration and its associated biological effect. These studies were conducted in collaboration with Dr Shantu Bhundoo (a close colleague within the research group conducting his MD) due to the need for immediate measurements conducted in parallel. Platelet aggregation measurements (LTA) and isometric tension studies were primarily conducted by Dr Bhundoo whereas I took primary responsibility for all biochemical analyses.

Detailed methodology is presented in III. General Methods. Briefly, for the LTA assay, platelet rich plasma (prp) was isolated from healthy subjects and added to a 96-well plate previously loaded with ADP/PBS and Th/Th-SNO/nitrite/GSNO placed for 10 min in the spectrophotometer. The increase in transmittance over time was calculated relative to that of ADP-induced aggregation. GSNO reflected 100 % RSNO yield.

In vessel-based studies, rabbit aortic rings were isolated and exercised as described before and pre-constricted with 1 μM PE prior to addition of Th/Th-SNO/nitrite. The maximal relaxation (Rmax) of Th-SNO was calculated as a percentage relative to PE-induced constriction. The relaxation of nitrite was determined over the same time and subtracted from Rmax of Th-SNO. In experiments with denuded vessels, the endothelium was carefully removed by gently rubbing the internal surface of the tissue ring with a wooden applicator before the experiment. Removal of endothelium was confirmed by the absence of a vasodilator response to Ach (10 μM) in pre-constricted vessels. In experiments, where ODQ (sGC inhibitor) was used, 10 μM ODQ was added to tissue baths for 0.5 h before pre-constricting

with PE. After thorough washing, aortic rings were pre-constricted again and relaxed with cumulative doses of GSNO (1 nM-10 μM) in order to 1) compare Rmax of drug-SNO with standard nitrosothiol and 2) estimate the intra-assay coefficient of variability.

2.5 Statistics and data analysis

The results are expressed as mean±SD and "n" represents the number of independent experiments. Concentration-responses to inhibitors of platelet aggregation or GSNO were fitted by nonlinear regression curves (sigmoidal dose-response) and the respective doses producing 50 % platelet inhibition (IC_{50}) or 50 % vessel relaxation (EC_{50}) were determined.

Differences between 2 groups were analysed by 2-tailed student's *t*-test (paired or unpaired where appropriate). For the comparison of more than two groups, one way ANOVA with Bonferroni post hoc test was applied where necessary. A p value of <0.05 was considered statistically significant.

All analysis was performed using GraphPad Prism® (version 5).

3. Results

3.1 Optimization of 2Cs method for detection of drug-SNO

Cu^{+}/Cys (2Cs) reagent allows for specific detection of RSNO species according to the chemistry presented earlier (see III. General Methods, page 88). Briefly, cleavage of NO from RSNO is catalysed by Cu^{+}, and Cys is used for oxidation of Cu^{2+} back to Cu^{+}. This reaction requires a neutral pH, therefore pH of 2 Cs reagent was adjusted to pH=7.2 before use and pH of drug-SNO preparations was adjusted to pH>7.2.

3.1.1 Modification of 2Cs reagent

It was noticed that some peaks obtained from drug-SNO preparations had irregular shapes (Figure 4.3), different from uniform and defined NAC-SNO peaks observed from the standards (see III. General Methods, pages 88-90). It was suspected these differences could have been caused by the changing pH of 2Cs reagent in the course of study or day, and to solve this another 2Cs reagent was prepared in PBS to buffer these changes. 2Cs prepared in PBS provided more consistent signals from drug-SNO (Figure 4.4). It was therefore used in all further studies including preparation of the standard curves (Figure 4.5).

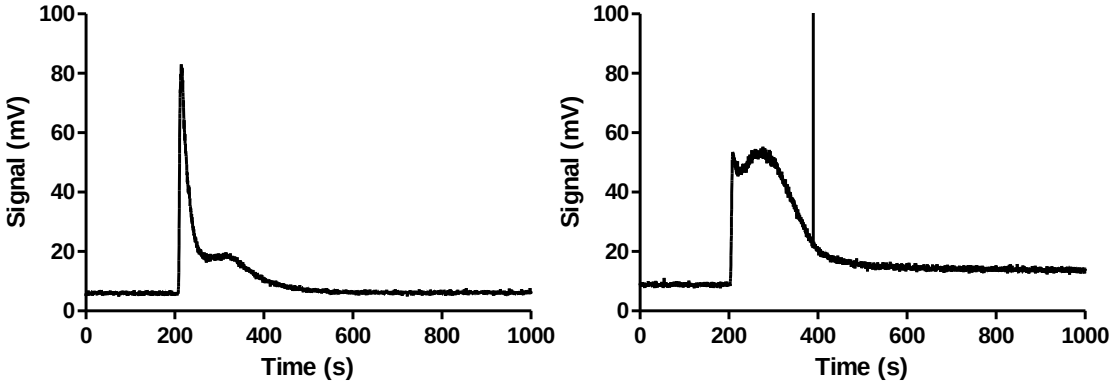


Figure 4.3. Drug-SNO signals detected using 2Cs reagent prepared in water.

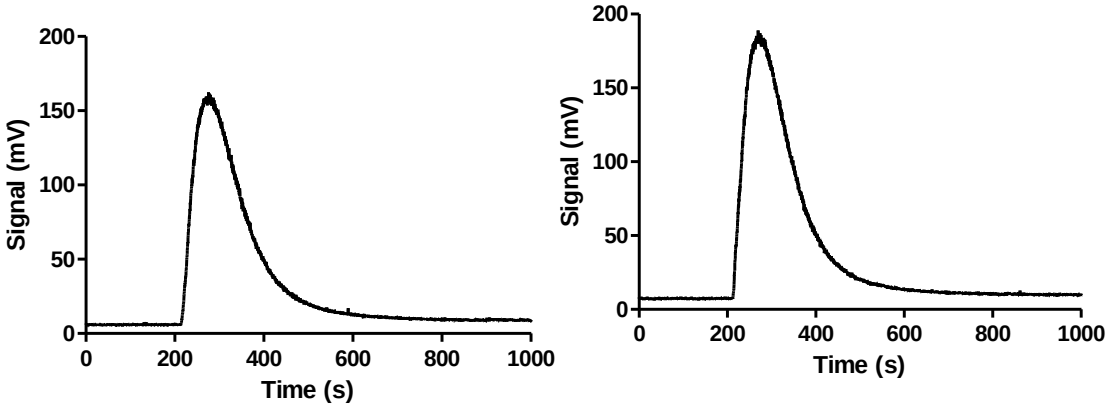


Figure 4.4. Drug-SNO signals detected using 2Cs reagent prepared in PBS.

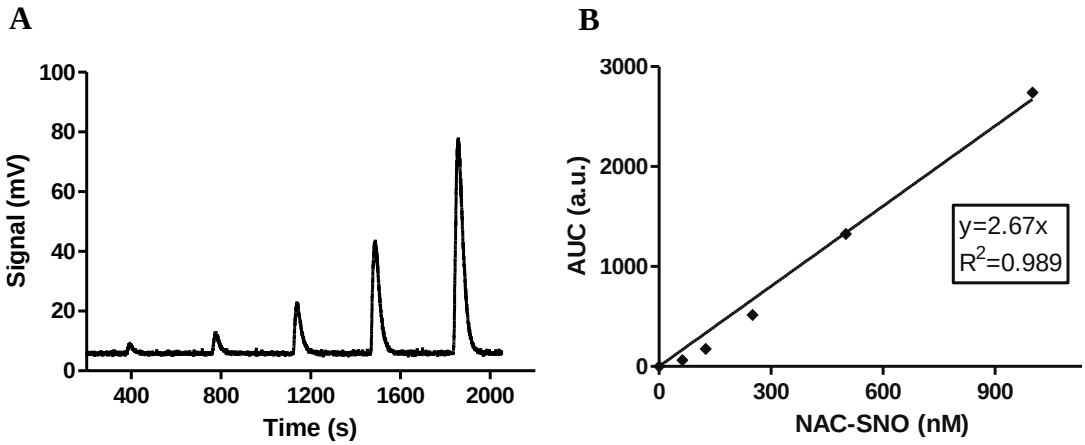


Figure 4.5. NAC-SNO signals detected using 2Cs reagent prepared in PBS (A) and used for preparation of standard curve (B).

3.1.2 Interaction of nitrite with 2Cs reagent

The 2Cs reagent is utilized across many fields and is universally accepted as a means for specific cleavage of NO from SNO species, however, to the best of our knowledge it was never tested with high mM concentrations of nitrite. Such excessively high nitrite concentrations were introduced in this study in order to improve drug-SNO yield. We found that 2Cs is capable of detecting NO from nitrite under these conditions and that the acidic environment and 10 min incubation at 37 °C utilized for the preparation of drug-SNO had an influence on the signal profile obtained (Figure 4.6). Therefore a stringent analysis was performed to ensure accurate RSNO reporting. The appropriate nitrite controls were performed on a daily basis and the respective AUC was subtracted from the total AUC generated by drug-SNO (Figure 4.7).

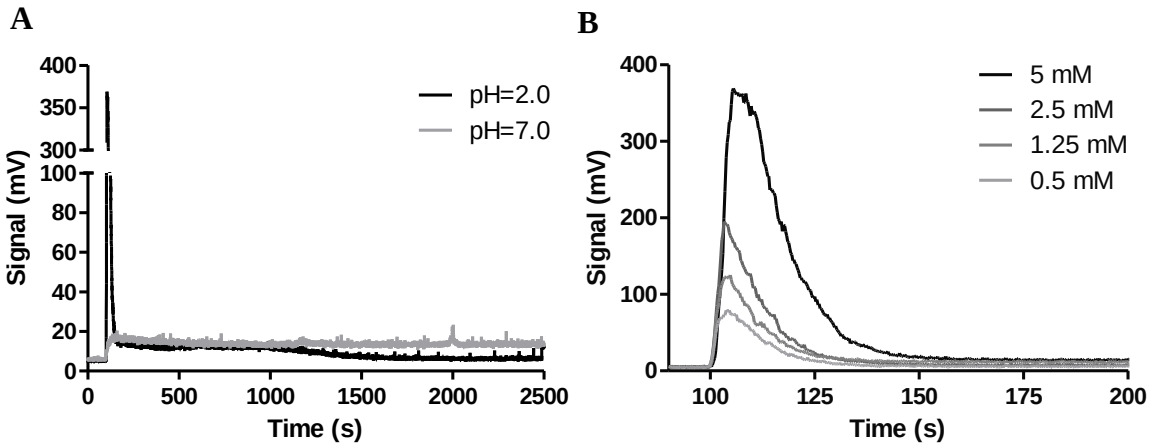


Figure 4.6. Nitrite signals detected using 2Cs reagent. (A) 5 mM nitrite was incubated in pH=2.0 or pH=7.0 at 37 °C for 10 min prior to neutralization and measurement on NOA; (B) different concentrations of nitrite were incubated in pH=2.0 at 37 °C for 10 min prior to neutralization and measurement on NOA.

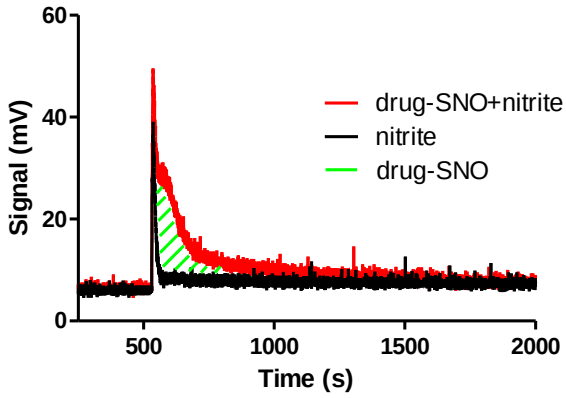


Figure 4.7. Typical signals detected from drug-SNO+nitrite and nitrite control. The absolute area of drug-SNO was calculated by subtracting the area generated by nitrite from the total area of the signal obtained from drug-SNO and unreacted nitrite.

3.2 Biochemical properties of drug-SNO

3.2.1 Properties of native drug preparations

Knowing that formation of RSNO depends on low pH and amount of free thiol available for the reaction with nitrite, we measured pH and the content of reduced thiol groups in all of the native drug preparations (Table 11). An important observation was that the pH of the aqueous solutions of thienopyridine was acidic, even without addition of HCl, with clopidogrel being the most acidic (pH=2.0), followed by prasugrel (pH=4.0) and ticlopidine (pH=5.0). The pH of aqueous ticagrelor was 7.0. The content of reduced thiol (drug-SH) varied across the different drug preparations. Ticagrelor-SH (Ticag-SH) was the lowest exhibiting 33.4± 4.6 nM/mM of ticagrelor. The respective Th-SH in ticlopidine, clopidogrel and prasugrel preparations were 66.7±1.8, 131.0±9.1 and 873.2±50.2 nM/mM of drug.

Drug	pH of water solution	Free thiol content (nM/mM of drug)
Ticlopidine	5.0	66.7±1.8
Clopidogrel	2.0	131.0±9.1
Prasugrel	4.0	873.2±50.2
Ticagrelor	7.0	33.4± 4.6

Table 11. pH and reduced thiol content of drug preparations. n=3 for ticlopidine, clopidogrel and prasugrel and n=1 for ticagrelor.

3.2.2 Detection of drug-SNO using NOA

Firstly, aqueous preparations of native drug (without pH enforcement) were mixed with 1 mM nitrite and analysed using OBC and 2Cs reagent (Figure 4.8). Prasugrel-SNO (pras-SNO) or clopidogrel-SNO (clop-SNO) formed readily, resulting in distinctive NOA signals above that of the nitrite control. Neither ticlopidine nor ticagrelor formed the respective drug-SNO derivatives in these conditions.

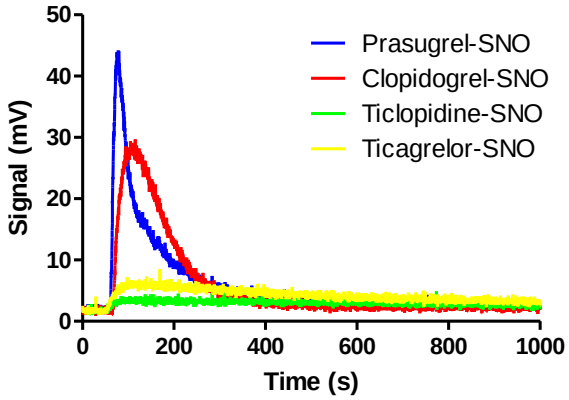


Figure 4.8. Typical NOA signals showing formation of drug-SNO by prasugrel, clopidogrel, ticlopidine and ticagrelor in their basal pH. Only prasugrel-SNO and clopidogrel-SNO produced clear signals above the background nitrite. Nitrite signals are not shown for simplicity.

3.2.2.1 Comparison of clopidogrel tablet with purified clopidogrel

Clopidogrel chemical was compared with clopidogrel tablet (*Plavix*) for potential to make clop-SNO. Both preparations generated clop-SNO but in a different manner and quantity (Figure 4.9). Clopidogrel chemical produced a sharp, intense peak whereas clopidogrel tablet resulted in a wide, low intensity peak. Furthermore, although equimolar concentrations of clopidogrel sulphate were used, clopidogrel tablet produced ~6 times more clop-SNO than clopidogrel chemical (as AUC were compared). None of the excipients of the thienopyridine tablets contained reduced thiol groups, which might have accounted for the detected RSNO species (see Shawmendra Bundhoo MD 2011). Although the reason for these discrepancies is not known, one can speculate that the tablet excipients, which are generally added to increase the absorption of the drug could have improved a poor drug solubility in water and thus increased the amount of clop-SNO.

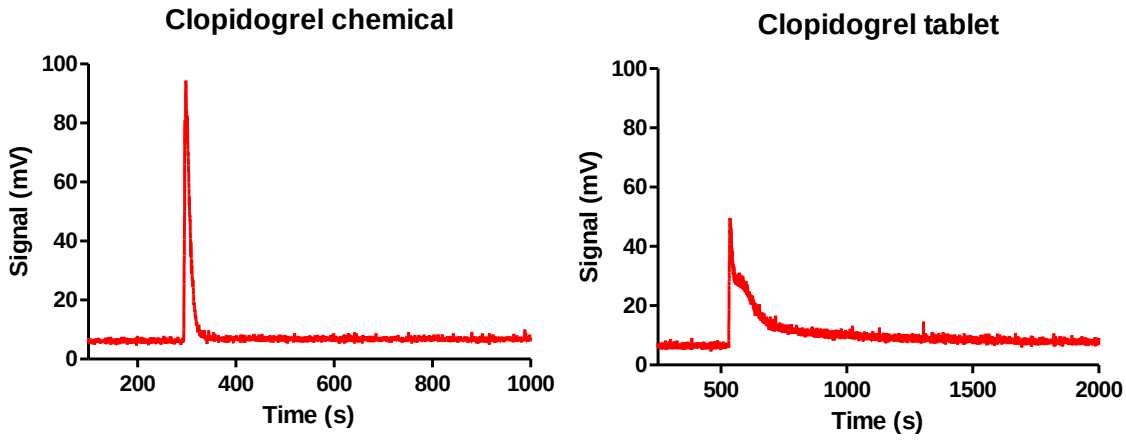


Figure 4.9. NOA signals generated by a chemical and tablet clopidogrel-SNO. The equivalent of 5 mM clopidogrel sulphate and 1 mM nitrite were used.

3.2.2.2 Confirmation of clop-SNO formation by spectrophotometry

RSNO exhibit UV and visual absorption maxima in the range 320-360 nm with additional peaks at around 545 nm and 510-520 nm[184]. Clopidogrel chemical or tablet (10mM) was mixed with 1 mM nitrite, incubated at 37 °C for 10 min and then filtered through a 0.22 μm filter. Absorption was determined at the native pH of clopidogrel solution in water, i.e. pH=2.0 (Figure 4.10) and after neutralization to pH>7.2 (Figure 4.11). Following this, samples at pH>7.2 were pretreated with mercuric chloride (8.9 mM $HgCl_2$) to remove the NO^+ group, thus confirming the presence of RSNO.

At pH=2.0 clop-SNO and nitrite resulted in multiple peaks with no clear and distinctive peak in the 320-360 region. Sample neutralization resulted in a smoother absorption shoulder with the highest absorbance for tablet clop-SNO, followed by chemical clop-SNO and nitrite. However, at pH>7.2 the absorption of particular clop-SNO preparations was not different from the respective parental clopidogrel solutions or after addition of mercuric ions. This suggests that spectrophotometry is not sensitive enough for detection of clopidogrel-SNO.

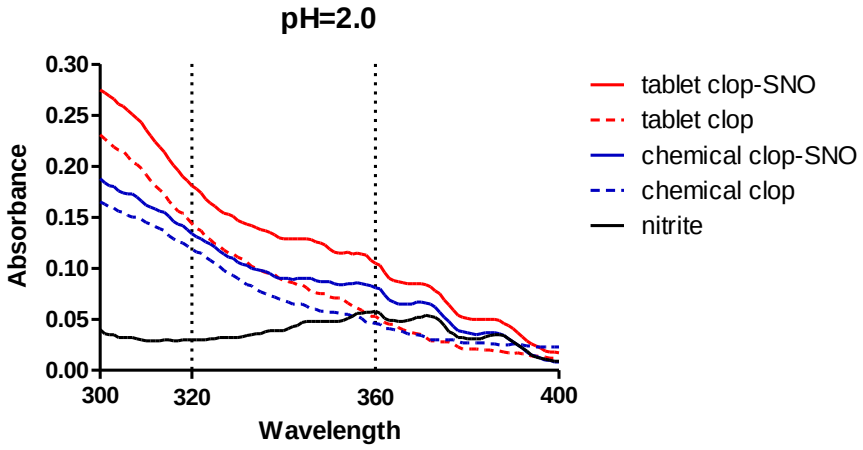


Figure 4.10. Spectrophotometric detection of clopidogrel-SNO in its basic pH=2.

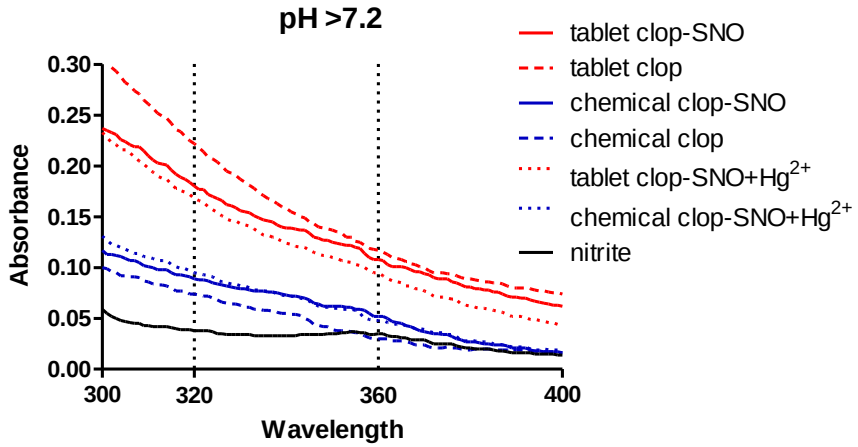


Figure 4.11. Spectrophotometric detection of clopidogrel-SNO after neutralisation.

3.2.3 Influence of pH on the formation of drug-SNO

Drug-SNO formation was dependent on the pH of the native solution. All drugs generated SNO in an acidic medium provided that the pH was ≤ 4.0. Although ticlopidine and ticagrelor did not generate SNO when in its native solution at a respective pH of 5.0 and 7.0, drug-SNO synthesis was observed when the pH was forced lower through addition of HCl. Both ticlopidine and prasugrel SNO synthesis

was maximal at pH 3.0 whereas clopidogrel and ticagrelor SNO synthesis was maximal at pH 2.0 (Figure 4.12).

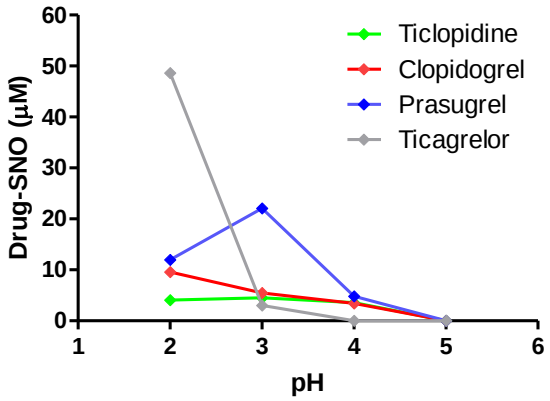


Figure 4.12. Typical pH dependence of drug-SNO formation.

3.2.4 Influence of nitrite concentration on the formation of drug-SNO

3.2.4.1 High nitrite concentration

Clop-SNO and pras-SNO formation increased with increasing nitrite concentration 1-100 mM (Figure 4.13). The amount of pras-SNO was greater than clop-SNO reaching statistical significance at 50 mM nitrite (856.0±172.2 vs. 458.5±48.4 µM, unpaired t-test, n=4, p=0.004) and at 100 mM nitrite (1413.0±215.5 vs. 535.4±57.9 µM, unpaired t-test, n=4, p<0.001). Based on these data, the maximal pras-SNO formation was up to 1 % of the parent drug.

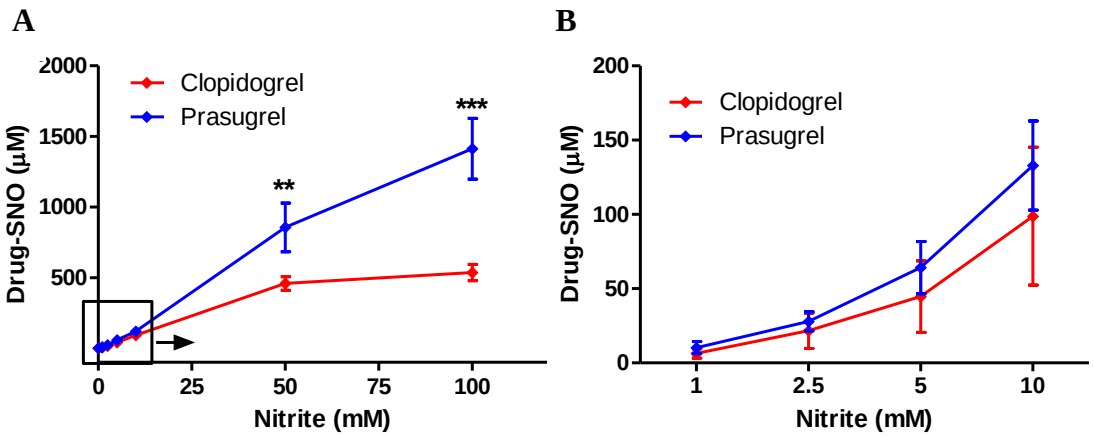


Figure 4.13. Dependence of high nitrite concentration on the clop-SNO and pras-SNO formation. (A) 1-100 mM nitrite, contains the insert (graph B); (B) 1-10 mM nitrite. Drugs were used at their basal pH. Unpaired t-test, n=4, **p<0.01, ***p<0.001.

In contrast to clop-SNO and pras-SNO, ticagrelor was first acidified to pH=2.0 in order to generate ticag-SNO and then various nitrite concentrations 0.25-2.5 mM were added (Figure 4.14). The formation of ticag-SNO was subsequently higher with increasing nitrite concentrations. Additionally, in comparison to clop-SNO formation at similar conditions (2.5 mM nitrite, pH=2.0), ticagrelor generated more –SNO derivatives (ticag-SNO=127.1±15.4 µM vs. clop-SNO=15.7±10.6 µM, n=2). Based on these data, ticag-SNO formation was up to 3 % and clop-SNO was up to 0.5 % of parental drugs.

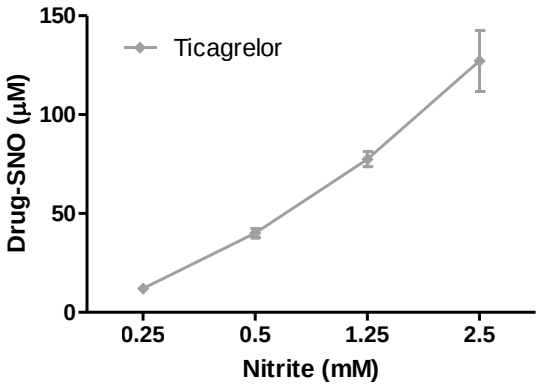


Figure 4.14. Dependence of high nitrite concentration on the ticagrelor-SNO formation. Ticagrelor was used at forced pH=2.0. n=2.

3.2.4.2 Physiological nitrite concentration

Given that the nitrite concentrations in stomach are typically in the order of 0.6 – 20 μM[185, 186], the possibility of drug-SNO synthesis was also studied at 10-1000 μM nitrite (Figure 4.15). When drug concentrations were adjusted according to the expected stomach concentrations after 1-tablet ingestion (ticagrelor 5.7 mM, clopidogrel 7.8 mM and prasugrel 0.9 mM) and pH of all drug preparations was fixed at pH=2.0, the formation of all drug-SNO derivatives was still apparent at lower levels of nitrite.

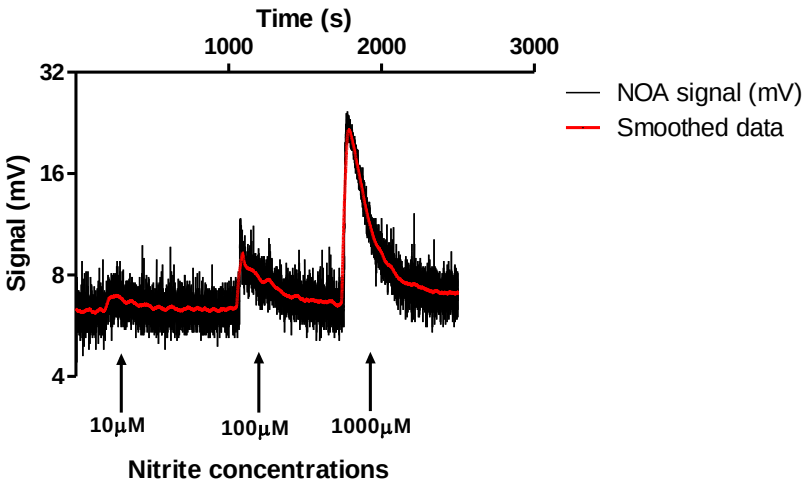


Figure 4.15. Representative clop-SNO signals generated by 10 mM clopidogrel and 10-1000 μM nitrite. Because no nitrite signal at a concentration <200 μM could be detected by 2Cs reagent, these raw traces represent only Th-SNO formation, confirming their synthesis at physiological nitrite concentrations.

3.2.5 Influence of incubation time on the formation of Th-SNO

Stability of Th-SNO was tested by increasing the incubation time of 10 mM clopidogrel and prasugrel with 10.1 mM nitrite (Figure 4.16). Clop-SNO synthesis was maximal when mixed with nitrite for 10 min but declined thereafter (t½~66min). Pras-SNO formation was sustained over a period of 2 h. The relative proportion of Clop-SNO remaining after 2 h was significantly lower when compared to pras-SNO (14.0±2.3 % vs. 117.0±21.9 %, unpaired t-test, n=3, p=0.008). All solutions were neutralized prior to 2Cs analysis.

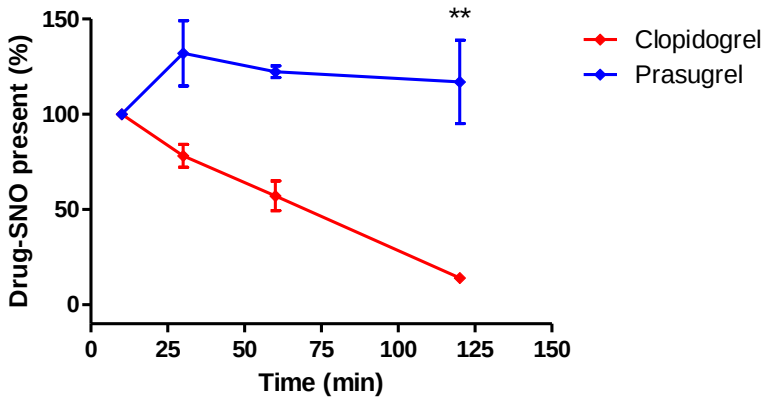


Figure 4.16. Proportion of formed Th-SNO remaining over time. 10 mM thienopyridine was mixed with 10.1 mM nitrite. Unpaired t-test, n=3, **p<0.01.

3.3 Biological properties of Th-SNO

Nitrosothiols have anti-platelet and vasodilatory properties primarily via donation of NO^+ and activation of sGC/cGMP pathways in platelets and smooth muscle. These two properties were tested for clop-SNO and pras-SNO. In order to relate the amount of Th-SNO made with its biological effect, LTA and isometric

tension studies, NOA analysis was run in parallel on the same samples. GSNO was used as a standard nitrosothiol and a positive control. Nitrite and basal drug suspensions (without SNO) were used as negative controls.

3.3.1 Inhibition of platelet aggregation

Clop-SNO and pras-SNO significantly inhibited the ADP-induced platelet aggregation in comparison to their parental forms (Figure 4.17). 20 mM thienopyridine preparations mixed with 20 mM nitrite were used to obtain the maximal inhibition of platelet aggregation. Clop-SNO inhibited 38.6±19.9 % of platelet aggregation in controls (paired t-test, n=8, $p<0.0001$) while pras-SNO inhibited 32.3±15.2 % of platelet aggregation in controls (paired t-test, n=8, $p=0.003$). In turn, 10 µM GSNO resulted in 53.7±16.3 % inhibition.

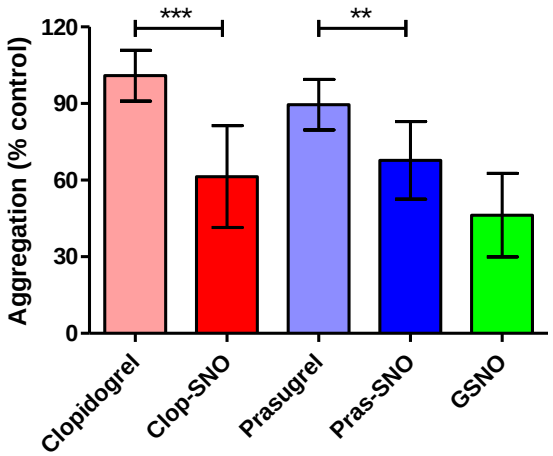


Figure 4.17. ADP-induced platelet aggregation of clop-SNO, pras-SNO and GSNO.
Paired t-test, n=8, **p<0.01, ***p<0.001.

To compare the effective inhibitory concentrations of Th-SNO and GSNO, and control for unreacted nitrite in Th-SNO mixture, concentration response curves were constructed using different concentrations of inhibitors (Figure 4.18). When normalized for the different SNO yields, clop-SNO and pras-SNO inhibited ADP-induced aggregation equally and similarly to GSNO (pras-SNO IC_{50}=7.9±2.1 μM vs. clop-SNO IC_{50}=10.6±2.8 μM vs. GSNO IC_{50}=9.8±4.6 μM, 1-way ANOVA, n=4, p=0.270), but were significantly different to that of nitrite alone (IC_{50}=91.7±51.9 mM, 1-way ANOVA, p=0.002).

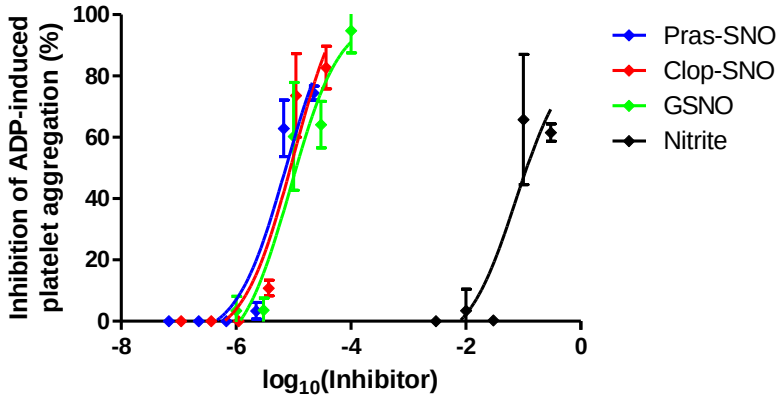


Figure 4.18. Concentration response curves of ADP-induced platelet inhibition for pras-SNO, clop-SNO, GSNO and nitrite.

3.3.2 Vasodilation of rabbit aortae

Pras-SNO and clop-SNO induced immediate relaxation of pre-constricted rabbit aortic rings and their relaxation profiles were different from the slow relaxation observed in the respective nitrite controls (Figure 4.19). The addition of the basal prasugrel and clopidogrel preparations followed by nitrite did not change nitrite-induced relaxation of pre-constricted vessels (not shown), confirming that Th-SNO is

made only when the drug preparations are mixed with nitrite prior to addition to the tissue bath.

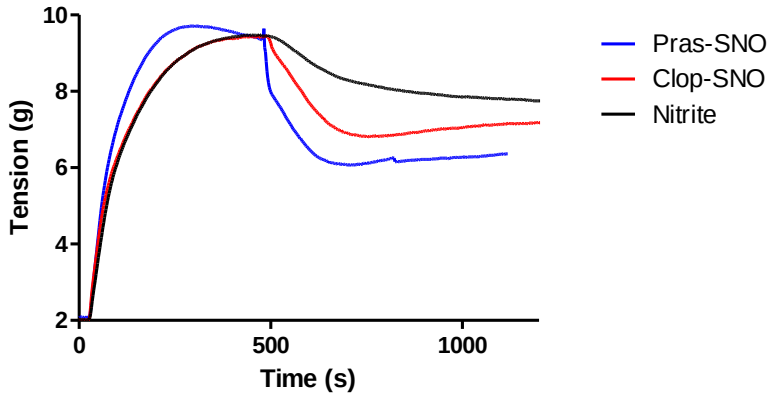


Figure 4.19. Relaxation profiles showing the difference between pras-SNO, clop-SNO and nitrite-induced responses of pre-constricted rabbit aortic rings.

Pras-SNO and clop-SNO induced greater relaxation when higher nitrite concentrations were used for synthesis (Figure 4.20), although this reached statistical significance comparing 1 mM and 10 mM nitrite only (clop-SNO Rmax=11.3±5.7 % vs. 33.0±13.3 %, unpaired t-test, n=5, p=0.010 and pras-SNO Rmax=14.4±7.8 % vs. 30.4±13.2 %, unpaired t-test, n=5, p=0.024). Clop-SNO and pras-SNO-induced vasodilation was not statistically different at any used concentration of nitrite.

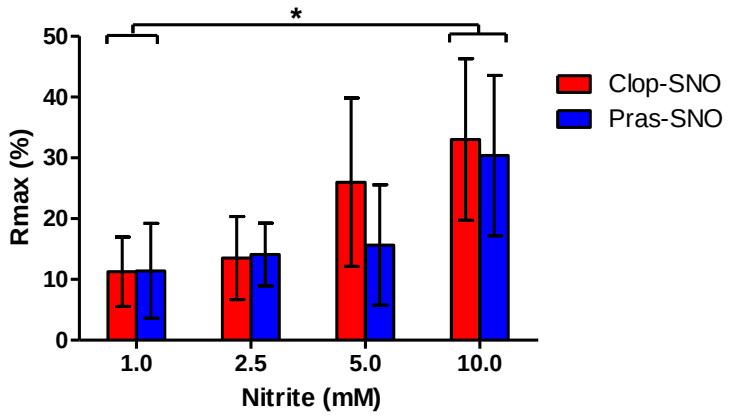


Figure 4.20. Maximal relaxation (Rmax) of pre-constricted aortic rings induced by clop-SNO and pras-SNO where Th-SNO yield was varied by increasing nitrite concentrations. Unpaired t-test, n=5, *p<0.05.

To compare the potency of Th-SNO-induced relaxations with a standard nitrosothiol GSNO, aortic rings previously used for Th-SNO-induced responses were washed, pre-constricted and subjected to different concentrations of GSNO. When normalized for the different SNO yields, clop-SNO and pras-SNO-induced relaxation was similar and equivalent to ~40 % of GSNO-induced relaxation (Figure 4.21).

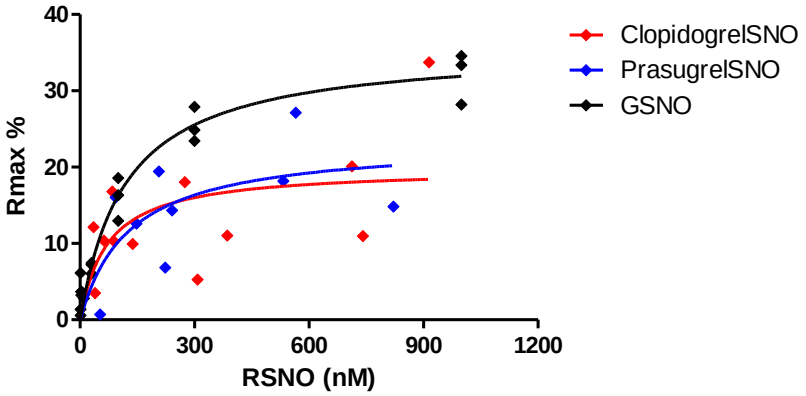


Figure 4.21. Potency of Th-SNO- and GSNO-induced relaxation of pre-constricted aortic rings when related to different RSNO content.

In order to understand this result, the extent of unreacted thienopyridine was considered. Taken into account that our Th-SNO synthesis resulted in ~1 % of Th-SNO with respect to the parental drugs, the unreacted thienopyridines (including Th-SH) had potential to recapture the relaxant NO^+, leading to only partial relaxation. In order to test this hypothesis, a mixture of GSNO/GSH was used in a separate set of experiments. As the concentration of GSH was increased, the relaxation induced by the same dose of GSNO was proportionally reduced (Figure 4.22). A similar trend was observed for Th-SNO/Th, suggesting the competition between reduced thiols (RSH) in the buffer and sGC in the smooth muscle for released NO^+.

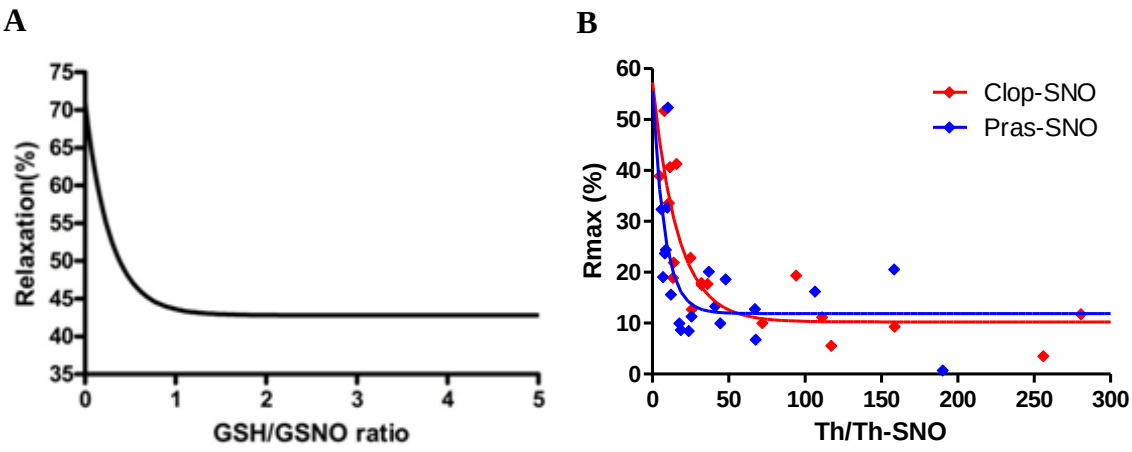


Figure 4.22. Rmax of pre-constricted aortic rings induced by GSNO (A) and clop-SNO and pras-SNO (B) in relation to GSH/GSNO (A) and Th/Th-SNO (B) ratio.

In order to find the mechanism of the clop-SNO- and pras-SNO-induced vasodilation, the role of endothelium and the involvement of sGC-dependent pathways were investigated (Figure 4.23). Denuded vessels produced a similar relaxation to both Th-SNO in comparison to normal vessels (clop-SNO –EC Rmax=63.1±13.4 % vs. clop-SNO Rmax=52.2±14.8 % and pras-SNO –EC Rmax=54.6±4.4 % vs. pras-SNO Rmax=44.4±5.9 %, ANOVA, n=5, ns), suggesting primarily that endothelium-independent signalling was involved. In turn, Th-SNO-induced relaxation in ODQ-treated vessels was significantly lower when compared with relative controls (clop-SNO +ODQ Rmax=7.9±13.1 % vs. clop-SNO and pras-SNO +ODQ Rmax=2.8±1.1 % vs. pras-SNO, ANOVA, n=5, $p<0.001$), implying a crucial role for sGC.

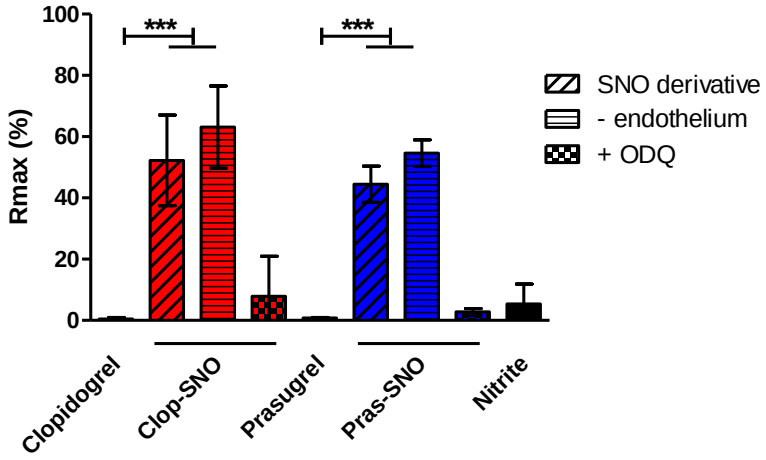


Figure 4.23. Involvement of endothelium and sGC-dependent pathways in the relaxation induced by clop-SNO and pras-SNO. Relevant nitrite controls were performed. A stimulation with clopidogrel and prasugrel alone did not induce any relaxation. ANOVA, n=5, ***$p<0.001$.

4. Additional data

4.1 Interaction of Th-SNO with human plasma

It was demonstrated by our group[187] that the SNO moiety from clopidogrel-SNO can be transferred to bovine serum albumin (BSA) or human plasma protein (primarily albumin) (Figure 4.24). This was clearly indicated by the transfer of SNO from later fractions of the G50 column (Clopidogrel-SNO) to early fractions (BSA/plasma) and confirmed by spectrophotometry and 2 C's analysis. These results suggest that clop-SNO similarly to other RSNO species participate in transnitrosation reactions in biological systems.

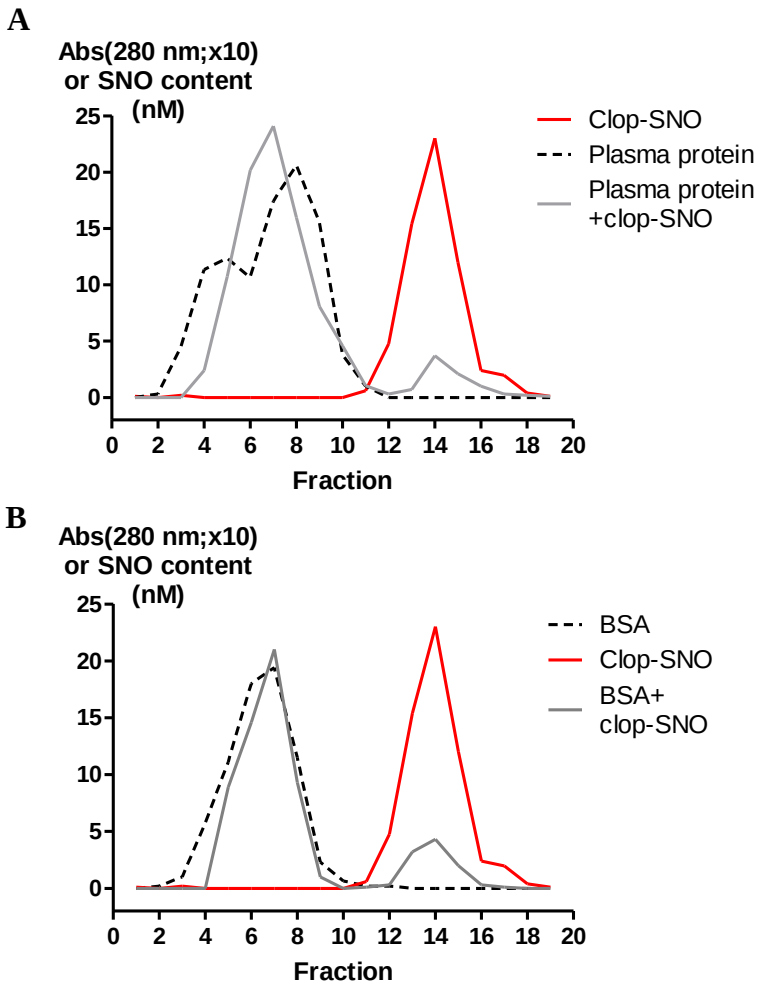


Figure 4.24. Typical experiment showing transfer of SNO from clop-SNO (~fraction 14) to plasma albumin (~fraction 7; A) or to BSA (~fraction 7; B).

4.2 Analysis of clopidogrel derivatives before and after addition of nitrite

In order to identify the components present in an aqueous preparation of purified clopidogrel sulphate and again following addition of nitrite, MS analysis was carried out (Table 12)[110]. Native clopidogrel (identified at m/z 322) was in largest abundance, followed by minor contributions (0.01%) at each of m/z 356 (active metabolite) and m/z 308 (carboxy metabolite), and 0.05% at m/z 341 (carboxy metabolite with open S-H group). Following addition of nitrite, significant peak

increases were observed at m/z 192 and m/z 370 amounting to a relative abundance each of 0.5% of the native clopidogrel m/z 322 peak.

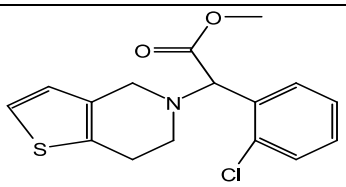
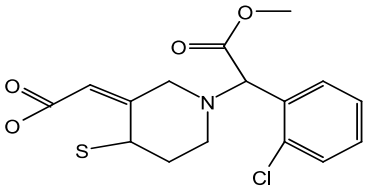
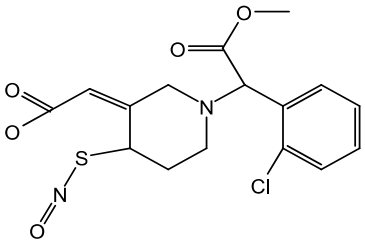
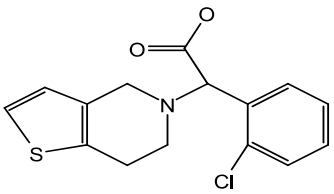
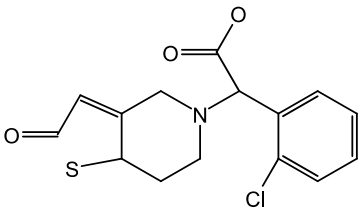
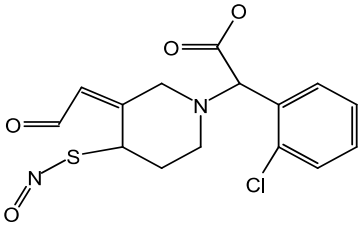
Clopidogrel species	**Structure**	**m/z**	**Relative contribution -nitrite (%)**	**Relative contribution +nitrite (%)**
Native clopidogrel		322	99.93	98.99
Active metabolite		356	Trace 0.01	None evident
Active metabolite-SNO		384/ 192	None evident	0.5
Carboxy metabolite		308	Trace 0.01	None evident
Carboxy metabolite (open thiol)		341	Trace 0.05	Trace 0.01
Carboxy metabolite-SNO		370	None evident	0.5

Table 12. Structure, anticipated m/z, and relative abundance of clopidogrel species detected prior to and following addition of nitrite. Adapted[110].

4.3 Comparison of different clopidogrel salts

Pharmaceutical companies market clopidogrel tablets made from different salts of clopidogrel. This has significant cost implication. Although in this thesis only those containing clopidogrel sulphate (clop-S) were studied, our group also investigated the nitrosation properties of other clinically relevant preparations, containing clopidogrel besylate (clop-B) or clopidogrel hydrochloride (clop-Cl)[110]. All clopidogrel salts produced the corresponding SNO derivatives after mixing with nitrite but there was a significant variation between drugs in terms of SNO yield and the relaxation profile induced in pre-constricted aortic rings (see Shawmendra Bundhoo MD 2011). In particular, clop-Cl-SNO seemed to be considerably different than other clopidogrel salts. It had the smallest SNO yield (Figure 4.25) and produced the most transient relaxation when accounted for the difference in concentration of SNO.

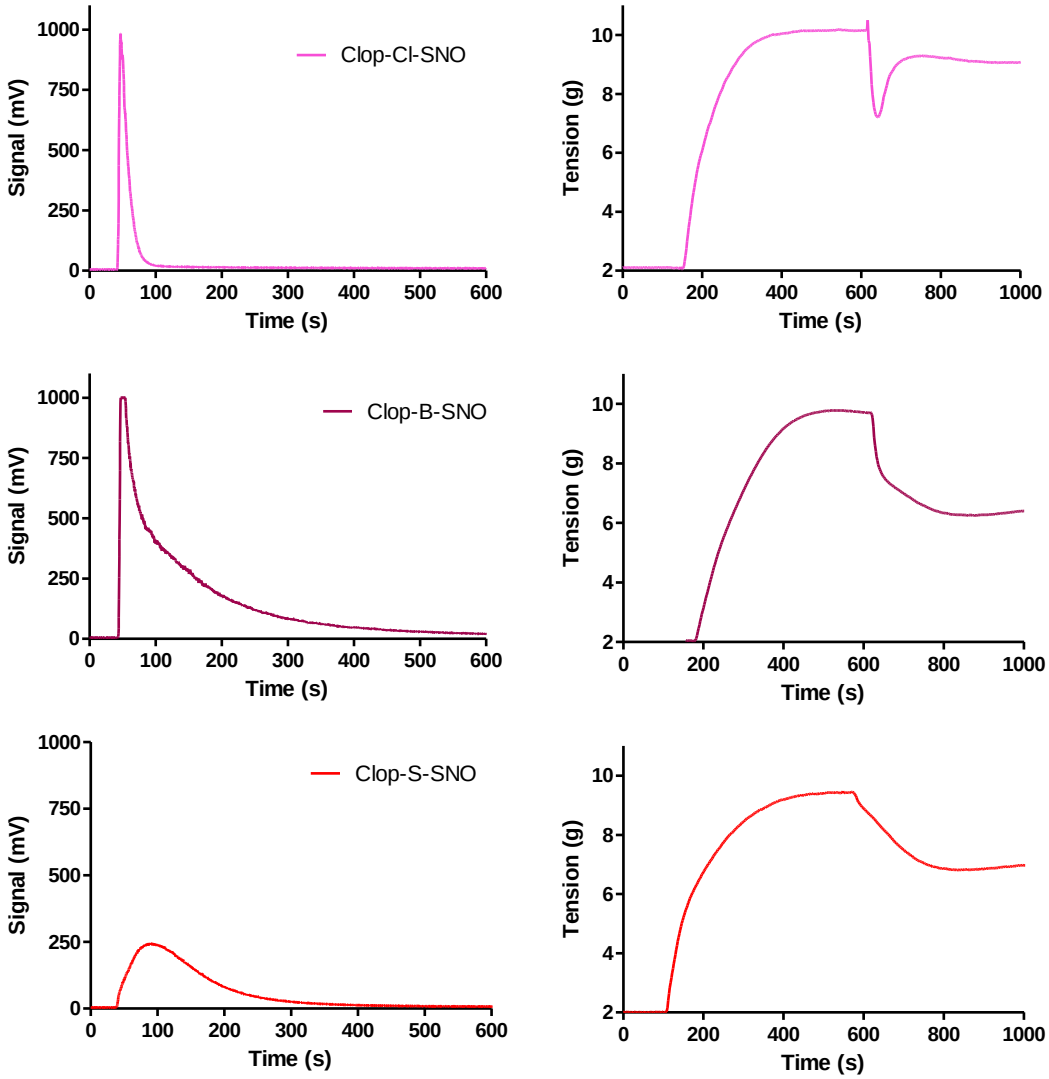


Figure 4.25. Comparison of typical signals obtained from different clop-SNO salts using OBC (left) and their typical relaxation profiles of pre-constricted rabbit aortae (right).

5. Discussion

Novel nitrosation properties of thienopyridines and ticagrelor were discovered in this study. The formation of *in vitro* synthesized SNO derivatives of drugs was dependent on the extent of free thiol, pH and nitrite concentration and was affected by the resulting stability of the SNO produced. Prasugrel exhibited the greatest SNO production, based largely on possessing more free thiol and stability of the resulting SNO over 2 h. Clopidogrel also readily formed SNO; despite having less free thiol in aqueous solution, the low pH of the clopidogrel solution may have favoured increased SNO formation. However, the resulting clop-SNO showed reduced stability (t½~66min) when compared to pras-SNO (stable for 240 min). Ticlopidine and ticagrelor did not readily form SNO when in solution unless the pH was artificially reduced further to induce more favourable conditions for SNO synthesis. Increased doses of nitrite resulted in proportionate higher concentration of drug-SNO and reached a plateau at 10 mM dose probably due to saturation of available thiols.

The biological effects of synthesized drug-SNO were similar to those expected from RSNO biochemistry and included inhibition of ADP-induced platelet aggregation, vasodilation of rabbit aortae and transnitrosation to albumin. Pras-SNO, clop-SNO and GSNO displayed the same capacity to inhibit platelet aggregation when related to the SNO content. Native forms of these thienopyridines were ineffective inhibitors of aggregation, whereas nitrite was a relatively poor inhibitor of platelet aggregation in agreement with previous studies. Pras-SNO and clop-SNO exhibited direct vasorelaxant properties, although only ~40 % of the maximal GSNO-induced relaxation. The apparent reduction in effectiveness of NO^+ released from Th-SNO could be explained in terms of unreacted thienopyridine in the sample 'recapturing' NO^+ before it could reach the smooth muscle. The basal forms of thienopyridines did not exhibit any vasomodulatory actions while nitrite induced minor and

relatively slow vasodilation, which was deducted from the total Th-SNO-induced responses reported in this chapter. The nitrovasorelaxation of clop-SNO and pras-SNO was mediated via donation of NO^+ and activation of classic sGC signal transduction independently of endothelium. Importantly, upon the interaction of clop-SNO with human plasma, a transfer of NO^+ from clop-SNO to albumin was observed, confirming its important transnitrosation capacity.

Significant differences were observed between the extent of free thiol detected in the thienopyridine/ticagrelor neutral solutions and the extent of drug-SNO formed (at acidic pH). For example, the data for ticagrelor shows only ~0.004 % free thiol of the parent drug at pH=7, whereas on addition of 2.5 mM nitrite at pH=2 up to ~ 3 % ticagrelor–SNO was synthesized. One possible explanation for these differences is an effect of acidic pH on nitrite and/or anti-platelet drugs' molecules, which could govern the availability of drug-SH. The potential influence of low pH on the chemical structure of ticagrelor might be particularly important not only for the drug-SNO formation but also for the efficiency in the inhibition of platelet aggregation.

The relevance of drug-SNO formation in humans is unknown. Certainly physiological conditions were provided in terms of using the relevant amount of commercially available tablets (1-10 mM in stomach) and adjusting for the appropriate pH corresponding to the stomach pH=1.5-3.5. Additionally, although most studies were conducted using exaggerated mM concentrations of nitrite, we were able to detect drug-SNO formation with 10 μM nitrite, which is in the range of 0.6-20 μM nitrite found in human stomach (Figure 2.26)[185, 186]. Direct measurement of drug-SNO in patient plasma would be ideal for the *in vivo* confirmation of drugs nitrosation but is unlikely due to possible absorption of SNO species by stomach and gastrointestinal tract and complex interactions of RSNO metabolism in the bloodstream. Furthermore the metabolism of thienopyridines in the liver might limit a detection of Th-SNO

to the enterohepatic circulation. In order to test whether hapatic metabolism has an effect on systemic levels of RSNO, SNO derivatives could be given intravenously.

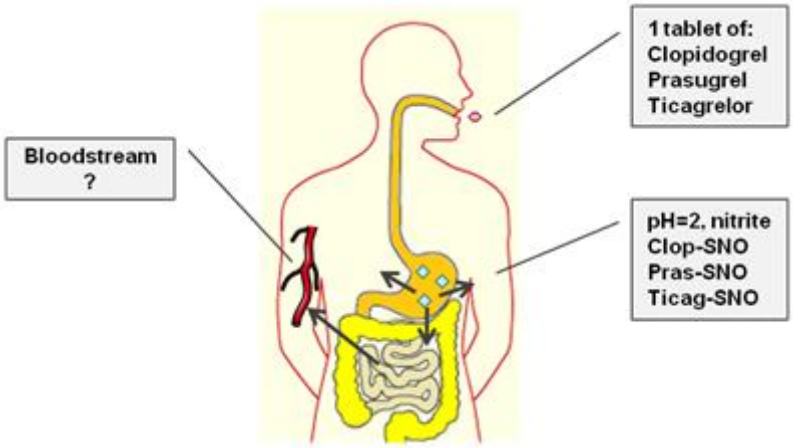


Figure 4.26. Simplified scheme of expected clop-SNO, pras-SNO and ticag-SNO formation in human stomach after ingestion of 1 tablet of clopidogrel, prasugrel and ticagrelor, respectively. The amount of SNO derivatives reaching the bloodstream is unknown and can be limited by tissue absorption, hepatic metabolism and transnitrosation reactions.

An interesting consideration, which was not taken into account in this study is the influence of disintegration time of tablets in stomach and/or gastrointestinal tract and the coating of tablets on the final drug-SNO formation. The tablet coating may partially protect $P2Y_{12}$ antagonists from the influence of stomach acid and subsequent generation of SNO derivatives. However, this could potentially be a limiting factor only for ticlopidine- and ticagrelor-SNO formation because aqueous clopidogrel and prasugrel readily formed RSNO in their normal acidic pH.

The *in vivo* formation of RSNO associated with anti-platelet therapy would be beneficial for patients suffering from endothelial dysfunction and reduced NO bioavailability. Transnitrosation between nitrosothiols and proteins prolongs the biological half life of NO via formation of dinitrosothiol iron complexes[188], which can be transported by circulation and

release NO on demand. Released NO may inhibit activation and aggregation of platelets or induce local vasodilation where endogenous NO production is impaired. It is important to say that because SNO (direct from thienopyridine or other sources) is unlikely to be released immediately and other counterregulatory physiological mechanisms can also be activated[65, 66], there is no risk of hypotensive effects. In support, clopidogrel therapy has never been associated with changes in systemic blood pressure.

The effectiveness of $P2Y_{12}$ antagonists in patients has largely been considered in terms of variation in patient metabolism and activation of the drug to its active form, yet the potential for direct drug-SNO formation could be important. It has been shown that the co-administration of some proton pump inhibitors to patients (such as omeprazole) reduce thienopyridine efficacy on platelet function[189, 190], a finding that to date has been attributed to competition for CYP450 metabolism[191, 192] but could clearly influence SNO formation by neutralising stomach pH. Remarkably, the extent of active thienopyridine found at peak in human plasma following oral loading is ~0.01 % of the inactive drug present at the same time[135], yet this is sufficient to produce irreversible clinically relevant inhibition of platelet activation. Based on the data we have shown, ~1 % of the parent drug can potentially be nitrosated, which could amount to significant levels systemically. As such, drug-SNO may not be detrimental in targeting $P2Y_{12}$ receptors, but could provide beneficial effects through parallel delivery of NO to platelets and blood vessels.

Summary:

- Novel nitrosation properties of thienopyridines were discovered without the need for prior metabolism to their active form. Unexpectedly, ticagrelor also formed SNO derivatives provided pH<4.

- The formation of newly synthesized drug-SNO species was dependent on the extent of free thiol, pH and nitrite concentration.

- The biological effects of SNO derivatives of drugs were similar to those expected from classical RSNO biochemistry and included inhibition of ADP-induced platelet aggregation, vasodilation of rabbit aortae and transnitrosation to albumin.

- Tablets containing different salts of clopidogrel have varying potential to make SNO species and have different profiles of drug-SNO-induced vasorelaxation.

- Future work could look at the effect of SNO derivatives on different platelet agonists (e.g. thrombin).

Part 3: Influence of clopidogrel therapy on NO production, metabolism and bioavailability in coronary artery disease (CAD) patients

Hypothesis: ***Clopidogrel increases NO bioavailability in blood in CAD patients.***

Specific aims of this chapter were:

- To determine whether a single loading or maintenance dose of clopidogrel influences levels of NO metabolites (nitrite, nitrate and RSNO), cGMP and oxidative and nitrosative stress markers in plasma.
- To investigate the relationship between vascular, oxidative and platelet-derived effects of clopidogrel on NO bioavailability in patients.

1. Introduction

Dysfunction of the endothelium is an early event in many if not all cardiovascular disease (CVD), including coronary artery disease (CAD)[5, 17, 25, 30]. A hallmark feature of endothelial dysfunction is deficiency in endothelial production of NO due to augmented oxidative stress[25, 193]. The reduced 'NO bioavailability' promotes abnormal vascular reactivity, inflammation and thrombosis leading to progression of disease and increased risk of cardiovascular events[30] (see I. General Introduction, pages 15-18). Clopidogrel treatment in CAD patients has been associated with increased FBF/FMD[157, 169, 170] whether administered pre or post percutaneous coronary intervention (PCI). Although, FMD is a technique which provides an index of NO-mediated vasodilation and is, therefore commonly used as a clinical marker of *in vivo* NO bioavailability, some evidence suggest it may not solely be related to NO[194].

The metabolism of NO in the human body is multi-factorial and it depends on diffusion rate, redox status and metal complexes within the vascular tissue and different compartments of blood (see Andrew Pinder PhD 2009). Different NO derivatives/metabolites such as nitrite[195, 196], S-nitrosothiols and S-nitrosoproteins (RSNO)[67, 197] and potentially nitrate[198] and nitrated lipids[199] participate in regulation of NO bioavailability in the circulation. The focus of this study was to assess the effect of clopidogrel therapy on the level and apportion of these NO metabolites in patients.

Endothelial nitric oxide synthase (eNOS) is the main source of NO in the vasculature[200] and its activity is considered directly proportional to the concentration of plasma nitrite, suggesting nitrite may reflect cardiovascular NO bioavailability[201, 202]. It was reported that 70 % of resting plasma nitrite is derived from eNOS activity in humans and other mammals[201]. Additionally plasma nitrite can be affected by diet and oxidative stress

limiting the amount of NO (see I. General Introduction, pages 15-18 and 25-27). In contrast, plasma nitrate levels are influenced by many eNOS-independent factors such as dietary nitrate ingestion and liver and kidney function[203, 204]. Importantly, NO metabolites including nitrite, nitrate and RSNO are now considered not only as a direct measure of a physiological activity of NO but also as a biologically active NO reservoir. Nitrite can regulate vascular tone and provide NO-mediated vascular protection under conditions of low oxygen[196]. Dietary nitrate can be reduced to nitrite by bacteria having nitrate reductase enzymes and residing in human saliva[56, 198]. Whether basal level of nitrate in human plasma is also bioconverted remains yet to be established. Another NO metabolite, RSNOs, possess very unique and versatile mechanisms of action. Apart from storage and release of NO on demand, it mediates S-nitosylation and transnitrosation of proteins, which influence downstream effects of NO signalling[68]. RSNO is less stable and occurs in human plasma in much lower quantities than nitrite (~10x less) or nitrate (~2000x less), making an accurate measurement of RSNO more challenging and often neglected. However, considering the novel nitrosation properties of pro-drug clopidogrel already identified *in vitro* (see IV. Results, pages 140-172), the enhanced formation of RSNO in patients receiving clopidogrel and the relevance to cardiovascular function was an exciting prospect worthy of investigating.

Platelets also produce NO, although in smaller amounts than endothelial cells[205, 206]. In addition, while endothelial-derived NO is known to work in a paracrine manner, i.e. diffuses to adjacent cells, platelet-derived NO is thought to exert its major effect within the cell it was produced, i.e. in an autocrine manner[205]. NO released by platelets has been recently shown to inhibit recruitment of platelets and leukocytes to the growing thrombus, where cells are in close proximity to each other[207, 208]. However the role of platelet-derived NO in regulating primary platelet aggregation remains controversial.

Clopidogrel therapy has been shown to reduce different pro-inflammatory markers in patients with stable CAD, ACS and/or undergoing coronary stenting[156, 157, 160-162, 209, 210]. Relatively little attention has been given to the influence of drug on oxidative stress, which is directly applicable to NO biovailability. Taking into account an inhibitory effect of pro-drug clopidogrel on vascular production of superoxide found in rabbit aortae (see IV. Results, pages 130-131), it was decided to explore possible anti-oxidant actions of clopidogrel in CAD patients.

Administering a loading dose of 600 mg clopidogrel to patients undergoing coronary stenting[128] is recognised as a standard clinical practice and the maximal inhibition of platelet aggregation occurs within 1-2 h after ingestion. A maintenance 75 mg dose of clopidogrel is recommended for all ACS patients[211-213]. Most of the recognised beneficial effects of clopidogrel have been studied in patients receiving clopidogrel chronically (for days/weeks/months). Therefore, aside from recruitment of CAD patients receiving chronic treatment with clopidogrel (at least 3 days), it was decided to investigate acute changes occurring within hours following a loading dose to clopidogrel naïve patients.

Previous *in vitro* studies from the first two results' chapters of this thesis were designed to study alternative and unknown effects of pro-drug clopidogrel on NO signalling. In order to relate our findings to a physiological/pathophysiological situation in patients, where clopidogrel is mainly used as a specific platelet $P2Y_{12}$ antagonist, the present investigation was conducted on CAD patients. The main goal was to investigate the effect of clopidogrel therapy on the level of circulating NO metabolites, particularly nitrite, nitrate and RSNO in addition to relevant selected markers of oxidative and nitrosative stress. The measurement of plasma cGMP was used as an index of vascular NO activity and in attempt to distinguish between vascular and platelet related effects, markers of platelet and endothelial activity were compared.

2. Methods

These patient studies were conducted in collaboration with Dr Shantu Bhundoo, a close colleague within the research group conducting his MD. Dr Bhundoo was specifically responsible for patient recruitment, consent and blood sampling. Due to the need for immediate measurements in parallel and the overall workload, platelet aggregation measurements (LTA) were primarily conducted by Dr Bhundoo whereas I took primary responsibility for biochemical analyses. The Results presented are those carried out by me other than where presented for comparison or background (and clearly stated otherwise).

2.1 Recruitment of patients and collection of blood samples

A prospective, single centre study was undertaken. A total of 58 patients undergoing PCI with stent implantation for stable angina or silent ischemia were enrolled after informed consent was obtained. The protocol was approved by the local ethics committee, and was in accordance with the declaration of Helsinki.

The first group of patients studied (36 subjects) were clopidogrel naive patients, who received 600 mg loading of clopidogrel. Blood samples were collected pre and 2 h post-loading with clopidogrel, through an intravenous cannula into vacutainers containing K_3EDTA or 3.2 % trisodium citrate (Vacuette Greiner Bio-One™). The platelet poor plasma (ppp) was isolated from K_3EDTA bottles by centrifugation immediately after collection (1500 g, 10 min, 4 °C), then snap frozen in liquid nitrogen and stored at -80 °C. Each sample was aliquoted into several tubes to avoid repeated freeze-thaw cycles. The ppp was used for measurement of NO metabolites, sP-selectin, cGMP and oxidative markers. The platelet rich

plasma (prp) was isolated from trisodium citrate bottles by centrifugation (100 g, 10 min, 25 °C) and used fresh for light transmission aggregometry (LTA) studies.

The second group of patients studied (22 subjects) were already on 75 mg clopidogrel maintenance dose for ≥3 days. Blood samples were collected and processed as in first group of patients.

It should be mentioned that initially it was calculated 22 people were to be recruited to the first and second group on the basis of power calculations (90%) for plasma nitrite levels in 12 patients pre and 2 h post-loading with clopidogrel (pilot study). However, standard deviation was higher in 22 subjects (40-50 %) than in 12 subjects (10-20 %) which influenced the power of the study (70 %). Therefore the power calculation was reassesed and an additional 14 patients were recruited to the first group.

Demographics and cardiovascular disease profiles of the CAD patients recruited are outlined below in Table 13. Specific recruitment criteria are presented in the results section (page 184).

	Clopidogrel Naive (N = 36)	**Clopidogrel for ≥3 days (N = 22)**
Age	61.8	58.1
Male (%)	85.7	73.7
BMI	29.5	27.1
Hypertension (%)	79.4	57.9
Treated hyperlipidaemia (%)	91.2	84.2
Current smoker (%)	14.7	26.3
Diabetes (%)	20.5	0.0
Family History (%)	61.8	57.9

Table 13. Demographics and cardiovascular disease profiles of the CAD patients recruited. Unpaired t-test was used to compare groups.

2.2 Assay techniques

Platelet aggregation was measured using LTA assay before and after loading with ADP and TRAP. Platelet activity was assessed on the basis of soluble P-selectin (sP-selectin) concentration. NO metabolites were determined using ozone-based chemiluminescence (OBC). sP-selectin and cGMP were measured using commercially available ELISA kits. Oxidative stress in plasma was assessed by total anti-oxidant capacity (TAC) assay, 3-Nitrotyrosine (3-NT) measurement by ELISA and reduced thiols measurement using a specific fluorescent probe.

Individual methods are described in more detail in II Methods.

2.3 Statistical analysis

Results are expressed as mean±SD and 'n' represents the number of assayed plasma samples of individuals. 'n' varies between assays within the same group of patients due to different amounts of frozen aliquots saved for analysis.

Differences between samples pre and post clopidogrel loading were analysed using paired 2-tailed student's *t*-test. Differences between samples pre clopidogrel loading and chronic treatment with clopidogrel were analysed by 1-sample t-test where the former was used as a hypothesised mean. A normal distribution between group differences was assumed on the basis of the interval data type and similar data size.

The association between variables was determined using Pearson's correlation coefficients. All analysis was performed using GraphPad® (version 5 or 6) software and $p<0.05$ was considered statistically significant.

3. Results

3.1 Patient recruitment criteria

Exclusion criteria were a crucial part of this patient study and were applied to all subjects (Table 14). Because the aim was to investigate both the short ("acute") and long-term ("chronic") effects of clopidogrel, patients attending for PCI and scheduled to receive a loading dose or currently receiving the maintenance dose of clopidogrel were engaged, respectively. Knowing, though, from previous biochemical analysis that different clopidogrel salts have varying potential to make RSNO (see IV. Results, pages 170-171), only those taking clopidogrel hydrogen sulphate (Plavix™) were included.

Major exclusion criteria were factors known to influence the amount of circulating NO metabolites, e.g. diet and organic nitrate therapy. Since we planned to measure the effect of clopidogrel on plasma levels of nitrite and nitrate, subjects were fasted for at least 6 h before the blood was taken and they were not taking any organic nitrates (e.g. GTN). Additionally, because drugs changing the stomach pH were anticipated to influence the potential *in vivo* production of RSNO, people receiving proton pump inhibitors or anti-histamines were excluded.

Another relevant issue was co-administration of drugs, which inhibit the activity and aggregation of platelets apart from clopidogrel. Thus, all patients on alternative anti-coagulant or anti-thrombin therapy were excluded. This involved patients receiving non-steroidal anti-inflammatory drugs (NSAIDs) in previous 7 days, as these have also been shown to affect platelet function[214].

Care was also taken to exclude people with ACS who have higher basal platelet activation than patients with stable disease[215], which could, in turn, interfere with the measurement of platelet inhibition by clopidogrel.

Finally, an important inclusion criterion was angiographically confirmed obstructive CAD. It is well documented that subjects with CAD suffer from endothelial dysfunction and reduced NO bioavailability. Thus, a positive impact of clopidogrel on vascular NO production and oxidative stress could be of considerable potential benefit.

All recruited patients were receiving a standard dose of aspirin (75 mg daily) as part of routine primary treatment for cardiovascular diseases.

Condition	**Inclusion**	**Cause**
Receiving or about to receive clopidogrel hydrogen sulphate (Plavix™)	Yes	Other clopidogrel salts have different nitrosation properties
Fasted for ≥6h	Yes	Influence of diet on levels of NO metabolites
Receiving organic nitrates	No	Influence on levels of NO metabolites
Receiving proton pump inhibitiors	No	Increase in stomach pH and influence on levels of RSNO
Receiving anti-histamines	No	Increase in stomach pH and influence on levels of RSNO
Receiving anti-coagulants	No	Influence on platelet inhibition
Receiving anti-thrombin therapy	No	Influence on platelet inhibition
Receiving non steroidal anti inflammatory drugs (NSAIDs) in previous 7 days	No	Influence on platelet inhibition
Acute coronary syndromes (ACS)	No	High basal platelet activity
Obstructive coronary artery disease (CAD)	Yes	Endothelial dysfunction
Receiving aspirin (75 mg/day)	Yes	Standard treatment

Table 14. Patient recruitment criteria on the basis of published data and previous results.

3.2 Confirmation of pharmacological actions of clopidogrel

In order to investigate the influence of clopidogrel therapy on NO bioavailability in CAD patients, we wanted to confirm in parallel its principal pharmacological target and effectiveness on platelets. Thus, we initially determined the effect of clopidogrel on ADP-induced aggregation in prp and the content of sP-selectin in ppp.

3.2.1 Inhibition of platelet aggregation

After 2 h-intake of a loading dose of clopidogrel, the ADP-induced aggregation was significantly inhibited by 49.6±31.9 % (pre-clopidogrel ΔTransmittance = 6.8±1.5 vs. post-clopidogrel ΔTransmittance = 3.4±2.3, paired t-test, n=8, p=0.006; Figure 5.1). Clopidogrel also inhibited significantly the thrombin-induced aggregation by 59.0+/-32.8 % (pre-clopidogrel ΔTransmittance = 6.9±3.3 vs. post-clopidogrel ΔTransmittance = 3.0±2.6, paired t-test, n=8, p=0.010), suggesting an influence of $P2Y_{12}$ blockage on thrombin-mediated aggregation. The platelet aggregation study was carried out on a randomised sample n=8 from the total cohort of 36 patients.

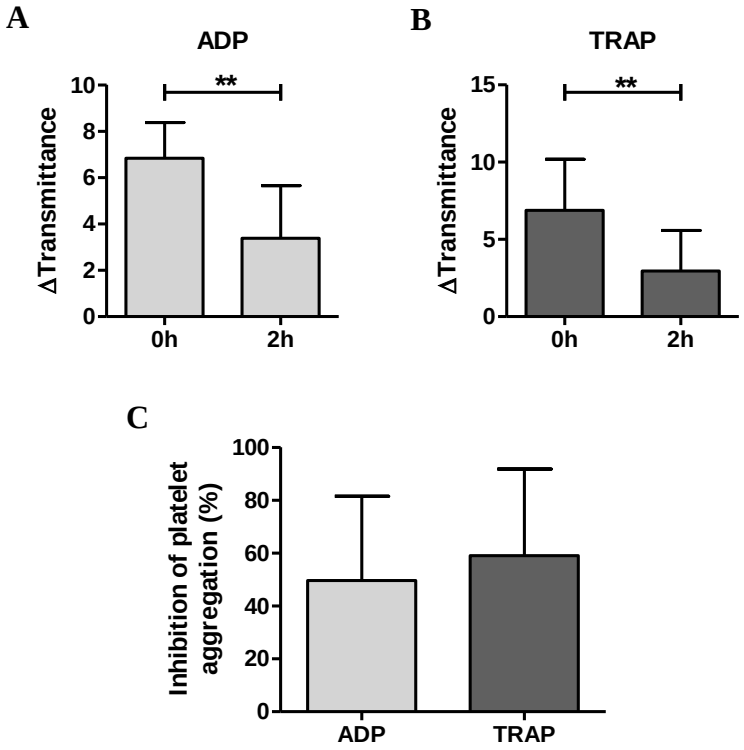


Figure 5.1. Inhibition of ADP- and TRAP-induced platelet aggregation by a loading dose of clopidogrel. (A, B) ΔTransmittance assessed by LTA; (C) % change (0-2h) of platelet inhibition. Paired student's t-test, n=8, **p<0.01.

3.2.2 Influence on the level of soluble P-selectin in plasma

Clopidogrel administered acutely reduced significantly levels of sP-selectin in ppp from 29.6±10.0 ng/ml to 26.7±8.2 ng/ml after 2 h of a single loading dose of clopidogrel (paired t-test, n=19, p=0.031; Figure 5.2). Levels of sP-selectin were not changed following at least 3 days of treatment (28.6±8.8 ng/ml (one sample t-test, n=14, p=0.688)).

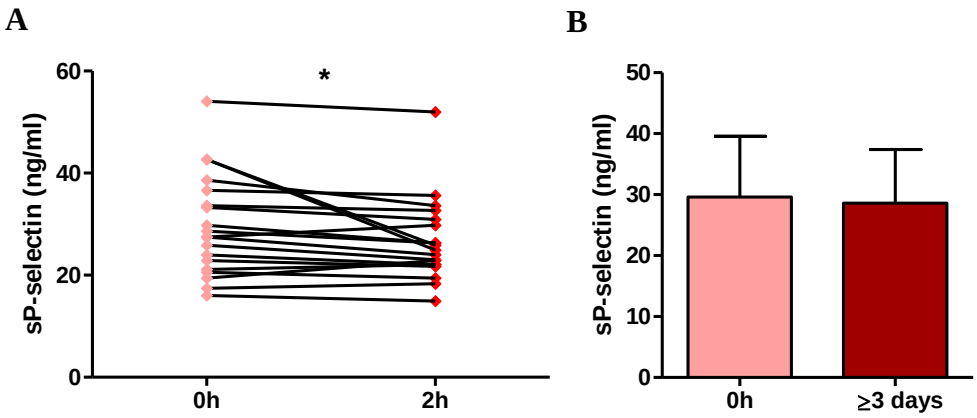


Figure 5.2. Plasma levels of sP-selectin in CAD patients (A) before (0h) and after (2h) receiving a loading dose of clopidogrel and (B) after at least 3 days of a maintenance therapy (≥3 days). (A) Paired t-test, n=19, *p<0.05; (B) one sample *t*-test, n=14 (≥3 days), p>0.05.

3.3 Influence of clopidogrel on NO metabolites

In order to determine the influence of acute and chronic therapy with clopidogrel on the vascular production of NO, the primary NO metabolites (namely nitrite, nitrate and RSNO) were measured in ppp.

3.3.1 Plasma nitrite

Clopidogrel significantly upregulated levels of nitrite (Figure 5.3). After 2 h treatment, plasma nitrite increased from 160.0±78.9 nM to 202.6±84.7 nM (paired t-test, n=36, p=0.015). In ≥3 days of treatement, nitrite levels were 254.3±139.4 nM (one sample t-test, n=22, p=0.005). Elevated amounts of plasma nitrite in ppp suggests the augmented endothelial production of NO or NO from another source in these patients.

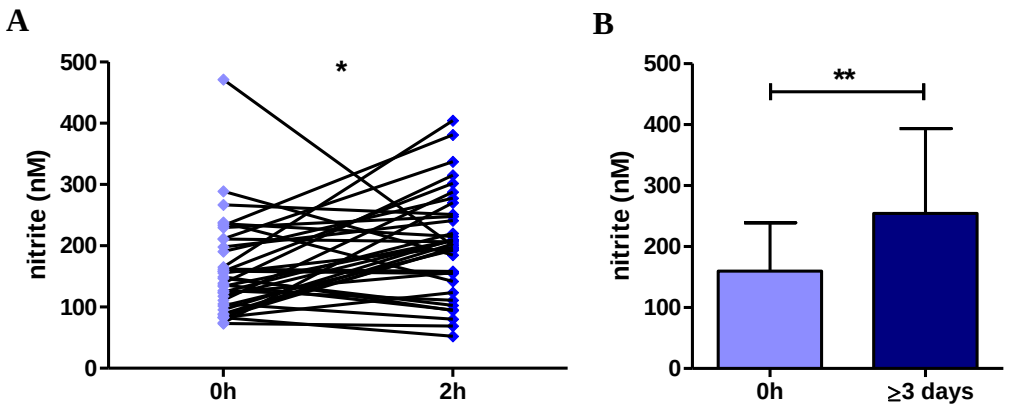


Figure 5.3. Plasma levels of nitrite in CAD patients (A) before (0h) and after (2h) receiving a loading dose of clopidogrel and (B) after at least 3 days of a maintenance therapy (≥3 days). (A) Paired t-test, n=36, *p<0.05; (B) one sample t-test, n=22 (≥3 days), **p<0.01.

3.3.2 Plasma nitrate

The increase in nitrite following initiation of clopidogrel treatment was not associated with increased levels of nitrate (Figure 5.4). In the acute setting, plasma nitrate declined significantly from 30.2±11.5 μM to 28.6±11.0 μM (paired t-test, n=36, p=0.025). After at least 3 days of treatment nitrate was 29.9±13.5 μM and unchanged from controls (one sample t-test, n=22, p=0.938).

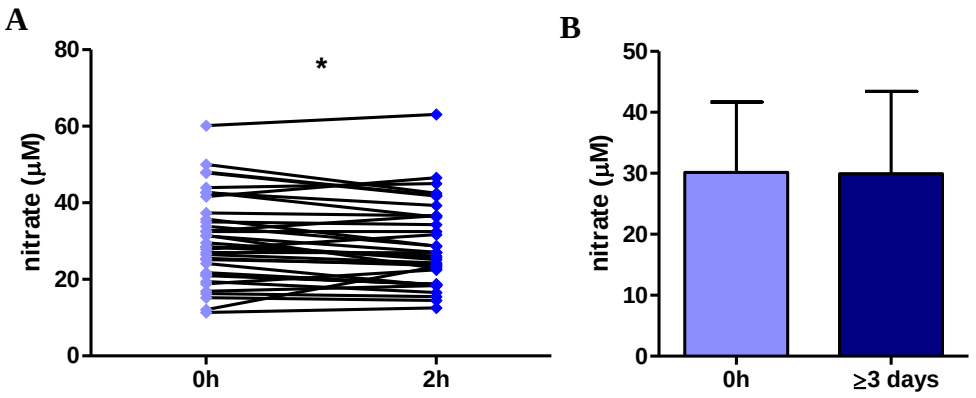


Figure 5.4. Plasma levels of nitrate in CAD patients (A) before (0h) and after (2h) receiving a loading dose of clopidogrel and (B) after at least 3 days of a maintenance therapy (≥3 days). (A) Paired t-test, n=36, *p<0.05; (B) one sample t-test, n=22 (≥3 days), p>0.05.

3.3.3 Plasma RSNO

On the basis of our previous results showing the potential for clopidogrel to make biologically active clopidogrel-SNO *in vitro* (see IV. Results, pages 140-172), we hypothesized that circulating levels of RSNO would increase following clopidogrel intake. However, RSNO levels remained unchanged after 2 h treatment with clopidogrel, (15.5±8.7 nM versus 14.1±5.4 nM (paired t-test, n=36, p=0.401)) and after at least 3-day treatment (14.2±9.2 nM (one sample t-test, n=22, p=0.504; Figure 5.5)).

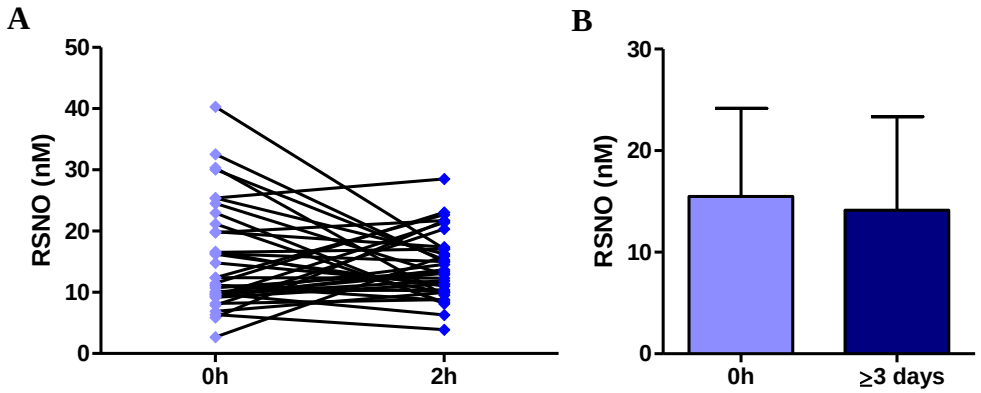


Figure 5.5. Plasma levels of RSNO in CAD patients (A) before (0h) and after (2h) receiving a loading dose of clopidogrel and (B) after at least 3 days of a maintenance therapy (≥3 days). (A) Paired t-test, n=36, p>0.05; one sample t-test, n=22 (≥3 days), p>0.05.

3.4 Plasma cGMP

Having shown the increase in plasma nitrite following clopidogrel, cGMP was also measured, as a marker of vascular NO activity. The level of cGMP in ppp was significantly upregulated (Figure 5.6). After 2 h treatment with clopidogrel, plasma cGMP increased from 215.2±127.0 pmol/ml to 234.6±109.0 pmol/ml (paired t-test, n=24, p=0.039) and was 281.7±70.8 pmol/ml after at least 3-day treatment (one sample t-test, n=19, p<0.001).

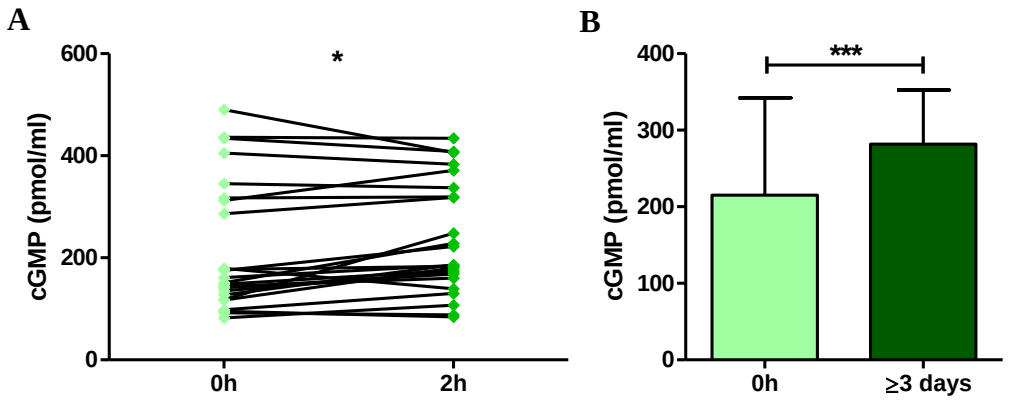


Figure 5.6. Plasma levels of cGMP in CAD patients (A) before (0h) and after (2h) receiving a loading dose of clopidogrel and (B) after at least 3 days of maintenance therapy (≥3 days). (A) Paired t-test, n=24, *p<0.05; (B) one sample t-test, n=19 (≥3 days), ***p<0.001.

3.5 Anti-oxidant actions of clopidogrel

Following on from our vascular experiments using rabbit aortae where we established an inhibitory effect of clopidogrel on vascular superoxide production (see IV. Results, pages 130-131), we also investigated whether a similar effect might be borne out in clopidogrel-treated CAD patients.

3.5.1 Direct anti-oxidant properties of clopidogrel

TAC assay did not reveal direct anti-oxidant properties of clopidogrel (Figure 5.7-8). While AAPH-induced loss in fluorescence was inhibited by common direct anti-oxidants such as trolox and tempol in a dose-dependent manner, clopidogrel did not provide similar protection.

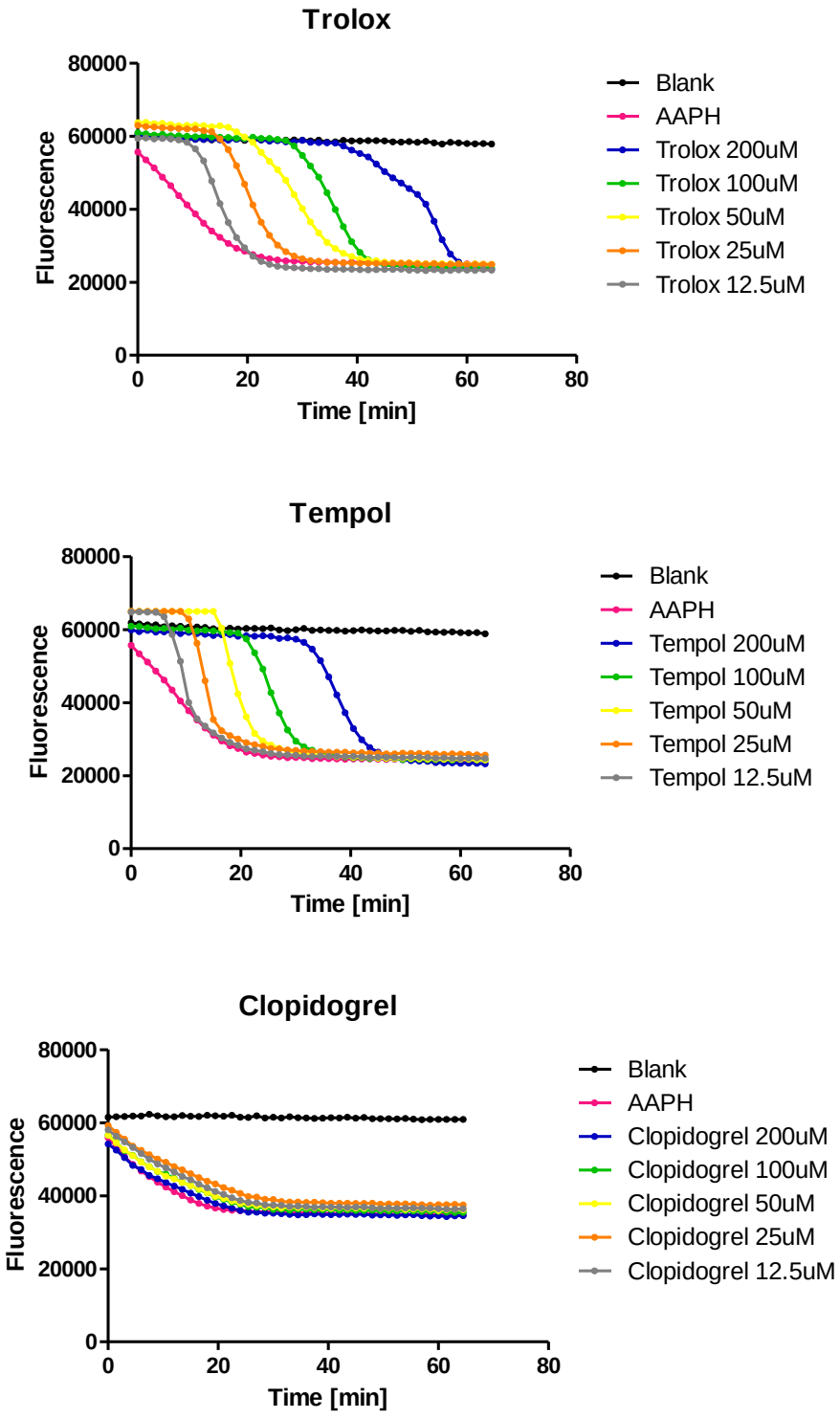


Figure 5.7. Fluorescent decay curves of fluorescein induced by AAPH in the presence of trolox, tempol and clopidogrel at different concentrations.

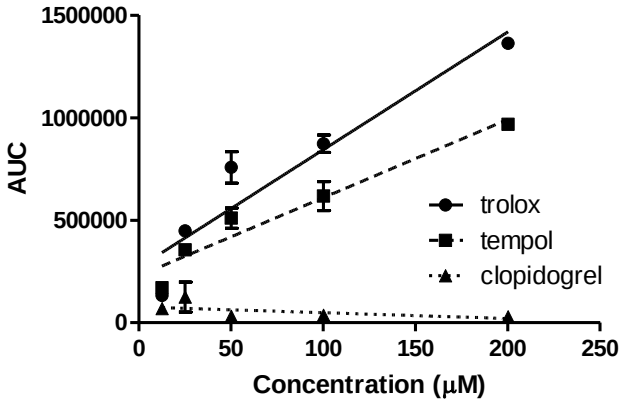


Figure 5.8. Linear plot of the net AUC vs. concentration of trolox, tempol and clopidogrel.

3.5.2 Influence of clopidogrel on the anti-oxidant status of plasma

Total anti-oxidant capacity (TAC) of ppp increased significantly from 60.7±11.5 %Tempol to 64.2±10.6 %Tempol after a single loading dose (paired t-test, n=25, p=0.037; Figure 5.9) and was not changed after a maintenance dose of clopidogrel: 62.1±4.2 %Tempol (one sample t-test, n=20, p=0.148). Results from TAC assay suggest that although clopidogrel is not an anti-oxidant itself, it was associated with an acute upregulation of the anti-oxidant defense mechanisms in plasma.

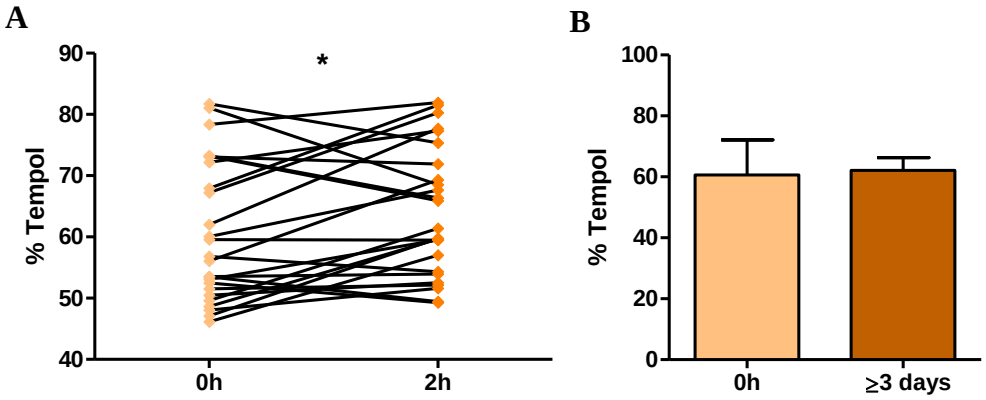


Figure 5.9. TAC of plasma from CAD patients (A) before (0h) and after (2h) receiving a loading dose of clopidogrel and (B) after at least 3 days of a maintenance therapy (≥3 days). (A) Paired t-test, n=25, *p<0.05; (B) one sample t-test, n=20 (≥3 days), p>0.05.

3.5.3 Influence of clopidogrel on nitrosative stress

Taking into account that clopidogrel therapy was associated with higher amount of circulating nitrite and increased anti-oxidant capacity, the effect on nitrosative stress was also investigated. 3-NT was measured in ppp as a marker of nitrosative stress (Figure 5.10). 3-NT was not significantly changed after a single loading dose (from 249.0±380.7 nM to 331.1±564.6 nM, paired t-test, n=12, p=0.278) or after a maintenence dose of clopidogrel (465.4±628.6 nM, one sample t-test, n=12, p=0.363). It is acknowledged however, this result was not confirmatory; there was high variation (0-1842 nM) between levels detected and some of them (n=8) could not be measured (see III. General Methods, page 94).

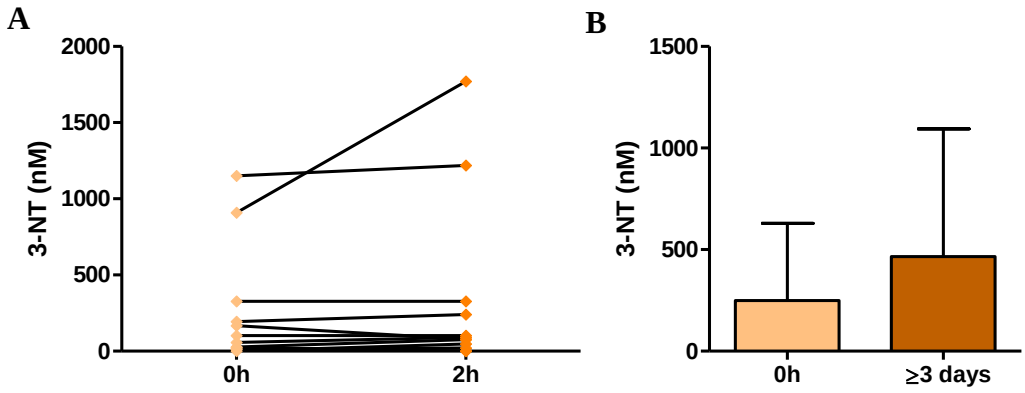


Figure 5.10. Plasma levels of 3-NT in CAD patients (A) before (0h) and after (2h) receiving a loading dose of clopidogrel and (B) after at least 3 days of a maintenance therapy (≥3 days). (A) Paired t-test, n=12, p>0.05; (B) one sample t-test, n=12 (≥3 days), p>0.05.

3.5.4 Influence of clopidogrel on the level of reduced thiols

The dynamic relationship between reduced (RSH) and oxidised (RSSR) redox state of proteins is regulated by reactive oxygen/nitrogen species (ROS/RNS) and correlates with the extent of various measures of oxidative stress[216, 217]. Hence, the amount of reduced thiols

present in plasma should reflect, in the most part, the global level of oxidative stress. In the short-term, levels of RSH did not change in comparison to control (364.6±34.2 μM vs. 355.2±29.5 μM; paired t-test, n=20, p=0.138) and in ≥3 days of treatment they reached significantly higher concentration: 389.7±25.1 μM (one sample t-test, n=14, p<0.001; Figure 5.11).

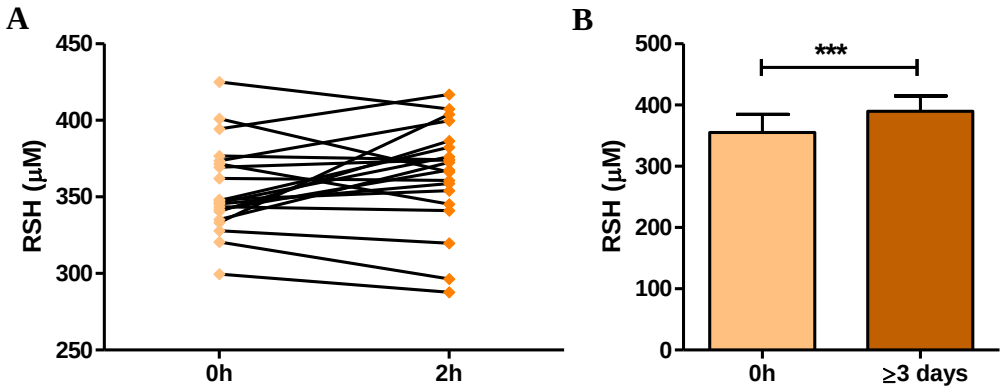


Figure 5.11. Plasma levels of RSH in CAD patients (A) before (0h) and after (2h) receiving a loading dose of clopidogrel and (B) after at least 3 days of a maintenance therapy (≥3 days). (A) Paired t-test, n=20, p>0.05; one sample t-test, n=14 (≥3 days), ***p<0.001.

3.6 Inter-relation between the various parameters measured

In an attempt to investigate potential relationships between vascular, oxidative and platelet-derived effects of clopidogrel on NO bioavailability, correlation analysis was performed for all measured parameters in plasma.

3.6.1 Relationship between different parameters in all groups of patients

Raw readings of all parameters from all patients (before, 2 h after receiving a loading dose of clopidogrel, as well as those on the maintenance dose) were compared (Table 15). This analysis was to provide the overall relationship between different parameters regardless the presence of clopidogrel.

	sP-selectin	**nitrite**	**nitrate**	**RSNO**	**cGMP**	**TAC**	**RSH**
sP-selectin							
nitrite	r=0.07 n=48 p=0.620						
nitrate	r=0.41 n=48 p=0.004	r=-0.04 n=67 p=0.765					
RSNO	r=0.08 n=48 p=0.590	r=-0.13 n=67 p=0.297	r=0.03 n=67 p=0.836				
cGMP	r=0.05 n=48 p=0.753	r=0.35 n=67 p=0.004	r=-0.11 n=67 p=0.361	r=0.08 n=67 p=0.533			
TAC	r=0.08 n=48 p=0.609	r=-0.27 n=67 p=0.028	r=0.22 n=67 p=0.078	r=-0.07 n=67 p=0.565	r=-0.53 n=67 p<0.0001		
RSH	r=0.03 n=50 p=0.820	r=0.37 n=48 p=0.011	r=0.04 n=48 p=0.778	r=-0.07 n=48 p=0.640	r=0.43 n=48 p=0.002	r=-0.24 n=48 p=0.098	

Table 15. Summary of the results of Pearson's correlations between parameters in all groups of patients. The Pearson's correlation coefficient (r), number of correlated samples (n) and the significance of correlation (p) have been reported. In red: significant correlations ($p<0.05$).

Nitrite and cGMP correlated with each other but did not correlate with sP-selectin (Figure 5.12). On the other hand, nitrite and cGMP correlated positively with RSH and negatively with TAC (Figure 5.13).

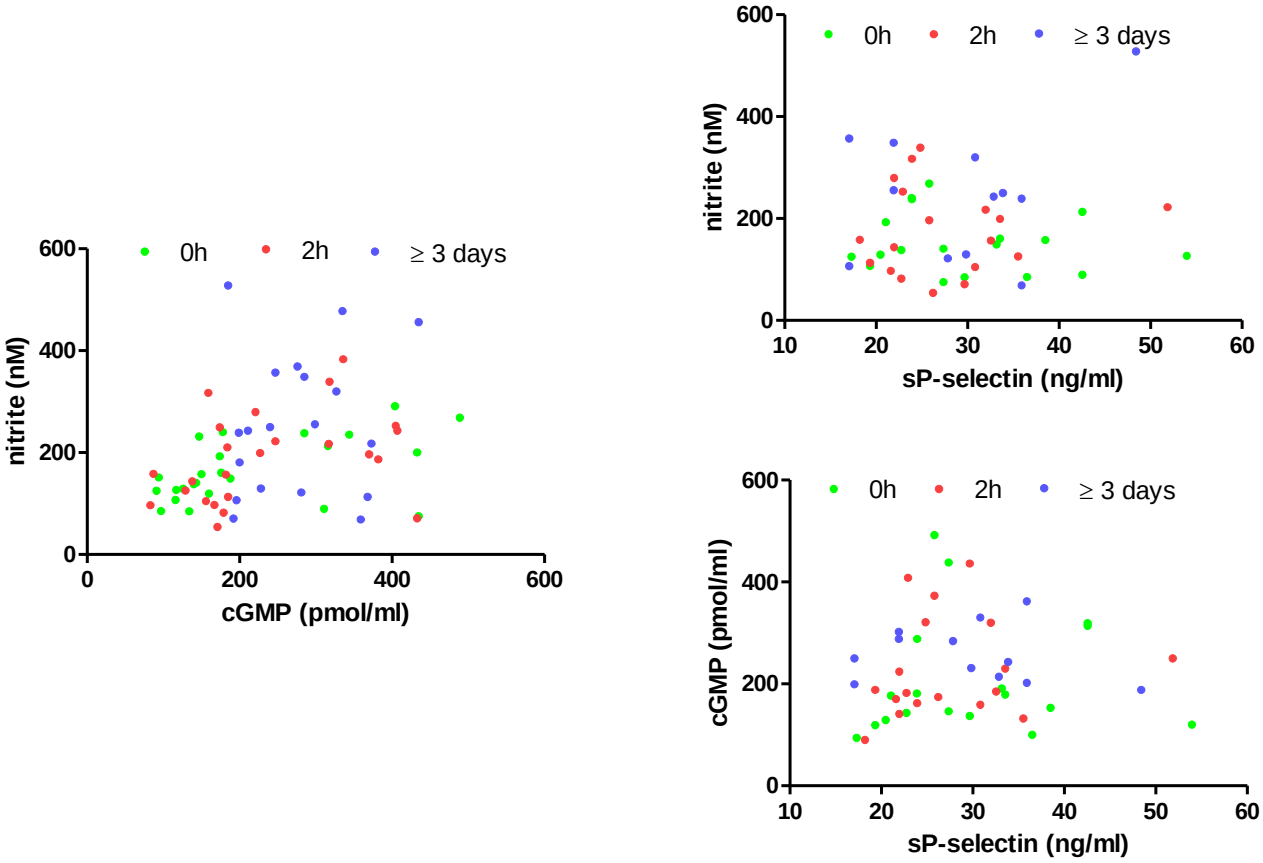


Figure 5.12. Pearson's correlations between nitrite, cGMP and sP-selectin in all groups of patients.

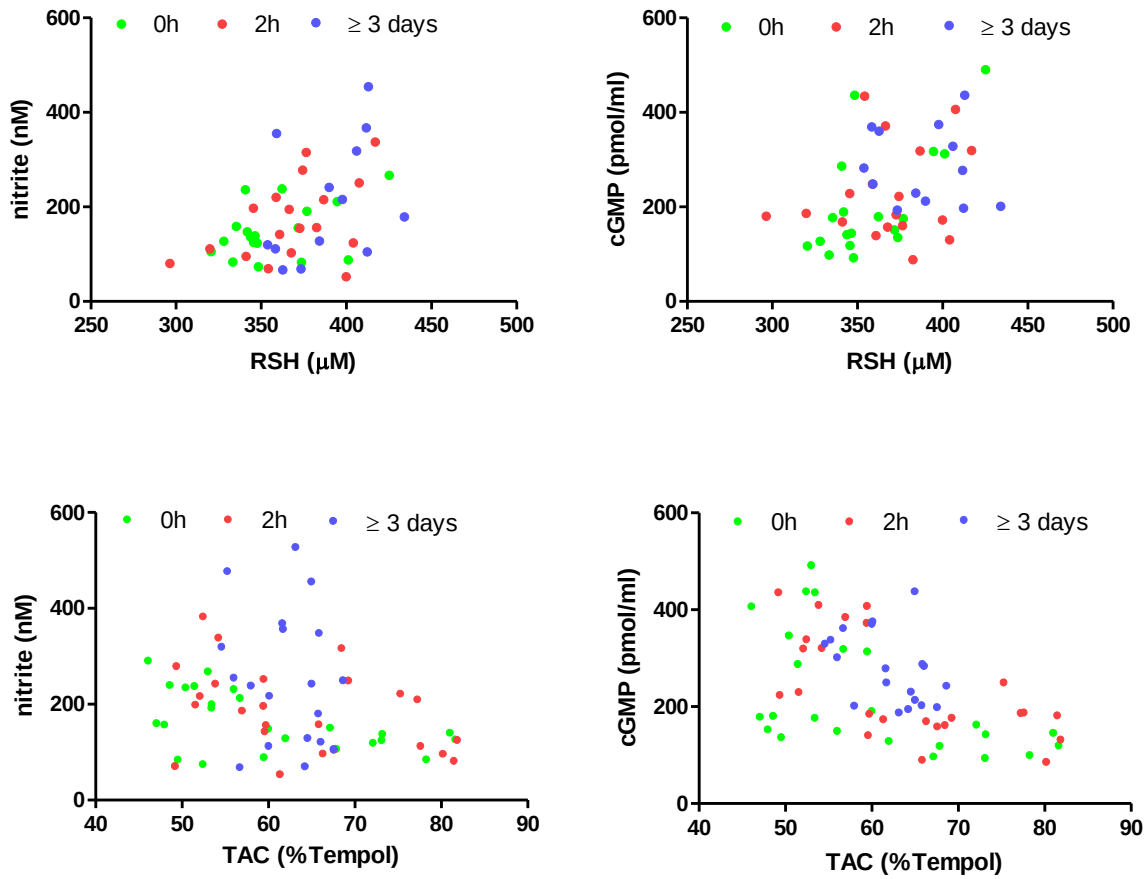


Figure 5.13. Pearson's correlations between nitrite, cGMP and RSH and TAC in all groups of patients.

3.6.2 Relationship between changes in different parameters following a single loading dose of clopidogrel

In order to relate changes in one parameter to another caused by the administration of clopidogrel, differences between clopidogrel naïve and the respective 2 h-post clopidogrel levels were compared (Table 16). This analysis was only performed within the group of patients receiving a loading dose of clopidogrel on the day of the study.

	ΔsP-selectin	Δnitrite	Δnitrate	ΔRSNO	ΔcGMP	ΔTAC	ΔRSH
ΔsP-selectin							
Δnitrite	r=-0.50 n=18 p=0.034						
Δnitrate	r=-0.53 n=18 p=0.022	r=0.32 n=24 p=0.134					
ΔRSNO	r=-0.46 n=18 p=0.054	r=0.15 n=24 p=0.478	r=0.42 n=24 p=0.042				
ΔcGMP	r=-0.01 n=18 p=0.965	r=0.33 n=24 p=0.115	r=0.20 n=24 p=0.361	r=-0.04 n=24 p=0.841			
ΔTAC	r=0.12 n=18 p=0.650	r=-0.62 n=24 p=0.001	r=-0.32 n=24 p=0.123	r=0.07 n=24 p=0.730	r=-0.16 n=24 p=0.448		
ΔRSH	r=0.27 n=18 p=0.276	r=0.06 n=18 p=0.807	r=0.16 n=18 p=0.534	r=-0.08 n=18 p=0.756	r=-0.13 n=18 p=0.593	r=-0.16 n=18 p=0.523	

Table 16. Summary of the results of Pearson's correlations between changes in parameters (Δ) after a loading dose of clopidogrel. The Pearson's correlation coefficient (r), number of correlated samples (n) and the significance of correlation (p) have been reported. In red: significant correlations ($p<0.05$).

The influence of clopidogrel on sP-selectin correlated significantly with changes in nitrite, nitrate and RSNO. Two extreme values of sP-selectin were identified (indicated by # on Figure 5.14) but were not significant outliers according to Grubbs' test, also called the (extreme studentized deviate) ESD method ($p>0.05$).

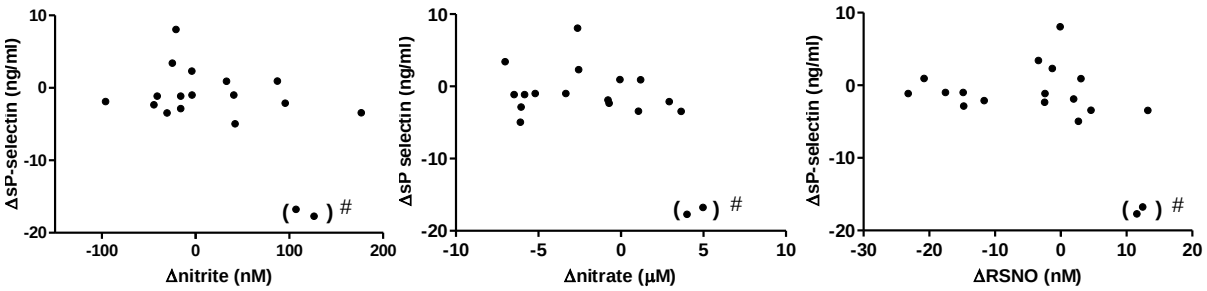


Figure 5.14. Pearson's correlations of changes in sP-selectin with nitrite, nitrate and RSNO after a loading dose of clopidogrel. # extereme values but not significant outliers (see text).

Interestingly, increased levels of nitrite after a loading dose of clopidogrel correlated inversely with increased levels of TAC in the same samples (Figure 5.15). It implies that the greater the increase in nitrite concentration following clopidogrel loading, the less TAC was upregulated.

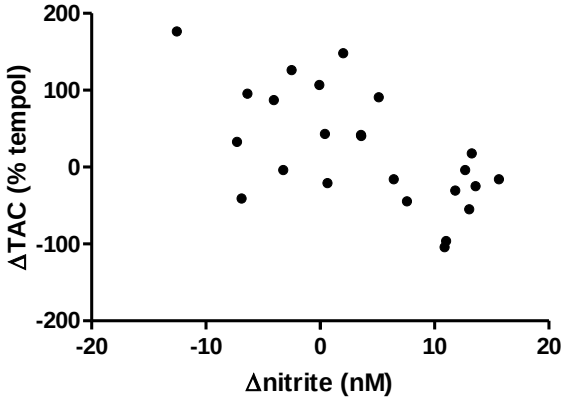


Figure 5.15. Pearson's correlation of changes in nitrite and TAC after a loading dose of clopidogrel.

4. Discussion

This observational study was designed to assess the potential influence of clopidogrel therapy on NO production, metabolism and bioavailability in CAD patients and to verify some of our previous findings *in vitro*. Patients receiving short- or long-term clopidogrel exhibit an increase in NO metabolites and effective vasodilation, as reflected by higher levels of nitrite and cGMP. The increased total anti-oxidant capacity of plasma in acute treatment group and augmented production of reduced thiols in the chronic treatment group also suggest a parallel positive influence on the anti-oxidant status of plasma.

A variety of methods have previously been utilized to assess platelet function and its inhibition during anti-platelet therapy. Among the available methods measuring aggregation of platelets, LTA has been the most widely used by researchers, whereas more recently VerifyNow^{TM} has been developed for more convenient clinical use[218]. My colleagues and I have found that a clopidogrel loading dose resulted in 49.6±31.9 % inhibition of ADP-mediated platelet aggregation assessed by LTA, and this was in close agreement with values reported by VerifyNow^{TM} assay (48.6±9.2 % - see Shawmendra Bundhoo MD 2011). These findings are also in line with published data, where 600 mg clopidogrel inhibited ~50 % of ADP-induced platelet aggregation[135], although it is acknowledged that the degree of inhibition by clopidogrel varies considerably between patients. The magnitude of platelet activation can also be measured using soluble markers of platelet activity such as sP-selectin[219, 220]. sP-selectin can be produced by both platelets and endothelial cells, although evidence suggest that the majority of circulating sP-selectin arises from activated platelets[221, 222]. Loading dose of

clopidogrel downregulated levels of sP-selectin after 2 h intake, which together with results from LTA and VerifyNow^{TM} assays imply that clopidogrel met its pharmacological purpose.

It is worth mentioning that 59.0+/-32.8 % TRAP-induced aggregation was also inhibited by clopidogrel. Inhibitory effect of $P2Y_{12}$ antagonists on PAR1 and PAR4-mediated thrombin signalling has been observed by others[223, 224] and led to reformulation of the reference channel in VerifyNow^{TM}[225].

An important consideration in the interpretation of results of this chapter is that there were two distinct clopidogrel-treated groups of CAD patients studied here. The first group involved individuals who received a loading 600 mg dose of clopidogrel for the first time, followed by evaluation of acute changes after 2 h ingestion. These people received the drug prior to PCI to protect them from peri-procedural complications. The second group involved those who were maintained on a daily 75 mg dose of clopidogrel for a minimum of 3 days. In this setting clopidogrel was used mainly to prevent vessel restenosis.

One of the main and novel findings of this work is the positive effect of clopidogrel on production of NO accompanied with augmented accumulation of cGMP in plasma (Table 17). Plasma nitrite, a marker of endothelial NO production and cGMP, a major secondary messenger of vascular relaxation were both significantly increased in the acute and chronic setting. It is very interesting considering that most of the beneficial effects of clopidogrel including reduced inflammatory markers and enhanced FMD were reported after prolonged anti-platelet therapy with clopidogrel. What is more, they have primarily been attributed to the inhibition of platelet activation and adhesion to the vascular wall leading to an improvement of vascular function over time, rather than the possibility of a platelet-

independent effect. However, in the light of our data, clopidogrel appears to be responsible for an improvement in production of NO and cGMP as early as 2 h after a loading dose, which points to an alternative route of action, possibly via direct interaction with vascular endothelium.

An increase in plasma nitrite and cGMP by clopidogrel is likely to be favorable for CAD patients with dysfunctional endothelium, where the classical L-arginine-eNOS-NO-sGC-cGMP pathway is dysregulated due to increased oxidative stress[5, 17, 25, 30]. Plasma nitrite levels reflect endothelial eNOS activity and is a marker of NO bioavailability[200, 201]. NO released by endothelium inhibits both platelet aggregation and adhesion as well as leukocyte recruitment and adhesion to sites of vascular injury, which could add to the anti-platelet and anti-inflammatory effects of clopidogrel mediated by direct blockage of $P2Y_{12}$ receptors. In addition, increased levels of plasma cGMP might be a consequence of enhancement of endothelium-dependent vasorelaxation mediated by NO[226].

In addition, under conditions where the endothelium is damaged or dysfunctional, circulating nitrite can serve protective roles, especially where lower pH or oxygen promote reduction of nitrite to NO by several mechanisms, including enzymatic reduction by deoxygenated hemoglobin or myoglobin, components of the mitochondrial respiratory chain, XO, eNOS, and cytochrome P450, as well as nonenzymatically by acidic disproportionation[198]. These mechanisms have been implicated in a variety of animal and human models of vascular injury. However, in our study the concentration of plasma nitrite is mainly taken to reflect the increased NO bioavailability and the potential advantageous effect of nitrite augmentation itself would be an interesting subject for further investigation.

	Groups									Effects			
	Pre			Post			Chronic			Post vs. pre		Chronic vs. pre	
	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean of differences	p-value	Difference between means	p-value
sP-selectin [ng/ml]	29.6	10.0	19	26.7	8.2	19	28.6	8.8	14	-2.9	$p < 0.05$	-0.7	$p > 0.05$
Nitrite [nM]	160.0	78.9	36	202.6	84.7	36	254.3	139.4	22	42.6	$p < 0.05$	94.3	$p < 0.01$
Nitrate [µM]	30.2	11.5	36	28.6	11.0	36	29.9	13.5	22	-1.6	$p < 0.05$	-0.2	$p > 0.05$
RSNO [nM]	15.5	8.7	36	14.1	5.4	36	14.2	9.2	22	-1.4	$p > 0.05$	-1.3	$p > 0.05$
cGMP [pmol/ml]	215.2	127	24	234.6	109.0	24	281.7	70.8	19	19.4	$p < 0.05$	67.5	$p < 0.05$
TAC [% Tempol]	60.7	11.5	25	64.2	10.6	25	62.1	4.2	20	3.5	$p < 0.05$	1.4	$p > 0.05$
RSH [µM]	355.2	29.5	20	364.6	34.2	20	389.7	25.1	14	9.4	$p > 0.05$	34.5	$p < 0.01$
3-NT [nM]	249.0	380.7	12	331.1	564.6	12	465.4	628.6	12	82.1	$p > 0.05$	193.8	$p > 0.05$

Table 17. Summary of effects of acute and chronic treatment with clopidogrel in CAD patients. In red: significant results.

Despite demonstrating nitrosation properties of clopidogrel *in vitro*, the level of RSNO in plasma was not increased in CAD patients receiving clopidogrel. Certainly all necessary conditions considered optimal for formation of RSNO were fulfilled. Briefly, clopidogrel acted as a donor of –SH moiety delivered in a form of active (0.01%) and inactive (1%) metabolites. The stomach and acidic clopidogrel provided an acidic pH environment (pH=2-3) and there was a potential source of nitrite ions in stomach (0.6-20 μM)[185, 186] or gastrointestinal tract. It is difficult to pin-point why the *in vitro* results were not translated to the patient setting. The lack of influence of an oral dose of clopidogrel on plasma level of RSNO might be explained by:

i) the labile nature of RSNO species, in terms of their sensitivity to temperature, light, and breakdown by transition metals[65], which would result in higer NO_2^- as an end product (as observed),
ii) generally low concentrations of RSNO in comparison to other NO metabolites in blood, which influence the detectability[34],
iii) tissue absorption and hepatic metabolism of clopidogrel as well as common transnitrosation reactions of RSNO, which could influence detectability [110, 187]
iv) the relatively low level of physiological nitrite[185, 186].

We found that a loading dose of clopidogrel increased the hydrophilic anti-oxidant activity of plasma against peroxyl radicals, whereas a maintenance dose resulted in a bigger plasma pool of reduced thiols. The mechanism for these observed changes is not known and requires further study. However, it was established using TAC assay that clopidogrel did not itself act as a direct scavenger of peroxyl radicals, but rather via regulation of enzymatic anti-oxidants. A positive correlation between cGMP, nitrite and reduced thiols also pointed to a

close relationship between these parameters. In turn, a negative correlation between nitrite, cGMP and TAC suggests the resolving mechanism is not straightforward.

The regulation of platelet-vascular wall interactions is shared between vascular endothelium and platelets, which makes a strict distinction between vascular- and platelet-originated effects a real challenge. Clopidogrel is an anti-platelet drug and yet there is substantial evidence in the literature and in this thesis (IV. Results Part 1 and 2) of additional effects on vasculature which are clearly platelet and/or $P2Y_{12}$ receptor independent. However, these additional effects are easier studied *in vitro* or in animal models than in humans. In patients, markers of platelet activation and aggregation are usually compared with markers of endothelial function in order to establish a relationship. It is commonly accepted that plasma nitrite reflects endothelial production of NO and plasma sP-selectin is mainly released by activated platelets. In our study the influence of clopidogrel on sP-selectin correlated significantly with changes in nitrite in acute setting suggesting that enhanced endothelial NO production by clopidogrel was related to the altered activation of platelets. However this relationship has not been confirmed in the chronic setting, where levels of sP-selectin were not changed and levels of nitrite were even higher than in former comparison.

The main limitations of this study is the relatively small number of recruited CAD patients receiving chronic treatment with clopidogrel and the fact that the study was not longitudinal, in that the same patients were not measured in all three groups. N=22 demonstrated a significant increase in nitrite, cGMP and reduced thiols but measurement of other markers (e.g. sP-selectin) could have been underpowered. In retrospect, the aggregation of platelets assessed by LTA should have been measured in all samples, which would have provided a more sensitive marker of platelet activation/aggregation as a functional test, rather than a biochemical assessment of sP-selectin.

Summary:

- Acute and chronic treatment of CAD patients with clopidogrel caused an increased endothelial production and vasoactivity of NO (as reflected by levels of plasma nitrite and cGMP, respectively).

- The level of NO_3^- in plasma was lower only in acute setting while RSNO was not changed in any CAD patients receiving clopidogrel.

- Clopidogrel therapy was associated with direct effects on plasma anti-oxidant and reduced thiol status, but more work is required to identify the precise signalling pathways involved.

V. GENERAL DISCUSSION AND CLINICAL RELEVANCE

My work focused on the influence of anti-platelet agents, in particular $P2Y_{12}$ antagonists on vascular NO metabolism and signalling. The rationale for addressing this is the equal importance of platelet activity and formation of endothelium-derived NO in the maintenance of vascular homeostasis. Platelets play a central role in the pathophysiology of atherothrombosis, which is a common consequence of many cardiovascular diseases. Endothelial derived NO is a key regulator of platelet aggregation, vascular relaxation and inflammation. Reduced endothelial NO production and bioavailability is the main determinant of endothelial dysfunction, which leads to the development of cardiovascular diseases and subsequent activation and aggregation of platelets. $P2Y_{12}$ antagonists target the ADP-induced activation and aggregation of platelets, which has been particularly efficient in treatment of patients with ACS and those undergoing coronary stenting as a secondary prevention strategy. However, therapies with different $P2Y_{12}$ blockers are often limited by drug metabolism, increased risk of bleeding or other side effects. Importantly, other beneficial effects of anti-platelet agents secondary to the main target have been noted, but these have been considered a result of the effects on platelets. Consequently, not much is known about their direct influence on vascular NO signalling, which might be a critical component for further improving patient outcomes and adjusting future treatment modalities.

In the first results chapter of this thesis the enhanced response to NO donors following incubation of rabbit aortic rings with pro-drug clopidogrel was observed. This effect was independent of inhibition of $P2Y_{12}$ receptors by clopidogrel in platelets or the vasculature. In the clinical setting, this might imply that patients receiving concomitant therapy with organic nitrates and clopidogrel could have an enhanced dilatory response than patients receiving organic nitrates alone. Clopidogrel loading has been recently associated with an enhancement

of brachial artery diameter when GTN was subsequently administered[161, 170]. This could be particularly important for patients requiring coronary stenting, where an accurate adjustment of coronary stents to the vessel diameter is crucial for clinical outcomes[227]. There is also longer term benefit for patients with existing endothelial dysfunction (reduced NO production or effectiveness) as a given amount of NO will have greater effect.

Results reported in the first results chapter also revealed anti-oxidant and anti-inflammatory effects of pro-drug clopidogrel when incubated with rabbit vessels for 3.5 h. The time necessary to observe these effects might have been the result of several factors including i) methods' sensitivity, ii) time required for genetic transcription and translation and/or iii) basal inflammation. With regards to the last point, it is possible that an induction of oxidative and inflammatory responses in the vascular tissue was needed in order to observe anti-oxidant and anti-inflammatory effects of clopidogrel – the model system we utilised (rabbit aortae) was essentially harvesting healthy tissue that may subsequently experience a degree of insult as a function of being kept under laboratory conditions, whereas future studies may show greater benefit comparing tissue from normal versus diseases animal models. In agreement with this, it has been noticed that clinical evidence of inflammation not only predicts major cardiovascular events in patients undergoing PCI but also defines the patients who will benefit from clopidogrel therapy[228]. For example, the post hoc analysis of CREDO trial revealed that treatment with clopidogrel was beneficial only in patients with high levels of inflammation markers i.e. high-sensitivity C-reactive protein (hs-CRP) and pregnancy associated plasma protein-A (PAPP-A)[209]. Furthermore, 1-year but not the 28-day treatment was sufficient to detect a significant reduction in the composite end point confirming the importance of time scale to the observed changes. The authors of this study questioned whether these anti-inflammatory effects of clopidogrel were the result of its anti-platelet effects alone. In our model, the anti-oxidant and anti-inflammatory effects of

clopidogrel could not be attributed to platelets pointing to the direct action of clopidogrel on vessels. It is relevant therefore that other invasive treatment strategies in CAD patients (e.g. revascularization, PCI) were more efficient among those who had evidence of inflammation on the basis of elevated Il-6 levels[229]. Likewise, the benefit of aspirin therapy for the primary prevention of MI was greatest in men with evidence of baseline inflammation[230].

The second results chapter of this thesis presented a discovery of novel nitrosation properties of thienopyridines and non-thienopyridines in an environment of nitrite ions and acidic pH (pH<4). All tested drugs were capable of the formation of drug-SNO derivatives but their amount was dependent on many factors such as chemical properties of pharmaceutical grade tablets, nitrite concentration available for the reaction and the stability of formed SNO complexes. At first, it was surprising to see that the pH of water solutions of $P2Y_{12}$ antagonist varied (from pH=2 of clopidogrel to pH=7 of ticagrelor) and that thienopyridine solutions contained reduced thiol groups even without prior metabolism. Despite this, we found that the low levels of reduced thiol in thienopyridine solutions detected was not sufficient to have biological action (in terms of inhibiting the ADP-induced aggregation or effects on vessel vasorelaxation). This is in complete agreement with the known pharmacology of these agents, which require hepatic metabolism to produce much higher amounts of active metabolites sufficient to exhibit biological effects. Importantly, we showed that the corresponding levels of SNO derivatives that could be formed under physiological conditions could certainly exert such activity.

In the presence of nitrite ions clopidogrel-SNO and prasugrel-SNO were formed readily in their basal pH and participated in all the reactions expected of RSNO species such as inhibition of platelet aggregation, vasodilation and transnitrosation. According to our expectations ticagrelor did not form SNO derivatives in its basal solution due to the very small detectable amount of reduced thiol group and a neutral pH. However, when pH was

lowered to pH=2-3, the formation of ticagrelor-SNO was also apparent. This unforeseen result might be important in terms of blocking platelet $P2Y_{12}$ receptors by ticagrelor. Whereas the exposure of the reduced thiol group in thienopyridines is desirable and increases their pharmacological activity, in ticagrelor this would alter its structure and could be detrimental to its specific interaction with $P2Y_{12}$ receptors. Certainly more studies are necessary to confirm the influence of acidity on the pharmacological efficiency of ticagrelor. One can speculate that the *in vivo* effect of stomach acid on ticagrelor would rather be small since the chemical is protected by tablet coating.

Although the drug-SNO formation was not confirmed *in vivo*, this might be due to overall low concentration and labile nature of RSNO species in comparison to other NO metabolites, especially in blood. Hypothetically, the oral intake of $P2Y_{12}$ antagonists could lead to the production of drug-SNO in the stomach or GI tract and increase the total pool of RSNO metabolites in blood, which would be beneficial for patients with reduced NO bioavailability. RSNO comprises an important part of NO signalling via protection of the NO moiety, which can then be released when required, but these effects are paralleled by the importance of RSNO in regulating S-nitrosylation and regulation of various proteins downstream from NO production.

In the third results chapter the positive influence of clopidogrel therapy on NO production and bioavailability in CAD patients was investigated. Plasma biomarkers reflecting endothelial release of NO and its main effector molecule cGMP were increased already after a 2 h loading dose and following a maintenance dose. This would imply that clopidogrel enhanced NO bioavailability and recovered, at least partially, endothelial function improving cardiovascular health and well-being in these patients. Although an early effect of clopidogrel on plasma nitrite and cGMP levels suggests the lack of platelet influence, the possible role of platelets remains to be confirmed. These findings could be important for

understanding the "rebound" phenomenon after withdrawal of clopidogrel, which has been reported in several studies[231-233]. The "rebound" effect occurs as a result of clopidogrel cessation when one or more parameters of either platelet activity or vascular inflammation is increased even further compared to before clopidogrel therapy was initiated. This effect has been observed in ACS patients undergoing PCI and/or coronary stenting in diabetic and non-diabetic populations and was associated with a clustering of adverse clinical events such as death and MI[231-234]. There is no clear indication whether "rebound" phenomenon is due to increased platelet activation resulting in pro-thrombotic and pro-inflammatory responses or due to independent augmentation of inflammation, which could be pro-thrombotic. In respect to our results, a premature cessation of clopidogrel could potentially deprive patients of a beneficial effect on endothelial NO, which in turn inhibits platelet activation and aggregation as well as having anti-inflammatory properties.

The increases in plasma nitrite measured as a result of clopidogrel treatment are particularly relevant to recent studies employing administration of nitrite, either i.v. or via dietary intake of nitrate, to alter vascular function and/or homeostasis. Nitrite itself is a relatively weak dilator, but it has been shown in animal and human models that in ischemic/hypoxic conditions it can be reduced to NO providing vasodilation, inhibition of platelet aggregation and cytoprotection[42-45, 198]. Additionally, novel anti-inflammatory pathways of nitrite have been revealed recently[235, 236]. In our laboratory a low dose sodium nitrite infusion (1.5 μM/min) for 20 min in patients with stable CAD increased plasma NO_2^- concentration from ~160 nM up to ~350 nM, which reduced inducible myocardial ischaemia without influencing normoxic vasculature[46]. Because a similar level of plasma NO_2^- concentration was observed in clopidogrel-treated CAD patients (254±140 nM), it is reasonable to assume that anti-platelet therapy with clopidogrel may protect patients from ischaemic events also by endogenous stimulation of NO production from the enhanced nitrite.

Although the advantage of clopidogrel pre-loading and treatment long-term has been demonstrated, the optimal duration and dosage of maintenance therapy is still a matter of debate[232]. Currently, 1-12 months of clopidogrel 75 mg is recommended for all ACS patients, depending on the indication, after which the clopidogrel is stopped. We have shown that plasma nitrite and cGMP levels were increased (accumulated) during clopidogrel treatment i.e. within minimum 3 days after intake. In order to establish the overall and the most optimal effect of clopidogrel on vascular NO signalling, further longitudinal studies using more specific time points throughout the entire treatment regimen and possibly after cessation of the drug preferably in the same people would need to be undertaken.

In summary, our studies using biochemical and biological *in vitro* and *in vivo* models show an influence of clopidogrel on vascular response to NO as well as NO production, metabolism and bioavailability. Some of these effects extended beyond the inhibition of platelet $P2Y_{12}$ receptors by clopidogrel increasing uncertainty about the precise mechanism by which clopidogrel achieves its anti-platelet effects. More research is required to identify the molecular pathways involved in the observed "side effects" not only to improve the understanding of the interpersonal variability in response to clopidogrel but also to develop better anti-thrombotic therapies for different individuals. Newer more potent anti-platelet agents such as prasugrel and ticagrelor already offer better inhibition of platelet aggregation but their influence on vascular health in long-term is not known and worth investigating. Furthermore because too efficient blockage of platelet aggregation is associated with life-threatening bleeding, the ultimate goal of new therapies should be an improvement of vascular function with adequate inhibition of platelet activity. Currently, because platelet activation is the only convenient clinical indication of risk, the drive is for more efficient platelet inhibition and the non-classical benefits of existing anti-platelet therapies are not being actively pursued.

Publications and presentations

1. Anderson RA, Bundhoo SS, **Sagan E**, Dada J, Harris RA, Halcox JP, Lang D, James PE. Direct Vasoactive Properties of Thienopyridine-Derived Nitrosothiols. TCT Congress 2010; Washington, USA: *Journal of American College of Cardiology*; 2010; 56; B26. Elsevier.

2. **Sagan E**, Bundhoo SS, Halcox JP, James PE. Interaction of Clopidogrel with vascular endothelium. Poster presented at BHF Sponsored Summer School in Vascular Biology, Bristol, July 2010.

3. **Sagan E**, Bundhoo SS, James PE. The vascular response to exogenous nitric oxide is enhanced by the anti-platelet drug, clopidogrel. Poster presented at Nitric Oxide Gordon Conference Ventura, USA. February 2011.

4. James PE, Bundhoo SS, **Sagan E**, Hassan N, Pinder AG, Rogers SC, Morris K, Anderson RA. Thienopyridine Derived Nitrosothiols. Poster presented at Nitric Oxide Gordon Conference Ventura, USA. February 2011.

5. Bundhoo SS, Anderson RA, **Sagan E**, Dada J, Harris RA, Halcox JP, Lang D, James PE. Direct Vasoactive Properties of Thienopyridine-Derived Nitrosothiols. *Journal of Cardiovascular Pharmacology*; 2011. In press. Lippincott Williams and Wilkins.

6. Bundhoo SS, Anderson RA, **Sagan E**, Hassan N, Pinder AG, Rogers SC, Morris K, James PE. Direct formation of thienopyridine-derived nitrosothiols - Just add nitrite!. *European Journal of Pharmacology*; 2011. In press. Elsevier.

7. Bundhoo SS, Anderson RA, **Sagan E**, Hassan N, Pinder AG, Rogers SC, Morris K, James PE. Direct formation of thienopyridine-derived nitrosothiols: A novel mechanism of action of an old drug. Abstract present at Cardiff Medical Society, University of Wales College of Medicine. April 2011.

8. Anderson RA, Bundhoo SS, **Sagan E**, Dada J, Harris RA, James PE. From thienopyridines to Nitrosothiols: A Novel Mechanism of Thienopyridines Activity: ACC Scientific Session 2011; New Orleans, USA: *Journal of American College of Cardiology*; 2011:57: 1918. Elsevier.

9. Anderson RA, Bundhoo SS, **Sagan E**, Dada J, Lang D, James PE. The formation of S-Nitrosothiols from Thienopyridines inhibit Platelet Aggregation without biotransformation: Novel mechanism of Action?: ACC Scientific Session 2011; New Orleans, USA: *Journal of American College of Cardiology*; 2011:57: 1917. Elsevier.

10. James PE, Bundhoo SS, **Sagan E**, Hassan N, Pinder AG, Rogers SC, Morris K, Anderson RA. Thienopyridine Derived Nitrosothiols. Poster presented at 13th UK Platelet Group Meeting, Cardiff. September 2011.

11. **Sagan E**, Bundhoo SS, Anderson RA, Halcox JP, James PE. Direct effects of platelet $P2Y_{12}$ receptor antagonists on Vascular Reactivity: a comparison of clopidogrel, prasugrel and cangrelor. Poster presented at 13th UK Platelet Group Meeting, Cardiff. September 2011.

12. **Sagan E**, Bundhoo SS, Anderson RA, Halcox JP, James PE. Direct effects of platelet $P2Y_{12}$ receptor antagonists on Vascular Reactivity: a comparison of clopidogrel, prasugrel and cangrelor. Poster presented at 26th Annual Postgraduate Research Day, Cardiff University, November 2011.

13. **Sagan E**, Bundhoo SS, Anderson RA, Halcox JP, James PE. The influence of antiplatelet therapy on the *in vivo* Nitric Oxide bioavailability. Poster presented at 7th International Conference on the Biology, Chemistry and Therapeutic Application of Nitric Oxide, Edinburgh, July 2012.

14. Bundhoo SS, **Sagan E**, James PE, Anderson RA. Clopidogrel results in favourable changes in Nitric Oxide metabolism in patients with stable coronary artery disease undergoing percutaneous coronary intervention and receiving chronic therapy. Manuscript in preparation.

15. **Sagan E**, Bundhoo SS, Francis SE, Halcox JP, James PE. Clopidogrel has direct, non-$P2Y_{12}$-mediated effects on vascular endothelium: Enhanced sGC-independent vasodilation to NO donors. Manuscript in preparation.

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